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Isolation of Milk-Clotting Enzyme from Transgenic Sheep Milk and Its Comparison with Calf Chymosin

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Abstract—Technology for preparation of chymosin from milk of transgenic sheep has been elaborated. Purification of the preparation by ion-exchange chromatography on aminosilochrom and biospecific chromatography on bacitracin-Sepharose yielded homogeneous active enzyme. Hydrolysis of protein substrates (hemoglobin, BSA, and sodium caseinate) by the transgenic sheep chymosin and stability of the enzyme at various values of pH were studied. Judging by the amino acid composition, the N-terminal sequence involving six amino acid residues, molecular mass, stability at various pH values, and the catalytic activity against the protein substrates, the transgenic sheep chymosin is identical to calf chymosin.

Key words: transgenic sheep chymosin, calf chymosin

Chymosin (EC 3.4.23.4) is the main digestive enzyme of newborn mammals. The enzyme belongs to the class of aspartate proteinases. Chymosin is secreted by stomach mucosa in the form of the inactive precursor prochymosin. Acid medium favors the conversion of prochymosin into chymosin by splitting off the N-terminal propeptide consisting of 42 amino acid residues [1].

Chymosin preferentially catalyzes the cleavage of the Phe105-Met106 bond in κ-casein, resulting in a decrease in stability of casein micelles and milk clotting [2]. The fact that chymosin possesses high milk-clotting activity and relatively low total proteolytic activity explains its wide use in cheese production. Besides, chymosin is used in the production of cottage cheese and protein paste. Chymosin serves as the basis for preparation of abomin, a medicine used in gastroenterology for treatment of gastrointestinal diseases.

The traditional source for isolation of chymosin is rennet bags of milky calves. In recent years, the supply of calf rennet bags is dramatically reduced, resulting in the emergence of a deficit of the starting material for production of chymosin. One solution to this problem is to obtain chymosin from transgenic animals producing the enzyme with milk. Under the direction of Prof. G. Brem (Institute of Animal Breeding and Genetics, University of Veterinary Sciences, Vienna, Austria) the gene construction boS1Cas-Chym of length ~14 kb in which the gene of cattle prochymosin is under control of the regulatory elements of the gene of cattle α_{S1}-casein was developed. Based on the gene construction, transgenic Romanov sheep were generated and the technology of obtaining chymosin preparation from milk of transgenic sheep was elaborated [3]. The chymosin preparation was used for producing several varieties of cheese that proved similar in gustatory and other characteristics to cheese produced using the enzyme preparation from calf rennet bags.

The goal of the present paper was to obtain a homogeneous preparation of milk-clotting enzyme from milk of transgenic sheep and to compare it with calf chymosin.

MATERIALS AND METHODS

Obtaining the preparation of milk-clotting enzyme from milk of transgenic sheep. Fresh and thawed milk of transgenic sheep was diluted with water at room temperature in the proportion 4 liters of water per liter of milk. The pH of whole milk was reduced from 6.5-6.7 to 4.5 by

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the addition of concentrated HCl. Within 10-15 min, the precipitate containing casein was separated. The pH of the serum was adjusted to 1.7 with concentrated HCl and fine-ground sodium chloride "Extra" was added in the proportion 250 g of salt per liter of serum. The mixture was thoroughly and slowly stirred until the salt dissolved. The precipitate formed was separated by filtration. The mass looking like paste was lyophilized. The yield of the enzyme mass was 50 g from 1 liter of milk.

Calf chymosin was isolated from a preparation produced by the Moscow Chymosin Factory using affinity chromatography on bacitracin-Sepharose and separated from admixed pepsin by ion-exchange chromatography on aminosilochrom C-80 [4].

Milk-clotting activity was determined as described in [4].

Bacitracin-Sepharose was synthesized by the method by Stepanov et al. [5]. The content of the ligand in the sorbent was 6.8 μmoles/ml.

Aminosilochrom C-80 was purchased from NPO Biolar (Olaine, Latvia) and contained 100 μmoles of amino groups per g of the substance.

Amino acid analysis was performed using a Hitachi-835 automatic amino acid analyzer (Japan) after hydrolysis of the protein in 5.7 M HCl at 105°C for 48 h. Before determining half-cystine, the protein was oxidized by the method by Moore [6].

