



Article

An Adapted High-Pressure Homogenization Workflow Improves Recombinant β -Casein Recovery from *Escherichia coli* Inclusion Bodies

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Abstract

The efficient extraction and purification of recombinant β -casein from *Escherichia coli* is a crucial step in bioprocess engineering. This study evaluates an adapted high-pressure homogenization (HPH) workflow for recombinant β -casein recovery and compares it with osmotic shock (OS) and standard HPH based on specific protein yield, estimated relative purity, and processing time. In contrast to standard HPH, which requires separate inclusion body recovery, washing, and subsequent denaturant-mediated solubilization, the adapted-HPH workflow integrates mechanical cell disruption with inclusion body recovery during homogenization. Standard HPH was evaluated in two downstream-processing runs, whereas adapted HPH and OS were each evaluated in three downstream-processing runs using separate aliquots from the same bioreactor biomass batch. Adapted HPH demonstrated higher specific yields (4.08 mg_{casein}/g_{CDW}) and required 40% less processing time compared to the OS and standard HPH methods. The specific yield obtained with adapted HPH was significantly higher than that obtained with standard HPH ($p = 0.026$), whereas the difference between adapted HPH and OS was not statistically significant ($p = 0.086$). Despite the inclusion body washing steps used for OS and standard HPH, adapted HPH achieved a slightly higher average purity than OS ($63.46 \pm 3.49\%$ versus $59.38 \pm 2.79\%$) and a purity comparable to standard HPH ($68.10 \pm 6.87\%$); however, these differences were not statistically significant. Overall, the comparative evaluation of these methods indicates that adapted HPH may provide a simplified alternative for recombinant β -casein recovery, although further validation with a larger number of independent experiments is required.



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Keywords: β -casein; high-pressure homogenization; osmotic shock; *Escherichia coli*; recombinant protein; bioprocess engineering

1. Introduction

Caseins are the major protein fraction of bovine milk and are widely used because of their nutritional and techno-functional properties. These proteins are secretory proteins that undergo post-translational modifications, including phosphorylation (α S1-, α S2-,

and β -casein) at serine residues and glycosylation (κ -casein) at threonine residues [1,2]. Among them, β -casein is an amphiphilic phosphoprotein with relevant emulsifying, foaming, and self-association properties, which supports its potential application in food and biotechnology-related products [3–7]. Moreover, β -casein serves as a precursor for bioactive peptides, some of which exhibit antihypertensive properties, contributing to its potential use in functional and health-promoting food products [7–11]. Recombinant production has therefore attracted interest as an alternative to conventional dairy-derived protein isolation. Recent developments in precision fermentation have expanded microbial production of animal-derived food proteins, including recombinant milk proteins, while highlighting challenges associated with host selection, production efficiency, downstream processing, and reproduction of native protein structures and post-translational modifications [12,13]. However, the heterologous production of β -casein remains challenging because its native properties are influenced by post-translational modifications, particularly phosphorylation, which are not reproduced in *Escherichia coli* [1,6].

E. coli is one of the most widely used hosts for recombinant protein production because of its rapid growth, well-characterized genetics, and high expression capacity [14]. Nevertheless, high-level expression frequently results in the formation of inclusion bodies (IBs), which require dedicated downstream-processing steps for protein recovery [15,16]. Previously, IBs were regarded as non-functional waste products that had to undergo complex unfolding and refolding procedures to recover active proteins [17,18]. Conventional IB processing typically includes cell disruption, separation of soluble and insoluble fractions, isolation and washing of IBs, denaturant-mediated solubilization, and subsequent purification and refolding [15,18,19]. Although these procedures can improve the enrichment of the target protein, they increase process complexity, handling time, and the number of unit operations.

Recent developments in IB bioprocessing have increasingly focused on controlling IB formation and physicochemical properties during upstream production while simultaneously optimizing early downstream operations, including cell disruption, IB recovery, washing, solubilization, and refolding [20].

High-pressure homogenization (HPH) is an established mechanical method for disruption of microbial cells and is commonly applied in recombinant protein recovery [18,21,22]. In conventional HPH-based IB processing, however, the target protein may be distributed between soluble and insoluble fractions after cell disruption, requiring separate processing streams and additional IB washing and solubilization steps [18,23,24]. This increases downstream-processing time and may contribute to product losses during repeated centrifugation, washing, and transfer operations. Recent approaches to IB downstream-process intensification have demonstrated that modification and integration of early processing operations can improve IB purity and subsequent protein recovery. For example, Klausser et al. (2026) recently demonstrated that integrating additional HPH cycles with IB washing increased IB purity and improved product concentrations after refolding, highlighting the potential of intensified HPH-based workflows for IB processing [25]. However, such intensified strategies generally retain distinct IB recovery, washing, and subsequent solubilization steps. In contrast, the adapted-HPH workflow evaluated in the present study integrates mechanical cell disruption with immediate denaturant-mediated treatment of the IB-containing material, thereby reducing the number of separate downstream-processing operations.

Osmotic shock (OS) represents a gentler non-mechanical alternative and has been used for selective release of cellular proteins from *E. coli* [26,27]. However, its suitability for the recovery of recombinant proteins accumulated in IBs is less straightforward because OS primarily induces membrane permeabilization rather than complete mechanical cell disruption. Consequently, additional IB isolation, washing, and denaturant-mediated solubilization may still be required, which can limit its practical advantages for IB-based processes.

In the present study, we investigated whether direct exposure of the biomass to denaturant during high-pressure homogenization could simplify recombinant β -casein recovery by integrating cell disruption and IB solubilization into a single workflow. This adapted-HPH strategy was compared with a conventional HPH-based process and an OS-based workflow using biomass obtained from the same fed-batch cultivation. The novelty of the present study, therefore, lies in evaluating this simplified HPH-based workflow for recombinant β -casein recovery and directly comparing its specific protein yield, estimated relative purity, and processing time with those of conventional HPH-based IB processing and OS, using biomass aliquots derived from the same bioreactor cultivation.

The study therefore addresses the following knowledge gap: although HPH and OS are established cell-disruption methods, their comparative performance for recombinant β -casein recovery from *E. coli* IBs, particularly with respect to process simplification, specific protein yield, estimated relative purity, and processing time, has not been systematically evaluated under a common biomass background. We hypothesized that integrating IB solubilization directly into the HPH step would reduce downstream-processing complexity and processing time while maintaining effective β -casein recovery. The objective of this work was therefore to evaluate the three workflows comparatively and to identify their respective advantages and limitations for recombinant β -casein recovery and pre-purification.

2. Materials and Methods

2.1. Chemicals and Materials

Chemicals used were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany) and Bio-Rad Laboratories, Inc. (Hercules, CA, USA). A purified standard of His₆-tagged β -casein was obtained following the cultivation of a recombinant *E. coli* BL21 (DE3) Gold strain $\Delta clpPQ$. The protein was isolated using immobilized metal affinity chromatography (IMAC) on an ÄKTA chromatography system (GE Healthcare Life Sciences, Chicago, IL, USA), using a HisPrep™ FF 16/10 column (20 mL) connected in series with a HisTrap™ HP column (5 mL) (GE Healthcare Life Sciences; Chicago, IL, USA). For detection via Western blot, an anti-His tag antibody from mouse (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was employed. To assess the characteristics of the recombinant protein, a bovine β -casein reference standard (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was used for comparative analyses.

2.2. Strains, Plasmids, and Cultivation

A codon-optimized gene encoding bovine β -casein was synthesized by Eurofins Genomics (Ebersberg, Germany), and the complete nucleotide sequence is provided in Figure S1. Briefly, the His₆-tagged gene sequence was cloned into the pET22b(+) plasmid system (Novagen, Merck Millipore, Darmstadt, Germany) (Table S1). This construct enables an N-terminal His₆-tag fusion and IPTG-inducible expression driven by the T7 promoter. The resulting recombinant plasmid was transformed into competent *E. coli* BL21 (DE3) Gold $\Delta clpPQ$ cells. The host strain carried a deletion of *ClpP* ($\Delta clpP$) and *ClpQ* ($\Delta clpQ$) to limit ClpP- and ClpQ-protease-mediated recombinant casein degradation and to enable intracellular casein production. Please see Table S2 for the primers used for cloning.

2.3. Expression of the Recombinant β -Casein

The pre-culture I was prepared in 100 mL shake flasks using 10% of the volume with LB medium supplemented with 100 μ g/mL of ampicillin as a selection marker and was cultivated at 37 °C and 120 rpm overnight in an incubator shaker (New Brunswick™/Innova® 44, Eppendorf AG, Hamburg, Germany). The pre-culture I was then transferred to 2 L shake flasks using 10% of the total volume with modified Riesenberg medium [28] in an OD₆₀₀ of 0.1 (pre-culture II). The modified Riesenberg minimal medium contained 97.7 mM KH₂PO₄,

37.8 mM $(\text{NH}_4)_2\text{SO}_4$, 30.3 mM $(\text{NH}_4)_2\text{HPO}_4$, and 8.9 mM citric acid monohydrate as buffering and nitrogen components. Glucose monohydrate was supplied at 25 g/L as the sole carbon source. For essential mineral supplementation, 4.9 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 10 mL/L of trace element solution (TES) were used. TES contained 22.9 mM Fe(III) Citrate, 7.6 mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 3.6 mM $\text{Zn}(\text{CH}_3\text{COO})_2 \cdot 2\text{H}_2\text{O}$, 0.9 mM $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 1.1 mM $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 2.5 mM $\text{Na}_2\text{-EDTA}$, 4.9 mM H_3BO_3 , 1 mM $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$. The medium pH was adjusted to 7.0 prior to sterilization by autoclaving.

Bioreactor cultivation was performed in a 42 L fermenter (ZETA GmbH, Graz/Lieboch, Austria) with modified Riesenbergs medium using a three-phase strategy. The cultivation process began with an initial batch phase containing 5 g/L glucose, followed by two fed-batch phases (FBI and FBII). The first was dedicated to biomass accumulation and the second for β -casein production. Fed-batch cultivation commenced upon glucose depletion, using a feed solution containing 50% (*w/w*) glucose. The first fed-batch phase employed an exponential feeding strategy with a μ_{set} of 0.3 until the culture reached an OD₆₀₀ of 40. Subsequently, the second fed-batch phase was initiated, during which β -casein expression was induced with 1 mM IPTG. At this stage, the μ_{set} was adjusted to 0.1, and the temperature was lowered to 30 °C to slow down the metabolic rate.

