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THE EFFECT OF LOW HEATING TEMPERATURES ON CASEIN WHEY PROTEINS

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V. Yukalo, Doctor of Biological Sciences, Professor
K. Datsyshyn, Candidate of Technical Sciences, Associate Professor
L. Storozh, Candidate of Technical Sciences, Associate Professor
Department of Food Biotechnology and Chemistry
Ternopil Ivan Puluj National Technical University
56 Ruska str, Ternopil, Ukraine, 46001

Correspondence:
K. Datsyshyn
E-mail: katkostyuk3103@gmail.com

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Abstract. In the work, a comparative analysis of the fractional composition and resistance to thermal denaturation of proteins of two samples of casein wheys was carried out: the laboratory one, which was obtained at a temperature of 18°C, and the production one – at a temperature of 46°C. In both cases, wheys were separated after isoelectric precipitation of casein complex proteins with hydrochloric acid. Express electrophoresis in a polyacrylamide gel showed an identical qualitative composition of major and minor proteins fractions in both samples. Also, no differences were found in the relative content of the main protein fractions of wheys – β-lactoglobulin (β-lg), α-lactalbumin (α-la), serum albumin (BSA) and immunoglobulins (Ig). To compare resistance to heat denaturation, samples of laboratory and production wheys were heated and held for 5 min at temperatures from 60°C to 100°C with an interval of 5°C. The precipitate of denatured proteins was isolated by centrifugation. The concentration of proteins in the supernatant was determined spectrophotometrically and the qualitative and quantitative composition of the main protein fractions was analysed by express electrophoresis. As a result, it was established that the concentration of undenatured protein in both wheys does not coincide in the temperature range of 70–90°C. Laboratory whey proteins were more resistant to heat denaturation. Moreover, these differences can be detected only with short-term (5 min) thermal denaturation. Long-term high-temperature heating (30 min) does not allow them to be detected. Electrophoretic analysis of the relative content of the main protein fractions showed that changes in stability occur due to two fractions – β-lg and BSA. The relative concentration of these fractions in the undenatured residue from production whey is < 3%, and in the residue from laboratory whey > 8% for BSA and > 24% for β-lg when heated to 90°C for 5 min. Long-term exposure of both wheys at 20°C before investigating their resistance to thermal denaturation may indicate that some of the changes in the stability of production whey proteins caused by preheating to 46°C are irreversible.

Key words: casein whey proteins, heating, thermal denaturation.

Introduction. Formulation of the problem

Casein whey is a valuable secondary raw material, which is balanced in the content of the main nutrients, in particular proteins. Moreover, a significant part of these proteins exhibits biological activity, and is also a source of numerous secondary bioactive compounds – peptides [1]. The biological effect of proteins and peptides of casein whey is important when using it for the production of baby food and special purpose products. Slight heating of milk during its skimming and acid precipitation of casein does not significantly affect the functional properties of whey – the amino acid content of its proteins and digestibility. However, such heat treatment can affect the native structure and, accordingly, the biological activity of casein whey proteins and peptides. When casein production technologies were developed, little was known about the biological activity of whey proteins and peptides. In addition, previously, the effect of high temperatures on whey proteins, which led to significant changes in functional properties, was mainly studied [2]. Therefore, the question of identifying the effect of low-temperature heating, which is used in the production of casein, on the properties of casein whey proteins is relevant today.

Analysis of recent research and publications

For many years, milk proteins have been a classic object for studying denaturation processes under the influence of such factors as pH, ionic strength, and temperature [3]. The change of these factors accompanies the production processes of dairy products, the formation of their rheological properties and organoleptic indicators [2]. In recent decades, studies of the milk proteins denaturation processes are closely related to the discovery of many types of biological activity inherent for these proteins, as well as the products of their proteolysis [1]. First of all, it concerns

the thermal denaturation of proteins, polypeptides and peptides of milk.

