JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

BIOLOGICALLY ACTIVE PROPERTIES OF HYDROLYSED AND FERMENTED MILK PROTEINS

Tatsiana M. Halavach[*1], *Natalia V. Dudchik*[2], *Ekaterina I. Tarun*[3], *Vasiliy G. Zhygankov*[4], *Vladimir P. Kurchenko*[1], *Roman V. Romanovich*[1], *Vladimir D. Khartitonov*[5], *Vladimir A. Asafov*[6]

Address(es):
[1]Belarusian State University, Faculty of Biology, Laboratory of Applied Biology, Nezavisimosti Av. 4, 220030 Minsk, Belarus, +375295641103.
[2]Scientific Practical Centre of Hygiene, Laboratory of Microbiology, Akademicheskaya Str. 8, 220012 Minsk, Belarus, +375295651234.
[3]International Sakharov Environmental Institute of Belarusian State University, Faculty of Environmental Medicine, Chair of Environmental Chemistry and Biochemistry, Dolgobrodskaya Str. 23/1, 220070 Minsk, Belarus, +375295007715.
[4]Scientific Practical Centre of Hygiene, Laboratory of Complex Problems of Food Hygiene, Akademicheskaya Str. 8, 220012 Minsk, Belarus, +375293733551
[5]All-Russian Research Institute of Dairy Industry, Laboratory of Technology of Milk Protein Concentrates, Food Additives and Derived Products, Lusinovskaya Str. 35/7, 115093 Moscow, Russia, +74992363223.
[6]All-Russian Research Institute of Dairy Industry, Sector of Functional Food and Feed Technology, Lusinovskaya Str. 35/7, 115093 Moscow, Russia, +74992360434.

*Corresponding author: halavachtn@gmail.com

doi: 10.15414/jmbfs.2020.9.4.714-720

ARTICLE INFO

Received 14. 3. 2019
Revised 29. 8. 2019
Accepted 2. 9. 2019
Published 3. 2. 2020

Regular article

OPEN ACCESS

ABSTRACT

A comparative analysis of protein and peptide profile, biological activities of hydrolysed bovine colostrum and whey was performed. It was found that the depth of proteolysis, qualitative and quantitative composition of protein component of samples determined the level of their antiradical, antimutagenic, antimicrobial and antigenic properties. SDS-electrophoresis of experimental samples revealed more extensive protein hydrolysis in the course of alcalase treatment than during enzymatic reaction with neutrase. Using fluorimetric method the influence of hydrolysis with endopeptidases and fermentation with *Lactobacillus acidophilus* on antioxidant properties of milk proteins was established. 1.7–5.5 times increase in antiradical activity of derived samples in comparison with native proteins was recorded. Reduction in mutation rate induced by whey hydrolysate in tested strain *Salmonella typhimurium* TA 98 ranged from 15.7 % to 49.2 %, whereas antimutagenic effect on strain TA 100 varied from 18.8 % to 52.1 %, exceeding the similar values shown by colostrum hydrolysates. Samples of colostrum and whey hydrolysed with alcalase are enriched with specific short-chain peptides which determine their relatively high antimutagenic and antiradical properties. Immunoprecipitation reaction demonstrated effective splitting of β-lactoglobulin by alcalase, resulting in production of hypoallergenic hydrolysates. It was found by impedimetric technique that neutrase-cleaved colostrum accounted for maximum inhibition of *Escherichia coli* ATCC 8739 (82 %) and *Staphylococcus aureus* ATCC 6538 (19 %). Samples of enzymatic hydrolysates of colostrum and whey proteins with confirmed antimicrobial, antimutagenic and antioxidant action were obtained. The use of hydrolysed and fermented colostrum with elevated antioxidant potential in special nutrition appears extremely promising.

Keywords: whey, colostrum, enzymatic hydrolysis, alcalase, neutrase, bacterial fermentation, biological activities

INTRODUCTION

Enzymatic hydrolysates of cow milk proteins possessing low allergenic potential and high nutritional value are added into the special infant, sport, and dietetic food formulas (**Clemente, 2000; El-Agamy, 2007; Tsabouri *et al.*, 2014**). Moreover, fermented dairy products (yogurt, kefir, sour cream, cheese) have a long application record as traditional ingredients of daily alimentary diet (**Tamime, 2002**). Beneficial physiological effect of protein hydrolysates and fermented foods is achieved by better digestion of peptides in gastrointestinal tract compared with native proteins and amino acids. It is also determined by a broad spectrum of biologically active properties (**Schaafsma, 2009; Raikos and Dassios, 2013; Sánchez and Vázquez, 2017**).

Casein and whey protein concentrates are major substrates for production of hydrolysates (**Clemente, 2000**). Bovine colostrum, or primary milk, also may be referred to promising sources of bioactive hydrolysates due to increased contents of immunoglobulins, whey proteins, and natural antioxidants (**Korhonen, 2011; Sacerdote *et al.*, 2013; Bagwe *et al.*, 2015; McGrath *et al.*, 2015; Dzik *et al.*, 2017**).

The ratio of casein to whey proteins in mature milk equals 4:1, versus 1:2 value in primary milk. Major whey proteins are β-lactoglobulin (β-lg), α-lactalbumin (α-la) and bovine serum albumin (BSA). In addition, bovine colostrum is distinguished by enhanced level of multifunctional glycoprotein – lactoferrin (LF) (**Park, 2015; McGrath *et al.*, 2015**). Among whey proteins β-lg and casein display maximum allergenic capacity. Enzymatic hydrolysis results in generation of hypoallergenic peptides following cleavage of antigenic determinant regions in protein macromolecules (**Heyman, 1999; Wal, 2004**). Complications in hydrolytic process are determined by problematic choice of most appropriate enzymes responsible for efficient splitting of whey proteins characterized by compact globular structure (**El-Agamy, 2007; Tsabouri *et al.*, 2014**).

