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Comparative evaluation of casein hydrolysates obtained by chymotrypsin and starter-culture hydrolysis under optimized conditions

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ABSTRACT

Hydrolysis parameters are critical in the production of protein hydrolysates, as they directly determine the degree of hydrolysis (DH), which in turn governs the resulting technological properties. The aim of the research is to conduct comparative studies of the properties of casein hydrolysates obtained using optimized hydrolysis parameters with chymotrypsin and acidophilic bacteria. The resulting hydrolysates were characterized for their chemical composition using standard methods, chemical structure by Fourier-transform infrared (FTIR) spectroscopy, particle size distribution using a particle size analyzer, and technological indicators, including oil-holding capacity, water-holding capacity, oil-emulsifying capacity, and solubility. The results indicated that the casein hydrolysate samples exhibited no significant differences in moisture content and only slight variations in protein content. FTIR spectral analysis revealed similar profiles across all samples, indicating the presence of the same key functional groups, albeit at different relative concentrations. These spectroscopic findings corroborated the degree of hydrolysis: the hydrolysate produced with chymotrypsin, which exhibited the highest DH, also demonstrated the greatest absorbance intensity. This suggests a more extensive accumulation of hydrolysis products, including peptides. Particle size analysis confirmed that this sample contained a larger proportion of smaller particles, with 93.67% of the particles measuring between 43 and 85.9 nm. The technological characterization revealed a clear dependence on the degree of hydrolysis. The sample with the highest DH (sample 6) displayed a lower oil-binding capacity (21.08%). Water-holding capacity did not vary substantially across samples, remaining relatively low (26.13% to 30.20%). Hydrolysates with a higher DH exhibited a reduced emulsifying capacity. Conversely, solubility increased progressively with increasing DH, ranging from 89.09% to 94.16%. In conclusion, these findings underscore that to obtain a protein ingredient with tailored techno-functional properties, it is essential to conduct controlled hydrolysis to a specific degree.

Keywords: casein, degree of hydrolysis, chymotrypsin, starter cultures, *Lactobacillus acidophilus*

INTRODUCTION

Milk protein consists of a diverse array of nitrogenous substances. The most significant of these are casein and whey proteins. Casein is the protein fraction that coagulates under the action of rennet enzymes to form curd, which subsequently becomes cheese. Whey proteins, on the other hand, do not coagulate under rennet action **[1]**. Casein, the principal protein fraction in bovine milk, makes up approximately 80% of the total nitrogen content and serves as a fundamental nutritional component **[2]**. Structurally, it is not a single homogeneous protein but a

complex family of phosphoproteins, including αs1-, αs2-, β-, and κ-caseins, that associate into supramolecular structures called casein micelles. These micelles also contain micellar calcium phosphate, which helps to maintain their integrity and stability **[3]**. This unique molecular structure, coupled with the lack of a stable tertiary structure in casein, makes it highly susceptible to proteolytic digestion. The structure and composition of casein micelles vary among different mammalian species, thereby influencing their digestibility, allergenic potential, and suitability for dairy applications, including cheese and yogurt production **[4]**. Casein is known for its biological value because it contains essential amino acids **[5]**. Casein also has high levels of phosphopeptides that enhance calcium absorption and have bioactive properties, making it a vital component in dairy foods and protein supplements **[6]**.

Casein hydrolysates are bioactive compounds produced through the enzymatic hydrolysis of casein. Enzymatic hydrolysis is the most common method for generating casein hydrolysates. Various proteases are used to degrade casein into smaller peptides. The selection of enzymes greatly affects the degree of hydrolysis, peptide profile, and bioactivity of hydrolysate products **[7]**.

Casein is recognized as an exceptionally suitable and well-characterized substrate for the production of protein hydrolysates through enzymatic hydrolysis. Controlled proteolysis of casein yields a diverse range of smaller peptides, many of which have significant biological activity beyond their inherent nutritional value. These so-called bioactive peptides are encoded within the native protein sequence and can be released by specific proteolytic enzymes. Consequently, casein hydrolysate is of considerable scientific and industrial interest as it represents a valuable source of functional ingredients with antioxidant, antihypertensive, immunomodulatory and mineral-binding properties. This makes it highly relevant for the development of functional foods and nutraceuticals **[2]**, **[7]**.

The production of bioactive peptides primarily relies on enzymatic hydrolysis and microbial fermentation. Enzymatic hydrolysis is a biochemical process whereby protein substrates are cleaved into amino acids and peptide fragments through the catalytic action of proteases **[8]**. This enzymatic approach is designed to simulate endogenous protein degradation pathways in the human body under *in vitro* conditions.

Microbial fermentation offers distinct advantages over conventional enzymatic hydrolysis, utilizing a single protease of defined specificity. Due to their vast metabolic plasticity, microorganisms not only synthesize an array of bioactive metabolites during fermentation but also function as highly efficient biocatalysts for modifying exogenous molecules. Endogenous metabolites secreted by microbial cells facilitate the selective biotransformation of proteinaceous substrates. These biocatalytic pathways result in profound structural alterations of the substrate, frequently leading to a significant enhancement of its biological potency **[9]**, **[10]**. Specifically, certain microorganisms possess sophisticated proteolytic systems that degrade milk proteins into peptides and amino acids, which serve as essential nitrogen sources for their growth and proliferation.

