Characteristics of proteolytic processes during the isolation of natural casein phosphopeptides

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Abstract

Introduction. The aim of this work is studying of the proteolytic processes features in the production of natural phosphopeptides of the milk casein complex.

Materials and methods. Casein substrate was isolated by isoelectric sedimentation from skimmed milk. Proteolysis was performed using pancreatin. The degree of proteolysis was determined spectrophotometrically by the absorption of low molecular weight products of proteolysis at λ = 280 nm. The yield of phosphopeptides was determined gravimetrically after precipitation with ethanol in the presence of calcium ions. Electrophoresis of phosphoproteins of the substrate and products of proteolysis was carried out in an alkaline system of homogeneous polyacrylamide gel in the presence of urea.

Results and discussion. Natural phosphopeptides were obtained by proteolysis of the phosphoproteins of the casein complex with pancreatin (E:S = 1:100) under physiological conditions (37° C, pH 7.9). At different stages of proteolysis, the yield of phosphopeptides and peptides soluble in 10% trichloroacetic acid was determined. Degree of proteolysis has increased monotonically throughout the studied period. The yield of phosphopeptides reaches its maximum in the 90th minute of proteolysis and then decreases continuously. The yield of phosphopeptides is lower than it was used proteolytic preparations of microbial origin. The results obtained by gel filtration and electrophoresis in a polyacrylamide gel indicate that a decrease in the yield of phosphopheptides after the 90th minute of proteolysis may be caused by the phosphopeptide's molecular weight decrease. Most of phosphopeptides obtained in the 90th minute of proteolysis have a molecular weight up to 2000 Da, which is characteristic for the already known biologically active phosphopeptides.

Conclusions. During the proteolysis of casein with pancreatin in physiological conditions, the total yield of proteolysis products increases monotonously. The yield of phosphopeptides has maximum. Gel filtration and electrophoresis data indicate that this is due to a decrease in the molecular mass.

Introduction

The proteins of milk casein complex are precursors of a large number of various biologically active peptides with difference action on various physiological systems of the organism [1, 2]. Among them, one of the most common and important is phosphopeptides [3]. Phosphopeptides are formed in the course of a normal digestion of a casein proteins complex in the intestine following the action of proteolytic enzymes of the pancreas. It is believed that their main function is the ability to bind ions of macroelements (calcium, magnesium, ferrum), as well as trace elements (zinc, nickel, cobalt, selenium) and to promote their adsorption in the gastrointestinal tract. Herewith casein phosphopeptides are able to form complexes with metal ions, resistant to the action of proteolytic enzymes [4, 5].

For the first time, the biological effect of phosphopeptides was described in 1950 [6]. It was an independent of vitamin D calcification of bones in children with rickets. In further studies, the positive effect of casein phosphopeptides on the absorption of calcium, zinc and iron ions has been established [2, 3]. The mechanism of action of phosphopeptides in adsorption of metal ions has not been finally established. This may be the formation with the participation of phosphopeptides of calcium-selective channels in the cytoplasmic membrane. Phosphopeptides can bind ions of calcium and transport by endocytosis. Also, the biological effect of phosphopeptides is associated with the prevention of teeth caries [7]. It has been established that phosphopeptides can participate in the restoration of the mineral composition of teeth, inhibit the adhesion of cariogenic bacteria to the surface of teeth, and contribute to the accumulation of bioavailable calcium ions.

Due to the biological effect of casein phosphopeptides, they are of considerable interest as ingredients for functional products [8]. For the production of phosphopeptides, the proteolysis of casein substrates is carried out. Proteases of animal, plant and microbiological origin are used as proteolytic enzyme [3, 9]. As a result, a large number of phosphopeptide preparations were obtained, which differ in their primary structure, molecular weight and properties. These differences may be reflected in the biological activity of phosphopeptides [4]. Obviously, the greatest value can have phosphopeptides, formed in conditions that reproduce the conditions of caseins proteolysis in the gastrointestinal tract. We have previously justified the choice of proteolytic enzyme and casein substrate, established their proportion to obtain natural phosphopeptides [10]. Taking into account the above mentioned information, the purpose of the work is to characterize proteolytic processes in the production of natural casein phosphopeptides.

Materials and methods

To obtain total casein, fresh cow's milk was used. Proteolysis of casein substrate was carried out with pancreatin, produced by PJSC "Tekhnoloh" (Ukraine). All electrophoretic buffers and gels were prepared using reagents of the company "Reanal" (Hungary).