Cow milk, cheese and fermented dairy products are readily available sources of bioactive peptides demonstrating hypotensive, immunomodulating, antioxidant, antimicrobial, opioid and other effects (**Mills *et al.*, 2011; Wada and Lönnerdal, 2014**). Bioactive peptides are generated by the impact of digestive enzymes on milk proteins, during treatment with purified proteases and fermentation with lactic acid bacteria (**Raikos, 2013; Mohanty *et al.*, 2016a**). Application of diverse proteolytic enzymes and probiotic microorganisms provides for production of hydrolyzed and fermented milk proteins with specific peptide and protein profile and characteristic bioactive properties (**Clemente, 2000; Madureira *et al.*, 2010; Sánchez and Vázquez, 2017**).

Radical-scavenging milk action is known to be determined by presence of casein, whey proteins and, to a lesser extent, by vitamin-mineral component (**Zulueta *et al.*, 2009**). Antioxidant activity (AOA) of native proteins and peptides is correlated with reducing properties of amino acid radicals (**Grażyna *et al.*, 2017**). Biological phenomenon of mutation suppression is expressed as reduced spontaneous and induced mutation rate under the influence of natural and synthetic compounds (**Horn and Vargas, 2003**). As a rule, antimutagenic potential of milk proteins and peptides is estimated in Ames test recording the frequency of reverse mutations in *Salmonella typhimurium* strains prototrophic for histidine (**Claxton *et al.*, 2010; Turbay et *al.*, 2012; Sah *et al.*, 2014**).

Antimicrobial peptides cause inhibition of growth or death of microbial cells (bacterial, fungal). Their amino acid composition, amphipathy, cation charge and size provide for the capacity to bind with anionic cell walls and insert into membrane bilayers of microorganisms (**Wada and Lönnerdal, 2014; Mohanty *et al.*, 2016a; Mohanty *et al.*, 2016b**).

It appears extremely relevant nowadays to formulate specialized foodstuffs with confirmed antioxidant potential, antimutagenic and antimicrobial action. The novelty of such studies is emphasized by collecting fresh data on biological activities of hydrolysed and enzymatically digested dairy proteins (whey and bovine colostrum) and by elucidating relationship between physical-chemical and functional characteristics of protein hydrolysates.

The purpose of this research was a comparative investigation of biological activities shown by enzymatic hydrolysates of whey and bovine colostrum as well as the fermented primary milk. Experimental samples of hydrolysed and fermented milk proteins served as objects of study. The subject of study was analysis of peptide and protein profile, radical-scavenging and antigenic properties, antimicrobial and antimutagenic effects of the experimental samples.

MATERIALS AND METHODS

Production of hydrolysate samples and their physical-chemical characterization

The samples of bovine milk, colostrum, and whey characterized in table 1 were used in further studies.

Table 1 Description of bovine milk, colostrum and whey protein samples engaged in experimental research

Sample name	Physical-chemical and microbiological characteristics	Manufacturer
Whey protein concentrate (specifications BY 100377914.550–2008)	Protein – 80.0 %	Dairy plant, Shchuchin, Belarus
Dry whole colostrum	Protein – 56.3 %	All-Russian Research Institute of Dairy Industry, Moscow, Russia
Dry defatted colostrum	Protein – 69.9 %	
Dry defatted fermented colostrum	Protein – 68.8 % *Lactobacillus acidophilus* strain 630 (acidophilic bacilli, non-viscous) Cell titer – 1×10^8 CFU/g	
Dry whole milk, top grade	Fat – 25 % Moisture – 4 %	Milk cannery, Glubokoye, Belarus

5 % solutions of native, defatted and fermented colostrum, 8 % solution of whey proteins were prepared in sodium phosphate buffer (pH 7.0 and 8.0, respectively). Serine protease (Alcalase® 2.4L, EC 3.4.21.62, derived from *Bacillus licheniformis*, enzyme activity 2.64 U/g, Sigma, USA) and metalloprotease (Neutrase® 0.8L, EC 3.4.24.28, derived from Bacillus amyloliquefaciens, enzyme activity 0.8 U/g, Sigma, USA) were used for hydrolysis of defatted colostrum and whey proteins. Hydrolysis was conducted at enzyme/substrate ratio 0.024 U/g for alcalase and neutrase, optimal pH value 8.0 for alcalase and 7.0 for neutrase action, temperature 50 ºC during 3–4 h. Filters Spin–X UF Concentrator 20 (Corning, UK) were applied to remove proteases and separate peptide fraction with molecular weight less or equal to 10 kDa.

The degree of milk protein hydrolysis was evaluated by SDS-electrophoresis in polyacrylamide gel (**Osterman, 1981**). The samples of colostrum and whey proteins were separated in 12 % and 16 % PAAG, respectively. Electrophoresis markers were Precision Plus Protein All Blue Standards (10–250 kDa range, Bio-Rad, USA) and Biotinylated Molecular Weight Marker (6.5–180 kDa range, Sigma, USA). Total nitrogen content in the initial samples and enzymatic hydrolysates was determined in accordance with ISO 8968–1:2014, solids ratio – according to ISO 6731:2010.

Assessment of antioxidant potential

Antioxidant activity of tested samples was estimated using the oxygen radical absorbance capacity (ORAC) method (**Ou *et al.*, 2001**). The investigators employed the procedure described by **Tarun (2014)**. The results of independent experiments (n=3) were averaged and reported as the mean value ± standard deviation. Significant differences between the compared data were established by confidence intervals technique.

