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ELECTROPHORETIC SYSTEMS FOR PREPARATIVE FRACTIONATION OF PROTEIN PRECURSORS OF BIOACTIVE PEPTIDES FROM COW'S MILK

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Abstract. The article considers the possibility of obtaining purified fractions-precursors of bioactive peptides from milk proteins by the method of preparative electrophoresis. To choose an electrophoretic system, a comparative study has been carried out of four methods of electrophoresis in polyacrylamide gel that are used to analyse milk proteins (disc-electrophoresis without disaggregating agents, and disc-electrophoresis in the presence of sodium dodecylsulfate in homogeneous and gradient gel, and electrophoresis in homogeneous gel with urea). Electrophoresis of the total milk protein has shown that none of these systems allows separating effectively all protein precursors of bioactive peptides. The next stage was obtaining two main groups of milk proteins – caseins and serum proteins for electrophoretic fractionation. With the help of analytical electrophoresis, it has been established that each of the obtained groups had a typical proteins composition. Then, the proteins groups obtained were fractionated by preparative electrophoresis using the four electrophoretic systems listed above. In this case, the casein proteins that differ in the primary structure (α_{S1}-, α_{S2}-, β-, and ϰ-caseins) can be effectively separated by preparative electrophoresis on the basis of a homogeneous gel system in the presence of urea. The composition of this electrophoretic system was simplified. Unlike the analytical variant of a homogeneous polyacrylamide gel system, the toxic 2-mercaptoethanol was excluded, and the urea concentration was reduced. For the fractionation of serum proteins, a disc-electrophoresis without disaggregating agents can be used as a basis. It allows obtaining the main precursors of bioactive peptides from milk serum proteins: β-lactoglobulin, α-lactalbumin, serum albumin, and immunoglobulins. The protein precursors obtained by preparative electrophoresis were used to develop the biotechnology of obtaining bioactive phosphopeptides and inhibitors of the angiotensin-converting enzyme.

Key words: preparative electrophoresis, milk proteins, bioactive peptides.

ЕЛЕКТРОФОРЕТИЧНІ СИСТЕМИ ДЛЯ ПРЕПАРАТИВНОГО ФРАКЦІОНУВАННЯ ПРОТЕЇНІВ – ПОПЕРЕДНИКІВ БІОАКТИВНИХ ПЕПТИДІВ З КОРОВ'ЯЧОГО МОЛОКА

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Анотація. У роботі розглянуто можливість отримання очищених фракцій-попередників біоактивних пептидів з протеїнів молока методом препаративного електрофорезу. Для вибору електрофоретичної системи проведено порівняльне дослідження чотирьох методів електрофорезу в поліакриламідному гелі, які використовуються для аналізу протеїнів молока (диск-електрофорез без дезагрегуючих агентів, диск-електрофорез у присутності додецилсульфату натрію в однорідному і градієнтному гелі та електрофорез в однорідному гелі з сечовиною). Електрофорез загального протеїну молока показав, що жодна із вказаних систем не дозволяє ефективно розділити всі протеїни-попередники біоактивних пептидів. Наступним етапом було виділення для електрофоретичного фракціонування двох основних груп протеїнів молока – казеїнів та протеїнів сироватки. Аналітичним електрофорезом встановлено, що кожна з виділених груп мала характерний склад протеїнів. Далі отримані групи протеїнів препаративно фракціонували за допомогою чотирьох перелічених електрофоретичних систем. При цьому ефективне розділення протеїнів казеїнового комплексу, які відрізняються первинною структурою (α_{S1}-, α_{S2}-, β- і ϰ- казеїни) було досягнуто препаративним електрофорезом на основі однорідної системи гелю у присутності сечовини. Склад цієї електрофоретичної системи був спрощений. Із однорідної системи поліакриламідного гелю, у порівнянні з аналітичним варіантом, було виключено токсичний 2-меркаптоетанол і зменшено концентрацію сечовини. Для фракціонування протеїнів сироватки молока взято за основу диск-електрофорез без дезагрегуючих агентів. Він дозволив отримати основні попередники біоактивних пептидів з протеїнів сироватки молока: β-лактоглобулін, α-лактоальбумін, альбумін сироватки та імуноглобуліни. Отримані препаративним електрофорезом протеїни-попередники використано при відпрацюванні біотехнології виділення біоактивних фосфопептидів та інгібіторів ангіотензин-перетворювального ферменту.

