IMPROVEMENT OF THE METHOD OF COMPARATIVE STUDY OF MILK WHEY PROTEINS ENZYMATIC HYDROLYSIS

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Abstract

Milk whey proteins are valueble nutritional ingredients with a number of health-beneficial properties. Whey proteins are also a source of bioactive peptides that can be released in the process of proteins enzymatic hydrolysis. In this connection, there often is a need to compare their proteolytic action on milk whey proteins. It is important to take into account the specificities of the composition and properties of milk whey proteins. The aim of the research was to improve the method of comparative study of milk whey proteins enzymatic hydrolysis. Casein and whey were obtained from fresh cow skimmed milk. The whey was separated by centrifugation after casein precipitation at the isoelectric point. The following enzyme preparations were used in the research: neutral protease, papain, trypsin, chymotrypsin and pancreatin. To select β-LG, gel filtration of the milk whey on the chromatographic column with Sephadex G-150 (Pharmacia) was used. The homogeneity of the received β-LG preparation was analyzed by express electrophoresis in the polyacrylamide gel plates (PAG). The preparation of general casein was isolated by repeated precipitation at the isoelectric point. The fractional composition of the casein substrate was analyzed by electrophoresis in the anode system of homogeneous PAG in the presence of urea. Quantitative treatment of electrophoregrams of the β-LG preparation was performed using the imread reading function. Determination of proteolytic activity of enzyme preparations was carried out according to the method of V. F. Selemenev [6].

In the course of the research, it was determined, that for the research of proteolysis under conditions of identical total proteolytic activity, the concentration of neutral protease should be increased by 1.02 times, papain – by 4.2 times, trypsin – by 2.8 times, pancreatin – by 2.12 times as compared to chymotrypsin. As a result, it has been shown that the use of β-lactoglobulin instead of serum albumin in spectrophotometric determinations allows obtaining more accurate values of the concentrations of whey protein and proteolytic products. In determining the ratio of enzyme : substrate it is advisable to take into account the general proteolytic activity of various enzyme preparations in comparative studies of whey proteins proteolysis with various enzyme preparations. These will simplify the methodology and reduce the time for objective evaluation of enzymatic preparations for proteolysis of milk whey proteins. In some cases, considering the specificity of proteases it could increase the yield of biologically active peptides.

Keywords: milk whey proteins, proteolysis, β-lactoglobulin, gel filtration.

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1. Introduction

One of the important ways of milk whey proteins processing is their proteolysis [1]. It is used for producing low-allergenic food mixtures, food products for children, athletes, as well as for the production of bioactive peptides [2, 3, 4]. Many proteolytic preparations of animal, plant and microbiological origin are tested in the process of development of the technology for

such products [5]. For this reason, there is often a need to compare their proteolytic effect on milk whey proteins. It is important to take into account these specificities of the composition and properties of milk whey proteins.

In many methods, the dependence of the optical density to the bovine blood albumin (BSA) concentration is used to determine the concentration of proteins and their products of splitting by spectrophotometry in the ultraviolet region [6]. In the case of determining the concentration of milk whey proteins, as well as products of their proteolysis, these results differ a lot from the actual ones. This is due to the significant difference in values of absorption coefficients for total milk whey proteins ($E_{280/1\ \%}$=12.3) and BSA ($E_{280/1\ \%}$=6.3) [7]. Much closer values of the absorption coefficient has the main protein of milk – β-lactoglobulin ($E_{280/1\ \%}$=9.6). A similar situation occurs when determining the concentration of products of milk whey proteins proteolysis. When the BSA calibration is used the results differ from the actual ones by almost 2 times.

