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MILK-COAGULATION AND PROTEOLYTIC ACTIVITY OF «GLEK» CARPATHIAN ENZYME PREPARATION

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The purpose of this work was to study milk-clotting and proteolytic properties of preparation «Glyeck». Using Soxlet method we showed that the preparation possesses a high milk-clotting activity, and this activity doesn't change a lot during the storing process (up to 2 years). This is due to two processes taking place simultaneously: decrease in milk-clotting activity of the preparation and reducing the water content in the preparation. Proteolytic activity of the preparation was investigated by both: the modified Benki method and electrophoresis in polyacrylamide gel alkaline system. Electrophoretically homogeneous fractions of α_{s1}-, β-, and κ-caseins were obtained to characterize the specificity of the proteolysis. The specificity of cleavage of casein fractions by «Glyeck» preparation was compared with this one of the rennet enzyme and pepsin. It was found that «Glyeck», rennet enzyme and pepsin actively split κ-casein. Preparation «Glyeck» showed low proteolytic activity to α_{s1}- and β-caseins that is typical for purified chymosin. Electrophoretic analysis of proteolysis products of casein fractions confirms the high specificity of «Glyeck» preparation towards κ-casein. Casein fractions such as α_{s1}- and β-casein were more resistant to this preparation. The results indicate that preparation «Glyeck» is a natural milk-clotting preparation with high quality and it is mainly consists of protelytic enzyme chymosin.

Key words: milk-clotting preparation «Glyeck», milk-clotting activity, proteolytic activity, casein fractions.

Enzyme coagulation of milk is an important process in the production of various types of cheese. This process can be attributed to food biotechnology. It reproduces coagulation of milk casein in the stomach of dairy calves. The biological significance of this phenomenon lies in the retention of milk protein casein complex in the stomach for better digestion [1, 2].

Casein coagulation occurs due to specific cleavage of κ-casein (peptide bond 105–106) using chymosin (EC 3.4.23.4). As a result a hydrophilic glycomacropeptide (κ-casein fragment 106–169) is formed. After losing glycomacropeptide the casein micelles coagulate thanks hydrophobic interactions, formation of calcium phosphate and hydrogen bonds [3]. With much less intensity chymosin also breaks down some peptide bonds of αs_1-, and β-casein (24 bonds of αs_1-casein and 9 bonds of β-casein). The cleavage of κ-casein, resulting in coagulation of milk and clot formation, is called specific proteolysis in cheese biochemistry. Splitting other casein fractions and κ-casein fragments is called nonspecific proteolysis [1]. Nonspecific proteolysis causes loss of proteins and occurrence of peptides with bitter taste. Therefore chymosin is the best suited agent for milk coagulation in cheese production. However, this enzyme is deficient because it is produced only from dairy calves stomachs. With the transition from milk to other feed calves begin to synthesize pepsin. Milk-clotting preparations containing pepsin have high non-specific proteolytic activity and lead to loss of the protein and occurrence of flavoring defects in cheese [4]. Attempts to find a cheap chymosin substitute have been lasted for many years. It was suggested pepsin from different animals, plant proteases, enzymatic preparations of microbial origin [2, 5]. But none of them could fully replace chymosin. In 80-th chymosin i-RNA was obtained and genetically engineered recombinant microorganisms that synthesize chymosin appeared. The properties of the obtained enzyme were similar to those of chymosin from the stomachs of calves [6, 7]. Enzyme preparations are widely used nowadays, but there are certain risks related to their origin from recombinant organisms.

That is why the traditional natural milk-clotting preparation, which is produced in farms of Ukrainian Carpathians, attracts

attention. This preparation is used there for a long time to produce soft cheese from sheep's and cow's milk. It is called «Glyeck» or «Glyeg». In 1955 professor Rudavska H. B. described the production process of cheese in the Carpathians using the preparation «Glyeck» [8]. According to the manufacturing technology of the preparation — it has to include chymosin as a milk-clotting enzyme. However, there has been no detailed research of composition, activity and specificity of enzyme preparation since those times.

Based on the foregoing, the purpose of our work was to study the biochemical characteristics of traditional milk-clotting enzyme preparation — «Glyeck».

