

LOW-ALLERGENIC HYDROLYSATES OF WHEY PROTEINS WITH NATURAL BIOACTIVE PEPTIDES

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Introduction. Formulation of the problem

Many children are allergic to whey proteins. The number of adults who, in some form, are intolerant of whey proteins is growing steadily too [1]. One of the most common ways to reduce the allergenicity of whey proteins is the use of their hydrolysates. Low-molecular-weight proteolysis products do not cause allergies. To obtain them, various enzyme preparations of animal, microbiological, and plant origin are used. Very often, these are active enzyme preparations with broad specificity for peptide bonds. Besides, it is known that digestion involving proteases of the gastrointestinal tract is accompanied by the formation of more than 200 natural bioactive peptides that affect various functions of the human body and have a positive effect on the immune, nervous, digestive, and cardiovascular systems [2]. However, when proteases

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Abstract. Hydrolysate of whey protein concentrate (WPC) has been obtained under conditions that ensure retention of natural bioactive peptides. Prior to this, the WPC was characterised by electrophoresis, which revealed the presence of major whey proteins that can cause allergies and be precursors of bioactive peptides. The electrophoretic studies have allowed establishing that by the 120th minute, the proteolysis of the main protein allergens was almost complete. That is why this sample of WPC hydrolysate was used for further studies. Sephadex G-50 gel filtration has shown that 23.4% to 27.5% of proteolytic products soluble in trichloroacetic acid are low-molecular-weight peptides with a molecular weight up to 1500 Da, while the control WPC sample contains less than 3 % of them. The hydrolysate obtained under physiological conditions was tested for allergenicity. The study was conducted in 18 rats divided into three groups. Animals of the first group (control) were given water, the second group whey protein concentrate, the third group pancreatin hydrolysate of whey proteins. According to the results of the experiment, the concentration of IgE in the 2nd group is significantly higher compared with the control (49%), and in the 3rd group, does not differ from the control values. To detect possible sensitisation in the experimental animals, we used the specific leucocyte agglomeration reaction, the leucocyte specific lysis reaction, the values of the change in the concentration of circulating immune complexes, and the neutrophil damage index. The studies have shown that in the animals receiving WPC hydrolysate, no signs of an allergic reaction were detected, while the animals sensitised with WPC developed type I hypersensitivity (by the value of the IgE content).

Key words: whey proteins, allergenicity, proteolysis, bioactive peptides.

of microbiological and plant origin are used, these peptides may not be formed. Thus, today's important problem is how to reduce the allergenicity of whey proteins without losing the bioactivity of their proteolytic products.

Analysis of recent research and publications

An allergic reaction to milk is a very common food allergy caused by various immune mechanisms due to specific protein components present in milk [3]. Milk allergy is the most typical allergic disease in infants and children, with a frequency of 2–3% [4,5]. Milk proteins are the first ones that most formula-fed babies come in contact with. As infants consume a lot of milk, the incidence of milk allergy is the highest in children under the age of two years [6]. Cow's milk contains at least 20 potentially allergenic proteins [7]. Most milk-allergic children are sensitive to more than

one allergen. Studies on milk allergy in adults are but fewer. Allergy to milk proteins in adults is much less common than in children, but its manifestations are more serious. The study by Stöger *et al.* [8] identified skin and airways as the main target organs in adults. Symptoms of gastrointestinal (mild to moderate) and cardiovascular (severe) damage were observed less frequently in adults than in children. General allergies and hypersensitivity to milk proteins are more common now than a few decades ago.

Cow's milk protein consists of approximately 80% of casein and 20% of whey proteins. The latter include β -lactoglobulin (β -LG), α -lactalbumin (α -LA), immunoglobulin (IG), serum albumin (BSA) and lactoferrin (LF). Caseins and certain whey proteins are major allergens in the development of milk allergies. It has been found that most milk-allergic patients have specific IgE antibodies against at least two or more antigens and that the antigenicity of whey protein is much higher than that of casein [7,9]. Allergies are most often caused by such whey proteins as β -lactoglobulin and α -lactalbumin. There are but a few studies that describe allergies to other whey proteins, such as immunoglobulin, serum albumin, or lactoferrin [10].