Preparation of samples for analysis of antimutagenic and antimicrobial effect

To get rid of microbiological contamination, ultrafiltrate samples of hydrolysed bovine colostrum (peptide fractions with molecular weight ≤10 kDa) and sample of whey hydrolysate were twice subjected to filtration through 0.45 μm polypropylene filters (Carl Roth, Germany). Due to partial loss of peptide fraction passed through polypropylene filters a sample of fermented colostrum was not engaged in further investigations of biologically active properties.

Determination of antimutagenic activity

Antimutagenic properties of whey hydrolysate and ultrafiltrates of colostrum hydrolysates were analyzed by the modified Ames test (**Dudchik, 2014; Golovach *et al.*, 2016**) using indicator strains *Salmonella typhimurium* TA 98 and TA 100. Direct-acting mutagens were introduced into the test system – ethidium bromide for strain *S. typhimurium* TA 98 and sodium azide for strain TA 100. Antimutagenic activity of whey hydrolysate was evaluated in concentration range 1.88–30.0 μg protein/plate, of colostrum hydrolysates – in the range 33–8700 μg protein/plate. Reduction of the mutation rate index (I_m, %) was calculated according to the proposed formula (1):

$$I_m = 100 - \frac{N_1}{N_2} \times 100 \quad (1),$$

where N_1 denotes the number of revertant colonies in the experiment, N_2 – the number of revertants in the positive control. Statistical significance of the results was checked by the Dunnett's multiple comparison test.

Investigation of antimicrobial action

Antimicrobial activity of whey hydrolysate and ultrafiltrates of colostrum hydrolysates was controlled by impedimetric method described by **Dudchik and Melnikova (2008), BY Patent No. 15228 (2010), and Dudchik and Shevlyakov (2016)**. The final concentration of active compounds varied from 0.2 to 0.3 mg protein/ml for colostrum hydrolysates and equaled 0.3 mg protein/ml for whey hydrolysate. Bacterial strains provided from Russian collection of industrial microorganisms – *Escherichia coli* ATCC 8739 and *Staphylococcus aureus* ATCC 6538 served as the test cultures. The studies were carried out using microbial analyzer BacTrac 4300 (SY–LAB, Austria).

Percentage of inhibition index (I, %) calculated according to the formula (2) was chosen as the quantitative criterion of antimicrobial activity:

$$I = \frac{IDT_2 - IDT_1}{IDT_1} \times 100 \quad \textbf{(2),}$$

where IDT_1 reflects the time of test culture growth detected in the control, h. IDT_2 – time of detected growth of test culture in the experiment, h. If I value is above zero, it may be deduced that the tested agent is distinguished by antimicrobial activity, in particular: I under 15 % indicates a weak antimicrobial action, I from 15 to 50 % signifies moderate antimicrobial potential, I values over 50 % testify to potent antimicrobial action. Test results are expressed as mean values for 3 independent experiments.

Analysis of antigenic properties

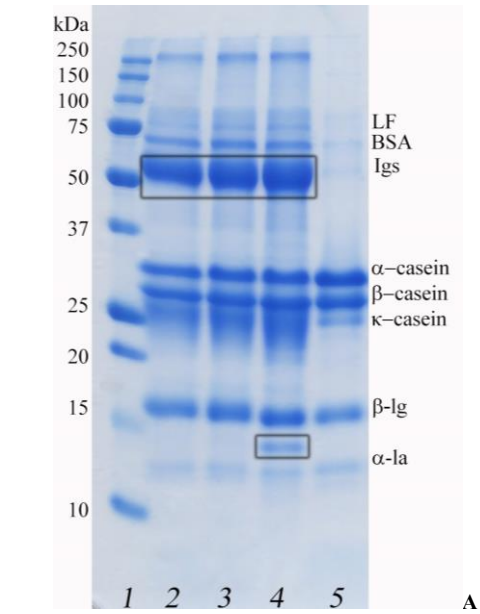
To assess antigenic properties of milk proteins and the derived proteolytic products we performed double radial immunodiffusion in agarose gel (according to Ouchterlony) using rabbit antisera (As) to β-lg and LF provided by Laboratory of Applied Biology (BSU, Belarus). The procedure is based on formation of heteromeric complexes [antigen–antibody] producing precipitate in the agarose gel as a result of reciprocal diffusion of As and components of protein or hydrolysate solution assayed for presence of bivalent antigenic determinants (**Frimel, 1987**). The amount of antigen added into the well to ensure generation of visible precipitate in the equivalence zone constituted 0.6/10.0 μg of β-lg/LF for control protein samples, 3.0/300.0 μg of β-lg/LF for native and hydrolysed colostrum.

RESULTS AND DISCUSSION

Using various methodological approaches, we examined protein components of whey and colostrum hydrolysates and studied their bioactive properties (antiradical potential, antimutagenic and antigenic effects). The influence of fermentation with *Lactobacillus acidophilus* (described in the Table 1) and enzymatic hydrolysis with serine protease (alcalase) and metalloprotease (neutrase) on antioxidant capacity of bovine colostrum was evaluated.

Peptide and protein profile

Molecular mass distribution of protein components in analyzed samples of whey and bovine colostrum was characterized by SDS-electrophoresis in polyacrylamide gel (SDS-PAGE). Elevated content of immunoglobulin fraction was shown for samples of native and fermented colostrum (Igs, molecular weight 50 kDa (Figure 1A: lanes 2–4, in the frame). The samples of native colostrum and milk were comparable in composition of casein fraction (19–25 kDa) and prevalent proteins of whey fraction (α-lg and β-lg, 14 and 18 kDa, respectively) (Figure 1A: lanes 2–5). In addition, intermediate product of bacterial proteolysis with molecular weight 15 kDa was found in fermented colostrum, as reflected in Figure 1A (lane 4, in the frame).