Materials and Methods

During our research we used the samples of milk-clotting enzyme preparation «Glyeck» which were stored nearly one month, one year, two years. The preparations were obtained in Kosiv region during 2013–2014. For comparison we used standard rennet enzyme which was manufactured in Moscow factory of rennet enzymes (Russia) and pepsin (Kaunas, Lithuania).

Total casein was obtained from fresh skim milk by isoelectric precipitation under condition of inactivation of natural proteases. Homogeneous fractions of αs_1-casein and β-casein were obtained by differential precipitation in the presence of urea and with ion-exchange chromatography purification with DEAE — Toyopearl-659M (Sigma-Aldrich). Homogeneous κ-casein was obtained by preparative electrophoresis in anodic system of polyacrylamide gel (PAAG). The conditions of preparative electrophoresis were described earlier [9]. Homogeneity of caseins and fractional composition of products division were analyzed by method of electrophoresis on the vertical plates of PAAG in the system of Stadiera. It was used the alkaline buffer gel system (pH-7.9), that included 25 mM of Tris, 27 mM of dietylbarbiturate,and 4.5 M of urea. Electrophoretograms were fixed and manifested using common methods. Fractional composition of milk-clotting preparations was analyzed with disc-electrophoresis on the PAAG plates under native conditions as it was described in [10]. Electrophoretic buffers and gels were prepared, using reagents of "Reanal (Hungary).

Milk-clotting activity by Sokslet method and proteolytic activity of enzyme preparations by Benki method were examined as previously described in [2]. Humidity of preparations was determined by drying them to constant mass at 100 °C. Total protein in preparations was determined by Keldal using the conversion coefficient 6.25.

Concentration of casein fractions was determined on a spectrophotometer SF-46 (λ = 280 nm). Absorbtion coefficients ($D^{1\%}_{1cm}$): 10.0 — for αs_1-casein; 4.6 — for β-casein; 9.6 — for κ-casein and 8.2 — for total casein.

Results and Discussion

We used for researches Enzyme milk-clotting preparations «Glyeck» that were stored under the same conditions during one month, one year and two years. The samples of preparations at different storage period are shown at Fig. 1.

All these preparations can be used for production of soft cheese, made from sheep's and cow's milk. The amount of preparation, which is used for milk clotting and clot obtaining, slightly depends on storage period. To clarify this phenomenon we determined the following indices for three groups of preparation (1 month, 1 year, 2 years): water content, proteins content and milk-clotting activity. Results are given at Table 1.

These results suggest the increase of milk-clotting activity during the first year of storage. The decrease was noticed only after two years. And only after two years it begins to decrease. Milk-clotting activity is relatively small in comparison with the standard rennet enzyme (100,000 u/g). Obviously this is due to the high water content, which gradually decreases throughout the storage period (from ~60% to 16%). Taking into account of water content the activity of fresh preparation relative to standard enzyme will be nearly 50,000 u/g. During storage period the activity of preparation decreases, based on the total protein, by more than 2 times for two years. But it doesn't change a lot in terms of the whole preparation. Such suitable properties of the

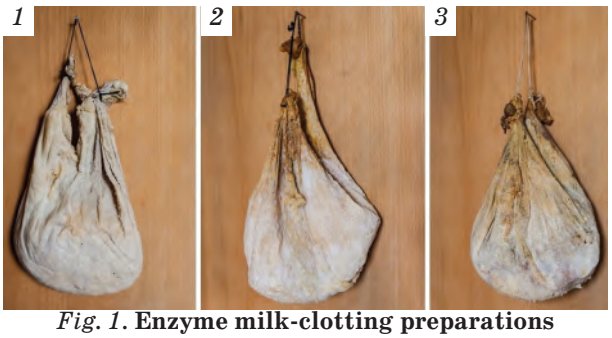

Fig. 1. **Enzyme milk-clotting preparations «Glyeck» at different storage period:**
1 — one month; *2* — one year; *3* — two years

Table 1. **Characteristic of milk-clotting preparations «Glyeck» of different storage time**

Storage time of preparations «Glyeck»	Total protein (g/100g of preparation)	Water content, (%)	Milk-clotting activity according to Soxlet (u/g of preparation)	Milk-clotting activity according to Soxlet (u/g of preparation protein)
1 month	11.0 ± 1.5	59 ± 8.3	5100 ± 580	45000 ± 740
1 year	17.0 ± 2.9	31.0 ± 3.4	5700 ± 450	33000 ± 5 600
2 years	25 ± 4.5	16 ± 2.5	4500 ± 580	18000 ± 4 900

Note: $P < 0.05$.

preparation were selected empirically on the base of conditions providing its manufacturing and storage.

