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GEL FILTRATION OF COW MILK WHEY PROTEINS

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Abstract. The work considers the isolation of homogeneous precursor proteins of biologically active peptides from milk whey by gel filtration, in conditions that maximally ensure the preservation of their structure, composition, and properties. Considering the range of molecular masses of the main precursor proteins, the sephadex G-100 was selected for the gel filtration. As a result of the gel filtration of milk whey on a column with this sephadex, three peaks have been obtained, one of which was asymmetric and divided into two sectors. In total, four sectors from the three peaks have been received. Further, an electrophoretic analysis in the polyacrylamide gel of the proteins composition of all sectors was carried out. Sector A (the first peak) included immunoglobulins, lactoferrin, serum albumin. Sectors B and C (the second peak) consisted of β-lactoglobulin and α-lactalbumin in different ratios. The components of sector D (the third peak) had a small molecular weight and did not contain proteins. The next stage of the work was obtaining homogeneous lactoproteins. To this end, another gel filtration of the combined fractions of sectors A, B, and C was performed. Each of the chromatographic peaks obtained was divided into three ranges for the analysis of the proteins composition. Analytical electrophoresis of the combined chromatographic fractions of each range has shown that in six ranges out of nine, homogeneous precursor proteins of bioactive peptides were present. As a result of this repeated gel filtration on sephadex G-100, two homogeneous fractions (β-lactoglobulin, immunoglobulins) were obtained, which together, based on the results of the three gel filtrations, composed 59% of the whole milk whey protein. The processing of electrophoregrams, with the use of the image reading function imread, has shown high homogeneity of the fractions obtained (immunoglobulins > 90%, and β-lactoglobulin >94%). These fractions were used to develop a biotechnology for obtaining and studying bioactive antihypertensive and bactericidal peptides from milk whey proteins.

Key words: gel filtration, milk whey protein, bioactive peptides.

ГЕЛЬ-ФІЛЬТРАЦІЯ ПРОТЕЇНІВ СИРОВАТКИ КОРОВ'ЯЧОГО МОЛОКА

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Анотація. У роботі досліджено виділення гомогенних протеїнів-попередників біологічно активних пептидів з сироватки молока, з використанням гель-фільтрації, в умовах, які максимально забезпечують збереження їхньої структури, складу і властивостей. Враховуючи діапазон молекулярних мас основних протеїнів-попередників, для проведення гель-фільтрації було вибрано сефадекс G-100. В результаті гель-фільтрації сироватки молока, на колонці з цим сефадексом було отримано три піки, з яких один асиметричний було розділено на два сектори. Всього отримано чотири сектори з трьох піків. Далі було проведено електрофоретичний аналіз в поліакриламідному гелі протеїнового складу всіх секторів. Сектор А (перший пік) включав імуноглобуліни, лактоферин, альбумін сироватки. Сектори В і С (другий пік) складалися з β-лактоглобуліну і α-лактальбуміну в різних співвідношеннях. Компоненти сектору D (третій пік) мали малу молекулярну масу і не містили протеїнів. Наступним етапом роботи було виділення гомогенних протеїнів сироватки. Для цього було проведено повторну гель-фільтрацію об'єднаних фракцій секторів А, В і С. Кожен з отриманих хроматографічних піків для аналізу протеїнового складу було поділено на три діапазони. Аналітичний електрофорез об'єднаних хроматографічних фракцій кожного діапазону показав, що в шести із дев'яти діапазонів були гомогенні протеїни-попередники біоактивних пептидів. В результаті проведеної таким чином повторної гель-фільтрації на сефадексі G-100 було отримано дві гомогенні фракції (β-лактоглобулін, імуноглобуліни), які разом за результатами трьох гель-фільтрацій становили 59% від всього протеїну молочної сироватки. Обробка електрофореграм з використанням функції зчитування графічних зображень imread показала високий ступінь гомогенності отриманих фракцій (імуноглобуліни >90% і β-лактоглобулін >94%). Ці фракції були використані для розробки біотехнології виділення і дослідження біоактивних антигіпертензивних і бактерицидних пептидів з протеїнів сироватки молока.

