2023 Т. 53 № 1 / Техника и технология пищевых производств / Food Processing: Techniques and Technology ISSN 2074-9414 (Print) ISSN 2313-1748 (Online)

https://doi.org/10.21603/2074-9414-2023-1-2422
https://elibrary.ru/GXNVFU

Original article
Available online at https://fptt.ru/en

Peptides of Trypsin Hydrolyzate in Bovine Colostrum

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Received: 30.06.2022
Revised: 08.09.2022
Accepted: 04.10.2022

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Abstract.
Bovine colostrum contains biologically active substances, e.g., immunoglobulins, peptides, and cytokines, which makes it a logical component of numerous functional products. Colostrum peptides also possess antimicrobial activity. This bioavailability increases during colostrum fermentation with proteolytic enzymes. The research objective was to describe peptides isolated from the trypsic hydrolyzate supernatant of bovine colostrum and to evaluate their antimicrobial and antifungal properties.
The supernatant of trypsin hydrolyzate of bovine colostrum was isolated by centrifugation at 3900 rpm for 7 min. The supernatant was separated by preparative chromatography. Its peptide composition was determined on a MALDI-TOF mass spectrometer, while the protein sequences were deciphered using the Mascot database. Proteins were precipitated with ammonium sulfate, and the antimicrobial activity was measured by the disk-diffusion method against gram-positive and gram-negative bacteria and dipoloid fungi. Strains were cultivated on a thick LB nutrient medium at 37°C. The antimicrobial activity was defined experimentally on Wistar rats infected intraperitoneally with *Salmonella enteritidis* 92.
The trypsin hydrolyzate supernatant of bovine colostrum revealed four peptides, one of which belonged to short peptides, while the remaining three belonged to polypeptides. The isolated peptides had different molecular weights of 8.4, 6.5, 13.0, and 8 kDa. The enzymatic hydrolyzate proved bactericidal against *Escherichia coli* and *Bacillus subtilis* and demonstrated antifungal activity against *Candida albicans*. When rats infected with *S. enteritidis* 92 were administered with trypsin hydrolysate, it promoted their survival, decreased LD_{50}, and increased the mean day of death period from 2 to 4 days.
The research proved the antimicrobial effect of colostrum peptides and suggested their immunotropic properties. The peptides obtained from the trypsin hydrolyzate supernatant of bovine colostrum can be recommended for functional food industry as part of antimicrobial products.

Keywords. Colostrum, milk protein, enzyme, hydrolysis, antimicrobial activity, antifungal activity, biologically active substances

For citation: Tikhonov SL, Tikhonova NV, Tursunov KhKh, Danilova IG, Lazarev VA. Peptides of Trypsin Hydrolyzate in Bovine Colostrum. Food Processing: Techniques and Technology. 2023;53(1):150–158. https://doi.org/10.21603/2074-9414-2023-1-2422

https://doi.org/10.21603/2074-9414-2023-1-2422
https://elibrary.ru/GXNVFU

Оригинальная статья
https://fptt.ru

Пептиды трипсинового гидролизата молозива коров

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Поступила в редакцию: 30.06.2022
Принята после рецензирования: 08.09.2022
Принята к публикации: 04.10.2022

