

Article

Use of *Chondrus crispus* Stakehouse Hydrolysate for Functional, Nutritional, and Potential Bioactive Health Benefits and Demonstration of Use as an Emulsifier in Selected Baked Goods

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Featured Application

The generated hydrolysate from the red seaweed *Chondrus crispus* has demonstrated techno-functional attributes with additional potential for use for its potential health benefits that include anti-inflammatory and anti-diabetic potential. The hydrolysate was used as an emulsifier ingredient in the development of prototype vegan fairy cakes.

Abstract

An increase in flexitarians, vegetarians, and vegan consumers coupled with soaring egg prices post covid has increased demand in the food industry for alternative ingredients, including emulsifier products. Plant-derived emulsifiers, including chickpea aquafaba and oat proteins, can imitate egg protein techno-functional attributes like foam formation and emulsification properties. Oat milk proteins provide less protein than soy or dairy and, as they are carbohydrate rich, can cause rapid blood sugar spikes. More alternative emulsifiers are required. This work describes the development of a red seaweed *Chondrus crispus* Stakehouse emulsifier ingredient applied in a vegan fairy cake recipe. The techno-functional attributes of the developed *C. crispus* hydrolysate were assessed using in vitro assays, and a prototype vegan fairy cake was made using the generated hydrolysate as an emulsifier. Product proximate qualities including protein, ash, and lipid content were compared to those of a traditionally made fairy cake. Results suggest that hydrolysis of *C. crispus* enables the extraction of 17.37% of available protein and makes the application of *C. crispus* in food products more attractive by improving emulsification properties. Moreover, novel bioactive peptides with sequences GIPDEWMGL, VLPSLPFM, GGPAGGGPAGGDAGLA, and AQVPPLPNMPRMPA were characterized and were shown to have in silico anti-inflammatory and anti-diabetic potential activities.

Keywords: *Chondrus crispus*; hydrolysate; emulsifiers; anti-diabetic; anti-inflammatory potential; bioactive peptides; alternative egg; vegan; alternative to oat protein

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1. Introduction

Proteins influence the functional characteristics of foods and significantly impact consumer acceptance of products. Proteins are essential ingredients in food processing and product development and frequently find applications as emulsifiers. Emulsifiers from natural sources including eggs, oats, milk, and soy are in demand currently and can command prices averaging €103/kg due to their clean label appeal and as they are considered natural ingredients [1]. Moreover, they have desirable functional properties, including emulsification, fat and water absorption, texture modification, colour control, and whipping abilities. However, nutritionally, plant protein-derived ingredients often lack selected essential amino acids, energy, and minerals like phosphorus and may contain anti-nutritional factors [2]. Therefore, the global demand for alternative protein ingredients, including those derived from marine resources, which do not rely on agricultural land for production, and which contain all essential amino acids, continues. The food industry's interest in seaweed proteins has surged, driven by the need to enhance product nutritional quality and functionality, especially in developing countries [3].

Seaweeds are consumed as food especially in Asian cultures but also in regions including Ireland, Brittany, Spain, Norway, Iceland, Hawaii, Peru, and Canada [4]. Indeed, several seaweeds including, but not limited to, the brown species *Ascophyllum nodosum*, *Saccharina japonica*, and *Laminaria hyperborea*, the red species *Porphyra* sp. and *Gelidium* sp., and the green species *Ulva intestinalis* are considered “not novel” according to the EU Novel Food Status Catalogue due to their history of consumption prior to May 1997 and are consumed as foods. Moreover, of the 250 seaweed species commercially utilized globally, 150 are favored for human consumption, and they serve primarily as sources of polysaccharides like agar, alginates, or carrageenan that are widely used in the food and pharmaceutical industries [4]. Seaweeds are low-calorie, high-nutrient value foods and contain vitamins, proteins, and minerals, including vitamins A, B1, B12, and C, as well as thiamine, riboflavin, β -carotene, tocopherols, and long-chain polyunsaturated essential fatty acids like eicosapentaenoic acid [5]. The bioavailability of seaweed proteins in many instances has not been determined, and there is a gap in knowledge in relation to the protein digestibility amino acid score (PDCAAS) and digestible indispensable amino acid score (DIAAS) values of seaweeds that is now only being addressed by researchers [6–8]. Seaweed is however, generally accepted as an excellent source of essential amino acids and unsaturated fatty acids.

Red seaweed has the most significant protein source, with reported protein levels ranging from 19% to 44% of dry weight (dw) depending on the species [9]. Conversely, green and brown seaweed species exhibit lower protein concentrations and are reported to contain 10–20% protein on a dry weight basis [10]. These protein levels rival or slightly surpass those found in legumes (20–30%), cereals (10–15%), or nuts (20–30%) [3]. However, digestibility scores measured using PDCAAS/DIAAS for seaweeds are generally lower than those of animal or soy proteins due to anti-nutritional factors including cell wall polysaccharides and phlorotannins in the case of brown seaweed-derived proteins. Despite the potential of seaweed-derived proteins, considerable challenges are associated with extracting proteins from seaweed, leading to a scarcity of studies on seaweed protein quality [11]. Surprisingly, there is a notable lack of research focused on the suitability of seaweed protein for human consumption. The extraction of proteins from seaweed presents challenges due to the presence of phenolic compounds and abundant polyanionic cell wall mucilage [12]. Phenolic compounds can disrupt native protein structures and can form covalent bonds with them, particularly in oxidative environments [13]. Moreover, the presence of cell wall mucilage leads to the formation of dense solutions, thereby hindering the extraction and purification of proteins [11,14]. In order to optimize the applicability of seaweed proteins as functional ingredients in innovative food formulations, it is

imperative to process whole biomass into high-protein products sustainably. One practical approach involves the generation of hydrolysates using combinations of carbohydrate degrading and proteolytic enzymes. Protein hydrolysates are pre-digested proteins broken down into smaller peptides and amino acids, manifesting improved solubility and bioavailability and faster absorption as well as enhanced health benefits due to the peptide content of the hydrolysates [15]. They often have improved solubility and dispersibility, facilitating their incorporation into diverse food matrices without affecting their texture or taste [16]. Furthermore, hydrolysis can remove undesirable flavors and odors associated with seaweed proteins, resulting in a more palatable product [17], and hydrolysis can also generate bitter-tasting peptides and peptides that can enhance the kukumi and umami flavors of seaweeds [18]. In addition, seaweed hydrolysates demonstrate enhanced techno-functional and bioactive properties including emulsification, foaming, and gelation, making them suitable for a broader range of applications in the food and beverage industries, as well as health benefits like anti-diabetic, antihypertensive, and anti-inflammatory benefits [18].

Chondrus crispus Stakehouse, commonly known as Irish moss, carrageen moss, or Mousse d'Irlande, has a rich history of human consumption dating back centuries [19]. *Chondrus crispus* has been employed as a thickening agent in soups, stews, and desserts, imparting a unique texture and a subtle briny flavor [20]. Beyond its culinary applications, this seaweed was used traditionally for its potential medicinal properties, believed to aid in digestion, soothe respiratory ailments, and even promote skin health [19]. Additionally, it is worth highlighting its protein content, which averages $27.2 \pm 1.4\%$, making it a valuable plant-based protein source for those interested in alternative protein sources [20]. The objectives of this work were to create a protein ingredient from *C. crispus* and incorporate this ingredient into a food product and determine the functional and potential bioactive health benefits of the hydrolysate ingredient. The objectives were achieved using hydrolysis combining carbohydrase and proteolytic enzymes, in vitro assessment of functional properties of the generated hydrolysates, and further characterization of the bioactive health potential of this marine-derived ingredient using mass spectrometry and in silico methods. Additional value for *C. crispus* biomass was created by identifying and characterizing the peptides generated following hydrolysis of the seaweed with these enzymes using MS and subsequent prediction of potential anti-inflammatory and anti-type-2-diabetes peptide sequences using in silico analysis. This work identifies the potential to use this *C. crispus* hydrolysate as a health food to replace eggs due to the protein, functional, and bioactive health benefits confirmed in the study. While *C. crispus* hydrolysates have been studied previously for their bioactivities, the specific combination of dual-enzyme hydrolysis for techno-functional improvement and application in a vegan cake, alongside peptide identification, is novel. This integrated approach where functionality, application, and peptide discovery are combined advances the field beyond previous studies that focused on only one aspect of *C. crispus* hydrolysate generation and characterization.

2. Materials and Methods

2.1. Biological Materials, Chemical Reagents, and Fairy Cake Ingredients

The red Irish seaweed (*Chondrus crispus* Stakehouse) was harvested off the West Coast of Ireland and supplied dried and milled by the Irish company SeaLac Ltd., (SeaLac Ltd., Sligo, Ireland). Samples were freeze-dried using a Cuddon Engineering Freeze drier (Cuddon Engineering, Marlborough, New Zealand), milled using a ball mill (Retsch, Dusseldorf, Germany) and vacuum-preserved until further analysis. To avoid physico-chemical modifications of the protein, all reagents used during these experiments were food-grade chemicals. Sunflower, rapeseed, and peanut oils from Musgrave House Ltd.

(Cork, Ireland) were purchased for use in this study from Supervalu supermarket (Ash-town, Dublin, Ireland).

Merck Ltd. (Dublin, Ireland) supplied the enzymes Viscozyme and Alcalase, hydro-chloric acid (HCl), and sodium hydroxide (NaOH). Deionized water obtained from a Mil-lipore nano-water purification and filtration system was supplied by Sigma-Aldrich (Dub-lin, Ireland). The Stuart SWB6D digital water bath and Stuart shaking incubator were sup-plied by Cole-Parmer (Saint Neots, UK).

The ingredients used to prepare the fairy cake were as follows: self-raising flour (Shackleton, Ireland), castor sugar (Supervalu, purchased locally, Dublin, Ireland), fresh whole eggs (Monaghan, Ireland), rapeseed oil, salt (purchased locally from Supervalu, Ashtown, Dublin, Ireland), sodium bicarbonate (Royal, baking powder, bought locally at Supervalu, Ashtown, Dublin, Ireland), and vanilla extract (purchased from Supervalu Ashtown, Dublin, Ireland) (Table 1).

Table 1. Fairy cake recipe.