One of the industrially important protein-containing products of milk processing is casein whey. It contains all milk proteins, except of the proteins of the casein complex and a very heterogeneous group of minor proteins of fat globules [4]. Its composition includes the following main proteins: β-lactoglobulin (β-lg), α-lactalbumin (α-la), blood serum albumin (BSA) or serum albumin and immunoglobulins (Ig). Thermal denaturation of these proteins determines the physical and chemical changes of casein whey during its heating. All these proteins differ significantly in their primary structure, spatial structure and, accordingly, resistance to thermal denaturation. Therefore, in order to find out the effect of heating on casein whey, a significant part of the research concerned the thermal denaturation of individual protein fractions of milk whey.

In general, whey proteins, unlike caseins, are not resistant to heat denaturation. According to various studies, changes in the structure and properties of whey proteins begin already at temperatures above 65°C. At temperatures above 70°C, these changes become irreversible and are fully completed at 100°C [4]. According to other studies, 10 minutes of heating at 95°C is enough to denature all whey proteins [5]. In this case, the degree of denaturation of whey proteins was determined by their solubility at pH 4.6 in a saturated NaCl solution. According to this criterion, sensitivity to heating increases in the following order: α-lactalbumin > β-lactoglobulin > serum albumin > immunoglobulins. According to differential scanning calorimetry, this order has the following form: immunoglobulins > β-lactoglobulin > α-lactalbumin > serum albumin. Moreover, denaturing changes in α-lactalbumin begin when the temperature reaches 62°C, and in β-lactoglobulin – at 75°C [6]. Also, the resistance of whey proteins to thermal denaturation depends on many factors: the method of heating, composition of the medium, pH, duration of heating, pretreatment of milk or whey [7,8].

In the case of casein whey, the effect on denaturation of a weakly acidic pH value of about 4.6, as well as preliminary low-temperature heating to 40–45°C during milk skimming and to 45–46°C during casein precipitation, is primarily relevant. The method of heating has a certain influence on the processes of whey proteins denaturation. In general, when considering β-lg, direct steam heating causes less denaturation compared to indirect heating. Depending on the pH, whey proteins are the least sensitive to heating at acidic pH values in the range of 2.5–3.5, as well as more than 6.5. The lowest resistance to thermal denaturation is manifested at pH values close to the α-la isoelectric point (pH 4.5), as well as in the range 5.8–6.2 [2, 4].

To understand the general picture of whey proteins thermal denaturation, it is important to consider the contribution of their main components to this process: β-lg, α-la, BSA and Ig. First of all, this applies to β-lg, which constitutes about 50% of milk whey proteins. According to the four-stage thermal denaturation scheme of β-lg [9], when its solution is heated to 40°C, its dimers transform into monomers (I stage). After achieving a temperature of 65°C, the tertiary and partially secondary structure of the β-lg molecule changes with increased availability of sulfhydryl groups of cysteine residues (stage II). The third stage (>70°C) is accompanied by the formation of intermolecular disulfide bonds and, accordingly, aggregates consisting of two or more β-lg molecules. During the fourth stage (>80°C), aggregation continues due to the formation of hydrophobic interactions between nonpolar amino acid residues. The first two stages are reversible, and changes in the structure fixed by covalent bonds and hydrophobic interactions of stages III and IV are irreversible. Thermal denaturation of β-lg is more active at pH values of about 4.5, where hydrophobic interactions play a greater role [10,11].

The initial temperature of denaturation of the second milk whey protein - α-la is 62°C [9]. However, it is the most heat-resistant whey protein. This is due to the presence of eight cysteine residues and, accordingly, four disulfide bonds in the primary structure of α-la. At the same time, there are no free sulfhydryl groups [12,13]. Such features of the primary structure of α-la provide it a high ability to reverse denaturation [9]. Analogous stages of α-la denaturation take place at much higher temperatures if compare α-la and β-lg, [14]. At the same time, irreversible changes in the spatial structure of α-la when heated even to 100°C can reach only 50%. Calcium ions also play an important role in the processes of thermal denaturation and renaturation of α-la [15]. Unlike β-lg, α-la exhibits the highest resistance to thermal denaturation at pH values of about 4.8. Further, it decreases when the pH changes to the acidic values.