Obtaining of total preparation of milk casein complex proteins

The preparation of total casein was obtained from skimmed milk by precipitation at pH 4.6. The separation of lipids from casein was carried out by centrifugation (4000 g, 10 minutes) in two steps at two different temperatures: 30 and 4° C, respectively. This allows more completely separation of milk lipids. The sedimentation of total casein at an isoelectric point was carried out with an addition of chloride acid solution (1 mol/dm^3). The resulting

precipitate was washed with distilled water and dissolved with sodium hydroxide (1 mol/dm^3). In this case, the pH value should not exceed 7.9 [10]. At higher values of pH the dephosphorylation of caseins may occur. Precipitation and dissolution procedure was repeated twice. The third precipitation was carried out with acetic acid at a pH of 4.0. The resulting total casein sediment has been incubated in acetic acid solution (pH 4.0) for 5 hours at a temperature of 4° C to inactivate the natural proteases of milk [11].

Proteolysis of total casein

Proteolysis of 9% total casein was carried out with pancreatin at physiological values of temperature and pH (37° C, pH 7.9). The ratio of "enzyme: substrate" was 1:100 [12].

Determination of casein substrate concentration and products of its proteolysis

The concentration of proteins in preparations of total casein and products of its proteolysis in hydrolysates was determined spectrophotometrically during absorption at the 280 nm by spectrophotometer СФ-46. In this case, the commonly accepted absorption coefficient ($D_{1cm}^{1\%}$) for total casein was 8.2 [13].

Gel filtration

Gel filtration of total casein and products of its proteolysis was carried out on columns (2 × 35 cm) from the liquid chromatography kit of the company "Reanal" (Hungary) filled with Sephadex G-25 (fine) of the company "Pharmacia" (Sweden). Preparation of sephadexes and a chromatographic column for gel filtration was carried out in accordance with the methodological recommendations of the "Pharmacia" company [14]. The samples were dissolved in a chromatographic buffer (0.005 Tris HCl, pH 7.9, 6 M urea) and centrifuged to remove insoluble fragments by T-24 centrifuge (15000 g, 20 minutes). 5 ml of sample was taken from the obtained supernatant and applicated on sephadex, equilibrated with a chromatography buffer. Gel filtration was carried out at an elution rate of 25 ml/h, selecting 5 ml of eluate. For obtaining of chromatograms the optical density at 280 nm was measured by a spectrophotometer СФ-46.

Electrophoresis

The analysis of total casein and hydrolysates was carried out in a device of the Stadier type on vertical plates of a homogeneous polyacrylamide gel in an alkaline electrophoretic system (pH 7.9), which included 4.5 M urea [15]. Electrophoretic buffers and plates of polyacrylamide gel were prepared using reagents of "Reanal" company (Hungary). Polyacrylamide gel was obtained by mixing solutions according to Table 1.

Also the electrode buffer and the buffer for dissolution of samples were prepared, according to table 2.

Electrophoregrams were fixed with 7% acetic acid and stained with 0.5% amidoblack 10B [16,17].

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. The reliability of the obtained results was taken at $p < 0,05$.

Table 1

Composition of polyacrylamide gel

Solutions	Ration in polyacrylamide gel (volume)	Component	Amount
Gel	1 part	Acrylamide	13,5 g
		N,N'- methylenebisacrylamide	0,75 g
		Water	up to 100 ml
Buffer for gel (pH 7.9)	2 part	Tris(hydroxymethyl) aminomethane	0,609 g
		Ethylenediaminetetraacetic acid (disodium salt)	0,2 mg
		Veronal	1,1 mg
		2- mercaptoethanol	1,0 ml
		Urea	54,0 g
		Water	up to 100 ml
Catalyst and initiator solution	1 part	Ammonium persulfate	selected experimentally
		N,N,N',N'-tetramethyl-ethylenediamine	0,05 ml
		Water	up to 10 ml

Table 2

Composition of electrode buffer and sample buffer

Buffer	Component	Amount
Electrode buffer (pH 7.9)	Tris(hydroxymethyl) aminomethane	6,09 g
	Ethylenediaminetetraacetic acid (disodium salt)	2,0 g
	Veronal	11,0 g
	Water	up to 1000 ml
Buffer for dissolution of samples (pH 7.9)	Tris(hydroxymethyl) aminomethane	30,5 mg
	Ethylenediaminetetraacetic acid (disodium salt)	10,0 mg
	Veronal	55 mg
	2- mercaptoethanol	0,25 ml
	Urea	13,5 g
	Water	up to 50 ml