Another important aspect when proteolysis of milk whey proteins is carried out is an objective comparison of the various proteolytic preparations activity. In many studies, in this case, only the ratio of the substrate: enzyme preparation is taken as the basis without taking into account their activity [8, 9]. Sometimes it is difficult to compare them due to the use of specific methods for various enzyme preparations [10]. It is obvious that in the comparative analysis it is necessary to use the amount of enzyme preparations, taking into account their total proteolytic activity. Accessible to many proteolytic enzymes, casein can be used as a substrate for this. The casein, prepared by Hammarsten, which is often used as a substrate, is badly soluble in water. More suitable is lyophilized casein, isolated during reprecipitation at the isoelectric point. Also, natural milk proteases may be present in the composition of casein preparations [11]. It is therefore advisable to predict a phase of natural proteases inactivation that may have influence on the course of proteolysis.

These will simplify the methodology and reduce the time for objective evaluation of enzymatic preparations for proteolysis of milk whey proteins. In some cases, considering the specificity of proteases, it could increase the yield of biologically active peptides.

2. Materials and methods

Enzyme preparations: neutral protease and papain from «Barrett industrial limited» company (Great Britain), trypsin and chymotrypsin from «Biozym» (Germany) and pancreatin from «Technolog» (Ukraine) company were used in the work.

For the production of whey and casein, fresh skimmed milk (18 °T) was used. Whey was separated by centrifugation (4000 g, 15 min) after casein precipitation at the isoelectric point (pH 4.6). The lyophilized casein substrate was isolated according to the following scheme (**Fig. 1**).Determination of enzyme preparations proteolytic activity was carried out according to the V. F. Selememev's method [6].

The isolation of β-LG was carried out by gel filtration of the milk whey on a column with the Sephadex G-150 from «Pharmacia» (Sweden) as described earlier [12].The columns (1.5×70 cm) from the «Reanal» (Hungary) company of liquid chromatography were used for gel filtration. The speed of the elution was set at 20 ml/h. The amount of taken fraction was 4 ml.

The homogeneity of the received β-LG preparation was analyzed by expressing electrophoresis in the polyacrylamide gel (PAG) plates. Gel's composition and conditions of electrophoresis are described in [13]. Gels were stained with 0.5 % solution of amido black 10 B and were kept in 7 % acetic acid.

The fractional composition of the casein substrate was analyzed by electrophoresis in the anode system of homogeneous PAG in the presence of urea. The composition of the PAG and the conditions for electrophoresis were described earlier [14].

Quantitative processing of β-LG preparation electrophoregrams was performed using the image reading function *imread* [15].

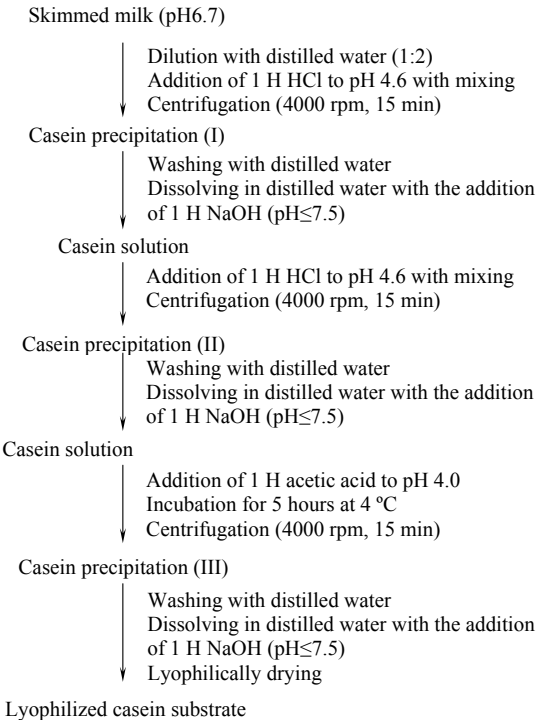

Fig. 1. Scheme of the casein substrate isolation

3. Results

The total casein preparation was isolated by the way of repeated precipitation in the isoelectric point. At the last stage of obtaining, the incubation in acetic acid at pH 4.0 for natural milk proteases inactivation was carried out. The fractional composition of the casein substrate proteins is shown on the electrophoregram (**Fig. 2**). The electrophoregram shows that the isolated substrate has a characteristic fractional composition of the milk casein complex proteins [11]. It is important that the obtained lyophilized preparation is well soluble in water.

