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# Efficient Enzymatic Hydrolysis of the Whey Protein Fraction from Bovine Milk

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Studies were conducted to optimize the hydrolysis of the major whey proteins with the serine proteases Alcalase and Protozyme, and of the immunoglobulin fraction of colostrum with Alcalase. The protein-peptide composition of the enzymatic hydrolysates of whey and colostrum was determined using high-performance liquid chromatography and electrophoresis data under denaturing conditions. The hydrolysis conditions, in conjunction with the application of a heat treatment of the substrate, were designed to enhance the efficiency of whey proteolysis using Protozyme. An increase in the proteolysis degree of the native immunoglobulin fraction of colostrum with Alcalase was also achieved. The results of the experimental work are intended for the production of a hypoallergenic protein component derived from bovine milk.
**Keywords**: Whey, colostrum, endopeptidase, hydrolysis, protein and peptide profile.

## INTRODUCTION

The enzymatic reaction of protein cleavage (proteolysis) is an important biochemical process for obtaining hydrolysates with varying proteolysis degree of substrates (Mora and Toldrá, 2023). The major biologically valuable and allergenic components of milk are casein, β-lactoglobulin (β-lg), α-lactalbumin (α-la), immunoglobulins (Igs), lactoferrin and bovine serum albumin (BSA) (Lajnaf *et al.*, 2023). They exert bioactivity directly or after cleavage into specific peptides during digestion in the gastrointestinal tract, as a result of milk fermentation by probiotic microorganisms, or by hydrolysis with proteases in vitro (Peighambardoust *et al.*, 2021). Thus, in reaction with proteolytic enzymes (proteases) from casein and whey fractions, bioactive peptides with antioxidant, antimicrobial, hypotensive, etc. effects are generated (Iwaniak *et al.*, 2024). It should be noted that proteolysis leads to the destruction of antigenic determinant regions, resulting in the formation of hypoallergenic peptides (Pang *et al.*, 2024). In this context, technologies have been developed to isolate bioactive peptides in the form of dietary supplements (Najafian, 2023).

Potentially allergenic milk proteins are cleaved with a wide range of proteases, which are enzymes of the hydrolase class that catalyze the hydrolytic breakdown of peptide bonds (Paiva *et al.*, 2022). The study of the features of dairy proteins hydrolysis with specific proteases is aimed at establishing the optimal parameters for obtaining hydrolysates with a predetermined peptide composition, hypoallergenic properties, and bioactivity as a food ingredient. Currently, the alkaline endopeptidase alcalase produced by *Bacillus licheniformis* is one of the most efficient proteases (Dos Santos Aguilar and Sato, 2018; Song *et al*, 2023). The relatively broad substrate and site specificity of Alcalase provides for the enzyme to be used for the hydrolysis of various protein substrates. Among the products of milk protein hydrolysis with Alcalase, peptides with a wide range of biological activities have been found (Tacias-Pascacio *et al.*, 2020). The alkaline endopeptidase Protozyme used in the present study is an analog of Alcalase. In the food industry, protease hydrolysis is completed by thermal (or pH) inactivation or fractionation of the hydrolysate to remove the protein fraction (Mora and Toldrá, 2023).

In the production of hydrolysates, the most available protein raw materials are those obtained from the whey processing (Dullius *et al.*, 2018). Colostrum, or first milk, is nutritionally superior to mature milk due to its higher content of biologically active ingredients, including a high concentration of immunoglobulin fraction. This makes it a promising raw material for the production of hydrolysates (Arslan *et al.*, 2021). Enzymatic hydrolysis is known to increase the bioactive potential of bovine colostrum proteins. A literature review was performed on the hydrolysis of colostrum proteins with different proteases (pepsin, trypsin, α-chymotrypsin, pancreatin, papain, Alcalase). It was found that the antioxidant activity of camel milk proteins increased after

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enzymatic hydrolysis with pepsin, trypsin, α-chymotrypsin, pancreatin, and papain (Oussaief *et al.*, 2019). In the following study the antioxidant properties of peptides obtained by hydrolysis of whey proteins from bovine colostrum with pepsin, and with pepsin and pancreatin were characterized. The maximum increase in antioxidant activity of the colostrum whey fraction was founded after hydrolysis with an enzymes complex, this effect was expressed in the proteolysis degree increase (Fajardo-Espinoza *et al.*, 2019). In addition, data on the stepwise hydrolysis of the whey fraction of bovine colostrum with Alcalase and Flavourzyme are reported (Chiang *et al.*, 2017). These works do not discuss the hydrolysis characteristics of each colostrum protein fraction with various proteases. This is important to improve the efficiency of the process. The present work is devoted to efficient cleavage of whole colostrum proteins with alcalase, especially the Ig component, which is the most resistant to proteolysis.

