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Methods of Production and Purification of Biologically Active Peptides.

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ABSTRACT

The main component of human health is nutrition, which is even more significant if a person is sick. There is a change of metabolic processes in humans with cancer, so they require a special nutritional diet. Probiotics and short peptides are an integral part of the diet in cancer patients. The maintenance of visceral protein pool is one of the goals of nutritional support in cancer patients. In modern blends for enteral support of cancer patients the protein component may be present in one of the following three types: native protein; whey/whole protein peptides in cow's milk; free amino acids. Conditions of enzymatic hydrolysis of milk proteins, providing for the receipt of biologically active peptides with directional effect, were optimized. Optimal parameters of the hydrolysis reaction, ensuring separation of the polypeptide chain into peptides and free amino acids, enzymatic system consisting of chymotrypsin or thermolysin at 37 ± 2 °C, with the enzyme-substrate ratio of 1:50 and the process duration of 12.00 ± 0.05 h were selected.

Keywords: milk protein, biologically active peptides, enzymatic hydrolysis, biotechnological methods.

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INTRODUCTION

Industrial production of healthy and balanced food products is one of the major problems facing humanity. In a difficult demographic situation in modern Russia, the development of efficient and cost-based approaches to accelerated health improvement of the population is becoming one of the highest priorities of scientific and applied research, which is reflected in the government-approved concept of public policy in the field of healthy nutrition of the Russian population in the period up to 2020. Large-scale introduction of functional foods with eubiotics in the everyday lives of humans, preserving and stimulating the natural defense mechanisms of the human body from the effects of adverse environmental factors of different nature, shall play an important role in this implementation [1-2].

The relevance of the research areas selected is also practically assured. In recent years, the concept of participation of human symbiotic microflora in maintaining the health and appearance of many diseases is gaining popularity. In this approach, the main purpose of research is the development of functional food products for nutritional support of cancer patients during rehabilitation [3].

Cancer, being the most important medical and social problem in most countries, including the Russian Federation, occupies a central place in the morbidity and mortality structure. About 10 million new cases of malignant tumors and more than 6 million deaths from various cancers are registered annually worldwide. In European countries, malignancies are the second largest cause of death after cardiovascular diseases. According to the World Health Organization (WHO), approximately one-third of all cancer deaths worldwide are related to malnutrition, and diet is ranked second after tobacco as a theoretically preventable cause of cancer [4].

In the last decade, humanity is beginning to change their attitude to cancer that significantly improved the results of its treatment and quality of life. In addition to the above, the maintenance therapy also plays an important role because it involves a variety of methods to ensure the functioning of the body of a cancer patient during treatment, as well as after it [5].

The aim of this work was the preparation and purification of bioactive peptides from milk protein.

OBJECTS AND METHODS OF RESEARCH

The following *reagents* are used in the performance of work: potassium hydrogenphosphate by GOST 2493-75; potassium dihydrogen phosphate by GOST 4198-75; casein by GOST R 53667-2009; Trypsin from porcine pancreas (Sigma, USA) with an activity of 250 u/mg dw of protein; chymotrypsin from bovine pancreas (Sigma, USA) with an activity of 50 u/mg dw of protein; thermolysin (Serva, Germany).

The tests are carried out in the Research Institute of Biotechnology "KemIFST". Sampling was performed in accordance with GOST 18321-73 and GOST 28495-90.

The control of parameters of the process for production of biologically active peptides is performed as follows.
The prebiotic properties of biologically active peptides were tested in accordance with Batch Culture Fermentation method *in vitro.*

Intercooling bioreactors (300 ml) were charged with 135 ml of previously sterilized basal medium for bacterial growth (2 g/l peptone water, 2 g/l of yeast extract, 0.1 g/l NaCl, 0.04 g/l K_2HPO_4, 0.04 g/l KH_2PO_4, 0.01 g/l $MgSO_4 \cdot 7H_2O$, 0.01 g/l $CaCl_2 \cdot 6H_2O$, 2 g/l $NaHCO_3$, 2 ml Tween 80, 0.02 g/l gemin, 0.5 g/l cysteine HCl, 0.5 g/l bile salts, pH 7.0) inoculated with 15 ml of biologically active peptides derived from milk proteins at pH of 7.0. Biologically active peptides were added to obtain final concentration of 1% (w/v). A control experiment was carried out without the addition of bioactive peptides isolated from milk proteins to the fermenter. The test was performed with constant stirring with a magnetic stirrer; the reaction temperature was 37 °C, pH 6.8. Anaerobic conditions were maintained by sparging the fermentor in oxygen free nitrogen at 5 ml/min. The samples (5ml) were taken every 0, 4, 8 and 24 hours for bacterial counting and analysis of fatty acids. The test was performed in three patterns.

