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Special Issue Reprint

Functionality and Food Applications of Plant Proteins

Edited by
Yonghui Li

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Editor

Yonghui Li

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Editor
Yonghui Li
Grain Science and Industry
Kansas State University
Manhattan
United States

Editorial Office
MDPI
St. Alban-Anlage 66
4052 Basel, Switzerland

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Contents

About the Editor

Yonghui Li

Dr. Yonghui Li is an Associate Professor of Grain Chemistry and the Director of the Wheat Quality Lab at Kansas State University. He also serves as a graduate faculty member of the Food Science Institute. He teaches undergraduate and graduate courses in Grain Analysis and Protein Chemistry and Technology. Dr. Li's research focuses on the structure, chemistry, modification, and functionality of grain proteins and bioactive peptides with the aim of developing high-quality, functional grain-based foods, ingredients, and nutraceuticals. Since joining the faculty in 2016, Dr. Li has received over USD 4.2 million in research funding, with USD 3.3 million awarded as the lead PI, from various federal, state, and industrial sources. Dr. Li has published 110 journal articles, 130 presentations, 3 book chapters, and 2 patents. He is an associate editor for both *Journal of Food Science* and *Cereal Chemistry* and serves on the editorial boards of several other international journals.

Preface to ”Functionality and Food Applications of Plant Proteins”

The demand for plant proteins continues to increase due to the growing global population, rising protein deficiency, and their versatile environmental, functional, nutritional, and health benefits. Plant proteins represent a more sustainable source to (partially) supplement costly animal-based foods, such as meat, egg, and dairy products. Protein functional properties, including solubility, emulsification, foaming, water retention, fat binding, viscosity, rheology, thickening, and gelling, are closely associated with protein behavior and application suitability in food items.

Many factors can influence protein functionality and application, such as protein sources, production methods, molecular structures, chemical properties, food formulations and environment, and food processing techniques. Physical, chemical, and biochemical methods have been investigated to further enhance protein functionality. The potential applications of plant proteins are diverse and include alternatives to meat, egg, and dairy products and ingredients, extenders in meat, poultry, and seafood, ingredients in bakery products, cereals, and snacks, beverages, confectionaries, and bioactive peptides, among others.

We have compiled 15 papers from the Special Issue of *Foods* entitled “Functionality and Food Applications of Plant Proteins”. These papers focus on the characterization, chemistry, interaction, processing, modification, functionality, and/or application of various plant proteins in relation to human food. This book is a reprint of that Special Issue, aiming to make the valuable insights and research presented in those papers more widely accessible to interested readers.

Yonghui Li
Editor

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Article

Salt Solubilization Coupled with Membrane Filtration-Impact on the Structure/Function of Chickpea Compared to Pea Protein

Brigitta P. Yaputri, Fan Bu and Baraem P. Ismail *

Food Science and Nutrition Department, University of Minnesota, 1334 Eckles Ave, Saint Paul, MN 55108, USA
* Correspondence: bismailm@umn.edu; Tel.: +1-612-625-0147

Abstract: The demand for pulse proteins as alternatives to soy protein has been steeply increasing over the past decade. However, the relatively inferior functionality compared to soy protein is hindering the expanded use of pulse proteins, namely pea and chickpea protein, in various applications. Harsh extraction and processing conditions adversely impact the functional performance of pea and chickpea protein. Therefore, a mild protein extraction method involving salt extraction coupled with ultrafiltration (SE-UF) was evaluated for the production of chickpea protein isolate (ChPI). The produced ChPI was compared to pea protein isolate (PPI) produced following the same extraction method in terms of functionality and feasibility of scaling. Scaled-up (SU) ChPI and PPI were produced under industrially relevant settings and evaluated in comparison to commercial pea, soy, and chickpea protein ingredients. Controlled scaled-up production of the isolates resulted in mild changes in protein structural characteristics and comparable or improved functional properties. Partial denaturation, modest polymerization, and increased surface hydrophobicity were observed in SU ChPI and PPI compared to the benchtop counterparts. The unique structural characteristics of SU ChPI, including its ratio of surface hydrophobicity and charge, contributed to superior solubility at both a neutral and acidic pH compared to both commercial soy protein and pea protein isolates (cSPI and cPPI) and significantly outperformed cPPI in terms of gel strength. These findings demonstrated both the promising scalability of SE-UF and the potential of ChPI as a functional plant protein ingredient.

Keywords: pea protein isolate; chickpea protein isolate; salt extraction coupled with ultrafiltration; scaled-up production; structural characteristics; functional properties

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1. Introduction

The demand for plant protein ingredients has considerably increased in recent years due to their low production cost, positive environmental impact, nutritional value, and health benefits. Accordingly, the plant protein ingredient market is expected to reach $3 billion by 2031 [1]. Yellow field pea (*Pisum sativum* L.) and chickpea (*Cicer arietinum* L.) protein ingredients are major contenders in the plant protein market, with an expected market share of $555 million by 2029 and $158 million by 2032, respectively [2,3]. Both pea and chickpea protein ingredients have been used as soy protein alternatives in high-protein food and beverages, including plant-based meat products [4,5].

Although pea and chickpea proteins have a similar profile to soy protein, both have relatively inferior functional properties, specifically gelation, emulsification, and solubility [6–9]. The functionality limitations hinder the expanded use of pea and chickpea proteins in various applications. The commercially available pea protein isolate generally has relatively poor functional properties due to excessive protein denaturation and polymerization attributed to harsh extraction and processing conditions [7,8,10]. Recently, it was shown that mild and controlled extraction and processing conditions can preserve pea protein structure and functionality [7].

While both the pea protein isolate (PPI) and the pea protein concentrate (PPC) are widely available commercially, chickpea protein in the market is mostly available in the form of chickpea protein concentrate (ChPC). Chickpea protein isolate (ChPI), on the other hand, is a rare commercial commodity. Both PPC and ChPC are produced by air classification that does not involve wet or thermal processing, contrary to the production of isolates. The most common commercial process for the production of plant protein isolates is alkaline extraction to separate the protein from the starch and fiber, followed by isoelectric point precipitation (AE-IEP) to isolate and purify the protein [11]. However, high alkalinity results in a high degree of protein denaturation and aggregation, which negatively impact functionality [7,8,12–14]. Mild extraction conditions, including salt solubilization coupled with membrane filtration, have been shown to preserve the protein structure and functionality [7]. While pea protein ingredient production has been studied extensively, limited research has been reported on the impact of extraction conditions on chickpea protein structural and functional properties at both bench-scale and under industrially relevant settings [9,15–18].

In our previous study, we determined that salt solubilization coupled with membrane filtration is an industrially feasible approach to producing a functional PPI [7]. Additionally, protein structural characteristics were preserved and functional properties were better than those of a commercial PPI produced following AE-IEP. To the best of our knowledge, there is no research on ChPI production following salt extraction coupled with ultrafiltration (SE-UF) at bench nor at a pilot scale. Most studies optimized ChPI production following AE-IEP [9,16–18], or alkaline extraction (at pH 9) coupled with ultrafiltration [18]. In another study, salt extraction coupled with dialysis was used to produce ChPI, which exhibited good functionality, yet was still inferior to soy protein [9]. The limited functionality could be attributed to the residual high-salt content that shielded the charge on the surface of the protein [19,20]. Ultrafiltration coupled with diafiltration or dialysis would be a more efficient strategy to remove excess residual salt. Therefore, SE-UF has the potential to produce ChPI with preserved structural characteristics and good functionality compared to currently available chickpea protein ingredients.

To evaluate the scalability and transferability of SE-UF for ChPI production, the objectives of this study were to (1) evaluate SE-UF conditions for the production of ChPI with acceptable purity and yield, (2) evaluate the scalability of the SE-UF process, and (3) determine the structural and functional properties of PPI and ChPI compared to commercial sources.

2. Materials and Methods

2.1. Materials

Yellow field pea flour (20% protein) and commercial pea protein concentrate (cPPC) (52.4% protein, 5.14% ash), FYPP-55, were provided by AGT Foods (Regina, SK, Canada). Defatted chickpea flour (26.8% protein), Artesa™ Chickpea Flour 20 M, and commercial chickpea protein concentrate (cChPC) (56.4% protein, 5.27% ash), Artesa™ Chickpea Protein, were provided by Nutriati (Henrico, VA, USA). Commercial soy protein isolate (cSPI) (90.1% protein, 4.16% ash), ProFam® 974, and commercial pea protein isolate (cPPI) (79.5% protein, 5.61% ash), ProFam® 580, were kindly provided by Archer Daniels Midland (ADM) (Decatur, IL, USA). Samples were stored at −20 °C when not in use. Vivaflow® ultrafiltration membrane crossflow cassettes (3 kDa cut-off) were purchased from Sartorius™ (Gottingen, Germany). SnakeSkin™ dialysis tubing (3.5 kDa cut off) and Sudan Red 7B were purchased from Thermo Fisher Scientific™ (Waltham, MA, USA). Precision Plus molecular weight marker, Criterion™ TGX™ 4–20% precast gels, Laemmli 4X loading buffer, 10X Tris/Glycine/SDS running buffer, and Imperial™ Protein Stain were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). 8-anilino-1-napthalenesulfonic acid ammonium salt (ANS), and 2-mercaptoethanol (BME) were purchased from Sigma-Aldrich (St. Louis, MO, USA). For size-exclusion high-performance liquid chromatography (SE-HPLC), SuperdexTM 200 Increase 10/300 GL Prepacked TricornTM Column, and gel

filtration LMW and HMW calibration kits were purchased from Cytiva (Marlborough, MA, USA). All other analytical-grade reagents and lab supplies were purchased from Sigma-Aldrich or Thermo Fisher Scientific.

2.2. Selection of Salt Solubilization Conditions for the Production of ChPI

In our previous study, the salt extraction conditions (0.5 M NaCl, 1 h of solubilization at room temperature) were selected for bench and pilot scale production of PPI based on protein yield and purity [7]. These salt extraction conditions were, therefore, used as the baseline for selecting the extraction conditions for ChPI production. Several studies reported that an elevated temperature could enhance protein solubilization, thereby contributing to a relatively higher protein yield [21,22]. Two temperatures (23 °C and 50 °C) and three salt concentrations (0.5 M, 0.75 M, and 1 M) were tested to determine the combination that might result in enhanced chickpea protein solubilization. In triplicate, chickpea flour was solubilized in 0.5, 0.75, or 1.0 M NaCl solution prepared with double deionized water (DDW) (1:20 w/v) and stirred for 1 h at its initial pH (~6.8) under room temperature (23 °C) or at 50 °C. The solution was then centrifuged at 5000× g for 10 min to separate the insoluble components and the supernatant was collected. The protein content of the supernatant was determined following the Dumas method (AOAC 990.03) using a LECO® FP828 nitrogen analyzer (LECO, St. Joseph, MI, USA), with a protein conversion factor of 6.25. Under all the tested conditions, up to ~65% of the protein in the starting flour was retrieved in the collected supernatant, with a slightly lower percent when 1.0 M NaCl was used. Therefore, the lowest salt concentration (0.5 M NaCl) coupled with solubilization at room temperature was selected for ChPI production.

2.3. Benchtop Production of PPI and ChPI

Benchtop production of PPI and ChPI was performed following the SE-UF method outlined by Hansen et al. [7] and the SE-UF conditions selected based on protein yield, respectively. Pea or chickpea flour was dispersed in a 0.5 M NaCl solution three times (1:20 w/v) and was stirred for 1 h at its initial pH (~6.8) and at room temperature (23 °C). The solution was then centrifuged at 5000× g for 30 min to precipitate insoluble components. The residual pellet was then lyophilized and its protein content was later determined for mass balance calculations. The supernatant, containing the solubilized protein, was collected, and the pH was adjusted to 7.0. Prior to ultrafiltration, a vacuum filtration step was included to filter the protein solution and remove small insoluble particles that could clog the ultrafiltration membrane. The solution was then subjected to crossflow ultrafiltration (UF) using the Sartorius Vivaflow® 200 system, followed by dialysis as described by Hansen et al. [7] to further remove residual salt and small molecular weight sugars to enhance protein purity. Components with a molecular weight larger than the membrane pore size (3 kDa) were retained and recirculated to the feed reservoir. Components smaller than 3 kDa passed through the membrane and were collected as permeate in the waste container. The protein solution was concentrated down to 50 mL. After concentration, the solution was diafiltered with 50 mL of DDW six times (300 mL total) to further purify the protein. At the end of diafiltration, the solution was concentrated down to 25 mL. To flush the remaining protein solution left on the membrane and increase protein yield, approximately 25 mL of DDW was pumped into the system. After filtration and dialysis, the samples were lyophilized. The protein yield and purity of PPI and ChPI were determined by the Dumas method. Ash content (AOAC method 942.05), moisture content (AOAC method 926.08), and fat content (AOAC method 922.06) were also determined.

2.4. Pilot Plant Scale-Up Production of PPI and ChPI

Pilot scale SE-UF was performed in the Joseph J. Warthensen Food Processing Center, University of Minnesota, to produce scaled-up (SU) isolates, SU PPI, and SU ChPI, following the process reported by Hansen et al. [7], with some modifications. To produce SU PPI and

SU ChPI, pea or chickpea flour was dispersed in water with 0.5 M NaCl (1:20 w/v) in a jacketed tank. The solution was stirred for 1 h at room temperature at its initial pH (~6.8). To separate the protein supernatant from the starch slurry, the solution was passed through a horizontal decanter centrifuge (Westfalia Separator AG, 1 gal/min, GEA Westfalia Separator Group Gmbh, Oelde, Germany) coupled with a desludging disc centrifuge (Westfalia SB7, 1 gal/min, GEA Westfalia Separator Group Gmbh, Oelde, Germany). The starch slurry was then re-solubilized in water with 0.5 M NaCl (1:5 w/v), stirred for 30 min, and passed through a second round of decanter and clarifier to optimize protein extraction. The protein supernatants from the first and second solubilization were combined and the pH was adjusted to 7.0 followed by ultrafiltration (3.5 kDa cut-off) to remove residual salt. To monitor salt removal and total loss, the total solids (TS) (%) of the permeate was regularly checked using a CEM AVC-80 Microwave Moisture/Solids Balance Analyzer (CEM, Charlotte, NC, USA). When the TS of the permeate reached 0.0%, the protein retentate solution was concentrated until its TS reached 8–10%, pasteurized, homogenized, and spray dried using an SPX Flow Anhydro Spray Dryer (9.5% TS, 180 °C inlet, 90 °C outlet, 9 L/h) with a wheel type atomizer (24,500 rpm) (SPX Flow Inc., Charlotte, NC, USA). The residual protein left on the membrane was flushed out and collected separately. Since the flushed protein only contained approximately 5% TS, an evaporation step was performed to concentrate the solution to 8% TS prior to pasteurization, homogenization, and spray drying. The non-evaporated protein retentate was referred to as "high solids" (HS), while the portion that underwent evaporation was referred to as "low solids" (LS). The spray-dried HS and LS were combined after structural characterization screening showed no significant differences between the two fractions. The protein, ash, moisture, and fat content of SU PPI and SU ChPI were determined as described above. SU isolates were stored at −20 °C when not in use.

2.5. Color Measurement

The color (L * a * b *) of benchtop, SU, and commercial protein samples was measured three times as described by Bu et al. [8] using a Chroma Meter CR-221 (Minolta Camera Co., Osaka, Japan). The total color difference (ΔE) between each produced isolate and its respective commercial ingredient was also calculated.

2.6. Protein Structural Characterization

2.6.1. Protein Profiling by Gel Electrophoresis

The protein profile of all samples was monitored using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as described by Laemmli [23] and modified by Boyle et al. [21]. Precision Plus™ MW standard and protein samples (5 µL, containing approximately 50 µg protein) in Laemmli buffer with and without a reducing agent (βME) were loaded onto Criterion™TGX™ 4–20% precast Tris-HCl gradient gel and electrophorized at 200 V. The gels were then stained with Imperial Protein Stain™, de-stained with DDW, and scanned using the Molecular Imager Gel Doc XR system (Bio-Rad Laboratories).

2.6.2. Protein Denaturation State

The denaturation temperature and enthalpy of all samples were determined, in triplicate, using DSC (Mettler Toledo, Columbus, OH, USA), following the method outlined by Bu et al. [8]. The thermograms were manually integrated using Mettler Toledo's STARe Software version 11.00.

2.6.3. Surface Properties of Protein Ingredients

To determine the surface charge, zeta potential (ζ) was measured, in triplicate, using a dynamic light scattering instrument (Malvern Panalytical, Malvern, UK) as described by Bu et al. [8], with no modifications. The surface hydrophobicity of all samples was deter-

mined using the spectrofluorometric method outlined by Boyle et al. [21] and modified by Bu et al. [8].

2.7. Protein Functional Characterization

2.7.1. Protein Solubility

The protein solubility of all protein samples was determined using the method outlined by Boyle et al. [21] and modified by Bu et al. [8]. Protein solutions (5% protein w/v) were prepared, in triplicate, at pH 7 and at pH 3.4 to assess the suitability for high protein neutral as well as acidic beverages. The protein solubility was measured at both room temperature and post-thermal treatment (80 °C for 30 min) using the Dumas method.

2.7.2. Gel Strength and Water Holding Capacity (WHC)

Thermally-induced gels were prepared as outlined by Bu et al. [8], with modifications in the protein concentration and heating time. In triplicate, 10 mL protein solutions (15% and 20% protein, w/v) were stirred for 2 h and adjusted to pH 7. The 15% protein solutions were heated for 10 min in a water bath at 95 °C (±2 °C), whereas the 20% protein solutions were heated for 20 min. After cooling, gel strength was measured using a TA-XT Plus Texture Analyzer (Stable Micro Systems LTD, Surrey, UK) following the same parameters outlined by Bu et al. [8]. The force (N) required to rupture the gel was recorded as gel strength. WHC of all samples (15% protein concentration, w/v) was measured as described by Boyle et al. [21], without modification.

2.7.3. Emulsification Properties

Emulsion capacity (EC, at 1% protein in DDW, w/v), activity index (EAI), and stability (ES) of all samples were determined at pH 7, in triplicate, according to the methods outlined by Boyle et al. [21] and Bu et al. [8].

2.8. Statistical Analysis

Analysis of variance (ANOVA) was determined using IBM® SPSS® Statistics software version 26 for Windows (International Business Machines Corp., Armonk, NY, USA). Tukey–Kramer multiple means comparison test was used to identify significant differences ($p \leq 0.05$) among the means of at least three samples. A student's unpaired t-test was used to test for significant differences ($p \leq 0.05$) between the means of the two samples.

3. Results and Discussion

3.1. Impact of Salt Extraction on the Protein Purity and Yield of Benchtop and Scaled-Up ChPI in Comparison to PPI

ChPI had a high protein purity (>90%) similar to that of PPI (Table 1). While Mondor et al. [18] utilized benchtop ultrafiltration to purify chickpea protein, the produced ChPI had lower protein purity (~84% on average), which could be attributed to limited protein solubility under the alkaline extraction conditions used in the absence of salt. In addition, the high MWCO membrane (50 kDa) used in their study likely led to a significant loss of protein. In another study by Karaca et al. [9], PPI and ChPI produced using salt solubilization coupled with dialysis on a bench scale had a lower protein purity (~81%) compared to the isolates produced in this study following SE-UF. Such a difference in protein purity could be attributed not only to the use of dialysis instead of ultrafiltration, but also to the use of a different salt type (K_2SO_4) at a low concentration (~0.3 M) for the solubilization of chickpea protein. Salt concentration and related ionic strength have a unique impact on protein solubilization based on the specific protein structure and surface charge. The chosen salt concentration should provide enough ions to stabilize the protein in the aqueous solution (salting in) [24].

Table 1. Protein, ash, and color (L * a * b * and ΔE) of benchtop and scaled-up pea and chickpea protein isolates (PPI, SU PPI, ChPI, and SU ChPI), as well as commercial SPI, PPI, PPC and ChPC.

Samples	Protein (%)	Ash (%)	Color			
			L *	a *	b *	ΔE [1]
cSPI	90.1 ± 0.07 [d2]	4.16 ± 0.14 [d]	86.7 ± 0.05 [e]	−0.23 ± 0.03 [e]	14.3 ± 0.08 [d]	
cPPI	79.5 ± 0.20 [e]	5.61 ± 0.03 [a]	86.7 ± 0.04 [e]	0.51 ± 0.02 [c]	17.3 ± 0.11 [b]	
cPPC	52.4 ± 0.18 [h]	5.14 ± 0.10 [bc]	92.2 ± 0.25 [b]	−1.21 ± 0.04 [g]	13.7 ± 0.17 [de]	
PPI	92.9 ± 0.17 [b]	2.14 ± 0.08 [e]	84.7 ± 0.03 [f]	1.07 ± 0.01 [b]	19.4 ± 0.04 [a]	3.00 ± 0.11 [B3]
SU PPI	90.9 ± 0.03 [d]	2.39 ± 0.09 [e]	88.4 ± 0.01 [d]	0.01 ± 0.03 [d]	12.5 ± 0.03 [f]	5.04 ± 0.07 [A]
cChPC	56.4 ± 0.23 [f]	5.27 ± 0.11 [ab]	92.9 ± 0.08 [a]	−0.62 ± 0.03 [f]	9.05 ± 0.10 [g]	
ChPI	91.9 ± 0.15 [c]	4.79 ± 0.07 [c]	85.2 ± 0.04 [f]	2.00 ± 0.05 [a]	15.2 ± 0.43 [c]	10.20 ± 0.28 [α4]
SU ChPI	94.0 ± 0.23 [a]	2.14 ± 0.06 [e]	90.9 ± 0.00 [c]	−1.02 ± 0.09 [g]	13.5 ± 0.06 [de]	4.91 ± 0.01 [β]

[1] Total color difference (ΔE) between each produced isolate and its respective commercial reference (cPPI and cChPC); [2] Lowercase letters denote significant differences among the means (n = 3) in each column, according to the Tukey–Kramer multiple means comparison test ($p < 0.05$); [3] Uppercase letters indicate significant differences between the ΔE of PPI and SU PPI in comparison to cPPI; [4] Greek alphabets indicate significant differences between the ΔE of ChPI and SU ChPI in comparison to cChPC, according to the student's unpaired *t*-test ($p < 0.05$).

While neither Mondor et al. [18] nor Karaca et al. [9] reported the protein yield, Espinosa-Ramírez et al. [25] reported chickpea protein extraction yields of up to 67%. The reported higher protein extraction yield compared to the obtained yield in this study (52%) is attributed to the high alkalinity of the solubilization solution (pH 9.5) used by Espinosa-Ramírez et al. [25], which was detrimental to protein functionality. While the chickpea protein yield obtained in this study is acceptable and comparable to what has been reported for pulse proteins [26], it is significantly ($p < 0.05$) lower than that obtained for PPI (64%), despite following similar extraction conditions. A significantly ($p < 0.05$) higher % residual protein (~34% of the original protein in the flour) was left in the pellet discarded during ChPI production compared to that (18% of the original protein in the flour) discarded during PPI production. This observation could be attributed to the content and structure of the starch [27] and fiber [28] in chickpeas compared to peas, which could have hindered protein solubilization and extraction efficiency.

The SU production of PPI and ChPI achieved similar protein purity to the benchtop counterparts (Table 1), with minor statistical differences. ChPI had a significantly higher ash content than SU ChPI, which could explain the slightly higher protein purity of SU ChPI. However, SU production of PPI and ChPI resulted in significantly ($p < 0.05$) lower protein yield, 59% and 41%, respectively, mostly due to losses during the decanting step. Nevertheless, SE-UF was successfully scaled up, for the first time, to produce ChPI with high protein purity and relatively low ash content, demonstrating the feasibility of production at an industrial scale. To improve the yield during scaled-up production, enhancement of the decanting step should be targeted in future trials.

3.2. Impact of Extraction Scale on the Color of PPI and ChPI Compared to Commercial Protein Ingredients

The SU PPI and SU ChPI were significantly lighter and more neutral in color compared to their benchtop counterparts (Table 1). Different drying methods (spray drying vs. freeze drying) used to produce SU and benchtop isolates could be mainly responsible for the color differences. The size and morphology of the particles could change the intensity and angle of the reflected light, resulting in different perceptions of color. In general, spray drying produces a more refined powder with a smaller particle size compared to freeze drying [29,30]. Spray-dried particles were reported to have a rounded morphology with the wrinkled surface, which enables the particles to reflect/scatter more light compared to freeze-dried particles that have a smooth surface, and plate-shaped morphology [30,31].

Commercial protein concentrates (cPPC, cChPC), on the other hand, exhibited a significantly lighter color than all the protein isolates, mostly attributed to the higher

starch content in the concentrates. When comparing PPI and SU PPI to cPPI, ΔE was modest. Therefore, it can be concluded that the SE-UF used in this study resulted in protein ingredients (SU PPI and SU ChPI) of a similar color profile to commercial counterparts, with slightly lighter and more neutral color compared to cPPI. This observation can be attributed to potentially less browning during the SE-UF process utilized in this study compared to the AE-IEP process used to produce cPPI.

3.3. Protein Profile of the Benchtop and Scaled-Up Isolates in Comparison to Commercial Samples

The protein profile of benchtop and scaled-up PPIs and ChPIs was compared to commercial samples under nonreducing and reducing conditions (Figure 1a,b). Under nonreducing conditions (Figure 1a, lanes 4–5), PPI and SU PPI had protein bands corresponding to lipoxygenase (~92 kDa), convicilin (~72 kDa), legumin (~60 kDa), vicilin (13–19, 30–35, and 50 kDa), and albumin (~10 kDa), similar to the pea protein profile reported in other studies [11,15,32–35]. Under reducing conditions, the disulfide linkages stabilizing the legumin monomers were cleaved, resulting in protein bands corresponding to the acidic and basic legumin subunits at ~40 kDa and ~20 kDa, respectively (Figure 1b, lanes 4 & 5). Similarly, under both nonreducing and reducing conditions (Figure 1, lanes 7 & 8), ChPI and SU ChPI had protein bands corresponding to the major protein components observed in PPI and SU PPI, in agreement with previous reports [25,36]. However, the bands corresponding to legumin monomers (under nonreducing conditions) and legumin acidic and basic subunits (under reducing conditions) were more intense than their counterparts in PPI and SU PPI, with a couple of additional variants that have slightly different molecular weights. A similar protein band pattern of legumin in chickpeas was also observed by Chang et al. [36], Wang et al. [37], Vioque et al. [38], and Papalamprou et al. [39].

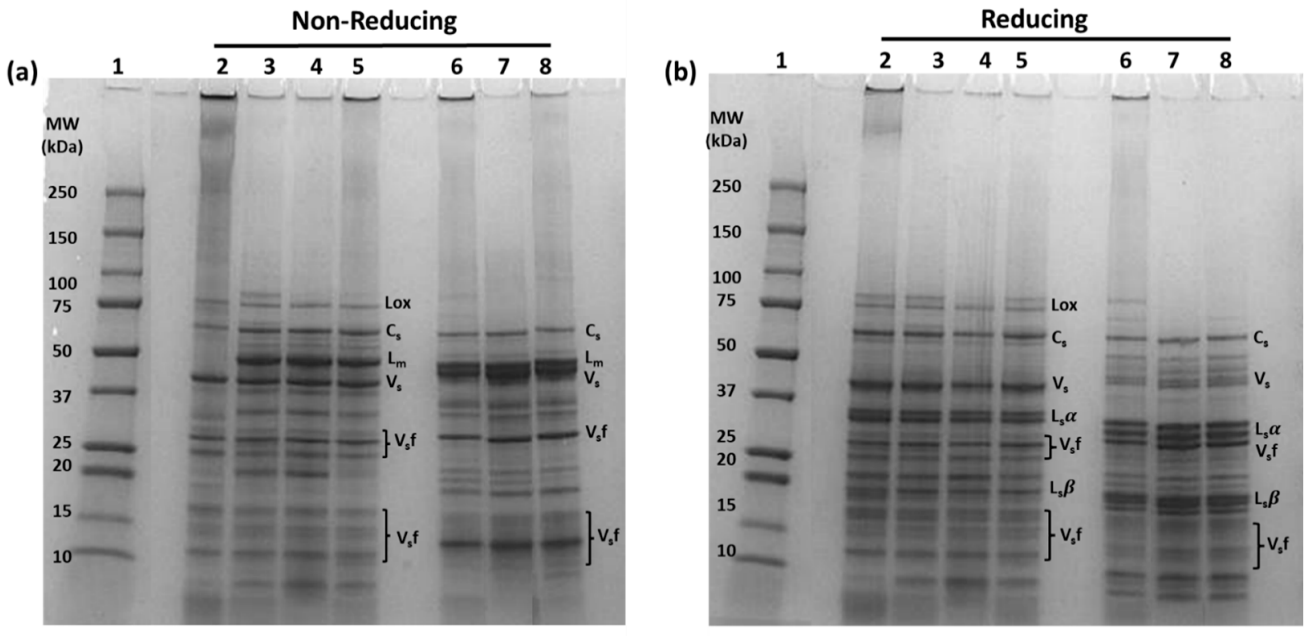

Figure 1. SDS-PAGE gel protein profile visualization of benchtop and scaled-up pea and chickpea protein isolates (PPI, SU PPI, ChPI, and SU ChPI), as well as commercial PPI, PPC, and ChPC under non-reducing (**a**) and reducing (**b**) conditions. Lane 1: Molecular weight (MW) marker; Lane 2, 3: cPPI and cPPC; Lane 4, 5: PPI and SU PPI, Lane 6: cChPC; Lane 7, 8: ChPI and SU ChPI. Lox: lipoxygenase; C_s: convicilin subunits; L_m: legumin monomer; V_s: vicilin subunits; $L_s\alpha$: legumin acidic subunits, $L_s\beta$: legumin basic subunits; V_sf: vicilin subunit fractions due to post-translational cleavages.

The protein profile of PPI and SU PPI, and that of ChPI and SU ChPI, were similar to cPPI and cPPC, and to cChPC, respectively. However, under nonreducing conditions, cPPI showed intense smearing in the upper region of its lane with no apparent legumin band at 60 kDa, indicating a high extent of legumin-involved polymerization (Figure 1a, lane 2). In contrast, there was no such smearing in cPPC (Figure 1a, lane 3). The air classification used to produce cPPC is a mild process [40,41] compared to the wet milling extraction process followed to produce cPPI. The use of a harsh alkaline extraction process induced protein denaturation and subsequent polymerization in cPPI, as was previously discussed by Hansen et al. [7]. Even under reducing conditions, dark bands and residual smearing persisted in the upper region of the cPPI lane (Figure 1a, lane 2), indicating the presence of high molecular weight (HMW) protein polymers that are stabilized by other types of covalent linkages, other than disulfide bonds. Irreversible covalent linkages induced by the Maillard reaction are commonly formed under excessive heat treatment and elevated pH [42–44]. These observations confirmed that the conditions used to produce PPI and ChPI at the bench as well as pilot scale were relatively mild, preventing the formation of large polymers that may negatively impact functionality.

However, there was mild smearing observed in the upper region of the SU PPI and SU ChPI lanes compared to those of PPI and ChPI (Figure 1a, lanes 5 & 8 vs. lanes 4 & 7), indicating the presence of some HMW legumin-involved polymers. Under the reducing condition, the smearing was resolved, indicating that the observed protein polymerization was mainly attributed to disulfide linkages (Figure 1b, lanes 5 & 8). The presence of such polymers was thermally induced during evaporation, pasteurization, and spray-drying steps of the scaled-up production. However, based on protein profiling (Figure 2), the formation of these polymers was mostly attributed to the evaporation step applied to the low solids (LS) fractions of SU PPI and SU ChPI. Dark smearing was noted in the upper region of the lanes of the LS fractions compared to those of the high solids (HS) fractions (Figure 2, lanes 3 & 5 compared to lanes 2 & 4), which was mostly resolved under reducing conditions (Figure 2, lanes 7 & 9). Spray-dried LS fractions were mixed with spray-dried HS fractions to produce the final SU isolate, thus explaining the presence of HMW polymers in both SU isolates.

In contrast to cPPC, cChPC had mild smearing in the upper region of its lane (Figure 1a, lane 6), similar to that noted for SU ChPI. Although cChPC was produced via air classification, the initial flour was defatted prior to air classification. The defatting process, while important to reduce the fat content of chickpea flour (7% to less than 1% fat), involves thermal desolventization, which will induce protein denaturation and subsequent polymerization. Having seemingly similar polymerization patterns, both cChPC and SU ChPI potentially may have similar protein functionality.

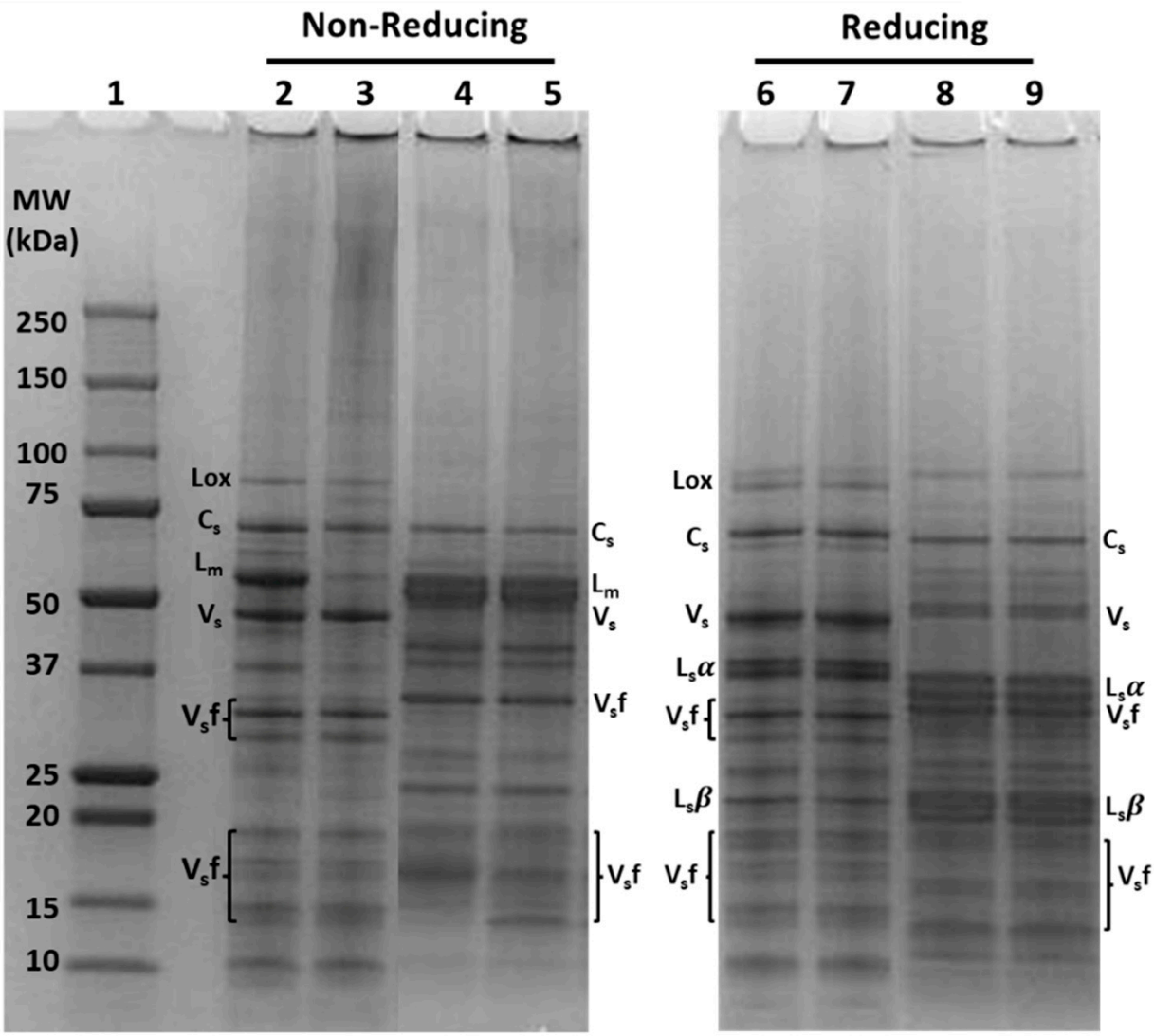

Figure 2. SDS-PAGE gel protein profile visualization of high solid (HS) and low solid (LS) fractions of SU PPI compared to that of cPPI and SU ChPI compared to that of cChPC under non-reducing (lane 2–5) and reducing (lane 6–9) conditions. Lane 1: Molecular weight (MW) marker; Lane 2, 6: SU PPI HS; Lane 3, 7: SU PPI LS; Lane 4, 8: SU ChPI HS; Lane 5, 9: SU ChPI LS. Lox: lipoxygenase; C_s: convicilin subunits; L_m: legumin monomer; V_s: vicilin subunits; $L_s\alpha$: legumin acidic subunits, $L_s\beta$: legumin basic subunits; V_sf: vicilin subunit fractions due to post-translational cleavages.

3.4. Protein Denaturation State of PPI and ChPI as Impacted by Extraction Scale and in Comparison to Commercial Protein Ingredients

The impact of the extraction scale (benchtop vs. scale-up) on the protein denaturation state of PPI and ChPI was evaluated in comparison to commercial protein ingredients (Table 2). Two distinct denaturation temperatures, corresponding to vicilin and legumin, were observed for benchtop and scaled-up PPI and ChPI, in agreement with previous studies [6,15]. Since the endothermic peaks of vicilin and legumin overlapped, as was also observed by others [39,45], both peaks were integrated as one peak and the total enthalpy of denaturation was obtained (Table 2).

Table 2. Denaturation temperature and enthalpy, surface hydrophobicity, and surface charge of benchtop and scaled-up pea and chickpea protein isolates (PPI, SU PPI, ChPI, and SU ChPI), as well as commercial SPI, PPI, PPC, and ChPC.

Samples	Denaturation Temperature and Enthalpy			Surface Properties	
	Denaturation Temperature (Td)		Total Enthalpy of Denaturation (ΔH)	Surface Hydrophobicity	Surface Charge
	°C		J g^{-1}	RFI	mV
cSPI	β-conglycinin *[1]	Glycinin *	*	10,820.3 ± 530.3 [b]	−41.3 ± 0.20 [a]
cPPI	Vicilin (7S) *	Legumin (11S) *	*	13,821.7 ± 434.4 [a]	−30.2 ± 0.13 [bc]
cPPC	85.5 ± 0.02 [a2]	94.4 ± 0.12 [b]	2.30 ± 0.04 [f]	7895.7 ± 271.8 [cd]	−27.9 ± 0.30 [cd]
PPI	82.6 ± 0.13 [b]	88.1 ± 0.03 [e]	10.9 ± 0.50 [b]	6564.4 ± 129.5 [d]	−26.2 ± 0.34 [d]
SU PPI	82.6 ± 0.13 [b]	89.9 ± 0.16 [d]	5.45 ± 0.07 [d]	14,199.7 ± 105.9 [a]	−27.2 ± 0.07 [d]
cChPC	81.5 ± 0.09 [c]	99.6 ± 0.02 [a]	3.77 ± 0.09 [e]	13,317.0 ± 450.4 [a]	−25.7 ± 0.33 [d]
ChPI	81.6 ± 0.08 [c]	89.9 ± 0.11 [d]	16.8 ± 0.54 [a]	4491.1 ± 157.9 [e]	−30.8 ± 0.15 [b]
SU ChPI	80.5 ± 0.07 [d]	90.8 ± 0.17 [c]	8.61 ± 0.14 [c]	8973.3 ± 186.5 [c]	−30.9 ± 0.21 [b]

[1] An asterisk (*) indicates the absence of endothermic peaks due to complete protein denaturation; [2] Lowercase letters indicate significant differences among the means (n = 3) in each column, according to the Tukey–Kramer multiple means comparison test ($p < 0.05$).

No apparent endothermic peak was observed for commercial protein isolates (cPPI and cSPI), indicating complete protein denaturation due to extensive wet processing conditions. In contrast, legumin and vicilin endothermic peaks were present in both cPPC and cChPC, which underwent air classification. As discussed, air classification is a milder process compared to wet milling [40,41]. However, the enthalpy of denaturation of the concentrates was significantly lower than that of the benchtop as well as the scaled-up PPI and ChPI samples. This observation could be attributed to matrix differences between isolates and concentrates, regardless of the extraction/fractionation conditions [46].

The presence of prominent endothermic peaks with a relatively high enthalpy of denaturation in the produced isolates compared to cPPI confirmed that the SE-UF process preserved the protein structure, limiting denaturation (Table 2) and consequent polymerization (Figure 1). Furthermore, ChPI produced on benchtop following AE-IEP [6,47] at high alkalinity had a considerably lower enthalpy (2.5–5.5 J/g) than that of both benchtop and scaled-up ChPI produced by SE-UF in this study. Similarly, benchtop and scaled-up PPI produced following SE-UF had higher denaturation enthalpy than their AE-IEP counterparts [7].

When comparing PPI to ChPI, both ChPI and SU ChPI had significantly higher denaturation enthalpy than PPI and SU PPI, respectively (Table 2). Chickpea protein had higher denaturation enthalpy than pea protein, regardless of the extraction scale, most likely due to its relatively higher legumin to vicilin ratio, as noted by SDS-PAGE (Figure 1). On the other hand, benchtop isolates had significantly higher denaturation enthalpy compared to their scaled-up counterparts (Table 2). This observation complimented the protein profiling observations (Figure 1), confirming that the thermal treatments (evaporation, pasteurization, and spray drying) during the scale-up production led to partial protein denaturation and consequent formation of HMW polymers. Partial denaturation may also impact the surface properties of the protein.

3.5. Protein Surface Properties of PPI and ChPI as Impacted by Extraction Scale and in Comparison to Commercial Protein Ingredients

Scaled-up isolates had significantly higher surface hydrophobicity than their benchtop counterparts (Table 2), attributed to the partial denaturation incurred during scaling-up production in the pilot plant, as discussed. Upon denaturation, surface hydrophobicity is expected to increase due to protein unfolding and exposure of buried hydrophobic

residues [48]. Enhanced surface hydrophobicity drives protein molecules into closer proximity, facilitating different forms of bonding, including disulfide linkages, as noted by SDS-PAGE (Figure 1, lanes 5 & 8 vs. 4 & 7).

Differences in surface hydrophobicity among the samples were also evaluated in comparison to commercial ingredients. While both SU PPI and cPPI had similar surface hydrophobicity, the latter was completely denatured (Table 2) and excessively polymerized (Figure 1a, lane 2). Maximum surface hydrophobicity is theoretically reached upon complete protein denaturation. However, the excessive polymerization of legumin in cPPI, as observed from protein profiling, likely reduced its measurable surface hydrophobicity. Polymerization of denatured proteins driven by hydrophobic interactions would bury the exposed hydrophobic groups, thus reducing measurable surface hydrophobicity [49]. Thus, due to differences in the extent of polymerization, SU PPI is expected to have better functionality than cPPI, despite having similar surface hydrophobicity. On the other hand, cPPC and benchtop PPI had similar surface hydrophobicity, and both were lacking in HMW polymers (Figure 1a, lanes 3 & 4). Meanwhile, cChPC had significantly higher surface hydrophobicity than both ChPI and SU ChPI. As discussed, this commercial sample was subjected to defatting prior to air classification, causing denaturation and thus exposure of the hydrophobic core.

In comparing the two different isolates, the pea protein isolate had significantly higher surface hydrophobicity than the chickpea protein isolate, regardless of the extraction scale. This observation could be attributed mostly to inherent differences among the species. The abundance of globulins compared to that of albumins, the ratio of 7S vicilin to 11S legumin, as well as the presence of different subunit variants could all contribute to differences in surface hydrophobicity [50,51]. Karaca et al. [9] similarly reported that PPI had higher surface hydrophobicity than ChPI. In soybeans, it is documented that 11S glycinin has higher surface hydrophobicity than 7S β-conglycinin [51]. In contrast, the higher abundance of 11S legumin in chickpeas compared to that in peas (Figure 1), did not contribute to higher surface hydrophobicity. This observation implied that variation in 11S amino acid sequence across species has a bigger impact on surface hydrophobicity than 7S/11S ratio.

Unlike surface hydrophobicity, variation in surface charge was limited across all pea and chickpea protein samples, with only a few minor statistical differences (Table 2). The extraction scale had no impact on the surface charge. However, ChPI and SU ChPI had a slightly but significantly higher net negative charge than PPI and SU PPI. While the surface charge of PPI was similar to previous reports [9,13,15], that of ChPI was higher than what was reported for both AE-IEP and salt-extracted ChPI [9]. This observation could be attributed to different extraction conditions (e.g., salt concentration, salt type), as well as the residual salt content in ChPI.

The surface hydrophobicity and charge of cSPI were also determined to better evaluate differences in functionality compared to pea and chickpea protein isolates. While cSPI had a relatively high surface hydrophobicity, it had a considerably higher net charge compared to all the samples. This balance between surface charge and surface hydrophobicity could explain the superiority of soy protein in certain functional properties as will be discussed.

3.6. Protein Solubility of PPI and ChPI as Impacted by Extraction Scale and in Comparison to Commercial Protein Ingredients

Protein solubility of benchtop and scaled-up PPI and ChPI in comparison to commercial samples was assessed before and after heat treatment at both neutral and acidic pH (Table 3). At pH 7 SU PPI had significantly lower protein solubility than benchtop PPI, most likely due to partial protein denaturation and aggregation induced by thermal treatments during scale-up production, as discussed. Heating (80 °C for 30 min) at pH 7 resulted in a significant decrease in protein solubility of PPI but had no impact on the solubility of SU PPI. Since benchtop PPI was significantly less denatured and had significantly lower surface hydrophobicity (Table 2) than SU PPI, the heat treatment could have caused denaturation and polymerization that resulted in a significant reduction in solubility.

Table 3. Protein solubility of benchtop and scaled-up pea and chickpea protein isolates (PPI, SU PPI, ChPI, and SU ChPI), as well as commercial SPI, PPI, PPC, and ChPC.

Samples	Percent Protein Solubility (5% Protein)			
	pH 7.0		pH 3.4	
	Non-Heated	Heated [1]	Non-Heated	Heated
cSPI	66.8 ± 0.40 [d2]	78.5 ± 0.39 [b*3]	24.9 ± 0.53 [c]	39.1 ± 0.11 [b*]
cPPI	29.5 ± 0.85 [e]	57.1 ± 0.64 [e*]	11.6 ± 0.49 [d]	17.5 ± 0.79 [c*]
cPPC	84.3 ± 0.17 [b]	67.4 ± 0.30 [cd*]	23.8 ± 0.92 [c]	20.2 ± 1.18 [c]
PPI	84.8 ± 0.22 [b]	57.4 ± 0.87 [e*]	82.7 ± 0.54 [a]	85.2 ± 0.12 [a*]
SU PPI	68.9 ± 0.42 [cd]	69.9 ± 0.90 [c]	71.7 ± 0.25 [b]	80.3 ± 0.48 [a*]
cChPC	70.7 ± 0.31 [c]	66.7 ± 0.47 [d*]	12.7 ± 0.20 [d]	16.2 ± 0.22 [c*]
ChPI	96.2 ± 0.09 [a]	94.1 ± 0.52 [a]	26.1 ± 0.58 [c]	34.5 ± 0.70 [b*]
SU ChPI	94.3 ± 0.69 [a]	92.5 ± 0.76 [a]	71.5 ± 3.00 [b]	82.8 ± 3.00 [a*]

[1] Heated at 80 °C for 30 min; [2] Lowercase letters denote significant differences among the means (n = 3) in each column, according to the Tukey-Kramer multiple means comparison test ($p < 0.05$); [3] An asterisk denotes significant differences between non-heated and heated samples ($p < 0.05$), according to the student's unpaired *t*-test ($p < 0.05$).

In contrast, SU PPI was already partially denatured and had HMW polymers, potentially explaining the lack of impact of heat treatment on protein solubility. In comparison, ChPI and SU ChPI exhibited the highest protein solubility at pH 7.0 among all samples, regardless of heat treatment. The relatively lower surface hydrophobicity to charge ratio of ChPI and SU ChPI compared to PPI and SU PPI could have contributed to the observed differences in solubility. Given that chickpea protein compared to pea protein has a relatively higher proportion of legumin, which has a higher denaturation temperature (>80 °C) than vicilin, heating of ChPI and SU ChPI did not have a significant impact on protein solubility at pH 7.

Although cSPI was completely denatured (Table 2), had a high degree of polymerization (Figure 1), and had a high surface hydrophobicity, it had an acceptable protein solubility at pH 7, which was attributed to its relatively high surface charge. In contrast, cPPI had the lowest solubility among all samples, due to being completely denatured, extensively polymerized, and having a high surface hydrophobicity to charge ratio compared to the other protein isolates. Given its mostly preserved protein structure, cPPC, on the other hand, had similar solubility to PPI and SU PPI. cChPC, however, had significantly lower solubility at pH 7 than ChPI and SU ChPI, due to protein denaturation and polymerization, as discussed.

At pH 3.4, the net charge of the protein would be lower than that at pH 7 since the protein is closer to its average isoelectric point (pH 4.5). Nevertheless, both PPI and SU PPI exhibited good solubility at pH 3.4 (Table 3), which is significantly higher than all commercial samples including cSPI. This observation confirmed that the scaled-up production of PPI following SE-UF was successful in preserving the protein structure resulting in superior solubility to cPPI produced following AE-IEP, similar to the findings of Hansen et al. [7]. In contrast, ChPI had considerably low solubility at pH 3.4, similar to that of cSPI, while SU ChPI had good solubility similar to that of PPI and SU PPI. This observation can be explained by the charge load on the protein. Since the net charge of the protein is relatively low when the pH is close to the pI, a slightly elevated salt content could have a "salting out" effect, thereby decreasing the solubility of the protein. The ash content of the benchtop ChPI was significantly higher ($p < 0.05$) than that of SU ChPI (4.79% vs. 2.14% ash), thus potentially contributing to the observed difference in solubility. Similarly, Carbonaro et al. [52] reported a significant impact of salt content on the solubility of chickpea proteins at pH 4. cChPC, on the other hand, had inferior solubility compared to ChPI and SU ChPI at pH 3.4. This observation is again attributed to a higher degree of protein denaturation, higher surface hydrophobicity, and lower surface charge

of cChPC compared to the produced isolates, in addition to the significantly higher ash content (Table 1). For the first time, this data confirmed that scaled-up production of ChPI following SE-UF can preserve protein structure and result in excellent solubility at both neutral and acidic pH, better than a commercial pea, chickpea, and soy protein ingredients.

3.7. Gelation of PPI and ChPI as Impacted by Extraction Scale and in Comparison to Commercial Protein Ingredients

Thermally-induced gels of all protein samples exhibited excellent WHC at 15% protein concentration (>98%) (Data not shown). Regardless of gel strength at 15 % protein, each of the protein samples formed a relatively stable protein network that had minimum syneresis, with no apparent impact of protein source, extraction conditions, or extraction scale. On the other hand, significant differences in gel strength were observed among the samples at both 15% and 20% protein concentration (Figure 3). Gel strength was determined at both 15 and 20% protein concentration since commercial pea protein isolate typically either forms a weak gel or does not form a gel at all at 15% [7].

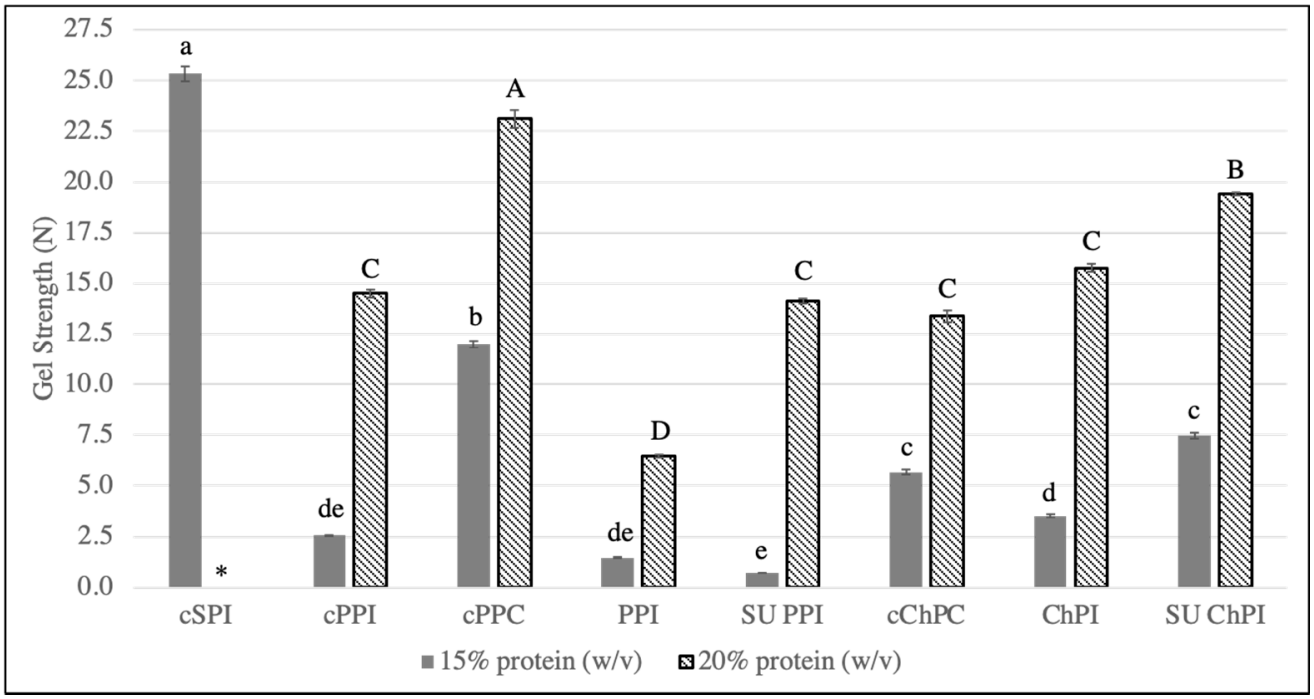

Figure 3. Protein gel strength of benchtop and scaled-up pea and chickpea protein isolates (PPI, SU PPI, ChPI, SU ChPI), as well as commercial SPI, PPI, PPC, and cChPC. An asterisk (*) denotes sample not analyzed due to high viscosity during sample solubilization. Error bars represent standard error (n = 3). Lowercase and uppercase letters above the bars denote significant differences among the samples evaluated at 15% and 20% protein concentration, respectively, according to the Tukey-Kramer multiple means comparison test ($p < 0.05$).

Among all protein ingredients, cSPI, at 15% protein concentration, had by far the highest gel strength, attributed to its good balance of protein-protein and protein-water interactions, as implied by its surface hydrophobicity to charge ratio. Another contributing factor is the higher 11S to 7S ratio and higher content of sulfhydryl groups in soy compared to pea and chickpea proteins [53]. In comparing chickpea to pea protein, ChPI and SU ChPI, specifically at 20% protein concentrations, had significantly higher gel strength than PPI and SU PPI, respectively. This observation again can be partially attributed to the higher 11S to 7S ratio in chickpeas compared to peas as evidenced by SDS-PAGE (Figure 1) and as previously reported [54]. In addition, the different 11S (legumin) variants (Figure 1, lanes 7 & 8) present in chickpeas could potentially have contributed to better

gelation properties. Further research is needed to differentiate the composition of 11S variants in chickpeas compared to peas.

Both SU PPI and SU ChPI, on the other hand, had significantly higher gel strength than their benchtop counterparts (Figure 3). The relatively higher surface hydrophobicity and partial denaturation (Table 2), as well as the presence of high molecular weight polymers (Figure 1) in scaled-up isolates potentially contributed to enhanced gel strength. Hydrophobic attractive forces will aid in bringing the protein molecules in closer proximity facilitating intermolecular disulfide linkages, thus strengthening the protein network. Compared to all the isolates, with the exception of cSPI, SU ChPI had the highest gel strength at both 15% and 20% protein. This observation confirmed that SE-UF can be scaled up to produce a chickpea protein isolate with better gelation potential than commercial pea protein isolate.

Among the commercial pea and chickpea protein samples, cPPC had significantly the highest gel strength at both 15% and 20% protein concentration. This observation is attributed mostly to the presence of starch, which acts as a good gelling agent [27,53]. During gel formation, heating the cPPC solution at 95 °C, above the gelatinization temperature of pea starch (64.2 °C) [55], contributed to enhanced gel strength. In contrast, cChPC did not outperform the protein isolates despite the presence of starch. The starch in cChPC was most likely pregelatinized and potentially retrograded [55]. The presence of pregelatinized starch, coupled with the denaturation state (Table 2), protein polymerization (Figure 1), and low solubility (Figure 3), had a compounded negative effect on the gel strength of cChPC. The impact of the processing steps employed during the production of cChPC needs to be investigated to identify the impact not only on the protein structure but also on the molecular characteristics of the residual chickpea starch [27] and fiber [28] in such a sample.

3.8. Emulsification Properties of PPI and ChPI as Impacted by Extraction Scale and in Comparison to Commercial Protein Ingredients

Minor statistical differences in emulsification properties were observed among the samples (Figure 4a–c). As expected, the EC of cSPI was superior among the protein isolates, with the exception of ChPI. This observation is attributed to a good balance between surface hydrophobicity and surface charge (Table 2). The EC of cPPC also was relatively high, which was attributed in part to the starch component [27]. On the other hand, the EC cChPC was comparable to that of cPPI.

While scaling up SE-UF production of PPI had no significant impact on EC, it did result in a significant decrease in EAI and ES. While partial denaturation aided in enhancing molecular flexibility and orientation at the interface, it could have contributed to attractive forces among the protein molecules on the interface, resulting in a slight reduction in emulsion stability. In contrast, all the measured emulsification properties of ChPI were adversely impacted by scaling up the extraction. The EC as well as ES of benchtop ChPI was the highest among the samples, owing to its well-preserved protein structure that had the highest enthalpy of denaturation, lowest surface hydrophobicity, and a relatively high surface charge compared to all pea and chickpea samples (Table 2). The shear induced by the homogenization employed during emulsion formation resulted in a partial unfolding of the native chickpea proteins in ChPI, allowing them to quickly migrate to the interface without precipitation, in contrast to already denatured and polymerized proteins. Withana-Gamage et al. [6] showed that benchtop ChPI had better emulsification properties (ES and EAI) than those of PPI, yet inferior to those of SPI, partially owing to the harsher extraction process (AE-IEP) that was adopted to produce ChPI. However, for a better understanding of the emulsification behavior of these proteins, an investigation of the molecular differences in the 11S and 7S proteins among chickpeas, pea, and soy is needed. Nevertheless, scaling up SE-UF production resulted in isolates of comparable emulsification properties to commercially available pea and chickpea protein ingredients (cPPI, cChPC), with SU PPI showing significantly higher EC.

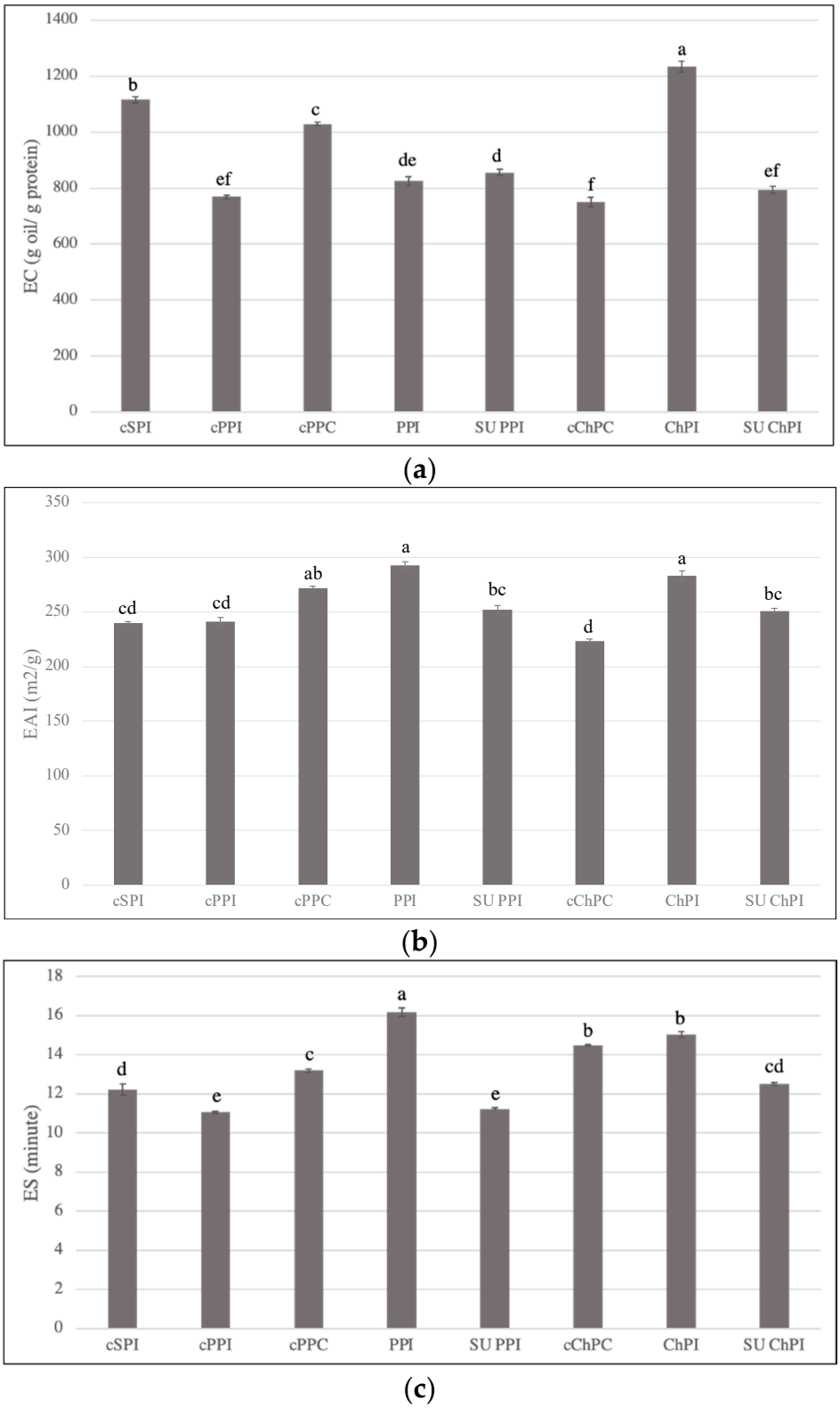

Figure 4. Protein emulsion capacity, EC, (**a**) emulsion activity index, EAI, (**b**) and emulsion stability, ES, (**c**) of benchtop and scaled-up pea and chickpea protein isolates (PPI, SU PPI, ChPI, SU ChPI), as well as commercial SPI, PPI, PPC, and cChPC. Error bars represent standard error (n = 3). Lowercase letters above the bars denote significant differences among the samples, according to the Tukey-Kramer multiple means comparison test ($p < 0.05$).

4. Conclusions

This study demonstrated that the selected SE-UF extraction conditions (0.5 M NaCl, 3.5 kDa cut-off membrane) can be successfully scaled up to produce ChPI and PPI with high protein purity, good protein yield, relatively preserved protein structure, and superior functionality to commercial counterparts (cChPC, cPPI). Specifically, this is the first study to evaluate the feasibility of scaling up the production of ChPI that had comparable or even better functional properties than both cSPI and cPPI. Specifically, SU ChPI had superior solubility at both neutral and acidic pH compared to cSPI and cPPI, and significantly outperformed cPPI in terms of gel strength. Accordingly, ChPI produced following the tested SE-UF process can be successfully incorporated in beverage applications and in food products requiring good gelling and water-holding properties. Additionally, the good gelation properties of SU ChPI could be leveraged for meat analogue applications. A comparative evaluation of the performance of both SU PPI and SU ChPI in various applications would be a natural follow-up study. Nevertheless, this work confirmed that SE-UF is scalable and thus should be commercially considered as an alternative protein extraction process for the production of pulse proteins with improved functional performance.

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Production of Plant-Based Seafood: Scallop Analogs Formed by Enzymatic Gelation of Pea Protein-Pectin Mixtures

Zhiyun Zhang [1], Kanon Kobata [1], Hung Pham [1], Dorian Kos [1], Yunbing Tan [1], Jiakai Lu [1] and David Julian McClements [1,2,*]

[1] Department of Food Science, University of Massachusetts, Amherst, MA 01003, USA; zhiyunzhang@foodsci.umass.edu (Z.Z.); kkobata@umass.edu (K.K.); hvpham@umass.edu (H.P.); dkos@umass.edu (D.K.); ytan@umass.edu (Y.T.); jiakailu@umass.edu (J.L.)
[2] 240 Chenoweth Laboratory, 102 Holdsworth Way, Amherst, MA 01003, USA
* Correspondence: mcclements@foodsci.umass.edu; Tel.: +1-(413)-545-2275

Abstract: This study investigated the possibility of using a phase separation, mixing, and enzymatic gelation approach to construct seafood analogs from plant protein-polysaccharide mixtures with properties mimicking real seafood. Heat-denatured pea protein (10%, *w*/*w*) and pectin (0–1%, *w*/*w*) were mixed to produce phase separated biopolymer blends. These blends were then subjected to mild shearing (350 rpm) to obtain fiber-like structures, which were then placed in molds and set by gelling the pea proteins using transglutaminase (2%, *w*/*w*). The appearance, texture, and cooking properties of the resulting scallop analogs were characterized and compared to those of real scallop. The presence of the pectin promoted the formation of a honeycomb structure in the scallop analogs, and microscopic orientation of the proteins was observed in the plane parallel to the applied shear flow. Lower pectin concentrations (0.5%, *w*/*w*) led to stronger gels with better water holding capacity than higher ones (1.0%, *w*/*w*). The appearance and texture of the plant-based scallop analogs were like those of real scallop after grilling, indicating the potential of using this soft matter physics approach to create plant-based seafood analogs. One of the main advantages of this method is that it does not require any expensive dedicated equipment, such as an extruder or shear cell technology, which may increase its commercial viability.

Keywords: plant-based foods; pea protein; pectin; thermodynamic incompatibility; transglutaminase

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1. Introduction

Consumers are increasing the number of plant-based foods in their diet due to environmental, health, and animal welfare concerns, including meat, seafood, egg, and dairy alternatives [1–3]. In this study, we focused on the development of a model plant-based seafood, as there is currently a lack of high-quality products in this area [4]. Seafood is an important source of protein in the human diet, as well as a good source of other health-promoting nutrients, such as omega-3 fatty acids, vitamins, and minerals. However, over-exploitation of wild seafood populations is depleting the oceans of these valuable resources [5,6]. Moreover, climate change is altering fish migration patterns, with profound effects on the fishing industry and coastal communities [7,8]. Wild seafood may also contain appreciable levels of toxins, especially mercury, persistent organic pollutants, and microplastics, which adversely affect human health [9–11]. Seafood extraction and processing have also been reported to be a significant contributor to greenhouse gas (GHG) emissions [12]. Finally, seafood, such as fish and shellfish, are a major source of allergens to a significant fraction of the population. The rapidly growing aquaculture industry alleviates some of these issues, but has its own challenges, including the need for protein-rich resources to feed the fish, as well as its propensity to cause pollution, such as eutrophication [13,14]. There are also substantial losses in aquaculture due to diseases, such as sea lice in farmed salmon, which contribute to food waste and economic losses estimated

to be around $6 billion per year [13]. Moreover, there are concerns that the antibiotics and pesticides used to tackle these diseases may contaminate fish and the environment. The availability of plant-based seafood analogs would help to reduce many these of these problems by creating an alternative to real seafood, thereby allowing existing seafood stocks to be managed more sustainably [15].

Plant-based foods have been fabricated using several processing technologies, including extrusion, shear cell, spinning, and 3D printing methods [16–23]. At present, extrusion is the most commonly used technology for the industrial production of plant-based foods because of its simplicity, versatility, and scalability [2,24]. In this approach, plant-based materials (usually proteins and polysaccharides) are heated and sheared under high pressure in a device that contains a barrel with a series of screws to mix and transport the materials. These processes change the solubility, conformation, and interactions of the proteins, which promotes the formation of protein aggregates. These aggregates are aligned in the direction of flow when the material passes through a long cooling die attached to the end of the extruder, leading to the creation of an anisotropic food matrix with meat-like structures and textures [2,25]. The shear cell technology also has potential to produce plant-based foods on a commercial scale [26,27]. Indeed, it has recently been adopted by a start-up company (Rival Foods) to produce plant-based meat analogs. This device has a cylinder-in-cylinder design, which consists of a heated stationary outer cylinder with a lid and a heated inner cylinder that is rotated via a drive shaft. Raw samples are pre-mixed and placed in the gap between the two cylinders. Unlike extrusion, the material deformation inside the shear device is well controlled and constant during the manufacturing process [26,27]. However, both extrusion and shear cell technologies require specialized equipment and high energy inputs, which limits their suitability for smaller companies and leads to some environmental concerns. A simpler, cheaper, and more energy-efficient means of creating plant-based meat and seafood products would therefore be advantageous.

Phase separation of protein-polysaccharide mixtures due to thermodynamic incompatibility can be used to create novel microstructures and textures in foods [28,29]. This approach is based on the fact that the free energy of a phase separated mixture of two types of biopolymers that repel each is lower than that of an intimate mixture [2]. The tendency for phase separation to occur is influenced by several factors, including the type and concentration of the biopolymers, as well as the pH and ionic strength of the surrounding solution [30]. After phase separation, the mixed biopolymer system can be stirred to form a "water-in-water" (w/w) emulsion, which consists of a dispersed phase rich in one kind of biopolymer and a continuous phase rich in the other kind of biopolymer. The droplets in w/w emulsions are characterized by a very low interfacial tension, which means they can be easily deformed and elongated into fiber-like structures by applying low shear stresses [18]. These structures can then be locked into place by promoting gelation of the dispersed and/or continuous biopolymer phase. This soft matter physics approach can therefore be used to create foods with meat-like structures and textures from plant proteins and polysaccharides.

In this study, the thermodynamic incompatibility approach was used to create seafood (scallop) analogs from plant proteins and polysaccharides. Commercial sea scallop analogs are already available, but most of them use fish or whey proteins as structuring agents. Those plant-based scallops that are on the market have protein concentrations (<2.5%) considerably below those of real scallops (10% to 12%), e.g., those sold by the Plant Based Seafood Company. These products tend to use starches and gums as structuring agents rather than proteins. In our study, we used pea protein and high methoxy citrus pectin as the protein and polysaccharide to formulate the scallop analog. These biopolymers were chosen because pea protein is not a major allergen and citrus pectin is a dietary fiber. Moreover, pea protein is a highly functional and affordable protein that can be obtained in sufficiently large quantities for commercial production. Similarly, citrus pectin is already widely used as a functional ingredient in the food industry and is also available at sufficiently large quantities for commercial applications. The pea protein concentration

was chosen to be close to that of a real scallop to match its nutritional content. The two biopolymers were mixed and blended to promote phase separation and fiber formation, placed in a mold, and then the pea proteins were crosslinked using a food-grade enzyme (transglutaminase) to lock the fiber structures in place and increase the gel strength. The structural and physicochemical properties of the plant-based scallops produced by this method were then compared to those of real sea scallops, including their microstructure, color, texture, water holding capacity, and cookability. To the best of our knowledge, this is the first study that has used a soft-matter physics approach to construct plant-based scallop analogs. One of the major potential advantages of this approach over existing methods of creating plant-based foods is that no expensive and energy-intensive structuring equipment is required, such as an extruder or a shear cell. Consequently, it may have considerable commercial potential for the production of these kinds of products. Moreover, it should be possible to use other combinations of proteins and polysaccharides to create these kinds of plant-based foods, which would lead to considerable flexibility in sourcing ingredients.

The knowledge gained from this study could be used by the food industry to create plant-based seafood analogs with improved quality, nutritional profile, and cooking properties. The availability of these products could facilitate the transition to a more sustainable and environmentally friendly food supply.

2. Materials and Methods

2.1. Materials

Native yellow pea flour was provided by Prof. Jiajia Rao, from North Dakota State University. Pectin from citrus peel (galacturonic acid $\geq$ 74.0% dried basis) was purchased from Sigma-Aldrich Co., Ltd. (St. Louis, MO, USA). ACTIVA RM transglutaminase (T-gase) preparation was purchased from Ajinomoto North America., Inc. (Chicago, IL, USA). Raw sea scallops were bought from a local grocery store (Stop & Shop, Amherst, MA, USA) and stored in a freezer (-20 °C) until used. Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Fisher Scientific (Waltham, MA, USA). The Bradford reagent used for the protein determination was obtained from the Bio-Rad company (Hercules, CA, USA).

2.2. Protein Extraction

Pea protein isolate (PPI) was extracted from yellow pea flour according to a method described previously, with some modifications [31]. Briefly, yellow pea flour (100 g) was dissolved in 1500 g of deionized water, and the solution was then adjusted to pH 9.0 using 6 N of NaOH. The alkaline protein solution was then continuously stirred using a magnetic stirrer at 500 rpm for 1 h at room temperature. The pH was checked every 15 min and adjusted back to 9.0 if necessary. Then, the solution was centrifuged at 5524$\times$ g for 20 min at 4 °C. The supernatant was filtered through a Whatman grade 1 (Whatman Grade 42, ashless, 90 mm diameter) using a bench-top vacuum and collected in a flask that was cooled down in an ice bath. The supernatant was then adjusted to pH 4.5 using 6 N of HCl followed by centrifugation at 5524$\times$ g for 20 min at 4 °C. The pellet from centrifugation was collected and re-suspended in water, and the solution was adjusted back to pH 7.0 using 1 N of NaOH. Powdered PPI was obtained by freeze-drying the pellet solution for 48 h.

2.3. Extracted Protein Concentration

The concentration of extracted pea protein was determined by the Bradford protein assay [32]. In brief, a standard curve was prepared using a series of bovine serum albumin (BSA) solutions of different protein concentrations (0 to 1000 μg/mL). For the test samples, 20 w/w% of pea protein stock solution was diluted 1000 times with deionized water. Then, 20 μL of diluted pea protein solution was vortex-mixed with 1 mL of Bradford reagent, incubated for 10 min, and the absorbance was measured at 595 nm using UV-visible spectrometer. The protein concentration was then estimated from the standard

curve. The test samples were prepared in duplicates and the blank consisted of deionized water. The protein concentration of the stock solution was assessed every time after overnight rehydration.

2.4. Differential Scanning Calorimetry Analysis

The thermal transitions of pea proteins dissolved in aqueous solutions were assessed by measuring changes in the heat flow with temperature using a differential scanning calorimeter (DSC 250, TA Instruments, New Castle, DE, USA). Pea protein solutions (20 w/w%) were placed in a high-volume aluminum pan that was then tightly sealed. Another empty high-volume aluminum pan was used as a reference. The weight of each test sample used in the DSC analysis was recorded. DSC measurements were performed by heating the samples from 10 to 130 °C at 3 °C/min under an inert atmosphere (400 mL/min of N2). The onset temperature (To), peak temperature (Tp), and enthalpy (ΔH) of the transitions were computed from the thermal curves using the instrument software (TRIOS 5.2). The same samples were then heated again under the same conditions to establish whether the thermal transitions were reversible.

2.5. Pea Protein-Pectin Gel (Scallop Analog)

Extracted pea proteins were rehydrated overnight to prepare 20 w/w% pea protein stock solutions. These stock solutions were then diluted to 10 w/w%, and the pH was adjusted back to 7.0. Ten grams of pea protein solution were dispensed into a 15 mL beaker (used as a scallop-shaped mold) and then heat-denatured and aggregated by holding at 95 °C for 30 min. This procedure was carried out to increase the effective molecular weight of the proteins, thereby reducing the entropy of mixing effects in the subsequent biopolymer mixtures. After cooling the heat-denatured pea proteins in an ice bath for another 30 min, different concentrations of pectin (0, 0.5, or 1.0 w_{pectin}/w_{total}%) were added and the mixtures were stirred at 500 rpm at room temperature for 60 min to ensure dissolution. Then, 2.0 $w_{T\text{-}gase}/w_{total}$% of transglutaminase (T-gase) was added to the biopolymer mixtures and the system was stirred for 30 min at 500 rpm at room temperature to promote enzyme dissolution. The stir bar was then removed, and the samples were incubated at 50 °C for 30 min to promote protein crosslinking, followed by 30 min of cooling in an ice bath. The gels formed were then gently removed from the beakers and placed onto petri dishes.

2.6. Fourier-Transform Infrared Spectroscopy (FTIR) Analysis

FTIR spectra were acquired using a Fourier Transform Infrared spectrophotometer (Shimadzu, Kyoto, Japan) equipped with an attenuated total reflectance (ATR) accessory under ambient conditions. The samples analyzed by the ATR-FTIR instrument were prepared according to a method described previously [33]. Briefly, freeze-dried powdered pea protein, pectin, or pea protein-pectin (uncooked scallop analog) were placed between two pieces of aluminum foil and then pressed into a small pellet. This pellet was then further pressed onto the germanium crystal surface using an ATR accessory to ensure good contact with the ATR crystal. The background signal was collected before each measurement. Each spectrum was the average of 32 scans in the wavenumber range from 4000 to 400 cm^{-1} at a 4 cm^{-1} resolution.

2.7. Texture Profile Analysis

A texture analyzer (TA.XT2, Stable Micro System, Surrey, UK) with a flat-ended cylinder probe (25 mm diameter) was used to characterize the mechanical properties of the scallop and scallop analog. Double compression was applied to all the samples and the texture profile analysis (TPA) parameters were calculated from the resulting stress-strain curves based on the methods described in a previous study [34]. In brief, a cylindrical test sample of fixed dimensions (4 cm diameter × 0.8 cm height) was placed on the instrument lower plate and the measurement probe was moved downward at a pre-speed

of 2 mm/s. When the probe first touched the surface of the test samples, their thickness was automatically recorded. The probe continued to press the samples to a final strain of 50% at a test speed of 2 mm/s. Then, the samples were allowed to recover for 15 s by removing the force of the probe that was applied on their surfaces. After that, the probe was then pressed onto the samples again, which resulted in a double compression, and then returned to its original position at a post-test speed of 2 mm/s. The trigger force was set to 0.049 N (5 g). The following parameters were then calculated from the texture analysis (TPA) profiles of each sample [34]:

Hardness: The hardness is a measure of the resistance of the sample to compression, which was taken to be the maximum force reached during the first compression of the sample (Fmax1).

Cohesion: The cohesion is a measure of how well the sample maintains its textural attributes after the first deformation, which was calculated as the ratio of areas under the curves for the second and first peaks in the TPA profile (A2/A1).

Springiness: The springiness is a measure of how well the sample springs back to its original dimensions after it has been deformed using a first compression, allowed to sit for 15 s, and then deformed again using a second compression. It is calculated as the ratio of the distances from the start of compression until the maximum is reached for peak 2 and peak 1 (D2/D1).

Chewiness: The chewiness is a measure of the energy required to chew solid foods, which is calculated as, Chewiness = Hardness $\times$ Cohesion $\times$ Springiness.

2.8. Scanning Electron Microscope Analysis

Both scallop and scallop analog were freeze-dried (Genesis Pilot Lyophilizer, SP Scientific, Stone Ridge, NY, USA) and then sputter-coated with gold [35]. All samples were examined by scanning electron microscopy (SEM) using a FEI Magellan 400 (FEI, Hillsboro, OR, USA) with an accelerating voltage of 5 kV under low vacuum conditions.

2.9. Other Physical and Functional Parameters

2.9.1. Water Holding Capacity

The water holding capacity of both scallops and scallop analogs were analyzed using a centrifugal method. A fixed amount (0.50 g) of each initial test sample was placed into a centrifuge tube and then centrifuged at 10,000 rpm at room temperature for 15 min. Any water released from the test samples was carefully removed using a pipette and their final weight was measured. The water holding capacity was calculated as follows:

$$\text{WHC (\%)} = (\text{Initial Weight [g]})/(\text{Final Weight [g]}) \times 100 \quad (1)$$

2.9.2. Colorimetric Analysis

The tristimulus color coordinates (L*, a*, b*) of the real scallop and scallop analog were measured using a colorimeter (ColorFlez EZ, HunterLab, Reston, VA, USA). The L* value describes lightness, the a* value describes redness/greenness, and the b* value describes blueness/yellowness (Commission Internationale de l'Eclairage, Vienna, Austria).

2.9.3. Cookability

The impact of pan frying on the structural and physicochemical properties of the real scallop and scallop analog was also tested. The samples were placed in a non-stick frying pan and heated on each side for 3 min, leading to a total cooking time of 6 min. The internal temperature was monitored with a 0.1 mm diameter copper-constantan thermocouple (Type-T). After cooking, the microstructure, texture and color of the scallop and scallop analog were measured.

2.10. Statistical Analysis

Triplicate analyses were performed for all measurements. Statistical analysis was conducted using Microsoft Excel 2019 software to determine p values using a student's t test. Significant differences ($p < 0.05$) between different group means were determined with the Tukey-Kramer HSD test.

3. Results and Discussion

3.1. Characterization of Extracted Pea Proteins

The molecular state of the extracted pea proteins (native or denatured) was determined using differential scanning calorimetry. The heat flow versus temperature profiles of pea protein solutions (20% w/w%) were measured when they were heated from 10 to 120 °C at a heating rate of 3 °C/min (Figure 1). The same sample was then cooled and heated again under the same conditions to establish whether any observed thermal transitions were reversible or irreversible. During the first scan, a major peak was observed at a temperature (Tpeak) around 85 °C, which was associated with an endothermic enthalpy change (H) of around 1.02 J/g. This endothermic peak was attributed to the thermal denaturation of the globulin fraction of the pea protein. Similar thermal denaturation temperatures have been reported for globulin pea proteins in other studies, e.g., 88 °C [36] and 86 °C [37]. Some researchers have reported two endothermic peaks for pea protein isolates during heating: one corresponding to the denaturation of the non-globulin fraction (around 67 °C) and another corresponding to the denaturation of the globulin fraction (around 85 °C) [38]. During the second scan of the pea protein solution, we found that the peak associated with the thermal denaturation of the proteins was greatly diminished (Figure 1), which suggested that most of the protein molecules had been irreversibly denatured during the first scan.

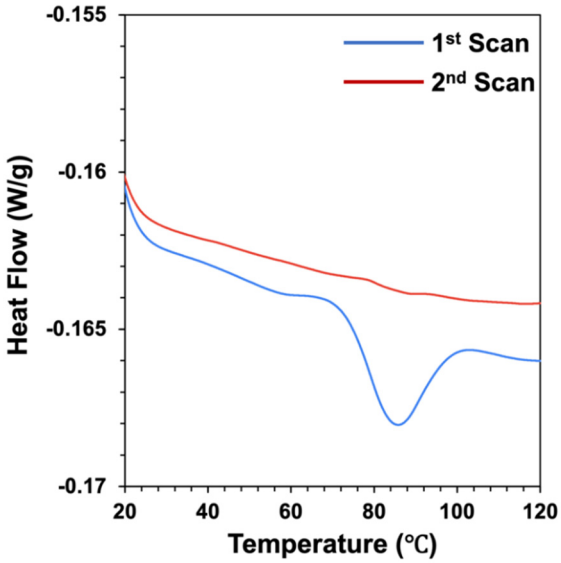

Figure 1. Heat flow versus temperature profiles of pea protein (20 wt%) when heated twice from 10 to 120 °C at 3 °C min^{-1}. The endothermic peak observed during the first scan suggests the proteins were originally in the native state, whereas the lack of peaks in the second scan suggests that they were irreversibly denatured by heating.

3.2. Preparation of Scallop Analogs

The series of steps used to prepare the plant-based scallops is shown schematically in Figure 2. Each major step in the process is described here with a discussion of the underlying physicochemical principles:

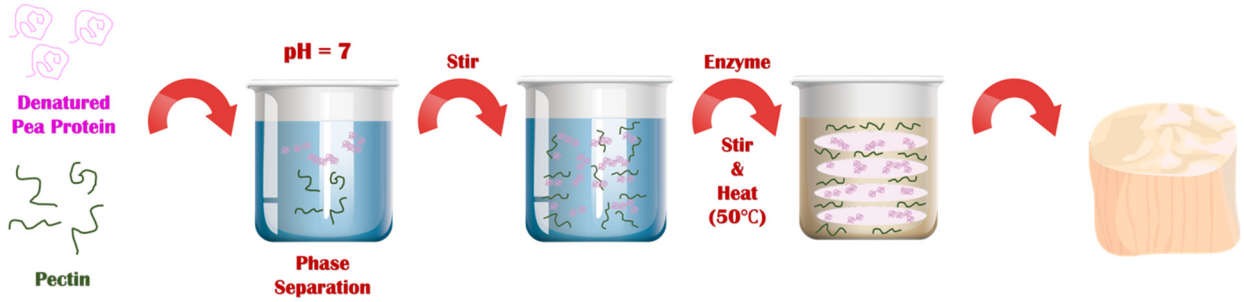

Figure 2. Scallop analogs can be formed through controlled phase separation, shearing, and gelling of mixtures of heat-denatured pea proteins and pectin.

First, a solution of native pea proteins was heated (95 °C, 30 min) above its thermal denaturation temperature to promote the unfolding and aggregation of the protein molecules. This step is required to increase the effective molecular weight of the proteins, thereby reducing the entropy of mixing effect that opposes phase separation. This step must be carried out under appropriate protein concentration, pH, and ionic strength conditions to ensure that the protein aggregates formed have appropriate dimensions. We found that heating a 10% (w/w) protein solution at neutral pH in the absence of salt was sufficient to achieve this goal. After formation, the solution of heat-denatured proteins was cooled to room temperature. The resulting solution was more viscous than the original solution but did not gel, which suggests that protein aggregates had been formed but they were not so large that they formed a network that occupied the entirety of the system.

Second, pectin was added to the heat-denatured protein solution at room temperature, and the system was mixed. In this study, we used a pea protein concentration of around 10% to mimic the protein concentration found in real scallop (10 to 12%). Several pectin concentrations (0, 0.5, and 1.0%) were used to assess the impact of polysaccharide addition on the microstructure and textural attributes of the scallop analogs. At sufficiently high biopolymer concentrations, Phase separation of mixed protein-polysaccharide systems is known to occur under similar biopolymer concentrations due to a phenomenon known as thermodynamic incompatibility [29,39,40].

Third, when a phase separated mixed biopolymer system is gently stirred, it forms a water-in-water (w/w) emulsion, in which the disperse phase droplets are enriched in one kind of biopolymer and the continuous phase is enriched in the other kind of biopolymer. Typically, the interfacial tension at the water-water interface is relatively low (~10^{-7} to 10^{-5} N/m), which means that the droplets are easily deformed and elongated by applying relatively mild shear forces [18]. This phenomenon has previously been used to create fibrous structures from soy protein/pectin mixtures by shearing them at high temperatures in a specialized shear cell device [17,18]. The biopolymer composite material formed consisted of pectin filaments embedded within a protein matrix. We therefore postulated that fiber-like structures would also be formed in the pea protein/pectin blends used in our study when the biopolymer mixture was sheared, which was supported by our microstructural analysis (see later).

Fourth, once the fiber-like structures were formed in the biopolymer mixture, they were locked into place by gelling the proteins using 2% transglutaminase. This food-grade enzyme induces protein crosslinking by catalyzing an acyl-transfer reaction between a γ-carbonyl group of a glutamine residue and an ε-amino group of a lysine residue [41,42]. It should be noted that microbial transglutaminase is widely used in the food industry as a crosslinking agent due to its relatively low cost and "Generally Recognized As Safe" labeling status [42,43].

Fifth, the scallop analogs formed by this process were removed from the glass beakers. These beakers were selected because they had similar dimensions to real scallops and could therefore be used as molds. In industry, molds with specific seafood-like shapes and sizes could be used to form other kinds of seafood.

3.3. Fourier-Transform Infrared Analysis

FTIR spectroscopy was used to provide information about the composition of the scallop analogs. As shown in Figure 3, bands were observed at wavenumbers of 1633, 1529, and 1389 cm^{-1}, which were consistent with the C=O, N–H, and C–N stretching/bending vibrations in amide I, II and III, respectively [44]. These bands were seen in both the pure pea protein and in the scallop analogs, which confirmed that the proteins were present within the scallop analogs. The strong band observed at 1012 cm^{-1} can be assigned to intermolecular hydrogen bonding of the pectin backbone [44]. This band was seen in both the pure pectin and the scallop analogs, which confirmed that the pectin was also present within the scallop analogs. Some new peaks were observed in the spectra of the scallop, which may have been due to the presence of water or due to changes in the molecular interactions in the system when the protein and polysaccharide molecules were mixed.

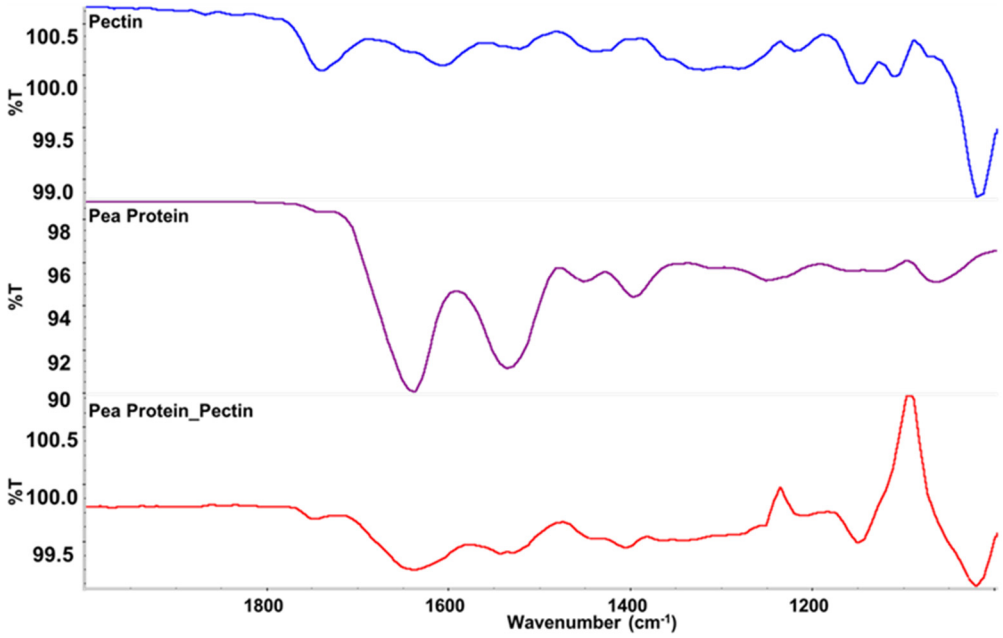

Figure 3. FTIR spectra of powdered pectin, pea protein, and scallop analogs. The scallop analogs consisted of 10% pea protein, 2% transglutaminase, and 0.5% pectin.

3.4. Textural and Water Holding Properties of Scallop and Scallop Analogs

Texture profile analysis was used to provide information about the impact of product formulation on the textural attributes of the plant-based scallops, as well as to compare their textural attributes to those of real scallops.

As shown in Figure 4, the hardness, springiness, and chewiness of the scallop analog constructed from 10% pea protein, 0% pectin, and 2% transglutaminase were not statistically different from those of real scallop. However, the cohesion of these scallop analogs was significantly higher than that of the real scallops. The hardness and chewiness of the scallop analogs increased when the pectin concentration was raised from 0 to 0.5% (w/w) but then decreased when it was further raised to 1.0% (w/w). These results suggest that low concentrations of pectin strengthened the texture of the uncooked scallop analogs, while high concentrations weakened it. We postulate that low pectin concentrations may have promoted phase separation of the protein-polysaccharide mixture, which increased the protein concentration in the continuous phase, thereby strengthening the gel matrix. Conversely, high pectin concentrations may have inhibited the molecular interactions between neighboring protein molecules. Similar effects have been reported in some other studies on protein-polysaccharide mixtures. For example, a study on ginkgo seed protein-pectin composite gels found that adding relatively low pectin concentrations (<0.5% w/w) strengthened the gels but adding a higher concentration (1% w/w) weakened them [45].

The springiness of the scallops and scallop analogs was relatively high (>95%) and did not depend on the pectin concentration used (Figure 4). This latter effect suggests that the incorporation of the pectin did not affect the ability of the scallop analogs to return to almost their original dimensions after the first compression.

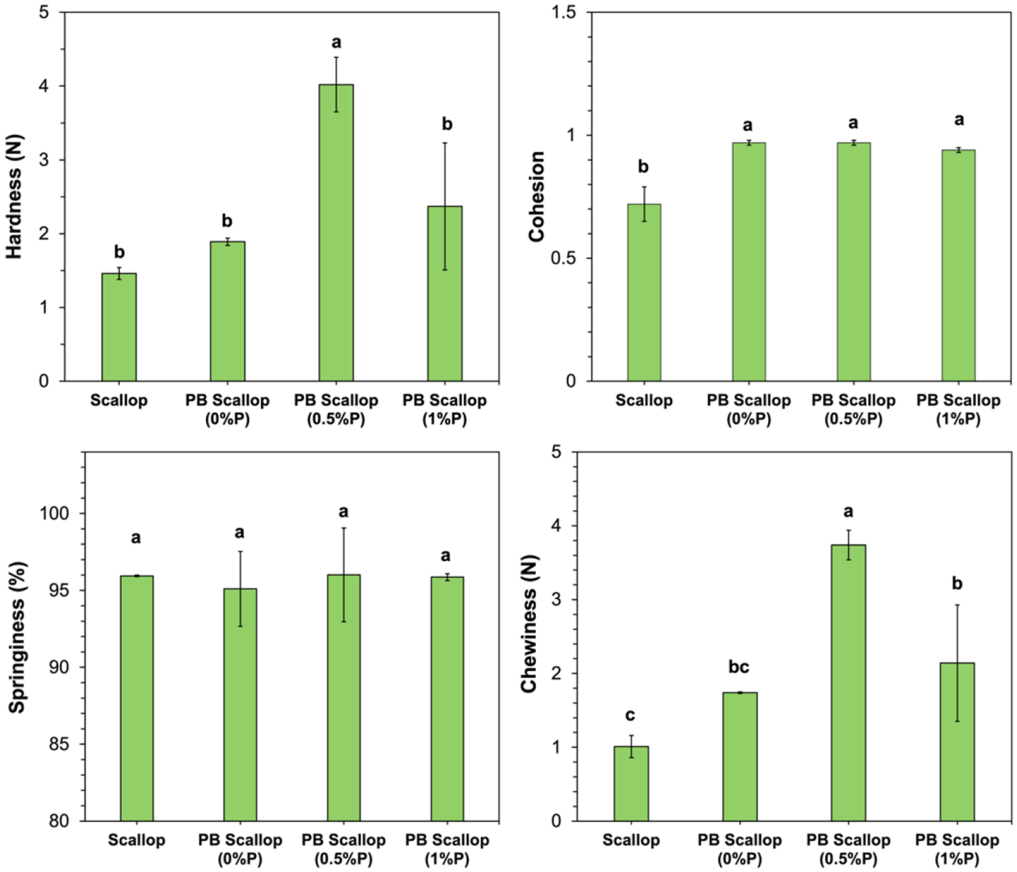

Figure 4. Textual profile analysis of raw scallop and uncooked scallop analogs containing different pectin concentrations. All scallop analogs contained 10% pea protein and 2% transglutaminase. Error bars represent the standard errors (n = 3), and similar letters mean no statistical difference between treatments ($p \leq 0.05$).

The water holding capacity (WHC) of the scallop and scallop analogs was also measured (Figure 5). The WHC of the scallop and scallop analog containing no pectin were quite similar, with no significant difference between them. The WHC of the scallop analogs decreased significantly with increasing pectin concentration, going from around 99.1% at 0% pectin to 94.1% at 1.0% pectin. In general, the WHC is a measure of the ability of a material to retain water when an external stress is applied, such as centrifugation [46,47]. The ability of porous food matrices to retain water can be attributed to the presence of a 3D network of entangled and crosslinked biopolymer molecules. Three main physicochemical processes typically contribute to the water holding properties of porous food matrices: (i) biopolymer-water mixing effects; (ii) ion distribution effects; and (iii) elastic deformation effects [47,48]. The biopolymer-water mixing effect depends on changes in the molecular interactions and entropy of the biopolymer and water molecules when they are combined. Consequently, it is governed by the type of molecular interactions (e.g., electrostatic, hydrogen bonding, and/or hydrophobic interactions) and contact area between the biopolymer and water molecules (which depends on the pore size of the biopolymer network). The ion distribution effect is mainly a result of concentration gradients between mineral ions inside and outside the biopolymer network, as this generates an osmotic pressure. These effects are therefore impacted by the tendency for counter-ions to accumulate around oppositely charged groups on the surfaces of biopolymer molecules in the

gel network. The elastic deformation effect results from the mechanical resistance of the biopolymer network to compression when an external force (such as centrifugation) is applied: the stronger the gel network, the greater the WHC.

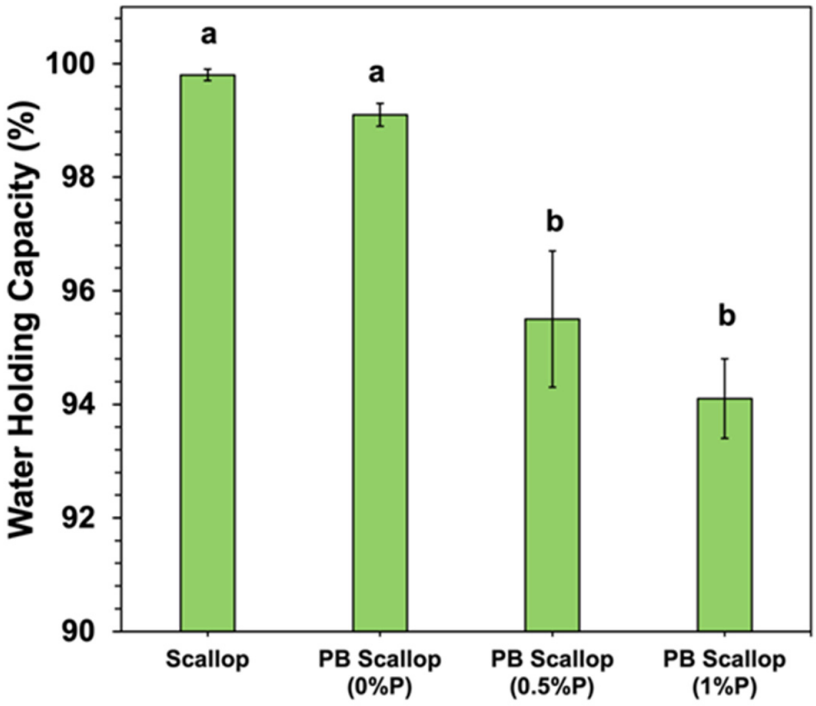

Figure 5. Water holding capacity of scallops and scallop analogs containing different pectin concentrations. All scallop analogs contained 10% pea protein and 2% transglutaminase. Error bars represent the standard errors (n = 3), and similar letters mean no statistical difference between treatments ($p \leq 0.05$).

The observed decrease in WHC with increasing pectin concentration therefore suggests that the presence of the polysaccharide impacted one or more of these physicochemical mechanisms. The TPA measurements showed that the addition of pectin increased the hardness of the scallop analogs (Figure 4), which suggests that elastic deformation effects were not responsible for the reduction in WPC. The presence of the pectin molecules increased the pore size of the biopolymer network (see later), which would have decreased the contact area between the protein molecules and water, thereby reducing the ability of the scallop analogs to retain water. The presence of the pectin may also have altered the balance of mineral ions inside and outside the gels, which would have altered the magnitude of the osmotic stress acting on the gels, thereby altering their WHC. Nevertheless, further research is needed to identify the precise physicochemical origin of these effects.

3.5. Microstructure

Scanning electron microscopy was used to provide insights into the microstructure of the scallops and scallop analogs (Figure 6A). The real scallop had a honeycomb structure, which is consistent with that reported previously for scallop adductor muscles [49]. Presumably, this structure was due to the presence of the muscle fibers in the scallop. During the dehydration process required to prepare the samples for SEM analysis, the fibers in the scallops may have separated from each other. In the absence of pectin, the scallop analogs had a much smoother microstructure than the real scallop (Figure 6B), which may have been because they only contained a network of closely packed globular pea protein molecules. As the pectin concentration was raised, the biopolymer network became more porous, and the pore size increased (Figure 6B–D). This effect may be due to the ability of the pectin molecules to promote phase separation of the pea protein-pectin mixtures, thereby leading to the formation of fiber-like structures when they were sheared during

the formation of the scallop analogs. The increase in pore size with increasing pectin concentration would account for the reduction in WHC when the pectin concentration was raised (Section 3.4). Overall, these results show that the microstructure of the scallop analogs is closer to that of the real scallops when pectin is incorporated into the system.