Our own experimental data has established the optimal conditions for the preliminary heat treatment of whey proteins (Halavach, 2022a). Furthermore, the features of Alcalase cleavage of the main protein fractions of whey and colostrum have been determined (Halavach, 2022b). Finally, their biologically active and antigenic properties have been characterized (Golovach *et al.*, 2016; Halavach *et al.*, 2020). At the same time, it is reasonable to search for promising proteases and subsequently optimize the conditions for hydrolysis of the target protein fractions.

The practical significance of the research is related to the actualization of the technology for obtaining partial enzymatic dairy hydrolysates of dairy proteins with the required peptide composition. This is achieved by selecting highly active proteases for efficient cleavage of whey proteins.

The aim of the present research is to optimize the hydrolysis conditions of the main whey proteins with Protozyme and to increase the hydrolysis efficiency of the immunoglobulin fraction from colostrum with Alcalase.

## MATERIALS AND METHODS

The hydrolysates were prepared using whey protein concentrate (80 % of protein, Schuchin Branch of Molochny Mir OJSC, Belarus), defatted dried colostrum (70 % of protein, All-Russian Research Institute of Dairy Industry, Russia), enzymes Alcalase (Alcalase® 2.4L FG, protease from *Bacillus licheniformis*, activity 2.4 EA/g, Novozymes A/S, Denmark) and Protozyme (protease from *Bacillus licheniformis*, activity 50,000 EA/g, Biopreparat, Russia), NaOH, phenylmethylsulfonyl fluoride (99% purity, Sigma, USA), distilled water.

The paper presents a modified methodology for obtaining hydrolysates proposed by previous studies (Golovach *et al.*, 2016; Halavach, 2022b). In the experiments with preliminary heat treatment of the substrate, a 5 % solution of milk proteins (pH 8.0 was adjusted with 1 M NaOH solution) was incubated in a water bath at 80 ºC for 10 min and cooled to the hydrolysis temperature. The enzymatic cleavage of milk proteins with Alcalase and Protozyme was carried out at an enzyme : substrate ratio of 1 and 5 %. The active acidity of the initial solution was 8.0 pH units, and the temperature reached 50 and 60 ºC for 2–3 hours. The enzymes were inactivated by adding the serine protease inhibitor PMSF at a final concentration of 0.1 mM or by thermal inactivation (85 ºC, 15 min). The resulting samples were stored at -20 °C for further analysis of protein and peptide composition.

The method of electrophoretic separation in 20 % polyacrylamide gel under denaturing conditions (SDS-electrophoresis) was used to analyze the protein and peptide profile of enzymatic hydrolysates (Osterman, 1984). PageRuler™ Prestained Protein Ladder 10–180 kDa marker (Thermo Fisher Scientific, USA) was used as a molecular mass ($M_W$) standard.

High-performance liquid chromatography (HPLC) was performed using acetonitrile (ACN, Sigma, USA) and trifluoroacetic acid (TFA, Fisher Scientific International, USA). β-lg (variants A and B, 90 % of protein), α-la (85 % of protein), BSA, 90 % of protein), casein (88 % of protein), and IgG (90 % of protein) purchased from Sigma (USA) were used as bovine milk protein standards.

HPLC analysis was performed on Agilent 1100 chromatograph (Agilent, USA) using Zorbax-300SB C8 column (4.6×250 mm, 5 μm, Agilent, USA). The column was equilibrated with 0.1% TFA aqueous solution.

Elution of whey protein samples (β-lg, α-la and BSA) was performed using ACN gradient (ACN–water–TFA = 95 : 5 : 0.1 mL/100 mL): 0–5 min, 5 %; 5–10 min, 5–10 %; 10–30 min, 10–40 %; 30–32 min, 40 %; 32–40 min, 40–50 %; 40–45 min, 50 %; 45–50 min, 50–10 %. Separation was carried out at room temperature with a flow rate of 1.0 mL/min for 50 min.

Elution of casein and Igs samples was performed using ACN gradient (ACN–water–TFA = 95 : 5 : 0.1 mL/100 mL): 0–5 min, 25 %; 5–10 min, 29–37 %; 10–12 min, 37–41 %; 12–14 min, 41–42,5 %; 14–16 min, 42,5 %; 16–17 min, 42,5–43 %; 17–19 min, 43–51 %; 19–21 min, 51–59 %; 21–23 min, 59–100 %; 23–26 min, 100 %; 26–28 min, 100–29 %; 28–33 min, 29 %. Separation was performed at 46 °C for 33 min at a flow rate of 1.0 mL/min. Detection was performed at 280 nm.

The amount of whey proteins, casein and Igs in hydrolysate samples was determined using calibration curves for standards. The degree of proteolysis (DP, %), or the proportion of cleaved milk proteins, was estimated using the formula:

$$DP=\frac{K\text{-}H}{K}\times 100$$

where K is the amount of protein in the control (without enzyme), mg/mL; H is the content of protein in the hydrolysate, mg/mL. In the case of whey hydrolysates, the degree of proteolysis was calculated as the ratio of the amount of cleaved main whey proteins (β-lg, α-la and BSA) to the sum of these proteins in the control sample. For colostrum experiments, DP was determined as the ratio of the amount of the cleaved Ig fraction to its content in the initial sample.