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RESULTS AND DISCUSSION

About two decades ago it became known that milk proteins are a major source of biologically active peptides. These peptides exert their specific biological actions only after the release from the polypeptide chain and standing in the free state. This aspect was studied since 1979, and numerous peptides were found and deciphered [6].

In recent years, the possibility of using biologically active peptides composed of specialized products and food supplements for the prevention of various diseases was discussed.

Currently, the main biotechnological methods are increasingly being applied to complex objects of natural origin and complex biochemical processes. One such process – the enzymatic hydrolysis of the polymer chains of polypeptides and proteins (proteolysis) – underlies a number of biological phenomena that manifest themselves in a wide range of degree of peptide bonds hydrolysis from limited proteolysis during activation of enzymes to deep hydrolysis. Proteolysis plays an important role in the modification of food proteins and the production of protein hydrolysates in the food industry. Obtaining of bioactive peptides by proteolysis from inactive protein precursors is a promising method for obtaining biopeptides.

Identification and selection of rational parameters for processing of biological systems, containing milk proteins, to obtain hydrolysates is the main processing task, the implementation of which creates an efficient technology for the production of bioactive peptide complexes based on milk proteins.

In this study, casein that is available and biologically valuable source of protein and the most adapted to the physiological characteristics of the organism in comparison with different proteins was used as a feedstock [6-7]. It is known that caseins, unlike some globular proteins, are well cleaved by proteinases in the native state, as in the native state they have a little ordered conformation similar to disorganized structure of denatured globular proteins [8-10]. This is due to very low content of α-coils and low structural organization of the main casein components of casein, which is caused by a high content of proline in the protein – from 8.5 to 16.0%, which seems to deform it into disordered coil.

Enzymatic hydrolysis of casein peptide bonds was carried out by proteolytic enzymes: trypsin, chymotrypsin and thermolysin. The process for producing bioactive peptides from milk proteins was carried out according to the scheme shown in Figure 1, comprising the following steps: acceptance, quality assessment, preparation of raw materials; phosphates introducing; casein dissolving; adjusting pH to 7.5; pasteurization at 74 ± 2 °C with an exposure of 15-20 seconds to kill microorganisms contained in the casein, cooling to a temperature of 37 ± 2 °C, introduction of an enzymatic system consisting of trypsin, chymotrypsin and thermolysin.

Enzymatic hydrolysis was carried out with the following parameters: temperature 37 ± 2 °C, duration 8.00 ± 0.05 hours, pH 7.5 ± 0.1, enzyme-substrate ratio 1:50. To avoid the development of extraneous microflora and odor prior to fermentation 0.5% solution of toluene was added, which is completely evaporated in the inactivation process. The resulting hydrolysate was heated to 95 ± 2 °C for 30 seconds in order to inactivate the enzymes complex and remove pathogenic microflora.

The next stage of the research was the study of the amino acid sequences in the fragments released in the selected hydrolysis parameters. Molecular weights of the extracted peptides were determined on MALDI-TOF mass spectrometer Reflect III (Bruker, Germany) equipped with a UV-laser with a wavelength of 336 nm. 2,5-dihydroxybenzoic acid (Sigma, Germany) in 20% acetonitrile, 0.1% TFA in a concentration of 10 mg/ml was used as a matrix. Procise cLC 491 (Applied Biosystems, USA) system of protein sequencing was used to determine the amino acid sequence of peptides. Phenylthiohydantoin derivatives of the amino acid residues were identified using 120A PTH analyzer (Applied Biosystems, USA). The results obtained are shown in Table 1.