Electrophoresis in polyacrylamide gel was carried out under non-denaturing conditions [7] or in the presence of SDS [8].

Amino acid sequencing of transgenic sheep chymosin was performed by the Edman method using a Knauer model 816 protein sequencer (Germany) equipped with an Applied Biosystems model 120A analyzer of PTH amino acids (USA) at the Engelhardt Institute of Molecular Biology (Russian Academy of Sciences, Moscow).

Isolation of milk-clotting enzyme from milk of transgenic sheep (transgenic sheep chymosin). The preparation of milk-clotting enzyme from milk of transgenic sheep (30 g) was stirred for 3 h with 300 ml of 0.1 M sodium acetate buffer, pH 5.4, at room temperature. Then the suspension was dialyzed against the same buffer at 4°C to remove salt. The buffer was changed several times. The suspension was centrifuged at 15,000 rpm (4°C; 30 min). The supernatant was filtered and placed on a column with 40 ml of aminosilochrom equilibrated with 0.1 M sodium acetate buffer, pH 5.4. The column was washed with 0.1 M sodium acetate buffer, pH 5.4, until the optical absorbance at 280 nm in the eluate was less than 0.1. The course of chromatography was followed by measuring optical absorbance at 280 nm and the milk-clotting activity. The fraction eluted in the free volume and solutions obtained by washing the column lacked the enzyme possessing the milk-clotting activity. The enzyme was eluted with 5 mM HCl. The pH of the enzyme solution obtained was 3.5. The pH of the solution was adjusted to 5.4 with 4 M sodium acetate buffer, pH 5.4. The enzyme solution was desalted on Sephadex G-25 (coarse) equilibrated with 0.1 M sodium acetate buffer, pH 5.4. Affinity chromatography on bacitracin-Sepharose was used for further purification of the enzyme. The enzyme solution in 0.1 M sodium acetate buffer, pH 5.4, obtained after desalting on Sephadex G-25, was placed on the column with bacitracin-Sepharose equilibrated with 0.1 M sodium acetate buffer, pH 5.4. The column was washed with 0.1 M sodium acetate buffer, pH 5.4, until the optical absorbance at 280 in the eluate was less than 0.1. Then 0.1 M sodium acetate buffer, pH 5.4, containing 1 M NaCl was used for washing the column. The fraction eluted in the free volume, solutions obtained by washing the column, and eluates obtained with 0.1 M sodium acetate buffer, pH 5.4, containing 1 M NaCl lacked the enzyme possessing the milk-clotting activity. The active enzyme was eluted with 10% isopropanol in 0.1 M sodium acetate buffer, pH 5.4, containing 1 M NaCl. The enzyme solution was desalted using Sephadex G-25 (coarse) equilibrated with water and lyophilized.

Determination of pH dependence of the proteolytic activity of transgenic sheep chymosin with hemoglobin as substrate. The following solutions were used: 0.06 M HCl, pH 1.3; 0.2 M sodium citrate buffer, pH 2.6, 3.0, 3.6; 0.2 M sodium acetate buffer, pH 4.0, 4.6, 5.0. Hemoglobin (Reakhim, Russia) was dissolved in 0.06 M HCl, pH 1.3 (the final concentration of hemoglobin was 2%). The solution was incubated at 20°C for 15 min. Then the pH was adjusted to 3.5 by the addition of 2 M KOH. The hemoglobin solution was poured into test tubes. Buffer (3 ml) with the specified pH value was added to each portion of hemoglobin (1 ml). The mixtures were incubated at 37°C for 5 min. The reaction was started by the addition of 0.1 ml of solution of the enzyme in water (pH 5.6; A_{280} = 0.34). The reaction mixtures were incubated at 37°C for 10 min, and then the reaction was stopped by the addition of 5 ml of 5% TCA solution. The precipitate was filtered through Filtrak-89 paper filter. Optical absorbance at 280 nm was measured in the filtrate. In the control experiment, 3 ml of the buffer with corresponding pH value was added to 1 ml of hemoglobin solution. After 10-min incubation at 37°C, 5 ml of 5% TCA solution and then 0.1 ml of the enzyme solution were added. Optical absorbance was measured at 280 nm. The control experiments were carried out at each pH value. Two parallel determinations of the enzymatic activity for each protein solution under study were performed, and the average value was used. Under the same conditions, we determined pH dependence of the proteolytic activity of calf chymosin by measuring the digestion of hemoglobin.