β -LG is an exogenous protein not found in human milk, but only in the milk of ruminants. It makes up about 7–12% of milk protein. However, it is highly antigenic and resistant to gastric acid, and causes allergies to milk after it enters the intestine [11]. Even very small amounts of β -LG can cause allergic reactions [12]. It is characterised by multiple linear epitopes that are bound by IgE immunoglobulins [13]. The fragment in β -LG f 124-135 is considered the main antigenic region, which has a large capacity for binding to specific IgE from human serum [14]. The incidence of antibodies to β -LG in milk allergy is 13% to 76%, according to different studies [10].

The second most common specific IgE produced in patients with milk allergy is α -LA [15]. The frequency of antibodies to α -LA in milk allergy ranges from 27.6% to 62.8%. Maynard, Jost, & Wal [16] showed that IgE bound to native α -LA and large peptides. This indicates the importance of conformational epitopes in the development of milk allergy. Hochwallner *et al.* [17, 18] identified six IgE-reactive peptides from α -LA.

Many studies have shown that technological factors (temperature, pH) do not allow significantly reducing the allergenicity of whey proteins. A more effective way, after complete replacement of allergens, is proteolysis of whey proteins, which leads to the cleavage of the corresponding epitopes [10]. Thus, allergenicity is partially reduced by proteolytic processes during production of fermented dairy products due to the action of proteolytic systems of lactic acid bacteria [19,20]. A decrease in the allergenicity of proteins depends on the degree of their hydrolysis. Partial hydrolysates containing peptides of

different sizes are characterised by residual antigenicity, which reaches 10^{-3} – 10^{-4} relative units. The residual antigenicity of hydrolysates with a high degree of hydrolysis is 10^{-4} – 10^{-6} relative units, which is by 104–106 times less than the antigenicity of native proteins [21].

Whey protein hydrolysates with a higher degree of proteolysis are used in special purpose products, children's and sports nutrition, diets of the elderly, weight loss programmes, nutrition of people with impaired digestive function of proteolytic enzymes, as well as in the manufacture of hypoallergenic dairy products. To produce these whey protein hydrolysates, proteases of microbiological, plant, and animal origin are used. Analysis of the technologies of products with whey protein hydrolysates shows that the effectiveness of enzyme preparations is assessed mainly by the degree of proteolysis, the price of the product (the use of cheap proteases with a wide specificity of action), technological and rheological characteristics, taste and smell. However, the conditions of proteolysis and the enzyme preparations used do not always reflect the physiological conditions of cleavage of these proteins in the gastrointestinal tract. In recent decades, it has been established that whey proteins are a source of more than 200 bioactive peptides (BAP) with antimicrobial, antihypertensive, immunomodulatory, antioxidant, anticarcinogenic effects [22]. The formation of natural BAP depends on the degree of proteolysis, the specificity of proteolysis of whey propeptides, which include β -LG, α -LA, LF, and IG. Proteolytic products of whey proteins can have different molecular weights. Those with high and medium molecular weights (more than 1500 Da) can contain epitopes that cause various allergic reactions [21]. On the other hand, these peptides have practically no biological effect. Low-molecular-weight peptides (up to 15 amino acid residues) are low-allergenic and can be bioactive. The vast majority of these natural bioactive peptides are only formed by the action of digestive proteases under conditions that reflect natural digestion. So, to achieve low allergenicity of hydrolysates and retain bioactive peptides in them, proteolysis of whey proteins should be used when digestive proteases act under conditions close to those in the gastrointestinal tract.

The purpose of the research is to obtain low-allergenic hydrolysates of whey proteins under conditions that result in the formation of natural bioactive peptides.

For this purpose, it is necessary to achieve the following **objectives**:

- using the pre-established conditions of proteolysis, to obtain a hydrolysate of whey protein concentrate, and to characterise the molecular weight distribution of peptides and polypeptides in it;
- to study how allergenic is the hydrolysate obtained under physiological conditions.