Results of quantitative data processing are presented as arithmetic mean ± half the width of the 95% confidence interval (n = 3). Statistical processing of the data was performed using R functions *aov*, *DunnettTest*, *t.test* and *TukeyHSD* from the *stats* (R Core Team, 2021) and *DescTools* (Signorell *et al.*, 2023) packages. Statistical differences between groups were considered significant at the $p<0.05$ level, adjusted for multiple pairwise comparisons.

## RESULTS AND DISCUSSION

***The hydrolysis of native and thermodenatured whey proteins with serine endopeptidases***

***The features of native whey proteolysis with Alcalase and Protozyme*:** A comparative analysis of the protein and peptide composition of partial enzymatic hydrolysates of whey proteins obtained with the serine endopeptidases Alcalase and Protozyme was carried out. In the first stage of the study, the hydrolysis of native whey was performed at a temperature regime optimal for Alcalase, in particular at 50 ºC, and the duration of the enzymatic reaction was 2 hours.

HPLC studies revealed significant differences in the profile of proteolysis products of native whey proteins with Alcalase and Protozyme, as shown in Figures 1 and 2. Thus, relatively lower amounts of β-lg and α-la were found in hydrolysates when Alcalase was used (Figure 1) than in samples obtained after cleavage with Protozyme (Figure 2). Moreover, the amount of hydrolyzed substrate at 1 % Alcalase content was higher than that at the maximum application of Protozyme (5 %).

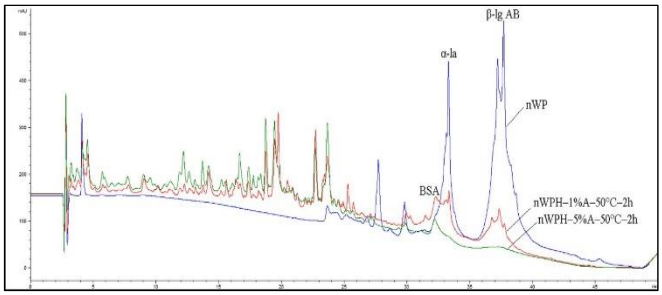



**Figure 1. HPLC profiles of native (nWP) and hydrolyzed whey proteins (nWPH) obtained with Alcalase (A) at enzyme concentrations of 1 % and 5 %, temperature of 50 °C and hydrolysis time of 2 hours .**

It should be noted that the high molecular weight fraction (BSA, $M_W$ 66.3 kDa) is resistant to the action of serine proteases. The application of 5 % Alcalase achieved the cleavage of the predominant whey proteins into peptides (β-lg, $M_W$ 18.4 kDa; α-la, $M_W$ 14.2 kDa), whereas in the case of Protozyme further optimization of the hydrolysis conditions is required.

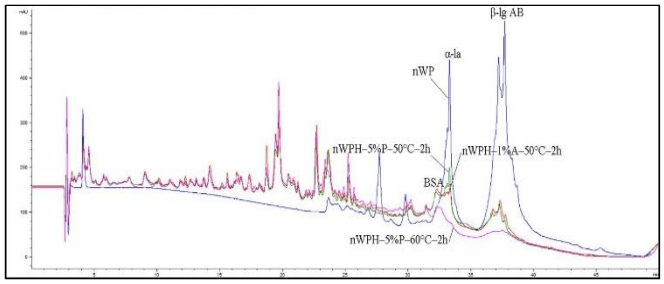



**Figure 2. HPLC profiles of native (nWP) and hydrolyzed whey proteins (nWPH) obtained with Alcalase (A) and Protozyme (P) at enzyme concentrations of 1 % and 5 %, temperatures of 50 and 60 °C, and hydrolysis duration of 2 hours**

***The effect of heat treatment of whey proteins on their proteolysis with Protozyme*:** Therefore, in the next step, the substrate was pretreated with heat (80 ºC, 10 min) and the enzymatic reaction with Protozyme (5 %) was carried out for 2 hours at elevated temperature (60 ºC). When the hydrolysis temperature was increased from 50 to 60 ºC, a significant increase in the extent of proteolysis was observed, up to the complete cleavage of β-lg and α-la into peptides, as shown in Figure 2. At the same time, native BSA was detected in the hydrolysate samples. The high molecular weight protein fraction (BSA) characteristic of hydrolysed native whey samples is not detected in hydrolysates of heat-treated whey proteins with Protozyme (Figure 3).

Thus, heat treatment of the protein substrate caused an increase in the degree of proteolysis (amount of hydrolyzed fraction). In addition, the elution profiles of the peptides overlapped, confirming the identity of the Alcalase and Protozyme hydrolysis sites (profiles of nWPH–1%A–50°C–2h and nWPH–5%P–50°C–2h in Figure 2).