Ingredient	Control Fairy Cake	Vegan Fairy Cake	Test Fairy Cake
Oatmilk/Seaweed hydrolysate		150 g (Oatmilk only)	150 g (Seaweed hydrolysate only)
Self-raising flour	150 g	150 g	150 g
Caster sugar	150 g	150 g	150 g
Butter	150 g		150 g
Eggs	2		
Rapeseed oil		60 g	60 g
Salt	0.5 g	0.5 g	0.5 g
Baking powder	1 g	1 g	1 g
Vanilla extract	1 g	1 g	1 g

2.2. Preparation, Extraction, and Enrichment of Hydrolysates from *Chondrus crispus*

The hydrolysis of dried and milled *Chondrus crispus* was performed in triplicate ($n = 9$) as a sequential process, starting with the carbohydrase enzyme (Viscozyme) to break down the seaweed's complex cell wall and facilitate the release of protein molecules. This was followed by the application of the protease enzyme (Alcalase), which facilitated the breakdown of proteins into smaller peptides and amino acids, enhancing their functional properties. Each step was conducted independently and replicated three times, following the procedure outlined below. Initially, 50 g of seaweed was combined with 500 milliliters of MilliQ deionized water. This mixture underwent heat treatment at 95 °C for 15 min in a Stuart SWB6D digital water bath (Saint Neots, UK) to deactivate inherent endogenous enzymes. After cooling to room temperature, the pH was adjusted to 5 using 0.1 M NaOH and 0.1 M HCl. Viscozyme was introduced at a concentration of 1% (w/v), followed by a 2 h incubation in a Stuart shaking incubator (Saint Neots, UK) set at 45 °C and 220 rpm. Hydrolysis was terminated by subjecting the mixture to 10 min of heating at 95 °C. The pH of the samples was then adjusted to 8–8.5 using 0.1 M NaOH and 0.1 M HCl. Subsequently, the samples underwent a second round of hydrolysis with Alcalase (1% w/v) under shaking incubation conditions at 55 °C and 220 rpm for 2 h. The enzyme was deac-tivated by heating at 95 °C for 10 min. The samples were then centrifuged at 8000× g for 30 min (Thermofisher Scientific Lynx 6000 centrifuge, Dublin, Ireland), followed by the separation of supernatant and residue fractions. Both the spent biomass and supernatants were frozen at −20 °C and later freeze-dried using an FD80GP freeze-drier (Cuddon Freeze Dry, Blenheim, New Zealand). The supernatant and spent biomass fractions were stored at −80 °C until further analysis.

2.3. Proximate Analysis

The proximate analysis of the *Chondrus crispus* whole biomass, hydrolysate, and spent biomass was carried out in triplicates ($n = 9$) (3×3 for each generated hydrolysate) and results expressed as mean values and standard deviations and results are reported in Table 2.

Table 2. Extraction yields obtained from independent hydrolysis of *Chondrus crispus* with Viscozyme and Alcalase, SD = (standard deviation, $n = 9$).

Biomass Type	Seaweed (<i>Chondrus crispus</i>)
Whole biomass (g)	50 ± 0.53
Supernatant/Hydrolysate (g)	9.71 ± 0.23
Hydrolysate yield (%)	19.42 ± 0.46

2.3.1. Ash Content

To assess the ash content of the whole biomass, hydrolysate, and spent biomass of *Chondrus crispus*, each sample was individually weighed into dried crucibles. Before initiating the ashing process, the samples were subjected to charring on a hotplate for a duration of 3 h at 100 °C. Subsequently, the pre-charred samples were transferred to a muffle furnace and exposed to ashing in air for 24 h at 540 °C. Following ashing, the samples were allowed to cool to room temperature within a desiccator. The percentage of ash was then determined utilizing the following equation:

$$\% \text{ Ash} = [(M_2 - M_0) / (M_1 - M_0)] \times 100,$$

M_0 is the mass of the dish (g), M_1 is the mass of the dish and test portion (g), and M_2 is the mass of the dish and ash portion (g).

2.3.2. Protein Content

The protein content assessment of all samples involved the determination of nitrogen content using the Dumas method with a LECO FP328 Protein analyzer (LECO Corp., Saint Joseph, MI, USA), in accordance with the protocols outlined in the Association of Official Analytical Chemists (AOAC) method 992.15 [21]. Prior to analysis, each sample was weighed into foil cups, and their respective weights were recorded. These samples were then analyzed using the LECO FP328 arranged in numbered carousels. The combustion and nitrogen quantification steps were executed utilizing a specific nitrogen conversion factor of 4.59 tailored for *Chondrus crispus*, established from the literature [22].

2.3.3. Lipid Content

The determination of total fat content followed a gravimetric procedure utilizing an ORACLE Rapid NMR Fat Analyzer (CEM Corp., Charlotte, NC, USA) in accordance with AOAC official method 2008.06 and AOAC PVM 1:2004, with adaptations drawn from the work of [23]. All samples were weighed on CEM glass fiber sample pads and subjected to overnight drying in an oven. After recording the weights of the dried samples, the pads containing the samples were enveloped in Trac Film. Subsequently, these wrapped samples were inserted into an ORACLE tube and subjected to heating for 30 min at 40 °C on a heat block before undergoing fat analysis using the ORACLE instrument.

2.3.4. Moisture Content

The total moisture content was assessed employing a LECO TGM 800 Moisture Analyzer (LECO Corp., Saint Joseph, MI, USA). Individual samples were weighed into foil cups, and their weights were recorded before drying in the LECO TGM 800, arranged in

numbered carousels. The decrease in mass of the sample following drying was utilized to calculate the moisture content, following the methodology previously outlined by [24].

2.3.5. Color Evaluation

The color of the seaweed hydrolysates and whole biomass was evaluated using six replicates ($n = 6$) using an UltraScan PRO Spectrophotometer (HunterLab, Murnau am Staffelsee, Germany) equipped with illuminant D65, diffuse 8° observer angle, and automated specular component. Results are expressed as mean values with standard deviations and are reported in Table 3. Calibration was performed using white and black standards (HunterLab, Murnau am Staffelsee, Germany) prior to all measurements. Results were recorded in the CIE Lab* system, where L^* indicates lightness, a^* denotes redness/greenness, and b^* represents yellowness/blueness.

Table 3. Color coordinates of *Chondrus crispus* hydrolysates and whole biomass (SD, standard deviation, $n = 6$). The values labeled with different lowercase letters in each row have significant differences ($p < 0.05$).

Color Coordinates	Whole Biomass	Hydrolysates
L^* value	51.02 ± 1.01^a	68.35 ± 0.29^b
a^* (red to green)	3.08 ± 0.46^a	4.44 ± 0.06^b
b^* (yellow to blue)	6.37 ± 0.66^a	11.60 ± 0.12^b
C^*	7.19 ± 0.51^a	12.14 ± 0.13^b
h°	62.89 ± 4.82^a	69.23 ± 0.18^b
ΔE^*_{ab}	18.34	

The chroma (C^*) and hue (h°) values were calculated using the following equation:

$$C^* = \sqrt{(a^*)^2 + (b^*)^2},$$

$$h^\circ = (\tan^{-1}) (b^*/a^*) \times 180/\pi$$

To assess the color disparity between the hydrolysates and the whole biomass, we computed ΔE^*_{ab} using the equation below. This metric quantifies the spatial gap between two colors within the CIELAB color space, delineated by their respective L^* , a^* , and b^* coordinates.

$$\Delta E^*_{ab} = \sqrt{(L^*_1 - L^*_2)^2 + (a^*_1 - a^*_2)^2 + (b^*_1 - b^*_2)^2}$$

L^*_1 , a^*_1 , and b^*_1 represent the color attributes of the hydrolysates, while those of the whole biomass are denoted by L^*_2 , a^*_2 , and b^*_2 for comparison purposes. A ΔE^*_{ab} value exceeding two is typically deemed perceptually significant.

2.3.6. Determination of pH and Water Activity (a_w)

The pH and water activity of the freeze-dried hydrolysate extract were assessed in six replicates ($n = 6$). The extract was reconstituted in distilled water at a concentration of 1% w/v , and the pH was measured using a pH meter (Orion model 420A, Thermo Orion, Cambridgeshire, UK). The water activity was determined using an AquaLab Water Activity System (Pullman, WA, USA) at a temperature of $8.17 \pm 0.07^\circ\text{C}$. Results are expressed as mean values with standard deviations reported.

2.4. Functional and Bioactive Properties

The functional properties of the freeze-dried *Chondrus crispus* hydrolysate, including water and oil holding capacities, as well as emulsion and foaming characteristics, were

evaluated in six replicates ($n = 6$), and results were expressed as mean values with standard deviations reported and are reported in Table 4.

Table 4. Water activity, pH, water holding capacity and oil holding capacity of *Chondrus crispus* hydrolysates (SD, standard deviation, $n = 6$). Same letters indicate no significant difference in the results ($p < 0.05$) for each row according to Tukey's test.

Water activity (a _w)		0.45 ± 0.007
pH		6.82 ± 0.01
Water Holding Capacity (WHC) (g of water/g of hydrolysate)		10.145 ± 0.221
Oil Holding Capacity (OHC) (g of oil/g of hydrolysate)	Sunflower oil	9.226 ± 0.084 ^a
	Peanut oil	9.175 ± 0.135 ^a
	Rapeseed oil	9.169 ± 0.137 ^a

2.4.1. Water (WHC) and Oil Holding Capacity (OHC)

The water-holding capacity (WHC) and oil-holding capacity (OHC) of the seaweed hydrolysates were determined following the method outlined by [25] with minor adjustments, and results were expressed as mean values with standard deviations. Specifically, 1 g of hydrolysate was combined with 10 mL of either distilled water or sunflower oil using a vortex mixer (Henry Troemner, Philadelphia, PA, USA), followed by centrifugation at 2200 g for 30 min. After centrifugation, the supernatants were carefully decanted, and the centrifuge tubes containing the sediment were weighed. WHC and OHC were quantified as the grams of water or sunflower oil retained per gram of hydrolysate, calculated using the following equation:

$$WHC \text{ or } OHC \text{ (g of water or sunflower oil per g of hydrolysate)} = W_2 - W_1 / W_0 \times 100$$

W_0 is the weight of the dry sample (g), W_1 is the weight of the tube plus the dry sample (g), and W_2 is the weight of the tube plus the sediment (g).