Results and discussion

Determination of the total yield of proteolysis products and phosphopeptides

When conducting proteolysis of total casein with pancreatin every 30 minutes, starting from the 30th from its beginning and up to the 180th minute inclusive, the samples were taken to determine the intensity of the process and the release of phosphopeptides. Soluble proteolysis products obtained after sedimentation uncleaved phosphoproteins with 10% trichloroacetic acid were diluted fortyfold with 5% acetic acid. For the obtained solutions,

the optical density was measured by a spectrophotometer СФ-46 at 280 nm. The results of proteolysis are shown in Figure 1 (1). Each point in the graph is the average value of three definitions. As can be seen from the graph, the degree of proteolysis monotonically increases throughout the period (up to the 180th minute). The slowdown in the intensity of proteolysis occurs after the 90th minute. Adamson and Reynolds have got similar results, obtained with enzyme preparations Novo trypsin and Pancreatin 4 NF [18].

Simultaneously, every 30 minutes of proteolysis samples have been taken for the extraction of phosphopeptides from the hydrolyzate. The basis was the methodology described earlier [19]. Herewith, the pH of the sampled hydrolyzate was adjusted to pH 4.6 with chloride acid solution of concentration 0.1 mol/dm^3. The unsolvated phosphoprotein and the large polypeptides sediment were separated by centrifugation at a centrifuge ОПН-8 (5000 rpm, 15 minutes). Then, 1 cm^3 of 10% $CaCl_2$ and 10 cm^3 of ethanol were added to 9 cm^3 of supernatant. The precipitate of phosphopeptides was centrifuged, washed with ethanol, dried to a constant weight and weighted. The results are shown in Figure 1 (2). The yield of phosphopeptides, obtained in physiological conditions, is lower than with the application of microbial proteolytic enzymes [9] and it approaches to the yield with trypsin using [19]. The analysis of the phosphopeptides extraction shows that after reaching a maximum (in the 90th minute), the value of the yield decreases. This occurs on the background of a further increase in a degree of proteolysis and may be due to a change in the phosphopeptides' molecular weight in the process of proteolysis [9, 18]. In this regard, to characterize the products obtained at various stages of proteolysis, we carried out gel filtration and electrophoresis.

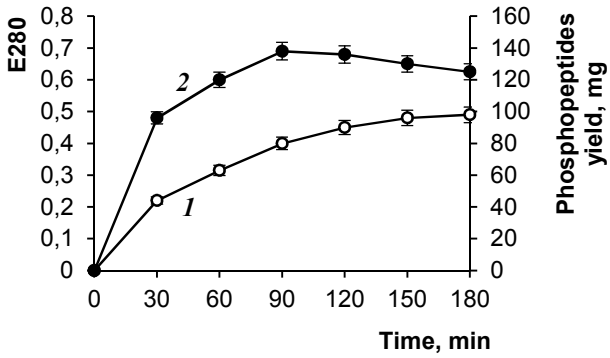

Figure 1. Proteolysis products (1) and phosphopeptides yield (2) in the process of proteolysis of the phosphoprotein substrate with pancreatin

Gel filtration and electrophoresis of proteolysis's products

Gel filtration on Sephadex G-25 allows estimating the molecular and weight distribution of proteolysis products in the range from 1000 to 5000 Da [14]. For gel filtration samples have been taken at different stages of proteolysis. The results of gel filtration are shown in Figure 2 and 3. For comparison, in each case, the chromatogram of the phosphoprotein substrate is shown in parallel. According to literature data, the molecular masses of the already known biologically active casein phosphopeptides do not exceed 2000 Da [8]. They are eluated in the 15th and subsequent chromatographic fractions, sampled during gel filtration. In the 90th minute (Figure 2), the greater part of proteolysis products, including phosphopeptides, have molecular weight up to 2000 Da. During further proteolysis (the 90th-180th minutes), the amount of low molecular weight peptides increases. At the same time, as can be seen from Figure 1 (2), the total amount of phosphopeptides up to the 90th minute does not change, and then begins to decrease.