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Table 1 – Characteristics of peptides resulting from casein hydrolysis by trypsin

Molecular weight, Da	The arrangement in the polypeptide chain	Amino acid sequence
Tryptic hydrolysate		
3,803	1-25	RELEELNVPGEIVESLSSSE ESITR
1,131	26-32	WTAQQKEL
1,463	33-48	FQSEEQQTEDELQDK
3,754	52-86	AQTQSLVYPFPGPIPNSLPQ NIPPLTQTPVVVPPF
3,668	87-118	LQPEVMGVSKVKEAMAPKHK EMPFPKYPVEPF
642	125-129	EIVPN
443	161-163	YPE
2,209	184-202	LLYEQPVLGPVGPPPIIV
585	203-209	GPFPIIV
Chymotryptic hydrolysate		
98	29	K
368	48-50	GKE
848	60-66	YPPPGPI
972	81-88	SSSEEIVP
426	92-94	KED
762	100-104	TGEDHN
5,172	114-169	YPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPWVMFPPQSVLSLSQSK
385	173-176	AYP
1,717	190-209	QEPVLGPVRGPFPIIV
Thermolysin hydrolysate		
1,802	26-32	INKKIEKF
390	30-32	IEK
2,329	33-51	QSEEQQQTEDELQDKIHPF
180	98-99	VK
293	106-107	KE
690	108-113	EMPFPK
585	145-148	QQKE
889	169-175	KVLPVPE
443	191-193	LLY
616	195-199	SDIPN
915	205-210	ENSEKTT

Note: A – alanine; N – asparagine; D – aspartic acid; Q – glutamine; E – glutamic acid; G – glycine; N – gistidin; I – isoleucine; L – leucine; K – lysine; P – proline; S – serine; T – threonine; W – tripotofan; Y – tyrosine; V – valine.

The analysis of Table 1 shows that all investigated fractions of trypsin, chymotrypsin and thermolysin casein hydrolysates contain in their structure some peptides consisting of amino acid residues in various amounts. Thus, in the tryptic hydrolysate of casein 9 peptides consisting of 3-35 amino acid residues were found. In the chymotrypsin hydrolysate of casein, 9 peptides consisting of 1-56 amino acid residues were found. In the thermolysin hydrolysate of casein, 11 peptides consisting of 2-19 amino acid residues were found.

The identification of peptide sequences obtained was performed by searching the NCBI (www.ncbi.nlm.gov) and SwissProt (www.expasy.ch) databases. Comparative results are summarized in Table 2.

Table 2 – Peptides with biological activity detected in the casein hydrolysates tested

Fragment	Enzyme used	Amino acid sequence in peptides	Function
26-32	Trypsin	Trp-Tre-Ala-Glu-Glu-Lys-Glu-Leu	Immunomodulatory
184-202	Trypsin	Leu-Leu-Tyr-Glu-Glu-Pro-Val-Leu-Gly-Pro-Val-Gly-Pro-Pro-Pro-Ile-Ile-Val	Immunomodulatory
60-66	Chymotrypsin	Tyr-Pro-Pro-Pro-Gly-Pro-Ile	Immunomodulatory
92-94	Chymotrypsin	Lys-Glu-Asn	Antioxidant
100-104	Chymotrypsin	Tre-Gly-Glu-Asn-His-Asp	Antitumor
106-107	Thermolysin	Lys-Glu	Antioxidant
169-175	Thermolysin	Lys-Val-Leu-Pro-Val-Pro-Glu	Immunomodulatory
191-193	Thermolysin	Leu-Leu-Tyr	Immunomodulatory

Table 2 shows that two biologically active peptides with immunomodulatory activity were detected in the trypsin hydrolysate of casein: Trp-Tre-Ala-Glu-Glu-Lys-Glu-Leu and Leu-Leu-Tyr-Glu-Glu-Pro-Val-Leu-Gly-Pro-Val-Gly-Pro-Pro-Pro-Ile-Ile-Val. The chymotryptic hydrolysate of casein contains three biologically active peptide with immunomodulatory (Tyr-Pro-Pro-Pro-Gly-Pro-Ile), antioxidant (Lys-Glu-Asn) and anticancer (Tre-Gly-Glu-Asn-His-Asp) activity. Casein hydrolysate, obtained by the action of thermolysin, contains three biologically active peptides, two of which are immunomodulatory (Lys-Val-Leu-Pro-Val-Pro-Glu and Leu-Leu-Tyr), and one – antioxidant (Lys-Glu).

The development of a method for isolation and purification of biologically active peptides contained in the casein hydrolysates is of great interest. To obtain purified biologically active peptides the following methods were tested in this study:

- ultrafiltration;
- gel filtration on Sephadex G-25;
- successive stages: ultrafiltration, preparative electrophoresis, reversed-phase high performance liquid chromatography.

