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ISOLATION OF κ-CN-1P AND β-CN-5P FRACTIONS FROM NATIVE CASEIN MICELLES

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Proteins of the casein complex of milk arouse considerable interest as the precursors of biologically active peptides which are capable of influencing various physiological systems of the body (digestive, nervous, cardiovascular, and immune). It has been established that various bioactive peptides are unevenly located in the structure of casein fractions. In this connection, there appeared a need to separate individual fractions of this protein for studying the pathways of formation and properties of bioactive casein peptides. To minimize negative effects of the purification procedure, we used the gel filtration on Sefarose 2B to produce native casein micelles and repeating filtration on Sephadex G-150 to separate individual fractions. As a result, according to electrophoretic analysis, casein micelles with a characteristic protein composition were obtained. Taking into account the similarity of the molecular weight of components for their dividing the repeated gel filtration was carried out with separating the chromatograms into sectors. The composition of the combined fractions of each sector was analyzed by electrophoresis. This approach allowed isolating two electrophoretically homogeneous proteins from native casein micelles – κ-CN-1P and β-CN-5P, as well as to obtain a substantially purified (> 83%) α_{S1}-CN-XP. Isolated caseins without the influence of extreme factors can be used to study natural bioactive peptides.

K e y w o r d s: native casein micelles, casein fractions, gel filtration.

Proteins of the milk casein complex were recently actively studied as precursors of bioactive peptides [1, 2]. Formation of such bioactive peptides is an additional function of natural food proteins of milk [3]. More than 300 biologically active peptides (BAP) which are formed as a result of casein proteolysis in the gastroenteric tract have been found to date. These peptides may affect the cardiovascular (casokinins, casoplatelins), nervous (antagonists and agonists of opiate receptors), immune (immunomodulators) and digestive (regulators of motility and secretion in the gastroenteric tract, metal-binding peptides) systems. New kinds of biological action of casein peptides are discovered [4, 5]. Casein belongs to heterogeneous proteins and consists of four basic (α_{S1}-CN, α_{S2}-CN, β-CN and κ-CN) and numerous minor fractions. It has been established that BAP are distributed unevenly in the composition of primary structure of various casein fractions [6].

Thus, it is necessary to use homogeneous casein fractions for further investigation of mechanisms of formation and biological effect of BAP. The available methods of isolation and purification of caseins are cumbersome, multistage and long-term ones [7, 8]. Therewith the effect of extreme factors is often used that may result in ruining the native structure and in changes of the chemical structure of casein micelles and separate casein fractions. Phosphoserine groups of caseins and carbohydrate components of κ-CN-1P fraction are especially sensitive. Such changes can affect specificity of proteolysis of casein substrates and, respectively, the possibility of BAP formation and their activity [7, 9]. In this connection it is necessary to fulfill the task of isolation of homogeneous casein fractions in conditions which could provide the maximum preservation of their structure and chemical composition.

On this basis gel filtration can be a promising method of casein fractionation. Its advantages in-

clude a possibility of protein fractionation in a wide range of pH, ion force and temperatures that allows re-creating conditions characteristic of milk. Owing to similarity of values of molecular masses of caseins, the successful isolation of only one electrophoretically homogeneous casein fraction (κ-CN-1P) is described in literature; this fraction forms high-molecular aggregates at the expense of disulphide bonds [7, 10]. The repeated gel filtration of denaturated acid casein proved to be more efficient [11].

The aim of this work was to isolate homogeneous κ-CN-1P and β-CN-5P fractions from the native casein micelles with the use of repeated gel filtration.