Cell cultures grown in a bioreactor were harvested and centrifuged at $25,000 \times g$ and 4 °C with a flow rate of 100 mL/min (Heraeus Contifuge Stratos, 3049 (HCT 22.300) rotor, Thermo Fisher Scientific GmbH, Waltham, MA, USA). Before purification, the cell pellets were frozen. The biomass used for all downstream-processing experiments originated from a single 42 L bioreactor cultivation. The harvested cell pellet was divided into separate aliquots to enable comparison of the three extraction workflows using the same biomass source and thereby minimize variation caused by differences between independent cultivation batches.

The conversion factor between OD₆₀₀ and cell dry weight (CDW) was determined using samples collected during the exponential growth phase of the bioreactor cultivation. At regular intervals, 10 mL cell suspension samples were collected, dried for 24 h at 110 °C, and weighed gravimetrically. The resulting CDW values were correlated with the corresponding OD₆₀₀ measurements by linear regression, yielding a conversion factor of 0.26 gCDW/L per OD₆₀₀ unit.

2.4. Standard Pressure Homogenizer Assisted Cell Lysis

This methodology was based on the purification described by Singh et al. [29]. To prepare the cell suspension for disruption and protein recovery, 50 g of bacterial cell biomass was resuspended in 300 mL of buffer 1 (Table S3, Figure 1). Cell disruption was carried out using a High-Pressure Homogenizer (APV 2000, SPX Flow Technology Germany GmbH, Norderstedt, Germany), where the suspension was subjected to four passes at a pressure range of 1300–1400 bars. To minimize thermal denaturation, the crude lysate from each cycle was collected in a pre-chilled beaker placed on ice. The lysate viscosity was assessed qualitatively during sample handling. When the lysate was considered too viscous for convenient handling and subsequent processing, additional buffer 1 (Table S3) was added incrementally in volumes of 50–100 mL. No quantitative viscosity threshold was defined or measured. Following homogenization, the lysate was centrifuged at $24,000 \times g$ and 4 °C for 30 min (Multifuge X3R, Thermo Fisher Scientific, Waltham, MA, USA). The resulting pellet was stored at −20 °C for subsequent recovery of the target protein. To prevent precipitation due to freeze–thaw cycles, the clarified supernatant was immediately filtered using MN 617 filter paper (Ø 185 mm) and processed for protein purification on the same day using an ÄKTA start chromatography system. Aliquots (1 mL) of both the crude lysate and the loaded sample were collected for subsequent protein quantification.

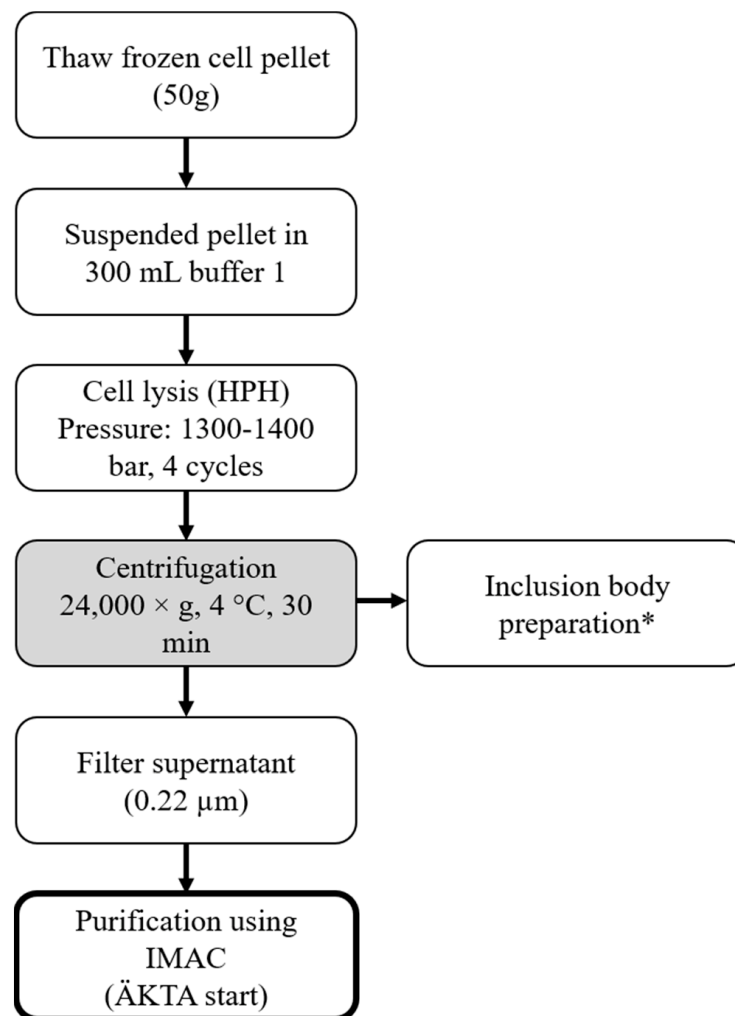


Figure 1. Standard high-pressure homogenization (HPH) workflow for cell disruption and fractionation during recombinant β -casein recovery. Cells are disrupted by HPH, followed by centrifugation to separate soluble and insoluble fractions. Subsequent inclusion body (IB) isolation and washing steps are required prior to denaturant-mediated solubilization and purification. *, Inclusion body preparation is detailed in Figure 2. Grey boxes indicate centrifugation steps. The bold outlined box indicates the final IMAC purification step performed using the ÄKTA chromatography system.

In addition, a sequential washing protocol adapted from the University of Cambridge [30] was applied (Figure 2). The membrane-associated proteins were removed by resuspending the cell pellet in 300 mL of buffer 2 (Table S3) and stirring for 45 min using a magnetic stirrer, followed by centrifugation (24,000 × g, 4 °C, 30 min). Then, nucleic acid was separated by washing with 300 mL of buffer 3 (Table S3) under identical conditions. Final contaminants were removed by a final washing step with buffer 1 (Table S3) for 1.5 h using a magnetic stirrer. To ensure complete dispersion, any residual pellet fragments after 45 min of resuspension were homogenized using a hand blender (ESGE Zauberstab, Berlin, Germany). The processed IBs, after centrifugation, were subsequently solubilized in buffer 4 (Table S3) for 1.5 h to facilitate protein denaturation and extraction. The resulting suspension was centrifuged under the same parameters, and the clarified supernatant was collected. Prior to purification using the ÄKTA start chromatography system, the supernatant was filtered through MN 617 filter paper ($\varnothing$ 185 mm) to remove insoluble debris.

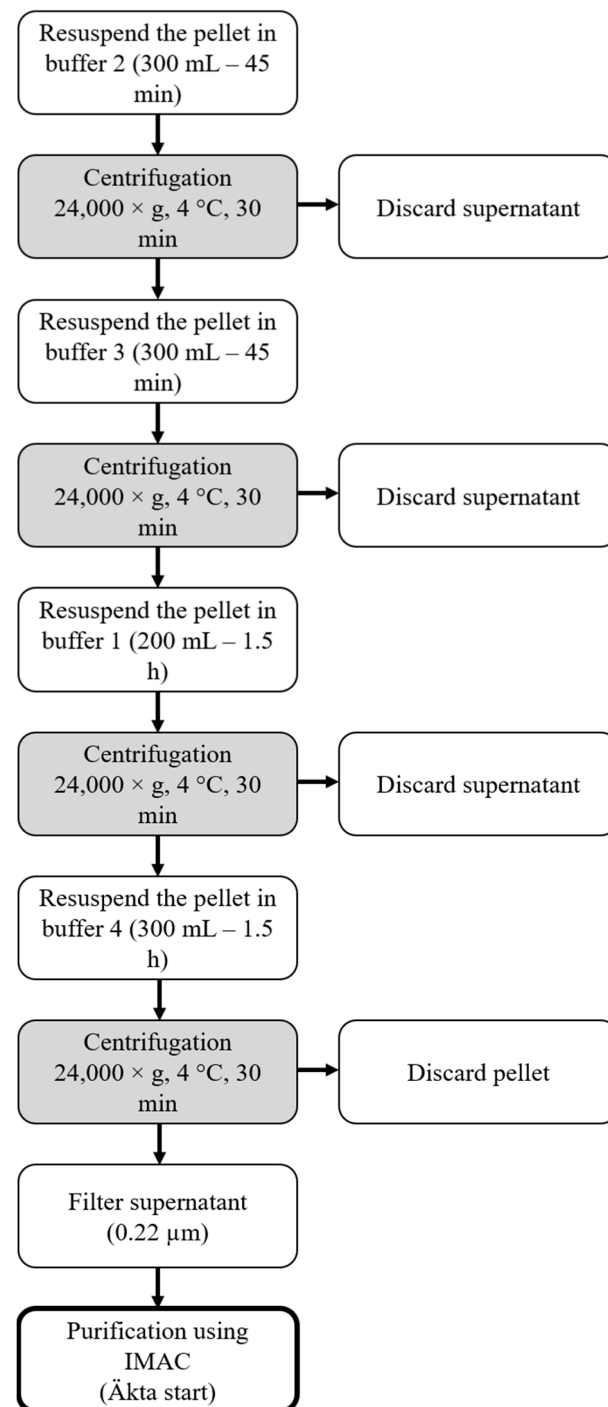


Figure 2. Inclusion body (IB) preparation and washing workflow adapted from the University of Cambridge protocol [30]. Following cell disruption and centrifugation, inclusion bodies are sequentially washed to remove membrane-associated proteins, nucleic acids, and residual cellular contaminants prior to denaturant-mediated solubilization. Optimizations introduced in this study aimed to improve β -casein recovery and process efficiency. Grey boxes indicate centrifugation steps. The bold outlined box indicates the final IMAC purification step per-formed using the ÄKTA chromatography system.

