Obtaining of β -LG, α -LA and BSA protein fractions from milk whey

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Abstract

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Introduction. The purpose of the work is to obtain the protein fractions β -LG, α -LA and BSA from the milk whey by preparative disc-electrophoresis.

Materials and methods. The whey was obtained from the fresh cow's milk after isoelectric precipitation of casein complex proteins. The concentration of whey proteins was determined on a spectrophotometer by the absorption at $\lambda = 280$ nm. Gel filtration was carried out on liquid chromatography columns. Sephadexes G-25 and G-100 were used for the gel filtration. The fractional composition and homogeneity of whey proteins were analyzed by disc-electrophoresis in the polyacrylamide gel slabs.

Results and discussion. A system of disc-electrophoresis in native conditions for acidic and neutral proteins has been selected for the preparative isolation of homogeneous fractions of bioactive peptides proteins-precursors from milk whey taking into account the effectivity and existence of denaturation factors. A sample of whey proteins, for preparative fractionation purified, from low molecular weight compounds had been obtained at the result of the gel filtration.

An optimal whey proteins sample amount should not exceed 10 ml to provide an effective separation in a modified electrophoretic chamber of Studier type apparatus. Too large sample amount does not allow to separate the whey proteins effectively and obtain the homogeneous fractions extraction. The duration of the extraction process of individual protein fractions after preparative disc-electrophoresis was about 55–60 min.

The degree of homogeneity of the obtained protein fractions was established with the help of analytical disc-electrophoresis. It was 95–97% for β -LG and BSA fractions and 89–91% for α -LA fractions. It has been calculated that the total yield of homogeneous milk whey protein fractions was nearly 49–61% (from milk whey proteins taken for separation).

Conclusions. The proposed variant of the disc-electrophoresis allows to isolate from milk whey the electrophoretically homogeneous fractions of β -LG, α -LA and BSA in native conditions. The duration of electrophoresis and proteins extraction is about 4 hours. The average yield of homogeneous fractions from whey proteins is $55 \pm 6\%$.

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Introduction

The main function of the milk proteins (caseins and whey proteins) is to provide mammals by the amino acid nutrition. Also, milk whey proteins perform a number of other important functions. Thus, β -lactoglobulin (β -LG) is involved in the transport of fatty acids and retinol, exhibits antioxidant effect. For α -lactoalbumin (α -LA) it is typical to take part in the synthesis of lactose and calcium transport, also it shows immunomodulatory and anticarcinogenic effects. Serum albumin (SA) performs the transport function. Lactoferrin (LF) is binding iron ions, shows antimicrobial and antioxidant effects [1]. In addition, in recent years it was found that part of milk whey proteins, like caseins, are precursors of bioactive peptides that can be created during the process of proteolysis [1, 2]. According to our calculations, 50% of amino acid residues of β -LG and more than 40% of α -LA are included to the composition of the bioactive peptides. To a lesser extent this concerns to lactoferrin (9.4%) and serum albumin (2.6%) [3]. Antihypertensive peptides, antagonists of opioid receptors, regulators of intestinal motility, immunomodulatory and antimicrobial peptides can be formed from different proteins of milk serum. New types of bioactive peptides that exhibit anticarcinogenic and antioxidant effect, reduce the level of cholesterol in the blood, regulate the appetite, affect on the calcium assimilation are intensively investigated [3,4].

Taken into attention the expressive fractional specificity of the formation of bioactive peptides, the actual for the research and for using this phenomenon is the obtaining of purified fractions of milk whey proteins. Today there are several laboratory and industrial techniques to obtain the main milk whey proteins: α -LA, β -LG, immunoglobulins (IG), LF. They are isolated from whey proteins concentrate and purified by the differential precipitation, filtration through the gel or membranes as well as by ion exchange chromatography [1,2,5]. By the combination of ultrafiltration, processing of permeate with trypsin and diafiltration, α -LA with a degree of purity about 90% was obtained [6]. β -LG (with degree of purification more than 95%) was obtained by Lozano et al [7] using differential ammonium sulfate precipitation and ion exchange chromatography. The main disadvantages of these methods are many stages, duration of procedure as well as the use of extreme pH, high concentrations of salts that can affect on the structure and chemical composition of serum proteins and reduce their yield. In addition, these methods do not always provide a high degree of protein fractions homogeneity.

Taking in to account the growing interest to the homogeneous milk whey proteins, their purification due to differences in electrophoretic mobility in a neutral environment may be promising [8]. This makes it attractive to use electrophoresis for milk whey proteins fractionation. Earlier in our laboratory, it was shown that a promising variant of electrophoresis for this may be disc-electrophoresis in PAG slabs in native conditions [9]. The influence of individual parameters of electrophoresis on the efficiency of proteins fractions separation [10] was established.