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Аннотация.
Молозиво коров из-за содержания биологически активных веществ, в частности иммуноглобулинов, пептидов и цитокинов, является перспективным сырьем для производства продуктов функциональной направленности. Пептиды молозива обладают антимикробным действием. Биодоступность действующих начал молозива повышается при его ферментации протеолитическими ферментами. Цель исследования – выделение и характеристика пептидов надосадочной жидкости трипсиного гидролизата молозива коров, а также оценка их антимикробной и противогрибковой активностей.
Для эксперимента использовали надосадочную жидкость трипсинового гидролизата молозива коров, выделенную методом центрифугирования при 3900 об/мин в течение 7 мин. Надосадочную жидкость разделяли методом препаративной хроматографии. Пептидный состав надосадочной жидкости ферментативного гидролизата определяли на МАЛДИ-ТОФ масс-спектрометре, расшифровку белковых последовательностей проводили с помощью базы данных Mascot. Для изучения белкового состава надосадочной жидкости гидролизата проводили осаждение белков сульфатом аммония. Антимикробную активность определяли диско-диффузионным методом. Культивирование штаммов бактерий проводили на плотной питательной среде LB при температуре 37 °C. Для оценки противомикробного действия пептидов провели эксперимент на крысах линии Вистар, инфицированных внутрибрюшинно *Salmonella enteritidis* 92.
В надосадочном трипсиновом гидролизате молозива коров выделили 4 пептида, один из которых относится к коротким пептидам, три – к полипептидам. Выделенные пептиды имели различную молекулярную массу – 8,4, 6,5, 13,0 и 18 кДа. Установлено, что ферментативный гидролизат надосадочной жидкости молозива коров обладал бактерицидным действием к грамотрицательной бактерии *Escherichia coli* и грамположительной бактерии *Bacillus subtilis*, а также антигрибковой активностью против *Candida albicans*. Введение крысам, инфицированным *S. enteritidis* 92, внутрь трипсинового гидролизата надосадочной жидкости молозива коров способствовало их выживаемости, снижению $ЛД_{50}$ и увеличению среднего срока гибели животных с 2 до 4 суток.
Полученные данные свидетельствуют об антимикробном действии пептидов молозива и возможных иммуннотропных свойствах. Практическая значимость проведенного исследования заключается в перспективности использования пептидов надосадочной жидкости трипсиного гидролизата молозива коров для производства продуктов функциональной направленности с антимикробными свойствами.

Ключевые слова. Молозиво, молочный белок, фермент, гидролиз, антимикробная активность, противогрибковая активность, биологически активные вещества

Для цитирования: Пептиды трипсинового гидролизата молозива коров / С. Л. Тихонов [и др.] // Техника и технология пищевых производств. 2023. Т. 53. № 1. С. 150–158. (На англ.). https://doi.org/10.21603/2074-9414-2023-1-2422

Introduction

Colostrum is a complex biological fluid. Główka & Woźniewicz believe that bovine colostrum is beneficial for the digestive, immune, and neuroendocrine systems and can boost physical performance in general because it contains growth factors, immunoglobulins, peptides, cytokines, lactoferrin, and hormones [1]. Athletes consume colostrum during high-intensity training because it is rich in immunoglobulins and can increase the buffer capacity of muscles. However, no scientific publications feature the exact recommended doses of colostrum that can increase physical performance.

Van Hese *et al*., Sukhikh *et al*., and Kharitonov *et al*. proved that dairy products and colostrum have a high content of immunoglobulins, minerals, vitamins, growth factors, and immune cells, as well as microRNAs [2–4]. A microRNA is a short non-coding RNA molecule that regulates post-transcriptional gene expression. MicroRNAs act as key regulators of various biological and developmental processes. Bovine colostrum MicroRNAs are signaling molecules. They are located inside extracellular vesicles that protect them from the harsh conditions of the gastrointestinal tract. As a result, they can get into the small intestine, where they are absorbed and enter the bloodstream. MicroRNAs stimulate the viability, proliferation, and activity of intestinal epithelial stem cells. In addition, they affect the entire immune system by differentiating B- and T-cells and controlling the production of interleukin by macrophages [2].

Chandwe & Kelly reported the anti-inflammatory activity of bovine colostrum in bowel diseases and infectious diarrhea [5].

Menchetti *et al*. and Playford *et al*. published *in vitro* and *in vivo* evidence of the beneficial effect of bovine colostrum on gastrointestinal diseases [6, 7].

More than half of all children with autism spectrum disorder have gastrointestinal comorbidities, e.g., chronic constipation, diarrhea, irritable bowel syndrome, etc. The severity of these symptoms correlates with the degree of gastrointestinal dysbacteriosis in the patient. Sanctuary *et al*. combined a probiotic (*Bifidobacterium infantis*) with a bovine colostrum product as a source of prebiotic oligosaccharides [8]. They assessed the efficacy of this preparation and the state of the gastrointestinal tract, microbiome, and immune factors in young gastrointestinal patients with autism spectrum disorder. The patients that received both preparations demonstrated a lower incidence of irritable gastrointestinal tract disorder, dysbacteriosis, and certain behavioral abnormalities. This improvement probably resulted from a lower production rate of IL-13 and TNF-α.