The evaluation of purity of biologically active peptides in all embodiments was performed using analytical protein electrophoresis, MALDI-TOF mass spectrometry, and analytical reverse phase HPLC.

The peptide mixture was ultrafiltered using MFU-R-45-300 (Russia) through membranes with a pore diameter of 10 and 15 kDa at pH of 6.0-6.5. The resulting filtrate was further subjected to sterilization, followed by condensation to a solids content of 50-55% and dried to a moisture content of 14-16%.

In the second purification method to 1 L of enzymatic casein hydrolysate was added 300 g of Sephadex G-25, and left for 10 minutes, then the mixture was centrifuged at 3000 rev/min for 20 minutes. The supernatant solution was removed; the swollen gel was eluted with distilled water and centrifuged again at 3000 rev/min for 20 minutes. The obtained liquid fractions were sterilized by filtration through fine-pore filters with a pore diameter of 0.2 µm. The fractions were freeze-drying to obtain dry fraction. The presence of low molecular weight peptides in the fraction was examined by polyacrylamide gel Laemmli electrophoretic technique.

The third peptide purification scheme was carried out as follows. In the first step, the casein enzymatic hydrolysates were ultrafiltered using MFU-R-45-300 (Russia). Further, for separation of proteins by preparative electrophoresis the resulting mixture, containing peptides and low molecular weight proteins with molecular weights of less than 10-12 kDa, was loaded onto an electrophoretic column (previously adding urea in the sample up to a concentration of 3 M). Preparative electrophoresis with continuous elution of proteins was performed in 12.5% polyacrylamide gel in acidic buffer system in the presence of urea in the Bio-Rad chamber (USA). Eluted protein fractions from the column were analyzed by analytical electrophoresis in the presence of sodium dodecyl sulfate. Further, the peptides contained in the protein fractions were separated using several consecutive cycles of reverse-phase high performance liquid chromatography (RP-HPLC) using LC-20 chromatograph (Shimadzu, Japan) eluting peptides in the acetonitrile concentration gradient using different

counterions. The fractions obtained after RP-HPLC were dried by centrifugation under vacuum using SpeedVac (Savant, USA).

The results of purification of peptides isolated from trypsin, chymotrypsin and thermolysin casein hydrolysates using different methods are presented in Tables 3-5.

Table 3 – Results of purification of biopeptid peptides isolated from casein hydrolysates and purified by ultrafiltration

Fraction number	Molecular weight, Da	Amino acid sequence	Purity, %
Tryptic hydrolysate			
1	1,131	Trp-Tre-Ala-Glu-Glu-Lys-Glu-Leu	45.0
2	2,209	Leu-Leu-Tyr-Glu-Glu-Pro-Val-Leu-Gly-Pro-Val-Gly-Pro-Pro-Pro-Ile-Ile-Val	52.5
Chem tryptic hydrolysate			
1	848	Tyr-Pro-Pro-Pro-Gly-Pro-Ile	48.7
2	426	Lys-Glu-Asn	43.9
3	762	Tre-Gly-Glu-Asn-His-Asp	44.6
Thermolysin hydrolysate			
1	293	Lys-Glu	38.5
2	889	Lys-Val-Leu-Pro-Val-Pro-Glu	47.2
3	443	Leu-Leu-Tyr	44.1

Table 4 – Results of purification of biopeptid peptides isolated from casein hydrolysates and purified by gel filtration using Sephadex G-25

Fraction number	Molecular weight, Da	Amino acid sequence	Purity, %
Tryptic hydrolysate			
1	1,131	Trp-Tre-Ala-Glu-Glu-Lys-Glu-Leu	56.8
2	2,209	Leu-Leu-Tyr-Glu-Glu-Pro-Val-Leu-Gly-Pro-Val-Gly-Pro-Pro-Pro-Ile-Ile-Val	64.5
Chymotryptic hydrolysate			
1	848	Tyr-Pro-Pro-Pro-Gly-Pro-Ile	55.0
2	426	Lys-Glu-Asn	58.9
3	762	Tre-Gly-Glu-Asn-His-Asp	60.2
Thermolysin hydrolysate			
1	293	Lys-Glu	49.3
2	889	Lys-Val-Leu-Pro-Val-Pro-Glu	62.1
3	443	Leu-Leu-Tyr	58.0

Table 5 – Results of biopeptid peptides purification isolated from casein hydrolysates and purified by a combination of ultrafiltration, preparative electrophoresis, reversed-phase high performance liquid chromatography