2.5. Adapted High-Pressure Homogenizer-Assisted Cell Lysis

In this adapted cell lysis method, 50 g of bacterial cell biomass was directly resuspended in 300 mL of buffer 4 (Table S3, Figure 3). With this change, the inclusion bodies were now solubilized directly from the beginning and were then expected to also be present in the supernatant of the disrupted cells after centrifugation, rather than in the pellets

as in the standard methodology. As previously, cell disruption was carried out using a High-Pressure Homogenizer (APV 2000, SPX Flow Technology Germany GmbH, Norderstedt, Germany), where the suspension was subjected to four passes at a pressure range of 1300–1400 bars. To minimize thermal denaturation, the crude lysate from each cycle was collected in a pre-chilled beaker placed on ice. The viscosity of the resulting lysate was assessed qualitatively during sample handling. When necessary to facilitate handling and subsequent processing, additional buffer 4 was added incrementally in volumes of 50–100 mL. No quantitative viscosity threshold was defined or measured. Following homogenization, the lysate was centrifuged at $24,000\times g$ and $4\text{ }^{\circ}\text{C}$ for 30 min (Multifuge X3R, Thermo Fisher Scientific, Waltham, MA, USA) to separate the soluble protein fraction from insoluble cellular debris. The resulting pellet was stored at $-20\text{ }^{\circ}\text{C}$ for subsequent recovery of the target protein. To prevent precipitation due to freeze–thaw cycles, the clarified supernatant was immediately filtered using MN 617 filter paper ($\varnothing$ 185 mm) and processed for protein purification on the same day using an ÄKTA start chromatography system. Aliquots (1 mL) of both the crude lysate and the loaded sample were collected for subsequent protein quantification.

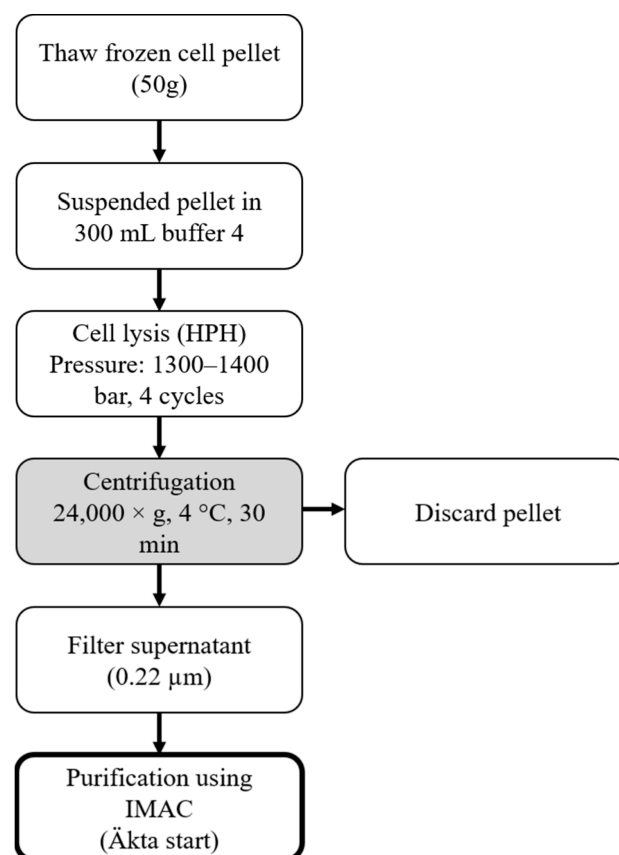


Figure 3. Adapted high-pressure homogenization (HPH) workflow for streamlined recovery of recombinant β -casein. The process integrates cell disruption with direct solubilization of inclusion bodies during lysis, followed by centrifugation and purification of a single protein stream. This approach eliminates separate inclusion body washing and parallel purification of soluble and insoluble fractions, reducing process complexity and overall processing time. Grey boxes indicate centrifugation steps. The bold outlined box indicates the final IMAC purification step performed using the ÄKTA chromatography system.

2.6. Osmotic Shock Assisted Cell Lysis

An aliquot of 50 g from the cell pellets was suspended in 300 mL of hypertonic osmotic buffer (Table S3, Figure 4) and incubated for 2 h. This treatment induced cellular

dehydration due to water efflux, resulting in cell shrinkage. Following the incubation, the cell suspension was centrifuged at $24,000 \times g$ and $4\text{ }^{\circ}\text{C}$ for 30 min (Multifuge X3R, Thermo Fisher Scientific, Waltham, MA, USA). The resulting pellet was then resuspended in 1 L of cold water and incubated for 30 min to 2 h to facilitate osmotic lysis, driven by rapid water influx and subsequent cell rupture. The lysed cell suspension was subjected to another round of centrifugation under the same conditions to separate the insoluble fraction [27].

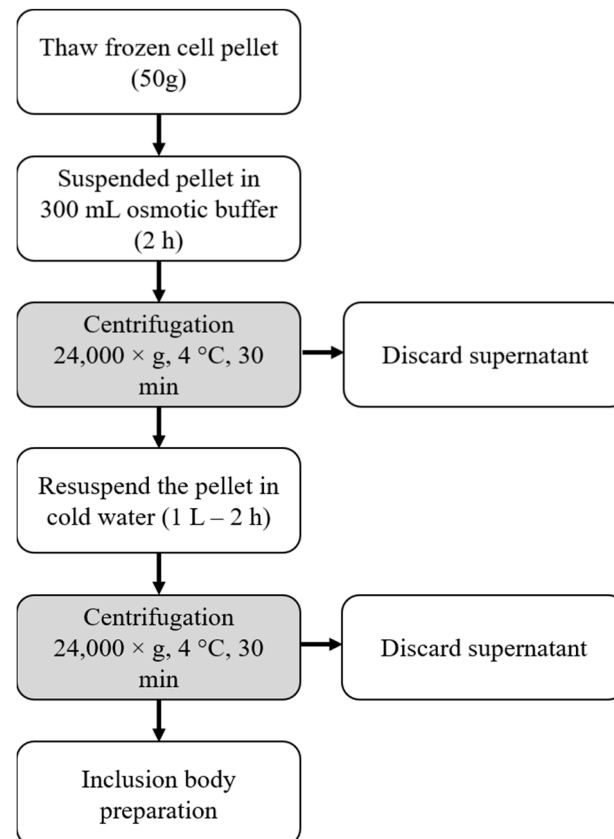


Figure 4. Osmotic shock (OS)-based downstream workflow for recombinant β -casein recovery. Cell permeabilization is achieved through sequential osmotic treatments, followed by centrifugation steps to recover the target protein predominantly from the soluble fraction. Grey boxes represent centrifugation steps.

To improve the purity of the obtained IBs, the same sequential washing protocol adapted from the University of Cambridge [30] was applied (Figure 2).

The OS workflow underwent minor procedural adjustments during the experimental series. In particular, equilibration and inclusion body solubilization times were extended in later runs to improve resuspension, cell lysis, reduce sample heterogeneity, and obtain material suitable for subsequent centrifugation and purification.

2.7. Immobilized Metal Affinity Chromatography

Recombinant His-tagged β -casein was purified using IMAC with an automated ÄKTA™ start chromatography system (Cytiva Europe GmbH, Freiburg, Germany) with UNICORN™ start 1.0 software (GE Healthcare, Piscataway, NJ, USA). A HisPrep™ FF 16/10 column (20 mL), prepacked with Ni Sepharose™ 6 Fast Flow affinity resin, was connected in series with a HisTrap™ HP column (5 mL), prepacked with Nickel Sepharose High Performance (HP) affinity resin, resulting in a total IMAC bed volume of 25 mL for selective capture of the His-tagged protein. Prior to sample loading, the column was equilibrated with five column volumes of buffer 1 (Table S3) [31]. The samples were loaded

onto the column at a low flow rate (0.5 mL/minute) to minimize the column back pressure. Following the loading step, any non-specifically bound proteins and impurities were washed out with approximately 10 column volumes of buffer 1 (Table S3). The β -casein-containing fraction was eluted using a one-step gradient (100%) of elution buffer (Buffer 5—Table S3) and detected at 280 nm. The standard-HPH workflow was evaluated in two downstream-processing runs, whereas adapted HPH and OS were each evaluated in three downstream-processing runs. Each run was performed using a separate biomass aliquot obtained from the same 42 L bioreactor cultivation. These runs therefore represent repeated downstream-processing experiments rather than independent biological cultivation replicates. The standard-HPH and adapted-HPH runs followed fixed method-specific conditions. In contrast, minor adjustments to equilibration and inclusion body solubilization times were introduced during the OS series.

2.8. Quantification of Intracellular β -Casein

The total protein concentration of the samples was estimated using the Bradford assay (Roti® Quant; Carl Roth GmbH Co. KG, Karlsruhe, Germany) [32]. A calibration curve was generated with bovine serum albumin fraction V (0.02–0.20 g/L), and all measurements were performed in triplicate. For SDS-PAGE analysis, samples were denatured in sodium dodecyl sulfate (SDS) and β -mercaptoethanol, then separated on 5–12% polyacrylamide gels using a Mini-PROTEAN Tetra Cell system (Bio-Rad Laboratories, Hercules, CA, USA). The PageRuler Plus prestained protein ladder (10–250 kDa; Thermo Fisher Scientific GmbH) served as the molecular weight marker. An in-house expressed and purified His₆- β -casein protein standard was included as a reference. Gels were stained with Coomassie dye (Roti-Blue quick; Carl Roth GmbH & Co. KG, Karlsruhe, Germany).

The relative abundance and purity of β -casein in the different fractions were estimated semi-quantitatively by densitometric analysis of the Coomassie-stained SDS-PAGE gels using GelAnalyzer software (Version 23.1.1; developed by István Lázár Jr. and István Lázár Sr., available at www.gelalyzer.com). Each lane and target band is selected manually to create an intensity profile plot. The raw volume (RV) of the whole lane and target band is determined by measuring the area under the curve (AUC) from the plotted intensity profile. The raw volume content (RVC) of the protein present in the sample was determined as Equation (1), and the percentage purity (PP (%)) of target protein relative to the total protein content was estimated as Equation (2). The concentration of β -casein ($C_{\beta\text{-CN}}$) and the amount of β -casein ($A_{\beta\text{-CN}}$) present in the sample were estimated with Equations (3) and (4), respectively. Where $C_{\beta\text{-CN}}$ and C_{TP} are the β -casein concentration and total protein concentration of samples measured in (mg/mL), respectively. $A_{\beta\text{-CN}}$ indicates the amount of β -casein (in mg) present in volume 'v' (in mL) of the sample.

$$\text{RVC} = \frac{\text{RV}_{\text{target band}}}{\text{RV}_{\text{whole lane}}} \quad (1)$$

$$\text{PP}(\%) = \text{RVC} \times 100 \quad (2)$$

$$C_{\beta\text{-CN}} = \text{RVC} \times C_{\text{TP}} \quad (3)$$

$$A_{\beta\text{-CN}} = C_{\beta\text{-CN}} \times v \quad (4)$$

The total yield of β -casein of an experiment was determined by taking the sum of the amount of β -casein present in each fraction of the experiment. Additionally, the specific yield of each experiment was calculated as the ratio of the total yield of β -casein to the weight of pellet used in the experiment. The purity of the total yield of an experiment was determined in terms of the weighted average purity (WAP) of all fractions obtained. It is calculated as the ratio of the product of the percentage purity and amount of β -casein of each fraction to that of the sum of the amount of β -casein of all the fractions (Equation (5)).