Bovine colostrum was reported to have a greater bioavailability when fermented with proteolytic enzymes, which increase the peptide yield. Jørgensen *et al*. tested the biological activity of peptides isolated from bovine colostrum on mouse intestinal cells (mIC(c12)) [9]. After protease treatment, colostrum peptides became more bioactive. They detected bioactive colostrum peptides mainly in the casein fraction (MALDI MS/MS).

Jørgensen *et al*. explained the beneficial effect of colostrum consumption by the presence of biologically active peptides obtained from intact proteins [9]. According to Korhonen, these peptides can be released during gastrointestinal digestion or colostrum fermentation [10]. Therefore, enzymatic hydrolysates of bovine colostrum are a potential source of biologically active native proteins and peptide fractions to be included in functional foods [11, 12].

Nowadays, dairy peptides are more than a quintessence of healthy nutrition: they have gained therapeutic value as well. For instance, colostrum peptides appeared to inhibit SARS-CoV2 [13]. Fermented colostrum, raw milk, and microfiltered milk from cows vaccinated against SARS-CoV-2 may even provide a short-term protection against SARS-CoV-2 [14]. Colostrum peptides also demonstrated a strong antimicrobial effect [15].

Enzymatic protein hydrolysis based on proteolytic enzyme preparations can be an effective production method for obtaining bioactive peptides [13]. Animal enzymes, e.g., trypsin, often participate in hydrolysis [14].

This research isolated peptides from the trypsic hydrolyzate supernatant of bovine colostrum to be described and tested for antimicrobial and antifungal activities.

Study objects and methods

We obtained trypsin hydrolyzate of bovine colostrum by the following technology. First, we removed the fat fraction by centrifugation at 3900 rpm for 10 min in a SM-12-06 centrifuge (TAGLER, Russia). After that, we introduced trypsin (Samson-Med, Russia) in the ratio of 0.15% to the colostrum weight into a phosphate buffer solution at pH 7.4. The solution consisted of disodium hydrogen phosphate dodecahydrate (Rosspolymer, Russia). The hydrolysis lasted for 12 h at 36°C. Eventually, the temperature was brought up to 75°C to inactivate the enzyme. Table 1 summarizes the physicochemical parameters of trypsin hydrolyzate of bovine colostrum.

The trypsin hydrolyzate supernatant of bovine colostrum was isolated by centrifugation at 3900 rpm for 7 min.

The supernatant was separated by preparative chromatography on silica gel, and the isocratic ratio of eluent PBS to EtOH was 9:1. The peptide composition of

Table 1. Physicochemical parameters of trypsin hydrolyzate of bovine colostrum

Таблица 1. Физико-химические показатели трипсинового гидролизата молозива коров

Indicator	Property
Mass fraction of protein, %	13.18 ± 0.09
Mass fraction of fat, %	0.15 ± 0.01
Mass fraction of ash, %	9.50 ± 0.02
Mass fraction of solids, %	24.17 ± 0.42
Density, g/cm^3	1.05 ± 0.03
Acidity, °T	4.78 ± 0.12

the trypsin hydrolyzate supernatant was determined on a MALDI-TOF mass spectrometer, while the protein sequences were deciphered using the Mascot database.

Proteins were precipitated with ammonium sulfate to allow for a more complete study of the protein composition of the hydrolyzate supernatant. After sedimentation, the samples were centrifuged at 3900 rpm for 7 min to collect the protein precipitate. The protein precipitate was purified from salts and inorganic impurities on a column with Amberlit XAD2 with eluent for buffer A of 10 mM (CH_3COONa), pH = 6, 10 mM (CH_3COONa) pH = 4, 10 mM (KCl/HCl), pH = 1.5. The salt gradient for buffer A+ was 0.2, 0.4, and 1% NaCl. We used the Bradford method to test fractions of each sample for protein. The resulting peptide fractions were separated by preparative chromatography on silica gel. The isocratic ratio of eluent PBS and EtOH was 9:1. The separation resulted in the mpT fraction, which was studied using the MALDI-TOF method.

