ISSN 0003-6838, Applied Biochemistry and Microbiology, 2018, Vol. 54, No. 6, pp. 569–576. © Pleiades Publishing, Inc., 2018.
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Biochemical Properties of Recombinant Chymosin in Alpaca (*Vicugna pacos* L.)

S. V. Belenkaya[a], A. P. Rudometov[a], D. N. Shcherbakov[a, b], D. V. Balabova[b, c], A. V. Kriger[c], A. N. Belov[c], A. D. Koval[c], and V. V. Elchaninov[c, *]

[a] *Vector State Research Center of Virology and Biotechnology, Koltsovo, 630559 Russia*

[b] *Altai State University, Barnaul, 656049 Russia*

[c] *Federal Altai Scientific Center for Agrobiotechnology, Siberian Research Institute for Cheese Making, Barnaul, 656016 Russia*

**e-mail: ve3636@yandex.ru*

Received March 22, 2018

Abstract—This paper discusses biochemical properties of the recombinant chymosin in alpaca (*Vicugna pacos*), which influence the production of rennet cheeses. These properties determine its value in the production of rennet cheeses. Recombinant bovine chymosins are used as a control. In comparison to them, the recombinant alpaca chymosin is characterized by a high specificity towards bovine κ-casein: the threshold for its thermal inactivation is 10–15°C higher and reaches 60°C. The nature of the relation of its specific activity to the pH and concentration of CaCl meets the requirements for the use of milk-clotting enzymes in the production of rennet cheeses.

Keywords: recombinant alpaca chymosin, biochemical properties, proteolytic activity, thermal stability

DOI: 10.1134/S0003683818060054

INTRODUCTION

Rennet which contains the aspartic endopeptidase chymosin (EC 3.4.23.4) is traditionally used for milk curdling in cheesemaking. The unique combination of biochemical properties of this enzyme, including its high milk-clotting activity (MCA) in the weakly acidic pH range, low total proteolytic activity (PA), moderate thermolability, and MCA dependence on the Ca^{2+} content enables production of cheese of the highest quality. Bovine chymosin (*Bos taurus* L.) is considered to be a standard milk-clotting enzyme (MCE) and can be used in the production of any kind of rennet cheese [1].

The steady growth of cheese production, depletion of the natural MCE resources, and prion disease epidemics in cattle have led to global rennet shortage, which resulted in a search for substitutes. Many prokaryotic and eukaryotic proteases capable of clotting cow's milk have been studied. However, due to a number of parameters negatively affecting cheese quality, and, in particular, excessive PA and high thermal stability, most of these enzymes are not used in cheesemaking [2–5].

One possible way to compensate for the rennet deficit is the development and implementation of recombinant chymosins in the cheese making process [3, 4, 6]. However, in order to successfully produce cheese, MCE should possess a set of biochemical properties capable of ensuring a high-quality final cheese product.

The most promising direction in the search for new milk coagulants would be study of chymosins in animals living in severe climatic conditions (short summer period, extreme temperatures, meager food resources, reduced partial oxygen pressure, etc.). One of the mechanisms of animal adaptation to unfavorable environmental conditions involves a decreased maturation period and the fastest possible transition from breastfeeding to self-feeding with roughage. In comparison to cow's milk, the milk of such animal species usually has a higher content of nutrients, including caseins [7–10], which implies the synthesis of highly efficient milk-clotting proteases in newborns' stomachs.

The main object of this study is the recombinant chymosin (rCh) of the alpaca (*Vicugna pacos* L.), an even-toed, ungulate, farm animal of the *Camelidae* family that inhabits the Andes (South America) at an altitude of 3500–5000 m above sea level (a.s.l.). The milk of *V. pacos* is 4.5–6.9% protein, which exceeds the average value for cow's milk (3.2–3.6%, respectively) [7, 9, 10]. The protein content of alpaca milk was found to depend on the severity of habitat conditions. For example, the protein content of the milk of the species living in the high-altitude areas of the Andes at 4000 m a.s.l., semideserts of Patagonia at

12 m a.s.l., and acclimatized areas of California (United States) is 6.9, 6.5, and 4.5%, respectively [7, 10].

