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A Comparative Study of Functional Properties of Calf Chymosin and Its Recombinant Forms

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Abstract—The action of calf chymosin obtained from transgenic sheep milk and the recombinant protein expressed in yeast *Kluyveromyces lactis* (Maxiren) on fluorogenic peptide substrates, namely Abz-A-A-F-F-A-A-Ded, Abz-A-A-F-F-A-A-pNA, Abz-A-F-F-A-A-Ded, Abz-A-A-F-F-A-Ded, Abz-A-A-F-F-Ded, Abz-A-A-F-F-pNA, and heptapeptide L-S-F-M-A-I-P-NH_2, a fragment of κ-casein (the native chymosin substrate), was investigated. It has been established that transgenic chymosin and recombinant chymosin (Maxiren) differ from the native enzyme in their action on low molecular weight substrates, whereas there was no difference in enzymatic action on protein substrates. Pepstatin, a specific inhibitor of aspartic proteinases, inhibits the recombinant chymosin forms less efficiently than the native enzyme. Perhaps this is associated with local conformational changes in the substrate binding site of recombinant chymosin occurring during the formation of the protein globule.

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Key words: chymosin, recombinant chymosin, fluorogenic substrates, pepstatin

Chymosin is one of proteolytic enzymes secreted by gastric mucosa of newborn mammals. Among enzymes from different animals, calf chymosin is the most studied protein. The amino acid sequence and three-dimensional structure are established, and the mechanism of action is discussed. Chymosin accounts for ~80% of the total proteolytic activity of abomasum (fourth stomach) of four-to-six month old calves. Chymosin content decreases with age, and pepsin begins to dominate. Contrary to most other aspartic proteinases of the digestive tract, the enzyme has narrow substrate specificity. It predominantly cleaves the Phe105–Met106 bond in milk κ-casein, thus destabilizing casein micelles and resulting in the formation of curd precipitate. Although milk-clotting activity of chymosin is high, its total proteolytic activity is rather low. This feature has enabled wide application of chymosin in food and medical industry. The enzyme is usually isolated from abomasum of newborn calves. Due to the limited production of abomasal enzyme, the search for alternative sources of chymosin is ongoing. One of the approaches to solve the problem is the production of recombinant enzyme. Transgenic animals were suggested for use as chymosin producers [1]. Preparation of chymosin from milk of transgenic animals is attractive because it is not necessary to isolate and purify the protein for further use in food industry, since it is already contained in its natural food product, milk.

As a rule, recombinant chymosin forms have only been characterized in terms of milk-clotting activity and interaction with antibodies. Physicochemical and enzymatic properties have not been thoroughly investigated. However, industrial implementation of recombinant enzymes as substitutes of abomasal chymosin requires more detailed investigation of the properties of these enzymes.

We previously described a method for isolation of milk-clotting enzyme from transgenic sheep milk [1]. It was shown that the isolated enzyme is identical to native calf chymosin by a number of parameters, including amino acid composition, molecular weight, amino acid sequence, hydrolysis of protein substrates, and stability at different pH values.

The aim of this work was further investigation of functional properties of recombinant chymosin analogs:

Abbreviations: Abz) *o*-aminobenzoyl; Ded) 2,4-dinitrophenylethylenediamine residue; pNA) *p*-nitroaniline residue.

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chymosin from transgenic sheep milk (transgenic chymosin) as well as chymosin expressed in *Kluyveromyces lactis* yeast (Maxiren preparation), and comparison of their properties with that of the native enzyme. The main attention is focused on investigation of enzyme interactions with low molecular weight peptide substrates and pepstatin, a specific inhibitor of aspartic proteinases.

MATERIALS AND METHODS

Calf chymosin was isolated from an industrial preparation of abomasal enzyme [2]. Chymosin from transgenic sheep milk was isolated as described in [1].

Isolation of recombinant chymosin from Maxiren preparation. One gram of Maxiren preparation (Gist-brocades, Netherlands) was stirred for 2 h at room temperature in 5 ml of 0.1 M sodium acetate buffer, pH 5.4. The resulting suspension was dialyzed against 0.1 M sodium acetate buffer, pH 5.4, and centrifuged (15,000*g*, 4°C, 30 min). The supernatant (9.5 ml) was applied to a column containing 5 ml of bacitracin-Sepharose (ligand content was 6.8 μmol/ml [3]) 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, and 1 M NaCl in 0.1 M sodium acetate buffer, pH 5.4, until A_{280} of the eluate was below 0.1. The enzyme was eluted with 10% solution of isopropanol in 0.1 M sodium acetate buffer, pH 5.4, containing 1 M NaCl. Chymosin-containing fraction was desalted on Sephadex G-50 (coarse) equilibrated with water and then lyophilized. The yield of the enzyme yield ~2 mg. The yield estimated by milk clotting enzyme activity [2] was 99%.