2.4.2. Emulsifying Activity and Emulsion Stability

The emulsifying activity (EA) of the hydrolysates was evaluated following the methodology established by [25], with minor adaptations, and results are expressed as mean values with standard deviations. Initially, the hydrolysate sample was diluted in Millipore ultrapure water (Millipore, Cork, Ireland) to a concentration of 1% w/v , and the pH was adjusted to pH 2, 4, 6, 8, and 10 using either 0.1 M NaOH or 0.1 M HCl. The protein solution underwent homogenization for 30 s at 14,000 rpm. Subsequently, to create the emulsion, commercial oils such as sunflower, rapeseed, and peanut oil were introduced into the aqueous phase containing the hydrolysate at a ratio of 3 parts oil to 2 parts protein solution. This addition of oil occurred in two stages: firstly, half of the oil volume was added and mixed for 30 s at 14,000 rpm, followed by the addition of the remaining oil volume and further mixing for 90 s at the same speed. The resulting emulsion was transferred to centrifuge tubes and centrifuged at 1100 g for 5 min, after which the volume of the emulsion layer was measured. The EA was then determined using the following equation:

$$EA \% = (V_E / V_T) \times 100$$

V_E is the volume of the emulsion layer after centrifuging (mL), and V_T is the total volume inside the tube (mL).

For emulsion stability (ES), the previously prepared emulsions were heated at 85 °C for 15 min in a water bath (Stuart model SWB6D, 6 L, Yorkshire, UK), cooled at room

temperature for 10 min, and centrifuged again at 1100 g for 5 min. The ES was expressed as the % of the emulsion layer remaining after centrifugation:

$$ES \% = (V_{\text{Emulsion after heating}} / V_{\text{Original emulsion}}) \times 100$$

2.4.3. Foaming Capacity and Stability

The hydrolysates' foaming capacity (FC) was assessed following the method established by [25], with slight adjustments, and results were expressed as mean values with standard deviations. Suspensions of hydrolysates were prepared at room temperature with a concentration of 1.5% *w/v*, and the pH was adjusted to 2, 4, 6, 8, and 10 using 0.1 M NaOH or 0.1 M HCl, respectively. The suspensions underwent homogenization using a T 25 digital ULTRA-TURRAX® homogenizer (IKA®, Staufen, Germany) at 10,000 rpm for 1 min, and the resulting foam volume was quantified using a graduated cylinder. The foaming capacity was determined by calculating the volume of foam produced as a percentage of the initial solution volume using the following equation:

$$FC \% = [(V_F - V_0) / (V_0)] \times 100$$

V_0 is the initial volume of protein solution before homogenization (mL), and V_F is the volume of foam generated after homogenization (mL).

The foam stability was determined by using the equation below that measures the decrease in volume of foam as a function of time up to a period of 15 min.

$$FS \% = (V_S / V_F) \times 100$$

V_S is the volume of protein solution after standing for 15 min (mL), and V_F is the volume of foam generated after homogenization (mL).

2.4.4. Mass Spectrometry in Tandem Analysis of Hydrolysate

An amount of 1 µL of the *C. crispus* hydrolysate sample was quantified by nanoDrop (A 280 nm, ϵ = 1 mg/mL). The volume corresponding to 190 ng was diluted in 20 µL with 0.1% formic acid (FA) and loaded in an Evotip pure tip (EvoSep ApS, Odense, Denmark) according to the manufacturer's instructions. Tandem mass spectrometry analysis (LC-MS/MS) was performed in a Tims TOF fleX mass spectrometer (Bruker, Billerica, MA, USA). The sample loaded in the Evotip pure was eluted to an analytical column (EvoSep 15 cm × 150 µm, 1.5 µm) by the EvoSep One system and solved with the 30 SPD chromatographic method defined by the manufacturer.

The eluted peptides were ionized in a captive spray with 1700 V at 200 °C and analyzed using ddaPASEF mode with the following settings: TIMS settings: mode: custom; 1/K0: 0.7–1.76 V.s/cm²; ramp time: 100 ms; duty cycle: 100%; ramp rate: 9.42 Hz; Ms averaging: 1; auto calibration: off. MS settings: scan: 100–1700 *m/z*; ion polarity: positive; scan mode: PASEF. MS/MS: number of PASEF ramps: 4; total cycle time: 0.5 s; charge minimum: 0 (unknown); charge maximum: 5; Scheduling: target intensity: 12,500, intensity threshold: 1000. Active exclusion: ON. The system sensitivity was controlled with 20 ng of HELA-digested proteins. A total of 4646 proteins identified with the 30 SPD gradient.

2.4.5. Protein Identification

MSFragger searches were performed (via FragPipe v24.0) to identify non-tryptic peptides. Single databases were generated at Rhodophyta and Uniprot-*Chondrus*. Every sample was analyzed using an adequate database.

2.4.6. In Silico Analysis of Identified Peptides to Predict Bioactivities

Following the identification of peptides (>2000) found within the *C. crispus* hydrolysate using MS, potential bioactivities were determined using an in silico approach previously used by our group in relation to characterization of peptides from hydrolysates of the microalga *Porphyridium* sp., mesopelagic fish [26,27], and the brown seaweed *Laminaria digitata* [28]. Briefly, the identified peptides (>2000) were assessed for their potential bioactivity (antimicrobial) nature using Peptide Ranker (accessed on 17 January 2025) based on amino acid charge and peptide structure, where a threshold value of 0.8 was applied. Peptides with a PeptideRanker value >0.8 are listed in Table 5 [26]. The anti-inflammatory predictive program PreAIP—<http://kurata14.bio.kyutech.ac.jp/PreAIP/> (accessed on 17 January 2025) [29] ranked peptides in terms of their potential anti-inflammatory potential by viewing the primary peptide sequence, as well as evolutionary and structural information of the peptide, through a random forest classifier [29]. Scores ≥ 0.468 indicate high confidence that a peptide is anti-inflammatory. Scores between 0.468 and ≥ 0.388 indicate medium confidence that a peptide is anti-inflammatory, while a score of 0.388 to 0.342 indicates that the peptide has a low probability of having anti-inflammatory activity. Peptides were identified as novel by performing a search in the database BIOPEP-UWM. The potential of peptides within the *C. crispus* hydrolysate to influence taste was determined using Umami-MRNN—<https://umami-mrnn.herokuapp.com/> (accessed on 17 January 2025) [30]. The program AntiDMPpred, available at <http://i.uestc.edu.cn/AntiDMPpred/cgi-bin/AntiDMPpred.pl> (accessed on 17 January 2025), was used to identify the anti-diabetes type 2 activity of peptides using a threshold of 0.6 [31]. Values with threshold figures greater than 0.6 have a higher probability of having anti-diabetic benefits.

2.5. Preparation of Fairy Cakes

For the preparation of vegan and control fairy cake samples (nine fairy cakes), the formulation outlined in Table 1 was adopted.

The oat milk portion in the formulation for the vegan formulation was substituted with seaweed hydrolysates for the test fairy cake based on their protein content. Oat milk typically contains around 1% protein [32], meaning that 150 mL of oat milk provides approximately 1.5 g of protein. The hydrolysates were included in the formulation based on their protein content. In a large mixing bowl, self-rising flour, seaweed hydrolysates, caster sugar, salt, and baking powder were sifted together. A mixture of rapeseed oil and vanilla essence was gradually added to 200 mL of water and then slowly incorporated into the dry ingredients, followed by thorough manual stirring. The oat milk or seaweed hydrolysate, used independently, combined with 60 g (mL) of rapeseed oil, was used to substitute for eggs and butter in the vegan and test fairy cakes, respectively. Water was added to the control fairy cake 150 mL. The resulting cake batter was evenly distributed into cake cases, filling them almost to the top. The cakes were baked in the center of a preheated conventional oven (Macpan MDBt8, Thiene, Italy) at 200 °C (180 °C fan) for 20–25 min or until golden brown and afterwards allowed to cool to room temperature.

For preparing nine fairy cake control samples, all the ingredients were mixed, followed by thorough manual stirring and even distribution into cake cases, which were filled almost to the top. The cakes were baked in the center of a preheated conventional oven (Macpan MDBt8, Thiene, Italy) at 200 °C (180 °C fan) for 20–25 min or until golden brown and afterwards allowed to cool to room temperature. The cakes were stored in PP (polypropylene) containers.

Table 5. Peptide sequences identified from *C. crispus* hydrolysate and in silico analysis predicted bioactivity results for selected peptides identified using mass spectrometry using different programs, including PeptideRanker <http://bioware.ucd.ie/~compass/biowareweb/>, accessed on 17 January 2025; PreAIP <http://ku-rata14.bio.kyutech.ac.jp/PreAIP/>, accessed on 17 January 2025; BIO-PEP-UWM <https://biochemia.uwm.edu.pl/biopep-uwm/>, accessed on 17 January 2025; AntiDMPpred available at <http://i.uestc.edu.cn/AntiDMPpred/cgi-bin/AntiDMPpred.pl>, accessed 17/01/2025; and Umami-MRNN <https://umami-mrnn.hero-kuapp.com/>, accessed 17 January 2025.