The goal of the work is to produce alpaca rCh, to conduct a comparative study of its biochemical properties versus recombinant bovine chymosins, which are essential for the production of rennet cheeses, and to assess the possibilities of its use in cheese making.

MATERIALS AND METHODS

Production of Recombinant Alpaca and Bovine Chymosins (rCh of Alpaca and Cow)

Standard genetic engineering techniques were used to produce rCh of cow (rCh Bos) and rCh of alpaca (rCh Vic) in the system of *Escherichia coli* Castellani and Chalmers [11]. The nucleotide sequences encoding the alpaca and bovine chymosins were retrieved from GenBank [https://www.ncbi.nlm.nih.gov/]. The codon optimization of the alpaca chymosin for the specific expression system was carried out with an online tool from Integrated DNA Technologies [https://eu.idtdna.com/ CodonOpt]. The nucleotide sequences were synthesized by the DNA-Synthesis company (Russia). The synthesized genes were cloned into the expression vector pET21a (Novagen, Merck, Germany) with the use of unique restriction sites. This resulted in the construction of recombinant plasmids pET_T7Vic and pET_T7Bos. The producing organisms containing the constructed plasmids were obtained via chemical transformation of the *E. coli* strain BL21(DE3) (Invitrogen Corp., USA) [11]. Individual colonies of this strain containing recombinant plasmids were cultured overnight in LB broth on an orbital shaker (180 rpm, 37°C). The inoculum was transferred at a 1 : 100 ratio into an Erlenmeyer flask with LB medium and grown to an optical density at 600 nm of 0.8. Isopropyl-β-D-1-thiogalactopyranoside inducer was then added to a final concentration of 1 mM, and the culture was further incubated in a shaker (180 rpm) at 25°C for 12 hours. The isolation of inclusion bodies, solubilization, semiaffinity purification, and refolding of target proteins were conducted with the previously described techniques [12]. Preparations of rCh of alpaca (rCh Vic) and rCh of cow (rCh Bos) were produced.

Study of Biochemical Properties

The total PA, MCA, and thermal stability, as well as the MCA dependence on pH and the Ca^{2+} content, were determined by previously published methods [13, 14]. Aqueous solutions (1%) of dry commercial recombinant bovine chymosin (rCh Bos-C) produced in the *Aspergillus niger* Tiegh system were used as a control. All preparations of the recombinant chymosins were normalized to MCA of rCh Bos having minimal initial activity. All experiments were carried out at least in triplicate; the standard Microsoft Excel package was used for statistical processing.

Milk-clotting activity. Solutions of a standard milk substrate (SMS) heated to 35°C were mixed with the studied MCE in a 10 : 1 ratio. A control sample of MCA-certified industrial rennet was used as a reference. The result was expressed in reference units (RU/mL).

Total proteolytic activity. A 1% solution of Hammerstein-grade casein in a 20 mM Na-phosphate buffer (pH 5.6) was used as a substrate. The investigated MCEs were introduced into the substrate solution in a 1 : 4 ratio and incubated at 35°C for 0 (control), 30, 90, and 180 min. The reaction was interrupted by adding trichloroacetic acid. The precipitates were filtered, and the optical density of the filtrate was measured at 280 nm with a "zero" point as a control. To assess the specificity of the rCh preparations, the D_{280} values of the samples incubated for 180 min were designated as the PA values. The specificity was defined as the ratio of MA to total PA (MA/PA).

Thermal stability. Aliquots of MCE were heated in the temperature range of 30–60°C for 30 min and then assessed for residual MCA. The MCA values obtained in the samples heated at 30°C were assigned 100%.

Dependence of milk-clotting activity on pH. The SMS solutions were adjusted to pH levels of 5.5, 6.0, 6.5, and 7.0, and the MCA of the studied preparations was then determined. The MCE activity at a pH of 5.5 was assigned as 100%.

Dependence of milk-clotting activity on the calcium chloride concentration. $CaCl_2$ was added to the SMS to a final concentration of 1–5 mM, and the clot formation time was measured therein. The values obtained in $CaCl_2$-free samples of SMS were taken as 100%.