Determination of pH dependence of proteolytic activity of transgenic sheep chymosin with BSA as substrate. BSA was purchased from Serva (Germany). BSA (600 mg)

was dissolved in 30 ml of water. The BSA solution was poured into test tubes. Buffer (3 ml) with specified pH value was added to each portion of BSA (1 ml). The mixtures were incubated at 37°C for 5 min. The reaction was started by the addition of 0.1 ml of the enzyme solution (1 mg/ml in 0.1 M sodium acetate buffer, pH 5.4). The solution was incubation at 37°C for 1 h. The reaction was stopped by the addition of 5 ml of 10% TCA solution. The precipitate was separated by 20-min centrifugation at 5000 rpm. Optical absorbance in the filtrate was measured at 280 nm. In the control experiment, 3 ml of the buffer with corresponding pH value was added to 1 ml of BSA solution. After 1-h incubation at 37°C, 5 ml of 10%-solution of TCA and then 0.1 ml of the enzyme solution were added. The precipitate was separated by centrifugation. Optical absorbance was measured at 280 nm. The control experiment was performed at each pH value.

Activity of transgenic sheep chymosin measured by digestion of sodium caseinate. Solution of sodium caseinate (1%) in 0.1 M sodium acetate buffer, pH 5.7, was incubated at 37°C for 10 min. The enzymatic reaction was started by the addition of solution containing 10 μg of the enzyme to 2 ml of sodium caseinate solution. The reaction mixture was incubated for 1 h, and the reaction was stopped by the addition of 4 ml of 5% TCA solution. In the control experiment, 0.1 ml of the enzyme solution was incubated at 37°C for 10 min. Then 4 ml of 5% TCA solution was added. After 1-h incubation, 2 ml of 1% sodium caseinate solution were added to the control test tubes. The precipitate was removed by filtration, and 5 ml of 0.5 M Na_2CO_3 solution was added to 1 ml of the filtrate. After the addition of 1 ml of Folin reagent [9], the mixture was thoroughly stirred. Then the mixture was incubated at room temperature for 20 min and optical absorbance at 670 nm was measured. The specific activity was calculated using the formula:

$$a_{sp} = 6.1(A_{670} - A_{contr})/(TE)tC,$$

where A_{670} and A_{contr} are the values of optical absorbance in the experiment and in the control, respectively, TE is the tyrosine equivalent (ml/μmole), t is time of the enzymatic reaction (min), and C is the concentration of chymosin in the sample (μg/ml). The tyrosine equivalent was defined as the value of A_{670} for a solution of tyrosine with concentration of 1 μmole/ml. The enzymatic activity of calf chymosin with sodium caseinate as a substrate was determined under the same conditions.

Determination of pH dependence of stability of transgenic sheep chymosin. To characterize the stability of transgenic sheep chymosin at various pH values, 0.1 ml of the enzyme solution (1 mg/ml) in 0.1 M sodium acetate buffer, pH 5.4, was added to 0.9 ml of 0.2 M sodium citrate buffer (pH 2.0, 2.6, 3.0, 3.6), or of 0.2 M sodium acetate buffer (pH 4.0, 4.6, 5.0, 5.6), or of 0.2 M sodium phosphate buffer (pH 6.0, 6.6, 7.0). The solutions were incubated at 37°C. Aliquots (0.01 ml) were withdrawn immediately after the addition of the enzyme and after 24- or 48-h incubation of the enzyme at 37°C. The milk-clotting activity was determined in the aliquots. The pH dependence of stability of calf chymosin was characterized in analogous fashion.

RESULTS AND DISCUSSION

To obtain a concentrated preparation of milk-clotting enzyme from milk of transgenic sheep, the pH of milk diluted 5-fold was adjusted to 4.5 using concentrated HCl. Under these conditions the most of the casein, which is characterized by isoelectric point at this pH value, is precipitated. After removing the casein, the filtrate was acidified to pH 1.7 to transform prochymosin to the active enzyme.