Two other main fractions of milk whey proteins – BSA and IgG are less resistant to heat. Their initial denaturation temperatures are 64^0C and 72^0C, respectively [9]. The course of thermal denaturation of BSA resembles the same of β-lg. However, this protein begins to form aggregates at a lower temperature than β-lg [16]. Irreversible changes during thermal denaturation of BSA occur at temperatures above 60°C. Immunoglobulins are the most sensitive to thermal denaturation in terms of solubility. According to scanning calorimetry, immunoglobulins are more stable than β-lg. But regardless of this, it is important that Ig loses its biological activity already at 72°C [17,18]. Also, the important minor protein of milk whey – lactoferrin (Lf) – begins to lose numerous biological activities at temperatures above 60–65°C [19].

So, the influence of temperature above 45–50°C on the structure and properties of milk whey proteins and, in particular, casein whey, is described in sufficient detail in the scientific literature. Therefore, the possible

influence of low temperatures (< 50°C) is actual, since such temperatures are used in the processes that precede the casein whey obtaining.

The purpose of the work is to establish the effect of low-temperature heating on the resistance of casein whey proteins to thermal denaturation. To achieve this goal, the following tasks must be solved:

- obtain a control sample of whey after low-temperature heating;
- characterize the peculiarities of the fractional composition of casein whey proteins after low-temperature heating;
- to conduct a comparative analysis of resistance to heat denaturation at different temperatures of casein whey proteins obtained without heating and with low-temperature heating.

Research materials and methods

Skimmed milk with an acidity of 18^0T and production casein whey (pH 4.6) from PJSC "Ternopil Dairy Factory" were used in the work.

Proteins of the casein complex were isolated from skimmed milk by isoelectric precipitation at pH 4.6 hydrochloric acid. Caseins were precipitated by centrifugation at 3000 rpm on an OPN-8 centrifuge. The precipitate was washed with distilled water and dissolved by adding 1 N NaOH with stirring at pH values less than 10. To purify the casein complex proteins from whey proteins and proteose-peptone fraction components, reprecipitation was carried out twice.

Laboratory whey samples were obtained after isoelectric precipitation of casein. When using whey proteins as a control during electrophoresis, they were purified from low-molecular-weight compounds and transferred to the environment of the electrophoretic buffer by gel filtration on a Sephadex G-25 column.

The concentration of caseins and whey proteins was determined by absorption at a wavelength of λ=280 nm on a SF-46 spectrophotometer. The protein concentration in the samples was calculated using the following absorption coefficients ($D_{1см}^{1\%}$): 12.3 for milk whey proteins and 8.2 for casein complex proteins [4]. Mathematical and statistical processing of research results was carried out using Microsoft Office Excel 2007 software packages. The fractional composition of whey proteins, the casein complex, as well as proteins of the undenatured residue after whey heating was analyzed by express electrophoresis in a polyacrylamide gel under native conditions [20]. Quantitative processing of electrophoregrams was carried out using the function of reading graphic images imread.

Results of the research and their discussion

To determine the effect of low-temperature heating on the fractional composition of casein whey, samples were taken from one batch of milk from the PJSC "Ternopil Dairy Factory". Casein was precipitated from one sample of such at the isoelectric point (pH 4.6) with hydrochloric acid at a temperature of 18°C in laboratory conditions. This whey was used as a control sample for further studies. The resulting casein was reprecipitated twice and used as a marker protein for electrophoretic analysis. The second whey sample was production casein whey from PJSC "Ternopil Dairy Factory" after casein separation using hydrochloric acid at a pH of 4.6 and heating to 46°C. The results of express electrophoresis in the native conditions of these samples are presented in Fig. 1.