Peptide Sequence	Protein & Accession Number	Peptide Ranker Score	Anti-Inflammatory Prediction with PreAIP RF (Combined Values)	Anti-Diabetic Potential Prediction with AntiDMPpred (tp 0.6)	Umami Prediction with Umami-MRNN	Novelty (Found in BIOPEP-UWM)
APPPPPAPF	Uncharacterized protein (R7QQW5_CHOCR)	0.97	Negative AIP (0.292)	No	non-umami	Novel
DDDPFGPF	Tyrosine-protein kinase ephrin type A/B receptor-like domain-containing protein (R7QEH3_CHOCR)	0.959	Negative AIP (0.299)	No	non-umami	Novel
VDPSPWPF	Cytochrome c oxidase subunit 3 (COX3_CHOCR)	0.945	Low confidence AIP (0.376)	No	non-umami	Novel
GMPSDDPFPF	Malate dehydrogenase (R7QPY8_CHOCR)	0.942	Low confidence AIP (0.372)	No	umami, predicted threshold: 39.25773mmol/L	Novel
SIPDGWMGL	Phosphoglycerate kinase (Q8GVE6_CHOCR)	0.939	Medium confidence AIP (0.443)	No	umami, predicted threshold: 31.829782 mmol/L	Novel
FDPVFIM	Potential copper-zinc superoxide dismutase, CuZn SOD2 R7QRY5_CHOCR)	0.933	Medium confidence AIP (0.436)	No	non-umami	Novel
SADDDPFGPF	Tyrosine-protein kinase ephrin type A/B receptor-like domain-containing protein (R7QEH3_CHOCR)	0.932	Negative AIP (0.299)	No	umami, predicted threshold: 27.236626 mmol/L	Novel
GGGAPFFPVA	SMP-30/Gluconolactonase/LRE-like region domain-containing protein (R7QCK1_CHOCR)	0.93	Low confidence AIP (0.367)	No	non-umami	Novel
GPGPGPGPGPGPV	Hint domain-containing protein (R7QIA6_CHOCR)	0.926	Negative AIP (0.241)	Predicted to be anti-diabetic	umami, predicted threshold: 15.696608 mmol/L	Novel
VGPPPPPPPP	C-CAP/cofactor C-like domain-containing protein (R7QP74_CHOCR)	0.923	Negative AIP (0.342)	No	non-umami	Novel
AMPPFPNPY	Uncharacterized protein (R7QQW5_CHOCR)	0.916	Medium confidence AIP (0.410)	No	non-umami	Novel
ADMGGMGF	NAD-dependent sugar epimerase (R7QUS9_CHOCR)	0.913	Medium confidence AIP (0.434)	No	umami, predicted threshold: 8.370863 mmol/L	Novel
SPPPPVDPF	Acetohydroxy-acid reductoisomerase (R7QNL4_CHOCR)	0.912	Negative AIP (0.317)	No	non-umami	Novel
AGPGPGPGPGPGPV	Hint domain-containing protein (R7QIA6_CHOCR)	0.911	Medium confidence AIP (0.444)	Predicted to be anti-diabetic	umami, predicted threshold: 22.584654 mmol/L	Novel
DDDFDNPL	catalase (R7QFJ3_CHOCR)	0.91	Low confidence AIP (0.349)	No	non-umami	Novel

FAGGPKIPF	Ascorbate Peroxidase, APX1(R7QH61_CHOCH)	0.906	Medium confidence AIP (0.392)	No	umami, predicted threshold: 38.71417 mmol/L	Novel
VGGDPSFGPF	NAD-dependent epimerase/dehydratase domain-containing protein (R7Q496_CHOCH)	0.9	Low confidence AIP (0.369)	No	umami, predicted threshold: 26.573849 mmol/L	Novel
GIPDEWMGL	Phosphoglycerate kinase (Q8GVE6_CHOCH)	0.9	High confidence AIP (0.469)	No	umami, predicted threshold: 36.3166 mmol/L	Novel
FDPVFIMN	Potential copper-zinc superoxide dismutase, CuZn SOD2 (R7QRY5_CHOCH)	0.9	Medium confidence AIP (0.436)	No	non-umami	Novel
VLPSLPFM	Animal heme peroxidase homologue (R7QJ77_CHOCH)	0.89	High confidence AIP (0.478)	No	umami, predicted threshold: 34.225975 mmol/L	Novel
SSPQFGFPT	SMP-30/Gluconolactonase/LRE-like region domain-containing protein (R7QCK1_CHOCH)	0.89	Negative AIP (0.335)	No	umami, predicted threshold: 36.78789 mmol/L	Novel
ALEPPPPVGPPGL	Ubiquitin carboxyl-terminal hydrolase (R7QGF5_CHOCH)	0.89	Medium confidence AIP (0.402)	Predicted to be anti-diabetic	umami, predicted threshold: 26.183607 mmol/L	Novel
GVSAPFDPF	Translation initiation factor eIF1 (R7QJC0_CHOCH)	0.88	Low confidence AIP (0.363)	No	umami, predicted threshold: 33.65273 mmol/L	Novel
YAMPPYAF	Photosystem I P700 chlorophyll a apoprotein A2 (M5DES3_CHOCH)	0.88	Low confidence AIP (0.379)	No	non-umami	Novel
SPDMPGYVGF	Uncharacterized protein (R7QQW5_CHOCH)	0.88	Low confidence AIP (0.364)	No	non-umami	Novel
DSDSDLFFG	Gal-2,6-Sulfurylases II (R7Q2Y4_CHOCH)	0.88	Low confidence AIP (0.361)	No	umami, predicted threshold: 29.827625 mmol/L	Novel
GGPAGGGPAGGDAGLA	Transitional endoplasmic reticulum ATPase (Valosin-containing protein), (R7QAA8_CHOCH)	0.87	High confidence AIP (0.480)	Predicted to be anti-diabetic	umami, predicted threshold: 13.955362 mmol/L	Novel
AQVPPLPNMPRMPA	Prohibitin (R7QRM0_CHOCH)	0.87	High confidence AIP (0.478)	Predicted to be anti-diabetic	umami, predicted threshold: 37.8484 mmol/L	Novel
DSDSDLFFGR	Gal-2,6-Sulfurylases II (R7Q2Y4_CHOCH)	0.87	Low confidence AIP (0.384)	No	umami, predicted threshold: 8.039184 mmol/L	Novel

2.6. Cake Analysis

The cake samples were analyzed in nine replicates ($n = 9$) on day 0 for baking loss (BL %), crust and crumb color, cake density, specific volume, and volume index. Additionally, moisture content, water activity, and pH of the prepared cake samples were assessed in triplicate ($n = 3$) over three consecutive days (days 0, 1, and 2) post baking.

2.6.1. Baking Loss (BL %)

The weight loss during baking was calculated on day 0 as the difference in the weight of the batter relative to the weight of the prepared cake by using the following equation [33]:

$$\text{BL \%} = [(W_{\text{Batter}} - W_{\text{Cake}}) / (W_{\text{Batter}})] \times 100,$$

where W_{Batter} is the batter weight before baking, and W_{Cake} is the cake weight after baking.

2.6.2. Crust and Crumb Color

The L^* , a^* , and b^* values of the crust and crumb of the cakes were determined using a Chroma meter CR-410 (Konica Minolta, Tokyo, Japan), which had been calibrated using a standardized white tile [33]. Three readings were recorded on the surface color (on the top region of the cake), and three readings were recorded on the crumb color of the cake. The ΔE values indicate the perceptibility of color differences: a value less than 1 means the difference is not easily visible, between 1 and 3 means it is not readily noticeable, and above three means the difference is apparent [34]. The overall color difference was calculated as follows:

$$\Delta E^*_{ab} = \sqrt{(L - L_{\text{Control}})^2 + (a - a_{\text{Control}})^2 + (b - b_{\text{Control}})^2},$$

2.6.3. Cake Density, Specific Volume, and Volume Index

The density of the prepared cake samples was calculated as the ratio between the weight of the cake and its volume (g/cm^3) [33]. The volume of the vegan fairy cakes was measured by the rapeseed displacement method [35]. The specific volume of the cake (cm^3/g) was determined by measuring the cake volume (cm^3) divided by the mass (g) [33]. The volume index of cakes was determined following the AACC method 10–91 [35]. Cakes were vertically sliced through their centers, and the heights of the samples were measured at three designated points (B, C, and D) across the cross-sections using a template. The volume index (cm) was then calculated using the following equation:

$$\text{Volume index} = B + C + D,$$

where C is the height of the cake at the center (cm), and B and D are the heights of the cake sample at the points 2.5 cm away from the center towards the left and right sides of the cake (cm), respectively.

2.6.4. Moisture Content, Water Activity, and pH

Crumb moisture was undertaken according to the AACC 44-15A method on days 0, 1, and 2 post baking. The crust was removed from the central two slices of the cake. The sample was cut into approximately 1 cm^3 cubes and allowed to dry in a Brabender oven ((Anton Paar TorqueTec, Duisberg, Germany) at 130°C for 60 min [36]. The moisture content was calculated using the below equation, where W_2 is the weight of the sample before drying (grams), and W_1 is the weight of the sample after drying (grams).

$$\text{MC \%} = [(W_2 - W_1) / (W_1)] \times 100,$$

For water activity, a section of the central region of the two center slices was crumbed, and the water activity of the cakes was determined using an Aqua Lab Lite (Decagon Devices, WA, USA) [37].

To measure the pH of the cake sample, 15 g of small pieces of cake were mixed with 150 mL of distilled water and vortexed for 3 min for cake pH measurements. The mixture was held at room temperature for 30 min to separate solid and liquid phases. The pH of the supernatant was measured using the pH meter mentioned above [38].

2.7. Statistical Analysis

Statistical analysis was conducted using one-way ANOVA followed by Tukey's HSD post hoc test to determine significant differences between means at a 95% confidence interval (GraphPad Prism 9.1.0, San Diego, CA, USA).

3. Results

3.1. Extraction Yield and Proximate Composition of Hydrolysates and Spent Biomass and Whole Biomass Fractions of *Chondrus crispus*

The objective of this study was to evaluate the feasibility of enzyme-assisted hydrolysis for extracting proteins and peptides from red seaweed in substantial quantities. This exploration aims to broaden the potential utilization of seaweed in food products and as functional ingredients. The quantity of hydrolysates (supernatant) obtained from 50 g of dried/milled *Chondrus crispus* following independent treatment with Viscozyme and Alcalase was 9.71 ± 0.23 g, respectively (Table 2). The percentage yield of the hydrolysates obtained was $19.42 \pm 0.46\%$ from the whole seaweed when measured on a dry weight basis. The hydrolysate yield from *Chondrus crispus* in our study significantly exceeded that reported by others [39]. Their work, which involved generating hydrolysates from *Palmaria palmata* using Chymotrypsin and a Tris-HCl buffer at pH 8 (30 °C for 24 h), achieved an average yield of just 12.50% on a dry weight basis.

The proximate compositions of *Chondrus crispus* whole biomass, spent biomass, and recovered hydrolysates are outlined in Figure 1.