Finally, the variability in the purity of the total yield of an experiment was assessed in terms of weighted standard deviation (WSD_P) (Equation (6)). Additionally, the repeatability constant of each method was calculated using Equation (7) to evaluate the consistency of each method. The weighted average purity is indicated by WAP; the fraction number by I ; the weighted standard deviation by WSD_P ; the repeatability coefficient by RC [33]; the standard deviation by SD.

$$WAP = \frac{\sum_{i=1}^n (PP_i \times A_{\beta-CN(i)})}{\sum_{i=1}^n A_{\beta-CN(i)}} \quad (5)$$

$$WSD_P = \sqrt{\frac{\sum_{i=1}^n A_{\beta-CN(i)} \times (PP_i - WAP)^2}{\sum_{i=1}^n A_{\beta-CN(i)}}} \quad (6)$$

$$RC = 1.96 \times \sqrt{2} \times SD \quad (7)$$

Western blotting was used only to confirm the presence of His-tagged β -casein in the analyzed fractions and should not be interpreted as evidence of protein purity, correct folding, native post-translational modification, or biological functionality. Accordingly, the reported purity values represent semi-quantitative estimates based on SDS-PAGE densitometry and should not be interpreted as a comprehensive characterization of residual host-cell proteins or other process-related impurities. For that, a nitrocellulose/filter membrane (0.45 μ m, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and a wet-type blotting system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used. The electro-transferred membrane was blocked using 10 mL of PBS buffer containing 0.1% (v/v) Tween20 and 3% (w/v) bovine serum albumin on a shaking platform for 1 h and was further incubated with 2 μ L of primary antibody (mouse anti-histidine tag antibody, clone AD1.1.10, Bio-Rad Laboratories, Inc., Hercules, CA, USA) on a shaking platform for 1 h. The membrane was subjected to washing with wash buffer for 5 min, followed by rinsing with water four times. Finally, the prepared membrane was incubated with 5 mL of Novex Chromogenic Substrate (Invitrogen Corporation, Waltham, MA, USA) for 3 min. The reaction was terminated by rinsing the membrane with deionized water. Coomassie-stained gels and Western blots were imaged using a gel documentation system (Quantum ST5; Vilber Lourmat Deutschland GmbH, Eberhardzell, Germany).

3. Results

3.1. Expression of Recombinant β -Casein in *E. coli*

Recombinant *E. coli* BL21 (DE3) Gold $\Delta clpPQ$ harboring pET22b(+)- β -casein was cultivated in a 42 L bioreactor using a three-phase strategy. During the batch phase, the culture reached a CDW of 10.8 g/L, accompanied by complete glucose depletion. After glucose exhaustion, the FBI phase was initiated with an exponential feed at $\mu = 0.3 \text{ h}^{-1}$ at 37 °C, resulting in an increase in biomass to 20.8 g/L by the end of the 10 h feeding period.

The FBII phase was started by the addition of IPTG (1 mM) and a temperature shift to 30 °C. A constant growth rate of $\mu = 0.1 \text{ h}^{-1}$ was maintained. During FBII, biomass plateaued, reaching 26.5 g/L at the end of the 3 h period. A transient increase in glucose concentration was observed immediately after induction (Figure S2). The detection and IMAC enrichment of soluble recombinant β -casein following cell lysis using the standard high-pressure homogenizer approach in experiments 1 and 2 are presented in Figures S3 and S4.

Cell pellets obtained at the end of cultivation were harvested by continuous centrifugation and stored at −20 °C until further use. These biomass samples were subsequently subjected to different cell disruption and purification approaches for β -casein recovery.

3.2. High-Pressure Homogenizer for Cell Disruption and β -Casein Recovery

The harvested cell pellets obtained from bioreactor cultivations were processed using the standard-HPH protocol. In addition, an adaptation of the protocol was also developed to improve time of work, protein recovery, and yields.

Both the standard- and adapted-HPH approaches led to efficient disruption of recombinant *E. coli* cells and the release of His₆-tagged β -casein. In the standard approach, the protein remained in the pellet inside the inclusion bodies; therefore, pellet wash and urea buffer were needed to release the protein to be purified. With the adapted approach, β -casein was immediately found in the supernatant after cell lysis using HPH, as urea was directly incorporated during the resuspension.

Across the two downstream-processing runs of the standard-HPH method, the elution chromatograms showed reproducible profiles with a distinct single peak corresponding to β -casein (Figures 5a and S5a). In the standard-HPH process, quantitative evaluation using GelAnalyzer revealed β -casein concentrations ranging from 2.13 to 2.89 mg/mL in the insoluble fractions, corresponding to average recoveries in insoluble fractions of 37.63 mg per run.

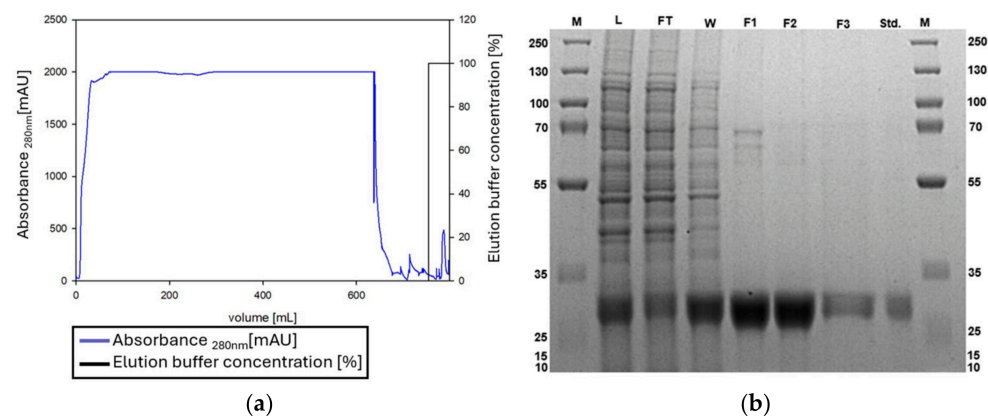


Figure 5. Detection and IMAC enrichment of insoluble recombinant β -casein after cell lysis by the standard High-Pressure Homogenizer approach from experiment 2. (a) The chromatogram displays the affinity chromatography addressing the His₆-tagged β -casein. The absorbance at 280 nm (blue line) and the elution buffer concentration (black line) are plotted. (b) SDS-PAGE showing the soluble protein fractions from load (L) as well as flow-through (FT), wash (W), elution fractions (F1–F3), purified His₆-tagged β -casein standard (Std.), and molecular weight marker (M).

In all the repetitions for the adapted method, the characteristic β -casein band (~25 kDa) was clearly visible both in the soluble fraction after lysis and strongly enriched in the elution fractions obtained by IMAC (Figures 6b and S6b and S7b). Western blot analysis confirmed the presence of His-tagged β -casein at the expected apparent molecular mass in these fractions, while weaker signals were detected in the flow-through and wash fractions (Figures 6c and S6c and S7c).

In addition, a higher protein concentration, ranging from 3.94 to 13.19 mg/mL, was found in the soluble fractions of the adapted-HPH process, resulting in an average protein recovery in the soluble fraction of approximately 202.73 mg per run. The average specific yield reached was 0.88 mg_{casein}/g_{CDW} and 3.83 mg_{casein}/g_{CDW} with a weighted average purity of 70.06% and 64.59% for the insoluble fraction of the standard HPH and soluble fraction of the adapted-HPH, respectively (Tables 1 and 2). Variation between replicates was low, as indicated by a weighted standard deviation of purity of approximately ± 4.10 and $\pm 2.92\%$. Total protein concentration was determined using the Bradford assay. The β -casein concentration, total amount of β -casein, and percentage purity (PP (%)) were calculated relative to the total protein concentration using GelAnalyzer software.

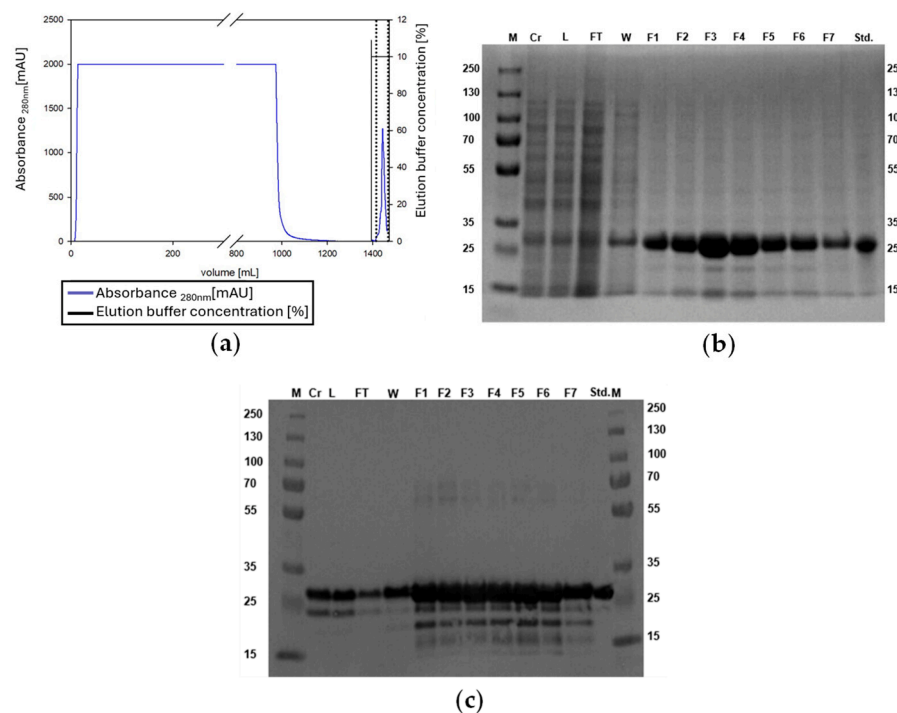


Figure 6. Detection and IMAC enrichment of soluble recombinant β -casein after cell lysis by the adapted High-Pressure Homogenizer approach from experiment 2. (a) The chromatogram displays the affinity chromatography addressing the His₆-tagged β -casein. The absorbance at 280 nm (blue line) and elution buffer concentration (black line) are plotted. The vertical dashed line indicates the transition from sample loading/washing to the elution phase. (b) SDS-PAGE and (c) Western blot analysis showing the soluble protein fractions from crude lysis (Cr) and load (L) as well as flow-through (FT), wash (W), and elution fractions (F1 to F7) from the IMAC process, marker (M) and standard (Std.). Equivalent amounts of total protein (6 μ g for all lanes) were loaded onto the gel, and protein bands were visualized by Coomassie brilliant blue staining. For the Western blot approach, an anti-His tag antibody was used.