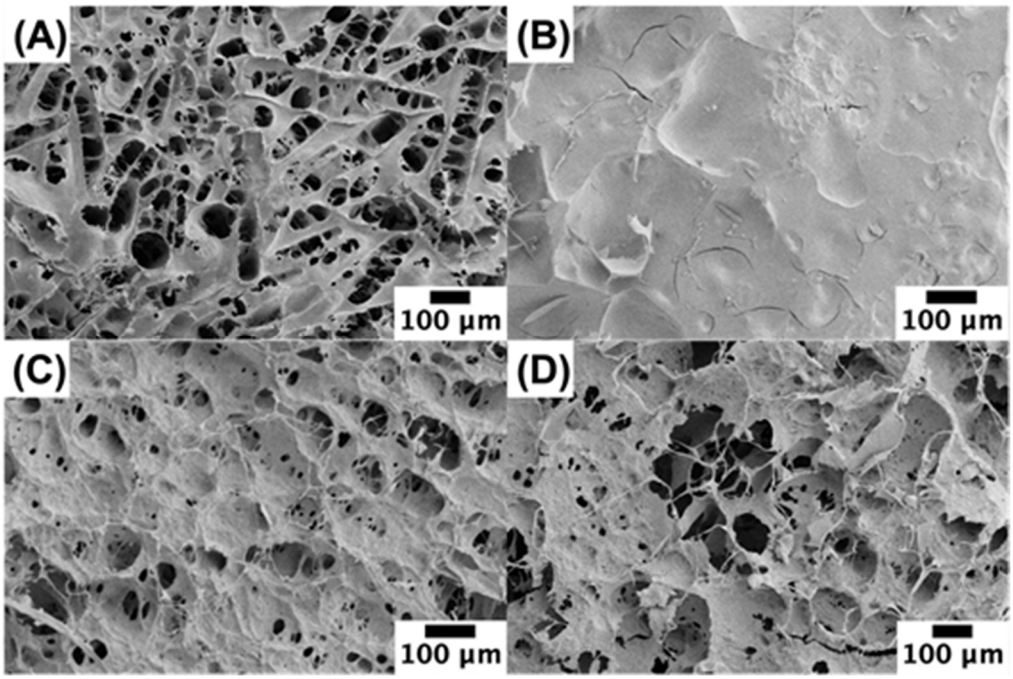

Figure 6. Scanning electron microscopy images of scallop (**A**) and scallop analogs containing 0% (**B**), 0.5% (**C**) and 1.0% (**D**) pectin. All scallop analogs contained 10% pea protein and 2% transglutaminase.

3.6. Color and Textural Properties of Scallop and Scallop Analog after Grilling

In these experiments, we compared the color and textural attributes of scallop analogs to those of real scallops after grilling. Scallop analogs containing 10% pea protein, 0.5% pectin, and 2% transglutaminase were selected for these studies because they had microstructures somewhat like real scallops. Moreover, the changes in physicochemical properties caused by grilling led to final products with textural attributes more like those of real scallops.

The surfaces of the real scallop turned golden brown after grilling for 3 min on each side (Figure 7). This color change can be attributed to the Maillard reaction, which is a complex series of non-enzymatic reactions between the ϵ-amino groups of proteins and the carbonyl groups of reducing sugars. The Maillard reaction is known to occur when fish, shellfish, shrimp, and squid are thermally processed, resulting in desirable flavors and colors during cooking [50,51]. Similar to real scallops, the surfaces of the plant-based scallops also became golden brown after grilling (Figure 7), which can be attributed to a Maillard reaction between the pea protein and pectin [52].

Further information about the appearance of the grilled scallops was obtained by colorimetric analysis (Table 1). There were no significant differences between the lightness (L* value), redness (a* value), and yellowness (b* value) of the real scallops and the scallop analogs. Both types of products had intermediate lightness values (53–55), moderate redness values (24–26), and low yellowness values (0.8 to 0.9). These results suggest that the appearance of real scallops could be closely matched using the plant-based scallop analogs developed in this study.

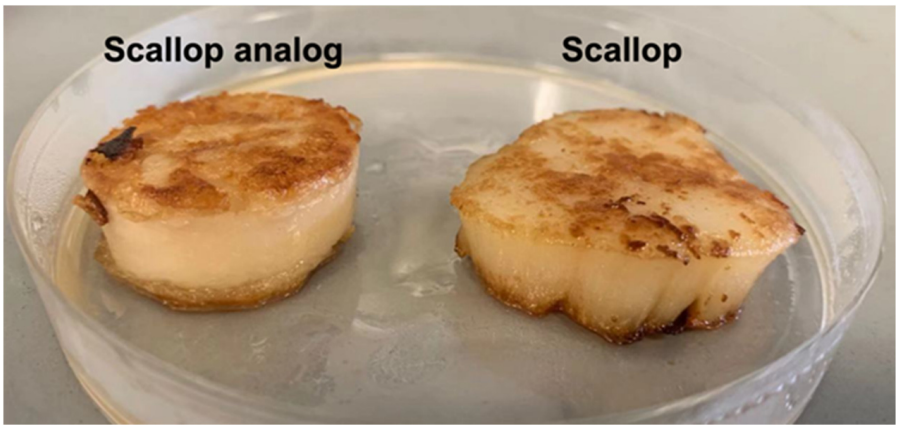

Figure 7. Scallop and scallop analog after grilling. The scallop analogs contained 10% pea protein, 0.5% pectin, and 2% transglutaminase.

Table 1. Colorimetric analysis of scallops and scallop analogs after grilling. The scallop analogs contained 10% pea protein, 0.5% pectin, and 2% transglutaminase. Error bars represent the standard errors (n = 3), and similar letters mean no statistical difference between treatments ($p \leq 0.05$).

	L	**a**	**b**
Scallop	52.5 ± 0.9 [a]	6.2 ± 0.8 [a]	26.3 ± 0.4 [a]
Scallop Analog	54.9 ± 2.2 [a]	6.5 ± 0.9 [a]	24.0 ± 2.0 [a]

The textural attributes of the real scallops and scallop analogs were also measured and compared after grilling. Compared to the uncooked versions, there was a large increase in the hardness and chewiness of both types of scallops after grilling. For instance, the hardness increased from 1.46 to 19.6 N for the real scallop and from 4.02 to 16.9 N for the plant-based scallop after grilling, while the chewiness increased from 1.01 to 15.4 for the real scallops and from 3.74 to 13.0 for the scallop analogs. For the real scallops, this effect can be attributed to unfolding and crosslinking of the protein molecules, as well as to moisture loss caused by the high temperature used during grilling, which increased the protein concentration and therefore the gel strength. For the plant-based scallops, the pea proteins were already thermally denatured prior to grilling, but the gel strength may still have increased due to the increase in protein concentration due to heat-induced moisture loss, as well as an increase in protein crosslinking caused by cooking. Interestingly, the relative increases in hardness and chewiness after grilling were greater for the real scallop (13- and 15-fold, respectively) than for the scallop analog (4.2- and 3.5-fold, respectively). This was one of the main reasons that 0.5% pectin was included within the scallop analogs (even though it led to harder gels before cooking). There was an increase in the cohesion of the real scallop after grilling (from 0.72 to 0.82) but a decrease for the scallop analogs (from 0.97 to 0.82), which suggests that cooking had different effects on their abilities to retain their shape after compression. Both the real scallop (96.4%) and scallop analog (93.6%) retained their high degree of springiness after grilling.

A direct comparison of the hardness, cohesion, springiness, and chewiness of the grilled real scallop and scallop analog showed they were not statistically different (Figure 8), which suggests their textural properties were similar. Nevertheless, further studies are still required to assess their mouthfeels and textures using sensory studies.

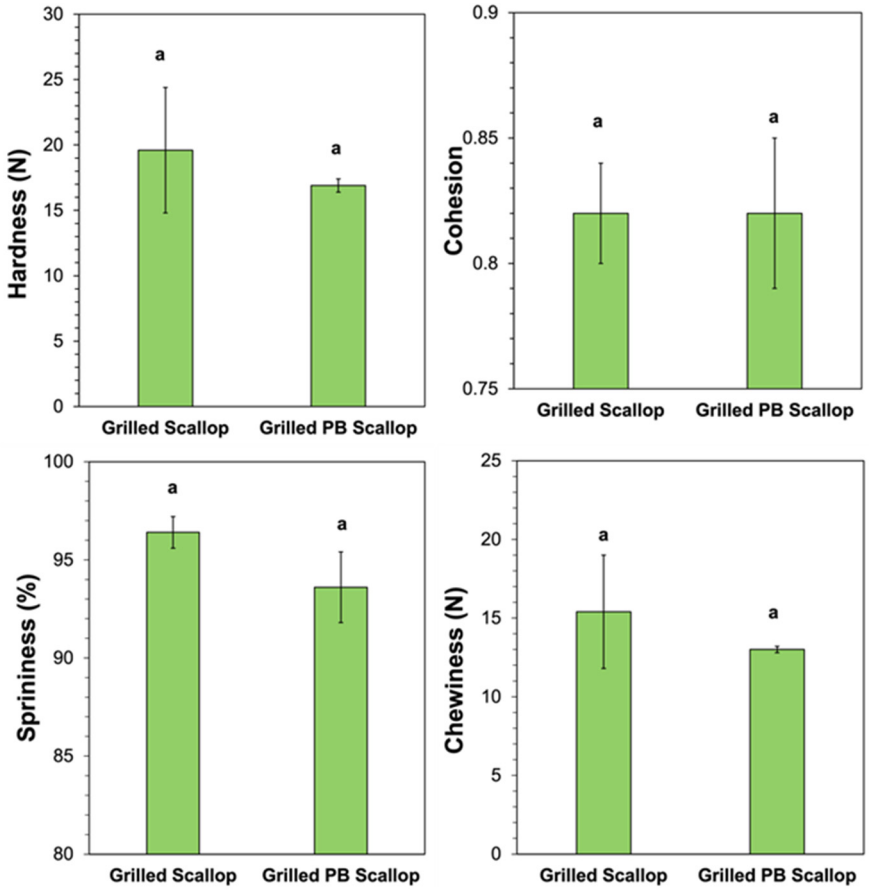

Figure 8. Textual profile analysis of scallop and scallop analog. The scallop analogs contained 10% pea protein, 0.5% pectin, and 2% transglutaminase. Error bars represent the standard errors (n = 3), and similar letters mean no statistical difference between treatments ($p \leq 0.05$).

4. Conclusions

In summary, we have shown that a soft matter physics approach can be used to produce scallop analogs based on thermal denaturation, phase separation, shearing, and enzymatic gelling of plant protein/polysaccharide mixtures under controlled conditions. Unlike conventional extrusion or shear-cell technologies, no specialized equipment (e.g., an extruder or pressurized high shear cell) is required to create the seafood analogs. The microstructure and physical properties of the scallop analogs could be controlled by adding different pectin concentrations. The gel strength increased upon the addition of a relatively low pectin concentration (0.5%, w/w) but decreased upon the addition of a higher concentration (1.0%, w/w). After grilling, the appearance and textural properties of the scallop analogs were very similar to that of the real scallop. Our results suggest that the method developed in this study may prove to be a simple and affordable means of producing plant-based seafood analogs. Nevertheless, further research is still required to fortify the seafood analogs with other nutrients, such as omega-3 fatty acids, vitamins, and minerals, as well as to test their sensory attributes using human studies. Numerous consumer studies have shown that the taste of food products is the main driver for consumer acceptance. Consequently, it will be important to compare the sensory attributes of the plant-based scallops developed in this study with those of real scallops, as well as to establish consumer acceptance and liking of these products. In future studies, we therefore intend to carry out this kind of sensory analysis.

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Conflicts of Interest: The authors declare that they have no conflict of interest in this work.

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Peptidomics Study of Plant-Based Meat Analogs as a Source of Bioactive Peptides

Shuguang Wang [1,2], Mouming Zhao [2], Hongbing Fan [1] and Jianping Wu [1,*]

[1] Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, AB T6G 2P5, Canada
[2] School of Food Science and Engineering, South China University of Technology, Guangzhou 510640, China
* Correspondence: jwu3@ualberta.ca; Tel.: +1-(780)-492-6885

Abstract: The demand for plant-based meat analogs (PBMA) is on the rise as a strategy to sustain the food protein supply while mitigating environmental change. In addition to supplying essential amino acids and energy, food proteins are known sources of bioactive peptides. Whether protein in PBMA affords similar peptide profiles and bioactivities as real meat remains largely unknown. The purpose of this study was to investigate the gastrointestinal digestion fate of beef and PBMA proteins with a special focus on their potential as precursors of bioactive peptides. Results showed that PBMA protein showed inferior digestibility than that in beef. However, PBMA hydrolysates possessed a comparable amino acid profile to that of beef. A total of 37, 2420 and 2021 peptides were identified in the gastrointestinal digests of beef, Beyond Meat and Impossible Meat, respectively. The astonishingly fewer peptides identified from beef digest is probably due to the near-full digestion of beef proteins. Almost all peptides in Impossible Meat digest were from soy, whereas 81%, 14% and 5% of peptides in Beyond Meat digest were derived from pea, rice and mung proteins, respectively. Peptides in PBMA digests were predicted to exert a wide range of regulatory roles and were shown to have ACE inhibitory, antioxidant and anti-inflammatory activities, supporting the potential of PBMA as a source of bioactive peptides.

Keywords: plant-based meat analogs; protein hydrolysates; nutritional property; peptide profile; bioactive assessment

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1. Introduction

A growing global population poses critical challenges in sustaining protein supply under already constrained resources and alarming concerns over climate change. Among various strategies towards sustainable protein production such as cellular agriculture (i.e., cultured meat), alternative proteins (i.e., terrestrial plant, insect and seaweed) and valorization of agricultural by-products [1,2], developing plant-based meat analogs (PBMA) is an attractive solution to replace traditional livestock production [3]. The market shares of alternative proteins remain low when compared with meat, even though governments and innovative companies increasingly advertise these alternatives to traditional meat products or dishes, such as plant-based burgers [4]. One major hurdle is consumer acceptance; in comparison, insects showed the lowest acceptance, followed by cultured meat, while terrestrial plant-based alternatives have the highest acceptance level [5]. The consumer acceptance of alternative proteins showed to be closely relevant to the drivers of taste and health, the color and aroma inherited, familiarity, food neophobia and disgust [1,2].

Since the successful launch of Beyond Meat and Impossible Meat, the market of PBMA has been on the rise; the global plant protein-based meat market is estimated to be approximately USD 21 billion by 2025 [6]. From a nutritional point of view, PBMA has unique advantages: its negligible cholesterol content, low fat content and high protein content with a well-balanced amino acids pattern [7,8]. McClements et al. reported that PBMA burgers contained fewer calories, cholesterol and fat than conventional beef burgers,

despite nearly equal protein content [9]. However, there are continuous debates over the health implications of PMBA due to the addition of additives and the use of highly processed ingredients [3,8]. The health benefits of plant foods are likely compromised in PBMA. There is a need to develop clean labels and minimally processed products. For instance, the clean-labelled ProDiem™Refresh Soy is characterized by its sustainable and optimized nutrients to simulate/fulfill a protein intake similar to egg/milk [10].

A wide range of alternative proteins is explored for use in PBMA, especially those from grains and legumes, such as soy, pea, wheat, mung and lentil [11]. However, terrestrial plant proteins commonly possess inferior digestibility to that of livestock proteins, which challenges the nutritional profile of protein in meat analogs [12]. For example, Xie et al. reported that real meat (pork and beef) exhibited higher digestibility than that of PBMA during simulated gastrointestinal digestion, and the digestibility of PBMA depends on the origin and structure of proteins as well as the method of protein processing [13]. Food proteins are known as good sources of bioactive peptides. Bioactive peptides usually consist of 2–20 amino acids in length that are encrypted in their parent proteins and can exert regulatory roles once released in certain scenarios, including the gastrointestinal tract [1]. Given its increasing role in human dietary patterns, it is imperative to understand the potential of PBMA as the precursor of bioactive peptides. For instance, Chen et al. showed the formation of higher molecular weight and higher hydrophobicity in PBMA-derived peptides (soy and wheat proteins) than in chicken breast [14]. Xie et al. reported a larger number of peptides were identified from real meat than those of PBMA after simulated gastrointestinal digestion [13].

However, PBMA used in previous studies was prepared experimentally; research on commercial PBMA, especially from Beyond Meat and Impossible Meat, two major producers, are rarely reported. Simultaneously, systematic studies on the gastrointestinal fate, especially peptide profile and bioactivities after gastrointestinal digestion of PBMA, are still insufficiently understood. Meanwhile, there is no doubt that the peptide fragments released from real meat and PBMA are diverse due to their different parent protein sequences. Thus, the potential health benefits of these peptide fragments released from real meat and PBMA will also differ. Additionally, peptidomics and bioinformatics are emerging tools for identifying and predicting peptide profiling, bioavailability and bioactivity of bioactive peptides [15]. Hence, exploration of the digestibility and peptide profile after gastrointestinal digestion with the aid of peptidomics and bioinformatics will facilitate our understanding of the potential health benefits of PBMA.

The purpose of this study was to compare the in vitro gastrointestinal digestion fate of beef and PBMA (from Beyond Meat and Impossible Meat) with a special focus on their potential as precursors of bioactive peptides through assessing digestibility and peptide profiles and evaluate the relationship between peptide features and biofunctions (angiotensin-converting enzyme (ACE) inhibition, antioxidant and anti-inflammation).

2. Materials and Methods

2.1. Materials

Cooked patties of beef hamburger and Beyond Meat hamburger were bought from A&W (Edmonton, Alberta, Canada), and cooked patties of Impossible Meat burger were bought from Burger King (Edmonton, AB, Canada). ACE (from rabbit lung), hippuryl-His-Leu (HHL), pepsin (porcine gastric mucosa), pancreatin (porcine pancreas), 2,4,6-trinitrobenzenesulfonic acid (TNBS), cytochrome C, aprotinin, vitamin B12, $(glycine)_3$, dithiothreitol (DTT) and angiotensin II (Ang II) were obtained from Sigma (Oakville, ON, Canada). Vascular smooth muscle A7r5 cell line was purchased from ATCC (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 4-(2-68 hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and non-essential amino acids (NEAA) were obtained from Gibco Invitrogen (Burlington, ON, Canada). Dihydroethidium (DHE) was purchased from Biotium (Fremont, CA, USA). Solvents used for UPLC were of chromatographic grade. Other chemicals applied were of analytical grade.

2.2. Preparation of Beef and PBMA Gastrointestinal Digests

The cooked beef patties and plant-based patties (Beyond Meat and Impossible Meat) in this study were bought from stores. Minced beef and PBMA were suspended in ddH_2O and then exposed to two-step simulated gastrointestinal digestion [16]. Briefly, beef and PBMA (5% protein, *w*/*v*) were hydrolyzed by pepsin (1% protease/substrate, *w*/*w* protein) at pH 2.0 and 37 °C for 2.0 h, and then the digests were adjusted to pH 7.5 for another 2.0 h of hydrolysis with pancreatin (1% protease/substrate, *w*/*w* protein). Hydrolysis was terminated by heating the slurry at 95 °C for 10 min to inactive the proteases. Subsequently, the mixtures were centrifuged (8000× *g*, 15 min, 4 °C) to collect the supernatants, which were filtered by qualitative filter paper before being lyophilized to obtain the hydrolysates including BfP (cooked beef-pepsin), BfPP (cooked beef-pepsin-pancreatin), ByP (cooked Beyond Meat-pepsin), ByPP (cooked Beyond Meat-pepsin-pancreatin), ImP (cooked Impossible Meat-pepsin) and ImPP (cooked Impossible Meat-pepsin-pancreatin).

2.3. Molecular Weight Distribution

The molecular weight distribution of beef and PBMA hydrolysates were performed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography according to the methods of Laemmli et al. [17] and Fan et al. [18], respectively. Briefly, for SDS-PAGE, beef and PBMA hydrolysates were initially dissolved in water at a concentration of 10 mg/mL and then diluted using a 2 × Laemmli sample buffer containing 5% β-mecaptoethanol at a volume ratio of 1:1. The prepared beef and PBMA hydrolysates were heated to 95 °C for 5 min before 20 µL of them were loaded to 16.5% Mini-Protean Tris-Tricine gel in a Mini-PROTEAN Tetra Cell with a PowerPac Basic electrophoresis apparatus (Bio-Rad, CA, USA) at a constant 150 V voltage. Gels were stained by Coomassie brilliant blue R250 dye and further destained by destaining buffer (ddH_2O:methanol:acetic acid = 5:4:1, *v*/*v*/*v*), and then were scanned through an Alpha Innotech gel scanner (San Leandro, CA, USA). On the other hand, the molecular weight distribution was analyzed by size exclusion chromatography connecting with an AKTA explorer 10XT system (GE Healthcare, Uppsala, Sweden) with a Superdex peptide 10/300 GL column. Beef and PBMA hydrolysates were dissolved in 30% ACN containing 0.1% TFA. Subsequently, 100 µL beef and PBMA hydrolysates at a concentration of 1 mg/mL were injected into the Superdex peptide 10/300 GL column and eluted at an isocratic gradient with a flow rate of 0.5 mL/min. Peaks were monitored at 220 nm. The molecular weight was calibrated by a protein marker mixture in SDS-PAGE, whereas aprotinin, cytochrome C, $(glycine)_3$ and vitamin B12 were used as molecular weight markers in size exclusion chromatography.

2.4. Degree of Hydrolysis (DH) and Amino Acid Compositions

The DH of beef and PBMA hydrolysates were evaluated using the TNBS method [19]. The amino acids analysis of beef and PBMA hydrolysates were determined according to the method of Zheng et al. [20].

2.5. Identification of Peptides by LC-MS/MS

The gastrointestinal-digested beef and PBMA hydrolysates were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) on an Atlantis dC_{18} UPLC column (Waters, Milford, MA, USA) using a nano-Acquity RP-UPLC system, coupled with a Micromass Quadrupole Time-of-Flight (Q-TOF) premier mass spectrometer (Bruker, Bremen, Germany), as previously described [16]. Solvents were chromatographic grade acetonitrile (mobile phase B) and H_2O (mobile phase A) containing 0.1% formic acid. The gradient program was set as 1%–60%–95% mobile phase B according to 0–2–40–55 min. Mass spectra were set in the positive-ion mode. The quadrupole ion energy was set at 4.0 eV, while the collision-inducing dissociation energy was set at 8–50 eV. The parameters for the ESI interface were as follows: 180 °C drying gas temperature, 8.0 L/min drying gas flow and 1.5 bar ESI nebulizer pressure. Data were interpreted by searching Mascot. The

major parent protein sequences of beef, pea, soy, mungbean, rice and potato were obtained from the UniProtKB [21].

2.6. ACE Inhibition Assay

ACE inhibition was measured by referring to the method of Wu et al. [22]. ACE, HHL, beef and PBMA hydrolysates were dissolved and diluted with 100 mM potassium phosphate buffer containing 300 mM NaCl (pH 8.3). Substrate HHL (50 μL, 5 mM) and beef/PBMA hydrolysate (10 μL) were initially mixed and preincubated at 37 °C for 5 min in a 2 mL polypropylene centrifuge tube, and then 20 μL of preincubated ACE (37 °C, 2 mU) was added and reacted for another half an hour by an Eppendorf Thermomixer R (Brinkmann Instruments, NY, USA). The reaction was terminated by further adding 1 M HCl (125 μL) and then analyzed using an UPLC system combined with an Acquity BEH C18 column (1.7 μm, 2.1 mm × 50 mm). Solvents were chromatographic grade acetonitrile (mobile phase B) and H_2O (mobile phase A) containing 0.05% formic acid. Samples (5 μL) were eluted at a flow rate of 0.245 mL/min, and the gradient program was set as 5%–60%–60%–5% B according to 0–3.5–4.2–5 min. Absorbance was monitored at 220 nm. Hippuric acid was identified and quantified through its standard curve. The IC_{50} value represents the concentration of PBMA hydrolysates when inhibiting ACE activity by 50%.

2.7. Desalting Protocol, Cell Culture and Cytotoxicity

Before incubation with A7r5 cells, beef and PBMA hydrolysates were desalted according to the method described previously by Fan et al. [18]. Briefly, beef and PBMA hydrolysates were dissolved in ddH_2O and then loaded into a Sep-Pak 35cc tC18 cartridge (Waters, MA, USA). Firstly, the cartridge was washed with ddH_2O at the volume of two column volumes for salt removal. Subsequently, ACN was added to wash the cartridge, and the ACN eluent was collected, vacuum evaporated and freeze-dried.

A7r5 cells were cultured with DMEM medium containing 10% FBS, 25 mM HEPES and 1% penicillin-streptomycin in a cell incubator at 37 °C, 5% CO_2 and 100% humidity. The culture media were changed every two days. The cytotoxicity of beef and PBMA hydrolysates against A7r5 cells was measured through an alamarBlue assay, as depicted by Fan et al. [18]. A7r5 cells were initially sown in a 96-well plate, and cells were treated with 1.0 mg/mL of beef and PBMA hydrolysates for 24 h when reaching 80% of confluency, and then the medium was replaced with 200 μL of 10% alamarBlue solution for another 4 h. Finally, the solution (150 μL) was transferred into an opaque 96-well plate for fluorescence signal detection, with an emission wavelength at 590 nm and excitation wavelength at 560 nm.

2.8. Superoxide Detection

Superoxide in A7r5 cells was investigated by the Dihydroethidium (DHE) staining method [23]. A7r5 cells were pre-incubated with hydrolysates (1.0 mg/mL) for 1 h before the addition of Ang II (1 μM) for 0.5 h. Subsequently, DHE (20 μM) was added and treated for another 30 min. After that, cells were triple-washed with non-phenol-red DMEM, and the fluorescence intensity was measured by an Olympus IX81 fluorescent microscope (Olympus, Tokyo, Japan). Each data was comprised of two or three random fields. The mean fluorescence intensity was obtained using ImageJ software (National institutes of health, Bethesda, MD, USA).

2.9. Western Blotting

A7r5 cells were pre-incubated with beef and PBMA hydrolysates (1.0 mg/mL) for 1 h before adding Ang II (1 μM) for 24 h. After the treatment, cells were scraped and lysed in boiling Laemmle's buffer containing 50 mM DTT and 0.2% Triton-X-100, and then cell samples were loaded onto a 9% separating gel and transferred to a nitrocellulose membrane for specific antibodies incubation. Bands of cyclooxygenase-2 (COX-2; Abcam, Toronto, ON, Canada) and inducible nitric oxide synthase (iNOS; BD Biosciences, San Jose, CA,

USA) were normalized to GAPDH (ab8245, Abcam). The fluorescent bands were visualized by adding corresponding secondary antibodies, and the signals were detected using Licor Odyssey BioImager (Licor Biosciences, Lincoln, NE, USA).

2.10. Statistical Analysis

SPSS 17.0 (SPSS Inc., Chicago, IL, USA) was applied to statistical treatment with ANOVA analysis followed by the Duncan post hoc test. Data were expressed as mean ± standard deviation. Differences were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Molecular Weight Distribution, DH and Amino Acid Compositions of Beef and PBMA Digests

Figure 1A shows that the pepsin and/or pancreatin treatments cause a substantial decrease/disappearance in the intensity of large-molecular-weight protein bands, which is due to the degradation of proteins into peptides/free amino acids. Likewise, the results of size exclusion chromatography further demonstrated that the small-molecular-weight fractions in beef and PBMA hydrolysates increased rapidly from gastric digestion to the intestinal digestion phase (Figure 1B), which dominated peptide composition in BfPP, ByPP and ImPP due to further extensive hydrolysis. Furthermore, DH data were consistent with the results shown in SDS-PAGE and size exclusion chromatography (Table 1). The DH of ByPP and ImPP increased gradually during in vitro digestion, being 4.92% and 6.09% after gastric digestion, and further increased to 7.94% and 7.48% after intestinal digestion, respectively. Beef hydrolysate had higher DH than PBMA throughout digestion. The gastrointestinal digestion fate of real meat and PBMA are hypothesized to be different due to the diversities in the structures and compositions of the raw material. Particularly, PBMA contains different sources of proteins as compared with real meat, as well as a variety of food additives which may affect protein digestion [24,25]. Moreover, the processing technologies in PBMA production may result in the formation of structures that negatively impact protein digestion. For instance, the dense mesh structure or aligned fibrils of proteins formation under the thermal–mechanical treatment largely impair the digestibility of proteins in PBMA [26]. A better swelling capacity of beef promotes penetration of gastrointestinal proteases, whereas the bulkiness of storage proteins, protein aggregates and the presence of antinutritional factors in beans limit the digestion of PBMA [27]. Our results are consistent with previous research. For instance, Xie et al. demonstrated that real pork and beef showed higher digestibility than PBMA [13], and the study of McClements et al. also reported the inferior digestibility of PBMA [8].

Amino acid composition is an indicator of the nutritional value of protein hydrolysates [28]. Essential amino acids (EAA) refer to amino acids which cannot be auto-synthesized by the human body, or the rate of synthesis is inadequate to meet the biological needs of the body. Thus, they need to be supplied by food protein intake. Normally, Val, Leu, Ile, Phe, Lys, His, Thr and Met are considered the eight EAA of individuals. Table 1 and Figure 2 shows that the total amino acid compositions of the three hydrolysates ranged from 67.56–87.64 g/100 g. Gastrointestinal digestion of beef had the highest content of amino acids, whereas ByPP and ImPP had a relatively low content of amino acids. However, the content of EAA in PBMA hydrolysate was comparable to the beef counterpart. The contents of EAA in ByPP and ImPP were 42.91 g/100 g and 41.60 g/100 g, whereas a higher value (46.04 g/100 g) was found in BfPP. EAA cannot be synthesized by mammals and must be obtained from food. EAA have important regulatory effects in many physiological events [29,30]. On the other hand, PBMA hydrolysates also contain a high level of non-EAA. Of which, Glu, Gly and Ala were abundant in beef hydrolysate, whereas Asp and Arg content was lower. In particular, no Glu was detected in PBMA hydrolysates. There is no compelling evidence to support that synthesis of non-EAA in the body could satisfy the requirement of physiological activities [31]. Thus, the content of non-EAA should still be taken into consideration when evaluating the nutritional value of proteins. From the

amino acids profile, PBMA hydrolysates were expected to possess comparable nutritional properties to that of beef hydrolysate.

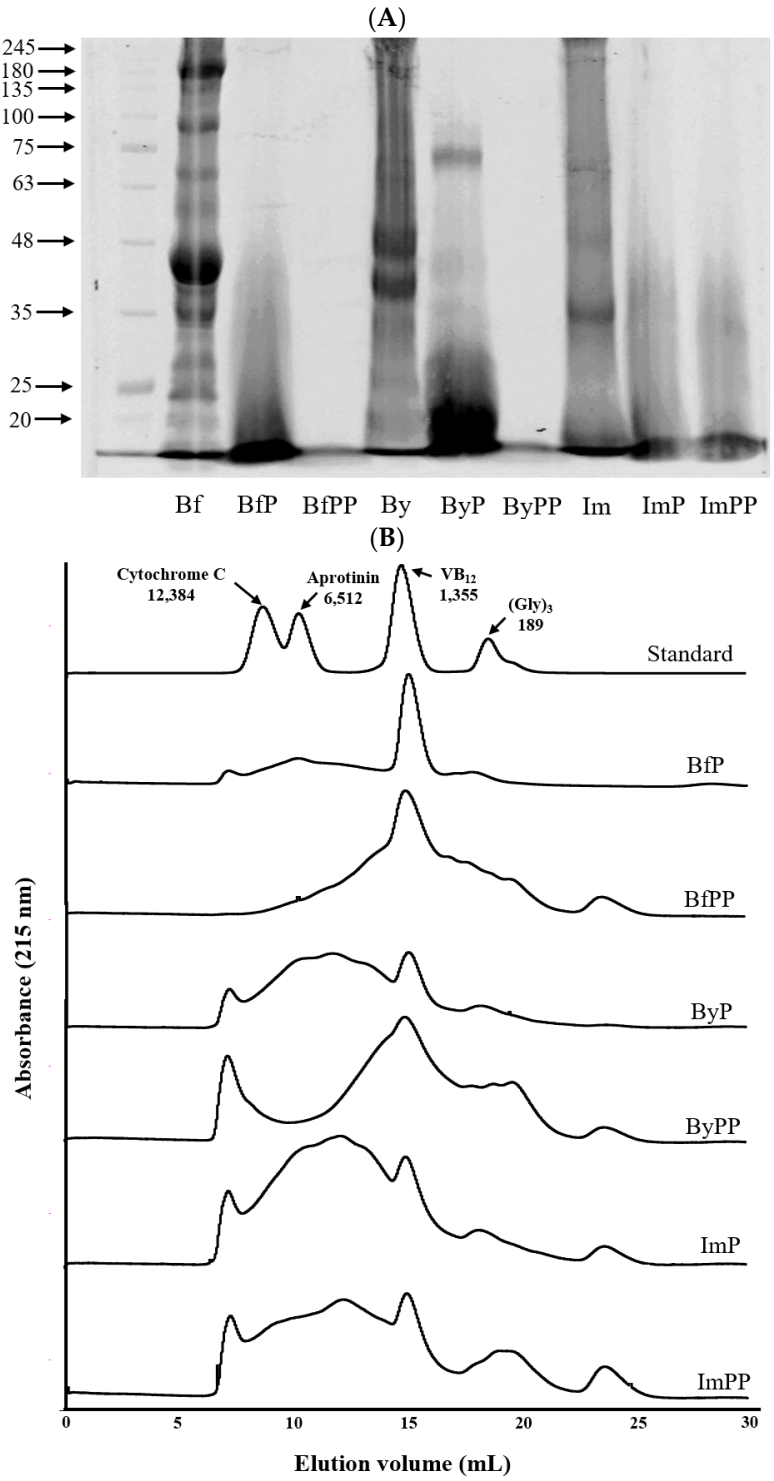

Figure 1. SDS-PAGE (**A**) and size exclusion chromatogram (**B**) of beef and PBMA hydrolysates. Bf = cooked beef patty; BfP = cooked beef patty after pepsin digestion; BfPP = cooked beef patty after pepsin and pancreatin digestion; By = cooked Beyond patty; ByP = cooked Beyond patty after pepsin digestion; ByPP = cooked Beyond patty after pepsin and pancreatin digestion; Im = cooked Impossible patty; ImP = cooked Impossible patty after pepsin digestion; ImPP = cooked Impossible patty after pepsin and pancreatin digestion.

Table 1. The degree of hydrolysis (DH) and amino acid compositions of beef and PBMA hydrolysates.

	BfP	BfPP	ByP	ByPP	ImP	ImPP
DH (%)	8.26 ± 0.67	10.66 ± 0.83	4.92 ± 0.11	7.94 ± 0.73	6.09 ± 0.59	7.48 ± 0.40
			Amino acids composition (g/100 g)			
Total amino acids	87.61 ± 1.51 ^{a}	87.64 ± 1.43 ^{a}	70.80 ± 1.26 ^{b}	75.11 ± 1.73 ^{c}	67.56 ± 1.20 ^{d}	69.35 ± 0.54 ^{d}
EAA	45.75 ± 0.84 ^{a}	46.04 ± 1.13 ^{a}	40.76 ± 0.53 ^{b}	42.91 ± 1.28 ^{c}	40.61 ± 0.71 ^{b}	41.60 ± 1.34 ^{b,c}

The data are represented as means ± SD; Values with different letters (a–d) within the same row indicate significant the differences.

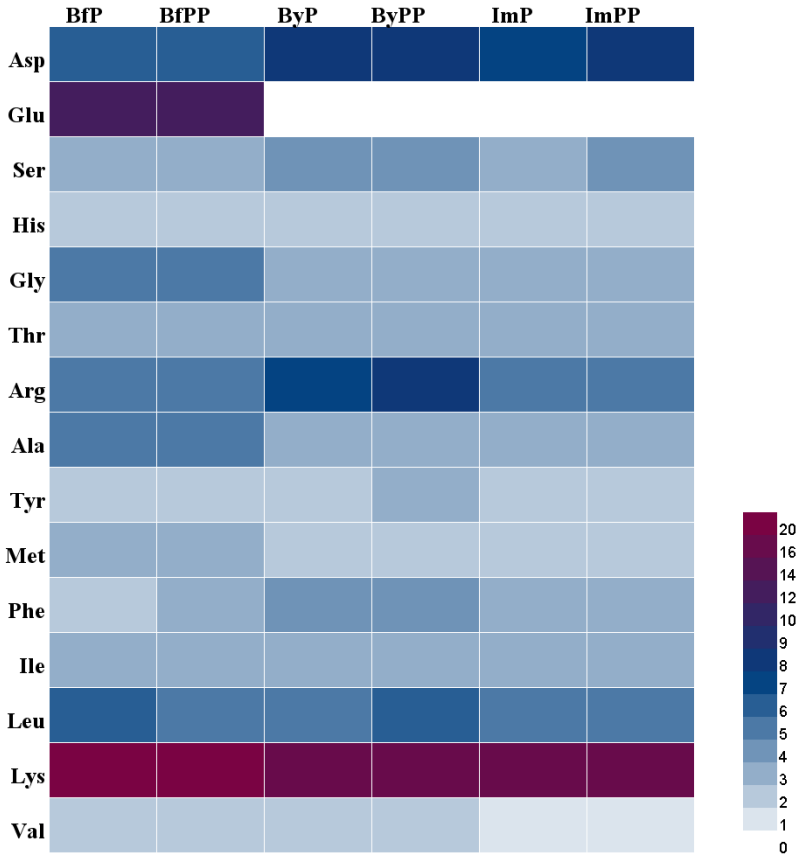

Figure 2. The amino acid compositions of beef and PBMA hydrolysates.

3.2. Effects of Gastrointestinal Digestion on Peptide Profiles of Beef and PBMA Hydrolysates

LC-MS/MS was used to identify the peptide profiles of beef and PBMA hydrolysates in this study, with the purpose of following the generation of peptides during in vitro gastrointestinal digestion and their relationship with bioactivities. To identify the potential bioactive peptides and predict their chemical properties, peptidomics and bioinformatics approaches were applied. Additionally, a peptide fragment may recur multiple times in its parent protein sequences, which can impact the theoretical content of peptides; therefore, this variation was also considered.

A total of 37, 2420 and 2021 peptides were identified in BfPP, ByPP and ImPP, respectively, indicating that gastrointestinal digestion had a significant impact on peptide release (Figure 3A). Among them, the abundant peptide fragments in ByPP were mainly derived from pea protein (81%), followed by rice protein (14%) and mung protein (5%). Almost all peptides identified in ImPP originated from soy protein. These results were consistent with the declaration of protein origins in their formulas. Even though beef hydrolysate had the highest DH, surprisingly, much fewer peptides were identified therein. This is probably because beef protein is more easily digested into free amino acids by gastrointestinal proteases or beef-derived peptides showing stronger hydrophilic properties, which were washed away from the reverse phase column prior to sequence identification. It is worth noting that the amino acid composition among

proteins largely dictates the extent of digestion, such as Phe, Tyr, Trp Lys, and Arg, which are the cleavage sites of gastrointestinal proteases [32]. Unfortunately, the peptide fragments released from in silico hydrolysis (pepsin and trypsin) in Supplementary Table S1 show a weak correlation with peptides identified by LC-MS/MS, suggesting the gaps between in silico hydrolysis and actual enzymatic hydrolysis. Particularly, in silico mimic hydrolysis is performed under ideal conditions where all proteins are fully digested, whereas the food matrix and processing conditions have a major impact on the digestibility of food proteins. Similarly, discrepancies between virtual and actual hydrolysis were also reported by others [33,34].

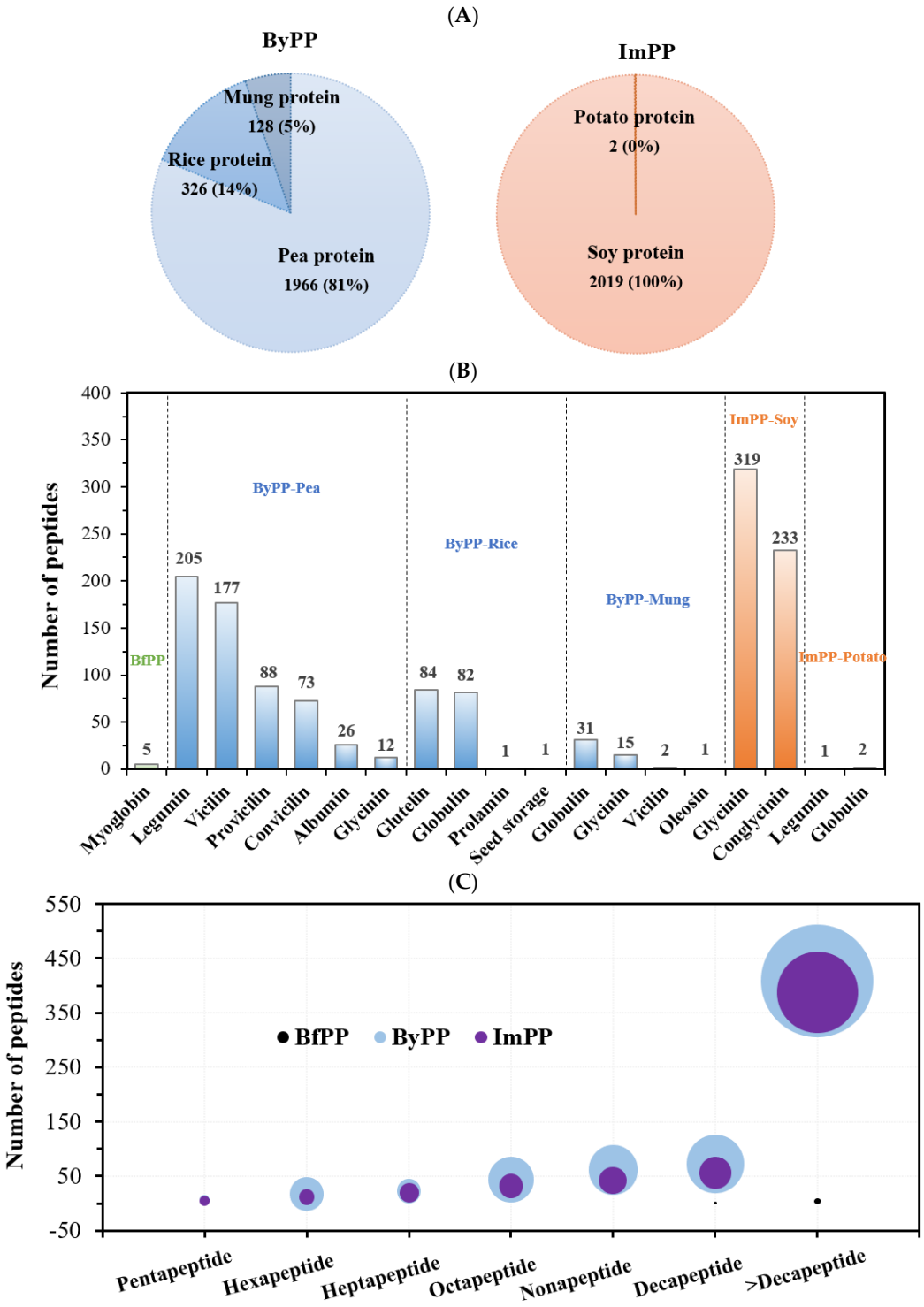

Figure 3. *Cont.*

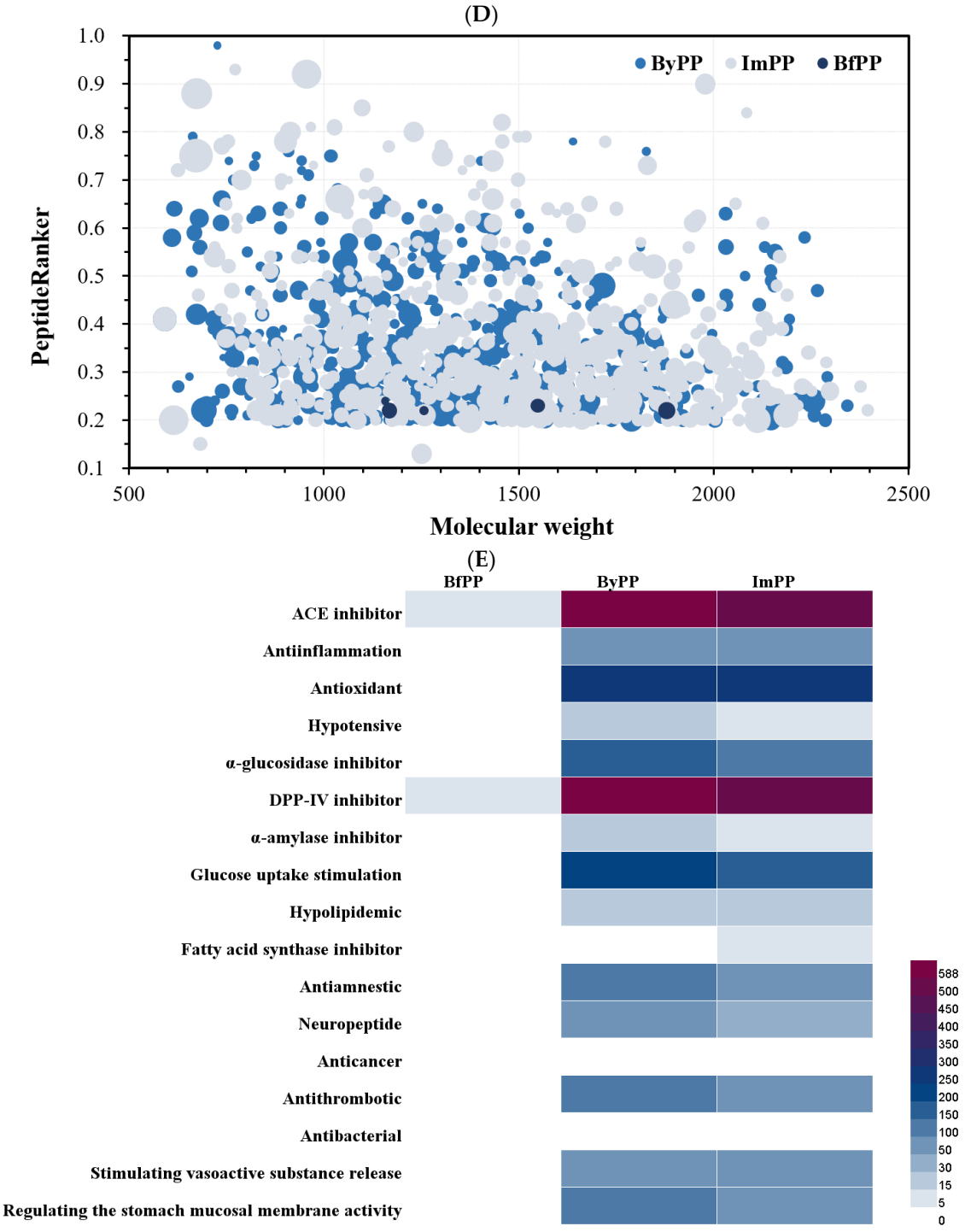

Figure 3. The peptide profile and virtual prediction of PBMA hydrolysates. (**A**) The distribution of peptides identified from BfPP, ByPP and ImPP. (**B**) Distribution of the potent peptides according to their origin proteins in BfPP, ByPP and ImPP. (**C**) Number of potent peptides fragment and their repetitions of each type released, and the bubble size represents the repeat numbers. (**D**) Molecular weight and PeptideRanker score of potent peptides, and the bubble size represents the CPPpred. (**E**) Heat map of the biological function of potent peptides from BfPP, ByPP and ImPP.

Generally, it is normally accepted that small peptides in protein hydrolysates possess better biological activities [16,24]. PeptideRanker is widely used to predict the potential bioactivity of peptides. A total of 5, 798 and 555 potent peptides were selected from BfPP, ByPP and ImPP based on the following filter conditions: peptide length < 20, molecular

weight <3 kDa and PeptideRanker scores >0.2. Parent proteins, peptide sequences, repeat numbers, PeptideRanker scores, CPPpred scores, potential bioactive peptides and biological function of these potent peptides are listed in Supplementary Tables S2–S7. Additionally, Figure 3B shows the distribution of selected peptides in each sample according to their protein origins. Globulin, including legumin and vicilin, is one of the major storage proteins in peas [10]. Almost half of the peptides that occurred in ByPP were from legumin and vicilin in peas. The remaining half was also derived from other storage proteins such as provicilin and convicilin in peas, glutelin and globulin in rice and globulin and glycinin in mung. On the other hand, peptides identified from ImPP were mostly derived from glycinin and conglycinin.

Additionally, the number of small peptides (peptide length < 10) released by gastrointestinal proteases were 222 and 166 in ByPP and ImPP, accounting for 35.24% and 29.96% of the total peptides identified, respectively. Peptides released in ByPP were repeated more frequently than those in ImPP. The potential bioactivities of peptides were predicted by calculating molecular weight, PeptideRanker scores and CPPpred scores. PeptideRanker is used to predict peptide bioactivities, and CPPpred predicts the ability of a peptide to go across the cell membrane [34]. As shown in Figure 3D, peptides in ImPP have higher PeptideRanker scores than those in ByPP. Additionally, most peptides in ByPP and ImPP had strong cell penetration capacity. These results indicated that gastrointestinal digestion could effectively release bioactive peptides from PBMA.

Recently, lifestyle-related chronic diseases have triggered a series of global public health concerns, leading to growing interest in researching food bioactives, including bioactive peptides, as alternatives for treatment. To further clarify and predict the potential biological functions of beef and PBMA hydrolysates, the screened peptides with active probability were compared to the reported active sequences in the BIOPEP database (Supplementary Tables S2–S7). Peptides shared the same sequence with the reported bioactive sequences in the BIOPEP database, implying that they exhibit the same biological functions. Bioactive peptides in PBMA hydrolysates were predicted to exert a wide range of regulatory roles, including amelioration of cardiovascular diseases (including hypertension, diabetes, obesity and hyperlipemia), antioxidation, anti-inflammation, anticancer and neuroprotection (Figure 3E). Taken together, our results suggest that PBMA is a good precursor of bioactive peptides with various biological functions.

3.3. ACE Inhibition, Antioxidant and Anti-Inflammation of Beef and PBMA Hydrolysates

After predicting the bioactivities of peptides identified from beef and PBMA digests, we further determined ACE inhibitory, antioxidant and anti-inflammatory activities. ACE is a target of blood pressure reduction [35], and amelioration of oxidative stress and inflammatory responses have been considered key preventive strategies against various chronic diseases [36–38].

Hypertension is widely known as a risk factor for cardiovascular diseases, and the renin–angiotensin system (RAS) plays a pivotal role in blood pressure regulation [39]. ACE activates the RAS and converts angiotensin (Ang) I into Ang II, which is a potent vasoconstrictor to trigger hypertension. Figure 4 shows in vitro ACE inhibition of beef and PBMA digests. ByPP showed the highest ACE inhibition, with an IC_{50} value of 0.16 ± 0.03 mg/mL, followed by that of ImPP and BfPP (IC_{50}: 0.20 ± 0.05 and 0.26 ± 0.05 mg/mL, respectively). Evidently, the results of ACE inhibition were consistent with the biological function prediction by in silico approach (Figure 3E). Similarly, a previous study also showed that PBMA-derived digests showed ACE inhibitory activity [13].

Oxidative stress triggers various kinds of damage to cells and further disrupts cellular function [40]. Sustained and aberrant oxidative stress contributes to vascular dysfunction, thereby causing hypertension, type 2 diabetes, atherosclerosis and other chronic diseases [41]. Vascular smooth muscle cells (A7r5) are a well-established model for evaluating health benefits, including relief of vascular dysfunction, anti-inflammation and antioxidation. In this study, antioxidant and anti-inflammatory activities in Ang II-induced

A7r5 cells were studied. All hydrolysates showed no cytotoxicity against A7r5 cells. Treatment of beef and PBMA hydrolysates significantly lowered superoxide levels in Ang II-stimulated A7r5 cells, especially for ByPP and ImPP (Figure 5). Fan et al. found that spent hen-derived peptides exhibited antioxidant effects by acting as direct radical scavengers or mediating endogenous antioxidant enzymes in Ang II-stimulated A7r5 cells [42]. Similarly, egg white-derived peptide IRW was also demonstrated to exhibit an antioxidant effect in A7r5 cells against Ang II stimulation [39]. In our study, the remarkable inhibition of superoxide generation ($p < 0.05$) in A7r5 cells indicated that PBMA was a good precursor of antioxidant peptides.

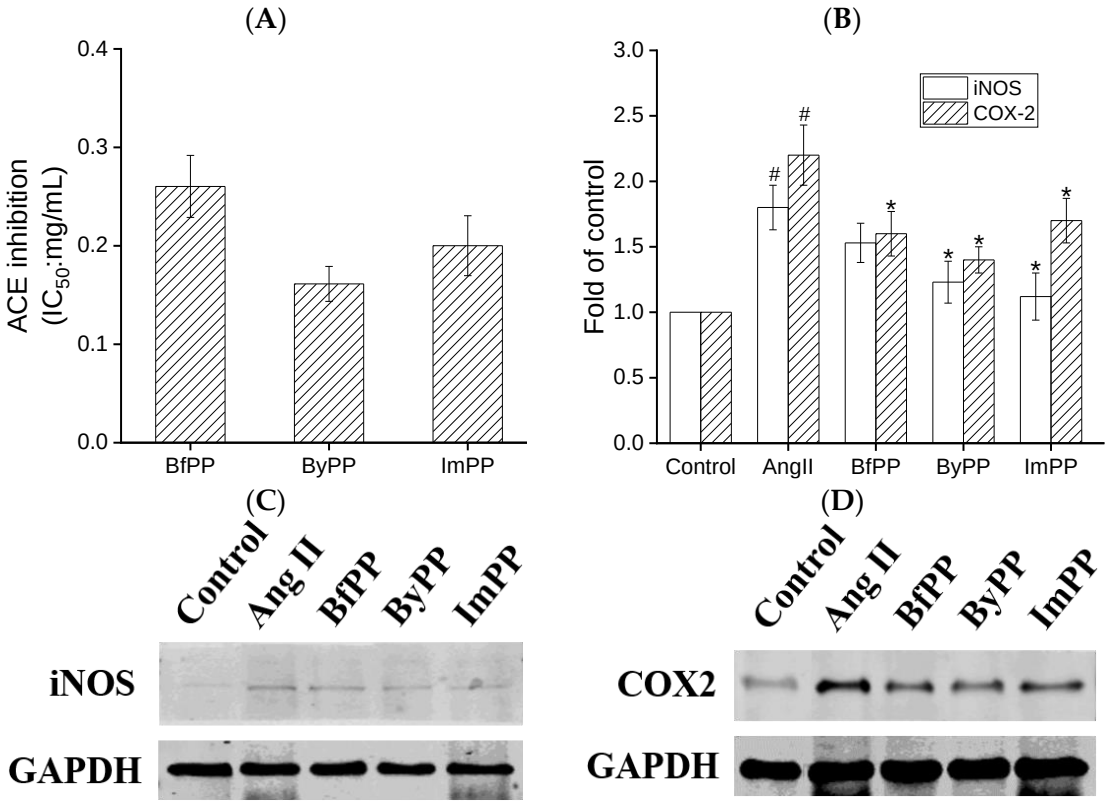

Figure 4. Effect of beef and PBMA hydrolysates prepared by gastrointestinal digestion on ACE inhibitory and anti-inflammatory activities. (**A**) In vitro ACE inhibition, and (**B**–**D**) the expressions of iNOS and COX-2 after co-treatment with 1 μM Ang II and hydrolysates for 24 h in A7r5 cells. The data are represented as means ± SD; # represents $p < 0.05$ vs. control group. * represents $p < 0.05$ vs. Ang II group.

Vascular inflammation is an underlying cause of hypertension and cardiovascular diseases. COX2 and iNOS are two proinflammatory mediators in vascular smooth muscle cells [38]; thus, the expression of these two proteins in A7r5 cells was detected to evaluate the anti-inflammatory activity of beef and PBMA hydrolysates. As shown in Figure 4, iNOS and COX2 expression levels surged in A7r5 cells upon Ang II insult ($p < 0.05$), whereas the hydrolysates treatment significantly inhibited their protein expressions. Similarly, peptides VVHPKESF and IRW could attenuate Ang II-induced inflammation in A7r5 cells [43,44]. These findings suggested the formation of anti-inflammatory peptides by gastrointestinal digestion from PBMA.

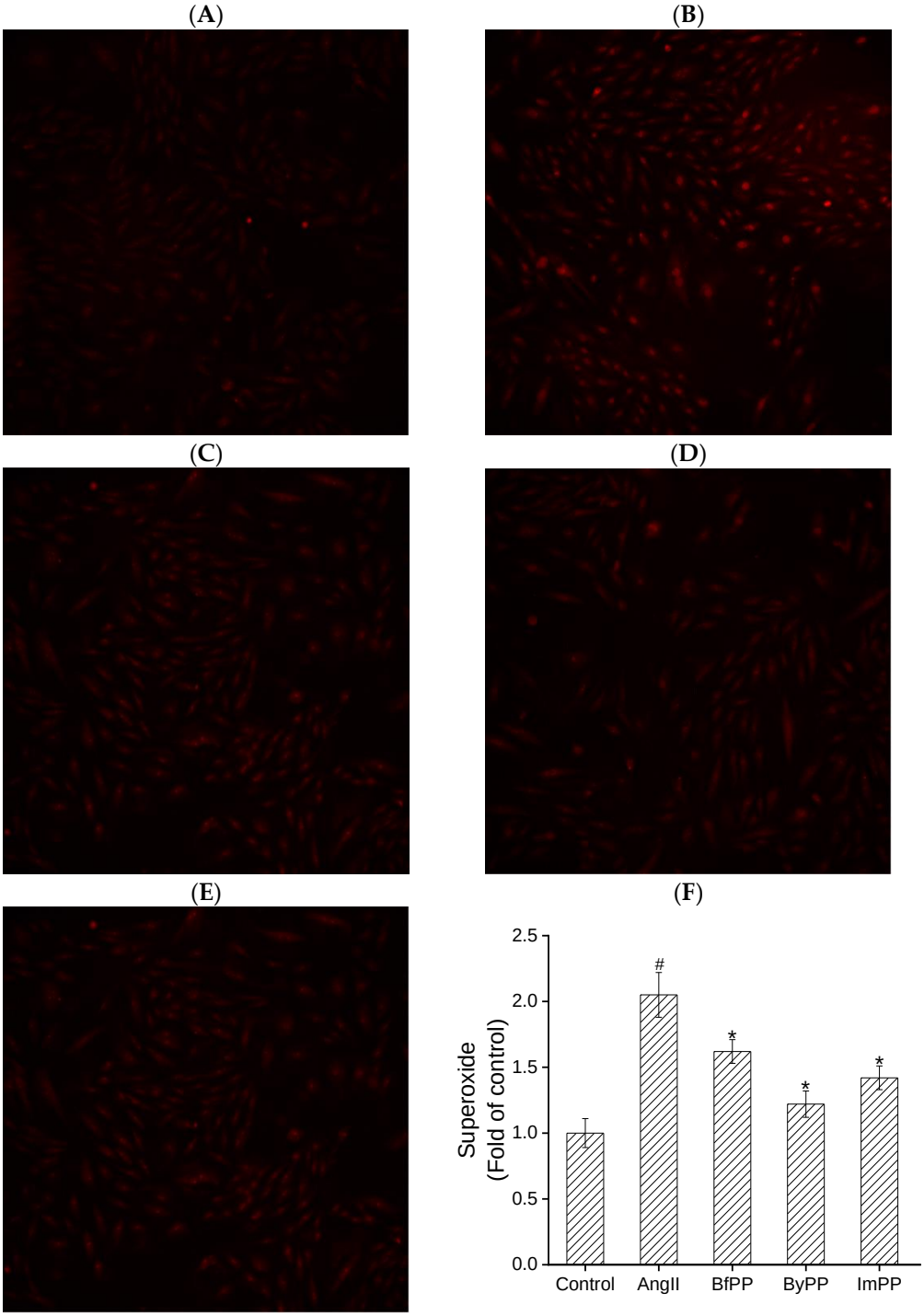

Figure 5. Effect of beef and PBMA hydrolysates prepared by gastrointestinal digestion on antioxidant capacity in A7r5 cells. (**A**) Control, (**B**) AngII, (**C**) BfPP, (**D**) ByPP and (**E**) ImPP, (**F**) oxidative stress in A7r5. The data are represented as means ± SD; # represents $p < 0.05$ vs. control group. * represents $p < 0.05$ vs. Ang II group.

4. Conclusions

This study mimicked the protein digestion of beef and PBMA through an in vitro gastrointestinal tract and further investigated the peptide profile and biological bioactivity by combining peptidomics, bioinformatics and wet lab experiments. Results obtained in SDS-PAGE, size exclusion chromatography and DH showed that gastrointestinal proteases were able to degrade beef and PBMA proteins. Notably, PBMA protein exhibited infe-

rior digestibility than that of beef, as reported previously. From the amino acids profile, PBMA hydrolysates were expected to possess comparable nutritional properties to beef hydrolysate. A total of 37, 2420 and 2021 peptides were identified in the gastrointestinal digests of beef, Beyond Meat and Impossible Meat, respectively. The astonishingly fewer peptides identified from beef digest is probably due to the near-full digestion of beef proteins. The analysis of peptide profiles indicated that PBMA could be considered a good precursor of bioactive peptides with widespread biological functions, including amelioration of cardiovascular diseases (including hypertension, diabetes, obesity and hyperlipemia), antioxidation, anti-inflammation, anticancer and neuroprotection. Furthermore, PBMA hydrolysates exhibited great ACE inhibition, antioxidant and anti-inflammation in test tube experiments and A7r5 cells. The current results underscored the promise of generating bioactive peptides from PBMA.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods12051061/s1. Table S1: Prediction of peptides released by gastrointestinal proteases through in silico hydrolysis of PBMA. Tables S2–S7: Potential bioactive peptides in BfPP, ByPP-pea protein, ByPP-rice protein, ByPP-mungbean protein, ImPP-soy protein and ImPP-potato protein, respectively.

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Physico-Chemical Properties and Texturization of Pea, Wheat and Soy Proteins Using Extrusion and Their Application in Plant-Based Meat

Delaney Webb, Hulya Dogan, Yonghui Li and Sajid Alavi *

Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506, USA
* Correspondence: salavi@ksu.edu

Abstract: Four commercial pea protein isolates were analyzed for their physico-chemical properties including water absorption capacity (WAC), least gelation concentration (LGC), rapid visco analyzer (RVA) pasting, differential scanning calorimetry (DSC)-based heat-induced denaturation and phase transition (PTA) flow temperature. The proteins were also extruded using pilot-scale twin-screw extrusion with relatively low process moisture to create texturized plant-based meat analog products. Wheat-gluten- and soy-protein-based formulations were similarly analyzed, with the intent to study difference between protein types (pea, wheat and soy). Proteins with a high WAC also had cold-swelling properties, high LGC, low PTA flow temperature and were most soluble in non-reducing SDS-PAGE. These proteins had the highest cross-linking potential, required the least specific mechanical energy during extrusion and led to a porous and less layered texturized internal structure. The formulation containing soy protein isolate and most pea proteins were in this category, although there were notable differences within the latter depending on the commercial source. On the other hand, soy-protein-concentrate- and wheat-gluten-based formulations had almost contrary functional properties and extrusion characteristics, with a dense, layered extrudate structure due to their heat-swelling and/or low cold-swelling characteristics. The textural properties (hardness, chewiness and springiness) of the hydrated ground product and patties also varied depending on protein functionality. With a plethora of plant protein options for texturization, understanding and relating the differences in raw material properties to the corresponding extruded product quality can help tailor formulations and accelerate the development and design of plant-based meat with the desired textural qualities.

Keywords: plant protein; functional properties; texturization; phase transition; meat analogues

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1. Introduction

The popularity of plant-based meat is soaring. A global interest in consuming protein sources which are perceived as ethical has been fostered in recent years, causing the rising interest in plant-based meat alternatives [1]. In the US, the increase in sales proves this; the plant-based meat market grew to USD 4.2 billion in 2020, a 24% increase from 2019 [2].

There are several types of plant-based meat including the traditional, gluten-based product seitan and newer extruded forms [3]. Among the latter, products that are extruded at a relatively low moisture level (30–40% wet basis) in order to generate the requisite mechanical energy in the process for protein cooking and cross-linking can be grouped together broadly as texturized vegetable protein. Although having fibrous layers like meat, these products are porous and are often further processed via milling, drying and/or rehydration before use [4,5]. Another category of extruded products, called high moisture meat analogs or HMMA, are processed at a much higher moisture level (example, 60–65% wet basis) in order to have a meat-like texture without the need for much further processing, and rely on a long slit die for cooling, layering and densification at the discharge end of the extruder [3,5]. While both single- and twin-screw technologies can be used to make

extruded plant-based meat, the HMMA products rely on the latter for the pumping capacity to move the product through the cooling die.

The plant-based market in 2019 relied heavily on soy, with soy-based products making up 48% of the plant-based meat market [6]. However, there is growing commercial investment in pea protein and large entities, including McDonald's and Beyond Meat besides many other companies, are entering this unique space. Thus, this alternative protein source is fast growing its market share [7]. Greenhouse gas emissions and land usage for production of pea protein are much lower (0.4 kg CO_2eq and 3.4 m^2, respectively, per 100 g) than what is required for beef (50 CO_2eq and 164 m^2 per 100 g), contributing to environmental ethics considerations and the rise of plant-based meats with pea ingredients [8,9]. Additionally, pea protein is attractive for companies to fulfill consumer desires for cleaner labels, as pea protein has low allergenicity and is non-GMO [9].

Although many companies use pea protein, different ingredient sources are associated with vastly different agronomic, isolation and commercial production conditions, leading to different raw material functionality [10]. Pea protein can be extracted in a wet environment or through dry fractionation and air classification. The more commonly used wet extraction process involves soaking yellow peas in water, crushing them, and separating the fiber and starch. The remaining protein is then placed in an alkaline solution for neutralization and extraction through the isoelectric point and then steam sterilized before being spray dried [11,12]. Isolating at a pH of 9 increases the aggregation of protein, decreases protein solubility and also the beaniness of the isolate compared to isolating at a pH of 8.5 [13]. A small adjustment in processing clearly leads to different protein functions.

For functional purposes, pea protein isolates may also be hydrolyzed with papain or bromelain enzymes or cross-linked with transglutaminase [11]. Some initial steps with the fermentation of pea protein for functional purposes have also been taken, though they have not been commercialized yet [14]. Prolonged heat treatment or exposure to high temperatures denatures the protein [15]. With varying heat and pH treatments, milling parameters, and hydrolysis, it is obvious that the function of pea proteins would vary greatly among suppliers. All of these differences are specific to the isolation process and are in addition to the differences that may exist prior to the isolation due to cultivar and environmental variances [16,17].

The goal of this study was to determine the raw material physico-chemical characteristics of multiple commercial pea protein isolates (water absorption, heat gelation, denaturation qualities, viscosity) and determine how those qualities may create unique opportunities for the extrusion-based textured product traits (water holding capacity, bulk density, layering, hardness, etc.) as well as to understand the relationships between protein properties and the internal structure of final product. The primary hypothesis was that protein physico-chemical properties, especially relating to hydration characteristics, can be an important determinant of end-product quality including cross-linking and layering. Soy and wheat gluten proteins, the conventional raw materials in plant-based meat, were also studied for comparison with pea proteins. The chemistry and functionality of these proteins have been reviewed previously [10]. Legumes such as soy and pea have a high concentration of globulin proteins, although the ratio of their legumin and vicilin fractions vary and that can dictate their functionality significantly. On the other hand, wheat gluten is mostly comprised of gliadin and glutenin proteins, leading to its unique properties. A relatively low moisture (30–40% wet basis) extrusion process, as described above, was used to texturize these proteins, with the products intended for longer storage before hydration for plant-based meat applications.

2. Materials and Methods

2.1. Materials

The purpose was to compare different commercial pea proteins, as well as compare different types of proteins. The plant proteins were selected based on market presence. Pea protein isolate was sourced from four separate companies (PP1–PP4). Soy protein isolate

and soy protein concentrate (SPC) were also obtained from a commercial source. Vital wheat gluten (VWG) was obtained from MGP Ingredients (Atchison, KS, USA) and hard red winter wheat flour was obtained from Hal Ross Flour Mill (Kansas State University, Manhattan, KS, USA).

A total of 8 treatments were tested in this study: 4 pea treatments, 2 wheat treatments, and 2 soy treatments. Treatment formulations are described in Table 1 and were created on the basis of protein content and prior knowledge of each protein to create a viable product. The composition of the main treatments of interest is shown in Table 2. Pea protein isolates had a protein content ranging from 80–83% db. VWG had a higher level of protein (86.7%), while the protein content of SPC was lower (70%). Wheat Mix and Soy Mix treatments were included to match the level of protein in the pea protein isolates to facilitate better comparison. For the Wheat Mix treatment, vital wheat gluten was diluted to roughly 80% protein with wheat flour, which had 12.5% protein, while in the Soy Mix treatment, soy protein isolate with 90% protein was added to SPC to increase the protein content to roughly 78%. The range of protein content of the final formulations (77.8–86.7%) was designed to be similar to that of animal meats such as chicken, fish and beef on a dry basis (77.7–86.7%) [18].

Table 1. Formulas (%) used in the extrusion treatments for plant-based meat.

Treatment	PP1	PP2	PP3	PP4	VWG	Wheat Mix	Soy Mix	SPC
Pea isolate 1	100							
Pea isolate 2		100						
Pea isolate 3			100					
Pea isolate 4				100				
Vital Wheat Gluten					100	90		
Wheat Flour						10		
Soy Protein Isolate							50	
Soy Protein Concentrate							50	100

Table 2. Composition of extrusion treatments as determined by proximate analysis and supplier specifications (%).

Component	PP1	PP2	PP3	PP4	VWG	Soy Mix
Protein	80.3	80.3	82.9	79.2	86.7	77.8
Carbohydrate	4.0	9.3	7.6	3.2	7.3	9.8
Fiber	4.0	0.2	1.0	2.7	0.2	1.8
Fat	6.0	0.5	0.4	6.0	0.9	0.6
Ash	1.6	4.1	5.3	4.1	0.4	4.6
Moisture	4.1	5.9	3.7	4.8	4.8	5.4
Total	100.0	100.0	100.0	100.0	100.0	100.0

2.2. Extrusion Parameters and Calculations

A ribbon blender (Wenger Manufacturing, Sabetha, KS, USA) was used to mix the soy and wheat treatments for 5 min. A pilot-scale (52 mm diameter, L/D ratio of 19.5), co-rotating twin-screw extruder (Model TX-52, Wenger Manufacturing, Sabetha, KS, USA) was used for texturization. Operating parameters for each treatment can be found in Table 3. The dry material feed rate was constant for all pea and wheat protein treatments at 50 kg/h. The dry feed rate was decreased to 40–45 kg/h for the soy treatments. The extruder screw speed was 450 rpm for pea and wheat treatments and 200–320 rpm for soy treatments. Water was added at a rate of 8 kg/h in the preconditioner for all treatments. Pea protein treatments received water in the extruder barrel at 8 kg/h, but wheat and soy treatments required 12–14 kg/h. A lower feed rate and/or screw speed, and also higher water input was required for wheat and soy protein treatments as described above because they tend to need less energy for texturization. High extrusion mechanical energy often leads to a less

than optimal product for these two protein types, and in the case of soy, burning. Steam injection was not used in any of the treatments. Four temperature zones were used at 40, 70, 90 and 110 °C from the inlet of the extruder barrel to the outlet.

Table 3. Extrusion parameters for each treatment. All parameters remained consistent for pea protein (PP) treatments, while optimization was required for Wheat Mix, vital wheat gluten (VWG), Soy Mix, and soy protein concentrate (SPC). The remaining variables were kept constant.

Extrusion Parameter	PP1	PP2	PP3	PP4	Wheat Mix	VWG	Soy Mix	SPC
Feed Rate (kg/h)	50	50	50	50	50	50	45	45
In-barrel moisture (%)	29.9	29.3	28.9	28.7	35.2	34.3	34.7	38.7
Screw Speed (rpm)	450	450	450	450	450	450	320	206
Venturi die size (in)	1/8	1/8	1/8	1/8	1/8	1/8	1/4	1/4

The screw profile was composed of double flighted elements of decreasing pitch, with two forward kneading blocks and four reverse kneading blocks dispersed throughout the profile, and a conical cut element at the end (Table 4). A 1/8″ (3.172 mm) venturi die (or back die) was used for all treatments, except soy treatments that used a 1/4″ (6.35 mm) venturi or back die to prevent burning. After the venturi die, a 11″ (27.94 cm) long Teflon spacer was placed, and then the final die plate which had two 1/4″ (6.35 mm) final circular die openings. Three hard knives were used with a knife speed of 250 rpm to cut the product. A sample was taken from the extruder, immediately milled to 0.18″ (4.6 mm) pieces (Comitrol, Urschel Laboratories Incorporated, Valparaiso, IN, USA) and frozen. Whole extrudate samples were dried at 200 °C for 12 min and cooled for 8 min in a dual-pass drier (Series 4800, Wenger Manufacturing, Sabetha, KS, USA). Dried extrudate samples were stored at room temperature.

Table 4. Extruder screw configuration. The two screws differed only in the first two feeding elements.

Left	**1**	**1**	**1**	**1**	**3**	**3B**	**1**	**1**	**4**	**4**	**5**	**6**	**5**	**6**	**5**	**5B**	**7**
Right	**2**	**2**	**1**	**1**	**3**	**3B**	**1**	**1**	**4**	**4**	**5**	**6**	**5**	**6**	**5**	**5B**	**7**
1	Full pitch, double flight																
2	Full pitch, single flight																
3	Forward kneading block																
3B	Forward kneading block, backward																
4	$\frac{3}{4}$ pitch, double flight																
5	Reverse kneading block																
5B	Reverse kneading block, backwards																
6	$\frac{1}{2}$ pitch, double flight, cut flight																
7	$\frac{3}{4}$ pitch, double flight, cut flight, cone																

Specific mechanical energy (SME) was calculated using the following formula:

$$\mathrm{SME}\left(\frac{\mathrm{kJ}}{\mathrm{kg}}\right) = \frac{\left(\frac{\tau-\tau_0}{100}\right) \times \frac{\mathrm{N}}{\mathrm{N_r}} \times \mathrm{P_r}}{\mathrm{m_f}} \quad (1)$$

where τ is the % torque, τ_0 is the no-load torque %, N is the measured screw speed in rpm, N_r is the rated screw speed (336 rpm), P_r is the rated motor power (22.4 kW) and m_f is mass flow rate in kg/s.

In-barrel moisture (IBM) content was calculated using the following equation:

$$\mathrm{IBM}(\%\mathrm{wb}) = \frac{(\mathrm{m_f} \times \mathrm{X_{wf}}) + \mathrm{m_{wp}} + \mathrm{m_{we}}}{\mathrm{m_f} + \mathrm{m_{wp}} + \mathrm{m_{we}}} \quad (2)$$

where m_f is the dry feed rate, X_{wf} is the moisture content of the dry feed material (expressed as wet basis fraction), m_{wp} is the water injection rate into the pre-conditioner in kg/h and m_{we} is the water injection rate into the extruder in kg/h. An IBM of roughly 29% was used for pea protein treatments, while 35–38% IBM was used for wheat and soy treatments.

2.3. Moisture Content

Moisture content was measured for raw ingredients, preconditioned treatments and extrudates (before drying), using the AACC 44–19.01 method. Triplicate samples of approximately 2 g were dried at 135 °C for 2 h for this procedure.

2.4. Raw Material Analysis

2.4.1. Particle Size Distribution

The particle size distribution of each treatment was determined in duplicate using an Air Jet Sieve e200LS (Hosokawa Alpine Group, Augsburg, Germany). A 100 g sample was placed on the smallest sieve with a negative pressure of 3400 Pa applied to the underside of the sieve to remove and transport particles finer than the screen into a collecting jar. The weight of the overs or remains on the screen were transferred to the next largest sieve and the process was repeated with progressively higher screen sizes until all material passed through. Sieves with 32, 53, 75, 106, 125, 150, 180, 212, 250 and 300 microns were used.

2.4.2. Water Absorption Capacity and Oil Absorption Capacity

Water absorption capacity (WAC) was measured as in a previous study described, but with modification [19]. Samples of 2.5 g were placed in centrifuge tubes with 30 mL of deionized water. To disperse the sample, the slurries were vortexed for 30 s. Samples were then allowed to sit at room temperature for 30 min with 2 additional agitations in that time. Samples were centrifuged at 3000× g for 30 min and the water was carefully decanted. WAC was calculated using the following equation:

$$\mathrm{WAC(g\ water/g\ protein)} = \frac{W_f - W_i}{W_i} \quad (3)$$

where W_f is the weight of the sediment and W_i is the initial weight of the sample.

Oil absorption capacity (OAC) was measured similarly, using the methods described with some modification [20]. Samples of 2.5 g were placed in centrifuge tubes with 30 mL of sunflower oil. Samples were shaken until the sample was dispersed and allowed to sit at room temperature for 30 min. Samples were centrifuged at 3000× g for 30 min and the oil was carefully decanted. The tubes were then inverted, allowing excess oil to drain for 20 min. OAC was then calculated using the following equation:

$$\mathrm{OAC(g\ oil/g\ protein)} = \frac{W_f - W_i}{Wi} \quad (4)$$

where W_f is the weight of the sediment and W_i is the initial weight of the sample. WAC and OAC were measured in triplicate for each sample.

2.4.3. Least Gelation Concentration

The least gelation concentration (LGC) of each treatment was obtained by dispersing different concentrations of pea and soy proteins (12–20% w/v) in 10 mL of DI water in 1 cm diameter test tubes. The solutions were then heated, uncovered, at 95–100 °C for 1 h, immediately cooled via a cold-water bath, and then kept at 4 °C for 2 h. Wheat proteins were not tested since they are hydrophobic in nature and clump upon the addition of water. The LGC was determined, after chilling, as the concentration that forms a stable gel that does not drop or run when the test tube is inverted.

2.4.4. Rapid Visco Analyzer Viscosity

A rapid visco analyzer (RVA) (RVA 4500, Perten Instruments, Waltham, MA, USA) was used to measure the pasting properties of each treatment using the AACC Method 76–21.02, as employed previously for flours [21]. Protein slurries at 15% solid concentration (w/v) were manually mixed so that protein clumps were better dispersed and there was reduced noise in the results plot. Slurries were placed in the RVA within 1 min of the initial mixing. Slurries were heated to 50 °C and held for 1 min, with initial stirring at 960 rpm for 10 s. For the remainder of the test, slurries were stirred at 160 rpm. Slurries were then heated to 95 °C at 12 °C/min, held for 2.5 min and then cooled again to 50 °C. Peak viscosity, time and temperature of peak viscosity, and end viscosity were measured and recorded. All RVA tests were conducted in triplicate.

2.4.5. Differential Scanning Calorimetry

Protein denaturation, as shown by enthalpy, was measured via differential scanning calorimetry (DSC) with a Q100 V9.9 Build 303 (TA Instruments, New Castle, DE, USA) and analyzed with the Universal Analysis Program, V4.5A (TA Instruments). Comparing the DSC results of raw commercial protein isolates can be helpful to understand the impact of isolation processing on denaturation. DSC was conducted according to Brishti et al. (2017) with a few modifications [20]. Raw samples of 8–10 mg dry matter were weighed into stainless steel, high volume, hermetically sealed pans. Samples were equilibrated to 20 °C and were heated to 250 °C at a rate of 10 °C/min. An empty pan served as a reference. The nitrogen purge flow was 50.0 mL/min. The start and peak denaturation temperature and enthalpy of denaturation were recorded. Tests were conducted in triplicate.

2.4.6. Molecular Weight

The molecular weight of each legume protein (raw and extruded) was qualitatively understood through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in non-reducing conditions. Extruded proteins were ground and sieved to less than 500 μm. Protein was extracted for an hour with deionized water (15 μg:1 mL) and then centrifuged for 5 min at 8000× g. The supernatant was then mixed with Laemmli buffer (2 supernatant: 1 buffer) and heated for 10 min in a boiling water bath. The 4x Laemmli sample buffer contained 277.8 mM Tris-HCl (pH 6.8), 44.4% (v/v) glycerol, 4.4% LDS and 0.02% bromophenol blue (Bio-Rad Laboratories, Inc, Hercules, CA, USA).

The prepared sample (12 μL) was pipetted into the gel lanes. Precision Plus Protein Standard (Bio Rad Laboratories, Hercules, CA, USA) was added at 5 μL and contained protein markers from 10–250 kDa. Electrophoresis was then conducted at 200 V, 25 mA, and 250 W to separate the proteins by molecular weight with 12% separating gel and 4% stacking gel. After electrophoresis, samples were fixed and stained using Brilliant Blue R concentrate. Samples were then destained overnight with 10% acetic acid, and further destained with deionized water.

2.4.7. Phase Transition Analysis

Phase transition analysis (PTA) was used to measure the raw material softening and flow point temperatures. The test was conducted on a Phase Transition Analyzer (Wenger Manufacturing) with samples hydrated to 24% [22]. Raw treatments (2 g) were compressed in the chamber with a blank die to 120 bars for 15 s. A pressure of 100 bars was applied as the sample was heated at a rate of 8 °C/min, with a starting temperature between 5–7 °C. After the softening point of the material was measured, a 2 mm capillary die was placed under the sample and compressed again to 120 bars for 15 s, with 100 bars of pressure thereafter. Wheat gluten treatments required using lower pressure; 75 bars of consistent pressure were used throughout the test. When material began to flow through the capillary die, the compressing rod displacement changed, showing the flow point, and the temperature was marked as the flow temperature.

Extruded material was also tested to determine changes in flow point temperature compared to the raw material. For this analysis, raw materials were extruded on a lab-scale, Micro-18 extruder (Micro-18, American Leistritz, Somerville, NJ, USA) in order to impart moderate shear energy for the initiation of protein crosslinking but not to the extent of macromolecular degradation typically observed in high-energy pilot-scale extrusion. A comparison of PTA flow temperature before and after moderate shear transformations can help understand the potential for protein networking prior to pilot scale extrusion. Raw materials were hydrated to 24% MC before extrusion and run at a 3.3 kg/h throughput and a screw speed at 550 RPM. The material was extruded through the barrel sections with temperatures of 30, 40, 55, 95, 120 and 140 °C. An oval die with width of 5.5 mm and length of 3.0 mm was used to make ropes of extrudate. The extrudate was not dried, was ground finer than 250 microns with a Wiley mill, hydrated to 24% moisture, and run on the PTA using the parameters described above. Both raw and extruded material PTA tests were conducted in triplicate.

2.5. Extrudate Analysis

2.5.1. Product Structure

The bulk density of extrudates was measured by measuring the mass of dried product filling a one-liter volume cup. The product's internal structure was captured via pictures of longitudinal (along the direction of extrusion) and of transverse or horizontal (perpendicular to the direction of extrusion) sections. Measurements were completed in duplicate.

2.5.2. Water Holding Capacity

Water holding capacity (WHC) is the measurement of water that is held within the structure of the final product, measured according to Kearns, Rokey and Huber (1989), with modifications [23]. Milled samples (15 g) were soaked in excess, room temperature water for 20 min, and then drained on a mesh screen for 5 min. Tests were conducted in triplicate. WHC was calculated using the following equation:

$$\text{WHC}(\%) = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100 \quad (5)$$

2.5.3. Textural Analysis

Hardness, springiness and chewiness characteristics were measured using a TA-XT2 Texture Analyzer (Texture Technologies Corp., Scarsdale, NY, USA), programmed for a two-cycle texture profile analysis (TPA) compression test [22]. Analysis was performed on ground and rehydrated product to understand the textural qualities of the uniform extrudate without any binders, and also on patties formed using binders. For the former, treatments were rehydrated to 60% moisture. A back-extrusion cup was utilized to contain 20 g of the sample that filled, approximately, up to 1 cm in height in the cup, depending on the product density. The two-cycle compression test involved compressing up to 70% of the total distance with a circular aluminum probe. Textural properties were measured for patties formed in accordance with guidelines from the American Meat Science Association [24]. Patties were made with 91.5 g of each treatment and pressed to 1 cm thickness. The formula for plant-based patties is shown in Table 5. Patties were pan-broiled with no oil until an internal temperature of 71 °C was reached. Patties were allowed to cool to room temperature and a 2.5 cm core was taken from the center of 10 patties. The two-cycle TPA compression test was conducted on the cores at room temperature, compressing them to 70% of the total distance with a circular aluminum probe. Patties based on ground chicken, beef and pork and a commercial plant-based meat product (Beyond Beef®, El Segundo, CA, USA) were also tested using TPA as benchmarks and for comparison with the textural attributes of products obtained in this study. All tests were replicated 10 times.

Table 5. Patty formulation for textural analysis. TVP refers to the extruded treatments as per Table 1.

Ingredient	Percentage
Textured Vegetable Protein (TVP)	59.25
Water	29.6
Pea Protein Isolate	1.5
Chickpea Flour	1.5
Sunflower oil	3
Methylcellulose	2.75
Salt	1
Beet Powder	0.75
Spices	0.65

2.6. Experimental Design and Statistical Analysis

A single factor experiment design structure was used with 8 treatments (or raw materials formulations), as described in Table 1 and Section 2.1. The independent variable was raw material formulation, while the dependent variables included raw material properties, processing characteristics such as SME and end-product attributes. The number of replicates is mentioned in the Methods sub-section corresponding to each test. All measurements were based on technical replicates, meaning that the same analytical procedure was applied to different samples, which, however, were not produced in replicate extrusion experiments. The extrusion treatments were not repeated, but for each treatment, different samples for any particular analysis were randomly selected from a group of extrudates weighing 10–16 kg and produced over a 15–20 min period. One-way ANOVA was performed to compare means and differences with SAS software (SAS, Cary, NC, USA). ANOVA was followed by Tukey's test to determine the significance of differences and control for Type 1 errors ($p < 0.05$).

3. Results and Discussion

In this section, a careful scientific analysis is presented on the linkages between the chemistry and the physico-chemical properties of plant proteins, and in turn the impact on extrusion characteristics and degree of cross-linking due to processing. Finally, all of these data have been tied together with end-product quality such as porosity, layering, water holding capacity and texture.

3.1. Raw Material Characteristics

3.1.1. Particle Size Distribution

Particle size can vary based on industrial processing due to varying temperatures, vaporization, and air–water interface which can cause the increased denaturation and aggregation of hydrophobic regions [14]. PP1 and PP3 had a significant portion of their particles under 75 microns, as did the soy treatments (Figure 1). PP2 and PP4, however, had a wider particle size distribution with substantial portions ranging from 75 to 180 microns. VWG had 47% under 75 microns, but the remaining portion (about 55%) was spread all the way to 250 microns. Thus, PP1 and PP3 had the most uniform particle size for pea treatments, while SPC and the soy mix also had a relatively uniform particle size.

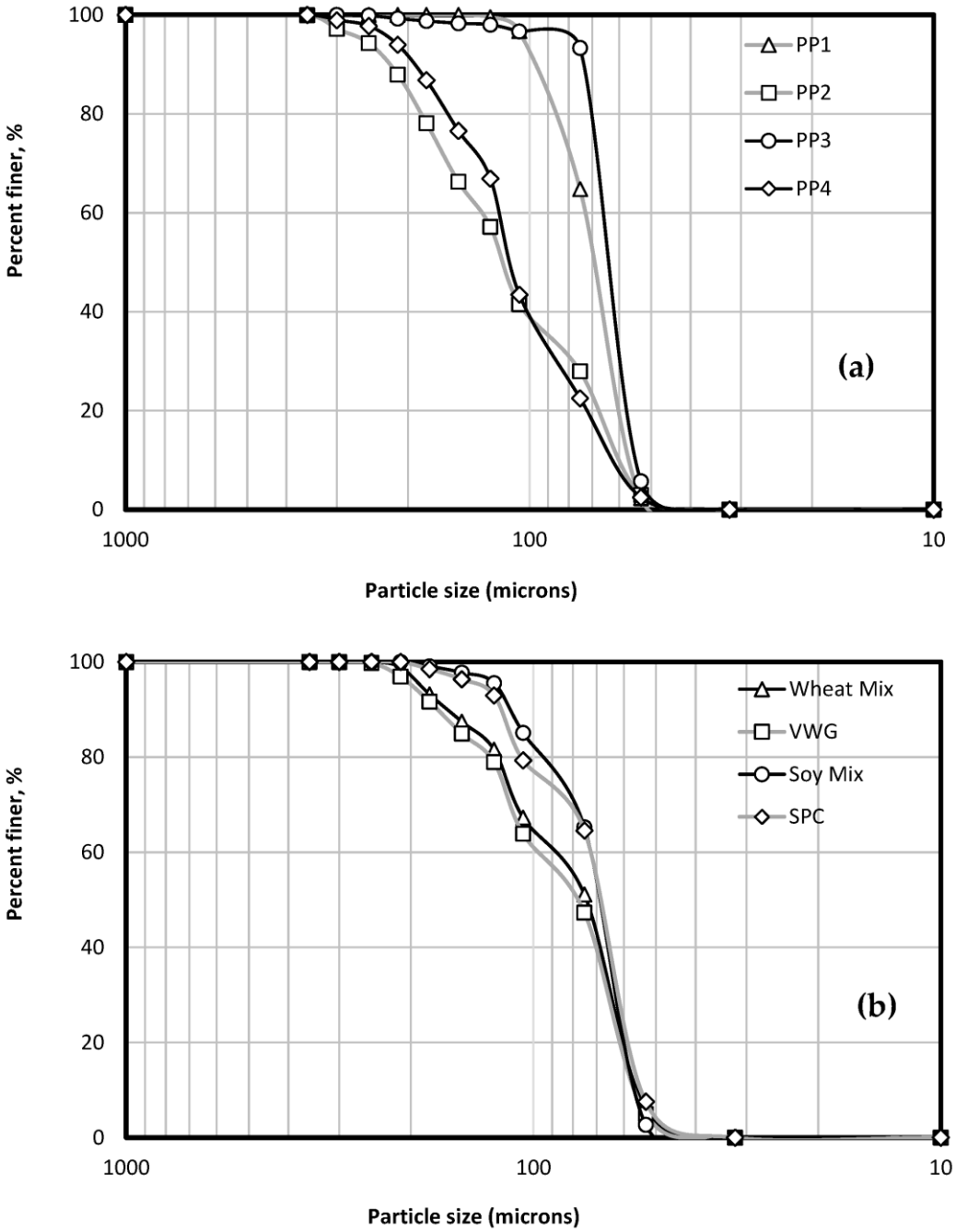

Figure 1. Particle size distribution of (**a**) pea protein and (**b**) wheat and soy treatments as the cumulative percentage of particles that passed through each sieve.

3.1.2. Water Absorption Capacity and Oil Absorption Capacity

The WAC and OAC of each treatment can be seen in Figures 2 and 3. The WAC and OAC of each of the pea treatments is within the same range as previously reported [25]. The differences between the pea proteins in their ability to absorb water at room temperature may be partially due to the hydrophilic versus hydrophobic nature of the proteins, which in turn depends on the protein sub-units, and also their structure, and any transformations occurring during the commercial production process. VWG and Wheat Mix displayed a relatively low WAC (1.4) due to the hydrophobic nature of wheat gluten. Unlike leguminous proteins such as those found in soy and yellow peas, which have albumins or globulins as the major protein fraction, wheat gluten contains prolamins and glutelins that are

soluble in alcohol and acid, respectively, rather than water or salt solutions [26]. The way gluten interacts with water is therefore quite different, and results in the low WAC observed. Soy Mix had the highest WAC of 4.2 as it comprised 50% soy protein isolate, which is typically highly water soluble. SPC had a moderate WAC (2.8), while the four pea protein isolates had a moderate to high WAC (2.7–3.8). Among the pea proteins, PP2 and PP4 had the highest WAC (3.8 and 3.6, respectively), which indicated a relatively high water solubility like soy protein isolate. On the other hand, PP1 and PP3 had a moderate WAC (2.7 and 2.8, respectively), pointing to a similar hydrophilicity and functionality to soy protein concentrate. This contrast between the four pea proteins is most probably due to differences in the isolation process during production, as they were obtained from four different commercial sources. The role of protein hydrophilicity versus hydrophobicity in the texturization process will be discussed in a later section.

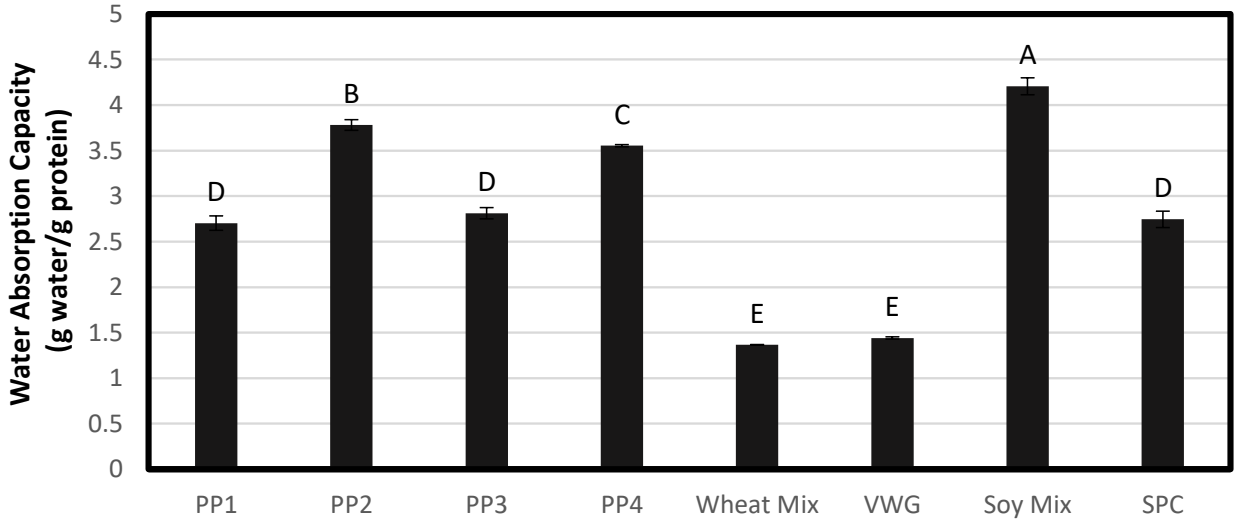

Figure 2. Raw material average water absorption capacity for all treatments. Bars denoted by the same letter are not significantly different ($p < 0.05$).

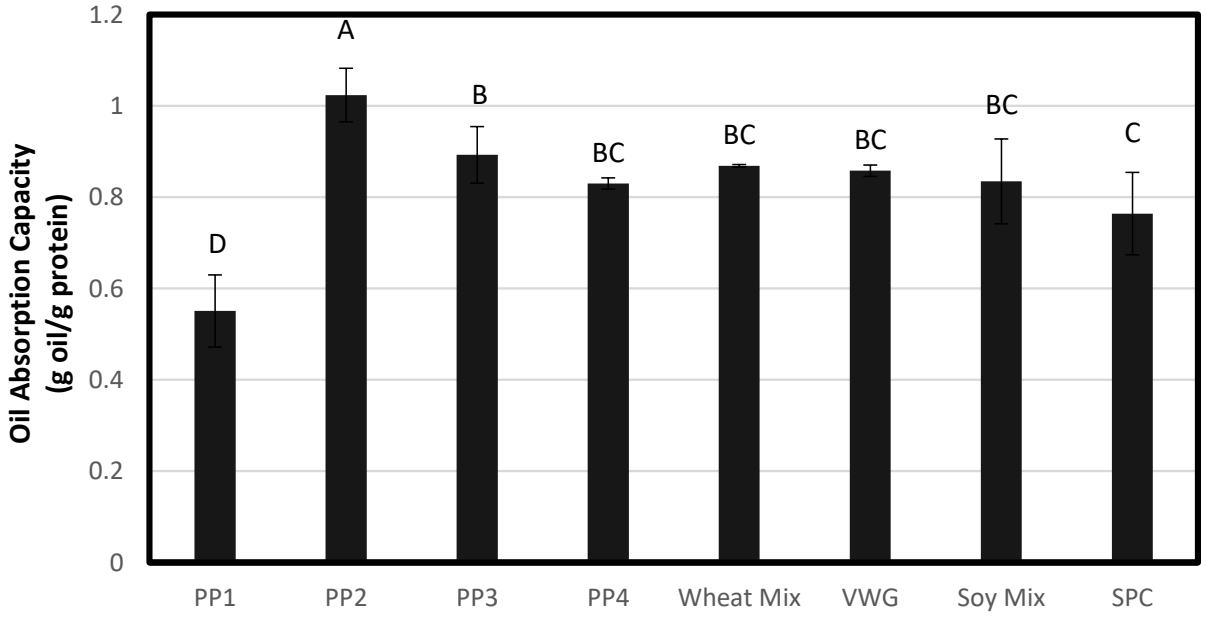

Figure 3. Raw material average oil absorption capacity for all treatments. Bars denoted by the same letter are not significantly different ($p < 0.05$).

PP2 had the highest OAC. Most proteins exhibited a similar OAC, but PP1 had a substantially lower OAC. Though gluten is a hydrophobic protein, it did not exhibit a higher OAC. Overall, the OAC of these proteins may be due to their physical attributes such as particle size, space between particles and agglomeration, rather than the affinity of the ingredients. Because PP2 had a greater particle size, it would be more agglomerated, with greater interstitial spaces and not as compact, and thus would be able to hold more liquid between the particles, while the small-particle-size PP1 would pack well and not hold much oil. PP2 and PP4 had a similar particle size distribution, but the higher water affinity of the former, as indicated by greater moisture content and WAC, caused more agglomeration of the particles, allowing a greater retention of oil.

3.1.3. Least Gelation Concentration

LGC is a test used to determine the heat-gelling properties of proteins [27,28]. Heat gelation is the ability of a protein to form a three-dimensional network through its denaturation and aggregation [29]. The structure is held by protein–protein interactions, bonds, and electrostatic forces. In a gel, the protein also interfaces with a solvent (water, in the case of the LGC test) held within the network [28]. LGC tests specifically for thermogelation, the same mechanism which occurs during extrusion, by determining the concentration at which a protein can form a gel in water after heating. Thus, unlike WAC, which is a cold-water solubility test, LGC is hot-water solubilization process. Measuring gelation properties is helpful as it can help to further characterize various proteins, and indirectly point to differences in their chemistry, structure and functionality. Moreover, the gelation of proteins under heat, pressure and shear is what helps create and solidify the fibrous and layered structure of meat analogs during extrusion [30].

Most PPIs had a relatively moderate heat-gelling ability, requiring at least 16% solids to gel (Table 6). The exception was PP1, which had a high heat-gelling ability or low LGC (14%). SPC also had a low LGC of 14%. Interestingly, Soy Mix had the least heat-gelling ability and required the highest solids concentration (18%) to create a firm gel, even though its protein content was similar to the pea proteins and higher than SPC. Thus, LGC was not a function of the content of protein, but more related to its chemistry and structure. For example, in a previous study, stronger gels came from pea proteins that were less fractionated [12].

Table 6. Least gelation concentrations for raw mixes based on pea and soy protein. The symbol '+' means gel was observed the given concentration and the symbol '−' means gel was not observed. Gluten not tested due to its hydrophobicity.

Treatment	12%	14%	16%	18%	20%
PP1	−	+	+	+	+
PP2	−	−	+	+	+
PP3	−	−	+	+	+
PP4	−	−	+	+	+
Soymix	−	−	−	+	+
SPC	−	+	+	+	+

It is also important to note that the protein isolation process manipulates the protein structure and processes vary throughout the industry [31]. Thus, proteins could be exposed to treatments that would allow for various gelation behaviors. The gelation concentration of globular proteins is affected by a number of factors, but especially by the pH and ionic strength the protein is exposed to, as well as enzymes and pressure treatment [32,33]. It is by various combinations of these treatments, too, that different proteins may test at the same LGC but for different molecular-level reasons. With the amount of factors that change protein properties, comparison between protein sources is difficult [33]. Higher solubility can be achieved through hydrolysis but results in a tradeoff of lower gel strength [33]. This could be an indication that some of the pea proteins may have been processed in a

way to increase solubility at the expense of gel strength, thus leading to a higher critical concentration of protein. Gelation properties as determined using the LGC test were further confirmed using RVA pasting properties, which is a rheological test discussed in the next section.

Despite the same LGC of PP1 and SPC, extrusion outcomes varied greatly in terms of internal structure and density (64 g/L and 253 g/L, respectively). Even within the same type of protein and with the same LGC, the final structure and density varied for PP2, PP3, and PP4 (65–172 g/L). LGC is a measure of gelation properties in a very dilute dispersion (12–20% solids), whereas protein network formation during extrusion-based texturization occurs in a much dryer processing environment (60–70% solids). Therefore, obvious differences exist between the two processes, including stronger protein interactions in the latter. Thus, LGC is not the sole determinant of product structure or extrusion characteristics, even though it is helpful in understanding differences between proteins. The role of heat gelation ability and LGC in the texturization process is discussed in more detail in a later section.