We use chromatographic methods to prepare the homogeneous enzyme from the concentrated milk-clotting enzyme preparation. Chromatography on biospecific sorbents is the efficient way to isolate enzymes. Previously, efficient biospecific sorbents for isolation of aspartate proteinases including calf chymosin were synthesized at Lomonosov Moscow State University (Department of Natural Compounds Chemistry, School of Chemistry) [10]. In the present paper, we used biospe-

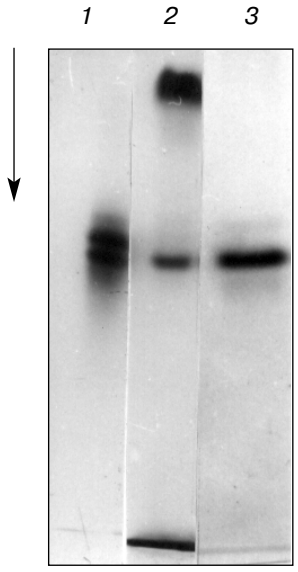

Fig. 1. Electrophoresis in 12% polyacrylamide gel under nondenaturing conditions (pH 5.4). The direction of electrophoresis is shown by the arrow. Lanes: *1*) calf chymosin; *2*) the transgenic sheep chymosin preparation after chromatography on aminosilochrom; *3*) transgenic sheep chymosin after chromatography on aminosilochrom and bacitracin-Sepharose.

Table 1. Isolation of transgenic sheep chymosin

Stage of purification	Total protein, A_{280}	Milk-clotting activity		Yield by activity, %
		specific, U/A_{280}	total, U	
Extract of the original preparation	V = 409 ml, turbid solution	4 900*	2 000 000	100
Ion-exchange chromatography on aminosilochrom C-80	V = 135 ml, turbid solution	12 500*	1 690 000	84
Desalting on Sephadex G-25	212	7 200	1 530 000	76
Affinity chromatography on bacitracin-Sepharose	28	51 300	1 440 000	70
Desalting on Sephadex G-25	20	62 500	1 250 000	62

* Activity is expressed in U/ml.

cific chromatography on bacitracin-Sepharose. Our results show that chromatography on bacitracin-Sepharose as a first stage of the purification procedure failed because of the complicated composition of the original preparation. Therefore, the first stage of purification was ion-exchange chromatography. Chromatography was carried out at pH 5.4, i.e., in the region of pH where chymosin is characterized by its highest stability. Under these conditions, the net charge of the chymosin molecule is negative (pI = 4.5 [10]). Therefore, we used the anionite γ-aminopropylsilochrom (aminosilochrom) that was successfully used for purification of aspartate proteinases [11]. The data on determination of the enzymatic activity showed that at pH 5.4 the enzyme is completely retained by the sorbent. Activity is eluted by 5 mM HCl with high yield. One could expect that ion-exchange chromatography would permit us to remove part of the admixed proteins that do not interact with the sorbent. However, according to the results of PAGE, the preparation obtained by ion-exchange chromatography still contained admixed proteins (Fig. 1).

The enzyme solution after ion-exchange chromatography was desalted on Sephadex G-25 equilibrated with 0.1 M sodium acetate buffer, pH 5.4, and placed on a column with bacitracin-Sepharose. Under these conditions, the enzyme was completely bound to the sorbent as indicated by the absence of milk-clotting activity in the fraction eluted in the free volume. Washing of the sorbent with 0.1 M sodium acetate buffer, pH 5.4, containing 1 M NaCl did not result in desorption of the enzyme. The enzyme possessing the milk-clotting activity was eluted with high yield (measured by activity) by 10% isopropanol in 0.1 M sodium acetate buffer, pH 5.4, containing 1 M NaCl. The enzyme solution was desalted on Sephadex G-25 and lyophilized. The results of chromatography are presented in Table 1. Figure 1 shows the results of electrophoresis of the preparation of transgenic sheep chymosin obtained in polyacrylamide gel under non-denaturing conditions. For comparison, electrophoresis of calf chymosin was carried out under the same conditions. The results of electrophoresis show that the preparation of transgenic sheep chymosin is heterogeneous and consists probably of isoforms A and B. There are indications in the literature of

