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Milk-clotting enzymes of various origin: prospects for application in cheese making

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The results of the comparative characteristics of rennet preparations have been presented. As the object of the study enzyme preparations possessing milk-clotting activity that are available in the Republic of Belarus have been considered. Indicators having important influence on the formation of a cheese curd were determined.
Keywords: rennet, cheese curd, physical and chemical properties.

В работе представлены результаты сравнительной характеристики ферментных препаратов сычужного действия. В качестве объекта исследования рассматривались доступные в Республике Беларусь ферментные препараты, обладающие молокосвертывающей активностью. Определялись показатели, оказывающие важное влияние на образование сырного сгустка.
Ключевые слова: сычужный фермент, сырный сгусток, физико-химические свойства.

According to the main Department of the processing industry of the Ministry of Agriculture and Food of the Republic of Belarus, the cheese market in the Republic of Belarus is constantly growing and developing branch. New types of cheese are being mastered; capacities for their production are increasing. The volume of production is 5 times the volume of consumption. In 2019 the Republic of Belarus exported cheese to 19 countries. In the structure of milk and dairy products supplies to the external market, cheeses account for almost 40 % of export earnings. The growth rate of cheese exports in 2019 was 118 %. The success of Belarusian dairy companies in the market is a combination of two factors: continuous work to improve the quality of products and affordable price for consumers [1].

According to the Republican unitary enterprise (RUE) «National Center for Marketing and Price Study» the main part of cheese is produced with the rennet. These enzymes are supplied by the Moscow rennet plant. There are also some European milk-clotting enzymes (from Denmark, Holland, Italy and other countries) which are supplied to Belarus by various intermediary firms [2]. Therefore, it is important to produce a domestic milk-clotting enzyme that will reduce the cost of purchasing rennet abroad.

The most famous enzyme traditionally used for milk coagulation is the rennet rennin or chymosin (EC 3.4.23.4). The enzyme belongs to the class of aspartate proteinases [3, p. 156], it is found in the juice of the fourth stomach of calves [4, p. 50], [5 p. 153].

The rapid expansion of dairy production and the shortage of animal rennet contributed to the emergence of new coagulants for milk of microbial and plant origin. Preparations of milk clotting enzymes can be conditionally divided into two groups: first – rennet; the second is rennet substitutes. The second group includes substitutes of animal, plant, microbial origin and recombinant chymosin.

Milk clotting enzyme preparations must meet the following basic criteria:

– strict compliance with the declared clotting activity of milk to the norm and, as a result, optimal consumption of preparations;

– high stability during long-term storage;

– optimal ratio of proteolytic and milk-clotting activity – one of the main characteristics that affect the quality of cheese;

– minimal non-specific proteolytic activity, which allows avoiding protein losses during cheese production, as well as the appearance of unpleasant tastes and consistency defects during cheese ripening.

Optimal thermal stability, allowing to strictly adhering to the main technological parameters of cheese production; – high purity of preparations [6 p. 50].

Not all milk-clotting enzymes meet these requirements and are therefore unsuitable for cheese making.

All commercial milk clotting enzymes are aspartate proteases (EC 3.4.23.) They specifically cleave the Phe105 – Met106 bond of bovine kappa casein.

The steady growth in global cheese production keeps the search for suitable rennet substitutes as the actual problem of science.

Fungi are an attractive source of various biologically active compounds [7, p. 1020]. Fungal enzymes are often produced in submerged conditions, but solid phase fermentation has also been used successfully. The cultivation conditions and the composition of the nutrient media strongly influence the ratio of milk clotting activity to the proteolytic activity of the enzyme [8, p. 997].

For each type of cheese, the use of one of the enzyme preparations offered by manufacturers should be theoretically and experimentally justified. Since in the Belarusian market there is a range of milk – clotting preparations that have different technological characteristics, we have attempted to give a comparative characteristic of rennet proteases presented on the Belarusian market of enzyme preparations.

Materials and methods. The following enzyme preparations were used in the work: rennet calf enzyme Renco; natural lamb enzyme Rennet; calf rennet E160; microbial fluid enzyme Maysan; microbial enzyme Rennet; enzyme preparation from the culture fluid of *Pleurotus ostreatus*.

Protein concentration was determined spectrophotometrically [9 p. 356].

Milk-clotting activity was determined according to the modified Pyatnitsky method. The unit of milk-clotting activity (MCA) was defined as the amount of enzyme that clots 100 ml of milk in 40 min at 35 C [10 p. 80].

The total proteolytic activity (PA) was determined by the lysis of gelatin in a thin layer of agar gel [11 p. 103]. For 1 unit of activity (E) of the enzyme was taken such an activity that leads to hydrolysis of the substrate in the area of the gel size 1 cm^2.

pH optimum of enzymes possessing milk clotting activity was measured at different pH values (from 3,5 to 7,5), using 0,2 M acetate buffer and 0,2 M phosphate buffer.