RESULTS AND DISCUSSION

Milk-clotting activity (MCA). The synthesized rCh Bos and rCh Vic were characterized by MCAs of 1978 ± 31 and 2014 ± 18 RU/mL, respectively. At the same time, the specific activity (MCA/mg protein) of rCh Vic and rCh Bos were, respectively, 10070 and 9890 RU/mg. The specific activity of rCh Bos-C was not calculated, since commercial preparations of rCh are contaminated with ballast proteins [1].

Total proteolytic activity (PA) and specificity. It is necessary to consider the proteolytic activity, along with its MCA, to assess the potential benefits of any new MCE. [15, 16]. Two types of PA are distinguished for the MCEs used in cheesemaking: the first type (1) refers to the specific activity or MCA, which is defined as the ability to hydrolyze one peptide bond Phe_{105}-Met_{106}, in the κ-casein molecules, which leads to clot formation; the second type (2) refers to the total PA, which characterizes the ability to hydrolyze any peptide bond in α-, β-, and κ-caseins of milk [16].

The high total proteolytic activity of MCE is considered to be an extremely negative factor in cheesemaking, because proteolysis products are lost in the whey and the cheese yield is decreased [17]. A coagulant with a broad proteolytic specificity retained in the cheese causes texture and flavor defects in cheeses which are ripened and stored for long periods [18]. The use of MCEs with a high PA impairs the technological properties of cheese whey used as a feedstock in the production of some dairy products. Therefore, an ideal MCE should possess the lowest possible total PA at the highest possible MCA [3, 16].

According to the classification proposed in [3], MCEs are arranged in a descending order of their specificity (defined as a MCA/PA ratio) as follows:

rCh of camel > rCh of cow, natural bovine chymosin > cow pepsin > mucorpepsins > endotiapepsin.

This series reflects the functional versatility of MCEs (the higher the specificity is, the more versatile the enzyme is) and indicates the increasing probability of developing flavor and texture defects in attempts to use a coagulant with a high total PA to produce cheeses for long-term ripening and storage.

The studied chymosins preparations differed in the accumulation dynamics of the enzymatic hydrolysis products of caseins versus the incubation time of the enzyme-substrate mixture (Fig. 1). It turned out that rCh Bos-C and rCh Bos had similar PA. The trend lines were parallel and almost coincident, and the equations describing them had the same slope and differed only slightly in coefficients of variation. At the same MCA values for rCh Bos-C, the rCh Bos, and rCh Vic, the proteolytic activity of rCh Vic (alpaca) was 2.9 times lower than that for the recombinant bovine enzymes (rCh Bos-C and the rCh Bos). The recombinant alpaca chymosin had a higher MCA to PA ratio than that of rCh Bos-C and the rCh Bos. This indicated a high specificity of rCh Vic and its similarity to rCh of another representative of the *Tylopoda* suborder—a one-humped camel (*Camelus dromedarius* L.) [6].

The specificity of chymosins of different mammalian species was compared with the method proposed in [6]. The data in Table 1 show that, according to the MCA to PA ratio, rCh Vic is superior to the cow, pig, buffalo, and goat enzymes but is inferior to the rCh of a one-humped camel. The data on MCA, total PA, and specificity of rCh of camel given in [6, 19] are contradictory, which may be due to the different methodological approaches for assessment of these parameters. At the same time, the data on the higher specificity of rCh of camel compared to rCh of cow [6] were confirmed in studies of the total PA of the enzymes directly in cheeses [20, 21].

The alpaca and camel chymosins are not the only MCEs that are superior to bovine chymosin by specificity. Due to their higher MCA, the buffalo and goat enzymes are characterized by a more favorable ratio of MCA to PA than that for cow rCh [19]. The porcine

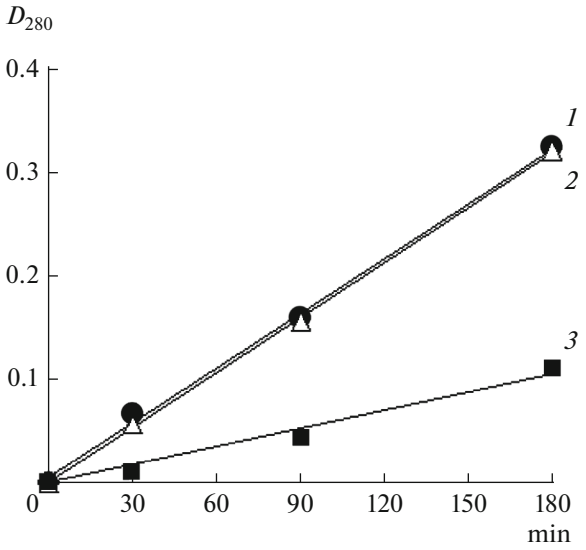

Fig. 1. Accumulation of proteolysis products (D_{280}) versus incubation time in the substrate mixture: (*1*) Bos-C rCh, (*2*) Bos rCh (2), and (*3*) Vic rCh.