We used the disk-diffusion method to define the antimicrobial activity of the trypsin hydrolyzate supernatant on gram-positive *Bacillus subtilis* and gram-negative *Escherichia coli*.

The strains were cultivated on solid and liquid media at 37°C. The solid LB nutrient medium consisted of 1.5% agar, 1% tryptone, 0.5% yeast extract, and 1% NaCl. The liquid LB nutrient medium included 1% tryptone, 0.5% yeast extract, and 1% NaCl.

The diffusion method was applied to determine the antimicrobial activity of the hydrolyzate. The test strain was sown on a lawn plate with agar nutrient medium, and the supernatant was placed on the lawn. A paper disk with a nutrient medium served as a control, and a disk with a standard antibiotic (Kanamycin) served as reference. The petri dishes were incubated at the optimal temperature for each test strain for 24.0 ± 0.5 h. The results depended on the presence and size (mm) of a microorganism-free area around the disk.

The antimicrobial activity of the peptides was tested on nine groups of Wistar rats. Each group included four three-month-old males. Group 1 consisted of control animals that received 0.4 mL of water per day intragastrically for 7 days. Groups 2, 3, 4, and 5 received 0.3 mL of the trypsin hydrolyzate supernatant of bovine colostrum every day for 7 days. One day after the introduction, groups 2-9 were injected intraperitoneally with a daily culture of *Salmonella enteritidis* 92. Groups 2 and 5 received 5 CFU, groups 3 and 6 – 100 CFU, groups 4 and 8 – 500 CFU, groups 5 and 9 – 5000 CFU. The rats were observed for 21 days after the infection. The efficacy of the preparation was defined based on the LD_{50} number of survivors and the mean day pf death period.

All animal procedures complied with the European Communities Council Directive 2010/63/EU on the welfare of experimental animals and were approved by the Ethics Committee of the Institute of Immunology and Physiology of the Ural Branch of the Russian Academy of Sciences.

Results and discussion

The fractionated supernatant of trypsin hydrolyzate (T) yielded three peptide fractions with different molecular weights: TT1, TT2, and TT3. Figure 1 shows a chromatogram for peptides.

Figures 2 show the mass spectra of peptide fractions TT1, TT2, and TT3 of the trypsin hydrolyzate supernatant of bovine colostrum.

Figures 2 demonstrate quite clearly that the peptides differed in molecular weight.

The molecular weight differed as follows: TT1 – 8.4 kDa, TT2 – 6.5 kDa, and TT3 – 13.0 kDa.

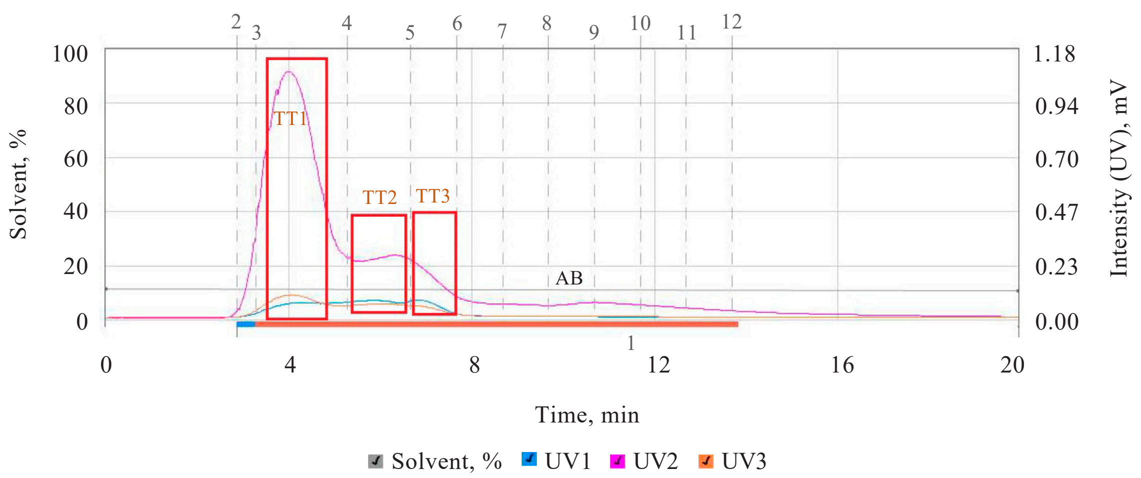

Figure 1. Supernatant of trypsin hydrolyzate of bovine colostrum: peptides T

Рисунок 1. Хроматограмма образца Т надосадочной жидкости трипсинового гидролизата молозива коров

