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Effect of hydrolysis degree with Alcalase on antioxidant and antigenic properties of whey and colostrum protein hydrolysates

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ABSTRACT

Enzymatic hydrolysis of dairy proteins is aimed to increase their nutritional value and reduce their allergenic properties. It is relevant to study the effects of enzymatic hydrolysis with highly active endopeptidase alcalase on the bioactivity of the whey and colostrum protein components. Dairy hydrolysates with various degree of Alcalase treatment were obtained in the present study. A comparative analysis of cleaved dairy proteins and their peptide fractions with a 5 and 10 kDa cutoff was performed. The protein and peptide composition of hydrolysates was determined using various techniques. The bioactivity of hydrolyzed whey and colostrum samples were estimated. The antioxidant activity of hydrolyzed and fractionated (5 kDa cutoff) whey samples increased by 5.91/6.62-fold and that of colostrum by 14.22/7.24-fold in fluorimetric/spectrophotometric assay, respectively. A significant decrease in the antigenic potential of milk peptides was observed with increasing degree of hydrolysis and subsequent ultrafiltration. Thus, bioactive dairy protein hydrolysates were obtained, which are applicable as a component of specialized foods with proven hypoallergenic properties and high antioxidant activity.

1. Introduction

Enzymatic hydrolysis is the most common method to improve the technical and functional properties of dairy proteins (solubility, viscosity, thermostability) and to reduce their allergenic potential by destroying the sites of allergenic epitopes in intact casein and whey protein molecules [1–3]. Bioactive peptides (BAP) with antioxidant, antimicrobial, hypotensive, and immunomodulatory activities produced by proteolysis of dairy raw materials have a significant potential as components of functional foods and therapeutic agents [4–7]. The technological process of preparing bioactive hydrolysates includes selection of substrate and enzyme(s), conditions of enzymatic reaction and the characterization of the proteolysis products [5,8,9].

Whey proteins are a versatile dietary ingredient for functional foods and also a potential source of BAP [10]. Studies aimed at research of bioactivities, functional properties and therapeutic applications of whey-based products are of great importance [11]. Along with this, bovine colostrum is a rich natural source of macro- and micronutrients, immunoglobulins, peptides with antimicrobial activity and growth factors [12,13]. Colostrum-based foods are used as a health-promoting and immunomodulating agents. In general, colostrum is a valuable raw material for the industry of novel dietary products and food supplements [14–16].

Industrial production of whey protein hydrolysates includes the following processing steps: obtaining of whey protein concentrate, protein hydrolysis by commercial enzymes, protease(s) inactivation, spray/lyophilic drying of the hydrolysate, and product packaging. It is possible to include additional steps in the technological process, such as protein pretreatment before hydrolysis, membrane separation [5,8]. Current industrial methods of peptide fractionation are membrane

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separation processes (ultrafiltration and nanofiltration). The advantages of membrane technologies are the affordable price of the target products and the improved functionality of the hydrolysates (high antioxidant activity, hypoallergenic properties) [17].

The dairy industry is a relevant application of proteolytic enzymes, that cleave dairy proteins to middle/short-chain peptides and amino acids [18,19]. The application of enzymes of microbial origin, in contrast to those from plant or animal sources, is most cost-effective on an industrial scale [20–22]. The best reported enzyme is Alcalase, an extracellular bacterial protease isolated from the culture medium of *Bacillus subtilis/B. licheniformis*. Relatively broad substrate range and site-specificity of Alcalase provide an advantage of enzyme using for cleavage of various protein substrates to achieve a high degree of proteolysis. Thus, Alcalase is actively used to reduce the allergenic potential of foods and increase their biological activity [18,20,22,23].

The diseases associated with oxidative stress and impaired quality of food products have potential to cause economic losses both in the field of health care and in the food industry. Therefore, it is advisable to actively include components with high antioxidant effect in the daily diet [22, 24]. The antioxidant activity (AOA) of food proteins depends on the structure, composition, hydrophobicity, and amino acid sequence [5,22, 25]. The antiradical effect of milk increases in 2–5 times after exposure to digestive enzymes due to the release of bioactive peptides [26]. The AOA of peptides is determined by the presence of specific amino acids (cysteine, methionine, lysine, histidine, tyrosine and tryptophan) in the peptide sequence. For example, the sulfhydryl group of cysteine interacts directly with radicals. The imidazole group of histidine is associated with metal ion chelation, and hydrogen transfer or trapping of radicals, whereas aromatic amino acids have hydrogen and electron donor properties [4,22,26]. The components of the casein fraction (α- and β-casein) are mainly responsible for the AOA of dairy raw materials, while in whey β-lactoglobulin and α-lactalbumin are the main source of bioactive peptides [25,27].

It is known that bovine milk contain nearly 30 potentially allergenic proteins and is responsible for up to 40% of food allergies [28]. The most common dairy allergens are the casein fraction (57% of milk allergy cases) and the major whey protein β-lactoglobulin (66%). α-Lactalbumin and bovine serum albumin causes allergy to a lesser extent (18%). Antigenic epitopes within protein macromolecules to which specific immunoglobulins (Igs) bind are the main targets of the immune response. Allergenicity reduction of the dairy protein component is achieved by cleavage of the sites of antigenic determinants as a result of physical treatment (heat, ultrasound and microwave treatment), as well as due to fermentation by lactic acid bacteria and enzymatic hydrolysis with various proteases, which is considered as the most effective method among them [3,28,29].

In general, the main area of dairy protein hydrolysates application is addition to specialized hypoallergenic infant diet [30]. According to the current scientific data, infant formulas based on native and hydrolyzed protein are safe, provide balanced nutrition and promote growth when breastfeeding is impossible or breast milk is partially replaced [31,32]. It is expected to expand the range of hydrolysate containing products for sports, clinical and pediatric nutrition [33,34].

The main features of whey and colostrum protein hydrolysis using different proteases and the bioactive effects of obtained hydrolysates were characterized according to the literature [35–42] and our own previous research [43–47]. The novelty of this work consists in a complex comparative study of the bioactive properties of enzymatic hydrolysates of whey and colostrum proteins, and in the determination of the effect of proteolysis with alcalase and subsequent ultrafiltration of hydrolysates on changes in the antioxidant and antigenic potential of peptide fractions. It is relevant to improve the technology for obtaining hypoallergenic hydrolysates of dairy proteins intended for the production of functional foods.

The aim of the present study is to characterize the molecular weight distribution of whey and colostrum protein hydrolysates obtained by Alcalase treatment with subsequent fractionation by ultrafiltration, and to study their bioactive properties (antioxidant activity, residual antigenicity).

2. Materials and methods

2.1. Enzymatic hydrolysis of whey and colostrum proteins

In the present study, whey protein concentrate with 80% protein content (Shchuchin Branch of Molochny Mir OJSC, Shchuchin, Belarus), skimmed bovine colostrum powder with 70% protein content (All-Russian Research Institute of Dairy Industry, Moscow, Russia), endopeptidase Alcalase® 2.4 L FG (food grade, activity 2.4 U/g, Novozymes, Copenhagen, Denmark), phosphate buffer (PBS, pH 7.4) and phenylmethylsulfonyl fluoride (PMSF, 99 % purity) from Sigma (St. Louis, MO, USA) were used.

Whey/colostrum protein solutions in 0.05 PBS (pH 7.4) with 5% mass concentration were prepared for enzymatic hydrolysis. The solutions were centrifuged at 10,000 rpm during 30 min to eliminate insoluble particles. The supernatant was used for hydrolysis. Enzymatic reaction was carried out at an enzyme dosage of 1% and 5%, a temperature of 50 °C, pH 7.4 during 2/3 h for whey/colostrum hydrolysis, respectively. PMSF was added at a final concentration of 0.1 mM for alcalase inactivation. Spin X UF500 centrifuge filters (Corning, London, England) with a 5 and 10 kDa cutoff were used for hydrolysates fractionation. Resulting samples were frozen at −20 °C for further analysis.

The mass fraction of total nitrogen (TN) and protein in whey and colostrum samples, their hydrolysates and ultrafiltrates were determined according to ISO 8968–1:2014 [48]. The mass fraction of α-amine nitrogen (AN) in hydrolysates was established according to GOST R 55, 479–2013 [49]. The hydrolysis degree (%) was defined as AN/TN ratio. The mass percentage of low-molecular-weight fraction (%) was calculated as the ratio of the protein amount in the filtrate (5/10 kDa) to the protein content in the initial hydrolysate.

Trichloroacetic acid (TCA, Pancreas, Barcelona, Spain) solution with 12% mass concentration was used to precipitate the high-molecular-weight protein component in the hydrolysate samples. 1000 μl of 12% TCA solution was added to 500 μl of test samples and incubated during 10 min at ambient temperature, then centrifuged at 13,000 rpm during 5 min. The protein content in hydrolysates and their supernatants was estimated by the method of F. Kalb et al. (1977) [50]. The mass proportion of low-molecular-weight fraction (%) was calculated as the ratio of the protein amount in the supernatant to the protein content in the initial hydrolysate.

2.2. Evaluation of the protein and peptide profile of hydrolyzed whey and colostrum

2.2.1. SDS-electrophoretic analysis

The method of electrophoretic separation in polyacrylamide gel (20%) under denaturing conditions (with sodium dodecyl sulfate, or SDS-electrophoresis) was used to determine the protein and peptide composition of native whey and colostrum samples, and also their enzymatic hydrolysates. The separation was performed according to the standard protocol [51]. PageRuler™ Prestained Protein Ladder 10–180 kDa marker (Thermo Fisher Scientific, Waltham, MA, USA) was used as a molecular weight standard.

2.2.2. High performance liquid chromatography (HPLC)

Acetonitrile (ACN, HPLC grade, Sigma Aldrich, St. Louis, MO, USA) and trifluoroacetic acid (TFA, mass spectrometry grade, Fisher Scientific International, Hampton, NH, USA) were used in the experimental work. β-Lactoglobulin (β-lg, genetic variants A and B, 90% of protein), α-lactalbumin (α-la, 85% of protein), bovine serum albumin (BSA, 90% of protein), casein (cas, 88% of protein), and immunoglobulin G (IgG, 90% of protein) from Sigma (St. Louis, MO, USA) were used as standards

for bovine milk proteins.

Agilent 1100 chromatograph (Agilent Technologies, Santa Clara, CA, USA) and Zorbax–300SB C8 column (4.6 × 250 mm, 5 μm, Agilent Technologies, Santa Clara, CA, USA) equilibrated with 0.1% aqueous TFA solution were used for the HPLC study.

Elution of whey proteins and their enzymatic hydrolysates (Paragraph 4.1.) was carried out according to the program presented below (Table 1). The separation was performed at ambient temperature at a flow rate of 1.0 mL/min for 50 min, and detection was performed at 214 nm.

Chromatographic separation of samples containing casein and IgG was performed using the protocol shown in Table 2. Separation was carried out at 46 °C at a flow rate of 1.0 ml/min during 33 min, and detection was performed at 214 nm. HPLC profiles were analyzed using specialized software ChemStation for LC 3D systems Rev.B.04.01 (Agilent Technologies, Santa Clara, CA, USA).

The amount of whey proteins, casein and immunoglobulin G in the hydrolysates were determined according to calibration graphs for protein standards. Proteolysis degree (*PD*, %), or the proportion of cleaved dairy proteins, was calculated with formula (1):

$$PD = \frac{C\text{-}H}{C} \times 100, \quad (1)$$

where C/H is protein amount in control/hydrolysate sample (mg/ml) respectively.

The *PD* for whey hydrolysates was calculated as the ratio of the amount of major whey proteins (β-lg, α-la and BSA) cleaved to the sum of the protein concentrations in the control sample. In experiments with colostrum, *PD* was determined as the ratio of the quantity of hydrolyzed immunoglobulin G to its content in the initial sample in experiments with colostrum.