Materials and Methods

Fresh defatted cow milk was used to isolate native casein micelles. Total casein was produced by reprecipitation in isoelectrical point with following inactivation of milk proteinase [12]. To identify proteins of casein complex the fractions α_{S1}-CN-8P and β-CN-5P were isolated by differential precipitation with following trimming by ion-exchange column chromatography with DEAE-cellulose (DEAE-52, Serva, Germany) [12]. Milk serum proteins were isolated from superprecipitation liquid after precipitation of caseins and purified again from low-molecular compounds by gel filtration in the corresponding chromatographic buffer on the columns with Sephadex G-25. Ion exchange chromatography and gel filtration were conducted on the columns from a set for liquid chromatography of firm Reanal (Hungary).

Concentration of milk serum proteins and caseins was determined with the help of spectrophotometer SF-46 (λ=280 nm). In so doing the following absorption coefficients were used: 8.2 – for total casein; 10.0 – for α_{S1}-CN-8P; 4.6 – for β-CN-5P and 12.3 – for total protein of milk serum.

The composition of proteins of casein complex and serum of milk, as well as homogeneity of certain fractions, were analyzed by the methods of electrophoresis. Casein was identified using alkaline buffer system in vertical plates of polyacrylamide gel (PAG). Disc-electrophoresis for acid and neutral proteins under non-denaturating conditions was used for milk serum proteins [13]. Electrophoresis in PAG plates was conducted by the Studier type apparatus, and in PAG cylinders – by the apparatus of Reanal type. Electrophoregrams were stained and fixed using generally accepted methods. Chromatographic and electrophoretic buffers and gels were produced of reagents from Reanal, Sigma and home reagents of high level purification. Densitometry of the obtained electrophoregrams was performed using the function of reading-out graphical images imread in the Matlab system.

Results and Discussion

Gel filtration of milk on the column with Sepharose 2B was conducted to isolate casein micelles in native state. It is known that this kind of sepharose allows fractionating protein molecules and their aggregates in the range of molecular masses from 7×10^4 to 40×10^6 Da. To provide stability of casein micelles the gel filtration was carried out in 0.01 M imidazole buffer (pH 6.7), which included 0.01 M $CaCl_2$. Calcium salt was introduced to compensate losses of Ca^{2+} ions by micelles during gel filtration. Ca^{2+} ions play an important role in forming supermolecular structures of casein – micelles which may have the mass from 10^6 to 10^9 Da. Gel filtration was conducted at 18 °C. At lower temperatures the fraction β-CN-5P leaves the composition of micelles [7]. A typical profile of chromatogram of micelle native casein is observed on the figure 1.1(1). To establish the limits of the range of fractions the authors have conducted gel filtration of milk serum proteins (Fig. 1.1(*2*)) and β-CN-5P (Fig. 1.1(*3*)). It is seen on the chromatogram that milk serum proteins and β-CN-5P are washed out of the column with volume close to the full column volume. Based on the results obtained the fractions with casein micelles marked over the chromatogram (A) were taken. Correctness of the chosen range is confirmed by electrophoretic analysis. The results of electrophoresis of combined fractions of the range A (electrophoresis in anode system of homogeneous PAG) have shown a characteristic electrophoregram for proteins of casein micelles according to modern classification [14]. The fractions of basic proteins of milk serum are absent on the electrophoregram (Fig. 1.2). Electrophoretic analysis of combined fractions of the range B (Fig. 1.3) shows availability of milk serum proteins (β-lactoglobulin (β-LG), α-lactalbumine (α-LA), serum albumine (BSA), fraction of immunoglobulines (IG)), as well as inconsiderable amount of casein fraction β-CN-5P, which partially leaves the composition of casein micelles.