The results of the quantitative analysis of the chromatographic profiles are presented in Table 1. In general, the whey protein hydrolysates with Alcalase contained (14.7±0.8)/(4.8±0.4) % native protein when 1/5 % of the enzyme was added, respectively, and (20.5±0.6) % in the case of Protozyme (5 %). When the hydrolysis temperature was increased to 60 °C with Protozyme (5 %), (95.2±0.4) % of the predominant whey proteins (β-lg and α-la) were hydrolysed, which is comparable to the hydrolysate obtained with Alcalase (5 %) at a reaction temperature of 50 °C.

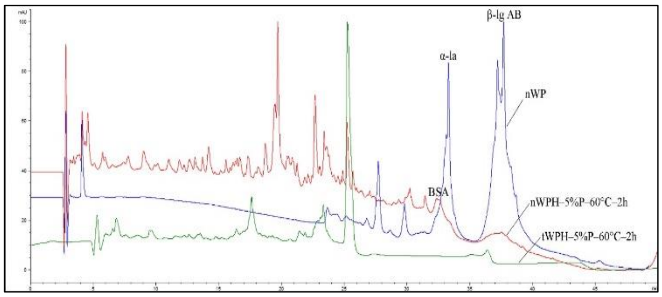



**Figure 3. HPLC profiles of native (nWP) and hydrolyzed native/ heat-treated whey proteins (nWPH/ tWPH) obtained with Protozyme (P) at an enzyme concentration of 5 %, temperature of 60 °C and hydrolysis time of 2 hours.**

**Table 1. Characterization of protein composition of native whey protein samples hydrolyzed with Alcalase and Protozyme .**

| Sample name | Protein composition, % | | | Degree of proteolysis (DP), % |
|---|---|---|---|---|
| | β-lg | α-la | BSA | |
| nWPH–1%A–50°C–2h | 9.8±0.6 | 12.1±1.3 | 100.0 | 85.3±0.8[a] |
| nWPH–5%A–50°C–2h | 0 | 0 | 100.0 | 95.2±0.4[b] |
| nWPH–5%P–50°C–2h | 15.2±0.7 | 19.7±1.5 | 100.0 | 79.5±0.6[c] |
| nWPH–5%P–60°C–2h | 0 | 0 | 100.0 | 95.2±0.4[b] |
| tWPH–5%P–60°C–2h | 0 | 0 | 0 | 100.0[d] |

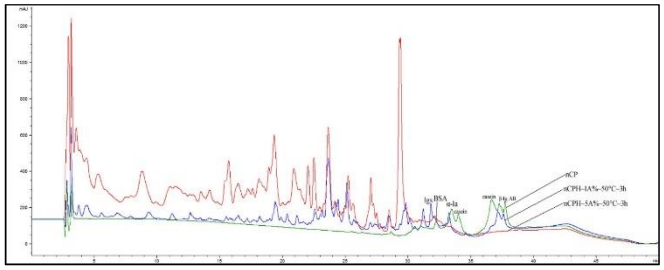
nWPH/tWPH – hydrolyzed native/heat-treated whey proteins obtained with Alcalase (A) and Protozyme (P) at enzyme contents of 1 % and 5 %, temperatures of 50 and 60 °C and hydrolysis time of 2 hours. Results are presented as arithmetic mean ± half the width of the 95 % confidence interval (n=3). Values without a common letter index (a–c) within the same column indicate significant differences ($p<0.05$)

It is therefore advisable to preheat whey proteins and increase the temperature of proteolysis with Protozyme from 50 to 60ºC to obtain hydrolysates in which the whole protein component is cleaved into a peptide fraction.

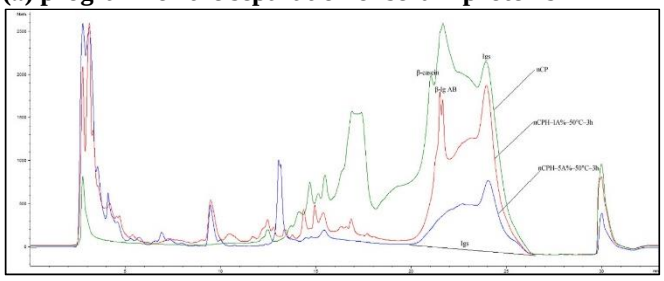
**The hydrolysis optimization of the immunoglobulin component from colostrum with Alcalase**

***The characteristics of native colostrum protein fractions cleavage*:** The comparative analysis of the protein and peptide composition of colostrum protein hydrolysates obtained using Alcalase (1 % and 5 %) was conducted. In the first stage of the research, the hydrolysis of colostrum proteins was carried out at a temperature regime optimal for the cleavage of whey proteins with Alcalase, specifically at 50 ºC for 3 hours.