3.1.4. Rapid Visco Analyzer Viscosity

Pea protein solutions displayed different behavior upon hydration, heating and low shear RVA testing. Average RVA curves are shown in Figure 4 and corresponding pasting property data are summarized in Table 7. Among pea proteins, only PP1 had an increase in viscosity during heating, with a peak viscosity of 1387 cP at 83 °C. PP1 was also the pea protein treatment with the highest heat gelation ability, having the lowest LGC that almost matched the concentration at which RVA tests were conducted (15%). Thus, both RVA and LGC data point to the same thermally induced gelation and swelling properties of PP1, where the heat allowed proteins to solubilize in water and increase the viscosity. All other pea proteins had peak viscosities prior to the commencement of heating (51–55 °C), demonstrating moderate to high cold-water swelling properties, unlike PP1. The pea protein PP2 had a very high peak viscosity of 2250 cP at the outset of testing and before heating, thus showing that it had instant hydration and swelling properties. PP4 also demonstrated quick swelling but a relatively lower peak viscosity (1257 cP) before heating. Both PP2 and PP4 also had the highest WAC among the pea proteins, as discussed earlier. On the other hand, PP3 demonstrated moderate cold-water swelling with a low peak viscosity of 460 cP. Both the heat-swelling PP1 and moderately cold-swelling PP3 had the lowest WAC among the pea proteins. Correlation analysis found a relationship between WAC and the peak viscosity ($0.7/p < 0.0001$), although it was moderate, possibly due to the low WAC of heat-swelling proteins. Similar findings have previously related the WAC and viscosity of pea proteins, which found that a pea protein that was able to absorb more water resulted in a higher viscosity [25].

Table 7. Means and standard deviations of RVA-based rheological measurements. Means in a column followed by the same letter are not significantly different ($p < 0.05$).

Treatment	Peak Viscosity (cP)	Time of Peak Viscosity (s)	Temperature of Peak Viscosity (°C)	Final Viscosity (cP)
PP1	1387 ± 157^{c}	223 ± 14^{c}	83 ± 3^{c}	195 ± 7^{b}
PP2	2250 ± 52^{a}	20 ± 0^{e}	51 ± 0^{e}	299 ± 3^{d}
PP3	460 ± 83^{de}	91 ± 6^{d}	55 ± 1^{d}	144 ± 21^{c}
PP4	1257 ± 38^{c}	75 ± 5^{d}	52 ± 1^{d}	856 ± 25^{d}
Wheat Mix	300 ± 11^{ef}	453 ± 2^{b}	91 ± 0^{b}	251 ± 8^{a}
VWG	211 ± 23^{f}	450 ± 2^{b}	91 ± 1^{d}	156 ± 14^{cd}
Soy Mix	1626 ± 94^{b}	56 ± 31^{d}	51 ± 1^{d}	403 ± 10^{d}
SPC	320 ± 47^{e}	552 ± 0^{a}	70 ± 0^{a}	580 ± 47^{d}

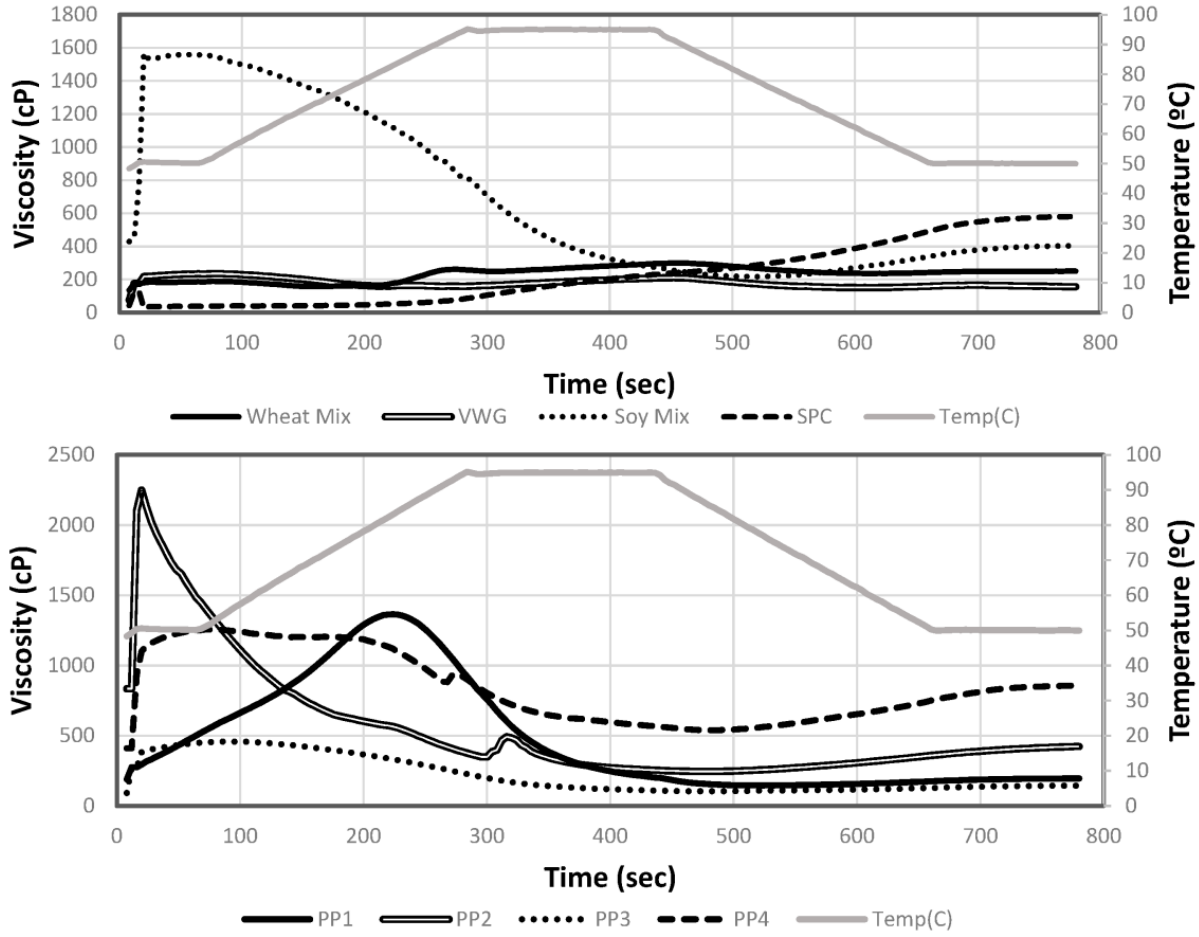

Figure 4. Rapid visco anlayzer (RVA) average viscosity curves for wheat and soy proteins (**top**) and pea proteins (**bottom**) using 15% solids concentration of raw materials.

Similarly, among the non-pea proteins, Soy Mix demonstrated instant hydration and cold-swelling properties with a peak viscosity of 1626 cP observed at 56 °C. Soy Mix also had the highest WAC among all proteins. This cold-water solubility was due to the soy protein isolate component. VWG and Wheat Mix exhibited no cold-water swelling during RVA testing and only a slight heat swelling. These two also had the lowest WAC among all the proteins. This reflects the hydrophobic nature of wheat gluten as discussed earlier. SPC exhibited heat-swelling properties, with RVA viscosity increasing steadily to 320 cP while the temperature remained higher than 70 °C, and further increasing to a final viscosity of 580 cP on cooling to 50 °C. The heat-induced solubilization and gelation properties of SPC could also be inferred from its low LGC as discussed earlier.

It is clear that RVA testing represents a combination of WAC and LGC, which are the cold-water solubility and heat gelation tests, respectively. Thus, a rheological test such as RVA in combination with WAC and LGC can be used as a useful set of physico-chemical analyses to characterize proteins by their cold-swelling and heat-swelling categories. PP2 and Soy Mix can be categorized as having high cold-water swelling properties, PP3 and PP4 as moderate cold-water swelling, and PP1, SPC, VWG and Wheat Mix as low cold-water swelling. On the other hand, PP1 and SPC can be categorized as having heat-swelling and -gelling properties. The relationship between these characteristics and texturization properties on extrusion are described in the extrudate analysis section.

It should be noted that the impact of starch, fiber and hydrocolloids on the pasting and gelling properties of raw materials can be significant, but the overall carbohydrate content is 10% or less for each ingredient investigated in this study, as can be seen from Table 2. Thus, the impact of these components is minimal as compared to the proteins that

are at the level of 80% or more. Thus, it is the proteins that primarily controlled the cold- and heat-swelling properties of the various materials in this study.

3.1.5. Differential Scanning Calorimetry

DSC thermograms showing start and peak temperature for the denaturation and enthalpy of denaturation for two representative ingredients are shown in Figure 5. No other thermal event was identified in any of the protein samples except for protein denaturation, as can be seen from the representative thermograms. The DSC thermograms of other samples were similar but had different values for these denaturation characteristics, as described below. The starting and peak temperature of denaturation were different among the pea proteins, ranging from 146–160 °C and 179–188 °C, respectively (Figure 6). Wheat treatments were lower than pea proteins in starting and peak temperatures of denaturation, while soy was higher. The enthalpy measured during denaturation varied as well (Figure 7). PP3 required the least amount of energy (12.6 J/g), while PP4 required the most (23.4 J/g). PP1 and PP2 required 19.4 and 17.0 J/g, respectively. The soy treatments required less energy to denature (10.9–15.1 J/g), while the wheat treatments required the highest (21.2–32.7 J/g). The heat denaturation properties of proteins are not clearly related to their cold- and hot-water swelling characteristics described earlier. Among pea proteins, PP1 required one of the highest enthalpies to denature, meaning it would require heat to denature, form a gel and build viscosity, as seen with RVA testing. PP4, though it also required a high enthalpy, had a much higher WAC, which led to a higher initial viscosity and thus no further increase in viscosity during heating.

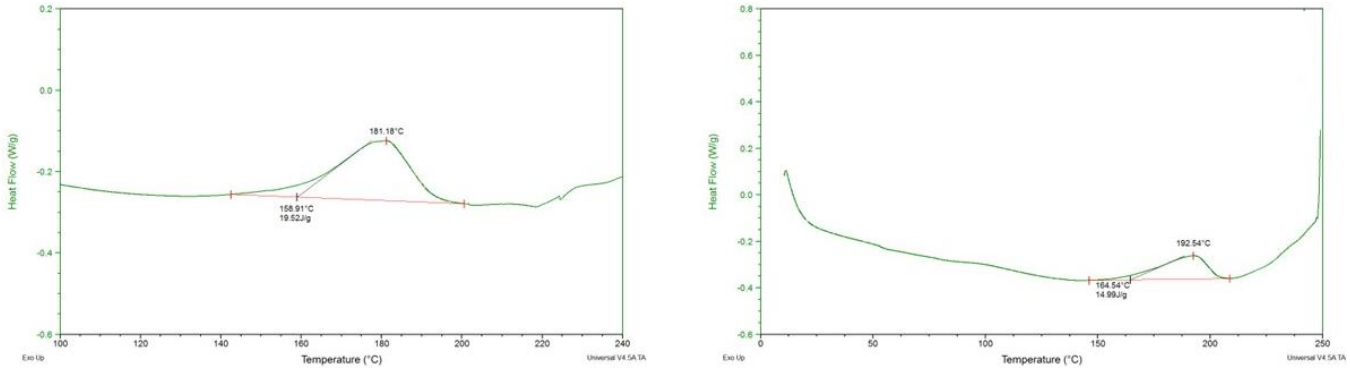

Figure 5. DSC thermograms showing start and peak temperature for protein denaturation and enthalpy for denaturation for two representative ingredients—PP1 (**left**) and soy mix (**right**).

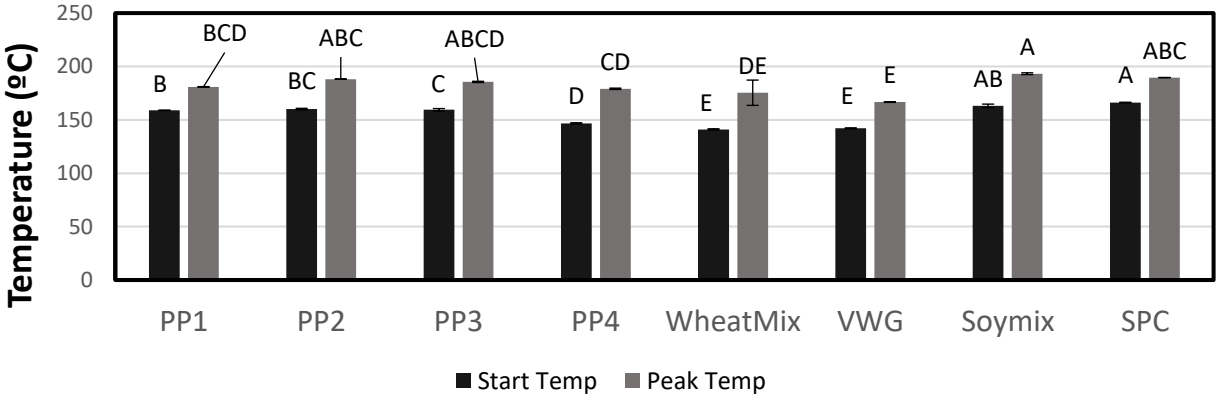

Figure 6. Mean starting and peak temperatures of denaturation for protein in each treatment as determined by DSC. Bars denoted by the same letter are not significantly different ($p < 0.05$).

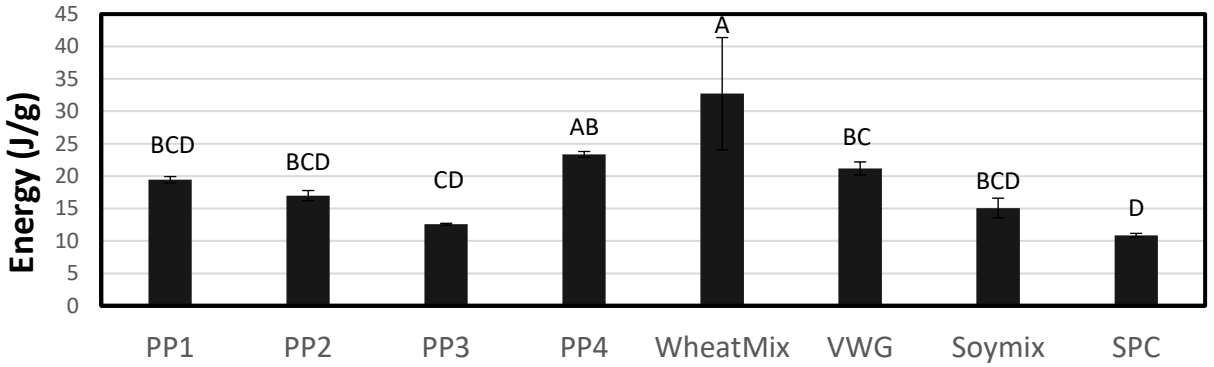

Figure 7. Mean energy required to denature proteins in the raw material as determined by DSC. Bars denoted by the same letter are not significantly different ($p < 0.05$).

These DSC results are not indicative of the properties of native proteins or their unfolded structures, as varying levels of denaturation might have occurred during the isolation and commercial production process of the different proteins. A previous study found that lower denaturation enthalpies are a result of harsher or longer thermal treatments [15]. Greater protein denaturation during isolation might explain the low enthalpy for PP3, due to the high prior unfolding of the protein structure, while PP4 may have the least denatured protein. This is, however, speculative, as information on the processing and isolation of proteins was not available. The relatively high enthalpies of VWG and Wheat Mix might be indicative of the relatively less aggressive wet milling process used in the isolation of wheat gluten.

3.1.6. Molecular Weight Analysis (SDS-PAGE)

Proteins differ based on their cultivar and extraction methods [14,34]. Having a molecular level understanding of proteins can be useful in understanding their gelation upon heating, and for pea proteins, this generally means understanding the solubility and the content of legumin, vicilin and convicilin. Legumin is attributed to disulfide bonding during gelation and texturization, due to the greater presence of sulfur-containing amino acids, but the gelation functionality of legumin can vary by variety [35]. Vicilin lacks the sulfur content of legumin, yet the convicilin subunit of vicilin still contributes to gelling. The core of convicilin is largely the same as vicilin, but convicilin is distinguished from vicilin because of a highly charged end of the protein which allows it to form the gel network [35].

Due to the lower intensity SDS-PAGE bands of unextruded PP1, this protein seems to be less soluble in water than the other proteins (Figure 8a). This observation is compatible with the lack of cold-water solubility of PP1 and its heat-swelling nature observed in the WAC, LGC and RVA tests. PP2 is the most soluble, as noted by the greatest intensity of the bands. The high solubility of PP2 gives an explanation of its high WAC and the RVA cold-swelling viscosity. After immediate hydration, PP2 is able to take in water and create viscosity, but upon heating and mild shear, the protein network begins to disintegrate and viscosity decreases. Unextruded PP3 and PP4 also exhibit relatively dark SDS-PAGE bands, although of less intensity than PP2, reflecting their low-to-moderate cold-swelling properties described earlier. Bands associated with a molecular weight higher than 70 kDa are present in PP2 but absent in PP3 and PP4. This could be the reason for the lower cold swelling and lower RVA peak viscosities observed in the latter. The unextruded Soy Mix had relatively high intensity bands at 70 kDa and above (Figure 8b), although distinct from PP2, which is consistent with its higher cold-swelling properties due to the soy protein isolate.

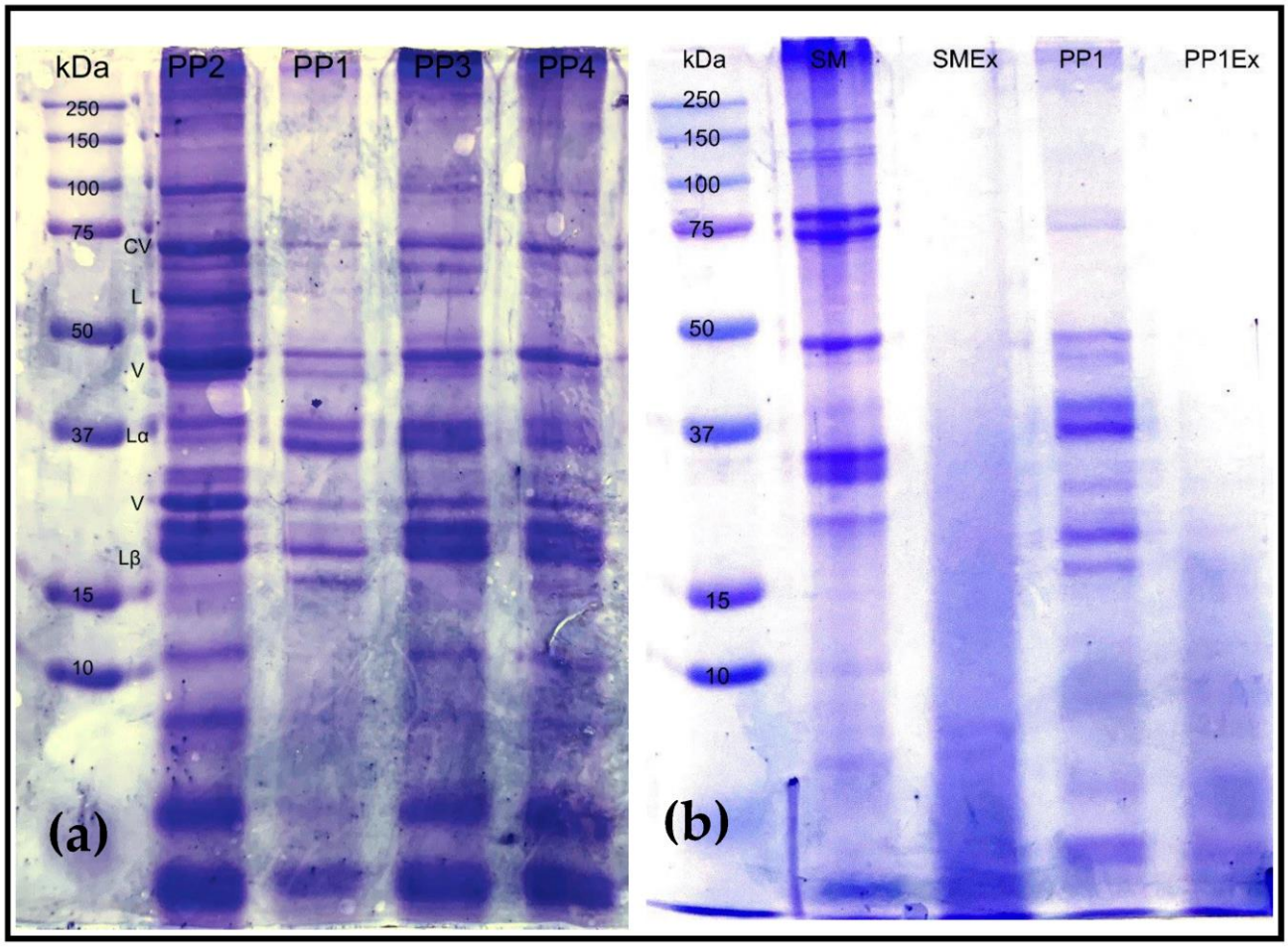

Figure 8. Non-reducing SDS-PAGE gel for raw pea proteins (**a**) and select raw and extruded proteins (**b**). Columns in (**a**) are the standard marker, PP2, PP1, PP3 and PP4. Note that PP2 comes before PP1. Columns in (**b**) are the standard marker, raw soy mix (SM), soy mix extruded (SMEx), PP1 and PP1 extruded (PP1Ex). CV, Convicilin; L, Legumin; V, Vicilin; Lα, acid subunit of legumin; Lβ, basic unit of legumin.

As can be further seen from Figure 8a, the more cold-soluble proteins (PP2-PP4) have 70 kDa bands of more intensity, indicating a higher presence of convicilin, and each of these had a slightly higher LGC, which could be due to the electrostatic repulsion preventing gelling and requiring slightly more protein to network. With a higher convicilin content, more N-terminus negative charges exist and therefore more repulsion occurs, requiring more protein to make a gel [30].

SDS-PAGE of select extrudates showed obvious change in the molecular weight and solubility of the proteins compared to that prior to extrusion (Figure 8b). No distinct bands were present after extrusion. During low-moisture extrusion, previous studies found that the vicilin protein was unaltered while legumin changed after extrusion, likely via aggregation and an increase in molecular weight [25,36]. Extrusion texturization is a continuous thermomechanical process that transforms globular proteins such as pea or soy proteins, or irregular plant proteins, such as wheat gluten, into meat-like fibrous structures. During this process, the moisturized protein matrix undergoes several physical, chemical and structural changes that significantly affect the textural quality of the extruded products. To form microscopic and macroscopic fibers, the proteins need to unfold, cross-link, and align themselves. Covalent bonds, such as peptide and disulfide bonds, and non-covalent interactions, including hydrogen bonding, hydrophobic interactions and ionic linkage, undergo alterations, and new bonding is formed through physical and chemical cross-

linking. These mechanisms led to the proteins in this study becoming aggregated and insoluble after extrusion, rendering a gel with no distinct bands.

3.1.7. Phase Transition Analysis

The various raw materials showed different phase transition behaviors (Figure 9). The temperature at which the material began to flow through the PTA capillary die was highest for soy and wheat proteins (64.5–76.8 °C), indicating a high resistance to flow. Among the pea proteins, the flow temperature was highest for PP3 (65.9 °C) and lowest for PP2 (49.2 °C). With a higher temperature required to achieve a flowable melt, it follows that more energy will be required to process the material in the extruder. The thermal energy required for raw materials to flow in the PTA mirrored the specific mechanical energy (SME) required for pilot-scale processing (Figure 10), as is discussed in the following section.

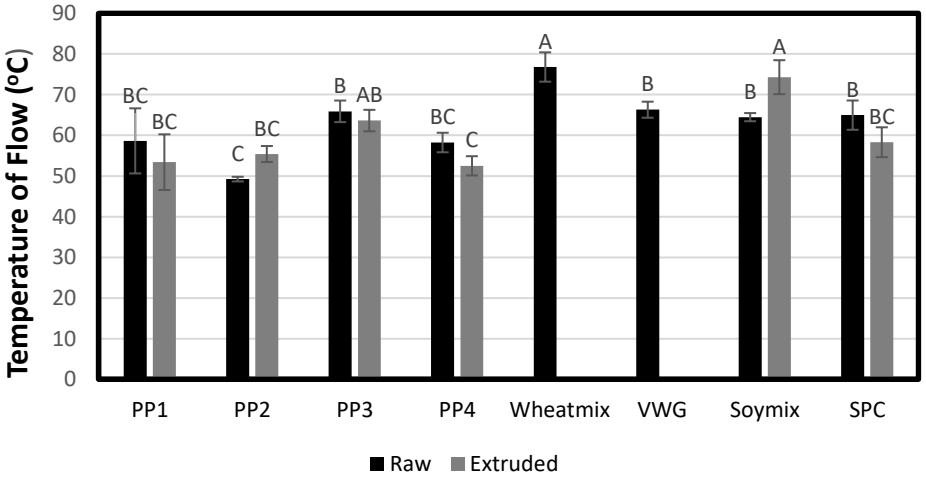

Figure 9. Flow temperature for raw material treatments and moderately sheared extrudate. Bars denoted by the same letter are not significantly different ($p < 0.05$). Wheat gluten was not able to be hydrated and extruded on the lab-scale extruder, thus no extruded PTA test was conducted on Wheat Mix and VWG.

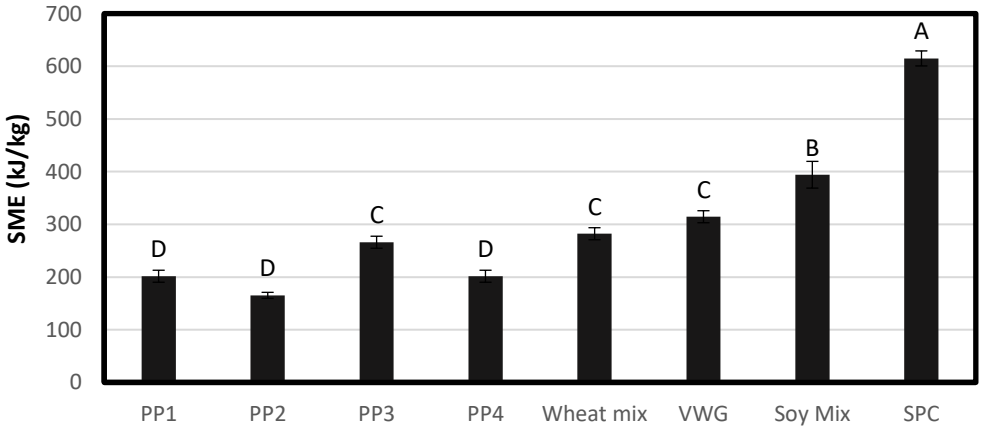

Figure 10. Mean SME required by each treatment during extrusion processing. Bars denoted by the same letter are not significantly different ($p < 0.05$).

The PTA flow temperature after moderate energy extrusion (using a lab-scale system) is also shown in Figure 9. The change in flow temperature between raw and extruded proteins can be a useful bench-top analysis tool for determining their cross-linking potential, as was described in a previous study by our research group [22]. In the current study, PP2 and Soy Mix were the two proteins that showed an increase in flow temperature (from 49.2 to 55.4 °C and 64.5 to 74.3 °C, respectively). This indicated an increase in resistance to

flow or viscosity due to high degree of protein networking or cross-linking induced by heat, shear and pressure during moderate extrusion. It is interesting to note that PP2 and Soy Mix also exhibited cold-water swelling properties as described earlier. Thus, cold-swelling proteins might intrinsically have a higher cross-linking potential. The relationship between protein cross-linking or networking potential is thus determined and product texturization and final quality is discussed in the extrudate analysis section. The protein networking induced in the other proteins due to moderate extrusion might be of a lesser degree and possibly dwarfed by any macromolecular degradation during the extrusion and grinding process, as can be seen from the slight reduction in flow temperature.

3.1.8. Extrusion Processing

Among pea proteins, SME was greatest for PP3 (266 kJ/kg), while PP2 had the lowest SME (165 kJ/kg) as can be seen from Figure 10. The highest SME was required for the processing of wheat and soy treatments (282–615 kJ/kg). These trends in general were similar to that of the raw material PTA flow temperature. Thus, the latter appeared to be a good indicator of extrusion SME, as has been shown previously as well [37]. Both PTA flow temperature and extrusion SME measurements are an aspect of the resistance to flow of raw materials. A raw material with a higher flow temperature requires more energy to flow, which means that the material will have greater resistance to flow during extrusion and require more SME for processing.

SME might also have some relation to the swelling or water hydration properties, as well as particle size and functionality of the proteins. For pea proteins, the SME has an inverse trend to their WACs. The more water the protein is able to hold, the less energy it requires to process. During extrusion, each pea protein was processed with the same amount of water. A reason for the low SME of PP2 (165 kg/kJ) may be the high solubility the protein has, which means it will not build viscosity as well, and it would require less energy to process. To attain the same SME with a more soluble protein such as PP2, a lower in-barrel moisture or IBM may be required, so that the melt can have greater viscosity. PP2 and PP4 had the largest particle size and the lowest SME of the pea protein treatments. In at least one previous study involving corn meal extrusion, a larger particle size was observed to have a lower SME due to easier flow [38]. SPC had the most water added to it during extrusion, which would generally plasticize the melt and reduce the viscosity and SME in the extruder. Still, the greatest SME was found in SPC (615 kg/kJ). This may be due to the presence of more functional, heat-swelling and viscosity-building proteins. Indeed, SPC has a higher heat-induced viscosity than most pea proteins and wheat treatments, as was observed in RVA data.

3.2. Extrudate Analysis

3.2.1. Visual Analysis

Pictures of product transverse (perpendicular to extrusion direction) and longitudinal (along extrusion direction) cross sections are shown in Figures 11 and 12. All extrudates were clearly texturized, as evident from their cohesive internal structure; however, they differ substantially in porosity and extent of layering. In general, a relatively porous structure was observed due to the product expansion that is typical for the low-moisture and higher-energy texturization process used in this study. Among pea proteins, the product based on PP2 displayed a very cellular, porous structure, followed by PP4 and PP3, with PP1 showing the least expanded internal structure. The longitudinal cross sections show some layering or lamination along the direction of extrusion, more prominently in PP1 and PP3. The porous structure of pea-protein-based products may be attributed to the strong protein–protein networking induced during extrusion, which led to 'film formation' and expansion. This high degree of extrusion-induced cross-linking is typical of pea proteins [22]. This was particularly a feature of PP2, which also showed a higher cross-linking potential in PTA analyses. It is inferred that the cold-swelling nature of PP2 promotes cross-linking to the extent that the product expands into a cellu-

lar structure after texturization rather than forming dense layers. It is expected that if a moderate quantity (10–20%) of starch or fiber is present to inhibit the protein cross-linking, a more fibrous or layered texture would result, as found in previous studies by our group [22,39]. Conversely, the low cold-swelling nature of PP1 (combined with heat swelling) and PP3 led to better layering and a denser product structure. Similarly, in non-pea proteins, the internal structures of VWG and Wheat Mix exhibited the least porosity, a fibrous structure with dense layering in both the horizontal and longitudinal cross sections. This is consistent with the very low cold-swelling properties and hydrophobic nature of wheat gluten. Moreover, gluten is naturally a fibrous-shaped protein, while both pea and soy are globular proteins [30]. Thus, wheat gluten texturizes into a fibrous structure more easily than the other proteins. Although not clearly visible from the pictures, the Soy Mix product had a more porous structure, while SPC was denser and layered. This is also consistent with the high cold-swelling properties of Soy Mix and particularly its soy protein isolate component, and the heat-swelling and low cold-swelling properties of SPC. These observations on visual structure are, however, not substantiated by objective measurements of features such as layers or cells.

To summarize, all proteins were texturized or cross-linked in the pilot-scale extrusion process. However, the high cold-swelling proteins (PP2 and Soy Mix) exhibited a greater degree of cross-linking, as was observed visually with a more cellular or porous structure, and confirmed by benchtop analyses using PTA data. Heat-swelling and/or low cold-swelling proteins (PP1, PP3, VWG, Wheat Mix and SPC) exhibited a lesser, yet optimum degree of cross-linking, leading to a denser, layered and fibrous structure. Moderate cold-swelling proteins (PP4) had a structure somewhere in between the two.

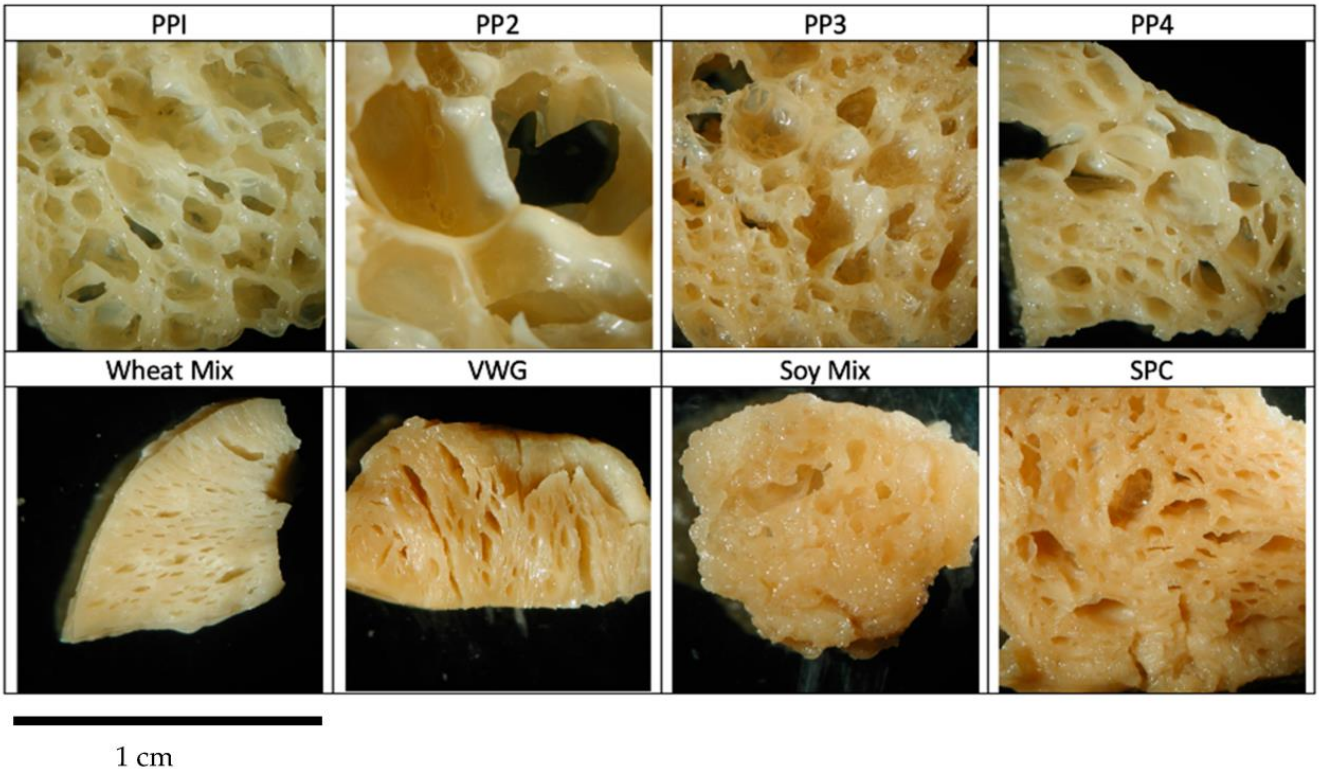

Figure 11. Horizontal cross sections of whole extrudate pieces. Horizontal cross sections are cut against the direction of flow from the extruder. Each image is approximately 1 cm across.

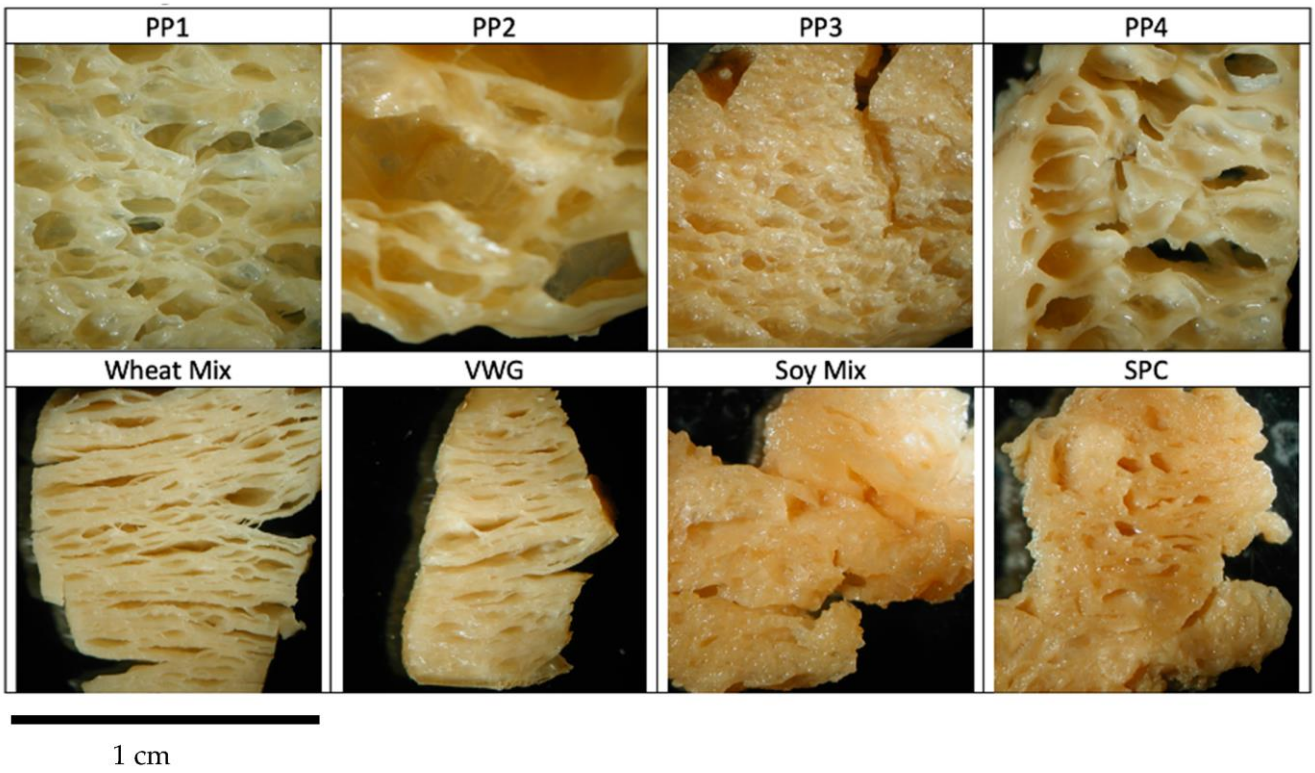

Figure 12. Longitudinal cross sections of whole extrudate pieces. Longitudinal pieces were cut along the direction of flow from the extruder. Each image is approximately 1 cm across.

3.2.2. Bulk Density

The bulk density of pea protein extrudates ranged from 58 to 143 g/L, and was generally lower than that of non-pea extrudates (146–268 g/L) (Figure 13). This corresponded well with the higher porosity observed visually, as bulk density is inversely proportional to the degree of expansion. PP2 product had the lowest bulk density of 58 g/L, which was consistent with its highly porous, cellular internal structure and the high cold-swelling and cross-linking potential of the PP2 protein. Within non-pea treatments, the wheat treatments (240–268 g/L) and SPC (258 g/L) had the highest bulk density, which was consistent with their denser, layered structure and the heat-swelling and/or low cold-swelling nature of gluten and SPC proteins. On the other hand, Soy Mix had a relatively low bulk density of 146 g/L due to higher cross-linking and the cold-swelling nature of soy protein isolate.

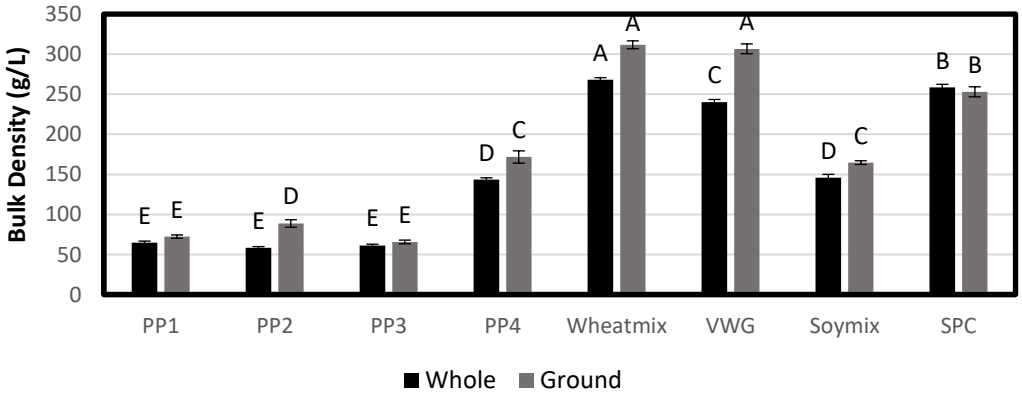

Figure 13. Average bulk density of whole extrudate and ground extrudate. Bars denoted by the same letter are not significantly different ($p < 0.05$).

In general, for the proteins studied, a higher SME resulted in a denser product. This was contrary to the usual observation for starch-based expanded extrudates, where higher mechanical energy input leads to a greater intensity of cooking and die temperatures and thus leads to more expansion and lower bulk densities [40]. The opposite trend in this study was due to the fact that the porosity of texturized protein extrudates was a function of the intensity of cross-linking, which in turn was more dependent on the nature and functionality of the protein than the energy input during extrusion.

3.2.3. Water-Holding Capacity

Texturized vegetable protein products typically have a layered yet porous structure [4]. Previous work has shown that the internal structure of extrudates and the bulk density have a great impact on the extrudate's WHC [22]. The open cell structure of some of the whole extrudates skewed the whole-product WHC trends, but ground-product WHC had a more consistent inverse relationship with bulk density, as has been observed previously (Figure 14). Extrudates with higher expansion and lower density resulted in higher ground WHC, as the micro-level porosity of the products was maintained even after grinding. For example, VWG, wheat mix and SPC had the lowest WHC (127–296%) and also the highest bulk density. These were also the products that exhibited relatively denser layering in the internal structure. A low WHC combined with high density can contribute to a lower sponginess of the hydrated product and a harder, more meat-like texture. On the other hand, pea protein treatments had a relatively high WHC (315–618%), which was consistent with their low bulk density. PP2 had the highest WHC of 618% and was also the most porous of all the products. Among non-pea treatments, Soy Mix had the highest WHC of 501% and also had the lowest density.

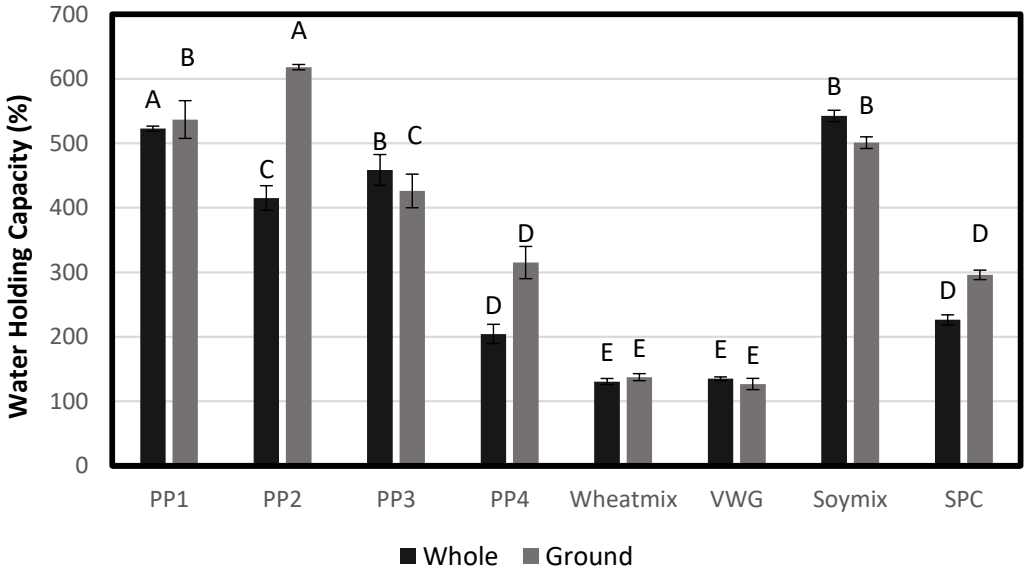

Figure 14. Water holding capacity (%) of whole extrudates and ground extrudates. Bars denoted by the same letter are not significantly different ($p < 0.05$).

3.2.4. Textural Analysis

The textural attributes of plant-based meat that mimics actual meat-like texture have not been extensively studied. Texture profile analysis (TPA) has been used to characterize plant-based meat, and in a very general sense, it was assumed that high hardness and chewiness and relatively low springiness could simulate meat muscle [22]. These primary TPA attributes for muscle-meat patties and plant-based meat products in this study are shown in Figure 15. The hardness of chicken, beef and pork patties ranged from 2804 to 4057 g (as compared to 4852 g for Beyond Beef®); springiness ranged from 0.77 to 0.84 (0.61 for Beyond Beef®); and chewiness from 1329 to 2333 (1083 for Beyond Beef®). Clearly

chewiness was one aspect where the commercial plant-based patty was found lacking. This was due to low springiness but also probably due to low cohesiveness, which is another attribute used for calculating chewiness besides hardness.

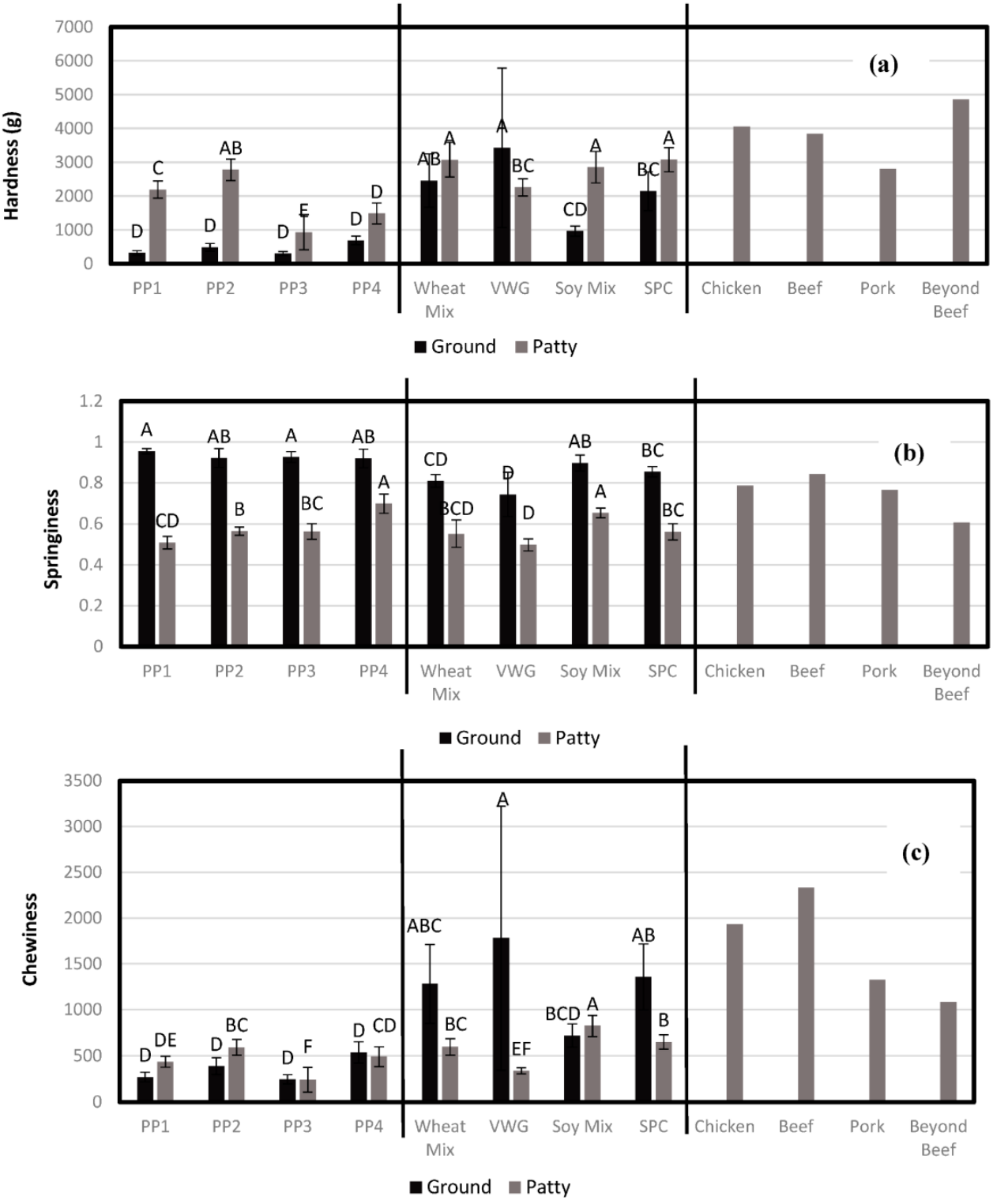

Figure 15. Mean hardness (**a**), springiness (**b**), and chewiness (**c**) of ground extrudate and patties made from the ground extrudate. Bars of the same color denoted by the same letter are not significantly different ($p < 0.05$).

Ground and hydrated pea-protein-based products (without binders) in this study did not compare well with the muscle meat or commercial plant-based meat (also based on pea protein) benchmarks, having very low hardness (304–692 g) and chewiness (246–538), and very high springiness (0.92–0.95). This was attributed to the high degree of cross-linking, porosity and WHC of these products. Similar results were found for whole hydrated pea-protein-based simulated meat products previously [22]. By adding binders (pea protein isolate, chickpea and methylcellulose), the corresponding patties showed an improvement in their textural attributes, with higher hardness (930–2772 g), similar or higher chewiness (242–594) and lower springiness (0.51–0.70). Most of these patty products still had too-low hardness and chewiness as compared to the benchmark products, except PP2, which interestingly, on the addition of binders, had the desired low springiness and also hardness similar to pork, which had the lowest value among all meats (2804 g). The chewiness of the PP2 patty was still much less than desired, but the use of a higher level of binders, or different binders, could possibly rectify that shortcoming.

Ground and hydrated non-pea-protein-based products (without binders) had much higher hardness (966–3433 g) and chewiness (721–1785), and lower springiness (0.74–0.90) than pea protein products, and compared reasonably well with chicken, pork and commercial plant-based meat benchmarks. The lowest hardness and chewiness and highest springiness was found for the Soy-Mix-based product, which was not surprising as that was associated with a higher level of cross-linking, porosity and WHC, as with pea protein products. As in the case of pea protein, the addition of binders in most cases led to an increase in hardness (2261–3078 g) and a decrease in springiness (0.50–0.65), but contrary to pea proteins, there was also a decrease in chewiness (340–825), which was not desirable for a meat-like texture. These products were nevertheless a better candidate for mimicking meat texture, and the use of different and better binders could potentially help in that regard to improve chewiness. Interestingly, Soy Mix treatment had the biggest improvement in hardness and even chewiness with the addition of binders, and its patty was closest to pork in texture. This, in combination with similar observations for PP2, pointed to the better compatibility of the set of binders used in the current study with these two cold-swelling proteins and the associated higher cross-linking, porosity and water-holding of the extruded products.

It should be noted that the TPA test for texture fails to account for mouthfeel, which can critically change the perception of the product. Sensory analysis to understand the mouthfeel differences in such products would be helpful, including attributes such as juiciness, cohesiveness of mass and surface properties (grainy, smooth, fibrous, lumpy, etc.), which can be expected to vary due to differences in porosity, layering and WHC. Though not many studies have been published on sensory analysis and the acceptance of plant-based meat products, in a study on high moisture extrusion (HME)-based soy and pea patties, sensory analysis data pointed to significant differences in the cohesiveness of mass, hardness and springiness of plant protein patties made with soy or pea protein, and a consumer study found that the overall texture of the soy patty, having greater hardness and springiness, was preferred to pea patties [41].

It should also be noted that hardness and chewiness data for ground VWG had high variation (standard deviation), since sample mass for all TPA tests were standardized to 20 g. As VVG samples had a very high bulk density compared to the other textured proteins, it resulted in a much thinner layer (low volume) sample as compared to other treatments. The lower thickness of the test samples led to inconsistent results and greater variability.

4. Conclusions

Pea proteins are innately different from wheat and soy proteins and thus resulted in different plant-based meat properties after low moisture extrusion. Formulations containing soy protein isolate and most pea proteins had highest cross-linking potential, required the least specific mechanical energy during extrusion and led to a porous and less layered, texturized internal structure. On the other hand, soy-protein-concentrate- and wheat-gluten-based treatments had a dense, layered extrudate structure. Protein characteristics including

water absorption capacity, least gelation concentration, rapid visco analyzer pasting profiles and phase transition flow temperature provided helpful information for understanding the functionality, texturization via extrusion and internal structure of the product. In turn, structural attributes such as porosity and layering and the use of binders significantly impacted the texture of plant-based meat patties including hardness, springiness and chewiness. This knowledge can be applied to tailor formulations and also the extrusion process to particular proteins and the desired product quality.

Author Contributions: D.W. collected experimental results, interpreted data, and wrote the manuscript. H.D. and Y.L. provided expertise in textural analysis and protein behavior as well as manuscript review and editing. S.A. was responsible for overall project coordination, handling the ingredient and equipment logistics, aiding in the interpretation of results, and editing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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Physicochemical and Functional Properties of Texturized Vegetable Proteins and Cooked Patty Textures: Comprehensive Characterization and Correlation Analysis

Shan Hong, Yanting Shen and Yonghui Li *

Department of Grain Science and Industry, Kansas State University, Manhattan, KS 66506, USA
* Correspondence: yonghui@ksu.edu; Tel.: +1-785-532-4061

Abstract: Rising concerns of environment and health from animal-based proteins have driven a massive demand for plant proteins. Textured vegetable protein (TVP) is a plant-protein-based product with fibrous textures serving as a promising meat analog. This study aimed to establish possible correlations between the properties of raw TVPs and the corresponding meatless patties. Twenty-eight commercial TVPs based on different protein types and from different manufacturers were compared in proximate compositions, physicochemical and functional properties, as well as cooking and textural attributes in meatless patties. Significant differences were observed in the compositions and properties of the raw TVPs ($p < 0.05$) and were well reflected in the final patties. Of all the TVP attributes, rehydration capacity (RHC) was the most dominant factor affecting cooking loss (r = 0.679) and textures of hardness (r = −0.791), shear force (r = −0.621) and compressed juiciness (r = 0.812) in meatless patties, as evidenced by the significant correlations ($p < 0.01$). The current study may advance the knowledge for TVP-based meat development.

Keywords: textured vegetable protein; meat analogs; physicochemical properties; rehydration capacity; patty textures

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1. Introduction

Recently, a massive demand for plant-protein-based diets has been appeared in consumers' perceptions, which is driven by the multifaceted pressures of animal protein production (e.g., environment, health, animal welfare and ethics issues) [1,2] as well as the high nutritional values and potential health benefits of plant proteins [3]. Plant proteins have been extensively involved in meat products as partial extenders or full replacements to enhance the properties of meat products or to imitate the meat-like texture and taste, thus expanding meat production [2,4]. Shen et al. incorporated a functionally enhanced pea protein, which was prepared through sequential enzymatic modification with protein glutaminase and conjugation with guar gum (pea-glutaminase-guar gum, namely PGG) in beef patties and found that the inclusion of 5% PGG effectively improved the cooking yield while decreasing hardness. The extended beef patty with softer and more tender texture may serve as a good option for elderly people [5].

Textured vegetable protein (TVP) is a processed plant product produced via texturization with fibrous textures, closely imitating the animal muscle meat [6]. TVPs have been derived from several grain proteins, with soy protein and wheat gluten being two of the most primary protein sources [7]. Soy protein has the advantages of its excellent nutritional attributes and highly similar appearance to meat [8]. However, the presence of allergenic protein and genetic modifications narrows down the application of soy protein [9]. Owing to the ability to form anisotropic meat-like structure, wheat gluten is popularly utilized in TVP manufacturing [10]. Nevertheless, the potential of allergy induction and the imbalanced composition of essential amino acids limit the popularity of wheat gluten [10]. By comparison, as a thriving alternative, pea protein is superior in hypoallergenic and

non-genetic modification [9]. Other plant proteins receiving increasing attention include chickpea protein, mung bean protein and peanut protein [11–13]. Mixed proteins are also processed to offset the imbalance of amino acids [1]. Different TVPs have been widely documented in meat products. For instance, Hidayat et al. studied the effect of TVP on the quality of beef sausages and found that different degrees of TVP substitution improved the water-holding capacity and cooking yield of beef sausages while maintaining a good sensory acceptance up to 30% of TVP replacement [14]. Previously, the integration of TVP in beef patties modified the hardness, cohesiveness and toughness [15,16].

One of the main challenges confronted by meat analogs is the texture. The quality of TVP can be highly affected by the sources and properties of raw materials. The physicochemical and functional properties of plant proteins that are important for texturization include protein solubility, emulsification, gelling ability, water and oil absorption capacity, among others [6,11]. Meanwhile, variations in the texturization process also make significant contributions to the final products. Different technologies have been explored, and some are now available to create the fibrous structures from plant-based proteins, such as fiber spinning, electrospinning, mechanical elongation method, shear cell technology and extrusion [7], of which, extrusion is the dominant approach. During extrusion, taking the low-moisture texturization as an example, moistened proteins are plasticized in the extruder barrel by the application of pressure, heat and mechanical shear. The plasticized mass is then pushed through the die openings during which the mass moisture partially evaporates, and the protein molecules align rapidly to generate fibrous textures [17]. The native structures of proteins are altered in response to the extrusion energy, leading to denaturation and conformation changes along with modifications in physicochemical and functional properties [6,11]. Therefore, manipulating the various extrusion conditions (moisture, temperature, pressure and shear) enables the fabrication of different TVPs with versatile structures and textures. Samard et al. found that TVPs manufactured at 50% moisture content and 130 °C die temperature possessed higher water absorption capacity and superior textural properties in terms of springiness, cohesiveness, chewiness and hardness compared with their counterparts produced at other conditions (40% moisture content and 150 °C die temperature) [18].

Despite the vast information on TVP production and application, little research has looked into the complete physicochemical and functional profiles of TVPs other than the native proteins and focused on how the properties of raw TVPs could be carried over into the end meat-like products. The hypothesis of this study was that the properties of TVP-based meat analogs were closely associated with and determined by the various physical and functional properties of the raw TVPs, and specific correlations existed between the properties of TVPs before and after formulating to patties. Thus, the objective of this research was to provide a comprehensive study on the physicochemical and functional properties of 28 commercial TVPs that are sourced from different protein types, then to evaluate the cooking and textural properties after formulating to the meatless patties, and finally, to establish the potential correlations between the upstream and downstream properties. The systematic study of a relatively high number of samples in the current research may help bridge the gaps between TVP properties and textures in the final plant-based meats, serving as a baseline knowledge to develop desirable plant-based meat analogs for the food industry.

2. Materials and Methods

2.1. Materials

A total of 28 textured vegetable proteins (labeled as 1–28), with samples 1–14 produced from soy protein, samples 15–21 from pea protein, samples 22–25 from wheat gluten, sample 26 from chickpea protein, sample 27 from pea/chickpea protein mixture and sample 28 from pea/navy bean protein mixture, were obtained from Amazon (Seattle, WA, USA) or other commercial sources. The sample selection mostly depended on the availability of TVP types. Methylcellulose and beetroot powder were purchased from Amazon. Coconut oil

and canola oil were purchased from a local grocery store. Other chemicals of analytical grade were obtained from Fisher Scientific (Waltham, MA, USA) unless otherwise stated.

2.2. Proximate Composition of TVP

TVP samples were ground into a fine powder using a coffee mill for 30 s. The protein content of TVP powder was determined following the combustion method (AACC Method 46-30.01) using a LECO analyzer with the nitrogen to protein conversion factor of 6.25. Moisture content (AACC Method 44-19.01) was measured as the weight loss of approximately 2 g of each powder that was dried at 135 °C for 2 h in an automatic oven (Isotemp Oven, Fisher Scientific, Waltham, MA, USA). Ash content (AACC method 08-01.01) was determined by incinerating around 3 g of sample powder in a furnace (Fisher Scientific, Waltham, MA, USA) at 575 °C overnight. The measurement of fat content was modified from a previous method [19]. Briefly, 2 g of each TVP powder was mixed with 30 mL ethyl ether with continuous shaking for 30 min at 250 rpm (Orbital Shaker Model 361, Fisher Scientific, Waltham, MA, USA). After centrifugation for 10 min at 10,000$\times$ g (Benchmark Hermle Z 366 K centrifuge, Hermle Labortechnik GmbH, Wehingen, Germany), the supernatant was collected in an aluminum dish pan and allowed to evaporate overnight in a fume hood to obtain the extracted fat. All the proximate compositions were analyzed in triplicate. Total carbohydrate content was determined by subtracting the total contents of protein, fat, ash and moisture from 100 percent, as in the following equation:

$$\text{Total carbohydrate (\%)} = 100 - (\text{protein} + \text{moisture} + \text{ash} + \text{fat})\ (\%) \quad (1)$$

2.3. Protein Solubility

Protein solubility was determined as previously reported with slight modifications [20]. The ground sample (1.5 g) was suspended in 30 mL of 0.5% w/v KOH followed by shaking for 20 min at 250 rpm and room temperature (RT). The supernatant was decanted after centrifugation at 10,000$\times$ g for 20 min (Benchmark Hermle Z 366 K centrifuge, Hermle Labortechnik GmbH, Wehingen, Germany). The extraction was repeated once, and the precipitate was freeze dried (Labconco FreeZone 4.5 Lite Benchtop FreezeDryer, Labconco Corporation, Kansas City, MO, USA). The lyophilized precipitate as the insoluble protein was then subjected to protein content analysis as described in Section 2.2. The protein solubility was calculated as the percentage of soluble protein to the total protein. The measurements were conducted in triplicate.

$$\text{Protein Solubility}\ (\%) = 100 - \frac{\text{wt of precipitate (g)} \times \text{ protein content in precipitate (\%)}}{\text{wt of TVP powder (g)} \times \text{ protein content in TVP (\%)}} \times 100 \quad (2)$$

2.4. Water Absorption Capacity and Oil Absorption Capacity

Water/oil absorption capacity (WAC/OAC) tests were performed following our previous method [5] with minor modifications. For WAC, 0.6 g (W_0) of ground sample was dispersed in 10 mL deionized (DI) water in a pre-weighed 15 mL centrifuge tube (W_1). The mixture was vortexed thoroughly and allowed to stand for 5 min at RT. After centrifugation for 30 min at 3000$\times$ g (Benchmark Hermle Z 366 K centrifuge, Hermle Labortechnik GmbH, Wehingen, Germany), the supernatant was discarded, and the tube with the residue was inverted to stand for 5 min before re-weighing (W_2). For OAC, 1 g (O_0) of each sample was mixed thoroughly with 10 mL canola oil in a pre-weighed 15 mL centrifuge tube (O_1). The mixture was allowed to stand for 30 min at RT before centrifugation at 3000$\times$ g for 30 min. After discarding the oil, the tube containing the protein sediment was inverted for 10 min to drain the excess oil followed by re-weighing (O_2). The WAC and OAC were expressed

as grams of water and oil absorbed per gram of sample using the following equations, respectively. Each sample was carried out in triplicate.

$$\text{WAC (g } H_2O\text{/g sample)} = \frac{W_2 - W_1 - W_0}{W_0} \quad (3)$$

$$\text{OAC (g oil/g sample)} = \frac{O_2 - O_1 - O_0}{O_0} \quad (4)$$

2.5. Viscosity

The viscosity characteristics of TVPs were measured on a Rapid Visco Analyzer (RVA) (RVA4500, Perten Instruments, Hägersten, Sweden) using the AACC method 76-21.02 (13 min procedure) with slight modifications. Approximately 7.0 g of each ground sample was placed in a canister and mixed with 25 mL DI water. The TVP powder slurry was heated to 50 °C and equilibrated for 1 min, followed by ramping up to 95 °C within 4 min while stirring at 960 rpm for the initial 10 seconds for thorough dispersion and at 160 rpm for the remaining RVA test. After holding for 3 min at 95 °C, the mixture was cooled to the initial 50 °C within 4 min and held for another 2 min. The peak time (the time at which peak viscosity occurred), peak viscosity (the maximum hot paste viscosity) and final viscosity (the viscosity at the end of the test after cooling to 50 °C and holding at this temperature) were recorded. Each sample was analyzed in duplicate.

2.6. Bulk Density

Dry TVP was filled in a 1 L graduated cylinder with gentle tapping twice to eliminate the interspace of the crumbles. The volume and the weight were recorded, and the bulk density was calculated as the weight per volume (g/L). Two measurements were taken for each sample.

2.7. Rehydration Capacity

Twenty grams of dry TVP was rehydrated in 300 mL DI water (1:15 solid to liquid ratio) for 2 h at room temperature (RT), followed by draining for 1 h on a mesh screen. The final weight was recorded to quantify the rehydration capacity (RHC) as follows. Each sample was conducted in triplicate.

$$\text{RHC (g } H_2O\text{/g sample)} = \frac{\text{weight after rehydration (g)} - \text{weight before rehydration (g)}}{\text{weight before rehydration (g)}} \quad (5)$$

2.8. Textural Properties of Rehydrated TVP

The textural properties of rehydrated TVPs were characterized by texture profile analysis (TPA) using a TA-XT Plus texture analyzer (Stable Micro System, Godalming, Surrey, UK) following our previous method [5]. Prior to measurement, dry TVP crumbles were hydrated in DI water at 1:15 mass ratio as described above. Approximately 15 g of each hydrated sample was transferred to a Petri dish for up to 1 cm height. TPA was performed by a two-compression test using a cylinder prober (2-inch diameter) at a strain compression rate of 50% with 20 g trigger force and a pre-test speed of 1.0 mm/s, a post-test speed of 5.0 mm/s and a test speed of 1.0 mm/s. The textural attributes of hardness (the peak force during the first compression), resilience (the ratio of the downstroke area to the upstroke area under the first compression peak), cohesiveness (the area under the first compression curve divided by the area under the second compression curve), springiness (the ratio of the time to reach the peak during the second compression over the time to reach the peak during the first compression) and chewiness (hardness × cohesiveness × springiness) were collected. Each TVP sample was conducted in four replicates.

2.9. Preparation of TVP Patties

Prior to formulation, dry TVPs were allowed to hydrate for 2 h followed by draining for 1 h at RT as described in Section 2.7. The drained TVP was ground for 30 s using a food processor (Ninja BL770 Mega Kitchen System, SharkNinja Operating LLC, Needham, MA, USA) to achieve uniform and smaller particles (2–3 mm). Thereafter, 100 g of the hydrated and processed TVP was mixed with 2 g methylcellulose, 1 g NaCl, 1 g beetroot powder and 20 g pre-melted coconut oil by hand thoroughly to obtain a homogeneous mixture. The mixtures of approximately 20 g weight were then formed into patties using a cylindrical mold, following which the patties were placed in a fridge (4 °C) for 30 min to solidify the shape. The patties were grilled on a non-stick plate without adding additional oil until the internal temperature reached 71 °C as measured by a probe thermometer and were allowed to cool for 40 min at RT before further analysis. The patty formulation was optimized and finalized during preliminary experiments and standardized by the authors.

2.10. Determination of Cooking Properties

Cooking loss was determined by the percentage weight difference of a patty before and after cooking using the following equation:

$$\text{Cooking loss } (\%) = \frac{\text{raw patty weight (g)} - \text{ cooked patty weight (g)}}{\text{raw patty weight (g)}} \times 100 \quad (6)$$

The diameter shrinkage of the patties was determined by random measurement of the diameter at three different locations of the raw and cooked patties and was expressed according to the following equation:

$$\text{Diameter shrinkage } (\%) = \frac{\text{raw patty diameter (mm)} - \text{ cooked patty diameter (mm)}}{\text{raw patty diameter (mm)}} \times 100 \quad (7)$$

The moisture content of both raw and cooked patties was measured as described in Section 2.2 by drying 2 g samples at 135 °C for 2 h. The moisture retention was then calculated as below:

$$\text{Moisture retention } (\%) = \frac{\text{Cooked patty weight (g)} \times \text{ moisture in cooked patty (\%)}}{\text{Raw patty weight (g)} \times \text{ moisture in raw patty (\%)}} \times 100 \quad (8)$$

For fat retention, both raw and cooked patties were freeze dried to remove the water, and the fat in lyophilized patties was then extracted and determined, as in Section 2.2. Fat retention was quantified according to the following equation:

$$\text{Fat retention } (\%) = \frac{\text{Cooked patty weight (g)} \times \text{ fat in cooked patty (\%)}}{\text{Raw patty weight (g)} \times \text{ fat in raw patty (\%)}} \times 100 \quad (9)$$

All cooking measurements were performed in four replicates per TVP treatment, except for cooking loss. Cooking loss was determined using eight different patties for each TVP.

2.11. Textural Property of TVP-Based Patty

Texture profile analysis of the cooked patties was carried out following the same procedure as in Section 2.8. Four patties from each TVP treatment were assigned for the determination of TPA.

2.12. Shear Force Measurement

The shear force test was performed using the same texture analyzer assembled with a Warner–Bratzler Shear Blade (Stable Micro System, Godalming, Surrey, UK). Cooked patties were cut into 2 cm wide strips (around 1 cm thickness) before being sheared straight through the perpendicular cooked patty surface at a test speed of 5 mm/s. The corresponding

force–distance curves were recorded. The shear force value was collected as the maximal peak force of shearing. Each TVP treatment was analyzed in four strip replicates.

2.13. Compressed Juiciness

The compressed juiciness of cooked patties was evaluated following a previous method with slight modifications [5]. Approximately 1 cm^3 cubes were taken from cooked patties and were placed between two filter papers, followed by pressing for 30 s at 1000 g force using a TA-4 probe (1–1/2-inch diameter acrylic cylinder, 20 mm tall) equipped on a Texture Analyzer (Stable Micro System, Godalming, Surrey, UK). The weight of the samples was recorded before and after the compression and used to calculate the compressed juiciness as follows. Four replicates were tested for each treatment.

$$\text{Compressed juiciness } (\%) = \frac{\text{weight of sample before pressing } (g) - \text{ weight of sample after pressing}(g)}{\text{weight of sample before pressing } (g)} \times 100 \quad (10)$$

2.14. Statistical Analysis

Data were analyzed using one-way ANOVA by the SAS University Edition (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test was used for mean comparisons, and $p < 0.05$ was considered significantly different. Least significant difference (LSD) values were calculated at 5% level of significance. Pearson correlation coefficients were determined to investigate the relationships among variables.

3. Results and Discussion

3.1. Proximate Compositions of TVP

Proximate compositions, including protein, moisture, ash, fat and total carbohydrate contents of TVPs, are presented in Table 1. As shown, TVP samples varied significantly in protein content among the diverse protein sources, with textured pea proteins overall having the highest protein amount (samples 15–21, 62.4 to 76.6%), which was closely followed by textured wheat gluten (samples 22–25, 64.4 to 72.1%) and textured mixed proteins (samples 27–28, 66.3 to 68.3%). Textured soy proteins (samples 1–14, 50.0 to 55.8%) and the textured chickpea protein (50.4%) were the lowest in protein content. Protein is the most paramount component of TVP. A protein content of 50–70% is generally required to form fibrous structures during extrusion [21]. In addition, soy, in comparison with other proteins, such as pea protein, is relatively easier to texturize when forming fibrous structures at lower protein content, as evidenced by the fact that many soy-based TVPs are made from protein concentrates, while pea TVPs are derived from protein isolates [22–24]. Overall, the wide range of protein concentration (50.0 to 76.6%) in the studied samples enabled the formation of fibrous textures.

A similar tendency was also observed in fat content. TVPs derived from pea proteins exhibited a substantially higher fat content (in an average of 6.0%) when compared with textured soy proteins (in an average of 2.7%) or wheat gluten (in an average of 2.8%). The textured mixed proteins located in a high range of fat content (samples 27–28, 6.0 to 6.6%) as well, while the textured chickpea protein (2.0%) was in the lowest range. On the contrary, textured soy proteins registered the highest ash content (5.6 to 7.1%), followed by the descending order of textured pea proteins (3.8 to 5.6%), textured mixed proteins (4.8 to 4.9%), textured chickpea protein (4.6%) and textured wheat gluten (2.4 to 3.0%). The higher ash content possibly arose from a higher amount of minerals in the raw materials prior to texturization. The moisture content of TVPs differed significantly from 4.8 to 8.5%, although with no specific tendency observed among the various protein sources, which might result from the differences in the extrusion conditions and the post-drying processes. The total carbohydrate content was found highly oppositely correlating with protein content ($r = -0.984$, $p < 0.01$, Table 2. The textured soy proteins (27.4–35.4%) and textured chickpea protein (34.9%) exerted the highest total carbohydrate content. TVPs sourced from

wheat gluten (14.7–22.0%) and mixed proteins (14.8–18.2%) contained considerably lower amounts of carbohydrate, whereas textured pea proteins were observed in the lowest place (4.8–20.9%). The variations in chemical compositions of the TVPs are likely responsible for the differences in the physicochemical and textural properties of TVPs before and after formulating to patties.

Table 1. Proximate composition (as-is wet basis) of different TVPs.

Sample [A]	Protein Content (%)	Moisture Content (%)	Ash Content (%)	Fat Content (%)	Total Carbohydrate (%)
1	51.1 ± 0.1 ^{no}	7.2 ± 0.08 ^{f}	6.1 ± 0.02 ^{g}	2.2 ± 0.3 ^{jkl}	33.4
2	51.4 ± 0.2 ^{mn}	8.1 ± 0.03 ^{bc}	6.0 ± 0.03 ^{h}	2.5 ± 0.1 ^{hi}	32.0
3	51.1 ± 0.0 ^{no}	7.1 ± 0.09 ^{g}	6.2 ± 0.01 ^{f}	2.3 ± 0.0 ^{ijk}	33.4
4	52.1 ± 0.1 ^{l}	6.5 ± 0.07 ^{k}	6.3 ± 0.01 ^{d}	2.2 ± 0.0 ^{klm}	32.9
5	51.3 ± 0.2 ^{mn}	6.1 ± 0.06 ^{m}	6.5 ± 0.04 ^{b}	2.2 ± 0.2 ^{ijk}	33.9
6	55.7 ± 0.1 ^{k}	7.3 ± 0.07 ^{e}	7.1 ± 0.01 ^{a}	2.4 ± 0.1 ^{hij}	27.4
7	50.5 ± 0.5 ^{pq}	7.8 ± 0.02 ^{d}	6.4 ± 0.00 ^{cd}	2.1 ± 0.1 ^{klm}	33.2
8	51.0 ± 0.0 ^{no}	7.2 ± 0.02 ^{f}	5.6 ± 0.04 ^{i}	7.9 ± 0.2 ^{b}	28.3
9	50.0 ± 0.0 ^{q}	6.9 ± 0.03 ^{h}	6.4 ± 0.02 ^{c}	2.2 ± 0.0 ^{ijk}	34.4
10	51.5 ± 0.0 ^{mn}	7.8 ± 0.01 ^{d}	6.3 ± 0.02 ^{e}	1.9 ± 0.2 ^{m}	32.5
11	51.7 ± 0.1 ^{lm}	8.1 ± 0.02 ^{bc}	6.3 ± 0.02 ^{e}	2.3 ± 0.0 ^{ijk}	31.6
12	50.5 ± 0.1 ^{p}	6.7 ± 0.08 ^{i}	6.5 ± 0.02 ^{b}	2.1 ± 0.0 ^{klm}	34.2
13	50.7 ± 0.0 ^{op}	6.6 ± 0.01 ^{j}	6.2 ± 0.06 ^{f}	2.4 ± 0.1 ^{hij}	34.1
14	50.5 ± 0.0 ^{p}	5.4 ± 0.02 ^{p}	6.2 ± 0.02 ^{f}	2.5 ± 0.0 ^{h}	35.4
15	76.6 ± 0.3 ^{a}	5.8 ± 0.01 ^{o}	3.9 ± 0.02 ^{o}	4.9 ± 0.2 ^{e}	8.8
16	74.0 ± 0.3 ^{d}	6.0 ± 0.02 ^{n}	5.6 ± 0.01 ^{i}	5.0 ± 0.1 ^{e}	9.4
17	74.1 ± 0.2 ^{cd}	7.3 ± 0.03 ^{e}	5.5 ± 0.03 ^{j}	4.8 ± 0.2 ^{e}	8.3
18	75.1 ± 0.4 ^{b}	6.2 ± 0.01 ^{l}	5.4 ± 0.01 ^{j}	8.5 ± 0.1 ^{a}	4.8
19	74.5 ± 0.1 ^{c}	7.0 ± 0.02 ^{g}	5.1 ± 0.00 ^{k}	8.1 ± 0.0 ^{b}	5.3
20	62.4 ± 0.2 ^{j}	6.9 ± 0.03 ^{h}	3.8 ± 0.03 ^{p}	6.0 ± 0.2 ^{d}	20.9
21	71.8 ± 0.1 ^{e}	8.1 ± 0.04 ^{bc}	4.9 ± 0.04 ^{l}	4.9 ± 0.2 ^{e}	10.2
22	66.1 ± 0.4 ^{h}	8.2 ± 0.04 ^{b}	2.4 ± 0.01 ^{t}	2.8 ± 0.1 ^{g}	20.5
23	70.5 ± 0.1 ^{f}	8.1 ± 0.03 ^{c}	2.6 ± 0.08 ^{s}	3.1 ± 0.1 ^{f}	15.7
24	72.1 ± 0.1 ^{e}	7.3 ± 0.03 ^{e}	3.0 ± 0.04 ^{q}	2.9 ± 0.1 ^{fg}	14.7
25	64.4 ± 0.3 ^{i}	8.5 ± 0.05 ^{a}	2.7 ± 0.03 ^{r}	2.4 ± 0.0 ^{hij}	22.0
26	50.4 ± 0.0 ^{pq}	8.1 ± 0.01 ^{bc}	4.6 ± 0.01 ^{n}	2.0 ± 0.2 ^{lm}	34.9
27	68.3 ± 0.1 ^{g}	5.5 ± 0.03 ^{p}	4.9 ± 0.03 ^{l}	6.6 ± 0.1 ^{c}	14.8
28	66.2 ± 0.2 ^{h}	4.8 ± 0.01 ^{q}	4.8 ± 0.04 ^{m}	6.0 ± 0.1 ^{d}	18.2
Ave. soy	51.4 ± 1.4 c	7.9 ± 0.8 b	6.3 ± 0.3 a	2.7 ± 1.5 b	32.6 ± 2.3 a
Ave. pea	72.7 ± 4.7 a	6.8 ± 0.8 b	4.9 ± 0.8 b	6.0 ± 1.6 a	9.7 ± 5.4 c
Ave. wheat	68.3 ± 3.6 b	8.0 ± 0.5 a	2.7 ± 0.2 c	2.8 ± 0.3 b	18.2 ± 3.6 b
Average [B]	60.2	7.0	5.3	3.8	23.8
LSD (5%) [C]	0.4	0.1	0.1	0.2	-

[A] Protein types of samples: 1–14 (soy protein), 15–21 (pea protein), 22–25 (wheat gluten), 26 (chickpea protein), 27 (pea and chickpea protein mixture), 28 (pea and navy bean protein mixture). Means with different superscript letters within the same column are significantly different ($p < 0.05$) among samples 1–28. Different lowercase letters indicate significant difference among means of soy, pea and wheat gluten samples within the same column ($p < 0.05$). [B,C] Average values of all samples and least significant difference (LSD) for comparison of different samples.

Table 2. Pearson correlation coefficients ® for the relationships between properties of TVPs and TVP-based patties.

	PC	MC	AC	FC	CC	BD	RHC	WAC	OAC	PS	PV	PT	FV	T-H	T-R	T-CO	T-S	T-CH
PC	1	−0.162	−0.611**	0.628 **	−0.984 **	−0.661 **	0.237	−0.073	0.711 **	−0.775 **	0.057	−0.652 **	−0.274	−0.237	0.386 *	0.057	0.431 *	−0.182
MC		1	−0.221	−0.380 *	0.164	0.094	−0.226	0.250	−0.394 *	−0.191	0.055	−0.294	−0.190	0.095	−0.016	−0.163	0.035	0.080
AC			1	−0.205	0.518 **	0.529 **	−0.160	0.180	−0.351	0.857 **	−0.096	0.620 **	0.416 *	0.340	−0.270	0.014	−0.403 *	0.305
FC				1	−0.732 **	−0.609 **	0.374 *	−0.383 *	0.852 **	−0.195	−0.201	−0.321	−0.259	−0.170	0.214	0.418 *	0.342	−0.102
CC					1	0.674 **	−0.258	0.099	−0.763 **	0.687 **	−0.008	0.633 **	0.277	0.208	−0.375 *	−0.122	−0.431 *	0.149
BD						1	−0.221	0.393 *	−0.552 **	0.591 **	0.225	0.300	0.509 **	0.141	−0.665 **	−0.607 **	−0.724 **	−0.002
RHC							1	0.032	0.362	−0.019	0.554 **	−0.190	0.404 *	−0.765 **	−0.149	0.062	0.107	−0.737 **
WAC								1	−0.337	0.023	0.621 **	−0.041	0.549 **	0.102	−0.113	−0.446 *	−0.082	0.049
OAC									1	−0.325	−0.071	−0.513 **	−0.249	−0.234	0.239	0.312	0.181	−0.188
PS										1	−0.133	0.615 **	0.388 *	0.203	−0.387 *	0.084	−0.414 *	0.170
PV											1	−0.199	0.778 **	−0.470 *	−0.190	−0.470 *	−0.131	0.537 **
PT												1	0.190	0.271	−0.091	0.166	−0.127	0.273
FV													1	−0.277	−0.368	−0.432 *	−0.336	−0.361
T-H														1	0.217	0.192	0.025	0.977 **
T-R															1	0.646 **	0.786 **	0.343
T-CO																1	0.634 **	0.335
T-S																	1	0.167
T-CH																		1

Table 2. *Cont.*

	CL	DS	MR	FR	CJ	P-H	P-R	P-CO	P-S	P-CH	SF
PC	−0.073	−0.047	0.046	−0.173	0.149	−0.102	0.577 **	0.519 **	0.515 **	0.434 *	−0.094
MC	−0.302	−0.305	0.222	0.148	−0.033	0.050	0.078	0.411 *	0.372	0.249	0.097
AC	0.111	−0.016	−0.357	0.189	−0.257	0.318	−0.382 *	−0.620 **	−0.636 **	−0.252	0.273
FC	0.104	0.099	−0.116	−0.111	0.153	−0.108	0.279	0.214	0.216	0.181	0.009
CC	0.063	0.056	0.003	0.150	−0.136	0.074	−0.564 **	−0.497 **	−0.488 **	−0.440 **	0.045
BD	0.076	0.032	−0.105	0.040	−0.105	0.023	−0.506 **	−0.514 **	−0.323	−0.444 *	−0.147
RHC	0.679 **	0.648 **	−0.290	−0.415 *	0.812 **	−0.791 **	−0.147	0.405 *	0.387 *	−0.412 *	−0.621 **
WAC	0.341	0.076	−0.508 **	−0.361	0.177	−0.068	−0.196	−0.023	0.018	−0.242	−0.204
OAC	−0.007	−0.005	−0.088	−0.071	0.071	−0.104	0.419 *	0.191	0.286	0.255	−0.112
PS	0.243	0.137	−0.319	0.123	−0.147	0.108	−0.604 **	−0.686 **	−0.624 **	−0.488 **	0.066
PV	0.605 **	0.478 *	−0.391 *	−0.601 **	0.579 **	−0.599 **	−0.168	0.295	0.220	−0.396 *	−0.587 **
PT	0.116	0.134	−0.143	0.065	−0.191	0.186	−0.414 *	−0.528 **	−0.663 **	−0.360	0.189
FV	0.660 **	0.456 *	−0.530 **	−0.552 **	0.368	−0.443 *	−0.497 **	−0.185	−0.200	−0.629 **	−0.479 **
T-H	−0.495 **	−0.622 **	0.051	0.347	−0.722 **	0.885 **	0.076	−0.458 *	−0.536 **	0.365	0.639 **
T-R	−0.074	−0.309	−0.155	0.028	−0.408 *	0.398 *	0.595 **	0.244	−0.013	0.606 **	0.321
T-CO	−0.035	−0.210	−0.015	0.252	−0.212	0.261	0.246	0.027	−0.148	0.299	0.293
T-S	0.126	−0.090	−0.056	−0.082	−0.006	0.079	0.416 *	0.446 *	0.218	0.385 *	0.089

Table 2. *Cont.*

	CL	DS	MR	FR	CJ	P-H	P-R	P-CO	P-S	P-CH	SF
T-CH	−0.497 **	−0.644 **	0.077	0.401 *	−0.719 **	0.889 **	0.128	−0.416 *	−0.520 **	0.410 *	0.673 **
CL	1	0.786 **	−0.655 **	−0.684 **	0.595 **	−0.618 **	−0.445 *	0.012	−0.054	−0.634 **	−0.528 **
DS		1	−0.333	−0.528 **	0.721 **	−0.714 **	−0.412 *	0.047	−0.050	−0.654 **	−0.489 **
MR			1	0.560 **	−0.074	0.118	0.257	0.199	0.230	0.327	0.157
FR				1	−0.437 *	0.537 **	0.390 *	−0.179	−0.079	0.466 *	0.405 *
CJ					1	−0.883 **	−0.312	0.470 *	0.454 *	−0.540 **	−0.653 **
P-H						1	0.387 *	−0.379 *	−0.443 *	0.639 **	0.778 **
P-R							1	0.477 *	0.404 *	0.888 **	0.246
P-CO								1	0.819 **	0.388 *	−0.215
P-S									1	0.280	−0.400 *
P-CH										1	0.543 **
SF											1

Abbreviations: PC, Protein content; MC, Moisture content; AC, Ash content; FC, Fat content; BD, Bulk density; RHC, Rehydration capacity; WAC, Water absorption capacity; OAC, Oil absorption capacity; PS, Protein solubility; PV, Peak viscosity; FV, Final viscosity; T-H, TVP hardness; T-R, TVP resilience; T-CO, TVP cohesiveness; T-S, TVP springiness; T-CH, TVP chewiness; CL, Cooking loss; DS, Diameter shrinkage; MR, Moisture retention; FR, Fat retention; CJ, Compressed juiciness; P-H, Patty hardness; P-R, Patty resilience; P-CO, Patty cohesiveness; P-S, Patty springiness; P-CH, Patty chewiness; SF, Shear force. * $p < 0.05$; ** $p < 0.01$.

3.2. Physicochemical Characteristics of TVP

Protein solubility commonly functions as a vital indicator of the degree of protein texturization [11]. Upon extrusion cooking, the protein is thermally denatured, with a series of unfolding and aggregation, leading to a decrease in soluble protein. Thus, lower solubility of textured proteins is usually observed compared with their native counterparts [11,20]. The soluble protein content of the studied TVPs ranged significantly between 43.0 and 90.3%, as shown in Table 3. An ascending trend was observed as follows: wheat gluten-based TVPs (samples 22–25, 43.0 to 48.5%) < pea-based TVPs (samples 15–21, 59.7 to 73.5%) < soy-based TVPs (samples 1–14, 74.9 to 90.3%). Meanwhile, the chickpea-based TVP (sample 9, 79.5%) exerted comparable solubility to soy-based TVPs, while the protein solubility of pea/chickpea- (sample 27, 68.6%) and pea/navy bean- (sample 28, 67.1%) mixed protein based TVPs fell within the range of pea-based TVPs. The differences of solubility among the various protein sources may arise out of their intrinsically different molecular structures, as well as varying degrees of protein denaturation during extrusions with diverse conditions.

Table 3. Physicochemical properties of TVPs.

Sample [A]	WAC (g/g)	OAC (g/g)	Solubility (%)	Pasting Property		
				Peak Viscosity (cP)	Peak Time (min)	Final Viscosity (cP)
1	2.1 ± 0.01^{ij}	0.69 ± 0.01^{t}	81.4 ± 0.2^{e}	544 ± 1^{q}	7.0 ± 0.0^{a}	1263 ± 17^{o}
2	2.1 ± 0.00^{fgh}	0.71 ± 0.01^{rs}	77.1 ± 0.6^{j}	651 ± 4^{p}	7.0 ± 0.0^{a}	1519 ± 4^{n}
3	2.2 ± 0.02^{efg}	0.77 ± 0.00^{l}	77.6 ± 0.1^{j}	2786 ± 4^{g}	7.0 ± 0.0^{a}	5832 ± 52^{d}
4	2.1 ± 0.05^{gh}	0.70 ± 0.01^{rs}	82.9 ± 0.1^{d}	2848 ± 9^{g}	7.0 ± 0.0^{a}	6049 ± 8^{c}
5	2.2 ± 0.03^{ef}	0.74 ± 0.02^{nop}	83.6 ± 0.1^{c}	2938 ± 9^{f}	7.0 ± 0.0^{a}	6674 ± 60^{b}
6	2.3 ± 0.01^{d}	0.78 ± 0.01^{k}	81.5 ± 0.2^{e}	1448 ± 16^{m}	7.0 ± 0.0^{a}	2415 ± 35^{k}
7	2.1 ± 0.02^{hi}	0.74 ± 0.01^{op}	80.5 ± 0.2^{f}	3168 ± 10^{e}	7.0 ± 0.0^{a}	6088 ± 16^{c}
8	2.0 ± 0.02^{l}	0.84 ± 0.00^{g}	85.0 ± 0.1^{b}	1703 ± 18^{k}	6.6 ± 0.1^{a}	2876 ± 31^{j}
9	1.9 ± 0.02^{l}	0.76 ± 0.00^{lm}	90.3 ± 0.1^{a}	2084 ± 16^{i}	7.0 ± 0.0^{a}	4615 ± 24^{g}
10	2.2 ± 0.00^{e}	0.69 ± 0.01^{st}	74.9 ± 0.3^{k}	502 ± 4^{q}	7.0 ± 0.0^{a}	1305 ± 3^{o}
11	2.2 ± 0.04^{fg}	0.76 ± 0.01^{lmn}	85.3 ± 0.1^{b}	2132 ± 17^{i}	7.0 ± 0.0^{a}	4962 ± 18^{f}
12	2.1 ± 0.02^{jk}	0.73 ± 0.01^{pq}	85.1 ± 0.1^{b}	2678 ± 29^{h}	7.0 ± 0.0^{a}	6901 ± 30^{a}
13	1.9 ± 0.02^{l}	0.72 ± 0.01^{qr}	78.2 ± 0.1^{i}	2152 ± 16^{i}	7.0 ± 0.0^{a}	4470 ± 39^{h}
14	2.0 ± 0.02^{k}	0.80 ± 0.00^{jk}	78.8 ± 0.3^{h}	2682 ± 21^{h}	7.0 ± 0.0^{a}	5240 ± 53^{e}
15	2.9 ± 0.05^{a}	0.92 ± 0.00^{d}	62.8 ± 0.3^{p}	4175 ± 159^{b}	4.8 ± 0.6^{de}	6957 ± 56^{a}
16	2.0 ± 0.01^{jk}	0.95 ± 0.01^{c}	60.6 ± 0.1^{q}	546 ± 1^{q}	5.0 ± 0.1^{bcd}	596 ± 3^{r}
17	2.6 ± 0.02^{b}	0.83 ± 0.01^{gh}	60.8 ± 0.2^{q}	3767 ± 1^{c}	4.5 ± 0.3^{ef}	4958 ± 59^{f}
18	1.5 ± 0.02^{q}	0.98 ± 0.01^{b}	68.0 ± 0.2^{n}	532 ± 1^{q}	7.0 ± 0.0^{a}	1024 ± 3^{p}
19	1.5 ± 0.01^{p}	1.04 ± 0.01^{a}	68.0 ± 0.0^{n}	1792 ± 4^{j}	2.2 ± 0.5^{g}	2969 ± 8^{i}
20	1.7 ± 0.01^{o}	0.97 ± 0.01^{b}	73.5 ± 0.0^{l}	659 ± 7^{p}	5.3 ± 0.0^{b}	848 ± 1^{q}
21	2.5 ± 0.04^{c}	0.82 ± 0.01^{hi}	59.7 ± 0.4^{r}	3488 ± 31^{d}	4.3 ± 0.0^{f}	5822 ± 30^{d}
22	2.1 ± 0.01^{ij}	0.86 ± 0.01^{f}	47.0 ± 0.2^{t}	4252 ± 52^{a}	4.7 ± 0.0^{de}	2424 ± 20^{k}
23	1.7 ± 0.02^{n}	0.76 ± 0.01^{lm}	48.5 ± 0.0^{s}	1061 ± 11^{o}	4.9 ± 0.0^{cde}	867 ± 4^{q}
24	1.7 ± 0.03^{o}	0.75 ± 0.01^{mno}	44.7 ± 0.6^{u}	1353 ± 8^{n}	5.2 ± 0.0^{bc}	1061 ± 10^{p}
25	2.2 ± 0.01^{e}	0.80 ± 0.01^{ij}	43.0 ± 0.4^{v}	2713 ± 1^{h}	4.8 ± 0.1^{cde}	1829 ± 8^{l}
26	1.8 ± 0.01^{m}	0.79 ± 0.00^{jk}	79.5 ± 0.1^{g}	1348 ± 6^{n}	4.2 ± 0.1^{f}	1581 ± 12^{m}
27	1.6 ± 0.02^{p}	0.90 ± 0.00^{e}	68.6 ± 0.2^{m}	1120 ± 0^{o}	7.0 ± 0.0^{a}	1783 ± 4^{l}
28	1.5 ± 0.02^{p}	0.92 ± 0.01^{de}	67.1 ± 0.3^{o}	1541 ± 13^{l}	7.0 ± 0.0^{a}	2913 ± 1^{ij}
Ave. soy	2.1 ± 0.1 a	0.74 ± 0.04 b	81.6 ± 4.1 a	2022 ± 925 a	7.0 ± 0.1 a	4300 ± 2037 a
Ave. pea	2.1 ± 0.6 a	0.93 ± 0.08 a	64.8 ± 5.2 b	2137 ± 1635 a	4.7 ± 1.4 b	3310 ± 2617 ab
Ave. wheat	1.9 ± 0.3 a	0.79 ± 0.05 b	45.8 ± 2.5 c	2345 ± 1461 a	4.9 ± 0.2 b	1545 ± 718 b
Average [B]	2.0	0.81	71.2	2024	6.0	3423
LSD (5%) [C]	0.04	0.02	0.5	70	0.4	59

Abbreviations: WAC, water absorption capacity of TVP powder; OAC, oil absorption capacity of TVP powder. [A] Protein types of samples: 1–14 (soy protein), 15–21 (pea protein), 22–25 (wheat gluten), 26 (chickpea protein), 27 (pea and chickpea protein mixture), 28 (pea and navy bean protein mixture). Means with different superscript letters within the same column are significantly different ($p < 0.05$) among samples 1–28. Different lowercase letters indicate significant difference among means of soy, pea and wheat gluten samples within the same column ($p < 0.05$). [B,C] Average values of all samples and least significant difference (LSD) for comparison of different samples.

A significantly negative relationship existed between the protein solubility and the protein content ($r = -0.775$, $p < 0.01$), as presented in Table 2. Indeed, a higher protein content could possibly contribute to a greater extent of protein denaturation during extrusion cooking, which resulted in an increase in protein texturization and insoluble proteins, thus lowering the solubility [11,18,20]. Moreover, the intermolecular disulfide bond was suggested as the major force being responsible for the fiber formation of TVP [25,26]. In contrast to legume proteins, wheat gluten contains relatively higher levels of methionine and cysteine [11]. Such sulfur-containing amino acid residues are likely to result in more disulfide cross linkages during texturization, which thereby lead to an increment of molecular weight and the insolubility of proteins [27]. This could possibly explain the lowest protein solubility of the textured wheat gluten samples (in an average of 45.8%) in the current study. However, the structures of extrudates are complex and are usually stabilized by the collective contributions of hydrophobic interactions, hydrogen bonds, disulfide bonds and their interactions [28]. Studies also showed that the importance of non-covalent bonds outweighed covalent bonds [28]. Overall, a lower protein solubility after extrusion is usually concluded as a greater protein denaturation and texturization.

WAC or OAC indicates the ability of a sample to absorb water or oil at the macromolecular level. The amphiphilicity of a protein enables its ability to interact with both water and oil [29]. As such, WAC and OAC are reliant on the availability of polar and non-polar amino acid residues, as well as the protein's micro- and macro-structures [29]. A lower presence of hydrophilic and polar amino acids over the surface of the protein molecule contributes to lower WAC, while higher availability of hydrophobic residues is responsible for higher OAC. Table 3 shows the WAC of TVPs varying from 1.5 to 2.9 g/g, being independent of protein types or protein contents but potentially associated with the available amounts of polar amino acids in each sample. Meanwhile, an improved entrapment of water has been reported as a consequence of the formation of a protein matrix that is induced by protein denaturation during extrusion [24]. In this study, the wheat-gluten-based TVPs may take great advantages of this phenomenon, as wheat gluten exerted statistically lower protein content but exhibited comparable WAC to that of pea-based TVPs (in an average of 2.1 and 1.9 g/g, respectively). Apart from proteins, the higher carbohydrate contents in the current extrudate samples may also play an important role in the WAC results, since more starch granules were able to absorb more water after gelatinization [9], which might account for the similar WAC of textured soy proteins (in an average of 2.1 g/g) to that of textured pea proteins, although the former were significantly low in protein content (Table 1).

It is worth noting that OAC was substantially greater for TVPs derived from pea proteins (samples 15–21, 0.82 to 1.04 g/g) than those made with wheat gluten (samples 22–25, 0.75 to 0.86 g/g) or soy proteins (samples 1–14, 0.69 to 0.84), which occurred possibly due to a higher content of hydrophobic amino acids in pea proteins (30.26 g/100 g protein) than in others (28.23 g/100 g protein for wheat gluten and 26.21 g/100 g protein for soy protein), as confirmed by Samard and Ryu [11]. Moreover, OAC was found to positively correlate with fat content ($r = 0.852$, $p < 0.01$) and protein content ($r = 0.711$, $p < 0.01$) of TVPs (Table 2). Joshi et al. [30] found that full-fat oilseed flours exhibited lower OAC than their defatted counterparts, as the removal of the fat greatly improved the protein proportion, thus allowing better capillary attraction between the protein and the oil [31]. However, a relatively higher fat content, which was not able to significantly lower the protein content, favored the OAC results in the current study, as the non-polar lipid may enhance the interactions with oil on the basis that protein was the predominant composition governing the OAC of the studied TVPs. On the other hand, WAC and OAC may associate with the extent of denaturation, as extrusion cooking results in the unfolding of proteins and the exposure of more hydrophobic sites [24]. Thus, increasing the protein concentrations may not only contribute to a higher amount of hydrophobic amino acids but is also potentially responsible for the greater extent of protein denaturation induced by extrusion, thereby introducing more available hydrophobic sites, which contribute to greater OAC values. Osen et al. [24] reported that extrusion heat treatment enhanced the

OAC of pea protein isolate due to the exposure of more hydrophobic sites. Meanwhile, the polar carbohydrates may, on the other hand, have a negative effect on the extent of interactions with oil, as shown an opposite relationship between carbohydrate content and OAC ($r = -0.763$, $p < 0.01$, Table 2). In summary, WAC and OAC are multifactor dependent, including protein composition, protein denaturation, as well as the extent of interactions with water and oil [24].

3.3. RVA Pasting Properties of TVP

Viscosity plays a crucial role in altering the flow behavior and the mechanical energy input in extrusion cooking [32]. In this study, RVA pasting profiles were obtained to understand the viscosity properties of proteins after texturization. As shown in Table 3, the TVPs behaved dramatically differently upon hydration, heating and cooling under a slow shear. During heating, all samples, regardless of the protein types, endured vast elevation in their viscosities, achieving significantly different peak viscosities ranging from 502 to 4252 cP. However, the peak viscosities were diminished to some extent from the shear in the case of textured wheat gluten (samples 22–25), as indicated by the lower final viscosities compared with their corresponding peak viscosities. Differing from this, the TVPs derived from other sources were increasing in viscosity throughout the holding and cooling, implying their better abilities against shear thinning, while forming viscous pastes or gels upon cooling, which may benefit the texture of the final products. The reduction in the final viscosity could possibly be related to the low protein solubility of textured wheat gluten (Table 3) on the basis of understanding that lower protein solubility is indicative of a more complete texturization, thus a higher denaturation degree, as stated earlier, and the already denatured proteins may have induced weaker protein–protein interactions upon heating, which weakened the resistance to shearing and thereby decreased the final viscosities.