chymosin is also superior to cow rCh by specificity, but it is four times inferior to it by MCA (Table 1). This means that, in order to coagulate the same volume of milk, it takes four times as much of the porcine chymosin than the cow's enzyme, which makes the industrial use of the porcine chymosin economically unfeasible.

Information on a preparation of lamb rCh was first published in 2001 [22]. The PA of lamb rCh was studied by the Kjeldahl method according to the changed ratio of nonprotein and total nitrogen. The total PA in cheeses produced using lamb rCh was 28% lower than that with cow rCh as a control. However, the study [22] does not provide any data on the content or activity of either chymosin in the studied cheeses. At the same time, it is known that up to 30% of the introduced MCE amount may be retained in cheese curds depending on the coagulant type, pH, temperature, and moisture content [23]. Thus, according to [24], the retention of cow rCh in Cheddar-type cheeses reaches 10–17.5% [24]. It can be assumed that the difference in the content of proteolysis products observed in the cheese is due to the different chymosin contents retained in cheese, rather than different PAs. The data [22] on the ratio of MCA to total P obtained from milk coagulation tests demonstrated that lamb rCh and bull rCh had almost the same specificity (Table 1).

Thus, according to the ratio of MCA to total PA, alpaca rCh is significantly superior to cow rCh and can be referred to as a high-quality, universal MCE suitable for the production of any kind of cheese.

MCA dependence on the concentration of calcium ions. Calcium ions promote rennet coagulation of milk. The native cow's milk contains about 30 mM

Table 1. Milk-clotting activity, total proteolytic activity, and specificity (MCA/PA) of different chymosins

Chymosin type	MCA, % of bovine chymosin MCA	PA, % of total PA of bovine chymosin	MCA/PA
Alpaca rCh (Vic rCh) *	102	34	3.0
Cow rCh (rCh Bos) *	100	100	1.0
Camel rCh **	170	25	7.0
Natural porcine Ch **	25	12	2.1
Cow rCh **	100	100	1.0
Lamb rCh ***	≈100*****	≈98*****	≈1.0*****
Cow rCh ***	100	100	1.0
Buffalo rCh ****	105	96	1.1
Camel rCh ****	99	110	0.9
Cow rCh ****	100	100	1.0
Goat rCh ****	148	106	1.4

* Cow and alpaca rCh expressed in *E. coli*.
** Cow and dromedary rCh expressed in *A. niger* [6].
*** Cow rCh expressed in *Kluyveromyces lactis* van der Walt, lamb rCh in *E. coli* [22].
**** Cow and dromedary rCh expressed in *A. niger,* buffalo and goat rCh in *P. pastoris* [19].
*****The exact measured values of PA and MCA are not available; the data is presented graphically.

calcium, and most of the calcium (~68%) is bound to casein micelles in the form of a colloidal or amorphous monohydrogen phosphate ($CaHPO_4$), and about 10% of the remaining 10 mM (*i.e.*, about 1 mM) of calcium is present in ionized form [25].

In the industrial production of most types of cheeses, milk undergoes a pasteurization step. The solubility of calcium salts of phosphoric acid falls with a temperature increase. Therefore, in the pasteurization process, some of the salts and calcium ions present in milk irreversibly precipitate in the form of insoluble calcium phosphate ($Ca_3(PO_4)_2$). This results in a decrease in the Ca^{2+} content and a prolonged duration of rennet coagulation. To eliminate this effect, 0.1–0.5 g/L (≈1–5 mM)) of $CaCl_2$ is added to the milk after pasteurization. The introduction of $CaCl_2$ improves the coagulation ability of pasteurized milk. First, Ca^{2+} can partly shield the negative charge on the surface of casein micelles, and the peptide bond Phe_{105}–Met_{106} of κ-caseins located in the hairy layer becomes more accessible for MCE. Secondly, Ca^{2+} participates in the formation of ion "bridges" between the destabilized micelles, which accelerates their aggregation and leads to the formation of milk clots [26]. The introduction of extra $CaCl_2$ is also used in cheesemaking to improve the coagulation properties and processability of rennet-lean milk [27].