Ключові слова : гель-фільтрація, протеїни сироватки молока, біоактивні пептиди.

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Introduction. Formulation of the problem

Proteins of milk whey are characterized by different types of biological activity. In recent years it has been established that the products of their proteolysis by gastrointestinal tract enzymes are also biologically active towards different physiological systems of the body. By now, more than a hundred bioactive peptides (BAP) from milk whey proteins have been discovered. They have a

positive effect on the immune, nervous, digestive, and cardiovascular systems. To obtain such peptides and create functional foods with a certain biological effect, it is necessary to use certain purified fractions of milk whey proteins. The existing methods of obtaining are either very complex and time-taking or do not provide proper purification. Often they are associated with using extreme factors that cause changes in the structure and chemical composition of lactoproteins.

Considering significant differences in the molecular masses of milk whey proteins, a promising method for their obtaining is gel filtration. This method allows fractionating in wide ranges of pH, ionic strength, and temperature, thus making it possible to reproduce the native conditions and retain the structure and chemical composition of proteins to the greatest possible extent. The results obtained by gel filtration can be used to isolate homogeneous precursor proteins of BAP from milk whey.

Analysis of recent research and publications

It has been established that, unlike caseins, milk whey proteins, besides their function of providing the body with amino acids, are characterized by different types of biological activity. These include immune protection (immunoglobulins); regulation of lactose synthesis in the mammary gland, transport of calcium ions, immunomodulatory and anti-carcinogenic action (α-lactalbumin); transport of fatty acids and retinol, antioxidant action (β-lactoglobulin); iron ions binding, antimicrobial and antioxidant action (lactoferrin) [1]. Besides, further studies have shown that the main milk whey proteins β-lactoglobulin (β-LG), α-lactalbumin (α-LA), serum albumin (BSA), lactoferrin (LF), and immunoglobulin (IG) are precursors of bioactive peptides. A lot of amino acid residues of the primary structure of β-LG (>50%) and α-LA (>39%) are part of different BAP [2,3]. Among BAP obtained from milk whey proteins, such peptides have been found as inhibitors of the angiotensin-converting enzyme (ACE), intestinal motility regulators, peptides with opioid, antimicrobial, immunomodulatory, and hypocholesterolemic effect, appetite regulators, and others [4,5].

Most bioactive peptides during proteolysis can be formed from the β-LG fraction. These include LGDT-1 (bactericidal action), β-lactosine B (ACE inhibitor), β-lactokinin (ACE inhibitor), and others. Some BAP from β-LG exhibit two or more different biological activities [6]. Thus, β-lactorfin is both an ACE inhibitor and an opiate agonist, and β-lactotensin regulates intestinal motility and can inhibit ACE activity. Among the BAP from α-LA, there are bactericidal, antihypertensive, and immunomodulatory peptides, but no peptides with hypocholesterolemic action and peptides that affect intestinal motility [2,3]. The highest specificity is that of lactoferrin BAP. With a few exceptions (lactofericin B), most lactoferrin BAP have bactericidal effects. Lactoferricin B is multifunctional. Besides being bactericidal, it is also immunomodulatory in action, affects the development of cancer cells, induces apoptosis in human cancer cells (THP-1) and inhibits the development of metastases from melanoma and lymphoma cells in mice [7]. From the serum albumin fraction, only two bioactive peptides – albutensin and serofin – have been obtained, which together amount less than 3% of the primary structure of BSA [2]. Albutensin belongs to multifunctional BAP. It is also an inhibits ACE and stimulates the intestine contraction. Serofin acts as a μ-type opioid receptor agonist.

This information indicates that there are quite a lot (over one hundred) of BAP, with different biological effects, that can be formed from milk whey precursor proteins. Among them, by the type of biological action, lactokinins are characterized most completely [8]. These peptides can inhibit ACE activity. The high activity of this enzyme is one of the causes of arterial hypertension, which is a major risk factor for the development of cardiovascular disease. ACE inhibitors are important in the treatment and prevention of hypertension. Lactokinins can penetrate the bloodstream and have an antihypertensive effect (but with a significant part of the lactokinins broken down by the proteases of the intestinal and blood epithelium and losing their activity). Another mechanism of vasodilatation by lactokinins is stimulating peripheral opioid receptors of the gastrointestinal tract [9]. In this case, BAP no longer need to penetrate the bloodstream. Several products based on antihypertensive peptides from milk whey proteins have been developed for hypertensive people. The most famous of them is Bio Zate of Davisco Company (USA). Its regular use allows lowering blood pressure by 8 mm Hg [10].