The dependence of enzyme stability on pH was determined by the method described by Mezina et al. [1].

Determination of temperature dependence of enzyme activity. Enzyme solution (50 μg/ml) in 0.1 M sodium acetate buffer, pH 5.6, was incubated at 28, 37, 40, 50, and 60°C for 15 min. After that, the milk-clotting enzyme activity was determined at 37°C as described in [2].

Amino acid analysis was performed using a Hitachi-835 automatic amino acid analyzer (Hitachi, Japan) after complete protein (48 h) or peptide (24 h) hydrolysis in 5.7 M HCl at 105°C.

Hydrolysis of fluorogenic peptide substrates. The following fluorogenic peptide substrates with internal quenching of fluorescence were synthesized in our laboratory previously [4] and used in this work:

Abz-A-A-F-F-A-A-Ded (I),

Abz-A-A-F-F-A-A-pNA (II),

Abz-A-F-F-A-A-Ded (III),

Abz-A-A-F-F-A-Ded (IV),

Abz-A-A-F-F-Ded (V),

Abz-A-A-F-F-pNA (VI).

Initial rates of substrate hydrolysis (V_0), kinetic characteristics, and the influence of pH on V_0 were determined as described in [2]. Hydrolysis time corresponding to the change in fluorescence after 10-20% hydrolysis of the substrate was 2-3 min. For all substrates, the concentration of transgenic chymosin and recombinant chymosin (Maxiren) was 14 nM.

Hydrolysis of L-S-F-M-A-I-P-NH_2 substrate. The amide of the corresponding heptapeptide was kindly provided by L. D. Rumsh (Institute of Bioorganic Chemistry, Russian Academy of Sciences).

Sodium acetate buffer (50 μl, 0.05 M, pH 4.7) and enzyme solution (5 μl, 0.4 μM, in 0.05 M sodium acetate buffer, pH 5.0) were added to 50 μl of 1 mM solution of substrate in 0.05 M sodium acetate buffer, pH 4.7. After 5, 10, 20, and 30 min the reaction was stopped by addition of 1.5 μl of 7% NaOH to 10 μl of the reaction mixture. Hydrolysis products were analyzed by HPLC using an Altex Model 110A liquid chromatograph (USA) and C_8 column (4 × 250 mm). Elution was performed using a 0-60% linear acetonitrile gradient in water. Elution rate was 0.8 ml/min, and elution time was 30 min. Products of substrate hydrolysis by calf chymosin were hydrolyzed with 5.7 M HCl at 105°C for 24 h. After that, the amino acid composition was determined as described above.

Pepstatin inhibition. A methanol solution of pepstatin with concentration of 3 mg/ml was prepared. This solution was used to prepare 0.06 and 0.006 mg/ml pepstatin solutions by dilution with 0.1 M sodium acetate buffer, pH 5.6. To 25 μg (0.7 nmol) of enzyme solution in 0.1 M sodium acetate buffer, pH 5.6, such amount of pepstatin solutions (at the concentration of 6 and 60 μg/ml) was added so that the resulting inhibitor concentration in the reaction mixture (total volume 1 ml) was 0.05, 0.075, 0.15, 0.25, 0.5, 1.0 μg/ml or 0.07, 0.11, 0.22, 0.35, 0.7, 1.4 nmol/ml, respectively. The mixture was incubated at 37°C for 30 min. After that, 50 μl aliquots were taken for determination of enzyme activity in the cleavage reaction with fluorogenic substrate Abz-A-A-F-F-A-A-Ded [2].

RESULTS AND DISCUSSION

Recombinant chymosin (Maxiren) was obtained from *K. lactis* cells transformed with chymosin cDNA [5]. The yeast *K. lactis* provides a number of advantages compared to other yeast species. In particular, *K. lactis* is not pathogenic for humans, which allows using chymosin expressed in this yeast in the food industry. *Kluyveromyces lactis* possesses effective ability to synthesize and secrete heterologous proteins. The stability of the protein expres-

sion system in *K. lactis* can be used to obtain extracellular chymosin in large quantities, thus promoting the development of large scale chymosin production. The resulting preparation was called Maxiren and is now recommended for use as a substitute for abomasal enzyme.