Colostrum is characterized by a polycomponent protein composition, which complicates the chromatographic separation of its fractions due to the co-elution of some proteins (Figure 4). It was shown that the efficiency of hydrolysis of the main colostrum proteins decreases in the sequence "casein – β-lg/α-la – IgG". In the hydrolysate with 5 % Alcalase content obtained at 50 ºC, proteolysis products of the minor whey proteins of colostrum (α-la and β-lg), partially hydrolyzed Ig G (Figure 4b) and its polypeptide with a retention time of 29 min, resistant to peptidase cleavage, were detected (Figure 4a).




**(a) program for the separation of serum proteins**




**(b) program for separation of casein and immunoglobulin fractions.**

**Figure 4. HPLC profiles of native (nCP), hydrolyzed colostrum proteins (nCPH) at Alcalase content of 1 % and 5 %, proteolysis temperature of 50 °C and hydrolysis time of 3 hours.**

At the same time, when 1 % of Alcalase was applied, a higher content of the native Igs fraction was detected (Figure 4b), together with the presence of partially hydrolyzed whey proteins (α-la and β-lg) (Figure 4a). Thus, the main characteristics of colostrum hydrolysates are mainly determined by the characteristics of Igs cleavage.

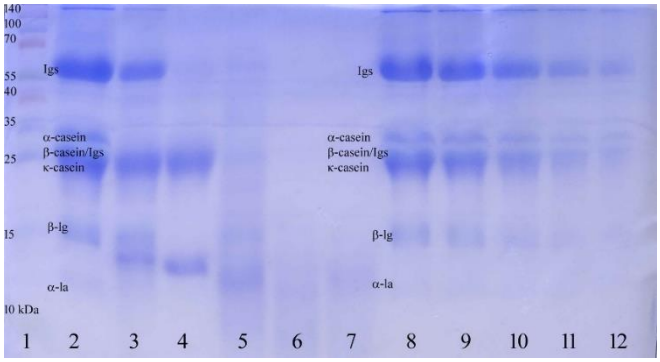
***The selection of immunoglobulin fraction proteolysis parameters*:** To optimize the cleavage of colostrum proteins with Alcalase, hydrolysis was carried out at a higher temperature (60 °C) and after thermal treatment of the colostrum samples (80 °C for 10 min). Aggregation of the protein fraction was observed after heating of the colostrum, since the Ig component is the most thermolabile ingredient of the first milk.

The SDS-electropherograms represent the profiles of colostrum hydrolysate samples obtained at enzyme concentrations of 1 and 5 % and a hydrolysis temperature of 60 °C. At 5 % Alcalase content, no high molecular weight protein fraction Igs (50 kDa) was detected, but an accumulation of its protease products with $M_W \approx 25$ kDa and 10 kDa$<M_W<$15 kDa was observed (Figure 5, lane 4). When 1 % of Alcalase was applied, native and cleaved Ig components were detected (Figure 5, lane 3).

Due to aggregation of thermodenatured colostrum proteins, electrophoretic separation of the corresponding hydrolysate (1 % Alcalase content, hydrolysis temperature of 60 °C) revealed a protein and peptide profile (Figure 5, lane 5), but when the protease content was increased to 5 %, the trace amounts of high molecular weight component were detected (Figure 5, lane 6). The protein fraction is absent in the profile of native colostrum hydrolysate, also obtained at 5 % enzyme : substrate ratio, but with thermal inactivation of the protease (85 °C, 15 min) as shown in Figure 5 (lane 7).

It should be noted that hydrolysates from thermodenatured colostrum are turbid, tend to aggregate and precipitate the protein component and are unsuitable for subsequent ultrafiltration (the presence of aggregates results in blocked filters). In addition, proteases can be inactivated by various methods – addition of inhibitors, thermodenaturation or removal during fractionation. Inhibitors cannot be used for food purposes, and filtration of turbid solutions is inefficient due to loss of milk protein. Importantly, Alcalase has a $M_W \approx 30$ kDa, similar to the denaturation and proteolysis products of IgG, which is also expected to result in protein loss when native colostrum protein hydrolysate is filtered. Thermal inactivation is the most reasonable and available approach.

According to the results of SDS-electrophoresis, fractions with $M_W \approx 25$ kDa and 10 kDa$<M_W<$15 kDa are present in the native colostrum hydrolysate (Figure 5, lane 4), which were detected in trace amounts after thermal inactivation of the protease (Figure 5, lane 7). This is due to the thermolability of the Ig component, which denatures upon heating of the hydrolysate, whereas Alcalase cleaves it due to the retention of residual proteolytic activity.