Extensive research has demonstrated that enzymatic hydrolysis of milk proteins by gastrointestinal enzymes is a critical biochemical process that liberates encoded peptide sequences with demonstrable biological activity **[11]**. These proteolytic events mediated primarily by enzymes such as pepsin, trypsin and chymotrypsin during digestion result in the cleavage of native protein structures into a diverse range of smaller peptide fragments. Many of these fragments have specific bioactive functions, including, but not limited to, antihypertensive effects through angiotensin-converting enzyme (ACE) inhibition, opioid agonism and antagonism, immune modulation, antimicrobial activity, and increased mineral binding and absorption **[12]**, **[13]**. The formation and physiological relevance of these bioactive peptides depend on multiple factors, including the specificity of proteolytic enzymes, the duration of hydrolysis, and the structural characteristics of precursor milk protein substrates. This highlights the intricate relationship between protein digestion and the generation of health-promoting compounds *in vivo* **[14]**.

The application of fermentation processes and directed enzymatic hydrolysis allows for the targeted degradation of casein molecules. These biotechnological methods facilitate the controlled cleavage of casein polypeptide chains, effectively converting native protein into casein hydrolysates. The resulting complex mixture of peptides, characterized by different molecular weights and functional properties, is determined by the specific proteolytic enzymes used, the degree of hydrolysis, and optimized reaction conditions, including pH, temperature, time, and enzyme-to-substrate ratio **[15]**.

The scientific literature notes that DH significantly impacts the processing properties of protein hydrolysates, primarily due to changes in molecular size and the accessibility of functional groups. Numerous studies show that increasing DH generally improves protein solubility by forming smaller, more hydrophilic peptide fragments and breaking down native protein aggregates **[16]**. However, the impact on other processing properties is often non-linear and dependent on the substrate. For example, water- and oil-holding capacities often exhibit an inverse relationship to DH, as proteolysis reduces protein's ability to form stable three-dimensional matrices for retaining water and lipids, while emulsifying properties are optimal at low to moderate levels of DH (less than 15%) where

a balance between increased solubility and structural flexibility exists, with sufficient peptide length for stabilizing the oil-water interface. Extensive hydrolysis can degrade these properties. This is supported by studies of β-lactoglobulin, which have shown that limited hydrolysis disrupts the specific hydrophobic binding sites necessary for optimal ligand interaction and functionality **[17]**. Consequently, the technological properties of protein hydrolysates depend on the degree of hydrolysis and should be adjusted depending on the purpose of the protein supplement.

Despite extensive research on casein hydrolysates, several aspects remain poorly understood. Although enzymatic hydrolysis using individual proteases and fermentation with unspecified microbial consortia have been studied separately, the scientific literature currently lacks a direct, side-by-side comparison of these two fundamentally different biotechnological approaches – namely, hydrolysis using a purified animal-derived protease (chymotrypsin) and fermentation using a target bacterial strain (*Lactobacillus acidophilus*) – under optimized and comparable conditions.

Most existing studies focus either on the specific peptide profiles of a single enzyme or on the general properties of fermented dairy products, without comparative evaluation of their chemical structure and effectiveness in modifying their techno-functional properties.

Therefore, the present study aims to expand our knowledge of the production and characterization of casein hydrolysates obtained using two different biotechnological approaches under comparable conditions. The novelty of this work lies in its straightforward, methodologically consistent comparative framework, which, for the first time, allows for a data-driven assessment of the relative merits of each approach. This comparison is crucial for determining which biotechnological strategy is more effective in producing hydrolysates with desired technological functional properties, thereby providing a rational basis for selecting production methods for specific applications in nutraceuticals and functional foods.

Scientific Hypothesis

Hydrolysates produced by chymotrypsin will exhibit smaller particle size, higher solubility, and lower oil-holding and emulsifying capacity than hydrolysates produced by starter-culture fermentation due to their higher degree of hydrolysis.

Objectives

The aim of the study is to compare the chemical structure, composition, particle size, and functional and technological properties of casein hydrolysates produced under optimized hydrolysis conditions using chymotrypsin and acidophilic bacteria.

MATERIAL AND METHODS

Samples

Samples description: The object of the study was casein hydrolysates obtained by hydrolysis of casein with the enzyme chymotrypsin and acidophilic bacteria. Casein was obtained from skim milk (0.05% fat content) by coagulating the protein with rennet (95% chymosin, 5% beef pepsin; Chr. Hansen, Denmark) at a rate of 20 ml per 100 liters of milk. For enzymatic hydrolysis of casein, the starter culture (BakZdrav, Russia) containing *Lactobacillus acidophilus* and *Streptococcus thermophilus* was used. Chymotrypsin (OOO Samson-Med, Russia) was also used for hydrolysis.

Samples collection: Casein samples were stored in a refrigerator at 3 ± 1 °C for no more than 12 hours before hydrolysis.

Samples preparation: Hydrolysis was carried out according to previously established optimal parameters **[18]**, and the process flow diagram presented in Figure 1. The designation of the obtained casein hydrolysate samples is given in Table 1. The DH values were also obtained in our previous optimization studies under identical conditions **[18]**.