Research materials and methods

Whey protein concentrate produced by the cheese factory *Buchatsky Syrzavod* (Buchach, Ukraine) according to TU U 15.5-00419880-XXX:2011 “Whey Protein Concentrate (WPC-UF). Technical specifications (WPC)” was used as a substrate for hydrolysis. Table 1 shows the physicochemical parameters of the whey protein concentrate.

Table 1 – Physicochemical parameters of the whey protein concentrate

Physicochemical parameters of the product	Value of the parameter
Mass fraction of moisture, %	7.85
Mass fraction of ash, %	4.80
Mass fraction of lactose, %	11.35
Mass fraction of fat, %	4.0
Solubility index, cm ³ of dry precipitate	0.3
Acidity, °T	16.8
Mass fraction of protein, %	72.0

The enzyme preparation Pancreatin manufactured by the PJSC *Tekhnolog* (Ukraine) was used for proteolysis.

Sephadex G-50 from *Pharmacia* (Sweden) was used for gel filtration. Gel filtration was performed in columns by the company *Reanal* (Hungary). Spectrophotometry of proteolytic products of whey protein concentrate was performed with a spectrophotometer SF-46. To determine the concentration (mg/cm³) of whey proteins and products of whey proteolysis, the known absorption coefficient ($D_{1\%}$) was used – 12.3. The concentration of proteins and proteolytic products in the chromatographic fractions was determined spectrophotometrically by absorption at the wavelength $\lambda = 280$ nm. The research results were processed mathematically and statistically using Microsoft Office Excel 2007 software packages.

The fractional composition of the WPC and proteolytic products was analysed by the rapid electrophoresis method in the anode system of homogeneous polyacrylamide gel (PAG) [23]. Quantitative processing of the electrophoregrams was performed using the function of reading graphical images imread [24].

The research on allergenicity was planned taking into account the recommendations in Paragraph 14 of the guidelines “Methodological and biological assessment of non-traditional food raw materials and new foods” (approved by Order of the Ministry of Health of Ukraine No. 204 of 09.07.97).

The experiments were conducted in 18 outbred male white rats. The animals were fed according to standard food and drinking diets. All rats were active sexually mature adults. The experimental work with animals complied with the national “General ethical principles of animal experiments” [25], which

are consistent with the provisions of European legislation [26].

Sensitisation of the experimental animals was performed by forced oral administration of 4 ml through a feeding tube 5 times a week for 4 weeks. The rats were preliminarily immunised by parenteral administration of a model protein antigen, ovalbumin. This protein is highly immunogenic for most mammalian species. To increase the intensity of the immune response, aluminium hydroxide was used as an adjuvant. Immunisation took place on the 1st day of the experiment. The rats were intraperitoneally administered 100 µg of ovalbumin adsorbed on 10 mg of aluminium hydroxide in 0.2 cm³ of normal saline [27].

To quantify the immune homeostasis of the experimental animals, circulating immune complexes (CIC) were determined spectrophotometrically [28], and the immunoglobulin E level in serum by enzyme-linked immunosorbent assay according to the instructions for the device STAT FAX PLUS-303.

Possible sensitisation in the experimental animals was established using a method of detecting the reaction of blood cells to the allergen in vitro: specific leucocyte agglomeration reaction (SLAR), specific leucocyte lysis reaction (SLLR), and neutrophil damage index (NDI).

The results were statistically processed using the Microsoft Excel software package. The compliance of the obtained data with the normal distribution law was checked according to the Shapiro–Wilk W test. The parametric data are described by Medium values (M) and standard deviations (SD), nonparametric with the help of the median (Me) and quartiles. If the data conformed to the normality of distribution, the reliability of the obtained differences of the values compared was assessed using single-factor analysis of variance (ANOVA). This was followed by Tukey’s HSD test and the Kruskal–Wallis H-test in cases where the distribution law deviated from normal. Changes with a significance level of more than 95% ($p < 0.05$) were considered significant.