We have applied affinity chromatography on a bacitracin-Sepharose column for purification of recombinant chymosin from Maxiren preparation. The results of purification have demonstrated that the preparation contains a significant amount of other proteins. The fraction containing the active enzyme comprised only up to 5% of the total amount of protein applied to the column. The specific milk-clotting activity of the chymosin increased 20-fold after purification. According to data of electrophoresis in non-denaturing polyacrylamide gel [6], the recombinant chymosin (Maxiren) obtained after affinity chromatography is homogeneous and obviously corresponds to the B-form of calf chymosin. The amino acid composition of chymosin isolated from Maxiren preparation corresponds to that of calf chymosin. According to our data, recombinant chymosin (Maxiren) has the same pH optimum and specific hemoglobin cleavage activity as the native enzyme and transgenic chymosin [1]. As reported in the literature [5], recombinant chymosin from *K. lactis* was characterized by peptide mapping, N-terminal amino acid sequence, and temperature stability. No difference was found between the characteristics of chymosin from *K. lactis* and the native enzyme.

Fluorogenic peptides I-VI with internal quenching of fluorescence were used as low molecular weight substrates. These substrates were successfully used previously for the determination of activity of a number of aspartic proteinases [4], including calf chymosin [2].

Initial rates of hydrolysis of fluorogenic substrate I by transgenic chymosin and recombinant chymosin isolated from the Maxiren preparation are linear functions of enzyme concentration in the range of 0.125-0.625 ng/ml. The same dependence is also typical for the native enzyme. Enzyme concentration of 7 nM was chosen for the reaction of hexapeptide I hydrolysis; for all other substrates, 14 nM.

The pH optimum for hydrolysis of substrate I by transgenic chymosin and chymosin from Maxiren preparation is 3.5-3.7 and matches the pH optimum for hydrolysis of this peptide by calf chymosin. However, the transgenic chymosin displays lower activity towards the studied substrates compared to the native enzyme (Table 1), as indicated by catalytic constants for cleavage reactions as well as by k_{cat}/K_m ratio (Table 1).

Transgenic chymosin did not catalyze the hydrolysis of tested tetrapeptides. Among pentapeptides, only substrate III was cleaved by transgenic chymosin, where the rate of hydrolysis and proteolytic coefficient were significantly lower than that of the native enzyme. Peptide IV, the best fluorogenic substrate for aspartic proteinases, was virtually not hydrolyzed by transgenic chymosin. Hexapeptide I was hydrolyzed by transgenic chymosin by one order of magnitude slower than by calf chymosin. Replacement of the Ded residue by pNA in this substrate resulted in complete inhibition of binding with transgenic chymosin. Thus, transgenic chymosin only cleaves fluorogenic substrates I and III, and the reaction is approximately one order of magnitude slower than in the case of the abomasal enzyme.

It was found that not only transgenic chymosin, but also chymosin from Maxiren preparation differ dramatically from the native enzyme by their action on fluorogenic substrates. Hexapeptide I and pentapeptide IV were cleaved by recombinant chymosin (Maxiren) by one order of magnitude less efficiently than by calf chymosin. However, the kinetic characteristics for the cleavage of pentapeptides III and IV were approximately the same, contrary to that for transgenic chymosin. Hexapeptide containing the pNA residue as a fluorescence quencher and tetrapeptide derivatives are not hydrolyzed by this enzyme. According to the obtained catalytic characteristics of fluorogenic substrate hydrolysis by the recombinant proteins, transgenic chymosin and chymosin from

Table 1. Hydrolysis of fluorogenic substrates by chymosins (0.1 M acetate buffer, pH 3.7, containing 0.5% dimethylformamide, ~20°C, [S] = 1-10 μM)