2.2.3. Mass spectrometric (MS) analysis

TFA (MS grade, Fisher Scientific International, Hampton, NH, USA), purified water (Honeywell Burdick & Jackson, Muskegon, MI, USA), calibration standard (Protein I/Standard II) and ionization matrix (α-cyano-4-hydroxycinnamic acid) from Bruker Daltonik (Bremen, Germany) were used for mass spectrometry analysis.

Defrosted samples (Paragraph 4.1.) were centrifuged at 10,000 rpm during 2 min, and 2% TFA solution was added to the supernatant at a volume ratio of 19:1. The resulting samples were centrifuged at 10,000 rpm for 2 min. The supernatant was demineralized and concentrated using pipette tips (Supel Tips C18) according to the instructions of the manufacturer (Supelco, Bellefonte, PA, USA). Sample eluate and matrix were spotted to the MALDI plate at a volume ratio of 1:1 and air-dried.

High-resolution mass spectrometer with a matrix-activated laser desorption ionization (MALDI) source in combination with a time-of-flight (TOF) mass analyzer (microflex LRFMALDI–TOF, Bruker Daltonics, Bremen, Germany) was used in the study. The resolving power was >15,000 FWHM at *m/z* 0.5–5.0 kDa in reflectron regime, the accuracy was up to 500 ppm in linear mode at *m/z* 0.005–0.1 kDa, 30 ppm in reflectron regime at *m/z* 0.5–5.0 Da. The software Compass for flexSeries 1.4 and flexControl Version 3.4 (Build 57), Bruker Daltonics BioTools 3.2 SR4 and Build 6.32 (Bruker Daltonik, Bremen, Germany) were applied for data evaluation.

Table 1
Separation conditions for whey proteins and their hydrolysates.

№ of stage	Time, minutes	Solution A, % (v/v)	Solution B, % (v/v)
1	5.0	95.0	5.0
2	10.0	90.0	10.0
3	30.0	60.0	40.0
4	32.0	60.0	40.0
5	40.0	50.0	50.0
6	45.0	50.0	50.0
7	50.0	90.0	10.0

Solution A – 0.1% TFA solution, Solution B – acetonitrile.

Table 2
Separation conditions for casein and immunoglobulin G fractions and their hydrolysates.

№ of stage	Time, minutes	Solution A, % (v/v)	Solution B, % (v/v)
1	5.0	71.0	29.0
2	10.0	63.0	37.0
3	12.0	59.0	41.0
4	16.0	57.5	42.5
5	17.0	57.0	43.0
6	19.0	49.0	51.0
7	21.0	41.0	59.0
8	26.0	0.0	100.0
9	33.0	71.0	29

Solution A – 0.1 % TFA solution, Solution B – acetonitrile.

2.2.4. Dynamic light scattering (DLS) analysis

Dairy protein hydrolysates and their ultrafiltrates (5 and 10 kDa cutoff) obtained as described in Paragraph 4.1 were applied in the experiment. Samples with protein concentration of 1 mg/mL were prepared in 50 mM PBS based on deionized water.

Samples were measured using the DynaPro® NanoStar® instrument (Wyatt Technology, Goleta, CA, USA). 50 μl of the sample was added to a disposable plastic cell. Experiments were performed at a cell compartment temperature of 25 °C, the number of measurements was 10, and the measurement time was 20 s. The molecular weight of the particles in the test samples was evaluated using the DYNAMICS 7.8.1 program from Wyatt Technology (Goleta, CA, USA).

2.3. Determination of antioxidant activity

2.3.1. Fluorimetric method

The reagents used for AOA estimation are fluorescein, ethylenediaminetetraacetic acid (EDTA), Mora salt, and phosphate buffered saline (PBS, pH 7.4) from Sigma (St. Louis, MO, USA), hydrogen peroxide (Acros Organics, Geel, Belgium).

2 μM FL solution, 50 mM EDTA solution, 250 mM Mora salt solution, 1.0 mM Mora salt (Fe^{2+}) solution with 1.0 mM EDTA, 10 mM H_2O_2 solution, 50 mM PBS (pH 7.4), and the experimental samples of hydrolysates (Paragraph 4.1) with protein concentration equal to 3–4000 μg/mL in 50 mM PBS were made.

The cuvette (optical path length 1 cm) was injected with 0.02 μM of FL solution in 50 mM PBS. Fluorescence studies were carried out on a RF–5301 PC fluorimeter (Shimadzu, Kyoto, Japan) at excitation wavelength (λ_{ex}) of 490 nm and emission intensity (λ_{em}) in the range of 495–600 nm (slit width – 5 nm). The maximum fluorescence intensity (*FI*) was registered at λ_{514}. The *FI* of the resulting peak was considered as the maximum value (100%).

The cuvette was supplemented with 0.02 μM FL solution in 50 mM PBS including 0.1 mM Fe^{2+} with 0.1 mM EDTA and 1.0 mM H_2O_2. Upon interaction of Fe^{2+} ions with H_2O_2 (Fenton reaction), the generated radicals suppressed the FL fluorescence. The received *FI* value was accepted as the minimum value.

The cuvette was loaded with 0.02 μM FL solution in 50 mM PBS containing 0.1 mM Fe^{2+} with 0.1 mM EDTA, 1.0 mM H_2O_2 and 0.3–400 μg/mL (per protein amount) of the test samples. The reaction was initiated with H_2O_2 inclusion at a final concentration of 1.0 mM. The resulting fluorescence peaks were percentage measured by taking the fluorescence of the control FL solution as 100%. The fluorescence intensity degree (*FID*, %) was estimated by formula (2):

$$FID = \frac{FI_{AO}}{FI_{control}} \times 100, \quad (2)$$

where $FI_{control}$ is the fluorescence intensity of the control FL sample (FL solution free of Fe^{2+}, EDTA, hydrolysate and H_2O_2), FI_{AO} is the

fluorescence intensity of FL solution with antioxidant (AO).

Graphs of *FID* dependence on protein amount in the experimental samples were generated. Based on the derived equation, the concentration of sample IC_{50} appropriate for 50% fluorescence inhibition was estimated.

2.3.2. ABTS method

The AOA measurement provided for the use of previously obtained cation-radical based on the diammonium salt of 2,2′-azino-bis [3-ethylbenzthiazoline-6-sulfonic acid] ($ABTS^{\bullet+}$) according to the modified method described in the article (Hernández-Ledesma et al.) [52].

ABTS, ammonium persulfate (≥98%), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, 97%), phosphate buffered saline (PBS, pH 7.4) manufactured by Sigma-Aldrich (St. Louis, MO, USA) were applied in the experiment.

The cation radical ($ABTS^{\bullet+}$) was prepared in reaction of 7 mmol ABTS stock solution with 2.45 mmol ammonium persulfate solution. This mixture was kept in the dark at room temperature for 12–16 h.

200 μl of $ABTS^{\bullet+}$ solution and 100 μl of 50 mM PBS were added to the well of a 96-well polystyrene plate (Sarstedt, Nümbrecht, Germany) and the optical density was measured at 734 nm on a SpectraMax M2 plate photometer (Molecular Devices, San Jose, CA, USA). The initial $ABTS^{\bullet+}$ solution was diluted with 50 mM PBS to achieve an absorbance level of 0.7 ± 0.02 units.

Calibration solutions of Trolox (0–100 μmol/mL), native proteins (whey protein concentrate, colostrum) and their hydrolysates/filtrates (0–750 μg/mL of protein; Paragraph 4.1) in 50 mM PBS were prepared. 100 μl of experimental samples or calibration Trolox solutions were added to a well of a plate containing 200 μl of diluted $ABTS^{\bullet+}$ solution, incubated in the absence of light during 30 min, and the optical density was measured at 734 nm.

The degree of cation radical reduction, or absorption inhibition (*I*, %), was calculated according to formula (3):

$$I = \frac{D_0 - D}{D_0} \times 100, \quad (3)$$

where D_0 is the optical density of the initial $ABTS^{\bullet+}$ solution, D is the optical density of the $ABTS^{\bullet+}$ solution after antioxidant addition.

Dependence of absorption inhibition (*I*, %) on the concentration of standard (Trolox) and protein content in the analyzed samples (μg/ml) was plotted. The sample concentration IC_{50} corresponding to 50% absorption inhibition was calculated according to the obtained equations.

Trolox Equivalent Antioxidant Capacity (*TEAC*, antioxidant activity index measured in μmol of Trolox per μg of protein) of the tested samples was determined according to formula (4):

$$TEAC = \frac{IC_{50}(Trolox)}{IC_{50}(protein)}, \quad (4)$$

where $IC_{50}(Trolox)$ is the concentration of 50% absorption inhibition when the standard (Trolox) was applied, $IC_{50}(protein)$ is the concentration of 50% absorption inhibition when samples of native proteins and their hydrolysates were added.

2.4. Evaluation of residual antigenicity

A kit for quantitative determination of β-lactoglobulin (β-lg) by competitive enzyme immunoassay (RIDASCREEN®β-Lactoglobulin, R4901) manufactured by R-Biopharm AG (Darmstadt, Germany) was used in order to evaluate the antigenic properties of experimental samples (Paragraph 4.1.). The kit is intended for the analysis of β-lg levels in hydrolyzed dairy products, including hypoallergenic infant formula. The test system provides detection of native and processed protein, and its fragments. The detection/quantification limit is 2.1/5.0 μg/g of β-lg.

According to the recommendations of the test system manufacturer, dilutions of native and hydrolyzed whey and colostrum proteins and corresponding ultrafiltrates (Paragraph 4.1) were prepared. The optical density of the target solutions was determined at a wavelength of 450 nm using a Multiskan Ascent microplate spectrophotometer (Thermo Labsystems, Waltham, MA, USA). RIDA®SOFT Win.net 1.103.0.0217 program (R-Biopharm AG, Darmstadt, Germany) with cubic spline function was used for experimental data processing.

Residual antigenicity (RA) was estimated as the proportion of β-lg concentration in the hydrolyzed sample to that in native milk sample and was presented as %.

2.5. Statistical data processing

In order to compare the means of factor's levels, a one-, two- or three-way analysis of variance (ANOVA) [53] with subsequent Dunnett's test (comparing several treatments with a control) [54], Student's *t*-test (matching the means of two groups) or Tukey's Honest Significant Difference (HSD) test (performing multiple pairwise comparisons) [55,56] were applied. R functions *aov*, *DunnettTest*, *t. test*, and *TukeyHSD* of the *stats* [57] and *DescTools* [58] packages were involved in the statistical analysis. Statistical differences between groups were significant at the $p < 0.05$ level with correction for multiple pairwise comparisons. Correlation analysis was performed using Pearson's Criterion [59].

Plots were created in Microsoft Office 2021 Excel program (MS Corporation, Shadeland, IN, USA). The data in tables and graphs are shown as the mean ± the half-width of 95% confidence interval (n = 3).

3. Results

3.1. Characterization of molecular weight profile of hydrolyzed whey and colostrum proteins

3.1.1. Electrophoretic separation of enzymatic hydrolysates

Experimental samples of whey and colostrum protein hydrolysates were obtained using highly active protease Alcalase at different mass ratios of enzyme to substrate (1 and 5%). SDS-electrophoretic analysis of the samples with different degree of Alcalase hydrolysis was performed as shown in Fig. 1.

The SDS-electrophoregram shows the typical protein composition of whey (Fig. 1, lane 2), in particular β-lactoglobulin (β-lg, M_W 18.4 kDa), α-lactalbumin (α-la, M_W 14.2 kDa), traces of bovine serum albumin (BSA, M_W 66.3 kDa) and lactoferrin (LF, M_W 76.8 kDa). A residual β-lg content was detected in the hydrolysate when 1% of Alcalase (per protein content) was added (Fig. 1, lane 3). In contrast increasing the enzyme concentration to 5% resulted in a practically complete cleavage of the major whey proteins (β-lg and α-la) into proteolysis products (Fig. 1, lane 4). At the same time, minor whey components (BSA and LF) resistant to Alcalase hydrolysis are removed by subsequent ultrafiltration.