In addition to milk-clotting activity the proteolytic activity is the important characteristic of milk-clotting preparations. As it was previously shown the proteolytic activity of preparation «Glyeck» in regarding to total casein was similar to this one of the rennet enzyme, moreover it was much lower than pepsin's activity. This may indicate that chymosin was the main component of the preparation. To verify this fact it was performed the proteolysis of main casein fractions by the modified method of Benki. 0.5% solution of casein fractions: αs_1, β- and κ-casein were used as substrates. Proteolytic preparations («Glyeck» — 1 month, rennet enzyme and pepsin) were taken in concentrations, which provided the same milk-clotting activity. Enzyme-substrate ratio for pepsin was 1:100. Concentration of other preparations was set so that they had the same milk-clotting activity. Proteolysis was performed at pH-7.2 and t° 35 °C. The products soluble in 12% trichloroacetic acid proteolysis products were determined spectrophotometrically at wavelength λ = 280 nm. However, this method was not sufficient to compare the proteolysis of the casein fractions because they differ significantly in their ability to absorb ultraviolet (see Matherials and Methods). Therefore we admitted in calculations that proteolysis products of each fraction have the same absorption coefficients ($D^{1\%}_{1cm}$) as the fractions themselves. Optical density (E_{280}) for α_{S1}-casein and for its proteolysis products was left unchanged, for β-casein the optical density was multiplied by a coefficient 2.17 ($D^{1\%}_{1cm\alpha_{s1}-CN}/D^{1\%}_{1cm\beta-CN}$), and for κ-casein by a coefficient 1.04 ($D^{1\%}_{1cm\alpha_{s1}-CN}/D^{1\%}_{1cm\kappa-CN}$). Results of proteolysis are given on graphics (Fig. 2, 4 and 6). Each point is the average of five measurements. As it can be seen all preparations actively cleave κ-casein. Higher values of pepsin proteolysis apparently achieved through non-

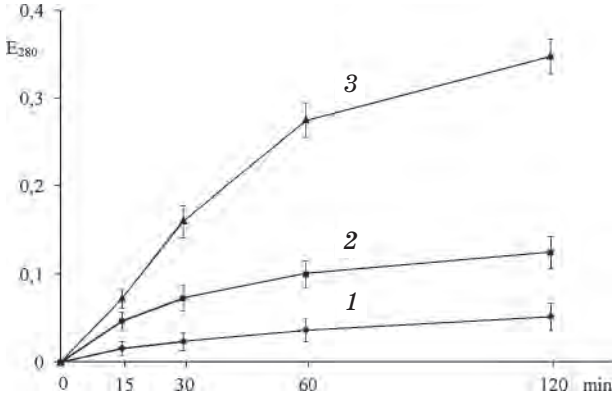

Fig. 2. **Proteolysis of α_{S1}-casein:**
1 — by milk-clotting preparation «Glyeck»; *2* — by rennet; *3* — by pepsin.

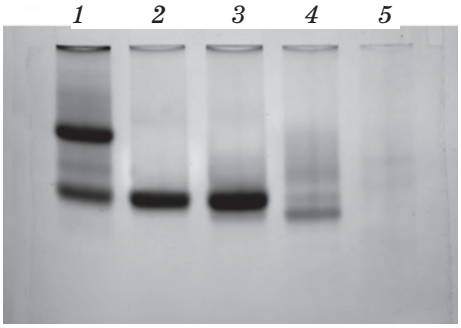

Fig. 3. **Electrophoretogram:**
1 — total casein; *2* — α_{S1}-casein; *3* — α_{S1}-casein after proteolysis by preparation «Glyeck»(60 min); *4* — α_{S1}-casein after proteolysis by rennet (60 min); *5* —α_{S1}-casein after proteolysis by pepsin (60 min).