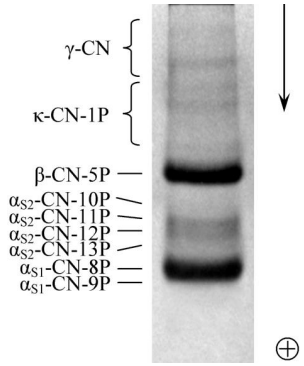

Fig. 2. Electrophoregram of casein substrate, obtained in the anode system of homogeneous PAG in the presence of urea

Taking into account the previously obtained results [12], chromatographic fractions from β-LG after the second gel filtration of milk whey on a column with Sephadex G-150 were combined and selected. The numbers of the combined fractions are shown in **Fig. 3, *a***. The results of the analysis in the combined fractions of β-LG for homogeneity are shown in **Fig. 3, *b***. The electrophoregram shows one band, which corresponds to β-LG in terms of electrophoretic mobility. The results of the densitometry of the obtained electrophoregram indicate a high degree of β-LG preparation homogeneity (**Fig. 3, *c***).

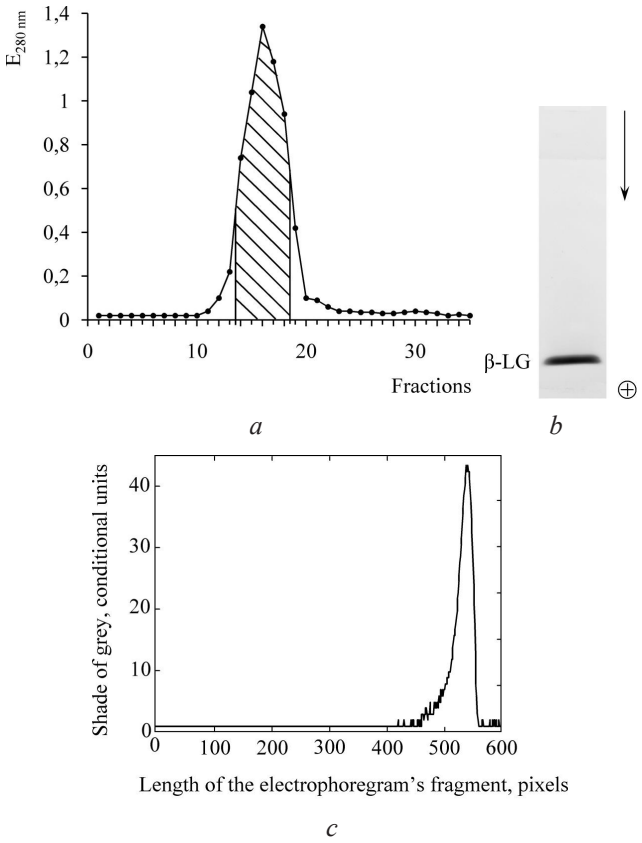

Fig. 3. The combined fractions of β-LG after the second gel filtration (*a*) of milk whey on the sephadex G-150. Electrophoregram (*b*) and densitogram (*c*) of the obtained β-LG preparation

To calculate the concentration of milk whey proteins and proteolytic products, a plot of dependence of optical density on the concentrations of β-LG, BSA and total milk whey protein was constructed (**Fig. 4**). Each point is the average value of three measurements. It can be seen, that the calibration chart of β-LG is much closer to the plot, constructed using a real sample of whey proteins.