As is indicated in literature [7, 10, 12] sephadex G-150 is the most efficient dextran gel for fractionation of caseins. The results of gel filtration of micellar casein on the column with Sephadex G-150 (fine) under the conditions without reducing agents

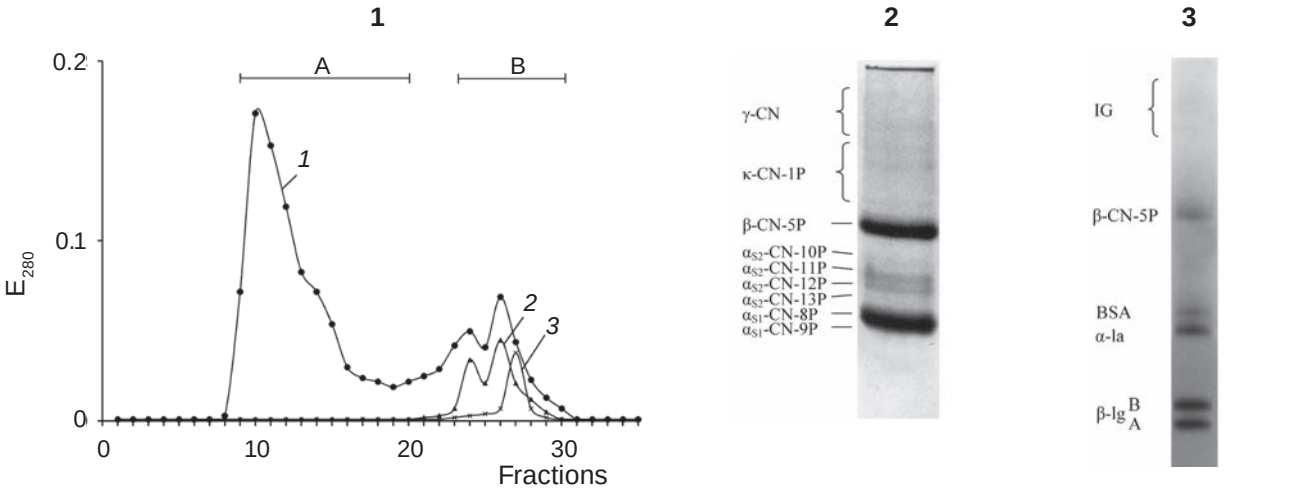

Fig. 1. Chromatograms of defatted milk (1.1), milk serum proteins (1.2), and casein fraction β-CN-5P (1.3), obtained with the help of gel filtration on sepharose 2B. Electrophoregrams of proteins of combined fractions of the range A (2) and the range B (3) after gel filtration of defatted milk on Sepharose 2B

[12] are shown on Fig. 2.1. Joint chromatographic fractions marked on chromatogram (I, II and III) were chosen to study protein content. The results of electrophoresis in anode system of PAG are shown on electrophoregram (Fig. 3(*2*, *3*, *4*)). The combined chromatographic fraction I includes κ-CN-1P. Its heterogeneity on the electrophoregram (about 5 bands) is determined by the difference in the number of oligosaccharide groups [1]. Chromatographic fraction II contains β-CN-5P and traces of α_{S1}-CN casein. The combined fraction III is mainly composed of α_{S1}-CN-8P casein and inconsiderable amount of β-CN-5P. Thus, homogeneous (by the initial structure) casein is contained only in the first fraction. That is in agreement with the already obtained results and is explained by formation of supermolecular structures (to 6 subunits) by κ-CN-1P caseins at the expense of disulphide bonds [14].

To isolate homogeneous caseins from the fractions II and III the authors repeated their gel filtration on sephadex G-150. The corresponding chromatograms are shown on Fig. 2(*2*) and 2(*3*). The chromatograms were conventionally divided into sectors A, B, C, D and E. Protein composition of the combined fractions of each sector was analyzed by electrophoresis (Fig. 3). Sectors A and B contain β-CN-5P casein. According to the data of densitometry the content of β-CN-5P casein in them exceeds 95%. Sectors C and D consist of a mix of α_{S1}-CN-XP and β-CN-5P fractions in various ratios. Sector C also in-