Substrate	Calf chymosin			Transgenic chymosin			Recombinant chymosin (Maxiren)		
	K_m, mM	k_{cat}, sec^{-1}	k_{cat}/K_m, $sec^{-1}\cdot mM^{-1}$	K_m, mM	k_{cat}, sec^{-1}	k_{cat}/K_m, $sec^{-1}\cdot mM^{-1}$	K_m, mM	k_{cat}, sec^{-1}	k_{cat}/K_m, $sec^{-1}\cdot mM^{-1}$
I	0.0070 ± 0.0020	1.6 ± 0.2	230	0.0056 ± 0.0009	0.16 ± 0.02	28	0.0056 ± 0.0012	0.12 ± 0.03	22
II	0.0040 ± 0.0007	1.4 ± 0.3	360	—	—	—	—	—	—
III	0.0018 ± 0.0004	0.14 ± 0.02	80	0.0078 ± 0.0012	0.06 ± 0.01	7	0.0021 ± 0.0005	0.06 ± 0.01	30
IV	0.0022 ± 0.0002	0.8 ± 0.1	370	0.0180 ± 0.006	0.02 ± 0.01	1.3	0.0035 ± 0.0006	0.11 ± 0.02	31
V	0.0030 ± 0.0003	0.27 ± 0.04	90	—	—	—	—	—	—
VI	0.0018 ± 0.0004	0.48 ± 0.09	270	—	—	—	—	—	—

Table 2. Hydrolysis of L-S-F-M-A-I-P-NH_2 by chymosins (0.05 M sodium acetate buffer, pH 4.7, 37°C, [S] = 0.5 mM, [E] = 20 nM)

Chymosin	Hydrolysis time, min	Amount of hydrolysis products in reaction mixture, %		
		LSF	$MAIPNH_2$	$LSFMAIPNH_2$
Calf	5	18	19	63
	20	37	44	19
	30	43	45	12
Transgenic	5	9	15	76
	20	28	37	35
	30	38	44	18
Recombinant (Maxiren)	5	9	11	80
	20	25	29	46
	30	32	37	31

Maxiren preparation exhibit similar behavior towards the studied compounds.

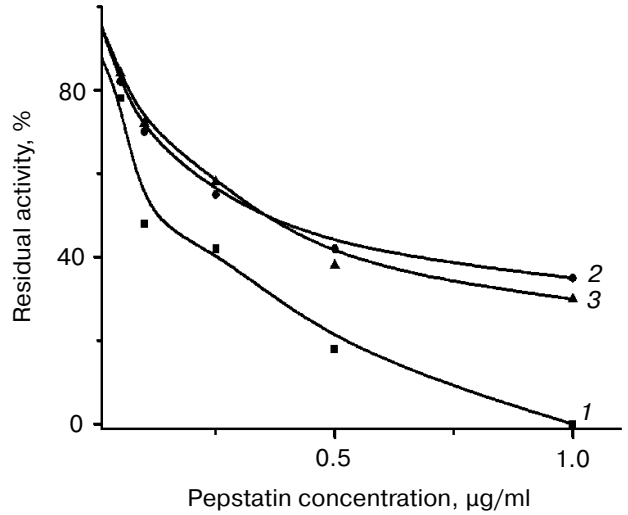
Thus, activity of calf chymosin towards the low molecular weight substrates is different from the corresponding activity of the transgenic chymosin and recombinant chymosin (Maxiren). In this connection, the question was raised of how the native enzyme and its recombinant forms will behave in regards to the fragment of native chymosin substrate (κ-casein), heptapeptide L-S-F-M-A-I-P-NH_2.

Chymosin cleaves the Phe105–Met106 bond in the κ-casein molecule. Kinetic parameters for hydrolysis of the whole κ-casein molecule are similar to those of pentadecapeptide $HPHPHL_{103}SFMAIP_{109}PKK$, which is a fragment of the κ-casein molecule and contains the scissile bond. As a substrate, we have chosen the pentadecapeptide fragment, heptapeptide 103-109, which is one of the best short substrates (κ-casein fragments) for chymosin according to Visser's data [7]. Using HPLC, we have demonstrated that the enzymes hydrolyzed the Phe–Met bond in the heptapeptide. The reaction of heptapeptide amide hydrolysis was monitored by depletion of initial amount of substrate and by accumulation of reaction products after 5, 20, and 30 min of hydrolysis (Table 2).

It was found that heptapeptide is most efficiently cleaved by native calf chymosin and less efficiently by transgenic chymosin and chymosin from Maxiren. The difference is especially noticeable after 30 min hydrolysis. Thus, difference in reaction rates of heptapeptide hydrolysis by the native enzyme and its recombinant forms exists, though it is not as dramatic as in the case of fluorogenic substrates. Apparently, this can be explained by the fact that amino acid sequence of heptapeptide amide is better corresponding to the requirements for chymosin specificity than the sequence of the fluorogenic substrates.