Lactorfins from lactoproteins belong to atypical opioid peptides [11]. Their action is similar to that of endogenous ligands, and its mechanism still lacks a full explanation. It has been shown that lactorfins can effect on the intestinal transport of electrolytes, inhibit intestinal peristalsis, act as analgesics, and influence the emotional state [12]. Immunomodulatory peptides (mainly from α-LA) effect on the activation of lymphocytes, antibody synthesis, the activity of natural killer cells, the regulation of the cytokines synthesis. These peptides are also able to increase the immunity of the cells of gastrointestinal mucosa and reduce allergic reactions [11]. A lot of antimicrobial peptides (mainly from LF and α-LA) are found among milk whey proteins [3,13]. These peptides can inhibit the development of many types of pathogenic microorganisms. It is important that lactic acid bacteria are resistant to their action. Several mechanisms of bactericidal and fungicidal action of antimicrobial peptides are suggested: destruction of the bacteria cell membrane; formation of additional ion channels in the membranes; release of lipopolysaccharides from the cell walls of Gram-positive bacteria; change in the ultrastructure of microscopic fungi [14]. Such promising types of the biological action of BAP as their effect on oxidation-reduction, lipid metabolism, appetite regulation, cytomudulatory and anti-carcinogenic action are still being studied [15,16].

Up today, despite a lot of information about the useful effects of BAP from milk proteins, just a few commercial functional products or ingredients have been produced on their basis. First of all, it is because the mechanisms of this additional function of milk whey pro-

teins need to be further studied in detail, and besides, accessible methods for obtaining BAP and their precursor proteins need to be developed. Taking into account the large number of different BAP, as well as the specific features of their distribution among the primary structures of milk whey proteins, it is necessary to isolate separate homogeneous precursor proteins. Also, in the process of obtaining and purifying the proteins fractions, it is necessary to minimize changes in their structure and chemical composition.

Existing methods for obtaining individual purified proteins from milk whey are complex, multi-stage, and unsuitable for scaling up and applying in industrial production [17-19]. It can result in denaturation of lactoproteins. In this regard, gel filtration can be a promising fractionation method. It is rarely used to obtain nutrients, but its use for the biologically active compounds can be quite reasonable. Gel filtration does not belong to high-resolution methods, but it has significant advantages [20]. These are the least possible damage to the molecules during fractionation, and the possibility of fractionation in wide ranges of pH, temperature, and ion composition. Thus, conditions are provided for the preservation of the structure and chemical composition of proteins. The properties of milk whey proteins, unlike those of caseins, allow effective gel filtration for their fractionation. These are: a big difference in the molecular weights – IG (>150000 Da), LF (76110 Da), BSA (66399 Da), β-LG (18363 Da) and α-LA (14178 Da); the typical globular structure of molecules; good solubility and no tendency aggregation in physiological conditions [6]. Gel filtration has been successfully used to isolate simultaneously native casein micelles, total lactoprotein, and low-molecular components of the proteose-peptone fraction. However, it is not effective enough to obtain purified individual proteins from milk whey [16]. To make gel filtration more effective, repeated separation is used, as well as separation of chromatographic peaks into sectors. This approach has allowed obtaining purified fractions of caseins, which are very similar in their molecular masses [21].

The **purpose** of our work is obtaining homogeneous precursors of bioactive peptides from milk whey proteins by gel filtration.

To achieve this goal, the following tasks must be solved:

– obtaining milk whey proteins after isoelectric precipitation of caseins;

– repeat gel filtration to isolate purified precursor proteins of BAP;

– analysis of the composition of proteins from chromatographic fractions and the individual sectors of chromatographic peaks.