1 – marker, 2 – nCP (50 mg/mL), 3 – nCPH–1%–60°C–3h, 4 – nCPH–5%–60°C–3h, 5 – tCPH–1%–60°C–3h, 6 – tCPH–5%–60°C–3h, 7 – nCPH–5%–60°C–3h–TI, 8 – nCP (40 mg/mL), 9 – nCP (30 mg/mL), 10 – nCP (20 mg/mL), 11 – nCP (10 mg/mL), 12 – nCP (5 mg/mL)

**Figure 5. SDS-electrophoresis of native (nCPH) and heated (tCPH) colostrum proteins treated with Alcalase (temperature of 60°C for 3 hours, 1/5 % of protease and its thermal inactivation – TI)**

Thus, the preliminary heat treatment of colostrum under the proposed conditions does not provide a hydrolysate with a stable dissolved protein fraction, which also complicates the subsequent ultrafiltration process. The separation of the Ig fraction is achieved by optimized Alcalase hydrolysis followed by thermal inactivation.

A comparative analysis of the enzymatic colostrum hydrolysates was then carried out using HPLC analysis. Figures 4 and 6 demonstrated the elution profiles of native and cleaved colostrum proteins obtained at different proteolysis temperatures (50 and 60 ºC). Previously, it was found that a 5 kDa cutoff fraction elutes from the column up to 23 min of colostrum hydrolysate separation, and in the case of a 10 kDa cutoff fractionation, up to 29 min of separation (Halavach *et al.*, 2023). According to previous studies, bovine milk casein is cleaved with Alcalase into low molecular weight peptides at an enzyme content of 0.25 % (Halavach, 2022b), which is significantly lower than the Alcalase concentration used in this experiment. The fractions with $M_W \approx 25$kDa and 10 kDa$<M_W<$15 kDa (Figure 5, lane 4) are derivatives of the Ig component of colostrum.

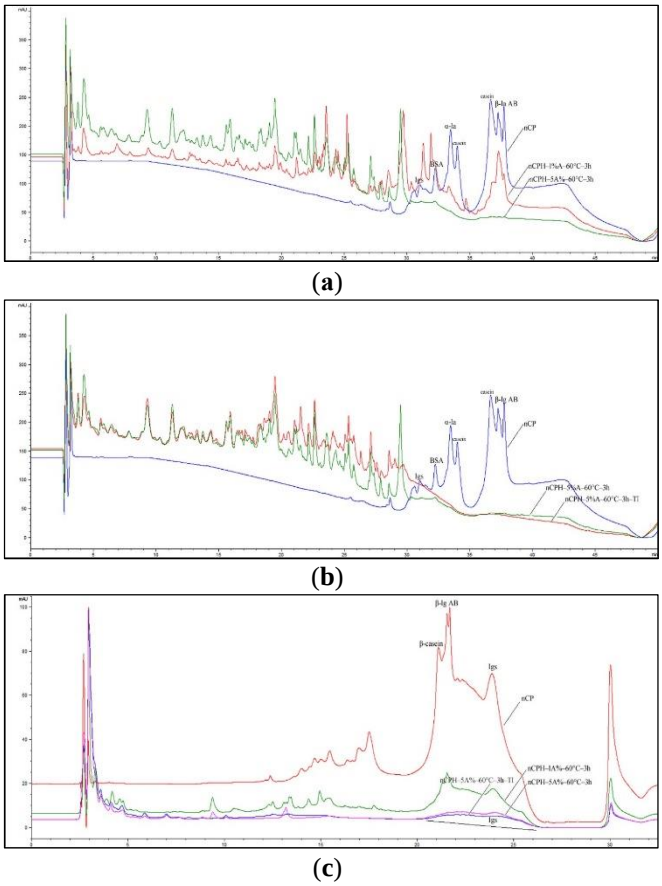
It should be noted that after thermal inactivation of the enzyme, the HPLC profile of this sample reveals only a minor peak with a retention time of 29 min, characteristic of samples where the Alcalase was inactivated by the addition of inhibitor (Figure 6b).

In general, it is advisable to increase the temperature of colostrum protein hydrolysis with Alcalase up to 60 °C, which enhances the proteolysis degree of the immunoglobulin fraction. Heat pre-treatment of the colostrum is not recommended due to aggregation of the protein component, which complicates the process of subsequent hydrolysis and micro/ultrafiltration. On the contrary, heat inactivation of Alcalase leads to cleavage of the residual Ig component.

The main significant difference with increasing the hydrolysis temperature up to 60 ºC is the higher proportion of cleaved Ig fraction, which is reflected in Figure 4b and 6c. At the same time, effective cleavage of α-la and β-lg and accumulation of characteristic products of Ig proteolysis was observed at Alcalase content of 5 % and hydrolysis temperatures of 50 and 60 ºC (Figures 4a, 6a and 6b).

***The quantitative estimation of the proteolysis degree in colostrum hydrolysates:*** Table 2 summarizes the results of the quantitative analysis of the Ig fraction content in the colostrum hydrolysates according to the chromatographic separation data. Thus, at 1/5 % Alcalase application and hydrolysis temperature of 60 ºC the cleaved colostrum samples contain (68,9±1,7)/(17,6±0,6) % of native immunoglobulins, which is 1,2/1,5 times less than in the corresponding samples cleaved at a lower temperature (50 ºC). In general, when the temperature of the enzymatic reaction was increased to 60°C and the Alcalase content was 5 %, up to (82.4±0.6) % of the protein component of the colostrum was hydrolyzed. At the same time, as a result of the

subsequent thermal inactivation of the enzyme, a complete cleavage of the Igs into the peptide fraction was observed.



