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A Study of Polyfunctional Properties of Biologically Active Peptides.

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ABSTRACT

The article focuses on the study of the impact of biotechnological processing on the process of the targeted hydrolysis of milk proteins with the purpose of obtaining biologically active peptides, as well as on the analysis of a number of properties thereof: immunomodulatory, cytotoxic, antioxidant and prebiotic. The authors examined the properties (immunomodulatory, cytotoxic, antioxidant, and prebiotic) of biologically active peptides, isolated from milk proteins. The study showed that all of the examined peptides exhibit antitumor properties; furthermore, with the increase of the concentration of biopeptides the survival rate of cancer cells of different lines is reduced.

Keywords: immunomodulatory properties, cytotoxic properties, antioxidant properties, prebiotic properties.

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INTRODUCTION

It is widely known that the development of a neoplastic process is accompanied by nutritional deficiency. According to the report of the ESPEN (European Society for Clinical Nutrition and Metabolism, 2000), the incidence of nutritional deficiency in cancer patients varies from 46 to 88%. A study conducted by the ECOG group in 2003, which included 3,047 cancer patients, found that the incidence of nutritional deficiency in case of tumor lesions of gastrointestinal tract (GIT) ranges from 70 to 83% [1]. Nutritional deficiency occurs most frequently in case of stomach and pancreatic cancer (75-80%), tumor localization in the lung, colon, prostate (54-64%), mammary gland, various sarcomas and hemoblastoses (31-40%). Nutritional deficiency, which reaches its maximum manifestation in the form of anorexia-cachexia syndrome, may be the immediate cause of death in 4 of 20 cancer patients [2]. The recent studies have shown that nutritional deficiency has a direct correlation relationship to the survival median, particularly in patients with colorectal cancer. It should be noted that the anorexia-cachexia syndrome worsens or develops during the combined modality treatment; this is the so-called iatrogenic anorexia-cachexia syndrome. During treatment, the loss of body weight exceeding 10% may occur in 45% of patients [3].

The regulation of the control of nutritional status and the timely nutritional support of cancer patients is confirmed by a number of studies. The main purpose of nutritional support in cancer patients is the maintenance of the visceral protein pool. In formulas, produced by various manufacturers for the enteral support of cancer patients, the protein component is usually represented by native protein, peptides of whey/whole cow's milk protein or free amino acids [4-5].

An important role in protein nutrition of cancer patients belongs to the biologically active peptides. Peptide complexes are short chains of amino acids, originally isolated from animal organs and tissues, and then artificially recreated in laboratory conditions.

The biological value of peptides consists in their ability to reverse functional disorders and hinder the development of pathological processes in those tissues and organs from which they were originally isolated. The area of biological activity of peptides is extremely wide. Peptides are able to affect the state of cardiovascular, reproductive, immune, endocrine, digestive and other systems, to change the energy metabolism in the body; however, they are particularly effective in regulating the functioning of the central nervous system. Peptides may also be multifunctional, i.e. one and the same peptide can modulate the functioning of many body systems. Moreover, the fragments of peptides, arising in the process of their breakdown in the body, may exhibit their own physiological activity [6-7].

The use of native protein in nutrient formulas for cancer patients should be deemed inappropriate, since its application requires meeting a number of conditions and, first of all, the normalization of the cavitary and parietal digestion. In addition, a large (as compared to other types of protein) mass of native protein reduces the tolerance level and significantly slows down the rate of absorption.

Formulas containing free amino acids may be preferable due to the low molecular weight of the main component, which is clearly indicative of the high speed of their absorption. But in the intestine, even under normal conditions, 2/3 of nitrogen is absorbed in the form of peptides (peptides have independent passive transport mechanisms, while free amino acids are transported by specific active transport systems), and in conditions of stress and developing starvation this figure is even higher for the absorbed peptides, which drastically reduces the efficiency of a nutrient formula based on free amino acids. There are some data indicating that formulas based on amino acids, as well as parenteral nutrition and starvation, result in the atrophy of the intestinal mucosa [8].

In turn, the main way of the intake of peptides in the intestine is the transcellular absorption through the villus membranes, either using shuttle molecules or by diffusion through lipid parts of membranes. Besides, it was noted that peptides are able to be absorbed, bypassing the intestinal villi (in particular, in the spots with mucosal defects). Besides, the absorption of peptides requires no enzymatic activity on the part of the pancreas. The currently available data suggest that a peptide diet more effectively supports the functioning of the liver, and therefore promotes the visceral synthesis of proteins [9].

Thus, the use of nutritional support in cancer patients should be timely, adequate and efficient. This approach makes it possible to protect a patient from a progressive loss of body weight, improve the quality of life, and ensure a more adequate treatment [10].