The characteristic protein composition of the colostrum is also reflected in the SDS-electrophoregram (Fig. 1, lane 5). The immunoglobulin fraction (Igs, mainly IgG) is represented by polypeptide chains with M_W of 25 and 50 kDa. Casein fraction (M_W 19.0–25.2 kDa), α-la, β-lg, traces of BSA and LF are also detected. The addition of Alcalase (1%) to whey results in partial cleavage of the major protein fractions in the hydrolysate Fig. 1, lane 7). Also, a residual amount of Igs and high-molecular-weight products of their partial cleavage (Fig. 1, lane 8) stable to proteolysis with Alcalase are detected in the hydrolysate at 5% enzyme concentration.

The basic properties of dairy protein hydrolysates are mainly determined by the features of Igs cleavage. The relatively high degree of whey protein hydrolysis is due to the efficient cleavage of the predominant whey proteins (α-la and β-lg) with Alcalase. Subsequent ultrafiltration is necessary to separate the high-molecular-weight protein and peptide fractions.

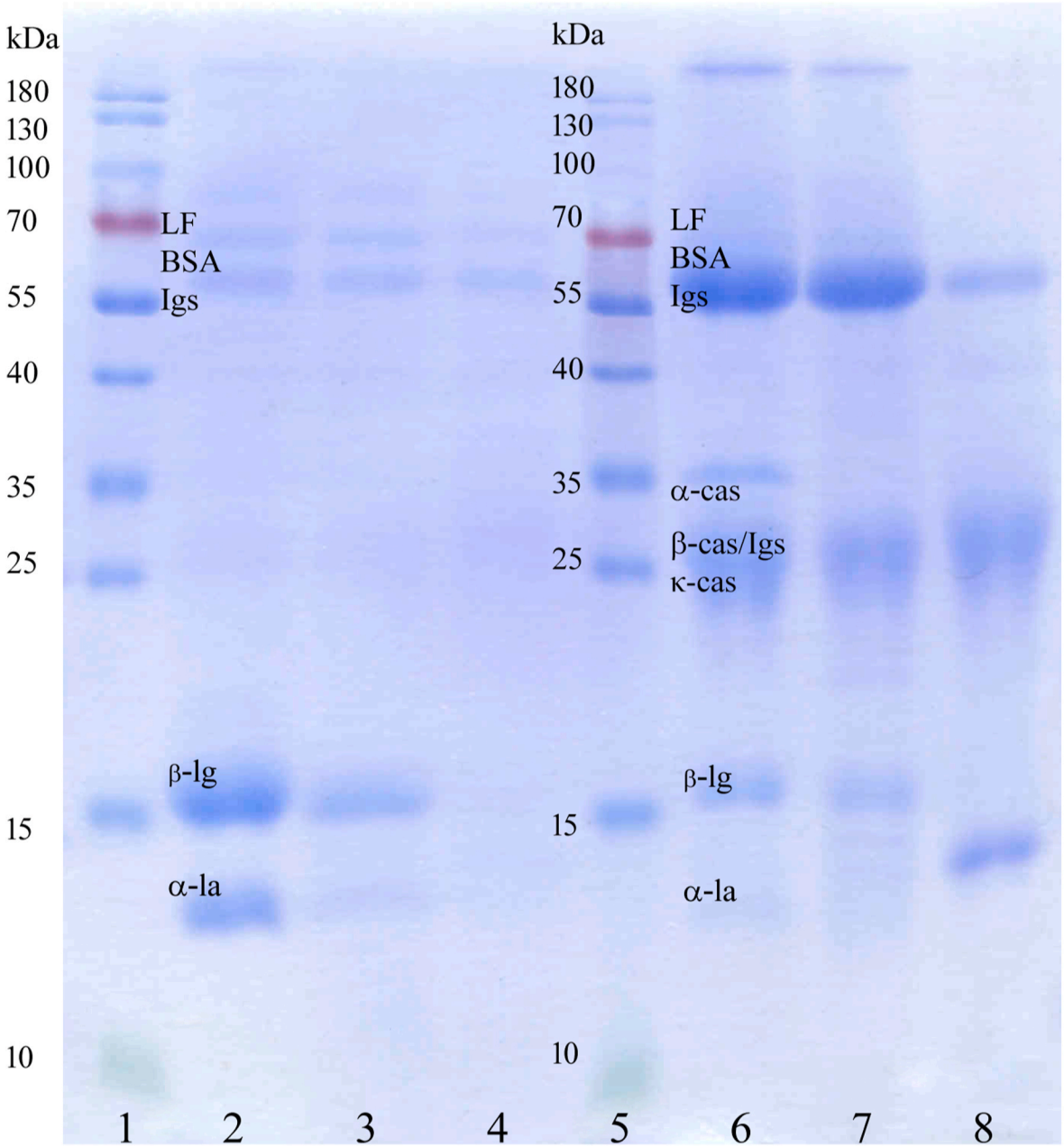

Fig. 1. SDS-electrophoregram of whey/colostrum proteins (WP/CP), whey/colostrum protein hydrolysates (WH/CH) obtained by adding of 1 and 5% of Alcalase. 1 – marker, 2 – WP, 3 – WH–1%, 4 – WH–5%, 5 – marker, 6 – CP, 7 – CH–1%, 8 – CH–5%, cas – casein.

3.1.2. High performance liquid chromatography (HPLC) analysis of dairy protein hydrolysates and determination of their peptide fraction content

Experimental samples of dairy protein hydrolysates (peptide fractions with a 5/10 kDa cutoff) were analyzed by HPLC method in order to determine their protein and peptide composition. Figs. 2–5 show HPLC profiles of initial whey and colostrum hydrolysates and their filtrates. Table 3 summarizes the results of quantitative analysis of the major whey and colostrum proteins in the hydrolyzed samples.

The chromatographic profiles of dairy protein hydrolysates before/after filtration were compared. It was found that the fraction with a 5 kDa cutoff elutes from the column up to the 23/25th minutes of separation of colostrum/whey hydrolysates. Proteolysis products with a 10 kDa cutoff fractionation elute from the column up to 29/30th minutes of separation of colostrum/whey samples (Figs. 3 and 5). Thus, as a result of fractionation, residual amount of native dairy proteins and polypeptides are removed from hydrolysates.

At the same time, the efficient cleavage of the major whey proteins (α-la and β-lg) with Alcalase was confirmed (Fig. 2), while BSA showed resistance to proteolysis and removed only after filtration (Fig. 3, Table 3).

In contrast, colostrum is characterized by a multicomponent protein composition, which complicates the chromatographic separation of its fractions, as coelution of several proteins is observed (Fig. 4). It has been shown that the hydrolysis efficiency of predominant colostrum proteins decreases in the range "casein – β lg – IgG". Proteolysis products of the major colostrum proteins, partially hydrolyzed IgG, and also its polypeptide with a retention time of 29 min, resistant to cleavage with endopeptidase, were found in the hydrolysate with 5% Alcalase content (Fig. 5). The accumulation of a polypeptide with M_W near to 15 kDa in the hydrolysate was confirmed by the data of SDS-electrophoretic analysis (Fig. 1, lane 8).

Quantitative analysis of protein and peptide profiles of enzymatic

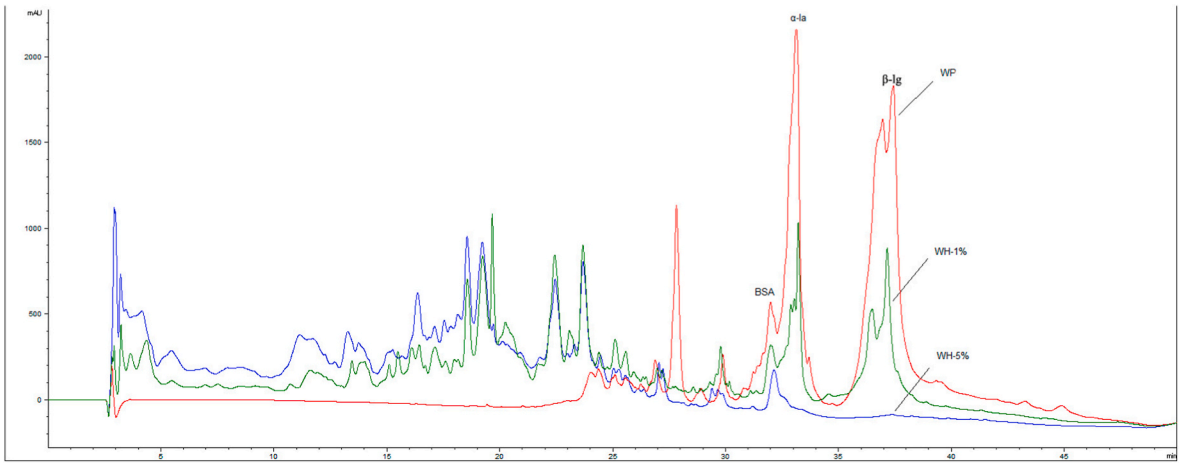

Fig. 2. HPLC profiles of native (WP) and hydrolyzed whey proteins at 1/5% Alcalase content (WH–1%/WH–5%).

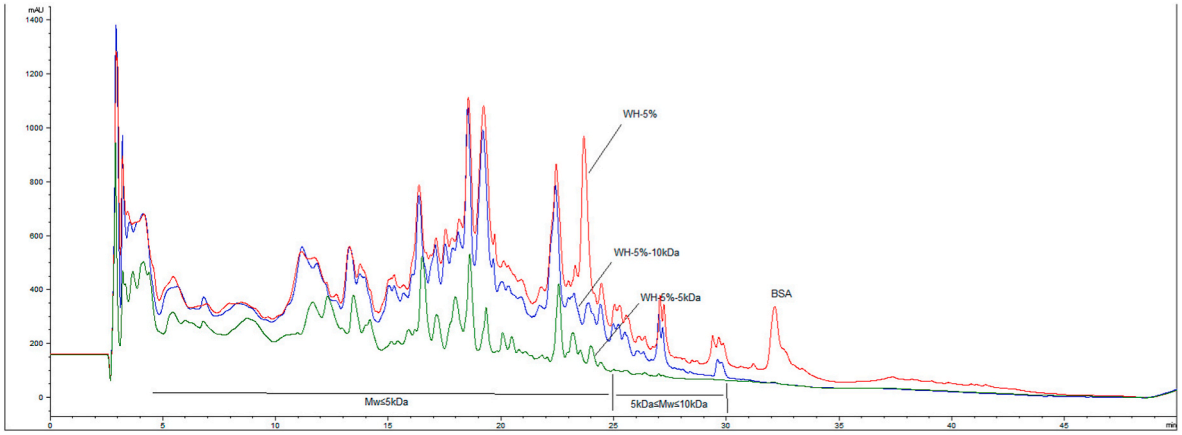

Fig. 3. HPLC profiles of hydrolyzed whey proteins at 5% Alcalase content (WH–5%) and their filtrates with a 5/10 kDa cutoff (WH–5%–5/10 kDa).

whey and colostrum hydrolysates with different degrees of hydrolysis was performed. Table 3 shows that the amount of hydrolyzed fraction increased when the Alcalase dose was increased from 1% to 5%.

The maximum content of peptides with a 5/10 kDa cutoff was determined in extensive whey protein hydrolysate (5% Alcalase content) and was (73.5 ± 1.3)/(81.6 ± 1.7)%. Thus, the filtrate with a 10 kDa cutoff of extensive whey protein hydrolysate contains 90% of the fraction with a 5 kDa cutoff. An increase in this low-molecular-weight fraction was observed along with an increase in the degree of proteolysis of whey and colostrum by 1.1- and 3.7-fold, respectively (Table 3). This is due to further cleavage of the peptide component in whey hydrolysates and also of the peptide and immunoglobulin fractions in the case of colostrum samples.