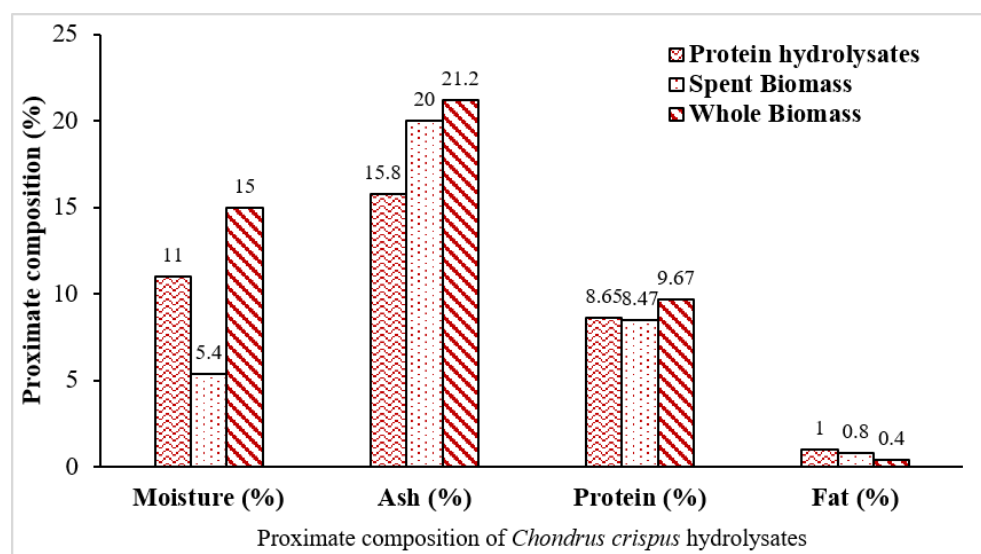


Figure 1. Compositional analysis values obtained for *Chondrus crispus* whole biomass, spent biomass, and supernatant fractions generated following Viscozyme and Alcalase enzyme hydrolysis and centrifugation steps. (SD, standard deviation, $n = 9$).

The recovered hydrolysate from *Chondrus crispus* exhibited a protein content of 8.65%, whereas the protein content for the spent and whole biomass fractions measured

8.47% and 9.67%, respectively. The nitrogen-to-protein conversion factor (NCF) of 4.59 was used to determine the protein content of all *C. crispus* fractions—whole biomass, hydrolysate, and spent biomass. The lipid content of the *Chondrus crispus* hydrolysate was 1%, and the moisture content was 11%. Additionally, the ash content of the hydrolysate was 15.8%. In terms of protein content, our findings align closely with a previous study conducted by [40], where a crude protein content of $13.94 \pm 0.94\%$ for *Chondrus crispus* hydrolysates was recorded. The efficiency of protein extraction, however, is influenced by the extraction method, harvesting location, prevailing weather conditions, and the maturity stage of the macroalgae [36]. In terms of protein quantification hydrolysis can generate amino acids (free and total), and this can impact protein quantification in samples, as described previously by Hayes [41] and Mahre [42] and, more recently, by the CEN/TC 454/WG 7 on saccharides, proteins, and amino acids [43].

3.2. Colour of Hydrolysates and Whole Biomass Fractions of *Chondrus crispus*

The color assessment of hydrolysates derived from *Chondrus crispus*, as well as the whole biomasses, was conducted by evaluating the L^* , a^* , and b^* values. L^* signifies the extent of color lightness, where a value of 100 indicates white, and 0 denotes blackness. Redness is indicated by $+a^*$, while $-a^*$ values indicate a greenish hue. Yellowness is denoted by $+b^*$, whereas $-b^*$ signifies blueness [44]. The chroma value (C^*) represents the degree of deviation from gray towards a pure chromatic color, while the hue value (h°) distinguishes one color from another, such as red, yellow, green, blue, or purple, based on their quality. The color values of *Chondrus crispus* hydrolysates were determined as $L^* 68.35 \pm 0.25$, $a^* 4.47 \pm 0.05$, $b^* 11.60 \pm 0.10$, $C^* 12.14 \pm 0.11$, and $h^\circ 69.23 \pm 0.17$ (Table 3). The ΔE^*_{ab} value denotes the difference in the color of the hydrolysates with respect to the whole biomass, and a value of 18.34 indicates that there was a significant difference between the colors perceptual to the human eyes.

To the best of our knowledge, no studies have explored the color attributes of *Chondrus crispus* hydrolysates. Interestingly, our findings reveal that the hydrolysates exhibited a perceptually distinct color compared to the whole biomass, with significantly higher values across all measured color attributes. This enhancement in lightness, yellowness, purity, and overall color distinction suggests that hydrolysis plays a crucial role in intensifying the visual characteristics of the resulting hydrolysates.

The striking color features observed in *Chondrus crispus* hydrolysates can be attributed to its unique light-absorbing pigments, which are bound to proteins and function in solar energy capture, leading to the formation of phycobiliproteins (PBPs) [45]. These PBPs—including allophycocyanin (APC), phycocyanin (PC), and phycoerythrin (PE)—are widely present in cyanobacteria and red seaweeds [46]. Beyond their role in pigmentation, PBPs exhibit bioactive properties, such as anti-inflammatory, hepatoprotective, and antioxidant effects [47]. Additionally, they have shown significant potential in photodynamic therapy for conditions like tumors and leukemia [48].

Assessing the color of food ingredients is essential for determining their compatibility in formulations, ensuring minimal impact on the final product's visual appeal. In this study, enzymatic hydrolysis not only enhanced the lightness and yellowness of the hydrolysates but also improved their potential for seamless integration into various food applications, making them a promising functional ingredient with both aesthetic and nutritional benefits.

3.3. Water Activity and pH of *Chondrus crispus* Hydrolysates

The a_w of the freeze-dried hydrolysates, measured at $8.17 \pm 0.07^\circ\text{C}$, was recorded as 0.45 ± 0.005 , while the pH of hydrolysate dispersion was determined to be 6.82 ± 0.01 .

(Table 4). The pH and water activity of the hydrolysates are critical physicochemical properties that define their stability during storage before further utilization by the industry and subsequent applications. Higher a_w levels in foods accelerate degradation due to biochemical and microbial changes, as water activity directly influences enzymatic and non-enzymatic reactions, microbial spoilage, and lipid oxidation [49]. Maintaining optimal a_w is, therefore, essential for preserving the quality and shelf life of hydrolysates, ensuring their suitability for various food formulations.

3.4. Water (WHC) and Oil Holding Capacity (OHC)

The WHC of the hydrolysates from *Chondrus crispus* was determined as 10.14 ± 0.221 g water/g hydrolysate. The oil holding capacity (OHC) of *Chondrus crispus* protein hydrolysates was evaluated with sunflower, peanut, and rapeseed oils, yielding values of 9.226 ± 0.084 , 9.175 ± 0.135 , and 9.169 ± 0.137 g of oil per gram of hydrolysates, respectively (Table 4).

Interactions between proteins, water, and oil are fundamental to food formulation and product stability. The capacity of proteins to bind and retain water and oil plays a crucial role in shaping the texture, consistency, and physicochemical characteristics of food products [50]. WHC and OHC are dictated by both intrinsic factors—such as amino acid composition, protein conformation, surface polarity, and secondary structure (β -sheet or α -helix)—and extrinsic factors, including enzyme type, hydrolysis conditions, and degree of hydrolysis [51].

High WHC is particularly beneficial in baked goods, where it delays retrogradation, preserves moisture, and enhances texture. Conversely, elevated OHC is essential in stabilizing emulsified products like cakes, mayonnaise, and salad dressings, preventing phase separation and improving flavor retention [52]. Proteins with superior WHC and OHC are highly desirable for bakery applications, meat formulations (as emulsifiers and partial meat substitutes), and other processed foods [50].

The WHC values obtained in this study were notably higher than those reported for *Chlorella* hydrolysates prepared with different proteases, which ranged between 1.7 and 5.1 g of water/g of hydrolysate [53]. Similarly, *Spirulina platensis* hydrolysates exhibited WHC values between 1.2 and 5.4 g of water/g of hydrolysate [54]. According to [55], enzymatic hydrolysis unfolds the protein structure in a controlled manner, exposing more polar groups ($-\text{COOH}$, $-\text{NH}_3$, and $-\text{OH}$), thereby enhancing interactions with water molecules and increasing WHC. In baked goods, this property is advantageous as it retains moisture without protein dissolution, contributing to improved viscosity, dough thickening, and reduced moisture loss in packaged products [56].

The ability of proteins to bind oil is affected by multiple physical and chemical factors, including the oil's viscosity, molecular weight, degree of saturation, polarity, and external conditions like temperature and pressure [57]. The OHC values of *Chlorella* hydrolysates produced using different proteases ranged from 4.73 to 5.7 g of oil/g of hydrolysate [50], while *Spirulina platensis* hydrolysates demonstrated OHC values between 5.3 and 7 g of oil/g of hydrolysate, with the highest OHC observed in Alcalase-treated samples [54]. Similarly, Xu and colleagues [58] highlighted the superior OHC of legume hydrolysates treated with Alcalase and bromelain, attributing this to increased exposure of hydrophobic amino acids. Nonpolar side chains, particularly aromatic and aliphatic groups, enhance fat absorption capacity through hydrophobic interactions [59], a property that is crucial for flavor retention and improving overall product palatability [60].

Given their high WHC and OHC, hydrolysates can serve as multifunctional ingredients in diverse food applications, enhancing both the structural and sensory attributes of formulated products.

3.5. Emulsifying Activity (EA) and Emulsion Stability (ES)

Understanding the emulsifying activity (EA) and emulsion stability (ES) of *Chondrus crispus* hydrolysates is key to positioning them as natural alternatives to synthetic emulsifiers in food applications. Their EA and ES were evaluated across pH 2–10 using sunflower, peanut, and rapeseed oils (Figure 2a,b). EA was highest at pH 2 and declined with increasing pH, ranging from $85.28 \pm 1.37\%$ to $5.23 \pm 1.31\%$ for sunflower oil, $85.64 \pm 1.34\%$ to $13.15 \pm 1.82\%$ for peanut oil, and $94.33 \pm 0.91\%$ to $13.91 \pm 0.98\%$ for rapeseed oil. Egg proteins show high emulsifying activity normally with reported values ranging from $>80\%$ (sunflower oil), $75\text{--}85\%$ (peanut oil), and $>85\%$ (rapeseed oil) at pH values ranging from 5 to 7. At pH values between 2 and 4, the figures reported for *C. crispus* protein hydrolysates in different oils fall within this range of EA % values and are comparable to egg protein. However, at pH values 6–10, the emulsifying activity of the *C. crispus* hydrolysates are much less than egg protein in terms of activity and stability.