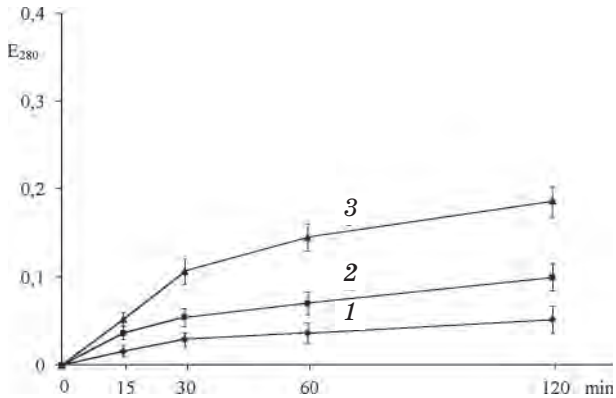

Fig. 4. **Proteolysis of β-casein:**
1 — by milk-clotting preparation «Glyeck»;
2 — by rennet; *3* — by pepsin

specific proteolysis of κ-casein (Fig. 6). Rennet enzyme preparation has also admixture of pepsin and therefore possesses higher proteolysis activity than «Glyeck». In relation to α_{s1}-casein and β-casein «Glyeck» showed low activity, that is typical for pure chymosin [1]. Rennet enzyme and especially pepsin cleave these fractions actively. To verify the specificity of proteolysis we selected samples for electrophoretic analysis of the proteolysis products. The results of electrophoresis are given at Fig. 3, 5 and 7. According to these data κ-casein was actively cleaved by all preparations. At the same time glycomacropeptide is absent, as in alkaline buffer system it is not included in the gel. According to electrophoresis data α_{s1}- and β-casein had no changes under the action of the preparation «Glyeck». At the same time rennet enzyme having the same milk-clotting activity showed the notable proteolysis of α_{s1}- and β-caseins. At Fig. 5 it is presented the typical peptides of β-casein, which are formed by the action of rennet enzyme and pepsin. The specificity of casein fractions proteolysis is associated with the appearance of the defect in cheese – its bitter taste. It takes place due to accumulation of bitter peptides. There are many hydrophobic amino acids in these peptides. Among the casein fractions the most hydrophobic is β-casein [11]. Its C-terminal site possesses the most pronounced hydrophobic properties. Peptides obtained from this site have extremely bitter taste. Another source of bitter peptides is α_{s1}-casein [4].

As «Glyeck» shows low activity towards α_{s1} and β-caseins, it can be concluded that it is a valuable natural milk-clotting preparation, which is close to chymosin according to its properties. On the other hand, «Glyeck» can be stored for a long time without significant changes in its milk-clotting activity.

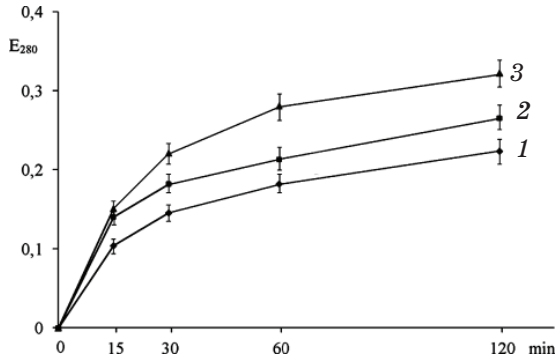

Fig. 6. **Proteolysis of κ-casein:**
1 — by milk-clotting preparation «Glyeck»; *2* — by rennet; *3* — by pepsin

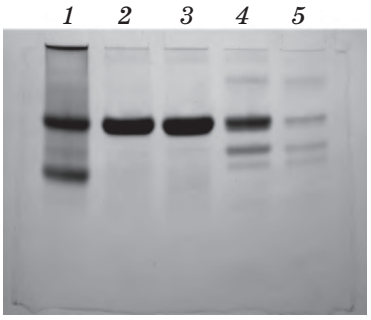

Fig. 5. **Electrophoretogram:**
1 — total casein; *2* — β-casein; *3* — β-casein after proteolysis by preparation «Glyeck»(60 min); *4* — β-casein after proteolysis by rennet (60 min); *5* — β-casein after proteolysis by pepsin (60 min)