The objective of the research was the study of immunomodulatory, cytotoxic, antioxidant and prebiotic properties of biologically active peptides.

RESEARCH OBJECTS AND METHODS

The cytotoxic properties of biologically active peptides are examined *in vitro* on cancer cell lines of: Burkitt lymphoma LBR2, human prostate cancer DU 145, human breast cancer MDAMB-231 and MCF7, hepatocellular carcinoma HepG2, brain cancer U-87, and human pancreatic cancer PANC-1. The count of the number of living cells in the wells after the end of the period of incubation with biologically active peptides is determined by the MTT-colorimetric method.

The cells are cultivated at 37°C and 5% of CO_2 in RPMI 1640 medium (PanEco, Russia), containing 10% of fetal bovine serum (HyClone Laboratories, Logan, UK), inactivated at a temperature of 56°C for 30 minutes, 2 mM of L-glutamine, 100 µg/ml of penicillin and 100 µg/ml of streptomycin sulfate (PanEco, Russia). The optical microscopy of cells is implemented using the AxioVision 4 system (Zeiss, Germany). Cell viability is determined by the exclusion of trypan blue (PanEco, Russia) in a Goryaev chamber.

Tumor cells in the logarithmic phase of growth are subcultured into flat-bottomed 96-well microplates ("Costar") in the amount of $5 \cdot 10^4$- $6.5 \cdot 10^4$ cells per well and preincubated during 24 hours before the addition of the tested biologically active peptides at 5% CO_2 and 37°C. The received cell cultures in a wide range of progressively decreasing concentrations are added into the wells with biologically active peptides (20 µl per 180 µl of cell suspension) and coincubated during 48 hours. 0.9% solution of sodium chloride is added into control wells in an adequate volume (20 µl). The number of living cells in the wells at the end of the incubation period is determined by the MTT-colorimetric method based on the ability of the dehydrogenases of living cells to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to violet crystals of formazan soluble in dimethylsulfoxide. The optical absorption of the stained dimethylsulfoxide solutions is measured by the microplate photometer Multiskan MS (Labsystem, Finland) at $\lambda = 540$ nm.

The cytotoxic properties of biologically active peptides are examined *in vivo* on the spleen splenocytes of Sprague-Dawley or Wistar rats. The result is determined by the complex parameters of the animal well-being, as well as by the number of living cells in the section of rat spleen and by the general appearance of the organ.

The peptide preparation is administered in several ways (intravenously, intraperitoneally, orally) as a single dose. The control group of animals receives 0.9% solution of sodium chloride in the same way. The total duration of observation of animals should be not less than 7 days. The death of animals, body weight, the presence or absence of possible clinical symptoms of intoxication, including the loss of coordination, as well as the pelage and skin status, coloring of mucous membranes, the position of the tail, should be registered on a daily basis. Splenocytes are taken from at least 5 surviving animals in 24 hours and 7 days, respectively. All the animals fallen within the observation period are subject to mandatory examination. After that, histological and cellular studies of both surviving and fallen animals are carried out. Animals are decapitated, the spleen is extracted, and cuts for histological examination are made with the subsequent homogenization. The splenocyte suspension in RPMI-1640 medium, containing 10% of fetal bovine serum (HyClone Laboratories, Logan, UK), inactivated at a temperature of 56°C during 30 minutes, 2 mM of L-glutamine, 100 µg/ml of penicillin and 100 µg/ml of streptomycin sulfate (PanEco, Russia) are filtered, treated with a 0.83% solution of NH_4Cl to lyse erythrocytes, centrifuged and resuspended in the same medium. After that, the coloring of the cells is analyzed.

For the histological study, a preparation is produced. Small pieces (0.5 x 1 x 1 cm) are cut from the corresponding organ and immersed into fixing fluid (formalin, methanol, etc.), usually for 24 hours. After the fixation, the samples are rinsed under running water during several hours. Then the samples are compacted so that they can be cut on the microtome. At the beginning of this procedure, the sample is dehydrated by a series of exposures to ethanol of different concentrations, each having a duration of 24 hours: 70%, 80%, 96%, 100%. After that, the samples are placed in the xylene-paraffin mixture, and then in the liquid paraffin for 1-2 hours at 52-56°C. After paraffin hardens, a block containing the sample is cut out from it, and a 5-15 nm thick section is

made on the microtome. Sections are put on the surface of warm water for their unfolding, and then on the slide plate. At the next stage, the paraffin is removed from the samples through a series of exposures lasting 2-5 minutes: xylene, ethanol 100%, 96%, 80%, 70%, 60%. For coloring, slides with sections are placed for a short time in the trypan blue solution, rinsed with water, dehydrated through a series of different ethanol concentrations (as described above), and then lightened up (in carbol-xylene and xylene) to remove excessive paint. At the final stage, a drop of immersion oil is applied to the preparation, which is then covered with a cover glass.