Despite distinct variations in viscosities, both peak viscosity and final viscosity were found positively correlating with WAC ($r = 0.621$ and 0.549, respectively, $p < 0.01$, Table 2). This finding is in line with previous studies, where a protein with higher WAC was able to absorb more water, which resulted in higher viscosities [24]. On the other hand, in contrast with pea or wheat gluten, textured soy proteins (samples 1–14) generally required a longer time (ranging from 6.6 to 7.0 min) to achieve peak viscosity, indicating that soy proteins need more time to hydrate and bind water and higher temperatures to denature before reaching the maximum viscosities. This result may be attributed to the relatively higher carbohydrate amount in such samples (Table 1), which may interfere with the hydration and swelling process of proteins, thus retarding the denature time ($r = 0.633$ between carbohydrate and peak time, $p < 0.01$, Table 2).

3.4. Bulk Density

The bulk density of TVP products interprets the overall expansion and changes in the protein network [12]. The studied TVP samples displayed a wide range in bulk density, as shown in Table 4, with sample 26 (chickpea protein) being the highest (453 g/L), and sample 27 (pea/chickpea mixture proteins) being the lowest (153 g/L). TVPs derived from soy proteins (samples 1–14) generally exhibited higher bulk density, going from 238 to 384 g/L, than those derived from pea proteins (samples 15–21, from 187 to 303 g/L) or wheat gluten (samples 22–25, from 211 to 222 g/L). Conventionally, higher protein content has been shown to undergo a higher degree of protein cross-linking and forming strong structures, which prevents expansion, thus increasing bulk density [33]. However, in this case, the different intrinsic properties of the raw material may make greater contributions to bulk density. As stated earlier, in contrast to other proteins, soy protein usually exerts a better ability to texturize and forms stronger structures, which result in a higher bulk density. Moreover, the wide spectrum of bulk density may also result from other extrusion variables, such as feed moisture, extruder barrel temperature and screw speed [12,13].

Table 4. Rehydration and textural properties of TVP.

Sample [A]	Bulk Density (g/L)	RHC (g/g)	Textural Property				
			Hardness (g)	Resilience (%)	Cohesiveness	Springiness (%)	Chewiness (g)
1	238 ± 6 ^{kl}	2.4 ± 0.03 ^{ij}	1061 ± 79 ^{d}	36.2 ± 0.5 ^{ab}	0.72 ± 0.02 ^{a}	100.1 ± 4.6 ^{abc}	878 ± 78 ^{c}
2	295 ± 10 ^{f}	1.5 ± 0.04 ^{n}	2428 ± 118 ^{a}	33.1 ± 1.2 ^{efg}	0.66 ± 0.01 ^{bc}	94.8 ± 2.1 ^{bcdefg}	1530 ± 53 ^{a}
3	317 ± 5 ^{e}	2.8 ± 0.05 ^{h}	975 ± 47 ^{e}	27.2 ± 0.5 ^{k}	0.65 ± 0.01 ^{bc}	88.1 ± 1.1 ^{ijk}	578 ± 10 ^{e}
4	353 ± 5 ^{c}	2.9 ± 0.01 ^{gh}	734 ± 26 ^{gh}	29.1 ± 1.7 ^{ijk}	0.61 ± 0.00 ^{fgh}	93.8 ± 3.4 ^{defghi}	367 ± 17 ^{hij}
5	356 ± 7 ^{c}	3.0 ± 0.08 ^{fg}	670 ± 15 ^{hijk}	29.1 ± 0.9 ^{ijk}	0.56 ± 0.01 ^{ij}	89.6 ± 2.4 ^{ghijk}	399 ± 14 ^{gh}
6	343 ± 3 ^{cd}	2.3 ± 0.04 ^{jk}	940 ± 47 ^{e}	32.1 ± 1.1 ^{fg}	0.63 ± 0.02 ^{cdef}	90.0 ± 2.1 ^{ghijk}	561 ± 0 ^{e}
7	330 ± 8 ^{de}	3.0 ± 0.07 ^{fg}	604 ± 44 ^{klm}	23.7 ± 0.8 ^{l}	0.59 ± 0.01 ^{hi}	85.3 ± 1.4 ^{k}	317 ± 12 ^{jk}
8	259 ± 11 ^{ik}	3.5 ± 0.09 ^{cd}	601 ± 15 ^{klm}	28.8 ± 0.5 ^{ijk}	0.70 ± 0.02 ^{a}	100.7 ± 1.5 ^{ab}	391 ± 43 ^{hi}
9	279 ± 5 ^{g}	3.8 ± 0.12 ^{b}	537 ± 38 ^{mno}	34.3 ± 0.2 ^{cde}	0.72 ± 0.01 ^{a}	98.7 ± 4.2 ^{abcde}	365 ± 8 ^{hij}
10	330 ± 3 ^{de}	1.8 ± 0.02 ^{m}	1618 ± 41 ^{b}	30.3 ± 1.0 ^{hi}	0.65 ± 0.02 ^{bc}	93.0 ± 7.2 ^{efghi}	1007 ± 55 ^{b}
11	325 ± 1 ^{e}	3.0 ± 0.03 ^{fg}	525 ± 37 ^{mno}	27.7 ± 0.5 ^{k}	0.64 ± 0.00 ^{bcde}	92.0 ± 4.6 ^{fghij}	343 ± 10 ^{hijk}
12	384 ± 14 ^{b}	3.0 ± 0.09 ^{fg}	679 ± 44 ^{hijk}	27.3 ± 1.2 ^{k}	0.59 ± 0.01 ^{hi}	85.0 ± 2.8 ^{k}	343 ± 22 ^{hijk}
13	354 ± 10 ^{c}	2.5 ± 0.06 ^{i}	842 ± 29 ^{f}	29.64± 1.3 ^{ij}	0.62 ± 0.02 ^{defg}	88.1 ± 0.9 ^{ijk}	459 ± 2 ^{fg}
14	319 ± 14 ^{e}	3.3 ± 0.05 ^{e}	623 ± 19 ^{jkl}	23.8 ± 0.4 ^{l}	0.63 ± 0.02 ^{cdef}	84.6 ± 2.9 ^{k}	320 ± 20 ^{ijk}
15	303 ± 9 ^{f}	3.5 ± 0.05 ^{d}	801 ± 36 ^{fg}	33.6 ± 0.5 ^{def}	0.62 ± 0.02 ^{defg}	98.2 ± 5.6 ^{abcde}	478 ± 17 ^{f}
16	247 ± 3 ^{jk}	2.0 ± 0.02 ^{l}	1258 ± 49 ^{c}	36.4 ± 0.7 ^{ab}	0.66 ± 0.01 ^{b}	95.9 ± 1.6 ^{abcdef}	815 ± 1 ^{d}
17	281 ± 6 ^{g}	3.8 ± 0.05 ^{b}	400 ± 16 ^{p}	22.0 ± 0.5 ^{m}	0.53 ± 0.02 ^{k}	87.0 ± 4.4 ^{jk}	208 ± 22 ^{l}
18	202 ± 9 ^{o}	3.1 ± 0.12 ^{f}	829 ± 35 ^{f}	35.7 ± 0.6 ^{bc}	0.71 ± 0.01 ^{a}	95.4 ± 1.5 ^{bcdefg}	572 ± 7 ^{e}
19	187 ± 12 ^{p}	3.2 ± 0.02 ^{e}	716 ± 32 ^{hi}	35.2 ± 1.2 ^{bcd}	0.70 ± 0.01 ^{a}	94.7 ± 2.1 ^{cdefgh}	470 ± 18 ^{f}
20	230 ± 5 ^{lm}	2.9 ± 0.07 ^{h}	638 ± 30 ^{ijkl}	28.4 ± 1.5 ^{jk}	0.64 ± 0.02 ^{bcdef}	89.0 ± 3.7 ^{hijk}	373 ± 7 ^{hij}
21	273 ± 2 ^{gh}	3.5 ± 0.06 ^{d}	510 ± 17 ^{no}	29.5 ± 0.2 ^{ij}	0.61 ± 0.02 ^{efgh}	98.1 ± 2.7 ^{abcde}	286 ± 2 ^{k}
22	216 ± 4 ^{mn}	3.6 ± 0.08 ^{c}	452 ± 38 ^{op}	37.5 ± 1.6 ^{a}	0.66 ± 0.02 ^{bc}	100.5 ± 1.2 ^{abc}	281 ± 25 ^{k}
23	222 ± 7 ^{mn}	2.8 ± 0.05 ^{h}	515 ± 30 ^{no}	31.7 ± 0.7 ^{gh}	0.64 ± 0.02 ^{bcd}	101.7 ± 1.5 ^{a}	340 ± 4 ^{hijk}
24	211 ± 8 ^{no}	2.2 ± 0.06 ^{k}	573 ± 27 ^{lmn}	32.6 ± 1.0 ^{fg}	0.59 ± 0.02 ^{gh}	97.0 ± 3.4 ^{abcdef}	335 ± 41 ^{hijk}
25	215 ± 9 ^{no}	2.8 ± 0.05 ^{h}	506 ± 34 ^{no}	36.9 ± 2.3 ^{ab}	0.64 ± 0.01 ^{bcd}	95.3 ± 1.6 ^{bcdefg}	325 ± 4 ^{ijk}
26	453 ± 11 ^{a}	2.9 ± 0.09 ^{gh}	621 ± 43 ^{jkl}	16.4 ± 0.5 ^{n}	0.56 ± 0.02 ^{j}	79.1 ± 3.1 ^{l}	276 ± 11 ^{k}
27	153 ± 1 ^{q}	4.2 ± 0.10 ^{a}	477 ± 22 ^{op}	31.9 ± 0.6 ^{gh}	0.72 ± 0.01 ^{a}	99.0 ± 1.9 ^{abcd}	332 ± 16 ^{hijk}
28	264 ± 3 ^{hi}	2.8 ± 0.03 ^{h}	695 ± 61 ^{hij}	28.3 ± 0.6 ^{jk}	0.65 ± 0.01 ^{bc}	93.6 ± 1.9 ^{defghi}	464 ± 64 ^{f}
Ave. soy	320 ± 41 a	2.8 ± 0.6 a	917 ± 522 a	29.5 ± 3.6 b	0.64 ± 0.05 a	91.7 ± 5.5 b	561 ± 350 a
Ave. pea	246 ± 43 b	3.1 ± 0.6 a	736 ± 277 a	31.5 ± 5.2 ab	0.64 ± 0.06 a	94.0 ± 4.4 ab	457 ± 200 a
Ave. wheat	216 ± 5 b	2.9 ± 0.6 a	512 ± 49.55 a	34.7 ± 3.0 a	0.63 ± 0.03 a	98.6 ± 3.0 a	320 ± 27 a
Average [B]	284	2.9	780	30.3	0.64	93.1	479
LSD (5%) [C]	13	0.1	74	1.6	0.02	5.0	61

Abbreviations: RHC, rehydration capacity of TVP. [A] Protein types of samples: 1–14 (soy protein), 15–21 (pea protein), 22–25 (wheat gluten), 26 (chickpea protein), 27 (pea and chickpea protein mixture), 28 (pea and navy bean protein mixture). Means with different superscript letters within the same column are significantly different ($p < 0.05$) among samples 1–28. Different lowercase letters indicate significant difference among means of soy, pea and wheat gluten samples within the same column ($p < 0.05$). [B,C] Average values of all samples and least significant difference (LSD) for comparison of different samples.

3.5. Rehydration Property

Water is critical in meat products to endow the appropriate texture and juiciness, so as to ensure customer acceptability. RHC, referring to the amount of water that could be held by the intact TVP upon rehydration, is an imperative factor affecting the meat-like texture of plant-based meat analogs [9]. In the current study, the RHC values of all samples were significantly different to each other, from 1.5 to 4.2 g/g, as demonstrated in Table 4. Differences in RHC are dependent on protein types, interactions between protein-water molecules, and water–water molecules [11,20] but are more closely related to the product structure, in particular, the porosity and air cell size [9,20]. Here, the external appearance and internal structure of TVPs after hydration are distinguished in Figures 1 and 2. As shown, the TVP samples showed porous structures with various sizes and numbers of air

cells, which may have resulted from the different degrees of expansion during extrusion. It is worth mentioning that the images were taken as their naturally displayed directions (longitudinal or horizontal cross sections of extrusion), since the current samples were commercially obtained and were difficult to cut purposely due to the limitation of their shape and size. This could explain why some TVPs exhibited more elongated cells, while others had pores with smaller diameters (Figure 2). Diverging from some previous studies that related a higher RHC to a lower bulk density, as products with low bulk density may possess higher porosity, which allows for faster water uptake and consequently leads to a better water-holding capacity [9,11], no such clear correlation occurred among the current samples. More compact products, such as samples 8, 14, 17, 21, 22, were also able to retain a great amount of water, as evidenced by the relatively high RHC values (3.3 to 3.8 g/g), while lower RHC also occurred in more porous and fibrous structures (samples 1, 2, 6, 10, 13, 16, 24, from 1.5 to 2.5 g/g). The inconsistence may be due to the difference in determining the RHC. The comparatively longer draining time in the current study (1 h) may permit more water to drain off from the more porous protein network, as a higher number of air cells is likely to result in easier water release caused by the gravitational force, whereas a shorter draining time possibly only allows water to drip and evaporate from the surface. In addition to the pore number, the size of the air space is also important to retain water [32].

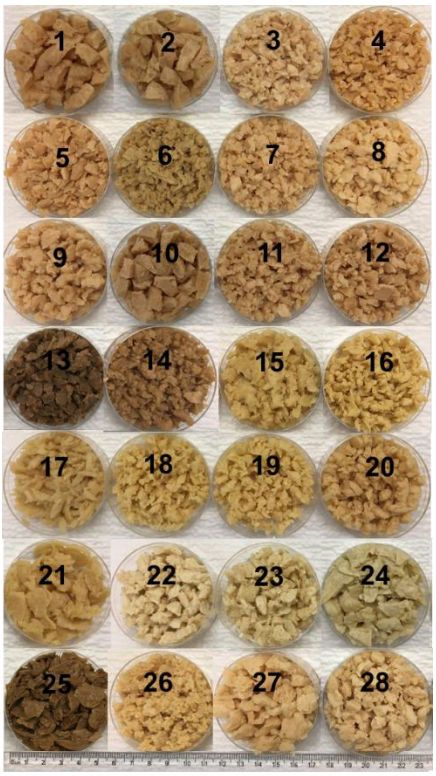

Figure 1. Visible appearance of different commercial TVPs. TVP types: 1–14: textured soy proteins; 15–21: textured pea proteins; 22–25: textured wheat gluten; 26: textured chickpea protein; 27: textured pea/chickpea mixed proteins; 28: textured pea/navy bean mixed proteins.

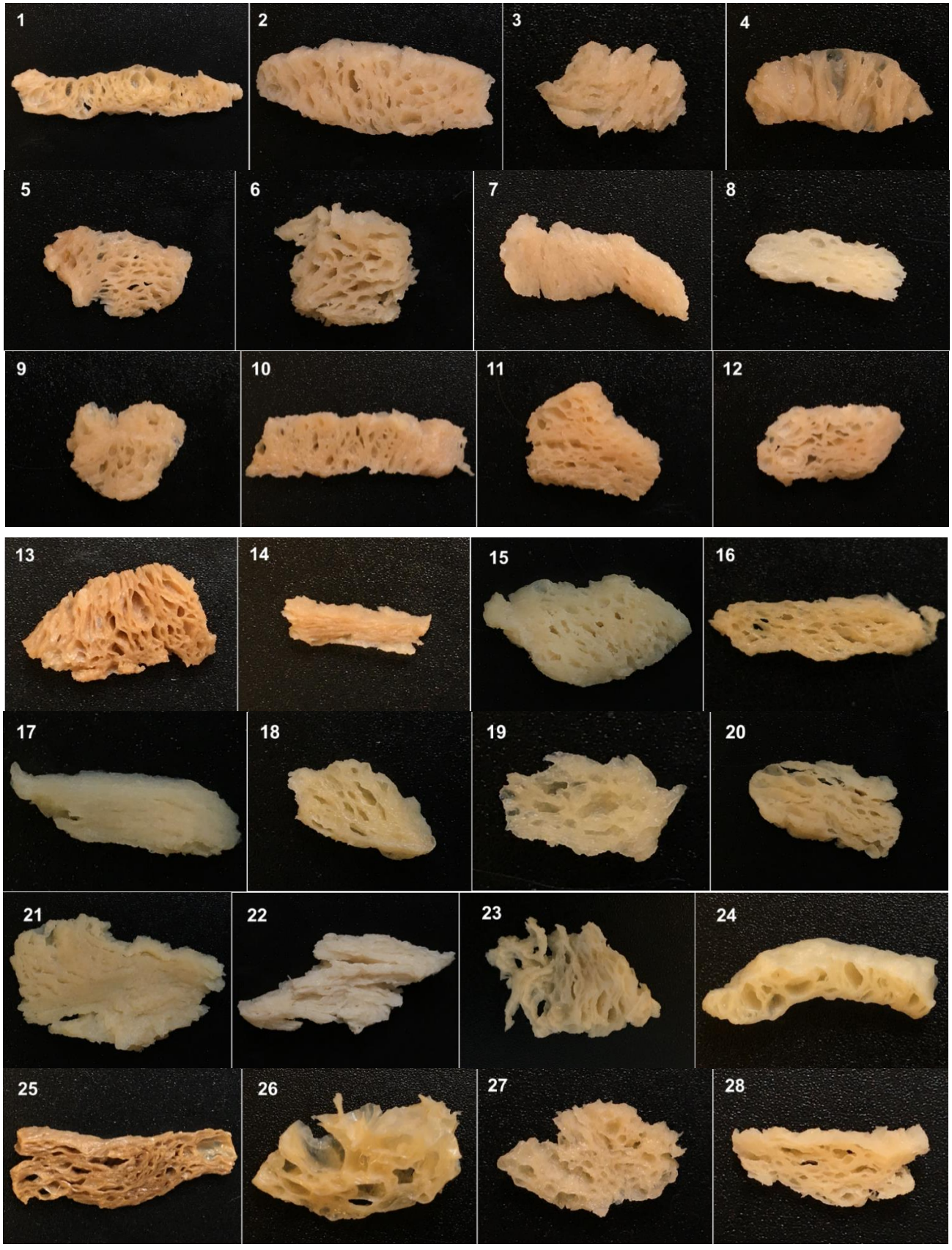

Figure 2. Structures of different commercial TVPs after hydration. TVP types: 1–14: textured soy proteins; 15–21: textured pea proteins; 22–25: textured wheat gluten; 26: textured chickpea protein; 27: textured pea/chickpea mixed proteins; 28: textured pea/navy bean mixed proteins.

3.6. Textural Properties of TVP

Texture is undoubtedly the most crucial attribute characterizing the quality of textured plant proteins, since a desirable texture that mimics the real meat is the main task of meat analogs. Table 4 shows the textural properties of hydrated TVPs in terms of hardness, resilience, cohesiveness, springiness and chewiness. Hardness is the maximum force required to attain a defined deformation [34]. It differed in a wide range, varying from 400 to 2428 g, among the studied samples (Table 4). Hardness may be indicative of the degree of protein texturization [20]. In this sense, a higher presence of protein content in the starting material is assumed to increase the degree of texturization and protein cross-linking, which prevents further expansion and leads to a higher hardness [20]. Webb et al. found that hardness decreased with the increasing inclusion of chickpea flour, from 10% to 30%, which interfered with the protein–protein interactions [9]. In addition, the hardness and RHC of TVPs appeared to be negatively correlated ($r = -0.765$, $p < 0.01$, Table 2), agreeing with some previous studies [9,26] that extensive hydration of TVP usually leads to a softer texture [35,36]. Additionally, the diversity of hardness may arise from the various processing variables. Rising barrel temperature and lowering feed moisture have been reported to associate with higher hardness [37]. Overall, it is rather difficult to manifest a clear clue addressing the wide range of hardness here, since all the studied samples came from different commercial sources and were made under diverse extrusion conditions.

Resilience measures how a sample recovers from deformation with regard to speed and forces. As shown in Table 4, resilience values extended from 16.4 to 37.5%, displaying no specific tendency among the protein sources, although being inversely correlated with bulk density ($r = -0.665$, $p < 0.01$, Table 2). Products with higher bulk density potentially possess more compact structures, which likely impair the resilience. Here, TVP samples exhibited relatively high springiness, going from 79.1 to 100.5%, suggesting good abilities of TVPs to regain their original form after compression. Likewise, springiness was negatively related to bulk density, with $r = -0.724$ ($p < 0.01$, Table 2). The lower bulk density benefits a higher porosity and loose structure, thereby enhancing the springiness. Cohesiveness indicates the strength of internal bonds and inter- and intra-actions constituting the product [34]. Samples exhibited a cohesiveness of 0.53 to 0.72 in the current study, which might be a response to the different degree of interactions formed during texturization and rehydration [12]. In addition, chewiness represents the energy necessary to masticate a solid product for swallowing [34]. As expected, the wide spectrum of chewiness (276 to 1530 g) positively corresponded with hardness ($r = 0.977$, $p < 0.01$). The lower chewiness may largely be a result of a higher RHC, which leads to a softer texture ($r = -0.737$ between chewiness and RHC, $p < 0.01$, Table 2).

3.7. Cooking Properties of TVP-Based Patties

The visible appearance of TVP-based patties before and after cooking is presented in Figure 3. It is worth mentioning that the patties in this study had the same formulation. In addition to the different types of TVPs, all the other ingredients (salt, pigment, binder, etc.) were added in the same amounts. Thus, the diverse properties of patties were assumed to result from the various properties of the TVPs. The effect of cooking on patties was investigated by measuring cooking loss, diameter shrinkage, moisture retention and fat retention. Cooking loss is an important parameter evaluating the textural and sensorial attributes of meat products with regard to juiciness, tenderness and also the yield of the final product [35]. It is mainly caused by the loss of liquid (moisture and fat) during the cooking process [38] and is linked to different variables, such as cooking time, temperature and method, type and amount of particular ingredients in the formulation [39,40].

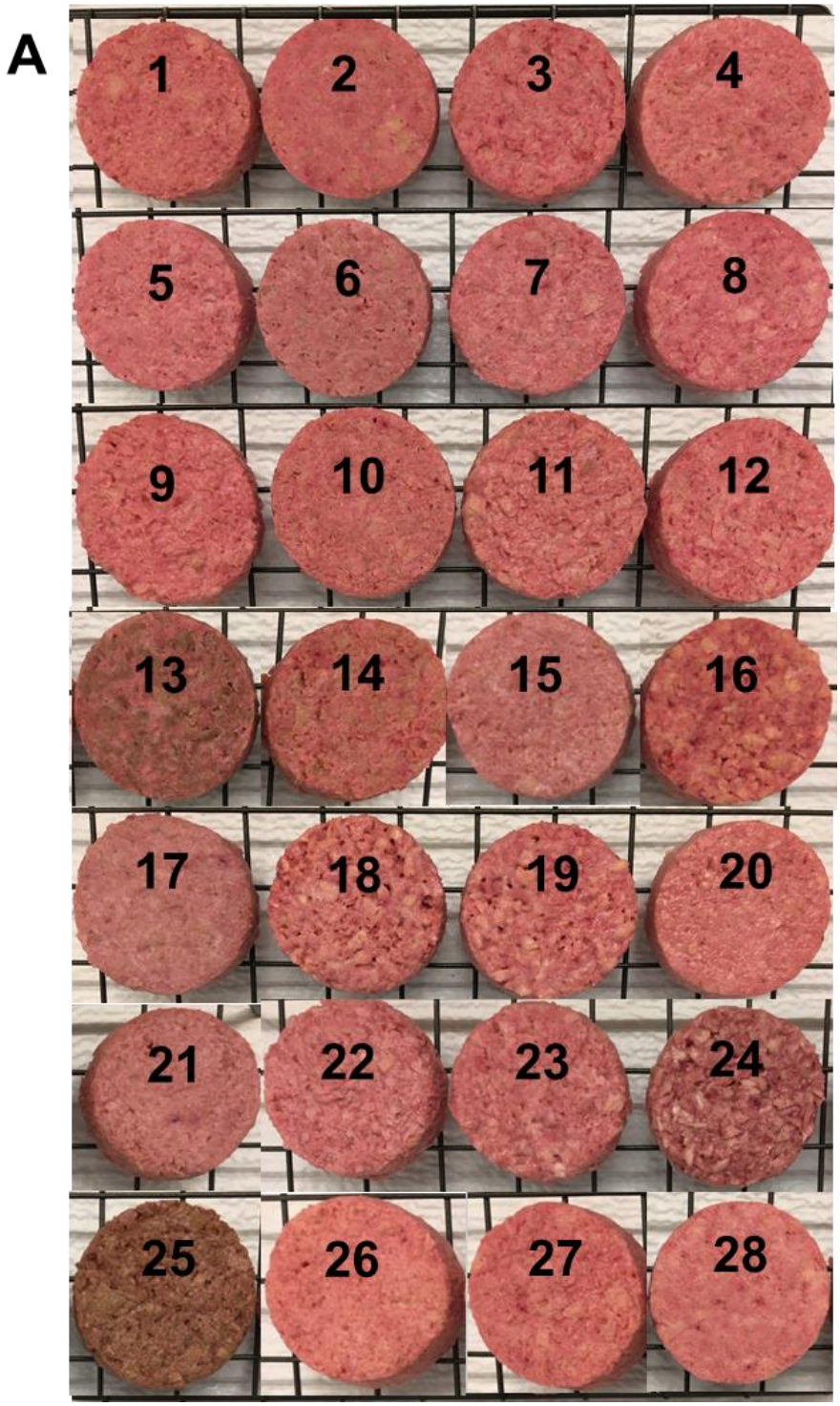

Figure 3. *Cont*.

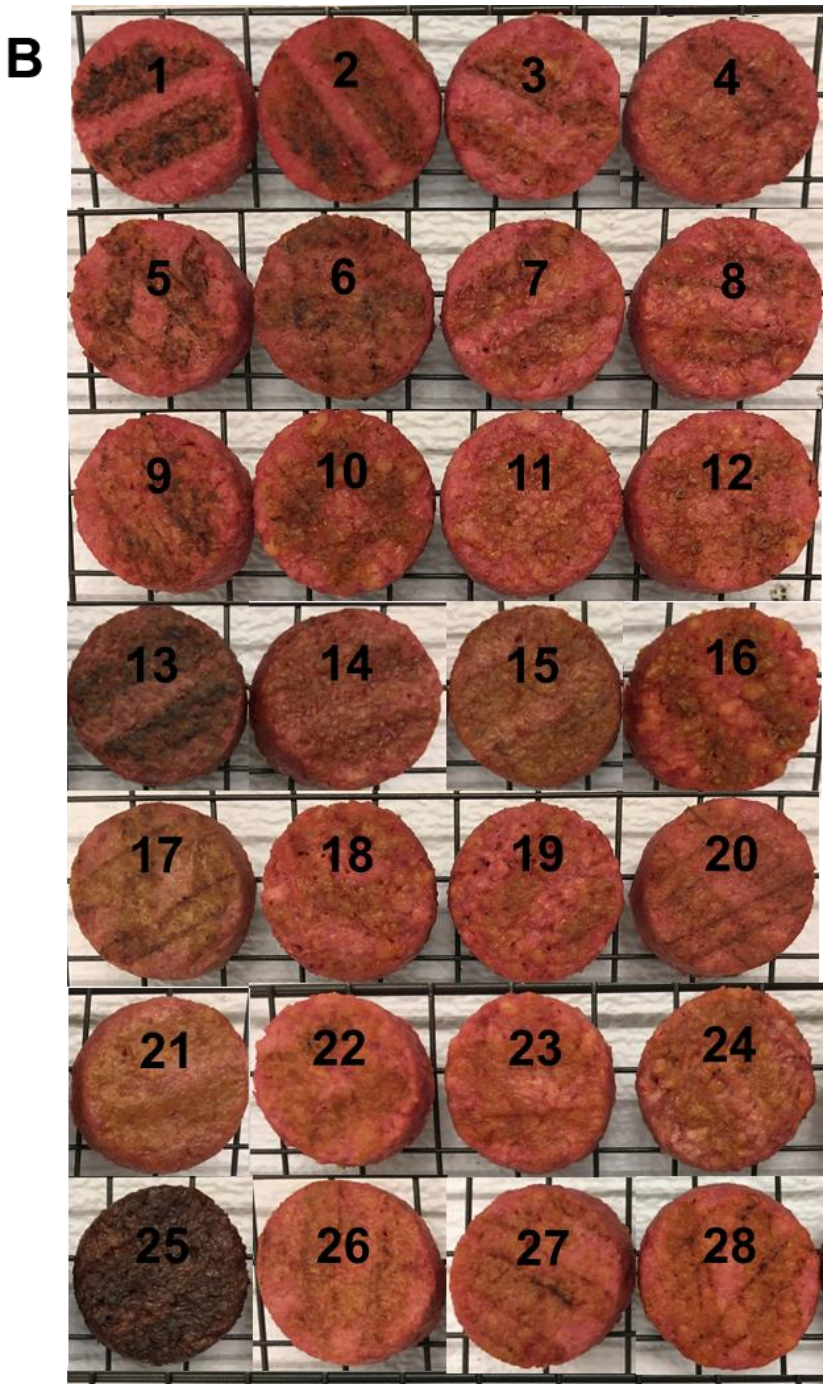

Figure 3. Pictures of TVP-based patties before (**A**) and after (**B**) cooking. Patty types: 1–14 produced from textured soy proteins; 15–21 from textured pea proteins; 22–25 from textured wheat gluten; 26 from textured chickpea protein; 27 made from textured pea/chickpea mixed proteins; and 28 made from textured pea/navy bean mixed proteins.

The cooking loss of TVP-based patties ranged vastly from 11.6 to 18.5% (Table 5), irrespective of protein types. A positive relationship was observed between the RHC of TVPs and the cooking loss, as stated in Table 2 (r = 0.679, $p < 0.01$). At higher RHC, a relatively higher amount of water was introduced to the meatless patty, causing the proportional decrease in solid content on the basis that the same total amount of hydrated TVP was incorporated. Upon heating, the hydrophobic residues in the proteins became exposed; the heated TVP consequently contributed less hydrophilic interactions with water, which resulted in a leakage of water, and thus, a high cooking loss [35]. On the other hand,

the methylcellulose in the formulation served as a binder that created a network upon protein hydration and helped combining the ingredients together [7]. It is supposed that the cage-like water molecules encircle the hydrophobic methyl residues of the methylcellulose polymer. Nevertheless, the increasing temperature disrupts the cage structure, causing the release of water [41]. In light of this, a higher cooking loss is likely to occur in patties formed by TVPs with higher RHC. This finding is in accordance with many other studies. Wi et al. [35] found a typical increase in cooking loss from 12.5 to 14.5% as the amount of water increased in meat analogs. The same trend was also reported by Sakai et al. [41], where the increasing amount of added water elevated the cooking loss.

Table 5. Cooking properties of TVP-based patties.

Sample [A]	Cooking Loss (%)	Diameter Shrinkage (%)	Moisture Retention (%)	Fat Retention (%)
1	14.7 ± 0.8 ^{ij}	6.4 ± 0.5 ^{jkl}	78.2 ± 0.6 ^{bcd}	89.0 ± 0.8 ^{b}
2	11.6 ± 0.6 ^{m}	4.4 ± 0.5 ^{n}	78.0 ± 1.1 ^{bcde}	84.4 ± 0.4 ^{cd}
3	14.8 ± 0.5 ^{hij}	7.3 ± 0.5 ^{fghi}	75.7 ± 0.0 ^{ghi}	83.1 ± 1.2 ^{de}
4	17.0 ± 0.6 ^{bc}	8.7 ± 0.9 ^{bc}	76.2 ± 0.7 ^{efghi}	80.0 ± 1.0 ^{gh}
5	16.7 ± 0.9 ^{c}	7.7 ± 0.6 ^{defgh}	74.8 ± 0.8 ^{ij}	79.9 ± 1.8 ^{gh}
6	14.9 ± 0.8 ^{hij}	6.9 ± 0.4 ^{hij}	73.6 ± 0.8 ^{j}	81.0 ± 0.8 ^{fg}
7	15.2 ± 0.5 ^{fghi}	7.7 ± 0.6 ^{defgh}	77.6 ± 1.3 ^{cdefg}	79.8 ± 1.0 ^{gh}
8	17.4 ± 0.4 ^{b}	9.0 ± 0.7 ^{ab}	75.5 ± 0.6 ^{hi}	79.4 ± 1.0 ^{gh}
9	16.4 ± 1.0 ^{cd}	7.4 ± 0.7 ^{efgh}	75.9 ± 1.5 ^{fghi}	80.9 ± 0.7 ^{fg}
10	12.3 ± 0.6 ^{l}	4.6 ± 0.3 ^{n}	78.1 ± 0.7 ^{bcde}	84.9 ± 1.4 ^{c}
11	13.7 ± 0.6 ^{k}	6.6 ± 0.5 ^{ijk}	77.8 ± 0.7 ^{cdef}	85.5 ± 0.4 ^{c}
12	15.5 ± 0.5 ^{efg}	7.8 ± 0.9 ^{defg}	76.3 ± 0.3 ^{defghi}	77.4 ± 1.5 ^{ij}
13	16.0 ± 0.6 ^{de}	8.2 ± 0.6 ^{bcde}	76.6 ± 1.6 ^{defghi}	80.6 ± 0.3 ^{fg}
14	15.9 ± 0.6 ^{def}	8.3 ± 0.7 ^{bcd}	78.0 ± 0.8 ^{cde}	78.8 ± 0.5 ^{hi}
15	18.5 ± 1.0 ^{a}	8.2 ± 0.7 ^{bcde}	73.2 ± 1.1 ^{j}	70.5 ± 0.4 ^{l}
16	11.6 ± 0.8 ^{m}	4.6 ± 0.4 ^{n}	77.7 ± 1.8 ^{cdef}	92.4 ± 0.4 ^{a}
17	15.6 ± 0.6 ^{efg}	9.5 ± 0.7 ^{a}	77.1 ± 0.7 ^{defgh}	80.0 ± 0.5 ^{gh}
18	13.7 ± 0.9 ^{k}	6.5 ± 0.8 ^{ijkl}	77.8 ± 1.1 ^{cdef}	85.1 ± 1.1 ^{c}
19	14.3 ± 0.7 ^{jk}	6.1 ± 0.5 ^{klm}	77.0 ± 1.1 ^{defgh}	82.8 ± 0.7 ^{e}
20	14.8 ± 0.7 ^{hij}	8.0 ± 0.7 ^{cdef}	76.0 ± 0.2 ^{fghi}	76.0 ± 0.5 ^{j}
21	15.9 ± 0.7 ^{def}	5.6 ± 0.3 ^{m}	75.4 ± 0.3 ^{hi}	74.3 ± 0.2 ^{k}
22	15.1 ± 0.5 ^{ghi}	7.7 ± 0.7 ^{defgh}	77.9 ± 1.0 ^{cde}	81.0 ± 0.5 ^{fg}
23	15.2 ± 0.4 ^{ghi}	7.9 ± 0.6 ^{cdef}	80.3 ± 0.3 ^{a}	81.8 ± 0.4 ^{ef}
24	12.2 ± 0.6 ^{lm}	7.0 ± 0.5 ^{ghij}	79.1 ± 0.3 ^{abc}	79.5 ± 0.6 ^{gh}
25	13.7 ± 0.7 ^{k}	5.7 ± 0.8 ^{lm}	77.7 ± 1.1 ^{cdef}	80.5 ± 1.3 ^{fg}
26	12.3 ± 0.3 ^{l}	5.9 ± 0.5 ^{klm}	80.5 ± 0.4 ^{a}	91.5 ± 0.6 ^{a}
27	15.8 ± 1.1 ^{defg}	8.2 ± 0.7 ^{bcd}	76.3 ± 0.9 ^{defghi}	82.7 ± 0.6 ^{e}
28	13.6 ± 0.5 ^{k}	6.5 ± 0.6 ^{ijkl}	79.8 ± 1.8 ^{ab}	81.1 ± 0.7 ^{fg}
Ave. soy	15.2 ± 1.7 a	7.2 ± 1.4 a	76.6 ± 1.4 b	81.8 ± 3.2 a
Ave. pea	14.9 ± 2.1 a	6.9 ± 1.7 a	76.3 ± 1.6 b	80.2 ± 7.4 a
Ave. wheat	14.0 ± 1.4 a	7.1 ± 1.0 a	78.8 ± 1.2 a	80.7 ± 1.0 a
Average [B]	14.8	7.1	77.1	81.6
LSD (5%) [C]	0.6	0.7	1.6	1.5

[A] Protein types of samples: 1–14 (soy protein), 15–21 (pea protein), 22–25 (wheat gluten), 26 (chickpea protein), 27 (pea and chickpea protein mixture), 28 (pea and navy bean protein mixture). Means with different superscript letters within the same column are significantly different ($p < 0.05$) among samples 1–28. Different lowercase letters indicate significant difference among means of soy, pea and wheat gluten samples within the same column ($p < 0.05$). [B,C] Average values of all samples and least significant difference (LSD) for comparison of different samples.

It is also interesting to note that patty cooking loss was positively associated with protein viscosities (r = 0.605 and 0.660 for peak and final viscosity, respectively, $p < 0.01$, Table 2). In this case, it might be hypothesized that the enhanced hydrophobic interactions induced by protein denaturation upon heating helped form a tighter network, which not

only increased the viscosity but also decreased the free space within the protein matrix, thus reducing water penetration and uptake and increasing the cooking loss.

Cooking causes meat shrinkage due to protein denaturation, change of structure, moisture loss and fat drainage [42]. As expected, the reduction in patty diameter was highly correlated with cooking loss (r = 0.786, $p < 0.01$, Table 2), together with a positive correlation with RHC (r = 0.679, $p < 0.01$, Table 2), which ranged from 4.4 to 9.5% (Table 5). This degree of shrinkage fell within the spectrum of 3.6–12.3% for commercial textured vegetable protein (C-TVP) and textured isolate soy protein (T-ISP) based patties, as reported by Bakhsh and others [43].

Proteins form a gel matrix during the cooking treatment, which is able to retain the essential components [42]. Moisture and fat retentions refer to the capabilities of a product to retain water and fat after cooking. They are crucial factors ensuring the sensory quality and acceptability of meat products. Table 5 displays the moisture retention of TVP-based patties varying from 73.2 to 80.5%, while the fat retention differs from 74.3 to 92.4%, being unaffected by protein sources. The diversity of these parameters was possibly derived from the different degrees of protein denaturation and the extent of the interactions between water/oil and the TVP structure [18]. Both moisture retention and fat retention were inversely related to cooking loss (r = -0.655 and r = -0.684, respectively, $p < 0.01$, Table 2), as a higher cooking loss usually occurs when a patty loses more fat or moisture [38,42]. In addition, the negative correlation between moisture retention and protein viscosity (r = -0.530, $p < 0.01$, Table 2) may again give an insight into the enhancement of the hydrophobic bindings, which allowed rising viscosity and retaining less moisture. Meanwhile, less fat was likely to be held due to less free space and enhanced rigidity of the protein gel, which may help explain the negative relationship between fat retention and pasting viscosity (r = -0.601 and r = -0.552 for peak and final viscosity, respectively, $p < 0.01$, Table 2). However, fat retention is a complex parameter, which may be associated with several other chemical and physical mechanisms [38].

3.8. Textural Properties of TVP-Based Patties

Table 6 shows the textural properties of cooked patties derived from different TVPs. While hardness in the patty form was highly related to that in the hydrated counterparts (r = 0.885, $p < 0.01$, Table 2), the former was generally greater than the latter (559 to 2767 g vs. 400 to 2427 g), which was possibly due to the methylcellulose binding during the patty formation and gelling during cooking that resulted in the compacting of the material. During the cooking process, methylcellulose gradually loses its hydrated water and is likely to bind together owing to the extensive hydrophobic interactions, which highly favors the thermal formation of gels [43]. The strong gels thereby toughen the texture of the final product. Consistent with TVP hardness, the hardness in cooked patties varied negatively with the RHC of TVPs (r = -0.791, $p < 0.01$, Table 2), since a higher water content commonly forms more softened meat analogs [35,36]. Meanwhile, when TVPs with higher RHC were incorporated, a relatively lower solid content was induced to the patty. The decrease in the solid amount may have caused the reduction in hardness as well. However, disagreeing with some previous studies [44], the hardness in the current patties was inversely associated with cooking loss (r = -0.618, $p < 0.01$, Table 2). It is possible that TVPs with a high RHC, although undergoing a higher cooking loss, as previously stated, may have still retained a relatively higher amount of water, and the softening effect caused by the residual water played a more important role than the toughening impact induced by the shrinkage, which thereby resulted in a lower hardness in such samples compared with those with a lower RHC but also lower cooking loss.

Table 6. Textural properties of TVP-based patties.

Sample [A]	Hardness (g)	Resilience (%)	Cohesiveness	Springiness (%)	Chewiness (g)	Shear Force (g)	Compressed Juiciness (%)
1	1554 ± 12 ^{e}	4.7 ± 0.1 ^{hijk}	0.20 ± 0.00 ^{ijk}	55.4 ± 1.2 ^{kl}	173 ± 7 ^{h}	432 ± 47 ^{cd}	7.1 ± 0.7 ^{hi}
2	2768 ± 47 ^{a}	5.3 ± 0.2 ^{fg}	0.20 ± 0.01 ^{jk}	50.1 ± 0.3 ^{m}	270 ± 12 ^{d}	527 ± 63 ^{b}	4.4 ± 0.5 ^{k}
3	965 ± 21 ^{ijkl}	4.1 ± 0.3 ^{lm}	0.20 ± 0.02 ^{jk}	53.2 ± 3.1 ^{lm}	95 ± 9 ^{kl}	255 ± 13 ^{ijkl}	8.9 ± 0.5 ^{cde}
4	1055 ± 60 ^{h}	4.6 ± 0.1 ^{jk}	0.20 ± 0.01 ^{ijk}	56.8 ± 0.9 ^{jkl}	134 ± 14 ^{ijk}	151 ± 23 ^{mn}	7.7 ± 0.4 ^{gh}
5	1249 ± 16 ^{g}	4.7 ± 0.2 ^{hijk}	0.20 ± 0.01 ^{jk}	56.2 ± 2.5 ^{kl}	136 ± 6 ^{ijk}	325 ± 34 ^{fg}	7.2 ± 0.5 ^{ghi}
6	1653 ± 139 ^{d}	5.0 ± 0.4 ^{gh}	0.21 ± 0.00 ^{hij}	57.6 ± 2.2 ^{jk}	197 ± 15 ^{fg}	441 ± 14 ^{cd}	5.8 ± 0.5 ^{j}
7	878 ± 6 ^{lm}	4.3 ± 0.3 ^{klm}	0.23 ± 0.00 ^{fgh}	67.9 ± 1.9 ^{efg}	141 ± 7 ^{ij}	354 ± 43 ^{f}	8.4 ± 0.4 ^{ef}
8	681 ± 18 ^{n}	5.0 ± 0.3 ^{ghi}	0.28 ± 0.01 ^{e}	69.4 ± 1.1 ^{ef}	130 ± 7 ^{jk}	318 ± 24 ^{fgh}	10.3 ± 0.5 ^{b}
9	1011 ± 78 ^{hij}	5.0 ± 0.2 ^{ghij}	0.28 ± 0.02 ^{e}	67.4 ± 2.3 ^{fg}	176 ± 15 ^{h}	285 ± 45 ^{ghi}	9.0 ± 0.4 ^{cde}
10	2457 ± 55 ^{c}	5.4 ± 0.4 ^{f}	0.20 ± 0.01 ^{ijk}	53.5 ± 2.7 ^{lm}	260 ± 11 ^{de}	809 ± 93 ^{a}	4.4 ± 0.5 ^{k}
11	929 ± 34 ^{jkl}	4.7 ± 0.2 ^{hijk}	0.22 ± 0.01 ^{ghi}	60.2 ± 1.6 ^{ij}	122 ± 5 ^{jk}	213 ± 45 ^{kl}	7.7 ± 0.7 ^{gh}
12	1020 ± 32 ^{hi}	4.6 ± 0.3 ^{ijk}	0.20 ± 0.01 ^{jk}	55.6 ± 2.3 ^{kl}	113 ± 6 ^{kl}	203 ± 36 ^{lm}	7.1 ± 0.5 ^{hi}
13	1388 ± 59 ^{f}	4.4 ± 0.2 ^{kl}	0.19 ± 0.00 ^{k}	50.9 ± 0.6 ^{m}	127 ± 5 ^{jk}	369 ± 28 ^{ef}	7.4 ± 0.3 ^{gh}
14	803 ± 27 ^{m}	4.5 ± 0.2 ^{kl}	0.24 ± 0.00 ^{fg}	62.1 ± 2.8 ^{hi}	125 ± 5 ^{jk}	230 ± 32 ^{ijkl}	10.3 ± 0.4 ^{b}
15	799 ± 21 ^{m}	4.5 ± 0.1 ^{kl}	0.20 ± 0.02 ^{ijk}	62.1 ± 1.3 ^{hi}	93 ± 5 ^{kl}	131 ± 20 ^{n}	8.9 ± 0.4 ^{cde}
16	2542 ± 42 ^{b}	11.5 ± 0.6 ^{a}	0.22 ± 0.01 ^{ghij}	71.3 ± 2.1 ^{de}	391 ± 9 ^{a}	415 ± 52 ^{de}	4.0 ± 0.2 ^{k}
17	559 ± 27 ^{o}	5.2 ± 0.3 ^{fg}	0.31 ± 0.03 ^{cd}	73.0 ± 0.7 ^{cd}	134 ± 12 ^{ijk}	266 ± 11 ^{hijk}	12.9 ± 1.0 ^{a}
18	1598 ± 85 ^{de}	8.2 ± 0.2 ^{c}	0.29 ± 0.01 ^{de}	65.0 ± 0.7 ^{gh}	308 ± 11 ^{b}	449 ± 10 ^{cd}	6.6 ± 0.5 ^{i}
19	1430 ± 67 ^{f}	7.1 ± 0.3 ^{d}	0.27 ± 0.02 ^{e}	65.5 ± 1.6 ^{gh}	294 ± 30 ^{bc}	490 ± 32 ^{bc}	6.7 ± 0.7 ^{i}
20	913 ± 51 ^{kl}	4.0 ± 0.1 ^{m}	0.20 ± 0.01 ^{ijk}	64.5 ± 1.7 ^{gh}	116 ± 6 ^{k}	235 ± 24 ^{ijkl}	7.6 ± 0.4 ^{gh}
21	596 ± 32 ^{o}	5.5 ± 0.2 ^{f}	0.38 ± 0.01 ^{a}	90.6 ± 0.5 ^{a}	193 ± 10 ^{gh}	69 ± 4 ^{o}	10.1 ± 0.5 ^{b}
22	887 ± 23 ^{l}	9.0 ± 0.5 ^{b}	0.39 ± 0.02 ^{a}	75.1 ± 1.2 ^{c}	275 ± 3 ^{cd}	212 ± 19 ^{kl}	9.2 ± 0.9 ^{cd}
23	887 ± 16 ^{l}	6.3 ± 0.3 ^{e}	0.32 ± 0.01 ^{bc}	75.6 ± 1.8 ^{c}	216 ± 4 ^{f}	224 ± 24 ^{jkl}	9.3 ± 1.0 ^{c}
24	1048 ± 46 ^{hi}	7.1 ± 0.1 ^{d}	0.34 ± 0.01 ^{b}	75.6 ± 1.6 ^{c}	271 ± 29 ^{d}	451 ± 19 ^{cd}	7.7 ± 0.7 ^{gh}
25	978 ± 71 ^{hijk}	7.2 ± 0.1 ^{d}	0.32 ± 0.01 ^{bc}	82.6 ± 1.3 ^{b}	245 ± 9 ^{e}	277 ± 17 ^{ghij}	7.9 ± 0.5 ^{fg}
26	891 ± 49 ^{l}	4.5 ± 0.2 ^{kl}	0.23 ± 0.01 ^{fgh}	79.9 ± 2.4 ^{b}	153 ± 19 ^{i}	151 ± 16 ^{mn}	8.5 ± 0.3 ^{de}
27	801 ± 11 ^{m}	5.2 ± 0.1 ^{fg}	0.25 ± 0.01 ^{f}	66.9 ± 3.1 ^{fg}	135 ± 11 ^{ijk}	264 ± 22 ^{hijk}	10.1 ± 0.4 ^{b}
28	964 ± 22 ^{ijkl}	4.5 ± 0.3 ^{kl}	0.20 ± 0.01 ^{ijk}	58.1 ± 2.8 ^{jk}	117 ± 4 ^{k}	264 ± 18 ^{hijk}	7.8 ± 0.6 ^{fg}
Ave. soy	1315 ± 618 a	4.7 ± 0.4 b	0.22 ± 0.03 b	58.3 ± 6.3 b	157 ± 53 b	351 ± 168 a	7.5 ± 1.8 a
Ave. pea	1205 ± 711 a	6.6 ± 2.6 a	0.27 ± 0.06 b	70.3 ± 9.8 a	218 ± 114 ab	294 ± 163 a	8.1 ± 2.8 a
Ave. wheat	950 ± 78 a	7.4 ± 1.1 a	0.34 ± 0.03 a	77.2 ± 3.6 a	252 ± 28 a	291 ± 11 a	8.5 ± 0.9 a
Average [B]	1189	5.6	0.25	65.1	184	315	8.0
LSD (5%) [C]	75	0.4	0.02	3.2	20	52	0.6

[A] Protein types of samples: 1–14 (soy protein), 15–21 (pea protein), 22–25 (wheat gluten), 26 (chickpea protein), 27 (pea and chickpea protein mixture), 28 (pea and navy bean protein mixture). Means with different superscript letters within the same column are significantly different ($p < 0.05$) among samples 1–28. Different lowercase letters indicate significant difference among means of soy, pea and wheat gluten samples within the same column ($p < 0.05$). [B,C] Average values of all samples and least significant difference (LSD) for comparison of different samples.

A moderately negative correlation existed between patty hardness and peak viscosity (r = −0.599, $p < 0.01$, Table 2). Given the above explanation, the comparatively higher water remainder in samples with high RHC may not only result in a tender texture of a patty, but also contribute to a relatively higher viscosity of the protein due to a higher retention of water during cooking. It is also worth noting that higher hardness was related to an increase in fat retention (r = 0.537, $p < 0.01$, Table 2). Barbut and Marangoni reported that oil droplets could help connect the protein–protein interactions due to their smaller size but larger surface area [45]. Therefore, an increasing oil globule in products with higher fat retention incremented such hydrophobic linkage and formed a more compact and firmer gel network among the protein matrix, thus enhancing the resistance to compression.

Differing from hardness, other textural attributes were all found to reduce in the patty form in contrast with the hydrated TVPs before binding (Tables 4 and 6). The resilience of the cooked patties remained with substantially lower values, going from 4.1 to 11.5% (Table 6). The observed lower results in patties made from textured soy proteins could be attributed to the better ability of soy protein to form strong structures, thus a more compact texture and higher bulk density ($r = -0.506$ between resilience and bulk density, $p < 0.01$, Table 2). Similarly, a dramatic decrease in springiness was observed, as most values ranged from 50 to 80% (Table 6), implying that they were more prone to be deformed in the patty form. Apart from a more compacted form induced by methylcellulose binding, the fat content introduced in the formulation that helped fill the interspace within the protein matrix may have also resulted in a reduction in springiness. Cohesiveness was similar to the above, in that there was a decline from the hydrated extrudates to the patty form (0.53–0.72 vs. 0.19–0.38). Cohesiveness is related to intermolecular attractions, which are able to hold the elements together [44]. In a food product, cohesiveness also represents the extent to which the food can be deformed before it ruptures [44]. Here, it may be more useful to regard the cohesiveness of the cooked patties as the strength to withstand fracture in a patty as an entirety rather than to disintegrate the TVP particles, which resulted in the difference before and after the formation of patties. As for chewiness, significantly lower values were found in the patty form (93 to 391 g) compared with those of hydrated TVPs before binding (208 to 1530 g). The lower force required to chew the cooked patties was possibly due to the protein denaturation caused by the cooking treatment, which altered the protein conformation and structure.

Shear force represents the maximal force needed to cut a patty, which can be interpreted as an indirect measurement of product tenderness [5]. Here, the shear forces ranged from 69 to 527 g among the studied patties. As reported elsewhere [43], shear force behaved in a significantly similar manner to hardness ($r = 0.778$, $p < 0.01$, Table 2), with RHC being the predominant affecting factor in the current study ($r = -0.621$, $p < 0.01$, Table 2). In this respect, a higher RHC of the TVP would be a favorable implication, achieving lower hardness and shear force, thus a softer and more tender texture.

Compressed juiciness refers to the percentage weight loss of cooked patties established in a compression test. As found in Table 6, juiciness in cooked patties varied significantly, from 4.0 to 10.3%. In general, TVPs with higher RHC yielded more juices when formulating a patty, as evidenced by a significantly positive correlation between the RHC and juiciness ($r = 0.812$, $p < 0.01$, Table 2). This phenomenon was inevitable due to a relatively higher amount of water left within the protein matrix, which was able to be squeezed out. Moreover, the compressed juiciness was negatively correlated to hardness, chewiness and shear force of patties ($r = -0.883$, -0.540 and -0.653, respectively, $p < 0.01$, Table 2), since a firmer structure was more capable of retaining fluid and more resistant to compression, thus imparting less juice [46]. Overall, the physicochemical and functional properties of the proteins, the ingredients in patty formulation, as well as the cooking process all play important roles in carrying over the TVP properties into the textures of final products.

4. Conclusions

Twenty-eight commercial textured vegetable proteins derived from different protein types and sources were comprehensively analyzed with respect to proximate compositions, physicochemical and functional properties of raw TVPs, alongside the cooking and textural characteristics of the final meatless patties. Significant correlations were established between the upstream and downstream attributes. Variations in chemical compositions were the basis contributing to different physicochemical and functional properties of TVPs. Protein content was found to be important in determining protein solubility ($r = -0.775$, $p < 0.01$), while fat content was crucial to OAC ($r = 0.852$, $p < 0.01$). Meanwhile, the WAC of the TVP powder played an important role in the pasting property ($r = 0.621$, and 0.549 for peak viscosity and final viscosity, respectively, $p < 0.01$). The bulk density of TVPs in this study was primarily determined by the intrinsic property of the material types rather than

other parameters. The diversity in the functional properties of TVPs resulted in various textures. As was found, higher RHC imparted lower hardness and chewiness of hydrated TVPs (r = −0.765 and −0.737, respectively, $p < 0.01$), while TVPs with lower bulk density exhibited higher resilience and springiness (r = −0.665 and −0.724, respectively, $p < 0.01$). The versatile attributes of raw TVPs were further carried over into the final patties. The cooking loss and textural properties (hardness, shear force and compressed juiciness) of meatless patties were predominantly associated with RHC (r = 0.679, −0.791, −0.621 and 0.812, respectively, $p < 0.01$). Aside from that, the pasting property of TVPs also served as an important indicator of patty attributes, as significant correlations occurred accordingly. Moreover, binders such as methylcellulose played important roles in integrating TVPs into the final products, causing significant differences in textures before and after binding. As such, targeting the texture of the final products depends not exclusively on the raw TVP but also on the binding system. The present study, for the first time, provided a systematic evaluation correlating the physicochemical and functional properties of raw TVPs to the cooking and textural properties of the final meatless patties. These findings may help provide a bottom-up insight for designing TVPs with various characteristics, which may benefit the final desirable meat analogs. Further studies, such as using other types of meat analogs and conducting consumer sensory evaluations, are suggested to further validate the importance of the correlations discovered in this study and unveil the possible associations between raw TVPs and sensory attributes, thus helping to improve consumer acceptability.

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Protein Nanofibrils from Fava Bean and Its Major Storage Proteins: Formation and Ability to Generate and Stabilise Foams

Anja Herneke [1,*], Christofer Lendel [2], Saeid Karkehabadi [1], Jing Lu [1] and Maud Langton [1]

[1] Department of Molecular Sciences, Swedish University of Agricultural Sciences (SLU), 750 00 Uppsala, Sweden
[2] Department of Chemistry, Royal Institute of Technology (KTH), 100 40 Stockholm, Sweden
* Correspondence: anja.herneke@slu.se

Abstract: Protein nanofibrils (PNFs) have potential for use in food applications as texture inducers. This study investigated the formation of PNFs from protein extracted from whole fava bean and from its two major storage proteins, globulin fractions 11S and 7S. PNFs were formed by heating (85 °C) the proteins under acidic conditions (pH 2) for 24 h. Thioflavin T fluorescence and atomic force microscopy techniques were used to investigate PNF formation. The foaming properties (capacity, stability, and half-life) were explored for non-fibrillated and fibrillated protein from fava bean, 11S, and 7S to investigate the texturing ability of PNFs at concentrations of 1 and 10 mg/mL and pH 7. The results showed that all three heat-incubated proteins (fava bean, 11S, and 7S) formed straight semi-flexible PNFs. Some differences in the capacity to form PNFs were observed between the two globulin fractions, with the smaller 7S protein being superior to 11S. The fibrillated protein from fava bean, 11S, and 7S generated more voluminous and more stable foams at 10 mg/mL than the corresponding non-fibrillated protein. However, this ability for fibrillated proteins to improve the foam properties seemed to be concentration-dependent, as at 1 mg/mL, the foams were less stable than those made from the non-fibrillated protein.

Keywords: plant protein; fava bean; amyloids; legumin; vicilin; 11S; 7S; microscopy; rheology

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1. Introduction

One suggested approach to lower the environmental impact of the food sector is to eat more locally produced plant-based proteins [1]. Fava beans can be cultivated in the Scandinavian climate, but are currently used primarily as animal feed [2]. Whole fava bean has a protein content of 19–39%, with the major proteins comprising two globulin fractions, legumin, and vicilin (11S and 7S) [3]. Fava bean has good potential for use as a locally produced protein source for humans in many different climate zones. However, convincing consumers to exchange animal-based products with plant-based alternatives is not always easy. Alternative products mimicking the appearance and texture of animal-based products can encourage consumers to eat more plant-based foods [4].

The ability to stabilise air bubbles is an important feature for the textural appearance of many food applications, such as bakery products, whipped cream, ice cream, and cheese [5]. By studying the foaming properties, conclusions can be drawn about an ingredient's function as a structure enhancer. Proteins have a long history of being used as foam stabilisers. Surface-active proteins can stabilise foams by decreasing the surface tension and forming thin interfacial films that capture air bubbles [6]. Small and flexible proteins usually have higher foaming capacity than large and rigid proteins due to their superior ability to reduce surface tension.

Protein nanofibrils (PNFs) from sustainable sources are of considerable interest to many researchers in material sciences due to the good mechanical properties of these

nano-scale fibrils [7]. These properties have also captured the interest of food scientists, who believe that PNFs can be used to create interesting texture profiles [8]. One of the most widely utilised methods for producing protein nanofibrils from food protein is to heat the protein at relatively high temperatures (70–90 °C) in acidic conditions [8]. These harsh conditions cause the proteins to unfold and hydrolyse into smaller peptides. The low pH makes the peptides positively charged, which generates repulsing forces [9]. Only highly ordered peptide assemblies, usually rich in β-sheets, can resist these repulsing forces and form PNFs. This results in a sample containing a mixture of peptides and mature fibrils.

In an early study by our group, we demonstrated that it is possible to produce PNFs from a broad range of plant-based proteins by exposing the protein to an acid environment and heat [10]. One of the plant-based PNFs characterised was produced from a whole protein isolate extracted from locally produced fava beans. When visualising the PNFs with atomic force microscopy (AFM), the result showed that depending on the protein source, the PNFs varied in morphology (being either curved or straight) and in length (~220–910 nm). The plant-based PNFs were compared with a well-studied animal-based protein (whey). Here, the result showed that whey formed considerably longer PNFs (several μm) than any of the PNFs from plant-based sources. We hypothesised that whey proteins superiority could be due to the protein isolate being very pure and consisting of a majority of two very small proteins (β-lactoglobulin and α-lactalbumin), with a molecular weight of 18.3 and 14.2 kDa [11]. The plant-based globulins investigated were approximately 10–20 times larger [12–16] than the major protein found in whey proteins. The fava bean protein used in this previous study was extracted in-house with isoelectric precipitation, generating a protein consisting of a mixture of the larger 11S globulins with a molecular weight of ~353 kDa and the smaller 7S globulins with a molecular weight of ~150 kDa [12]. By separating the two globular fractions and comparing the PNF-forming ability with the whole fava bean protein isolate, further information can be revealed about the impact of protein size on PNF formation.

Fibrillated protein from whey/β-lactoglobulin [17,18] and 11S from soy [19] have been reported to give higher foam volume and stability than non-fibrillated protein from the same sources. In the studies using fibrillated β-lactoglobulins by Peng et al. (2017) and 11S from soy by Wan et al. (2021), the fibrils were isolated by filtration to remove or separate unconverted peptides. Peng et al. (2017), showed that the PNFs produced from β-lactoglobulin formed more stable foams than the non-fibrillated protein, even at concentrations as low as 1 mg/mL, especially at a pH close to the isoelectric point (IP) of the protein. On the other hand, Wan et al., (2021) showed that isolated PNFs from the fractionated soy-11S globulin formed less stable foams than samples containing mixtures of PNFs and peptides or the peptides alone. The reason for these conflicting results is not fully understood. In a recently published study from our group, we investigated the foaming properties of curved PNFs from mung bean [20]. Here, we found that fibrillated protein formed more stable foams than non-fibrillated protein from the same source at in the pH range of 4–9. When separating the PNFs and peptides, we observed that at a pH that was close to the IP of the original protein and at a concentration of 1 mg/mL, the sample with isolated PNFs formed more stable foams than the sample consisting of separated peptides. There is still a need to further investigate if and how PNF can be used to stabilise foams, which will generate a better understanding about the future of plant-based PNFs as a texturing ingredient in food applications.

The aims of the present study were: (i) to characterise and compare the PNFs generated from fava bean protein isolate and the two major globular fractions (11S and 7S) in fava bean; and (ii) to investigate the foaming properties of these PNFs. The PNFs were detected using thioflavin T (ThT), a fluorescing dye with a specific affinity to the β-sheet-rich structure of PNFs [21], which were visualised by AFM imaging. Foaming properties such as foam capacity, stability, and volume of half-life were investigated for non-fibrillated and fibrillated proteins at two different concentrations (1 and 10 mg/mL) after the adjustment of the pH to 7. The only difference in the preparation of the samples was that the fibrillated

proteins had been heated at 85 °C for 24 h. These results will fill a knowledge gap on how PNF morphology and concentration influence foaming properties.

2. Materials and Methods

Fava beans (*Vicia faba* L. var. *Gloria*) were kindly provided by the RISE (Research Institutes of Sweden). Hydrochloric acid (HCl) was procured from VWR International (Paris, France), sodium chloride (NaCl) from VWR (Darmstadt, Germany), and thioflavin T (ThT) from Sigma (New Delhi, India).

2.1. Extraction of Protein from Fava Bean, 11S, and 7S

2.1.1. Fava Bean Protein Isolate

The whole fava bean protein isolate was produced according to the method previously described by Herneke et al., (2021) [10] with some minor adjustments. In brief, the fava beans were dehulled and milled in an ultra-centrifugal mill with a 500 µm mesh. The flour was dispersed in deionised water and diluted at a ratio of 1:10, and the pH was adjusted to 8.0 using 2 M NaOH (Figure 1a). The mixture was then stirred for 1 h, followed by centrifugation at 3700× *g* for 30 min. The supernatant was collected, the pH was changed to 4.0 using 2 M of HCl and incubated with continuous stirring for 1 h, and the supernatant was then centrifuged at 3700× *g* for 15 min. The pellet was collected and washed in deionised water at a ratio of 1:10 and the pH was adjusted to 4, and it was centrifuged at 3700× *g* for 15 min. The pellet was collected and freeze-dried.

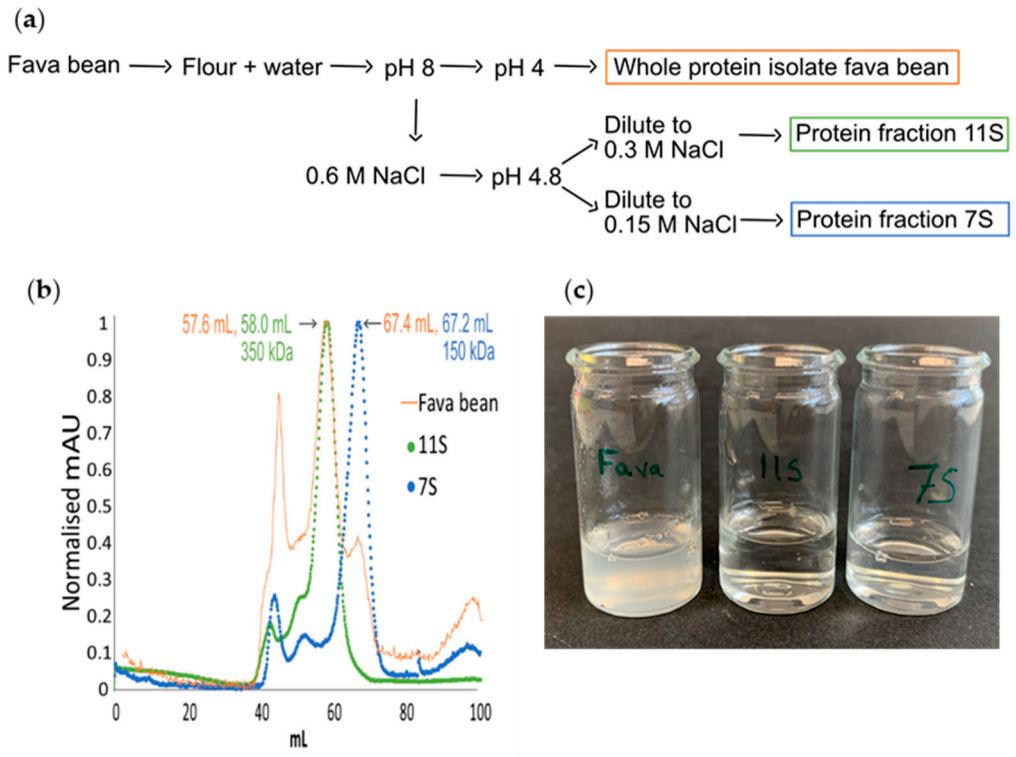

Figure 1. (**a**) Flow chart showing how whole protein isolate and its major globular fractions 11S and 7S were extracted from fava bean using the pH shift method and different concentrations of NaCl. (**b**) Results of normalised size exclusion chromatography of whole protein isolate from fava bean (orange) and fractions 11S (green) and 7S (blue). (**c**) Visual appearance at concentration 10 mg/mL and pH 2 of fibrillated protein from (left to right) fava bean, fraction 11S, and fraction 7S.

2.1.2. 11S and 7S

The 11S (legumin) and 7S (vicilin) fractions were extracted from whole fava beans using a similar protocol to the one described by Suchkov et al. (1990) [22] with some modifications (Figure 1a). The flour was dispersed in deionised water and diluted at a ratio

of 1:10 (*w*/*w*) at 20 °C. The pH of the mixture was adjusted to 8.0 using 2 M of NaOH (Figure 1a). The mixture was then incubated with stirring at room temperature (20 ± 2 °C) for 1 h, followed by centrifugation (Sorval Lynx 4000l, Thermo Scientific, Waltham, MA, USA) at 3700× *g* for 30 min. NaCl at a final concentration of 0.6 M was added to the supernatant, and the pH was adjusted to 4.8 with 1 M of HCl and then centrifuged at 5000× *g* (20 °C, 20 min). Both the supernatant (i) and pellet (ii) were saved.

(i) The supernatant was diluted to double the volume with deionised water and centrifuged at 1000× *g* for 15 min. The supernatant was saved overnight at 4 °C, followed by another round of centrifugation at 1000× *g* (15 min). The obtained supernatant was diluted with cold distilled water to double the volume and centrifuged again at 1000× *g* for 15 min, and the pellet was collected and freeze-dried (7S).

(ii) The pellet was dissolved in 1000 mL of 0.6 M NaCl solution and stirred to complete dissolution, followed by centrifugation at 5000× *g* (30 min). The supernatant was diluted with distilled water to double the volume. The solution was then left at room temperature, and the sediment was collected and freeze-dried (11S).

2.1.3. Protein Content

Using the Kjeldahl method and a conversion factor of 5.4 [23], the crude protein content was determined to be 78.0% for FPI, 85.8% for 11S, and 80.0% for 7S.

2.2. Size Exclusion Chromatography

A small amount of each protein isolate of 11S and 7S was dissolved in 25 mM of bicine buffer (pH 9.0, 500 mM NaCl). The protein solution was thoroughly vortexed and run through a PD-10 column using the same buffer. Using an Äkta explorer (GE, healthcare) and UV absorbance of A280 nm, 1 mL of the protein solution obtained was loaded onto a Superdex-200 Hiload 16/600 size exclusion column, and the separation started at a flow rate of 1 mL/min.

2.3. Preparation of Protein Nanofibrils from Fava Bean, 11S, and 7S Protein Isolate

Each protein was dissolved in 0.01 M of HCl to a final concentration of 50 mg/mL. The samples were centrifuged at 3700× *g* for 15 min and filtered through a 0.45 µm nylon syringe filter (Merck Millipore, Dublin, Ireland). Protein content was estimated by dry weight measurement after drying at 105 °C for 3 h. The concentration was adjusted to 10 mg/mL using 0.01 M of HCl; the pH was adjusted to 2, and the samples were incubated in an oven at 85 °C for 24 h without stirring. After heating, the fibrillated samples were cooled on ice and then stored at 4 °C. PNF detection was performed directly after the fibrillated samples were cooled, and the foaming experiments were conducted within a week after preparation.

2.4. Thioflavin T Fluorescence

A thioflavin T (ThT) fluorescence assay was performed according to the method previously described by Herneke et al., (2021) [10]. In brief, a 100 µL sample was mixed with 900 µL of 50 µM ThT working solution in phosphate buffer and incubated at room temperature for 20 min before testing. The fluorescence was measured using a multi-mode microplate reader (Polarstar Omega, BMG Labtech, Germany) at an excitation wavelength of 440 nm and emission wavelength of 480 nm.

2.5. Atomic Force Microscopy

An atomic force microscopy analysis was conducted using a Bruker Dimension FastScan instrument operating in fast scan mode. The samples were diluted between 1:50 and 1:500 in 0.01 M of HCl, and a 10 µL aliquot was applied on a freshly cleaved mica surface and dried in air. FastScan B cantilevers (Bruker, tip radius = 5 nm, spring constant = 2 N/m, peak force amplitude = 150 nm) were used for the experiments, and the micrographs were analysed with the Gwyddion 2.48 tool (http://gwyddion.net/, accessed

on 29 June 2022). All samples were measured in at least duplicates in several different locations on the mica plate. The surface that was measured was 3 × 3 μm.

2.6. Rheological Measurements

Steady shear measurements of fibrillated samples from whole fava bean protein isolate, 11S, and 7S at 10 mg/mL and pH 2 and 7 were carried out using the same method previously described in Herneke et al. (2023) [20]. The viscosity (η) was recorded during the shear rate from 0.1/s to 500/s on a Discovery HR-3 hybrid rheometer (DHR3) (TA Instruments, USA) with a cone plate geometry of 40 mm diameter, cone angle 2°, and 51 μm gap. All measurements were carried out at 25 °C and were repeated at least three times.

The power law model, Equation 1, was used to fit the viscosity result from fava bean, 7S, and 11S at pH 2 and 7.

$$\eta = K\dot{\gamma}^{n-1} \quad (1)$$

η is the shear viscosity, $\dot{\gamma}$ is the shear rate, K is the consistency index, and n is the flow behaviour index. The power law parameters were obtained in the analyse function in TRIOS (TA instrument), and none of the parameters obtained had a fitting of the model (R^2) below 0.89.

2.7. Zeta Potential

Measurements of the zeta potential were carried out using a Z-sizer (Malvern Instruments). Each sample was measured in triplicates, maintaining an attenuation between 7 and 9, and the temperature was 25 °C. All samples were measured at a concentration of 1 mg/mL.

2.8. Foaming Properties

To investigate the foaming properties, 10 mL aliquots of 1 or 10 mg/mL of each of the non-fibrillated and fibrillated samples (see Section 2.2) were transferred to 50 mL beakers. The pH of the samples was adjusted to 5 (1 mg/mL) and 7 (1 and 10 mg/mL) with 0.1 and 2 M of NaOH. The probe S25N (IKA®, Staufen, Germany) was immersed in the solution, the homogeniser (IKA ® Ultra Turrax T25) was started, and the speed was gradually increased from 8000 rpm to 13,500 rpm. The solution was allowed to foam for a total of 5 min, the foam volume (FV) was recorded, and foaming capacity (FC, %) was determined using the equation:

$$FC = (FV_i/V_i) \times 100 \quad (2)$$

where FV_i is the volume of foam at time zero and V_i is volume of the protein solution before whipping.

Foam stability (FS) was calculated based on foam volume after 30, 60, 120, and 360 min standing at room temperature using the equation:

$$FS = (FV_t/FV_i) \times 100 \quad (3)$$

where FV_t is the volume of foam at time t.

Foam height was calculated according to the exponential decay law as:

$$H(t) = H(0)\exp(-\lambda t) \quad (4)$$

where H(t) is foam height at time t, H(0) is initial foam height at time t = 0, and λ (lamda) is a decay constant. This exponential relationship can be converted to a linear equation by taking the natural logarithm of foam height ln(H(t) versus time:

$$\ln(H(t) = \ln(H(0)) - \lambda t \quad (5)$$

Half-life ($t_{1/2}$) of the foams was then determined using the equation:

$$t_{1/2} = \ln(H(t_{1/2}) - \ln(H(0))/-\lambda \quad (6)$$

2.9. Confocal Microscopy

The microstructure of foams made from non-fibrillated and fibrillated samples were detected using the method described by Herneke et al. (2023) [20] with minor modifications. A confocal laser scanning microscope (CLSM; Zeiss LSM 780, Jena, Germany) was used, equipping an inverted Zeiss Axio Observer and supersensitive GaASp detector. The protein distribution in the samples of foams created from non-fibrillated and fibrillated FPI, 11S, and 7S was detected by staining with the fluorescence dye ThT (3 mM, dispersed into phosphate-buffered saline 10 mM PBS, pH 6.8). The stained samples were placed on a concave microscope slide for observation. An Argon operated at 488 nm excitation wavelength and emission wavelengths between 500 nm and 530 nm was used to detect ThT fluorescence. All images were acquired using a C-Apochromat 63x oil immersion objective (1.32 NA) at an image resolution of 1024 × 1024 pixels.

3. Results and Discussion

3.1. Characterisation of Protein and Nanofibrils

Three different protein isolates were investigated: the whole protein from fava bean isolate (FPI) and its major globular fractions 11S and 7S. All three proteins were extracted with isoelectric precipitation with the addition of NaCl to collect protein fractions 11S and 7S (Figure 1a). Size exclusion chromatography confirmed that the whole protein isolate from fava bean (Figure 1b, orange) contained both 11S (arrow at 57.6 mL) and 7S (arrow at 67.4 mL). The protein extracted with a final concentration of 0.3 M NaCl was collected at 58.0 mL (Figure 1b, green), and the protein extracted with a final concentration of 0.15 M NaCl was collected at 67.2 mL (Figure 1b, blue). The elution volumes of 11S and 7S suggest molecular weights of 350 kDa and 150 kDa, respectively, which are almost identical to the previously reported values (355 kDa and 150 kDa, respectively) [12].

When the three protein isolates at pH 2 were heated at 85 °C for 24 h, the whole protein isolate had a much cloudier appearance than the samples containing fibrillated 11S and 7S proteins (Figure 1c). This cloudiness was most likely because the whole protein isolate contained starch, fibres, and ash residues [24].

Thioflavin T (ThT) dye is commonly used to detect amyloid-like protein nanofibrils due to its strongly enhanced fluorescence when bound to β-sheet-rich structures [21]. Figure 2 shows the average fluorescence recorded in the ThT assay for fava bean and fractions 11S and 7S before (Protein) and after incubation at 85 °C (PNFs). The increase in fluorescence after fibrillation was greatest for the 7S fraction, with an average increase of 60%, followed by whole fava bean, with an average increase of 56%. For fraction 11S, no increase was detected after fibrillation. As mentioned above, extracted fava bean protein consists of a mixture of 11S and 7S (see Figure 1b), indicating that the increase in fluorescence after fibrillation of the whole bean sample was probably due to the 7S fraction (Figure 2). A similar lack of increase in fluorescence after fibrillation for fraction 11S has been previously observed by our research group for protein isolates extracted from oat and rapeseed, which mainly consist of 12S globulins [10,14,15]. However, the AFM analyses revealed fibril structures in the samples, indicating that ThT assay cannot be used as a stand-alone detection method for PNFs.

The AFM results confirmed that heated proteins from fava bean, 11S, and 7S at pH 2 were able to form PNFs (Figure 3a–c′). All PNFs that formed from the three protein isolates had a similar morphology with a straight semi-flexible structure. However, fibrillated samples from the 7S protein appeared to have a higher density of PNFs when analysed with AFM. Most PNFs in the fibrillated 7S samples were short (~100–200 nm) but with some longer fibrils co-existing in the same samples. The longest fibril measured was 2.2 μm.

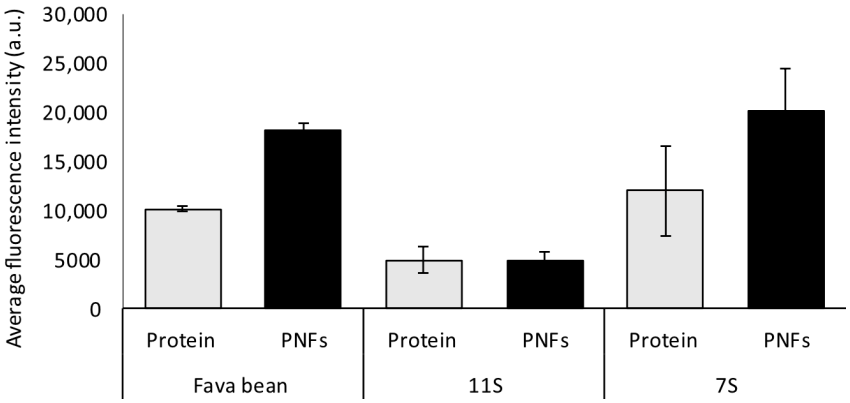

Figure 2. Average fluorescence intensity of non-fibrillated (protein) and fibrillated (protein nanofibrils, PNFs) samples from fava bean protein isolate and fava bean globular fractions 11S and 7S at pH 2 and concentration 10 mg/mL. The error bars refer to the standard deviation.

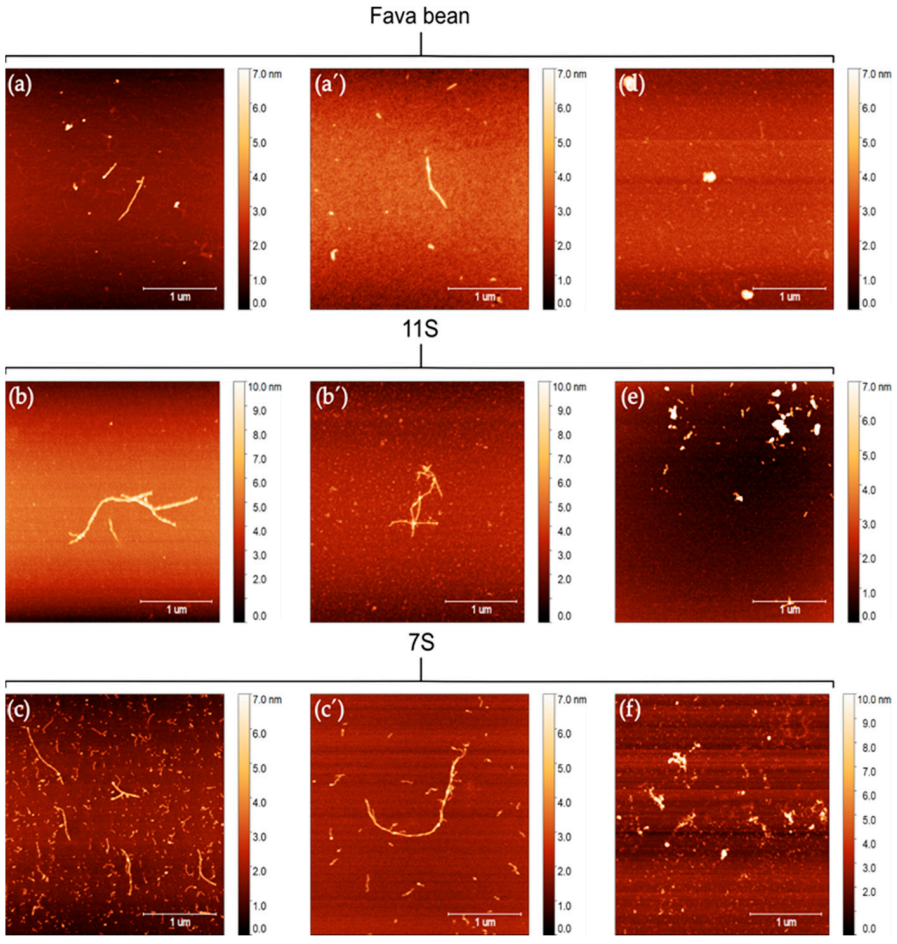

Figure 3. Atomic force microscopy images of two separate batches of fibrillated protein at pH 2 from (**a**–**a′**) fava bean isolate, (**b**–**b′**) fava bean globular fraction 11S, and (**c**–**c′**) fava bean globular fraction 7S. Atomic force micrographs of fibrillated protein from (**d**) fava bean isolate, (**e**) 11S, and (**f**) 7S after pH adjustment to 7.

Based on the results from the ThT assay (Figure 2) and AFM analysis (Figure 3), it can be concluded that 7S globulins from fava bean are superior to 11S globulins in forming PNFs. This agrees with earlier findings on PNF formation from the 7S and 11S globular fractions in soybean [25]. Using several different methods such as the ThT assay, Congo read spectroscopy assay, circular dichroism spectroscopy, and SDS-gel separation

techniques, that study confirmed that when heated at pH 2, the protein from the soybean 7S fraction more readily formed PNFs than the 11S fraction under the same conditions. The reason for 7S being superior to 11S globulins in forming PNFs is not fully understood. Tang and Wang speculated that it might be because 7S contains more charged amino acids, which hydrolyses into peptides more easily at low pH and high temperature [25]. The 11S globulin structures also have a higher denaturation temperature than the 7S structures, which might contribute to their ability to form PNFs [26]. Unfolding proteins by heating them above their denaturing temperature increases the ability of hydrolysis to occur by exposing previously buried residues [8]. For 11S from fava bean, the thermal denaturation midpoint is reported to lie at 85 °C, while the corresponding denaturation midpoint for 7S is reported to lie at 76.5 °C at low ionic strength (μ = 0.08) [26]. In the present study, PNF formation was investigated at 85 °C, and hence 7S had more optimal conditions for PNF formation, which might have generated a larger population of unfolded and hydrolysed 7S protein compared with 11S protein.

When the pH of the fibrillated samples was increased to 7, the fibrillary structure degraded in all samples (Figure 3d,e). For the fibrillated fava bean protein, the samples mainly contained globular aggregates (Figure 3d). Both 11S and 7S seemed to have some shorter fibrils, which grouped into larger aggregates in some cases (Figure 3e,f). These results show that the PNFs from whole fava bean and fractions 11S and 7S are less stable at pH 7 than at pH 2. Fibrillated proteins from whey, soybean, and mung bean have also been shown to be less stable at pH 7 [20,27,28]. However, the PNFs from those sources did not fragment as much as the fava bean 11S and 7S PNFs, so our results in this regard were unexpected.

The flow consistency index and flow behaviour index (Table 1) for fibrillated fava bean, 11S, and 7S at pH 2 and 7 were obtained by fitting the apparent viscosity (Figure 4a) with the power law equation (see method section).

Table 1. The flow consistency index (K) and flow behaviour index (n) for fibrillated protein from fava bean, 11S, and 7S at pH 2 and pH 7.

Sample	pH	K (Pa.s)	n
Fava bean	2	0.217 ± 0.016	-0.770 ± 0.021
	7	0.009 ± 0.001	-0.293 ± 0.081
11S	2	0.087 ± 0.063	-0.727 ± 0.005
	7	0.011 ± 0.047	-0.360 ± 0.129
7S	2	0.076 ± 0.012	-0.720 ± 0.029
	7	0.013 ± 0.007	-0.424 ± 0.014

Fibrillated fava bean protein had the highest apparent viscosity at pH 2 (Figure 4), which was probably due to residues of polysaccharides in the protein isolate [24] generating aggregates (see Figure 1). This was also confirmed with a higher consistency index for fibrillated fava bean than 11S or 7S at pH 2 (Table 1). When the pH was increased from 2 to 7, the viscosity of all fibrillated samples dropped (Figure 4), which was also correlated with a lower consistency index for all samples at pH 7 (Table 1). All samples at both pH 2 and pH 7 had a shear thinning behaviour, which was confirmed with a negative flow behaviour index (Table 1). This was probably because the PNFs were degraded to smaller particles (see Figure 3d–f). In an earlier study by our group investigating the viscosity of PNFs generated from mung bean protein [20], it was found that those PNFs had a distinctly different morphology than the PNFs obtained from fava bean-based proteins in the present study, having a curved structure instead of straight. The apparent viscosity of PNFs generated from mung bean (Figure S1 in Supplementary Materials (SM)) under the same conditions as applied here (10 mg/mL, pH 2) showed that the fibrillated mung bean protein had around 1.7–6.3 times higher apparent viscosity than the fibrillated protein from fava bean and its globulin fractions. This was also confirmed with the fibrillated mung bean samples having a higher consistency index (Table S1) than the fibrillated fava

bean, 11S, and 7S (0.386 vs. 0.217, 0.087, and 0.076 respectively) at pH 2. Similar differences in the viscosity profile have been reported for curly and straight PNFs produced from β-lactoglobulin, where the curved PNFs had higher viscosity than the straight PNFs at the same concentration and conditions [29]. Z potential measurements of both fibrillated and non-fibrillated proteins varied between 20.3 and 25.5 mV at pH 2, −5.5 and −26.9 mV at pH 5, and −13.8 and −21.3 mV at pH 7 (Figure 4b). The fibrillated samples had a similar Z potential at the respective pH, as earlier observed for fibrillated whey protein [30]. No differences could be observed for the samples containing fibrillated and non-fibrillated proteins from the globular fraction 7S and 11S compared with whole fava bean isolate. Based on this observation, the salt added during the protein extraction of 11S and 7S did not affect the charge of fibrillated or non-fibrillated protein. The higher viscosity for fibrillated samples at pH 2 compared with pH 7 (Figure 4a) might also be correlated to the high Z potential observed at low pH (Figure 4b). An increase in Z potential indicates a higher degree of repulsion between particles, resulting in a larger interaction size of particles, causing the particles to move less freely, and thus increasing viscosity.

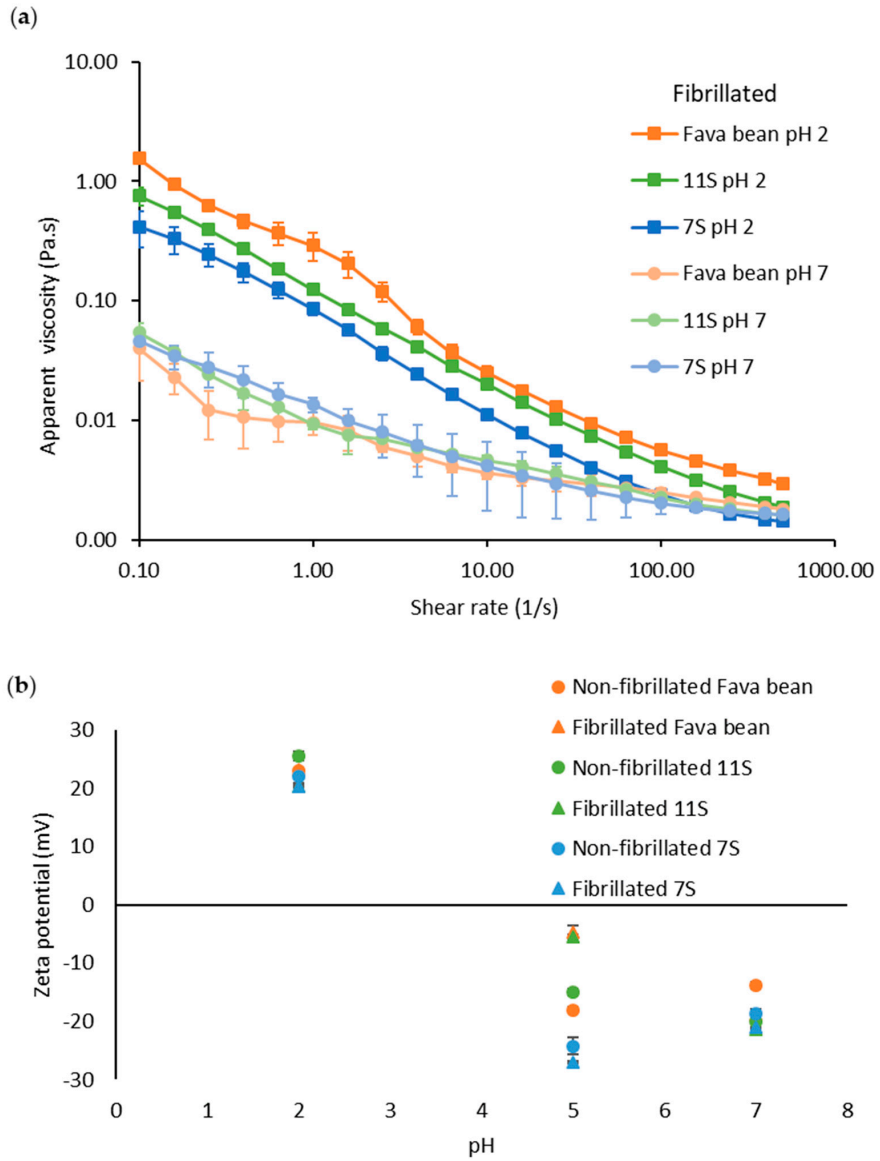

Figure 4. (**a**) Apparent viscosity versus shear rate (0.1–500 s^{-1}) for fibrillated protein from fava bean, 11S, and 7S at pH 2 (squares) and pH 7 (circles). All samples were at a concentration of 10 mg/mL. (**b**) Z potential of non-fibrillated and fibrillated fava bean (orange), 11S (green), and 7S (blue) protein at pH 2, 5, and 7. The error bars in figure (**a**,**b**) refer to the standard deviation.

3.2. Foaming Properties

The foaming properties (foaming capacity, foam stability, half-life) of non-fibrillated (protein) and fibrillated (PNF) fava bean, 11S, and 7S were evaluated over a time interval of 15–360 min at pH 7 (Figure 5a,b). Both the non-fibrillated and fibrillated samples were able to form foams after mixing (Figure 5a). However, the increase in foam volume for the fibrillated fava bean and 7S samples was 177% higher, and the increase for the 11S samples was 102% higher than for the non-fibrillated samples. In addition, the fibrillated samples generated much more stable foams than the non-fibrillated samples (Figure 5b,c). In particular, the non-fibrillated fava bean samples were unstable over time. Interestingly, the foams made from fibrillated 11S protein had the longest half-life among all samples investigated (Figure 5c).

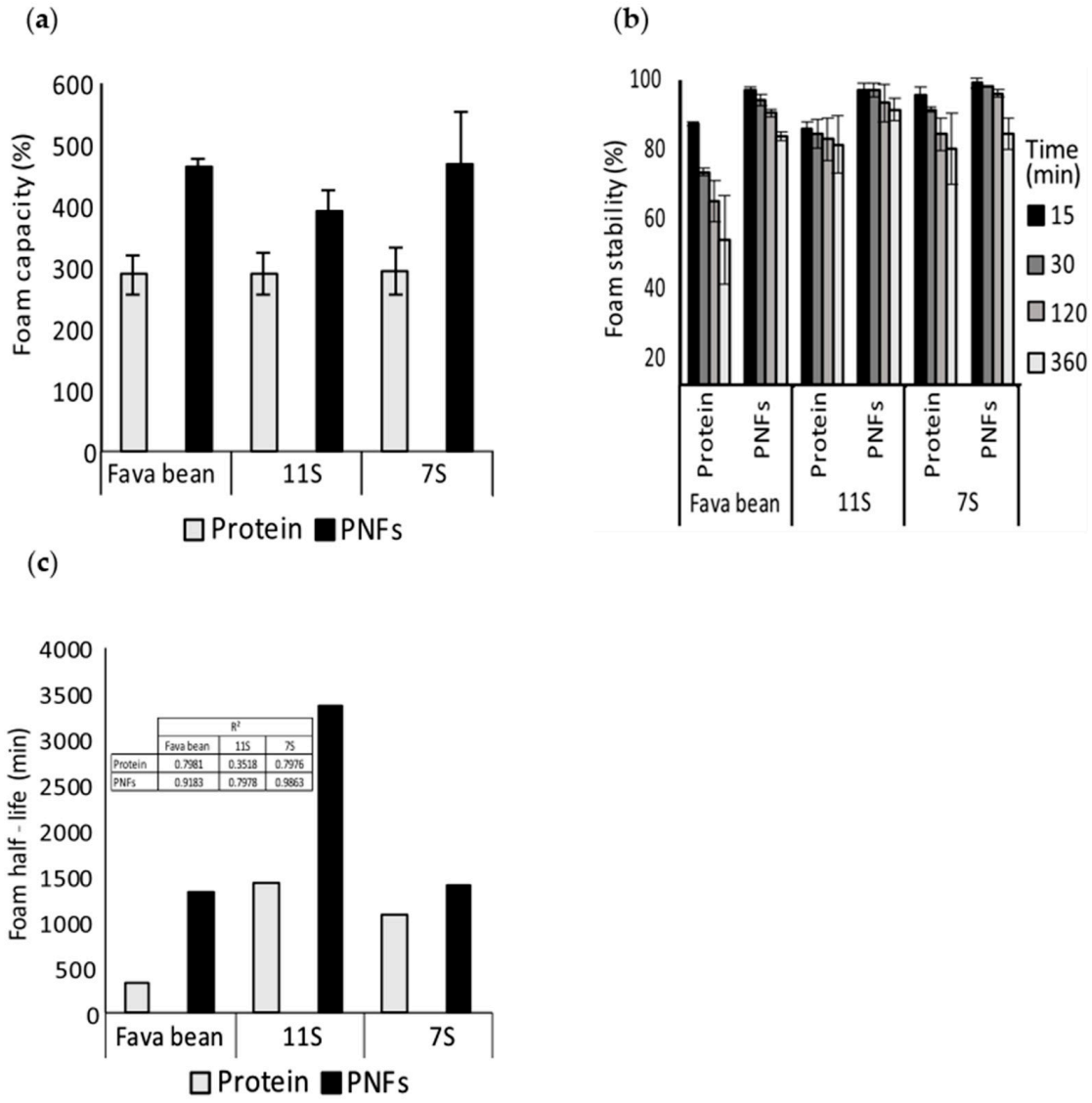

Figure 5. (**a**) Foaming capacity, (**b**) foam stability, and (**c**) foam half-life of non-fibrillated (protein) and fibrillated (protein nanofibrils, PNFs) protein from fava bean and its globular fractions 11S and 7S. The foams were generated at pH 7 and had a concentration of 10 mg/mL. The error bars in figure (**a**,**b**) refer to the standard deviation, and the insert in (**c**) shows the model fit (R^2).

To investigate whether these improved foaming properties persisted at very low concentrations for fibrillated protein, foams produced from non-fibrillated (protein) and fibrillated (PNFs) fava bean, 11S, and 7S proteins were diluted to a final concentration of 1 mg/mL at pH 7 (Figure 6a,b). The foaming capacity was found to be almost identical for

all three protein isolates irrespective of whether the samples were fibrillated (Figure 6a). However, the foams produced from fibrillated protein were less stable than those from the corresponding non-fibrillated protein (Figure 6b,c). Interestingly, the foam created from fibrillated 11S protein was the least stable, contradicting the observations made at higher concentrations. This indicates that the ability of the fibrillated proteins to stabilise foams was concentration-dependent.

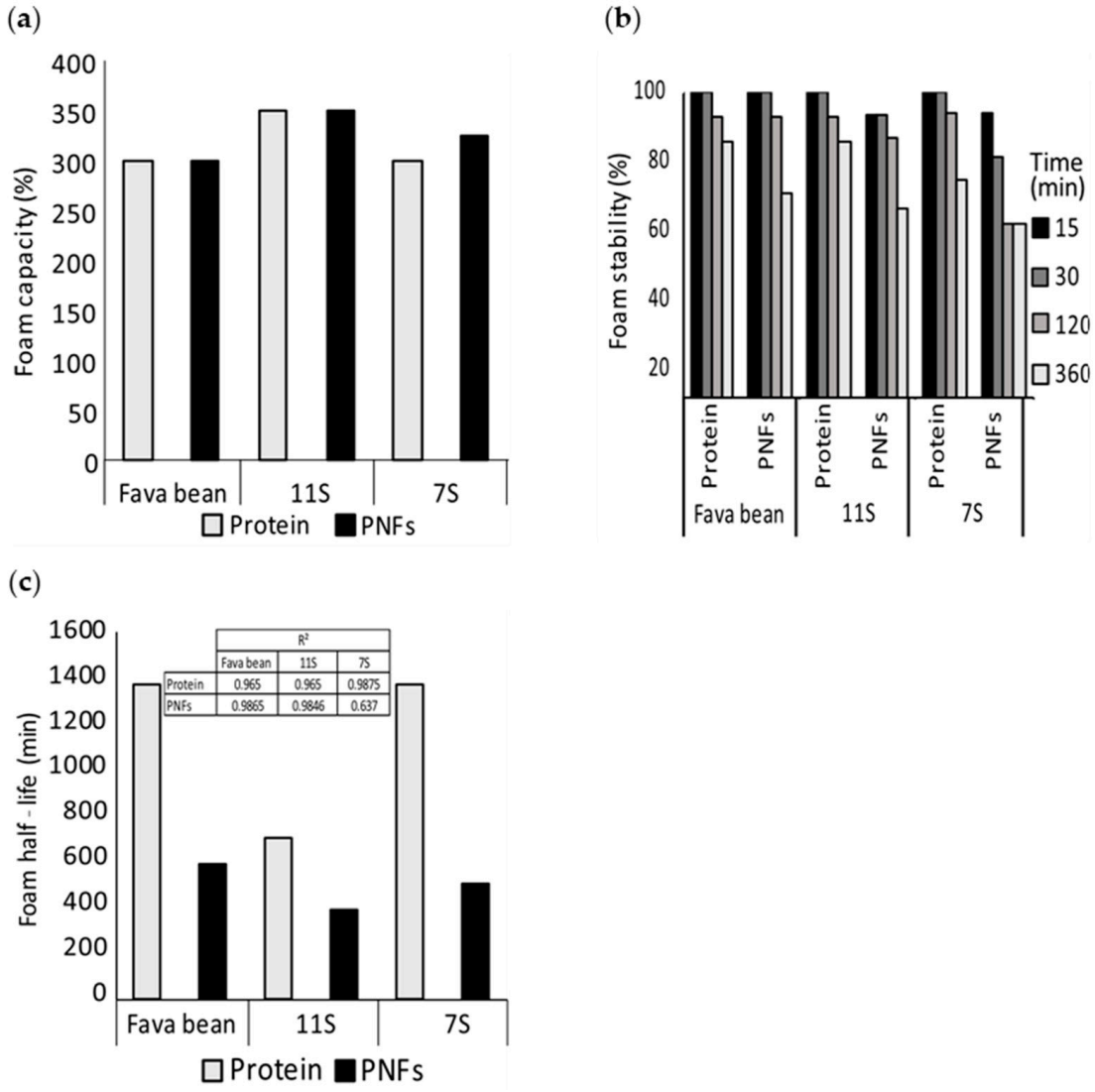

Figure 6. (**a**) Foaming capacity, (**b**) foam stability, and (**c**) foam half-life of fibrillated and non-fibrillated protein from fava bean and its globular fractions 11S and 7S. The foams were generated at pH 7 and concentration of 1 mg/mL. The insert in (**c**) shows the model fit (R^2).

This was confirmed by confocal microscopy of the foam structure (Figure 7). The non-fibrillated samples seemed to have a more substantial fluorescent film of air bubbles and a more uniform distribution of small and large bubbles. Only some of the peptides formed during incubation at low pH and high temperature contribute to the fibril formation [31]. When investigating the foaming properties of fibrillated 11S protein from soybean as a mixture and when separated into a pure fibril and pure peptide fraction, Wan et al. (2021) concluded that the peptides, and not the PNFs, contributed to the stability of the foams [19]. In contrast to the earlier study by our research group [20], we found that when fibrillated mung bean protein was separated into a pure fibril fraction and a pure

peptide fraction, the pure PNFs still formed more stable foams at pH 5 than the peptide fraction at a concentration of 1 mg/mL.