Ключові слова: препаративний електрофорез, протеїни молока, біоактивні пептиди.

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Introduction. Formulation of the problem

The promising direction of using milk proteins is obtaining natural biologically active peptides (BAP) from them and creating products on their basis that can positively affect the cardiovascular, nervous, immune, and digestive systems of the organism. The difficulty of extracting BAP from milk proteins for manufacturing such products is because there are too many of them (more than 400 different BAP), and they are unevenly arranged in the primary structure of protein fractions. Also, peptides with similar effect from different fractions can differ in their primary structure, activity, and mechanism of influencing the biological function. In addition, some fractions contain very few sequences that correspond to the structure of certain BAP. That is why, to isolate such peptides, it can be helpful to carry out limited proteolysis not of the total milk protein, but of the purified fractions – precursors of BAP.

Existing methods of extracting protein fractions from milk do not provide a sufficient degree of homogeneity or are multi-stage and laborious tasks. Often they can only be used to extract one or a few fractions. In this regard, it is topical to research the methods of obtaining and purifying certain milk proteins. One of such methods can be preparative electrophoresis. The advantages of electrophoresis are high resolution, one-stage purification, the reusability of an electrophoretic system. The obtained results of selecting the electrophoretic system can be used to isolate homogeneous protein precursors of bioactive peptides from milk.

Analysis of recent research and publications

By now, many studies have shown that milk proteins are precursors of biologically active peptides that can influence various physiological systems of the body [1-3]. In particular, among the identified ones, there are agonists and antagonists of opiate receptors (casomorphins, casoxins, lactorphins), angiotensin converting enzyme (ACE) inhibitors (casokinins, lactokinins, lactosins), antithrombotic peptides (casoplatelins), immunomodulatory peptides, mineral binding peptides (casophosphopeptides), and peptides that inhibit the development of pathogenic microorganisms. Studies of BAP from milk proteins are actively conducted nowadays, and new activity types are detected. Thus, in Hans Meisel's laboratory, anti-inflammatory peptides were obtained among the proteolytic products of milk proteins [4]. It was shown that casophosphopeptides, besides the assimilation of bivalent metal ions, can inhibit the growth of cancer cells of the glioblastoma and human adenocarcinoma [5]. In some cases, it has been proved that BAP from milk proteins can simultaneously have several different biological actions. Also, in a number of papers, biological activity is shown at the level of milk proteins [6]. They affect the assimilation of nutrients and the appetite, the development of the gastrointestinal tract, protect from pathogenic bacteria and viruses. It should be noted that, despite a large number of studies in this area, in many cases, the mechanism of biological action of bioactive peptides and milk proteins is not finally established and needs further study.

The existence of so many BAP (more than 300 in the structure of caseins, and more than 100 in the structure of milk serum proteins) is not accidental. These peptides occupy a significant part of the primary structure of milk proteins (about 50% in β-lactoglobulin, and more than 70% in caseins) [7]. BAP from milk proteins are often resistant to proteolytic enzymes in the digestive tract and blood. In new-borns, and sometimes in adults, BAP can exert biological action not only in the gastrointestinal tract, but it can also penetrate the bloodstream. Such properties of natural food proteins, which milk proteins also belong to, have allowed formulating the concept of an additional function of natural food proteins. This function offers certain advantages and plays a positive role at the early stages of the development of the organism [3].

Though there is a lot of information on BAP made from milk proteins, their effect and role are not taken into account in determining the biological value of dairy products, in particular, hydrolyzates of milk proteins. Also, there have only been a few attempts to use BAP from milk proteins in creating functional products and ingredients. These include the mineral-binding ingredients "Capolac" (Sweden) and CE90SRR (the Netherlands), a functional drink that promotes the assimilation of minerals "Tekkotsu Inryou" (Japan), antihypertensive functional products – "Calpis" (Japan) and "Evolus" (Finland), inhibitors of the angiotensin-converting enzyme "TensVida" (the Netherlands) [8,9]. One of the reasons for the limited use of BAP is the absence of effective methods for obtaining the protein precursors of bioactive peptides from milk. BAP is obtained from total milk protein, or from two main groups of milk proteins, which are the casein complex proteins and serum proteins. Each of these groups is a complex mixture of proteins, and therefore obtaining BAP preparations requires the use of complex purification methods, which makes them expensive and ineffective. Another reason that inhibits the use of BAP is their uneven distribution among milk proteins. There are no peptides of any kind of biological action that would be uniformly represented in all fractions. The analysis of BAP from casein complex proteins showed their fractional specificity [3,9]. Thus, ACE inhibitors and mineral binding peptides are most often formed from α_S- and β-caseins. Peptides that affect the secretion of digestive juices and the processes of blood coagulation are exclusively characteristic of $\varkappa$-casein. Also, it is mostly opiate receptor antagonists that are formed from this casein fraction. At the same time, opiate receptors agonists are formed only from α_{S1}- and β-caseins. The most specific precursor of peptides is α_{S2}-casein. Bactericidal, mineral binding peptides and ACE inhibitors can mainly be formed from it. Taking into account the above-mentioned, it may be af-