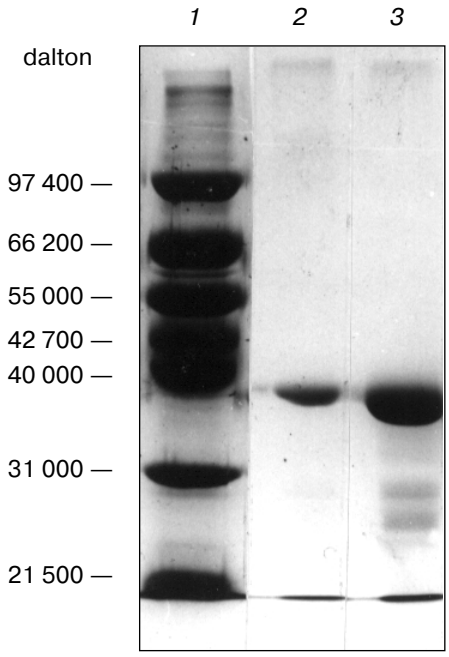

Fig. 2. Electrophoresis in 12.5% polyacrylamide gel in the presence of SDS (pH 8.8). Lanes: *1*) the mixture of standard proteins; *2*) the purified preparation of transgenic sheep chymosin; *3*) calf chymosin.

Table 2. Amino acid composition of transgenic sheep chymosin

Amino acid	Number of residues per molecule	
	calf chymosin [12]	transgenic sheep chymosin
CysOH	6	7-8
Asx	36	36
Thr	23	22
Ser	31	33
Glx	33	39
Pro	15	16-17
Gly	28	32
Ala	15	19
Val	26	22
Met	8	6
Ile	19	16
Leu	23	24
Tyr	19	16
Phe	17	15
Lys	9	11
His	5	4-5
Arg	6	6

heterogeneity of chymosin [12]. Forms A and B differ in the nature of amino acid residue in position 243 (numeration taken as for the sequence of pig pepsin). Forms A and B contain Asp and Gly in this position, respectively. Our preparation of transgenic sheep chymosin is homogeneous under the conditions of electrophoresis and corresponds probably to the A form of calf chymosin, which has a higher negative charge than that for chymosin B. Figure 2 shows the results of electrophoresis of transgenic sheep chymosin and calf chymosin in the presence of SDS. Transgenic sheep chymosin lacked impurities of proteins with other molecular mass. The mobility of transgenic sheep chymosin was identical to that for calf chymosin. Relying on the electrophoretic data, the molecular mass of the transgenic sheep chymosin was calculated. The value of the molecular mass obtained (35,500 daltons) is coincident with the corresponding value calculated from the primary sequence (35,604 daltons [12]). Protein bands with lower molecular masses particularly detectable in the preparation of calf chymosin are in all likelihood the result of cleavage of the enzyme during preparation of the sample for electrophoresis [12]. The amino acid composition of transgenic sheep chymosin is generally coincident with that for calf chymosin (Table 2). The sequence of six residues from the N-terminus of the molecule was determined using the automatic method by Edman: Gly-Glu-Val-Ala-Ser-Val-. The sequence is identical to the analogous sequence for calf chymosin [12].