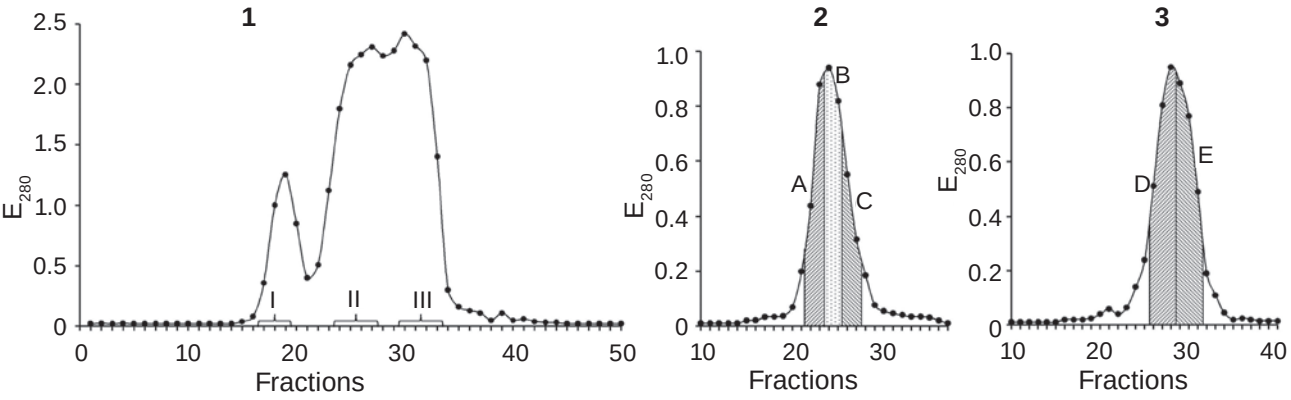

Fig. 2. Chromatogram of proteins of native casein micelles obtained with the help of gel filtration on Sephadex G-150 (1). Chromatograms of proteins of combined fractions of the sector II (2) and sector III (3) after repeated gel filtration on Sephadex G-150

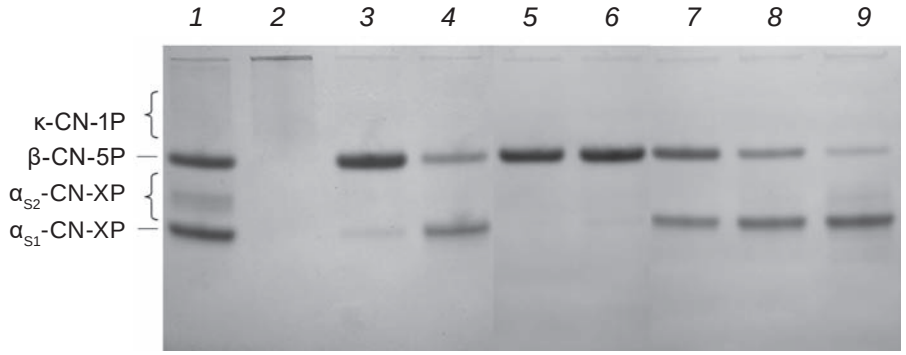

Fig. 3. Electrophoregrams of: casein (1); combined fractions of sectors I (2), II (3) and III (4) after gel filtration of proteins of native casein micelles on Sephadex G-150; combined sectors A (5), B (6), C (7), D (8) and E (9) after repeated gel filtration of sectors II and III on Sephadex G-150

cludes α_{S2}-CN-XP caseins. Molecules of this casein have two residues of cystein, but, judging from the position on the chromatogram, they do not form intermolecular disulphide bonds. Fraction α_{S2}-CN-XP has the biggest molecular mass (25 226 Da) among caseins, however it is eluted between caseins β-CN-5P (23 983 Da) and α_{S1}-CN-8P (23 615 Da). Maybe it is connected with the highest hydrophility of α_{S2}-CN-XP [15] that results in its delay in the column during gel filtration. The last sector (E) contains α_{S1}-CN-XP (86%) and traces of β-CN-5P casein. By the data of three gel filtrations used for fractionation of casein micelles the total output of purified fractions (sectors I, A, B, and E) was 31 ± 4% ($P \leq 0.05$) of the total content of protein.