The content of low-molecular-weight protein component in the supernatant obtained after precipitation of the high-molecular-weight fraction using trichloroacetic acid was determined. As the amount of Alcalase added to the samples of hydrolyzed whey and colostrum increased, the proportion of the target fraction became 1.9 and 2.4 times higher, respectively (Table 3). It is supposed that part of the intermediate proteolysis products and hydrophobic peptides precipitates. This phenomenon may cause a relatively small content of the low-molecular-weight fraction in the target product. There is an obvious trend of peptide fraction increasing with a higher proteolysis degree of dairy proteins.

The results of correlation analysis show a positive correlation between the peptide fraction amount and the hydrolysis degree (AN/TN). Thus, the maximum hydrolysis degree equals to (24.4 ± 1.9)% was determined in the sample of hydrolyzed whey with 5% enzyme addition. The maximal value of AN/TN in hydrolyzed colostrum is equal to (21.0 ± 2.2)%. The maximal peptide fraction content in WH and CH is equal to (81.57 ± 1.74) and (55.46 ± 2.50)% respectively (Table 3).

In general, the data obtained by chromatographic analysis are in agreement with the results obtained by SDS-electrophoretic analysis. The data obtained prove the different substrate specificity of Alcalase in relation to the main proteins, and the importance of Alcalase application followed by ultrafiltration for obtaining of whey and colostrum protein hydrolysates with high hydrolysis degree.

3.1.3. Mass spectrometric (MS) study of whey and colostrum peptide fractions

Enzymatic hydrolysates of dairy proteins and the corresponding ultrafiltrates (peptide fractions) were analyzed by mass spectrometry to determine their molecular weight distribution. Figs. 6 and 7 show the MS profiles of filtrates (10 kDa cutoff) of whey and colostrum protein hydrolysates obtained with dosage of Alcalase 1 and 5%, respectively.

Comparative analysis of hydrolyzed whey samples and peptide composition shown that increasing of protease concentration leads to formation of proteolysis products with lower molecular mass. The maximum value of *m/z* in filtrates of the whey hydrolysates obtained with addition of 1 and 5% of protease was equal to 7812 and 3872, respectively. These data indicate a higher degree of hydrolysis of the whey sample with 5% of Alcalase compared to the samples containing 1% of the enzyme (Fig. 6B).

Similar results were obtained for samples of hydrolyzed colostrum.

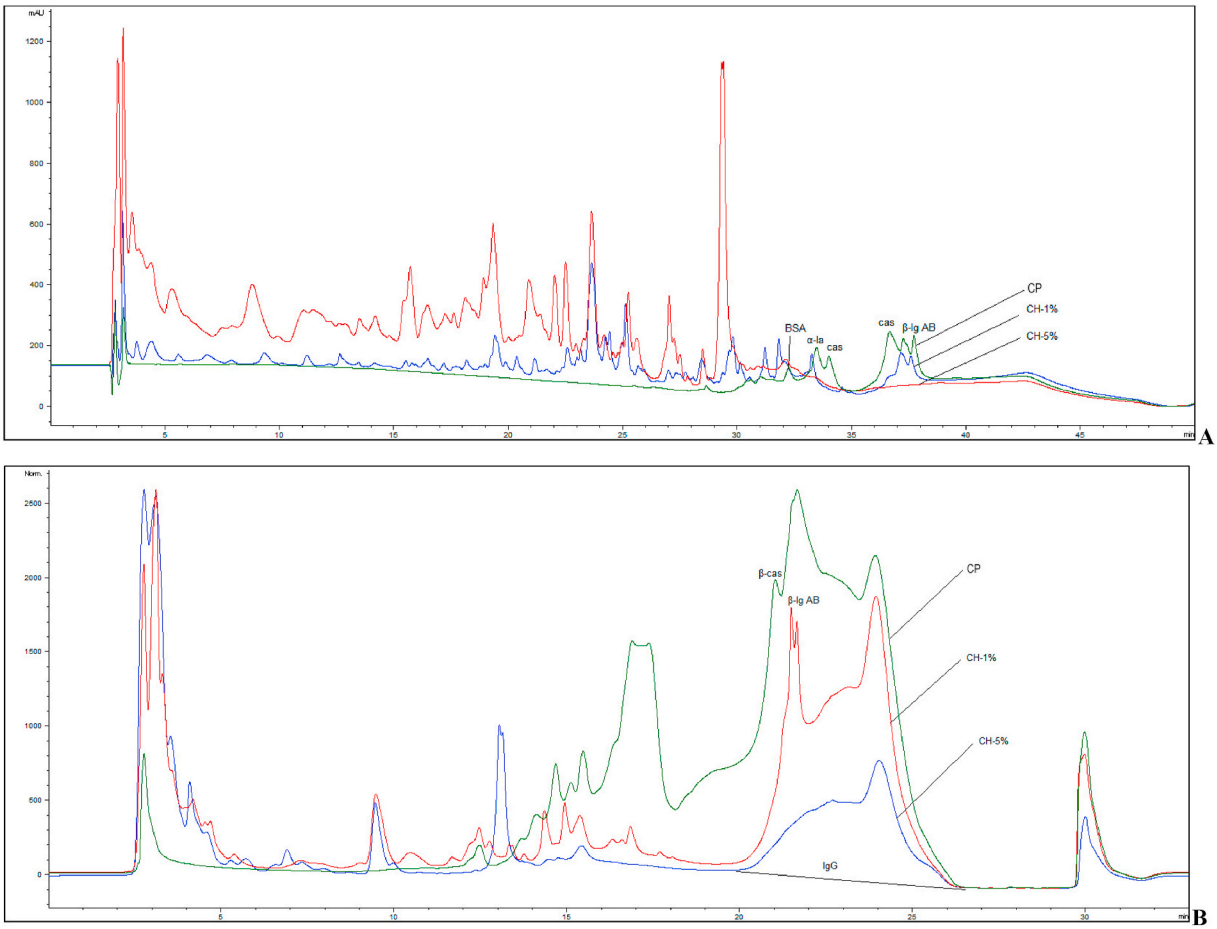

Fig. 4. HPLC profiles of native (CP) and hydrolyzed colostrum proteins at 1/5% Alcalase content (CH–1/5%). Cas – casein. Profiles were obtained using different programs to separate whey proteins with peptides (**A**) and casein fraction with IgG (**B**).

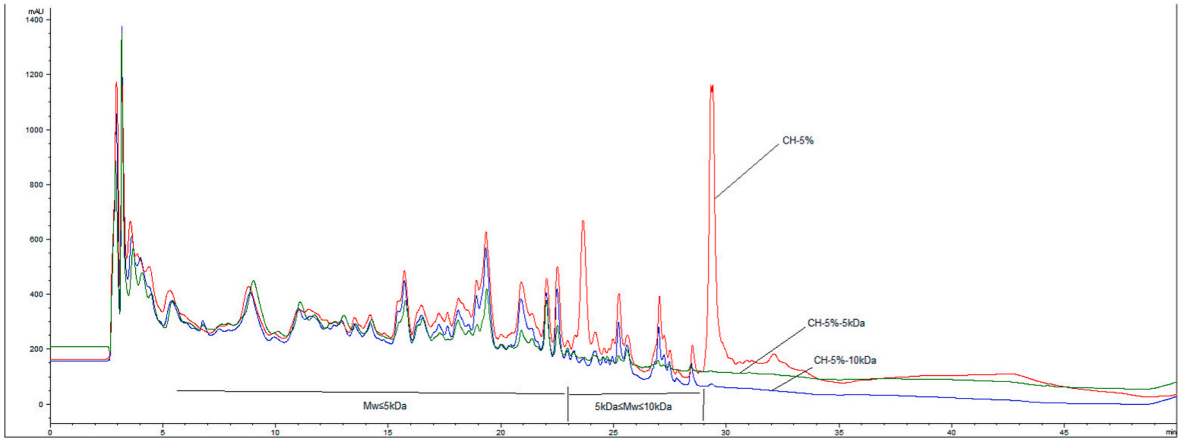

Fig. 5. HPLC profiles of hydrolyzed colostrum proteins at 5% Alcalase content (CH–5%) and their filtrates with a 5/10 kDa cutoff (CH–5%–5/10 kDa).

Thus, the MS spectra of hydrolyzed colostrum filtrates with addition of 1 and 5% of Alcalase revealed peaks with *m/z* values up to 5656 and 3332, respectively (Fig. 7). Peptides with maximum M_W are detected in trace amounts in all samples shown in Figs. 6 and 7.

Peptides with maximum *m/z* values of 2676 and 2186 were detected in the samples of whey and colostrum hydrolysates filtrates (5 kDa cutoff) with 5% Alcalase content (Fig. 8). The data obtained indicate that filtration with a 5 kDa cutoff results in production of permeate containing peptides with M_W less than 3 kDa. Proteolysis products with M_W greater than 3 kDa are present in trace amounts. The MALDI–TOF MS data (Figs. 6–8) do not evaluate the percentage content of peptides with $M_W < 1$ kDa, especially di- and tripeptides. The proportion of the peptide component was determined by estimating the amount of protein in the corresponding ultrafiltrates and initial hydrolysates (Table 3).

According to the data of total protein determination, the filtrate (10 kDa cutoff) of extensive whey protein hydrolysate after fractionation contains 90 % of the fraction with $M_W \leq 5$ kDa (Table 3). Based on mass-spectrometry data, this fraction is represented by a mixture of peptides

Table 3
Characteristics of the peptide composition of whey and colostrum protein hydrolysates with different degree of hydrolysis (according to HPLC data).

Sample name	Content of native milk proteins[1,2], %		Content of cleaved milk proteins (proteolysis degree)[2], %	Molecular weight distribution of filtrates[3], %		Content of low-molecular-weight fraction[4], %	Hydrolysis degree (AN/TN), %
	β-lg α-la BSA	IgG		5 kDa cutoff	10 kDa cutoff		
WH–1%	9.1 ± 0.4 10.8 ± 0.3 100	n/d	86.1 ± 0.2 [a]	51.0 ± 2.7[a,A]	65.6 ± 3.7[a,B]	27.4 ± 0.1[a]	19.0 ± 1.8[a]
WH–5%	0 0 100	n/d	95.2 ± 0.3 [b]	73.5 ± 1.3 [b,A]	81.6 ± 1.7 [b,B]	51.4 ± 0.2 [b]	24.4 ± 1.9 [b,d]
CH–1%	n/d	80.2 ± 5.3[a]	19.8 ± 5.3[c]	8.7 ± 1.2[c,A]	16.6 ± 1.2[c,B]	18.0 ± 0.1[c]	13.0 ± 0.6[c]
CH–5%	n/d	26.0 ± 2.8 [b]	74.0 ± 2.8 [d]	48.8 ± 1.7[a,A]	55.5 ± 2.5 [d,B]	42.4 ± 0.9 [d]	21.0 ± 2.2[a,d]

WH – whey proteins hydrolysate; CH – colostrum proteins hydrolysate; 1/5% – hydrolysis at 1/5% Alcalase content. [1] – estimation for the main whey proteins (β-lg, α-la and BSA) and the prevalent protein fraction of the colostrum (IgG); [2] – data according to HPLC results; [3] – data on protein determination in ultrafiltrates; [4] – data following the results of the low-molecular-weight fraction precipitation with trichloroacetic acid; n/d – not determined. The values represent the mean ± the half-width of 95% confidence interval (n = 3). Means without a common letter within the same column (a–d) and row (A,B) indicate significant difference at $p < 0.05$.