Table 1. Summary of experiments using standard High-Pressure Homogenizer.

Standard High-Pressure Homogenizer Methodology							
Experiment	Fractions	Weight of Pellet (g)	Yield of β -Casein (mg) *	SY (mg/g) ¹	WAP (%) ²	WSD _P ³	
1	Soluble	52.24	n.d. **	n.d. **	n.d. **	n.d. **	
	Insoluble	43.86	31.94	0.73	72.96	0.9	
	TOTAL		31.94	0.61	72.96	0.9	
2	Soluble	50.65	2.94	0.06	5.52	1.5	
	Insoluble	42.53	43.31	1.02	67.16	4.30	
	TOTAL		46.25	0.91	63.24	15.62	
Average	Soluble	51.45	1.47	0.03	5.52 ⁴	1.50 ⁴	
	Insoluble	43.20	37.63	0.88	69.62 ⁴	4.38 ⁴	
	TOTAL		39.10	0.76	67.21 ⁴	12.94 ⁴	

* Yields of β -casein and SY were calculated as described in Section 2.8; ** n.d., not determined; ¹ SY, specific yield, calculated as described in Section 2.8; ² WAP, weighted average purity (%), Equation (5); ³ WSD_P, weighted standard deviation, Equation (6); ⁴ related to the total number of fractions. Bold TOTAL values represent the combined β -casein yield, specific yield (SY), weighted average purity (WAP), and weighted standard deviation (WSD) calculated from the soluble and insoluble fractions of each experiment.

Only minor amounts of β -casein were detected in the insoluble fractions of the adapted-HPH method (Figures S8–S10) and in the soluble fractions of the standard-HPH method (Figures S3 and S4), confirming that the majority of the product was recovered from the soluble phase and insoluble phase, respectively, under the chosen conditions. When data from all experiments from each approach were combined, the mean specific yield obtained

with standard HPH and adapted HPH was 0.76 ± 0.21 mg/g and 4.08 ± 1.44 mg/g, and the mean purity was $68.10 \pm 6.87\%$ and $63.46 \pm 3.45\%$, respectively (Tables 1 and 2).

Table 2. Summary of experiments using adapted High-Pressure Homogenizer.

Adapted High-Pressure Homogenizer Methodology						
Experiment	Fractions	Weight of Pellet (g)	Yield of β -Casein (mg) *	SY (mg/g) ¹	WAP (%) ²	WSD _P ³
1	Soluble	53.77	294.17	5.47	67.89	6.64
	Insoluble	10.06	8.26	0.82	36.49	N/A **
	TOTAL		302.43	5.62	67.03	8.31
2	Soluble	50.36	191.79	3.81	63.53	5.14
	Insoluble	12.46	2.07	0.17	41.78	5.54
	TOTAL		193.85	3.85	63.30	5.61
3	Soluble	55.49	122.23	2.20	62.35	8.64
	Insoluble	27.94	31.35	1.12	51.10	2.34
	TOTAL		153.58	2.77	60.05	9.01
Average	Soluble	53.21	202.73	3.82	65.40 ⁴	7.11 ⁴
	Insoluble	16.82	13.89	0.70	47.74 ⁴	6.40 ⁴
	TOTAL		216.62	4.08	64.27 ⁴	8.29 ⁴

* Yields of β -casein were calculated as described in Section 2.8; ** N/A, Not Applicable; ¹ SY, specific yield, calculated as described in Section 2.8; ² WAP, weighted average purity (%), Equation (5); ³ WSD_P, weighted standard deviation, Equation (6); ⁴ related to the total number of fractions. Bold TOTAL values represent the combined β -casein yield, specific yield (SY), weighted average purity (WAP), and weighted standard deviation (WSD) calculated from the soluble and insoluble fractions of each experiment.

3.3. Evaluation of Osmotic Shock for Cell Disruption and β -Casein Recovery

Osmotic shock also enabled efficient recovery of His₆-tagged β -casein from *E. coli* cells, although overall yields were lower than those achieved with adapted HPH. As in the adapted-HPH experiments, in the OS approach, a band at approximately 25 kDa was visible in the relevant SDS-PAGE fractions, and Western blotting confirmed the presence of His-tagged β -casein at the corresponding position (Figures 7b,c, S11b,c and S12b,c). The chromatographic profiles obtained during IMAC purification showed well-defined elution peaks, indicating successful binding of the target protein to the Ni²⁺-Sepharose resin (Figures 7a, S11a and S12a).

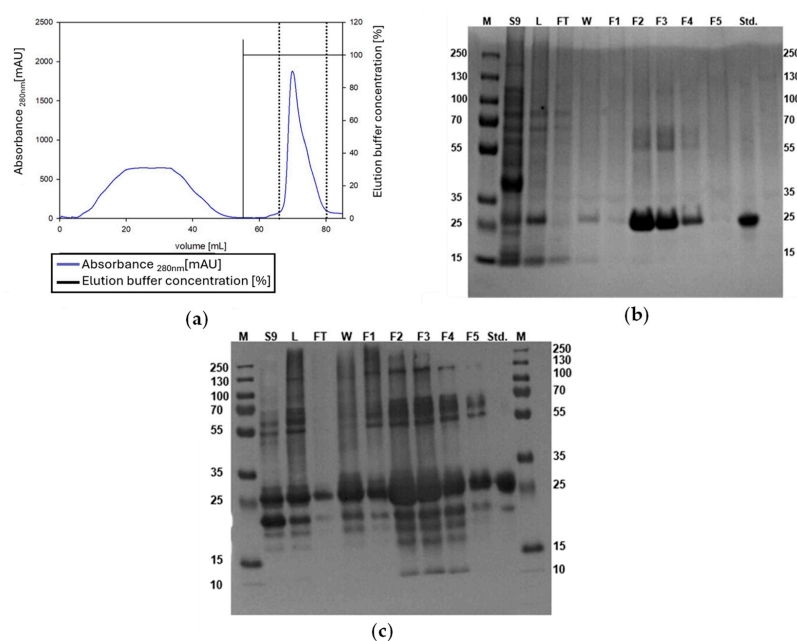


Figure 7. Detection and IMAC enrichment of soluble recombinant β -casein after cell lysis by the osmotic shock approach from experiment 2. (a) The chromatogram displays the affinity chromatography addressing the His₆-tagged β -casein from the experiment. The absorbance at 280 nm (blue line) and the elution buffer

concentration (black line) are plotted. The vertical dashed line indicates the transition from sample loading/washing to the elution phase. (b) SDS-PAGE and (c) Western blot analysis showing the soluble protein fractions from urea-solubilized inclusion bodies (S9) and load (L) as well as flow-through (FT), wash (W), and elution fractions (F1 to F5) from the IMAC process, marker (M) and standard (Std.). Equivalent amounts of total protein (6 µg for all lanes) were loaded onto the gel, and protein bands were visualized by Coomassie brilliant blue staining. For the Western blot approach, an anti-His tag antibody was used.

Quantitative analysis of the elution fractions revealed β -casein concentrations ranging from 2.81 to 6.21 mg/mL, corresponding to average total β -casein protein recoveries of 115.92 mg per run. The specific yield averaged 2.25 mg_{casein}/g_{CDW}, with a weighted average purity of 58.42% (Table 3). In comparison to standard HPH, adapted HPH displayed approximately 82% higher total yield, whereas standard HPH showed approximately 66% lower yield compared to OS. β -Casein was recovered in all three OS experiments, as supported by the chromatographic profiles, SDS-PAGE, and Western blot analyses (Figures 7a,b, S11a,b and S12a,b). However, because equilibration and solubilization times were adjusted during the experimental series, these experiments should be carefully interpreted.

Table 3. Summary of experiments using osmotic shock.

Experiment	Osmotic Shock Methodology				
	Weight of Pellet (g)	Yield of β -Casein (mg) *	SY (mg/g) ¹	WAP (%) ²	WSD _P ³
1	51.42	50.03	0.97	62.58	5.98
2	51.21	121.17	2.37	58.11	6.54
3	51.70	176.57	3.42	57.45	6.45
Average	51.44	115.92	2.25	58.42 ⁴	6.64 ⁴

* Yields of β -casein and SY were calculated as described in Section 2.8; ¹ SY, specific yield, calculated as described in Section 2.8; ² WAP, weighted average purity (%), Equation (5); ³ WSD_P, weighted standard deviation, Equation (6); ⁴ related to the total number of fractions. Bold values summarize the overall results across the three osmotic shock experiments.

Procedural adjustments to equilibration and solubilization times across the OS series were accompanied by progressive increases in protein recovery. Compared with experiment 1, the specific yields obtained in experiments 2 and 3 were approximately 144% and 253% higher, respectively. In addition, extending the solubilization time from approximately 1 h in experiment 2 to approximately 2 h in experiment 3 was accompanied by a further increase in specific yield. These observations suggest that longer equilibration and solubilization times may favor cell disruption and protein diffusion from inclusion bodies. However, because the parameters were modified during the experimental series rather than evaluated in a separate controlled optimization study, their individual contributions cannot be determined conclusively.

Across the three OS method-development experiments, the mean specific yield was 2.25 ± 1.23 mg_{casein}/g_{CDW} (Table 3).

4. Discussion

4.1. Physiological Shifts and Production Dynamics During High-Cell-Density Fed-Batch Cultivation

The three-phase fed-batch strategy successfully supported high-cell-density growth of *E. coli* BL21 (DE3) Gold Δ clpPQ and enabled induction of β -casein expression under controlled physiological conditions. The batch phase ensured rapid biomass formation prior to glucose depletion, whereas the exponential feeding phase (FBI) effectively sustained growth, as indicated by stable cultivation performance without visible stress signals. Transition to the production phase (FBII) combined IPTG induction with a reduced temperature and controlled growth rate, a strategy widely applied to mitigate metabolic burden

and improve protein folding in T7-based expression systems. This approach is well supported by studies demonstrating that post-induction temperature reduction enhances solubility and decreases inclusion body formation [34,35]. Lowering temperature decreases translational demand, slows aggregation kinetics, and promotes correct folding of recombinant proteins [36,37]. Temperature downshifts are widely recognized to mitigate the stress associated with T7-driven expression by reducing translational burden and slowing protein aggregation kinetics, which supports proper folding and reduces inclusion body formation [35,38,39].