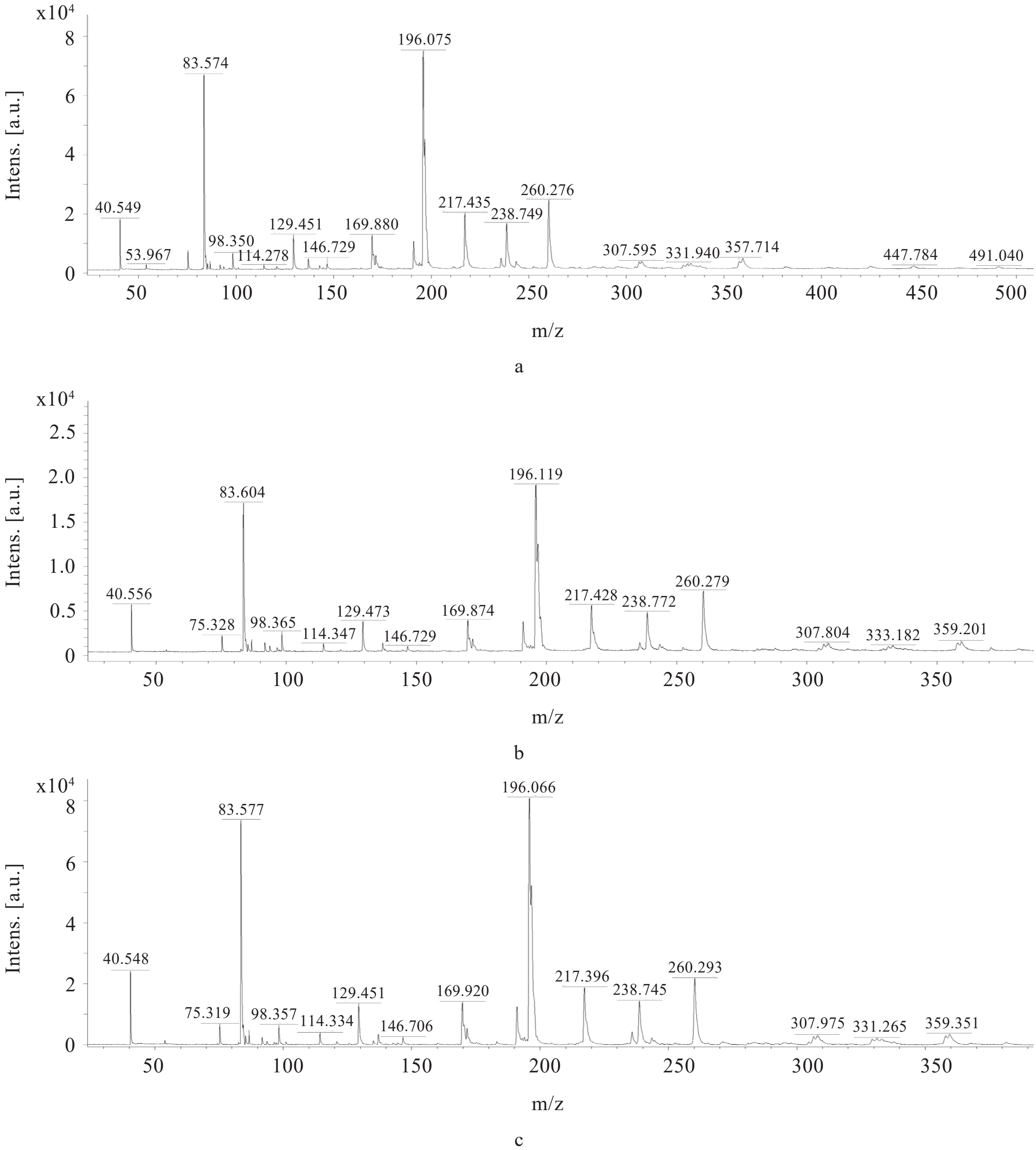

Figure 2. Mass spectrum: Samples TT1 (a), TT (b), and TT3 (c)