Fraction number	Molecular weight, Da	Amino acid sequence	Purity,%
Tryptic hydrolysate			
1	1,131	Trp-Tre-Ala-Glu-Glu-Lys-Glu-Leu	86.7
2	2,209	Leu-Leu-Tyr-Glu-Glu-Pro-Val-Leu-Gly-Pro-Val-Gly-Pro-Pro-Pro-Ile-Ile-Val	92.4
Chem tryptic hydrolysate			
1	848	Tyr-Pro-Pro-Pro-Gly-Pro-Ile	88.9
2	426	Lys-Glu-Asn	95.4
3	762	Tre-Gly-Glu-Asn-His-Asp	92.3
Thermolysin hydrolysate			
1	293	Lys-Glu	85.2
2	889	Lys-Val-Leu-Pro-Val-Pro-Glu	97.6
3	443	Leu-Leu-Tyr	94.1

From Tables 3-5 it follows that the most effective cleaning of the biologically active peptides isolated from enzymatic hydrolysates of casein is achieved by using multi-step purification: ultrafiltration, preparative electrophoresis, and reversed-phase high performance liquid chromatography. In this case, the degree of purity of biologically active peptides isolated from tryptic hydrolysate of casein, is in the range of 86.7-92.4%; from chymotrypsin casein hydrolysate – in the range of 88.9-95.4%; from thermolysin hydrolysate – in the range of 85.2-97.6%.

Based on the results for the isolation and purification of bioactive peptides from enzymatic hydrolysates of casein, the following scheme is recommended: successive stages of ultrafiltration ising MFU-R-45-300, preparative electrophoresis in 12.5% polyacrylamide gel in Bio-Rad chamber and reverse-phase high performance liquid chromatography using LC-20 chromatograph.

CONCLUSIONS

1. Conditions of enzymatic hydrolysis of milk proteins, providing for the receipt of biologically active peptides with directional effect, were optimized. It was found that for the hydrolysis with a high degree of protein cleavage (85-98%), but low yields of soluble nitrogenous compounds (26-30%) trypsin and chymotrypsin are the most specific enzymes, and for the hydrolysis with an average degree of protein cleavage (52%) and high yield of nitrogen compounds (65%) the thermolysin enzyme preparation is most specific. Optimal parameters of the hydrolysis reaction, ensuring separation of the polypeptide chain into peptides and free amino acids, enzymatic system consisting of chymotrypsin or thermolysin at 37 ± 2 °C, with the enzyme-substrate ratio of 1:50 and the process duration of 12.00 ± 0.05 h were selected.
2. The amino acid sequences in the fragments released during hydrolysis established parameters were studied. It is shown that all investigated fractions of trypsin, chymotrypsin and thermolysin casein hydrolysates contain in their structure peptides consisting of amino acid residues in various amounts.
   Two biologically active peptides with immunomodulatory activity were detected in the trypsin hydrolysate of casein: Trp-Tre-Ala-Glu-Glu-Lys-Glu-Leu and Leu-Leu-Tyr-Glu-Glu-Pro-Val-Leu-Gly-Pro-Val-Gly-Pro-Pro-Pro-Ile-Ile-Val. The chymotryptic hydrolysate of casein contains three biologically active peptides with immunomodulatory (Tyr-Pro-Pro-Pro-Gly-Pro-Ile), antioxidant (Lys-Glu-Asn) and anticancer (Tre-Gly-Glu-Asn-His-Asp) activity. Casein hydrolysate, obtained by the action of thermolysin, contains three biologically active peptides, two of which are immunomodulatory (Lys-Val-Leu-Pro-Val-Pro-Glu and Leu-Leu-Tyr), and one – antioxidant (Lys-Glu).
3. The optimum method for isolation and purification of biologically active peptides contained in the casein hydrolysates was selected. For the isolation and purification of bioactive peptides from enzymatic hydrolysates of casein, the following scheme is recommended: successive stages of ultrafiltration using MFU-R-45-300, preparative electrophoresis in 12.5% polyacrylamide gel in Bio-Rad chamber and reverse-phase high performance liquid chromatography using LC-20 chromatograph. In this case, the degree of purity of biologically active peptides isolated from tryptic hydrolysate of casein is in the range of 86.7-92.4%; from chymotrypsin casein hydrolysate – in the range of 88.9-95.4%; from thermolysin hydrolysate – in the range of 85.2-97.6%.

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