In connection with characteristics found for hydrolysis of low molecular weight substrates by calf chymosin and its recombinant forms, we investigated the inhibition of these enzymes by pepstatin, a specific inhibitor of aspartic proteinases. The results of multiple studies indicate that pepstatin is bound in the active site of aspartic proteinases and is an analog of the intermediate formed during hydrolysis of the peptide bond [8]. Inhibition was tested at fixed enzyme concentration and various concentrations of inhibitor. The residual activity was determined by the cleavage of fluorogenic substrate I. The pattern of dependence of enzyme activity on pepstatin concentration is identical for both native and recombinant enzymes. However, there is a difference in inhibitor binding by the various enzymes. In the presence of pepstatin, the native chymosin loses its activity faster than the other enzymes (see figure). Transgenic chymosin and recombinant chymosin (Maxiren) exhibit similar behavior. They lose their activity at a slower rate than the native enzyme for all pepstatin concentrations studied. Calf chymosin retains 15% of its initial activity at equimolar enzyme/inhibitor ratio (which corresponds to pepstatin concentration of 0.5 μg/ml) and is completely inhibited by a two-fold excess of pepstatin. Transgenic chymosin and chymosin from Maxiren retain 40 and 35% of their activities in the presence of equimolar amount of pepstatin, respectively. The complete inhibition of transgenic chymosin and recombinant chymosin (Maxiren) is not observed even in the presence of a two-fold excess of the inhibitor. Under these conditions, the enzymes retain 29 and 25% of their activities, respectively. This fact indicates a difference between the native enzyme and its recombinant analogs on the molecular level.

In our previous work [1], we reported that transgenic chymosin has essentially the same pattern of dependence of enzyme stability on pH as does its native analog. The same is also true for chymosin from Maxiren preparation. The enzymes display maximal stability at pH 2.0-6.0



Inhibition of calf (*1*), transgenic (*2*), and recombinant Maxiren (*3*) chymosins by pepstatin. The reaction was performed in 0.1 M sodium acetate buffer, pH 5.6. Activity was measured with substrate I; [S] = 5 μM

under incubation at 37°C for 24 and 48 h. A certain loss of activity occurs already at pH 3.5, and more profoundly at pH 7.0. These data are in agreement with the literature data for calf chymosin [9]. However, it should be noted that at pH 3.5 transgenic chymosin and chymosin from Maxiren preparation are inactivated slightly faster than the native enzyme. After the incubation for 48 h at 37°C and pH 3.5 the native chymosin retains 56% of its initial milk-clotting activity, chymosin from Maxiren preparation retains 50%, and transgenic chymosin retains 43%. Significant difference between the stability of the native enzyme and its recombinant analogs is observed at pH 7.0. After 24 h of incubation at pH 7.0, calf chymosin retains 30% of its activity, whereas transgenic chymosin and chymosin from Maxiren preparation are completely inactivated.

All three enzymes are active at 25-50°C. Calf chymosin and chymosin from Maxiren preparation lose less than 10% of milk-clotting activity after incubation for 15 min at temperatures below 50°C, whereas transgenic chymosin is inactivated by 17% under the same conditions. Calf chymosin loses 20% of its activity after incubation for 15 min at 50°C, chymosin from Maxiren preparation 24%, and transgenic chymosin 33%. Consequently, transgenic chymosin loses activity at 25-50°C slightly faster than abomasal enzyme and recombinant chymosin (Maxiren). All of the enzymes are inactivated at 60°C.

In this way, it has been established that transgenic chymosin and recombinant chymosin (Maxiren) differ from native calf chymosin by their action on low molecular weight substrates (fluorogenic derivatives of peptides I-VI and κ-casein fragment, heptapeptide L-S-F-M-A-I-P-NH_2), whereas no difference is observed for high molecular weight substrates. Recombinant chymosins hydrolyze fluorogenic substrates by one order of magnitude slower than the native enzyme (estimated by k_{cat}/K_m ratio). We assume that it is associated with local conformation changes in substrate binding sites of the recombinant chymosin analogs that occur during the formation of three-dimensional enzyme structure under non-natural conditions. This assumption is confirmed by the action of transgenic chymosin and recombinant chymosin (Maxiren) towards aspartic proteinase inhibitor, pepstatin. Thus, slow inhibition of recombinant chymosin forms is apparently associated with weak binding of pepstatin in the substrate binding site. The results on enzyme stability also confirm this assumption.

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