Рисунок 2. Масс-спектры образцов TT1 (a), TT (b) и TT3 (c)

Table 2 shows the amino acid sequence for each sample. Peptide TT3 consisted of nine amino acids (nonapeptides) and belonged to short peptides. Peptides TT1 and TT2 had 17 amino acids and belonged to polypeptides. Ko *et al*. identified TT1 peptide as the NCI_CGAP_Brn23 peptide *Homo sapiens* c DNA clone [17]. In fact, it is similar to TR: O35085 O35085 ARX HOMEOPROTEIN. This peptide is believed to affect the prenatal development of the central nervous system. Peptides TT2 and TT3 have never been identified.

This research featured the peptide composition of proteins precipitated with ammonium sulfate in the trypisine hydrolyzate supernatant of bovinecolostrum.

Figure 3 demonstrates a chromatogram of the peptide composition of proteins precipitated by ammonium sulfate of hydrolysate trypisine supernatant.

Table 2. Protein sequences of peptide samples TT1, TT2, and TT3 of trypsin hydrolyzate supernatant of bovine colostrum

Таблица 2. Белковые последовательности пептидов TT1, TT2 и TT3 надосадочной жидкости трипсинового гидролизата молозива коров

Peptide sample	Amino acid sequence
TT1	EGKSPRQ CLK SR G RK GY
TT2	PK CD YKRRS GP ALR TAK
TT3	LARKTSK IK

Note: A – alanine; D – aspartic acid; Q – glutamine; E – glutamic acid; G – glycine; I – isoleucine; L – leucine; K – lysine; P – proline; S – serine; T – threonine; Y – tyrosine; R – arginine; D – aspartic acid.

Примечание: A – аланин; D – аспарагиновая кислота; Q – глутамин; E – глутаминовая кислота; G – глицин; I – изолейцин; L – лейцин; K – лизин; P – пролин; S – серин; T – треонин; Y – тирозин; R – аргинин; D – аспаргиновая кислота.

When we precipitated supernatant proteins of bovine trypisine hydrolysate with ammonium sulfate, it yielded a TT4 peptide with the following amino acid sequence: EK LAKNK LAR GLK RK. The peptide had a molecular weight of 18.0 kDa and was identified as CO950255 protein (sus scrofa) with unknown functions.

We isolated four peptides: one short peptide and three polypeptides. All the samples had different molecular weights.

Our data were consistent with those obtained by Poirier *et al*., who detected biologically active peptides in the trypsin hydrolyzate of bovine colostrum [18]. They stimulated the proliferative activity of the T84 human intestinal epithelial cell line with pepsin and trypsin hydrolyzate of bovine colostrum. Therefore, bovine colostrum peptides are potentially bioactive substances that are able to restore the gastrointestinal tract during infections [19].

Birkemo *et al*. were the first to describe peptides with antimicrobial activity in fresh bovine colostrum [20]. They used chromatography to isolate three peptides of fresh colostrum and described their antimicrobial activity against *Escherichia coli* DH5alpha. The first two peptides were casecidin 17 and casecidin 15. They were identical to the sequences at the C-terminus of bovine beta-casein (YQEPVLGPVRGPFPIIV and YQEPVLGPVRGPFPI). Casecidin 17 and casecidin 15 had molecular weights of 1881.00 and 1669.06 Da, respectively. The third peptide was isracidin with a mass of 2763.80 Da and the sequence of RPKHPIKHQGLPQEVLNENLLRF. Casecidin 17 and casecidin 15 shared the same minimal inhibition concentrations against *E. coli* DPC6053: 0.4 mg/mL. Structural modeling showed that the amphiphilic structures also had identical inhibitory and structural properties. The minimal inhibition concentration of isracidin against *E. coli* DPC6053 was 0.2 mg/mL [20].

In this research, we studied the antimicrobial properties of the trypsin hydrolyzate supernatant of bovine colostrum on gram-positive and gram-negative bacteria, as well as on the *Candida albicans* diploid fungus. *E. coli* and *Bacillus subtilis* were selected as test bacterial strains.