Furthermore, recent work demonstrates that controlling post-induction specific growth rate is critical to preventing overflow metabolism and preserving metabolic capacity for recombinant protein synthesis. Kumar et al. showed that excessively high post-induction μ leads to substrate accumulation, reduced productivity, and rapid decline in cell physiological performance, whereas slower μ values ($0.02\text{--}0.08\text{ h}^{-1}$) maximize recombinant protein formation [40].

4.2. Streamlined HPH Workflow Reduces Processing Time Without Major Purity Loss

In the standard-HPH-based downstream process (Figure 1), high-pressure cell disruption is performed before the denaturant-mediated solubilization of IB. Also, it demands the purification of both soluble and insoluble fractions for the complete isolation of the target protein. The necessity of working with the soluble fraction (obtained before IB solubilization) is due to the release of soluble proteins present in IBs into the binding buffer during cell lysis at high pressure [23,24]. In the standard methodology, purification and analysis of both the fractions require a minimum of 5 to 6 days, resulting in increased processing time. Eggenreich et al., Palmer & Wingfield, Ban et al., and Pieracci et al. also mentioned the multiple downstream processing steps involved in conventional recombinant protein purification processes [18,19,41,42]. These steps include separation of IBs from cell debris, washing of IBs, solubilization and purification of target protein from IBs as well as purification of soluble fraction. In the adapted-HPH workflow (Figure 3), most of the recovered β -casein was detected in the soluble fraction, whereas the contribution of the insoluble fraction varied among the three downstream-processing runs. A paired two-tailed *t*-test did not detect a statistically significant difference between the fraction-specific yields ($p = 0.099$). However, given the limited number of paired observations, this result cannot be interpreted as evidence of equivalence or as proof that the insoluble fraction makes no relevant contribution. Omission of the insoluble purification stream should therefore be considered a potential process-simplification strategy that requires confirmation through additional experiments and an appropriately designed equivalence or non-inferiority analysis.

This adaptation reduced the total purification time to 2–3 days, compared to 5–6 days required in the general methodology, corresponding to an overall 40% improvement in time efficiency relative to the adapted-HPH workflow. The major contributor to this reduction was the elimination of the IB-washing step and the direct solubilization of IBs in urea during cell lysis. However, bypassing the IB-wash step also introduced some drawbacks. The presence of unremoved cell debris and contaminants increased the impurity load entering the chromatography columns, which may have contributed to column clogging and to the loss of target protein in the flow-through and wash fractions, as observed in Figures 6b and S7b [31,43].

Overall, the standard methodology (with an IB-wash step) yields slightly higher product purity. However, this advantage comes with major drawbacks, including reduced product yield, negative effects on subsequent downstream operations [16,18,44], and an increase in processing time and cost due to the additional washing step and

the need to purify both soluble and insoluble fractions. These trends are clearly visible in the experiments conducted for standard HPH. Although purity obtained with the standard method ($68.1 \pm 6.87\%$) was marginally higher than that of the adapted-HPH process ($63.46 \pm 3.49\%$), this difference was not statistically significant (Welch's *t*-test, $p = 0.253$). In contrast, the adapted-HPH method achieved markedly higher product yields ($4.08 \text{ mg}_{\text{casein}}/\text{g}_{\text{CDW}}$) compared to the standard approach ($0.76 \text{ mg}_{\text{casein}}/\text{g}_{\text{CDW}}$), as shown in Tables 1 and 2.

Given these trade-offs, it is important to evaluate whether the modest and statistically insignificant increase in purity offered by the standard method justifies the additional processing burden [45]. Furthermore, assessing how the purity level obtained with the adapted methodology influences refolding efficiency would provide a more comprehensive understanding of overall process performance.

4.3. Method Development of the OS Workflow: Effects of Extended Equilibration and Solubilization Times

The OS experiments combined methodological development with performance evaluation. Equilibration and solubilization times were adjusted across the experimental series to improve cell lysis and protein recovery. Consequently, the individual OS runs were not performed under fully identical conditions, and the observed differences among them cannot be attributed exclusively to random experimental variability.

The overall performance of the OS method was improved by modifying the time required for suspending the cell pellet in the lysis step and IBs in the solubilization step. The procedural adjustments were followed by the failure of an experiment, where the final resuspension of pellets in binding buffer containing urea turned out to be highly viscous.

Urea is known to destabilize double-stranded nucleic acid structures, including DNA, by forming hydrogen bonds with the nitrogenous bases, which can disrupt the stability of the DNA double helix [46,47]. Consequently, the high viscosity of the final suspension after IB preparation and solubilization is due to the breakage and release of genomic DNA. This led to the optimization of equilibration time of the cell pellet in step 1 and step 3, where it was increased from less than 30 min to a minimum of 2 h for enhancing cell lysis. The exposure of cells to a concentration gradient for a longer time could facilitate osmosis and the development of stress required for damaging the membrane [48]. The procedural adjustments resulted in a 144% increase in specific yield for experiment 2 and a 253% increase in specific yield for experiment 3, compared to experiment 1. This finding was supported by the study conducted by Rathore et al., where a 28% increase in product recovery was reported by increasing the equilibration time [48]. Additionally, the time optimization of solubilization from slightly higher than 1 h to approximately 2 h in experiment 3 compared to experiment 2 has also resulted in around a 44% increase in specific yield. The procedural adjustments were supported by the findings of Walther et al., where the rate-controlling step in IB solubilization is diffusion of proteins from the inner core of IBs through the outer layer [24]. For the effective diffusion of proteins, sufficient solubilizing agents must penetrate the IBs through pores to initiate solubilization, which requires time. Moreover, the significance of the time of contact of IBs with denaturant for efficient solubilization was also reported in the University of Cambridge protocol [30]. Overall, the later OS experiments suggest that longer equilibration and solubilization may improve cell disruption and protein recovery. However, because these adjustments were introduced during the experimental series and were not evaluated in a separate controlled optimization design, their effects should be regarded as preliminary.

4.4. Comparative Analysis of Cell Lysis Methods

The efficiency of cell lysis was primarily evaluated based on the specific yield of the target protein, as this metric directly reflects the effectiveness of protein release relative to biomass. This supports the hypothesis that the adapted-HPH method, owing to its operational simplicity and integrated solubilization strategy, can achieve higher specific yields compared to both standard HPH and OS. Consistent with this expectation, substantial differences were observed among the three approaches, with average specific yields of 0.76 mg_{casein}/g_{CDW} for standard HPH, 4.08 mg_{casein}/g_{CDW} for adapted HPH, and 2.25 mg_{casein}/g_{CDW} for OS (Tables 1–3). Statistical analysis confirmed that the difference between standard HPH and adapted HPH was significant (*t*-test, *p* = 0.026), supporting the conclusion that adapted HPH is expected to provide superior protein recovery.

Adapted HPH showed a higher mean specific β -casein yield than the OS experiments under the conditions tested. The comparison should be interpreted as descriptive and exploratory rather than as evidence of statistically demonstrated superiority. A confirmatory comparison would require both methods to be evaluated using predefined, unchanged protocols and an adequate number of independent runs. In this case, the best-performing experiment and the mean yield obtained with adapted HPH exceeded the corresponding OS values by approximately 64% and 87%, respectively, indicating a numerical trend rather than statistically confirmed superiority (Figure 8b,c). The limited number of independent experiments restricts the statistical power of this comparison, and additional independent downstream-processing runs will be required to determine whether the observed difference is reproducible.

Mechanistically, Walther et al. demonstrated that inclusion body (IB) solubilization is diffusion-limited, with proteins gradually migrating from the IB core toward the surface [24]. This behavior is reflected in the OS results, where extended solubilization times led to increased yields. In contrast, the adapted-HPH workflow bypasses separate IB isolation and prolonged solubilization, as IBs are mechanically disrupted and directly exposed to urea during high-pressure cell lysis. This enables immediate recovery of solubilized protein by centrifugation and IMAC. Conventional workflows, by comparison, typically involve IB isolation, washing, and subsequent solubilization prior to purification [18,19,41,42]. Eggenreich et al. reported that eliminating IB washing increased product yield at the expense of purity [18], a trend that is also observed here, with standard HPH showing lower yield but slightly higher purity compared to adapted HPH.

The high specific yield achieved by adapted HPH despite a shorter urea contact time than OS may reflect the combined effects of intensive mechanical cell disruption and immediate exposure of the released IB-containing material to denaturant. HPH is recognized as a critical unit operation in IB processing because homogenization conditions can influence cell disruption, release of intracellular material, IB recovery, particle characteristics, and subsequent separation and purification steps [18,49]. Eggenreich et al. (2020) demonstrated that HPH conditions substantially influence early downstream processing of bacterial inclusion bodies and can affect IB recovery and purity [18]. More recently, Klausser et al. (2026) showed that integrating additional HPH cycles into IB washing altered IB-processing performance, increased IB purity, and resulted in higher product concentrations after subsequent refolding [25]. These findings support the concept that HPH can influence not only cell disruption but also the downstream processability and physicochemical properties of IB-containing material. However, the specific effects of the adapted HPH procedure used here on β -casein IB particle size, morphology, aggregation state, and solubilization kinetics were not directly characterized and therefore require further investigation. Consequently, adapted HPH enables improved protein recovery while substantially reducing overall processing time.