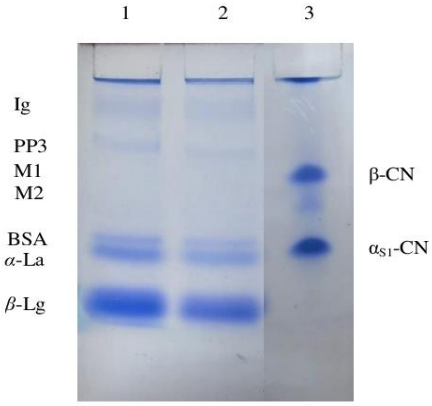

Fig. 1. Electrophoregrams of control laboratory whey (1), production casein whey (2) and casein preparation (3). M1 and M2 are unidentified minor fractions

Proteins of casein complex are absent in both whey samples, as can be seen on the electrophoregram. Also, the qualitative fractional composition of whey proteins in both samples is identical. However, the bands of minor proteins, as M1 and M2, are less expressed in the production whey sample. For more accurate detection of quantitative differences in the composition of protein fractions, their densitometry was carried out (Tables 1 and 2). In general, it can be noted that the quantitative differences for the main fractions (β-lg, α-la, BSA, Ig) are insignificant.

Selected samples of casein whey (laboratory and production) were further tested for resistance to thermal denaturation by holding for 5 min at temperatures from 60 to 100°C with an interval of 5°C. Denatured proteins were precipitated by centrifugation (3000 rpm), and the supernatant was filtered. Further, it was experimentally determined that it needs to be diluted 30 times for spectrophotometry at a wavelength of 280 nm. It is such dilution that allows to obtain optical density values in the range from 0.2 to 0.6 and provides a direct dependence of optical density values from protein concentration. The results of research on the resistance of laboratory and production casein wheys to thermal denaturation are shown in Fig. 2.

Table 1 – The content of the main protein fractions in the residue of undenatured proteins of laboratory casein whey after short (5 min) heating at 70, 80, 90 and 100°C (M±m, n=5)

Whey protein fraction	The content of protein fractions (%)				
	Control laboratory whey	Holding of laboratory whey for 5 minutes at temperature:			
		70°C	80°C	90°C	100^0C
β- lactoglobulin	39.0±4.5	43.1± 3.9	42.4±3.6	Traces *	Traces *
α- lactalbumin	17.1±2.1	19.3±1.2	23.9±1.5	43.5±2.2	35.06±1.58
Serum albumin	9.8±1.2	5.6±0.5	4.0±0.3	Traces *	Traces *
Immunoglobulins	16.5±1.4	5.5±0.3	Traces *	Traces *	Traces *

* less 3%

Table 2 – The content of the main protein fractions in the residue of undenatured proteins of production casein whey after short (5 min) heating at 70, 80, 90 and 100°C (M±m, n=5)

Whey protein fraction	The content of protein fractions (%)				
	Control laboratory whey	Holding of production whey for 5 minutes at temperature :			
		70°C	80°C	90°C	100°C
β- lactoglobulin	41.1±4.0	43.3±4.1	50.1± 4.5	24.2±1.4	Traces *
α- lactalbumin	17.3±1.9	19.4±1.6	28.3±2.3	29.3±1.6	36.4±1.5
Serum albumin	10.2±1.4	7.9±0.4	8.5±0.6	8.3±0.7	8.9±0.5
Immunoglobulins	17.1±1.0	5.7±0.5	Traces *	Traces *	Traces *