Results of the research and their discussion

Whey protein concentrate was used as a substrate for proteolysis. It contains all the major whey proteins that are precursors of natural bioactive peptides (BAP). Prior to the experiments, the WPC was characterised using electrophoresis to determine the fractional composition of whey proteins. The results of rapid electrophoresis in the anode system of homogeneous PAG are presented in Fig. 1.

For comparison, whey isolated from fresh skimmed milk after isoelectric precipitation of casein was used. The results obtained indicate that the WPC contains all major proteins that can cause allergies and are precursors of natural bioactive peptides (BAP). According to densitometry, the ratio of protein fractions in WPC is close to their ratio in whey.

Proteolysis of the WPC with pancreatin was performed under physiological conditions (temperature 37°C, pH 7.9), which provide the formation of more than 90% of all known natural BAP [2] and can lead to lower allergenicity. The previously substantiated enzyme to substrate ratio (1:20) was used in the research [22,29,30]. During proteolysis, samples were taken from the reaction medium, and the content of TCA-soluble protein cleavage products (those soluble in trichloroacetic acid) was analysed by absorption at 280 nm. The course of WPC proteolysis is shown in

Fig. 2 (1). The graph makes it clear that proteolysis is the most intensive in the first 60 minutes and usually ends by the 120th minute. Electrophoretic analysis of the reaction mixture sampled at different stages of proteolysis shows that in the 120th minute of proteolysis, the cleavage of the main whey proteins is complete (Fig. 2.2).

Taking these results into account, we took a WPC hydrolysate sample in the 120th minute of proteolysis for a more detailed study of proteolytic products and of their allergenicity.

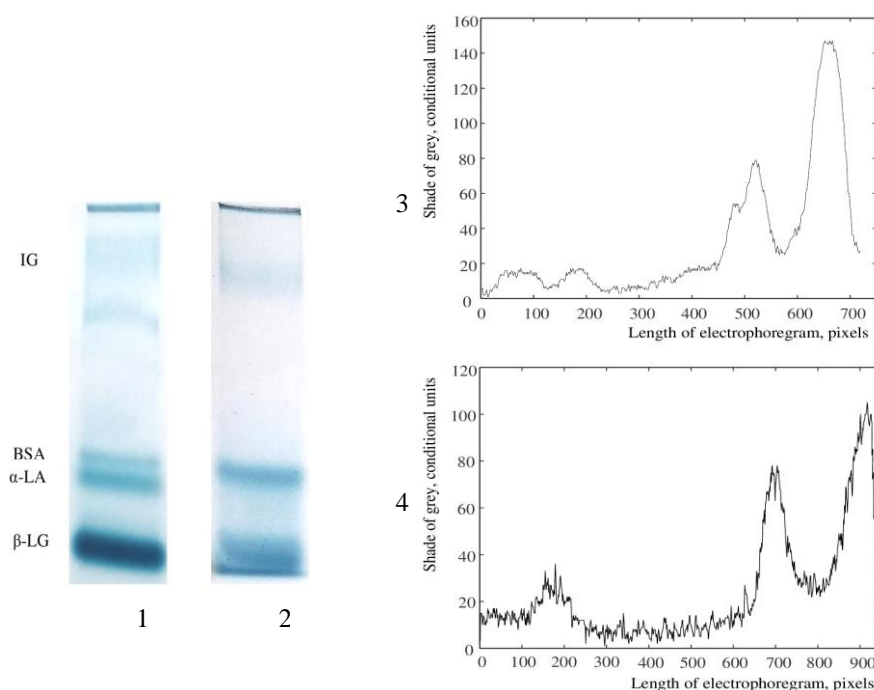


Fig. 1. Electrophoregram of proteins of milk whey (1) and WPC (2). Densitograms of the electrophoregram of whey proteins (3) and whey protein concentrate (4)