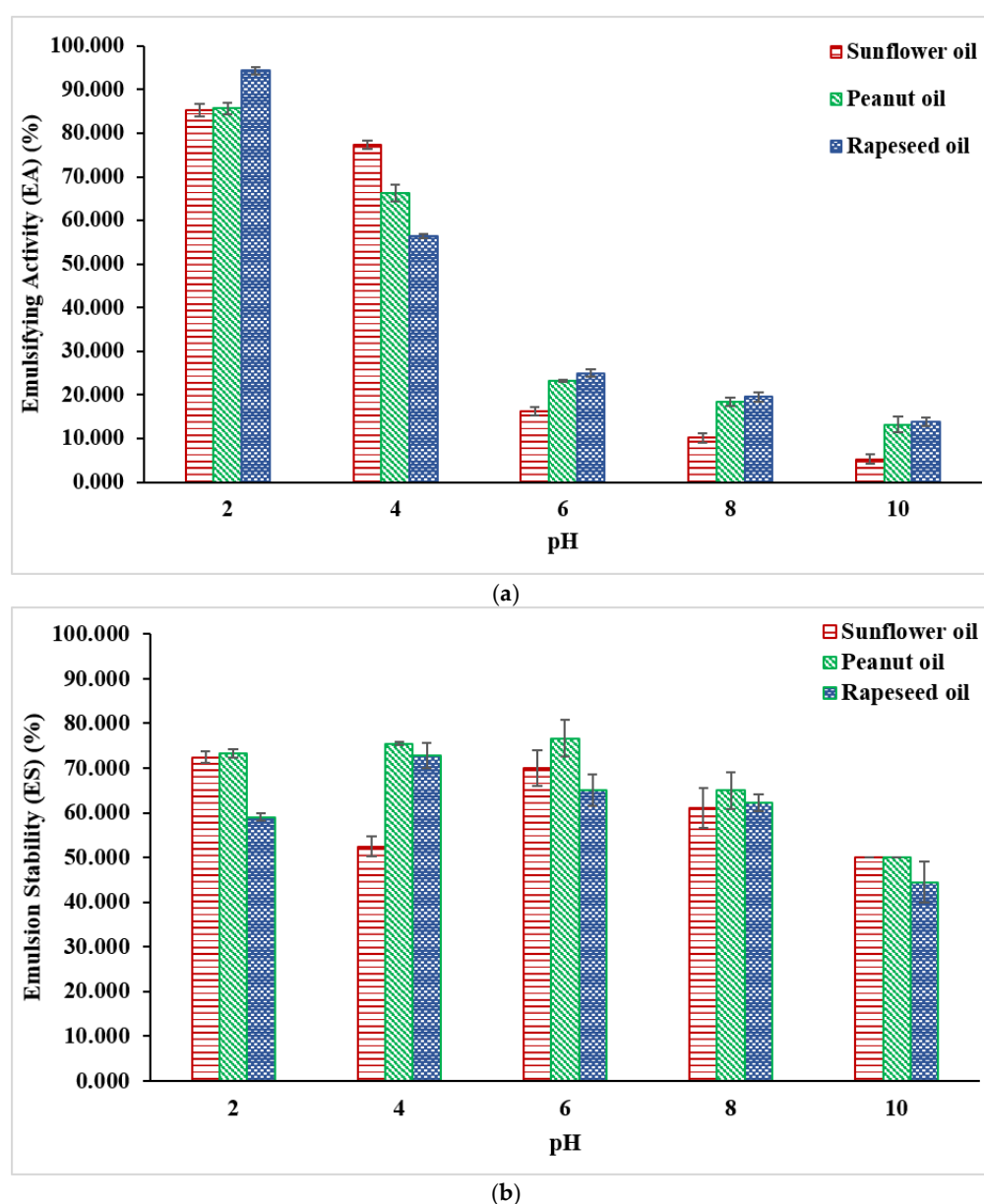


Figure 2. (a): Emulsifying activity of *Chondrus crispus* protein hydrolysates. (SE, standard error, $n = 9$). (b): Emulsion stability of *Chondrus crispus* protein hydrolysates. (SE, standard error, $n = 9$).

Emulsification—a key functional property of proteins in food formulations—arises from their amphiphilic nature and is essential in products like sauces, baked goods, and spreads. Enzymatic hydrolysis enhances this property by exposing hydrophobic groups, improving protein interaction with both oil and water phases. Optimal emulsification relies on a balanced distribution of hydrophilic and hydrophobic regions within the protein. The process occurs in two stages: initial droplet disruption and deformation, followed by stabilization through the alignment of surface-active agents at the newly formed oil–water interface [61]. Protein adsorption at the oil–water interface follows a diffusion-controlled mechanism, which is more effective at lower protein concentrations. However, higher protein concentrations may activate an energy barrier, hindering diffusion and subsequently reducing emulsifying activity [62,63]. The emulsification activity (EA) of *Chondrus crispus* hydrolysates varied significantly across oils and pH levels. EA peaked at pH 2 for all oils, with rapeseed showing the highest value (94.33%), followed by peanut (85.64%) and sunflower oil (85.27%). At pH 4, notable differences emerged in sunflower (77.36%), peanut (66.27%), and rapeseed (56.41%). EA was lowest at pH 10, ranging from 5.23% to 13.91%. These trends align with prior findings: [64] reported 20.11% EA for *Chlorella pyrenoidosa* hydrolysates in krill oil at pH 6.6, while [65] documented EA values of 20% and 25% for *Spirulina platensis* hydrolysates using pepsin and pancreatin at pH 6, respectively. Variations in EA are likely attributed to shifts in the hydrophobic–hydrophilic balance of amino acid residues with changing pH [63].

Emulsion stability (ES) of *Chondrus crispus* hydrolysates was significantly influenced by oil type and pH, with values ranging from $72.43 \pm 1.28\%$ to 50% for sunflower oil, $73.25 \pm 1.01\%$ to 50% for peanut oil, and $59.04 \pm 0.85\%$ to $44.44 \pm 4.54\%$ for rapeseed oil (pH 2 to 10). Sunflower and peanut oils showed consistently higher stability, particularly between pH 2 and 6. These trends align with [65], who reported ES indices of 75% (pepsin) and 25% (pancreatin) hydrolysates at pH 6 in *Spirulina platensis*. Emulsion stability is governed by factors such as pH, droplet size, net charge, interfacial tension, viscosity, and protein conformation [66]. The high thermal stability observed post heating likely results from protein dissociation into more lipophilic subunits, enhancing interactions with the lipid phase [67].

These findings underscore the potential of *Chondrus crispus* hydrolysates as effective natural emulsifiers, offering stable performance across diverse food systems. Their ability to enhance emulsification without synthetic additives makes them a promising clean-label ingredient for functional food innovation.

3.6. Foaming Capacity (FC) and Stability (FS)

The foaming properties of *Chondrus crispus* hydrolysates varied notably across pH 2–10 (Figure 3). Foam capacity (FC) showed a pH-dependent trend, peaking at $20.20 \pm 2.88\%$ at pH 10 and declining to $15.55 \pm 1.81\%$ at pH 2, with intermediate values at pH 4 ($10.26 \pm 2.09\%$), pH 6 ($12.12 \pm 2.47\%$), and pH 8 (9.11%). Foam stability (FS) remained consistently high, reaching $89.32 \pm 1.19\%$ at pH 6, followed by $88.45 \pm 0.18\%$ at pH 2, $88.89 \pm 2.27\%$ at pH 8, $83.33 \pm 1.94\%$ at pH 10, and $79.21 \pm 2.93\%$ at pH 4.

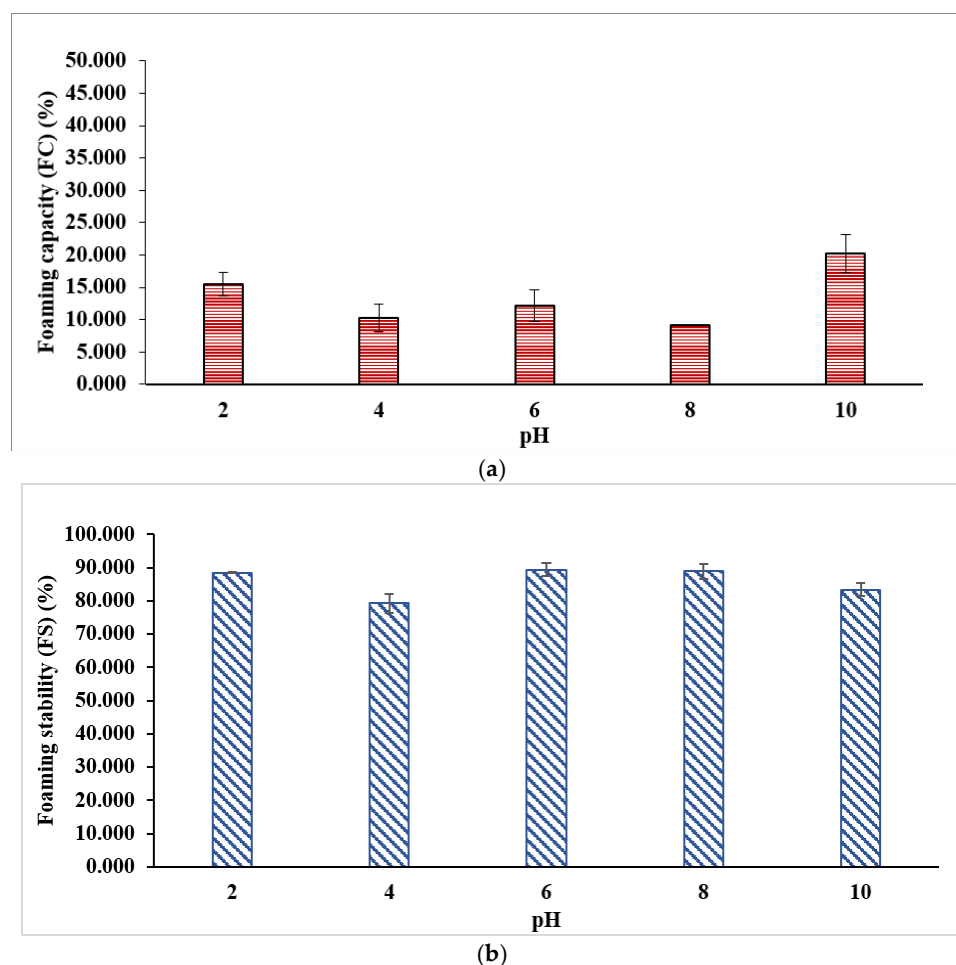


Figure 3. (a): Foaming capacity (%) of *Chondrus crispus* protein hydrolysates. (SE, standard error, $n = 9$). (b): Foam stability (%) of *Chondrus crispus* protein hydrolysates. (SE, standard error, $n = 9$).