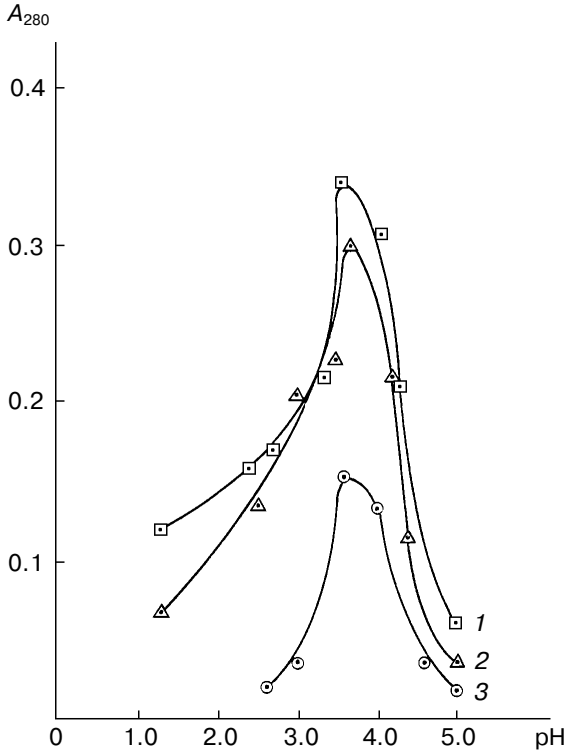

Fig. 3. pH dependence for the activities of calf chymosin and transgenic sheep chymosin: *1*) hydrolysis of hemoglobin by calf chymosin; *2*) hydrolysis of hemoglobin by transgenic sheep chymosin; *3*) hydrolysis of BSA by transgenic sheep chymosin. A_{280} is the optical absorbance of hydrolyzates at 280 nm after precipitation by trichloroacetic acid.

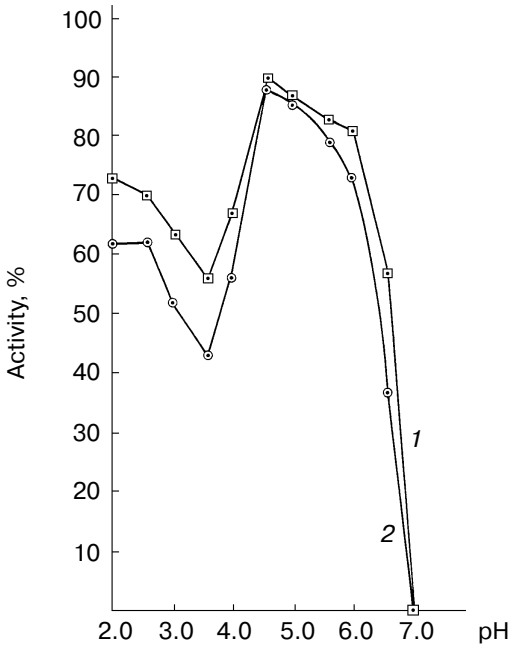

Fig. 4. pH dependence of stability of transgenic sheep chymosin: residual milk-clotting activity after 24-h (*1*) and 48-h (*2*) incubation at 37°C.

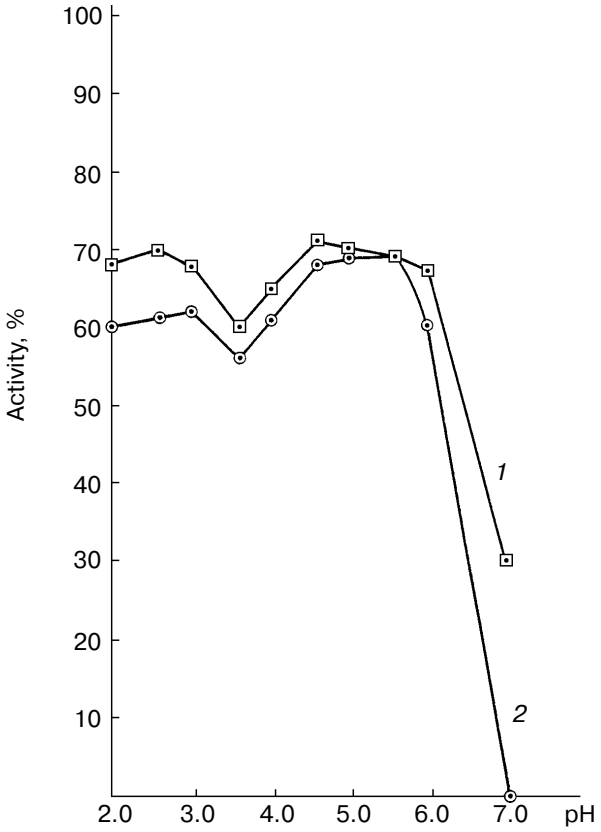

Fig. 5. pH dependence of stability of calf chymosin: residual milk-clotting activity after 24-h (*1*) and 48-h (*2*) incubation at 37°C.

Thus, the analytical data obtained indicate that the preparation of chymosin isolated by us from milk of transgenic sheep is homogeneous.

Then we studied the digestion of some protein substrates (hemoglobin, BSA, and sodium caseinate) by the transgenic sheep chymosin and compared its proteolytic activity with that for calf chymosin.