The purpose of this work is to isolate the homogeneous protein fractions β -LG, α -LA and BSA from milk whey by disc-electrophoresis in native conditions.

Materials and methods

Materials

Whey proteins were isolated from the cow's fresh skimmed milk. The whey was separated by repeated isoelectric point precipitation of casein complex proteins. Reagents from the "Reanal" (Hungary) company and high-purity domestic production reagents were used in this work.

Determination of the milk whey protein concentration

The concentration of the milk whey proteins, as well as the concentration of individual milk protein fractions, were determined spectrophotometrically by absorption at $\lambda = 280$ nm on a spectrophotometer SF-46. In this case, previously used absorption coefficients ($D_{cm}^{1\%}$) were used: 12.3 – for the total milk whey protein; 9.6 – for β -LG; 20.1 – for α -LA; 6.7 for the BSA and 13.6 for the IG [8].

Gel filtration

Gel filtration of milk whey was carried out on columns (2×35 cm) from the liquid chromatography kit of «Reanal» (Hungary) company. Sephadex G-25 from «Pharmacia» (Sweden) was used for gel filtration [11]. Sephadex was equilibrated with the electrode buffer for disc-electrophoresis before the application into the column. Whey samples before the application into the column were centrifugated (centrifuge T-24 10000 g, 20 minutes) to remove insoluble components. 5 cm³ of supernatant was taken for gel filtration and applied on sephadex. The elution speed was set on 20 cm³/h. 5 cm³ of eluate was taken. The optical density in chromatographic fractions was determined at $\lambda = 280$ nm for constructing the chromatogram.

Homogenous β -LG fractions and the IG fraction obtained by repeated gel filtration of the milk whey on Sephadex G-100, as previously described, were used to identify the electrophoretic fractions [12].

Disc-electrophoresis

The fractional composition and homogeneity of milk whey proteins were analyzed by disc-electrophoresis in the slabs of polyacrylamide gel (PAG). The analytical system of disc-electrophoresis for acidic and neutral proteins in native conditions was used as the basis [13]. Gels were stained and fixed by conventional methods. Electrophoresis was performed on a modified Studier type apparatus [14]. Stacking and separating PAG were prepared as shown in Tables 1 and 2.

Composition of the separating PAG

Table 1

Solutions	Part of the solution in the PAG (volume)	Component	Amount
Gel components	2	Acrylamide	15 g
		N,N'-methylenebisacrylamide	0,4 g
		water	to 50 cm ³
Buffer for gel and catalyst	1	1 H HCl	24 cm ³
		Tris (hydroxymethyl) aminomethane	18,3 g
		N,N,N',N' tetramethylethylenediamine	0,115 cm ³
		Water	to 50 cm
Initiator solution	5	Ammonium persulphate	are selected experimentally
		Water	to 25 cm ³

Table 2

Composition of the stacking PAG

Solutions	Part of the solution in the PAG (volume)	Component	Amount
Gel components	2	Acrylamide	5 g
		N,N'-methylenebisacrylamide	1,25 g
		Water	to 50 cm ³
Buffer for gel	1	1H H ₃ PO ₄	12,8 cm ³
		Tris (hydroxymethyl) aminomethane	2,85 g
		Water	to 50 cm ³
Initiator and catalyst solution	1	Ammonium persulphate	are selected experimentally
		N,N,N',N' tetramethylethylenediamine	0,115 cm ³
		Water	to 5 cm ³
Sucrose solution	4	Saccharose	20 g
		Water	to 50 cm ³

Also, an electrode buffer was prepared as shown in Table 3. Before use, the buffer was diluted 10 times with distilled water.

Table 3

Composition of the electrode buffer

Solution	Buffer components	Amount
Electrode buffer (pH 8,3)	tris (hydroxymethyl) methylamine	6 g
	glycine	28,8 g
	Water	to 1000 cm ³

Statistical analysis

The statistical analysis of the obtained results and the graphical representation of the experimental data were carried out using the Microsoft Excel 2007 program. The accuracy of the obtained results was provided by triple repetition of the experiments.

Results and discussion

Whey protein fractions separating in disc-electrophoresis system

For analytical fractionation of whey proteins, the most commonly used are two types of disc-electrophoresis. This is disc-electrophoresis recommended by the American Dairy Science Association Committee on the Nomenclature, Classification, and Methodology of

Milk Proteins in the sodium dodecylsulfate (SDS) [8]. Also, disc-electrophoresis in native conditions for acidic and neutral proteins is used [13]. Taking into account, that the denaturation of proteins occurs in the presence of SDS, the disc-electrophoresis system in the native conditions (Figure 1) have been taken as the basis for whey protein fractions separating. As can be seen from the electrophoregram, the system allows effective separation of the main milk whey proteins fractions. These are β -LG (A and B variants), α -LA, BSA, as well as the heterogeneous group of immunoglobulins (IG) and proteose-peptone fraction (PPF).