Research materials and methods

Milk whey proteins were isolated from fresh skimmed milk by precipitating casein complex proteins at an isoelectric point. In some cases, for analytical purposes, milk whey proteins, after isoelectric precipitation of caseins, were purified from low molecular compounds and transferred to the appropriate electrophoresis buffer by gel filtration on Sephadex G-25 (Pharmacia) [22].

The concentration of milk whey proteins was determined spectrophotometrically at the wavelength λ=280 nm. To calculate the concentration, the previously established absorption coefficients were used ($D_{1cm}^{1\%}$): 12.3 for the total milk whey protein; 19.6 for β-LG, and 20.1 for α-LA [6]. Gel filtration of skimmed milk and milk whey proteins was performed on the dextran gels (the "Pharmacia" company) on the columns from a liquid chromatography kit (the "Reanal" company). The size of the column was 1.5 cm×70 cm, the volume of the column was 120 ml, the volume of the fractions was 4 ml.

To analyze the protein composition of chromatographic fractions and evaluate the homogeneity of milk whey proteins in the selected sectors of chromatographic peaks, disc-electrophoresis in the native conditions was used [23]. Electrophoresis in polyacrylamide gel (PAG) plates was performed on a modified apparatus of the Studier type, and in PAG columns, on the apparatus of the "Reanal" company. The gels were coloured and fixed by traditional methods [24]. Quantitative processing of electrophoregrams was carried out by constructing the densitograms with the image reading function imread [25].

Results of the research and their discussion

The analysis of the molecular masses of the major BAP precursors from milk whey proteins has shown that their values are within the range 14000 to 161000 Da [16]. This fractionation range can be provided by two types of dextran gels: G-100 (5000–150000 Da) and G-150 (5000–300000 Da) [20]. Sephadex G-100 was chosen for the first gel filtration of milk whey, because it provides better separation in the range of low molecular weights (<50000 Da). This range includes the two major precursors of BAP – β-LG and α-LA [6].

The results of milk whey gel filtration on Sephadex G-100 are shown in Fig. 1 (1). On the chromatogram, there are three peaks, the second of which is asymmetric and obviously consists of two. The first peak is eluted with a volume close to the free volume of the column (high molecular fractions of proteins), and the third – with a volume equal to the total volume of the column (low molecular components). Due to the shape of the second peak, it was divided into two parts to determine the protein composition. As a result, the chromatographic fractions of the four sectors, which were marked on the chromatogram: A, B, C, and D, were combined for the analysis. The combined fractions were lyophilically dried and analysed by disc disc-electrophoresis. As a control, a preparation of total milk whey protein was used. It was obtained after the precipitation of casein from skimmed milk and separation of low molecular components by gel filtration on the sephadex G-25. The proteins concentration in the samples of milk whey and its fractions for the electrophoresis was set 0.5% by spectrophotometry with the use of appropriate absorption coefficients at the wavelength λ=280 nm.

The results of the electrophoresis are shown in Fig. 1 (2). The characteristic separation of the control preparation of milk proteins in this electrophoretic system (track 1) can be seen in the electrophoregram.

The first chromatographic fraction (sector A) consists of high molecular weight IG, and besides, contains LF and BSA. The combined fractions of sectors B and C include β-LG and α-LA in different ratios. Components of sector D consist of proteose-peptone fraction (PPF) compounds that are not fixed in the PAG. It should be noted here that during gel filtration of milk whey, proteins, along with their fractionation, are also purified from low molecular components. At the same time, this low molecular fraction is a valuable source of many natural BAP of milk [26]. Consequently, the first gel filtration has resulted in obtaining no homogeneous lactoprotein.