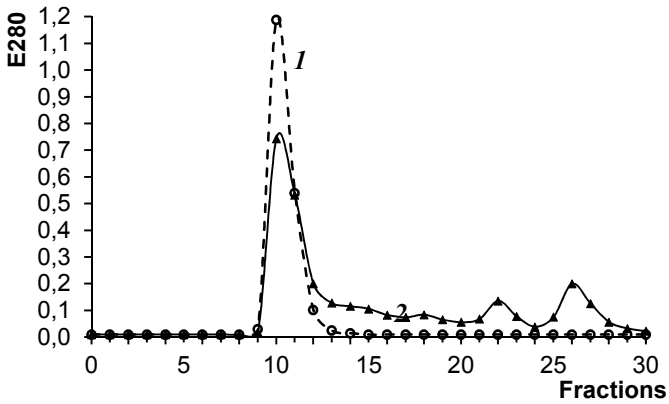

Figure 2. Chromatogram of the phosphoprotein substrate (1) and its hydrolyzate (2) obtained in the 60th minute of proteolysis with pancreatin

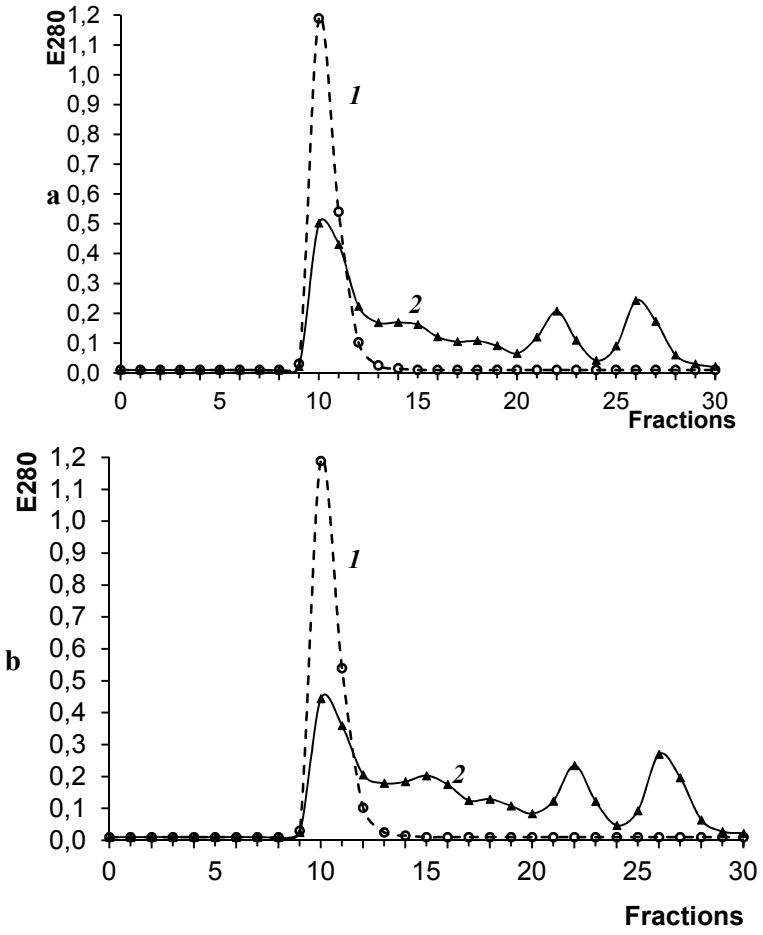

Figure 3. Chromatogram of the phosphoprotein substrate (a.1 and b.1) and its hydrolyzate, obtained in the 90th (a.2) and 120th minutes (b.2) of proteolysis with pancreatin

The samples for gel filtration were selected according to the scheme (Figure 4):

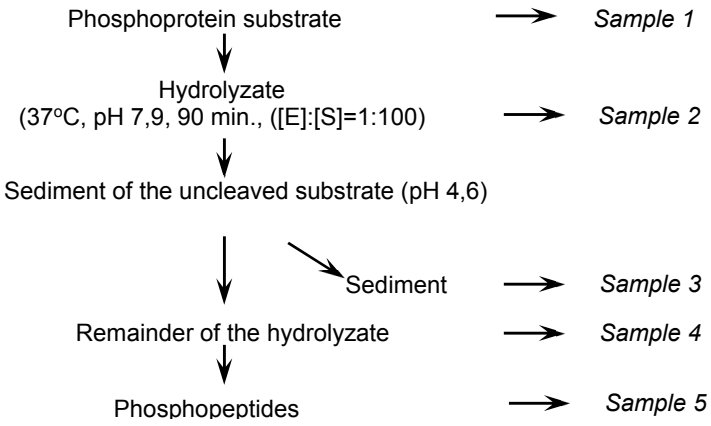

Figure 4. Scheme of sample selection for gel filtration.

The Figure 5 shows the chromatograms of the hydrolyzate obtained in the 90th minute under physiological conditions (37° C, pH 7.9), as well as the uncleaved substrate, sedimened at pH 4,6, the residue of the hydrolysate and the isolated phosphopeptides.