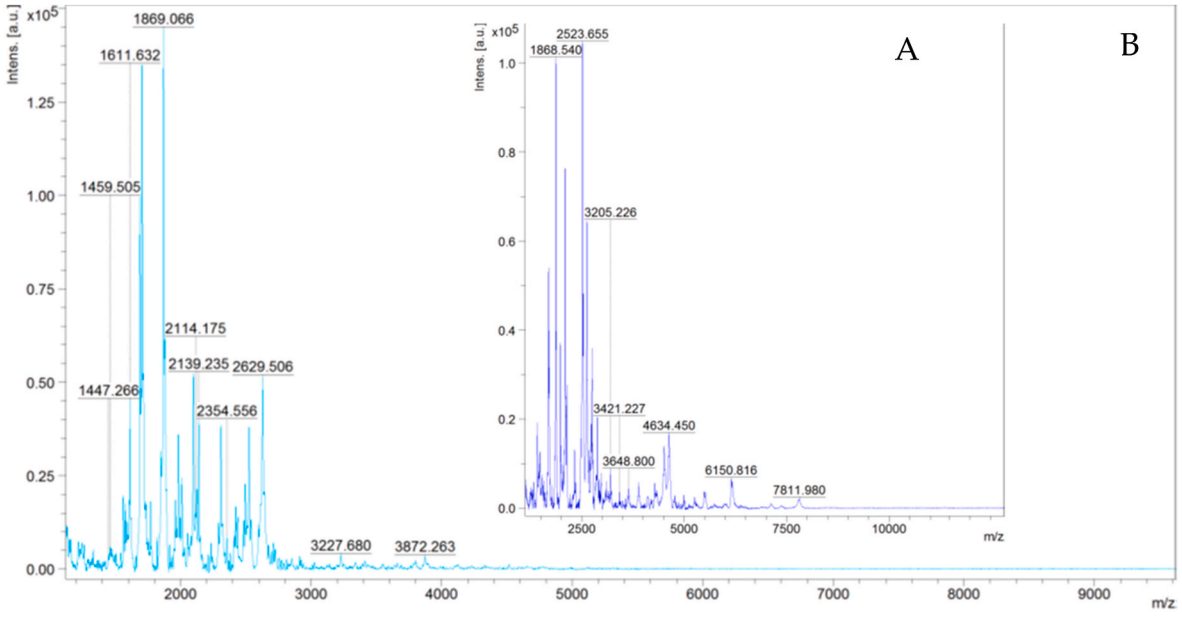

Fig. 6. MALDI–TOF MS profiles of filtrates (10 kDa cutoff) of hydrolyzed whey proteins at 1 % (**A**) and 5 % (**B**) Alcalase content.

with $M_W < 3$ kDa (Figs. 6–8).

It should be noted that proteolysis products with a lower molecular weight were detected in hydrolyzed colostrum compared to whey hydrolysates. This is due to the efficient cleavage of casein fraction with Alcalase and release of low-molecular-weight peptides. At the same time, the whey fraction is relatively resistant to proteolysis, which is confirmed by the data obtained from SDS-electrophoretic analysis (Fig. 1).

3.1.4. Assessment of molecular weight distribution of filtrated hydrolysates with dynamic light scattering (DLS) analysis

DLS method was used to determine the molecular weight distribution of filtrates (5 kDa cutoff) of whey and colostrum hydrolysates with different degree of hydrolysis. Comparative analysis of hydrolysate samples is presented in Table 4.

The fractions (5 kDa cutoff) of hydrolyzed whey and colostrum samples include a spectrum of peptides close in size, as evidenced by the relatively high degree of heterogeneity (relative polydispersity) of the system (Table 4). The particle size in the hydrolysates, and consequently their M_W, was found to be lower with increasing amount of added enzyme (1 or 5% Alcalase content). At the same time, filtrates (5 kDa cutoff) of whey protein hydrolysates at 1/5% Alcalase amount contain a fraction with a higher molecular weight (up to 3.8/2.3 kDa) compared to that of the colostrum sample (up to 1.7/1.4 kDa). It should be noted that the usage of ultrafiltration with a 5 kDa cutoff provides obtaining the fraction where M_W of peptides is less than 3 kDa, which is crucial for the preparation of extensive protein hydrolysates.

In general, the results of the DLS method correlate with the data of electrophoretic analysis, HPLC and mass spectrometry (Figs. 1–8), which indicate an increase in the degree of proteolysis with higher concentration of Alcalase, as well as suggest a greater degree of colostrum protein hydrolysis.

3.2. Antioxidant activity of hydrolyzed and fractionated dairy proteins

The antioxidant activity (AOA) of native whey and colostrum

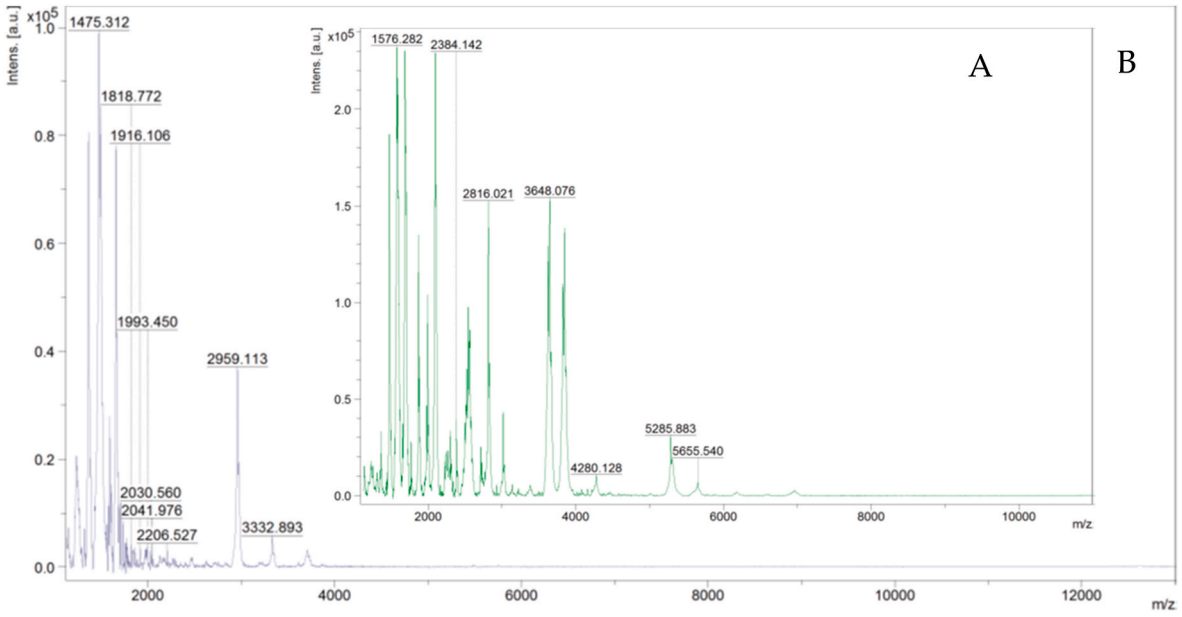

Fig. 7. MALDI–TOF MS profiles of filtrates (10 kDa cutoff) of hydrolyzed colostrum proteins at 1 % (**A**) and 5 % (**B**) Alcalase content.

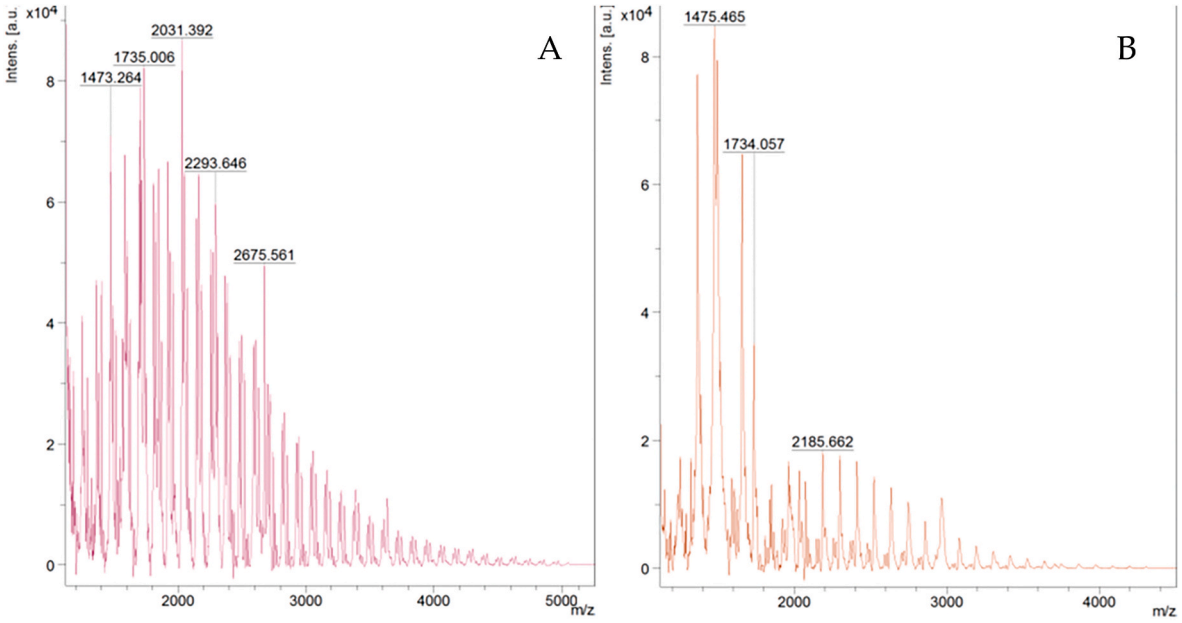

Fig. 8. MALDI–TOF MS profiles of filtrates (5 kDa cutoff) of hydrolyzed whey (**A**) and colostrum (**B**) proteins at 5% Alcalase content.

proteins, their enzymatic hydrolysates and filtrates (5/10 kDa cutoff) was studied by fluorimetric and spectrophotometric methods (ORAC and ABTS assays, respectively).

Fluorescence intensity of fluorescein (FL) was determined as a function of native and hydrolyzed dairy proteins in the test samples. Protein concentrations of the experimental samples corresponding to 50 % recovery of FL fluorescence at addition of components with antioxidant activity (IC_{50}) were calculated.

AOA measurement also involved the previously obtained cation radical $ABTS^{\bullet+}$ (blue colored solution), which is converted to its colorless neutral form upon its reduction. Trolox, a water-soluble derivative of vitamin E, was used as an antioxidant standard. The IC_{50} sample concentration corresponding to 50 % inhibition of $ABTS^{\bullet+}$ absorbance upon addition of Trolox or experimental samples was calculated.

In accordance with the fluorimetric analysis data a significant increase in radical-scavenging properties of dairy protein hydrolysates, in particular, their ability to recover the FL fluorescence intensity was revealed after proteolysis with Alcalase and fractionation (Table 5). As a result of hydrolysis at 1/5% Alcalase concentration, the AOA rate of hydrolyzed whey and colostrum increased by 1.74/2.54 and 1.86/3.29 times, respectively. Thus, the increase in the hydrolysis degree of protein substrates with Alcalase was associated with an enhancement of their radical-scavenging potential.

A significant increase in the AOA of peptide fractions was found after filtration. The antiradical effect of whey hydrolysate filtrate with a 10/5 kDa cutoff and 5 % Alcalase dosage increased additionally by 1.66/2.33 times. The radical-scavenging effect of the similar colostrum filtrate samples with a 10/5 kDa cutoff increased by 1.53/2.01 times compared to the initial hydrolysate. Thus, the experimental data indicate an AOA increase when samples are enriched with low-molecular-weight

Table 4
Characterization of the molecular weight distribution of filtrates (5 kDa cutoff) of whey and colostrum protein hydrolysates.