firmed that the use of preparations of homogeneous fractions of milk proteins could greatly simplify obtaining specific BAP.

Known methods for industrial obtaining of protein fractions from milk in most cases do not provide a high degree of purification (~ 60–70%). This is due to the fact that such fractions were used to enrich certain foods or regulate the physical and chemical properties of food masses [10]. For example, the β-CN-5P protein was obtained to be used as an emulsifier or foaming agent in food systems. The β-CN-5P and ϰ-CN-1P fractions were used to create a baby formula based on cow's milk, because human milk mainly contains these factions. The protein fraction ϰ-CN-1P is used to stabilize casein micelles in food systems. In all these cases, a high degree of purification of protein fractions is not necessary. Also, a method based on low-temperature disaggregation of casein micelles was used for preparative selection of β-CN-5P [11]. The release of β-CN-5P is due to its high hydrophobicity. Further ultrafiltration allows separating partially purified β-CN-5P from aggregates of other fractions. Of course, in these cases, the degree of purification of fractions is low and does not exceed 70%. According to other authors, the higher yield of the β-CN-5P fraction can be achieved by using cold extraction of milk proteins after their processing by milk-coagulation preparations. However, the preparations of β-casein obtained by such methods contain a significant amount of products of proteolysis [2,10]. Enriched ϰ-CN-1P is obtained by extraction from the micelles after heating them at neutral pH values. The heating in this case can lead to the formation of complexes with milk serum proteins. Such preparations with a small degree of purification meet the needs of food industry, where they are used as viscosity regulators, foaming agents, as stabilizers, emulsifiers, etc. However, they cannot be used to obtain BAP.

Several industrial and laboratory methods have been developed to obtain highly purified fractions of milk serum proteins. Differential precipitation, various types of filtration and preparative chromatography are used to purify the proteins [12-14]. By the combination of ultrafiltration, diafiltration, and limited proteolysis by trypsin, α-lactalbumin (*α*-LA) with a high degree of purification was obtained [15]. Using differential ammonium sulfate precipitation and ion exchange chromatography, a preparation containing about 95% of β-lactoglobulin (β-LG) was obtained [16]. Such preparations can be used to produce BAP. However, the significant disadvantages of these methods are a long, multi-staged procedure and, consequently, a high cost of protein preparations. Also, these methods include using extreme values of pH and ionic strength, that may affect the structure and chemical composition of proteins and BAP derived from them.

In this regard, the method of electrophoresis is attractive for obtaining purified precursors of BAP from milk proteins. Moreover, it is analytical electrophoresis that has been chosen to analyse and identify milk proteins and to be the basis for their modern classification [17]. The advantages of electrophoresis include high resolution, the possibility of one-stage purification, and repeated use of the electrophoretic system.

The **purpose** of the presented work is the selection of an electrophoretic system for preparative fractionation and obtaining purified fractions of BAP precursors from milk proteins.

To achieve this goal, the following main **tasks** need to be solved:

- to conduct a comparative analysis of existing analytical electrophoretic systems used for fractionation and analysis of milk proteins;
- to suggest an effective preparative electrophoretic system for obtaining the main BAP precursors from milk proteins.

Research Materials and Methods

To obtain proteins of casein complex and serum, fresh skim milk was used. A preparation of total casein was obtained by re-precipitation at an isoelectric point with the following inactivation of naturally occurring proteases of milk. Serum proteins were obtained from the supernatant after precipitating the caseins and then purified from low molecular weight compounds by gel filtration in an appropriate electrophoretic buffer on the columns with Sephadex G-25 (*Pharmacia*) [18].