Temperature optimum of the activity of milk-clotting enzymes was determined after incubating the reaction mixture at different temperatures in the range from 25 to 45°C

The total proteolytic activity (PA) was determined by the lysis of gelatin in a thin layer of agar gel. For 1 unit of activity (E) of the enzyme was taken such an activity that leads to hydrolysis of the substrate over a gel area of 1 cm^2.

The pH optimum of enzymes with milk clotting activity was measured at various pH values (from 3,5 to 7,5) using 0,2 M acetate buffer and 0,2 M phosphate buffer.

The temperature optimum for the activity of milk coagulation enzymes was determined after incubation of the reaction mixture at various temperatures in the range from 25 to 45°C.

The effect of $CaCl_2$ concentration on MCA: enzyme activity was measured adding in milk various concentrations of $CaCl_2$ (from 2,5 to 30 mM).

The cheese curd formation was carried out in accordance with GOST 92225.

Results and discussion. The aim of the work was to study the activity of milk-clotting enzymes of different origin, which are used for the cheese production. The following preparations were chosen:

1. Rennet calf enzyme Renco. Natural rennet calf enzyme, a liquid salt solution comprising at least 92 % chymosin as the active component. Made in New Zealand («Renco»).

2. Natural lamb enzyme Rennet. This product is used for the production of fresh and medium-ripening cheese with a sweet and spicy taste. It is also very suitable for hard cheeses with a long ripening period. Made in Italy (Caglio Bellucci s.lr.).

3. Calf rennet E160. (In the form of solution). The preparation is suitable for the production of any type of cheese – hard cheeses, semi-hard cheeses, soft and blue cheeses,as well as cottage cheese. Composition: Chymosin 80 %, Pepsin 20 %. Made in Italy (Caglio Bellucci s.lr.).

4. Microbial enzyme Rennet. Milk-clotting enzyme preparation is the microbial coagulant and is produced on the basis of Rhizomucor miehei. All ingredients used in every stage of the manufacturing process are not animal-based. It is used for the production of fresh and medium-ripening cheeses. Enzyme composition: *chymosin: 75 % – pepsin: 25 %.* % . Made in Italy ((Caglio Bellucci s.lr.).

5. Liquid microbial enzyme Mayasan. This food microbial enzyme is produced by controlled fermentation of Rhizomucor miehei. It is used for the preparation of soft cheeses, feta cheese, as well as semi-hard and hard cheese with a ripening period of 3–6 months. Made in Turkey (Istambul, MAYASAN GIDA SAN).

6. An enzyme preparation developed at the Department of Biotechnology of the EE «Polessky State University» on the basis of the culture fluid of *Pleurotus ostreatus*.

Each preparation has the declared clotting activity, these data were compared with the results determined experimentally, at standard conditions according to GOST10 288-2001 «Preparations of milk clotting enzymes. Technical conditions». The results of these studies are presented in table 1.

Table 1 – Milk-clotting activity of commercial enzyme preparations

Trade mark	Milk-clotting activity, arbitrary units	
	The declared	Installed
Renco	580	580
Rennet	280	260
Rennet rhizomucor	700	750
E160	150	280
Mayasan	10000	1000

However, the claimed MCA data do not always coincide with those obtained experimentally. The experimental data results are presented in table 2.

Table 2 – MCA data of the studied enzyme preparations

Enzyme prepation	Volume, ml	Protein, mg/ml	Activity, units		Specific activity, unit/mg protein		MCA/PA
			MCA	PA	MCA	PA	
Renco	10	10	580	497	58	49,7	1,17
Rennet	10	10	260	265	26	26,5	0,98
Rennet rhizomucor	10	10	750	430	75	43	1,7
E160	10	10	280	191	28	19,1	1,5
Mayasan	10	10	1000	418	100	41,8	2,4
Enzyme preparation from *P. ostreatus*	1	1,07	81,08	6,32	75,78	5,91	13,7

The highest specific MCA (100 units/mg protein) contains preparation Mayasan, however, this preparation has high proteolytic activity, which is undesirable for rennet. It is known that with a high proteolytic activity of an enzyme preparation, not only clot formation is observed, but also its further hydrolysis. This leads to the appearance of bitter peptides and makes such an enzyme preparation not very suitable for use in cheese making [12, p. 49].

The enzyme preparations from *P. ostreatus* and Rennet Rhizomucor have almost identical specific MCA (about 75 U / mg protein), however, the PA index in the case of Rennet Rhizomucor is significantly higher than that of the enzyme preparation from *P. ostreatus*.