An increase in the calcium chloride content by 1–5 mM leads not only to a higher MCA but a higher total PA of the enzyme as well, especially in the coagulation step. According to a study [28], the enzymatic activity of camel rCh increased from 20 to 100% in the range of 0–20 mM $CaCl_2$, and the maximum MA was observed at 20–40 mM $CaCl_2$. Thus, in the case of MCEs with a high sensitivity to the Ca^{2+} content, the risks and negative effects of increasing the total PA should be accounted for. In view of the above, cheesemakers tend to use the minimum required doses of $CaCl_2$ in the production of cheese from pasteurized milk. The MCE sensitivity to the Ca^{2+} content, which is equivalent to or less than the sensitivity of the reference enzyme (bovine chymosin), is a positive factor, because it provides the opportunity to vary the introduced amount of calcium chloride without concerns for significant changes in MCA and PA.

In comparison to rCh Bos and rCh Bos-C, the specific activity of alpaca rCh is less sensitive to the increase in the Ca^{2+} content in the milk substrate. If the content of the introduced $CaCl_2$ increases from 1 to 5 mM, the MCA of alpaca rCh increased by 19–52%. In the same concentration range of calcium chloride, the MA of rCh Bos and rCh Bos-C increased by 27–66 and 28–68%, respectively (Fig. 2). At 3 mM calcium chloride (most commonly used in cheesemaking), the coagulation activity of Vic rCh increased by 42%, and the activity of Bos-C rCh and Bos rCh increased by 54 and 55%, respectively. These data were consistent with the results obtained for the rCh of other species. Thus, it was shown [22] that lamb rCh exhibited moderate sensitivity to the Ca^{2+} content; it was close to the sensitivity of cow rCh. At 0–2 mM $CaCl_2$ and a pH of 6.6, the coagulation activity of rCh of camel was 10% higher than MCA of cow rCh [6].

The results showed that when the $CaCl_2$ concentration increased from 1 to 5 m*M*, both the Bos-C and rCh Bos rCh demonstrated almost the same dynamics in increased MCA. Compared to the reference Bos-C rCh, the coagulation activity of Vic rCh turned to be

8–14% less sensitive to variations in the same concentration range of calcium chloride in milk. This made it possible to conclude that the sensitivity of alpaca rCh to the content of calcium ions completely met cheesemaking requirements.

Thermal stability. Along with the total PA, thermal stability is an essential biochemical characteristic of MCEs. In the process of milk coagulation under the action of chymosin, αs- and β-caseins are not hydrolyzed. The proteolytic degradation of para-κ-casein, αs1-, αs2-, and β-caseins by the enzyme retained in the curd begins later in the process of cheese ripening [20, 23]. The hydrolysis of caseins results in the formation of peptides and amino acids, as well as products of their degradation and biochemical modification, which influence the development of physicochemical and sensory properties of cheeses. That is why the data on the thermal stability of the used MCE make it possible to control both the proteolysis degree and the ripening time by adjusting the temperature of cheese curd treatment.

It was shown [6] that the total PA of rCh of cow and camel increased with an increased temperature. The maximum nonspecific PA revealed for dromedary and cow rCh was at 55 and 52.5°C, respectively. Thus, a thermostable MCE can lead to an increase of undesirable PA in the steps of cheese curd treatment, which is associated with increased heating temperatures.