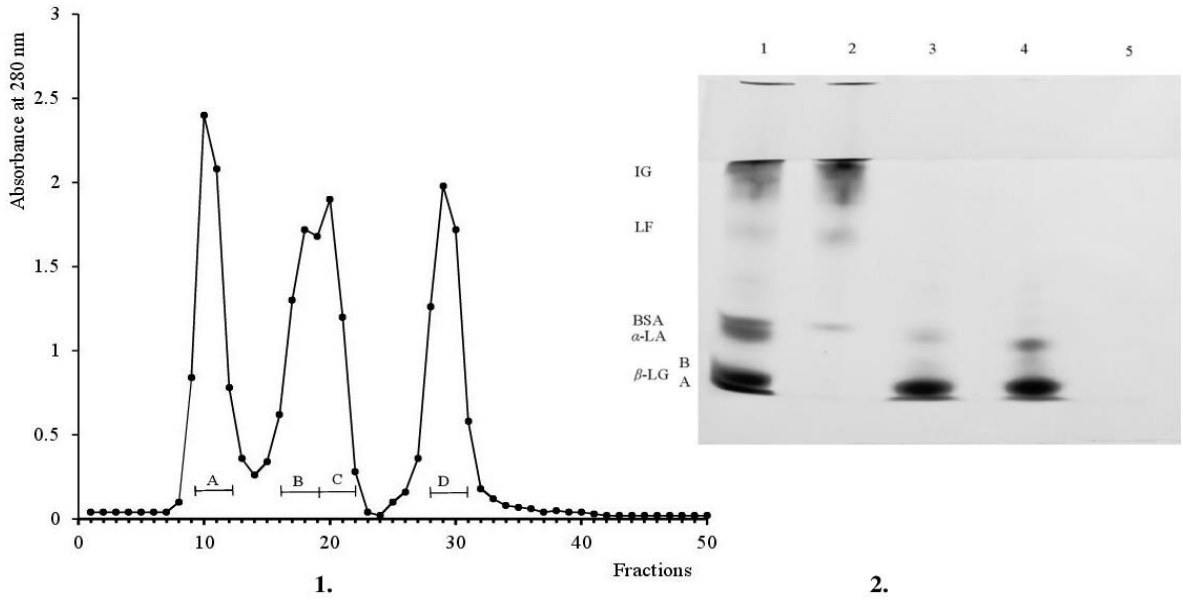


Fig. 1. Chromatogram (1) of milk whey proteins obtained as a result of gel filtration on Sephadex G-100. Electrophoregram (2) of the total milk whey protein (2.1) and the combined fractions of sector A (2.2), sector B (2.3), sector C (2.4), and sector D (2.5).