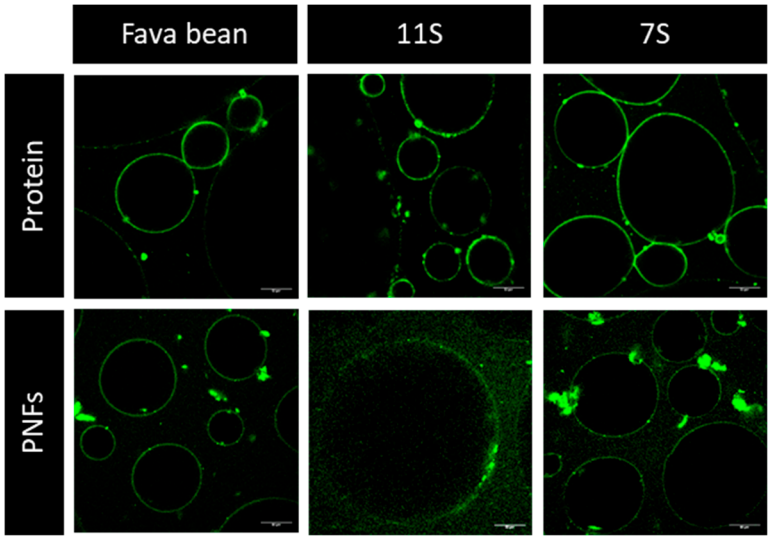

Figure 7. Confocal images (staining with thioflavin T fluorescent dye) of bubbles stabilised by non-fibrillated (protein) and fibrillated (protein nanofibrils, PNFs) proteins from fava bean and its globular fractions 7S and 11S. Scale bar 50 μm.

However, it is still not fully understood how curved PNFs contribute to stabilising the foams at low concentrations. A possible explanation is that curved PNFs increase the bulk viscosity in the solution's continuous phase and thereby sterically hinder bubble rupture. At a pH close to the isoelectric point, the viscosity increased even more due to the aggregation of the PNFs, which probably explains why the foam produced from pure mung bean fibrils was most stable at pH 5 [20]. The fibrillated protein from fava bean, 11S, and 7S did not form stable foams at this low concentration (1 mg/mL), even at pH 5 (Figure S2 in SM). The less stable foams probably developed because the straight PNFs generated from fava bean and its major globulin fractions (11S and 7S) were not sufficiently viscous to help stabilise the continuous phase between the bubbles at these low concentrations. Additionally, it cannot be excluded that the peptides in the fibrillated fava bean, 11S, and 7S samples had an impact on the superior foaming properties at the higher concentration (10 mg/mL), as previously observed for fibrillated samples from soy 11S [19]. The PNFs produced from fava bean and its globular fraction might be too inflexible to stabilise the interfacial film of the air bubbles. At the lower concentration (1 mg/mL), the peptides might be too diluted to maintain the stabilising effect observed at higher concentrations. To summarise, based on the results from this study, our previous study about curved PNFs from mung bean, and the study conducted by Wan et al. (2021) about PNF from soy 11S [19], it appears that no general conclusions can be drawn about plant-based PNFs foam stabilisation ability. PNFs will have different morphology (straight/curved) depending on the protein sources. Our findings indicated that curved PNFs are superior in their foam stabilising ability because of their ability to form a more viscous sample.

The non-fibrillated proteins had approximately the same foaming capacity and foam stability/half-life at low concentrations (Figure 6a–c) as at high concentrations (Figure 5a–c). Similarly, a previous study investigating the foaming properties of a fava bean protein isolate at pH 7 and concentrations from 0.1–3% (w/v) observed that higher concentrations did not give any significant improvement in foaming capacity or stability [32]. In contrast, the foams generated from non-fibrillated protein in the present study had higher foaming capacity. This might be due to the differences in the method used for foam formation or sample preparation. The only difference in treatment between the non-fibrillated and fibrillated proteins examined in this study was that the non-fibrillated proteins were not

heated. The initial adjustment of the pH to 2 might have caused the partial unfolding of the native protein structure, generating smaller and more flexible structures and exposing hydrophobic sites that could help stabilise the air/liquid interface [33].

4. Conclusions

This study showed that whole protein isolates from fava bean and its two major globulin fractions, 11S and 7S, form straight semi-flexible PNFs at pH 2 when heated at 85 °C for 24 h. Based on the data from the ThT assay and AFM imaging, the 7S fraction forms PNFs more easily than the 11S fraction. The PNFs formed in all fibrillated samples were fragmented when the pH was increased from 2 to 7. At a concentration of 10 mg/mL, the fibrillated protein from all three fractions (whole fava bean, 11S, 7S) formed more voluminous and more stable foams than the non-fibrillated proteins. Fibrillated proteins probably stabilise foams due to their ability to increase the viscosity of the continuous phase, as indicated by the finding that foam stability decreased when diluting the fibrillated proteins to 1 mg/mL, which was not the case for the non-fibrillated proteins. It cannot be excluded that the peptides within the fibrillated samples also contributed to the improved foaming properties at the higher concentration. The results from this study and our earlier study on foaming properties of mung bean-based PNFs show that curved PNFs are superior to straight semi-flexible PNFs in their ability to stabilise foams at low concentrations (1 mg/mL). The results from these studies have generated a greater understanding of how plant-based PNFs with different morphologies can contribute to stabilising future food applications. The introduction of stable air bubbles in food products are important for the appearance and mouthfeel of many food applications. Today, animal proteins are superior to plant-based proteins in the aspect of creating stable bubbles in food. Here, we show that by reconstructing the plant proteins into PNFs, the ability to stabilise air bubbles dramatically increases, generating new insight into how plant protein can be used to create suitable food applications.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/foods12030521/s1: Figure S1: Viscosity as a function of shear rate (0.1–500/s) for fibrillated protein from mung bean at pH 2 and at a concentration of 10 mg/mL. Table S1: The flow consistency index (K) and flow behaviour index (n) for fibrillated protein from mung bean at pH 2. Figure S2: (**A**) Foaming capacity, (**B**) foam stability, and (**C**) foam half-life of fibrillated and non-fibrillated proteins from fava bean and its globular fractions 11S and 7S. The foams were generated at a pH of 5 and concentration of 1 mg/mL. The insert in (**C**) shows the model fit (R^2).

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Improving the Aromatic Profile of Plant-Based Meat Alternatives: Effect of Myoglobin Addition on Volatiles

Jolien Devaere [1], Ann De Winne [1], Lore Dewulf [1], Ilse Fraeye [1], Irena Šoljić [2], Elsa Lauwers [2], Andy de Jong [3] and Hermes Sanctorum [3,*]

[1] KU Leuven Technology Campus Ghent, Gebroeders De Smetstraat 1, 9000 Ghent, Belgium; jolien.devaere@kuleuven.be (J.D.); ann.dewinne@kuleuven.be (A.D.W.); lore.dewulf@kuleuven.be (L.D.); ilse.fraeye@kuleuven.be (I.F.)
[2] Paleo b.v., 12 Rue des Pr. Jeener et Brachet, 6041 Gosselies, Belgium; irena.soljic@paleo-taste.com (I.Š.); elsa.lauwers@paleo-taste.com (E.L.)
[3] Paleo b.v., Meilrijk 98, 3290 Diest, Belgium; andy.dejong@paleo-taste.com
* Correspondence: hermes.sanctorum@paleo-taste.com

Abstract: Market demand for palatable plant-based meat alternatives is on the rise. One of the challenges is formulating products with sensorial characteristics similar to conventional meat. In this study, the effect of myoglobin on the aromatic profile of plant-based meat alternatives was assessed. Plant-based burgers were made with soy-textured protein, supplemented with three levels of myoglobin (0, 0.5 and 1.0%, the latter two mimicking endogenous myoglobin levels in meat), and grilled for 12 min at 250 °C. To evaluate the aromatic profile of the compounds, raw and grilled samples were subjected to headspace solid-phase microextraction (HS-SPME) followed by gas chromatography-mass spectrometry (GC-MS). Principal component analysis (PCA) analysis was then performed to visualize the interaction between grilling and myoglobin addition, and the effect exerted on the resulting aromatic profile. Myoglobin significantly affected several classes of volatile compounds, either by itself or in conjunction with grilling. A notable increase in aldehydes and a decrease in hydrocarbons were noted after adding myoglobin. As expected, an increase in pyrazines was observed after grilling. The results suggest myoglobin positively influences the aromatic profile of plant-based meat alternatives, contributing to a profile closer to the one of conventional meat.

Keywords: myoglobin; plant-based meat alternatives; HS-SPME-GC-MS; volatiles; PCA-analysis; aroma; Maillard reaction; lipid oxidation; aldehydes; pyrazines

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1. Introduction

Meat consumption has a long history in human evolution, likely going back to the earliest known human-like ancestor living 5–7 million years ago [1]. Environmental but also health and animal welfare concerns regarding conventional meat production and consumption are the main drivers for developing meat alternatives [2–5]. To appeal to the largest consumer segment that is not committed to vegetarian or vegan diets, the food industry is placing extra emphasis on introducing meat alternatives to consumer markets. One of the aims is formulating plant-based products with similar sensorial (texture, color, flavor) [6,7] and nutritional characteristics to conventional meat [7].

Flavor and aroma are complex attributes of meat palatability. Cooking meat involves a series of reactions resulting in the development of various volatile compounds. These include Maillard reactions, lipid oxidation, interactions between Maillard reaction products and lipid oxidation products, and thiamine degradation [8]. Hundreds of volatile compounds result from these reactions, e.g., aldehydes, alcohols, carboxylic acids, ketones, pyrazines and esters. Saturated and unsaturated aldehydes, especially those containing 6–10 atoms of carbon, are a major contributor to the volatile profile and flavor development of cooked meat [9,10]. Roast flavors in foods are usually associated with the presence of

heterocyclic compounds such as pyrazines, thiazoles and oxazoles. In well-done grilled meat, pyrazines are reported to be the major class of volatiles [11].

The inherent flavor and aromas of a meat product can be influenced by its lipid content [12] and pH [13], the diet, age and gender of the animal [14], and the presence of myoglobin [15]. Myoglobin is a globular heme protein found in muscles, capable of reversible oxygen binding via a heme-bound iron atom [16]. It is important for the sensory quality of meat and has been associated with a serum-like taste and metallic mouthfeel of beef [17]. The close relationship of heme proteins (hemoglobin and myoglobin) with lipid oxidation during cooking has been extensively studied [18–20]. Myoglobin has long been proposed to contribute to aroma development by catalyzing lipid oxidation reactions [21,22]. Lipid oxidation products in turn promote myoglobin oxidation and alter its stability [23].

Upon cooking, myoglobin unfolds, exposing the heme cofactor. The cofactor then catalyzes a series of reactions that transform amino acids, nucleotides, vitamins and sugars in the meat into a variety of flavor and aroma compounds characteristic of the complex aromatic profile of cooked meat. Consistent with the important role of heme iron in meat aroma, a plant heme protein, is now commercially used to optimize flavor in ground beef analogues intended to be cooked [24]. This heme protein, called Leghemoglobin, is originally found in the root nodules of leguminous plants.

Some published studies assess the safety, toxicity, and allergy potential of plant heme-protein in plant-based meat alternatives [25,26]. However, to our best knowledge, there is no publicly available data on the influence of myoglobin, the muscle heme protein, on the aromatic profile of meat alternatives. In the present proof-of-concept study, we used gas chromatography-mass spectrometry (GC-MS) to assess the effect of myoglobin on the formation of volatile compounds. The perspective here is to evaluate the potential of fermentation-derived, animal-free myoglobin as functional ingredient for plant-based meat alternatives. The information presented here could therefore be of value to plant-based food manufacturers interested in ameliorating the sensory properties of their products and formulating a product closer to conventional meat.

2. Materials and Methods

2.1. Preparation of Meat Alternatives (Raw and Grilled) with Addition of Commercial Myoglobin

Plant-based burger alternatives were produced using 57.5% (m/m) of reverse osmosis (RO) water, 25% (m/m) textured soy protein (TSP) (Fuji Oil, Ghent, Belgium), 15% (m/m) sunflower oil (Vandemoortele, Ghent, Belgium), 1% (m/m) methylcellulose (50D, Snick Euroingredients, Ruddervoorde-Oostkamp, Belgium) and 1.5% (m/m) table salt, with the on top addition of either 0%, 0.5% or 1% (m/m) of commercial bovine Mb (Tebu-bio, Boechout, Belgium). First, TSP was hydrated in water (45%) for 30 min at 15 °C. Next, methylcellulose, salt and sunflower oil were added, after which the mixture was grinded using a plate with 4 mm openings (meat mincer, Minerva Omega Group, Bologna, Italy). Then, the Mb was dissolved in water (12.5%) and mixed with the batter. Raw hamburgers (30 g, Ø 6 cm) were shaped manually and baked for 12 min at 250 °C in an oven (Rational Climaplus Combi CPC 61, Paal, Belgium).

2.2. HS-SPME-GC-MS Analysis

The volatile compounds of burger samples were isolated by means of headspace solid-phase microextraction (HS-SPME) using a Gerstel MPS2 autosampler fitted with a 50/30 µm Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/Carboxen/PDMS) SPME fiber (Supelco, Bellefonte, PA, USA). Prior to analysis, the SPME fiber was conditioned for 30 min at 270 °C, according to the manufacturer's instructions.

Three grams (± 0.1%) of sample material was transferred into 20 mL glass headspace vials, sealed with aluminum crimp caps lined with PTFE/silicone septa and stored in a cooled tray at 4 °C until analysis. Samples were equilibrated at 45 °C for 20 min with intermittent agitation at 250 rpm (5 s on/2 s off). During the last five minutes of incubation,

the SPME fiber was conditioned at 270 °C and then immediately exposed to the vial headspace for 40 min at 45 °C to extract the headspace volatiles.

Extracted compounds were subsequently separated and analyzed using an Agilent 7890A/5975C GC-MS system equipped with an Agilent HP-1ms capillary column (30 m × 0.25 mm × 0.25 µm). The SPME fiber was desorbed for 5 min in the GC inlet at 250 °C using a 0.75 mm ID HS-SPME liner (Supelco, Bellefonte, PA, USA), in splitless mode. An initial oven temperature of 35 °C was held for 5 min and increased at 4 °C/min to 215 °C, followed by a second ramp of 7 °C/min to a final oven temperature of 250 °C, which was held for 5 min. Helium (99.9999%) was used as a carrier gas at a constant flow rate of 1.2 mL/min.

The MS detector was operated in Electron impact ionization (EI) mode with an ionization energy of 70 eV. The source and quadrupole temperatures were set to 230 and 150 °C, respectively. Mass ranges were scanned between 40 and 250 m/z.

2.3. Data Analysis

Volatile organic compounds (VOC) were tentatively identified by matching mass spectra with MS data libraries (NIST08, WILEY275) and by comparing their linear retention indices (LRI) with the literature. LRI are calculated using Van Den Dool and Kratz's equation for temperature programmed GC conditions, in which t_x is the retention time of compound "x", and t_n and t_{n+1} are the retention times of n-alkanes (C_6–C_{16}) with carbon number "n" eluting before and after compound "x":

$$(LRI)_x = 100n + 100 \times \frac{t_x - t_n}{t_{n+1} - t_n} \quad (1)$$

Identified VOCs were classified according to their chemical nature: organic acids, alcohols, aldehydes (saturated, unsaturated, branched and cyclic), hydrocarbons, ketones, phenols, pyrazines and others. The peak areas of individual compounds as well as the total peak areas of each class of compounds are reported as area units (AU) × 10^3 (mean ± standard deviation) of HS-SPME-GC-MS analysis performed in triplicate.

The effect of Mb addition (% Mb), grilling (G) and their interaction (% Mb × G) on individual volatiles or groups of compounds was statistically analyzed through two-way ANOVA using IBM SPSS 27. In case of a significant interaction, the interaction term was further interpreted using one-way ANOVA followed by a post-hoc Tukey's honestly significant difference (HSD) test. A significance level of $p < 0.05$ was employed.

Principal component analysis (PCA) was carried out using The Unscrambler X (v. 10.5.1) on a multivariate dataset containing peak areas of all identified compounds in each analyzed burger. Data were pre-processed by mean-centering and scaling to unit variance prior to analysis. The first two principal components (PCs) were considered to visualize the interaction between grilling and myoglobin enrichment of meat alternatives, and the resulting aromatic profile.

3. Results and Discussion

3.1. Volatile Profile

A total of 40 volatile compounds were identified in the analyzed samples (Table 1), of which 3 appeared to be exclusive to grilled samples: furfural, furfuryl alcohol and pyrrole. The volatile profile of raw meat alternatives (RMA), without addition of myoglobin (Mb), consists mainly of hydrocarbons and 2-pentylfuran, which account for 34.0 ± 6.7% and 29.7 ± 2.2% of the total peak area respectively, followed by alcohols (13.7%) and unsaturated aldehydes (12.0%). Grilling the plant-based meat alternatives (GMA) is associated with a significant decrease in alcohol and cyclic aldehyde content, in favor of the formation of branched aldehydes, ketones, phenols and most notably pyrazines. Despite this shift in volatile fractions, the grilling process does not significantly affect the total peak area of volatiles present in RMA compared to GMA. However, addition of myoglobin (Mb) leads to significant ($p < 0.05$) formation of various odor-active volatile compounds. Statistical

analysis further indicates a significant interaction between effects of Mb addition and grilling (%Mb × G) on the total peak area, suggesting that a synergetic relationship exists between both factors.

A total of 11 saturated and unsaturated aldehydes are identified in the plant-based meat alternatives, supplemented with Mb. Most of these aldehydes are typically associated with (auto-)oxidation of unsaturated fatty acids in food matrices [27]. Due to their low odor thresholds, aldehydes are known to greatly impact the aroma of meat and meat products. Of all classes of chemical compounds, the peak area of saturated aldehydes displays the most pronounced increase with rising Mb concentrations, making them the dominant fraction of the volatile profile in Mb-enriched samples. In RMA + 1.0%Mb and GMA + 1.0%Mb, they respectively make up 36.9 ± 1.0% and 30.2 ± 1.3% of the total peak area. Hexanal is the most abundant aldehyde in the analyzed samples. In high concentrations, it imparts an unpleasant rancid odor, but at low levels it is characterized by a pleasant, grassy aroma and generally contributes to a desired aroma in meat products [28,29]. RMA contains high initial levels of hexanal (10.6 ± 1.3% of the total peak area), which may originate from the soy protein used to prepare the burgers. This aldehyde is reported as the predominant volatile compound in soybeans, accounting for 40.9% of the total volatile profile of the ingredient [30]. Grilling found to reduce the amount of hexanal in GMA. Hexanal can also be formed as an oxidation product of linoleic acid and is often considered as an excellent indicator of lipid oxidation in meats and meat products rich in n-6 polyunsaturated fatty acids [31]. Addition of Mb in both RMA + Mb and GMA + Mb leads to considerable increases in hexanal content which greatly exceeds the decrease caused by the grilling process in GMA. Additionally, the highest peak areas for hexanal are found in GMA + Mb. This suggests that the addition of Mb has a significant ($p < 0.01$) influence on the degree of lipid oxidation in RMA + Mb and GMA + Mb, which is further amplified by the heat of the grilling treatment. Further research is required to determine whether these hexanal concentrations exceed rancid odor thresholds or remain within a desirable range. Among other identified aldehydes, heptanal and nonanal (saturated aldehydes derived from oleic acid), and unsaturated aldehydes exhibit similar, increasing tendencies in relation to Mb concentration. Branched aldehydes (2- and 3-methylbutanal), on the other hand, originate from proteolysis and degradation of amino acids [27], and exhibit an opposite behavior, whereby they decrease as the Mb content increases in both raw and grilled samples.

The volatile profile of meat products and the analyzed plant-based meat alternatives appears to differ fundamentally in terms of hydrocarbon content, which represents a substantial portion of the total peak area in RMA (34.0 ± 6.7%) and GMA (32.9 ± 2.1%). Hexane is the most abundant hydrocarbon observed in all analyzed samples. While it is not naturally present in soybeans [30,32], it may be a residue from lipid extraction using hexane as a solvent during the production of soy protein [33]. HS-SPME-GC-MS analysis of raw ingredients, performed under identical conditions to those of the sample analysis, indicated that hexane was the most abundant volatile compound in the TSP used to prepare the plant-based burgers (data not shown). In meat matrices, hydrocarbon compounds are generally reported in low levels and are not considered to contribute significantly to meat-like aromas [34–38]. They are considered to originate from the thermal oxidative decomposition of lipids, catalyzed by heme compounds such as hemoglobin and myoglobin [6]. Research data on aroma development during the heat treatment of meat products is limited and not conclusive regarding hydrocarbon contents. Contrary to the understanding of how they are formed, short-chain carbohydrates (<C14) are mostly reported to degrade or to be unaffected during the cooking of beef and pork [7,10]. In GMA grilling does not affect the hydrocarbon content either. On the other hand, addition of Mb is found to reduce the amount of hydrocarbon compounds (except octane) to 13.7 ± 1.1% and 6.9 ± 0.7% of the total peak in RMA + 1.0%Mb and GMA + 1.0%Mb, respectively. Further research is required to determine whether excess hydrocarbons pose challenges with regards to flavor in meat alternatives, but if so, the addition of Mb offers potential to reduce hydrocarbon levels and better mimic the natural volatile profile of meat.

Table 1. Peak areas of volatiles components (average AU × 10^3 ± standard deviation × 10^3, n = 3) identified in raw and grilled meat alternatives enriched with varying concentrations of commercial myoglobin (Mb). Significance of main effects of % Mb, grilling (G) and their interaction (% Mb × G) following two-way ANOVA analysis are indicated (NS = not significant). In case the interaction term was found to have a significant effect, Tukey's HSD test was conducted instead of two-way ANOVA of the main effects (- = not analyzed), in which case values within the same row not sharing the same subscript were found significantly different between treatments at $p < 0.05$.

N°	Compound	Unknown LRI [1]	Reference LRI [1]	Raw Meat Alternatives (RMA)			Grilled Meat Alternatives (GMA)			2-Way ANOVA Significance (Alpha = 0.05)		
				RMA	RMA + 0.5%Mb	RMA + 1.0%Mb	GMA	GMA + 0.5%Mb	GMA + 1.0%Mb	% Mb	G	% Mb × G
	Acids			**204.67 ± 37.24**	**163.53 ± 34.15**	**304.27 ± 50.84**	**235.46 ± 160.10**	**1093.37 ± 1199.55**	**568.79 ± 377.98**	**NS**	**NS**	**NS**
1	Acetic acid	625	625	179.22 ± 33.03	118.01 ± 49.49	208.00 ± 46.91	212.77 ± 172.16	309.64 ± 101.23	263.82 ± 173.09	NS	NS	NS
2	Octanoic acid	1162	1169	5.52 ± 4.95	17.15 ± 8.81	34.27 ± 21.24	8.47 ± 7.44	101.15 ± 149.02	42.01 ± 17.96	NS	NS	NS
3	Nonanoic acid	1258	1268	19.93 ± 8.77	28.36 ± 10.44	62.00 ± 50.16	14.23 ± 12.45	682.58 ± 1015.61	262.95 ± 214.72	NS	NS	NS
	Alcohols			**3685.55 ± 524.65**	**4684.63 ± 591.67**	**5586.83 ± 163.30**	**1545.67 ± 228.85**	**3364.99 ± 174.73**	**4244.33 ± 318.35**	**0.000**	**0.000**	**NS**
4	Furfuryl alcohol	837	826	n.d. ^{a}	n.d. ^{a}	n.d. ^{a}	68.35 ± 36.08 ^{a,b}	89.64 ± 35.55 ^{b,c}	155.34 ± 36.25 ^{c}	-	-	0.030
5	1-Hexanol	855	850	2976.13 ± 416.67	2089.81 ± 442.57	1950.16 ± 274.80	1116.23 ± 209.27	727.07 ± 96.73	386.26 ± 46.11	0.001	0.000	NS
6	1-Octen-3-ol	966	983	709.42 ± 109.36	2594.82 ± 310.85	3636.68 ± 399.18	361.09 ± 24.91	2548.28 ± 164.25	3702.73 ± 301.48	0.000	NS	NS
	Branched Aldehydes			**5.15 ± 8.91 ^{a}**	**14.28 ± 24.73 ^{a}**	**9.76 ± 16.90 ^{a}**	**506.23 ± 21.30 ^{c}**	**192.92 ± 48.22 ^{b}**	**199.87 ± 41.69 ^{b}**	-	-	**0.000**
7	3-Methylbutanal	625	622	5.15 ± 8.91 ^{a}	9.66 ± 16.73 ^{a}	5.73 ± 9.93 ^{a}	143.27 ± 15.05 ^{c}	66.47 ± 10.18 ^{b}	63.01 ± 8.83 ^{b}	-	-	0.000
8	2-Methylbutanal	635	627	n.d. ^{a}	4.62 ± 8.00 ^{a}	4.03 ± 6.98 ^{a}	362.96 ± 9.83 ^{c}	126.45 ± 38.42 ^{b}	136.86 ± 34.70 ^{b}	-	-	0.000
	Cyclic Aldehydes			**1146.19 ± 107.85**	**1742.39 ± 299.87**	**2177.20 ± 300.31**	**699.86 ± 105.60**	**1167.18 ± 107.89**	**1368.04 ± 25.49**	**0.000**	**0.000**	**NS**
9	Furfural	799	802	n.d. ^{a}	n.d. ^{a}	n.d. ^{a}	16.15 ± 27.97 ^{a}	106.27 ± 52.19 ^{b}	148.46 ± 35.45 ^{b}	-	-	0.005
10	Benzaldehyde	922	921	1146.19 ± 107.85	1742.39 ± 299.87	2177.20 ± 300.31	683.71 ± 78.49	1060.91 ± 68.71	1219.58 ± 60.78	0.000	0.000	NS
	Saturated Aldehydes			**3193.57 ± 343.90 ^{a}**	**10,662.48 ± 1011.73 ^{b}**	**13,330.93 ± 481.47 ^{c}**	**1556.37 ± 173.13 ^{a}**	**11,275.58 ± 921.93 ^{b}**	**14,951.23 ± 41.23 ^{c}**	-	-	**0.002**
11	Hexanal	775	771	2834.01 ± 321.13 ^{b}	10,340.95 ± 916.87 ^{c}	12,848.83 ± 420.92 ^{d}	1201.11 ± 176.28 ^{a}	10,800.63 ± 911.84 ^{c}	14,297.20 ± 11.47 ^{d}	-	-	0.002
12	Heptanal	877	874	151.18 ± 16.64 ^{a,b}	114.66 ± 63.14 ^{a}	165.32 ± 14.29 ^{a,b}	124.27 ± 7.53 ^{a}	213.31 ± 7.45 ^{b,c}	253.48 ± 9.39 ^{c}	-	-	0.004
13	Nonanal	1081	1083	198.36 ± 24.19	206.87 ± 44.04	316.78 ± 63.56	212.23 ± 8.77	261.64 ± 23.14	400.55 ± 26.92	0.000	0.012	NS
14	Decanal	1183	1203	10.02 ± 9.15	n.d.	n.d.	18.77 ± 2.25	n.d.	n.d.	0.000	NS	NS
	Unsaturated Aldehydes			**64.91 ± 11.69 ^{a}**	**1713.19 ± 333.87 ^{b}**	**3028.08 ± 716.24 ^{c}**	**111.56 ± 3.34 ^{a}**	**2412.52 ± 257.87 ^{b,c}**	**4291.35 ± 375.27 ^{d}**	-	-	**0.046**
15	2-Heptenal	928	951	n.d. ^{a}	910.59 ± 130.13 ^{b}	1482.92 ± 264.78 ^{c}	n.d. ^{a}	1135.85 ± 136.92 ^{b,c}	1984.58 ± 147.95 ^{d}	-	-	0.036
16	2-Octenal	1030	1061	34.27 ± 1.89 ^{a}	310.70 ± 64.42 ^{b}	563.49 ± 167.38 ^{c}	29.13 ± 8.54 ^{a}	396.22 ± 62.10 ^{b,c}	908.21 ± 79.95 ^{d}	-	-	0.010

Table 1. *Cont.*

N°	Compound	Unknown LRI [1]	Reference LRI [1]	Raw Meat Alternatives (RMA)			Grilled Meat Alternatives (GMA)			2-Way ANOVA Significance (Alpha = 0.05)		
				RMA	RMA + 0.5%Mb	RMA + 1.0%Mb	GMA	GMA + 0.5%Mb	GMA + 1.0%Mb	% Mb	G	% Mb × G
17	(E,E-)-2,4-Nonadienal	1182	1199	8.68 ± 7.53	373.74 ± 113.42	719.18 ± 210.16	n.d.	338.64 ± 40.71	735.01 ± 87.60	0.000	NS	NS
18	2-Decenal	1233	1256	0.85 ± 1.47	23.09 ± 8.46	55.54 ± 19.43	1.31 ± 2.27	30.08 ± 3.60	74.49 ± 8.48	0.000	NS	NS
19	(E,Z-)-2,4-Decadienal	1263	1268	n.d. ^{a}	19.97 ± 7.63 ^{a,b}	48.12 ± 15.24 ^{b}	n.d. ^{a,b}	56.37 ± 4.00 ^{c}	81.44 ± 9.77 ^{c}	-	-	0.004
20	(E,E-)-2,4-Decadienal	1283	1288	18.65 ± 4.85 ^{a}	66.21 ± 26.82 ^{a,b}	133.86 ± 34.23 ^{b}	79.56 ± 2.31 ^{a,b}	441.46 ± 44.42 ^{c}	472.97 ± 57.88 ^{c}	-	-	0.000
21	2-Undecenal	1334	1350	2.47 ± 2.61	8.89 ± 4.20	24.96 ± 10.36	1.56 ± 1.37	13.90 ± 2.04	34.65 ± 3.66	0.000	NS	NS
	Alkanes			**9537.27 ± 3932.97**	**4757.54 ± 1085.68**	**4946.03 ± 605.80**	**9807.61 ± 521.13**	**4839.94 ± 633.10**	**3430.74 ± 447.39**	**0.000**	**NS**	**NS**
22	2-Methylpentane	<600	553	577.36 ± 431.74	262.44 ± 111.86	290.99 ± 30.33	602.01 ± 74.63	321.95 ± 91.95	232.19 ± 28.72	0.020	NS	NS
23	3-Methylpentane	<600	590	1091.26 ± 558.45	521.94 ± 142.57	562.94 ± 91.92	1107.55 ± 86.87	531.33 ± 112.32	375.19 ± 63.76	0.001	NS	NS
24	Hexane	600	600	5502.04 ± 1770.46	2556.70 ± 468.23	2619.76 ± 338.67	6235.53 ± 306.44	2686.46 ± 304.45	1835.29 ± 209.84	0.000	NS	NS
25	Methylcyclopentane	618	618	1125.23 ± 673.07	687.57 ± 146.88	658.26 ± 54.99	807.21 ± 93.13	443.10 ± 75.67	338.51 ± 78.14	0.032	NS	NS
26	Cyclohexane	647	647	1159.83 ± 485.84	693.62 ± 196.39	760.50 ± 92.52	712.66 ± 63.45	395.53 ± 44.83	322.60 ± 47.96	0.013	0.003	NS
27	Octane	800	800	81.56 ± 20.14 ^{a}	35.28 ± 30.63 ^{a}	53.59 ± 6.93 ^{a}	342.65 ± 6.78 ^{b}	461.57 ± 23.92 ^{c}	326.96 ± 30.81 ^{b}	-	-	0.000
	Ketones			**625.06 ± 72.65**	**715.46 ± 134.18**	**796.79 ± 18.06**	**2708.99 ± 53.96**	**2488.64 ± 514.06**	**3340.68 ± 361.13**	**0.021**	**0.000**	**NS**
28	2-Heptanone	868	870	582.86 ± 66.40	458.72 ± 83.76	434.12 ± 59.12	2674.78 ± 53.78	2088.24 ± 518.36	2723.19 ± 399.77	NS	0.000	NS
29	2,3-Octanedione	963	966	42.21 ± 6.30 ^{a}	256.74 ± 53.32 ^{b}	362.67 ± 41.37 ^{c}	34.21 ± 0.97 ^{a}	400.41 ± 22.52 ^{c}	617.49 ± 39.43 ^{d}	-	-	0.000
	Phenols			**12.62 ± 3.70**	**n.d.**	**0.19 ± 0.33**	**185.83 ± 60.22**	**169.72 ± 31.93**	**310.78 ± 105.83**	**NS**	**0.000**	**NS**
30	Guaiacol	1056	1052	0.57 ± 0.99 ^{a}	n.d. ^{a}	0.19 ± 0.33 ^{a}	52.99 ± 32.95 ^{a,b}	94.46 ± 29.05 ^{b}	205.96 ± 46.20 ^{c}	-	-	0.001
31	2-Methoxy-4-vinylphenol	1070	1060	12.05 ± 2.83	n.d.	n.d.	132.85 ± 50.37	75.26 ± 11.17	104.82 ± 61.59	NS	0.000	NS
	Pyrazines			**300.22 ± 28.26 ^{a}**	**270.90 ± 60.00 ^{a}**	**285.85 ± 34.30 ^{a}**	**3557.19 ± 948.44 ^{b}**	**4717.95 ± 1057.69 ^{b}**	**7969.41 ± 1309.12 ^{c}**	-	-	**0.001**
32	Methylpyrazine	794	796	21.31 ± 20.28 ^{a}	51.44 ± 20.80 ^{a,b}	36.19 ± 31.53 ^{a}	495.34 ± 123.11 ^{b,c}	802.87 ± 226.49 ^{c}	1266.36 ± 305.34 ^{d}	-	-	0.006
33	2,5-Dimethylpyrazine	882	884	101.22 ± 4.32 ^{a}	79.74 ± 17.63 ^{a}	91.92 ± 2.71 ^{a}	1264.96 ± 366.65 ^{b}	1649.87 ± 388.37 ^{b}	2938.08 ± 510.88 ^{c}	-	-	0.001
34	2-Ethyl-6-methylpyrazine	971	970	24.39 ± 4.90 ^{a}	20.31 ± 2.23 ^{a}	23.61 ± 1.16 ^{a}	321.32 ± 100.02 ^{b}	410.41 ± 112.05 ^{b}	710.70 ± 145.65 ^{c}	-	-	0.005

Table 1. *Cont.*

N°	Compound	Unknown LRI [1]	Reference LRI [1]	Raw Meat Alternatives (RMA)			Grilled Meat Alternatives (GMA)			2-Way ANOVA Significance (Alpha = 0.05)		
				RMA	RMA + 0.5%Mb	RMA + 1.0%Mb	GMA	GMA + 0.5%Mb	GMA + 1.0%Mb	% Mb	G	% Mb × G
35	2-Ethyl-5-methylpyrazine	973	973	57.26 ± 10.19 ^{a}	41.63 ± 7.28 ^{a}	51.90 ± 7.34 ^{a}	647.55 ± 134.84 ^{b}	850.09 ± 167.10 ^{b}	1390.69 ± 158.40 ^{c}	-	-	0.000
36	2,5-Dimethyl-3-ethyl-pyrazine	1053	1053	89.18 ± 11.76 ^{a}	77.79 ± 13.32 ^{a}	79.78 ± 2.08 ^{a}	701.87 ± 196.78 ^{b}	853.54 ± 179.08 ^{b}	1392.55 ± 198.91 ^{c}	-	-	0.002
37	2-Methyl-3,5-diethyl-pyrazine	1132	1138	6.87 ± 4.10 ^{a}	n.d. ^{a}	2.46 ± 4.26 ^{a}	126.15 ± 28.06 ^{b}	151.17 ± 27.13 ^{b}	271.03 ± 57.79 ^{c}	-	-	0.002
	Others			**8461.00 ± 1206.46 ^{b}**	**5849.08 ± 417.59 ^{a}**	**5719.21 ± 224.49 ^{a}**	**8937.64 ± 28.79 ^{b}**	**8661.31 ± 152.26 ^{b}**	**8847.58 ± 969.56 ^{b}**	-	-	**0.009**
38	Pyrrole	727	731	n.d.	n.d.	n.d.	280.33 ± 67.51	232.82 ± 117.28	270.24 ± 71.31	NS	0.000	NS
39	2-Pentylfuran	978	994	8001.63 ± 1252.79 ^{b}	5354.32 ± 177.22 ^{a}	5136.56 ± 258.42 ^{a}	7706.66 ± 314.55 ^{b}	7365.33 ± 194.02 ^{b}	7196.63 ± 798.75 ^{b}	-	-	0.011
40	Maltol	1276	1272	459.38 ± 88.24	494.76 ± 242.44	582.65 ± 118.56	950.65 ± 222.92	1063.15 ± 22.85	1380.71 ± 107.83	0.024	0.000	NS
	Total volatiles			**27,236.22 ± 6183.06 ^{a}**	**30,573.47 ± 3231.45 ^{a,b}**	**36,185.15 ± 2222.29 ^{b}**	**29,852.42 ± 480.67 ^{a,b}**	**40,384.13 ± 1512.33 ^{c}**	**49,522.78 ± 1936.45 ^{d}**	-	-	**0.035**

[1] Linear retention index (LRI) based on 30 m HP-1ms column. Reference values obtained by comparison with sources from the literature in the NIST Chemistry WebBook (https://webbook.nist.gov/chemistry, accessed on 20 May 2022) using similar columns and similar temperature programs.

All six pyrazine compounds identified in the analyzed samples have previously been reported in beef, pork, chicken and mutton [34]. Pyrazines are derived from Maillard reactions and their presence in meat matrices is mainly associated with roasted aromas [39–41]. Small amounts of pyrazines are initially present in RMA, and do not vary with Mb addition. They are likely formed during the extrusion-cooking process in the production of TSP. Temperatures at which soy protein are heated during extrusion generally range from 120 to 180 °C, at which point pyrazines can be formed via the Maillard reaction [42–44]. HS-SPME-GC-MS analysis of raw materials (data not shown) confirmed the presence of all six pyrazines in TSP, and similar findings have previously been reported in the literature [45]. Grilling is expected to increase the pyrazine content in plant-based meat alternatives considerably, but Mb supplementation in GMA is found to further stimulate pyrazine formation significantly ($p < 0.05$). Interactions are known to occur between products derived from lipid-oxidation and intermediates of the Maillard reaction [46,47]. The pyrazine fraction of the total volatile profile increases from 11.9 ± 3.0% in GMA to 16.1 ± 2.3% in GMA + 1.0%Mb. Other Maillard-related compounds, such as furfural and furfuryl alcohol [48], are found to exhibit similar patterns. These compounds are not detected in RMA and display a rising trend in function of Mb concentration in combination with grilling. Mb addition increases pyrazine formation in grilled samples, possibly by enhancing the Maillard browning reaction, thereby improving the desired roasted aromatic profile in plant-based meat alternatives.

Beyond aldehydes, hydrocarbons and pyrazines, other aromatic compounds found in meat alternatives include organic acids, alcohols, ketones, phenols and others (pyrrole, 2-pentylfuran and maltol). The amounts of acidic compounds display large fluctuations between the different repetitions, but no significant changes are observed as a function of the grill treatment or the addition of Mb. All remaining volatile compounds are generally found to increase with rising Mb concentrations, except for 2-heptanone, 2-methoxy-4-methylphenol and pyrrole.

3.2. Multivariate Analysis

The results from Table 1 revealed that the total peak areas of the different meat alternatives were significantly influenced by both addition of Mb and grilling as well as their interaction. PCA is conducted to visualize the relationship between plant-based meat alternatives in terms of their volatile profile (Figure 1). The score plot (Figure 1A) shows that the first two principal components (PC) explained 79.8% of the total variability. All VOCs (n = 40) are shown in the plane of the first 2 PC (PC1 and PC2); the circles indicate if variables are reconstructed at the 50% (inner circle) and 100% (outer circle) of the total explained variance (Figure 1B).

The first PC, accounting for 52% of variance, separates GMA + Mb samples from GMA, RMA and RMA + Mb. Additional contribution of PC2, explaining 28% of variance, drives scores upwards as a function of increasing Mb concentration and down as a result of grilling. In the resulting score plot, raw and roasted samples are clearly separated by a diagonal line. Grilling causes a strong increase in pyrazine content, which is characterised by a shift towards the lower left. Additionally, the samples migrate along the separation line towards the upper right corner as a function of Mb addition. Evidence for a significant interaction between Mb addition and grill treatment is apparent from the fact that the direction of RMA and RMA + Mb shifts to their corresponding GMA and GMA + Mb scores are not parallel, and from the greater distance between GMA and GMA + Mb clusters, compared to RMA and RMA + Mb. The addition of Mb is necessary for aroma precursor formation whereas grilling is mainly responsible for transforming these precursors into volatile components. While most volatile components clearly play a role, acids (1–3) appear to be less important. GMA + Mb were mainly described by saturated (11–13) and unsaturated aldehydes (15–21) and pyrazines (32–37). As explained above, RMA are characterized by mainly (branched) hydrocarbons (22–26), 1-hexanol (5) and decanal (14).

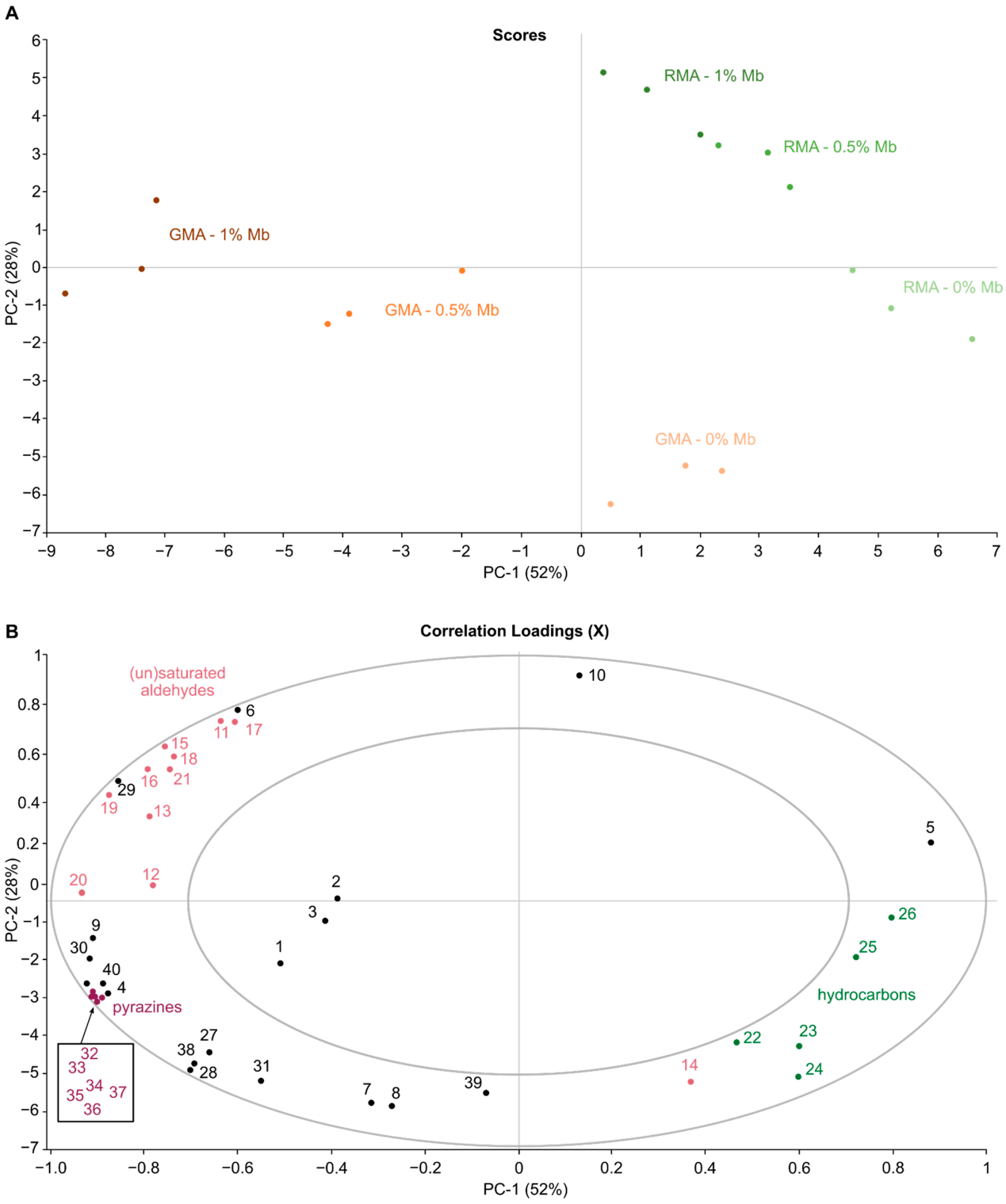

Figure 1. PCA scores (**A**) and correlation loadings (**B**) plots of PC1 and PC2 for volatile compounds in raw and grilled plant-based burgers enriched with varying concentrations of myoglobin. Volatiles (loadings) are denoted by their numbers in Table 1 [Acids (1–3), Aldehydes (7–21), Hydrocarbons (22–27), Pyrazines (32–37)].

4. Conclusions

Formulating meat alternatives that are attractive for a large consumer segment is essential to meet the ever-growing global protein demand. The results from the present study indicate that supplementing meat alternatives with myoglobin has the potential to enhance the volatile profile in a desirable way.

5. Patents

Data included in this manuscript are part of an international patent application No. PCT/EP2021/087884.

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Effect of Different Extraction Methods on Physicochemical Characteristics and Antioxidant Activity of C-Phycocyanin from Dry Biomass of *Arthrospira platensis*

Qian Chen [1], Shuhui Li [2], Hua Xiong [1] and Qiang Zhao [1,*]

[1] State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, China; ncuspychenqian@163.com (Q.C.); huaxiong100@126.com (H.X.)
[2] Jiangxi Academy of Agricultural Sciences, Nanchang 330200, China; linlish1023@163.com
* Correspondence: qiangzhao@ncu.edu.cn or qiangzhao1221@yahoo.com; Tel./Fax: +86-791-86634810

Abstract: The effect of four different extraction methods on physicochemical characteristics and functionalities of chloro-phycocyanin (CP) was investigated. Swelling (S-CP), freezing and thawing (4FT-CP), ultrasonication with freezing and thawing (4FT+U-CP), and the high-pressure cell disruption (HPCD-CP) process affected CP differently, thus resulting in different levels of solubility, DPPH scavenging activity, ABTS scavenging activity, and reducing power. Among the four CPs, HPCD-CP had the highest CP content (15.3%), purity (1.66 ± 0.16), and ΔE value but the lowest Δb value. The ζ potential of HPCD-CP (−38.8 mV) was the highest, but the average particle size of 4FT+U-CP (719.1 nm) was the highest. UV-Vis absorption spectra and fluorescence spectra illustrated that high-pressure cell disruption-assisted extraction had more profound impacts on the microenvironment of tetrapyrrole chromophores, the environment of aromatic amino acids, and the phycocyanobilin of CP. Furthermore, HPCD-CP and 4FT-CP showed higher solubility and antioxidant activities than S-CP, especially 4FT+U-CP. The results obtained in this study demonstrate that HPCD technology could obtain a food-grade C-phycocyanin product with higher CP concentration, purity, solubility, and antioxidant activity.

Keywords: C-phycocyanin; high-pressure cell disruption; antioxidant activity; extraction method; *Arthrospira platensis*

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1. Introduction

Spirulina (*Arthrospira platensis*) is a blue-green alga rich in protein (60–70%, including phycobiliproteins). It is a supplement containing various kinds of essential amino acids, vitamins, minerals, chlorophyll, carotenoids, ascorbic acid, and phenolic compounds, thus playing a significant role in scavenging free radicals and preventing oxidative stress-related diseases [1–4]. Phycobiliproteins (PBPs) are composed of proteins and phycobilins and are covalently bound with open-chain tetrapyrrole chromophores of phycobilins via the cysteine amino acid of proteins [5,6]. Chloro-Phycocyanins (CP, blue, λ_{max} = 610–620 nm), phycoerythrins (PE, pink-purple, λ_{max} = 540–570 nm), allophycocyanins (APC, bluish-green, λ_{max} = 650–655 nm), and phycoerythrocyanins (PEC, orange, λ_{max} = 560–600 nm) are the four subclasses of PBPs. Commonly used as a blue pigment, CP constitutes the main part of PBPs in *Arthrospira platensis* when compared with PE, PEC, and APC, and it can be applied in many ways, such as in natural pigments for food and cosmetics and fluorescent tags in biomedical research [7]. In addition, owing to its high antioxidant activity against hydroxyl radicals [8] and free-radicals [9], it has been used as an agent anti-inflammatory and potential therapeutic agent for oxidative stress-induced diseases [2].

Nowadays, there are many studies aimed at CP extraction from Spirulina with different methods of cell disruption, such as thawing and homogenizing [10], freezing and thawing [11], supercritical CO_2 extraction followed by the electrocoagulation method [12],

high-pressure cell disruption [13], and ultrasound-assisted extraction [14]. Chittapun, Jonjaroen, and Charoenrat [15] indicated that the freezing and thawing technique showed better performance in terms of CP concentration than the pulsed electric field technique, while CP obtained from the pulsed electric field technique showed a higher purity. Li et al. [16] investigated the high-pressure process, pulsed electric field, and ultrasonication process techniques and concluded that most phycocyanins could be released from the broken small particles by ultrasonic waves and pass through the cell walls of Spirulina. It is worth noting that high-pressure cell disruption was superior to bead-beating for the extraction of CP [17]. Many of the reported processes to date are expounded in terms of recovery yield, purity indices, as well as the yield of CP from different primary extraction methods. However, as far as we know, there are few studies on the influences of different extraction methods of CPs on their functional and physicochemical properties [18].

Hence, in this work, we investigated and compared the effects of four extraction methods, namely the swelling process (stirred continuously at 25 °C for 12 h, S-CP), freezing and thawing process (freezing at −20 °C for 12 h and thawing to 27 °C for 12 h, 4 cycles, 4FT-CP), freezing and thawing combined with ultrasonication process (ultrasonication at 40% for 30 min after freezing and thawing process, 4FT+U-CP), and high-pressure cell disruption process (70 MPa for 1 cycle, HPCD-CP), on the physicochemical characteristics and antioxidation of CP from dry biomass of *Arthrospira platensis*. In this study, high-pressure cell disruption (HPCD) was developed as a new method using a mechanical process in the production of C-phycocyanin from the dry biomass of *Arthrospira platensis*. The findings of this study also provide more evidence to support further evaluation of the use of mechanical extraction (for other microalgae protein) for future applications in the functional food formulation industry.

2. Materials and Methods

2.1. Materials

Dry biomass of *Arthrospira platensis* was supplied by Jiangxi Zhongzao Biotechnology Co., Ltd (Ruijin, China). and stored at −20 °C. Petroleum ether (boiling range was 30~60 °C) and ammonium sulfate ($(NH_4)_2SO_4$) were bought from Xilong Scientific Co., Ltd. (Guangdong, China). Two, 2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the reagents applied were of analytical grade.

2.2. Primary Extraction of C-Phycocyanin

2.2.1. Pretreatment of *Arthrospira platensis*

Dry biomass of *Arthrospira platensis* was defatted three times with petroleum ether (60–90 °C) at a ratio of 1:10 (*w*/*v*). After decanting the supernatant, the Arthrospira platensis biomass was air-dried in a fume hood for two days, so the residual petroleum ether was allowed to evaporate.

2.2.2. Swelling Extraction Process

The swelling extraction process was performed based on the method described by Li et al. [16] with slight modifications. Predetermined weights of defatted *Arthrospira platensis* were dispersed in distilled water at a ratio of 1:20 (S/L) and stirred continuously for 12 h at room temperature in the dark. The supernatant and pellet were separated after being centrifuged in a centrifuge (LXJ-IIB, Anting Scientific Instrument Factory, Shanghai, China) at 4500 rpm for 30 min. Lastly, the supernatant was kept for purification, named S-CP.

2.2.3. High-Pressure Cell Disruption

The high-pressure cell disruption extraction process was performed by the method of Drévillon et al. [19]. Predetermined weights of defatted *Arthrospira platensis* were dispersed in distilled water at a ratio of 1:20 (S/L) and stirred continuously for 4 h at room temperature

in the dark for pre-soaking. Then, the slurry was subjected to high-pressure cell disruption with a high-pressure cell disruptor (TS-series 1.1 kW model, Constant Systems Ltd., South Easton, UK) at 70 MPa for 1 cycle. Lastly, the supernatant, named HPCD-CP, was obtained by centrifugation and kept for purification.

2.2.4. Freezing and Thawing

The freezing and thawing extraction process was accomplished according to Antecka et al. [20]. After pre-soaking in distilled water, the predetermined weights of defatted *Arthrospira platensis* were dispersed at a ratio of 1:20 (S/L). Then, the slurry underwent 4 freeze-thaw cycles. In each cycle, the slurry was continuously frozen at a certain temperature of -20 ± 2 °C for 12 h, followed by thawing for 12 h at room temperature. Lastly, the supernatant, named 4FT-CP, was obtained by centrifuge and kept for purification.

2.2.5. Ultrasonication with Freezing and Thawing

Ultrasonication with the freezing and thawing extraction process was accomplished based on the work performed by Tavanandi et al. [21]. Predetermined weights of degreased *Arthrospira platensis* were dispersed in distilled water at a ratio of 1:20 (S/L) for pre-soaking. Then, referring to our previous pre-experiment, the slurry was subjected to ultrasonication at an amplitude of 40% for 30 min (KQ-800KDE, Kunshan Ultrasonic Instrument Co., Ltd., Suzhou, China) after 4 freeze-thaw cycles. Lastly, the supernatant, named 4FT+U-CP, was obtained by centrifugation and kept for purification.

2.3. Purification of CP

The purification of CP was accomplished based on the method of Patel et al. [22] with minor modifications. The crude CP from *Arthrospira platensis* was enriched first by salting it out with solid ammonium sulfate at 25% (w/v) for 4 h. The slurry was centrifuged at 4500 rpm for 30 min, then the supernatants were recovered and further precipitated by adding solid ammonium sulfate to 50% (w/v) saturation and allowing the slurry to stand overnight at 4 °C. The precipitated proteins, containing mainly CP, were collected by centrifugation at 4500 rpm for 30 min. The isolated protein was re-suspended in distilled water and then centrifuged again at 8000 rpm for 20 min (Hitachi high-speed refrigerated centrifuge, Himac CR21N, Tokyo, Japan). Lastly, the supernatants were dialyzed (8000–12,000 Da) at 4 °C to remove the solid ammonium and freeze-dried (LGJ-18 Vacuum freeze dryer, Songyuanhuaxing Technology Develop Co., Ltd., Beijing, China) for storage. The protein content of CP was determined using the Kjeldahl method.

2.4. Color Measurement

A CIE-Lab color scale was used for measuring the spent biomass of CP by a colorimeter (HP-2136 Portable Colorimeter, Puxi Shanghai, China), according to the method of Tavanandi et al. [21]. The sample solution was placed in a 20 mL glass colorimetric bottle with distilled water as a standard [23]. All these processes were performed on white A4 paper three times. The brightness of the color was denoted by L*, where the number "0" represented black and "100" represented white. The +a* value indicated that the sample was red, while −a* was used to indicate green. +b* indicated that the sample color was yellow, while −b* indicated blue [24]. The ΔE*, ΔL*, Δa*, and Δb* values were read directly from the instrument.

2.5. UV-Vis Spectra

The absorption spectra of CP were measured on a UV-Vis spectrophotometer (TU-1900, Puxi General Instrument Co., Ltd., Beijing, China). The spectral scanning was performed in a wavelength range from 250 nm to 700 nm. The absorbance at λ = 620 nm, 652 nm, and

280 nm was extracted to calculate the CP, APC, and total protein concentration. Bennett and Bogorad [25] determined the CP concentration via the following equation:

$$\text{CP (mg/mL)} = [A_{620} - 0.474(A_{652})]/5.34 \quad (1)$$

The purity of CP was expressed as P (purity) and calculated as follows:

$$P = \frac{A_{620}}{A_{280}} \quad (2)$$

where A_{620} represents the absorption of phycocyanin, while A_{280} represents the absorption of total protein.

The CP content of the samples and the yield were evaluated using the following equations (sample concentration was 1 mg/mL):

$$\text{CP content (\%)} = \left[\frac{\text{CP (mg/mL)}}{\text{Sample content (mg/mL)}}\right] \times 100 \quad (3)$$

$$\text{Yield (\%)} = \frac{\text{Dry CP powder from primary extraction methods (g)}}{\text{Dry biomass of } \textit{Arthrospira platensis} \text{ (g)}} \times 100\% \quad (4)$$

2.6. Fluorescence Spectra

The intrinsic fluorescence of the samples was measured by a fluorescence spectrophotometer (F-7000, Hitachi, Kyoto, Japan) at a concentration of 1 mg/mL. When the sample was excited at 280 nm and 580 nm, the emission band was recorded. We set a 5 nm slit for emission.

2.7. Fourier Transform Infrared Spectroscopy (FTIR)

The infrared spectra of freeze-dried CP were measured with KBr pellets on an FTIR spectrophotometer (Nicolet 5700, Thermo Fisher, Boston, MA, USA). The spectra were scanned with a wavenumber range between 400–4000 cm^{-1}, at a resolution of 4 cm^{-1}.

2.8. Thermogravimetric Analysis (TGA)

The measurements were performed according to Lemos et al. [26] via a pre-calibrated Perkin Elmer thermogravimetric analyzer (TGA 4000, Perkin Elmer, Waltham, MA, USA). Conditions for the use of a platinum crucible were as follows: an approximately 5 mg sample mass was heated from 30 °C to 800 °C at a heating rate of 10 °C/min with a nitrogen flow rate of about 40 mL/min.

2.9. ζ Potential and Particle Size

The ζ potential and particle size distribution of the samples were detected by a Nano ZS90 Malvern Zetasizer (Malvern Instrument, Malvern, Worcestershire, UK). The samples were dispersed in distilled water at a concentration of 1 mg/mL. Each measurement was performed three times.

2.10. Protein Solubility

The protein solubility measurement was conducted according to the method of Bera and Mukherjee [27] with some modifications. To better determine the impact of the pH on the functional properties of CP, the pH of CP dispersion in distilled water (l mg/mL) was increased from 2.0 to 12.0 (intervals of 1.0). The sample solutions were stirred at room temperature for 2 h and then centrifuged at 8500 rpm for 10 min. The content of the protein in the supernatant was measured using a bicinchoninic acid (BCA) protein quantification

assay (Thermo Fisher Scientific, Darmstadt, Germany). The protein solubility (%) was calculated by the following equation:

$$\text{Solubility } (\%) = \frac{\text{Protein concentration in the supernatant } (\text{mg/mL})}{\text{Sample concentration } (\text{mg/mL})} \times 100\% \quad (5)$$

2.11. Antioxidant Activity

2.11.1. DPPH Scavenging Activity

The DPPH radical scavenging activity was determined using the previously reported method [9]. An amount of 100 µL of distilled water, with 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg of the sample solutions, was placed in a 96-well plate and then mixed with a 100 µL of DPPH ethanol solution (0.1 mmol/L). The sample containing a DPPH solution without the sample served as a control. A blank sample containing a sample solution with ethanol was also prepared. The mixture was incubated at 37 °C for 30 min and measured at 517 nm. The DPPH scavenging activity of the sample was evaluated using the following equation:

$$\text{DPPH radical scavenging activity } (\%) = \frac{A_{control} - A_{sample} + A_{blank}}{A_{control}} \times 100\% \quad (6)$$

where $A_{control}$ indicates the absorbance control group; A_{sample} indicates sample absorbance rate; A_{blank} indicates blank absorbance.

2.11.2. ABTS Scavenging Activity

The ABTS radical scavenging activity was assessed by the inhibition percentage of the ABTS radical, as described by Wang et al. [28]. 200 µL of diluted ABTS radical solution was blended with 10 µL of 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/mL of sample solutions. After 6 min incubation at 37 °C, the absorbance against the corresponding blank was measured at 734 nm. The solution containing ABTS solution without sample served as a control. The ABTS scavenging activities of the samples were evaluated using the following equation:

$$\text{ABTS scavenging activity } = \frac{A_{control} - A_{sample} + A_{blank}}{A_{control}} \times 100\% \quad (7)$$

where $A_{control}$ and A_{sample} stand for the absorbance without/with sample, respectively; A_{blank} indicates the absorbance of blank group.

2.11.3. Reducing Power

The reductive capacity was evaluated by the method previously reported by Liu et al. [9] with slight modifications. Two milliliters of phosphate-buffered saline (PBS, pH 6.6, 0.2 mol/L) and one milliliter of a potassium ferricyanide solution (1%, w/v) were added to one milliliter of 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg/mL of the sample solution and incubated at 50 °C for 20 min. Then, 2 mL of a trichloroacetic acid solution (10%, w/v) was added to the mixture to terminate the reaction. An amount of 2 mL of distilled water and a 0.5 mL ferric chloride solution (0.1%, w/v) were added to the mixture and reacted for 10 min. The absorbance was recorded at 700 nm against a blank containing all reagents except the sample.

2.12. Statistical Analysis

All assays were performed on three samples, with the results recorded as a mean ± standard deviation, and the significant differences ($p < 0.05$) of data were processed using Tukey's test by analysis of variance (ANOVA) from Origin 2018 software (OriginLab Corporation, Northampton, MA, USA).

3. Results and Discussion

3.1. Yield, Purity, and Color of CP

Table 1 shows the CP content, protein content, and yield. The CP yields were 15.9% for S-CP, 5.80% for HPCD-CP, 9.80% for 4FT-CP, and 15.92% for 4FT+U-CP. According to the result, the 4FT+U-CP had the highest efficiency of extraction, while the HPCD-CP presented the lowest CP sample yield; this was consistent with the study of Tavanandi et al. [21], as ultrasonication presented a stronger ability to break down the *Arthrospira platensis* cell walls [16]. During ultrasonication, intense local shock waves, corresponding to thousands of atmosphere pressure, were produced to destroy the cell walls. In Figure 1A, three peaks at 350 nm, 375 nm, and 424 nm were also present in the spectra of the ultrasonicated sample; this is because more of the other compositions were obtained in crude 4FT+U-CP. In addition, the extraction methods also had a certain effect on the CP content of the samples ($p < 0.05$). HPCD-CP had the highest CP content (15.3%) but the shortest extraction time compared to S, 4FT, and 4FT+U. The results of Li et al. [16] indicated that most CPs can be obtained in about 3 h, so a longer extraction time may cause the leakage of excessive substances from the Spirulina cells. For CP, the ratio of the active substance to the total quantity of protein (A_{620}/A_{280}) is defined as purity. Purity 0.7 is considered food-grade, 3.9 reactive-grade, and above 4.0 analytical-grade [29]. As shown in Figure 1B, the purities of all samples were over 0.7, so, in the case of foods, these extraction methods, with a two-step solid ammonium sulfate purification process, were proved to be industrially applicable. The purity of HPCD-CP (1.66 ± 0.16) and the ΔE (Figure 1C) were the highest, while the Δb value was the lowest, which means that the color of HPCD-CP is significantly bright blue ($p < 0.05$). At the same time, the color of HPCD-CP also proved that both the purity and CP content results of HPCD-CP were the highest.

Table 1. The CP content, protein content, and yield of CPs obtained by different extraction methods in wet mass.

	CP Content (wt%)	Protein Content (wt%)	Yield (%)
S-CP	10.9 ± 0.00^{c}	79.1 ± 0.7^{b}	15.9
HPCD-CP	15.3 ± 0.00^{a}	77.8 ± 0.2^{b}	5.8
4FT-CP	13.6 ± 0.53^{b}	83.7 ± 2.2^{a}	9.8
4FT+U-CP	12.2 ± 0.45^{c}	81.1 ± 0.5^{ab}	15.92

Different superscript letters in the same column indicate significant differences ($p < 0.05$).

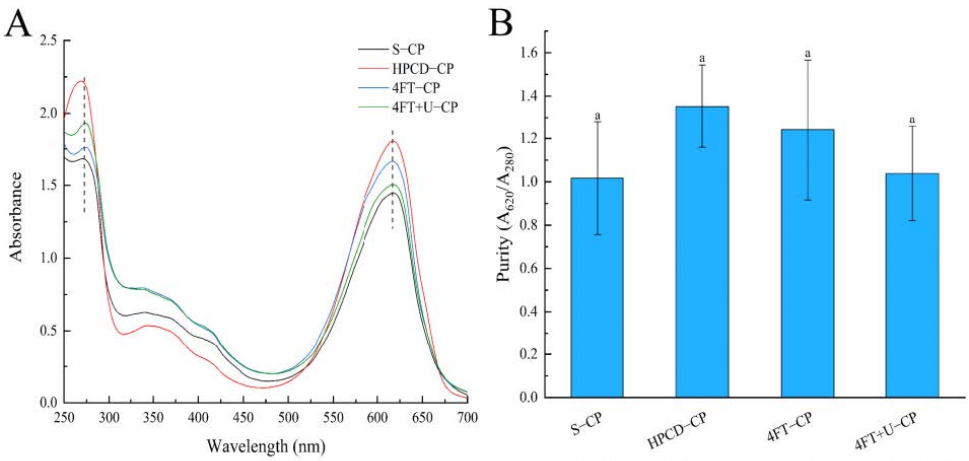

Figure 1. *Cont.*

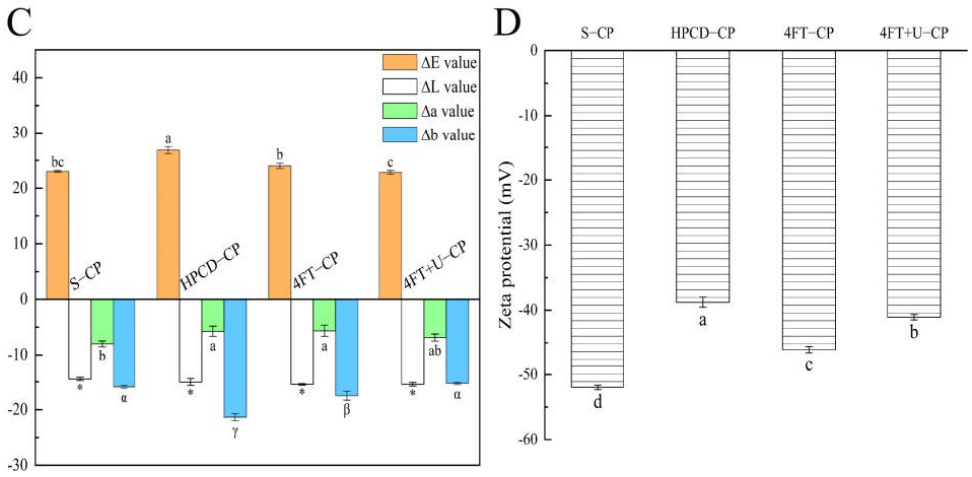

Figure 1. Absorption spectra (**A**), CP purities (**B**), color analysis (**C**), and zeta potential (**D**) of CPs obtained by different extraction methods. Different lowercase letters or single asterisks indicate significant differences ($p < 0.05$).

3.2. ζ Potential and Particle Size

The ζ potential of the CPs extracted from different methods are shown in Figure 1D. According to the figure, the ζ potential of the CPs decreased in the following order: HPCD-CP (−38.8 mV) > 4FT+U-CP (−41.1 mV) > 4FT-CP (−46.1 mV) > S-CP (−52.0 mV). The average particle size of the CPs extracted from different methods are presented in Figure 2A in the following decreasing order: 4FT+U-CP (719.1 nm) > HPCD-CP (654.2 nm) > 4FT-CP (536.3 nm) > S-CP (451.4 nm). An increase in average particle size was observed with a further increase in the ζ potential; this can be interpreted as if a sample has a high ζ potential (either positive or negative), providing further away from the zero point so it can be electrically stable. When the ζ potential is low, a sample solution will be unstable (tend to coagulate or flocculate easily) [30]. S-CP had the lowest ζ potential and particle size, significantly different ($p < 0.05$) from those of 4FT+U-CP and HPCD-CP. It can be suggested that extraction with mechanization may result in an increase in the protein particle size, especially under low intensities [31,32].

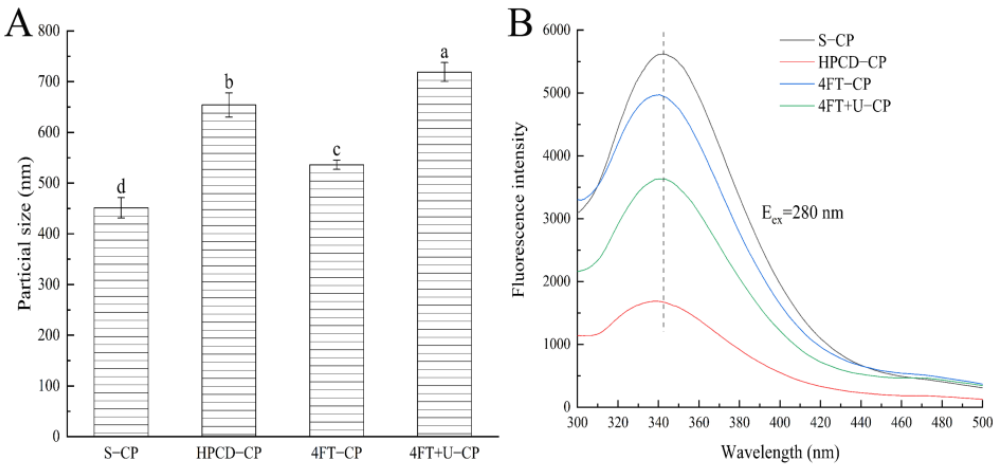

Figure 2. *Cont.*

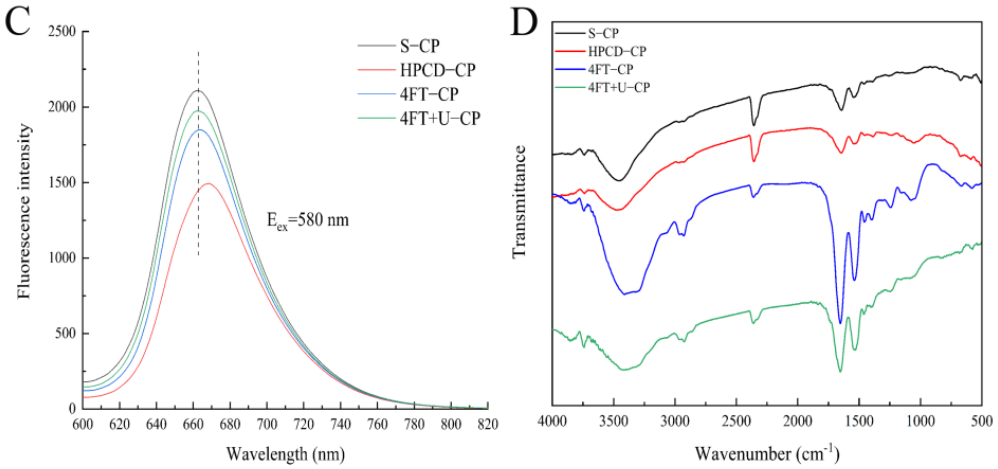

Figure 2. Average particle size (**A**), emission fluorescence spectra (using two different excitation wavelengths: 280 nm (**B**) and 580 nm (**C**)), and FTIR spectra (**D**) of CPs obtained using the different methods. Different lowercase letters indicate significant differences ($p < 0.05$).

3.3. Spectrophotometric of CP

Figure 1A shows the UV-visible absorption spectra of the CPs. The CPs displayed three relatively strong absorption peaks located at 280 nm, 350 nm, and 620 nm, similar to the maximum absorption of aromatic amino acids, denatured phycocyanin, or the unbound phycocyanobilin chromophore in its cyclic conformation and protein–pigment complex, respectively [22]. The absorption intensity, at 620 nm, increased in the following order: S-CP < 4FT+U-CP < 4FT-CP < HPCD-CP; this is similar to the previous results of the CP content. According to the intensity at 350 nm of the samples, S-CP was lower than 4FT-CP and 4FT+U-CP but higher than HPCD-CP, indicating that the microenvironments of the tetrapyrrole chromophores of 4FT-CP and 4FT+U-CP are more hydrophobic, followed by S-CP. The increased intensity at 350 nm (compared to HPCD-CP) might be related to the fact that there are more denatured phycocyanin or unbound phycocyanobilin chromophore caused by a longer extraction time of S-CP, 4FT-CP, and 4FT+U-CP [16,22].

As shown in Figure 2B, there was a blue shift (compared to the emission maximum of the S-CP result) from 344 nm to 338 nm in the other three CPs upon the excitation of 280 nm, indicating that the microenvironment around the aromatic amino acids was more hydrophobic [33]. Zhou et al. [34] found that an ultrasound treatment on egg white protein increased the number of hydrophobicity groups. The HPCD-CP exhibited a strong emission peak at 669 nm upon excitation at 580 nm (Figure 2C), and there was a blue shift of the fluorescence emission wavelength in the other three CPs, indicating that the microenvironment of phycocyanobilin was more hydrophobic. Additionally, the largest decrease in fluorescence intensity was observed in HPCD-CP. The reason for the changes in the protein molecules might be the different production conditions of different methods, such as production time, process, and intensity.

Three typical protein bands of amide I (1600–1700 cm^{-1}), amide II (1500–1580 cm^{-1}), and amide III (1200–1400 cm^{-1}) were observed by FTIR spectroscopy [35]. Shown in Figure 2D are the strong absorption peaks at 1648 cm^{-1}, 1648 cm^{-1}, 1658 cm^{-1}, and 1649 cm^{-1}, pertaining to S-CP, HPCD-CP, 4FT-CP, and 4FT+U-CP, respectively. Basically, C=O tensile (amide I), and the α-helix corresponds to 1660–1650 cm^{-1} separately. The absorption peaks of the 4FT-CP and 4FT+U-CP samples at 3400–3500 cm^{-1} had a blue shift, and the peak shape became wider, indicating that the amide carbonyl group vibrated along the protein backbone. Table 2 shows the estimation of the secondary structure in the amide I region of the CPs obtained by different methods. The content of the random coil was 22.46% for HPCD-CP but without the α-helix. The contents of the α-helix were 19.16%, 28.31%, and 28.66% for S-CP, 4FT-CP, and 4FT+U-CP, respectively. The structure of CPs may be disintegrated and transformed to an ordered structure during swelling,

repeated freezing and thawing, and the ultrasonic extraction process. Thus, these findings confirm that different extraction methods have a significant effect on the protein secondary structure of CPs.

Table 2. Estimation of secondary structure in amide I region of CPs obtained by the different methods.

Sample	Area (%)			
	α-Helix	β-Sheet	β-Turn	Random Coil
	1660–1650 cm^{-1}	1640–1600 cm^{-1} / 1690–1670 cm^{-1}	1670–1660 cm^{-1} / 1700–1690 cm^{-1}	1650–1640 cm^{-1}
S-CP	19.16	54.14	26.70	0
HPCD-CP	0	55.46	22.08	22.46
4FT-CP	28.31	49.50	22.19	0
4FT+U-CP	28.66	49.84	21.50	0

3.4. Thermogravimetric Analysis (TGA)

Figure 3 shows that the TGA pyrolysis characteristics of the CPs extracted by different methods can be divided into three stages. In the first stage, most of the adsorbed water and bound water began to become lost at temperatures of 55–150 °C [36]. The greatest reduction in the biomass was observed in the second stage, at temperatures of 150–600 °C, at which the main organic compounds of the microalgal biomass, such as lipids, proteins, and carbohydrates, decompose, which is why this stage is also known as the active pyrolysis zone. In the third stage, thermally stable compounds decomposed at 600–800 °C and formed biochar. The CPs' TGA curves showed a biomass loss of 1–5% in the first stage, decomposition of 59–75% in the second stage, and 8–18% in the last stage. The microalgal biomass decomposition in the second stage was lower than that which was previously reported [37]. A TGA spectroscopic analysis of all CPs suggested that the devolatilization peak, at 311–325 °C, showed the maximum decomposition of CP. Another important peak, to the left of the main peak, was also observed at 271–282 °C. The first and second stage temperatures of S-CP were 70.8 °C and 325.9 °C; those of 4FT-CP, HPCD-CP, and 4FT+U-CP were 5.25 °C and 4.84°C, 10.76 °C and 14.72 °C, and 15.72 °C and 8.34 °C lower than S-CP, respectively. Temperature changes at all peaks showed differences in microalgal biomass, similar to the results of Pandey, Srivastava, and Kumar [38].

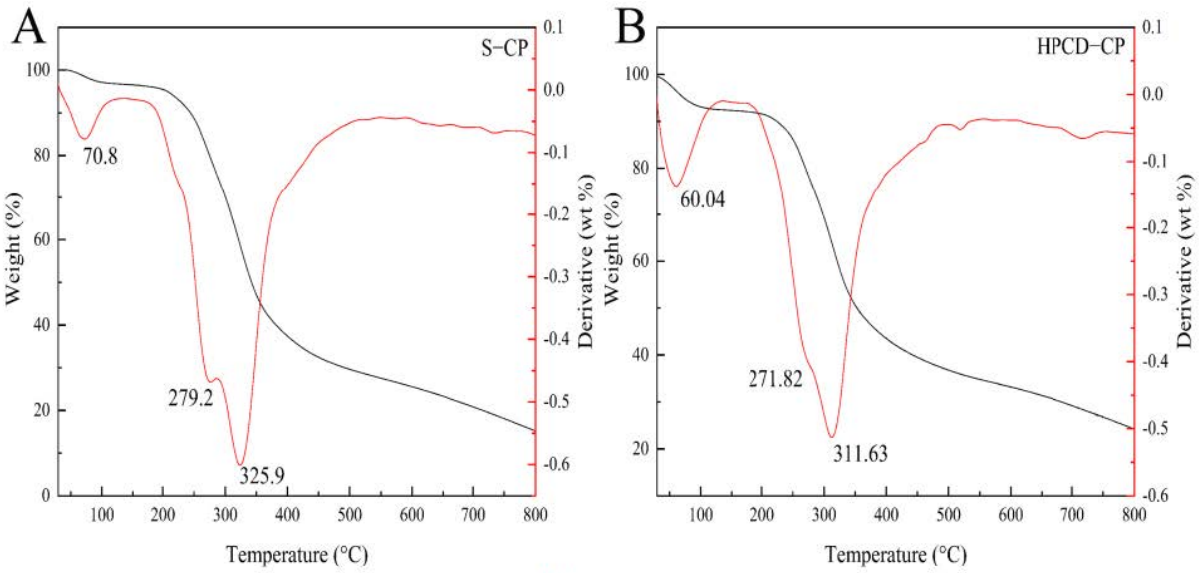

Figure 3. *Cont.*

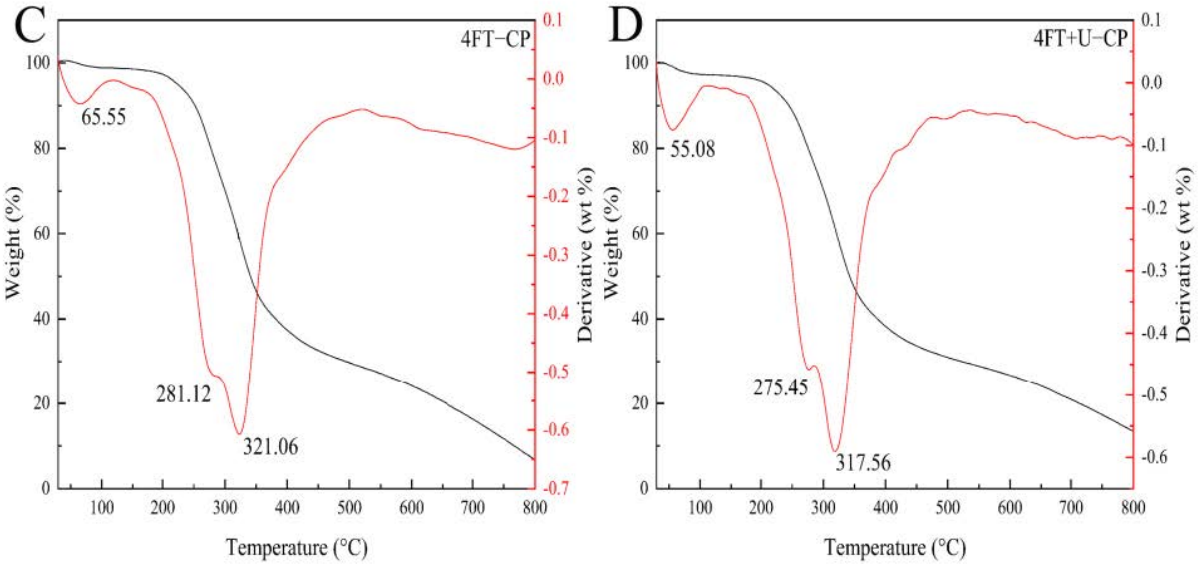

Figure 3. TGA of S-CP (**A**), HPCD-CP (**B**), 4FT-CP (**C**), and 4FT+U-CP (**D**) (protein concentration used was 1 mg/mL).

The temperatures at the three main phases of the S-CP and 4FT-CP samples were higher than those of the other two samples. Zhang et al. [39] reported that the ultrasonic treatment could disrupt the inherent structure and lead to poor thermal stability. Other work also indicated that water evaporation and weight loss can be influenced by the content of the protein [40]. Thus, the reason for the difference in the temperatures of the CPs may be determined by two factors: **(i)** the CPs obtained through high-pressure cell disruption and ultrasound, assisted by the freezing and thawing process, were extracted by mechanization, and **(ii)** the significantly different amount of the protein of these purified CP samples [41].

3.5. Solubility of CP

The effect of different extraction methods and pH on protein solubility are shown in Figure 4A. These CPs from different extraction methods had the minimum solubility near pH 4 (<10%), and their solubility was higher at pH values below and above four, which corresponds to their isoelectric point [42]. Similar results have been found for casein, soybean meal, mung bean protein, and other microalgal proteins; for instance, Nannochloropsis oculate and Spirulina LEB 18, with minimal solubility near the isoelectric point of pH 4–5 [43,44]. The solubility of 4FT-CP increased in the range of pH 4–8, decreased in the range of pH 8–10, and had the maximum solubility (82.2 $\pm$ 1.6%) at pH 11. In addition, the solubility of 4FT-CP was higher than that of the other three CPs between pH 7 and pH 9 and at pH 11. Furthermore, its high solubility in the pH 6–9 range is very similar to that of common vegetable protein drinks, which is at about 6.8 to 7.0 [45], indicating that CPs with different extraction methods may be applicable in food processing.

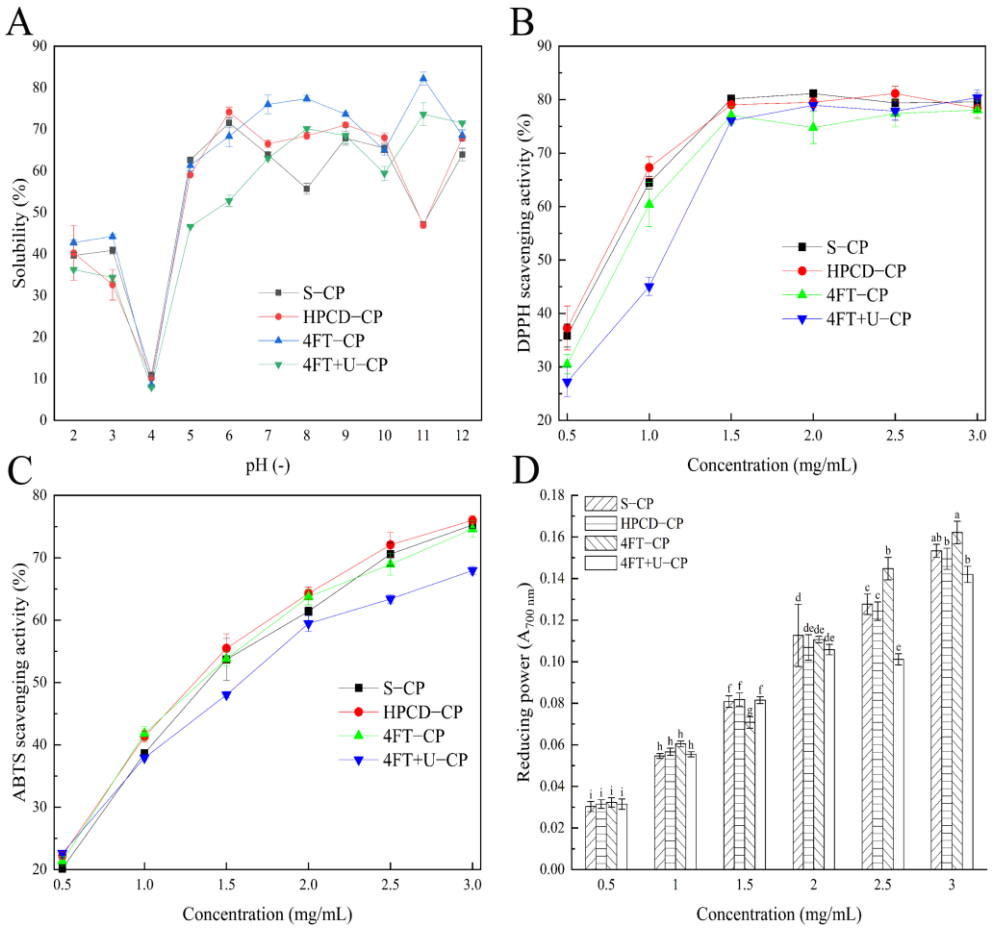

Figure 4. Solubility (**A**), free radical scavenging activities, and total reducing power of CPs obtained using different methods: DPPH radical scavenging activity (**B**), ABTS radical scavenging activity (**C**), and total reducing power (**D**). Different lowercase letters indicate significant differences ($p < 0.05$).

3.6. Antioxidant Activity of CPs

3.6.1. DPPH Radical Scavenging Activities

The DPPH radical scavenging effects of the CPs extracted from distinct methods are shown in Figure 4B. The sample concentration that can inhibit 50% of free radicals was defined as IC_{50}. The IC_{50} value of HPCD-CP (0.68 mg/mL) CP was slightly lower than those of 4FT-CP (0.79 mg/mL) and S-CP (0.71 mg/mL) but significantly lower than that of 4FT+U-CP (0.92 mg/mL). Obviously, we can see from the results that the CPs obtained through different extracted methods displayed an increasing radical scavenging activity with an increase in the CP purities, as previously reported [46]. When the concentration is at 1 mg/mL, the DPPH radical scavenging capacity of HPCD-CP (67.32%) was significantly higher than those of the other CP samples.

3.6.2. ABTS Radical Scavenging Activities

According to the analysis in Figure 4A, the IC_{50} values of the four CP samples are between 1.28 mg/mL and 1.51 mg/mL. The IC_{50} value of the 4FT+U-CP sample (1.51 mg/mL) was obviously higher than that of the other CP samples. It is concluded that ultrasound may destroy free radical scavenging components in biological systems [47]. Figure 4C shows the effect of the ABTS radical scavenging activity on the CPs. The ABTS radical scavenging ability of the four CP samples was similar when the concentration was at 0.5 mg/mL. The free radical scavenging activities of ABTS were 75.26% for S-CP, 76.01% for HPCD-CP, 74.62% for 4FT-CP, and 67.96% for 4FT+U-CP at a concentration of 3 mg/mL. In conclusion, the ABTS radical scavenging activity of the samples was significantly lower

than the DPPH radical scavenging activity. This finding may be due to the difference in scavenging reactions that occurred in the aqueous phase of ABTS and the organic phase of DPPH [46].

3.6.3. Reducing Power

Figure 4D shows the reducing capacity of the CPs. It can be seen from the figure that the reducing power of the sample increased with the increase of the sample concentration ($p < 0.05$). The reducing power of 4FT-CP (A_{700nm} = 0.071) was significantly lower than those of the other CP samples when the concentration was at 1.5 mg/mL, while the value of the 4FT-CP (A_{700nm} = 0.061) was slightly higher than those for the other three CP samples (A_{700nm} = 0.055–0.057) at a concentration of 1 mg/mL. In general, all of these CP samples exhibited a remarkable ability to capture radicals ($DPPH^+$, $ABTS^+$) and reduce ferric to ferrous ions.

4. Conclusions

In the present study, we explored the impact of different processes for the extraction of phycocyanin from the dry biomass of *Arthrospira platensis* on the physicochemical properties and antioxidant activities of CPs. According to the results of this study, the freezing and thawing technique, combined with ultrasonication, showed the best performance than the other three methods in terms of yield (15.92%) and average particle size (719.1 nm), while the high-pressure cell disruption process was better than the others in obtaining a product with a higher CP concentration (15.3%), purity (1.66 $\pm$ 0.16), ζ potential (-38.8 mV), DPPH (IC_{50} = 0.68 mg/mL), and ABTS (IC_{50} = 1.28 mg/mL) radical scavenging activity. Phycocyanins extracted by different methods have different secondary and tertiary structures. In all the methods, the high-pressure cell disruption process could be used as an effective method to obtain food-grade C-phycocyanin. The understanding of the effects of extraction methods on their properties could assist in selecting the appropriate extraction method to optimize the utilization of CP fraction as an alternate functional food for the food and drug industry.

Author Contributions: Q.C. was in charge of conceptualization, methodology, investigation, data curation, visualization, and writing—original draft preparation; S.L. helped with the writing—review & editing; H.X. provided resources, and Q.Z. was responsible for the conceptualization, resources, supervision, data curation, writing—review and editing, and project administration, etc. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: Data is contained within the article and available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

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Review

Enzymatic Hydrolysis of Pulse Proteins as a Tool to Improve Techno-Functional Properties

Martin Vogelsang-O'Dwyer [1], Aylin W. Sahin [1], Elke K. Arendt [1,2,*] and Emanuele Zannini [1]

[1] School of Food and Nutritional Sciences, University College Cork, T12 YN60 Cork, Ireland; m.vogelsangodwyer@umail.ucc.ie (M.V.-O.); aylin.sahin@ucc.ie (A.W.S.); e.zannini@ucc.ie (E.Z.)
[2] APC Microbiome Ireland, University College Cork, T12 YT20 Cork, Ireland
* Correspondence: e.arendt@ucc.ie

Abstract: Pulse proteins are being increasingly investigated as nutritious and functional ingredients which could provide alternatives to animal proteins; however, pulse protein ingredients do not always meet the functionality requirements necessary for various applications. Consequently, enzymatic hydrolysis can be employed as a means of improving functional properties such as solubility, emulsifying, foaming, and gelling properties. This review aims to examine the current literature regarding modification of these properties with enzymatic hydrolysis. The effects of enzymatic hydrolysis on the functionality of pulse proteins generally varies considerably based on the enzyme, substrate, processing steps such as heat treatment, degree of hydrolysis, and pH. Differences in protease specificity as well as protein structure allow for a wide variety of peptide mixtures to be generated, with varying hydrophobic and electrostatic properties. Typically, the most significant improvements are seen when the original protein ingredient has poor initial functionality. Solubility is usually improved in the mildly acidic range, which may also correspond with improved foaming and emulsifying properties. More work should be carried out on the potential of enzymatic hydrolysis to modify gelation properties of pulse proteins, as the literature is currently lacking. Overall, careful selection of proteases and control of hydrolysis will be necessary to maximize the potential of enzymatic hydrolysis as a tool to improve pulse protein functionality and broaden the range of potential applications.

Keywords: pulse proteins; enzymatic hydrolysis; hydrolysate; protease; functional properties; plant protein

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1. Introduction

There is currently a need to accelerate the development and utilisation of plant-based protein sources, with the end goal of providing alternatives to traditional animal-derived foods. Growing global population and protein demand, awareness of the negative environmental consequences of animal-based food production, as well as ethical and health concerns, are contributing to the increasing interest in the development of plant-based foods, and it has become clear that a dietary transition away from animal protein is needed for sustainability and food security [1,2]. It is now recognised that growing protein-rich plant crops for animal feed is in many cases less efficient and sustainable than direct consumption of plant proteins by humans [3], which incentivises further development and exploitation of plant protein sources, such as pulses. Pulses are leguminous seeds including various peas, beans, chickpeas, lentils, and lupins, generally considered separately from oilseed legumes such as soybeans and peanuts [4,5]. They are typically starch-rich crops with a relatively high protein content, although they are usually lower in protein than soybeans. The dominant protein fractions in pulses are globulins (salt soluble proteins) and albumins (water soluble proteins). Typically, globulins are present in higher amounts than albumins; however, the relative amounts can vary considerably between different

pulses, and also due to variety and cultivation conditions, and the albumin/globulin ratio has been reported as high as ~0.5 [6–9]. The albumins are mainly composed of metabolic proteins and enzymes, and pea albumins include PA-2, PA-1, lipoxygenase, protease inhibitors, and lectins [7,8]. Globulins, on the other hand, are comprised of storage proteins. The two main globulin fractions in pea and other pulse proteins are referred to as legumin and vicilin, and a third fraction, convicilin, may also be present. In general, proteins from different pulses show structural similarities. Legumin is a hexamer with a molecular weight of ~340–360 kDa, whereas vicilin is a trimer with a molecular weight of ~175–180 kDa [10]. Different structural and surface properties of legumin and vicilin can correspond to differences in functionality (e.g., solubility and emulsifying properties); therefore, the legumin:vicilin ratio, which can vary considerably between different pulses and varieties, is an important consideration [11,12]. In addition, the protein composition can be altered with processing, for example, some removal of albumins is likely during isoelectric precipitation [4].

Pulses are increasingly being explored as a nutritious and sustainable source of plant protein. The protein content for most pulses is in the range of ~15–30% of dry matter [13]. This could be considered relatively high (e.g., compared to cereals); however, concentration/isolation steps are required to produce high protein ingredients [14]. Dry processing by milling and air classifying can be used to produce protein concentrates with protein content up to ~70%, depending on the pulse used [15]. Protein isolates with higher protein content (often > 80%) can be produced using aqueous extraction followed by techniques such as isoelectric precipitation or ultrafiltration [16,17]. Pulse protein isolates and concentrates have generated much interest due to their good techno-functional properties. Pea protein ingredients are important in the food industry and are used in a variety of plant-based products, whereas other pulse protein sources are receiving increasing attention for their potential (e.g., faba bean and lentil) [18–21]. Pulse proteins have shown good promise in plant-based alternatives, and could potentially prove to be useful alternatives for milk, egg, and meat protein, as well as soy protein. Examples include milk alternatives produced with pea, lupin, or lentil protein, as well as meat alternatives produced with pea or faba bean protein concentrate [17,22–24].

Depending on the application, certain functional properties may be required, such as solubility, emulsifying, foaming, and gelling ability, or a combination of these. Wide variability in these properties has been observed depending on various factors such as protein source, processing, and environmental conditions [10,16]. In addition, due to differences in structure, it remains challenging to replicate the functionality of animal proteins with plant-derived proteins. For example, the fibrous structure of muscle tissue cannot be easily mimicked using globular plant proteins, and also the unique 'random coil' structure of caseins and the casein micelle structure are important for the textural properties of dairy products such as cheese and yoghurt [25]. When formulating plant-based products there is often a gap between the required functionality versus the functionality provided by protein ingredients. Furthermore, the solubility of pulse proteins is particularly poor under mildly acidic conditions, in the pH region near their isoelectric point, as their solubility is typically influenced to a large degree by electrostatic repulsion [10]. Partial enzymatic hydrolysis is a method which has in many cases been shown to improve solubility and other techno-functional properties of proteins, especially in cases where the proteins showed poor functionality to begin with [26,27].

In addition, hydrolysis can also potentially provide the benefit of improved digestibility, for example, enzymatic hydrolysis of lentil protein was found to increase in vitro protein digestibility [28]. Enzymatic hydrolysis is often preferred to chemical hydrolysis as it does not require harsh conditions, is easier to control, and retains the nutritional quality of the protein [29]. As pulse protein ingredients become more widely available and utilised in the food industry, knowledge of the tools and strategies to improve their functionality will be essential in order to broaden the range of applications; therefore, this review aims to focus on current knowledge of the effects of enzymatic hydrolysis on important techno-functional

properties of high protein ingredients from pulses, and its potential for improving these properties. Currently, the literature regarding the influence of enzymatic hydrolysis on pulse protein techno-functional properties has not been reviewed. As high-protein pulse ingredients are growing in interest, importance, and variety, it will be important to assess and improve our understanding of techniques such as enzymatic modification.

2. Proteases

Proteases (peptidases) are enzymes that cleave peptides and proteins in the presence of water by hydrolysis. Proteases may be classified in various ways. Based on positional specificity, they are divided into two main groups, endo- and exopeptidases. Endopeptidases act on internal bonds of polypeptides, whereas exopeptidases cleave near the ends at the C- or N-terminus; thus, endopeptidases cleave proteins to peptides of various sizes, whereas exopeptidases liberate either a single amino acid residue, a dipeptide or a tripeptide, depending on the type [30,31]. In addition, proteases are classified according to the main chemical group responsible for catalysis at the catalytic site. They include serine proteases, cysteine proteases, threonine proteases, aspartic proteases, glutamic proteases and metalloproteases [30,31]. Furthermore, proteases may be classified according to their origin (i.e., microbial, plant, or animal derived). The majority of industrially used enzymes are of microbial origin [30], and microbially derived alternatives are now available for some traditionally animal-derived proteases [32].

Importantly, proteases exhibit sequence specificity, exhibiting a preference for specific amino acids next to the peptide bond to be hydrolysed, corresponding to the amino acid sequence near the enzyme's catalytic site [31]. This is shown schematically in Figure 1. Amino acid residues at the catalytic site of the protease correspond to specific amino acids in the protein substrate, in each case labelled according to their proximity to the peptide bond to be hydrolysed, and directionally towards the C- or N-terminus; therefore, wide variability in peptides generated can be expected with different enzyme and substrate combinations. In addition, some proteases exhibit broad specificity, whereas others show narrower specificity [28]. Although a protease may be able to hydrolyse multiple peptide bonds, the rate of cleavage may be very different depending on the specific bond [33]. Various food-grade proteases have been utilised to produce hydrolysates of pulse proteins, examples of which are shown in Table 1. Endoproteases are typically used to produce protein hydrolysates, sometimes in combination with exoproteases. Commercial enzyme preparations may contain mainly one protease, or a mixture of proteases. Alcalase is an example of a commonly used serine endoprotease, with broad specificity. It has been well studied and is mainly composed of Subtilisin A (Subtilisin Carlsberg), originating from *Bacillus lichenformis* [34]. Other serine endoproteases include Savinase, trypsin, and chymotrypsin. Trypsin shows narrowly defined specificity and cleaves next to lysine and arginine, whereas chymotrypsin is non-specific, although it preferentially hydrolyses next to certain amino acids, including tryptophan, tyrosine, phenylalanine, and leucine. Neutrase is an example of a zinc metalloprotease, derived from *Bacillus amyloliquefaciens* [35]. Papain and bromelain are cysteine endoproteases derived from papaya latex and pineapple stem, respectively [35]. Flavourzyme is a widely used exoprotease preparation, an enzyme mixture originating from *Aspergillus oryzae*. It contains various exopeptidases and endopeptidases [29]; however, the key enzyme activity according to the manufacturer is that of aminopeptidase, liberating amino acids from the N-terminal. As the name suggests, a major function of Flavourzyme is to improve sensory characteristics, although it has also been shown to modify techno-functional properties [35,36].

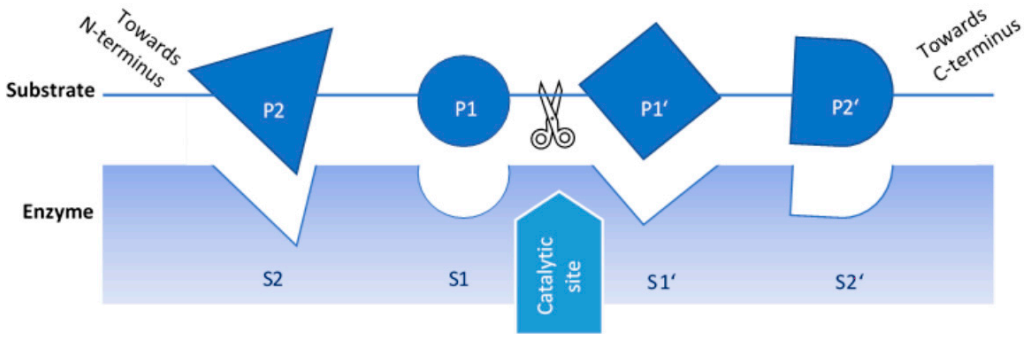

Figure 1. Schematic representation of protease sequence specificity. Adapted from Rawlings and Barrett [31], permission obtained.

Table 1. Some commonly used proteases for food protein hydrolysis.