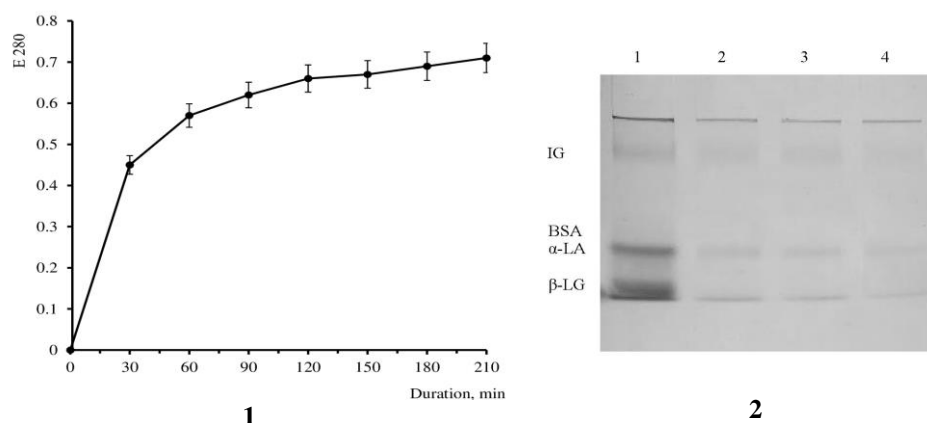


Fig. 2. Proteolysis of whey protein concentrate using pancreatin: 1 – concentration of TCA-soluble proteolytic products; 2 – electrophoregrams of the reaction mixture after 0 min (1), 60 min (2), 120 min (3), and 180 min (4) of proteolysis

An important characteristic of whey protein hydrolysates is molecular weight distribution. This characteristic is closely related to allergenicity and the formation of BAP among proteolytic products. To determine the molecular weight distribution, we performed gel filtration of the TCA-soluble proteolytic products (120 min) on a column with Sephadex G-50. WPC solution was used as a control. The results of gel filtration are shown in Fig. 3. To estimate the content of peptides and polypeptides, the chromatograms were divided into sectors taking into account the elution volume (I, II, and III). The molecular weight of protein compounds of the first sector is $>30,000$ Da, that of proteins and polypeptides of the second sector is 1500 to 30,000 Da, and that of peptides of the third sector is <1500 Da. For quantitative characteristics, the fractions of the sectors were combined, and the total content of protein compounds was determined spectrophotometrically. According to the results of three gel filtrations, it has been found that 23.4% to 27.5% of TCA-soluble proteolysis products are peptides with a molecular weight of up to 1500 Da (in the control WPC, they make up $<3\%$). Polypeptides with a molecular weight of $1500 < M < 30000$ Da make up 69.1% to 73.65% (63.1%–66.3% in the control), and there is less than 2% of proteins and polypeptides with $M > 30000$ Da (29.2% to 33.7% in the control). Thus, in the hydrolysate obtained, a significant part of

the proteolytic products are of low molecular weight (<1500 Da) and can contain natural BAP, taking into account the specificity of the proteases used and the proteolysis conditions.

The hydrolysate obtained under physiological conditions (120 min) was tested for allergenicity. The study was performed in rats divided into 3 groups: 1st group – control (were given water), 2nd group – animals that were given the whey protein concentrate (WPC); 3rd group – animals that received pancreatin hydrolysate of the whey protein concentrate produced under physiological conditions.

Throughout the experiment, the dynamics of the experimental animals' weight did not differ from this parameter in the control group, and no significant differences among the experimental groups were found either.

According to the results of the experiment, the IgE concentration in the 2nd group is significantly higher compared with the control (49%), and in the 3rd group, it does not differ from the control values (Table 2). Comparison of the obtained data with the reference values of total immunoglobulin E in healthy rats' serum has shown significant deviations of the values of this parameter [31] in the group receiving WPC. These results indicate the development of IgE-mediated allergic response in animals of the experimental group that were sensitised with WPC.