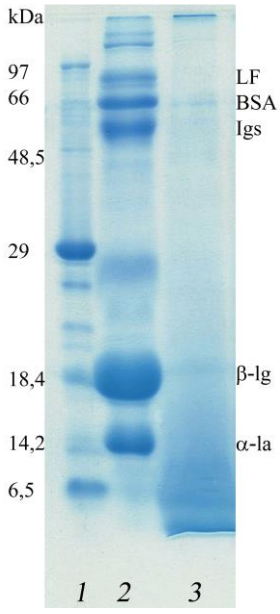


Figure 1 SDS-electrophoregrams of native and fermented colostrum samples (A), enzymatic hydrolysates of colostrum (B) and whey (C):
A: 1 – marker, 2 – native colostrum, 3 – defatted colostrum, 4 – fermented defatted colostrum, 5 – whole milk
B: 1 – marker, 2 – defatted colostrum (control, alcalase-free), 3 – defatted colostrum hydrolysate (alcalase treatment), 4 – filtrate of colostrum hydrolysate (alcalase treatment), 5 – defatted colostrum (control, neutrase-free), 6 – defatted colostrum hydrolysate (neutrase treatment), 7 – filtrate of colostrum hydrolysate (neutrase treatment)
C: 1 – marker, 2 – whey (control, alcalase-free), 3 – whey protein hydrolysate (alcalase treatment)

Numerous products of Igs fraction partial proteolysis were revealed, cleavage of casein and whey proteins α-la and β-lg was detected in alcalase-derived colostrum hydrolysate (Figure 1B: lane 3). On the contrary, colostrum hydrolysate resulting from neutrase application retained native Igs and partial splitting of casein and β-lg was recorded (Figure 1B: lane 6). Ultrafiltrates of hydrolysates contained a low molecular weight fraction of peptides not identified by SDS-PAGE technique (Figure 1B, lane 4 and 7).
Electrophoregram presented in Figure 1C (lane 2) illustrates typical make-up of milk whey: domination of proteins β-lg and α-la, moderate amounts of BSA, LF, Igs and traces of casein. Alcalase-mediated hydrolysis of milk whey led to almost complete proteolysis of β-lg, α-la and minor proteins to intermediate peptides (Figure 1A: lane 3).
In general, electrophoretic analysis of the samples of native, defatted and fermented colostrum indicated superior ratio of Ig fraction over that in the whole milk. Proteolytic product with molecular weight 15 kDa was found in fermented colostrum sample. Alcalase cleavage was distinguished by higher degree of protein hydrolysis than enzymatic reaction with neutrase. According to the results of total protein determination in native and treated samples, alcalase-digested colostrum hydrolysate comprised 29.1±1.2 % of fraction with molecular weight ≤10 kDa, whereas the similar value for neutrase-generated product equaled 32.6±1.0 %.
Added to comparable ratio of low molecular weight fraction in both hydrolysates, alcalase due to its broad site specificity ensures splitting of protein substrates (casein, β-lg and α-la) into short-chain peptides. It should be noted that whey hydrolysate contains around 98 % of molecular weight fraction ≤10 kDa while the percentage for hydrolysed colostrum is 3.4 times lower. This could be explained by the presence of proteolytic products from Ig fraction with molecular mass over 10 kDa. It may be deduced that alcalase-degraded whey sample is characterized by greater extent of protein substrate hydrolysis than enzymatically treated colostrum.

Antioxidant properties

Radical-scavenging activity of hydrolysed whey and colostrum samples was evaluated using ORAC procedure. AOA of experimental samples was assessed via their ability to bind free radicals, resulting in retarded free radical oxidation of fluorescein (FL). According to the obtained data, the graphs correlating intensity of fluorescence with concentration of solids in analyzed samples were plotted. Rising inhibition of FL free radical oxidation from 20 % to 90 % was recorded when the samples were fed into the system in the range 0.1 to 1000 μg/ml. IC_{50} concentration of the sample corresponding to 50 % suppression of fluorescence was determined. Significant increase of AOA in comparison with native proteins was established for samples of whey and colostrum hydrolysates as well as for

fermented primary milk. The top radical-neutralizing effect was demonstrated in experiments with ultrafiltrates of hydrolysates.

Protein content in samples of native and hydrolysed colostrum constituted 28.7–33.9 mg/ml as compared to 8.7–10.7 mg/ml for ultrafiltrates of hydrolysates. Noteworthy that percentage of protein in initial defatted colostrum and hydrolysates reached 50.8–58.1 % of total solids, whereas in ultrafiltrates of hydrolysates it was only 23.6–30.5 %. The protein ratio in dry whole colostrum amounted to 56.3 %, in defatted and fermented samples – 68.8–69.9 %. As to whey assays, the protein portion in whey protein concentrate and the respective enzymatic hydrolysate surged up to 88.4-90.2%, while protein/solids ratio climbed even higher – to 95.2-97.1 % mark. Taking all this into account, AOA level was calculated relative to solids and protein concentrations to estimate contribution of protein and non-protein components.

Table 2 provides experimental data evaluating antiradical activity of native and hydrolysed whey and colostrum samples as well as the sample of colostrum fermented with *Lb. acidophilus*.