(**a**)

(**b**)

(**c**)

**a** and **b** – program for the separation of serum proteins, **c** – program for separation of casein and immunoglobulin fractions.

**Figure 6. HPLC profiles of native (nCP), hydrolyzed colostrum proteins (nCPH) at 1 and 5 % Alcalase content, temperature of 60 °C for 3 hours and thermally deactivated (TI)**

**Table 2. Characterization of the protein composition of colostrum hydrolysates (by HPLC).**

| Sample name | Igs content, % | Degree of proteolysis (DP), % |
|---|---|---|
| nCPH–1%–50°C–3h | 80.2±5.3 [a] | 19.8±5.3 [a] |
| nCPH–5%–50°C–3h | 26.0±2.8 [b] | 74.0±2.8 [b] |
| nCPH–1%–60°C–3h | 68.9±1.7 [c] | 31.1±1.7 [c] |
| nCPH–5%–60°C–3h | 17.6±0.6 [d] | 82.4±0.6 [d] |
| nCPH–5%–60°C–3h–TI | 0 [e] | 100 [e] |

nCPH – hydrolyzed native colostrum proteins obtained with Alcalase (at enzyme contents of 1 and 5 %, hydrolysis temperatures of 50 and 60 °C for 3 hours and thermal inactivation of protease – TI). Results are expressed as arithmetic mean ± half the width of the 95 % confidence interval (n=3). Values without a common letter index (a–e) within the same column indicate significant differences ($p<0.05$)

Consequently, it is reasonable to increase the hydrolysis temperature of first milk with Alcalase from 50 to 60 ºC at an enzyme : substrate ratio of 5 %, which provides an increase in the cleavage efficiency of the immunoglobulin fraction.

## DISCUSSION

According to previous studies, the conditions of whey protein hydrolysis were optimized using highly active proteases of animal (trypsin) and bacterial (Alcalase, thermolysin) origin. The bacterial endopeptidase Alcalase was identified as the most effective and available protease approved for nutritional purposes (Halavach, 2022a; Halavach, 2022b). The protein and peptide profiles of whey protein hydrolysates with average and extensive degree of hydrolysis were characterized (Halavach, 2022a; Halavach *et al.*, 2023). The influence of the proteolysis degree of whey and colostrum with Alcalase on the biologically active properties (antioxidant activity, antigenic properties, antimicrobial action) of the corresponding hydrolysates and their peptide fractions was determined (Golovach *et al.*, 2016; Halavach *et al.*, 2020; Halavach *et al.*, 2023).

In addition, the options of preliminary heat treatment of whey proteins were previously adjusted to achieve complete cleavage of whole whey allergen proteins with Alcalase (Halavach, 2022a). In particular, thermodenaturation of whey proteins at 80 °C (10 min, pH 8.0) followed by proteolysis with Alcalase (enzyme : substrate ratio – 1 : 100) resulted in a 6.3 % increase in the release of the peptide fraction ($M_W \leq 10$ kDa) and a significant increase in the hydrolysis degree of protein substrates.

A method to obtain whey protein hydrolysate by proteolysis with Alcalase and filtration with a 20 kDa cutoff is known (Sviridenko *et al.*, 2018). The authors reported a significant reduction in antigenicity of the peptide fraction obtained. However, the capacity of the separation filter is not sufficient to remove the residual amount of uncleaved whey proteins, since the $M_W$ of the major whey proteins β-lg/α-la is 18.4/14.2 kDa respectively. Another method involves the addition of Alcalase or Protamex and filtration (10 kDa cutoff), with a peptide fraction yield of 80 % (Prosekov *et al.*, 2014). The proposed method provided a greater amount of peptide fraction by cleaving all whey proteins with Protozyme, including BSA, that is stable to proteolysis with serine endopeptidases.

In the present work, thermodenaturation of whey proteins and cleavage with Protozyme under optimized conditions provided hydrolysates with a proteolysis degree comparable to that of samples cleaved with Alcalase (Figures 2 and 3, Table 1). This will expand the range of applications for Protozyme in the production of protein hydrolysates with a high degree of hydrolysis.

In addition to whey, which is in high demand, bovine colostrum is a potential functional ingredient for a wide range

of food products (cheeses, yogurts, dried and ready-to-drink beverages, etc.) (Playford and Weiser, 2021; Poonia and Shiva, 2022). IgG content (>50 g/L) in the first milk is considered a quality marker. In the technological process of milk-based foods, it is usually subjected to high-temperature short-term pasteurization (72 °C, 15 s) or periodic pasteurization (60–63 °C, 30–60 min). It was demonstrated that heat treatment at 72 °C is critical for the alteration of the secondary structure of IgG molecules. Conversely, the loss of immunoreactivity has been observed at 73 °C. (Playford and Weiser, 2021; Chatterton *et al.*, 2020).