The immunomodulatory properties of biologically active peptides are tested in accordance with the method based on *in vitro* identification of surface lymphocyte markers using the flupenthixole-labelled antibodies and flow cytofluorometry. To this end, the composition of lymphocytes is determined on the basis of the CD nomenclature, according to which a specific number is assigned to each molecule, regardless of its chemical structure. The presence of a molecule on the surface is designated by "+", its absence is designated by "-".

To determine the immunomodulatory properties, the immunophenotyping method is used: the cells are stained by CD-specific antibodies, conjugated with fluorescent dyes. The binding of the labelled antibodies to the cell is determined by the type of CDs expressed by the cell. After such coloring, the analysis of the fluorescence of cells is carried out using flow cytofluorometry.

Before the beginning of the work, a lysing solution is prepared (in the amount of 450 µl per a test tube) and diluted 10-fold with deionized water. This solution ensures hypotonic lysis of erythrocytes; at the same time, the environment conditions remain mild enough to maintain the viability of ~ 92% of leukocytes. Falcon 12 × 75 mm test tubes are prepared and labelled A and B.20 µl of CD3/CD8/CD45/CD4 antibody solution is added to the test tube A, 20 µl of CD3/CD16, CD56/CD45/CD19 antibody solution is added to the test tube B. After that, 50 µl of blood is added; in doing so, the contact of blood with test tube walls should be avoided. The compounds are mixed and left in a dark place at room temperature for 15 minutes. After that, 450 µl of lysing solution is added. The compounds are mixed and left in a dark place at room temperature for 15 minutes. The samples are analyzed within 24 hours after staining.

For the analysis using the flow cytofluorimeter, the number of collected events is set to be equal to 20,000. For the analysis of the test tube A, the following channels are used: FSC – Forward Scatter, SSC – Side Scatter, FL1-H – CD3 FITC, FL2-H – CD8 PE, FL3-H – CD45 PerCP FL4-H – CD4 APC. The results are collected in the charts: FSC vs SSC, CD45 PerCP vs SSC, CD3 FITC vs CD8 PE, CD8 PE vs CD4 APC. For the analysis of the test tube B, the following channels are used: FSC – Forward Scatter, SSC – Side Scatter, FL1-H – CD3 FITC, FL2-H – CD16,56 PE, FL3-H – CD45 PerCP, FL4-H – CD19 APC with charts FSC vs SSC, CD45 PerCP vs SSC, CD3 FITC vs CD16,56 PE, CD16,56 PE vs CD19 APC.

According to the results of the research, the percentage of immune cells in the control sample and in the experiment is calculated, and the conclusion on the immunomodulatory properties of the studied peptide is drawn.

The antioxidant properties of biologically active peptides are analyzed according to the method based on the determination of the capacity of the absorption of oxygen radicals (of Trolox equivalent).

The antioxidant activity of bioactive peptides is measured according to the method described by Kao et al. (1995). The original β-phycoerythrin solution is prepared by diluting 1 mg of substance in 5.6 ml of phosphate buffer (0.2 M, pH 7.0). The original solution is stored in the refrigerator. The working solution is prepared by mixing 300 µl of the original solution with 13.4 ml of the phosphate buffer immediately before use. The solution of 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) is prepared before starting the test. For this purpose, 60 mg of AAPH is weighed and dissolved in 5 ml of phosphate buffer. The solution of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) is prepared by dissolving 5 mg of substance in 200 ml of 0.2 M phosphate buffer as a basic solution (100 µm). To get the working solution, 1 ml of original solution is mixed with 9 ml of phosphate buffer. The original solution is stored at 2°C. The phosphate buffer is prepared by mixing 0.75 M of K_2HPO_4 and NaH_2PO_4 solutions in 61.6:38.9 volume ratio. Then the mixture is diluted with distilled water 1:9, bringing the pH to 7.0. The working solution (0.2 M) is stored at 2°C.

To evaluate the antioxidant activity of biologically active peptides, the aliquots of samples are diluted (100 ×), and a control buffer is added to the reaction mixture; the degree of the protection against oxidation by β-phycoerythrin is quantified by the measurement of relative fluorescence at 595 nm and at 535 nm during the 70-minute period.