Data from the literature data indicate that the pH optimum for digestion of hemoglobin by calf chymosin is at 3.5 [13]. Our data show that the pH optimum for transgenic sheep chymosin and calf chymosin are indistinguishable (pH optimum 3.6-3.7; Fig. 3). The specific activities of calf chymosin and transgenic sheep chymosin with hemoglobin as the substrate at the optimum pH value were 8.5 and 8.8 U/A_{280}, respectively. The pH optimum for hydrolysis of BSA by transgenic sheep chymosin was at 3.5 (Fig. 3). The same pH optimum was observed for calf chymosin [13]. The specific activity of calf chymosin in the reaction of digestion of sodium caseinate (a natural substrate of the enzyme) was found to be 670 μmoles/min per g of protein. A similar value was obtained for transgenic sheep chymosin: 533 μmoles/min per g of protein. Also, the milk-clotting activities of transgenic sheep chymosin and of calf chymosin are of the same order.

Data presented in Figs. 4 and 5 show that the maximum stability of transgenic sheep chymosin and calf chymosin is observed in the pH interval from 2.0 to 6.0. Incubation of the enzymes at 37°C for 24 and 48 h results in substantial loss of enzymatic activity at pH 3.6 and 7.0. These results are coincident with the literature data for calf chymosin [13]. Inactivation of chymosin at pH 3.5 is probably due to autolysis, since this pH is most favorable for digestion of protein substrates by chymosin.

The main result of the present paper is the elaboration of a technique for preparation of chymosin from milk of transgenic sheep. Purification of the preparation by ion-exchange and biospecific chromatography yielded the homogeneous active enzyme. Based on the data on electrophoresis under non-denaturing and denaturing conditions, the results of determination of molecular mass, amino acid composition, and N-terminal sequence, and the study of stability at various values of pH and characteristics of hydrolysis of protein substrates, it is concluded that the milk-clotting enzyme isolated from milk of transgenic sheep is identical to calf chymosin.

REFERENCES

1. Foltmann, B. (1981) *Essays Biochem.*, **17**, 52-84.
2. Jolles, J., Alais, C., and Jolles, P. (1968) *Biochim. Biophys. Acta*, **168**, 591-593.
3. Prokof'ev, M. I., Mezina, M. N., Ermolaev, V. I., Demidovich, S. S., and Chernyh, V. Ya. (1999) *Zootekhniya*, No. 10, 28.
4. Starovoitova, V. V., Filippova, I. Yu., Lysogorskaya, E. N., Oksenoit, E. S., and Lavrenova, G. I. (2000) *Biochemistry (Moscow)*, **65**, 713-717.
5. Stepanov, V. M., Rydenskaya, G. N., Yanonis, V. V., Ostoslavskaya, V. I., Gonchar, M. V., Kotlova, E. K., and Strongin, A. Ya. (1987) *Bioorg. Khim.*, **4**, 1256-1263.
6. Moore, S. (1963) *J. Biol. Chem.*, **238**, 235.
7. Abdel Malak, C. A., Abou El Adab, I. F. G., Vukashinovich, V., Zalunin, I. A., Timokhina, E. A., Lavrenova, G. I., and Stepanov, V. M. (1996) *Comp. Biochem. Physiol.*, **113B**, 57-62.
8. Laemmli, U. K. (1970) *Nature*, **227**, 680-685.
9. Block, R., and Bolling, D. (1949) *Amino Acid Composition of Proteins and Food Products* [Russian translation], Izd-vo Inostrannoi Literatury, Moscow, p. 123.
10. Stepanov, V. M., Lavrenova, G. I., Adli, K., Gonchar, M. V., Balandina, G. N., Slavinskaya, M. M., and Strongin, A. Ya. (1976) *Biokhimiya*, **41**, 294-302.
11. Solov'eva, T. A., Belyaev, S. V., and Stepanov, V. M. (1977) *Khim. Prirod. Soedin.*, 398-403.
12. Foltmann, B. (1992) *Scand. J. Clin. Lab. Invest.*, **52** (Suppl. 210), 65-79.
13. Foltmann, B. (1966) *Compt. Rend. Trav. Lab. Carlsberg*, **35**, 143-229.