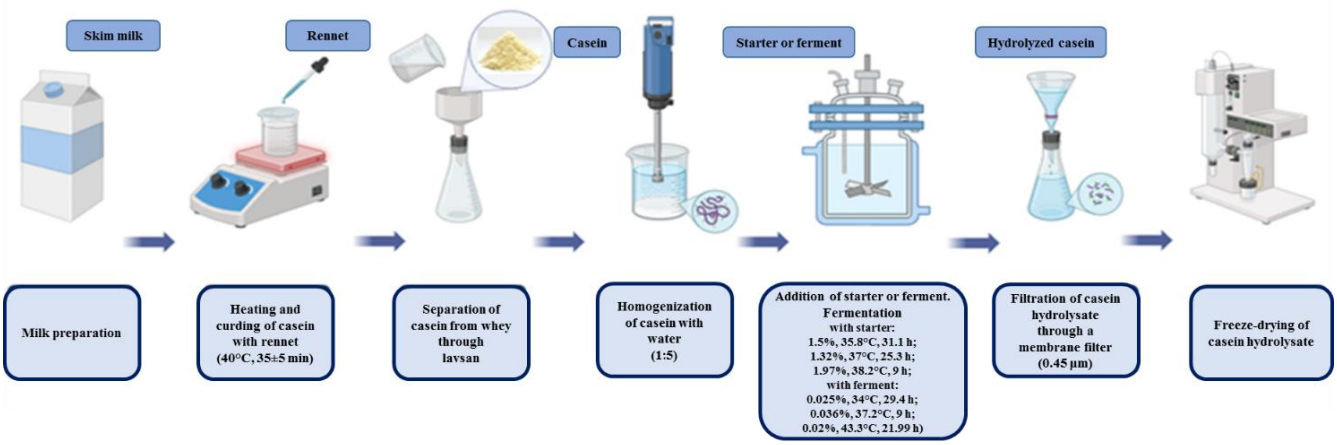

Figure 1 Flow chart for producing casein hydrolysates.

Table 1 Designation of samples prepared according to optimal hydrolysis parameters **[18]**.

Sample	Sample producing parameters	Degree of hydrolysis, %
1	S–1.5–35.8–31.1	17.33
2	H–0.025–34.0–29.4	20.90
3	S–1.32–37.0–25.3	16.94
4	H–0.036–37.2–9.0	21.30
5	S–1.97–38.2–9.0	17.45
6	H–0.02–43.3–21.9	28.60

Note: Designation: S, H – fermentation with starter (S) or chymotrypsin (H), respectively; starter/enzyme concentration (%) – temperature (°C) – fermentation duration (h).

Number of samples analysed: 6.

Chemicals

The following reagents were used in the experiments: ethyl alcohol 95% (OOO Gipppokrat, Russia), sulfuric acid chemically pure 95–97% (AO Vekton, Russia), potassium sulfate analytical grade (AO Vekton, Russia), copper sulfate (II) 5-aqueous, analytical grade f.0.1 (AO Vekton, Russia), hydrogen peroxide (AO Vekton, Russia), sodium hydroxide analytical grade (AO Vekton, Russia), boric acid chemically pure (AO Vekton, Russia), hydrochloric acid titer standard 0.1 n (Uralkhiminvest. Russia), Tashiro indicator, Eureka PH13-0.1-100et solution, 0.1% (in total), in ethanol (NPK Eureka, Russia), potassium bromide, analytical grade (AO Vekton, Russia).

Animals, Plants and Biological Materials

In this study, we used a bacterial starter culture to obtain casein hydrolysate by microbial fermentation. The bacterial count in the starter culture (Limited Liability Company BakZdrav, Russia) containing *Lactobacillus acidophilus* and *Streptococcus thermophilus* was $2 \cdot 10^8$ CFU/g.

Instruments

Shimadzu IRAffinity-1S spectrophotometer (Shimadzu, Kyoto, Japan), Nanotrac Ultra particle size analyzer (Microtrac Inc., USA), homogenizer Stegler S-10 (Langfang Zhihang Science Instrument Co., Ltd., Китай), thermostat TV-20-PZ-K (Kasimovsky PZ, Russia), centrifuge TsLU-1 Orbita (NPO Vetinstrument, Russia), Freeze-drying chamber ABAT LF-06P (ChuvashTorgTechnika, Russia).

Laboratory Methods

Determination of chemical structure: The chemical structure of the casein hydrolysates was characterized using Fourier transform infrared (FTIR) spectroscopy. Spectra were acquired using a Shimadzu IRAffinity-1S spectrophotometer (Shimadzu, Kyoto, Japan) over a wavenumber range of 650–1650 cm^{-1}.

Determination of particle size: The average particle size of the hydrolysates was determined by measuring the mean hydrodynamic diameter (nm) via laser diffraction analysis. Measurements were performed using a Nanotrac Ultra particle size analyzer (Microtrac Inc., USA).

Data acquisition and spectral processing were carried out using LabSolutions IR software (Version 2.2, Shimadzu, Japan). Identification of spectral peaks was performed by correlating the observed absorption bands with the characteristic vibrational frequencies of specific chemical bonds and functional groups.

Determination of chemical composition: The chemical composition of the hydrolysates was determined in quintuplicate following the official procedures of the Association of Official Analytical Chemists. Total nitrogen

content was quantified via the Kjeldahl method, and the crude protein concentration was subsequently calculated using a conversion factor of 6.38 (AOAC Methods 991.20, 992.15). Moisture content was assessed gravimetrically in accordance with AOAC Method 950.46 B.

Determination of technological indicators: The oil-holding capacity (OHC) of the hydrolysate was assessed using a gravimetric/volumetric method based on the principle of oil absorption and separation. A 2.5 g sample of the hydrolysate was accurately weighed into a graduated centrifuge tube. Subsequently, 15 mL of refined vegetable oil (density: 0.63 g/mL) was added. The mixture was homogenized using an overhead homogenizer at 1800 rpm for 1 min to ensure uniform dispersion of the sample in the oil. The resulting dispersion was allowed to stand for 30 min at ambient temperature to facilitate maximum oil absorption. Following the incubation period, the mixture was subjected to centrifugation at 4000 rpm for 15 min to separate the unabsorbed oil.

The OHC was determined volumetrically. After centrifugation, the total volume of the mixture and the volume of the free (unabsorbed) oil layer were recorded. The OHC, expressed as a percentage, was calculated using the formula (1):

$$OHC = \frac{(30 - V) \cdot \rho}{C} \cdot 100 \quad (1)$$

Where: V – is the volume of oil remaining unsorbed, mL; ρ – is the relative density of the oil, g/ mL; C – is the mass of the hydrolysate sample, g.