In this analysis, the overall capacity of the absorption of oxygen radicals (of Trolox equivalent) is directly proportional to the area under the kinetic curve of the built relative values of fluorescence and time. To correct any deviations, caused by instruments, reagents, or any other conditions of the analysis, the value of the analyzed samples was expressed with a reference to the corresponding amounts and concentrations of Trolox, and the values were presented in the form of Trolox equivalents (Cao *et al.*, 1995). All the reactions are carried out in a 48-well plate. To each well of the plate, 20 µl of the corresponding sample and 160 µl of the working solution with β-phycoerythrin are added. Immediately before the beginning of the measurement, 20 µl of AAPH is added to each well to initiate the reaction. The plate is closed and placed in the analyzer. In the analysis, the capacity of the compounds for the protection of β-phycoerythrin against oxidation is controlled using a decay curve. Quantification was accomplished by identifying a clean protection zone in accordance with the curve of the decay of β-phycoerythrin in the presence of AAPH.

To calculate the oxygen radical absorption capacity, the following equation is used (Cao *et al.*, 1998):

RESULTS AND DISCUSSION

With regard to the prospects of the use of biologically active peptides, isolated from milk proteins, as one of the components of functional food products for the rehabilitation of oncological patients, the study of cytotoxic (antitumor), antioxidant, prebiotic and immunomodulatory properties of bioactive peptides is of special interest.

The antitumor properties of biologically active peptides, isolated from milk proteins, were examined *in vitro* on cancer cell lines of: Burkitt lymphoma LBR2, human prostate cancer DU 145, human breast cancer MDAMB-231 and MCF7, hepatocellular carcinoma HepG2, brain cancer U-87, and human pancreatic cancer PANC-1. The number of living cells in the wells after the end of the period of incubation with biologically active peptides is determined by the MTT-colorimetric method.

The results of the study of the antitumor properties of biologically active peptides, isolated from milk proteins, are presented in Table 1.

Table 1 – The results of the study of the antitumor properties of biologically active peptides, isolated from milk proteins

Biologically active peptide in different concentrations, wt%	Cell survival rate, % for various cell lines						
	LBR2	DU 145	MDAMB-231	MCF7	6HepG2	U-87	PANC-1
Trypsin hydrolysate							
Trp-Tre-Ala-Glu-Glu-Lys-Glu-Leu							
0.005							
0.010	91.7±4.6	88.0±4.4	83.4±4.2	89.6±4.5	77.6±3.9	90.4±4.5	74.0±3.7
0.100	80.3±4.0	75.4±3.8	72.0±3.6	61.3±3.1	49.9±2.5	65.5±3.3	43.2±2.2
	66.7±3.3	62.3±3.1	60.2±3.0	38.7±1.9	32.4±1.6	58.9±2.9	26.5±1.3
Leu-Leu-Tyr-Glu-Glu-Pro-Val-Leu-Gly-Pro-Val-Gly-Pro-Pro-Pro-Ile-Ile-Val							
0.005	87.8±4.4	76.0±3.8	79.5±4.0	95.6±4.8	79.4±4.0	89.0±4.4	92.9±4.6
0.010	70.3±3.5	46.5±2.3	41.1±2.0	81.3±4.1	47.0±2.3	77.5±3.9	81.1±4.0
0.100	56.2±2.8	30.3±1.5	27.2±1.4	64.0±3.2	29.8±1.5	55.3±2.8	60.4±3.0
Chymotrypsin hydrolysate							
Tyr-Pro-Pro-Pro-Gly-Pro-Ile							
0.005	83.4±4.2	94.0±4.7	79.9±4.0	92.1±4.6	75.5±3.8	93.7±4.7	80.4±4.0
0.010	50.2±2.5	76.5±3.8	48.7±2.4	79.0±4.0	44.2±2.2	68.0±3.4	47.8±2.4
0.100	33.1±1.7	53.0±2.7	31.0±2.0	57.6±2.9	26.6±1.3	55.2±2.8	28.6±1.4
Lys-Glu-Asn							
0.005	92.0±4.6	81.2±4.1	95.6±4.8	77.6±3.9	79.0±4.0	94.0±4.7	92.0±4.6