In *Chondrus crispus* hydrolysates, FC was strongly pH-dependent, peaking at pH 10 ($20.21 \pm 2.88\%$) and reaching a minimum at pH 8 (9.09%). No significant differences were observed among FC values at pH 2 ($15.55 \pm 1.81\%$), 4 ($10.25 \pm 2.09\%$), and 6 ($12.12 \pm 2.47\%$) (Figure 3). The drop in FC at pH 8 likely corresponds to the protein's isoelectric point, where net charge is minimal [65]. Supporting this, [66] reported FC values of $6.4 \pm 1.0\%$ to $55.0 \pm 2.6\%$ for *Enteromorpha* protein concentrates across pH 2–12, attributing the enhanced foaming at alkaline pH to increased net protein charge, reduced hydrophobic interactions, and greater molecular flexibility—factors that facilitate rapid diffusion to the air–water interface and improved foam formation [67].

Foam stability (FS), defined as the percentage of foam retained over time, varied significantly with pH for *Chondrus crispus* hydrolysates. FS peaked at pH 6 ($89.32 \pm 1.92\%$) and 8 ($88.88 \pm 2.26\%$) (Figure 3), likely due to increased charge density that enhances electrostatic repulsion and minimizes bubble coalescence [67]. These values were substantially higher than the FS reported for protein concentrates from *Enteromorpha* species—ranging from 4.4% to 39% across pH 2–12 under zero salt conditions [66]. They attributed this to the foam stability generally being influenced by the protein film's water-retention capacity and electrostatic interactions [68].

The promising techno-functional properties of *Chondrus crispus* hydrolysates indicate their potential as functional ingredients in baked goods. Accordingly, they were applied as an egg replacer (emulsifier) in the preparation of vegan fairy cakes, as detailed in Section 3.8.

3.7. Predicted Bioactivities of *C. crispus* Hydrolysate Peptides

The bioactivities of identified *C. crispus* hydrolysate peptides with sequence homology greater than 85% with the parent protein were predicted using in silico analysis, and results are shown in Table 5. Two peptide sequences, GGPAGGGPAGGDAGLA and AQVPPLPNMPRMPA, derived from the proteins Transitional endoplasmic reticulum ATPase (Valosin-containing protein) (accession number R7QAA8_CHOCH) and Prohibitin (accession number R7QRM0_CHOCH), were predicted to be antimicrobial with Peptide Ranker (scores of 0.87 for both peptides), anti-inflammatory (scores of 0.480 and 0.478, respectively, for GGPAGGGPAGGDAGLA and AQVPPLPNMPRMPA) with PreAIP, and anti-diabetic with AntiDMPpred and a threshold probability of 0.6. In addition, both peptides were identified as having the potential to cause umami flavor. The peptide GIPDEWMGL, having sequence homology with the protein Phosphoglycerate kinase (accession number Q8GVE6_CHOCH), was predicted to be very bioactive (0.9) using Peptide Ranker and has predicted anti-inflammatory activity (0.469 value determined for the peptide using PreAIP). However, it was not identified as having the potential to inhibit enzymes important in the development of T2D. Another peptide, with the sequence VLPSLPFM, having sequence homology with the protein Animal heme peroxidase homologue (accession number R7QJ77_CHOCH), is predicted to be highly bioactive with Peptide Ranker (0.89) and anti-inflammatory (value of 0.478 with PreAIP) but also was not predicted to have T2D prevention qualities. However, both peptides are predicted to cause umami flavor. The predicted bioactivities determined herein for these peptides suggest that these peptides are ideal candidates for further study in vitro and in vivo as potential health-beneficial ingredients/compounds, but chemical synthesis and confirmation of predicted bioactivities are required.

In total, over 2000 peptides were identified from the *C. crispus* hydrolysate, and Table 5 lists those predicted to be most bioactive using in silico analysis. The peptide AQVPPLPNMPRMPA derived from the protein Prohibitin is predicted to be anti-inflammatory and anti-diabetic and to cause umami (Table 5). Prohibitin has been described in animals, unicellular eukaryotes, plants, and fungi. Prohibitins are positive regulators of cell proliferation in plants. The peptide AQVPPLPNMPRMPA was not found in the literature or BIOPEP-UWM and warrants further research based on predicted bioactivities. GGPAGGGPAGGDAGLA, the peptide corresponding to amino acid sequences of a valosin protein, also was predicted to have anti-inflammatory, anti-diabetic, and umami benefits. The peptide GIPDEWMGL, derived from a Phosphoglycerate kinase protein, was also predicted to have anti-inflammatory and umami activities. These peptides warrant further research, including chemical synthesis and confirmation of observed bioactivities in vitro, but this work is beyond the scope of this paper. Peptide AQVPPLPNMPRMPA includes the well-known anti-hypertensive peptide VPP at position 3–5 [69–72]. For example, the peptide sequence GLA, found at the C-terminal end of peptide GGPAGGGPAGGDAGLA, predicted to have anti-inflammatory benefits, has sequence homology with the peptide RLARAGLAQ (BIOPEP-UWM entry number 9812), identified by [73] as having Cyclooxygenase 1 and 2 inhibitory activities, linked to prevention of pain and inflammation. The peptide GIPDEWMGL has at the N-terminal end G a non-polar but not strongly hydrophobic amino acid, followed by I, a hydrophobic amino acid, and P, a hydrophobic amino acid. The C-terminal end has L, a hydrophobic amino acid. The peptides have a net negative charge overall, and the C terminal is not polar in this case.

3.8. Baking Loss and Color of the Developed Vegan Cakes

The baking loss for the control sample was $8.92 \pm 0.63\%$, whereas the vegan fairy cake incorporating hydrolysates exhibited a higher baking loss of $11.72 \pm 0.13\%$ (Table 6). In

terms of crust color, the control sample had L^* , a^* , and b^* values of 49.50 ± 0.33 , 17.52 ± 0.15 , and 37.54 ± 0.22 , respectively. Its crumb displayed L^* , a^* , and b^* values of 68.02 ± 0.42 , 2.70 ± 0.09 , and 38.03 ± 0.21 , respectively, with a total color difference (ΔE^*ab) of 10.16. In contrast, the vegan fairy cake formulated with seaweed hydrolysate had a crust with L^* , a^* , and b^* values of 56.23 ± 0.22 , 10.03 ± 0.26 , and 36.17 ± 0.36 . The crumb of this sample showed L^* , a^* , and b^* values of 59.93 ± 0.37 , 3.25 ± 0.06 , and 29.71 ± 0.30 , respectively, with a ΔE^*ab of 11.61.

Table 6. Baking loss (%), color (L^* , a^* , b^* and ΔE^*ab), cake density (g/cm^3), specific volume (cm^3/g), and volume index (cm) of control and formulated vegan fairy cakes containing hydrolysates ($\pm$ SE, standard error, $n = 9$).

Cake Characteristics			Control Cake	Vegan Cake
Baking loss (%)			8.92 ± 0.63	11.72 ± 0.13
Color characteristics	L*	Crust	49.50 ± 0.33	56.23 ± 0.22
		Crumb	68.02 ± 0.42	59.93 ± 0.37
	a*	Crust	17.52 ± 0.15	10.03 ± 0.26
		Crumb	2.70 ± 0.09	3.25 ± 0.06
	b*	Crust	37.54 ± 0.22	36.17 ± 0.36
		Crumb	38.03 ± 0.21	29.71 ± 0.30
ΔE*ab			Crust (10.16) & Crumb (11.61)	
Cake density (g/cm³)			0.51 ± 0.01	0.61 ± 0.02
Specific volume (cm³/ g)			1.95 ± 0.02	1.66 ± 0.04
Volume index (cm)			9.66 ± 0.13	9.21 ± 0.18

Chondrus crispus hydrolysates were utilized as an egg replacer in the preparation of vegan fairy cakes, and their performance was evaluated alongside control cakes over three consecutive days after baking. One critical factor in cake quality is the baking loss percentage, which represents the weight lost during baking, mainly due to moisture evaporation. This loss is significant for several reasons: It directly influences the cake's texture and moisture content—too much loss can result in a dry, crumbly texture, while too little can lead to a dense, overly moist cake. Additionally, baking loss affects the cake's volume and density, ensuring proper rise and crumb structure. Moreover, the level of baking loss can impact the shelf life of the cake; excessive loss may lead to faster staling, while insufficient loss could result in a shorter shelf life due to excess moisture. Therefore, understanding and managing baking loss is essential to producing cakes with desirable characteristics and maintaining freshness.

As shown in Table 6, the vegan fairy cake made with *Chondrus crispus* hydrolysates as an emulsifier experienced a baking loss of $11.72 \pm 0.13\%$. In contrast, the control cake, which used egg as the conventional emulsifier, had a lower baking loss of $8.92 \pm 0.63\%$.

In exploring aquafaba powder, lentil protein, and citric acid as egg replacers in gluten-free cake formulations, Silva and colleagues [74] reported baking losses ranging from 12.27% to 15.20%, notably higher than the control cake made with egg, which had a baking loss of $10.40 \pm 0.80\%$. A similar comparison by Aslan and co-workers [75] assessed chickpea aquafaba as an egg replacer, reporting a baking loss of 13.74% for the eggless formulation, compared to 12.72% in the egg-based control.

Beyond texture and moisture, cake color—particularly of the crust and crumb—is a key quality attribute. In this study, vegan cakes showed a darker crust and a slightly redder crumb compared to controls, likely due to phycobiliproteins (PBPs) and phenolic compounds present in the hydrolysates. Overall, the color characteristics of the eggless cakes were deemed highly appealing. A related study [76] assessing angel food cakes and muffins formulated with lentil protein as an egg/milk substitute found a marked reduction in

L*, a*, and b* color values, alongside a significant rise in ΔE^*ab values—18.9 for the crust and 21.2 for the crumb—indicating noticeable color changes. The use of enzymatically generated lentil hydrolysates in functional bakery goods was investigated previously [77], and a progressive decline in L* values in the resulting muffins was reported, indicating a darkening effect.