The water-holding capacity (WHC) was determined similarly by adding water to the hydrolysate instead of oil.

The oil-emulsifying ability (OEA) of the casein hydrolysate was evaluated using a centrifugation-based method. A 1.0 g sample of the hydrolysate was dispersed in 50 mL of distilled water and homogenized using a laboratory blender for 1 min to ensure complete dissolution or uniform dispersion. Subsequently, 50 mL of refined sunflower oil was added to the aqueous dispersion, and the mixture was emulsified in the blender for an additional 5 min at ambient temperature to form a stable oil-in-water emulsion.

The resulting emulsion was then transferred to centrifuge tubes and subjected to centrifugation at 4000 rpm for 5 min to separate the emulsified and non-emulsified phases. The OEA, expressed as a percentage, was calculated using the formula (2):

$$OEA = \frac{V_e}{V_o} \cdot 100 \quad (2)$$

Where: Ve – is the volume of the emulsified layer, mL; Vo – is the total volume of the mixture, mL.

To determine the solubility (S), a portion of hydrolysate weighing 5 g was dissolved in 50 mL of distilled water, heated in a thermostat to 24°C, and stirred in a laboratory mixer for 90 seconds. Then the solution was transferred to test tubes and centrifuged at 4000 rpm for 5 minutes. Then the top layer of liquid was decanted and the mass of the test tube with precipitate was measured. Solubility (S) was calculated by the formula (3):

$$S = 100 - \left[\frac{M_s - M_o}{m} \cdot 100\% \right] \quad (3)$$

Where: Ms – is the mass of the solution in the test tube, g; Mo – is the mass of the precipitate in the test tube, g; m – is the mass of the hydrolysate sample, g .

Description of the Experiment

Study flow: In the first stage of the experiment, casein hydrolysates were obtained from casein using the technology shown in Figure 1. Three hydrolysate samples were obtained by acidophilic bacteria hydrolysis, and three more samples were obtained with chymotrypsin hydrolysis. Then, the average samples from each hydrolysate sample were collected for further study. In the next stage, the protein and moisture contents of the samples, as well as their chemical structures and particle sizes, were determined. Finally, technological indicators (oil-holding capacity, water-holding capacity, oil-emulsifying capacity, and solubility) were measured. The obtained data were processed and analyzed statistically, and the hypothesis was tested for reliability.

Quality Assurance

Number of repeated analyses: Five technical replicates were performed for each experimental condition.

Number of experiment replication: Three independent biological replicates (n = 3) were produced for each treatment condition. For each biological replicate, five technical replicates (n = 5) were measured for every functional assay. Thus, each reported value represents the mean of 15 individual measurements (3 biological × 5 technical).

Reference materials: Industrially produced skim milk with a fat content of 0.05% was used to obtain casein. The milk and resulting casein were stored in a refrigerator at 4°C. The starter culture was stored under the conditions specified by the manufacturer (BakZdrav, Russia) to preserve bacterial activity. Six hydrolysate samples were obtained for the experiments using the described process flow chart, with the experiment repeated five times. All chemicals used were of analytical grade from certified suppliers. The equipment used had manufacturer's certificates, and the measuring instruments had verification confirmation.

Calibration: Each instrument was calibrated before each experiment, and calibration checks were performed regularly to ensure measurement accuracy.

Laboratory accreditation: The study was conducted in the laboratory of the Department of Food and Biotechnology at South Ural State University (national research university) (Russian Federation).

Data Access

Data are available from the corresponding author upon reasonable request.

Statistical Analysis

All results are expressed as the mean ± standard deviation (SD) of three independent measurements. Statistical significance was set at $p \leq 0.05$. Experimental data from five replicate experiments were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's honest significant difference (HSD) post hoc test for multiple comparisons. The statistical analyses were performed using the open-source web-based platform described in reference **[19]**.

Reporting and transparency statement

This experimental study did not involve randomization or blinding. All samples were included in the analysis, ensuring comprehensive results. The sample size (n = 3 biological replicates, n = 5 technical replicates per biological replicate) was determined empirically based on previous optimization studies and standard practices in the field.

RESULTS AND DISCUSSION

The chemical composition and properties of hydrolysates depend on the degree of hydrolysis, which reflects the proportion of cleaved peptide bonds in the protein substrate molecule as a result of the action of proteolytic enzymes **[20]**, **[21]**.

The infrared spectroscopy results presented in Figure 2 reveal some differences in the biochemical composition of the studied samples. Variations in absorption intensity between samples are noted and are associated with both the chemical composition of the substrate and the different concentrations of substances with specific chemical bonds formed during hydrolysis. Analysis of the spectral patterns reveals similarities among all samples and the presence of the same main functional groups, but with different relative concentrations. Figure 2 shows that the samples exhibit different transmittance values, confirming differences in the formation of key chemical groups induced during enzymatic hydrolysis.

Sample 6 has the highest concentration of IR-absorbing compounds, while sample 1 has the lowest, consistent with the degree of hydrolysis. Sample 6 exhibits an intense, broad absorption band in the region of C=O stretching and N–H bending vibrations (≈1550–1650 cm^{-1}), indicating a significant peptide content in the hydrolysate. Sample 4 also has a strong signal in this region, unlike samples 1, 2, and 3, which showed significantly weaker absorption, indicating a lower peptide concentration. Similar results were obtained in a study of squid hydrolysates, which showed a strong absorption band at 1650 cm^{-1}, which is characteristic of peptide bonds. The research team thus confirms the occurrence of protein hydrolysis **[22]**.