The casein fractions α_{S1}-CN-8P and β-CN-5P were obtained by differential precipitation in the presence of urea and purified by ion exchange chromatography on DEAE-cellulose columns (DEAE-52, *Serva*). The homogeneous fraction ϰ-CN-1P was obtained by gel filtration on Sephadex G-150 (fine) columns. The milk serum proteins fractions α-LA and β-LG were obtained by gel re-filtration on Sephadex G-100 (fine). Ion exchange chromatography and gel- filtration were carried out on the columns from a liquid chromatography kit by the company *Reanal* [18].

The concentration of milk proteins was determined spectrophotometrically at the wavelength $\lambda = 280$ nm. The absorption coefficients used were: 8.2 for general casein; 10.0 – for α_{S1}-CN-8P; 4.6 – for β-CN-5P; 9.6 for ϰ-CN-1P; 12.3 – for total protein of milk whey; 20.1 for α-LA, and 9.6 for β-LG [17].

To analyze the composition of milk proteins and to select an electrophoretic system for obtaining fractions, we used disc-electrophoresis in the presence of sodium dodecyl sulphate (SDS) with gradient and homogeneous separating polyacrylamide gel (PAG), disc-electrophoresis in native conditions, and electrophoresis with homogeneous PAG in the presence of urea [19]. Electrophoreses in PAG plates were carried out on a modified Studier type apparatus made in our laboratory, and those in PAG columns were carried out on an apparatus from the *Reanal* company. The electropherogrammes were stained and fixed by conventional methods [20]. Quantitative processing of electropherogrammes was carried out by constructing densitograms using the image reading function *imread*.

Results of the research and their discussion

To analyse milk proteins, different electrophoretic systems are used (disc-electrophoresis in native conditions, disc-electrophoresis in homogeneous or gradient gel with SDS, electrophoresis in homogeneous gel in the presence of urea). All considered, electrophoreses of skimmed milk proteins, casein complex proteins, and milk serum proteins have been carried out using all of the systems listed above.

Of the three variants, disc-electrophoresis in native conditions (Fig. 1(2)) has appeared the most effective. The main fractions of caseins – α_{S1}-CN and β-CN – as well as both variants (A and B) of the main protein of milk serum β-LG, can be identified on the skim milk proteins electropherogramme. The second important α-LA serum protein is masked by the wide band of α_{S1}-CN protein. Other fractions are difficult to identify. It is also known that in conditions of the native system for disc-electrophoresis, caseins can form aggregates with different molecular weights, which complicates the analysis of electropherogrammes. The two widespread, effective for many proteins, variants of disc-electrophoresis in the presence of SDS in homogeneous and gradient gel turned out to be hardly suitable for the analysis of milk proteins.