It should be noted that the alkaline endopeptidase Alcalase efficiently cleaves the main whey proteins (β-lg and α-la), while proteolysis of BSA is achieved after heat treatment of the substrate at 80 °C (10 min, pH 8.0). Furthermore, BSA is not degraded with this enzyme when heated to 70 °C (Halavach, 2022a).

In this study, heat treatment of colostrum (80 °C, 10 min, pH 8.0) had a negative effect on its technological properties. Heating led to denaturation and subsequent irreversible aggregation of the protein component, and impeded access to proteolysis sites. Immunoglobulins are unstable (thermolabile) in an alkaline environment (pH 8.0), which is optimal for Alcalase hydrolysis. In general, heat pre-treatment of the colostrum was ineffective in increasing the proteolysis degree of Ig fraction with Alcalase.

Alcalase is known to maintain its stability at the enzymatic reaction temperature of 50–60 °C (Tacias-Pascacio *et al.*, 2020). Under the experimental conditions, hydrolysis of native colostrum was carried out at 60 °C, the optimal temperature for the retention of proteolytic activity of Alcalase, together with the absence of denaturation processes in the Ig fraction. At 50 °C, proteolysis of casein and minor whey proteins (β-lg and α-la) was observed, whereas cleavage of the predominant Ig component was enhanced by increasing the hydrolysis temperature to 60 °C (Figures 4 and 6, Table 2).

Along with this, during thermal inactivation of the enzyme (at 85 °C for 15 min), heating above 70 °C causes denaturation of the Ig component and loss of its immunoreactivity (Chatterton *et al.*, 2020; Playford and Weiser, 2021). Under these conditions, Alcalase possesses residual proteolytic activity that results in cleavage of the denatured protein. When the hydrolysate temperature reaches 85 °C, the thermostable protease loses its activity.

There are a number of papers on the production of hydrolysates from colostrum using a variety of proteolytic enzymes (pepsin, trypsin, α-chymotrypsin, pancreatin, papain, Alcalase) (Chiang *et al.*, 2017; Oussaief *et al.*, 2019; Fajardo-Espinoza *et al.*, 2019). At the same time, the specifics of the cleavage of casein and whey fractions of colostrum are not the subject of these works. This should be considered when obtaining hypoallergenic bioactive hydrolysates. The present study investigated the possibility of hydrolyzing the colostrum protein component with alcalase. The necessity of using thermal inactivation of protease to increase the proteolysis degree of the Ig fraction was revealed.

In general, the present work has provided new data on the characteristics of the cleavage of native and heat-treated whey proteins with the alkaline bacterial endopeptidase Protozyme. The conditions for the whey hydrolysis with Protozyme were optimized to obtain parameters characteristic of Alcalase. At the same time, the use of preliminary heat treatment of colostrum is not recommended due to the thermolability of the immunoglobulin fraction. The optimization of proteolysis conditions with Alcalase and the introduction of a thermal inactivation stage for the enzyme resulted in an increase in the hydrolysis degree of the Ig component of bovine colostrum. This process will enable the production of colostrum proteins hydrolysates with a high hydrolysis degree and bioactive potential, resulting in the release of specific peptides.

***Conclusions*:** The protein and peptide compositions of partial enzymatic protein hydrolysates of whey and colostrum obtained with the serine endopeptidases Alcalase and Protozyme were characterized by SDS-electrophoresis and HPLC. Complete cleavage of the predominant whey proteins (β-lg and α-la) into the peptide fraction was found when the enzymes were added at a level of 5 % of the substrate content at a hydrolysis temperature of 50/60 °C for Alcalase/Protozyme, respectively. In addition, cleavage of the minor high molecular weight whey fraction (BSA) with Protozyme is achieved by preheating the whey. The maximum proteolysis degree of the native immunoglobulin fraction of colostrum with Alcalase (82.4 %) is achieved at 5 % enzyme content and a hydrolysis temperature of 60 °C. As a result of the subsequent thermal inactivation of the protease, this colostrum hydrolysate contains proteolysis products with $M_W$<10 kDa.

This study presents the first experiments on parameter selection for hydrolysis of whey proteins with Protozyme in order to achieve parameter values corresponding to highly active protease Alcalase. This is the inaugural study to optimize the hydrolysis of the Ig fraction of bovine colostrum, which is the most resistant to proteolysis.

In general, samples of partial enzymatic hydrolysates of dairy proteins were obtained, which are potential hypoallergenic ingredients for food products. Enzymatic hydrolysates of whey and colostrum proteins are promising substitutes for the native protein component in infant formulas, functional foods (sports, dietary and gerodietary nutrition) or nutraceuticals.

The bioactivities (antimutagenic and antimicrobial effects) and antigenic properties of bovine milk hydrolysates will be the focus of subsequent studies.

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