Sample 6 also retains a high plateau-like absorption in the C-O/C-N stretching region (≈1000–1300 cm^{-1}), consistent with a diverse and complex mixture of organic molecules. The remaining samples exhibit lower, more distinct characteristics, with sample 1 being the least complex in terms of organic-molecule composition.

In the C-O and C-N stretching region (≈650–1000 cm^{-1}), the differences between the samples are less pronounced, indicating the presence of some common molecular components or structural similarities. Moreover, analysis of this absorption region reveals the presence of aromatic rings. Their identification by C=C stretching vibrations at 1582 cm^{-1}, as well as C-H at 756 cm^{-1} and 856 cm^{-1}, has been reported in the literature. López-Medina

et al. noted from their research that such features indicate the presence of aromatic amino acids (phenylalanine, tyrosine or tryptophan), which impart biological activity to protein hydrolysates **[23]**.

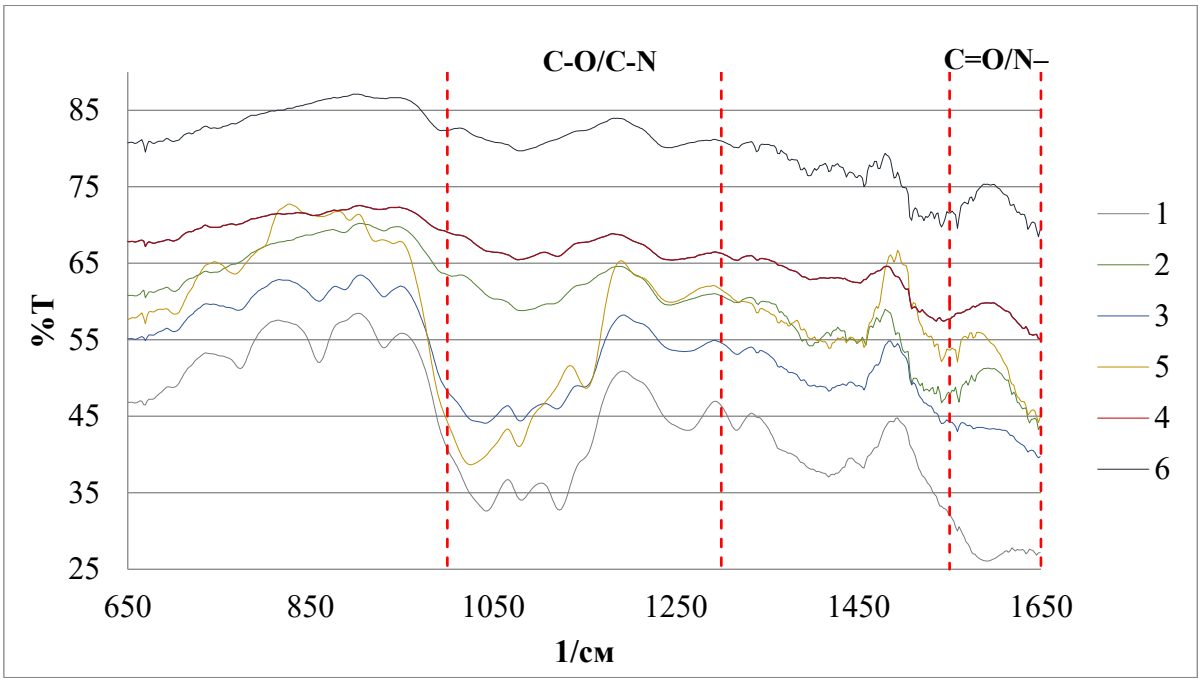

Figure 2 IR spectra of casein hydrolysate samples.

Thus, the IR analysis results demonstrate compliance with the degree of hydrolysis determined during optimization of the hydrolysis parameters. The sample with the highest degree of hydrolysis (sample 6) demonstrated the highest absorption capacity, indicating intensive accumulation of hydrolysis products, including peptides.

The results of determining the average particle size, shown in Figure 3, confirm the dependence between particle size and degree of hydrolysis.

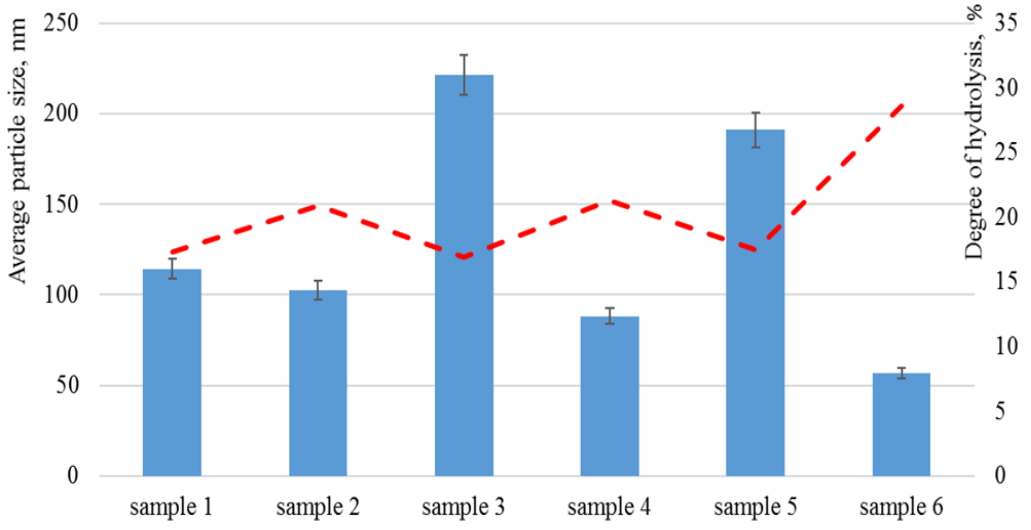

Figure 3 Average particle size in a dispersed system and the degree of hydrolysis.