0.010	69.4±3.5	52.3±2.6	75.4±3.8	47.4±2.4	53.5±2.7	74.3±3.7	71.2±3.6
0.100	47.8±2.4	32.5±1.6	52.0±2.6	29.4±1.5	30.2±1.5	51.5±2.6	49.9±2.5
Tre-Gly-Glu-Asn-His-Asp							
0.005	85.3±4.3	88.2±4.4	70.0±3.5	96.5±4.8	69.8±3.5	75.5±3.8	87.6±4.4
0.010	67.0±3.3	62.0±3.1	45.5±2.3	79.0±4.0	42.0±2.1	48.9±2.4	65.0±3.2
0.100	43.2±2.2	41.2±2.1	28.8±1.4	66.3±3.3	25.6±1.3	29.0±1.4	44.2±2.2
Thermolysin hydrolysate							
Lys-Glu							
0.005	78.6±3.9	92.1±4.6	76.9±3.8	90.0±4.5	87.5±4.4	75.5±3.8	88.4±4.4
0.010	49.0±2.4	77.6±3.9	47.0±2.3	78.3±3.9	71.1±3.6	46.2±2.3	75.0±3.8
0.100	30.7±1.5	48.0±2.4	29.5±1.5	51.1±2.6	48.7±2.4	28.8±1.4	49.9±2.5
Lys-Val-Leu-Pro-Val-Pro-Glu							
0.005	89.5±4.5	77.0±3.9	79.4±4.0	92.0±4.6	81.2±4.1	94.6±4.7	95.0±4.8
0.010	71.5±3.6	45.3±2.3	46.7±2.3	77.4±3.9	53.4±2.7	75.4±3.8	73.2±3.7
0.100	53.2±2.7	27.5±1.4	31.2±1.6	54.6±2.7	30.8±1.5	48.9±2.4	46.5±2.3
Leu-Leu-Tyr							
0.005	78.5±3.9	76.0±3.8	79.6±4.0	92.4±4.6	95.0±4.8	93.4±4.7	90.7±4.5
0.010	44.2±2.2	45.2±2.3	47.8±2.4	78.0±3.9	72.6±3.6	74.0±3.7	72.6±3.6
0.100	31.3±1.6	29.0±1.4	30.5±1.5	55.3±2.8	53.1±2.7	48.9±2.4	50.3±2.5

The data of Table 1 indicate that all of the studied peptides have antitumor properties, and with the increase of the concentration of biopeptides the survival rate of cancer cells of different lines is reduced. The biologically active peptide of the composition Trp-Tre-Ala-Glu-Glu-Lys-Glu-Leu, isolated from trypsin hydrolysate, is characterized by the most pronounced cytotoxic activity in relation to the cell lines MCF7 (the survival rate of cancer cells at 0.1 wt% peptide concentration amounts to 38.7%), 6HepG2 (the survival rate of cancer cells at 0.1 wt% peptide concentration amounts to 32.4%) and PANC-1 (the survival rate of cancer cells at 0.1 wt% peptide concentration amounts to 26.5%). The biologically active peptide of the composition Leu-Leu-Tyr-Glu-Glu-Pro-Val-Leu-Gly-Pro-Val-Gly-Pro-Pro-Pro-Ile-Ile-Val, isolated from trypsin hydrolysate, is characterized by the most pronounced cytotoxic activity in relation to the cell lines DU 145 (the survival rate of cancer cells at 0.1 wt% peptide concentration amounts to 30.3%), MDAMB-231 (the survival rate of cancer cells at 0.1 wt% peptide concentration amounts to 27.2%) and 6HepG2 (the survival rate of cancer cells at 0.1 wt% peptide concentration amounts to 29.8%).

The biologically active peptide of the composition Tyr-Pro-Pro-Pro-Gly-Pro-Ile, isolated from chymotrypsin hydrolysate, is characterized by the most pronounced cytotoxic activity in relation to the cell lines LBR2 (the survival rate of cancer cells at 0.1 wt% peptide concentration amounts to 33.1%), MDAMB-231 (the survival rate of cancer cells at 0.1 wt% peptide concentration amounts to 31.0%), 6HepG2 (the survival rate of cancer cells at 0.1 wt% peptide concentration amounts to 26.6%) and PANC-1 (the survival rate of cancer cells at 0.1 wt% peptide concentration amounts to 28.6%).

The biologically active peptide Leu-Leu-Tyr, isolated from thermolysin hydrolysate, is characterized by the most pronounced cytotoxic activity in relation to the cell lines LBR2 (the survival rate of cancer cells at 0.1 wt% peptide concentration amounts to 31.3%), DU 145 (the survival rate of cancer cells at 0.1 wt% peptide concentration amounts to 29.0%) and MDAMB-231 (the survival rate of cancer cells at 0.1 wt% peptide concentration amounts to 30.5%).

The antioxidant properties of biologically active peptides, isolated from milk proteins, were analyzed according to the method based on the determination of the capacity of the absorption of oxygen radicals (of Trolox equivalent).

The obtained results imply that all of the discussed peptides, isolated from trypsin, chymotrypsin and thermolysin casein hydrolysates, are characterized by pronounced antioxidant properties: the value of trolox equivalent for the biopeptide of the composition Trp-Tre-Ala-Glu-Glu-Lys-Glu-Leu ranges from 487 to 1,345; for the biopeptide of the composition Leu-Leu-Tyr-Glu-Glu-Pro-Val-Leu-Gly-Pro-Val-Gly-Pro-Pro-Pro-Ile-Ile-Val – from 505 to 1,287; for the biopeptide of the composition Tyr-Pro-Pro-Pro-Gly-Pro-Ile – from 490 to 1,400; for the biopeptide of the composition Lys-Glu-Asn – from 513 to 1,454; for the biopeptide of the composition Tre-Gly-Glu-Asn-His-Asp – from 475 to 1,325; for the biopeptide of the composition Lys-Glu – from 521 to 1,500;

for the biopeptide of the composition Lys-Val-Leu-Pro-Val-Pro-Glu – from 509 to 1,498; for the biopeptide of the composition Leu-Leu-Tyr – from 501 to 1,432.