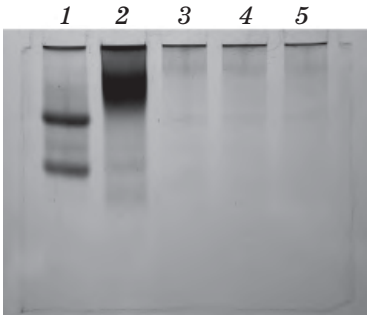

Fig. 7. **Electrophoretogram:**
1 — total casein; *2* — κ-casein; *3* — κ-casein after proteolysis by preparation «Glyeck»(60 min); *4* — κ-casein after proteolysis by rennet (60 min); *5* — κ-casein after proteolysis by pepsin (60 min)

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МОЛОКОЗСІДАЛЬНА ТА ПРОТЕОЛІТИЧНА АКТИВНІСТЬ КАРПАТСЬКОГО ЕНЗИМНОГО ПРЕПАРАТУ «ГЛЄК»

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Метою роботи було дослідження молокозсідальних і протеолітичних властивостей препарату «Глєк». Результати дослідження методом Сокслета показали, що препарату притаманна висока молокозсідальна активність, причому вона майже не змінюється в процесі зберігання (до двох років). Це відбувається завдяки одночасному зменшенню молокозсідальної активності під час зберігання та зменшенню вмісту води у препараті. Протеолітичну активність досліджували модифікованим методом Бенкі, а також електрофорезом у лужній системі поліакриламідного гелю. Для характеристики специфічності протеолізу було виділено електрофоретично гомогенні фракції α_{S1}-, β-, і к-казеїнів. Специфічність розщеплення казеїнових фракцій препаратом «Глєк» порівнювали із сичужним ензимом та пепсином. У результаті встановлено, що «Глєк», сичужний ензим і пепсин активно розщеплюють к-казеїн. Стосовно α_{S1}- і β-казеїнів «Глєк» показав низьку протеолітичну активність, що характерно для очищеного хімозину. Дані електрофоретичного аналізу продуктів протеолізу казеїнових фракцій підтверджують високу специфічність препарату «Глєк» стосовно к-казеїну. Фракції α_{S1}- і β-казеїнів є відносно стійкими до дії цього препарату. Отримані результати свідчать, що з протеолітичних ензимів «Глєк» містить у своєму складі переважно хімозин і є високоякісним природним молокозсідальним препаратом.

Ключові слова: молокозсідальний препарат «Глєк», молокозсідальна активність, протеолітична активність, казеїнові фракції.

МОЛОКОСВЕРТЫВАЮЩАЯ И ПРОТЕОЛИТИЧЕСКАЯ АКТИВНОСТЬ КАРПАТСКОГО ЭНЗИМНОГО ПРЕПАРАТА «ГЛЕК»

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Целью работы было исследование молокосвертывающих и протеолитических свойств препарата «Глек». Результаты исследования методом Сокслета показали, что препарату присуща высокая молокосвертывающая активность, причем она практически не изменяется в процессе хранения (до двух лет). Это происходит благодаря одновременному уменьшению молокосвертывающей активности при хранении и уменьшению содержания воды в препарате. Протеолитическую активность исследовали модифицированным методом Бэнки, а также электрофорезом в щелочной системе полиакриламидного геля. Для характеристики специфичности протеолиза было выделено электрофоретически гомогенные фракции α_{S1}, β- и к-казеинов. Специфичность расщепления казеиновых фракций препаратом «Глек» сравнивали с сычужным энзимом и пепсином. В результате установлено, что «Глек», сычужный энзим и пепсин активно расщепляют к-казеин. По отношению к α_{S1}- и β-казеинам «Глек» показал низкую протеолитическую активность, что характерно для очищенного химозина. Данные электрофоретического анализа продуктов протеолиза казеиновых фракций подтверждают высокую специфичность препарата «Глек» относительно к-казеина. Фракции α_{S1}- и β-казеинов относительно устойчивы к действию этого препарата. Полученные результаты свидетельствуют, что из протеолитических энзимов «Глек» содержит в своем составе преимущественно химозин и является высококачественным природным молокосвертывающим препаратом.

Ключевые слова: молокосвертывающий препарат «Глек», молокосвертывающая активность, протеолитическая активность, казеиновые фракции.