Table 2 Characterization of antioxidant activity of native and hydrolysed whey and colostrum samples plus the fermented colostrum sample

Sample name	IC_{50}, μg solids/ml	IC_{50}, μg protein/ml
Milk whey (alcalase-free control)	85.6±4.2	83.1±4.1
Whey protein hydrolysate (with alcalase)	31.1±2.2	29.6±2.1
Whole bovine colostrum	116.8±0.6	70.4±0.3
Defatted colostrum (control, no acidophilic bacilli)	156.4±2.2	119.4±1.6
Fermented defatted colostrum (with acidophilic bacilli)	87.2±4.7	66.1±3.6
Defatted colostrum (alcalase-free control)	180.0±2.2	99.5±1.2
Defatted colostrum hydrolysate (with alcalase)	35.7±1.5	18.1±0.7
Filtrate of colostrum hydrolysate (alcalase-treated)	25.4±2.3	6.2±0.5
Defatted colostrum (neutrase-free control)	199.5±7.7	104.0±4.0
Defatted colostrum hydrolysate (with neutrase)	105.7±4.6	61.4±2.7
Filtrate of colostrum hydrolysate(neutrase-treated)	56.2±1.6	17.1±0.5

The obtained IC_{50} values evidence reduction of antioxidant activity of defatted colostrum (control, no acidophilic bacillus) in comparison with whole colostrum 1.3 and 1.7 times relative to the contents of solids and protein, respectively. Decrease of antioxidant action of skim milk was observed by **Zulueta *et al.* (2009)**. Depletion of fatty constituent comprising lipid-soluble vitamins and hydrophobic low molecular weight protein component brings down radical-reducing capacity of defatted colostrum.

In case of fermented colostrum IC_{50} values proved 1.8 times lower than the similar parameters of initial defatted substrate. Increase of radical-scavenging activity of fermented colostrum is correlated with cleavage of protein fraction by *Lb. acidophilus* proteolytic system as vividly demonstrated by the results of SDS-electrophoretic analysis (Figure 1A, lane 4).

IC_{50} values of defatted colostrum samples intended for hydrolysis reached 180–199.5 μg/ml for solids and 99.5–104.0 μg/ml for protein. Distinctions in AOA levels of colostrum samples are caused by peculiarities in preparation of solutions, namely partial loss of protein fraction following centrifugation. Enzymatic hydrolysis of colostrum with alcalase and neutrase raised its antioxidant capacity 5.0/5.5-fold and 1.9/1.7-times, respectively, relative to solids/protein contents. Antiradical potential of protein substrates tended to rise with growing extent of their hydrolysis.

Ultrafiltration of samples resulted in 1.4 and 2.9 times increment of antioxidant activity in alcalase hydrolysates of bovine colostrum relative to solids and protein content, respectively. In case of neutrase hydrolysis the respective AOA indexes rose 1.9 and 3.6 times. It should be noted that ultrafiltration upgrades the samples with peptide fraction contributing to counter-radical activity. Compared to native bovine colostrum antioxidant activity of ultrafiltrate of alcalase hydrolysate increased 7.1/16.0 times and AOA of neutrase hydrolysate stepped up 3.5/6.1 times relative to solids/protein contents. A sample of fermented defatted colostrum is similar in antioxidant capacity to neutrase-derived hydrolysate sample – 66.1±3.6 and 61.4±2.7 μg protein/ml, respectively.

Comparative analysis of radical-scavenging properties of alcalase hydrolysed whey and colostrum samples was performed. After enzymatic reaction with alcalase antioxidant effect of whey increased 2.8 times. A comparable level of antioxidant activity was shown by whey and colostrum hydrolysates in terms of IC_{50} calculated for solids – 31.1±2.2 and 35.7±1.5 μg/ml, respectively. 1.6 times surplus of radical-scavenging activity was recorded in hydrolysed colostrum over whey protein hydrolysate as indicated by IC_{50} protein values.

Sah *et al*. (2014) investigated antioxidant and antimutagenic properties of peptide fractions recovered from yogurt samples obtained by fermentation of milk with associations of probiotic lactic acid bacteria *Lb. acidophilus* (ATCC® 4356™), *Lb. casei* (ATCC® 393™) and *Lb. paracasei* subsp. *paracasei* (ATCC® BAA52™). AOA was evaluated via reduction of cation-radical ABTS derived from 2,2′-azino-bis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt. In experiments where peptide fractions varied in degree of hydrolysis from 5.38 to 11.51 %, the corresponding IC_{50} values ranged from 2.43 to 1.63 mg/ml. The increased extent of milk protein cleavage was directly correlated with rise in radical-scavenging activity.

The study of **Peng *et al.* (2010)** has revealed proton donor capacity of whey protein alcalase hydrolysates and their ability to inhibit lipid oxidation. Using ORAC method **O'Keeffe and FitzGerald (2014)** also observed enhanced antiradical potential in whey proteins split with alcalase, neutrase, flavourzyme and Corolase PP. **Ripollés *et al.* (2016)** found in test system with 2,2-diphenyl-1-picrylhydrazil (DPPH) upsurge of antioxidant activity of skim milk and butter milk upon hydrolysis with alcalase, Prolyve and Corolase PP. The top counter-radical effect was recorded for Corolase PP-exposed samples.

Hernández-Ledesma *et al*. (2007) applied $ABTS^{\bullet+}$–trolox system to evaluate radical-reducing activity of ultrafiltrates (3 kDa) of eight hydrolysed infant formulas differing in casein–whey protein ratio. Following hydrolysis with pepsin and pancreatin and subsequent ultrafiltration IC_{50} values reached 60.11–198.11 μg protein/ml. **Oh *et al.* (2013)** also reported different levels of antioxidant activity in native milk proteins and alcalase hydrolysates. After reduction of ABTS-radical IC_{50} values for intact and hydrolysed whey protein concentrate equaled 395±3.8 and 2.1±0.3 μg/ml, respectively, and in case of sodium caseinate the respective values were 47.2±0.8 and 1.17±0.03 μg/ml. In general, alcalase hydrolysis raised considerably antiradical capacity of both protein substrates.