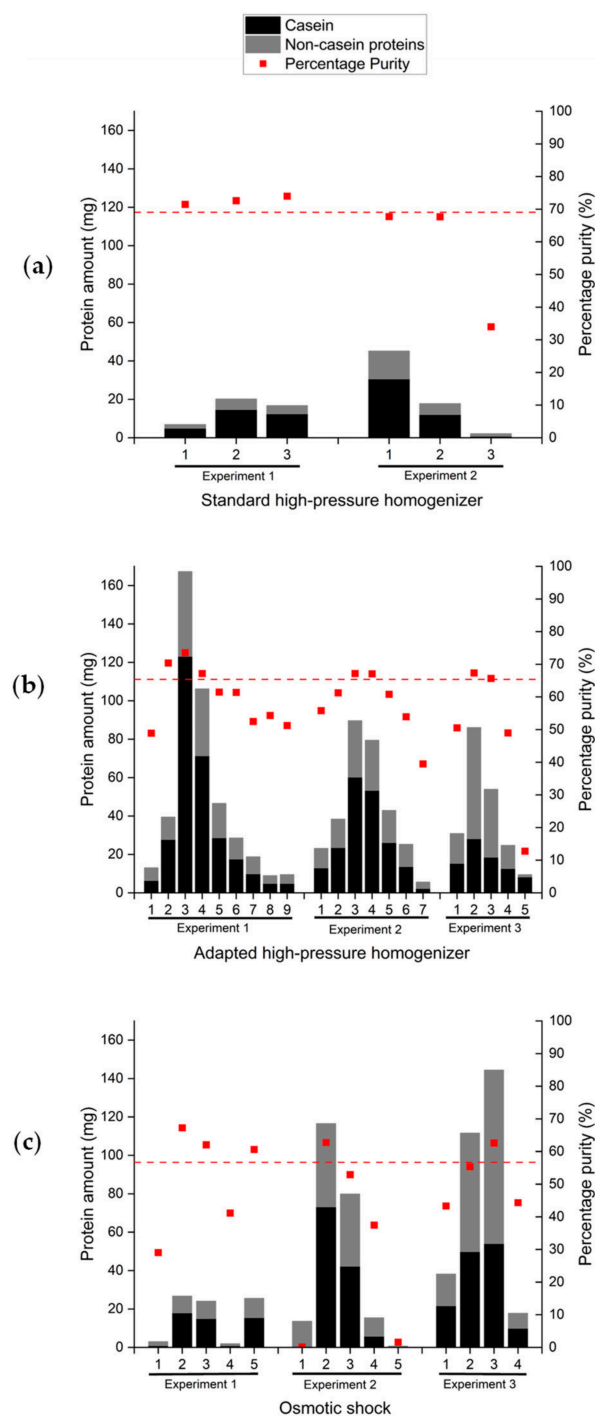


Figure 8. Fraction-wise distribution of β -casein and non-casein proteins obtained during downstream processing using the three evaluated workflows. Black bars represent the amount of β -casein, while gray bars represent non-casein proteins stacked on top of the β -casein bars; the total bar height therefore corresponds to the total protein amount in each fraction. Red square markers indicate the estimated percentage purity of each fraction, and the red dashed line represents the weighted average purity for the respective method. Panels are arranged from top to bottom as follows: (a) standard high-pressure homogenization, (b) adapted high-pressure homogenization, and (c) osmotic shock. Fractions are grouped according to the corresponding downstream-processing experiment. The numbers on the x-axis denote the sequential downstream-processing fractions collected within each experiment, as described in the respective workflow.

The absence of an inclusion body washing step in the adapted-HPH workflow and the direct solubilization of IBs during high-pressure cell lysis may also influence specific yield

due to the increased presence of host-cell impurities in the solubilisate. Previous studies have shown that high-pressure homogenization can cause micronization of cell debris and release of membrane-associated proteins, which may interfere with column binding capacity and lead to target protein losses in the flow-through and wash fractions [16,31,43,50]. Such effects are consistent with the protein losses observed in Figures 6b and S7b, highlighting a trade-off between process simplification and impurity load in the adapted-HPH approach.

4.5. Trends in β -Casein Purity Following High-Pressure Homogenization and Osmotic Shock

The lower product purity obtained with the OS approach cannot be explained solely by experimental execution or parameter selection. Rather, it reflects a structural limitation of the workflow when applied to processes relying on inclusion bodies. OS primarily induces membrane permeabilization instead of complete mechanical disruption, which can result in heterogeneous cell breakage and incomplete release of inclusion bodies. As a consequence, the subsequent addition of denaturant for protein solubilization may simultaneously promote further cell disruption, leading to the uncontrolled release of intracellular components. This sequential and partially overlapping release of target protein and host-cell impurities reduces the selectivity of the separation step. Therefore, the purity constraints observed for OS are best interpreted as a workflow-dependent limitation in the context of inclusion body recovery, rather than as a consequence of insufficient process optimization.

Osmotic shock differs fundamentally from mechanical cell disruption because it relies on alterations of the bacterial envelope rather than complete mechanical cell breakage. Vázquez-Laslop et al. (2001) demonstrated that osmotic shock of *E. coli* can produce a molecular-sieve-like release mechanism in which proteins and protein complexes are differentially released according to their native size, whereas larger macromolecular assemblies tend to remain associated with the cells [51]. This selective release mechanism is commonly exploited for recovery of periplasmic and soluble recombinant proteins while limiting the simultaneous release of intracellular cellular components [26,27]. However, its application to proteins accumulated predominantly as intracellular inclusion bodies is less straightforward because IBs represent aggregated protein particles rather than freely soluble protein molecules [15,17,20].

Accordingly, recovery of IB-associated β -casein using OS likely depends not only on the initial osmotic treatment but also on subsequent recovery, washing, and denaturant-mediated solubilization of the insoluble material [15,17,20]. The progressive increase in β -casein recovery observed during the OS series may therefore reflect improvements in several sequential steps, including osmotic treatment, recovery of insoluble material, and prolonged IB solubilization. Because these parameters were modified during method development rather than evaluated independently, the present data do not allow their individual contributions to be resolved. These considerations suggest that OS may offer selective advantages for soluble or periplasmic protein recovery, whereas IB-based processes generally require additional downstream operations for the isolation and solubilization of the aggregated target protein.

In addition, the quality of the isolated protein is one of the significant factors in recombinant protein production regardless of its final application. Purity of the IB preparation can influence subsequent solubilization and refolding performance because host-cell proteins, nucleic acids, membrane-derived material, and other co-solubilized impurities increase the complexity of the refolding environment and may promote competing intermolecular interactions and aggregation. Consequently, early downstream-processing steps that improve IB purity can have effects that extend beyond the initial purification stage. Klausser et al. (2026) recently demonstrated this relationship experimentally by showing that an HPH-based intensification of IB washing increased IB purity and was associated with higher

achievable product concentrations following refolding [25]. Similarly, studies of IB-derived recombinant proteins have emphasized that solubilization and pre-refolding purification conditions strongly influence subsequent refolding yield and recovery of biologically active protein. Therefore, although the adapted-HPH workflow in the present study achieved comparable semi-quantitative purity with fewer processing steps, the consequences of this purity level for β -casein refolding efficiency, aggregation, and final soluble recovery remain unknown and require direct experimental evaluation.

OS would generally be expected to yield higher protein purity than adapted HPH because it incorporates dedicated IB preparation and washing steps. However, the experimental results did not follow this anticipated trend, as the average weighted purity of protein obtained with adapted HPH (63.46%) was slightly higher than that of OS (59.38%) (Figure 8b,c). Generally, IBs are subjected to washing steps to enhance the purity of recombinant protein, as it assists in removing contaminating proteins and cellular components [15,52–55]. Similar to the findings from these studies, the IB wash conducted for OS experiments was also effective in removing cellular contaminants (Figure S13). However, despite the different IB wash, the purity of the isolated protein is still less than that of the protein isolated using adapted HPH. The addition of denaturant for IB solubilization prior to complete cell lysis will result in breakage of cells and release of genomic DNA. This phenomenon is clearly explained in Figure S14. The number of non-target bands present in lane S10 is considerably less than that of lane S12. Hence, it could be assumed that the resuspension of the pellet in binding buffer containing urea led to the breakage of unlysed cells, resulting in the release of genomic DNA and non-target proteins into the load. Consequently, the limited lysis efficiency of OS may contribute to the reduced purity of the isolated protein, despite the presence of dedicated IB washing steps. However, this step is methodologically crucial in processes based on inclusion bodies, as complete dissolution of the proteins is required. In contrast, the slightly higher purity obtained using adapted HPH, despite the absence of washing steps, may be attributed to the formation of smaller cell debris, which can be more efficiently separated from inclusion bodies by centrifugation [16,18,56]. Eggenreich et al. also reported a comparable purity of the product obtained from IBs that were subjected to washing and those that were not [18].

The comparable purity of standard HPH relative to OS (one-tailed *t*-test, $p = 0.15$) and adapted HPH (two-tailed *t*-test, $p = 0.5$) is indeed interesting. A one-tailed test was applied for the comparison between OS and standard HPH because OS was expected to yield higher purity due to its dedicated IB preparation steps. In contrast, no directional assumption was made for the comparison between adapted HPH and standard HPH, justifying the use of a two-tailed test. Despite the selective release mechanism of OS and sharing an IB preparation workflow similar to standard HPH, the purity values did not differ significantly. These findings are also evident in the SDS-PAGE gel images (lanes containing load) of standard HPH, adapted HPH, and OS (Figures S5b, S6b, S7b, S11b and S12b), where the number of bands in the load of adapted HPH is more than that of standard HPH and OS.

The reported purity values should therefore be interpreted as semi-quantitative estimates derived from SDS-PAGE densitometry. Comprehensive characterization of residual host-cell proteins and other process-related impurities would require orthogonal analytical methods such as RP-HPLC or LC-MS/MS and was beyond the scope of the present study.

Likewise, the SDS-PAGE and Western blot results demonstrate recovery and enrichment of His-tagged β -casein but do not provide comprehensive characterization of the structural or functional quality of the recovered protein. In particular, correct folding, self-association behavior, phosphorylation state, residual host-cell protein content, residual DNA, endotoxin burden, and biological or techno-functional activity were not assessed. Because *E. coli* does not reproduce the native phosphorylation pattern of bovine β -casein,

additional structural, physicochemical, and functional analyses will be required before conclusions regarding product quality or application suitability can be drawn [6].

From an application perspective, the simplified adapted-HPH workflow may provide advantages by reducing the number of downstream-processing steps required for β -casein recovery. However, because the protein is recovered from inclusion bodies under denaturing conditions, subsequent removal of urea and recovery of a soluble and functionally appropriate protein remain essential steps before its suitability for food applications can be established. In particular, the influence of the adapted workflow on β -casein folding, self-association, aggregation behavior, and techno-functional properties, such as emulsifying and foaming capacity, was not evaluated in the present study. Therefore, although the simplified workflow facilitates recombinant β -casein recovery, additional refolding, structural, and techno-functional characterization will be required to determine its suitability for specific food applications.