The lowest MCA values are shown for Rennet and E160 preparations (26 and 28 U/ mg protein correspondingly), which can negatively affect the cheese quality and significantly increase the time for its formation.

Influence of temperature. In order to study the physicochemical properties of milk-clotting preparations, the effect of temperature on the MCA was investigated. The effect of temperature on the catalytic activity of enzymes possessing MCA showed a typical activity-temperature dependence of the studied enzymes. The increased milk clotting activity at a higher temperature can be explained by the aggregation of proteins and molecular rearrangements in the protein structure [13 p. 345]. As it is shown in figure 2, for all enzyme preparations MCA was observed in the entire investigated temperature range from 25 to 45^0 C. At the same time, the temperature optimum of the preparations is different.

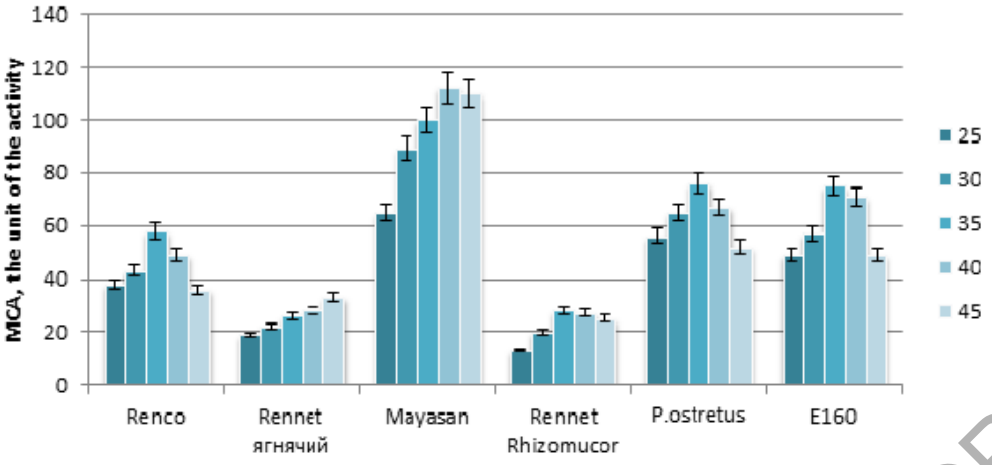

Figure 1 – Effect of temperature on enzyme preparations

It was shown the different temperature optima for the studied enzymes, mainly because of their structural peculiarities. Conformational changes in the protein structure at high temperatures can make it vulnerable to proteolysis, since unfolding of the protein can present new cleavage sites to enzymatic hydrolysis [14, p. 395], [15, p. 483], [16, p. 1902]. For enzyme preparations from Rennet Rhizomucor, *P. ostreatus*, E160 and Renco, the temperature optimum is at 35^0 C. This value is slightly lower than for the enzymes from Maysan and lamb Rennet. This is in accordance with literature data concerning temperature influence on the activity of enzyme preparations possessing MCA [17, p. 597].

Effect of pH. All enzyme preparations retained MCA in the pH range from 4 to 9. The pH optima of enzyme preparations from different origins are somewhat different (see figure 2).

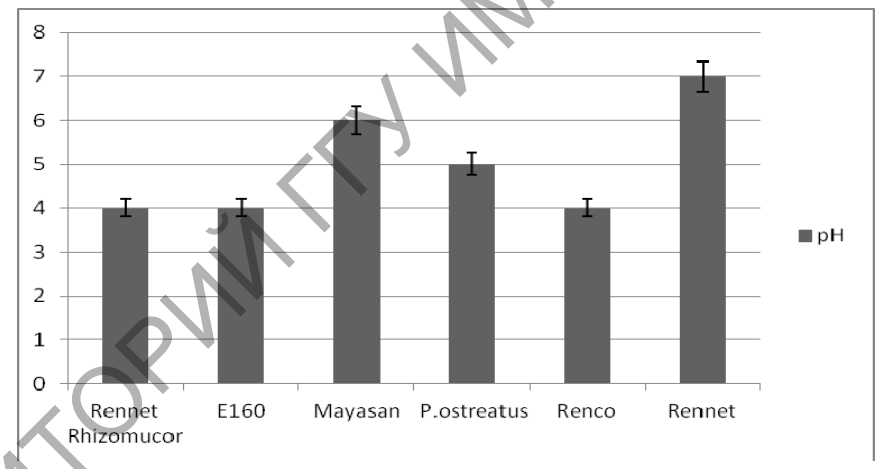

Figure 2 – pH optima of enzyme preparations from different origins

According to Demir [18, p. 286], enzymes with high pH optima are more profitable in food production, because the forming clots are bad and weak at a lower pH of milk. The range of stability of milk-clotting proteinases is in the pH range from 3,5 to 7,5 [19, p. 527]. These data are in accordance with our results.