Figure 1. Analytical disc-electrophoresis of milk whey proteins (1) and chromatographic fraction I after whey gel filtration on sephadex G-25 (2)

Before preparative electrophoretic fractionation low molecular weight compounds were separated from the milk whey by gel filtration on a column with Sephadex G-25 [11]. Sephadex was equilibrated with the electrode buffer for disc-electrophoresis. The results of gel filtration are shown in Figure 2. The combined fractions of the chromatographic peak I were selected for the preparative electrophoresis. The analytical disc-electrophoresis of this peak testifies that the proteins composition does not change at the result of gel filtration comparing with the milk whey (Figure 2).

Figure 2. Chromatogram of milk whey after gel filtration on Sephadex G-25

The concentration of PAG was reduced by 0.5%, in order to reduce the electrophoresis duration and the extraction process alleviation. Preparative electrophoresis testing was performed on a modified Stadler type apparatus [13]. The design of the electrophoretic chamber and former were modified in such way that they can provide an effective separation of the milk whey sample. The electrophoresis duration has been reduced up to 2 hours and the volume of whey can be increased to 10 cm³ as a result of these modifications.

The amount of whey in the sample for each electrophoretic chamber was determined experimentally. Too large sample amount does not allow to separate the whey proteins effectively. In this case, the electrophoregram has the following form (Figure 3a). Densitogram (Figure 3b) testifies about the impossibility of homogeneous fractions extraction from such gel. While using the optimal amounts of milk whey, the electrophoregram has such form (Figure 4). Comparing with the analytical variant (Figure 1) there are no significant differences. It may be noted only the union of two fractions of β -LG-A and β -LG-B into a single line.

Figure 3. Electrophoregram (a) and densitogram (b) of milk whey proteins in the preparative variant of disc-electrophoresis. Excessive amount of milk whey.

Figure 4. Electrophoregram (a) and densitogram (b) of milk whey proteins in the preparative variant of the disc-electrophoresis. The optimal amount of milk whey.

Identification and extraction of main fractions of the milk whey proteins

After completion of the electrophoresis, we remove the gel slab from the chamber and cut the side strips with a width of about 1 cm and place them in a solution of fresh dye for 1 minute. After intense destaining during 15 min, the main fractions of the milk whey proteins can be identified on the strips. Then, the strips were applied to the unstained part of the gel plate for the identification and cutting out areas with certain fractions [15]. In this case, we remove gel fragments that contain β -LG, α -LA, BSA and IG. The duration of the gel strip staining process, identification of fractions and cutting of the main plate of the gel was less than 30 minutes. In special studies, we have shown that for such period in the gel without fixation, the milk whey proteins do not mix due to diffusion. Next, obtained gel fragments with the appropriate fractions were crushed and protein extraction with an electrode buffer (1:5) was performed. During the extraction the samples were selected and the yield of proteins was controlled by the absorption at $\lambda = 280$ nm. The results are shown in Figure 5. Each point on the chart is the average value of the three definitions. According to the results presented in Figure 5, we can conclude that extraction is mostly completed after 60 min.

Figure 5. The course of extraction of separate fractions of milk serum proteins from polyacrylamide gel:

1 – β -lactoglobulin, 2 – immunoglobulins, 3 – α -lactalbumin, 4 – serum albumin

Degree of homogeneity of whey protein fractions

The fractions obtained after extraction were analyzed in the same electrophoretic system (analytical version) for homogeneity (Figure 6) [13]. It is visible on the electrophoregram,

that β -LG, BSA and IG fractions are electrophoretically pure and only α -LA fractions content β -LG bands. A heterogeneous fraction of IG is also separately isolated. The total protein yield was determined in the obtained electrophoretically pure fractions based on the three preparative electrophoresis results. The concentration of the proteins in whey and individual fractions was determined spectrophotometrically [8]. The yield of purified fractions was nearly $55\pm 6\%$ (from milk whey proteins taken for separation). Losses can be explained by incomplete extraction for 60 minutes, as well as by the fact that only separate sectors were taken for the extraction, and not the whole gel.

Figure 6. Electrophoregram of milk whey proteins and obtained fractions.
1 – proteins of milk whey, 2 – β -lactoglobulin, 3 – α -lactalbumin,
4 – serum albumin, 5 – immunoglobulins

Conlusion

1. The proposed version of the disc-electrophoresis in the PAG slabs allows isolating the electrophoretically homogeneous fractions of β -LG, α -LA and BSA from milk whey in native conditions.
2. The duration of electrophoresis and proteins extraction is about 4 hours.
3. The average yield of homogeneous fractions from proteins is $55\pm 6\%$. The obtained fractions are the major precursors of BAP amount the serum proteins and can be used to study the ways of their formation.

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