According to the literature sources (**Hernández-Ledesma *et al.*, 2007; Peng *et al.*, 2010**; **Sah *et al*., 2014; O'Keeffe and FitzGerald, 2014; Oh *et al.*, 2013; Ripollés *et al.*, 2016**) and our experimental findings the level of antioxidant activity was found to be determined by the extent of proteolysis as well as by the quantitative and qualitative composition of protein constituent. Research results have shown increase in antioxidant activity of whey and colostrum cleaved with alcalase by 2.8 and 5.5 times, respectively (calculated relative to protein content). The maximum radical-scavenging effect was established for ultrafiltrate of colostrum hydrolysate derived from alcalase treatment. The similar AOA level was detected in colostrum fermented with acidophilic bacilli and hydrolysed with neutrase.

Antimutagenic effect

Antimutagenic properties of test sample of whey protein hydrolysate and colostrum hydrolysate samples (peptide fractions with molecular mass≤10 kDa) were studied. It was found that products of whey and colostrum proteolysis in concentration range 1.88–30.0 and 33–8700 μg protein per plate, respectively, did not display bacteriostatic or bactericidal effect on test model cultures *S. typhimurium* TA 98 and TA 100, which might lead to false positive results. Statistically significant reduction of induced mutation rate was registered in all experimental variants with test samples of hydrolysates. The revealed distinctions in the number of revertants in the control and the experiment were statistically significant ($p<0.05$) upon introduction of the examined hydrolysate samples into the test system as reflected in Tables 3 and 4.

The most dramatic drop in mutation rate was pointed out during previous study in experiments with test specimen of whey hydrolysate, constituting 15.7–49.2 % for strain *S. typhimurium* TA 98 and 18.8–52.1 % for strain TA100 (**Golovach *et al.*, 2016**). In case of alcalase-promoted colostrum proteolysis the induced mutation rate fell by 10–29.6 % for strain *S. typhimurium* TA 98 and 12.5–32.4 % for strain TA 100. For neutrase-derived colostrum hydrolysate antimutagenic effect ranged from 8.4 to 15.6 % in test system with *S. typhimurium* TA 98 and from 7.9 to 12.6 % in experiment with strain TA 100. It should also be noted that according to SDS-electrophoresis data (Figure 1) whey and colostrum samples cleaved with alcalase are distinguished by more extensive hydrolysis of protein substrates than those produced in enzymatic reaction with neutrase.

Table 3 Evaluation of antimutagenic activity of test sample of whey hydrolysate produced by alcalase treatment

Sample amount, μg protein/plate	Decrease of mutation rate, %	
	S. typhimurium* TA 98	***S. typhimurium* TA 100****
30	**49.2**	**52.1**
15	**45.0**	**49.0**
7.5	**39.3**	**37.1**
3.75	**23.0**	**25.1**
1.88	**15.7**	**18.8**
0	–	–

Note: Mutagens – ethidium bromide* and sodium azide**, 10 μg per plate. Strain response to mutagen action varied within the standard range

Table 4 Evaluation of antimutagenic activity of test sample of colostrum hydrolysate produced by alcalase and neutrase treatment

Sample amount, μg protein/plate	Decrease of mutation rate, %			
	S. typhimurium* TA 98		***S. typhimurium* TA 100****	
	alcalase treatment	**neutrase treatment**	**alcalase treatment**	**neutrase treatment**
8700	29.6	15.6	32.4	12.6
2175	23.6	14.4	29.1	11.3
544	21.2	12.0	20.0	10.9
136	15.6	10.0	18.0	8.4
33	10.0	8.4	12.5	7.9
0	–	–	–	–

Note: Mutagens – ethidium bromide* and sodium azide**, 10 μg per plate. Strain response to mutagen action varied within the standard range

Our experimental data agree well with the results of other researchers. For instance, **Turbay *et al.* (2012)** explored biologically active properties of α and β-casein fermented with thermophilic lactic acid bacterium *Lactobacillus delbrueckii* ssp. *lactis* CRL 581. Mutagenic action of 4-nitroquinoline-1-oxide on *S. typhimurium* TA 98 and TA 100 cultures diminished when α and β-casein hydrolysates were supplied into the test system, and antimutagenic effect of hydrolysates tended to rise with increase in percentage of proteolysis.

Sah *et al.* (2014) investigated antimutagenic and antioxidant capacity of peptide fractions recovered from samples of yogurt prepared by fermentation of milk with combinations of probiotic lactic acid bacteria. Sodium azide in dose 0.1–5.0 μg per plate was fed into the test system as mutagenic agent. For peptide fractions showing degree of hydrolysis 5.38–11.91 % antimutagenic effect totaled 15.87–26.35 %, while the respective IC_{50} values reached 2.43–1.63 mg/ml. Greater extent of milk protein cleavage contributed to enhanced antimutagenic and radical-scavenging activity.

Previous reports (**Turbay *et al.*, 2012; Sah *et al.*, 2014**) and our experimental findings corroborate the direct relationship between the depth of protein hydrolysis and the level of antimutagenic activity. More potent antimutagenic potential was demonstrated by alcalase-derived hydrolysates because this enzyme splits milk proteins into short-chain peptides.