Sample name	Radius of particles[1], nm	Molecular weight (M_W) of particles[1], kDa	Range of revealed M_W^2, kDa	Relative polydispersity[3], %
WH–1%–5 kDa	0.8	1.8	0.7–3.8	43.0
WH–5%–5 kDa	0.7	1.7	1.4–2.3	48.6
CH–1%–5 kDa	0.6	1.0	0.8–1.7	65.1
CH–5%–5 kDa	0.6	0.9	0.7–1.4	41.8

WH – whey proteins hydrolysate; CH – colostrum proteins hydrolysate; 1/5% – hydrolysis at 1/5% Alcalase content; 5 kDa – filtration with a 5 kDa cutoff. [1] – value for predominant particles; [2] – prevailing peptide fraction. [3] – heterogeneity degree of the system by M_W (the ratio of the standard deviation to the mean value, %).

Table 5
AOA of hydrolyzed dairy proteins and corresponding filtrates according to ORAC assay.

Sample name	$IC_{50(Fl)}$, μg (protein)/mL	IC_{50} (native proteins)/IC_{50} (hydrolysate)
WP	$81.6 \pm 2.1^{a, \alpha}$	1.00^{a}
WH–1%	46.9 ± 1.3^{b}	1.74 ± 0.01^{b}
WH–1%–10 kDa	23.5 ± 0.8^{c}	3.47 ± 0.20^{c}
WH–5%	32.1 ± 1.6^{d}	2.54 ± 0.18^{d}
WH–5%–10 kDa	19.4 ± 0.6^{e}	4.21 ± 0.23^{e}
WH–5%–5 kDa	$13.8 \pm 0.7^{f, \gamma}$	5.91 ± 0.16^{f}
CP	$56.9 \pm 0.9^{A, \beta}$	1.00^{A}
CH–1 %	30.6 ± 0.7^{B}	1.86 ± 0.02^{B}
CH–1%–10 kDa	13.5 ± 0.5^{C}	4.23 ± 0.12^{C}
CH–5%	17.3 ± 0.5^{D}	3.29 ± 0.05^{D}
CH–5%–10 kDa	11.3 ± 0.5^{E}	5.05 ± 0.21^{E}
CH–5%–5 kDa	$8.6 \pm 0.3^{F, \delta}$	6.62 ± 0.22^{F}

WP – whey proteins; WH – whey proteins hydrolysate; CP – colostrum proteins; CH – colostrum proteins hydrolysate; 1/5% – hydrolysis at 1/5% Alcalase content; 5/10 kDa – filtration with a 5/10 kDa cutoff. The values represent the mean ± the half-width of 95% confidence interval (n = 3). Means without a common letter (a–f, A–F, α–δ) within the same column indicate significant difference at $p < 0.05$.

peptides.

It should be noted that the antioxidant effect of native colostrum exceeds the values established for whey sample. Maximum AOA was determined for filtrates (5 kDa cutoff) of whey/colostrum hydrolysates with 5 % Alcalase content, whose IC_{50} reaches 13.8/8.6 μg (protein)/mL, that corresponds to an increase of AOA by 5.91/6.62 times.

According to the results of spectrophotometric analysis, proteolysis with Alcalase and subsequent filtration caused a significant increase in the AOA of dairy protein hydrolysates, exactly their ability to recover the $ABTS^{\bullet+}$ cation radical (Table 6).

Proteolysis with Alcalase at an enzyme level of 1/5% resulted in a 3.01/4.21- and 1.19/2.89-fold increase in the antioxidant activity of whey and colostrum proteins, respectively. Thus, similar to ORAC method, the higher degree of proteolysis determines the AOA increase of hydrolysate samples. The difference is that the radical-scavenging effect of colostrum hydrolysates increases less compared to the whey samples. Subsequent fractionation of whey and colostrum hydrolysate (5% Alcalase content) resulted in an additional 1.29/3.38-fold and 1.66/2.50-fold increase in the AOA of peptide fractions with 10/5 kDa cutoff, respectively. Thus, the increase of AOA was confirmed with the increase in the short-chain peptide concentration.

Moreover, the antiradical effect of native colostrum was greater than

Table 6
AOA of hydrolyzed dairy proteins and their filtrates in accordance with ABTS assay.

Sample name	$IC_{50(ABTS)}$, μg (protein)/mL	TEAC, μmol (Trolox)/μg (protein)	IC_{50} (native proteins)/IC_{50} (hydrolysate)
WP	$83.7 \pm 2.6^{a, \alpha}$	$0.23 \pm 0.01^{a, \alpha}$	1.00^{a}
WH–1%	28.2 ± 2.2^{b}	0.69 ± 0.05^{b}	3.01 ± 0.25^{b}
WH–1%–10 kDa	19.5 ± 0.2^{c}	0.99 ± 0.01^{c}	4.34 ± 0.03^{c}
WH–5%	20.1 ± 0.3^{d}	0.96 ± 0.01^{d}	4.21 ± 0.06^{d}
WH–5%–10 kDa	15.5 ± 0.8^{e}	1.25 ± 0.06^{e}	5.45 ± 0.24^{e}
WH–5%–5 kDa	$5.9 \pm 0.2^{f, \gamma}$	$3.26 \pm 0.08^{f, \gamma}$	14.22 ± 0.32^{f}
CP	$63.1 \pm 1.4^{A, \beta}$	$0.31 \pm 0.01^{A, \beta}$	1.00^{A}
CH–1 %	53.2 ± 2.2^{B}	0.36 ± 0.02^{B}	1.19 ± 0.05^{B}
CH–1%–10 kDa	16.0 ± 0.6^{C}	1.21 ± 0.04^{C}	3.94 ± 0.22^{C}
CH–5%	21.8 ± 0.3^{D}	0.89 ± 0.01^{D}	2.89 ± 0.07^{D}
CH–5%–10 kDa	13.2 ± 1.0^{E}	1.47 ± 0.11^{E}	4.80 ± 0.37^{E}
CH–5%–5 kDa	$8.7 \pm 0.1^{F, \delta}$	$2.22 \pm 0.02^{F, \delta}$	7.24 ± 0.17^{F}

WP – whey proteins; WH – whey proteins hydrolysate; CP – colostrum proteins; CH – colostrum proteins hydrolysate; 1/5% – hydrolysis at 1/5% Alcalase content; 5/10 kDa – filtration with a 5/10 kDa cutoff. The values represent the mean ± the half-width of 95% confidence interval (n = 3). Means without a common letter (a–f, A–F, α–δ) within the same column indicate significant difference at $p < 0.05$.

that of whey (Table 6). The highest level of AOA was achieved after filtration of whey/colostrum hydrolysates (5% Alcalase amount) with a 5 kDa cutoff, when the IC_{50} was 5.9/8.7 μg (protein)/mL (with a 14.22/7.24-fold increase in AOA). In general, the ABTS assay showed a greater antioxidant effect for the above-mentioned sample of whey filtrate than for the corresponding colostrum samples. The differences detected by fluorimetric and spectrophotometric methods might be related to the peculiarities of the molecular mechanisms of antioxidant action implementation in the compared test systems.

The AOA value was also determined in TEAC units relative to the standard (Trolox), as reported in Table 6. The antiradical activity of filtrates with a 5/10 kDa cutoff was comparable or higher than that of the standard sample, which work out at 0.99–3.26 μmol (Trolox)/μg (protein).

In general, the different AOA value of native and hydrolyzed whey and colostrum proteins is determined by the degree of their hydrolysis. Hydrolysis degree depends on the dosage of added Alcalase, mode of the filtration used to hydrolysates enrichment with low-molecular-weight peptides, as well as the composition of the protein substrate (the ratio of casein and whey protein fractions), and the presence of non-protein antioxidant components. The ORAC/ABTS assay revealed an increase in the AOA of dairy proteins with the growth of Alcalase hydrolysis degree and enrichment with low-molecular-weight peptide fraction after filtration.

3.3. Antigenic properties of hydrolyzed dairy proteins

The antigenic properties (or the capacity to bind with antibodies) of whey and colostrum samples, their enzymatic hydrolysates and filtrates with a 5/10 kDa cutoff were studied. The residual amount of the main milk allergen (β-lg) in dairy proteins hydrolysates and corresponding fractions was determined using a competitive enzyme-linked immunosorbent assay (ELISA).

Proteolysis of whey and colostrum with Alcalase resulted in a significant decrease in the concentration of native β-lg with an increase in the enzyme dose. Filtration causes a further reduce in the residual antigenicity (RA) of the samples due to the removal of trace amounts of

undegraded β-lg, as well as products of its partial proteolysis containing antigenic determinants. The filtrate containing peptide fraction with $M_W < 3$ kDa (Table 7) is characterized by the lowest RA level among whey samples, corresponding to 0.047 ± 0.002 % (more than 2000-fold decrease in RA). No β-lg was detected in the colostrum hydrolysate sample subjected to filtration with a 5 kDa cutoff.

Thus, application of Alcalase at an enzyme dose of 5 % followed by filtration (5 kDa cutoff) result in the maximal RA reduction in whey and colostrum hydrolysates. The higher antigenicity level of samples containing whey proteins is due to the relatively high initial content of β-lg (50 % of total whey protein). The different antigenicity level of native and hydrolyzed samples of whey and colostrum can be explained by the peculiarities of the qualitative and quantitative composition of their proteins (the content of casein and whey fractions), the amount of introduced highly active protease and the value of molecular weight cutoff during hydrolysates fractionation.

4. Discussion

Dairy proteins are cleaved into peptides with a wide range of bioactive functions (antimicrobial, antioxidant, opioid, immunomodulatory activity) during technological processing and digestion in the gastrointestinal tract [4–7]. A systematic review of bioactive peptides derived from dairy raw materials was performed, and a database was created to target functions of bioactive peptides search. As a result, 47 peptides with AOA were characterized, including 32 from bovine milk proteins (68%) [60].

Enzymatic hydrolysates contain a mixture of peptides differing in amino acid chain length, hydrophobicity, and charge. Separation processes aim to obtain peptide fractions with a targeted range of physicochemical and functional properties. In particular, a mixture of peptides is separated during ultrafiltration according to their molecular weight. Ultrafiltration membranes are available with different molecular weight cutoff values (0.5–100 kDa). Typical strategies for peptide separation by this technique include the recovery of retentate (concentrate) or permeate (ultrafiltrate) to obtain peptide fractions with the desired molecular weight [61]. The classification of hypoallergenic infant formulas is based on the molecular weight distribution of peptides. Partially hydrolyzed mixtures for allergy prevention mainly include peptides with M_W 3–10 kDa (medium hydrolysis degree), whereas formulas for therapeutic diets based on extensive hydrolysates are represented by a peptide fraction with $M_W < 3$ kDa (extensive hydrolysis degree) [3,62]. Extensive protein hydrolysates for therapeutic products usually contain about 90% of the fraction with $M_W < 3$ kDa [63].

Particular attention is focused on the selection of a highly active endopeptidase to obtain bioactive hydrolysates, with enzyme preparations of microbial origin predominantly used [18–20]. The active use of commercially available protease Alcalase in the production of bioactive peptides has been known since 2010. Peptides with antioxidant, hypotensive, metal binding, antidiabetic, anti-inflammatory, and antimicrobial activities were found among the hydrolysis products of this endopeptidase. Alcalase is one of the most effective proteases for the hydrolysis of wide range of protein sources, especially food industry by-products [21–23].

Alcalase is a registered trademark of Novozymes Corp. It is a liquid enzyme preparation with the “food grade” specification containing 9% of protease extract from *Bacillus licheniformis*. The Alcalase application results in production of hydrolysates with hydrophobic properties and a predominance of low-molecular-weight peptides due to the wide range of potential hydrolysis sites (peptide bonds with Phe, Trp, Tyr, Glu, Met, Leu, Ala, Ser, and Lys) [22,23,64].