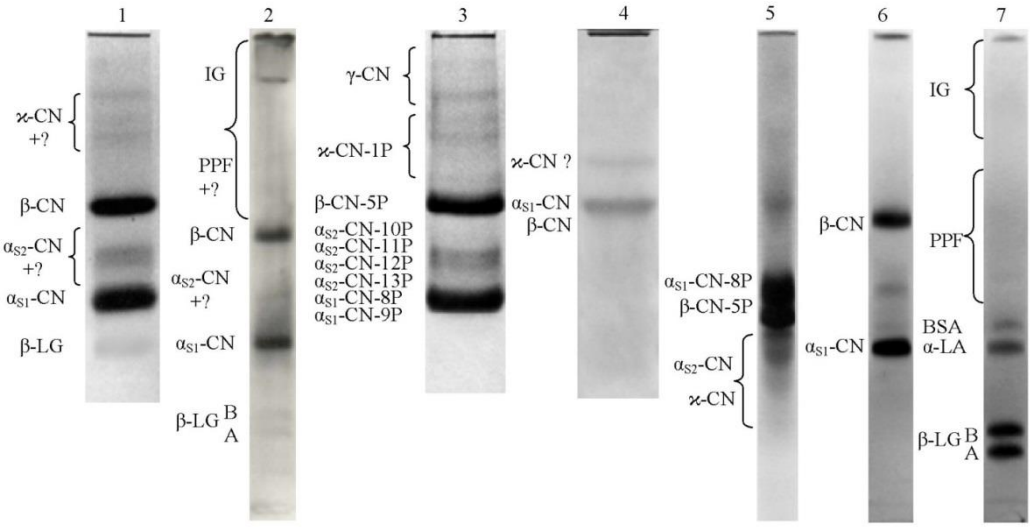

Fig. 1. Electropherogrammes of milk proteins obtained in the anode system of homogeneous PAG in the presence of urea (1), and by disc-electrophoresis in native conditions (2). Electropherogramme of casein complex proteins obtained in the anode system of homogeneous PAG in the presence of urea (3); disc-electrophoresis in the presence of SDS in gradient PAG (4) and in homogeneous PAG (5); disc-electrophoresis in native conditions (6). Disc-electrophoresis of milk serum proteins in native conditions (7). Electropherogrammes 1, 3, 4 were obtained on the PAG plates, and 2, 5, 6, 7 – in the PAG columns.

First of all, this is due to an anomalous interaction of caseins with SDS. That is why, the molecular weights of individual casein fractions calculated by the results of this electrophoresis can differ by 5000–10000 Da from the real ones, and the α_{S1}-CN-8P fraction (M=23615 Da) has a lower electrophoretic mobility than the β-CN-5P fraction (M=23983 Da). The second reason is the close values of the molecular masses of the main protein fractions of the casein complex, which leads to their coincidence on the electropherogrammes (Fig. 1(4,5)). These systems can be effectively used to analyze individual proteins. Besides, SDS can cause the denaturation of milk serum proteins that are not denatured in the native system.

Many of these disadvantages can be avoided if we use an electrophoretic system with a homogeneous PAG in the presence of urea. The results of the analysis of skimmed milk proteins in such system are presented on an electropherogramme (Fig. 1(1)). The system makes it possible to identify all major fractions except α-LA, which is similar in its electrophoretic mobility to the α_{S1}-CN-8P fraction. Common zones are also formed by α_{S2}-CN and BSA fractions, as well as by γ-CN and IG fractions. The results obtained indicate that none of the electrophoretic systems in question can be equally efficient in separation of all the major milk proteins at the same time.

The best results have been obtained by the electrophoretic analysis of two separate large groups of milk proteins – caseins and serum proteins. In particular, all the fractions of caseins can be identified in the anode electrophoretic system of homogeneous PAG in the presence of urea (Fig. 1(3)). Systems of this type are recommended by the International Committee on the Nomenclature and Methodology of Milk Proteins for identification of casein complex proteins [17]. Native disc-electrophoresis is only reliable in obtaining the α_{S1}-CN-8P and β-CN-5P fractions (Fig. 1(6)). Other electrophoretic fractions on the electropherogramme can be the result of caseins aggregation [2]. Electro-

phoresis of milk serum proteins in homogeneous PAG is ineffective, as wide protein bands are formed. This can be due to the denaturation effect of urea and the absence of a concentration effect. The best result for serum proteins has been shown by disc-electrophoresis in native conditions (Fig. 1(7)). On the electropherogramme, the main fractions of β-LG (A and B variants), α-LA, BSA, and IG are clearly separated. Lactoferrin in this system is part of the composition of the protease-peptone fraction (PPF). On the basis of these electrophoretic studies, it can be concluded that for preparative fractionation, it is suitable to use separately the proteins of the casein complex and those of the serum, but not the total milk protein. Moreover, different electrophoretic systems are appropriate for them.

For the preparatory variant of electrophoresis, a Studier type apparatus [20] for vertical electrophoresis was modified. The changes to the electrophoresis chamber has allowed a hundredfold increase of the number of proteins in the sample to be divided. Besides, the composition of electrophoretic systems was made as simple as possible. From the homogeneous PAG system, as compared with its analytical variant, toxic 2-mercaptoethanol was eliminated, and the urea concentration was reduced. In the system of native disc-electrophoresis, the concentration of PAG was reduced by 0.5%. The results of preparative electrophoreses of proteins of the casein complex and milk serum are shown in Fig. 2. An analysis of the electropherogrammes and densitograms obtained confirms the high effectiveness of separating the major groups of milk proteins in these systems. On the electropherogrammes, one can identify the zones ϰ-CN, β-CN-5P, α_{S2}-CN, α_{S1}-CN, IG, α-LA, and β-LG.