Antimicrobial action

Antimicrobial properties of experimental samples of colostrum hydrolysates (peptide fractions with molecular weight ≤10 kDa) and whey protein hydrolysate toward Gram-negative test strain *E. coli* ATCC 8739 and Gram-positive test strain *S. aureus* ATCC 6538 were examined in this study.

Time lag in growth of test cultures after supplementation of the medium with hydrolysate samples was assessed because at the stage of retarded growth (lag), when the cells adapt to the environment, it is possible to most vividly assess the inhibitory effect of the studied compounds (**Dudchik and Melnikova, 2008; BY Patent No. 15228, 2010; Dudchik and Shevlyakov, 2016**). The augmented detection time index (IDT) was recorded in the course of test culture development on the medium with milk peptides as compared with the control.

Table 5 presents data illustrating antimicrobial activity of colostrum hydrolysates and whey protein hydrolysate against test cultures. In particular, lag in growth of *E. coli* ATCC 8739 equalled 2.9 and 4.1 h upon introduction into the medium of alcalase and neutrase colostrum hydrolysates, respectively, evidencing clear-cut antimicrobial action (inhibition degree I over 50 %). Moderate effect (I in the range 15 to 50 %) was shown by peptides released from whey proteins. Addition of the peptides into the cultural medium provoked 1.7 h growth lag of Gram-negative bacterium. With respect to *S. aureus* ATCC 6538 the impact of tested hydrolysate samples was relatively weak (9–19 % growth inhibition), reflected also as the increased IDT parameter – 0.6–1.2 h longer than the control analog.

Table 5 Antimicrobial activity level of colostrum and whey protein hydrolysates regarding test strains *E. coli* ATCC 8739 and *S. aureus* ATCC 6538

Sample description	**Index of inhibition degree (I) toward test strains**	
	E. coli ATCC 8739	*S. aureus* ATCC 6538
Ultrafiltrate of colostrum hydrolysate (alcalase)	57 % (strong antimicrobial action)	9 % (weak antimictobial action)
Ultrafiltrate of colostrum hydrolysate (neutrase)	82 % (strong antimicrobial action)	19 % (moderate antimictobial action)
Hydrolysate of whey proteins (alcalase)	34 % (moderate antimictobial action)	13 % (weak antimictobial action)

In general, both colostrum and whey protein hydrolysates show more pronounced antagonistic activity against Gram-negative test culture *E. coli* ATCC 8739 as compared with Gram-positive *S. aureus* ATCC 6538. In accordance with experimental data antimicrobial activity of studied samples diminishes in the following sequence: colostrum hydrolysate (neutrase), colostrum hydrolysate (alcalase), whey protein hydrolysate (alcalase).

When comparing biological activity of peptides released during milk protein hydrolysis catalyzed by bacterial proteases, it appears that more potent antioxidant and antimutagenic effect was triggered by alcalase. On the contrary, maximum inhibition of test strains *E. coli* ATCC 8739 and *S. aureus* ATCC 6538 was stated for colostrum exposed to neutrase treatment.

This phenomenon may be interpreted by the distinctions in protein composition of colostrum and whey and by different substrate affinity and cleavage site specificity of hydrolytic enzymes. According to **Sánchez and Vázquez (2017)** and **Mohanty *et al.* (2016b)** antimicrobial peptides of casein fraction display bioactivity toward both Gram-positive and Gram-negative species, while application of peptides derived from whey proteins is more preferential against Gram-positive bacteria. Noteworthy, that peptide kappacin (f 106–169) isolated from pepsin hydrolysate of κ-casein is distinguished by antimicrobial action on gram-positive bacteria, whereas kappacin (f 42–49) recovered from trypsin hydrolysate of κ-casein possesses a broad spectrum of antimicrobial activities (**Mohanty *et al.*, 2016b**).

The above-mentioned surveys (**Sánchez and Vázquez, 2017; Mohanty *et al.*, 2016a,b**) emphasized that antimicrobial peptides were produced mainly by degradation of milk proteins with enzymes of gastrointestinal tract (pepsin, chymosin, trypsin, chymotrypsin) and by fermentation with probiotic lactic acid bacteria. The present research provides a comprehensive analysis of antimicrobial action, antioxidant and antimutagenic potential of hydrolysates resulting from cleavage of colostrum and whey proteins with bacterial enzymes (alcalase and neutrase).

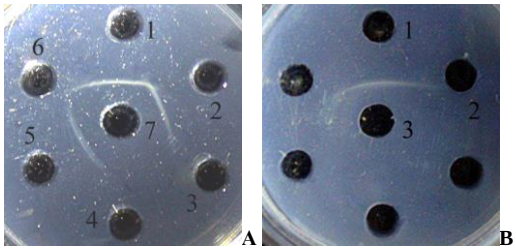
Fermented colostrum was not engaged in comparative examination of antimutagenic and antimicrobial properties due to the partial loss of peptide fraction in the process of sample preconditioning. Owing to the relatively low degree of hydrolysis of colostrum proteins under the influence of *Lb. acidophilus* and resistance of β-lg to bacterial proteolysis, corroborated by SDS-electrophoresis data, the sample of fermented colostrum was not subjected to immunochemical tests. Investigation of antimicrobial and antimutagenic characteristics of post-fermentation colostrum remains the subject of additional study.

Antigenic properties

Antigenic properties of one of the key milk allergens (β-lg) after hydrolysis of colostrum and whey with enzymes alcalase and neutrase were estimated. In addition, the level of the valuable protein lactoferrin was traced prior to and upon completion of colostrum hydrolysis using immunochemical analysis.