Hydrolysis of whey and colostrum proteins was performed in the present study using 1% and 5% dosage of Alcalase. Alcalase was recommended to be added in the amount of 1% of the protein substrate following our own previous studies [46,47], for the production of hydrolysates with an average hydrolysis degree, whereas the production of hydrolysates with an extensive hydrolysis degree required an increase in the Alcalase concentration up to 5%.

Studies on obtaining extensive whey protein hydrolysate with Alcalase has been reported [35]. A 20% solution of whey proteins (pH 8.0) was hydrolyzed with Alcalase at a temperature of 45 °C and an enzyme:protein ratio of 1:10 during 5 h. According to high-performance size exclusion chromatography data, the hydrolysate contained 80% of the fraction with $M_W < 2$ kDa and about 10% with $M_W > 10$ kDa. The method [37] includes hydrolysis with Protamex or Alcalase (4% per whey dry matter) at pH 7.5–8.0, temperature of 40–55 °C for 4 h, and subsequent usage of membranes with a 10 kDa cutoff was reported. A peptide fraction yield of 80% was obtained with a significant reduction in RA compared to native whey proteins in this study. Along with this, hydrolysis of natural bovine milk with Alcalase and separation of the supernatant after centrifugation also resulted in the predominance of the fraction with $M_W < 3$ kDa in the hydrolysate (according to gel permeation chromatography data) [29].

According to the results of fractionation (Table 3) and MS analysis (Figs. 6–8), samples containing 90/88% of the fraction with $M_W < 3$ kDa were obtained after hydrolysis of whey/colostrum with Alcalase (5%) and ultrafiltration (10 kDa cutoff), which corresponds to the requirements for extensive protein hydrolysates [3,62,63]. Filtration (5 kDa cutoff) allows additional removal of the potentially allergenic fraction with 10 kDa $< M_W < 3$ kDa. Along with this, filtrate (10 kDa cutoff) of whey hydrolysate with Alcalase (1%) was obtained, which is categorized as a protein component with a medium degree of hydrolysis.

The antioxidant activity of dairy proteins was evaluated by various in vitro techniques: 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical assay, Trolox equivalent antioxidant capacity (TEAC) assay, ferric-reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) [27]. Due to the complex nature of antioxidants (AOs), none of the methods is completely standardized and validated. Methods for the AOA analysis are divided into three classes based on the radical scavenging mechanism, namely hydrogen atom transfer (HAT), single electron transfer followed by proton transfer (SET–PT), and sequential proton loss by electron transfer (SPLET) [22,24,65].

The preferred HAT method is the ORAC assay. Oxidative degradation induces the loss of fluorophore fluorescence. The protective role of AOs by hydrogen donation involves the inhibition of fluorophore oxidation, leading to fluorescence recovery [65]. The ORAC assay is applied for the determination of both hydrophilic and lipophilic antioxidants. Single electron transfer based methods, particularly TEAC assay, rely on the involve non-competitive single electron transfer from antioxidant to

Table 7
Antigenicity evaluation of native and hydrolyzed whey and colostrum proteins and filtrates of the corresponding hydrolysates.

Sample name	Residual antigenicity (RA), %	RA (native proteins)/RA (hydrolysate)
WP	100^{a}	1.00^{a}
WH–1%	24.9 ± 1.8 ^{b}	4.02 ± 0.31 ^{b}
WH–1%–10 kDa	1.60 ± 0.08^{c}	62.4 ± 3.1^{c}
WH–5%	5.80 ± 0.22 ^{d}	17.3 ± 0.7 ^{d}
WH–5%–5 kDa	0.047 ± 0.002^{e}	2139 ± 76^{e}
CP	100^{A}	1.00^{A}
CH–1%	93.7 ± 1.6 ^{B}	1.07 ± 0.02 ^{B}
CH–1%–10 kDa	1.86 ± 0.02 ^{C}	53.9 ± 0.7 ^{C}
CH–5%	0.68 ± 0.05^{D}	147 ± 11^{D}
CH–5%–5 kDa	0.0 ^{E}	–

WP – whey proteins; WH – whey proteins hydrolysate; CP – colostrum proteins; CH – colostrum proteins hydrolysate; 1/5 % – hydrolysis at 1/5 % Alcalase content; 5/10 kDa – filtration with a 5/10 kDa cutoff. The values represent the mean ± the half-width of 95% confidence interval (n = 3). Means without a common letter (a–e; A–E) within the same column indicate significant difference at $p < 0.05$.

oxidant (radicals, metals and carbonyl groups). The reducing power of AOs is quantified by measuring the change in spectrophotometric absorbance. The cation-radical is formed in the TEAC system as a result of the ABTS oxidation. The colored $ABTS^{\bullet+}$ reacts rapidly with AO and its reduction leads to decrease of coloration. The degree of decolorization is calculated relative to the standard (Trolox) [24,52].

In the present study, ORAC and TEAC assays were performed to evaluate the AOA of whey and colostrum protein hydrolysates. According to the review information, dairy proteins represent the most widely studied and rich source of bioactive peptides hidden in the structure of casein and whey proteins [25]. In particular, following casein derived peptides with pronounced AOA have been identified: Glu–Leu, Tyr–Phe–Tyr–Pro-Glu–Leu, Phe–Tyr–Pro-Glu–Leu, Tyr–Pro--Glu–Leu, and Pro-Glu–Leu, indicating the importance of the Glu–Leu sequence for antioxidant action [66]. The amino acid composition analysis of the peptides consists in the composition of whey proteins hydrolysates revealed that the AOA is resulted from the predominance of His and hydrophobic amino acids. Thus, hydrolyzed whey proteins have the potential of effective antioxidants as hydrogen donors, metal ion chelators, and radical stabilizers [67]. Several structural characteristics (chemical nature, charge, basicity, aromaticity, and hydrophobicity) of amino acids primary sequence influence on the three-dimensional conformation of the peptide and, consequently, its biological function. Thus, Met and Cys include the sulfhydryl group (sulfoxide formation in contact with radicals), Tyr and Phe involve phenolic groups (electron/hydrogen donating ability), Pro includes an electron-rich nitrogen-containing pyrrolidone ring, His contains imidazole ring associated with the chelating activity and the ability to scavenge lipid radicals [4,22,25]. Many BAPs possess antioxidant properties due to their interaction with reactive forms of oxygen and nitrogen. In addition to BAPs, milk contains some naturally occurring AOs, such as vitamins (C, A and E) and enzymes (superoxide dismutase, glutathione peroxidase and catalase) [24,26].

In accordance with our own studies [47], the antioxidant activity of whey and colostrum was determined. The higher AOA of colostrum compared to whey was shown, which may be explained by both a different ratio of whey:casein fractions and the presence of non-protein AOs [4,25]. It was established in the present study that the antiradical effect of colostrum was 1.33/1.43 times greater than whey according to data obtained by TEAC/ORAC methods, respectively (Tables 5 and 6).

The most widely used method for obtaining of antioxidant peptides from dairy proteins is enzymatic hydrolysis [4,24–27]. In particular, gastrointestinal enzymes (pepsin and trypsin), commercial proteases of microbial origin, and proteases combinations are applied for dairy proteins hydrolysis. It was established that peptides obtained with Alcalase possess relatively high antioxidant properties [24,27,40,41,68].

In particular, hydrolysis of preheated whey protein isolate with Alcalase caused an increase in DPPH reducing activity from 11.4 to 62.9%. Greater inhibition of the cation radical $ABTS^{\bullet+}$ (by 35.5%) showed for whey hydrolysate obtained with Alcalase compared to the treatment with bacillolysin, Neutrase, and Protamex [27]. Besides, the antioxidant activity of milk enriched with whey protein hydrolysates was studied by the ABTS assay. The Trolox equivalent of whey protein hydrolysate obtained with Flavourzyme and Alcalase were equal to (0.81 ± 0.04) and (1.16 ± 0.05) respectively. These values were higher than Trolox equivalent of native protein, which was equal to (0.19 ± 0.01) [68]. In another study, Alcalase hydrolysate was more active in reducing the cation radical $ABTS^{\bullet+}$, in comparison with hydrolysates prepared with trypsin, Novo pro–D and Flavourzyme [69]. Peptides with high AOA were obtained by the whey proteins hydrolysis using combining of Alcalase and other proteases (Alcalase–Protamex, Alcalase–Protease A 2SD, Alcalase–Flavourzyme, and Alcalase–ProteAXH) [42].

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 AOA of camel milk proteins increased after enzymatic hydrolysis with pepsin, trypsin, α-chymotrypsin, pancreatin, and papain according to DPPH, ABTS, and FRAP assays. Pancreatic hydrolysates had the greatest antioxidant effect [40]. 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 AOA (TEAC and FRAP assay) of the colostrum whey fraction was founded after hydrolysis with an enzymes complex, this effect was expressed in the proteolysis degree increase [41]. In addition, data on the stepwise hydrolysis of the whey fraction of bovine colostrum with Alcalase and Flavourzyme are reported. In vitro inhibition of DNA damage and oxidation of low-density lipoproteins was observed when the hydrolysate of the colostrum whey fraction was administered [70].

Our own previous studies [43,44] showed an increase in the AOA of enzymatic hydrolysates of whey proteins obtained using Alcalase, as well as an increase in the antimutagenic and antimicrobial activity of the peptides. In addition, cleavage of whey proteins with the serine proteases Alcalase and trypsin resulted in a 5.0 and 3.6-fold increase in the antiradical activity of the hydrolysates, respectively [47]. According to the data of the comparative analysis of bovine colostrum samples hydrolyzed with Alcalase and Neutrase, a positive correlation was found between the degree of hydrolysis and the AOA level. Consequently, the antiradical effect of colostrum proteins after Alcalase/Neutrase hydrolysis increased by 5.5/1.7-fold, which is due to the relatively high hydrolysis degree of colostrum proteins with Alcalase [45].

According to the ORAC and ABTS assays performed in the present study (Tables 5 and 6), an increase in Alcalase content leads to higher AOA of hydrolyzed whey and colostrum samples. Thus, according to literature sources and our own research, the level of antiradical effect is determined by the choice of highly active endopeptidase and positively correlates with the degree of proteolysis.

In another study whey protein concentrate was hydrolyzed by Alcalase, Neutrase, Corolase and Flavourzyme, followed by ultrafiltration through membrane filters with 0.2 μm, 10 kDa, 5 kDa and 1 kDa cutoffs. According to the ORAC assay, the obtained hydrolysate fractions had significantly higher AOA than the non-treated whey. Furthermore, the maximum effect was observed for filtrates with $M_W < 1$ kDa compared to the peptide fraction with $M_W < 5$ kDa [38]. In case of colostrum samples, pepsin and pancreatic hydrolysates were separated by ultrafiltration into three fractions with $M_W < 10$, $10 < M_W < 30$ and $M_W > 30$ kDa. The highest AOA values were obtained for proteolysis products with $M_W < 10$ kDa [41].

In accordance with experimental data, the enrichment of hydrolyzed whey and colostrum samples with low-molecular-weight peptides by ultrafiltration (5 and 10 kDa cutoffs) leads to an increase in their radical scavenging properties. Based on fluorimetric/spectrophotometric methods, the AOA of hydrolyzed and fractionated (5 kDa cutoff) whey samples increased by 5.91/6.62-fold, whereas that of colostrum was 14.22/7.24-fold higher, respectively. The ORAC method revealed a more pronounced increase in the antiradical effect (ability to recover FL fluorescence) of hydrolyzed colostrum samples compared to those of hydrolyzed whey (Table 5). On the contrary, in the TEAC system, the ABTS reducing effect of hydrolyzed whey (relative to the native whey proteins) exceeds the values obtained for the corresponding of colostrum samples (Table 6). The revealed differences in the AOA level of hydrolysates in relation to native proteins using spectrophotometric and fluorimetric methods are apparently due to the different contribution of processes related to hydrogen donation (ORAC assay) and/or electron transfer (TEAC assay) from antioxidants (hydrolysates) to free radicals [22,24,52,65].