* less 3%

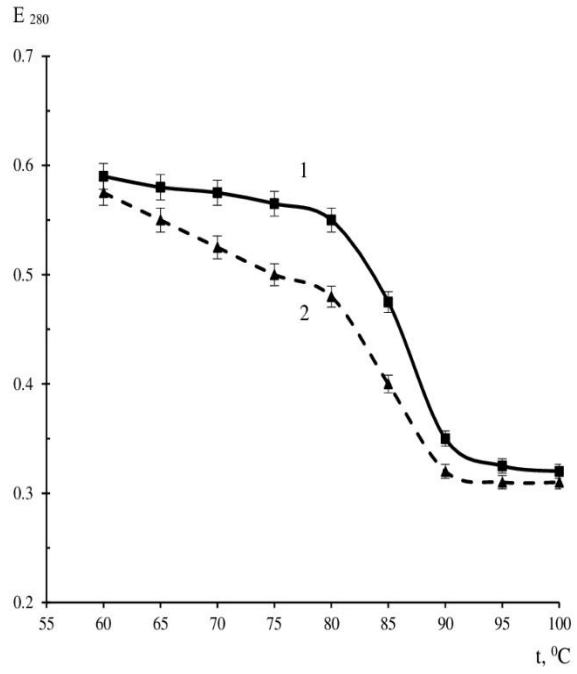

Fig. 2. The change of optical density of the solution of undenatured proteins of laboratory (1) and production (2) casein wheys after short-term (5 min) heating

As can be seen in the graphs, the values of the concentration of undenatured protein for both whey samples do not coincide. The difference is especially noticeable in the temperature range from 70 to 90°C. In general, laboratory whey proteins are more resistant to heat denaturation under such conditions. It is obvious that low-temperature heating of production whey to 46°C during casein separation can cause the first stages of denaturation changes in the protein structure. The possibility of such changes was indicated in publications [9,13]. Such changes can reduce resistance to high-temperature thermal denaturation. Taking into account the holding duration of production casein whey at room temperature (3–4 hours) before research conducting – they may be irreversible.

Taking into account the heterogeneous composition of casein whey proteins, an electrophoretic analysis of the undenatured protein residue was performed after heating for 5 minutes at 70, 80, 90 and 100°C. Typical electrophoregrams of undenatured proteins of both types of whey are shown in Fig. 3 and 4.

According to the qualitative composition of the protein fractions, it is possible to note the absence (traces) of β-lg in the production whey after heating at 90 and 100°C. On the electrophoregram of laboratory whey a band that corresponds to β-lg after heating at 90°C is clearly visible. Also, on the electrophoregram of laboratory whey, unlike production one, the BSA band remains after heating at 90°C and to a lesser extent at 100°C. Qualitative changes of other fractions during heating are similar for both whey samples. This concerns to Ig, α-la and PP3. Moreover, the coloration intensity of the PP3 band fractions does not change starting from a temperature of 70°C.

Quantitative assessment of the undenatured main protein fractions (β-lg, α-la, BSA, Ig) ratio based on densitometry of five electrophoregrams of each type of casein whey after short-term heating at 70, 80, 90 and 100°C is shown in table. 1 and 2. The dependence of the relative content of the main protein fractions in the undenatured residue on the heating temperature is complicated by a simultaneous decrease in the content of minor proteins and polypeptides. They also

gradually lose their solubility (except for the proteose-peptone fraction components) [4]. However, in general, they show greater resistance to heating and their content in the undenatured residue at high temperatures (80-100°C) is more than 50% of all proteins.

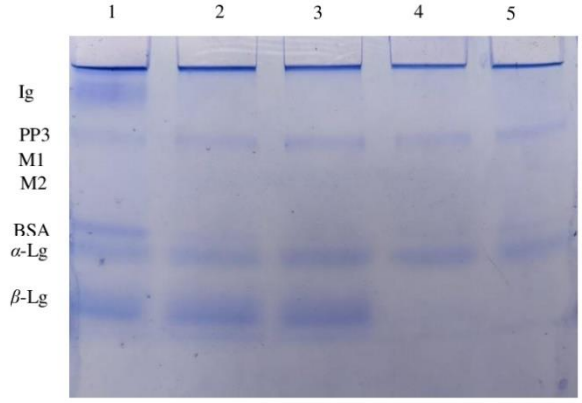

Fig. 3. Electrofogram of control laboratory casein whey proteins (1) and undenatured proteins of the same whey after heating (5 min) at 70^{0}C (2), 80^{0}C (3), 90^{0}C (4) and 100^{0}C (5)