Table 3 summarizes the antimicrobial and antifungal activity of the trypsin hydrolyzate supernatant of bovine colostrum.

Table 3 illustrates the effect of trypsin hydrolyzate on the survival of Wistar rats infected with *Salmonella enteritidis* 92.

According to Table 4, the trypsin hydrolyzate had a bactericidal effect against gram-negative *E. coli* (lysis

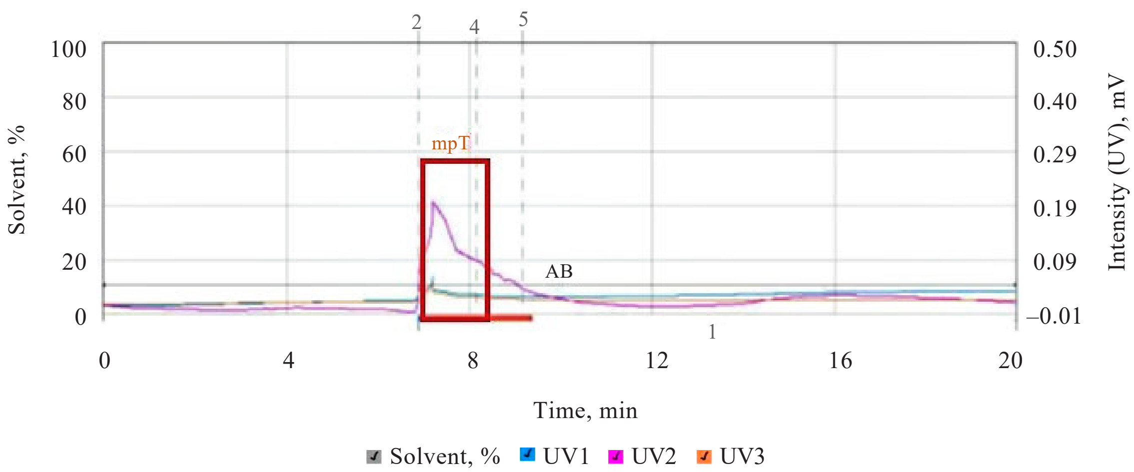

Figure 3. Peptides during the precipitation of proteins from trypisine hydrolysate supernatant of bovine colostrum with ammonium sulfate

Рисунок 3. Хроматограмма пептидов при осаждении сульфатом аммония белков надосадочной жидкости триписинового гидролизатата молозива коров zone diameter – 3 mm) and gram-positive *B. subtilis* (lysis zone diameter – 5 mm), as well as antifungal activity against *C. albicans*.

Table 4 demonstrates the effect of trypsin hydrolyzate on the survival of rats infected with *S. enteritidis* 92.

Rats that received trypsin hydrolyzate supernatant of bovine colostrum had a better survival rate against *S. enteritidis* 92. Therefore, the colostrum peptides possessed antimicrobial properties. Group 4 rats, which received 500 CFU of *S. enteritidis*, had two survivors (50%), while Group 7 had only one (25%). The LD_{50} for Wistar rats with *S. enteritidis* was 100 CFU, but those injected with trypsin hydrolyzate had a LD_{50} of 500 CFU of *S. enteritidis*. The mean day of death value was lower by 50% in the infected animals treated with hydrolysate. It was two days in Group 9 infected with 5000 CFU of *S. enteritidis*. In Group 5, where the rats received the same dose of *S. enteritidis* and were treated with hydrolysate, it was three days.

Our results were consistent with the data obtained by Sears *et al*., who proved that bovine colostrum is a practical and effective prophylactic against gastrointestinal diseases [21]. Travelan is a commercial drug known to prevent diarrhea caused by enterotoxigenic *E. coli*. It possesses official clinical efficacy against *Salmonella*. However, its immune components and antimicrobial activity are yet to be identified [21].