The prebiotic properties of biologically active peptides, isolated from milk proteins, were analyzed in accordance with the method of Batch Culture Fermentation *in vitro* (fermentation of bacteria in one cycle). The prebiotic index (PI) was determined on the basis of the numerical change of two bacterial groups (bifidobacteria, lactobacilli).

The analysis of the obtained results showed that in the presence of the analyzed biopeptides in the process of fermentation the number of bifidobacteria and lactobacilli increases dramatically. The growth of bifidobacteria is most affected by the peptides of the composition Lys-Glu, Lys-Glu-Asn and Lys-Val-Leu-Pro-Val-Pro-Glu, the growth of lactobacilli – by the peptides of the composition Lys-Val-Leu-Pro-Val-Pro-Glu, Lys-Glu and Leu-Leu-Tyr.

For each of the studied biopeptide, the prebiotic index (PI) was calculated. The obtained results are shown in Table 2.

Table 2 – Prebiotic index of biologically active peptides, isolated from milk proteins

Peptide composition	PI
Trp-Tre-Ala-Glu-Glu-Lys-Glu-Leu	0.99
Leu-Leu-Tyr-Glu-Glu-Pro-Val-Leu-Gly-Pro-Val-Gly-Pro-Pro-Pro-Ile-Ile-Val	1.05
Tyr-Pro-Pro-Pro-Gly-Pro-Ile	1.21
Lys-Glu-Asn	1.13
Tre-Gly-Glu-Asn-His-Asp	0.95
Lys-Glu	1.75
Lys-Val-Leu-Pro-Val-Pro-Glu	1.56
Leu-Leu-Tyr	1.62

Table 2 shows that the value of the prebiotic index for the discussed biologically active peptides, isolated from milk proteins, varies within the range from 0.95 to 1.75; therefore, all the peptides have demonstrated a prebiotic effect on the microflora of the gastrointestinal tract. However, the most pronounced prebiotic properties are characteristic for such biopeptides as Lys-Glu (PI = 1.75), Leu-Leu-Tyr (PI = 1.62) and Lys-Val-Leu-Pro-Val-Pro-Glu (PI = 1.56).

The immunomodulatory properties of biologically active peptides, isolated from milk proteins, were evaluated *in vivo* on the basis of the number of antibody-forming cells (AFC) in the mouse spleen in relation to sheep erythrocytes (SE) in the modification of A.J. Cunningham [9]. An experiment was carried out on white outbred Wistar rats weighing 180-200 g. The T-cell immunity was evaluated by the simulation of delayed contact hypersensitivity (DH) to sheep erythrocytes (on the basis of the difference of the oedematous and healthy ears of mice). Sheep erythrocytes in a dose of $5 \cdot 10^8$ were used for immunization. Biologically active peptides were administered in a dose of 20 mg/kg during 7 days. The comparator agent was Oxymethyluracil in a dose of 50 mg/kg. The functional state of phagocytes was assessed on the basis of the ability of leukocytes to absorb latex particles by the morphological method in examining the smear stained using the Romanowsky-Giemsa staining technique. The intensity of phagocytosis was evaluated on the basis of the absolute phagocyte count, proceeding from the phagocytic number, phagocytic index and the total number of leukocytes per 1 l of blood.

The results of the evaluation of the impact of biologically active peptides on the humoral immune response to phagocytosis are presented in Table 3.

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Table 3 – Impact of biologically active peptides, isolated from milk proteins, on the humoral immune response (the formation of antibody-forming cells in the spleen of mice and delayed hypersensitivity to sheep erythrocytes)