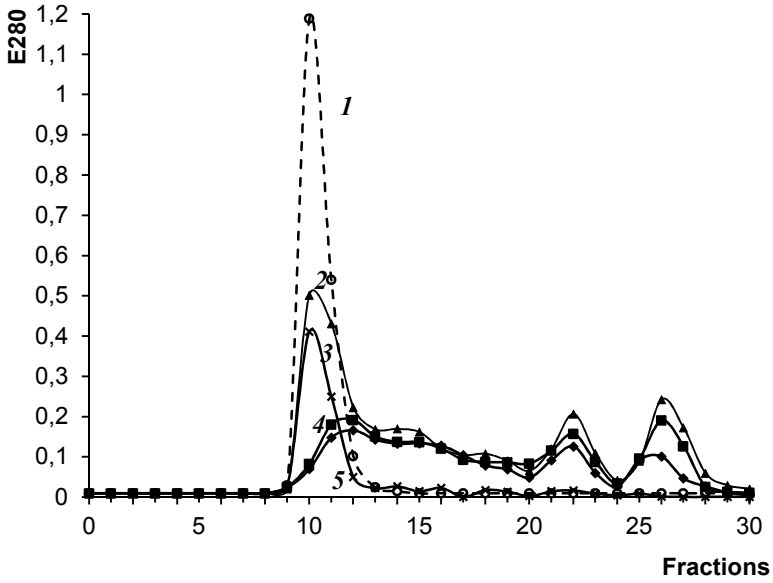

Figure 5. Chromatogram of phosphoprotein substrate (1), and products of its proteolysis, obtained in the 90th minute of proteolysis: 2 - hydrolyzate; 3 - uncleaved substrate; 4 - remainder of the hydrolyzate; 5-phosphopeptides

According to the gel filtration data at pH 4.6, polypeptides with a molecular weight greater than 5000 Da are sedimented from hydrolyzate (Figure 5). Scientific investigations confirm that under such conditions non-hydrolyzed proteins, as well as polypeptides with a large molecular weight are isolated [1, 13]. The chromatogram of the hydrolyzate residue (Figure 5 (4)) has a similar profile to the chromatogram of phosphopeptides (Figure 5 (5)). Moreover, among the remainder of the hydrolyzate, phosphopeptides present a larger part. This fact is consistent with published dates [18-20].

In addition to gel filtration, electrophoresis was used to characterize the hydrolyzate (Figure 6). The obtained results indicate that after the 15th minute, the main substrate phosphoproteins (α_{S1}-CN-8P, α_{S2}-CN-XP, β-CN-5P) are cleaved to form low molecular weight and separate macromolecular products that are retained in a polyacrylamide gel. In the 90th minute, high molecular peptide fractions are absent (Figure 6). Similar results with a large number of various low molecular weight phosphopeptides are described in other scientific researchs [21, 22].

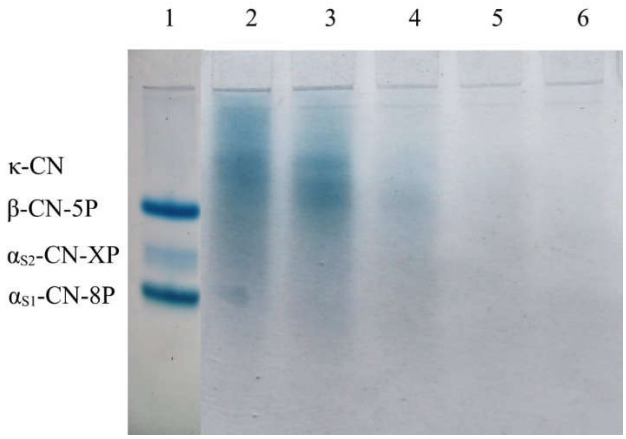

Figure 6. Electrophoregram of phosphoprotein substrate (1) and products of its proteolysis obtained in the 15th (2); 30th (3); 60th (4); 90th (5th); 120th (6) minutes

Conclusions

During the casein substrate proteolysis with pancreatin (E:S ratio 1:100) under physiological conditions (37° C, pH 7.9), the total yield of peptides that are soluble in 10% trichloroacetic acid and phosphopeptides does not coincide. The total amount of proteolyctic products monotonously increases throughout the whole period of proteolysis. The yield of phosphopeptides reaches a maximum in the 90th minute of proteolysis with a further decrease. The results of gel filtration and electrophoresis indicate that this fact is associated with a decrease in the molecular weight of phosphopeptides.

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