The proposed variant of repeated gel filtration permits isolating three basic proteins (κ-CN-1P, β-CN-5P and α_{S1}-CN) from the native casein micelles. The total output of purified fractions is 31 ± 4%. Caseins isolated without using the extreme factors may be used for studying the ways of formation and properties of native bioactive casein peptides, i. e., phosphopeptides, casokinins, casomorphines, morphiceptin, neocasomorphine (from β-CN-5P fraction) and casoxines and casoplatelines (from κ-CN-1P fraction).

ВИДІЛЕННЯ κ-CN-1P І β-CN-5P ФРАКЦІЙ З НАТИВНИХ КАЗЕЇНОВИХ МІЦЕЛ

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Протеїни казеїнового комплексу молока становлять значний інтерес як попередники біологічно активних пептидів, здатних впливати на різні фізіологічні системи організму (травну, нервову, серцево-судинну, імунну). Встановлено, що різні біоактивні пептиди нерівномірно розміщені у структурі казеїнових фракцій. У зв'язку з цим для дослідження шляхів утворення і властивостей біоактивних казеїнових пептидів виникла необхідність виділення окремих казеїнових фракцій. Для мінімізації негативного впливу процедури виділення нами було використано гель-фільтрацію на сефарозі 2В для отримання нативних казеїнових міцел і повторну гель-фільтрацію на сефадексі G-150 для виділення окремих казеїнових фракцій. Було одержано, за даними електрофоретичного аналізу, казеїнові міцели з характерним складом

протеїнів. Враховуючи подібність молекулярних мас для виділення окремих фракцій, проведено повторну гель-фільтрацію з поділом хроматограми на сектори. Склад об'єднаних фракцій кожного сектору аналізували за допомогою електрофорезу. Такий підхід дозволив виділити два електрофоретично гомогенні протеїни з нативних казеїнових міцел – к-CN-1P і β-CN-5P, а також значною мірою очищений (>83%) α_{S1}-CN-XP. Одержані без впливу екстремальних факторів казеїни можуть бути використані для дослідження природних біоактивних пептидів.

Ключові слова: нативні казеїнові міцели, казеїнові фракції, гель-фільтрація.

ВЫДЕЛЕНИЕ к-CN-1P И β-CN-5P ФРАКЦИЙ С НАТИВНИХ КАЗЕИНОВЫХ МИЦЕЛЛ

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Протеины казеинового комплекса молока представляют значительный интерес как предшественники биологически активных пептидов, способных влиять на различные физиологические системы организма (пищеварительную, нервную, сердечно-сосудистую, иммунную). Установлено, что различные биоактивные пептиды неравномерно размещены в структуре казеиновых фракций. В связи с этим для исследования путей образования и свойств биоактивных казеиновых пептидов возникла необходимость выделения отдельных казеиновых фракций. Для минимизации негативного влияния процедуры выделения нами были использованы гель-фильтрация на сефарозе 2В для получения нативных казеиновых мицелл и повторная гель-фильтрация на сефадексе G-150 для выделения отдельных казеиновых фракций. Было получено, по данным электрофоретического анализа, казеиновые мицеллы, с характерным составом протеинов. Учитывая сходство молекулярных масс, для выделения отдельных фракций проведена повторная гель-фильтрация с разделением хроматограммы на секторы. Состав объединенных фракций каждого сектора анализировали с помощью электрофореза. Такой подход позволил выделить два электрофоретически гомогенные протеины с нативных казеиновых мицелл – к-CN-1P и β-CN-5P, а также в значительной степени очищенный (> 83%) α_{S1}-CN-XP. Полученные без влияния экстремальных факторов казеины могут быть использованы для исследования природных биоактивных пептидов.

Ключевые слова: нативные казеиновые соединения, казеиновые фракции, гель-фильтрация.

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