The particle size distribution shown in Figure 4 shows that in hydrolysate samples 4 and 6, the largest part is made up of particles with sizes of 60.8–121.5 (91.15%) and 43–85.9 nm (93.67%), respectively. Of the hydrolysates obtained by fermentation of casein with acidophilus bacillus, the smallest particle size was found in sample 1, the majority of which were particles with sizes from 72.3 to 144.5 nm (81.24%). The results of assessing the size of the molecules of milk protein concentrate hydrolysates by Alcalase, Flavourzyme, and Protamex showed that with varying degrees of hydrolysis from 9.9 to 15.7%, the particle size fluctuated within the range of 183.3–286.9 nm ($p < 0.05$), and the solubility reached 91.1% (hydrolysis for 180 min with Alcalase) **[24]**.

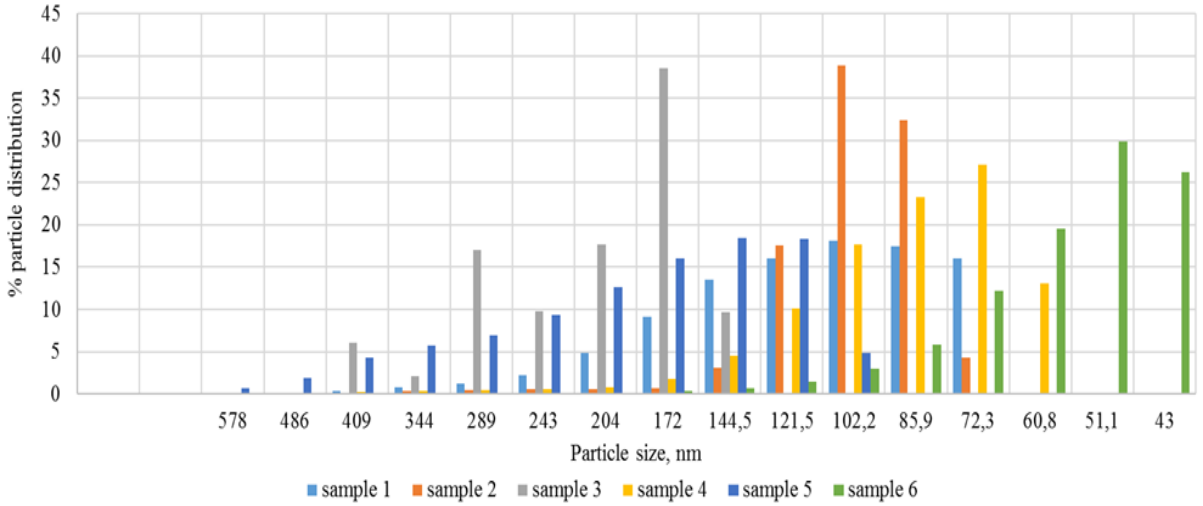

Figure 4 Particle size distribution in casein hydrolysates.

In analysing the hydrodynamic parameters of macromolecules, Wilkins and colleagues **[25]** identified a statistical relationship between the hydrodynamic radius of a protein in solution and the length of its polypeptide chain. During fermentation, the casein polypeptide chain is cleaved by proteolytic enzymes produced by *Lactobacillus acidophilus* into simpler peptide molecules with smaller hydrodynamic diameters. The results demonstrate the high proteolytic activity of this bacterial culture on the casein substrate, which is only slightly lower than that of purified chymotrypsin. The slight difference in proteolytic efficiency between bacterial fermentation and purified enzyme can be explained by several interrelated factors. First, chymotrypsin is the only serine protease with a clearly defined substrate specificity, preferentially hydrolyzing peptide bonds formed by carboxyl groups of amino acids such as tyrosine, tryptophan and phenylalanine **[26]**, **[27]**.

This ensures rapid and predictable degradation of casein. In contrast, *L. acidophilus* produces a complex array of cell envelope proteinases (CEPs) and intracellular peptidases, including serine and metalloproteinases with broader and partially overlapping specificities **[28]**, **[29]**.

Although this multi-enzyme system is highly efficient at meeting bacterial metabolic needs, its activity can be modulated by factors such as substrate availability, spatial organization of enzymes within the cell wall and potential feedback inhibition by hydrolysis products **[28]**.

Secondly, the kinetic parameters of the two systems differ fundamentally. Chymotrypsin acts as a free enzyme in solution, providing unimpeded access to casein micelles and rapid digestion. In contrast, the bacterial proteolytic system requires physical contact between the bacterial cell and the substrate. Most proteolytic activity is associated with the cell wall, which imposes diffusion limitations and results in a lower initial hydrolysis rate than that of soluble enzymes. However, *L. acidophilus* can compensate for these limitations through the synergistic action of multiple proteases and continuous enzyme regeneration during bacterial population growth, thereby maintaining relatively similar efficiency levels between the two systems.

Third, the observed reduction in hydrolysate particle size reflects not only the depth of proteolysis but also specific cleavage zones. Chymotrypsin, with its narrow substrate specificity, generates more uniformly sized particles, whereas the combined action of bacterial proteases and peptidases yields a heterogeneous mixture of presumably oligopeptides and free amino acids **[30]**.

The ability of *L. acidophilus* to achieve a degree of proteolysis through a dedicated digestive enzyme underscores its potential as a powerful biocatalyst for the controlled production of casein hydrolysates **[31]**, **[32]**.

The chemical composition and technological properties of casein hydrolysates are presented in Table 2.