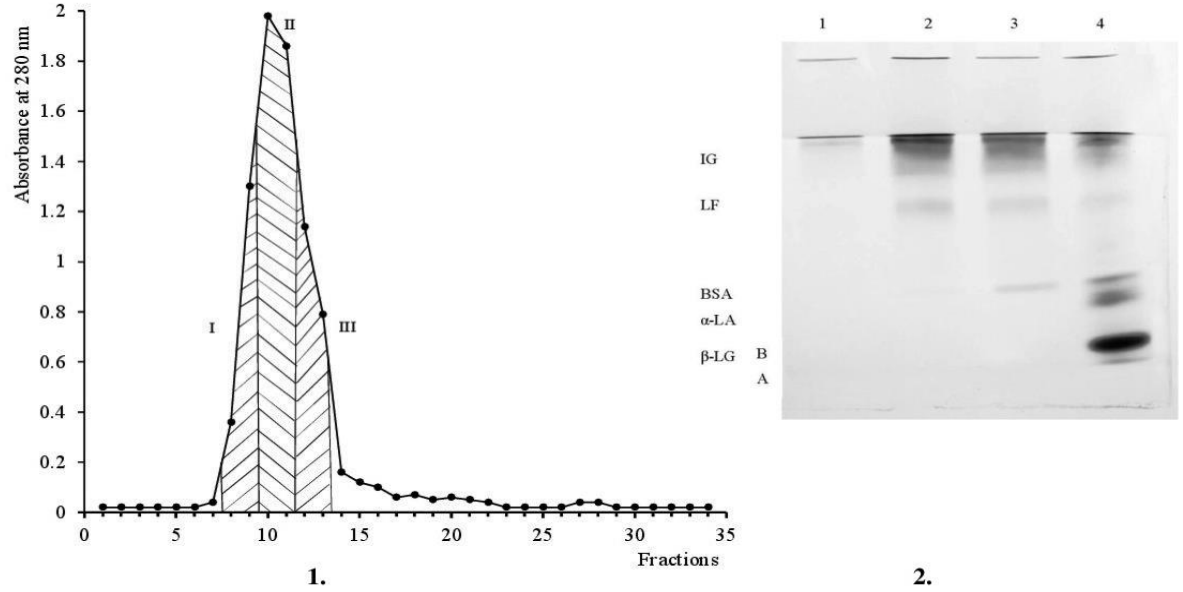


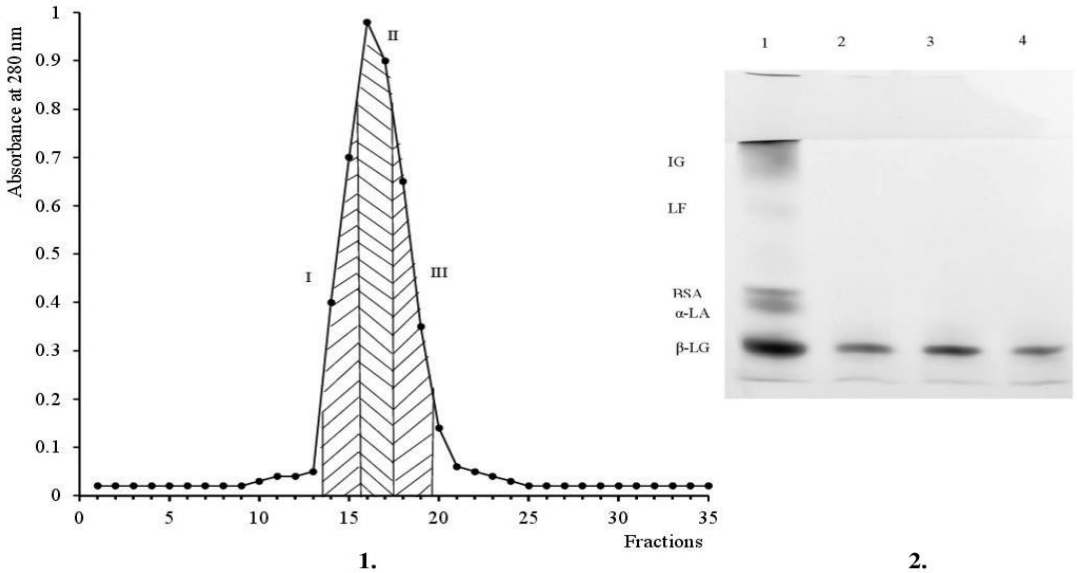
Fig. 2. Chromatogram (1) of the second gel filtration of the combined fractions of sector A on Sephadex G-100. Electrophoregram (2) of total milk whey protein (2.4) and combined fractions in ranges I (2.3), II (2.2), and III (2.1).

For the second gel filtration, samples of the combined fractions of sectors A, B, and C were taken. The results of gel filtration of the proteins of the sector A fractions are shown in Fig. 2 (1). To analyse the protein composition, the chromatographic peak obtained was divided into three ranges (I, II, and III). The combined fractions of these ranges were analysed by disc-electrophoresis (Fig. 2.2). In range I, only the immunoglobulins with

the highest molecular weight (IG M and IG A) were detected. Range II includes all immunoglobulins and, besides, the LF fraction. Range III includes, additionally, the BSA fraction.