Peptide	Dose, mg/kg	Delayed hypersensitivity to sheep erythrocytes	The total number of AFC in the spleen	The number of AFC per 1 mln splenocytes
Trp-Tre-Ala-Glu-Glu-Lys-Glu-Leu	20.0	28.7±4.3	36,788±6,622	365.1±54.8
Leu-Leu-Tyr-Glu-Glu-Pro-Val-Leu-Gly-Pro-Val-Gly-Pro-Pro-Pro-Ile-Ile-Val	20.0	32.4±4.9	37,023±6,664	372.0±55.8
Tyr-Pro-Pro-Pro-Gly-Pro-Ile	20.0	30.6±4.6	38,546±6,938	375.3±56.3
Lys-Glu-Asn	20.0	31.8±4.8	36,400±6,552	357.0±53.6
Tre-Gly-Glu-Asn-His-Asp	20.0	29.4±4.4	36,777±6,620	362.7±54.4
Lys-Glu	20.0	33.0±5.0	37,145±6,686	373.1±56.0
Lys-Val-Leu-Pro-Val-Pro-Glu	20.0	31.6±4.7	38,241±6,883	374.0±56.1
Leu-Leu-Tyr	20.0	32.9±4.9	36,315±6,537	354.2±53.1
Oxymethyluracil	50.0	31.4±4.7	85,205±6,816	462.5±92.5
Control sample	–	46.5±7.0	28,955±7,239	195.6±35.2

Table 4 – Impact of biologically active peptides, isolated from milk proteins, on phagocytosis

Peptide	Dose, mg/kg	Time, days	Phagocytic number, %	Phagocytic index	Absolute phagocyte count
Trp-Tre-Ala-Glu-Glu-Lys-Glu-Leu	20.0	8 16	1.95±0.29 5.43±0.81	2.24±0.24 2.16±0.32	5.10±0.97 1.95±0.20
Leu-Leu-Tyr-Glu-Glu-Pro-Val-Leu-Gly-Pro-Val-Gly-Pro-Pro-Pro-Ile-Ile-Val	20.0	8 16	1.88±0.28 5.55±0.83	2.20±0.33 2.11±0.32	5.17±1.03 2.04±0.41
Tyr-Pro-Pro-Pro-Gly-Pro-Ile	20.0	8 16	1.90±0.29 5.38±0.81	2.22±0.33 2.14±0.32	5.33±1.07 2.10±0.42
Lys-Glu-Asn	20.0	8 16	2.01±0.30 5.40±0.81	2.25±0.34 2.15±0.32	5.22±1.04 1.90±0.38
Tre-Gly-Glu-Asn-His-Asp	20.0	8 16	1.98±0.30 5.49±0.82	2.18±0.33 2.13±0.32	5.25±1.05 2.07±0.41
Lys-Glu	20.0	8 16	1.85±0.28 5.45±0.82	2.19±0.33 2.09±0.31	5.40±1.08 2.15±0.43
Lys-Val-Leu-Pro-Val-Pro-Glu	20.0	8 16	1.92±0.29 5.51±0.83	2.20±0.33 2.12±0.32	5.36±1.07 1.99±0.40
Leu-Leu-Tyr	20.0	8 16	2.05±0.31 5.47±0.82	2.23±0.33 2.11±0.32	5.30±1.06 2.08±0.42
Oxymethyluracil	50.0	8 16	3.25±0.52 4.05±0.65	2.22±0.22 2.67±0.53	7.01±1.05 3.25±0.65
Control sample	–	8 16	1.25±0.43 2.85±0.40	4.02±0.68 3.58±0.26	3.62±0.90 1.33±0.23

Table 4 shows that all the discussed biologically active peptides, isolated from milk proteins, cause an increase in the number of antibody-forming cells in the spleen of rats 1.25-1.33 times as compared to the control sample. In terms of the number per 1 mln splenocytes, the studied biologically active peptides provide augmentation of antibody response. The data of Table 2.3 also indicate that biologically active peptides, isolated from milk proteins, weaken the delayed hypersensitivity to sheep erythrocytes, which can be explained by the presence of the anti-inflammatory effect of the studied peptides.

Along with the activation of immune reactions in case of various liver damages, the oppression of some immunological parameters can be observed (reduction of the functional activity of phagocytes, a decrease in the content of T-lymphocytes). For the evaluation of the phagocytic activity, the absolute phagocyte count is most informative. In case of the administration of biologically active peptides, isolated from milk proteins, to laboratory animals, an increase in the number of phagocytizing cells (4.05-5.55) as compared to the control sample (2.85%) was observed during 16 days. The activity of phagocytes, which was evaluated on the basis of the average number of particles ingested by one cell (phagocytic index) in animals treated with the studied peptides, was similar to the phagocytic index in animals treated with Oxymethyluracil (2.13 and

2.67, respectively). On the basis of the analysis of the absolute phagocyte count, it was found that all of the studied biologically active peptides, isolated from milk proteins, have an ability to stimulate phagocytosis.

Thus, the conducted studies made it possible to optimize the conditions of the enzymatic hydrolysis of milk proteins, ensuring the generation of targeted biologically active peptides and the study of properties of bioactive peptides: immunomodulatory, cytotoxic, antioxidant, prebiotic.