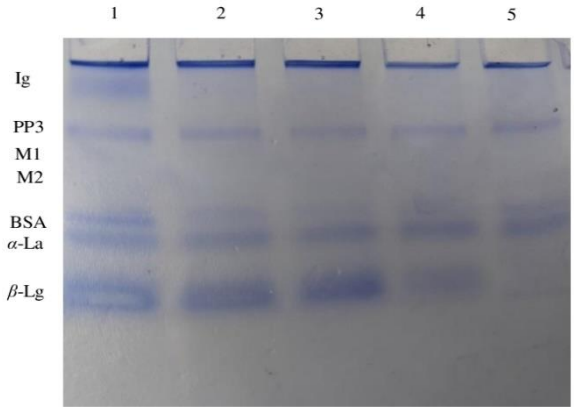

Fig. 4. Electrofogram of production casein whey proteins (1) and undenatured proteins of the same whey after heating (5 min) at 70°C (2), 80°C (3), 90°C (4) and 100°C (5)

When considering individual fractions, it is possible to note the similarity of the relative content of immunoglobulin and α-lactalbumin in the undenatured residues of both types of casein whey (tables 1 and 2). A certain increase in the relative content of α-la (43.5±2.2%), when the production whey was heated to 90°C (Table 2), is explained by the almost complete absence of undenatured β-lg under these conditions. The higher resistance of α-la to heating was shown earlier [14]. The BSA of the laboratory whey was more resistant to heating (>80°C) compared to the BSA of the production casein whey. The biggest differences were established for β-lg. This fraction is present in significant amounts (24.2±1.4%) in the undenatured residue of laboratory whey after heating for 5 minutes at 90°C. In the production whey under such conditions, β-lg is practically absent (Table 2).

The influence of preliminary low-temperature heating on the resistance of casein whey to heat denaturation can be detected only with short-term heating to high temperatures. During long-term heating of both samples of casein whey in the range from 60 to 100°C, no differences in the concentration of undenatured proteins were found (Fig. 5). It can mean that long-term heating at high temperatures cancels differences in the properties of whey proteins caused by previous low-temperature heating.

The obtained results also indicate that both reversible and irreversible changes in the structure of milk whey proteins occur during low-temperature heating. Reverse changes do not affect the resistance of whey proteins to high-temperature denaturation. Since we found such an effect, part of the changes in the structure of whey proteins can be attributed to irreversible changes in the case of preliminary low-temperature heating to temperatures below 50°C.

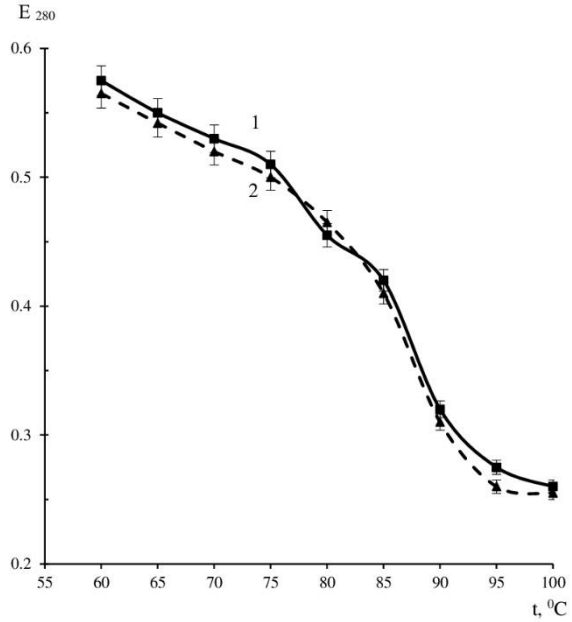

Fig. 5. The change of optical density of the solution of undenatured proteins of laboratory (1) and production (2) casein wheys after long-term (30 min) heating

Approbation of research results. The obtained results are used at PJSC "Ternopil Dairy Factory" in the processing of casein whey, as well as in the laboratory of milk biochemistry of the Ternopil Ivan Puluj National Technical University in the isolation of biologically active proteins and peptides from milk whey.