Similarly, Majzoobi and co-workers [78] observed that incorporating soy protein isolate into sponge cake formulations diminished crust lightness, attributing the darkening to Maillard reactions between free amino groups and reducing sugars during baking, while noting that crumb color is primarily determined by the ingredient composition.

3.9. Density, Specific Volume, and Volume Index of the Developed Vegan Cakes

Table 6 outlines relevant data pertaining to cake density, specific volume, and volume index of the developed vegan cakes in comparison to the egg-containing control cake samples. The control cake had a density of 0.51 ± 0.01 g/cm³, a specific volume of 1.95 ± 0.02 cm³/g, and a volume index of 9.66 ± 0.13 cm. In comparison, the cake with seaweed hydrolysates exhibited a density of 0.61 ± 0.02 g/cm³, a specific volume of 1.66 ± 0.04 cm³/g, and a volume index of 9.21 ± 0.18 cm.

Density and volume are essential quality indicators for consumers when evaluating cakes, especially eggless varieties. The results showed that vegan fairy cakes made with seaweed hydrolysates achieved a texture comparable to the control cakes but that they were slightly less light and fluffy compared to the control cakes, based on specific volume values reported. A low specific volume means it is heavy and dense. While there was a slight increase in density and a minor decrease in specific volume, the volume index remained unchanged, which suggests that incorporating hydrolysates helped maintain the cake's overall structure while contributing to a slightly denser texture. These findings highlight its potential as a viable alternative to traditional formulations, striking a balance between texture and structure in egg-free cakes.

In line with these findings, Anwar and Soliman [79] reported similar outcomes in their study on whole-meal chia flour in eggless cakes. They noted a slight increase in cake density and a reduction in specific volume, reinforcing the idea that plant-based protein alternatives can influence cake texture without compromising its overall structure. Similarly, Mohammadi [77] observed a 10% decrease in the specific volume of muffins made with lentil hydrolysates.

During the baking process, the production of steam and CO₂ from the leavening agent plays a crucial role in forming air cells that contribute to the cake's spongy texture and low density [80]. The hydrolysates in the vegan cakes exhibited a remarkable ability to retain moisture, highlighting their capacity to absorb and hold onto water molecules effectively. While this water retention is beneficial for the cake's moisture content, it may also reduce the water available for steam production during baking, potentially limiting the formation of air cells and contributing to a slight reduction in specific volume.

On the other hand, Lin and colleagues [81] found a significant decrease in the specific volume of eggless cakes made with isolated pea proteins. They attributed this reduction to insufficient aeration during mixing, which hindered the expansion of the batter during baking. The volume index of a cake, which reflects its overall size and internal structure, was comparable between the control and the vegan cakes. A higher volume index typically signals a lighter, more aerated cake, while a lower value suggests a denser, more compact product. In this study, the volume index of the vegan cakes was on par with the control cakes, indicating that the use of hydrolysates did not negatively impact the cake's rise.

Similar trends were observed by Anwar and Soliman [79] when they used whole-meal chia flour in eggless cakes, noting a slightly denser structure in the flour-based cakes

compared to their egg-containing counterparts. These results suggest that plant-based protein alternatives can successfully maintain a cake's volume while subtly affecting its texture. Protein hydrolysates emerge as a promising and innovative solution for producing high-quality, egg-free baked goods without sacrificing the key attributes of traditional cakes.

Moisture Content, Water Activity, and pH of the Developed Vegan Cakes

Cake samples were analyzed in triplicate over three days post baking for moisture content, water activity (a_w), and pH (Table 7). On day 0, the vegan cake with hydrolysates showed higher moisture ($29.34 \pm 0.18\%$) and lower a_w (0.754 ± 0.004) and pH (7.25 ± 0.00) compared to the control ($26.03 \pm 0.41\%$, 0.810 ± 0.001 , and 7.84 ± 0.01 , respectively). Similar trends persisted through days 1 and 2, with both samples showing gradual moisture loss and rising a_w and pH values. Notably, the vegan cake consistently retained more moisture and had lower a_w and pH across all days.

The cake samples were analyzed in triplicate for moisture content, water activity, and pH over three consecutive days following baking. On day 0, the moisture content of the vegan cakes was found to be $29.34 \pm 0.18\%$, notably higher than the control, for which a value of $26.03 \pm 0.41\%$ was recorded. Preserving the freshness and sensory appeal of baked goods relies on reducing the factors that contribute to degradation during storage, particularly retrogradation. This process involves the gradual movement, redistribution, and eventual loss of water within the cake matrix [82,83]. The vegan cakes maintained a higher moisture content, likely due to the superior moisture retention capabilities of the hydrolysates used. Similar trends were observed previously [33], where a significant reduction in moisture content in soy protein isolate and polysaccharide-based eggless cakes compared to controls were noted. Likewise, formulations using isolated pea protein also exhibited lower moisture retention values [81].

Water activity (a_w), a critical determinant of food shelf life, strongly influences microbial growth and overall product stability [84]. Consistently, the control cakes exhibited a higher water activity than the vegan cakes over the three days. This difference can be attributed to the high water-holding capacity of the hydrolysates, which limits the availability of free water. Comparable results were observed in a study by Anwar and Soliman [79], where the water activity of a control cake was measured at 0.808 ± 0.007 , whereas the eggless version made with whole meal chia flour showed a reduced water activity of 0.766 ± 0.007 .

Table 7. Moisture content (%), water activity, and pH of control and formulated vegan fairy cakes containing hydrolysates (SE, standard error, $n = 9$).

Days	Moisture Content (%)		Water Activity (a_w)		pH	
	Control	Vegan	Control	Vegan	Control	Vegan
0	26.03 ± 0.41	29.34 ± 0.18	0.810 ± 0.001	0.754 ± 0.004	7.84 ± 0.01	7.25 ± 0.02
1	24.63 ± 0.60	28.15 ± 0.13	0.817 ± 0.003	0.759 ± 0.005	7.85 ± 0.02	7.30 ± 0.41
2	24.06 ± 0.08	27.20 ± 0.07	0.822 ± 0.003	0.762 ± 0.005	7.89 ± 0.00	7.31 ± 0.32

The pH level of a cake plays a significant role in influencing various chemical reactions that occur during baking, such as leavening, browning, and flavor development. It is a critical factor for ensuring the desired texture, taste, and overall quality of the final product. The pH of the developed vegan cakes was found to be slightly lower than that of the control cakes, which could have impacted the flavor profile and the chemical environment of the cake. Typically, the pH of cakes falls within a neutral to slightly alkaline range, which is ideal for achieving optimal flavor, texture, and quality.

4. Discussion

The percentage yield of the hydrolysates obtained was $19.42 \pm 0.46\%$ from the whole seaweed when measured on a dry weight basis. This value compares favorably with values reported by Cain and colleagues previously [8]. The hydrolysate generated here had a protein content of 8.65%, a lipid content of 1%, and ash content of 15.8%, and the results align closely with values obtained for *C. crispus* hydrolysate previously generated [40] where the protein content was reported as $13.94 \pm 0.94\%$. The ash content reported herein for the hydrolysate exceeds that normally acceptable for human foods (5–15% ash content) and could be reduced using washing steps before further post-harvest processing or use of xylanase enzymes [85,86]. In terms of techno-functional benefits, the WHC values for the hydrolysate are greater than those reported for *Chlorella* [50] or *Spirulina platensis* hydrolysates [54]. WHC helps retain moisture and improves viscosity and dough thickening in baked goods. The *C. crispus* hydrolysate maintained emulsions best in sunflower and peanut oils.

Following MS characterization, several novel peptide sequences were identified, and in silico analysis determined that four of these peptides had a high probability of having anti-inflammatory and anti-type 2 diabetes health benefits. These peptides include peptide 1 with the sequence GIPDEWMGL, peptide 2 with the sequence VLPSLPFM, peptide 3 with the sequence GGPAGGGPAGGDAGLA, and peptide 4 with the sequence AQVP-PLPNMPRMPA. These peptides contain hydrophobic amino acids and are low in molecular weight. Peptide 1 is nine amino acids in length, has a molecular weight of 1016.46 Da and a net charge of -2 , is rich in bulky side-chain aromatic amino acids including tryptophan and asparagine, and also contains proline (P), isoleucine (I), and leucine (L) at the C-terminal end of the peptide. Anti-inflammatory peptides usually contain hydrophobic amino acids (including phenylalanine, leucine, isoleucine, proline, tryptophan, methionine, and valine) at the N-terminal end of the peptide and polar at the C-terminal end [84], similar to Peptide 1, which has isoleucine, proline, tryptophan, and methionine in its sequence. Peptide 2, with the sequence VLPSLPFM, contains valine, leucine, and proline at the N-terminal end of the peptide and has a molecular weight of 902.4919. Peptides 3 and 4 are also rich in hydrophobic and aromatic amino acids and have low molecular weights, characteristics of anti-diabetic and anti-inflammatory peptides [87,88].

5. Conclusions

This research highlights the potential of *Chondrus crispus* Stakehouse hydrolysate as a sustainable, marine-derived protein ingredient with strong techno-functional properties that can act as an emulsifier and as an egg and oat protein replacer in baked goods and has identified potential for use as an anti-inflammatory and anti-diabetic ingredient. Through enzymatic hydrolysis, a high-protein seaweed hydrolysate was generated, exhibiting improved emulsifying performance compared to the unprocessed biomass. Its successful incorporation as an egg substitute in a vegan fairy cake formulation demonstrates its viability as a clean-label, plant-based alternative for baked goods, particularly within vegan and allergen-conscious markets. Beyond its functionality, in silico analysis of the hydrolysate revealed peptides with predicted bioactive properties, including anti-inflammatory and anti-type-2 diabetes potential. These findings suggest that *C. crispus* hydrolysates may serve dual roles—as a functional ingredient and as a source of health-promoting bioactive peptides—making it attractive for future development of functional or health-oriented food products. Moreover, the bioactive potential identified supports further exploration into the nutraceutical and therapeutic applications of seaweed-derived protein hydrolysates and peptides. This work contributes to the broader movement toward sustainable, plant-based protein innovation and underscores the untapped

potential of red seaweeds in the development of multifunctional food ingredients tailored to meet both functional and health-driven consumer needs.

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