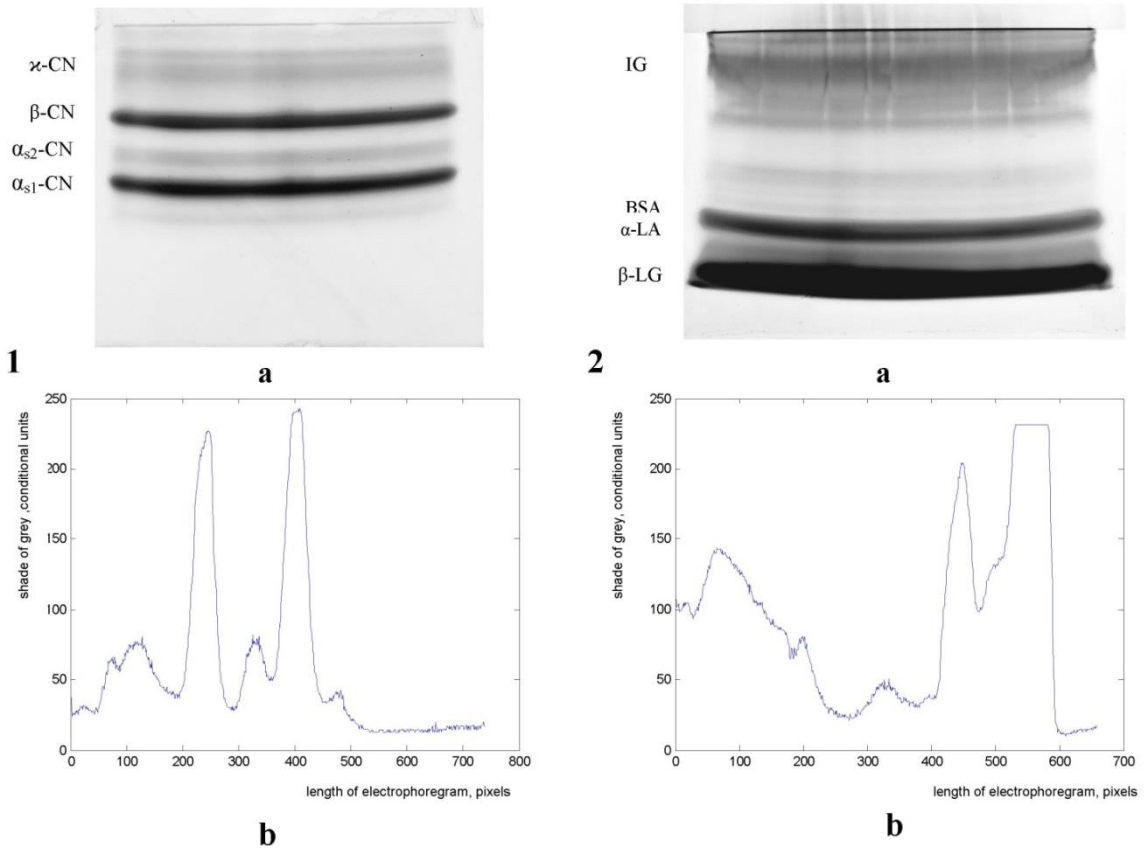

Fig. 2 Electropherogramme (1a) and densitogram (1b) of casein complex proteins, obtained by the preparative variant of electrophoresis in homogeneous PAG. Electropherogramme (2a) and densitogram (2b) of milk serum proteins, obtained by preparative disc-electrophoresis in native conditions.

All listed protein groups, except α-LA, are heterogeneous, but, with the exception of IG, they have the same primary structure. That is, they can be used as precursors of definite BAP. It will permit simplifying the purification scheme of BAP of certain action from protein precursors. Studies by Japanese scientists who obtained BAP from total casein using preparative electrophoresis also confirm the advantages of individual fractions [21].

Approbation of the research results

The results obtained are implemented in the Milk Biochemistry Research Laboratory of Ternopil National Technical University. The purified fractions of the protein precursors of biologically active peptides, as well as bioactive phosphopeptides, have been isolated from milk. New types of the biological activity for some phosphopeptide preparations have been discov-

ered in cooperation with the Institute of Cell Biology of the National Academy of Sciences of Ukraine [5].

Conclusions

The obtained research results allow to conclude:

- the preparative electrophoretic fractionation of milk proteins it is appropriate to conduct separately for casein complex and milk serum proteins;

- the effective separation of the casein complex proteins of milk can be achieved by preparative electrophoresis on the basis of a homogeneous PAG system in the presence of urea;

- disc-electrophoresis in the native conditions can be taken as the basis for the fractionation of the milk serum proteins.

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