The presence of bivalent antigenic determinants of β-lg was controlled experimentally in colostrum and whey hydrolysates (Figure 2). Precipitate was produced in the reaction of rabbit antiserum with defatted colostrum (Figure 2A: 2) and whey (Figure 2B: 1) as well as neutrase-derived colostrum hydrolysate

(Figure 2A: 5). However, β-lg was split by alcalase and hence was not revealed immunochemically (Figure 2A: 3, Figure 2B: 2) as indicated by SDS-electrophoresis data (Figures 1B and C: lane 3). Filtrates of colostrum hydrolysates are devoid of bivalent antigenic determinants of β-lg which were either split enzymatically or removed during ultrafiltration (Figure 2A: 4, 6).
In compliance with the previous studies (**Halavach *et al.*, 2015**) and novel experimental findings it may be assumed that application of highly active serine protease (alcalase) ensures production of hypoallergenic enzymatic hydrolysates of bovine colostrum and whey.



A **B**

Figure 2 Analysis of antigenic capacity of colostrum (A) and whey(B) hydrolysates in the reaction of double radial immunodiffusion:
A: 1 – β-lg (protein-antigen, control), 2 – defatted colostrum (enzyme-free control), 3 – hydrolysate of defatted colostrum (alcalase-derived), 4 – filtrate of colostrum hydrolysate (alcalase-derived), 5 – colostrum hydrolysate (neutrase-promoted), 6 – filtrate of colostrum hydrolysate (neutrase-promoted), 7 – rabbit antiserum against β-lg
B: 1 – whey (enzyme-free control), 2 – whey hydrolysate (alcalase-produced), 3 – rabbit antiserum against β-lg

Figure 3 Evaluation of LF levels in alcalase and neutrase colostrum hydrolysates:
1 – LF (protein-antigen, control), 2 – defatted colostrum (enzyme-free control), 3 – hydrolysate of defatted colostrum (alcalase-promoted), 4 – filtrate of colostrum hydrolysate (alcalase-promoted), 5 – colostrum hydrolysate (neutrase treatment), 6 – filtrate of colostrum hydrolysate (neutrase treatment), 7 – rabbit antiserum against LF

The double radial immunodiffusion procedure detected lactoferrin in samples of defatted and hydrolysed defatted colostrum (Figure 3: 2, 3 and 5). According to SDS-electrophoresis data trace amount of LF was revealed in colostrum hydrolysate produced by neutrase (Figure 1C: lane 6) in contrast to alcalase action leading to LF decomposition (Figure 1C: lane 3). Evidently under the impact of serine protease the product of partial LF proteolysis is generated. It carries bivalent antigenic determinants resulting in formation of precipitate in the course of immunodiffusion reaction.

CONCLUSION

Comparative investigation of bioactive properties of enzymatic hydrolysates of bovine colostrum and whey derived with the aid of serine protease (alcalase) and metalloprotease (neutrase) as well as fermented colostrum was carried out. Enzymatic hydrolysate of milk whey is represented by peptide component with molecular mass ≤10 kDa whereas high molecular weight fraction of partly decomposed immunoglobulins was detected in colostrum hydrolysates. It was shown that antimutagenic effect and the level of antioxidant activity depended both on the depth of proteolysis and protein composition of tested samples.
Hydrolysis of colostrum with serine protease (alcalase) and subsequent ultrafiltration of the hydrolysate raised radical-scavenging activity of the resulting samples 5.5 and 16.0 times, respectively, while the corresponding values for neutrase-cleaved colostrum were 1.7 and 6.1 times (relative to protein content). In parallel, antioxidant capacity of hydrolysed whey proteins rose 2.8 times. Likewise antioxidant potential of fermented colostrum increased 1.8 times in comparison with native substrate. Elevation of proteolysis degree promoted antiradical action of peptide fraction. Thus, fermentation, hydrolysis with endopeptidases and the following ultrafiltration yield protein-derived components varying in the level of antioxidant activity.
According to SDS-electrophoresis and immunoprecipitation data alcalase proved more effective proteolytic agent than neutrase, accounting for the lower allergenicity of the former peptide fraction. Serine protease owing to wide substrate and site specificity splits casein, β-lg and α-la into short-chain peptides. One of the principal cow milk allergens – native β-lg was not detected in alcalase-hydrolysed whey and colostrum samples. Immunochemical analysis revealed lactoferrin in samples of native and hydrolysed colostrum.
The most pronounced antimutagenic effect was expressed by whey hydrolysate – 15.7–49.2 % for strain *S. typhimurium* TA 98 and 18.8–52.1 % for strain TA 100. Analysis of peptide fraction resulting from alcalase hydrolysis demonstrated decline in mutation rate 10.0–29.6 % in test system with *S. typhimurium* TA 98 and 12.5–32.4 % for strain TA 100, exceeding thereby the parameters shown by neutrase hydrolysis products.
Antimicrobial properties of peptides derived from colostrum and whey proteins against test strains *E. coli* ATCC 8739 and *S. aureus* ATCC 6538 were studied. The potent antimicrobial action toward *E. coli* ATCC 8739 was recorded for alcalase and neutrase colostrum hydrolysates (57 % and 82 %, respectively), whereas moderate effect (34 %) was achieved for peptides from alcalase hydrolysates of whey proteins. It should be noted that index of *S.aureus* ATCC 6538 inhibition did not exceed 19 %.
Results of our experiments confirm that the samples of colostrum and whey protein hydrolysates are the sources of bioactive peptides with antioxidant, antimutagenic and antimicrobial potential. Introduction of colostrum and whey protein hydrolysates as proteinaceous ingredients into special food formulas (infant, dietetic, and sport rations) appears an extremely promising application area.

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