Our research revealed four antimicrobial peptides in the trypsin hydrolyzate supernatant of bovine colostrum. They possessed bioprotective properties against pathogens, which was consistent with other studies on the antimicrobial and immunotropic effects of bovine colostrum. Anderson *et al*. argue that bovine colostrum increases the protective barrier of the small intestine and positively affects colon peristalsis [22]. However, for colostrum to be used as a functional product, it must remain bioactive after processing. The integrity of the intestinal protective barrier and the antimicrobial properties of colostrum increased because of the short peptides that were released during enzymatic processing. In [22], proteolytic enzymes had a beneficial effect on the integrity of the small intestine barrier, which is consistent with our research results. Indeed, the peptides can be used to develop functional foods aimed at improving intestinal health.

Playford *et al*. reported that orally-administered bioactive peptides have potential clinical benefits [23]. However, their applicability is limited by the proteolysis with gastric and pancreatic enzymes. As oral antimicrobial agents, bioactive peptides have to be combined with substances that ensure their proper digestion. In [23], the authors turned to casein and/or soy flour with high protease inhibiting activity to provide the stability of bovine colostrum peptides in laboratory rats. Soy and, to a lesser extent, casein increased the biostability of colostrum peptides in relation to digestive enzymes.

Table 3. Antimicrobial and antifungal activity of the trypsin hydrolyzate supernatant of bovine colostrum

Таблица 3. Антимикробная и противогрибковая активность надосадочной жидкости трипсинового гидролизата молозива коров

Sample	Lysis diameter, mm		
	Escherichia coli ATCC 25922	*Bacillus subtilis*	*Candida albicans*
Trypsin hydrolyzate supernatant of bovine colostrum	3	5	7
Control	0	0	0
Antibiotic	25	26	0

Table 4. Effect of trypsin hydrolyzate on the survival of rats infected with *Salmonella enteritidis* 92

Таблица 4. Влияние трипсинового гидролизата надосадочной жидкости молозива коров на выживаемость крыс линии Вистар, зараженных *Salmonella enteritidis* 92

Group	Surviving animals, g	Mean day of death, days
Group 1 (control)	4	–
Group 2 (5 CFU *Salmonella enteritidis* + hydrolyzate)	4	–
Group 3 (100 CFU *Salmonella enteritidis* + hydrolyzate)	4	–
Group 4 (500 CFU *Salmonella enteritidis* + hydrolyzate)	2	4
Group 5 (5000 CFU *Salmonella enteritidis* + hydrolyzate)	0	3
Group 6 (5 CFU *Salmonella enteritidis*)	4	–
Group 7 (100 CFU *Salmonella enteritidis*)	2	2
Group 8 (500 CFU *Salmonella enteritidis*)	1	2
Group 9 (5000 CFU *Salmonella enteritidis*)	0	2

Conclusion

We isolated peptides from the trypsin hydrolyzate supernatant of bovine colostrum and investigated their antimicrobial and antifungal activities. The trypsin hydrolyzate was preliminarily fractionated by centrifugation. Three peptide fractions (TT1, TT2, and TT3) differed in amino acid sequences and molecular weights. One of the selected peptides was classified as short because it had nine amino acids in its composition, while the other two belonged to polypeptides and consisted of 17 amino acids. Sample TT1 was identified as the NCI_CGAP_Brn23 peptide, which is known to affect prenatal development of the central nervous system. Peptides TT2 and TT3 were not identified. We isolated a polypeptide with a molecular weight of 18.0 kDa from the ammonium sulfate-precipitated trypsin hydrolyzate supernatant of bovine colostrum. The peptide was identified as CO950255 protein sus scrofa, but its functions remained unknown. The peptides demonstrated activity against gram-positive bacteria *Escherichia coli*, gram-negative bacteria *Bacillus subtilis*, and diploid fungus *Candida albicans*. We infected laboratory Wistar rats with various doses of *Salmonella enteritidis* 92, and the orally-administered trypsin hydrolyzate promoted their survival, as well as reduced the LD_{50} and the mean day of death period. The research proved the antimicrobial and immunotropic properties of the peptides obtained from the trypsin hydrolyzate of bovine colostrum.

Contribution

All authors provided critical feedback and helped shape the research, analysis and manuscript.

Conflict of interest

The authors declared no conflict of interests regarding the publication of this article.

Критерии авторства

Авторы в равной степени принимали участие в исследовании и оформлении рукописи.

Конфликт интересов

Авторы заявляют об отсутствии конфликта интересов.

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