The results indicate that the samples do not differ significantly in moisture content, with minor differences in protein content. However, samples with a higher predicted degree of hydrolysis (samples 6 and 4) tend to have a higher protein content in the hydrolysate than the other samples.

The OHC largely depends on molecular size. Large peptides can form a network that retains oil droplets. During hydrolysis, peptides become shorter and more hydrophilic due to an abundance of terminal charged groups **[33]**. Short peptides cannot form a spatial network to effectively retain oil, leading to a decrease in the OHC. This is confirmed by study results showing that the sample with the highest degree of hydrolysis (sample 6) had the lowest oil-binding capacity – 21.08% ($p < 0.05$), which was 38.6% lower than the OHC for sample 3, which had a degree of hydrolysis of 16.94%.

Table 2 Chemical composition and technological properties of casein hydrolysates.

Indicator	Sample of casein hydrolysate					
	1	2	3	4	5	6
Mass fraction of protein, %	76.09 ± 1.326^{b}	78.30 ± 1.269^{ab}	74.88 ± 0.523^{b}	80.27 ± 1.562^{ab}	77.81 ± 1.373^{ab}	82.59 ± 1.810^{a}
Mass fraction of moisture, %	6.86 ± 0.013^{a}	6.61 ± 0.048^{d}	6.74 ± 0.013^{bc}	6.77 ± 0.018^{ac}	6.83 ± 0.017^{ab}	6.69 ± 0.013^{cd}
OHC, %	31.54 ± 0.681^{b}	26.23 ± 0.401^{c}	34.31 ± 0.551^{a}	25.75 ± 0.449^{c}	29.49 ± 0.551^{b}	21.08 ± 0.496^{d}
WHC, %	26.81 ± 0.319^{b}	27.9 ± 0.462^{b}	26.13 ± 0.404^{b}	28.05 ± 0.564^{b}	27.74 ± 0.500^{b}	30.2 ± 0.592^{a}
OEA, %	61.90 ± 1.041^{a}	44.42 ± 0.826^{b}	64.74 ± 1.123^{a}	34.29 ± 0.558^{c}	61.11 ± 1.242^{a}	27.83 ± 0.489^{d}
S, %	91.17 ± 1.602^{ab}	92.87 ± 1.136^{a}	89.09 ± 1.758^{b}	93.63 ± 1.038^{a}	91.40 ± 1.223^{ab}	94.16 ± 1.452^{a}

Note: Values are means ± SEM, n = 5 per treatment group. Means in a row without a common superscript letter differ ($p < 0.05$) as analyzed by one-way ANOVA and the TUKEY test.

These findings are consistent with previously reported data on casein hydrolysates. Research by Roman and Sgarbieri (2005) demonstrated that increasing the degree of hydrolysis of enzymatically coagulated bovine casein from 5.7% to 35.8% resulted in a progressive decline in water retention capacity, while hydrolysates with higher DH (12.8%, 20.1%, and 35.8%) completely lost their ability to form emulsions **[34]**. Similarly, a study on sodium caseinate hydrolysis showed that all hydrolysates exhibited poor emulsifying and foaming properties compared to the unmodified protein, despite achieving high initial foam levels **[35]**. This confirms that the loss of functional properties is directly correlated with the extent of proteolytic degradation.

More recent work by Ewert et al. using a thermolysin-like peptidase revealed that longer, more hydrophobic peptides capable of self-assembling into network-like supramolecular particles were preserved only up to a DH of 2.2%. At this limited DH, emulsion half-life increased by 400% and foam half-life by 31% compared to untreated sodium caseinate. However, at higher DH (8.5%), the hydrolysates became less hydrophobic, formed smaller spherical supramolecular structures, and phase separation occurred within minutes **[36]**. This mechanistically explains why our sample with the highest DH exhibited the lowest OHC: extensive hydrolysis disrupts the supramolecular network essential for oil droplet entrapment.

Hydrolysates bind moisture in a slightly different way: the cleavage of peptide bonds during hydrolysis leads to the release of reactive amino and carboxyl groups. Partial hydrolysis disrupts the protein's quaternary and tertiary structures, exposing hydrophilic amino acid residues **[37]**. At high hydrolysis rates, when short peptides and free amino acids are formed in large quantities, the water-holding capacity of the water-holding gel may decrease due to the high solubility of the resulting products. Peptides dissolve in the solution, and the ability to retain water as a gel is lost. The weakening of hydrophobic interactions in the system in the presence of casein hydrolysates may also be due to the high content of phosphopeptides and glutamic acid, which are charged **[38]**. In the samples studied, this indicator did not vary significantly, ranging from 26.13% to 30.20%. A similar relationship was observed in the study of egg albumin hydrolysates: as hydrolysis time increased, and thus the degree of destruction, the water-holding and fat-holding capacities of the hydrolysates decreased **[39]**.

Padial-Dominguez et al. demonstrated that emulsifying properties reach a maximum within certain ranges of the degree of hydrolysis (DH): optimal emulsifying capacity of soy protein hydrolysates was observed at a DH of 6.5%, whereas excessive hydrolysis (above 14%) worsened emulsifying capacity **[40]**. This trend is confirmed by our studies, in which hydrolysates with a higher degree of hydrolysis (samples 4 and 6) showed lower emulsifying capacity than the sample with the lowest degree of hydrolysis (sample 3) – 64.74% ($p < 0.05$). Kaur et al. also confirmed this phenomenon, demonstrating a negative dependence of emulsifying properties on increasing DH in milk protein hydrolysates **[17]**. Scientific publications report that peptides in hydrolysates can exhibit emulsifying properties due to their surface interactions with oil droplets during homogenization. The resulting surface layer prevents the oil droplets from coalescing **[41]**. It has been noted that the surface hydrophobicity of peptides formed during hydrolysis affects the reduction of interfacial tension. This suggests that the emulsifying properties of protein hydrolysates are influenced by their peptide composition **[42]**, **[43]**. The decrease in emulsifying capacity with increasing degree of hydrolysis can be explained by a loss of stability during emulsion formation due to the inability of short peptides to form a strong interfacial film **[44]**. Oil droplets coalesce, and the emulsion breaks down. Meanwhile, medium-sized peptides (2–5 kDa) diffuse to the oil-water interface faster than native proteins, accelerating the formation of the primary emulsion layer. Partial hydrolysis produces peptides with an optimal ratio of hydrophobic and hydrophilic regions, which effectively reduce surface tension.