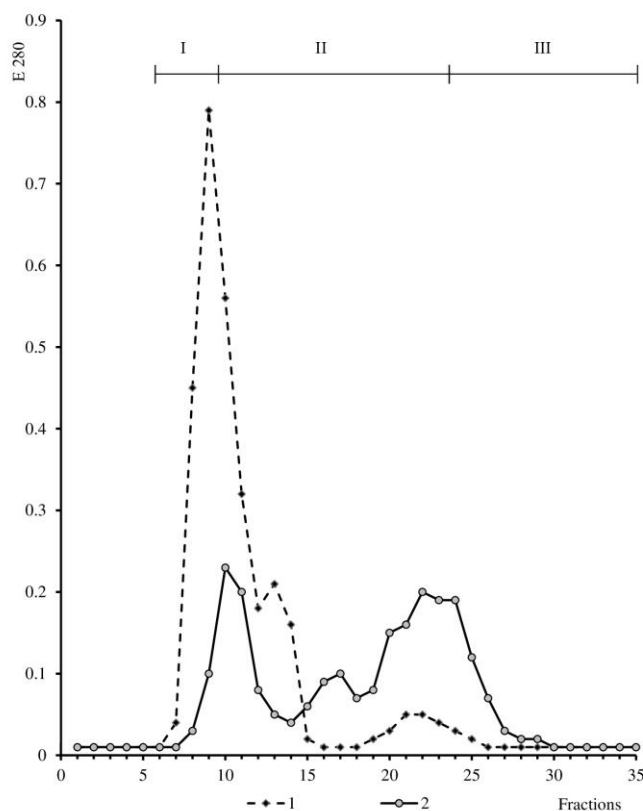


Fig. 3. Chromatogram of the whey protein concentrate (1) and proteolytic products (2) obtained by gel filtration on Sephadex G-50

Table 2 – Characteristics of the humoral component of the immune system of rats acted upon by the WPC and the WPC hydrolysate (n = 6)

Parameters	Control animals	Experimental animals	
		WPC	WPC hydrolysate
IgE, IU d/ml	1.3±0.09	1.94±0.36*	1.57±0.14
CIC, cu	40 [25%–38.50; 75%–40.75]	49 [25%–47.50; 75%–49.75]	49 [25%–41.50; 75%–49.75]

Note: *p<0.05₁₋₂

Both in the group of animals sensitised with the WPC and in the group of animals sensitised with the WPC hydrolysate, no statistically significant changes in the concentration of CIC were detected, as compared with the control, although there is a general tendency for their increase in the two experimental groups. The CIC is the product of the interaction of immunoglobulins with the corresponding specific antigen (allergen). The formation of antigen-antibody immune complex is a component of the process, which is accompanied by changes in the autoantigenic structure of tissue proteins and is aimed at neutralising and eliminating these antigens. A further direction of this process due to the influence of the preparations under study can probably be found out after longer allergen load of these preparations on the experimental animals.

The phenomenon of agglomeration is the first phase of an allergic reaction of cells. The results of the SLAR test indicate no delayed-type allergic reaction after administration of the preparations under study (Table 3). No significant differences have been found in the average group values of leucolysis of the experimental and control groups. Exceeding its critical level of 10% was not observed. Still, in the group which was treated with the WPC, 33% of the experimental animals showed a tendency for an individual increase in this parameter. That is, under the conditions of our experiment, the cellular component of the immune system did not react to influence of the WPC hydrolysate. By the results of the SLLR test, slight disruptions of immunological homeostasis

have been noted in the experimental animals of the WPC group.

The neutrophil damage index test (NDI test) is a generally accepted reliable method to diagnose changes in the structure of the nucleus and cytoplasm of neutrophils that appear in vitro under the action of an allergen [32]. This characteristic had no significant differences in its average values in all experimental groups. Yet it should be noted that in the group of animals sensitised with WPC, the critical value of spontaneous cell destruction 0.05 was exceeded in 16% of individuals. This tendency is due to the effect of the allergen on the increase in neutrophilic granulocytes with amoeboid activity and indicates specific sensitisation of the body.

Thus, the results of studying allergenicity have shown that sensitising animals with WPC per os led to the development of type I hypersensitivity in the animals of group 2 in terms of the IgE content and showed weak allergenic potential by the RSL and NDI tests. In animals of the group sensitised with WPC hydrolysate, no statistically significant changes in the parameters under study have been observed, and no signs of sensitisation detected.