The data in [6] were consistent with the results obtained in [20], which showed that the proteolysis intensity in Reggianito cheeses depended on the heat stability of the used coagulant and temperatures of cheese curd heating. In these studies, the authors investigated thermolabile enzymes with low total PA (cow rCh and camel rCh). It is known that chymosins attack the molecules of αs1-casein at the Phe_{23}-Phe_{24} bond. This results in the formation of two peptide products, αs1 (f1-23) and αs1-I (f24-199), which are used as markers of total PA in cheeses. In the production of Reggianito-type cheeses with cow and camel rCh, it was shown that the content of αs1-casein proteolysis products, in particular, polypeptide αs1-I (f24-199), significantly decreased when the curd heating temperature increased from 50 to 56°C. This occurs due to the complete MCE inactivation at elevated curd heating temperatures. When a more thermostable camel rCh is used [6, 19], the intensity of proteolysis in cheeses with a heating temperature of 56°C was higher than that with cow rCh [20]. Thus, camel rCh, which has a fourfold lower level of total proteolysis than cow rCh but exceeds it in terms of heat stability [6], exhibited a higher nonspecific PA in maturing and stored cheeses.

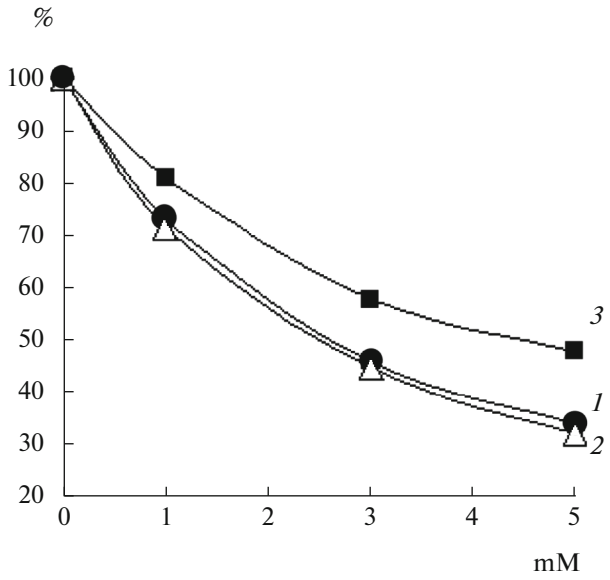

Fig. 2. Milk-clotting activity (%) versus concentration of calcium chloride (mM): (*1*) Bos-C rCh, (*2*) Bos rCh, and (*3*) Vic rCh.

It should be noted that the data on chymosin heat stability is ambiguous even for recombinant enzymes of the same species expressed in different producing organisms (Table 2). Thus, it was shown that the thresholds for complete thermal inactivation of camel rCh expressed in higher fungi (*A. niger)* [6] and yeast *(Pichia pastoris* Phaff*)* [28] differed by 10°C. Natural chymosins have a wider range of thermal stability than their genetically modified counterparts.

Figure 3 shows the results of studies on the thermostability of the rCh of Vic, rCh Bos-C, and rCh Bos. The thermostability threshold of rCh Bos-C was 45°C.

Table 2. Thermal stability of natural and recombinant chymosins of different genesis [6, 17, 18, 26]

Chymosin type, producer	Range of thermal stability	Comments
Natural bovine chymosin, *B. taurus taurus*	20–60°C	At 60°C—50% MCA
Cow rCh, *A. niger*	5–52.5°C	Complete inactivation at >55°C
Camel rCh, *A. niger*	5–55.0°C	Complete inactivation at 60°C
Camel rCh, *P. pastoris*	20–50°C	Complete inactivation at 50°C
Lamb rCh, *E. coli*	25–50°C	At 50°C—30% MCA
Goat rCh, *P. pastoris*	30–50°C	At 50°C—45% MCA
Natural goat chymosin, *Capra hircus* L.	30–60°C	At 60°C—40% MCA

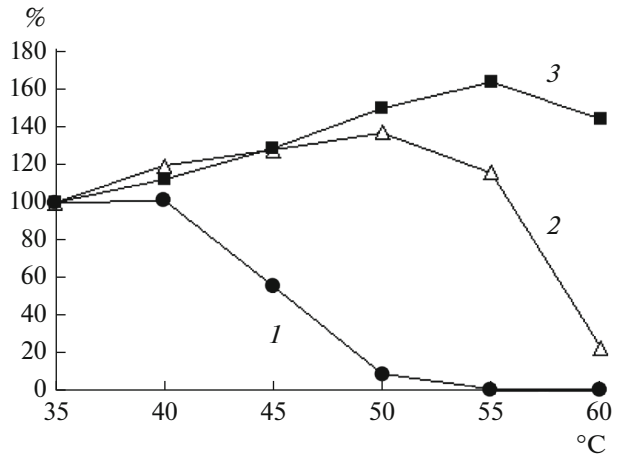

Fig. 3. Milk-clotting activity (%) versus enzyme heating temperature (thermal stability): (*1*) Bos-C rCh, (*2*) Bos rCh, and (*3*) Vic rCh.