It has been noted that the practical aspects of casein's application are related to its technological properties, particularly its solubility, which increases with enzymatic hydrolysis **[45]**. The research results showed that solubility increased with increasing degree of hydrolysis – from 89.09% in sample 3 to 94.16% in sample 6 ($p < 0.05$). Literature has noted an increase in the solubility of whey protein concentrate hydrolysates to 97% with

a degree of hydrolysis of 15% **[17]**. Our results are also consistent with the arguments of other authors confirming the inverse dependence of solubility on particle size **[46]**.

It is important to note that the functional properties of casein hydrolysates are not determined solely by the degree of hydrolysis. The specificity of the proteolytic enzyme preparation and the resulting peptide profile also play critical roles. Research by Rajarathnam et al. (2016) showed that sodium caseinate hydrolysates produced with four different enzyme preparations (Alcalase, Prolyve, FlavorPro Whey, and pepsin) at similar DH exhibited significantly different nitrogen solubility indices over the pH range of 2.0–8.0 **[47]**. This indicates that the enzyme's cleavage pattern determines the size, charge distribution, and hydrophobicity of the peptides, which in turn influence their functional performance.

Similarly, a study by Ye et al. confirmed that casein hydrolysates with the same degree of hydrolysis (DH) exhibited similar molecular weight distributions, regardless of the hydrolysis conditions (temperature and enzyme/substrate ratio). However, different proteases produced significantly different peptide profiles at the same DH **[48]**. This emphasizes the importance of selecting the appropriate enzyme system for the desired functional outcome.

The observed relationship between DH and functional properties has important practical implications for various applications. For example, in applications that require gel formation, texture modification, or fat stabilization, such as meat products and bakery fillings, hydrolysates with a low DH (typically between 5–10%) are preferred, as they retain a sufficient molecular size for network formation. On the other hand, for applications where high solubility and rapid digestibility are prioritized, such as sports nutrition, medical foods, and infant formulas, higher DH values (between 15–25%) may be more appropriate, despite the loss of certain functional properties.

Additionally, at higher levels of hydrolysis, more short peptides with strong biological activity are produced, which could be incorporated into the composition of bioactive films and packaging materials.

Thus, targeted hydrolysis should be performed at the required depth to obtain a protein ingredient with superior technological properties. The optimal degree of hydrolysis ranges from 5–15% **[49]**. At these values, the protein is already sufficiently fragmented for high solubility and rapid adsorption, but still retains sufficient chain size to retain moisture and fat and stabilize the emulsion. The research results confirm a deterioration in technological parameters such as oil-holding and emulsifying capacity for the studied hydrolysates as solubility increases with increasing degree of hydrolysis.

It can also be observed that the values of the indicators are close to the expected degree of hydrolysis and vary among hydrolysates produced by hydrolysis with chymotrypsin and those produced by acidophilic bacteria.

Limitations

The main limitation of this study is that the test samples were obtained from milk from one specific manufacturer, which may limit the generalizability of the results to hydrolysates obtained from casein in milk from other manufacturers. Furthermore, the experiment was conducted under controlled laboratory conditions (with the stated hydrolysis process parameters set to an accuracy of 0.1 °C and 0.1 h), which may not fully reflect industrial-scale processing. Another limitation is the fact that, in this experiment, DH was not measured directly. Instead, the values of the degree of hydrolysis were obtained by optimizing process parameters in a previous study **[18]**. Another limitation is that no enzyme inactivation was performed after chymotrypsin fermentation, which may affect the interpretation of hydrolysate properties. Further studies on the amino acid and peptide composition of casein hydrolysates are needed to better understand the mechanisms underlying these observations. They are planned for future research.

CONCLUSION

For the research, casein hydrolysates were produced through fermentation with chymotrypsin and acidophilic bacteria under pre-determined optimal hydrolysis conditions. The chemical composition, chemical structure, particle size, and technological properties of the resulting samples were then analyzed. The IR analysis results were consistent with the degree of hydrolysis calculated during the determination of optimal hydrolysis parameters. Samples with the highest degree of hydrolysis, achieved by chymotrypsin hydrolysis, demonstrated the highest absorption capacity, indicating intensive accumulation of hydrolysis products, including peptides. Analysis of spectral patterns showed similarities among all samples, with key functional groups present, though in different relative concentrations. The particle size assessment revealed that the hydrolysates from casein hydrolyzed with chymotrypsin had a higher proportion of the smallest particles. This finding was consistent with the IR analysis and the estimated degree of hydrolysis. Hydrolysates with a higher degree of hydrolysis had lower emulsifying capacities, although solubility increased with increasing hydrolysis. This suggests that targeted hydrolysis to a specific depth is necessary to achieve the desired technological properties of a protein ingredient.

Future planned research will focus on determining the peptide composition and evaluating the bioactive properties of hydrolysates produced from casein. Based on the obtained technological parameters, we plan to study the effects of casein hydrolysates on various food systems, including bioactive films and coatings for food products.

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