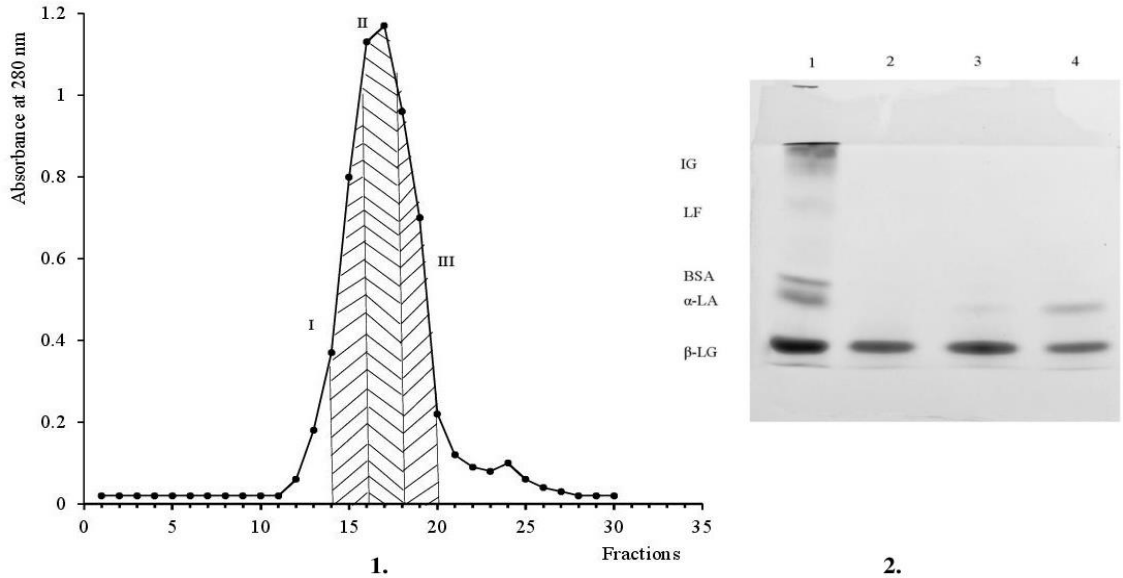
By the results of the disc-electrophoresis, all the three ranges (I, II, III) of the second gel filtration of the combined sector B fractions consist of homogeneous β-LG (Fig. 3). The degree of homogeneity was determined by the area of the peaks on the densitogram, as described earlier [25]. For β-LG, the homogeneity was more than 94%.

The chromatogram of the second gel filtration of the sector C proteins and the character of selecting samples for electrophoretic analysis are shown in Fig. 4.1. As seen in the electrophoregram (Fig. 4.2), range I of this chromatographic peak includes homogeneous β-LG too, and range II, besides β-LG, contains traces of α-LA. Range III includes a mixture of α-LA and β-LG (Fig. 4.2).



1. **2.**

Fig. 3. Chromatogram (1) of the second gel filtration of the combined sector B fractions on Sephadex G-100. Electrophoregram (2) of the total milk whey protein (2.1) and the combined fractions of ranges I (2.2), II (2.3), and III (2.4).



1. **2.**

Fig. 4. Chromatogram (1) of the repeated gel filtration of the combined sector C fractions on Sephadex G-100. Electrophoregram (2) of the total milk whey protein (2.1) and the combined fractions of ranges I (2.2), II (2.3), and III (2.4).

Thus, as a result of the first gel filtration of milk whey, three chromatographic peaks have been obtained, none of which, even after dividing into four sectors (A, B, C, and D), has any homogeneous protein fractions. The second gel filtration, with ranges of chromatographic peaks isolated, has allowed obtaining electrophoretically homogeneous IG and β-LG fractions. Processing the electrophoregrams with the image reading function im-

read has shown high homogeneity of the IG (>90%) and β-LG (>94%) fractions obtained.

According to the results of the three repeated gel filtrations, 59% of proteins in the composition of homogeneous fractions have been isolated. An important thing is that the main precursor of the BAP, β-LG [2], has been obtained. In order to separate homogeneous α-LA, in the future, it may be practical to use sephadexes G-50 or G-75 for the repeated gel filtration of sector C fractions. Another approach may consist in a second gel filtration in a weak acid medium (pH<5.5), where β-LG molecules form octamers [6].

Validation of the research results. The milk whey proteins fractions obtained are used in the laboratory of milk biochemistry of the Ternopil Ivan Puluj National Technical University for limited proteolysis as precursors of bioactive peptides, in particular, antihypertensive and bactericidal ones.

Conclusion

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

– gel filtration on the Sephadex G-100 can be used to isolate the precursor proteins of natural bioactive peptides from milk whey;

– it is practical to carry out the second gel filtration with dividing chromatographic peaks into sectors to increase the separation efficiency of milk whey proteins;

– by the second gel filtration on Sephadex G-100 two electrophoretically homogeneous fractions have been obtained, which make up 59% of the total protein of milk whey.

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