Field testing of the research results. The whey protein hydrolysate obtained according to the technology developed was tested at the cheese factory *TOV Buchatsky Syrozavod*. The low-allergenic whey protein hydrolysate obtained under physiological conditions was used to develop technologies of special purpose products *Milk beverage with WPH*, *Cheese paste with WPH*, and *Soya product with WPH*.

Table 3 – Results of in vitro allergy tests in rats sensitised with the WPC and the WPC hydrolysate (n=6)

Parameters	Control animals	Experimental animals	
		WPC	WPC hydrolysate
SLLR H %	0/6 7.08±1.9	2/6 8.33±1.4	0/6 6.2±2.0
SLAR H %	0/6 1.1 [25%–1.10; 75%–1.1]	0/6 1.3 [25%–1.30; 75%–1.40]	0/6 1.2 [25%–1.20; 75%–1.20]
NDI, H %	0/6 0.04±0.01	1/6 0.04±0.02	0/6 0.03±0.005

Note: H – in the numerator, the number of animals with a positive result, in the denominator – all in the experiment

Conclusion

As a result of the research, the following conclusions can be drawn:

- at physiological values of the temperature (37°C) and pH (7.9) and with the enzyme to substrate ratio 1:20, pancreatin hydrolysate of whey protein concentrate was obtained under conditions used to obtain natural bioactive peptides;
- by electrophoretic and chromatographic studies, it has been found that this hydrolysate of whey proteins does not contain allergenic whey proteins, has the

degree of hydrolysis 71%, and contains about 25% of low-molecular-weight peptides ($M < 1500$ Da);

- the hydrolysate of whey proteins obtained under physiological conditions was tested for allergenicity, and it has been established that in animals of the group sensitised with WPC hydrolysate, there were no statistically significant changes observed in the parameters under study and no signs of sensitisation detected, which indicates the reduced allergenicity of this hydrolysate.

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НИЗЬКОАЛЕРГЕННІ ГІДРОЛІЗАТИ БІЛКІВ СИРОВАТКИ МОЛОКА З ПРИРОДНИМИ БІОАКТИВНИМИ ПЕПТИДАМИ

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Анотація. У роботі отримано гідролізат концентрату сироваткових білків (КСБ) в умовах, що забезпечують збереження природних біологічно активних пептидів. Попередньо КСБ охарактеризовано з допомогою електрофорезу і встановлено наявність у ньому основних білків сироватки, котрі можуть викликати алергію та бути попередниками біологічно активних пептидів. За допомогою проведених електрофоретичних досліджень, встановлено, що до 120-ї хвилини протеоліз основних білків-алергенів практично повністю завершується. Тому для подальших досліджень було використано цей взірць гідролізату КСБ. За допомогою проведеної на сефадексі G-50 гель-фільтрації, показано, що серед розчинних у трихлороцтовій кислоті продуктів протеолізу від 23,4% до 27,5% становлять низькомолекулярні пептиди з молекулярною масою до 1500 Да, у той час як у контрольному КСБ <3%. Отриманий у фізіологічних умовах гідролізат було досліджено на алергенність. Дослідження проводили на 18 щурах, котрі були розділені на три групи. Тварини першої групи відносились до контрольної і отримували воду, другої – концентрат сироваткових білків, третьої – панкреатинний гідролізат білків сироватки. За результатами експерименту концентрація IgE в 2-й групі має вище значення порівняно з контролем (на 49%), а в 3-й групі не відрізняється від контрольних значень. Для виявлення можливої сенсibiлізації у експериментальних тварин використовували реакцію специфічної агрегації лейкоцитів, реакцію специфічного лізису лейкоцитів, значення зміни концентрації циркулюючих імунних комплексів та показник пошкодження нейрофілів. За результатами досліджень встановлено, що у тварин, що отримували гідролізат КСБ, ознак алергічної реакції не виявлено у той час, як у тварин сенсibiлізованих КСБ спостерігався розвиток гіперчутливості I типу за показником вмісту IgE.

Ключові слова: протеїни сироватки, алергенність, протеоліз, біоактивні пептиди.