At this temperature, the enzyme lost >60% of its coagulation ability, and it lost more than 90% of the initial MCA at 50°C. In terms of thermostability, the synthesized Bos rCh was markedly superior to the control preparation Bos-C rCh. At heating temperatures in the range of 35–50°C, the residual MCA of Bos rCh did not decrease (moreover, it increased by 37%). The thermal inactivation of Bos-C rCh began at 55°C, and it lost more than 75% of the initial MA at 60°C.

The alpaca rCh had the highest thermal stability, and its MCA gradually increased by 1.6 times with a heating temperature increase from 35 to 55°C. A descending trend in the *Vic* rCh coagulation ability was observed only at 60°C. Thus, the thresholds for thermal inactivation (T°C at which the chymosin MCA began to decrease) of the rCh of Bos-C, rCh Bos, and rCh Vic were 45, 55, and 60°C, respectively.

It can be assumed that the high thermal stability revealed for *Bos* and *Vic* rCh is due to the peculiarities of their expression in the prokaryotic system. It is known that folding and posttranslational modification of de novo–synthesized recombinant proteins proceeds in prokaryotes and eukaryotes in different ways [28], and this may be the reason for the observed differences in the heat stability of rCh Bos-C produced in the eukaryotic system of *A. niger*, and Bos and Vic rCh expressed in the prokaryotic system of *E. coli*. Thus, in terms of thermolability, alpaca rCh was significantly inferior to the control Bos-C rCh. On the one hand, the high thermal stability significantly limits the practical application of the alpaca-derived coagulant and indicates its use only in the production of cheeses that ripened and stored for short periods. On the other hand, it cannoy be ruled out that the very low total alpaca rCh PA is capable of at least partially mitigating the negative effect of its high thermal stability.

MCA dependence on the substrate pH. The production process of most types of cheeses involves the step of milk pretreatment before the MCE is added to the milk. A lactic-acid bacterial concentrate is added to raw milk with a pH of about 6.7. The mixture is then incubated at 32–35°C for 30–40 min. The developing microflora of the starter culture begins to metabolize lactose to lactic acid, and, as a result, the mixture pH begins to decrease. Most often, the MCE is introduced into the mixture at a pH of 6.5–6.6. In this regard, the ability to effectively coagulate milk in the weakly acidic pH range is one of the main technological requirements for the MCE.

Even slight fluctuations in the pH values of the milk mixture lead to a significant change in the balance of forces stabilizing casein micelles, which affects their biochemical properties and the rennet clotting time. If the H^+ content is increased, the clotting time depends not only on the activity of MCEs, which have an optimum pH range of 4.5–5.5 [http://www.brenda-enzymes.org/enzyme.php?ecno = 3.4.23.4], but also on the electrostatic and hydrophobic properties of casein micelles. Milk acidification decreases the negative charge of caseins as the pH approaches the pI values of these proteins. The decreased net negative charge reduces the electrostatic forces of intermicellar repulsion and simultaneously enhances the casein-casein hydrophobic interactions, accelerating the clot formation [3, 26]. When the pH values increase and move away from the pI of caseins, their net negative charges increase. As a result, the electrostatic forces of intermicellar repulsion are strengthened, which prevents casein micelles from approaching each other closely. At the same time, the hydrophobic casein–casein interactions are weakened. The cumulative effect of these physicochemical changes leads to delayed curd formation. In view of the above, the increasing pH of the milk mixture in the range of 5.0–7.0 should lead to increased rennet clotting time [26].

It turned out that Bos-C and Bos rCh had the same trend of MCA changes versus the substrate pH (Fig. 4). Both preparations showed a high activity in the pH range of 5.5–6.0. As the pH approached neutral values, their specific activity began to decline rapidly. At a pH of 7.0, the milk coagulation activity of Bos-C and Bos rCh decreased by more than 96% of the baseline values. The results were consistent with the data of studies of cow rCh [6, 19], in which a similar MCA dependence on the substrate pH was observed.