CONCLUSIONS

- The authors examined the immunomodulatory, cytotoxic, antioxidant, and prebiotic properties of biologically active peptide, isolated from milk proteins. It was determined that all of the examined peptides exhibit anticancer properties; furthermore, with the increase of the concentration of biopeptides the survival rate of cancer cells of different lines is reduced. The biologically active peptide of the composition Trp-Tre-Ala-Glu-Glu-Lys-Glu-Leu, isolated from trypsin hydrolysate, is characterized by the most pronounced cytotoxic activity in relation to the cell lines MCF7, 6HepG2 and PANC-1. The biologically active peptide of the composition Leu-Leu-Tyr-Glu-Glu-Pro-Val-Leu-Gly-Pro-Val-Gly-Pro-Pro-Pro-Ile-Ile-Val, isolated from trypsin hydrolysate, is characterized by the most pronounced cytotoxic activity in relation to the cell lines DU 145, MDAMB-231 and 6HepG2. The biologically active peptide of the composition Tyr-Pro-Pro-Pro-Gly-Pro-Ile, isolated from chymotrypsin hydrolysate, is characterized by the most pronounced cytotoxic activity in relation to the cell lines LBR2, MDAMB-231, 6HepG2 and PANC-1.

- The biologically active peptide of the composition Lys-Glu-Asn, isolated from chymotrypsin hydrolysate, is characterized by the most pronounced cytotoxic activity in relation to the cell lines DU 145, MCF7 and 6HepG2. The biologically active peptide of the composition Tre-Gly-Glu-Asn-His-Asp, isolated from chymotrypsin hydrolysate, is characterized by the most pronounced cytotoxic activity in relation to the cell lines MDAMB-231, 6HepG2 and U-87. The biologically active peptide of the composition Lys-Glu, isolated from thermolysin hydrolysate, is characterized by the most pronounced cytotoxic activity in relation to the cell lines LBR2, MDAMB-231 and U-87. The biologically active peptide of the composition Lys-Val-Leu-Pro-Val-Pro-Glu, isolated from thermolysin hydrolysate, is characterized by the most pronounced cytotoxic activity in relation to the cell lines DU 145, MDAMB-231 and 6HepG2. The biologically active peptide of the composition Leu-Leu-Tyr, isolated from thermolysin hydrolysate, is characterized by the most pronounced cytotoxic activity in relation to the cell lines LBR2, DU 145 and MDAMB-231.

- It was shown that all of the discussed peptides, isolated from trypsin, chymotrypsin and thermolysin casein hydrolysates, are characterized by pronounced antioxidant properties: the value of trolox equivalent for the biopeptide of the composition Trp-Tre-Ala-Glu-Glu-Lys-Glu-Leu ranges from 487 to 1,345; for the biopeptide of the composition Leu-Leu-Tyr-Glu-Glu-Pro-Val-Leu-Gly-Pro-Val-Gly-Pro-Pro-Pro-Ile-Ile-Val – from 505 to 1,287; for the biopeptide of the composition Tyr-Pro-Pro-Pro-Gly-Pro-Ile – from 490 to 1,400; for the biopeptide of the composition Lys-Glu-Asn – from 513 to 1,454; for the biopeptide of the composition Tre-Gly-Glu-Asn-His-Asp – from 475 to 1,325; for the biopeptide of the composition Lys-Glu – from 521 to 1,500; for the biopeptide of the composition Lys-Val-Leu-Pro-Val-Pro-Glu – from 509 to 1,498; for the biopeptide of the composition Leu-Leu-Tyr – from 501 to 1,432.

- The analysis of the prebiotic activity of the discussed peptides showed that the growth of bifidobacteria is most affected by the peptides of the composition Lys-Glu, Lys-Glu-Asn and Lys-Val-Leu-Pro-Val-Pro-Glu, the growth of lactobacilli – by the peptides of the composition Lys-Val-Leu-Pro-Val-Pro-Glu, Lys-Glu and Leu-Leu-Tyr. It was shown that the value of the prebiotic index for the discussed biologically active peptides, isolated from milk proteins, varies within the range from 0.95 to 1.75; therefore, all the peptides are characterized by a high prebiotic effect on the microflora of the gastrointestinal tract.

- In the course of the study of the immunomodulatory properties of biologically active peptides *in vivo* it was found that all the discussed biologically active peptides, isolated from milk proteins, cause an increase in the number of antibody-forming cells in the spleen of rats 1.25-1.33 times as compared to the control sample. In terms of the number per 1 mln splenocytes, the studied biologically active peptides provide augmentation of antibody response. On the basis of the analysis of the absolute phagocyte count, it was found that all of the studied biologically active peptides, isolated from milk proteins, have an ability to stimulate phagocytosis.

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