Enzyme Preparation	Main Activity	Origin
Alcalase	Serine endoprotease; broad specificity, preferentially hydrolyses peptide bonds at the C-terminal side of hydrophobic residues	*Bacillus lichenformis*
Trypsin	Serine endoprotease; specific for peptide bonds at the C-terminal side of Lys and Arg residues	Bovine/porcine pancreas
Chymotrypsin	Serine endoprotease; preferentially hydrolyses peptide bonds at the C-terminal side of Tyr, Phe, Trp and Leu residues	Bovine/porcine pancreas
Savinase	Serine endoprotease, broad specificity	*Bacillus lentus*
Protamex	Broad specificity endoprotease	*Bacillus* sp.
Corolase 2TS	Metallo endoprotease	*Bacillus thermoproteolyticus*, *Bacillus stearothermophilus*
Neutrase	Metallo endoprotease	*Bacillus amyloliquefaciens*
Pepsin	Aspartic endoprotease, broad specificity	Bovine/porcine gastric mucosa
Papain	Cysteine endoprotease, broad specificity	Papaya latex
Bromelain	Cysteine endoprotease, broad specificity	Pineapple stem
Flavourzyme	Exo and endoprotease mixture. Includes aminopeptidases, carboxypeptidases, and endoproteases	*Aspergillus oryzae*

Certain environmental conditions are required for effective hydrolysis depending on the protease. In particular, each protease demonstrates temperature and pH optima, as well as a range for each in which the protease is active [26]. Above a certain temperature, denaturation will occur, deactivating the enzyme. Protease activity is sensitive to pH, due to the functional groups involved in the hydrolysis reaction. Generally, serine proteases show highest activity at alkaline pH, cysteine proteases around neutral pH, and aspartic proteases acidic pH [33]. Additionally, protease selectivity should be considered. The rate of hydrolysis of a specific cleavage site can be influenced by various factors, including other amino acids near the cleavage site, pH, temperature, and accessibility of the cleavage site [37,38]; therefore, hydrolysis conditions (e.g., pH, can influence the hydrolysate properties in addition to the rate of hydrolysis).

3. Production of Protein Hydrolysates and Assessment of the Extent of Hydrolysis

There are various ways in which enzymatic hydrolysis can be applied to pulse proteins to improve functionality. Typically, a dispersion of protein isolate or concentrate is prepared, incubated under specific conditions with protease(s), and then dried to produce a protein hydrolysate powder [39,40]. Other approaches are also possible, such as incorporating an enzymatic hydrolysis step during food product production or during protein extraction from seed material. Due to the high cost of enzymes, immobilisation methods for enzymes have also been developed, which allows them to be recovered after hydrolysis [41,42]. In addition to batch processes, continuous methods have been developed which allow for lower costs and decreased product variability [43]. Generally, in laboratory-scale studies, a protease is added to a protein dispersion at a specific dosage, and hydrolysis is carried out with controlled temperature and pH, until a specific time or degree of hydrolysis has been reached [44,45]. If pH is not controlled, changes in pH may occur during hydrolysis, depending on the initial pH environment. If pH is above the pKa of the amino groups, newly released carboxyl groups and amino groups will both be deprotonated, with the net effect of releasing protons and lowering pH, whereas if the pH is below the pKa of the carboxyl groups, both the amino and carboxyl groups will be protonated; therefore consuming protons, with the effect of raising pH [27]. After the required hydrolysis duration, the enzyme is usually deactivated by denaturation with a heat treatment step. At laboratory scale, the hydrolysate is typically freeze dried prior to analysis, although this is not always the case. Other steps can include centrifugation (e.g., in some cases, the hydrolysate is centrifuged and only the soluble fraction is recovered) [46]. Such differences in processes should be taken into account as they may have a significant influence on the structural and functional properties of the hydrolysates; with centrifugation, a certain fraction of the proteins/peptides would be excluded, and functionality may also be affected by the drying method [47]. The requirement for enzyme deactivation (typically by heat treatment) is an important disadvantage of enzymatic hydrolysis, due to the harsh conditions and extra energy input required. It is important to consider the effects of the heat-treatment step on protein properties, as structural changes such as unfolding and protein–protein aggregation may influence functionality [48,49]. Many studies make comparisons between hydrolysates and an untreated protein ingredient; however, this does not account for the enzyme deactivation heating step, and significant functional differences have been found between untreated protein isolates/concentrates, and those which have been subjected to the same conditions as the hydrolysates but without the addition of enzymes [45,50]. Additionally, pre-treatments can be applied, which can influence proteolysis, and potentially modify the functionality of hydrolysates. One potential method is initial heat treatment before hydrolysis to induce unfolding of proteins and expose previously buried peptide bonds [26,33]. High-pressure processing has also been explored as a pre-treatment. Al-Ruwaih et al. [51] and Ahmed et al. [52] used this method before the hydrolysis of kidney bean and lentil protein hydrolysates, respectively, resulting in significant differences in functional properties of the hydrolysates.

The degree of hydrolysis, defined as the percentage of peptide bonds hydrolysed relative to the untreated protein substrate, is commonly used to measure the extent of enzymatic hydrolysis; however, there is no standard method for degree of hydrolysis, and the different techniques that are commonly used can yield varying results; therefore, a direct comparison between studies is usually not possible. In addition, some methods may be more suitable for particular substrates or protease types [53]. The various methods and the principles behind them have been reviewed by Rutherfurd [54]. The methods that are mainly used are based on various principles, including base consumption needed to maintain pH (pH-stat method), changes in osmolality (osmometric method), determination of free amino groups (o-phthalaldehyde (OPA) method, trinitrobenzenesulfonic acid (TNBS) method, and formol titration method), and solubility of amino acids and small peptides in trichloroacetic acid (soluble nitrogen-TCA method). In general, measuring the degree of hydrolysis is helpful, as differences in functionality are often found

depending on degree of hydrolysis (e.g., a particular functionality might be increased up to a certain degree of hydrolysis, but then decrease on further hydrolysis); however, the degree of hydrolysis alone does not provide specific information on structural changes [27]; therefore electrophoresis, most often in the form of SDS-PAGE, is usually used to gain more specific information on the degradation of proteins during hydrolysis. This allows the approximate molecular weight distribution to be visualised, showing the extent of degradation for different protein fractions along with the appearance of smaller peptides within a certain range. Electrophoresis is particularly useful not just for showing the overall extent of degradation, but also differences in molecular weight distribution, which can provide key information regarding the specificity of the proteases in relation to different protein fractions [35,50]. In addition, size exclusion chromatography can be used to assess peptide size distribution, and is capable of detecting smaller peptides which fall below the sizing range of electrophoresis. Furthermore, liquid chromatography followed by mass spectrometry can be used to separate and identify peptide fractions.

4. Solubility

Solubility is usually considered to be a critical functional property of protein ingredients. Many food applications require high solubility, and the ability of proteins to contribute other functionalities such as foaming, emulsifying, and gelling is typically dependent on their initial solubilisation [42,55,56]. Solubility is also important for high protein beverages such as milk alternatives, where sedimentation of insoluble protein particles may be undesirable [24]. One of the disadvantages of plant proteins in general is poor solubility, especially compared with animal proteins such as whey or egg proteins. This can limit the ability of the proteins to act as functional ingredients. Pulse proteins often exhibit better solubility around neutral pH compared to other plant proteins, such as cereal proteins [56]; however, they are generally poorly soluble in the mildly acidic range, near the isoelectric points of the main protein fractions [14,45]. Above the isoelectric point, proteins carry a net negative charge, while they carry a net positive charge below their isoelectric point. The repulsive forces between similarly charged proteins is an important factor for protein solubilisation. Near the isoelectric point, the net charge is negligible and the proteins are prone to precipitation. This generally narrows the range of suitable applications, and even near neutral pH (away from the isoelectric point), pulse proteins may be inadequately soluble in some cases. It has been suggested that commercial protein isolates often demonstrate relatively poor solubility compared with those produced at laboratory scale, attributable to denaturation during processing [57,58].

The solubility of proteins depends on the balance of protein–protein and protein–water interactions, including repulsive and attractive forces. Native globular proteins are typically folded in a conformation where more hydrophobic regions are buried at the centre, whereas more hydrophilic regions are exposed at the surface. Protein structure, and the proportion of polar and non-polar groups exposed to the surface, governs solubility in a given environment [25,59]. Repulsion due to similarly charged proteins promotes solubility, whereas hydrophobic interactions between proteins promotes aggregation and lower solubility [6,27,55]. Both intrinsic and extrinsic environmental factors influence solubility [60]. Protein solubility is usually assessed by centrifuging a protein dispersion, measuring the protein concentration of the supernatant, and expressing it as a percentage of the initial dispersion concentration. It can be difficult to compare directly between studies due to differences in methods, including centrifugation conditions [61]. Aside from the protein's intrinsic structural properties, the dispersion preparation method/conditions (e.g., homogenisation vs stirring) can have a major impact on solubility values that should not be overlooked [24,62]. Enzymatic hydrolysis generates a variety of smaller peptides, decreasing molecular weight, and at the same increasing the exposure of both hydrophobic regions and ionisable groups. These structural changes often lead to differences in solubility upon hydrolysis [26,27].

Table 2 shows an overview of the effects of enzymatic hydrolysis on the solubility of pulse protein isolates and concentrates at various pH values. Although the results vary considerably, in most cases, increased solubility is seen in the mildly acidic range near the isoelectric point, whereas outside this range, solubility may increase, but a decrease is also often observed. A typical 'u-shaped' pH-dependent solubility curve for pulse proteins is shown in Figure 2, along with two different solubility profiles, which might be expected for hydrolysates. The effect of enzymatic hydrolysis on solubility on a given protein ingredient may vary depending on different factors, including the protease, time/degree of hydrolysis, and environmental conditions. In addition, for the same protease, differences can be seen between substrates (e.g., different pulse types or different varieties); thus, a wide variety of outcomes may be expected with different enzyme and substrate combinations, as well as other factors, such as hydrolysis time and environmental conditions. Differences are often observed based on degree of hydrolysis. Mokni Ghribi et al. [46] found that solubility of chickpea protein treated with Alcalase increased with an increasing degree of hydrolysis across a broad pH range. Betancur-Ancona et al. [63] observed a similar trend with *Phaseolus lunatus* hydrolysates produced with Alcalase or Flavourzyme. In contrast, other studies have found more varied effects, with the increasing degree of hydrolysis not necessarily accompanied by an increase in solubility [35,42,45]. For a given protein substrate and conditions, choice of protease is important if maximum solubility is desirable.

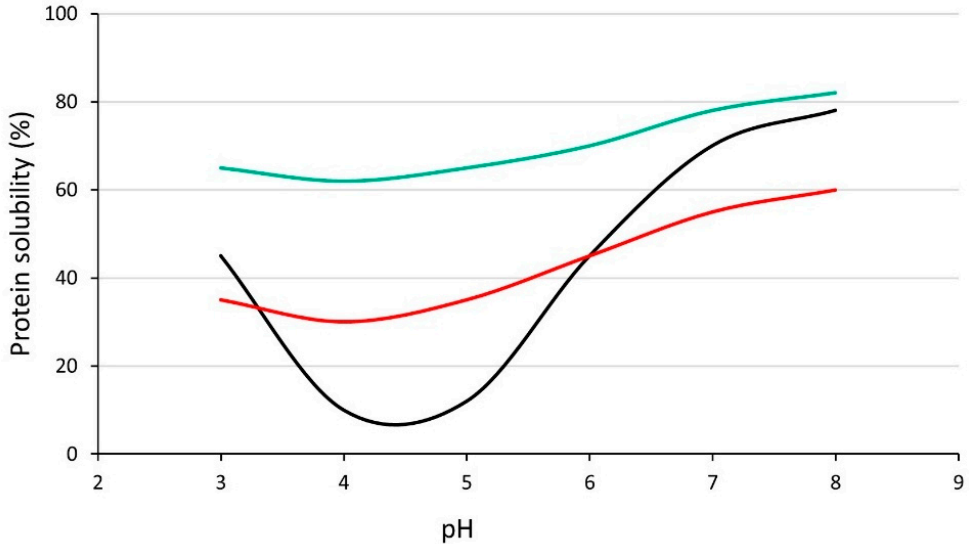

Figure 2. Generalised pH dependent solubility curves showing a typical solubility profile for non-hydrolysed pulse protein and two potential profiles for solubility after hydrolysis. Black: typical pattern for non-hydrolysed pulse proteins; red: hydrolysate with improved solubility near isoelectric point but otherwise reduced solubility; green: hydrolysate with improved solubility across the pH range.

As previously mentioned, changes in solubility have been attributed to decreased molecular weight and an increase in both hydrophobic patches and ionisable groups. Due to differences in specificity between proteases, the hydrolysis products for a given substrate can be very different with regard to these properties [59]. García Arteaga et al. [35] compared the impact of hydrolysis with 11 different proteases on the solubility of pea protein isolate and found major differences depending on the protease applied. At pH 4.5 the solubility of the original isolate was very low at 2%. The least effective protease was found to be chymotrypsin, with little or no improvement at 15 or 30 min hydrolysis. The most effective was Esperase after 120 min hydrolysis, increasing solubility to 71%. At neutral pH, solubility decreased from 51% for the untreated isolate to as low as 24% depending on hydrolysis time with Flavourzyme or chymotrypsin, whereas solubility of 78% was reached with 120 min hydrolysis with Esperase. SDS-PAGE revealed some major differences in

molecular weight distribution between the hydrolysates of different proteases, illustrating the differences in specificity leading to peptide mixtures with varying solubility. The study of Barac et al. [50] showed considerable variability in solubility of pea protein isolate with different combinations of pea variety, protease, hydrolysis time, and pH. With papain treatment in particular, major differences in solubility were found between hydrolysates of the two pea varieties tested (L1 and Maja). The authors attributed this to differences in legumin and vicilin content between the varieties, as papain preferentially targeted vicilin and acidic subunits of legumin. The lower solubility of the Maja hydrolysates was attributed to a higher legumin content, and therefore, more hydrophobic peptides and free sulfhydryl groups which promote the formation of insoluble aggregates.

Several studies have assessed changes in surface properties upon hydrolysis of pulse proteins, including surface hydrophobicity, and surface charge (zeta-potential). Surface charge is important as electrostatic repulsion promotes solubility of proteins. At the same time, increased exposure of hydrophobic groups could promote aggregation and reduced solubility. Zhang and Motta [45] found that hydrolysis of the Great Northern bean and navy bean hydrolysates with Alcalase or papain resulted in either increased, unchanged, or decreased surface hydrophobicity at neutral pH; however, the heat-treated control showed higher hydrophobicity compared with the hydrolysates. Interestingly, the solubility of hydrolysates at this pH was not different compared with the untreated protein concentrates. Konieczny et al. [64] hydrolysed pea protein enriched flour with trypsin, Savinase, papain, or pepsin to various degrees of hydrolysis, and found that all hydrolysates had higher surface hydrophobicity and zeta-potential, and lower solubility compared to the untreated ingredient.

It might be expected that hydrolysis should expose previously buried hydrophobic groups, and therefore, higher surface hydrophobicity; however, it can also lead to lower surface hydrophobicity. This has been attributed to aggregation due to hydrophobic interactions, effectively re-burying hydrophobic groups [48]. Hydrolysis has been found to result in more negative surface charge, corresponding to a shift in isoelectric point to slightly lower pH [44,46]; however, compared with intact proteins, the solubility of hydrolysates tends to vary less with changes in pH. Although the impact of enzymatic hydrolysis on pulse protein solubility can vary significantly depending on enzyme and substrate combinations, the greatest increases are usually observed near the isoelectric point. Increased solubility at an acidic pH can be particularly useful for acidic products where high solubility is necessary, for example, faba bean protein hydrolysates have been used to fortify apple juice, in a pH range where the original protein extract was poorly soluble [65].

Table 2. Overview of the effects of enzymatic hydrolysis on solubility, with various pulse protein sources and proteases.

Reference	Protein Source	Protease	Effect on Solubility
Barać et al. [66]	Pea protein isolate	Chymosin	Increased at pH 3; increased/no difference at pH 5 depending on HT; decreased at pH 7; increased at pH 8
Barac et al. [50] *	Pea protein isolate (L1)	Papain	Increased at pH 3 and 5; increased/decreased at pH 7 depending on HT; increased at pH 8
		S. griseus protease	Increased at pH 3 and 5; decreased at pH 7 and 8
	Pea protein isolate (Maja)	Papain	Increased at pH 3 and 5; increased/decreased at pH 7 depending on HT; decreased at pH 8
		S. griseus protease	Increased at pH 3, 5 and 7; increased/decreased at pH 8 depending on HT

Table 2. *Cont.*

Reference	Protein Source	Protease	Effect on Solubility
Betancur-Ancona et al. [63]	*P. lunatus* protein isolate	Alcalase	Increased at pH 2, 4, 6, 8 and 10
		Flavourzyme	Increased/no difference at pH 2 depending on HT; increased at pH 4 and 6; increased /no difference at pH 8 and 10 depending on HT
Eckert et al. [39]	Faba bean protein isolate	Pepsin	Increased at pH 5 and 7
		Trypsin	Increased at pH 5 and 7
		Flavourzyme	Increased at pH 5 and 7
		Neutrase	Increased at pH 5 and 7
García Arteaga et al. [35]	Pea protein isolate	Alcalase	Increased at pH 4.5; increased/no difference at pH 7 depending on HT
		Papain	Increased at pH 4.5; no difference at pH 7
		Esperase	Increased at pH 4.5 and pH 7
		Bromelain	Increased at pH 4.5; decreased/no difference at pH 7 depending on HT
		Trypsin	Increased at pH 4.5; increased/no difference at pH 7 depending on HT
		Chymotrypsin	Increased/no difference at pH 4.5 depending on HT; decreased at pH 7
Klost and Drusch [44]	Pea protein concentrate	Trypsin	Decreased/no difference at pH 3 depending on DH; increased at pH 4, 5, and 6; decreased at pH 7
Konieczny et al. [64]	Pea protein-enriched flour	Trypsin	Decreased at pH 4, 7 and 10
		Savinase	Decreased at pH 4, 7 and 10
		Papain	Decreased at pH 4, 7 and 10
		Pepsin	Decreased at pH 4, and 7; decreased/no difference at pH 10 depending on DH
Mokni Ghribi et al. [46]	Chickpea protein isolate	Alcalase	Increased at pH 2, 4, 6, 8, 10 and 12
Schlegel et al. [40]	Lupin protein isolate	Alcalase	Increased at pH 4, 5 and 6; no difference at pH 7, 8 and 9
		Papain	Increased at pH 4, 5 and 6; no difference at pH 7, 8 and 9
		Neutrase	Increased at pH 4, 5 and 6; no difference at pH 7, 8 and 9
		Protease N-01	Increased at pH 4 and 5; no difference at pH 6, 7, 8 and 9
		Flavourzyme	Increased at pH 4 and 5; no difference at pH 6; decreased at pH 7, 8 and 9
		Protamex	Increased at pH 4, 5 and 6; no difference at pH 7, 8 and 9
		Corolase 7089	Increased at pH 4, 5 and 6; no difference at pH 7, 8 and 9
		Pepsin	Increased at pH 4, 5 and 6; no difference at pH 7, 8 and 9
		Corolase N	Increased at pH 4, 5 and 6; no difference at pH 7, 8 and 9

Table 2. *Cont.*

Reference	Protein Source	Protease	Effect on Solubility
Segura-Campos et al. [36]	Cowpea protein concentrate	Alcalase	Decreased at pH 2; increased at pH 4 and 6; decreased at pH 8 and 10
		Flavourzyme	Increased at pH 2, 4, 6, 8 and 10
Xu et al. [67]	Chickpea protein isolate	Alcalase	Increased at pH 2, 4, 7 and 9
		Flavourzyme	Increased at pH 2, 4, 7 and 9
Yust et al. [42]	Chickpea protein isolate	Alcalase	Increased/no difference at pH < 4 depending on DH; increased at pH ~4–6; increased/decreased at pH 7 depending on DH; increased at pH 8, 9 and 10
Zhang and Motta [45] *	Great Northern bean protein concentrate	Alcalase	Decreased/no difference at pH 3 depending on DH; increased at pH 4, 5 and 6; no difference at pH 7
		Papain	Decreased at pH 3; increased at pH 4, 5 and 6; no difference at pH 7
	Navy bean protein concentrate	Alcalase	Increased/no difference at pH 3 depending on DH; decreased/no difference at pH 4 depending on DH; increased at pH 5 and 6; no difference at pH 7
		Papain	Increased at pH 3; no difference at pH 4; increased at pH 5 and 6; no difference at pH 7

This table is intended as an overview only—methodology, data representation, and statistics can vary between studies, making direct comparisons difficult. HT: hydrolysis time; DH: degree of hydrolysis. * Compared with thermally treated control.

5. Emulsifying Properties

Many proteins are useful as emulsifiers due to their structure and amphiphilic properties [68]. Various foods consist of oil in water emulsions, such as milk, mayonnaise, and dressings, or water in oil emulsions, such as margarine. Ideally, small emulsifier-coated droplets are dispersed in the continuous phase and should be resistant to aggregation and separation. Proteins stabilise emulsions by reducing the interfacial tension between the two immiscible phases, thus lowering the overall free energy [69]. The balance of hydrophobic and hydrophilic properties of proteins are important in determining their effectiveness as emulsifiers. The protein should possess good solubility in water and be capable of rapid migration to, and adsorption at, the oil–water interface during homogenisation [69]. Once at the interface, globular proteins may structurally rearrange in a conformation where more hydrophilic regions extend to the water phase, whereas more hydrophobic regions extend into the oil phase [68,70]. Emulsion stability depends on protein–protein interaction to form a strong viscoelastic layer at the interface. At the same time, electrostatic repulsion is generally important for prevention of droplet aggregation and phase separation. Overall, as well as solubility, an appropriate balance and distribution of hydrophobic and hydrophilic regions is required [68,69].

Pulse proteins such as lentil, lupin, pea, and chickpea have been shown to be useful emulsifiers for various applications, including milk alternatives and salad dressings [24,71,72]; however, enzymatic hydrolysis could potentially be a useful tool to modify emulsifying properties where improvement is required. By decreasing molecular weight and exposing hydrophobic regions, controlled hydrolysis can potentially deliver an improved ability to form and stabilise emulsions [27]. Emulsifying properties of protein ingredients can be examined using various methods. Emulsifying activity and stability indices are often measured using the turbidimetric method of Pearce and Kinsella [73]. Other approaches include measuring the maximum amount of oil capable of being emulsified with a defined protein dispersion before phase inversion [40]. Emulsion stability can be assessed in terms

of separation rate or cream layer height [72,74]. In addition, particle size measurements provide useful information on emulsifying properties [44,45]. Caution should be exercised when comparing studies, as there are often major differences in methods of emulsion preparation and composition, as well as analytical methods.

Table 3 shows the effects of hydrolysis on emulsifying properties of various pulse protein ingredients, using various proteases. Somewhat similarly to solubility, the effects can vary considerably with enzyme, substrate, degree of hydrolysis, and pH. Emulsifying properties often improve near the isoelectric point, along with increased solubility, but this is not always the case. Avramenko et al. [48] found that lentil protein hydrolysates produced with trypsin had lower emulsifying activity and emulsion stability indices than the untreated protein, regardless of degree of hydrolysis. At the same time, the hydrolysates had lower surface hydrophobicity (possibly due to aggregation), greater surface charge and lower interfacial tension. It was suggested that the reduced surface hydrophobicity negatively influenced the emulsifying properties. Barac et al. [50] found that the effect of hydrolysis on the emulsifying activity and emulsion stability indices of pea protein was dependent on the protease, pea variety, pH environment, and hydrolysis time. It was suggested that where reductions in emulsifying properties were observed, the formation of high molecular weight inflexible aggregates could be a key factor. García Arteaga et al. [35] found that hydrolysis either improved or had no significant effect on the emulsifying capacity of pea protein, depending on the enzyme. The highest emulsifying capacity was observed for trypsin and chymotrypsin hydrolysates.

Numerous studies show that emulsifying properties of pulse protein hydrolysates can vary considerably according to the degree of hydrolysis [39,45,46,63], and in many cases, the emulsifying properties seem to be more sensitive than solubility to degree of hydrolysis. The formation of small oil droplets and resistance to flocculation/coalescence is important for avoidance of phase separation in oil in water emulsions. Tamm et al. [7] investigated the impact of a trypsin or Alcalase hydrolysis of pea protein concentrate on emulsion characteristics. They found that the Alcalase hydrolysis had a negative effect, especially at higher degrees of hydrolysis where emulsions separated quickly. In contrast, trypsin hydrolysates generally resulted in improved emulsions with increasing degree of hydrolysis, with smaller droplet sizes, stronger interfacial film formation, and higher zeta potential. Klost and Drusch [44] assessed the droplet size and zeta-potential of emulsions stabilised with pea protein concentrate, either untreated or hydrolysed with trypsin, as a function of pH. Especially with higher degree of hydrolysis, they found that the emulsions were less stable when they were away from the isoelectric point compared to the control. Larger droplets were likely due to flocculation, which are also visible in micrographs. Overall, they hypothesised that for the hydrolysates, hydrophobic interactions were dominant over electrostatic repulsion across the pH range. Zhang and Motta [45] prepared hydrolysates of Great Northern bean or navy bean protein concentrate, using Alcalase or papain. They found that emulsions prepared with hydrolysates generally had smaller droplet size compared with those prepared with the untreated concentrates, and for all samples there was little or no increase in droplet size over an 8 day period. For the Alcalase hydrolysates of both the Great Northern bean and navy bean protein, the smallest droplet size was observed with the highest degree of hydrolysis, whereas for the papain hydrolysates, the smallest droplet size was observed for the low and intermediate degree of hydrolysis. Interestingly, heat-treated controls (i.e., non-hydrolysed samples otherwise subjected to the same conditions as the hydrolysates) formed emulsions with smaller droplet sizes compared with those of the untreated ingredients. This also corresponded with higher surface hydrophobicity and lower surface tension, which underlines the fact that processing steps such as heat treatments can significantly impact protein structure and functionality and should not be overlooked.

It is evident that careful control of hydrolysis is often necessary to achieve improved emulsion stability. In addition, heat stability of emulsions is an important and sometimes overlooked consideration, as many products will require a heat treatment step to ensure

microbial stability. In one study, hydrolysis of chickpea protein isolate with Alcalase improved emulsion heat stability only at the lowest degree of hydrolysis tested, and otherwise resulted in a considerably lower stability [42]. In a similar study, hydrolysis of chickpea protein isolate with Flavourzyme resulted in a slightly increased or decreased heat stability of emulsions depending on the degree of hydrolysis [41].

In general, increased hydrophobicity resulting from exposure of hydrophobic groups has been recognised as an important factor in improving the emulsifying properties of pulse proteins [45,48,75]. At the same time, this may lead to aggregation and impaired emulsifying ability [48,50]. It is evident that for a given protein ingredient, careful choice of protease and hydrolysis conditions will be necessary in order to generate peptides with the specific properties favouring formation of stable emulsions (i.e., size, amphiphilic properties, and molecular flexibility). As peptides in a certain size range are required to form a stable viscoelastic film at the oil-water interface, excessive hydrolysis can lead to reduced emulsion stability [22,39,43,53]. Moreover, loss of amphiphilicity could occur; therefore, the high variability found in studies is not surprising, due to the very diverse potential for different peptide mixtures. In particular, differences in protein composition (e.g., between different varieties) can have a major influence and should not be overlooked [50].

Another consideration is the type of emulsion product of interest, as different applications may have very different characteristics, and therefore, different emulsification requirements and challenges. For example, salad dressings may have a low protein/oil ratio, acidic pH, and high viscosity, whereas high-protein milk alternatives would likely have a higher protein/oil ratio, neutral pH, and low viscosity. Many studies use fundamental tests to predict functionality which may not always be relevant for specific applications.

Table 3. Overview of the effects of enzymatic hydrolysis on emulsifying properties from various protein sources and proteases.

Reference	Protein Source	Protease	Effect on Emulsifying Properties
Ahmed et al. [52]	Lentil protein isolate	Alcalase	EAI: decreased; ESI: decreased
Al-Ruwaih et al. [51]	Kidney bean protein isolate	Alcalase	EAI: increased (but decreased for high pressure treated sample) ESI: decreased
Avramenko et al. [48]	Lentil protein isolate	Trypsin	EAI: decreased; ESI: decreased
Barać et al. [66]	Pea protein isolate	Chymosin	EAI: increased at pH 3; increased/no difference at pH 5 depending on HT; increased/decreased at pH 7 depending on HT; decreased at pH 8 ESI: decreased at pH 3; increased/decreased at pH 5 depending on HT; increased/no difference at pH 7 and 8 depending on HT
Barac et al. [50]	Pea protein isolate (L1)	Papain	EAI: increased at pH 3, 5, 7, and 8 ESI: increased at pH 3; decreased/no difference at pH 5 depending on HT; increased at pH 7 and 8
		S. griseus protease	EAI: increased at pH 3, 5, 7 and 8 ESI: increased at pH 3; decreased at pH 5; increased at pH 7 and 8

Table 3. *Cont.*

Reference	Protein Source	Protease	Effect on Emulsifying Properties
	Pea protein isolate (Maja)	Papain	EAI: decreased at pH 3; increased/decreased at pH 5 and 7 depending on HT; increased/no difference at pH 8 depending on HT ESI: increased/decreased at pH 3 and 5 depending on HT; decreased at pH 7 and 8
		S. griseus protease	EAI: increased/decreased at pH 3, 5, 7, and 8 depending on HTESI: increased at pH 3 and 5; increased/decreased at pH 7 depending on HT; decreased at pH 8
Betancur-Ancona et al. [63]	*P. lunatus* protein isolate	Alcalase	EC: decreased at pH 2, 4, 6, 8, and 10 ES: decreased at pH 2; increased at pH 4; decreased at pH 6, 8, and 10
		Flavourzyme	EC: increased at pH 2; no difference at pH 4; increased/no difference at pH 6 depending on HT; increased at pH 8 and 10ES: No difference at pH 2; increased at pH 4 and 6; decreased/no difference depending on HT at pH 8 and 10
Eckert et al. [39]	Faba bean protein isolate	Pepsin	Decreased EAI and ESI
		Trypsin	Increased/decreased EAI and ESI depending on HT
		Flavourzyme	Decreased EAI; increased ESI
		Neutrase	No difference in EAI, increased ESI
García Arteaga et al. [35]	Pea protein isolate	Alcalase	EC: no difference
		Papain	EC: no difference
		Esperase	EC: increased/no difference depending on HT
		Bromelain	EC: no difference
		Trypsin	EC: increased
		Chymotrypsin	EC: increased
Konieczny et al. [64]	Pea protein-enriched flour	Trypsin	EAI: increased at pH 4; increased/decreased at pH 7 depending on DH; increased at pH 10 ESI: decreased at pH 4, 7, and 10
		Savinase	EAI: increased/decreased at pH 4 depending on DH; decreased at pH 7; increased at pH 10 ESI: decreased at pH 4, 7, and 10
		Papain	EAI: decreased at pH 4, 7, and 10 ESI: increased at pH 4; decreased at pH 7 and 10
		Pepsin	EAI: decreased at pH 4, 7, and 10 ESI: decreased at pH 4, 7, and 10
Mokni Ghribi et al. [46]	Chickpea protein isolate	Alcalase	EAI: increased/decreased depending on DH ESI: decreased/no difference depending on DH

Table 3. *Cont.*

Reference	Protein Source	Protease	Effect on Emulsifying Properties
Schlegel et al. [40]	Lupin protein isolate	Alcalase	EC: decreased
		Papain	EC: decreased
		Neutrase	EC: decreased
		Protease N-01	EC: no difference
		Flavourzyme	EC: decreased
		Protamex	EC: decreased
		Corolase 7089	EC: no difference
		Pepsin	EC: no difference
		Corolase N	EC: no difference
Wani et al. [76]	Kidney bean protein isolate (French Yellow)	Papain	EAI: increased/decreased at pH 3 depending on HT; decreased at pH 5; increased at pH 7 ESI: increased/no difference at pH 3 and 5 depending on HT; no difference at pH 7
	Kidney bean protein isolate (Contender)	Papain	EAI: increased at pH 3, 5 and 7 ESI: decreased/no difference at pH 3 depending on HT; increased/no difference 5 depending on HT; decreased at pH 7
	Kidney bean protein isolate (Master Bean)	Papain	EAI: increased at pH 3, 5 and 7 ESI: increased/no difference at pH 3 depending on HT; decreased at pH 5 and 7
	Kidney bean protein isolate (Local Red)	Papain	EAI: increased at pH 3, 5 and 7 ESI: no difference at pH 3; increased/no difference at pH 5 and 7 depending on HT
Wani et al. [77]	Black gram protein isolate (Mash 1-1)	Papain	EAI: increased at pH 3, 5 and 7 ESI: increased/no difference at pH 3 depending on HT; increased at pH 5; decreased/no difference at pH 7 depending on HT
	Black gram protein isolate (PU-19)	Papain	EAI: increased/decreased at pH 3 and 5 depending on HT; increased/no difference at pH 8 depending on HT ESI: increased/decreased at pH 3 and 5 depending on HT; decreased at pH 8
	Black gram protein isolate (T-9)	Papain	EAI: increased/decreased at pH 3 depending on HT; increased at pH 5; increased/decreased at pH 7 depending on HT ESI: increased at pH 3 and 5; increased/no difference at pH 7 depending on HT
Xu et al. [67]	Chickpea protein isolate	Alcalase	EAI: increased; ESI: increased
		Flavourzyme	EAI: increased; ESI: increased

This table is intended as an overview only—methodology, data representation, and statistics can vary between studies, making direct comparisons difficult. HT: hydrolysis time; DH: degree of hydrolysis; EAI: emulsifying activity index; ESI: emulsion stability index; EC: emulsifying capacity; ES: emulsion stability.

6. Foaming Properties

Foams can be described as dispersions of gas bubbles, surrounded by a liquid or solid continuous phase [78]. Foam formation and stability are key properties for many food applications, including meringues, cakes, ice cream, frothed milk beverages, whipped toppings, and mousses [2,78,79], many of which involve proteins as surfactants. Proteins can stabilise foams by reducing interfacial tension, aligning and forming a viscoelastic

layer at the air–water interface. The molecular properties of proteins required to produce stable foams are somewhat similar to those required for emulsions (e.g., appropriate amphiphilicity, flexibility, solubility and size); therefore, enzymatic hydrolysis is often a useful tool for the modification of foaming properties.

Foaming properties are typically measured in terms of foaming capacity (the amount of foam produced relative to starting volume), and foam stability (the proportion of foam remaining after a specified time). Other characteristics such as foam density and texture may also be of interest. Some pulse proteins already display high foaming capacity and stability, and in such cases enzymatic treatment may not be useful for enhancing these properties; however, others with poor foaming properties might be significantly improved.

Table 4 shows examples of the effect of enzymatic hydrolysis on foaming properties of various pulse proteins. As with emulsifying properties, there can be considerable variability in the effects of hydrolysis on foaming properties depending on enzyme, substrate, degree of hydrolysis, and pH [50]. Hydrolysis can be useful for improving foaming capacity near the isoelectric point, which can be related to increased solubility. For example, Eckert et al. [39] found that the foaming capacity of faba bean protein increased to varying degrees at pH 5 with pepsin, trypsin, Flavourzyme, or Neutrase, whereas at pH 7, foaming capacity was either increased or unchanged depending on the protease and hydrolysis time. At both pH 5 and 7, pepsin hydrolysis for 15 min resulted in the highest foaming capacity. At the same time, decreased foaming capacity may also correspond to decreased solubility after hydrolysis [64]. Similarly to emulsions, at a higher degree of hydrolysis, decreased foam stability may be observed. Even though the peptides may have good solubility and migrate quickly to the air/water interface, they may be too small to form and maintain a strong interfacial film [80]. This was observed in the studies of Ahmed et al. [52] and Al-Ruwaih et al. [51] where Alcalase hydrolysis led to an increased foaming capacity but decreased foam stability for lentil and kidney bean protein, respectively. Betancur-Ancona et al. [63] found that hydrolysis of *Phaseolus lunatus* protein isolate with Flavourzyme increased foaming capacity and foam stability across a range of pH values. Alcalase hydrolysis, on the other hand, resulted in lower foaming capacity across the pH range, which was attributed to a higher DH compared with Flavourzyme, and also decreased or increased stability depending on hydrolysis time and pH.

Overall, similarly to emulsifying properties, enzymatic hydrolysis has great potential for tailoring the foaming properties of pulse protein ingredients, but at the same time, it may be difficult to predict and requires careful optimisation (i.e., choice of protease and hydrolysis conditions). As with emulsification, foam stabilisation also requires peptides with specific properties. There seems to be wide variation in the foaming properties of pulse protein ingredients, thus many are already effective foaming agents, and in those cases, hydrolysis can reduce foaming capacity, and quite often foam stability; however, in some cases, hydrolysis may be very useful for improving these properties, and may prove useful in providing effective alternatives to animal proteins used for foam formation (e.g., egg proteins).

Table 4. Overview of the effects of enzymatic hydrolysis on foaming properties from various protein sources and proteases.

Reference	Protein Source	Protease	Effect on Foaming Properties
Ahmed et al. [52]	Lentil protein isolate	Alcalase	FC: increased; FS: decreased
Al-Ruwaih et al. [51]	Kidney bean protein isolate	Alcalase	FC: increased (but decreased for high pressure treated sample) FS: decreased
Barać et al. [66]	Pea protein isolate	Chymosin	FC: increased at pH 3, 5, and 7; increased/decreased at pH 8 depending on HT FS: increased/decreased at pH 3 and 5 depending on HT; increased/no difference at pH 7 depending on HT; decreased at pH 8
Barac et al. [50]	Pea protein isolate (L1)	Papain	FC: increased at pH 3, 5, 7, and 8 FS: increased/decreased at pH 3 and 5 depending on HT; increased at pH 7; increased/decreased at pH 8 depending on HT
		S. griseus protease	FC: increased at pH 3 and 5; increased/decreased at pH 7 depending on HT; decreased at pH 8 FS: decreased at pH 3 and 5; no difference at pH 7; decreased at pH 8
	Pea protein isolate (Maja)	Papain	FC: increased at pH 3, 5, and 7; decreased/no difference at pH 8 depending on HT FS: increased at pH 3, 5, 7 and 8
		S. griseus protease	FC: increased/decreased at pH 3, 5, and 7 depending on HT; decreased at pH 8 FS: increased/no difference at pH 3 depending on HT; increased/decreased at pH 5 and 7 depending on HT; increased at pH 8
Betancur-Ancona et al. [63]	*P. lunatus* protein isolate	Alcalase	FC: decreased at pH 2, 4, 6, 8, and 10 FS: increased/decreased at pH 2 and 4 depending on HT; increased at pH 6 and 8; increased at pH 10
		Flavourzyme	FC: increased at pH 2, 4, 6, 8, and 10 FS: increased at pH 2, 4, 6, 8, and 10
Eckert et al. [39]	Faba bean protein isolate	Pepsin	FC: increased at pH 5 and 7 FS: no difference at pH 5; increased/no difference at pH 7 depending on HT
		Trypsin	FC: increased at pH 5; increased/no difference at pH 7 depending on HT FS: no difference at pH 5; increased at pH 7
		Flavourzyme	FC: increased at pH 5 and 7 FS: no difference at pH 5; increased at pH 7
		Neutrase	FC: increased at pH 5; increased/no difference at pH 7 depending on HT FS: increased/decreased at pH 5 and 7 depending on HT

Table 4. *Cont.*

Reference	Protein Source	Protease	Effect on Foaming Properties
Konieczny et al. [64]	Pea protein-enriched flour	Trypsin	FC: decreased at pH 4, 7, and 10 FS: decreased/no difference at pH 4 depending on DH; increased/no difference at pH 7 depending on DH; increased at pH 10
		Savinase	FC: decreased at pH 4, 7, and 10 FS: decreased/no difference at pH 4 depending on DH; increased at pH 7; increased/no difference at pH 10 depending on DH
		Papain	FC: increased/decreased at pH 4 depending on DH; decreased at pH 7 and 10 FS: no difference at pH 4; increased/no difference at pH 7 depending on DH; increased at pH 10
		Pepsin	FC: increased no/difference at pH 4 depending on DH; decreased at pH 7; decreased/no difference at pH 10 FS: increased at pH 4, 7, and 10
Schlegel et al. [40]	Lupin protein isolate	Alcalase	FC: increased; FS: no difference
		Papain	FC: increased; FS: no difference
		Neutrase	FC: increased; FS: no difference
		Protease N-01	FC: increased; FS: no difference
		Flavourzyme	FC: no difference; FS: no difference
		Protamex	FC: increased; FS: no difference
		Corolase 7089	FC: increased; FS: no difference
		Pepsin	FC: increased; FS: no difference
		Corolase N	FC: increased; FS: no difference
Yust et al. [42]	Chickpea protein isolate	Alcalase	FC: increased; FS: increased
Yust et al. [41]	Chickpea protein isolate	Flavourzyme	FC: increased; FS: increased/no difference depending on DH

This table is intended as an overview only—methodology, data representation, and statistics can vary between studies, making direct comparisons difficult. HT: hydrolysis time; DH: degree of hydrolysis; FC: foaming capacity; FS: foam stability.

7. Gelation and Rheological Properties

Gelation is important for various foods, including processed meats/meat alternatives, cheese, yogurt, tofu, and desserts. There is now considerable interest in formulating plant-based alternatives to products such as meat and cheese, which require certain textural properties that proteins could potentially contribute to [22,25,81]. Pulse proteins can play a functional role in gelled products; however, it can be difficult to mimic the structural and textural properties of the original products. Gelation of proteins can occur when the proteins unfold, allowing interaction to form a three-dimensional crosslinked network capable of binding water. A critical concentration must be reached before gelation can occur. Proteins in gel structures can be linked by both non-covalent (electrostatic, hydrogen bonds, hydrophobic) and covalent interactions (disulphide bonds) [61,78]. Most often, heat-induced gelation is studied; however, gelation may also be induced or aided by other means including pH changes (usually acid gels), changing ionic strength, high-pressure processing, or enzymatic crosslinking [27,78,82].

Many pulse proteins can form gels; however, they can be relatively weak (e.g., when compared to soy protein gels, attributable at least in part to the lower prevalence of sulfhydryl groups, and consequently, fewer disulphide bonds in the final gels) [83,84].

There is relatively little literature available on the impact of enzymatic hydrolysis on the gelation of pulse proteins; however, for other proteins such as whey and soy, various effects have been observed with enzymatic hydrolysis. Hydrolysis can result in increased gel strength, decreased gel strength, or no gel formation, depending on factors such as protease, degree of hydrolysis, and pH [85,86]. In some cases, limited hydrolysis may lead to improved gelling ability. It is possible that exposure of reactive groups during limited hydrolysis could allow for increased protein–protein interaction during heating, and structural changes could alter the type of network formed. At the same time, above a certain degree of hydrolysis, peptide sizes tend to be too small to form a continuous network, and gelation is impeded [27,59].

Felix et al. [87] examined the impact of hydrolysis with trypsin on the heat gelling properties of pea protein concentrate, at pH 2, 6.5, and 8. The mechanical spectra revealed little impact of hydrolysis on gel strength at low degrees of hydrolysis. At higher degrees of hydrolysis, however, gel strength was reduced at pH 8 compared to pH 6.5. All gels were very weak at pH 2 regardless of treatment. Some differences were apparent between samples regarding the type of gel interactions. Different contributions of ionic bonds, hydrogen bonds, hydrophobic interactions, and disulphide bonds could be seen, depending on hydrolysis time as well as pH. Pea protein gel characteristics have been shown to be highly dependent on pH, and to a lesser extent on ionic strength [88]. Klost et al. [89] prepared fermentation-induced gels from pea protein concentrate, and hydrolysates thereof, to investigate the impact of hydrolysis on the gel rheological properties. The hydrolysates were produced with Protamex, trypsin, or Alcalase. The Alcalase treated sample was unable to form a gel due to the low molecular weight of the peptides. Gels prepared with Protamex or trypsin showed very little difference in rheological properties compared to the unhydrolyzed sample; however, hydrolysis did modify the interaction between protein fractions, with trypsin promoting increased involvement of vicilin in the gel structure. Guldiken et al. [84] compared the heat-gelation properties of faba bean, lentil, and yellow pea protein concentrates, and found that gelation properties were influenced by hydrophobicity and legumin:vicilin ratio; therefore, it may be useful to consider enzyme specificity in relation to these properties.

Due to the relatively small amount of literature available on the effect of hydrolysis on pulse protein gelation, it is difficult to grasp an overall picture of its potential. As previously mentioned, pulse protein gels can be relatively weak, and may be more suitable for softer gelled applications (e.g., yogurts, soft cheese alternatives, or desserts). Hydrolysis could potentially be used to alter gel characteristics to improve texture; however, it is clear that the extent of hydrolysis may need to be very limited to avoid impaired network formation. In addition, it may be expected that for protein ingredients with very poor solubility, in some cases, hydrolysis could improve solubility, and therefore, gelation potential.

As well as gelation, enzymatic hydrolysis could be used to modify the rheological properties of pulse proteins in liquid systems. Enzymatic hydrolysis of protein dispersions can often result in decreased viscosity. Hydrolysis with Alcalase was found to reduce the viscosity of lentil protein [52] and kidney bean protein [51] dispersions. Bajaj et al. [90] examined the effect of hydrolysis with various proteases on pea protein dispersions with high initial viscosity, to reduce viscosity and facilitate microencapsulation of flaxseed oil. They found a considerable reduction in viscosity with most of the treatments. Viscosity reduction with enzymatic hydrolysis could be particularly useful for high-viscosity pulse protein ingredients, for example, in nutritional beverage applications where high protein content but low viscosity is required. At the same time, bitterness could present difficulties for such products.

8. Sensory Considerations

Although protein ingredients can provide essential functionality to food products, they may be of limited use if they contribute undesirable sensory attributes. One of the key limitations of protein hydrolysates generally, is the generation of bitter peptides; therefore,

reduction or elimination of bitterness in hydrolysates has become an important concern for the food industry [91,92]. Bitter peptides can be generated with hydrolysis of many food proteins; however, some are particularly susceptible (e.g., casein) [93]. Much work has focused on dairy and soy proteins hydrolysates; however, bitterness is an important concern for various hydrolysates from various protein sources including pulses. Depending on the type of food product, some level of bitterness may be desirable or acceptable; however, if the level of bitterness is excessive, sensory quality is reduced [93–95]. This may be particularly important for high protein beverage applications [91]. As well as peptides, free amino acids can elicit taste, including bitter, umami, sweet, and sour [95].

It is generally accepted that the perception of bitterness is related to the generation of small peptides with a high proportion of hydrophobic side chains [31,89]. Hydrolysis exposes hydrophobic residues which were previously buried in the intact protein [87]. Although these peptides tend to be more hydrophobic, it is difficult to use this property alone to predict the level of bitterness. As well as the proportion of certain amino acid residues, the amino acid sequence, peptide length, and terminal amino acids, affect bitterness level [95,96]. Several important factors which influence the bitterness of protein hydrolysates should be considered. These include the composition and hydrophobicity of the substrate, the protease(s) used, the degree of hydrolysis, any separation steps such as filtration or centrifugation, and other components which could mask bitterness [33].

In sensory evaluations, a bitter substance is typically used as a reference, such as a caffeine solution or a bitter hydrolysate solution. Cho et al. [91] investigated the relationship between peptide properties and the bitterness of two commercial soy protein hydrolysates. They fractionated the hydrolysates based on molecular weight and found for both hydrolysates that the 5–10 kDa fractions had the highest bitterness, with bitterness decreasing towards relatively higher or lower molecular weight fractions. Interestingly, they did not find a correlation between hydrophobicity of the fractions (based on amino acid composition) and bitterness.

Various studies have shown that bitterness is influenced by the protease used and degree of hydrolysis. Seo et al. [97] used taste dilution analysis (taste threshold concentration) to assess differences in bitterness in soy protein hydrolysates. At a constant degree of hydrolysis, they found the highest bitterness for Alcalase hydrolysate, and the lowest for Flavourzyme hydrolysate. Intermediate values were found for Neutrase, Protamex, papain, and bromelain. This illustrates the importance of protease specificity in relation to the peptide mixture produced and corresponding bitterness, for a given substrate. Humiski and Aluko [92] compared the bitterness of pea protein hydrolysates produced with different proteases. They found the highest bitterness for the hydrolysate produced with Alcalase, followed by Flavourzyme, trypsin, chymotrypsin, and papain hydrolysates. The authors suggested that the higher bitterness of the Alcalase hydrolysate could be related to its broad specificity and preference for cleaving near hydrophobic residues and that the release of free amino acids by Flavourzyme could have contributed to increased bitterness. García Arteaga et al. [35] measured bitterness of pea protein isolate hydrolysed with various protease preparations, with a hydrolysis time of 15 min or 120 min. Significantly higher bitterness was found, compared with the unhydrolysed protein isolate for Alcalase at 15 min hydrolysis, Esperase at 120 min hydrolysis, and Savinase at 15 min and 120 min hydrolysis. This also corresponded with a relatively higher degree of hydrolysis for these proteases, which are known to exhibit broad specificity. No significant differences were found compared with the protein isolate for the other proteases, which included Flavourzyme, Neutrase, Protamex, trypsin, chymotrypsin, papain, bromelain, and Corolase 7089. Schlegel et al. [40] compared sensory properties of lupin protein hydrolysates using nine different protease preparations. The hydrolysate prepared with Alcalase was rated as being extremely bitter, and was the only hydrolysate for which bitterness was significantly higher than the untreated protein isolate. In a similar study, Meinlschmidt et al. [98] assessed the bitterness of soy protein hydrolysates produced with various proteases and different hydrolysis times. The intensity of bitterness varied with the protease as well

as hydrolysis time. Alcalase and Corolase 2TS hydrolysates had a significantly higher bitterness compared with the protein isolate at all hydrolysis times, whereas for most of the other proteases, a significantly higher bitterness was only found for certain hydrolysis times. Generally, it was found that the relationship between hydrolysis time and bitterness varied with the protease; for example, with Neutrase, bitterness was only significant after longer periods of hydrolysis, whereas with papain, hydrolysates had a significantly higher bitterness compared with the protein isolate at 10 and 30 min, but not at 60 and 120 min.

Combinations of different proteases have also been explored in relation to bitterness. Schlegel et al. [99] hydrolysed lupin protein isolate with various combinations of proteases, using either one or two step hydrolysis. Significantly higher bitterness compared with the protein isolate was not found for any of the combinations, with the exception of Alcalase and papain. Meinlschmidt et al. [100] hydrolysed soy protein isolate with various enzyme combinations, in one or two steps. Two combinations resulted in significantly higher bitterness compared with soy protein isolate—Alcalase, Neutrase, and Flavourzyme, and also for the same combination but with the addition of Corolase 7089. Interestingly, two of the combinations provided hydrolysates with a significantly lower bitterness than the protein isolate—Neutrase, Corolase 7089, and Flavourzyme, as well as the combination of papain and Flavourzyme.

The tendency towards bitterness of hydrolysates produced with Alcalase, or other subtilisins, is thought to be related to their broad specificity and preference for cleaving next to hydrophobic amino acid residues, which are then positioned as terminal residues in the peptides. Rezvankhah et al. [101] produced hydrolysates from lentil protein concentrate using either Alcalase, Flavourzyme, or a sequential hydrolysis with Alcalase followed by Flavourzyme. Bitterness, umami, and sweetness of the protein isolate and hydrolysates were assessed in an umami soup consisting of water, salt, and monosodium glutamate. Increased bitterness was only perceived for the Alcalase hydrolysate. Increased umami was apparent for all the hydrolysates, but particularly for the Alcalase and Alcalase/Flavourzyme hydrolysates. Increased sweetness was found for the Flavourzyme and Alcalase/Flavourzyme hydrolysates. It seemed that Flavourzyme was effective in eliminating the bitterness related to the Alcalase hydrolysis, as well as increasing sweetness perception. Although increased bitterness is by far the most prominent sensory impact of hydrolysis, other sensory attributes may also be affected to some extent. For example, Schlegel et al. [40] found that hydrolysis with pepsin significantly reduced the oatmeal-like retro-nasal attribute of lupin protein isolate.

Various approaches have been applied to reduce bitterness, which could be used in the preparation of pulse protein hydrolysates to improve sensory properties. These include complexation of bitter peptides with activated carbon, removal using hydrophobic column absorption, and exopeptidase treatment [96]. Application of exopeptidases is the most widely used approach for the debittering of hydrolysates. The terminal amino acids of peptides have a significant impact on bitterness; therefore removal of these residues with aminopeptidases or carboxypeptidases may allow reduced bitterness, while also retaining functionality [96,102]. At the same time, it is possible for a reduction in peptide length to contribute to reduced bitterness [96]. Ewert et al. [102] investigated the use of four different aminopeptidases for debittering a caseinate hydrolysate. These included three aminopeptidases, which they produced using *Lactobacillus* species, as well as Flavourzyme with its endoprotease activity reduced using ultrafiltration. Depending on the aminopeptidase used and its specificity, the treatment either reduced bitterness without impacting the functional properties, or improved functionality of the hydrolysate without affecting bitterness. In addition, release of free amino acids can increase the umami taste in hydrolysates. Großmann et al. [103] hydrolysed pea, soy, or canola protein using different commercial proteases which contain exoprotease activity. The specific activity of the proteases could be correlated to the free amino acid profile. Some significant differences were found between the hydrolysates for umami and bitter taste, depending on the protease. These properties also varied to some extent depending on the substrate.

In addition, the presence of other taste components may help to mask or reduce the perception of bitterness, such as umami or acids [33]; therefore, the acceptability of bitterness in hydrolysates may depend on the particular application. Bhaskar et al. [104] incorporated horse gram flour hydrolysate in the instant soup, and found that although panellists were able to identify the soup with hydrolysate from the control soup, there was no significant difference in preference between the two. Overall, various approaches can be taken to mitigate bitterness and improve sensory properties, especially careful selection of protease and control of hydrolysis, as well as application of exoproteases. It may be challenging to optimise the hydrolysis for both functionality and sensory quality simultaneously.

9. Future Outlook

Many studies have investigated enzymatic hydrolysis as a means of improving the functionality of pulse proteins, with varying results depending on different factors as previously discussed; however, further studies relating structural and surface properties to hydrolysate functionality would be useful. In addition, further research on factors such as pre/post hydrolysis treatments, ingredient processing, and their relationship to hydrolysate functionality should be carried out. In particular, work on the optimisation of enzyme inactivation conditions is largely missing from the literature; this could prove very useful in future studies regarding pulse protein hydrolysates. As pulse protein ingredients are increasingly being investigated and developed (e.g., with processing improvements), it is likely that more functional ingredients will be available and suitable for a range of applications; however, poor functionality of commercial ingredients may still be an issue, which is less evident in lab-scale ingredients, largely due to harsh processing conditions, leading to denaturation and aggregation. Enzymatic hydrolysis could potentially provide major functional improvements for such ingredients. Furthermore, there are many commercial protease preparations available that have not yet been investigated for the production of pulse protein hydrolysates, which could prove valuable. Moreover, novel techniques such as the 'activity fingerprint' have been described, which can provide rapid and detailed information on protease specificity using synthetic substrates; this could allow the prediction of hydrolysate composition and characteristics [32]. Technology such as protease engineering could provide even more options [105].

10. Conclusions

Enzymatic hydrolysis shows excellent potential as a tool for improving the functional properties of pulse protein ingredients where they are found to be lacking; however, it is difficult to predict the outcome of hydrolysis on ingredient functionality. Differences in protease specificity allow for a wide variety of peptides with differing properties in the hydrolysates. In addition, various other factors need to be considered, including protein substrate composition/structure, hydrolysis conditions/degree of hydrolysis, pre- and post-hydrolysis treatments, and target pH environment. Further investigations into the relationship between structure and surface properties and corresponding functionality will be useful. Improvements in solubility are often most effective where the protein substrate demonstrates poor initial solubility. In addition, enzymatic hydrolysis usually reduces the pH sensitivity of solubility, and the most significant relative increases are observed near the isoelectric point, which greatly increases the potential for the use of pulse proteins in acidic foods and beverages. Overall, in many cases, different functionalities can be improved with careful selection of proteases and control of hydrolysis. In addition, more research should be done on the effects of enzymatic hydrolysis on gelling properties as this is an essential functionality for many applications, and currently lacking in the literature. Furthermore, the effects of heat treatment and other treatments on functionality should be given more attention, especially in relation to food product processing. Sensory issues, especially bitterness, can be a limiting factor for the use of pulse protein hydrolysates. More work should be carried out to explore the relationship between hydrolysis and bitterness, specifically for various pulse proteins, along with debittering techniques. Overall, pulse

protein ingredients will likely have an important role in satisfying global protein demand, in a more sustainable and economical manner. Modifications such as enzymatic hydrolysis can potentially be very useful as a means of increasing their utility, especially for plant-based food and beverage products.

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The Protein Composition Changed the Quality Characteristics of Plant-Based Meat Analogues Produced by a Single-Screw Extruder: Four Main Soybean Varieties in China as Representatives

Bo Lyu [1,2,3], Jiaxin Li [1,2], Xiangze Meng [1,2], Hongling Fu [1,2], Wei Wang [2,4], Lei Ji [1,2], Yi Wang [1,2], Zengwang Guo [2,3,*] and Hansong Yu [1,2,*]

1 College of Food Science and Engineering, Jilin Agricultural University, Changchun 130118, China; michael_lvbo@163.com (B.L.); li1997jiaxin@163.com (J.L.); mxz625797@163.com (X.M.); 15764381475@163.com (H.F.); jilei0616@163.com (L.J.); wangyi284419@163.com (Y.W.)
2 Division of Soybean Processing, Soybean Research & Development Center, Chinese Agricultural Research System, Changchun 130118, China; wangwei19936@163.com
3 College of Food Science, Northeast Agricultural University, Harbin 150030, China
4 Jilin Provincial Agricultural Products Processing Industry Promotion Center, Changchun 130022, China
* Correspondence: gzwname@163.com (Z.G.); yuhansong@163.com (H.Y.)

Abstract: Plant-based meat analogues (PBMs) are increasingly interesting to customers because of their meat-like quality and contribution to a healthy diet. The single-screw extruder is an important method for processing PBMs, and the characteristics of the product are directly affected by the composition of the raw materials; however, little research focuses on this issue. To explore the effect of protein composition on the quality characteristics of PBMs produced by a single-screw extruder, four soybean varieties used in China (Heihe 43 (HH 43), Jiyu 86 (JY 86), Suinong 52 (SN 52), and Shengfeng 5 (SF 5)) were selected. The 11S/7S ratios for these varieties ranged from 1.0: 1 to 2.5: 1 in order to produce PBMs with different protein compositions. The structure, processing, nutrition, and flavor characteristics were explored to analyze their differences. The results showed that protein composition affected the structure of PBMs, but the correlation was not significant. Meanwhile, a lower 11S/7S ratio (HH 43) did not prove to be a favorable characteristic for the processing of PBMs. From the perspective of nutrition and flavor, it seems acceptable to use a moderate 11S/7S ratio (JY 86 and SN 43) to produce PBMs. This study proved that the protein composition of raw materials affects the characteristics of PBM products produced by a single-screw extruder. To produce PBMs of higher quality, soybeans with a markedly different 11S/7S ratio should not be selected.

Keywords: extrusion technology; textured soy protein; protein subunit composition; processing applicability; plant-based meat analogues

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1. Introduction

With improvements in living standards, great changes have taken place in people's dietary structures, including the increased intake of animal-derived foods [1]. However, the increase in the intake of animal-derived foods and the decrease in vegetarian intake is one of the causes of many diseases, such as intestinal and cardiovascular diseases [2,3]. This result may be caused by the excessive intake of fats, drug residues, or other factors [4,5]. To maintain the excellent taste and good processing characteristics of meat products while preventing the potential health risks caused by the excessive intake of animal-based food, plant-based meat analogues (PBMs) came into being. The intake of PBM products not only does not cause health risks but also can reduce land use and resource consumption [6,7]. PBMs can also meet the requirements of modern people for food diversity [8]; this has gradually increased the acceptability of PBM products, as well as the demand for them [9].

An important aspect of PBM products is the fact that the process of manufacturing textured plant protein mainly depends on the changes in protein structure caused by the high temperature and pressure produced by screw extruders [10]. Mainstream textured plant protein production equipment includes the twin-screw extruder and the single-screw extruder [11]. The twin-screw extruder is widely used because of its excellent processing capacity; as a result, there are more theoretical studies based on it [10,12]. However, the single-screw extruder is also used on a large scale because of its lower cost and ability to process a wider variety of raw materials, such as insoluble dietary fiber, starch, etc. [13]. Therefore, consumers may encounter products manufactured using two different kinds of extrusion technologies in the market at the same time. Certainly, there are differences in the quality of the products because of the differences in chemical cross-linking and molecular aggregation [14].

Soybean protein is considered to be a good choice for producing PBMs because of its excellent gelation, superior nutritional value, low cost, and safety as a raw material [15]. The composition of soybean protein affects the processing and nutritional characteristics of soybean products directly [16]. In particular, the proportions of soy glycinin (11S) and soy β-conglycinin (7S) directly affect the key processing characteristics, such as gel and foaming properties [17,18]. It is generally believed that high 7S content is related to hydration characteristics, such as emulsification and foaming properties [17,19]. A high 11S content means a higher protein structural strength, as manifested in characteristics such as gelation [20]. However, the relationship between them is not strictly linear. There is reason to believe that the texture of PBMs is also related to the gel properties of soybean protein [21]. However, no study has compared the properties of PBM products produced using different varieties of soybean with different protein compositions, and no study has demonstrated what kind of protein composition is more suitable for PBM products produced by single-screw extruders. Previous studies on PBMs tend to analyze the composition of soybean protein isolate (SPI). Currently, under the guidance of "Whole-Soybean Processing", a great deal of PBMs are processed directly from defatted soybean powder. As such, we should pay more attention to the protein composition of soybean. In addition, soybean protein should be perceived as a safe raw material for producing PBM products. Soybeans used in the production of protein products are required not to use transgenic soybeans in many countries [22–24]; similar regulations also avoid some of the risks of processing livestock products, such as hormones [25]. Although soybean protein has a certain potential to aggravate food sensitivities, high temperatures and pressures, such as those the single-screw extruder provides, can reduce the allergen content [26], which can improve the acceptability of soybean products. Therefore, soybean protein can be regarded as the best raw material for the production of PBMs.

In this study, to explore the potential impact of the soybean protein composition of the raw materials on PBM characteristics, we selected four soybean varieties, representative of the main planting varieties in China, with different compositions and ratios of 11S and 7S (11S:7S = 1, 1.5, 2, 2.5), which were processed into defatted soybean flours (DSFs) with different protein compositions. A single-screw extruder was used to produce textured soybean protein as a representative of PBM products. The structure (basic composition, sulfhydryl content, secondary structure, functional group composition, and microstructure), processing characteristics (water-absorption capacity, water-holding capacity, water-swelling capacity, tensile strength, breaking elongation, and texture characteristics), nutritional characteristics (dietary fiber, reducing sugar, phytic acid, trypsin inhibitor, plant lectin, amino acid composition, and isoflavone content) and flavor characteristics were used to measure the quality of PBM products, to determine the impact of different protein compositions on their quality.

2. Materials and Methods

2.1. Selection of Soybean Varieties and Preparation of Defatted Soybean Flour (DSF)

By searching the database of the China Agriculture Research System (CARS): Soybean Processing Division, we selected four kinds of soybeans with different protein subunit compositions as the experiment raw materials: Heihe 43 (HH43), Jiyu 86 (JY 86), Suinong 52 (SN 52), and Shengfeng 5 (SF 5), for which the ratios of 11S/7S were 1.0, 1.5, 2.0, and 2.5, respectively. All four varieties are under large-scale cultivation in China. After soybean dehulling, the oil was removed by an oil press, and then defatted soybean flour (DSF) was obtained after crushing (oil content <7%). A SH-28 single-screw extruder (Shandong Yuya Soybean Machinery Manufacturing CO., Ltd., Zaozhuang, China) was used to produce PBMs under the following conditions: the ratio of DSF to water was 2:3; the temperature was 240 °C in the first zone, 220 °C in the second zone, 200 °C in the third zone, and 180 °C in the fourth zone; the screw rotated at 70 rpm.

2.2. Analysis of Soybean Protein Composition by SDS-PAGE

The analysis of the soybean protein composition was measured according to the method described by Song et al. [27]. The presence and absence of glycinin and β-conglycinin subunits in the soybean seeds were confirmed by SDS-PAGE. The total seed proteins were extracted from a small portion of cotyledon tissues with an SDS sample buffer (2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 5 M urea, and 62.5 mM Tris aminomethane) and then centrifuged at 15,000× g. Then, 10 μL of the supernatant was separated on 4.5% stacking and 12.5% separating polyacrylamide gels and stained with Coomassie Brilliant Blue R250. The gels were scanned by an Image Lab 3.0 (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and the number of protein subunits was quantified according to the gray value.

2.3. The Structure of PBMs

2.3.1. The Basic Composition of Four Kinds of PBMs

The moisture, protein, ash, and fat content of four kinds of PBMs were determined according to the AOAC Official Method (AOAC 2007.04).

2.3.2. The Sulfhydryl Content of Four Kinds of PBMs

Four kinds of PBMs were crushed into 80 mesh after being fully dried, and a method employing Ellman's reagent (10 mm DTNB, 0.2 mm EDTA) was used to determine the content of SH in the samples [28]. The preparation of Tris-Glycine buffer was as follows: 0.086 M tris + 0.09 M Glycine + 4 mM EDTA, pH = 8.0. The samples were dispersed in the Tris-Glycine buffer to obtain 2 mg/mL solutions. Then, 0.03 mL Ellman's reagent was added to 3 mL solution, and the solution was mixed immediately and stored for 15 min at room temperature before measuring the absorbance at 412 nm. A buffer solution without a protein sample was used as a reagent blank.

2.3.3. Circular Dichroism Spectrum (CD)

The crushed PBM samples (80 mesh) were prepared with 1 mM phosphate-buffered saline (PBS, pH = 7.0) into a 1 mg/mL solution, placed in a 1 mm optical path length quartz cell, and measured with a J-810 CD spectrometer (JASCO, Tokyo, Japan). The sensitivity was set to 2 mdeg/cm. The 185–260 nm CD spectrum was recorded at 20 °C.

2.3.4. Fourier Transform Infrared Spectroscopy (FT-IR)

Fourier transform infrared spectroscopy (FT-IR) analyses of four kinds of PBMs were performed using a Nicolet iS5 spectrometer (Thermo Fisher, Waltham, MA, USA). The dried samples were mixed with KBr powder (1:100, w/w) after being crushed into 80 mesh, and the spectra were read over the range of 4000–400 cm^{-1} with a resolution of 4 cm^{-1}.

2.3.5. Scanning Electron Microscopy (SEM)

The PBMs were cut into strips of appropriate size for sample pre-treatment. The samples were immersed in a glutaraldehyde solution (2.5%, pH = 7.2–7.4) for 24 h. After they were fixed, the samples were washed with a phosphate buffer (pH = 7.2) 3 times before eluting with 30%, 50%, 70%, 85%, 95%, and 100% ethanol solutions. The samples were made into 2 mm × 2 mm flakes, as thin as possible, after freeze-drying in order to observe the microscopic appearance of four kinds of PBMs using a SU8020 scanning electron microscope (SEM; Hitachi, Tokyo, Japan) after spraying with a gold–palladium alloy. The scanning images were captured at accelerating voltages of 5 kV and photographed at magnifications of 5000X (scale bar 10 μm).

2.4. The Processing Characteristics of PBMs

2.4.1. Water-Absorption Capacity (WAC), Water-Holding Capacity (WHC), and Water-Swelling Capacity (WSC)

WAC: After recording the weight of the fully dried sample (M1), it was soaked in 60 °C water for 5 h, then drained for 6 min. The samples were weighed (M2).

$$WAC = (M2 - M1)/M1 \times 100\% \quad (1)$$

WHC: 1.00 g crushed sample (M1) and 20 mL of water were mixed in a dry centrifuge tube (M0). The sample was kept at RT for 24 h and centrifuged at 4000 rpm for 20 min. The supernatant was removed, and the weight (M2) was recorded.

$$WHC = (M2 - M0)/M1 \times 100\% \quad (2)$$

WSC: 1.000 g crushed, fully dried sample (M) and 10 mL of water were mixed in a dry centrifuge tube and kept at RT for 24 h. The volume of the sample was recorded (V).

$$WSC = V/M \times 100\% \quad (3)$$

2.4.2. The Tensile Strength (TS) and Breaking Elongation (BE) of Four Kinds of PBMs

The WDW-200H electronic tensile testing machine (Hongtuo, Dongguan, China) was used to analyze the tensile strength of four kinds of PBMs. The experiment conditions were as follows. The wet PBM was cut to 10 cm × 6 cm for testing; the initial clamping distance was 40 mm, and the tensile speed was 5 mm/s. The following values were recorded: the maximum tension at break (P), the cross-sectional area of samples (S), the elongation at break (δL), and the original length (L). Values were calculated for the tensile strength (TS) and breaking elongation (BE).

$$TS\ (MPa) = P/S \quad (4)$$

$$BE\ (\%) = \delta L/L \times 100\% \quad (5)$$

2.4.3. Texture Profile Analysis (TPA)

The texture characteristics of four kinds of PBMs were analyzed by a Texture Analyzer (TA.new plus, Isenso, Shanghai, China). The samples were cut into a square with a length of 10 mm, and the conditions were as follows. The detection mode was TPA mode with the P/36R detector; the rate before the test was 2 mm/s; the rate during the test was 1 mm/s; the rate after the test was 2 mm/s; the compression degree was 50%.

2.5. The Nutritional Characteristics of PBMs

2.5.1. The Dietary Fiber, Reducing Sugar, Phytic Acid, Trypsin Inhibitor, Plant Lectin, and Isoflavone Content of Four Kinds of PBMs

The dietary fiber, reducing sugar, phytic acid, and isoflavone content of four kinds of PBMs were determined according to the AOAC Official Methods (AOAC 2017.16, AOAC 945.66, and AOAC 986.11). The amounts of trypsin inhibitor and plant lectin (Soybean agglutinin, SBA) found in the four kinds of PBMs were determined using the

Trasylol Elisa kit (Ml064289, Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) and the SBA Elisa kit (Ml002453, Enzyme-linked Biotechnology Co., Ltd., Shanghai, China).

2.5.2. The Amino Acid Composition

The analysis of the amino acid composition of four kinds of PBMs was performed according to the method described by Song et al. [27]. After the PBMs were fully dried, a meal was prepared by mill grinding through a 0.25-mm sieve and thoroughly mixing. Total amino acids were obtained by the hydrolysis of seed meal with an excess of 6 M HCl for 22 h in sealed evacuated tubes at a constant boiling temperature (110 °C). An L-8800 amino acid analyzer (Hitachi, Tokyo, Japan) was used to determine the amino acid compositions of the hydrolysates. The amino acid composition was expressed as relative content (%) on a dry basis.

2.6. The Flavor Characteristics of PBMs

The volatile flavor compounds found in four kinds of PBMs were measured by a 6890N-5975B Gas Chromatography-Mass Spectrometry workstation (GC-MS; Agilent, Palo Alto, CA, USA).

Sample pre-treatment was as follows. The samples were sealed in head-space bottles and warmed at 80 °C in a water bath for 30 min. A solid-phase microextraction needle (SPMEN; 100 μL PDMS; SUPELCO, Bellefonte, PA, USA) was used for extraction for 30 min at 80 °C before desorption for 5 min.

The experiment conditions for GC-MS were as follows. Chromatographic column: HP-5MS (30 m × 0.25 mm × 0.25 μm); split ratio: no split; carrier gas flow rate: 1.2 mL/min; injection port temperature: 250 °C; scanning mode: full scan; ion source temperature: 230 °C; quadrupole temperature: 150 °C; temperature program: initial temperature of 50 °C for 2 min, raised to 180 °C at the rate of 5 °C/min for 5 min, then raised to 250 °C at the rate of 10 °C/min for 5 min.

The mass spectra were searched using the NIST database to identify the volatile components in the samples, and the relative content of each component was analyzed by the area normalization method.

2.7. Statistical Analysis

All determinations were conducted at least three times, and all results were expressed as mean ± standard deviation ($x \pm$ SD). One-way analysis of variance (ANOVA) and Duncan's test were used to analyze the differences in the properties of four kinds of PBMs using IBM SPSS 25.0 (SPSS Inc., Chicago, IL, USA); $p < 0.05$ was considered significant, and all results were expressed as mean ± standard deviation ($x \pm$ SD). All statistical graphs were produced with Origin Pro 2018 (GraphPad Software Inc., San Diego, CA, USA).

3. Results and Discussion

3.1. Effect of Protein Composition on the Structural Characteristics of PBMs

The basic compositions of the four kinds of PBMs are shown in Table 1. Among them, the protein (49.18%) and oil contents (4.17%) of SF 5 were slightly higher than those of the other varieties. The SDS-PAGE spectra of four kinds of PBMs are shown in Figure 1a, and the quantitative results of different protein subunits are shown in Table 2. The content and ratio of 11S/7S differed significantly among the four kinds of PBMs, of which SF 5 showed the highest ratio of 11S/7S (2.50), and HH 43 showed the lowest (1.05). This result is consistent with the protein composition of the corresponding soybeans in the CARS database, which met the requirements of the experiment.

Table 1. The basic components of four kinds of plant-based meat analogues (PBMs) (%).

	Heihe 43	Jiyu 86	Suinong 52	Shengfeng 5
Protein	46.76 ± 0.07 ^{c}	46.68 ± 0.07 ^{c}	48.10 ± 0.03 ^{b}	49.18 ± 0.04 ^{a}
Oil	4.02 ± 0.01 ^{b}	3.94 ± 0.04 ^{c}	3.97 ± 0.01 ^{c}	4.17 ± 0.01 ^{a}
Ash	5.28 ± 0.01 ^{b}	4.92 ± 0.07 ^{d}	5.19 ± 0.05 ^{c}	5.46 ± 0.01 ^{a}
Moisture	7.26 ± 0.01 ^{b}	6.91 ± 0.06 ^{c}	7.77 ± 0.01 ^{a}	6.24 ± 0.01 ^{d}

Different lowercase letters indicate a significant difference ($p < 0.05$).

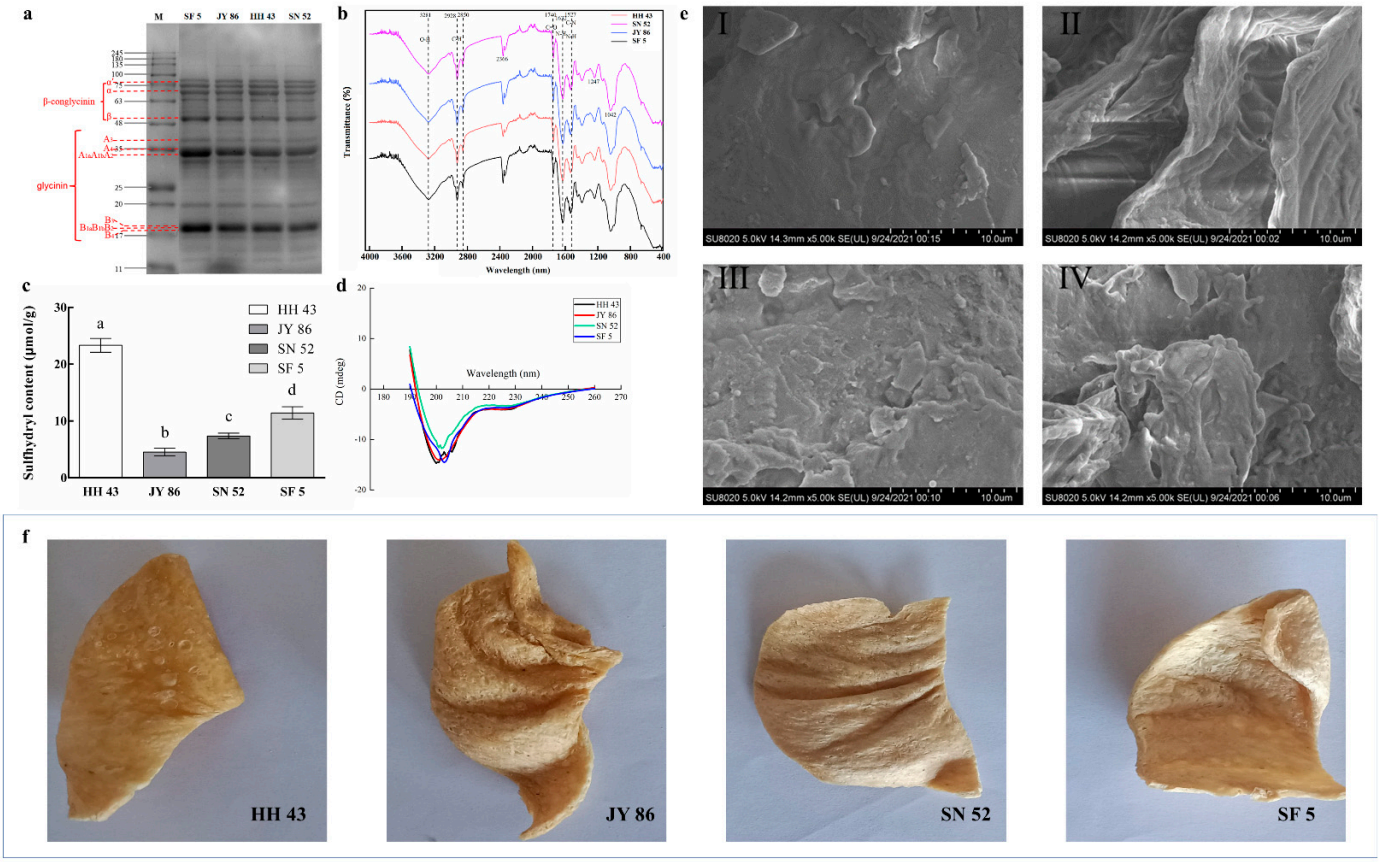

Figure 1. Structural characteristics of four kinds of plant-based meat analogues (PBMs): (**a**) SDS-PAGE of four kinds of PBMs; (**b**) FTIR spectra of four kinds of PBMs; (**c**) Free sulfhydryl content of four kinds of PBMs; (**d**) CD spectra of four kinds of PBMs; (**e**) The microstructure of four kinds of PBMs [I: HH 43; II: JY86; III: SN 52; IV: SF 5]; (**f**) The outward appearances of four kinds of PBMs. Different lowercase letters indicate a significant difference [$p < 0.05$]). HH 43: Heihe 43, JY 86: Jiyu 86, SN 52: Suinong 52, SF 5: Shengfeng 5.

The FTIR spectra of four kinds of PBMs are shown in Figure 1b. As shown, the significant absorption peaks were located at 3281 cm^{-1}, 2928 cm^{-1}, 2850 cm^{-1}, 2366 cm^{-1}, 1740 cm^{-1}, 1632 cm^{-1}, 1527 cm^{-1}, 1247 $cm^{-1,}$ and 1042 cm^{-1}; the spectra of the four kinds of PBMs showed minor differences. The spectra are determined by the combination of protein and dietary fiber in PBMs. The secondary structure of the protein was determined based on the amide I band analysis (1700–1600 cm^{-1}) [29], but there are also some functional groups similar to soybean dietary fiber in other components, such as some aldehyde and carboxyl groups [30]. Thus, the functional group composition of the four PBMs displayed little difference.

Table 2. Subunit composition of the proteins in plant-based meat analogues (PBMs) (%).

Soybeans	α′	α	β	7S	A_3	$A_{1a}A_{1b}A_2A_4$	$B_{1a}B_{1b}B_2B_3B_4$	11S	11S/7S
Heihe 43	13.53 ± 1.25 ^{b}	14.26 ± 0.63 ^{b}	7.98 ± 0.05 ^{c}	35.77 ± 1.66 ^{b}	3.03 ± 0.33 ^{d}	17.39 ± 1.24 ^{c}	17.37 ± 0.82 ^{b}	37.79 ± 0.43 ^{d}	1.05 ± 0.05 ^{d}
Jiyu 86	11.88 ± 0.85 ^{a}	12.02 ± 0.85 ^{a}	9.83 ± 0.47 ^{a}	33.73 ± 1.32 ^{a}	4.69 ± 0.27 ^{c}	27.48 ± 0.67 ^{b}	20.70 ± 1.73 ^{b}	52.87 ± 2.24 ^{b}	1.56 ± 0.20 ^{c}
Suinong 52	9.69 ± 0.85 ^{b}	8.90 ± 0.57 ^{c}	8.08 ± 0.35 ^{c}	26.67 ± 0.46 ^{c}	4.66 ± 0.18 ^{b}	24.65 ± 0.55 ^{c}	22.19 ± 0.09 ^{b}	51.50 ± 0.61 ^{c}	1.94 ± 0.05 ^{b}
Shengfeng 5	9.62 ± 0.85 ^{c}	8.80 ± 0.85 ^{b}	9.88 ± 0.14 ^{b}	28.30 ± 0.14 ^{b}	5.44 ± 0.30 ^{a}	37.85 ± 1.53 ^{a}	27.30 ± 0.34 ^{a}	70.59 ± 2.07 ^{a}	2.50 ± 0.11 ^{a}

Different lowercase letters indicate a significant difference ($p < 0.05$).

The effect of the protein composition on free sulfhydryl (SH) content in PBMs is shown in Figure 1c. The SH content seems to have no obvious correlation with protein composition: HH 43 showed the highest, and JY 86 was the lowest. The SH content of 11S is higher than that of 7S [31]. However, in a mixed system, more 11S converts SH to disulfide bonds [32]. Therefore, this result might be caused by the mixed nature of the system and the protein denaturation process of PBMs. Studies have shown that the SH content in textured protein is related not only to raw materials but also to extrusion temperature, protein denaturation, and other factors [33,34]. Studies have shown that, with the extrusion process, the degree of protein cross-linking increases, the proportion of high-molecular-weight protein subunits increases, and the small-molecular-weight subunits decreases, resulting in a lower SH content [35]. Therefore, the high SH content does not mean that the texture characteristics of the PBMs were better; this needs to be discussed comprehensively in combination with future research.

The CD spectra of four kinds of PBMs are shown in Figure 1d. Combined with the analysis of the amide I band (1700–1600 cm^{-1}) in FTIR, the secondary structure compositions of the proteins in the four kinds of PBMs are shown in Table 3. There were significant differences in the protein secondary structures of the four kinds of PBMs. Compared to conventional soybeans or soy protein isolate (SPI), the ratios of the various secondary structures were also different [29,36]. The secondary structures of soybean proteins of different varieties should be very different, but the differences between them are significantly smaller after being processed into PBM. This shows that the screw extrusion process rearranges the secondary structures of the proteins, which should be regarded as the key factor for changing the secondary structures of proteins, rather than choosing different soybean varieties. In proteins, the existence of α-helix and β-sheet is mainly maintained by hydrogen bonds, while β-turn depends on the amino acid residues with charge [37]. This result shows that the extrusion process strengthens the rigidity of the protein structures, reduces the exposure to amino acid residues, and makes the protein structures more stable. A similar result has been found in other studies [38,39].

Table 3. Changes in the secondary structure of four kinds of plant-based meat analogues (PBMs) (%).

	α-Helix	β-Sheet	β-Turn	Random Coil
Heihe 43	24.49 ± 0.19^{a}	45.72 ± 0.31^{a}	17.50 ± 0.32^{b}	12.31 ± 0.15^{a}
Jiyu 86	25.30 ± 0.19^{c}	44.98 ± 0.34^{c}	17.29 ± 0.31^{c}	12.54 ± 0.15^{b}
Suinong 52	24.79 ± 0.16^{b}	45.20 ± 0.32^{b}	17.32 ± 0.31^{c}	12.66 ± 0.14^{c}
Shengfeng 5	24.44 ± 0.18^{a}	45.12 ± 0.34^{a}	17.36 ± 0.31^{a}	12.26 ± 0.16^{c}

Different lowercase letters indicate a significant difference ($p < 0.05$).

The outward appearances of the four kinds of PBMs are shown in Figure 1f. As shown, the appearances of the four kinds of PBMs were not very different; among them, the surface of HH 43 was slightly dense. It should be noted that the wrinkles on the surface of PBMs result from the shear caused by the grinding head changing between different stages, rather than any difference in the apparent structure of the PBM itself. Figure 1e shows the differences in the microstructures of the four kinds of PBMs. JY 86 has an obvious lamellar structure and flat surface (II), the surface of HH 43 is uneven with torn lamellae (I), SN 52 has a smooth surface, less clearance, and an irregular shape (III), and SF 5 has the most obvious structure and is dense with an irregular shape (IV).

In summary, protein composition did affect the structural properties of the PBMs, but the correlation was not significant.

3.2. Effect of Protein Composition on the Processing Characteristics of PBMs

The water absorption capacity (WAC, WHC, and WSC) of the four kinds of PBMs is shown in Figure 2a. As shown, JY 86 showed the highest WAC (275%); a lower or higher ratio of 11S/7S results in a lower WAC. Meanwhile, WHC and WSC had a positive correlation with the 11S/7S ratio, wherein a higher content of 11S led to a higher WHC and WSC. Excellent

water adsorption capacity could make PBMs more like meat [40]. Therefore, the higher WHC (274%) and WSC (135%) might give SF 5 superior processing characteristics.

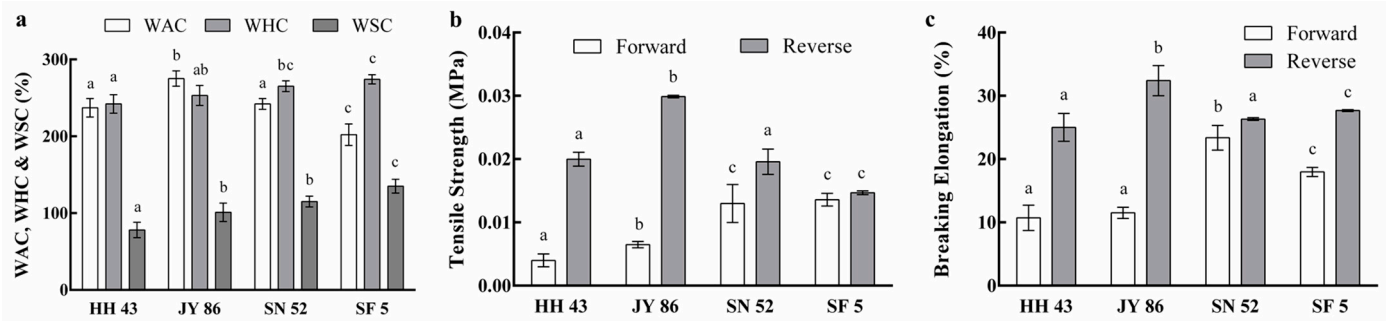

Figure 2. Processing characteristics of four kinds of plant-based meat analogues (PBMs): (**a**) WAC, WHC, and WSC of four kinds of PBMs; (**b**) TS of four kinds of PBMs; (**c**) BE of four kinds of PBMs. Different lowercase letters indicate a significant difference [$p < 0.05$]). HH 43: Heihe 43, JY 86: Jiyu 86, SN 52: Suinong 52, SF 5: Shengfeng 5, WAC: Water-Absorption Capacity, WHC: Water-Holding Capacity, WSC: Water-Swelling Capacity, TS: Tensile Strength, BE: Breaking Elongation.

Tensile strength (TS) refers to the maximum tensile capacity that food can bear and represents the toughness of food. Breaking elongation (BE) is the maximum length change of food before fracture and represents the elasticity. The results are shown in Figure 2b,c. The trends for TS and BE were similar: SN 52 (11S/7S = 2.0) showed the highest forward strength (TS = 0.013 MPa, BE = 23.38%) and JY 86 (11S/7S = 1.5) showed the highest reverse strength (TS = 0.030 MPa, BE = 32.41%). The forward strength represents the tightness of the textured structure of the PBM, while the reverse strength represents the strength of the force between proteins. Therefore, JY 86 might have better toughness and elasticity. In addition, this result seems to be opposite to that of free SH content in Figure 1c, in that the PBM with the lowest SH content showed the best elasticity and toughness; this proves that a high SH content does not mean that the texture characteristics of a PBM are better.

The texture characteristics of four kinds of PBMs were determined by a texture analyzer, and the results are shown in Table 4. The results show that the five indexes were directly proportional to the 11S content; that is, SF 5 showed the best texture characteristics. Among them, the resilience and the springiness increased gradually as 11S content increased, but there was no significant difference ($p > 0.05$), whereas the hardness, adhesiveness, and chewiness were significantly different among different varieties ($p < 0.05$). This showed that 11S globulin plays an important role in the extrusion process.

Table 4. Texture characteristics of four kinds of plant-based meat analogues (PBMs).

	Heihe 43	Jiyu 86	Suinong 52	Shengfeng 5
Resilience	0.32 ± 0.03^{a}	0.35 ± 0.02^{a}	0.35 ± 0.03^{a}	0.39 ± 0.04^{a}
Springiness	0.80 ± 0.02^{a}	0.83 ± 0.03^{a}	0.87 ± 0.04^{a}	0.92 ± 0.09^{a}
Hardness (g)	606.29 ± 21.74^{b}	658.03 ± 19.11^{a}	693.82 ± 18.88^{c}	748.10 ± 20.38^{d}
Adhesiveness (g·sec)	0.15 ± 0.02^{ab}	0.22 ± 0.06^{a}	0.25 ± 0.01^{b}	0.29 ± 0.01^{c}
Chewiness	510.34 ± 12.66^{b}	554.46 ± 12.11^{a}	578.33 ± 13.53^{c}	634.01 ± 10.91^{d}

Different lowercase letters indicate a significant difference ($p < 0.05$).

The 11S content is closely related to the textural properties of soy products. For protein gel especially, there is a correlation between 11S and textural properties [41]. Increasing the 11S content could improve the texture quality of protein products to a certain extent, which is attributable to the formation of disulfide bonds [42]. A study by Zheng et al. showed that a higher β sheet content and a high ratio of 11S/7S increases the quality of soy protein gel, and disulfide bonds might be one of the reasons [43]. In addition, in the process of soy texturization, the processing conditions also strengthen the texture characteristics of

the product, such as temperature and pressure, among others. Research has shown that, in conventional soy product processing, adjusting the pressure and temperature of raw material processing directly affects the processing characteristics of the products [44]; this is caused by changes in the solubility, conformation, and protein aggregation of 11S. in the same is true of the extrusion process. During heating, individual subunits within globulins undergo dissociation, unfolding, and reaggregation to render them more functional by virtue of qualities such as higher gelation [45]. In essence, textured protein is another form of gelation, which also requires the rearrangement of different protein subunits. The higher 11S content also leads to the higher strength of the protein aggregates [46], which better withstand high pressure [47]. Therefore, the excellent texture characteristics of SF 5 may be due to the high 11S content; meanwhile, the high temperature and high pressure provided by the extrusion process enhance the rearrangement of the protein subunits and the formation of the spatial structure.

3.3. Effect of Protein Composition on the Nutritional Properties of PBMs

We chose to use the content of dietary fiber, reducing sugar, phytic acid, phytohemagglutinin, trypsin inhibitor, isoflavone, and amino acid composition to analyze the nutritional characteristics of PBMs comprehensively. The results are shown in Figures 3 and 4 and Table 5.

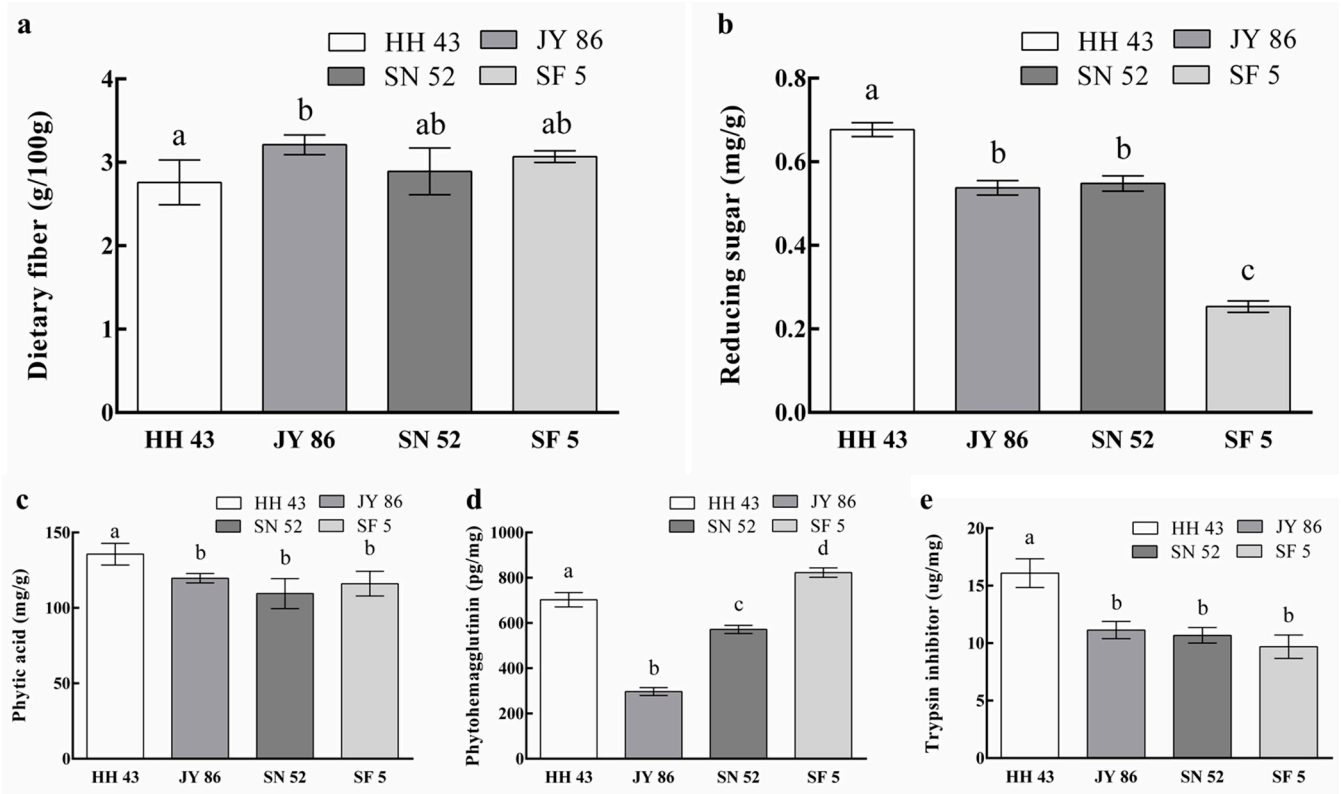

Figure 3. Nutritional properties of four kinds of plant-based meat analogues (PBMs): (**a**) The dietary fiber content; (**b**) The reducing sugar content; (**c**) The phytic acid content; (**d**) The phytohemagglutinin content; (**e**) The trypsin inhibitor content. Different lowercase letters indicate a significant difference [$p < 0.05$].

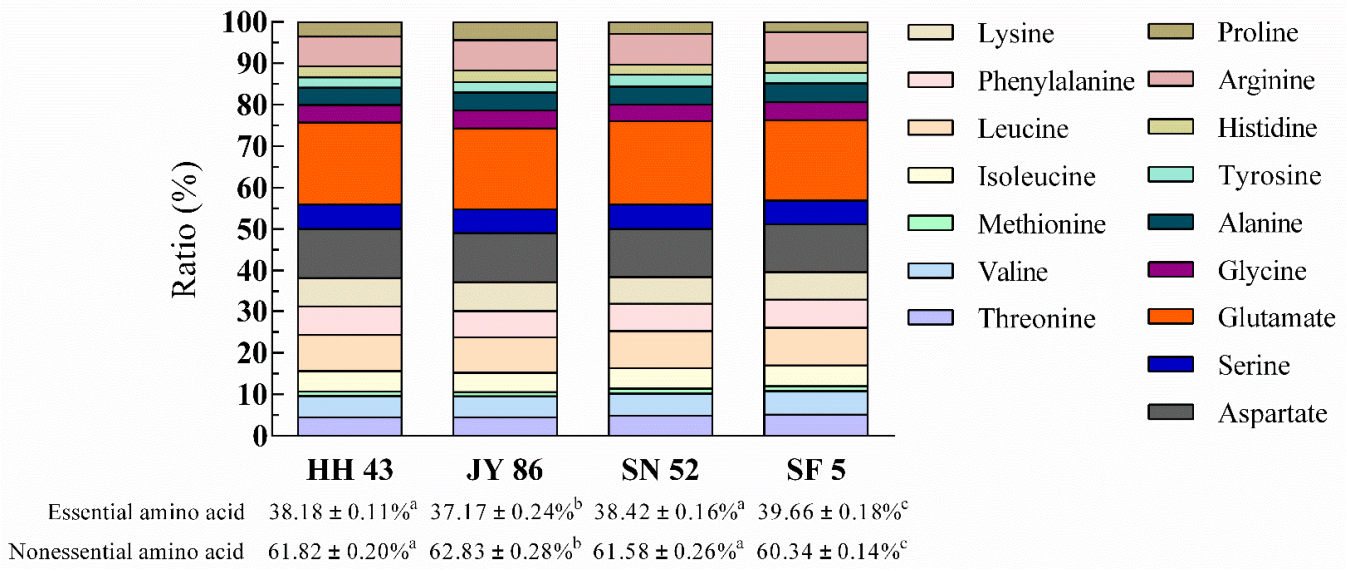

Figure 4. Composition of amino acids (%). Different lowercase letters indicate a significant difference ($p < 0.05$).

Table 5. Composition and content of soybean isoflavones (ng/g).

	Heihe 43	Jiyu 86	Suinong 52	Shengfeng 5
Daidzin	0.413 ± 0.03 ^{b}	0.456 ± 0.02 ^{a}	0.418 ± 0.02 ^{b}	0.389 ± 0.03 ^{c}
Glycitin	0.113 ± 0.03 ^{b}	0.106 ± 0.02 ^{b}	0.118 ± 0.03 ^{b}	0.236 ± 0.05 ^{a}
Genistin	0.911 ± 0.07 ^{a}	0.821 ± 0.08 ^{a}	0.877 ± 0.08 ^{a}	0.668 ± 0.05 ^{b}
Daidzein	0.019 ± 0.01 ^{a}	0.017 ± 0.01 ^{a}	0.024 ± 0.01 ^{a}	0.018 ± 0.01 ^{a}
Glycitein	0.432 ± 0.06 ^{a}	0.302 ± 0.04 ^{b}	0.254 ± 0.05 ^{c}	0.312 ± 0.03 ^{b}
Genistein	0.117 ± 0.01 ^{a}	0.095 ± 0.01 ^{b}	0.136 ± 0.01 ^{a}	0.098 ± 0.00 ^{b}
Total	2.005 ± 0.13 ^{a}	1.797 ± 0.16 ^{b}	1.827 ± 0.15 ^{c}	1.721 ± 0.10 ^{d}

Different lowercase letters indicate a significant difference ($p < 0.05$).

The dietary fiber and reducing sugar content of four kinds of PBMs are shown in Figure 3a,b. In PBMs, neither have a strong relationship with protein composition, in theory. Dietary fiber in soybean products is derived from cellulose, hemicellulose, and lignin in soybean [30], while reducing sugar is derived from the destruction of polysaccharides, including dietary fiber, during extrusion [48].

The findings for three antinutritional factors in PBMs are shown in Figure 3c–e. As these are substances that have negative effects on processing and nutritional characteristics, a lower content of these antinutritional factors in PBMs may improve the quality. The results show that there was no significant relationship between the antinutritional factor levels and the protein composition, with the exception of trypsin inhibitors. HH 43 showed the highest levels of phytic acid (135.62 mg/g) and trypsin inhibitors (16.09 μg/mg), and SF 5 showed the highest phytohemagglutinin content (822.80 pg/mg). Overall, the levels of antinutritional factors for JY 86 and SN 52 were slightly lower.

Trypsin inhibitors mainly exist in 2S globulins [49]. However, excessive intake leads to the decline of protein digestion, absorption, and utilization in the intestine [50]. Phytohemagglutinin mainly exists in 7S [51] and may cause a decrease in digestive ability [52]. Compared with the two other antinutritional factors, the content of phytic acid depends more on the existence of enzymes necessary for its biological process. The key enzymes in phytic acid biosynthesis, myo inositol-3-phosphate synthase (MIPS) and phosphatidylinositol kinase (IPK), are mainly located in 7S [53]. However, the molecular weight of many enzymes is still uncertain and complex [54], and the effect of the 11S/7S ratio on phytic acid metabolism cannot currently be determined. However, the content of antinutritional factors

in soybean products changes significantly with the processing process [55]. Therefore, the extrusion process may greatly change the content of antinutritional factors in PBMs.

The composition of isoflavones and the levels found in the four kinds of PBMs are shown in Table 5. Similar to other nutrients, there was no significant correlation between isoflavone content and protein composition. HH 43 showed the highest total isoflavone content (2.005 ng/g), and SF 5 showed the lowest (1.721 ng/g). During extrusion, the bioactivity and stability of isoflavones in PBMs are affected by the processing conditions [56,57]. Isoflavones may degrade, especially at high temperatures or high pressure [58]. One study showed that isoflavones transform into daidzein groups under high temperatures [59]. However, in this study, the daidzein, glycitein, and genistein levels were significantly lower than that of daidzin, glycitin, and genistin, which means that the isoflavones were significantly transformed during extrusion.