The influence of calcium. There are numerous data that calcium at a sufficiently high concentration is an important component in the formation of milk clot. The hardness of the cheese can be increased up to 81 % by the addition of about 10 mM $CaCl_2$, but higher concentrations of calcium chloride may decrease the hardness of the final product [20, p. 1393]. The use of high concentrations of calcium chloride can have negative consequences in cheese production. High calcium concentrations change the cheese fusibility, which causes a number of problems in cheese production. As it is shown in figure 3, the maximum MCA of the enzyme from *P. ostreatus* in our study was the highest when calcium chloride was added to the substrate (milk) at a final concentration of 10 mM, which is consistent with the literature data.

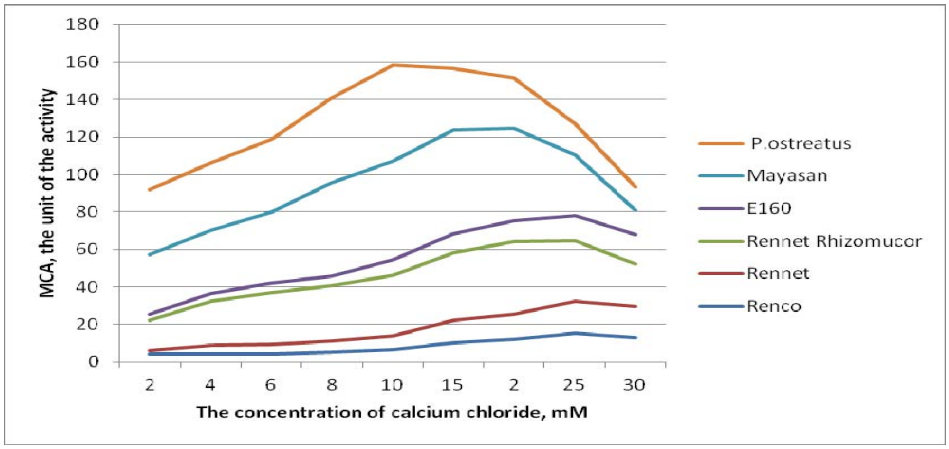

Figure 3 – Effect of calcium chloride concentration on enzyme preparations

For the Rennet Rhizomucor, E160, Rennet and Renco preparations, the highest MCA was obtained by adding 25 mM calcium chloride in final concentration that may have negative effect on cheese curd formation.

Cheese curd. According to GOST 92225, the quality of milk or enzyme suitability for cheese production are estimated by the nature of the formed cheese curd. The data of cheese curd quality with the studied enzyme preparations are presented in table 3.

Table 3 – Quality of the cheese curd

Enzyme preparation	Clot characteristic	Serum characteristics
Renco	The clot has a delicate, uniform consistency that meets the requirements for soft cheeses.	The serum does not have a bitter taste, it is transparent.
Rennet	The torn clot is soft to the touch with breaks. This clot is of satisfactory quality.	The serum is turbid.
Rennet rhizomucor	The clot has a delicate, uniform consistency that meets the requirements for soft cheeses.	The serum does not have a bitter taste, it is transparent.
E160	The torn clot is soft to the touch with breaks. This clot is of satisfactory quality.	The serum is turbid.
Mayasan	The clot is elastic to the touch, without eyes in the longitudinal section	The serum has a slightly bitter taste
Enzyme preparation from *P. ostreatus*	The clot has a creamy hue, uniform throughout the mass. The clot was elastic to the touch, without eyes in the longitudinal section. It has a light mushroom aroma.	The serum does not have a bitter taste, it is transparent.

Thus, the best cheese curd was obtained using the enzyme preparations of Renco, Rennet Rhizomucor, Mayasan and the enzyme preparation *P. ostreatus*. However, when using the Mayasan preparation, on serum had a slightly bitter taste, which was associated with the high proteolytic activity of the preparation.

Conclusion. This study was carried out to assess the competitiveness of milk-clotting enzymes of different origins. We have previously developed a scheme for the isolation and purification of the milk clotting enzyme from the culture liquid of *Pleurotus ostreatus*. This preparation has similar characteristics with these ones which are available in the market of Republic of Belarus: high milk clotting activity, the similar value of pH optimum (5,0) and temperature optimum (35°C), similar effect of 10 mM calcium chloride. The enzyme from *Pleurotus ostreatus* forms the cheese curd of high quality. This preparation also has its own peculiarities: a high ratio of milk-clotting and proteolytic activity, which is a good indicator for the formation of a cheese curd. Also, the cheese curd formed with the enzyme from *Pleurotus ostreatus* has a slight mushroom aroma.

Thus, it is possible to propose the enzyme preparation from the culture liquid of *Pleurotus ostreatus* for use in the dairy industry at the stage of cheese curd formation.

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