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Conclusions

As a result of the research, the following conclusions can be made:

– low-temperature heating up to 50°C during production of casein whey leads to a decrease in the resistance of its proteins to high-temperature denaturation in the temperature range (70–100°C) (5 min) in comparison with whey proteins obtained at 18°C in laboratory conditions. Long-term high-temperature treatment of wheys does not allow such changes to be detected.

– electrophoretic analysis of the relative content of the main protein fractions of the undenatured residue after high-temperature heating showed that changes in stability occur due to β-lactoglobulin and serum albumin (BSA). The relative concentration of these fractions in the undenatured residue from production whey is <3%, and in the residue from laboratory whey >8% for BSA and >24% for β-lg when heated to 90°C for 5 min.

– long-term holding of both wheys at 20°C before the study of their resistance to heat denaturation shows that some of the changes during the previous low-temperature heating to 50°C can lead to irreversible changes in the structure and properties of casein whey proteins.

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ВПЛИВ НИЗЬКИХ ТЕМПЕРАТУР НАГРІВАННЯ НА БІЛКИ КАЗЕЇНОВОЇ СИРОВАТКИ

В. Юкало, доктор біологічних наук, професор, *E-mail*: yukalo2007@gmail.com
К. Дацишин, кандидат технічних наук, доцент, *E-mail*: katkostyuk3103@gmail.com
Л. Сторож, кандидат технічних наук, доцент, *E-mail*: lstorozh@gmail.com
Кафедра харчової біотехнології і хімії
Тернопільський національний технічний університет імені Івана Пулюя
вул. Руська, 56, м. Тернопіль, Україна, 46001

Анотація. У роботі проведено порівняльний аналіз фракційного складу і стійкості до теплової денатурації білків казеїнових сироваток: лабораторної, яка була отримана при температурі 18°C, і виробничої – при температурі 46°C. В обох випадках сироватки відділяли після ізоелекричного осадження білків казеїнового комплексу хлороводневою кислотою. Експрес-електрофорез в поліакриламідному гелі показав ідентичний якісний склад основних і мінорних білків в обох сироватках. Також не виявлено відмінностей у відносному вмісті основних білкових фракцій сироваток: β-лактоглобуліну (β-lg), α-лактальбуміну (α-la), альбуміну сироватки (BSA) та імуноглобулінів (Ig). Для порівняння стійкості до теплової денатурації зразки лабораторної і виробничої сироваток нагрівали і витримували 5 хв при температурах від 60°C до 100°C з інтервалом 5°C. Осад денатурованих білків відділяли центрифугуванням. Концентрацію білків в супернатанті визначали спектрофотометрично і аналізували якісний та кількісний склад основних білкових фракцій експрес-електрофорезом. У результаті встановлено, що концентрація неденатурованого білка в обох сироватках не співпадає в діапазоні температур 70–90°C. Більш стійкими до теплової денатурації виявились білки лабораторної сироватки. Причому ці відмінності можна виявити лише при короткотривалій (5 хв) тепловій денатурації. Довготривале високотемпературне нагрівання (30 хв) не дозволяє їх виявити. Електрофоретичний аналіз відносного вмісту основних білкових фракцій показав, що зміни у стійкості відбуваються завдяки двом фракціям – β-lg і BSA. Відносна концентрація цих фракцій в неденатурованому залишку з виробничої сироватки < 3%, а в залишку з лабораторної сироватки > 8% – для BSA і > 24% для β-lg при нагріванні до 90°C протягом 5 хв. Тривале витримування обох сироваток при 20°C перед дослідженням їх стійкості до теплової денатурації може свідчити, що частина змін у стійкості білків виробничої сироватки, які спричинені попереднім нагріванням до 46°C, є незворотними.

Ключові слова: протеїни казеїнової сироватки, нагрівання, теплова денатурація.