The amino acid compositions and contents for four kinds of PBMs are shown in the Supplementary Materials (Table S1) and in Figure 4. Generally, the amino acid compositions of the four kinds of PBMs displayed little difference, but SF 5 was dominant with a higher content of essential amino acids (39.66%). Among all amino acids, the glutamate content was the highest, and it was higher in SN 52 than in the other three (20.08%). As an amino acid that can enhance flavor [60], the high glutamate content may change the flavor of PBMs; analysis of flavor characteristics is required. In addition, we found that the levels of many amino acids increased with a higher amount of 11S, such as threonine, alanine, and arginine; however, some decreased, such as aspartate. This may be due to the differences in amino acid composition between 11S and 7S [61].

Briefly, soybean varieties with a low 11S/7S ratio should not be selected if nutritional characteristics are used as the standard to judge the quality of PBMs.

3.4. Effect of Protein Composition on the Flavor of PBMs

The results pertaining to the flavor compounds for the four kinds of PBMs are shown in the Supplementary Materials (Tables S2–S5). Additionally, we plotted the results in Figure 5 according to the classification of substances. As shown, the relative amounts of flavor components in different kinds of PBMs were different, but the main components in all cases were aldehydes, alcohols, alkanes, etc. SN 52 had the highest aldehyde content, and HH 43 had the lowest; the highest alcohol compound content was found in SF 5, and the lowest was SN 52; SN 52 had the highest alkane content, and HH 43 had the lowest. Some of the flavor substances, including acetic acid, hexanal, benzaldehyde, 1-octene-3-alcohol, 2,4-decadienal, trans-2-heptenenal, etc. are defined as typically negative flavor components of soybean products [62], and acetic acid and hexanal are considered to be the main contributors to the beany flavor [63]. In this study, SN 52 showed the lowest beany flavor (10.52%), which means that it should be more accepted by consumers.

There is a relationship between flavor and the protein composition of soybean products, but it is very complex, and even the preparation process is closely related to the flavor [64]. Similarly, the flavor characteristics of PBMs are closely related not only to the composition and structure of the proteins but also to the processing and the characteristics of the products [39]. Compared to protein composition, we believe that the structure of PBMs has a greater impact on flavor. In this study, the structural characteristics of SN 52 demonstrated average features (all indexes were in the middle reaches), which may mean that the soybean varieties suitable for preparing PBMs are not those varieties with a notable difference in their 11S/7S ratios (too high or low). In addition, whether the bad flavor of PBMs can be reduced by adjusting the processing parameters is an important research direction.

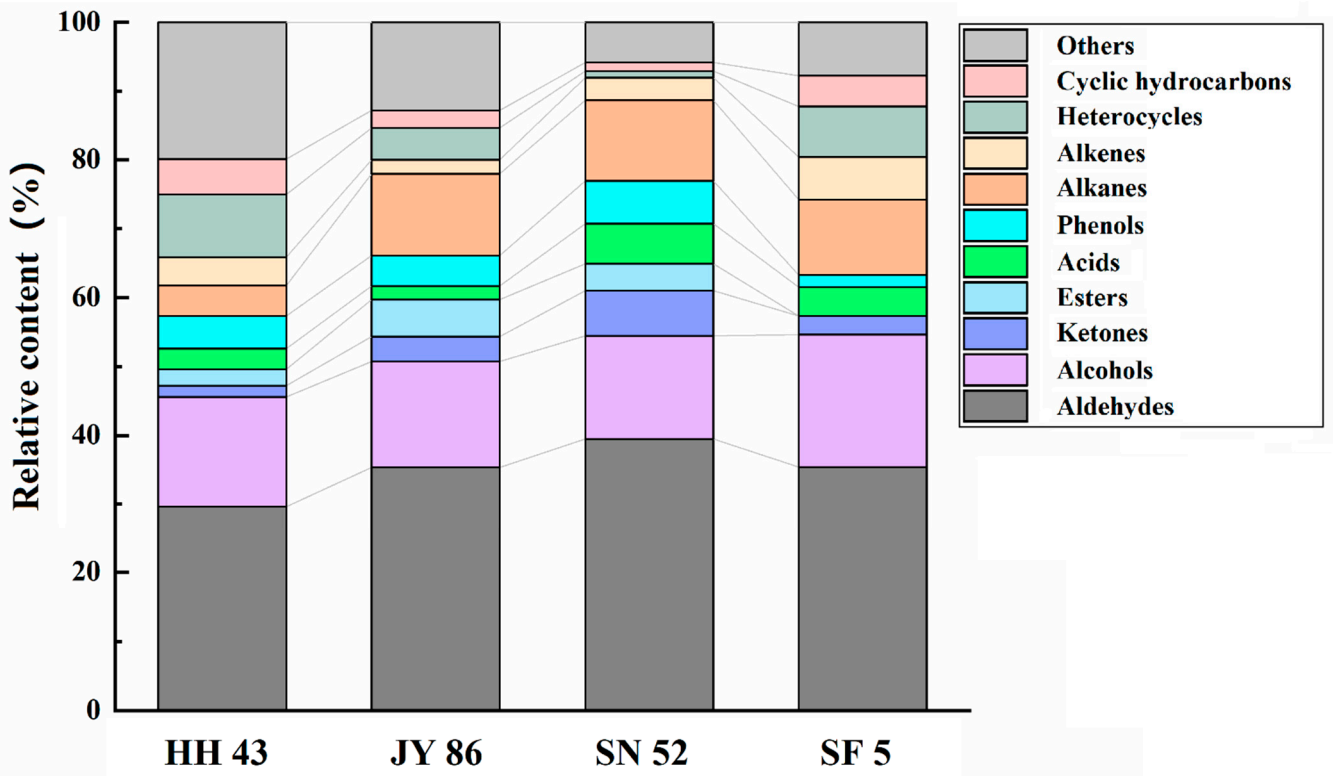

Figure 5. Flavor components of four kinds of plant-based meat analogues (PBMs).

4. Conclusions

In this study, defatted soybean powder was prepared from four of the main soybean varieties in China, and four kinds of PBMs with different protein compositions were prepared. According to our analysis of the structure, processing characteristics, nutritional characteristics, and flavor characteristics, it may be advisable to prepare PBMs using a soybean with a moderate 11S/7S ratio (1.5:1 to 2.0:1) in order to achieve better quality characteristics. In addition, although the protein composition affects the quality characteristics of PBMs significantly, alterations in the processing process may cause even greater changes, which may play a key role in actual production. This study provides a basic theory for selecting the raw materials for PBMs, and in-depth research would be conducive to further development, including the manufacturing of special equipment, special-use soybean varieties for PBMs, bioactive substances—PBM complex reconstruction, and products with different processing applications. However, these potential applications require a more in-depth study of the production mechanisms of PBMs, including not only the selection of raw materials but also the transformation of bioactive substances, the conformational transition of proteins at different stages, the effects of different additives on the characteristics of PBMs, and even methods of sensory gaining. The above problems need further research to promote the improved development of PBM products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods11081112/s1, Table S1: The amino acid composition of four kinds of plant-based meat analogues (PBMs) (%); Table S2: Volatile flavor compounds of the PBM produced by Heihe 43; Table S3: Volatile flavor compounds of the PBM produced by Jiyu 86; Table S4: Volatile flavor compounds of the PBM produced by Suinong 52; Table S5: Volatile flavor compounds of the PBM produced by Shengfeng 5.

Author Contributions: B.L.: conceptualization, software, writing—original draft; J.L.: visualization, software, writing—original draft; X.M.: methodology; H.F.: investigation; W.W.: methodology; L.J.: visualization; Y.W.: investigation; Z.G.: funding acquisition, project administration; H.Y.: funding

acquisition, project administration. All authors have read and agreed to the published version of the manuscript.

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Review

Applications of Plant Protein in the Dairy Industry

Ge-Ge Hu [1], Jing Liu [1], Yi-Hui Wang [1], Zhen-Nai Yang [1,*] and Hong-Bo Shao [2,3,*]

[1] Beijing Advanced Innovation Center for Food Nutrition and Human Health, Beijing Engineering and Technology Research Center of Food Additives, Beijing Technology and Business University, No. 11 Fu-Cheng Road, Hai-Dian District, Beijing 100048, China; 2050201003@st.btbu.edu.cn (G.-G.H.); 2030201010@st.btbu.edu.cn (J.L.); 2030201018@st.btbu.edu.cn (Y.-H.W.)

[2] Jiangsu Key Laboratory for Bioresources of Saline Soils, Jiangsu Synthetic Innovation Center for Coastal Bio-Agriculture, Yancheng Teachers University, Yancheng 224002, China

[3] Salt-Soil Agricultural Center, Institute of Agricultural Resources and Environment, Jiangsu Academy of Agriculture Sciences (JAAS), Nanjing 210014, China

* Correspondence: yangzhennai@th.btbu.edu.cn (Z.-N.Y.); shaohongbochu@126.com (H.-B.S.); Tel.: +86-10-68984870 (Z.-N.Y.)

Abstract: In recent years, a variety of double protein dairy products have appeared on the market. It is a dairy product made by replacing parts of animal protein with plant protein and then using certain production methods. For some countries with limited milk resources, insufficient protein intake and low income, double protein dairy products have a bright future. More and more studies have found that double protein dairy products have combined effects which can alleviate the relatively poor functional properties of plant protein, including solubility, foaming, emulsifying and gelling. In addition, the taste of plant protein has been improved. This review focuses on the current state of research on double protein dairy products. It covers some salient features in the science and technology of plant proteins and suggests strategies for improving their use in various food applications. At the same time, it is expected that the fermentation methods used for those traditional dairy products as well as other processing technologies could be applied to produce novelty foods based on plant proteins.

Keywords: plant protein; double protein dairy; process flow; production; health effects; taste; flavor

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1. Introduction

Protein is the material basis of all life and plays a very important role in maintaining the normal growth, metabolism and immune regulation of the body. According to the source of intake, protein can be divided into animal protein and plant protein. Animal protein is rich in a lot of essential amino acids, but excessive intake of animal protein will greatly increase fat intake. Saturated fatty acids as the main component will lead to increased plasma cholesterol levels, which is not conducive to human health [1]. Due to the improvement of people's living standards and the gradual increase in protein intake, the supply of animal protein is not enough to meet people's growing demand [2]. Plant protein has the advantages of abundant resources, being cheap and easy to obtain, having no cholesterol, and it can prevent diseases [3]. It is a green and safe food raw material, which can make up for the insufficient supply of animal protein.

In 2006, the "Shanghai Declaration" was released at the "Second China Soybean Food Industry Roundtable Summit" held in Shanghai, which was the first time that China proposed the concept of "double protein". The concept of "double protein" and the "double protein" strategy were put forward for the first time, emphasizing the combination of soy protein and milk protein to meet the health needs of comprehensive protein supplementation. It is pointed out that efforts should be made to develop new nutritional and healthy foods such as nutritionally fortified foods and double protein foods. With people's further

awareness of nutrition and health, high-nutrition and high-protein products are more and more attractive to consumers [4].

Dairy products are liquid or solid products made from milk as the main raw material through heating, drying and fermentation processes. Dairy products are rich in protein, fat and carbohydrates, which are easy to digest and absorb. In particular, it contains vitamins and calcium, which are an excellent source of nutrients needed by the human body. As a nutritious and comprehensive ideal food, dairy products occupy a very important position in the dietary structure [5]. Due to the existence of healthy long-chain unsaturated fatty acids, the development of dairy products based on plant protein endows dairy products with new nutrition and health care connotations. It not only realizes the functionalization and popularization of dairy products, but also promotes the diversified development of the dairy processing industry.

2. Double Protein Dairy

2.1. Double Protein Yogurt

Yogurt is a fast acid-producing product, which is made from raw cow (goat) milk or milk powder after high-speed homogenization, sterilization and fermentation. Due to its unique flavor and texture, it is currently the most widely distributed and consumer favorite fermented dairy product in the world. Double protein yogurt uses plant protein and animal protein as the main nutritional bases. Through probiotic fermentation, it has a unique flavor and high nutritional value, which helps to improve nutrition and improve human health.

During the fermentation process of yogurt, the performance of the starter greatly affects the quality of yogurt. Therefore, the development of probiotic strains with excellent fermentation performance is the key to the preparation of a highly active starter [6]. At present, dairy production enterprises mainly use *Streptococcus thermophilus* and *Lactobacillus bulgaricus* as starters for producing yogurt. In recent years, research on plant protein yogurt starter has been increasing. In the fermentation of suitable strains of plant-based yogurt, one or more functional strain combinations can be included.

Sertovic et al. [7] mixed *Streptococcus thermophilus*, *Lactobacillus bulgaricus* and *Lactobacillus acidophilus* to ferment milk and soymilk, and they found that the acidity of fermented milk was higher than that of fermented soymilk. This showed that the optimal starter for soy protein yogurt was slightly different from that of ordinary milk yogurt. Havas et al. [8] fermented pure soymilk using human-derived bacteria *Bifidobacterium* B3.2 and *Bifidobacterium* B7.1. The results showed that the acid-producing ability of the two kinds of bifidobacteria in soymilk was similar to that of cow's milk, and there was no unpleasant odor. Li et al. [9] found that both *Lactobacillus plantarum* fs-4 and *Lactobacillus casei* 05-20 had protease activity. They could utilize nutrients such as sugar and protein in peanut milk and were suitable for fermenting peanut milk. The obtained peanut yogurt was white in color and had a peanut and milk flavor. Wan et al. [10] used rice-milk double-protein yogurt fermented by *Lactobacillus furfur* or *Lactococcus lactis* subsp. *lactis*. When the degree of hydrolysis was 7.5%, the sensory score was the highest, which showed that the color was bright, the curd state was stable, the taste was fine and there was no bad flavor. When the degree of hydrolysis exceeded 7.5%, the bitterness of the product became heavier. The main reason was that the rice protein was hydrolyzed to a certain extent and produces bitter peptides. Wang [11] found that mixed strain fermentation had a better effect on the overall quality of coconut yogurt than single strain fermentation. When the addition amount of coconut milk was 30% and the addition amount of whey protein and sodium caseinate was 2%, the starter was mixed and fermented with *Streptococcus thermophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei* and *Lactobacillus plantarum* in the same proportion. Coconut yogurt was the best quality.

Taking peanut yogurt as an example, peanuts are rich in protein and essential amino acids, and the nutritional composition of peanut protein and animal protein is similar. If the peanut milk is simply fermented with lactic acid bacteria, the fat content in the peanut

kernel is too high, which will lead to oil circles. The high fiber content in peanuts will also affect the formation of peanut yogurt gel. Therefore, milk powder can be added to the peanut yoghurt, so that the peanut yoghurt gel can be formed stably. Peanut yogurt is made of peanuts and milk or milk powder as the main raw materials. After lactic acid bacteria fermentation, it has the characteristics of complementary animal and plant protein and reasonable nutritional structure [12]. The process flow of peanut milk and peanut yogurt is shown in (Figure 1). The researchers studied the ratio of peanut protein and milk or milk powder and obtained peanut yogurt with unique flavor and good taste.

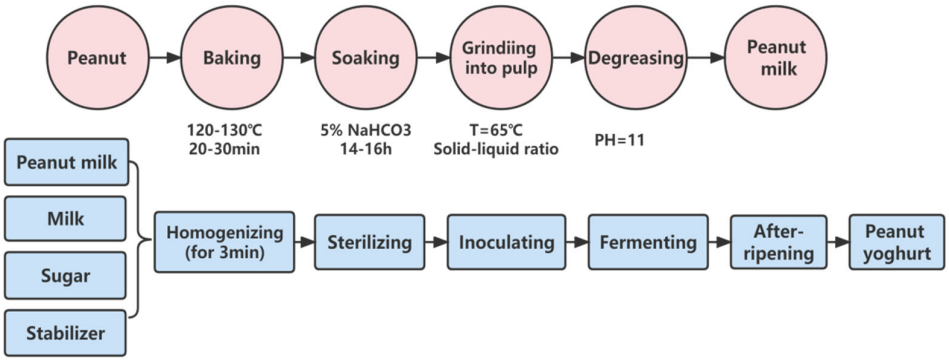

Figure 1. Process flow chart showing some common methods and steps used for production of peanut protein and peanut yogurt.

Qin et al. [13] reported the best production process parameters for peanut protein yogurt: the ratio of peanut protein powder to water was 1:9.4, pure milk 15%, whey protein powder 2%, white sugar 10%, starter 3% and fermentation time 7.4 h. The obtained sensory score was 38.02% higher than that before the previous optimization. Compared with the conventional nutritional content of commercially available yogurt, peanut protein yogurt was found to have at least 81.07% higher protein content, at least 25.08% lower fat content, and no significant difference in acidity. According to the acidity, taste and product status, Yang et al. [14] found that the optimal inoculum of lactic acid bacteria-fermented peanuts was about 3%, and the mass ratio of peanut milk, white granulated sugar and milk powder was 90:5:3.5. The obtained peanut yogurt had both the rich aroma of peanut and the flavor of yogurt, and the curd was dense and shiny. Tong [15] found that the ratio of peanut protein to milk protein was 1:2, and then added 0.02% konjac flour and 0.1% soybean polysaccharide for compounding. The peanut yogurt was glossy, evenly curd, moderately sweet and sour, and had a peanut flavor. Ma et al. [16] found that the optimal formula for sprouted peanut yogurt was 8% sugar and 2% sprouted peanut freeze-dried powder. Sprouted peanut yogurt had higher protein content than regular yogurt and slightly lower moisture content. Fang et al. [17] selected *Lactobacillus Delbruckii* as the strain for fermentation, and used purple potato, peanut milk and milk powder as the main raw materials. The peanut and purple potato were organically fermented, and the nutrients of the two were retained. The prepared purple potato peanut yogurt had a mellow taste, full color and greatly improved antioxidant activity. Cao et al. [18] used shelled ginkgo nuts and peanuts as the main raw materials to determine the optimal formula of ginkgo peanut yogurt. The mass ratio of ginkgo to peanut was 1:6, the volume ratio of peanut milk to milk was 6:4, the added sugar was 7% and the inoculum of *Bacillus bulgaricus* and *Streptococcus thermophilus* was 3%. It was possible to obtain nutritious yogurt with a unique taste and delicate organization.

At present, in addition to the scientific research and manufacturing of double-protein yogurt with peanuts as raw materials, double-protein yogurt with beans, nuts, cereals and other plants as raw materials are also emerging one after another. Bruckner-Guhmann et al. [19] reported that the gel strength and elasticity of fermented milk added with oat protein were lower than those of pure fermented milk. However, the sensory evaluation results showed that the fermented milk containing oat protein had better taste, more

delicate texture, and had a significant oat flavor. Su et al. [20] mixed pecan milk and milk to develop pecan milk yogurt. Pecan milk yogurt had higher antioxidant properties than regular yogurt. Total solids, fat, crude protein, and amino acids were also higher. Appearance and flavor scores were not significantly different from regular yogurt, but texture scores were higher. Li et al. [21] developed coagulated pea yogurt with pea protein powder and milk as the main raw materials. When the added amount of pea protein powder was 6%, the obtained yogurt had moderate acidity and good quality characteristics. The sensory evaluation was the highest, with a mixed aroma of pea protein powder and yogurt. Chang et al. [22] fermented quinoa milk with 5% puffed quinoa flour and milk as raw materials. At this time, the consistency and acidity of quinoa yogurt had reached the optimal level. Compared with ordinary yogurt, 30 new flavor substances were detected in quinoa yogurt, including six plant-derived terpene compounds and alpha-terpineol. Therefore, the addition of puffed quinoa powder made yogurt have certain advantages in terms of nutrition and flavor. Yang et al. [23] found that the fat content of quinoa yogurt was much lower than that of ordinary yogurt, but the protein content was higher than that of ordinary yogurt. At the same time, it was rich in 8 essential amino acids, among which the content of essential amino acids such as isoleucine and leucine were significantly higher than other yogurt. Gao et al. [24] found that adding about 0.2% soybean and corn combination peptide and 87.5% trehalose to milk powder, the obtained soybean and corn combination peptide fermented milk had the best quality. The smell was refreshing, the taste was delicate, the ingredients were uniform and it had the nutritional and health care functions of soybean polypeptide and rice polypeptide.

Therefore, combining the advantages of plant protein and yogurt can not only enrich the product variety of yogurt products, but also more in line with contemporary people's pursuit of health and nutrition.

2.2. Double Protein Beverages

With the development of science and technology, people's pursuit of health is getting higher and higher. The development of beverages has entered a new stage, from the original scale growth to the quality upgrade. As a result, the market share of carbonated beverages has continued to decline, and the consumption trend of healthy and natural beverages such as plant protein beverages, fruit and vegetable juices and tea beverages has risen.

Today, plant-based cereal and nut beverages are the newcomers to the dairy industry, but there are some technical challenges in making new dairy products from cereals and nuts. Compared with natural milk, some grains and nuts are rich in starch and fiber. The suspension stability in milk beverages is poor, so the phenomenon of particle suspension and stratification precipitation is easy to occur. In addition, the taste of beverage products is light and bitter. In order to solve these problems, researchers have conducted related research in recent years, including adding stabilizers and thickeners to stabilize product quality; adding flavor substances to improve taste; enriching and strengthening nutrients to improve nutritional value, etc.

Yang et al. [25] studied the compounding scheme of emulsion stabilizer in oat milk beverage. Orthogonal test results showed that the compound stabilizer ingredients included 0.3% microcrystalline cellulose, 0.012% carrageenan, 0.10% mono- and diglyceride fatty acid esters and 0.06% sodium stearoyl lactylate. The stabilizer could effectively suspend product particles and had a good effect of controlling product fat floating. Li et al. [26] determined that the optimal ratio of peanut pulp and milk was 1:2, the stabilizers were sucrose fatty acid ester (SE) 0.05%, glycerol monostearate (GMS) 0.1%, carboxymethyl cellulose sodium (CMC-Na) 0.025%. The produced beverage had good stability. Han et al. [27] found that the addition of walnut juice was 15%, the addition of peanut juice was 25%, the addition of milk was 30%, and the addition of sucrose was 6%. The developed compound milk beverage had the best taste flavor. Under the optimal process conditions, when sodium alginate, gum arabic and CMC were selected as stabilizers, the precipitation

rate of the composite beverage decreased and its stability was the best. Huang et al. [28] added 0.04% pectin, 0.02% gellan gum and 0.6% CMC in the production of fermented walnut milk beverages, which not only solved the problem of layered precipitation, but also gave the beverage rich taste.

Enzymatic hydrolysis is the use of amylase or protease to enzymatically hydrolyze macromolecular substances such as starch and protein in grains or nuts under certain conditions. This will refine the granules in the drink, decompose some insoluble starch and protein into soluble sugar, dextrin, polypeptide and amino acid, thereby improving the stability of the drink [29]. Hou et al. [30] used oat as the main raw material and added 0.15% α-amylase for enzymatic hydrolysis. Then, added 1.50% whole milk powder, 3.0% white sugar, 0.15% citric acid, 0.10% pectin and 0.05% xanthan gum to develop the best production process of a new type of cereal beverage. Li et al. [31] used walnut pulp and pea milk as the main raw materials, the addition of milk was 10%, and the addition of white sugar was 3%. The amount of α-amylase added was 0.4%, and the enzymatic hydrolysis was carried out at 70 °C for 3 h. The walnut and pea milk produced under this condition was stable and had the aroma of walnut kernels and peas.

In addition, homogenization can make fat globules smaller. The miniaturization and homogenization of suspended particles can prevent the separation of finished fat and the precipitation of protein particles, thereby improving the emulsification and stability of liquid grain dairy products. Two important parameters of homogenization are homogenization pressure and homogenization temperature. Ma et al. [32] determined the optimal homogenization conditions in the stability study of black glutinous rice milk beverage, that is, homogenized twice under the conditions of 60 °C and 20–30 MPa. When the homogenization temperature was too high, the protein in the system might denature and cause flocculation. When the homogenization pressure exceeded 40 MPa, the number of collisions of suspended particles in the system increased, resulting in polymerization, which eventually led to an increase in the precipitation rate of the system.

From the perspective of raw materials, the taste of beverages can be improved by adding natural raw materials. Zheng et al. [33] used barley and buckwheat as raw materials. Passion fruit juice (10%), xylitol (10%) and citric acid (0.05%) were added to enhance the taste, and the resulting compound grain beverage was rich in aroma and sweet in taste. Wang et al. [34] used walnut juice and milk as the main raw materials. With the addition of 10% macadamia juice, the drink tasted best and had a special aroma of walnuts and macadamia nuts. Zhang et al. [35] used peanut, wolfberry and milk as raw materials, white granulated sugar and xanthan gum as ingredients to develop peanut and wolfberry milk. The study found that the best roasting temperature for peanuts was 120 °C, and the best roasting time was 20 min, the peanuts had the strongest aroma.

From the perspective of preparation, suitable flavor substances can be derived by means of fermentation. Tavares et al. [36] pointed out that organic acids such as lactic acid and acetic acid are released during fermentation and refrigeration of corn beverages fermented with probiotics and yeast. Maintaining the pH of beverages at around 4.0 had an important impact on food safety, taste and aroma. Tue et al. [37] dried and ground germinated brown rice into powder, and fermented after adding honey, corn germ oil and yeast. After adding milk, white sugar and citric acid, it was homogenized to make a brown rice enzyme milk drink with unique flavor.

The double protein beverage has rich raw material resources, meets the individual needs of consumers, conforms to the development trend of market consumption and has a certain health care value, so it has broad development prospects.

2.3. Double Protein Cheese

Cheese is made from cow or goat milk. Adding an appropriate amount of starter and rennet can make the protein coagulate, discharge part of the whey, and finally ferment and mature after a certain period of time. During the stage of cheese fermentation, proteins and fats are enzymatically decomposed into tiny substances that are easily absorbed in

the human digestive system, which improves the absorption and utilization rate of cheese. Therefore, it has the reputation of milk gold in the industry.

In recent years, a mixed cheese has appeared on the market, which is a cheese made by replacing part of the protein in animal milk with protein extracted from plants. Using plant protein to replace part of animal protein can not only reduce the cost of cheese production, but also improve the nutritional value of cheese. In the cheese research and development field, the experiment of replacing part of animal protein with plant protein has become a new research and development direction [38].

Among them, mixed soybean cheese is the most studied double protein cheese. Soybean contains 35~40% protein, which is a high-quality source of plant protein. It has high nutritional value, contains various amino acids and unsaturated fatty acids necessary for the human body, and is also rich in minerals and vitamins. Soy protein plays an important role in the diet structure of many countries. The development of mixed soybean protein cheese can not only reduce the production cost of cheese, alleviate the shortage of milk source, but also promote the deep processing of soybean and increase the added value of soybean products.

Under the same ripening conditions, compared with ricotta cheese, mixed soybean cheese has larger pores and looser texture. Large particles of soy protein can reduce curd stability and affect the compactness of the casein structure [39]. Therefore, the current research on the quality of mixed cheese mainly focuses on the addition amount of soymilk, the processing method and the improvement of production technology.

Yang [40] reported that when the content of soybean protein isolate was controlled at 4%, the muted taste of mixed cheese was greatly reduced, and the milky aroma was stronger. When the content of soy protein was more than 4%, the taste of milk cheese became rougher, the aroma of milk decreased, and the aroma of soy increased (Figure 2). Zhao et al. [41] found that the addition of soymilk resulted in a higher yield of Mozzarella mixed cheese and significantly reduced the fat content and firmness of the cheese samples. However, the addition of soymilk also made the cheese waterier, especially when the addition exceeded 10%. Its stretchability was significantly reduced, which was detrimental to its application on pizza. Bai et al. [42] found that with the increase in black soybean milk addition in the range of 2–6%, the water activity of cheese increased, the pH decreased, the hardness, elasticity, adhesiveness and chewiness increased. Based on the analysis of each index, the cheese made with black soybean milk 4% had a special flavor and proper indexes including color, texture, protein degradation and so on.

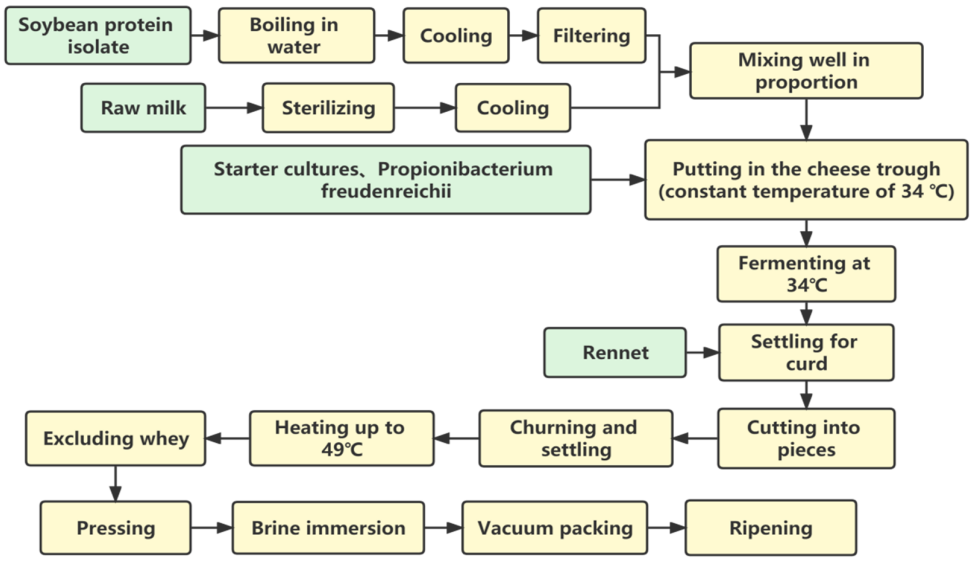

Figure 2. Process flow chart showing the production of mixed soybean cheese using soybean protein isolate and milk.

On the premise of not reducing the cheese yield, the addition of enzymatically hydrolyzed soymilk has a better effect on improving the texture of the mixed cheese. Li et al. [43] found that after adding soymilk and enzymatically hydrolyzed soymilk to cheddar cheese, the moisture content increased, and the fat content decreased significantly. As cheese matures, hardness and cohesion increased, while elasticity decreased. However, the cheese made by adding soymilk had poor shape and brittleness. Adding enzymolyzed soymilk could improve this phenomenon, and the protein structure formed by adding enzymolyzed soymilk was more compact. Han et al. [44] used 0.3% papain to hydrolyze soybean milk for 15 min before processing, which could significantly reduce the particle size of soybeans, reduce product hardness and smear work. Adding complex emulsified salt (sodium citrate: sodium tripolyphosphate: sodium hexametaphosphate) to the spread-type mixed soybean cheese could significantly improve the fineness and stability of the product.

Some researchers had pointed out that an important reason why consumers do not accept blended cheeses containing soymilk was its soy flavor [45]. At present, there are three main methods to remove the beany smell. The first is to discover and cultivate new soybean varieties through the improvement of raw materials; the second is to reduce the beany smell during processing by inactivating or inactivating the activity of lipoxygenase in soybeans; the third is to improve storage conditions [46]. Ali et al. [47] used protease and peptidase to produce flavored enzyme-modified cheese. The results showed that after enzymatic hydrolysis, the contents of amino acids, free fatty acids and volatiles in cheese were significantly increased, and the sensory properties were significantly improved. Han et al. [44] found that compared with ordinary refining, the soymilk obtained by anaerobic refining had lower overall volatile flavor substances, especially beany flavor substances. The types and contents of beany flavor components in the spread-type soybean cheese prepared by anaerobic refining were significantly reduced, and the sensory evaluation was higher.

At present, in addition to soy cheese, other plant-based mixed cheeses are also emerging. Shi et al. [48] developed a hazelnut processed cheese with an optimal dosage of 30% hazelnut, and the emulsifier included 1.2% sodium citrate and 1.2% compound phosphate. The prepared processed cheese had a sweet taste, fine texture and mellow hazelnut aroma. Wu [49] reported that almond pulp and milk were mixed at 45:55%, 5% starter was added for fermentation, 0.8% rennet and 0.06% CaCl2 were added for processing. The almond cheese with milky white color, smooth and uniform, moderate sour and sweet, and rich flavor could be obtained. Tian et al. [50] developed a fermented spread walnut cheese with walnut kernels as the main raw material. The additions of lipase and flavor protease were 0.2%, and the additions of whey protein, cream, and sucrose were 1.56%, 0.81%, and 6.37%, respectively. Walnut cheese was full of flavor, high nutritional value, and had better sensory qualities. Zhang [51] used red dates and skim milk as raw materials, added starter and rennet, and made red date cheese through curdling. The experiment found that adding 4% of jujube puree, the curd time was relatively short and the curd strength was the greatest. At this time, the cheese was rich in flavor, pure in frankincense, and of the best quality. Chen [52] invented a preparation method of whole grain cheese with mild flavor and tender taste. Purple potato, sweet potato, wheat, red bean and oat were milled to make multigrain juice, mixed with skim milk, maltose, fructo oligosaccharide and honey, and then inoculated with lactic acid bacteria to ferment the curd.

At present, the research on double protein cheese is very extensive and in-depth, and the scientific research results have been applied to actual production. The development of various forms of cheese is of great benefit to the cultivation of the cheese market. The growing double protein cheese has great development potential and wide application prospects in the dairy industry.

2.4. Calf Double-Protein Milk Replacer

In order to wean the calves early, the calves should be fed with milk replacer (also known as artificial milk) instead of regular milk about 10 days after birth. At present, the use of milk replacer to cultivate and implement the early weaning technology of sucking calves has become a common technical means in the world dairy farming industry. Milk replacer raw materials are mainly composed of dairy by-products such as skim milk, whey protein concentrate, dry whey, etc. [53]. With the deepening of research and the development of milk replacer processing technology, low-cost and high-quality plant protein has become the main research direction for the development of milk replacer protein sources. High-quality plant protein and high proportion of milk protein have obvious effects on preventing and reducing calf diarrhea, and also on increasing daily weight gain of calves. Good economic benefits have been achieved by saving feeding costs [54].

The most widely used plant protein in calf milk replacer is soybean protein, wheat protein, rice protein, etc. Plant protein sources are rich and high in crude protein. The crude protein content of feed-grade soybean protein isolate, wheat hydrolyzed protein and rice protein provided in China can reach about 90%, 85% and 65%, respectively. Different sources of plant protein have different nutritional characteristics because of their different protein components and amino acid compositions and have different effects on the growth function of sucking calves [55]. Although plant protein has a slightly poorer amino acid balance, certain functional amino acids are abundant in plant protein. For example, wheat protein is rich in glutelin, accounting for 30% of the total amino acid [56]. In addition to synthesizing proteins to meet the needs of animal growth and maintenance, these functional amino acids are also necessary for the synthesis of various biologically active substances.

In the past studies, plant protein mainly had adverse factors such as poor solubility, low digestibility, poor amino acid balance and containing anti-nutritional factors [57]. However, with the development of science and technology, it is now possible to remove anti-nutritional factors through modification and processing, add different plant proteins to achieve amino acid balance, and add some enzymes or probiotics to improve the digestibility of plant proteins in animals (Table 1). Therefore, after using soybean protein isolate, wheat hydrolyzed protein and rice protein isolate as the protein source of milk replacer to partially replace milk-derived protein to feed calves, it can achieve a feeding effect similar to that of milk-derived protein [58].

Table 1. Main functional properties of plant protein components.

Classification	Main Source	Advantages	Disadvantage	Technical Transformation	References
Globulin	Soybean	– Balanced composition of amino acid. – Good solubility.	– High content of antigen protein, which affects the intestinal health of young animals.	– Modification to inactivate antigenic proteins.	[59–61]
Gliadin	Corn, Wheat, Sorghum	– High content of glutamine, which can repair the intestinal mucosa of young animals.	– The increase in feed viscosity with the increase in gliadin content. – Not easily digested by animal endogenous digestive enzymes.	– The control of addition amount. – Directed enzyme digestion or microbial fermentation.	[62–64]
Glutelin	Rice	– Balanced composition of amino acid. – Easily digested by animal endogenous digestive enzymes.	– Low solubility in aqueous solution.	– Modification	[65–67]
Albumin	Corn, Wheat	– Balanced composition of amino acid.	– Existence of trypsin inhibitors and allergens.	– Modification – Gene modification	[68–70]

The broad concept of "double protein" does not mean that there can only be one type of plant-derived protein added. In terms of human nutrition, the purpose of proposing the "double protein" project is to balance the diet, optimize the dietary structure, and improve the nutritional status. Therefore, adding two or more plant-derived proteins into milk replacer is particularly important for balancing amino acids and optimizing the dietary structure of livestock [71]. More and more studies have found that milk replacer composed of a variety of plant proteins have a combined effect, and the feeding effect is better than the combination of a single plant-derived protein and milk-derived protein.

Huang [72] reported that the feeding combination of 30% milk protein + 23.4% soybean protein concentrate + 23.3% rice protein isolate + 23.3% peanut protein concentrate was more in line with the nutritional needs of calves. For the same 30% milk protein retention, Liu et al. [73] found that the combined milk replacer with 40% soybean protein isolate, 10% wheat hydrolyzed protein and 20% rice protein isolate could give calves better growth performance. Raeth et al. [74] found that when 50% of milk-derived protein was replaced by soy protein isolate and wheat hydrolyzed protein in the same proportion, it would cause a decrease in daily weight gain and feed efficiency of calves. Studies had shown that high proportions of gliadin and glutelin in calf milk replacer can lead to reduced growth performance. However, as long as no less than 40% of retained colostrum was added or no less than 60% of whey protein was added, the normal growth and development of calves could be ensured.

Sucking calves are in the stage of rapid growth and development, and the level of energy intake directly affects the growth rate and nutrient metabolism of the body. It is very important to ensure an appropriate energy supply for sucking calves. Different protein sources of milk replacer have different energy utilization rates in animals. Compared with milk-derived protein, plant protein can reduce the metabolic rate of energy, nitrogen, calcium and phosphorus in calves. However, the effect of plant protein on the metabolic rate of energy, nitrogen, calcium and phosphorus in calves has a downward trend with the increase in age, and the adaptability of calves to plant protein is also continuously improved [72,75]. This is because plant protein contains a certain amount of fiber and rich nutrition, which has a significant promoting effect on the development of digestive organs such as the rumen and intestinal tract of the calf, and it lays a good foundation for the high production performance in the later period [76].

There were also differences in the effects of protein sources in milk replacers on the immune function of calves. Huang et al. [72] found that from the serum IgG, IgA, IgM and 1L-2 levels of calves, the stress caused by milk-derived protein, soybean protein and rice protein to calves was significantly lower than that of wheat protein and peanut protein. At the same time, compound plant protein could also increase the body's deposition of nitrogen by increasing the levels of GH and IGF-1 in serum and improve the ability of tissue growth and development [73].

With the in-depth research on the development of plant protein, the development of milk replacer with plant protein as the protein source has a very good prospect. However, research on double-protein milk replacer is not sufficient, and other nutrients other than protein and amino acids have not been systematically studied. There is also a lack of relevant reports on the specific effects of feeding double-protein milk replacer on the microecology of the calf's digestive tract and digestion and absorption function, as well as on the subsequent production performance. Therefore, more research is needed to reveal the nutritional potential of double-protein milk replacers, so as to provide more of a theoretical basis for precision feeding in the breeding stage of calves.

2.5. Other Double Protein Dairy Products

Cereal milk powder is more nutritious and functional than milk powder, and supplements the dietary fiber needed by the human body without changing the taste of milk powder. Cereal milk powder can be consumed in breakfast or other meals as a staple functional beverage. When drinking milk on an empty stomach for breakfast, the protein in

the milk will be converted into sugar to release energy, resulting in a waste of protein. The grain-added milk can be eaten directly as a staple food, so that the protein can be absorbed well, and the convenience and staple food of dairy products can be realized. Wang [77] invented a method for preparing oat milk powder. After the oat flakes were extruded by a twin-screw extruder, milk powder, xylitol and fructo oligosaccharide were added in a certain proportion to make oat milk powder. Zhang [78] invented a preparation method of corn milk powder. The corn flour was puffed and then pulverized, and 30% of the puffed corn flour, 68% of the milk powder, 1.8% of the sugar and 0.2% of the edible essence were mixed in a mixer to make the corn milk powder.

According to the growth and development characteristics of infants and young children, timely and reasonable addition of complementary foods plays an important role in promoting the healthy development of infants and young children. Rice protein is a recognized hypoallergenic protein, and various clinical studies have also shown that rice protein can be used as a hypoallergenic protein resource, especially suitable for infant food ingredients. Liu et al. [79] obtained a series of formulas through orthogonal experiments based on the nutritional characteristics of infants and young children in different periods. Taking 100 g as the standard, the nutritional rice flour for infants and young children in 0–6 months contained 46 g of rice flour, 41 g of first-stage milk powder, 7.2 g of vegetable and fruit powder, 3.6 g of FOS, and 2 g of various trace elements. Nutritional rice flour for infants aged 6–12 months contained 46 g of rice flour, 32 g of second-stage milk powder, 12.6 g of multigrain flour, 6.3 g of FOS, and 3 g of various trace elements.

Ice cream is a popular dairy product for summer, and the production of new, safe and healthy ice cream has become an industry trend. Wu et al. [80] used flaxseed meal as raw material, extracted flax protein with enzyme preparation, added flax protein content of 3%, skim milk powder content of 13%, cream content of 15% and sucrose content of 16%. The finished product was light brown, with pure fragrance and fine texture. It could be seen that adding flax protein can improve the quality of ice cream. Zheng et al. [81] used USPI-PLW to replace part of the milk powder. Compared with ordinary low-fat ice cream, the expansion rate of ice cream was increased by 94.84%, the melting rate of ice cream was reduced by 26.86%. The product was in a stable condition, with a good appearance and smooth taste, which made its flavor more popular.

3. Perspectives for the Future

Based on the current research and development status of double-protein dairy products, several suggestions are put forward on the research and development ideas of this category of products in China in the future, which should be followed as below:

1. Increase the construction of high-quality beans and grain raw material bases to produce high-quality plant protein raw materials. On the premise of not destroying the taste, try to ensure the integrity and availability of raw materials, avoid waste of resources, and improve the nutritional quality of products.
2. Make full use of compound biological enzymatic hydrolysis preparation technology and probiotic fermentation technology. Accelerate the screening and development of probiotic strains, starters and probiotic preparations suitable for various plant-based fermentations, create natural and green manufacturing technologies, and improve the flavor and nutritional quality of products.
3. Strengthen research on product stability, and continuously improve related key production technologies and equipment, such as starch modification technology, pulsed electric field, ohmic heating, high- and ultra-high-pressure homogenization. Research and develop new production technology and equipment and adopt more advanced cold sterilization and aseptic packaging methods to improve product stability and extend shelf life.
4. Appropriately increase plant-based raw materials such as vegetables and Chinese herbal medicines with the same origin of medicine and food to enrich product types. In particular, new products with special flavor and nutritional and health characteristics

can be purposefully developed for different consumer groups. For infants and young children, fruit and vegetable raw materials rich in vitamins and minerals can be developed and strengthened; for middle-aged and elderly people, some medicinal and food homologous ingredients rich in antioxidants and anti-aging substances can be added.

4. Conclusions

Concerns about environmental impact and sustainability, animal welfare and personal health issues have fueled consumer demand for plant protein. However, the transition towards greener diets is being hampered by the poor acceptance of vegan foodstuffs among consumers. Mixed animal/plant products to familiar dairy products offer a new field of innovation. Therefore, plant-based proteins were used in a variety of dairy products. This comprehensive review presents the research and application of plant protein in the dairy industry, a distinctive and interesting topic for researchers in food technology, nutrition and dietetics. The continuous development of new blended products and the expansion of the application of plant protein in dairy products can promote a greater role of plant protein in human society. The current focus is on possible ways to improve nutritional properties through processing, such as the use of enzymes, the selection of raw materials based on their protein quality, advanced processing and technological interventions. There is also a need to ensure the palatability and acceptability of double-protein dairy for the population.

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Review

Novel Protein Sources for Applications in Meat-Alternative Products—Insight and Challenges

Marcin A. Kurek *, Anna Onopiuk, Ewelina Pogorzelska-Nowicka, Arkadiusz Szpicer, Magdalena Zalewska and Andrzej Półtorak

Department of Technique and Food Development, Institute of Human Nutrition Sciences, Warsaw University of Life Sciences, Nowoursynowska 159c Street 32, 02-776 Warsaw, Poland; anna_onopiuk@sggw.edu.pl (A.O.); ewelina_pogorzelska_nowicka@sggw.edu.pl (E.P.-N.); arkadiusz_szpicer@sggw.edu.pl (A.S.); magdalena_zalewska@sggw.edu.pl (M.Z.); andrzej_poltorak@sggw.edu.pl (A.P.)
* Correspondence: marcin_kurek@sggw.edu.pl

Abstract: Many people are increasingly interested in a vegetarian or vegan diet. Looking at the research and the available options in the market, there are two generations of products based on typical proteins, such as soy or gluten, and newer generation proteins, such as peas or faba beans, or even proteins based on previously used feed proteins. In the review, we present the characteristics of several proteins that can be consumed as alternatives to first-generation proteins used in vegan foods. In the following part of the work, we describe the research in which novel protein sources were used in terms of the product they are used for. The paper describes protein sources such as cereal proteins, oilseeds proteins coming from the cakes after oil pressing, and novel sources such as algae, insects, and fungus for use in meat analog products. Technological processes that can make non-animal proteins similar to meat are also discussed, as well as the challenges faced by technologists working in the field of vegan products.

Keywords: protein; meat analog; texture; insect protein; algae protein

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1. Introduction

As consumer awareness of the environmental impact of food production increases, so does the consumption of products derived entirely from plants. This has to do with the narrative that meat production requires extensive land and water resources, negatively impacting the terrestrial and aquatic biodiversity and emitting greenhouse gases [1]. More and more people are also avoiding plant-based products, which are very interesting in terms of nutritional value, vitamins, micro and macro elements, and the ability to counteract some of the diseases prevailing among the civilization, due to their cholesterol and blood-pressure lowering properties [2]. This state of affairs influences the substantial growth of the meat analog market, which today is increasing expansively.

Most meat analogs are based on two proteins: soy and gluten. Soy protein is a good alternative to meat in terms of amino acid composition and textural properties [3]. However, the use of soy is quite controversial among consumers due to genetically modified (GM) crops. There is a study showing that 55% of consumers are opposed to GM foods and nearly 60% do not trust GM scientists [4]. Some consumers are strongly against GM soy application in feed for animals [5]. In contrast, the use of gluten ensures that a proper network is built in the product, but it is a fairly high allergenic raw material and may be avoided by some people [6]. This is because more people are diagnosed with celiac disease as well as gluten intolerance. Although important scientific advances have been made in the understanding of the pathologic mechanisms behind nonceliac gluten sensitivity, this disorder is still a matter of active debate in the scientific community [7]. More and more importance is being given to searching for alternative novel protein sources that can be used in meat analog products.

It is also worth noting that the cost of producing plant protein is significantly lower than the cost of producing animal protein. Of course, this translates into technological properties, but there are a number of methods that try to prevent this, such as protein texturization [8]. According to World Bank reports, there is an increase in demand for animal protein that cannot be met, hence the need for more intensive work on new sources of protein [9].

In 2020, the market value of plant-based-meat worldwide was estimated to be worth USD 6.67 billion. This figure is estimated to steadily increase over the next few years and reach roughly 16.7 billion in 2026. This is also influenced by the opinion on the safety of animal-based proteins, which are linked to epidemics such as mad cow disease, swine influenza, or avian flu that appear from time to time [10]. For this reason, this literature review is a systematization of knowledge in the utilization of novel protein sources according to origin—plant, microbial, fungal, insect, and algae.

2. The Function of Protein in Meat-Alternative Products

Proteins play an important role in human nutrition by providing building materials essential for both growth and cell regeneration. First of all, most of the meat alternative food sources from which proteins can be obtained differ in the composition of proteins themselves, as well as their amino acid profiles. Most often, meat alternatives do not use only protein isolates; concentrates or powders derived from plants, such as dietary fiber, vegetable fat, or carbohydrates, may also be included in the formulation. For many years, plant protein was considered to have lesser nutritional value, but this trend is now reversing [11].

Concerning nutritional values, it is worth noting that plant protein is not consumed as an individual ingredient but in a group with other ingredients. Therefore, it is not easy to control the potential effects of different nutrients from different meat alternative foods and attribute the observed benefits solely to the protein content. Furthermore, specific sources of plant and animal protein in the diet have been shown to have different health effects. Thus, general statements about plant or animal protein may be too simplistic, and effects may depend on the dietary matrix and accompanying nutrients.

Regarding nutritional properties, it is worth focusing on the fact that the meat analog has similar or comparable nutritional value to an identical meat-based product. As a rule of thumb, if a product has more than 30% protein with low-fat content, it can be considered a good meat substitute [12]. At the same time, it is also worth noting that substitutes or alternatives to meat products should be characterized by their similarity to meat protein digestibility-corrected amino acid score (PDCAAS) [13]. Supplementation or obtaining iron or vitamin B_{12} from other sources is crucial if meat is excluded from the diet entirely.

Protein has a number of technological functions specific to a particular protein origin and degree of concentration—depending on whether it is a formulation, concentrate, or isolate. These characteristics include solubility, thermal stability, emulsification, flavor binding capacity, and digestibility score [14]. These characteristics are directly linked to the technological and functional roles in creating meat analog products.

From a technological point of view, it is not really possible to create a direct alternative to meat protein solely from the plant-based protein (except for the use of cultured meat target) product. This is due to aspects such as the reconstruction of the fiber network, which would reflect the myofilaments that are crucial for shaping tenderness and juiciness. Therefore, product development research on plant-based alternatives has largely been limited to restructured (or reconstructed) products [15]. This makes the alternative meat products mainly belong to two groups ranked by particle size. These can be divided into coarse-particle products, such as burgers, patties, sausage, meatballs, nuggets, etc., and fine-particle products, which are highly homogenized products that often have emulsion properties.

Many of the proteins are used in meat applications as ingredients due to their properties of functioning as a water binding agent. Several proteins are often combined together

for better results, such as pea protein isolate–wheat gluten blends and soy protein isolate–wheat gluten blends [16]. In terms of whole meat protein substitution, soy protein, which consists of albumin and globulin fractions, is the most common. In addition to soy protein, pea protein is also common. Most proteins derived from legumes possess the gel-forming ability, which is crucial because viscoelastic gel structure plays a major role in adhering particles, immobilizing fat, and entrapping water within the matrix of emulsion-type alternative protein products [17].

A widely used protein in meat alternative applications is gluten, which imparts the appropriate chewiness to products. The elasticity and extensibility properties of gluten are ascribed to two major protein fractions: glutenins and gliadins, which specifically influence the structure of meat products [18].

However, it is worth noting that soy is often associated negatively among consumers due to its strong association with genetic modification. At the same time, gluten is a highly allergenic protein and is not tolerated by people struggling with celiac disease.

3. Protein Sources and Their Roles in Meat-Alternative Products

3.1. Legume Proteins

In recent years, consumers have paid particular attention to plant-based diets. This is due to the increasing public awareness of the health-promoting effects of bioactive compounds from plants on human health and the willingness to reduce meat consumption for environmental reasons [19]. Of particular importance in the diet are legumes, whose effect on inhibiting diseases has been scientifically confirmed. These plants belong to the group of annual plants of the Fabaceae family of legumes [20]. Their edible part is the fruit, or the so-called pods, which are eaten whole or partially depending on the species and the degree of maturity of the fruit. The seeds of leguminous plants are characterized by their high nutritional value. Compared to other plants, they are distinguished by a fairly high protein content, ranging from 20% to 35% on average, depending on the type, growing conditions, and degree of maturity. Legume seeds are a rich source of dietary fiber, vitamins, minerals such as magnesium, iron, zinc, potassium, and phosphorus, and compounds with high antioxidant potential [21]. The seeds of these plants are low in saturated fats and, like all plant foods, are free from cholesterol [22,23]. A legume-rich diet improves bowel function and benefits hormonal balance [24]. Legume seed protein differs from cereal grain protein in amino acid composition—a significantly higher proportion of lysine (especially peas) and threonine, whereas the factor limiting its biological value is the insufficient content of sulphur amino acids (methionine and cystine) and tryptophan. In the protein of legume seeds, two fractions are distinguished: albumin and globulin. Albumins make up 10–25% of the total protein, can be soluble in water, and are mainly found in the germinal part. They are structural and enzymatic proteins, forming complex linkages with carbohydrates, lipids, and nucleic acids. The more albumin a seed contains, the greater its nutritional value. Globulins are soluble in dilute solutions of neutral salts. Different legume species provide varying amounts and qualities of protein to organisms [25]. Edible legume species include peas, lentils, lupins, chickpeas, broad beans, and mung beans.

Peas are an excellent source of protein and are exceptionally high in lysine and threonine, as well as other essential amino acids. They have a low glycemic index. Numerous scientific studies show that peas play a large role in preventing colon cancer and help treat breast cancer, pancreatic cancer, prostate cancer, lung cancer, and leukemia. Lentil seeds are also full of nutritional value because they contain 9/100 g protein and 0.4/100 g fat in edible parts; they are rich in iron, phosphorus, magnesium, and B vitamins. There are many types of lentils, including red, brown, green, yellow, and black lentils, among others [26].

Lupin seeds are another type of legume protein source whose nutritional value of low-alkaloid varieties is comparable to soybeans. Among legumes, lupin seeds contain the most protein (up to 46%) and the least undesirable non-nutrients. Due to the presence of functional components, they have potent health-promoting properties. They show antioxidant and hypocholesterolemic activity, have a low glycemic index, increase the bioavailability of

minerals, and have anti-allergic and anti-inflammatory effects [27]. Lupine seeds, as well as soybeans, can be used in the production of both traditional and functional foods.

Proteins from other legumes are also used in the production of meat analogs. In recent years, many studies have been conducted on the possibility of using chickpea, faba bean, and mung bean proteins in the production of meat analogs [28]. One of these is the study by Bühler et al., in which the researchers subjected faba bean protein concentrate to heating [29]. This led to modifications in the water holding capacity and solubility of the protein, achieving properties similar to soy protein concentrate, which is used in most meat analogs. This study showed that the choice of ingredients for meat analogs should depend both on the protein content and source and its nutritional value, but also on its thermal processing history, which can have a decisive influence on its technological properties. Among the previously noted three species of legumes, chickpea is the most consumed by consumers [30]. Chickpea protein is characterized by good properties in terms of texture, ability to bind water and oil, and ability to gel. The ability to stabilize emulsions and foam comparable to soy protein isolate and whey proteins is also an important property of chickpea protein. Moreover, chickpea protein isolate shows the ability to absorb more fat and a similar amount of water compared to soy protein isolate [31]. An essential advantage of chickpea is its positive effect on the color of the meat analog. Studies have shown that partial replacement of textured vegetable protein with chickpea flour significantly increased the color acceptability of meatless nuggets. The reason for this is the carotenoids contained in chickpeas [32].

Faba bean proteins may be a promising ingredient for producing meat analogs. Like chickpea proteins, they are excellent in stabilizing emulsions and foam, but to a lesser extent than soy protein isolate [32]. This is a limiting factor for using faba bean proteins as an ingredient in meat analogs. Many factors influence the technological utility of plant proteins. Thus, it is possible to improve the functionality of legume proteins as a result of appropriately selected parameters of production and processing processes. The study showed that dry fractionation enhanced the properties of protein-rich faba bean flour compared to faba bean protein isolate produced by acid extraction. Dry fractionation produced proteins with higher solubility at pH 7. The gelling and foaming abilities were also improved [28]. Faba bean proteins have been successfully used in the production of meat analogs by wet spinning, shear cell technology, and high moisture extrusion methods [33].

Mung bean proteins are also growing in popularity as an ingredient in meat analogs. The mung bean is a plant valued for both its nutritional value and functional properties. It is characterized by high protein (25–28%) and low fat content (1–2%). A significant amount of proline, glutamic acid, arginine, leucine, and phenylalanine is present in mung bean protein [34]. The limiting amino acid in mung bean protein is leucine. Notably, the digestible indispensable amino acid score (DIAAS) for this protein is 86, compared to 91 for soybean protein and 70 for pea protein. Mung bean protein is composed mostly of globular proteins, resulting in good gelling properties [35]. Like chickpea and faba bean proteins, mung bean proteins show the ability to stabilize foams and emulsions. Mung bean proteins are, therefore, used to obtain a balanced amino acid profile and desirable textural properties of meat analogs because they have globulins (60%, vicilin-type 8S with MW 26–60 kDa), albumins (25%, MW 24 kDa), and other globulins including basic-type 7S and legumin-type11S [17,34].

3.2. Oilseeds Proteins

In recent years, many oilseeds have been used as sources of protein in the food industry. The whole seeds and meals obtained from them are a valuable source of proteins with a well-balanced profile of essential amino acids with sulphur-containing amino acids. Their antioxidant, antihypertensive, and neuroprotective properties make them a valuable and functional alternative source of protein, e.g., in the baking and meat industry. The oil plants used as a source of protein include, inter alia, soybean, chia seeds, evening primrose,

flaxseed (brown), hemp seeds, milk thistle, nigella seeds, pumpkin seeds, rapeseed, sesame, safflower, glandless cottonseed, and sunflower seeds [36].

In addition, these proteins complement desirable functional properties when added to certain foods; this applies to whipping capacity, viscosity, emulsifying capacity, and water and oil binding capacity. Rapeseed and soybean protein isolates have a higher whipping capacity than sunflower, peanut, sesame, cottonseed, and safflower. Furthermore, the addition of sugar improves the whipping properties of the oilseed proteins. In contrast, if the oilseed proteins are heated, the whipping ability is reduced. Of the oilseed proteins, soy protein has the greatest emulsifying power. The emulsifying properties of heat-treated oilseed proteins are similar or better than that of animal proteins. Cotton seed protein has a very high water and oil binding capacity. However, the water-binding capacity of the oilseed proteins gradually decreases with increasing heating time at 100 °C. In contrast, heated oilseed proteins have an oil binding capacity similar to or better than that of unheated proteins [37]. In addition, the low allergenicity of pumpkin and hemp seeds or the potential non-allergy of evening primrose, milk thistle, black cumin, and chia compared to legume proteins makes it possible to use them as functional ingredients in newly developed food products [36].

Among the many benefits of proteins obtained from oil plant seeds, one should also remember the dangers of plants such as rapeseed that contain, in addition to many nutrients beneficial for the human diet, toxic erucic acid and sulfur compounds—glucosinolates, which are a component of the protein fraction [38]. To reduce the amount of anti-nutritive compounds (including glucosinolates, sinapin, and phytic acid) from proteins derived from oilseeds, innovative extraction methods are used.

3.3. Cereal and Pseudocereal Proteins

In grain-based proteins used in meat analogs, wheat, oats, or rice are used. The most common is wheat protein, which is gluten, due to its viscoelastic properties [1]. Other wheat proteins are not as often used as a base for creating meat alternative products, but due to their properties, they are fairly well distributed as structuring agents, even in true meat products.

A study conducted by de Angelis et al., indicated that oat protein isolates produced a rather good sensory effect when combined with pea protein [39]. However, the positive sensory properties were only observed after the extrusion process, which positively affected quenching the pea odor. The oats themselves were regarded by consumers for the pleasant smell but still far from being meaty.

Both legumes and cereals proteins contain pretty significant amounts of phytic acid, which is judged to be anti-nutritional by being strongly element restrictive. Some researchers have additionally introduced enzymatic activity and fermentation to reduce the phytic acid content of meat analog extrudates. The results were quite promising, but too much enzyme activity can end up degrading macromolecules, thus making it difficult to maintain an appropriate texture [40].

A very promising raw material for creating meat analogs is rice, an established low allergenicity raw material and, in particular, is presented as an alternative to soy. Raw rice was reported to be more allergenic than cooked rice, even though some allergens are heat stable and proteolysis resistant [41]. Currently, rice flour is being used as a substitute for fat while taking advantage of its water-binding properties in meat products. The use of 4–6% rice flour effectively increases the firmness of sausage-type meat products while being highly acceptable to consumers [42].

Cereals that are high in protein are pseudo-cereals like amaranthus and quinoa. Amaranth and quinoa grains are equally good as cereals and legume seeds because of their high content of lysine, arginine, tryptophan, and other sulphur-containing amino acids. Amaranth is an example of a plant with a high protein content of up to 14%. Some difficulty in obtaining protein is the isolation of starch in the case of amaranth [43]. Amaranth itself

also has a flavor that consumers may not fully accept. However, amaranth has already been successfully used as a binding agent in sausage formulations.

Another type of pseudo-cereal used in meat products is quinoa, a raw material with approximately 8% protein but a very high nutritional value containing all nine essential amino acids. The use of quinoa in meat products improved its water-holding capacity, reduced its toughness, and positively affected the sensory experience [44,45]. Further work on protein concentration and isolation from quinoa may lead to a good base combination for creating legume-based meat analogs.

When using grain-based proteins, they must be proteins with a fairly good amino acid profile. At the same time, existing concentrates or formulations have a widely accepted flavor and are not treated negatively. The ability to bind water means that in the future, they can be used as additional proteins in the composite to create meat analogs [30].

3.4. Algae Proteins

Algae, or photosynthetic eukaryotes, are distinguished as microalgae and seaweed. Microalgae is a huge group covering almost 200,000 species [46]. Out of this group, several species have been tested for a variety of purposes: food additives, cosmeceuticals, animal feed, or wastewater treatment. Foods obtained or formulated with the addition of algae are included in the definition of novel foods in the Novel Foods Regulation (EU). Microalgae is a promising novel ingredient that might be applied in the formulation of meat analogs. The growth rate of microalgae cultivars is superior to other crops used as sources of plant proteins. The estimated microalgae yield of dry biomass reaches 15–30 tonnes annually per unit area compared to 1.5–3.0 tonnes for soybeans. Microalgae and seaweed also contain more protein per unit area (4–15 tonnes/Ha per year and 2.5–7.5 tonnes/HA per year, respectively) in comparison to soybean (0.6–1.2 tonnes/Ha per year) or wheat (1.1 tonnes/Ha per year) [47]. Depending on strain and cultivation conditions, microalgae can produce up to 70% of proteins in cells compared to 30–40% for soybeans. Even more important from the quantity of protein occurring in algae is its quality. The nutritional quality of protein is determined by the composition of amino acids and the amount of essential amino acids. Two most dominant microalgae species on the market, Spirulina (*Arthrospira*) and Chlorella, are characterized by the higher than standard (100) essential amino acids index (102.6 and 107.5, respectively). Those values are similar to casin milk protein and higher than soybean meal [48]. There are microalgae of good essential amino acids (EAAs) balance. For instance, *Chlorella* contains 7 EAAs, comparable to beef but with a slightly lower level of cysteine and methionine. However, in most algae species, lysine and tryptophan are limiting amino acids [49]. Further, for brown algae except for the two noted above, also lysine, while for red species, leucine and isoleucine occur in low concentrations. In the case of seaweed, cysteine is most limiting, whereas glutamic acid and aspartic acid are most abundant [50].

Proteins acquired from microalgae exhibit techno-functional potentials such as high solubility and capacity to emulsify and form gels and foam. Solubility of *Chlorella protothecoides* proteins at pH 2–12 is estimated to be approximately 84.3%. For comparison, soybean protein (glycin) at pH 4.5–6.0 is soluble at less than 20% [51]. In turn, emulsifying and foaming are comparable to soy and whey proteins. Some species, such as *Chlorella vulgaris*, have even higher emulsifying properties. Algae proteins are also considered to be safe as food components. Those properties drew the scientific community's attention towards using algae proteins as a substitute for animal protein. Palanisamy et al., (2019) observed that adding *Spirulina* (*Arthospira platensis*) flour at a level of 30% to lupin protein-based meat decreased in vitro protein digestibility from 82% to 76.5%. However, it was reversed partially by changing the process parameters [52]. Based on the data, the authors stated that *Spirulina* increased nutritional (higher antioxidant activity and phenolic content) and physico-chemical properties of the meat analog. Other studies revealed that adding spirulina at higher concentrations gives the product dark color, musty odor, and intensive earthy flavor [53]. Nonetheless, also in this study, setting the proper process conditions—

low moisture content with high temperature and screw speed—enables partly replacing soy with spirulina in meat substitute and obtaining a product of decent flavor quality. Even though methods to produce microalgae rich in proteins on a large scale were invented about 50 years ago, still today, there exists only a few novel products formulated based on them. There are several reasons for that. First of all, algae dry matter contains 10% of the cellulosic cell wall, which is not digested and utilized by humans and non-ruminant animals. Thus, it is required to use various extraction and purification methods, thus increasing the costs of microalgae biomass application and limiting its use to high-value industries. Furthermore, algal protein concentrates are characterized by green and yellow colors and an unattractive fishy odor. Those attributes negatively influence consumers' perception of meat analogs formulated with algae addition. Sensory experiments showed that the product acceptance decreases with the increase in algae content [54]. Lowering prices was the only way to make eating meat substitutes with algae content more attractive [53]. Some researchers suggest that familiarity with food influences buying behavior and that algae meat analogs are unattractive for consumers because they are still unfamiliar to them. Nonentheless, to date, there is a lack of ideas for how to positively affect consumers' attitudes toward algae meat substitutes.

3.5. Insect Proteins

Insects are common food for 2 billion people in 119 countries across the globe [55]. There are over 2000 edible species. The most consumed insects that are used as protein sources are Coleopatra Beetles (31%), Lepidoptera Caterpillars (18%), Hemynoptera, wasps, bees, and ants (14%). However, those are still novel foods for Western countries. This is slowly changing due to growing need for alternative sources of proteins, production of which would be more sustainable. Studies on the life cycle assessment of Hermetia illucens performed by Smetana et al., (2019) revealed that insect protein concentrates had a lower environmental footprint than animal proteins but higher than plant proteins [56]. In accordance with studies conducted by Mason et al., (2018), the production of one gram of beef requires 21 times more water (16.8 g) than the production of the same amount of protein from cricket (0.7–0.8 g) [57].

Insects are a good source of proteins. The average content of proteins in them is 40% and ranges from 20% up to 70% depending on the species. Three species that are widely bred in Europe (Tenebrio molitor, Gryllodes sigillatus, Schisocerca gregaria) are considered to have the biggest potential as food components in the European Union and contain 52.35, 70, and 76% of proteins, respectively [58]. The amount and quality of proteins within the same species vary greatly depending on diet, metamorphic stage, or habitat. However, protein content is also often overestimated due to the presence in insects of a non-protein nitrogen. It has been estimated that up to 26% of whole larvae nitrogen may be non-protein [59]. Insect proteins are more digestible (76–98%) than plant proteins (lentils 52%) and slightly less digestible than animal proteins (95% egg protein, 98% beef protein) [60]. The essential amino acids' score for insects ranges from 46% to 96%, which greatly exceeds the lowest recommended level for human diets (>40%). The quantity of the same amino acids is even higher in insects than those from plant and animal protein sources [61]. Insect proteins have high threonine and lysine content but low levels of methionine or tryptophan. Proteins acquired from insects are characterized by a low level of solubility ranging from 3% to 45%. However, the solubility may be improved by enzymatic hydrolysis. For instance, the major solubility improvement of migratory locust protein was observed to be 10–22% and up to 55%. Along with solubility, authors also observed higher emulsifying activity of approximately 54%, enhanced foam ability of approximately 326%, and improved oil banding capacity [62]. Thus, the application of insect proteins is recommended for foods that do not require high solubility, such as meat analogs. Furthermore, insect proteins are especially recommended as an addition to plant meat analogs to improve its protein profile. Smetana et al., observed that using the highest temperature of a barrel extruder (170 °C) made it possible to introduce 40% of insect protein to a soy-based meat analog,

keeping its optimal meat-like texture [63]. In turn, Kim et al., (2022) performed studies on the usage of insect proteins along with textured vegetable proteins to produce restructured jerky analogs [64]. In conclusion, the authors of the studies stated that it is possible to produce meat analog combining both of those proteins to get tender jerky of high nutritional value. There is also a study aiming to partially replace meat protein (10%) with insect flour (*Tenebio molitor* or *Bombyx mori*). The results of this experiment indicate that even though they obtained high-value emulsion sausages, those were harder than control meat samples. However, consumer safety is also an issue. There is a risk of an allergic reaction after consuming insect proteins, which contain tropomyosine and arginine kinase—two major proteins responsible for allergic reactions. Furthermore, insect-derived food and feed might be contaminated chemically with heavy metals and biologically with spore-forming bacteria [65].

3.6. Edible Fungus Proteins

Mushrooms have been classified into a separate kingdom because of their different cellular organization, and they do not belong to either animals or plants [66]. Fresh edible fungus has about 90% water, and the remaining 10% dry matter is composed of 8–40% protein, 3–28% carbohydrate, 3–32% fiber, 2–8% fat, and 8–10% ash, varying with the mushroom species and other factors [67]. Yu et al., (2020) examined 23 edible mushrooms and determined their protein content. It was found that the protein content in edible mushrooms was approximately 8.5–36.9%, which was much higher than that of vegetables, fruits, and grains. The higher protein content was found in *Trichloma* (36.87%), and Tremella had the lowest protein content (8.46%). Other more popular mushrooms like *Shiitake*, *Lentinus Edodes*, *Volvariella Volvacea*, and *Boletus* had a protein content of 15.38%, 11.59%, 10.24%, and 12.16%, respectively. Fungus proteins are gaining more and more popularity all over the world. As meat production has a significant impact on the environment, it is important to find a cheap, alternative, and less resource-intensive source of protein to partially replace meat or meat products. Mushrooms cannot be considered as a significant source of proteins compared to meat sources, even though they are a part of human nutrition mainly because of their taste [68]. Other authors, however, believe that mushrooms may play an important role in meat analogs by providing nutrients and promoting the development of sensory properties such as appearance, texture, and taste of the product [69]. The use of mushrooms as an alternative source of protein in the human diet is not a new concept. Edible mushrooms can be treated as a functional food due to their nutritional value. The use of edible mushrooms has been used in meat products as meat substitutes or fillers to improve the physicochemical and sensory characteristics and their nutritional value. The production of mycoprotein products is based on submerged fermentation of fungi in a liquid culture medium [70]. The mycoprotein production is based on the continuous fermentation of the filamentous fungus *Fusarium venenatum* on a glucose substrate, which allows the production of a high-protein, low-fat food ingredient [71]. They are usually grown in bioreactors with a high metabolic rate. Miller and Dwyer (2001) assessed the tolerance of humans to mycoprotein, and the results demonstrated that mycoprotein is well tolerated by humans and has an extremely low allergenic potential [72]. Singh et al., (2021) indicate that the mycoprotein of some fungi is a good source of protein. Still, due to its low digestibility, it is rarely used to prepare meat analogs, although *Fusarium venenatum* is cultivated to derive mycoprotein and prepare meat substitutes [1]. The mycoprotein may have a meat-like texture and flavor. Some researchers argue that proteins produced using mycoproteins have structures similar to muscle fibers of meat and claim that mycoproteins can be considered as an alternative source of the food protein. Due to their functional properties, it is possible to use them in new attractive health-promoting food products. The use of biotechnological methods for their production creates an opportunity to reduce production costs and improve the sensory and nutritional properties [73]. The harvested mycoprotein can be used to prepare vegetarian sausages, burger patties, or minced cutlets. Other mushroom-based meat substitutes are produced from *Aspergillus oryzae*. Filamentous

mushrooms are used in most mushroom-based meat products because their long fibers create a meat-like texture. Denny et al., (2008) stated that the mycoprotein may have a meat-like texture and flavor and is the main component of various mycoprotein products, including minced meat, chicken pieces, burgers, sausages, nuggets, fillets, ready to eat meals, cakes, and pies [71]. In many Asian countries, *Monascus purpureus*—treated with yeast produce red rice—and *Aspergillus oryzae*—fermented with soy—is used in hamanato, miso, and shoyu. Nowadays, in the European market, Quorn™, a meat substitute originated in Great Britain, is sold. Quorn™ contain mycoprotein derived from the *Fusarium venenatum* filamentous fungus [74]. Mushrooms and fermented products have a meaty taste, a long shelf life, good nutritional values, and reduced cooking time, so they can be a new generation of plant proteins in the future. All real products in which the novel sources of proteins were used are summarized in Table 1.

4. Processing of Proteins Applied in Meat-Alternative Products

Textured vegetable protein (TVP) was one of the first ingredients used in the production of meat analogs. The TVP production technology was developed in the 1970s, and it was then that this type of product was introduced to the market for the first time. Initially, TVP was used as a filler in various conventional food products. In the following years, the development and production technology of meat substitutes based on TVP began. The primary raw material for TVP production is soy proteins, although other ingredients such as cotton, wheat, and corn are also used. Nevertheless, the TVP consists mainly of processed dried soy flour to give it a spongy texture and is flavored to improve the meat-like sensory properties. TVP is produced in the extrusion process (Figure 1). High-temperature (120–200 °C) and high-pressure (20 MPa) processing of the raw material make it possible to obtain products of various shapes (such as cubes or stripes), sizes, colors, and textures [17,75].

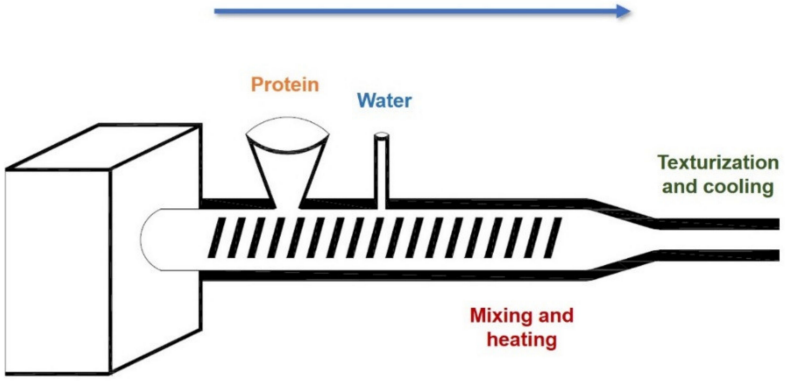

Figure 1. Process of texturization of proteins for application in meat analog production.

In the 1980s, the fiber spinning technique began to be used to produce meat analogs. In this method, the alkaline protein solution was forced through the matrices into the acidic coagulation base. This led to the precipitation of fibers that were mixed with bonding materials. However, the process was very complex, required a highly concentrated protein solution, and had lower yields than large-scale production costs [76].

Currently, the main technology for producing this type of product is thermoplastic extrusion. Extrusion used in recent years is a method characterized by high efficiency and allows for the reduction of the energy cost of production. Skimmed vegetable proteins are made with the addition of water, salts, carbohydrates, lipids, flavors, and other functional additives. The mass is then put on the extruder screw where the product structure is shaped under the influence of high temperature and pressure [77].

Table 1. Summary of real products where the novel sources of proteins were applied.

Type of Proteins	Source of Protein	Type of Product	Characteristic Traits	Reference
Legume	Faba bean	Texturized product after high-moisture extrusion (HME)	The best parameters of HME: 130 and 140 °C, water:product ratio = 4 and feed rate 11 rpm (1.10 Kg/h), good bite-feeling, good elasticity/firmness, positive sensory attributes	[78]
	Mung bean	Texturized product after extrusion cooking	Optimized extrusion parameters: 49.33% feed moisture, 80.66 rpm screw speed and 144.57 °C barrel temperature, partial protein unfoldment, fibrous structure, high retention of amino acids	[34]
Oilseeds	Soy protein Isolate–gluten	Couette cell product	More layered and fibrous structured products, formation of anisotropic structures in the microscale	[79]
	Lima bean and African oil bean seed	Texturized vegetable protein (TVP)	Higher overall acceptance than cooked meat, Concentrations of essential amino-acids range between 0.90 and 7.3% with a near absence of anti-nutritional factors (0.0022–1.0008) g/kg	[75]
Cereal and pseudocereal	Pea protein dry-fractionated, pea protein isolated, soy protein isolated and oat protein	Extrudates from twin-screw extruders	Lower water absorbtion for samples with oat protein; intense odor and taste profile for samples with pea protein dry-fractionated and oat protein	[39]
	Oat protein concentrate and pea protein isolate	Texturized product after extrusion cooking	Extruded product with minimum recommended amounts of essential amino acids for adults but lower content of phytic acid 1.5%	[40]
	Rice flour	Meat-based sausages	Lower cooking loss and better emulsion stability for the samples with rice flour	[42]
	Black quinoa	Bologna-type sausage	Better emulsion stability, lower water activity and lipid oxidation values	[44]
Algae	Spirulina platensis flour	Lupin protein based meat analogs	Improved physico-chemical and nutritional properties	[52]
	Spirulina	Spirulina-soy extrudate for pasta filling	Decreased liking of product with higher content of soy-spirulina filling	[54]
Insects	*Alphitobius diaperinus*	Insect based meat analog	Hardness texture and protein composition similar to meat	[63]
	Mealworms	Restructured jerkey analog	Similar texture and nutrient quality to animal meat	[64]
Edible fungus proteins	Filamentous fungus *Fusarium venenatum*	Quorn™ meat substitute or cooking ingredient	A meat-like texture and flavour, high-fibre, low-fat food ingredient, an average protein content of 45%	[71,74]
	Aspergillus oryzae fermented with the soybean	used in hamanato, miso, and shoyu	5–10% protein content, meaty flavour, long-shelf life	[1]
	Lentinus edodes, *Coprinus comatus* and *Pleurotus ostreatus*	Mushroom-based meat sausage Analog	Texture and flavour close to beef, a satisfactory level of consumer acceptability	[69]

To meet the expectations of customers, the production of meat analogs focuses on obtaining acceptable sensory characteristics such as taste, smell, color, and consistency. Two methods of extracting proteins used for the production of meat analogs are known as 'dry' and 'wet' extrusion. Unfortunately, "dry extrusion" (humidity <30%) does not allow for obtaining a sensorially acceptable product. In contrast, 'wet extrusion' (humidity 40–80%) enables the production of meat analogs of premium quality. The preparations obtained through this method are characterized by a consistency resembling real meat, and their appearance and mouthfeel are similar to cooked meat [52]. Due to the use of high moisture extrusion (HME), it is possible to produce from raw materials with low solubility, and in addition, this method is more economically viable [80]. Meat analogs produced by HME from soy protein are the most common [78], but using this method, it is possible to obtain high-moisture meat analogs (HMMAs) from plants, such as hemp, yellow pea, lentils, and faba bean [64,81,82].

The latest technical solutions are based on Couette thermostatic shear, in this case, the suspension of proteins and gluten gels in a linear flow. Because of this process, it is possible to obtain a product characterized by a fibrous structure. Moreover, shear-induced structuring with a high-temperature shear matrix created fibrous protein structures. The developed closed-chamber rheometer allows you to control thermal and mechanical stresses. Due to this, it is possible to obtain conditions similar to extrusion [79].

5. Challenges for Protein Applications in Meat-Alternative Products

Although the current development trend is towards developing foods for vegetarians, almost every product has similar challenges. These are not only technological but also consumer or even sociological challenges.

From a sensory point of view, achieving a viable alternative to a meat product is quite difficult because the specificity of meat in terms of amino acid structure, peptide sequences, and intermolecular connections is very specific and impossible to counterfeit. Sensory properties and, in particular, mouthfeel are influenced by a texture with very low granularity that is able to bind water. In order to maintain these characteristics, plant proteins must be subjected to several different structuring processes, such as thermomechanical extrusion or shear. Despite the use of a number of methods that alter the structure of plant proteins or increase water-holding capacity, there are still many difficulties that need to be solved. One of them is juiciness, which is a specific characteristic of meat, resulting from water absorption and the form of water-binding with proteins and in their fibers. Hence there is currently no possibility to replace meat with proteins of the same or similar texture.

Although plant proteins are the most common alternative to meat proteins, they have a particular taste that is different from meat. For example, in legume-protein products, an aftertaste derived from a characteristic beany odor is thought to be related to the secondary lipid oxidation products [83].

In addition to texture and palatability issues, meat products are characterized by a red, reddish, or pink color that, for obvious reasons, is impossible to achieve without the use of colorants. Unfortunately, the problem is present because many consumers interested in vegetarian products are consumers who avoid additives, which further increases the technological difficulty [84]. It is the lack of a clean label that makes consumers uncomfortable with meat protein product alternatives. Vegetarian products that are alternatives to meat protein products often contain a very high amount of ingredients like preservatives, stabilizers, colorants, or thickeners [17].

The protein alternative must also be a nutritional alternative, which is understood by adequate nutrient density. Unfortunately, because protein source alternative products are highly processed products, their nutritional value is not the same as meat products produced directly from raw meat. This is mainly because the protein used to produce the alternatives is already processed by heat and other methods. There is still no clear confirmation whether replacing meat protein with vegetable protein does not negatively impact human health by reducing the supply of heme protein, zinc, or selenium, which

are characteristic of products based directly on meat. Meat processing methods and meat alternatives such as grilling, roasting, frying, and baking are considered methods that can lead to increased concentrations of carcinogenic substances such as heterocyclic aromatic amines [85]. However, using polyphenolic substances in plant-based products is easier than adding to meat products, which may lead to reduced HAA formation [19].

6. Conclusions

Today, more and more consumers are turning to vegetarianism or looking for products that are not based on animal products. This is understandable from the point of view of worldview, religion, or often just the search for new tastes. In most meat analogs, we encounter proteins of soy origin and wheat origin, like gluten. Unfortunately, both of these proteins are allergenic, and additionally, soy is associated with GMO crops, which are also negatively perceived by some people. The development of a range of meat analog products is possible by using novel sources of protein as well as methods of processing. These can be raw materials rich in protein, such as legumes, or by-products of various processes, as in the case of oilseeds cakes. Novel sources of protein are algae, insects, and fungus. With texturization technology, it is possible to obtain a product of sufficient quality in terms of texture. At the same time, it is worth bearing in mind that it is almost impossible to obtain the texture of meat, so analogs can only be suitable analogs of meat products after processing.

The increase in demand for plant-based protein will certainly be seen in future years as we look for new sources of protein to meet the needs of a growing population. In developed countries, more consumers are turning to vegetarianism and veganism, which will also contribute significantly to the demand for such products. However, a certain unmatched element will be the elaboration of not only the nutritional, but more importantly the physical and technological properties that meat protein possesses. Some hope is offered by zoonotic sources such as insects and from single-celled organisms such as algae because of their easy modification.

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Review

Effect of Fractionation and Processing Conditions on the Digestibility of Plant Proteins as Food Ingredients

Andrea Rivera del Rio, Remko M. Boom and Anja E. M. Janssen *

Food Process Engineering, Wageningen University, 6700 AA Wageningen, The Netherlands; andrea.riveradelrio@wur.nl (A.R.d.R.); remko.boom@wur.nl (R.M.B.)
* Correspondence: anja.janssen@wur.nl

Abstract: Plant protein concentrates and isolates are used to produce alternatives to meat, dairy and eggs. Fractionation of ingredients and subsequent processing into food products modify the techno-functional and nutritional properties of proteins. The differences in composition and structure of plant proteins, in addition to the wide range of processing steps and conditions, can have ambivalent effects on protein digestibility. The objective of this review is to assess the current knowledge on the effect of processing of plant protein-rich ingredients on their digestibility. We obtained data on various fractionation conditions and processing after fractionation, including enzymatic hydrolysis, alkaline treatment, heating, high pressure, fermentation, complexation, extrusion, gelation, as well as oxidation and interactions with starch or fibre. We provide an overview of the effect of some processing steps for protein-rich ingredients from different crops, such as soybean, yellow pea, and lentil, among others. Some studies explored the effect of processing on the presence of antinutritional factors. A certain degree, and type, of processing can improve protein digestibility, while more extensive processing can be detrimental. We argue that processing, protein bioavailability and the digestibility of plant-based foods must be addressed in combination to truly improve the sustainability of the current food system.

Keywords: plant protein digestibility; protein isolates; protein concentrates; alternative fractionation; heat treatment; protein modifications; in vitro protein digestion

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1. Introduction

The current food production system is not sustainable [1]. The largest environmental impact can be attributed to the production of animal-based protein [2]. One of the measures proposed by Willett et al. [3] to reduce this negative impact is to lower our consumption of foods of animal origin and to increase that of plant-based foods. To facilitate this transition, plant-based alternatives to meat, dairy and eggs are continuously introduced in the market. With the increase in flexitarian diets, there is a growing demand in the vegetarian and vegan food space [4]. Nevertheless, the extensive transformation and purification of the ingredients, in addition to the lower protein yield from crop to food product, limits the sustainability potential [5,6].

Generally, plant proteins present less favourable techno-functional properties compared to milk proteins, particularly those depending on solubility such as gelling, emulsifying and foaming properties [7]. In addition, it is not clear yet whether plant- and animal-based proteins can be interchangeable from a nutritional point of view. The dietary requirement of indispensable amino acids (AA) can be satisfied by proteins from various crops [8,9]. Antinutritional factors, digestibility and bioavailability must also be considered when assessing the nutritional quality of proteins. The in vivo protein digestibility-corrected amino acid score (PDCAAS) [10] and in vitro digestibility [11] of some protein-rich ingredients and whole foods have been reported. Furthermore, the effect of domestic and industrial processing on digestion of proteins from legumes consumed as a whole food or flour, i.e., not as a protein-rich ingredient, has been reviewed [12].

Plant proteins are diverse, and most constitute a mixture of various protein units, each with its own properties. For instance, varieties of the same legume species have different globulin to albumin ratios. Globulins have been found to be more susceptible to hydrolysis by digestive enzymes [13,14]. Moreover, 7S and 11S globulin-rich protein fractions from hemp protein isolate (PI) presented different in vitro digestion profiles [15]. Yang et al. [16] found that higher proportions of β-7S subunits had a detrimental effect on the in vitro digestibility of soybean PI. Protein concentrates (PC) from different cultivars of the same species can present different structural, thermal, techno-functional properties and nutritional value, such as the indispensable AA content and digestibility, as was found for rice and millet proteins [17,18]. Meanwhile, different varieties of lupin and sorghum differ in composition and structural properties but are digested to a similar extent [19,20]. This already suggests that the digestibility of proteins from different plant sources might not be affected in the same way by a given type of processing.

The objective of this article is to review the large body of data on the digestion of protein-rich ingredients and on how processing, before, during or after the extraction of the ingredient, may alter it. We recognize the breadth of protocols used to simulate digestion as well as the methods used to describe or quantify the extent of it (Figure 1). As these confounding factors contribute to variations in results, we limited this review to studies that compare some treatment or processing to a control and noted the effect on protein digestibility of a given ingredient.

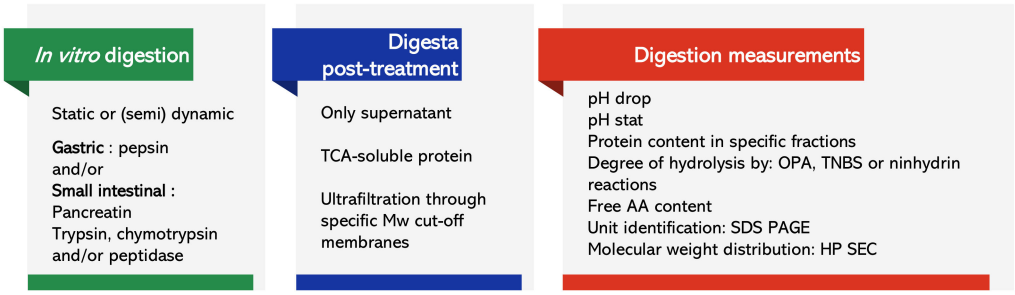

Figure 1. Characteristics of in vitro assays, treatment of digesta and description or quantification of *digestibility* in the studies reviewed. AA, amino acid; HP SEC, high performance size exclusion chromatography; Mw, molecular weight; OPA, *o*-phthalaldehyde; SDS PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TNBS, trinitro-benzene-sulfonic acid.

There is some disparity in the number of studies favouring some types of processing over others, as well as some crops over others. Moreover, the wide range of digestion assays makes it relatively futile to quantitatively compare results from different studies. We therefore present a narrative review with elements of a systematic one, instead of a full systematic review with meta-analysis.

2. Method and Definitions

Review characteristics: The search query used in Scopus was: ("protein" W/6 digest*) AND "in vitro" AND "human" AND ("gastric" OR "intestinal" OR "gastrointestinal" OR "pepsin" OR "trypsin"). In PubMed, the MeSH terms for "plant proteins, dietary" and "digestion" were also included. From the results, the works considering some measure of digestibility or protein hydrolysis by digestive enzymes, simulating some physiological condition(s), were included. Studies on whole foods or flours were not considered, as these sources have been studied elsewhere. Articles studying feed, e.g., for ruminal digestion, emulsions, animal-sourced foods or proteins, and works dealing with allergenicity or immunoreactivity were excluded.

The term "protein digestibility" is used rather ambiguously throughout the reviewed literature. By definition, digestibility is the proportion of an ingested food or nutrient

that can be absorbed into the bloodstream or body. However, it is also used to describe protein degradability, i.e., the proportion of intact protein remaining, the resulting degree of hydrolysis (DH) or the proportion of low molecular weight peptides resulting after the action of digestive enzymes. Other measurements of digestibility are listed in Figure 1 and details of the digestion assays and measurements for each of the studies reviewed are listed in the Supplementary Materials.

Figure 2 presents a scheme of the different processes reviewed. Throughout the text, "conventional aqueous fractionation" refers to milling, optional defatting for oil-containing seeds, alkaline extraction, centrifugation, isoelectric precipitation, centrifugation, washing and freeze drying, as it is mostly performed in laboratory setting, or spray drying, more common in commercially available ingredients. Table 1 summarizes the effects on digestibility of the more commonly studied processes for different plant sources.

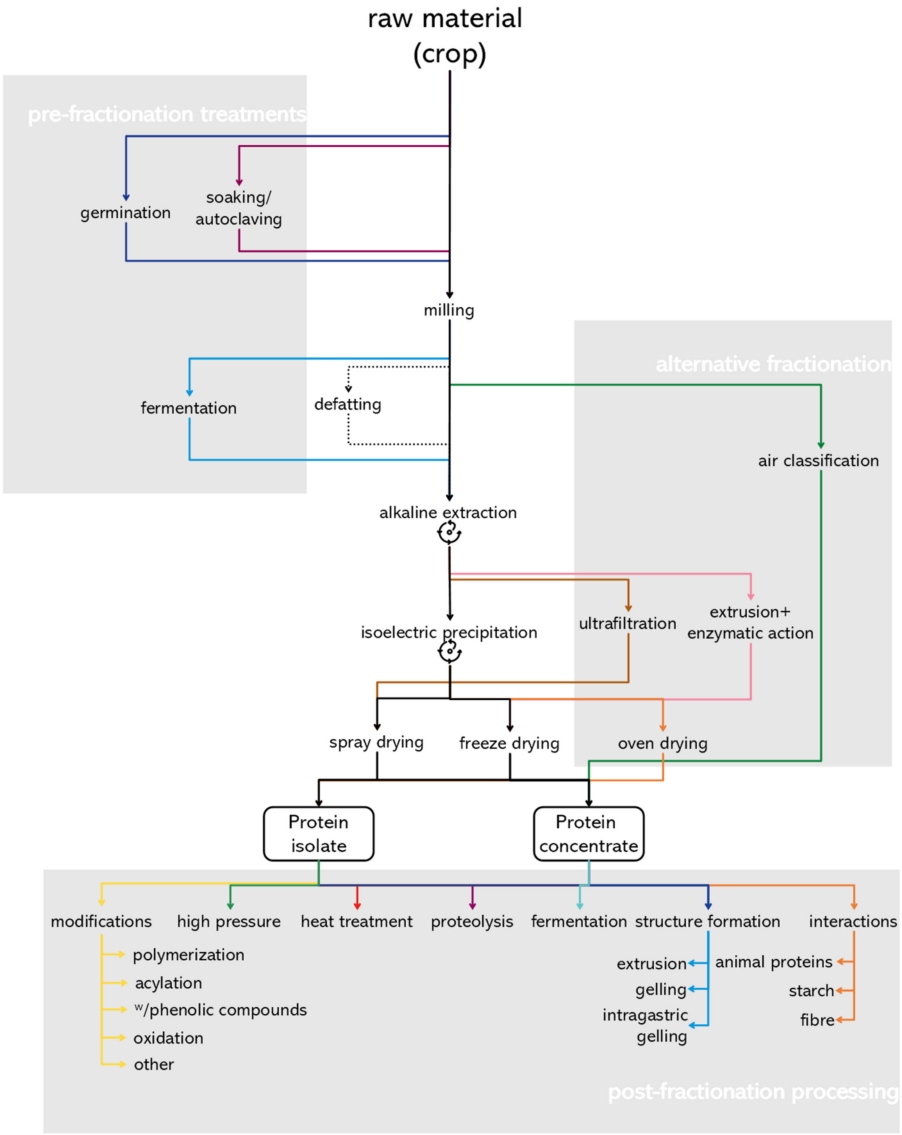

Figure 2. Overview of the processing steps before, during and after fractionation of plant proteins from the studies included in this review. Colours indicate the different routes for processing, the conventional route for aqueous fractionation is presented in black, and ⟳ represents centrifugation after alkaline extraction and isoelectric precipitation.

Table 1. Overview of the effect of different types of processing before, during or after protein fractionation from different crops. 🔴, 🟡 negative; 🟢, 🟢 positive; or 🔵 neutral effect on protein digestibility. Only processes or ingredients with more than one study reporting on the effect of processing on digestibility were included in this table.

Crop	Pre-Fractionation	Fractionation				Processing								Nutrient Interactions Starch
	Germination	Dry Fractionation	Alkaline Treatment	Fermentation	Enzymatic Hydrolysis	Heating	High Pressure	Polymerization	Acylation	Phenolic Compounds	Oxidation	Gelling	Extrusion	
soybean	🟢🔴	-	🔴 also post-fractionation	🟢	🔵🟢	🟢🔴	-	🔴	🟢 hydrolysate	🟢🔴	🟢🔴	🔴	🟢	-
black bean	🟢	-	-	-	-	-	-	🔴	-	-	-	-	-	-
chickpea	-	-	-	-	🔵	🟢	-	-	-	-	🔴	-	-	-
fava bean	-	🟡	-	-	-	🔴 legumin	-	-	-	-	-	-	-	-
lentil	-	🔵	-	-	🔴	🟢 globulin	-	-	-	-	-	-	-	-
lupin	-	-	🔴	🟢 pre-fractionation	-	🟡🟢	-	-	-	-	-	-	-	-
maize	-	-	-	-	🟢	-	-	-	-	-	-	-	🟢	-
mung bean	-	-	-	-	-	🟢	-	-	🟢	-	-	-	-	-
navy bean	-	-	🔴	-	-	🟢	-	-	-	-	-	-	-	
quinoa	-	🟢	🔵	-	-	🔴	-	-	-	-	-	-	-	🔴
rapeseed	-	-	🔴	-	🟢	-	-	-	-	-	-	-	-	-
red kidney bean	-	-	-	-	-	🔵	🔴	🟢	🟢	-	-	-	-	-
rice	-	-	🔴	-	🟢	-	-	-	-	-	-	-	-	-
yellow pea	-	🔴	-	🟡🟢	-	🔵	🟢	-	-	🔴	-	🔵	🟢	🔴

3. Ingredient Preparation

3.1. Pre-Fractionation Treatment

Most commonly, seeds are milled into a flour or grits prior to alkaline extraction. Soaking seeds at high temperatures, before milling for conventional aqueous fractionation, was shown to improve the in vitro digestibility of soybean and cowpea PI. In the work of Wally-Vallim et al. [21], PI from soybean seeds soaked at 40 °C was more digestible than at 60 °C. The in vitro gastric digestibility was improved by longer soaking times for both temperatures. It was argued that at 40 °C, proteins were partially denatured, while at 60 °C the 7S fraction was completely denatured, and protein structures had rearranged. Meanwhile, PI from soaked and autoclaved cowpea seeds was more extensively hydrolysed by pepsin–pancreatin than that from raw seeds [22].

Some studies explored the effect of germination prior to fractionation of soybean and black bean. A direct relation between the germination time and the extent of hydrolysis achieved by digestive enzymes was observed [23,24]. Concurrently, the trypsin inhibitory activity (TIA) was reduced by germination, associated to protease-catalysed hydrolysis of lectins and trypsin inhibitors. Aijie et al. [25] found a similar relation; however, the DH decreased, and the TIA increased for the longest germination times, which they explained by a resynthesis of trypsin inhibitors by photosynthesis. For black soybean, an inverse relation was observed: the PI produced from non-germinated seeds yielded the largest proportion of low molecular weight peptides [26]. It was hypothesised that these small peptides were used for tissue formation during germination.

Solid state and submerged fermentation of milled lupin with different strains of *Pediococcus* prior to subsequent conventional aqueous fractionation improved the in vitro protein digestibility in the PI compared to the non-fermented control [27]. At the same time, the fermentation reduced the content of trypsin inhibitors. No clear relation can be drawn between the type of fermentation and digestibility, as many different lupin hybrid lines and strains of *Pediococcus* were studied.

3.2. Conventional Protein Fractionation

After a defatted meal has been obtained, alkaline extraction is the first step in conventional aqueous fractionation. Higher protein purities, at the expense of lower yields, can be obtained with increasingly higher concentrations of a strong alkali, typically NaOH. Alkaline treatment has been associated with the formation of lysinoalanine and AA isomerisation in rice residue PI, reducing the in vitro digestibility and absorption in a rat model [28]. Protein extracted from defatted lupin meal at acidic pH (pH 2) was more readily and extensively digested than that extracted at neutral or alkaline pH (pH 8.5) conditions, using an in vitro digestion assay [29]. The extraction pH was thought to induce different structural conformations and extents of denaturation. Nevertheless, Ruiz et al. [30] did not find a significant effect on the in vitro gastric digestion of quinoa PI extracted at pH 8 to 11.

Either PC or PI can be obtained from the conventional fractionation process. Commercial PC and PI have been used in in vivo rat assays, showing a small variation in PDCAAS, the true or standard ileal digestibility, of soybean ingredients [31,32]. Meanwhile, the in vitro gastric digestibility of commercial soybean PI remained unchanged after long-term storage at freezing and high temperatures [33].

3.3. Alternative Protein Fractionation Strategies

Modifications to the conventional aqueous fractionation process have been proposed to improve the purity, yield or techno-functional properties of the ingredients obtained. Conventionally, alkaline extraction is performed with NaOH, with the pH adjustment for isoelectric precipitation performed with HCl. Chamba et al. [34] proposed the use of alkaline ash from burnt green and purple amaranth and lemon juice as “natural” alternatives to the more commonly used chemicals to isolate soybean protein from full fat and defatted flour. The PDCAAS was slightly higher for the material extracted with “natural” chemicals, while

no significant difference was observed between the in vitro pepsin–pancreatin digestibility of "natural" and conventional chemicals. The use of conventional chemicals was somewhat more effective at reducing the content of antinutritional factors such as trypsin inhibitors and phytic acids in PI. Na_2SO_3 has been used to extract proteins and to prevent oxidative darkening of the PI, from lupin and chickpea. The digestibility of Na_2SO_3-extracted lupin PI was higher than the conventionally fractionated ingredient [35]. However, for chickpea PI, the digestibility from both extractions did not differ [36].

Ultrafiltration has been used as an alternative to isoelectric precipitation. The TIA was similarly reduced by either process for soybean PI [37]. The extent of hydrolysis achieved with pepsin–pancreatin digestion, as well as the reduction of the TIA, was comparable for brown lentil PI separated by ultrafiltration and for conventional isoelectric precipitation [38].

The effect of different drying methods on the protein digestibility was studied for buckwheat and hempseed PI. Tang [39] showed that freeze drying, compared to spray drying, produces buckwheat PI that is better digestible by pepsin–trypsin. However, when alkaline extraction was assisted by ultrasonic treatment instead of by just mechanical stirring, freeze- and spray-dried PI were equally digestible. Meanwhile, Lin et al. [40] compared vacuum oven, oven or freeze drying of hempseed PC. In this study, freeze drying also produced better digestible PC compared to drying at higher temperatures, which was attributed to the formation of poorly digestible Maillard products during oven or vacuum oven drying.

Enzyme-assisted fractionation paired with extrusion has been presented as an environmentally friendly alternative to conventional aqueous fractionation [41]. Oil and protein were simultaneously extracted from soybean flakes that were extruded and treated with a bacterial endoprotease under alkaline conditions to obtain oil-, fibre- and protein- and sugar-rich fractions. Extrusion or enzyme action during processing did not alter the pepsin digestibility of the resulting ingredients, although some techno-functional properties were improved. Extrusion and α-amylase-catalysed starch liquefaction were used to concentrate proteins from white sorghum [42]. While the moisture content in the barrel during extrusion influenced the in vitro gastric digestibility, no effect from α-amylase action was observed. Nevertheless, the sorghum PC showed lower digestibility than sorghum flour. This was attributed to re-aggregation during the boiling step that was used for enzyme inactivation.