This paper presents a study characterizing the effect of several relevant factors on the AOA level of hydrolysates, in particular different protein sources (whey, colostrum), applied endopeptidase dose and ultrafiltration with different M_W cutoffs.

It is noted that immunochemical methods with high specificity and sensitivity provide direct detection and quantification of analyte traces in a complex food matrix [71]. Among the available immunochemical approaches, enzyme-linked immunosorbent assay (ELISA) techniques are the most widely used. Therefore, in the present study, a competitive ELISA was used for the analysis of hydrolyzed β-lg.

The high allergenicity of the major whey protein (β-lg) is related to its resistance to proteolysis in the acidic environment of stomach, its ability to pass through the intestinal mucosa and subsequently transfer into breast milk [72]. Sensitisation to β-lg is due to the presence of numerous continuous epitopes along the entire length of the molecule, as determined by overlapping peptides [73–78]. IgE-binding peptides of β-lg were identified and classified according to the intensity and frequency of response in direct or competitive ELISA. The main epitopes were fragments Val_{41}–Lys_{60}, Tyr_{102}–Arg_{124}, and Leu_{149}–Ile_{162}, which were detected in 92, 97, and 89% of serum samples, respectively. Another group includes the fragments Leu_1–Lys_8 and Ala_{25}–Arg_{40}, which were detected in 58 and 72% of the test serum samples, respectively. The third group includes the peptides Gly_9–Lys_{14}, Ile_{84}–Lys_{91} and Val_{92}–Lys_{100}, detectable in >40% of serum samples tested. The peptide Val_{41}–Lys_{60} forms a protruding loop between β-fold structures on the surface of the native molecule. Tyr_{102}–Arg_{124} is stabilized by hydrogen and disulfide bonds. Va_{l92}–Ser_{100} is localized in the hydrophobic core of the molecule, inaccessible in the native state [72]. Three B cell epitopes of β-lg corresponding to the Leu_{95}–Pro_{113}, Ile_{12}–Ser_{27}, and Thr_{124}–Lys_{135} regions were identified [74]. Among trypsin hydrolysis peptides, one functional bivalent B cell epitope localized in the C-terminal region of β-lg (Leu_{149}–Ile_{162}) was found [75]. It forms a flexible loop hidden inside the native β-lg molecule. Three T cell epitopes of β-lg (Ser_{30}–Lys_{47}, Thr_{97}–Leu_{117}, and Ala_{142}–Ile_{162}) are localized in the region of β-folded structure [76]. The maximal T cell response is triggered by the epitope formed by Thr_{97}–Leu_{117}. It is hidden inside the β-lg molecule and orientated towards the globule surface. In general, 11 antigenic determinants are located on the surface of the β-lg protein globule and one is hidden inside the molecule [77]. Fifty-seven overlapping decapeptides corresponding to the surface regions of the β-lg protein globule were synthesized. According to the experimental data, 7 IgE and 6 IgG binding epitopes of β-lg were detected in patients with persistent bovine milk allergy [78]. The highly active endopeptidase Alcalase possesses broad site-specificity (it performs hydrolysis on peptide bonds containing Phe, Trp, Tyr, Glu, Met, Leu, Ala, Ser, and Lys) [23,35,64]. It should be noted that 7 IgE and 6 IgG binding epitopes of β-lg have been identified [78] that contain Alcalase cleavage sites, potentially resulting in an effective reduction in the antigenicity of hydrolyzed β-lg.

Several technological approaches have been proposed to reduce the allergenic potential of food proteins, in particular enzymatic hydrolysis with a commercial proteases [29,64] or protease complexes [79,80], and also a combination of hydrolysis with preheat substrate treatment [46,81] or fermentation by lactic acid bacteria [7], and hydrolysis under high hydrostatic pressure [82] or ultrasound [83].

Thus, hydrolysates of whey proteins obtained with Alcalase in free and immobilized forms possessed different physicochemical properties (molecular weight distribution) and residual antigenicity. It was found that free Alcalase cleaved the major whey allergens (α-la and β-lg) more efficiently [64]. In another study on the results of enzymatic hydrolysis of dairy proteins with different proteases (Flavourzyme, papain, Alcalase), it was reported that the antigenicity of the samples was reduced to 69.00–81.27% compared to the native substrate [29]. At the same time, a protease mixture (Protamex and Alcalase) was used to produce cheese whey hydrolysates with lower bitterness. The hydrolysate obtained using the Protamex:Alcalase complex (3.5:0.5) had twice the antioxidant activity and 11 times lower residual antigenicity (in the β-lg detection test system) [79]. A decrease in the allergenicity of casein derived from bovine milk was observed as a result of sequential hydrolysis with chymosin and papain. Thus, an increase in the degree of proteolysis with the combination of proteases leads to a decrease in the allergenic properties of hydrolyzed casein [80]. According to our previous experimental data [46], the combined treatment of the serine proteases Alcalase and trypsin caused an increase in the degree of hydrolysis of whey proteins and consequently to a RA decrease in partial proteolysis products.

Heat pretreatment of protein substrates was reported to increase the efficiency of hydrolysis of milk allergen proteins with plant peptidases. Whey proteins were more resistant to proteolysis than the casein fraction of milk [81]. Previous studies also found a decrease in the residual antigenicity of whey proteolysis products with Alcalase after thermodenaturation of whey proteins followed by ultrafiltration with a 3 and 10 kDa cutoff [46]. However, additional experimental data were required to evaluate the effect of the hydrolysis degree with Alcalase and ultrafiltration on the antigenicity level of dairy peptides, as shown in the present study. According to the literature sources, hypoallergenic dairy protein peptides are also obtained by hydrolysis with trypsin and fermentation by lactic acid bacteria [7].

A method for the preparation of hypoallergenic whey protein hydrolysate by substrate treating with pepsin under high pressure (400 MPa, 37 °C, 30 min) was proposed. Hypoallergenic properties of the experimental hydrolysate sample were confirmed by laboratory studies on BALB/c mice [82]. In order to reduce the antigenicity of whey protein hydrolysate, a preliminary ultrasonic impact on the protein and further enzymatic hydrolysis were also considered. Ultrasound treatment provided an increase in the concentration of low-molecular-weight fraction in hydrolysate samples. According to the data of direct ELISA, this resulted in the maximum decrease in the RA level of α-la and β-lg (88.46%) [83].

In the present study, competitive ELISA demonstrated a decrease in RA of β-lg in hydrolyzed whey and colostrum as a result of increased Alcalase content (1 and 5%). The greatest RA lowering was observed after ultrafiltration of hydrolysates. Thus, the RA of the peptide whey fraction ($M_W < 3$ kDa) was 0.047% of the initial protein, whereas no antigenic determinants of β-lg were detected in the colostrum filtrate (Table 7).

It is possible to obtain whey protein hydrolysate by proteolysis with Alcalase and ultrafiltration with a 20 kDa cutoff [39]. The authors reported a significant antigenicity reduction of the obtained peptide fraction. However, the separation filter capacity (20 kDa cutoff) is not sufficient to remove the residual amount of uncleaved whey proteins, since the M_W of the main whey proteins β-lg/α-la is 18.4/14.2 kDa. Another method [37] involves the addition of Alcalase or Protamex and ultrafiltration (10 kDa cutoff). According to the test results, the yield of the peptide fraction reached 80% and the mass fraction of whey antigens in the hydrolysate was equal to 4.3×10^{-5} relative units (rel. un.) in the case of Alcalase and 3.3×10^{-5} rel. un. in that of Protamex, indicating a significant reduction in antigenicity. Moreover, there is a known method of whey protein hydrolysate production with a high degree of hydrolysis, which involves hydrolysis with Flavourzyme and subsequent ultrafiltration (2 and 5 kDa cutoffs) [36]. The process allows the production of hydrolysate with residual antigenicity (mass fraction of whey antigens) of at most 1.0×10^{-5} rel. un.

Following the present study and patent data [36,37], ultrafiltration with a ≤10 kDa cutoff is an effective method for removing residual uncleaved milk allergens and their partial proteolysis products that can interact with antibodies.

5. Conclusions

This study focused on the characteristic of hydrolysis of the whey and colostrum proteins with a highly active endopeptidase Alcalase. It was found that the major whey proteins (α-lactalbumin, β-lactoglobulin) and casein were efficiently cleaved with Alcalase, whereas bovine serum albumin and high molecular weight cleavage products of the immunoglobulin fraction were removed by subsequent ultrafiltration of the hydrolysates.

According to the mass spectrometric analysis, reverse-phase high-performance liquid chromatography, dynamic light scattering and electrophoretic analysis in polyacrylamide gel under denaturing conditions, the protein and peptide composition of hydrolysates and their peptide fractions with a 5 and 10 kDa cutoffs was determined. The amount of hydrolyzed fractions increased with higher Alcalase dosage (1 and 5%). The maximum content of peptides with $M_W < 3$ kDa equals to (73.5 ± 1.3)% is established in extensive hydrolysate of whey proteins in accordance with mass-spectrometry and high-performance liquid chromatography data. The higher content of the fraction with $M_W < 3$ kDa was revealed when more enzyme added along with increasing of proteolysis degree of whey and colostrum proteins. This effect is associated with further cleavage of peptide and immunoglobulin components. Colostrum hydrolysates are characterized by the presence of peptides with lower M_W, resulting from the efficient casein cleavage with Alcalase into a low-molecular-weight fraction.

The antioxidant activity level of native, hydrolyzed and fractionated whey and colostrum samples was determined. The ORAC and ABTS assays suggested an increase of dairy proteins AOA with a higher degree of proteolysis and after ultrafiltration of hydrolysates (enrichment with low-molecular-weight peptide fractions). The maximum effect was observed for filtrates (5 kDa cutoff) in the composition of whey/colostrum hydrolysates with 5% Alcalase content (5.91/6.62-fold increase in AOA) when fluorescein fluorescence was restored. The highest AOA level was obtained in the case of ABTS-radical scavenging after filtration of whey/colostrum hydrolysates (5 kDa cutoff) containing 5% Alcalase (14.22/7.24-fold increase in AOA). In contrast to the ORAC assay, the $ABTS^{\bullet+}$ system reported higher antioxidant activity for the whey hydrolysate filtrate than that of colostrum. The differences in AOA levels between ORAC and ABTS assays are explained by the different mechanisms of antiradical action.

Following competitive ELISA, whey and colostrum proteolysis with Alcalase decreases the amount of the main allergen of bovine milk (β-lg). Filtration eliminates residual quantity of native β-lg and its immunoreactive peptides. The minimum level of residual antigenicity in whey samples is achieved by filtrate containing peptide fraction with $M_W < 3$ kDa (RA reduction of more than 2000 times). No β-lg was detected in a similar ultrafiltrate of the colostrum hydrolysate, due to the its relatively high initial content in whey. Thus, the application of Alcalase (5% of substrate weight) and filtration (5 kDa cutoff) determined the maximum RA decrease of whey and colostrum hydrolysates.

Hypoallergenic enzymatic hydrolysates of whey and colostrum proteins with high antioxidant activity are recommended as an ingredient of functional foods.

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CRediT authorship contribution statement

Tatsiana M. Halavach: Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Conceptualization. **Vladimir P. Kurchenko:** Writing – review & editing, Supervision, Project administration, Conceptualization. **Ekaterina I. Tarun:** Validation, Investigation, Formal analysis, Conceptualization. **Alexey V. Yantsevich:** Validation, Software, Methodology, Investigation, Formal analysis. **Veronika V. Shchur:** Validation, Software, Methodology, Investigation, Formal analysis. **Vasili G. Tsygankow:** Supervision, Data curation. **Aleksey D. Lodygin:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Data curation. **Ivan A. Evdokimov:** Supervision, Funding acquisition, Data curation. **Natasa Poklar Ulrih:** Supervision, Funding acquisition, Data curation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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