In the case of alpaca rCh, the dynamics of the MCA decrease with increasing pH differed from the relevant dynamics for Bos-C and rCh Bos rCh. At a pH of 6.0, the difference in the MCA values of alpaca rCh and recombinant bovine chymosins reached 55–60%. After a sharp decline in MCA at a pH of 6.0, the decrease in activity slowed; at a pH of 6.5, the difference in the rates of milk coagulation induced by Vic rCh and recombinant bovine chymosins was not more

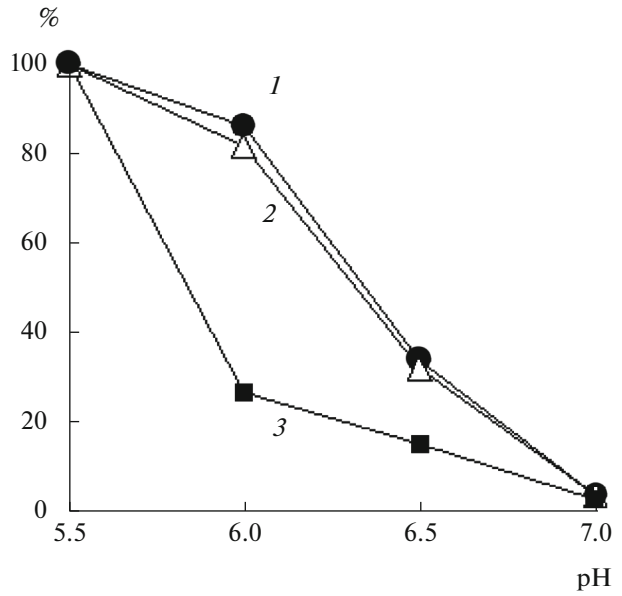

Fig. 4. Milk-clotting activity (%) versus pH of milk substrate: (*1*) Bos-C rCh, (*2*) Bos rCh, and (*3*) Vic rCh.

than 19%. The ±20% variation of the enzyme MCA from the activity of cow rCh is permissible and does not limit the use of the coagulant in cheesemaking. At a pH of 7.0, Vic, rCh Bos-C, and rCh Bos rCh almost completely lost their MCA.

The dependence of the MCA dynamics on the substrate pH for alpaca rCh is very similar to that for camel rCh, which currently assumes the role of a substitute for bovine chymosin. According to the data of [28], the coagulation activity of rCh of camel at pH 6.0 and 6.5 was, respectively, about 18 and 16% of the maximum values observed at pH 5.0.

The results led to the conclusion that alpaca rCh is able to coagulate cow's milk effectively at a pH of 6.5. Based on this criteria, alpaca rCh can be used in cheesemaking without restrictions.

Thus, recombinant alpaca chymosin was prepared, and its biochemical properties important for the production of rennet cheeses were studied. The specificity of Vic rCh action in relation to bovine κ-casein was three times superior to that of the reference cow rCh. According to its specificity, alpaca rCh can be considered a high-quality, versatile, milk-clotting enzyme. According to the MCA sensitivity to calcium chloride and pH, it meets the requirements of the cheese making industry. Compared to the control commercial cow rCh, the preparation of alpaca rCh had a higher heat stability, which limits the possibilities of its use in cheesemaking. The combination of its biochemical properties means that Vic rCh can be applied in the production of cheeses with short-term ripening and storage. It can be assumed that the high thermal stability of Vic rCh, which is a negative feature from a practical point of view, is due to the low level of posttranslational modification of the enzyme expressed in *E. coli*. Should this assumption be correct, then changing the prokaryotic producer to a eukaryotic expression system may result in changes to the biochemical properties. If the thermal stability of alpaca rCh is decreased by 10–15°C, this enzyme could be transferred to the category of versatile milk coagulants, which would allow it to be considered a highly effective substitute for cow rCh.

ACKNOWLEDGMENTS

The work was financially supported by the Russian Foundation for Basic Research (project no. 17-44-220540 r_a) and the Foundation for Assistance to Small Innovative Enterprises in Science and Technology in the framework of the Program UMNIK (project no. 12050GU/2017).

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. This article does not contain any studies with animals performed by any of the authors.

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Translated by M. Romanova