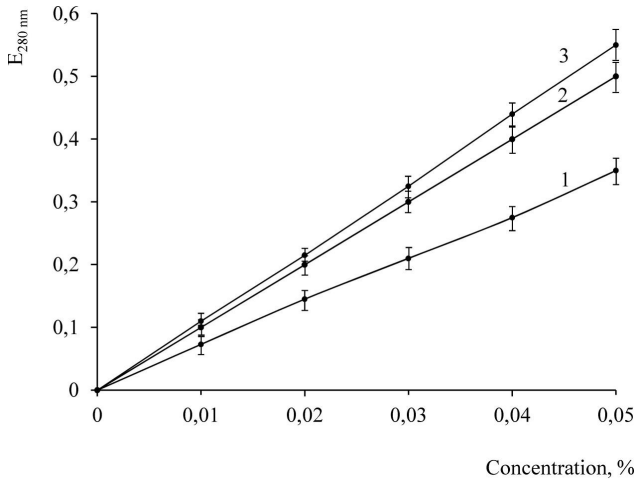

Fig. 4. The dependence of the optical density (λ=280) on the solution of BSA (1), β-LG (2) and total whey protein (3) from their concentration

The results of casein substrate proteolysis products' optical density dependences on the concentration of proteolytic preparations (pancreatin, chymotrypsin, trypsin, papain and neutral

protease) are shown on **Fig. 5**. Then, for each preparation, indicator b which is proportional to the proteolytic activity, according to the method of V. F. Selemenev, was determined [6]. It was as follows: for chymotrypsin – 5.67, for neutral protease – 5.55, for pancreatin – 2.67, for trypsin – 2.02 and for papain – 1.35. That is, the total proteolytic activity of chymotrypsin is greater than that of the neutral protease in 5.67/5.55=1.02 times; for pancreatin in 5.67/2.67=2.12 times; for papain in 5.67/1.35=4.2 times and for trypsin in 5.67/2.02=2.8 times.

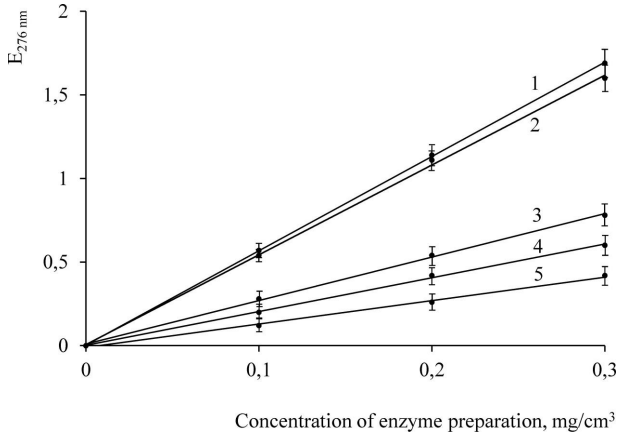

Fig. 5. Dependences of the proteolytic products' optical density (λ=276) on the concentration of enzyme preparations: chymotrypsin (1), neutral protease (2), pancreatin (3), trypsin (4), papain (5)

Thus, for conducting studies of proteolysis in the conditions of identical total proteolytic activity, the concentration of neutral protease should be increased by 1.02 times, papain – by 4.2 times, trypsin – by 2.8 times, pancreatin – by 2.12 times, comparing with chymotrypsin. That is, if the ratio of the enzyme : substrate is 1:50 1 part of chymotrypsin and 50 parts of the substrate should be used. Whereas, to provide a similar ratio for pancreatin the ratio 2.12:50 should be used. Similarly the amount of other enzyme preparations is calculated. The ratio of activity must be set for each match of enzyme preparations.

4. Conclusions

The use of β-lactoglobulin instead of blood serum albumin in spectrophotometric determinations allows obtaining more accurate values of the milk whey proteins concentrations and the products of their proteolysis. β-lactoglobulin, obtained by repeated gel filtration on sephadex G-150, can be used for calibration plot construction.

It is advisable to take into account the total proteolytic activity of enzyme preparations in comparative studies of milk whey proteins proteolysis, when determining the ratio of enzyme: substrate. This will allow obtaining more objective data when choosing a proteolytic preparation. However, this does not give an idea of the hydrolyzed peptide bonds amount. In the future, characterizing the milk whey proteins proteolysis, it is advisable to take into account the total proteolytic activity and the amount of hydrolyzed peptide bonds.

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