4.6. Comparative Evaluation of Process Robustness and Considerations for Scale-Up

Adapted HPH and standard HPH showed standard deviations in specific yield of 1.44 and 0.21 $\text{mg}_{\text{casein}}/\text{g}_{\text{CDW}}$, respectively. However, these values were derived from only three and two downstream-processing runs, respectively, all performed using separate aliquots from a single bioreactor biomass batch. They therefore describe variability in downstream processing under the tested conditions but do not capture biological variability between independent cultivations.

Repeatability could not be formally assessed for the OS workflow because equilibration and inclusion body solubilization times were adjusted during the experimental series. The observed standard deviation of 1.23 $\text{mg}_{\text{casein}}/\text{g}_{\text{CDW}}$ consequently reflects both run-to-run variation and procedural changes and cannot be interpreted as evidence of the reproducibility of a fixed OS protocol. For the same reason, direct statistical comparisons of variance or repeatability between OS and the HPH workflows were not considered appropriate.

A similar limitation applies to product purity. The observed standard deviations were 3.49% for adapted HPH, 2.79% for OS, and 6.87% for standard HPH. These values are presented descriptively only. In particular, the variability observed for OS may partly reflect the procedural adjustments introduced during the method-optimization series, whereas the standard HPH estimate is based on only two runs. Additional experiments using predefined, unchanged protocols and biomass from independent bioreactor cultivations would be required to establish meaningful differences in reproducibility among the workflows.

In pilot- and large-scale processing, the time efficiency of the cell lysis method is crucial, as it is directly related to the cost of processing. Based on the conditions investigated in this study, the adapted-HPH workflow required approximately two days, whereas OS and standard HPH required approximately five days. This corresponds to a reduction in overall processing time of approximately 40%. Although shorter processing times may reduce operational requirements, the associated effects on production costs, productivity, and scale-up were not directly evaluated in the present study.

In large-scale operations, the size of the downstream processing method is determined after knowing the amount of product produced in a single bioreactor run and the available processing time [57]. Although high-pressure homogenization is widely applied for recombinant protein recovery, the transferability of the adapted-HPH workflow to larger biomass quantities was not experimentally investigated in the present study. Therefore, dedicated scale-up studies will be required to determine whether the observed process advantages are maintained at industrially relevant scales [22]. One of the modifications mentioned by Inguva et al. (2024) in order to obtain the desired particle size was modifying the size and shape of the homogenizing valve [49]. Even though it could result in increased

capital costs, it could be considered as a one-time investment. On the other hand, the lower energy consumption, less complexity in the method, and moderate capital requirement are the major advantages of the osmotic shock method [45]. In the OS series, longer equilibration and solubilization times were accompanied by increased protein recovery. However, because these parameters were adjusted during the experimental series rather than evaluated in a separate controlled optimization design, a direct causal relationship cannot be established; hence, a larger amount of cell pellet would require longer duration and necessitate larger volumes of water and chemicals, which could lead to increased processing time and operational cost [58,59]. Both OS and standard HPH included additional inclusion body preparation and washing steps that increased workflow complexity relative to adapted HPH. Nevertheless, the economic consequences of these differences were not quantified and would require a dedicated process-economic analysis. Overall, the adapted-HPH workflow demonstrated reduced processing time and lower downstream complexity compared with OS and standard HPH under the conditions tested. These operational characteristics may facilitate future scale-up; however, dedicated scale-up experiments and process-economic analyses are required before conclusions regarding industrial suitability can be drawn.

4.7. Time Efficiency and Process Simplification

The downstream processing of recombinant proteins produced as inclusion bodies is generally considered challenging because it involves multiple steps, including inclusion body isolation, washing, solubilization, and refolding [18]. Although these procedures may improve product purity, they also increase processing time and cost, which represents an important limitation for large-scale production. In the present study, the adapted HPH workflow (Figure 3) reduced processing time by integrating cell disruption and inclusion body solubilization, thereby eliminating several steps required in the conventional workflows shown in Figures 1 and 4.

In the adapted-HPH workflow, the majority of β -casein was recovered from the soluble fraction, whereas the contribution of the insoluble fraction varied among the three downstream-processing runs. However, given the limited number of paired observations, this result cannot be interpreted as evidence of equivalence or as proof that the insoluble fraction makes no relevant contribution. A direct comparison between the complete adapted-HPH workflow and a soluble-fraction-only process, supported by additional replication and an appropriately designed equivalence or non-inferiority analysis, would be required before elimination of the insoluble purification stream can be recommended.

Because β -casein was recovered from inclusion bodies under denaturing conditions, the implications of this process simplification for overall process performance also depend on downstream refolding efficiency, which was not evaluated in the present study. If future experiments confirm that processing of the insoluble fraction provides no practically relevant additional recovery, omission of this stream could further simplify the workflow and shorten processing time. This would address a common limitation of conventional HPH-based inclusion body processing, in which target protein may be distributed between soluble and insoluble fractions, requiring both streams to be processed separately [23,24].

In contrast, the OS and standard-HPH workflows required approximately five days for cell disruption, inclusion body preparation, solubilization, and purification, whereas the adapted-HPH workflow was completed in approximately two days. Under the conditions evaluated, the adapted-HPH workflow therefore reduced the overall processing time by approximately 40% compared with OS and standard HPH, primarily by eliminating separate inclusion body washing and prolonged solubilization steps.

5. Conclusions

The adapted-HPH method achieved the highest mean specific β -casein yield among the evaluated workflows. The average specific yields obtained with adapted HPH, standard HPH, and OS were 4.08 ± 1.44 , 0.76 ± 0.21 , and 2.25 ± 1.23 mg/g_{CDW}, respectively. The yield obtained with adapted HPH was significantly higher than that obtained with standard HPH ($p = 0.026$). In comparison with OS, adapted HPH showed a higher mean yield; however, this difference did not reach statistical significance ($p = 0.086$) and should therefore be interpreted as a numerical trend under the tested conditions.

The average product purities obtained with adapted HPH, standard HPH, and OS were $63.46 \pm 3.49\%$, $68.10 \pm 6.87\%$, and $59.38 \pm 2.79\%$, respectively. Although adapted HPH showed a slightly higher mean purity than OS and a slightly lower mean purity than standard HPH, the differences were not statistically significant. These findings indicate that the simplified adapted-HPH workflow did not result in a statistically demonstrable loss of purity under the conditions evaluated.

In addition, the reduction in processing time of the adapted-HPH workflow represents a practical process advantage and may support future scale-up studies. However, industrial suitability cannot be concluded without validation at larger scale and a dedicated process-economic assessment.

The interpretation of the comparative results is limited by the small and unequal numbers of downstream-processing runs and by the use of biomass derived from a single bioreactor cultivation. Furthermore, downstream refolding efficiency was not assessed. The present results therefore do not establish equivalence between the soluble and insoluble fractions of adapted HPH and do not support definitive omission of the insoluble purification stream. Moreover, the present study focused on downstream recovery rather than comprehensive characterization of the recombinant β -casein product; therefore, protein sequence identity, phosphorylation status, folding, aggregation behavior, solubility after denaturant removal, and functional properties remain to be evaluated in future studies. Additional experiments using biomass from independent cultivation batches, together with an appropriately designed equivalence or non-inferiority analysis and an assessment of refolding performance, are required to validate this potential process simplification.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/applmicrobiol6080090/s1>, Table S1. Bacterial strains and plasmids used. Table S2. Primers used for cloning. Table S3. Buffers used for protein purification. Figure S1. Codon-optimized β -casein gene sequence for heterologous expression. Figure S2. Development of bioreactor process for T7-induced β -casein production in recombinant *E. coli* BL21 (DE3) Gold $\Delta clpPQ$. Figure S3. Detection and IMAC enrichment of soluble recombinant β -casein after cell lysis by the standard High-Pressure Homogenizer approach from experiment 1. Figure S4. Detection and IMAC enrichment of soluble recombinant β -casein after cell lysis by the standard High-Pressure Homogenizer approach from experiment 2. Figure S5. Detection and IMAC enrichment of insoluble recombinant β -casein after cell lysis by the standard High-Pressure Homogenizer approach from experiment 1. Figure S6. Detection and IMAC enrichment of soluble recombinant β -casein after cell lysis by the adapted High-Pressure Homogenizer approach from experiment 1. Figure S7. Detection and IMAC enrichment of soluble recombinant β -casein after cell lysis by the adapted High-Pressure Homogenizer approach from experiment 3. Figure S8. Detection and IMAC enrichment of insoluble recombinant β -casein after cell lysis by the adapted High-Pressure Homogenizer approach from experiment 1. Figure S9. Detection and IMAC enrichment of insoluble recombinant β -casein after cell lysis by the adapted High-Pressure Homogenizer approach from experiment 2. Figure S10. Detection and IMAC enrichment of insoluble recombinant β -casein after cell lysis by the adapted High-Pressure Homogenizer approach from experiment 3. Figure S11. Detection and IMAC enrichment of soluble recombinant β -casein after cell lysis by the Osmotic Shock approach from experiment 1. Figure S12. Detection and IMAC enrichment of soluble recombinant

β -casein after cell lysis by the Osmotic Shock approach from experiment 3. Figure S13. SDS–PAGE analysis of inclusion body (IB) preparation during Osmotic Shock (OS) in experiment 1. Figure S14. SDS–PAGE gel image of the supernatant of final IB wash (S10), suspension in binding buffer containing urea (S11), and its supernatant (load) (S12) of experiment 3 after Osmotic Shock (S: refers to step).

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Abbreviations

The following abbreviations are used in this manuscript:

$A_{\beta\text{-CN}}$	Amount of B-casein
AUC	Area Under the Curve
C_{TP}	Total Protein Concentration
$C_{\beta\text{-CN}}$	Concentration of B-casein
Cr	Crude lysis
CDW	Cell Dry Weight
FBI	Fed-Batch Phase I
FBII	Fed-Batch Phase II
FI	Elution Fractions; I defines the number of the fraction
FT	Flow-through
HPH	High-Pressure Homogenizer
I	Fraction Number
IB	Inclusion body
IMAC	Immobilized Metal Affinity Chromatography

L	Load
M	Protein Marker
OS	Osmotic Shock
PP (%)	Percentage Purity
RC	Repeatability Coefficient
RV	Raw Volume
RVC	Raw Volume Content
SD	Standard Deviation
Std.	Standard
SY	Specific Yield
W	Wash
WAP	Weight Average Purity
WSD _p	Weight Standard Deviation

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