Air classification is a dry fractionation technique. The digestibility of pea, lentil and fava bean PC obtained from air classification were compared to that of NaCl-extracted PI from aqueous fractionation in a mice study [43]. Overall, the digestibility of the PC was lower than that of the PI, most significantly for pea. Likewise, air-classified fava bean PC was less extensively hydrolysed during pepsin–pancreatin digestion than a PI from isoelectric precipitation and spray drying [44]. Further, the TIA from the initial flour was maintained in the air-classified ingredient and significantly reduced in the conventionally produced PI. Conversely, air jet-sieved quinoa PC was slightly more extensively hydrolysed by pepsin than a conventional aqueous-fractionated PI [45]. We hypothesize that the protein denaturation achieved through heating during spray drying facilitates the access of digestive enzymes to the cleavage sites within the proteins.

4. Post-Fractionation Processing

Protein ingredients are further processed into finished products. The effects of different protein steps (fermentation, ultrasound treatment, heating, protein modification, among others) have been researched on PI and PC from various crops. Ultrasonic treatment of fava bean PI dispersions slightly reduced the in vitro digestibility [46].

Fermentation of commercial pea PC with *Lactobacillus plantarum* had a positive effect on the in vitro protein digestibility and a reduction of antinutritional factors, phenols, tannins, chymotrypsin and trypsin inhibitors. Nevertheless, the in vitro PDCAAS was negatively impacted. This was explained by the catabolism of sulphur-containing AA by the lactic acid bacteria [47]. Similarly, *L. plantarum*-fermented soybean PI released more

free AA than the non-fermented control, in a dynamic in vitro gastrointestinal digestion assay [48]. Additionally, protein aggregation was observed in the gastric phase only for the non-fermented PI, as well as a higher proportion of high molecular weight peptides at the beginning of the intestinal phase.

4.1. Proteolysis

Protein hydrolysis has mixed effects on protein digestibility. For soybean protein, hydrolysis by immobilized trypsin improved or had no effect on the extent of digestion [49]. In this study, pre-digested proteins were better digestible under infant gastric condition, simulated by a less acidic pH (pH 4) compared to adult models. Meanwhile, a soybean protein pepsin–hydrolysate was as digestible as the intact PI, in a different infant model with reduced digestive enzyme concentration, compared to an adult model [50].

A series of studies investigated the effect of the co-ingestion of soybean PI and dietary actinidin from green kiwifruit extract on the protein digestion. From an in vitro pepsin–pancreatin assay, some subunits such as the 11S basic polypeptide showed some effect of the actinidin; however, no overall effect on the protein degradability was observed [51]. From an in vivo rat study, the presence of actinidin in the diet showed no significant effect on the true ileal digestibility of soybean PI [52]. Gastric chyme samples from a subsequent rat study were analysed for their true gastric total protein digestion [53]. The presence of actinidin here improved the gastric digestibility of the PI. Meanwhile, actinidin had a positive effect on the digestibility of zein but had virtually no effect on the digestibility of wheat gluten. These studies highlight the relevance of the type of assay and measure of digestion to assess the effect of processing or modification on plant protein digestibility.

Green lentil PI from conventional aqueous fractionation was hydrolysed with acid protease, actinidin, bromelain and papain, prior to in vitro digestion [54]. Intact proteins proved to be better hydrolysable than the protein hydrolysates. Nevertheless, as a net result, more low molecular weight peptides were produced from the protein hydrolysates than from intact PI.

Hydrolysis positively affected the digestibility of rapeseed and rice bran PI. Fibre and protein from a rapeseed PI that was obtained by membrane processing were hydrolysed [55]. The true digestibility of the hydrolysate was higher than the intact PI, as shown by a rat assay. As a result, the PDCAAS of the hydrolysate was also higher, compared to the original ingredient. Similarly, for progressively higher degrees of hydrolysis, a papain–hydrolysate of rice bran PC was more extensively digested than the intact PI by pepsin–pancreatin digestion [56].

Chickpea protein hydrolysis did not alter the digestion. Neither alcalase, flavourzyme [57], trypsin, papain nor pepsin [58] changed the extent of protein digestibility in in vitro assays. Nevertheless, the TIA was significantly reduced by the hydrolysis [57].

4.2. Heat Treatment

The process step most studied in terms of its effect on protein digestibility is heat treatment. Different conditions as well as different crops have been studied with positive, neutral or negative effects of heating on protein digestibility.

It is commonly thought that a certain extent of heat induced protein denaturation improves the digestibility, while more extensive heat treatment would induce protein aggregation which would, in turn, reduce the digestibility. The work of Tian et al. [59] demonstrates the relation between heating time and temperature, and the extent of pepsin-catalysed hydrolysis of soybean PI. Dispersions heated at 85 °C for 15 min presented the highest DH, while those heated at 70 or 100 °C were hydrolysed to a significantly lesser extent. In terms of time, PI heated at 85 °C for 20 min showed the highest DH compared to those heated for 10 or 60 min. Overall, all heated samples were more extensively hydrolysed than the unheated control.

Soybean is one of the crops most widely studied in terms of the effect of heat treatment on protein digestibility. Studies have shown improvement but also reduction of protein

digestibility as a result of heat treatment. The in vitro pepsin–pancreatin digestibility of soybean PI was improved by relatively short heating for 15 min at 95 to 121 °C [60–62]. β-conglycinin is known to be less susceptible to pepsin-catalysed hydrolysis than glycinin. Nevertheless, the gastric digestibility of both fractions was improved by heat treatment [60]. In this study, heating induced protein aggregation as well as pepsin during the gastric phase. The TIA of germinated soybean PI was reduced by the heat treatment [25]. Conversely, the apparent digestibility of heated, spray-dried and autoclaved pastes of soybean PI, determined in a rat assay, was significantly lower than that of non-autoclaved pastes [63]. Besides the heat treatment during drying, these pastes were autoclaved for up to four hours, highlighting that extensive heat treatment, both in time and temperature, has a detrimental effect on protein digestibility.

Heat treatment does not affect the digestibility of different pulse protein ingredients in the same way. Heating at 95 °C for 30 min improved the pepsin–trypsin digestibility of mung bean PI, reduced it for red bean PI, and did not change it for red kidney bean PI [64]. A larger extent of aggregation in heated mung bean PI was reported than in red kidney bean PI. It was suggested that the presence of basic, hydrophobic and uncharged polar AA influences the thermal and structural stability of proteins, and thus the tendency to aggregate when heated. Meanwhile, the in vitro digestibility of lupin and winged bean PC was improved by heating in a boiling water bath for up to 30 min [65,66]. The trypsin and chymotrypsin inhibitory activity of the freeze-dried winged bean PC was inactivated by heat treatment [66].

Likewise, the digestibilities of individual protein fractions from different crops are not modified in a similar manner upon heating. Vicilin-like proteins from chickpea and common bean are both resistant to gastric digestion; however, the digestibility of the former was improved by autoclaving, while for the latter, it was reduced [67,68]. Furthermore, chickpea albumin, 11S and total globulin digestibility increased, as a result of heat treatment [68]. Conversely, native protein fractions from fava bean were better digestible than those that denatured after autoclaving [67].

One might expect that preventing heat-induced aggregation would lead to a positive effect on protein digestibility. This was observed for lentil globulins which were unsusceptible to heat-induced aggregation, given that disulphide interactions were not observed [69]. Nevertheless, the negative charge of a protein fraction from common bean made the protein less prone to aggregation and yet less digestible than its unheated, less negatively charged, counterpart [67]. Based on the effect of heating on the electric charge of proteins and peptides, the latter study suggested that protein electronegativity and hydrophobicity were associated with protein aggregation and digestibility.

Net-zero effects may result from concurring events improving and reducing the DH achieved by digestive enzymes. Commercial soybean and pea PI dispersions heated at 90 and 120 °C for 30 min did not show different DH during in vitro gastric digestion compared to their unheated counterparts. Upon close inspection of the soluble and sedimented tailings, we found that heating improved the solubility of the commercial PI, and that the proteins separated into this fraction could be more extensively hydrolysed than those in the sedimented fractions [70].

Meanwhile, for dry-fractionated ingredients, heat treatment has shown to reduce the gastric digestibility of lupin and quinoa proteins. More small peptides (<3 kDa) were released from the unheated and heated at 60 °C dispersions of air-classified lupin PC than the dispersion heated at 90 °C [71]. A similar trend was observed for dry fractionated quinoa PC, with unheated and heated at 60 °C dispersions being more extensively hydrolysed than dispersions heated at 90 and 120 °C [45,72]. Similarly, quinoa PI from conventional aqueous fractionation showed lower DH with increasingly higher heating temperatures [30].

As previously discussed, alkaline heat treatment is generally detrimental for protein digestibility. Heating at higher pH reduced the in vitro protein digestibility of globulins from navy bean [73], of soybean PI [74] and rapeseed PC [75]. These results were confirmed

for spray-dried soybean PI by an in vivo rat study [76]. For the most part, the limited digestibility can be attributed to the formation of lysinoalanine at high pH [74–76].

Thus far, we discussed studies on so-called moist heating, but the environment during heating does influence the protein digestibility. Sathe, Iyer and Salunkhe [14] compared dry and moist heating of navy bean PC and PI extracted with Na_2CO_3, as well as water-extracted albumins and NaCl-extracted globulins. The DH achieved with trypsin-α-chymotrypsin-peptidase was improved more significantly by moist than by dry heating. Similarly, boiling, microwaving, autoclaving, and dry or oven heating improved the digestibility of sweet potato PC [77]. Autoclaved dispersions presented the highest DH by pepsin–pancreatin digestion, followed by microwave and, lastly, dry heating. The PDCAAS determined in a rat assay was improved for autoclaved PI compared to the unheated ingredient. As previously reported, the TIA was reduced by all types of heat treatments studied.

These observations give a sense of the optimum range of heat treatment to improve the protein digestibility; more heating can negatively impact the digestibility (Table 1). The appropriate heat treatment would then depend on the ingredient source, the type of protein fraction, the type and conditions of heating.

4.3. High Pressure Processing

Laguna et al. [78] conducted a comprehensive study on the effect of heating and high pressure processing at two different pH (3.6 and 6.2) of commercial pea PI on its in vitro digestibility. For the most part, high pressure processing improved the gastric digestibility of pea protein. Samples prepared at a higher pH were more digestible than those at pH 3.6. Autoclaving did not alter the protein digestibility at either pH, which shows that the effect of pressure cannot be explained by denaturation, similar to that during heating. High pressure processing followed by a 30 min, 80 °C heat treatment at pH 3.6 reduced the protein digestibility. In contrast, high pressure processed red kidney bean PI presented a significantly lower in vitro digestibility by trypsin [79]. This was attributed to the generally low digestibility of phaseolin, particularly when aggregated. In this case, we may conclude that the protein source, as much as the processing steps, influences the digestibility of proteins.

5. Crosslinking, Complexation and Other Modifications

Forming protein complexes with other proteins or other compounds can be an unintended consequence of combining materials in one matrix or can be intentionally induced to achieve certain functions, such as colon-targeted drug delivery [80] or to confer an added nutritional benefit [81].

5.1. Transglutaminase-Catalysed Polymerization

Phaseolin from *Phaseolus vulgaris* L. was cross-linked by microbial transglutaminase [80]. Its isopeptide bonds made phaseolin more resistant to pepsin and trypsin action, especially for pepsin. Similarly, the pepsin–trypsin digestibility was reduced for native and heated crosslinked proteins from soybean PI, while it was improved by heat treatment alone [82]. While a single protein source was used in this study, covalent crosslinks were identified between β-conglycinin and acidic subunits of glycinin. In contrast, positive effects on the trypsin digestibility as a consequence of crosslinking by transglutaminase have been reported in red kidney bean PI [83]. The digestibility increased in crosslinked protein with longer crosslink reaction times, which was attributed to protein unfolding and denaturation of the vicilin unit.

Limited protein degradation by pepsin–pancreatin was observed for soybean PI polymers and heteropolymers with whey PI or casein, compared to the untreated PI [84]. Furthermore, soybean PI heteropolymers were more resistant to in vitro digestion compared to the whey PI-casein heteropolymer. This was attributed to reduced accessibility for enzymes to the peptide bonds, due to blockage of lysine residues and steric hinderance.

Likewise, soybean PI–bovine gelatine composites showed lower pepsin–trypsin digestibility than the PI [85]. Trypsin-catalysed hydrolysis, prior to in vitro digestion, increased the digestibility slightly but it remained significantly lower for the untreated PI.

Glycation and crosslinking soybean PI with chitosan, or oligo-chitosan with transglutaminase improved the pepsin–trypsin digestibility [86,87]. The crosslinked soybean PI was more digestible than the untreated PI in both pepsin and pepsin–trypsin digestion assays.

To assess the effect of Maillard reaction products, crosslinked commercial soybean PI was heated with D-ribose or sucrose [88]. Crosslinking had a negative effect on in vitro protein digestibility, particularly at longer transglutaminase incubation times. Overall, sucrose-containing samples were more digestible than ribose-containing samples. AA loss was reported as a consequence of crosslinking, most significantly of lysine.

Therefore, the effect of transglutaminase-catalysed crosslinking on protein digestibility depends on the extent to which cleavage sites become exposed or buried within the structure of the crosslinked protein. Furthermore, AA bioavailability could also decrease as a result of this processing step.

5.2. Acylation

Acylation of proteins can result in techno-functionality, such as solubility and emulsifying activity [89]. Mung bean PI was acylated with succinic and acetic anhydrides [90]. The trypsin–pancreatin digestibility was improved by acylation, probably due to protein unfolding. Acetylation was reported to reduce antinutritional factors (phytic acid, tannins and trypsin inhibitors) to a greater extent than succinylation. Similarly, acetylated and succinylated red kidney bean PI were more digestible by trypsin than their untreated counterpart [89]. This was attributed to increased protein solubility and protein unfolding.

The improved digestibility due to acylation observed with these ingredients was also reported for a soybean PI hydrolysate [91]. The in vitro digestibility was significantly higher for succinylated soybean PI hydrolysates compared to the non-succinylated control. The authors also attributed this effect to protein dissociation or unfolding, and an increase in solubility. de Regil and Calderón de la Barca [92] assessed the in vivo digestibility of a soybean protein hydrolysate enzymatically bound by chymotrypsin to methionine methyl-ester using a rat study. There was no significant difference between the apparent digestibility of modified soybean PI hydrolysate and the control with free methionine. Nevertheless, the protein efficiency ratio was significantly higher for the modified ingredient.

Again, protein unfolding is related to an improvement of its digestibility, as was also observed with thermal denaturation. Moreover, peptides of lower molecular weight and, perhaps as a result, increased solubility would generally result in better digestibility, unless opposed by other cross-effects.

5.3. Complexation with Phenolic Compounds

The digestibility of thermally denatured soybean PI was significantly improved, mostly by pepsin, when complexed with anthocyanins from black rice extract [62]. It was suggested that the network formed by the complex promotes enzymatic action is made possible by changes in the secondary structure; again, (partial) unfolding then facilitates the digestion. In a similar manner, soybean PI–curcumin complexes were more extensively hydrolysed than the non-complexed PI, particularly by pepsin, in a sequential pepsin–pancreatin in vitro digestion assay [60]. Heating before complexation did not influence the extent of digestion of the proteins. Furthermore, the typically pepsin-resistant β-conglycinin unit was completely degraded when it was part of the curcumin nanocomplex. Budryn et al. [93] studied soybean PI–hydroxycinnamic acids complexes, either individual 5-caffeoylquinic acid, caffeic acid or ferulic acid, combined in green coffee extract or encapsulated in β-cyclodextrin. The reduction in average molecular weight after pepsin-(trypsin-chymotrypsin) digestion was greater for the complexes than for the untreated PI. It was suggested that interactions and exposure of hydrophobic AA were responsi-

ble for the enhanced digestibility, although proteases might also interact directly with hydroxycinnamic acids.

In contrast to the positive effects of anthocyanins and hydroxycinnamic acids, protein–polyphenol complexes reduce the digestibility of pea and soybean PI. Nine commercial pea PI with different physical and chemical characteristics were used to form complexes with polyphenols from cranberry pomace [81]. For some PI, no significant differences were found in the pepsin digestion of non-complexed and complexed proteins; however, all complexed isolates were less extensively hydrolysed by pancreatin digestion. The digestion rate was inversely related to the particle size of the PI. Similarly, soybean PI complexed at 70 or 121 °C with polyphenols and flavonoids from black soybean seed coat extract, was less extensively hydrolysed by pepsin–trypsin than the non-complexed ingredient [61]. Moreover, the DH was further reduced by increasing extract concentrations used to produce the complexes. Extract–enzyme or extract–protein interactions were thought to alter the digestive enzymes' conformation, rendering them inactive for protein hydrolysis. In a rat assay, the true nitrogen digestibility was reduced for soybean PI that was complexed with both chlorogenic acid and quercetin [94]. The PDCAAS was significantly reduced for derivatized protein with lysine being the limiting AA.

Yang et al. [95] proposed a multistep process to produce a fermented soybean milk enriched with isoflavone aglycone. More intact proteins remained after pepsin–trypsin hydrolysis of the soybean PI–isoflavone complex, than of the PI. The isoflavone probably inhibited the protease activity. Nevertheless, heated and fermented soybean PI–isoflavone were more extensively hydrolysed than their unheated or non-fermented counterparts.

Phenolic compounds can modify the conformation not only of the proteins but also of the digestive enzymes. Changes in protein conformation can have a positive or negative effect on protein digestion. The former, if unfolding leads to the exposure of cleavage sites, or the latter, if it leads to steric hinderance surrounding the cleavage sites. Furthermore, phenolic compound could also act as inhibitors when bound to the digestive enzymes.

5.4. Protein Oxidation

Zhao et al. [96] found that a certain extent of protein oxidation had a positive effect on the soybean protein gastric digestibility as a result of protein unfolding, particularly for glycinin. However, severe treatments, i.e., by lipoxygenase-catalysed linoleic acid oxidation [96] or by incubation with 2,2′-azobis (2-amidinopropane) dihydrochloride [97], had a negative impact. In the latter study, the action of the radical-generating compound did not affect the gastric digestion, but it reduced the DH by pancreatin in the intestinal phase. This effect was directly influenced by increasing concentrations of the compound in the system. It was shown that oxidation can degrade several AA and induce protein aggregation. Sánchez-Vioque et al. [98] attributed a reduction in digestibility of chickpea legumin mixed with linolenic acid, to protein oxidation or non-covalent protein–lipid interactions. Meanwhile, no clear relation between carbonyl content, from oxidation products, and extent of hydrolysis in the gastric phase has been observed in thermomechanical processed soybean PC and PI [99].

5.5. Other Modifications

Soybean PI incubated with malonaldehyde, a lipid peroxidation product, was subjected to in vitro pepsin–pancreatin digestion [100]. β subunits of β-conglycinin were somewhat degraded by pepsin but they became more resistant to pancreatin digestion with increasing malonaldehyde concentration. The availability of indispensable and total free AA after digestion decreased in modified soybean PI.

Soybean PI, cottonseed PC and peanut PC formed complexes with glucose or sucrose [101]. In vitro digestibility was reduced by longer heating times to form the complexes. Protein–glucose complexes were less digestible than the sucrose complexes. Further, available lysine was reduced with heat treatment.

Lastly, soybean PI was incubated with phytase from *Aspergillus niger* to obtain ingredients with different phytate contents [102]. Phytate content, parallel to TIA, was inversely related to pepsin–pancreatin digestibility.

Repeatedly, we find that any process or modification that would induce a certain degree of unfolding will generally facilitate digestion, but extensive unfolding leading to aggregation will result in slower or reduced digestion. Furthermore, modification of AA, particularly of lysine, will often lead to their reduced bioavailability. Finally, processes that reduce or inactivate antinutritional factors, such as phytate or protease inhibitors, will also improve or facilitate the digestion of proteins.

6. Structure Formation

6.1. Extrusion and Texturization

The in vitro digestibility of yellow pea and soybean PC can be improved by extrusion. The barrel temperature and screw speed are positively related to the protein digestibility of air-classified pea PC, while the moisture content has a negative influence on its digestibility [103]. Soybean PC, maize meal and cassava root starch were mixed and extruded [104]. The samples extruded at the highest temperature, moisture content and screw speed were the most digestible. The TIA, phytic acid and cyanide contents were reduced by extrusion; however, the tannin content was not reduced. Higher temperatures during extrusion led to more digestible proteins, which opposes the observations from moist heat treatments (Section 4.2), the reason is not fully understood and requires further research.

Duque-Estrada, Berton-Carabin, Nieuwkoop, Dekkers, Janssen and van der Goot [99] explored the effect on in vitro gastric digestibility of high temperature shearing of soybean protein ingredients, as well as the relevance of structure and size reduction in the digestibility. Sheared samples were cut into small pieces or ground into finer particles. Pepsin-catalysed hydrolysis was faster for unheated dispersions, followed by ground matrices. Cut samples were more slowly and less extensively hydrolysed than the other physical states.

The work from Li et al. [105] shows how the formation of rice glutelin fibrils through heat treatment under acidic conditions makes the protein more resistant to pepsin–pancreatin digestion.

6.2. Pre- and Intra-Gastric Gelation

Opazo-Navarrete et al. [106] related the mechanical strength and porosity of heat-induced gels of soybean PI and pea PC to their gastric digestibility. No significant differences were observed between gels pre-heated at different temperatures. Soybean protein gels were less extensively hydrolysed than the control consisting of a protein dispersion, unlike pea protein gels that were hydrolysed to a similar extent as the dispersion.

Pressure-induced gels from air-classified lentil and fava bean PC were more digestible than heat-induced gels under in vitro gastric conditions [107]. It was suggested that the network of pressure-induced gels allowed for a similar extent of access to pepsin as in concentrated protein dispersions. Meanwhile, both treatments changed the structure of the 55 kDa fractions to be better digestible in the gastric phase. The TIA was more significantly reduced by heating than by pressurization.

Soybean PI coagulates formed with $MgCl_2$ or glucono-δ-lactone were more digestible than gels prepared with transglutaminase [108]. This was attributed to the covalent isopeptide bonds formed by transglutaminase that cannot be degraded during in vitro gastrointestinal digestion. In contrast, the non-covalent bonds formed during coagulation by $MgCl_2$ or glucono-δ-lactone could be broken during digestion. Soybean PI and glycerol films were prepared with ferulic acid, tannin, corn starch or H_2O_2 at pH 7 to 10 [109]. The gastric digestibility of the films was significantly lower than that of the PI in a dispersion, except for the films prepared with corn starch, which were digested to a similar extent as the control. Lysine availability was also lower in the films. Ferulic acid and tannins were thought to form crosslinks with AA, while H_2O_2 could have oxidized certain AA. Lastly,

films formed at pH 9 and 10 were less digestible than at pH < 8.5. This was attributed to AA isomerisation and crosslinking at high pH.

In a simulated gastric environment, dispersions of soybean PI and negatively charged polysaccharides (xanthan gum, carrageenan [110] or alginate [111]) self-assemble into a hydrogel. The pepsin-catalysed hydrolysis of the gels was slower even at low polysaccharide contents, compared to the single PI. Hu, Chen, Cai, Fan, Wilde, Rong and Zeng [110] similarly found that soybean PI–carrageenan gels were digested more slowly than those with xanthan gum, due to the more compact and dense gel network in the former.

Generally, structure formation led to a slower and sometimes lower extent of hydrolysis by digestive enzymes compared to liquid dispersions. This is explained by physical hinderance surrounding the cleavage sites. Therefore, looser structures as weaker gels allow for a better digestibility than tighter structures. Further, covalent crosslinking inhibits protein unfolding, while non-covalent bonds can dissociate, especially at lower pH in the stomach, and thus allow for faster digestion. As heat treatment is often required before gelation, antinutritional factors, such as trypsin inhibitors, can also be inactivated.

7. Macronutrient Interactions

Proteins are almost never processed or consumed on their own. The effect of the interaction of proteins with other macronutrients on protein digestion is not fully understood, but there are some general directions suggested.

7.1. Animal- and Plant-Based Protein Hybrid Foods

Reconstituted beverages containing the combination of bovine milk PC and soybean, pea or rice PI showed an improved in vitro DH and PDCAAS of blends compared to individual plant proteins [112]. However, this was not observed in solid matrices. Proteins from pea PI, rice protein or lentil flour were enzymatically bound to beef chuck ground meat using transglutaminase [113]. The cooked restructured beef steaks were digested using the INFOGEST 2.0 model with expectorated boluses. No outstanding differences were observed in the peptide size distribution in the digestates of the samples with different treatments. Lentil-enriched steaks released the highest amounts of free isoleucine, lysine, phenylalanine and valine. Protein (re-)aggregation was observed after in vitro gastrointestinal digestion.

7.2. Starch

Oñate Narciso and Brennan [114] found a relationship between the amylose content of starch with protein digestion. Pea PI was combined with starch from basmati and glutinous rice, with high and low amylose to amylopectin ratios, respectively. All proteins from the samples prepared with glutinous rice starch were degraded after pepsin–pancreatin digestion, but the vicilin and legumin acidic subunit from basmati rice starch samples remained after digestion. The authors proposed that the proteins were embedded into the amylose network. Similarly, quinoa protein from aqueous or dry fractionation was combined with starch-rich fractions from dry or mild aqueous fractionation, which after heating showed lower DH from in vitro gastric digestion than starch-free, unheated protein dispersions [45,72]. This reduction directly related to the heating temperature and was thus probably associated to starch gelatinization. Therefore, embedding the protein in a gelatinized starch gel does reduce the digestibility, probably due to the inaccessibility of the gel for the enzymes.

7.3. Fibre

The DH obtained by pepsin digestion of dry-fractionated quinoa PC was slightly reduced in quinoa fibre-containing unheated and heated dispersions [72]. The effect of fibre on quinoa protein gastric digestion was not as significant as for starch. Fibre seemed to counter the low hydrolysis induced by starch gelatinization. The fibre does not form a gel that is difficult to penetrate for enzymes but may induce somewhat better mixing due to the higher viscosity.

8. Conclusions

Plant proteins have the potential to provide all indispensable amino acids. However, as described at length, processing and plant protein digestibility are strongly related.

Heating and soybean are the process step and crop most researched, respectively, reflective of their ubiquity in the production of plant-based food products. Moderate heating may enhance the digestibility by inducing partial unfolding of the proteins, thereby rendering them better accessible for the proteases. However, extensive heating induces aggregation, which makes the cleavage sites less accessible. Similar effects are seen with other types of treatments. Acylation of protein-rich ingredients improved their digestibility, probably also due to partial unfolding. Meanwhile, alkaline treatment, during or after fractionation, consistently reduces the digestibility of different crops, since it strongly changes the structure of the protein and induces AA isomerisation. Again, we see an optimum in the severity of the treatments for digestibility. It is however clear that the exact impact depends on the origins of the proteins.

Ultimately, it is desirable to attain an overarching relationship between the digestibility and the modifications resulting from processing. This review can serve as a guide when considering a certain processing step in the production of plant-based alternatives to animal-sourced products. There are ample opportunities for further research of unexplored processes for promising crops and vice versa, to truly consider the use of plant protein-rich ingredients in food products as a transition pathway to a more sustainable food system.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/foods11060870/s1, Table S1: Summary of digestion assays, sample treatment and measurement from studies investigating the effect of processing on protein digestion. References [21–114] are cited in the Supplementary Material.

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Adsorption of Quercetin on Brown Rice and Almond Protein Matrices: Effect of Quercetin Concentration

Mirela Kopjar [1,*], Ivana Buljeta [1], Ina Ćorković [1], Anita Pichler [1] and Josip Šimunović [2]

[1] Faculty of Food Technology, Josip Juraj Strossmayer University, F. Kuhača 18, 31000 Osijek, Croatia; ivana.buljeta@ptfos.hr (I.B.); ina.corkovic@ptfos.hr (I.Ć.); anita.pichler@ptfos.hr (A.P.)
[2] Department of Food, Bioprocessing and Nutrition Sciences, North Carolina State University, Raleigh, NC 27695-7624, USA; simun@ncsu.edu
* Correspondence: mirela.kopjar@ptfos.hr; Tel.: +385-3122-4309

Abstract: Plant-based proteins are very often used as carriers of different phenolic compounds. For that purpose, complexation of quercetin with almond and brown rice protein matrices was investigated. The amount of protein matrices was constant, while the concentration of quercetin varied (1 mM, 2 mM or 5 mM) during complexation. Dried complexes were investigated for quercetin amount (HPLC analysis) and antioxidant activity (DPPH, FRAP and CUPRAC methods). Additionally, complexation was proven by DSC and FTIR-ATR screening. An increase in the concentration of quercetin in the initial complexation mixture resulted in the increase in the adsorption of quercetin onto protein matrices. For the brown rice protein matrices, this increase was proportional to the initial quercetin concentration. Adsorption of quercetin caused the change in thermal stability of microparticles in comparison to corresponding protein matrices that have been proven by DSC. FTIR-ATR analysis revealed structural changes on microparticles upon adsorption of quercetin.

Keywords: quercetin; almond protein matrix; brown rice protein matrix; HPLC; antioxidant activity; DSC; FTIR-ATR

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1. Introduction

A growing area of interest in the field of polyphenols is their interactions with other components present in the food matrix, including proteins, carbohydrates and lipids [1]. Proteins as building blocks have both nutritional and functional properties and are an important dietary source of amino acids [2]. As an outcome of interactions between polyphenols and proteins, various complexes can be formed, which consequently cause changes in antioxidant properties of polyphenols and affect the functional, structural and nutritional properties of proteins [3]. Flavonoids are an important subgroup of polyphenols and quercetin, as a member of the flavonol subclass of flavonoids is found in various fruits, vegetables and tea [4]. The intake of quercetin is related to the assembly of beneficial health properties such as antioxidant, anti-inflammatory and anti-viral properties, improvement in cardiovascular health [5] and reducing the intensity of the symptoms and negative predictors of COVID-19 [6], which is a hot topic nowadays. Its anticancer effect was also established in numerous studies [7,8]. For the aforementioned reasons, the involvement of quercetin in the human diet is strongly recommended and it is proposed as an excellent ingredient for functional foods [9]. On the other hand, its broader application is limited because of insufficient water solubility and chemical instability. The application of an adequate delivery system is one way to achieve stabilization and improvement of the health benefits of quercetin. The "Delivery by design" approach leads to the expansion of the search for effective delivery systems of bioactives in order to encapsulate them; on the one hand for their protection from environmental factors and on the other hand to control their release under defined conditions. Possibilities for the application of delivery systems can be various from food, agrochemical, pharmaceutical, cosmetic to personal care industries [10].

There are various types of delivery systems, and each one has specific advantages and disadvantages; thus, the selection should be based on the field of utilization [11]. Both animal-based and plant-based protein matrices were used for complexation with polyphenols. The most used animal-based protein matrices were whey, gelatin, milk proteins, bovine serum albumin [12–16]. Applications of plant-based protein matrices are gaining higher attention and proteins from different sources such as sunflower, legume seed, corn kernels, quinoa, wheat, rice, peas, hemp, almond and pumpkin [17–28] have been used for the preparation of complexes which can be further used as functional food additives. As a result of consumers' awareness of the importance of a healthy and balanced diet, functional foods are becoming more popular. In addition, convenience is becoming a very important element in the selection of foods. A very busy and mobile lifestyle, the search for simple meal preparation and easier consumption, healthy snacking options in as well as outside of homes are also some of the emerging trends among consumers [3,17,19,29] that need to be addressed by the food industry.

The aim of this research was to prepare microparticles from brown rice or almond protein matrices and quercetin in order to investigate whether different concentrations (1 mM, 2 mM or 5 mM) of quercetin in the initial mixture had an impact on its adsorption on proteins. Determination of quercetin concentration using high-performance liquid chromatography (HPLC) and antioxidant activities of prepared microparticles were performed. Additionally, DSC and FTIR-ATR screening of microparticles were performed. DSC analysis was conducted to evaluate the effect of quercetin interactions with proteins on the thermal stability of protein matrices, while screening of IR spectra was performed as proof that interactions between quercetin and protein matrices occurred.

2. Materials and Methods

2.1. Materials

Almond protein matrix was purchased from Raab Vitalfood GmbH (Rohrbach, Germany) and brown rice protein matrix was from Kernnel premium (Zagreb, Croatia). Quercetin, trolox and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate was obtained from Kemika (Zagreb, Croatia). Neocuproine, copper (II) chloride and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) were obtained from Acros Organics (Geel, Belgium). Orthophosphoric acid (HPLC grade > 85%) was obtained from Fisher Scientific (Loughborough, UK), while methanol (HPLC grade) was purchased from J.T. Baker (Deventer, The Netherlands). Iron (III) chloride hexahydrate, ethanol, sodium acetate and ammonium acetate were purchased from Gram-mol (Zagreb, Croatia).

2.2. Preparation of Protein/Quercetin Microparticles

The microparticles were formulated by the complexation of protein matrices (constant amounts; 5%) with 20 mL of quercetin ethanol solution (1 mM, 2 mM or 5 mM). Two protein matrices were used (both are usually used as dietary supplements), brown rice with approximately 85% of proteins (7.7% of carbohydrates and 5.1% of lipids), and almond with approximately 50% of proteins (fibers 17%, 9% of carbohydrates and 11% of lipids). The preparation method was adapted from other studies [21,27,28]. The protein matrix was weighed and added to the quercetin solution. In order to prepare protein/quercetin microparticles, obtained mixtures were mixed on a magnetic stirrer for 15 min at room temperature. During that time, part of quercetin was adsorbed onto the protein matrix and the other part remained in the solution. Afterward, well-homogenized mixtures were centrifuged for 15 min at 4000 rpm in order to remove the quercetin that did not adsorb onto the protein matrix and remained in the supernatant. The supernatant was discarded and the wet–solid phase that represented adsorbed quercetin onto protein matrix was collected. After air-drying, protein/quercetin microparticles were obtained in the form of dried powder.

2.3. Extraction of Quercetin from Protein/Quercetin Microparticles

Quercetin was extracted from obtained protein/quercetin microparticles. Microparticles were weighted (0.15 g), 10 mL of acidified methanol (methanol:HCl ratio was 99:1) was added and the obtained mixture was well homogenized. Extraction was conducted at room temperature for 24 h. After that time, mixtures were filtered to obtain clear extracts which were immediately utilized for the determination of the amount of quercetin and antioxidant activities.

2.4. Reverse-Phase High Performance Liquid Chromatography (RP-HPLC)

A RP-HPLC system (1260 Infinity II; Agilent technology, Santa Clara, CA, USA) was used for the evaluation of the amount of quercetin. The system was equipped with a DAD (diode array) detector, a quaternary pump and a column (poroshell 120 EC-C 18; 4.6 × 100 mm, 2.7 μm). Two mobile phases were used; mobile phase A was orthophosphoric acid (0.1%) and mobile phase B was methanol (100%). The gradient that was applied for separation was described in previous studies [27,28]. The injection volume of the extract was 10 μL, under the flow rate of 1 mL/min at room temperature. A calibration curve for quercetin was constructed in the range from 5 to 150 mg/L. UV/Vis spectra was screened in the range from 190 to 600 nm, and quercetin was determined at 360 nm. Duplicate evaluations were conducted.

2.5. Antioxidant Activity

DPPH, CUPRAC and FRAP methods were utilized for the evaluation of antioxidant activities of microparticles extracts. Details for these methods were previously given by Buljeta et al. [30]. Assays were performed in triplicate and results were presented as micromoles of Trolox equivalent per 100 g of sample (μmol TE/100 g).

2.6. Differential Scanning Calorimetry (DSC)

For the DSC scanning of microparticles, a differential scanning calorimeter (Mettler Toledo 822, Mettler Toledo, Greifensee, Switzerland) was applied. In a 40 μL aluminum pan, 7 ± 0.2 mg of microparticles was weighed. The aluminum pan was covered and then inserted into the oven of the DSC. Screening of microparticles was performed from 25 °C to 140 °C. Firstly, samples were left for 4 min at 25 °C. Afterwards, the temperature was increased at a rate of 5 °C/min up to 140 °C, where samples were also left for 4 min. Duplicate screenings were conducted.

2.7. Fourier-Transform Infrared Spectroscopy-Attenuated Total Reflectance (FTIR-ATR)

The IR spectra of protein matrices and protein matrices loaded with quercetin were recorded using FTIR-ATR (Cary 630 FTIR spectrometer, Agilent Technology, Santa Clara, CA, USA), equipped with software MicroLab Expert. Samples were screened in the interval from 4000 cm^{-1} to 600 cm^{-1}.

2.8. Statistical Analysis

STATISTICA 13.1 (StatSoft Inc., Tulsa, OK, USA), the software program, was utilized for analyzing the obtained results. Variance analysis (ANOVA) and Fisher's least significant difference (LSD) with significance defined at $p < 0.05$ were selected for statistical evaluation of the results, which were presented as mean value ± standard deviation.

3. Results

3.1. Quercetin Amount and Antioxidant Activity of Protein/Quercetin Microparticles

The amounts of quercetin and antioxidant activities of protein/quercetin microparticles are given in Table 1. Comparison of almond protein/quercetin (AP/Q) microparticles and brown rice/quercetin (RP/Q) microparticles showed that RP/Q microparticles had a higher amount of quercetin than AP/Q microparticles prepared with the same initial concentration of quercetin, indicating that the brown rice protein matrix had a higher

affinity towards quercetin. Additionally, it can be observed that an increase in concentration of quercetin in the initial solution for complexation, resulted in an increase in the amount of quercetin. Results were compared in order to investigate whether this increase in quercetin amount was proportional to the initial concentration of quercetin. Amounts of quercetin on AP/Q microparticles were 60.18, 98.38 and 196.34 mg/100 g for AP/Q_1, AP/Q_2 and AP/Q_5 (i.e., 1 mM, 2 mM and 5 mM of quercetin in initial solution), respectively. Results indicated that with the double increase in the initial quercetin, the resulting concentration increase was 1.6 times, and for the five times increase in the initial quercetin concentration, the resulting increase was 3.7 times higher. For RP/Q microparticles, a different trend was observed. Amounts of quercetin on microparticles were 108.24, 226.50 and 506.98 mg/100 g for RP/Q_1, RP/Q_2 and RP/Q_5 (i.e., 1 mM, 2 mM and 5 mM of quercetin in initial solution), respectively. Results indicated that with a double increase in initial quercetin concentration, the increase was 2.1 times and with a five times increase in initial quercetin concentration, the resulting increase was 4.7 times higher. Interesting data were obtained by comparing the amount of the quercetin on microparticles to the initial amount of quercetin, i.e., calculating the adsorption efficiency of protein matrices towards the quercetin. For the almond protein matrix, as was expected, a lower efficiency was determined than for the brown rice protein matrix. Even though the quercetin amount increased on A/Q microparticles with the initial quercetin amount, a decrease in adsorption efficiencies were observed; 20%, 16.3% and 13% for AP/Q_1, AP/Q_2 and AP/Q_5, respectively. For RP/Q microparticles, a slightly different trend was obtained, i.e., for RP/Q_1 and RP/Q_2, adsorption efficiencies were 36.8% and 37.5%, and for RP/Q_5, 33.6%.

Table 1. Amount of quercetin (mg/100 g) and antioxidant activity (μmol TE/100 g) of protein/quercetin microparticles.

Microparticles	Q Amount	DPPH	CUPRAC	FRAP
Almond protein matrix				
AP/Q_1	60.18 ± 0.17^{a}	29.38 ± 0.95^{a}	106.39 ± 0.93^{b}	0.66 ± 0.04^{a}
AP/Q_2	98.38 ± 1.21^{b}	30.63 ± 0.21^{a}	125.42 ± 1.91^{c}	1.02 ± 0.01^{b}
AP/Q_5	196.34 ± 1.45^{d}	32.18 ± 0.00^{b}	192.75 ± 1.14^{e}	2.34 ± 0.08^{c}
Brown rice protein matrix				
RP/Q_1	108.24 ± 1.75^{c}	$31.12 \pm 0.47^{a,b}$	84.38 ± 1.91^{a}	1.20 ± 0.02^{b}
RP/Q_2	226.50 ± 3.17^{e}	34.22 ± 0.12^{c}	161.59 ± 1.19^{d}	2.78 ± 0.04^{c}
RP/Q_5	506.98 ± 0.42^{f}	40.40 ± 0.52^{d}	414.52 ± 1.03^{f}	8.35 ± 0.19^{d}

Q—quercetin; AP—almond protein matrix; RP—brown rice protein matrix; 1, 2 and 5—represent concentration (mM) of initial quercetin solution; data in one column labeled with different letters statistically differ.

For the evaluation of antioxidant activities of microparticles, three methods were selected: DPPH, FRAP and CUPRAC methods. Values for antioxidant activities obtained with all methods followed the amount of quercetin, i.e., an increase in quercetin amount caused an increase in antioxidant activity. However, a trend of the proportional increase in antioxidant activity with the quercetin amount was not observed for all methods. DPPH antioxidant activities for AP/Q microparticles were from 29.38 to 32.18 μmol TE/100 g, while for RP/Q, microparticles values were slightly higher, from 31.12 to 40.40 μmol TE/100 g. Values of antioxidant activities were much higher with the CUPRAC method and they ranged from 106.39 to 192.75 μmol TE/100 g for AP/Q microparticles and from 84.30 to 414.52 μmol TE/100 g for RP/Q microparticles. With the FRAP method, the lowest values of antioxidant activities were obtained. For AP/Q microparticles, they ranged from 0.66 to 2.34 μmol TE/100 g, and for RP/Q microparticles, from 1.20 to 8.35 μmol TE/100 g.

3.2. Temperature of Denaturation of Protein Matrices and Protein/Quercetin Microparticles

The results of the DSC determination of denaturation temperatures of protein matrices and protein/quercetin microparticles are presented in Table 2. Both protein matrices had similar values of denaturation temperature at 85.25 °C. When comparing the obtained microparticles, AP/Q microparticles had lower denaturation temperatures (around 83.5 °C) than the corresponding protein matrix, while RP/Q microparticles had it higher (from 85.78 to 86.72 °C). Additionally, a difference in the enthalpy of denaturation was observed. For RP/Q complexes the enthalpy of denaturation increased for 3 J/g, while for AP/Q complexes, the increase was for 1.5 J/g.

Table 2. Temperatures of denaturation (T_d) of protein matrices and protein/quercetin microparticles.

Samples	T_d (°C)
Almond protein matrix	
100%	85.24 ± 0.07 [b]
AP/Q_1	83.71 ± 0.28 [a]
AP/Q_2	83.27 ± 0.25 [a]
AP/Q_5	83.24 ± 0.29 [a]
Brown rice protein matrix	
100%	85.26 ± 0.05 [b]
RP/Q_1	85.78 ± 0.12 [c]
RP/Q_2	86.70 ± 0.14 [d]
RP/Q_5	86.72 ± 0.22 [d]

Q—quercetin; AP—almond protein matrix; RP—brown rice protein matrix; 1, 2 and 5 represent concentration (mM) of initial quercetin solution; data in column labeled with different letters statistically differ.

3.3. FTIR-ATR Spectra of Protein Matrices and Protein/Quercetin Microparticles

Changes in IR spectra that were obtained by FTIR-ATR screening of protein/quercetin microparticles prepared with different initial concentrations of quercetin were the same, so only one IR spectra of microparticles was presented (the one obtained with 5 mM of quercetin). Figure 1 represents the IR spectra of the almond protein matrix and almond protein/quercetin microparticle. Comparing those two spectra, changes in protein structure after the adsorption of quercetin were established. In two regions, one from 3500 cm^{-1} to 3000 cm^{-1} and another one from 1650 cm^{-1} to 600 cm^{-1}, the intensity of the protein spectra was lower than for the microparticle. However, in the region from 3000 cm^{-1} to 2800 cm^{-1} and for the band at 1745 cm^{-1}, a reverse tendency was observed. The region from 3500 cm^{-1} to 3000 cm^{-1} can be assigned to amide A, N-H stretching coupled with hydrogen bonding. Additionally, in this region, the band at 3004 cm^{-1} assigned to the C-H bond disappeared after quercetin adsorption. In the region from 3000 cm^{-1} to 2800 cm^{-1}, two bands were detected, one at 2922 cm^{-1} and another one at 2855 cm^{-1} both assigned to CH_2 stretching. A band at 1745 cm^{-1} can be assigned to the C=O band of polysaccharides. Additionally, a change in the Amid I structure of protein occurred. A band at 1632 cm^{-1} shifted to 1625 cm^{-1} after the adsorption of quercetin. One additional change caused by quercetin adsorption was a loss of shoulder at 1141 cm^{-1} (assigned to C-O band of oligosaccharides) on the protein matrix.

Figure 2 represents IR spectra of other set of samples, i.e., the brown rice protein matrix and brown rice protein/quercetin microparticle. Even though results of the amount of adsorbed quercetin showed that RP/Q microparticles had a higher amount of this phenolic, structural changes were not so pronounced as for AP/Q microparticles. Two bands that were detected at 2922 cm^{-1} and 2855 cm^{-1}, both assigned to CH_2 stretching, after adsorption of quercetin, shifted to 2929 cm^{-1} and 2875 cm^{-1}. Another change was

detected at 1737 cm^{-1} (assigned to C=O band of polysaccharides) that disappeared after the adsorption of quercetin.

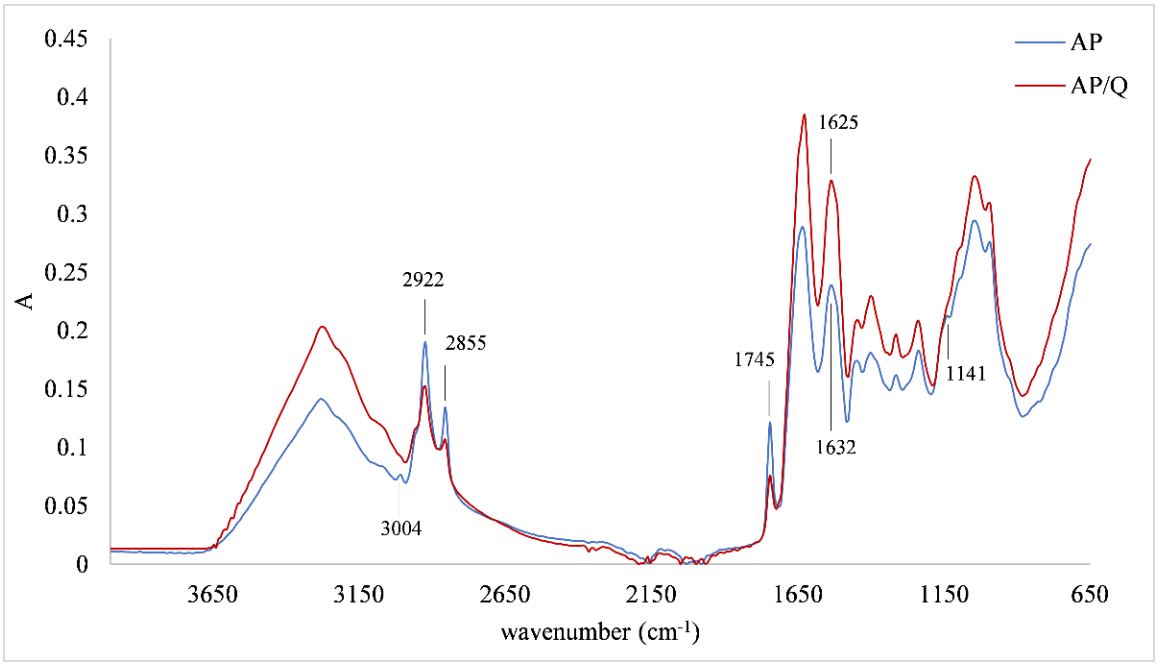

Figure 1. IR spectra of almond protein matrix (AP) and almond protein/quercetin microparticles (AP/Q).

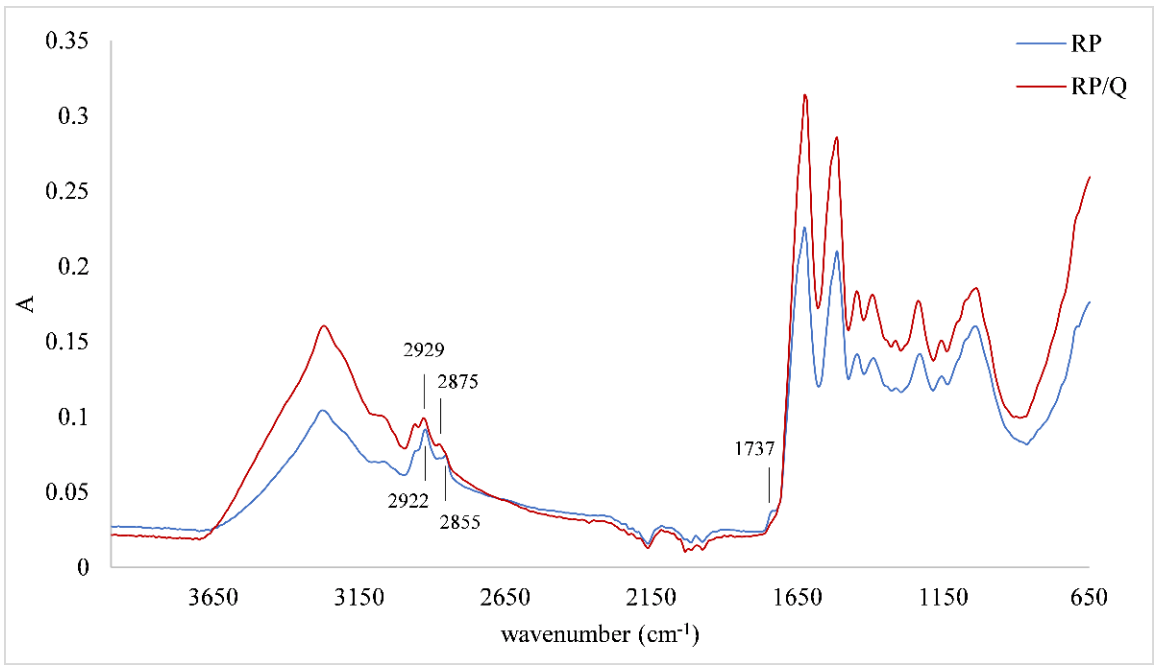

Figure 2. IR spectra of brown rice protein matrix (R) and brown rice protein/quercetin microparticles (RP/Q).

4. Discussion

There have been many different instrumental techniques used for the characterization of protein/phenolics complexes [31]. In this study, we applied HPLC, DSC and FTIR-ATR analyses for evaluation of the adsorption of quercetin on selected protein matrices.

Interactions that are created between phenolics and proteins upon their complexation depend on the structure of both compounds, as well as complexation conditions [32,33]. Similar protein matrices for the adsorption of different phenolics were used in other studies. Adsorption of glucosyl-hesperidin on pea, almond, pumpkin and brown rice protein matrices revealed that glucosyl-hesperidin was determined in the highest amount on pea protein microparticle, followed by almond, brown rice and in the lowest amount on pumpkin protein microparticles [27]. For the adsorption of cinnamic acid on pea, almond and pumpkin protein matrices, a different trend was observed; hence, cinnamic acid had the highest affinity for pumpkin and the lowest for almond protein matrices [28]. The investigated protein matrices differ in the protein content; pea and brown rice protein matrices had 85% of protein content, while the almond and pumpkin had 50%. Other organic molecules such as polysaccharides can be incorporated in different types of protein matrices [3,27,28,31,34], consequently having an effect on the adsorption of phenolics onto them. As a result of the encapsulation of cranberry phenolics onto different protein matrices with 50% of proteins (medium roast peanut or defatted soy flours) to protein matrices with over 70% of proteins (pea, soy or hemp protein isolates), a non-linear trend was achieved when protein amount and the adsorption capacity for phenolics were put in correlation [17]. In that study, cranberry phenolics had the highest affinity towards defatted soy and medium roasted peanut flours as well as towards hemp protein isolate. Additionally, a mentioned non-linear trend was obtained in another study, which deals with the adsorption of blueberry anthocyanins on matrices with lower amounts of proteins such as corn flour (5.3%), brown rice flour (8.6%), white whole-wheat flour (13%) and defatted soy flour (47%) [21]. Generally looking, the reactivity of phenolics towards proteins is correlated with two main factors, one is the number of hydroxyl groups and the other is hydroxyl groups position in phenolics structure [32]. The binding capacity of some phenolics (quercetin, kaempferol, myricetin, flavone apigenin, chlorogenic acid, caffeic acid, gallic acid) towards soy protein revealed that among those phenolics, the highest affinity had gallic acid, followed by chlorogenic acid and quercetin [32]. Comparison of the binding capacity of chlorogenic acid, ferulic acid, gallic acid, catechin, quercetin and apigenin towards albumin and globulin was conducted. Authors have determined that quercetin and catechin had equal binding capacity towards albumin; it was lower than for chlorogenic and gallic acid but higher than for apigenin and ferulic acid. The binding capacity of quercetin towards globulin was lower than for chlorogenic acid, catechin and gallic acid, but higher than for apigenin and ferulic acid [33]. Different studies emphasized that covalent and/or non-covalent interactions can occur between phenolics and proteins [34–36]. Non-covalent ones include interactions through hydrogen bonds, hydrophobic association, van der Waals forces and electrostatic attraction. However, as the most important non-covalent interactions for the complexation of proteins with phenolics, hydrophobic interaction and hydrogen bonds were pointed out [37]. Sui et al. [38] studied the binding of anthocyanins with soy protein isolate while increasing the amount of anthocyanins in the initial mixture and determined that the increase in binding of anthocyanins was proportional to their increase in the initial mixture. We obtained similar results in our study for the brown rice protein matrix that contained a higher protein content, which could be the reason for more pronounced hydrophobic interactions.

Numerous methods for evaluation of the antioxidant activity of foods, dietary supplements and nutraceuticals are available in the literature and can be applied. We selected DPPH, FRAP and CUPRAC methods, which are based on different mechanisms of action. The DPPH method is based on the reaction of radicals with hydrogen-donating antioxidants, which leads to the formation of the non-radical form. One of the characteristics of the DPPH radical is its selectivity in the reaction with hydrogen donors [39]. From our results, it can be observed that with the increase in quercetin amount, DPPH antioxidant activity increased; however, this increase was not proportional to the increase in quercetin amount on microparticles. Results of the other two methods that were used better followed the trend of quercetin amount on microparticles. In CUPRAC assay, the reduction of

Cu(II) to Cu(I) by antioxidants is measured spectrophotometrically, while the reduction of Fe(III) complex to Fe(II) caused by the presence of antioxidants is assessed by FRAP [40,41]. According to our results, microparticles had a significantly higher capability of reduction of Cu(II) to Cu(I) than of Fe(III) to Fe(II).

Interactions between phenolics and proteins can cause a change in the denaturation temperature of the corresponding protein matrix. Usually, this parameter is used for the prediction of thermal stability of formulated protein/phenolic complexes [32]. When the denaturation temperature of formulated protein/phenolic microparticles is higher than the denaturation temperature of the corresponding protein matrix, the formulated microparticle is more stable than the protein matrix and vice versa [42]. From our results, it can be concluded that the adsorption of quercetin on the brown rice protein matrix resulted in its thermal stabilization. However, quercetin affected the almond protein matrix differently, i.e., it caused the decrease of thermal stability. Results of the other studies showed positive, negative or no effect on proteins stability depending on types of proteins and phenolics. The increase in thermal stability was achived when cinnamic acid was adsorbed on the almond protein matrix, while adsorption of the same phenolic acid onto the pea and pumpkin protein matrix had the opposite effect [27]. Green tea polyphenols caused a decrease of the thermal stability of β-lactoglobulin and egg albumen [42,43]. Complexes of soy protein with quercetin, myricetin or phenolic acids had higher stability than protein alone, while flavone, apigenin or kaempferol did not affect the stability of the mentioned protein [32]. The adsorption of chlorogenic acid on lysozyme, bovine serum albumin and α-lactalbumin also caused the increase of thermal stability of those proteins [44,45]. A conjugate of (−)-epigallocatechin gallate and zein had higher thermal stability than pure zein, while conjugates of zein and quercetagetin or chlorogenic acid had similar denaturation temperatures as protein alone [46]. A decrease of thermal stability was also observed upon the adsorption of raspberry juice phenolics onto brown rice proteins [26].

Structural changes of proteins upon adsorption of phenolics depend on protein structure that can be proved by a recoding of the IR spectra of microparticles and their comparison with the protein matrix. On both types of microparticles (AP/Q and RP/Q), a difference at the amide A region (3500 cm^{-1} to 3000 cm^{-1} assigned to N-H stretching coupled with hydrogen bonding) was observed, which could be an indication of non-covalent interactions between proteins and phenolics, i.e., indication of hydrogen bonding or hydrophobic association [46]. These interactions were probably involved in the adsorption of quercetin onto two selected matrices in our study. Alternation in IR spectra of proteins in the region of 3000 to 2800 cm^{-1} (assigned to CH_2 antisymmetric and symmetric stretching vibrations) are an indicator of the existence of hydrophobic contact in the protein/phenolic complexes [47]. Hasni et al. [47] determined these changes in the complexes between α-caseins and β-caseins with tea phenolics. Based on the shifting of the protein antisymmetric and symmetric CH_2 stretching vibrations, they proposed the existence of hydrophobic interactions throughout phenolics rings and hydrophobic pockets in caseins. Considering these results, we can also assume that those hydrophobic interactions occurred between the quercetin and protein matrices used in this study. In RP/Q microparticles, we also observed a shift of bands in this region that can be connected to hydrophobic interactions, as proposed before. On the other hand, on IR spectra of AP/Q microparticle, a change in intensity of bands in this region occurred, i.e., upon adsorption of quercetin, a decrease of the band intensity of protein occurred, suggesting a similar mechanism of interactions. Additionally, these different changes in protein matrix structure could be explained by the fact that the brown rice protein matrix had higher (85%) while almond had lower (50%) protein content; therefore, shifting was more pronounced on RP/Q microparticles. In addition, according to the quercetin amount adsorbed on protein matrices, these interactions were more pronounced for RP/Q microparticles since they contained a higher amount of this phenolic compound and its adsorption was proportional to initial concentration. Probably, these hydrophobic interactions as well as the higher amount of adsorbed quercetin were the reason for the improvement of thermal stability of RP/Q microparticles. Additionally,

there was no change in IR spectra in Amid I structure, which could also lead to higher thermal stability of RP/Q complexes.

5. Conclusions

Functional ingredients with a broad range of applications are becoming more and more popular. Two plant-based protein matrices, brown rice and almond, were chosen for complexation with quercetin. The brown rice protein matrix had a higher affinity for quercetin than almond; thus, it would be a more efficient carrier of this phenolic compound. Additionally, it was observed for the brown rice protein matrices that with the increase in quercetin concentration in the initial mixture, the amount of quercetin on microparticles proportionally increased. This trend was not observed for the almond protein matrix. Additionally, brown rice protein microparticles were thermally more stable in comparison to the corresponding protein matrix, while the reverse trend was determined for almond protein microparticles.

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Ultrasonic Extraction of Bioactive Compounds from Green Soybean Pods and Application in Green Soybean Milk Antioxidants Fortification

Noppol Leksawasdi [1], Siraphat Taesuwan [1,2,3], Trakul Prommajak [4], Charin Techapun [1], Rattanaporn Khonchaisri [2], Nattha Sittilop [2], Anek Halee [5], Kittisak Jantanasakulwong [1,6], Suphat Phongthai [1,2], Rojarej Nunta [7], Maneerat Kiadtiyot [2], Arisa Saefung [2] and Julaluk Khemacheewakul [1,2,3,*]

1 Agro-Bio-Circular-Green and Bioprocess Research Cluster, Faculty of Agro-Industry, School of Agro-Industry, Chiang Mai University, Chiang Mai 50100, Thailand; noppol.l@cmu.ac.th (N.L.); siraphat.t@cmu.ac.th (S.T.); charin.t@cmu.ac.th (C.T.); jantanasakulwong.k@gmail.com (K.J.); su.phongthai@gmail.com (S.P.)
2 Division of Food Science and Technology, Faculty of Agro-Industry, School of Agro-Industry, Chiang Mai University, Chiang Mai 50100, Thailand; rattanaporn.khon@gmail.com (R.K.); nattha.sittilop@gmail.com (N.S.); jennymaneerat11@gmail.com (M.K.); pedfung48@gmail.com (A.S.)
3 Cluster of Innovative Food and Agro-Industry, Chiang Mai University, Chiang Mai 50100, Thailand
4 Division of Food Safety, School of Agriculture and Natural Resources, University of Phayao, Phayao 56000, Thailand; tpromjak@gmail.com
5 Division of Food Science and Technology, Faculty of Science and Technology, Kamphaeng Phet Rajabhat University, Kamphaeng Phet 62000, Thailand; nek_ha@hotmail.co.th
6 Division of Packaging Technology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai 50100, Thailand
7 Division of Food Science and Technology, Faculty of Science and Technology, Lampang Rajabhat University, Lampang 52100, Thailand; quan_rn@hotmail.com
* Correspondence: julaluk.kh@cmu.ac.th; Tel.: +66-991414244

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Abstract: Green soybean (*Glycine max* L.) pods (GSP) are agro-industrial waste from the production of frozen green soybean and milk. These pods contain natural antioxidants and various bioactive compounds that are still underutilized. Polyphenols and flavonoids in GSP were extracted by ultrasound technique and used in the antioxidant fortification of green soybean milk. The ultrasound extraction that yielded the highest total polyphenol content and antioxidant activities was 50% amplitude for 10 min. Response surface methodology was applied to analyze an optimum ultrasonic-assisted extraction (UAE) condition of these variables. The highest desirability was found to be 50% amplitude with an extraction time of 10.5 min. Under these conditions, the experimental total phenolic content, total flavonoid content, and antioxidant activity were well matched with the predicted values ($R^2 > 0.70$). Fortification of the GSP extracts (1–3% *v*/*v*) in green soybean milk resulted in higher levels of bioactive compounds and antioxidant activity in a dose-dependent manner. Procyanidins were found to be the main polyphenols in dried GSP crude extracts, which were present at a concentration of 0.72 ± 0.01 mg/100 g. The addition of GSP extracts obtained by using an ultrasound technique to green soybean milk increased its bioactive compound content, especially procyanidins, as well as its antioxidant activity.

Keywords: green soybean; ultrasonic extraction; bioactive compounds; antioxidant; dairy product; fortification; pod; *Glycine max* L.

1. Introduction

Free radicals shorten the shelf-life of food products as well as increase oxidative stress within cells, a recognized pathologic pathway of several chronic diseases [1]. During the past two decades, the utilization of either bioactive compounds or natural antioxidants in food products or by-products through both non-biological [2] or biological means [3–6] has gained considerable interests due to the participating roles of these compounds in

absorption and neutralization of free radicals, thereby slowing down the autoxidation process. The benefits of natural antioxidants were due not only to their biological values, but also to their economic impact, as most of them can be extracted from food by-products and under-exploited plant species [7].

Legumes are an excellent source of bioactive compounds with antioxidant capacity such as flavonoids, anthocyanin, and other phenolic compounds. Recent studies showed that a high intake of legumes protected the human body against oxidative damage and reduced the risk of type 2 diabetes mellitus [8]. Green soybean (*Glycine max* L.) is a legume that has been shown to exhibit strong antioxidant activity. Peiretti et al. [9] stated that black and green soybean exhibited comparatively higher FRAP values than yellow soybean. Hence, many researchers try to use green soybean as an alternative supplement source of bioactive compounds in food products such as butter cake [10] and cookies [11]. Green soybean pods (GSP), a by-product of green soybean processing, potentially contain natural antioxidants, but research on the antioxidant capacity of these GSP is still limited.

Ultrasonic assisted extraction (UAE) has been widely used to extract antioxidants from plants. High-frequency ultrasonic waves induce contraction and expansion cycles that subsequently cause cavitation, breakage of plant cell walls, and infiltration of solvents into cells. The extraction rate and yield of UAE are influenced by several factors, including ultrasonication time and amplitude [12]. Viell et al. [13] compared homogenizer-assisted extraction with UAE for flavonoid content from teff grains (*Eragrostis tef* (Zucc.) Trotter). Under optimal conditions, the total flavonoid content and the antioxidant capacity were significantly higher when UAE was used. Crupi et al. [14] stated that UAE offers efficiency and reproducibility advantages compared to conventional techniques due to their time-saving, ease of procedure, and environment-friendly properties, as well as yielding a cost-effective output of high-quality phenolic extracts. Therefore, UAE could be an alternative green technology over conventional methods (e.g., distillation, maceration, and Soxhlet), which use high temperatures and concentrations of organic solvents [15]

The objectives of the present work were (1) to optimize the UAE process with water as eco-friendly solvent to obtain GSP extracts enriched in antioxidants in the classes of total content of phenolic and flavonoid; (2) to investigate the antioxidant capacity of green soybean milk supplemented with GSP extracts; and (3) to identify some specific antioxidants in the GSP extracts. The novelties of this study were the use of a green technology in the extraction of natural antioxidants from GSP by-products and the demonstration of real-world application in green soybean milk fortification, which will encourage technology adoption by the industrial sector.

2. Materials and Methods

2.1. Materials

Whole green soybeans (*Glycine max* L.) were obtained from Lanna Agro Industry Co., Ltd. (LACO, Chiang Mai, Thailand). GSPs were separated from whole beans and washed using tap water. The pods were further cut into small pieces of approximately 1 cm squares using a stainless-steel knife and oven-dried at 60 °C for 24 h in a hot-air oven (Memmert UF 110, Schwabach, Germany) until moisture content decreased below 10% [16]. Dried GSPs were ground to fine powder using an electric chopper (Model DPA130, Tefal, France), and the powder was sieved through a 20-mesh sieve. Finally, the dried powder samples were packed in vacuum polyethylene bags and stored at 4 °C before use. All chemicals used were analytical grade.

2.2. Ultrasonic-Assisted Extraction

Ultrasonic-Assisted Extraction (UAE) was carried out according to our previous study, Zhou et al. [17], and Sharayei et al. [18], with some modifications. Specifically, 5 g of dried GSP powder was placed in a 250 mL beaker and extracted with 100 mL distilled water using an ultrasonic probe (VX500, Hartford, CT, USA) with a maximum power of 500 W at 20 kHz frequency. The ultrasonication extraction process was carried out in an ice-water

bath to prevent heating of samples for three different durations (10, 15, and 20 min) and three different amplitudes (30, 40, and 50%) [19]. The mixtures were centrifuged at 3000× *g* for 15 min at 4 °C (Nüve NF400R, Ankara, Turkey), and the supernatant was filtered through filter paper (Whatman No. 1, Wallingford, UK). The filtered extracts were collected in a centrifuge tube and kept at −18 °C until further analysis.

2.3. Preparation of Green Soybean Milk

Fresh green soybean seeds (500 g) were soaked in 2000 mL of tap water for 1 min. The beans were then ground and blended in 1000 mL of drinking water using a blending machine (HR2602, Philips, Ningbo, China) at medium speed until homogeneous (approximately 10 min). The mixture was filtered using a muslin cloth to obtain green soybean milk, to which the GSP extracts were added at the concentration levels of 0 (control), 1, 2, and 3% (*v*/*v*). The green soybean milk was then boiled at 95 °C for 20 min before being immediately placed in an ice bath. The cooled samples were then stored at −18 °C until further analysis.

2.4. Determination of Total Phenolic Compounds

Total phenolic compounds were analyzed using the Folin–Ciocalteu method, with some modifications [17]. A 500 µL properly diluted sample or standard solutions of varying concentrations were mixed with 2.5 mL of 1:10 Folin–Ciocalteu:water solution and then thoroughly mixed. After incubation for 8 min at room temperature, 2 mL of 7.5% (*w*/*v*) Na_2CO_3 solution was added, and the mixture was immediately mixed and incubated for 2 h. Absorbance was read at 765 nm on a spectrophotometer (G10S UV-Vis, Thermo Fisher Scientific, Waltham, MA, USA). Measurements were conducted in quadruplicate. One mg/mL gallic acid was used as the standard, and the total phenolic compounds of the samples were expressed in mg gallic acid equivalent (GAE) per g sample (mg GAE/g).

2.5. Determination of Total Flavonoid Content

Samples (0.25 mL) were mixed with 1.25 mL of distilled water and 75 µL of 5% $NaNO_2$ solution and incubated for 6 min at room temperature. The mixture was then combined with 150 µL of 10% $AlCl_3$ and 500 µL of 1 M NaOH and brought to 275 mL with distilled water. The solution was mixed thoroughly and left for 5 min at room temperature. Its absorbance was recorded using a spectrophotometer at 510 nm. Catechin equivalents (CAE) per g of sample (mg CAE/g) were used to express total flavonoid contents [20].

2.6. Determination of Antioxidant Activity

2.6.1. DPPH Free Radical Scavenging Activity

DPPH (2,2-diphenyl-1-picryl-hydrazyl radical) solution was used to estimate antioxidant activity according to the method described by Lu et al. [21], with minor modifications. Briefly, 150 µL of samples were mixed with 3 mL of 0.6 mM DPPH. The mixture was incubated for 30 min in the dark to allow for complete reaction. Absorbance values of each sample and control (distilled water) were read using a spectrophotometer at 517 nm. The antioxidant activity of each sample was expressed as µmol of Trolox equivalent per g of sample (µmol Trolox/g).

2.6.2. FRAP Free Radical Scavenging Activity

The Fe^{2+} chelating activity of the samples was measured by the method of Sharma et al. [22], with minor modifications. The ferric reducing antioxidant power (FRAP) reagent was prepared by mixing 25 mL of 0.3 M acetate buffer (pH 3.6/22.8 mM sodium acetate trihydrate) with 2.5 mL of 0.01 M 2,4,6-Tripyridyl-s-Triazine solution and 2.5 mL of 0.02 M $FeCl_3.6H_2O$. An amount of 150 µL of the sample or the control (methanol) was then added to 2850 µL of the FRAP reagent. The reaction mixture was incubated at room temperature in the dark for 30 min, and absorbance was assessed at 593 nm. The antioxidant activity of each sample was expressed as µmol Trolox/g.

2.7. Sensory Evaluation of Green Soybean Milk

One hundred untrained panelists (20–40 years of age) were recruited from the Division of Food Science and Technology, Faculty of Agro-industry, Chiang Mai University, Thailand. The samples were coded with a three-digit random number and presented to the panelists. Water was provided for rinsing the mouth between samples. The panelists were asked to provide acceptance scores for color, texture, aroma, sweet taste, salt taste, and overall acceptability based on a standard nine-point hedonic scale (9 = like extremely, 8 = like very much, 7 = like moderately, 6 = like slightly, 5 = neither like nor dislike, 4 = dislike slightly, 3 = dislike moderately, 2 = dislike very much and 1 = dislike extremely) [23].

2.8. Quantification of Phenolic Compounds by HPLC

The phenolic compounds (procyanidins, quercetin, glycitin, daidzein, genistin, and linalool) in GSP extracts and green soybean milk were analyzed by high-performance liquid chromatography (HPLC), as previously reported [17,24], with some modifications. Briefly, an Agilent HPLC system (Agilent Technologies, Santa Clara, CA, USA), consisting of a binary pump and a photodiode-array detector equipped with an Agilent Zorbax C18 (4.6 × 250 mm, 3.5 μm) column was employed. The mobile phase consisted of solution A (0.1% *v/v* trifluoroacetic acid) and solution B (pure methanol), which were used to create gradients according to the following program: 0 min, 15% B; 5 min, 25% B; 9 min, 55% B; 12 min, 75% B; 15 min, 75% B; 18 min, 15% B. The flow rate was 0.8 mL/min, and the injection volume was 5.0 μL for procyanidins, quercetin, glycitin, daidzein, and genistin. The program for linalool was 0–20 min, 85% B. The detection wavelength was set at 260 nm for procyanidins, quercetin, glycitin, daidzein, and genistin, and at 210 for linalool. The result was expressed as mg of procyanidins, quercetin, glycitin, daidzein, genistin, and linalool equivalent per 100 g of sample.

2.9. Statistical Analysis

Amplitude and exposure time variables were analyzed using two-way analysis of variance for each of the four measures of ultrasonic performance. Comparison among different proportions of GSP supplements in green soybean milk on the antioxidant and sensory characteristic were analyzed using one-way analysis of variance. Significant difference ($p < 0.05$) among samples were followed by Duncan's new multiple range post-hoc analysis. All of the above analyses were conducted using SPSS for Window version 16. Data were reported as mean values ± standard deviation. Response surface methodology was applied to analyze an optimum UAE condition using Design Expert version 6.0.11 (Stat-Ease, Minneapolis, MN, USA).

3. Results

3.1. Bioactive Components

Total phenolic contents varied from 85.9 to 107 mg GAE/g across different ultrasonic processing conditions (Table 1). A significantly ($p < 0.05$) higher total phenolic content of 107 ± 0.5 mg GAE/g was obtained using the highest amplitude (50%) for 10 min. Amplitude had a significant effect ($p < 0.05$) on total phenolic content at 10 min extraction time. A higher amplitude creates higher thermal energy to break the plant cellular structure. Increased permeability of cell walls and membranes and the breakdown of secondary metabolites from matrix interactions (polyphenols with lipoproteins) caused enhancement of polyphenols solubility and mass transfer. Thus, a higher ultrasonic amplitude increased extraction efficiency and yielded greater amounts of bioactive compounds [24,25]. Our previous studies also found the same extraction efficiency of phenolics and flavonoid content from green soybean pods, which was achieved using either water or ethanol solution as the extracting solvent ($p > 0.05$). Water was thus indicated to be an efficient solvent in the ultrasound-assisted extraction of green soybean pods. This might be due to the most abundant group of phenolic compounds in the pods being water-soluble. It is worth pointing out that phenolic compounds can be extracted from green soybean

pods from a cheap and broadly accessible solvent, which is likewise safe to humans and to the environment. The duration of extraction also influenced polyphenol yields. Increased extraction time from 10 to 20 min at 50% amplitude resulted in significant ($p < 0.05$) reductions in total phenolic content. A longer exposure time could increase solvent temperature beyond optimal levels, resulting in the degradation of thermo-sensitive compounds presented in the GSP samples. Evidently, the extraction condition of 10 min at 50% amplitude was deemed optimal for phenolic content.

Table 1. Total phenolic (mg GAE/g) and flavonoid (mg CAE/g) content and antioxidant activity based on DPPH and FRAP (%) of ultrasound-assisted green soybean pod extracts as a function of time and the ultrasonic amplitude level.

Time (min)	Amplitude (%)	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg CAE/g)	Antioxidant Activities (μmol Trolox/g)	
				DPPH	FRAP
10	30	85.9 ± 0.9^{d}	6.19 ± 0.1^{c}	24.4 ± 0.1^{cd}	41.4 ± 0.1^{e}
	40	91.5 ± 1.1^{c}	6.56 ± 0.3^{bc}	24.5 ± 0.1^{c}	44.0 ± 0.1^{c}
	50	107 ± 0.5^{a}	8.94 ± 0.1^{a}	25.6 ± 0.1^{a}	46.8 ± 0.1^{a}
15	30	90.9 ± 1.1^{c}	8.50 ± 0.2^{a}	22.8 ± 0.1^{f}	43.4 ± 0.1^{d}
	40	93.2 ± 1.0^{c}	8.69 ± 0.3^{a}	22.8 ± 0.1^{f}	45.5 ± 0.1^{b}
	50	103 ± 0.5^{b}	8.75 ± 0.1^{a}	25.2 ± 0.1^{b}	45.5 ± 0.1^{b}
20	30	90.6 ± 0.9^{c}	5.75 ± 0.1^{c}	23.0 ± 0.1^{f}	40.4 ± 0.1^{f}
	40	91.3 ± 1.1^{c}	7.44 ± 0.2^{b}	23.7 ± 0.1^{e}	40.2 ± 0.1^{g}
	50	90.1 ± 1.1^{cd}	7.38 ± 0.1^{b}	24.1 ± 0.1^{d}	40.4 ± 0.1^{f}

Data are expressed as means ± standard deviation ($n = 3$). Different letters (a–g) in the same column represent statistically significant difference ($p < 0.05$). DPPH = 2,2-diphenyl-1-picryl-hydrazyl radical; FRAP = ferric reducing antioxidant power.

Flavonoids have been shown to improve blood lipid profiles; enhance immunity; and have antioxidant, antibacterial, and antitumor properties [26]. Table 1 shows that amplitude and time affected flavonoid content. The highest amount of total flavonoid extracted (8.94 ± 0.1 mg CAE/g) under 50% amplitude was significantly ($p < 0.05$) higher than 30 and 40% within the first 10 min of extraction. In addition, the use of 30–50% amplitude for 15 min extraction time did not show significant difference ($p > 0.05$) in total flavonoid content (8.50–8.75 mg CAE/g) when compared to the maximum concentration of total flavonoid content. However, the extraction efficiency showed a decreasing trend when the extraction time was enhanced from 15 to 20 min (5.75–7.44 mg CAE/g).

When considering the effect of amplitude, a higher amplitude resulted in a higher flavonoid content in the 10–20 min extraction groups. Loss of flavonoids at longer extraction times was due to overheating by the ultrasound treatment, which especially affected the heat-sensitive flavonoids [27]. Flavonoids (e.g., rutin) were more sensitive to thermal degradation than phenolic acids. The concentration of rutin from olive leaves using ultrasound-assisted extraction was 2.11 ± 0.1 mg/g during a longer extraction time of 21 min, which was lower than the extraction time of 7 min (2.22 ± 0.1 mg/g) [28]. According to Bi et al. [29], the gradual increase in the bioactivity of the extract with time may be attributed to the fact that polyphenols, and other bioactive compounds, were still bound within the cell matrices during the early stage of extraction. A sufficient time was thus required to allow for their release. The subsequent decrease in bioactivity might be due to the longer time of exposure to ultrasonic conditions, inducing the degradation or oxidation of these bioactive compounds. Based on these findings, sonication for more than 15 min was found to be unsuitable, as there was not a great amount of total phenolic and flavonoid content extracted by increasing the time interval. It was also clear from the results that the extraction condition of 10 min at 50% amplitude was deemed optimal for both total phenolic and flavonoid content.

3.2. Antioxidant Activity

DPPH assay has been used widely and is a popular technique to assess the free radical scavenging activity of different plant extracts. DPPH free radical reduction was determined by the decrease in its absorption at 517 nm when the color of the DPPH assay solution changed from purple to light yellow. The scavenging potential of plant extract antioxidants corresponds to the degree of the discoloration [30]. The highest ($p < 0.05$) antioxidant activity based on DPPH (25.6 ± 0.1 μmol Trolox/g) and FRAP (46.8 ± 0.1 μmol Trolox/g) were obtained in the extract in which the highest content of total phenolics and flavonoid content were also obtained at 50% amplitude and 10 min extraction time. As shown in Table 1, by increasing the ultrasound amplitude, the antioxidant capacity was increased in all sonication times. It is well known that amplitude plays an important role in the intensification of the extraction due to its impact in cavitation. Some authors have found that high percentage of ultrasound amplitude can result in the breakage of bonds in the polyphenolic bonds [15]. However, increasing the time of sonication from 10 to 20 min resulted in a decrease of the antioxidant capacity. These results were consistent with an earlier report by Wang et al. [31], who found no increase in the total content of phenolic, flavonoid, and antioxidant activity with extraction time beyond 15 min when extracting blueberry leaves using ultrasonic extraction. It was evident that, for some plant materials, excessive extraction duration in water may cause degradation of some target compounds, resulting in reduced contents. According to the results from Muflihah et al. [32], a longer extraction time exhibited a negative effect of lower antioxidant from *Zingiberaceae* herbs, which was presumably due to the resultant prolonged heat exposure leading to the decreasing amount of targeted antioxidant compounds. The antioxidant activity of GSP extract was related to their chemical composition, primarily attributed to their richness in total phenolic content and total flavonoid content. The variation trend of FRAP values was consistent with the total phenolic contents. These results were in accordance with Hassan et al. [33], who observed that the phenolic contents of brown seaweed extract using UAE with a working frequency fixed at 42 kHz and a power of 100 W had a close correlation with FRAP antioxidant.

The relation between UAE conditions and response variables could be fit with quadratic and linear models, as follows:

$$\text{Total phenolics} = 28.63 + 7.31T + 0.13A - 0.11T2 + 0.026A2 - 0.11TA \ (p < 0.0001, R^2 = 0.90)$$

$$\text{Total flavonoids} = -11.69 + 2.11T + 0.16A - 0.064T2 - 0.0056TA \ (p < 0.0001, R^2 = 0.73)$$

$$\text{DPPH} = 36.28 - 0.87T - 0.36A + 0.025T2 + 0.0055A2 \ (p < 0.0001, R^2 = 0.86)$$

$$\text{FRAP} = 1.83 + 3.85T + 0.73A - 0.11T2 - 0.0025A2 - 0.027TA \ (p < 0.0001, R^2 = 0.99)$$

where T is extraction temperature and A is ultrasonic amplitude. All models were significant ($p < 0.0001$) with a minimum R^2 of 0.73. The response surfaces of these variables are shown in Figure 1. The total phenolic content, total flavonoid contents, DPPH, and FRAP increased slowly with the increase of amplitude at a fixed extraction time and nearly reached a peak at the highest amplitude tested. As presented in the three-dimensional plots for antioxidant contents of DPPH and FRAP (Figure 1C,D), the extraction process variables effected the extraction of antioxidants in a similar way to the case of total content of phenolic and flavonoid. This was due to the fact that the antioxidant activities of GSP extract were closely associated with the bioactive compounds. Optimization criteria were set at maximum for all response variables. The highest values occurred with 50% amplitude and extraction time of 10.5 min, which yielded a total phenolics content of 106.5 mg GAE/g, total flavonoids of 8.54 mg CAE/g, DPPH scavenging activity of 25.6 μmol Trolox/g, and FRAP of 46.7 μmol Trolox/g. The models were verified by extraction using the optimal conditions. The actual and predicted response values were not significantly different, indicating that the models were suitable for predicting the extraction parameters within the studied range (Table 2). A total energy consumption of

24.15 kWh was also calculated based on the voltage and electrical current used by the system during processing time. Additionally, specific energy consumption was calculated based on the energy needed to obtain the unit weight of bioactive compounds [34,35]. For the optimal condition of 50% amplitude and 10.5 min extraction time, the specific energy consumption was 0.23 ± 0.01 kWh/mg GAE/g sample for the phenolic content and 2.85 ± 0.02 kWh/mg CAE/g sample for the flavonoid content.

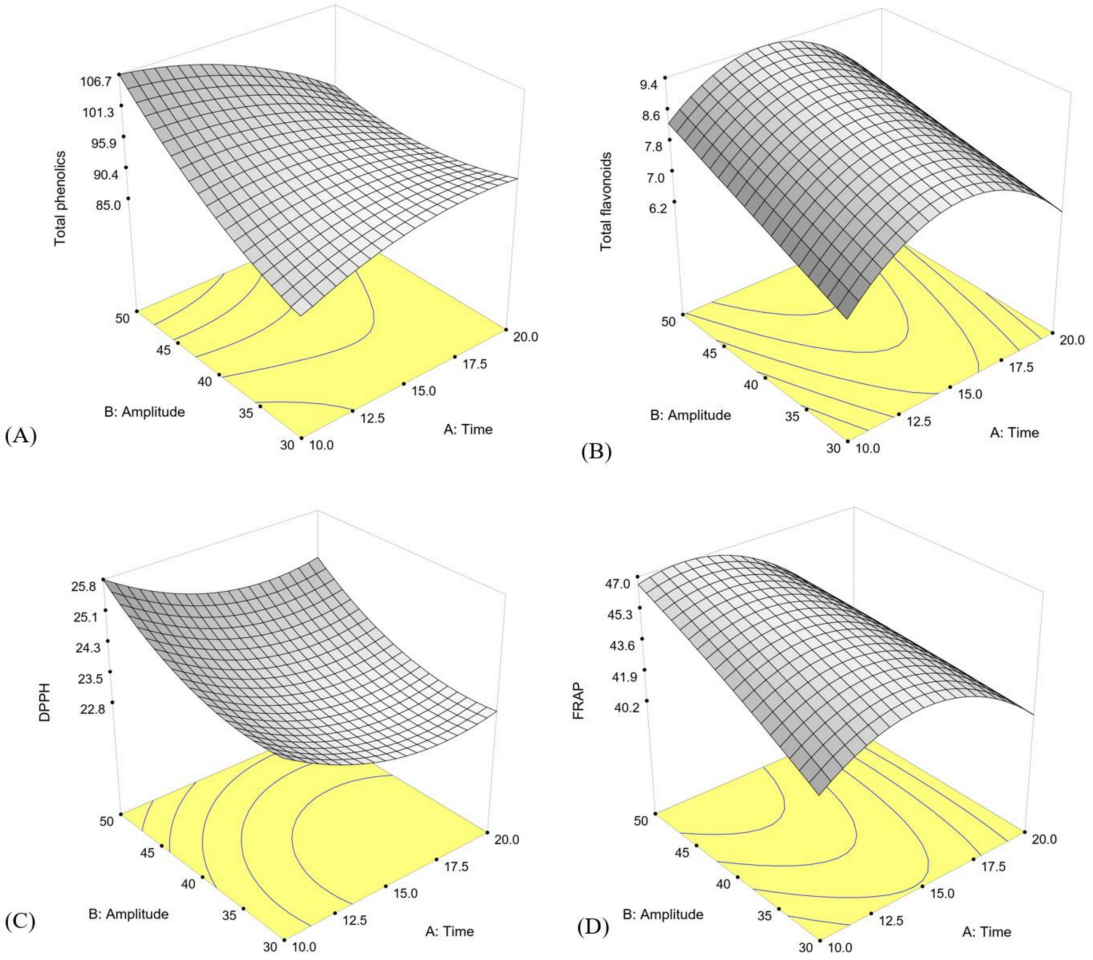

Figure 1. Response surfaces of (**A**) total phenolic compounds, (**B**) total flavonoids, (**C**) DPPH, and (**D**) FRAP as a function of UAE time and amplitude.

Table 2. Actual and predicted response values at the optimal conditions.

Responses	Predicted Value	Actual Value
Total phenolic content (mg GAE/g)	106.5	104.8 ± 2.44
Total flavonoid content (mg CAE/g)	8.54	8.48 ± 0.12
DPPH (μmol Trolox/g)	25.6	23.79 ± 0.61
FRAP (μmol Trolox/g)	46.7	45.82 ± 0.62

3.3. Evaluation of Antioxidant and Sensory Properties of Green Soybean Milk Fortified with GSP Extracts

Bioactive compounds comprise an excellent pool of molecules for the production of nutraceuticals, functional foods, and food additives [36]. The pods of green soybean waste were collected from shelling process before the seed was ground for milk production. The GSP extracts produced from the optimized UAE were used as natural antioxidants in term of food additive for improving the oxidative stability in green soybean milk. Phenolic and flavonoid contents as well as the antioxidant activity of GSP-fortified

green soybean milk are shown in Table 3. Among all samples, the 3% fortified milk sample had the highest ($p < 0.05$) total phenolic (136 ± 0.5 mg GAE/g) and total flavonoid (109 ± 0.5 mg GAE/g) content and the highest ($p < 0.05$) DPPH (176 ± 1.9 μmol Trolox/g) and FRAP (248 ± 0.3 μmol Trolox/g) antioxidant activity. In a similar study, Dabija et al. [37] revealed that the fortification of yogurt with hawthorn (*Crataegus monogyna*) extracted in increasing concentration levels (0.25, 0.50, 0.75, and 1% (w/w)) could promote the higher total phenolic content (3.46, 3.88, 4.22, 4.34 mg GAE/mL, respectively) and DPPH activity (19.23, 21.60, 32.02, and 33.38%, respectively). Lee et al. [38] also found that the increase in concentration level of *Inula britannica* flower extract for cheese fortification from 0.25 to 1.0% (w/v) caused an increase of total phenolic content and DPPH of from 54.8 to 70.8 mg GAE/g and from 53.3 to 79.1%, respectively. It was thus evident that the bioactive compounds and antioxidant activities of green soybean milk could be enhanced by pod-extracted fortification compared to milk alone.

Table 3. The content of phenolic and flavonoid, antioxidant activities of green soybean milk fortified with pod extract.

Pod Extract in Green Soybean Milk (%)	Total Phenolic Content (mg GAE/g)	Total Flavonoid Content (mg CAE/g)	Antioxidant Activities (μmol Trolox/g)	
			DPPH	FRAP
0 (Control)	81.3 ± 0.8 ^{c}	42.0 ± 0.9 ^{c}	53.2 ± 1.2 ^{d}	239 ± 0.4 ^{b}
1	115 ± 2.5 ^{b}	85.3 ± 1.2 ^{b}	125 ± 1.1 ^{c}	240 ± 0.2 ^{b}
2	114 ± 2.8 ^{b}	85.7 ± 1.1 ^{b}	132 ± 1.9 ^{b}	240 ± 0.1 ^{b}
3	136 ± 0.5 ^{a}	109 ± 0.5 ^{a}	176 ± 1.9 ^{a}	248 ± 0.3 ^{a}

Data are expressed as means ± standard deviation ($n = 3$). Different letters (a–d) in the same column represent statistically significant difference ($p < 0.05$).

The sensory evaluation of fortified green soybean milk was conducted by 100 untrained panelists on a 9-point structured scale, with 9 being the best and 1 the worst quality. All sensory attributes were in the range of 5–8, indicating that all formulae were at least moderately acceptable (Table 4). The addition of 3% (v/v) GSP extracts resulted in higher aroma (6.24 ± 1.6), sweetness (5.88 ± 1.6), and saltiness (5.94 ± 1.7) ratings, and a lower color (7.28 ± 1.2) rating, compared to the control formula. The lower appearance rating may be attributed to the intense green color of the product due to the addition of the GSP extract, which increased the green color, but reduced the luminosity of the milk. More intense green color was not generally well-accepted by consumer. Tamer et al. [39] reported that lemonade with 5% (v/v) green tea was rated lower in terms of color compared to control samples (0%). In addition, Farhan et al. [40] found that yogurt fortified with mint leave extracts had a lower color score than the control. Although color was directly related to consumer acceptability of the product [41], overall acceptability scores did not significantly ($p > 0.05$) differ between the 3% formula and the control. In fact, the color of the 3% (v/v) GSP-fortified milk, which was supposedly the greenest, was accepted equally to the color of the control. The panelists preferred the 3% formula the most, even more than the 2% formula. Based on the favorable sensory and antioxidant results, the 3% (v/v) GSP-fortified milk was selected for quantification of phytochemicals by HPLC.

3.4. Quantitative Analysis of Phytochemicals Composition

Quantification of phytochemical contents (procyanidins, quercetin, glycitein, daidzein, genistin, and linalool) in crude GSP extracts and green soybean milk with and without the addition of crude GSP extracts were determined using HPLC (Figure 2A–D). The most abundant phytochemicals in crude GSP extracts were procyanidins (0.72 ± 0.01 mg/100 g), followed by linalool (0.69 ± 0.11 mg/100 g) and quercetin (0.47 ± 0.02 mg/100 g). The procyanidin content in the crude GSP extracts in the present study was higher than lentils (0.5 mg/100 g) [42]. Compared to GSP, greater amounts of phytochemicals were observed in green soybean milk with crude extracts, especially procyanidins (3.89 ± 0.04 mg/100 g), linalool (2.79 ± 0.01 mg/100 g), glycitein (1.36 ± 0.01 mg/100 g), and quercetin (1.14 ± 0.01 mg/100 g) (Figure 3).

Table 4. Sensory analysis of green soybean milk fortified with GSP extracts. Product preference was evaluated using a 9-point hedonic scale.

Green Soybean Pod Fortification (%)	Color	Texture	Aroma	Sweetness	Saltiness	Overall
0 (Control)	7.39 ± 1.2 ^{a}	6.75 ± 1.5 ^{a}	6.01 ± 2.0 ^{a}	5.74 ± 1.8 ^{b}	5.34 ± 2.0 ^{b}	6.46 ± 1.7 ^{ab}
1	7.24 ± 1.1 ^{ab}	6.67 ± 1.5 ^{a}	6.20 ± 1.6 ^{a}	6.07 ± 1.7 ^{ab}	5.90 ± 1.7 ^{a}	6.46 ± 1.5 ^{ab}
2	7.16 ± 1.4 ^{b}	6.37 ± 1.5 ^{b}	6.01 ± 1.7 ^{a}	6.12 ± 1.7 ^{a}	5.76 ± 1.8 ^{a}	6.17 ± 1.5 ^{b}
3	7.28 ± 1.2 ^{ab}	6.96 ± 1.7 ^{a}	6.24 ± 1.6 ^{a}	5.88 ± 1.6 ^{ab}	5.94 ± 1.7 ^{a}	6.54 ± 1.4 ^{a}

Data are expressed as means ± standard deviation (n = 100). Different letters (a,b) in the same column represent statistically significant difference ($p < 0.05$).

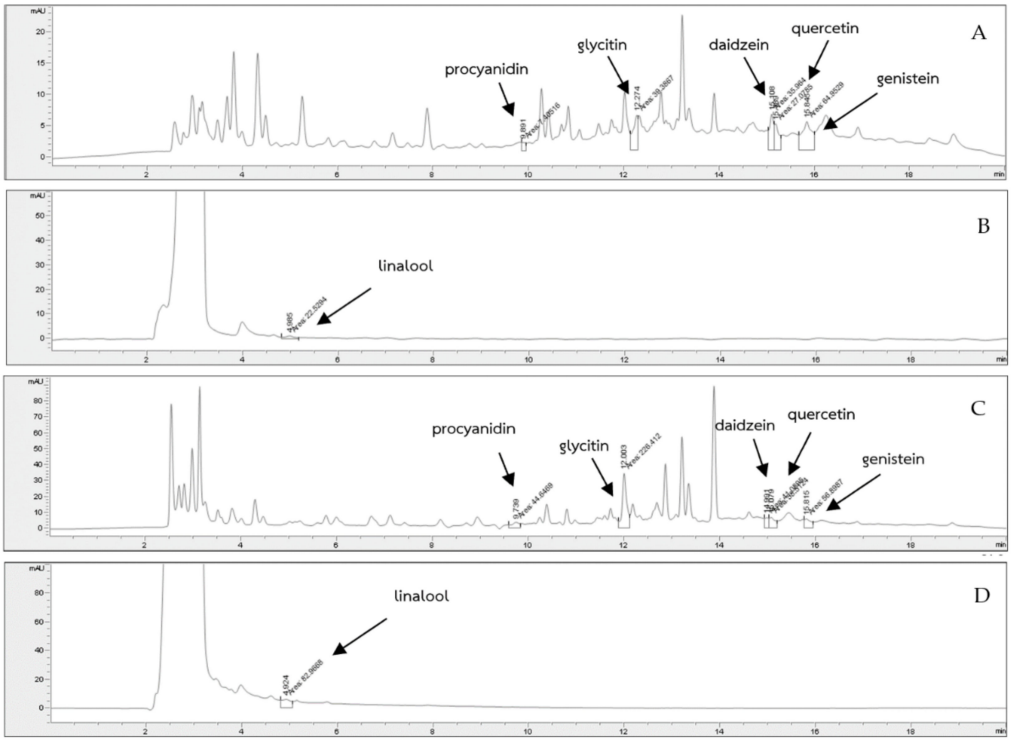

Figure 2. HPLC chromatogram of phenolic compounds in crude GSP extracts (**A**,**B**) and GSP-fortified green soybean milk (**C**,**D**).

These results were not surprising because bean seeds are nutrient- and antioxidant-rich [43]. Hence, the green soybean milk had more content of these phytochemical groups than the pod-extracted sample. Nonetheless, the GSP extracts contained greater amounts of daidzein and genistein than the green soybean milk. Avanza et al. [8] compared the content of polyphenols from cowpea seeds and pods in the extracts of water by pressurized liquid extraction. Although the result showed a higher polyphenol content in pods than in seeds, there were remarkable differences between the analyzed flavonoid groups. Cowpea seed extracts exhibited higher content on quercetin, procyanidin, and other tetrahydroxylated flavonoids compared to pod extracts. Regarding pod extracts, gallic and ferulic acids, and o-hydroxybenzoic acid, were in greater abundant. These results might be due to the different groups of polyphenol compounds (flavonoid and phenolic acid) existing naturally in legume pods and seeds.

Procyanidins are a subclass of flavonoids found in commonly consumed foods, such as fruits, vegetables, legumes, grains, and nuts, which have attracted increasing attention due to their potential health benefits [44]. In addition to antioxidant properties, procyanidins have been reported to exhibit anticancer [45], anti-infectious, anti-inflammatory, cardio-protective, antimicrobial, antiviral, antimutagenic, wounding healing, antihyperglycemic,

and anti-allergic activities [46]. Moreover, polyphenol compounds such as quercetin were reported to have neuroprotective properties attributed to their inhibiting activity against enzyme acetylcholinesterase [47]. Other polyphenols, such as genistein, daidzein, and glycitein were main phytoestrogens in the form of isoflavones. Phytoestrogens can also suppress the clinical symptoms of menopause caused by a decrease in the production of endogenous estrogen. Several studies have proven the protective effects of phytoestrogens on cardiovascular disease, which can decrease total cholesterol and improve heart function [48]. Linalool was present at rather high concentration levels in the pod extract. This kind of phytochemical is acyclic monoterpene, which is an important odorous constituent in a series of plant aromas. Linalool and linalool-rich essential oils are also known to exhibit various biological activities, such as antimicrobial, anti-inflammatory, anticancer, and antioxidant properties. In fact, several in vivo studies have confirmed various effects of linalool on the central nervous system [49]. The fortification of green soybean milk with GSP extracts enhanced both the antioxidant activity and the phytochemical content and variety of the products; thus, its increasing nutritional values.

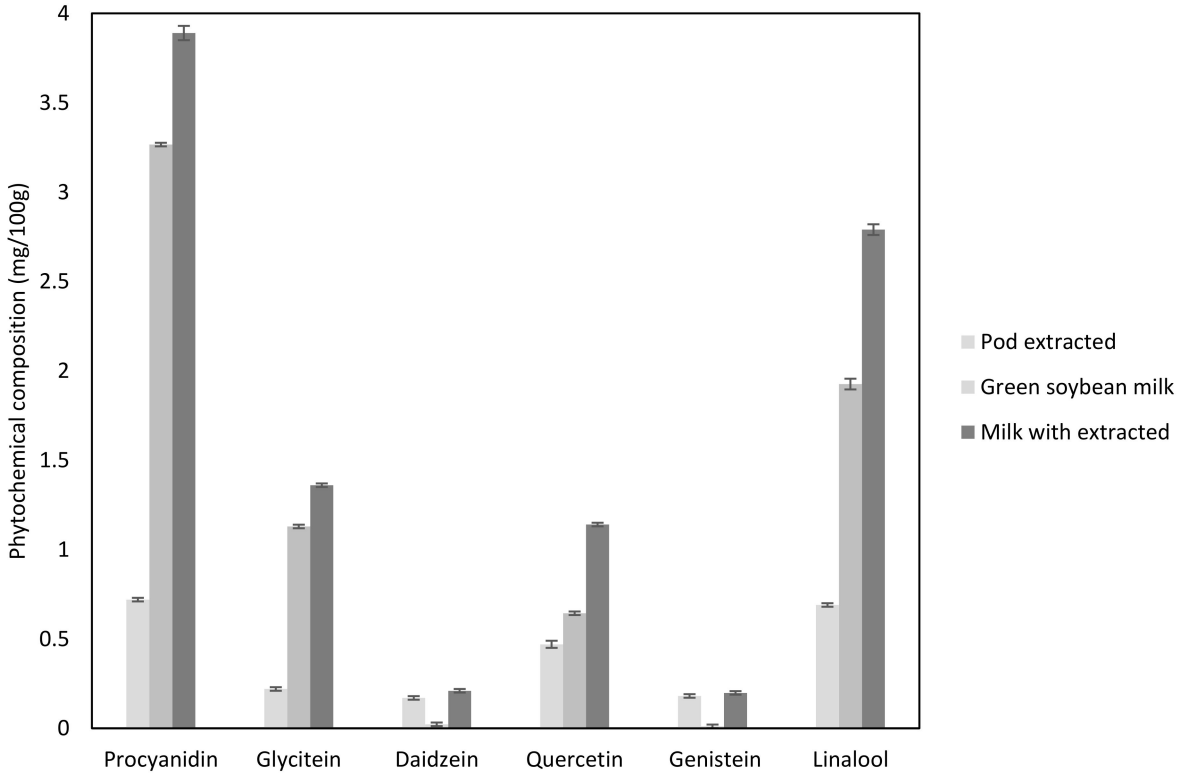

Figure 3. Phytochemical composition of crude GSP extracts and green soybean milk.

4. Conclusions

The optimal conditions for the UAE of GSP extract are 50% amplitude for 10.5 min. Green soybean milk samples containing 3% GSP extract have the highest phenolic and flavonoid content, antioxidant activity, and overall acceptability compared to the control formula. Procyanidins were found to have the highest concentration level of green soy bean pod extract among five phytochemical analyses. Study results suggest that GSP extracts are a potential source of natural antioxidant, pharmaceutical, and functional ingredients in food industries.

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