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Effect of divalent metal ions on milk-clotting activity of a mutant variant of recombinant reindeer chymosin (*Rangifer tarandus*)

Abstract

The effect of divalent metal ions on the milk-clotting activity of a mutant variant of recombinant chymosin of reindeer (*Rangifer tarandus*) with a point amino acid substitution S198R was studied. Commercial recombinant chymosin of cow and commercial recombinant chymosin of single-humped camel were used as comparison enzymes. Findings showed that the effect on the coagulation activity of chymosins, metal cations may be divided into two groups: stimulating (Mg^{2+}, Mn^{2+}, Ca^{2+}) and inhibitory (Cu^{2+}, Ni^{2+}, Co^{2+}). Ba ions demonstrated a multidirectional effect: they suppressed the activity of the reindeer enzyme and stimulated the coagulation ability of the comparison enzymes. The authors of the article suggest that differences in the degree of suppression or stimulation of the milk-clotting activity of different genetically engineered chymosins by the same metals are associated with the peculiarities of the distribution of surface charges in the molecules of individual enzymes.

Keywords: chymosin, coagulating milk, amino acid

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Introduction

Aspartate endopeptidase chymosin (EC 3.4.23.4) is widely used for milk coagulation in the production of rennet cheeses. Chymosin is synthesized in the stomachs of most species of newborn mammals. The coagulation ability of chymosin is based on proteolytic activity, due to that the enzyme hydrolyzes the F105-M106 bond in the kappa-casein molecule (k-CN) that leads to the formation of a milk clot.[1]

The value of chymosin for cheese making is due to a unique combination of its biochemical properties: high specificity, moderate thermal stability and technologically optimal dependence of milk-clotting activity (MA) on pH and $CaCl_2$ concentration. When introduced into milk, chymosin hydrolyzes the F105-M106 bond in the k-CN molecule at a high rate, almost without affecting other peptide bonds of caseins and whey proteins. This selectivity of proteolytic activity underlies the high specificity of chymosin and distinguishes it from many other proteases capable of coagulating milk.[1-3]

At the end of the XX century, the growth in the production of rennet cheeses, combined with the scrapie epidemic, led to the formation of a global shortage of chymosin-containing milk-clotting enzymes. Recombinant DNA technologies have been actively used to solve this problem.[4]

Recombinant chymosin of the cow (*Bos taurus*) was the first milk-clotting enzyme obtained by genetic engineering methods and embedded in cheese-making.[5-7] Later, recombinant chymosin of the single-humped camel (*Camelus dromedarius*) was synthesized.[8] Currently, both of these enzymes are widely used in the cheese-making.[9-10] Despite the fact that recombinant cow chymosin (rChn-Bos) and recombinant camel chymosin (rChn-Cam) are considered as high-quality milk coagulants, each of them has disadvantages. Recombinant cow chymosin is inferior to recombinant camel chymosin in the ratio of milk-clotting activity and proteolytic activity, but has a technologically more advantageous range of thermal stability.[11] Thus, the search for new milk-clotting enzymes that could surpass recombinant cow chymosin and recombinant camel chymosin is a topical biotechnological challenge.

During the search for thermolabile milk-clotting enzymes for cheese-making, the staff of the State Research Center of Virology and Biotechnology VECTOR obtained an engineering version of recombinant chymosin of reindeer (*Rangifer tarandus*) with a point amino acid replacement S198R (rChn-Rta-S198R). The side group S198 participates in the formation of a stabilizing hydrogen bond with E261. In order to reduce the thermal stability of the enzyme, the residue S was replaced with R. The replacement of S198R also leads to an increase in the total positive charge of the surface of rChn-Rta-S198R and should accelerate its binding to k- CN. The enzyme was developed in the yeast expression system of the *Kluyveromyces lactis*, strain GG799 ("NEB", USA).

The biochemical characterization of any new recombinant chymosin should include the study of its MA in the presence of divalent metal ions (Me^{2+}). This is due to that Me^{2+} can significantly affect the structure of allosteric and active centers, as well as the rate of formation and decay of enzyme-substrate complexes. It is known that with an increase in the concentration of Ca^{2+} in milk, in the range of 1-4 mM, the duration of the formation of a milk clot, under the action of various chymosins, is significantly reduced.[11] The introduction of $CaCl_2$ into pasteurized or rennet-flaccid milk is widely used in cheese-making to reduce the duration of coagulation and save milk-clotting enzyme.[12,13] Besides the calcium, MA of recombinant chymosin is also affected by other Me^{2+}.[14-16] Data on the effect of Me^{2+} on MA can be used in the preparations of liquid milk-clotting enzymes, for example.

The purpose of this work is to study the effect of divalent metal cations on the MA of an engineering variant of recombinant chymosin of reindeer with a point amino acid substitution S198R.

Materials and methods

The total MA of recombinant chymosin was determined by the method.[17] The milk-clotting activity was expressed in arbitrary units (AU) per 1 ml (AU/ml). The rChn-Rta-S198R, diluted with 20 mM Na-acetate buffer (pH 5.8) to MA about 1300 AU/ml, was used in the work.

The comparison enzymes were dry recombinant chymosin of cow ("Maxiren 1800", "DSM Food Specialties", France) and dry recombinant chymosin of single-humped camel ("Chy-Max M", "Chr. Hansen", Denmark). To prepare for research, solutions of recombinant cow chymosin (rChn-Bos) and recombinant camel chymosin were prepared in a 20 mM Na-acetate buffer (pH 5.8). All the studied recombinant chymosin preparations were normalized according to MA rChn-Rta-S198R.

Pasteurized cow's milk was used as a substrate. To prepare for work, NaN_3 was added to the milk to a concentration of 0.02 % and the pH was adjusted to 6.5 by 2.0 M HCl. To study the Me^{2+} impact on MA of recombinant chymosin were used 1.0 M solutions of the following compounds: $MgCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, $CoCl_2 \cdot 6H_2O$, $CuCl_2 \cdot 2H_2O$, $BaCl_2$, $MnCl_2 \cdot 4H_2O$, $CaCl_2$.

Enzyme solutions containing 10 mM Me^{2+} were prepared. To do this, 5 mkl of a Me^{2+} solution was added to 0.5 ml of the recombinant chymosin. To the control sample was added 5 mkl of distilled water instead of a Me^{2+} solution.

Conducting an analysis. The substrate (1.25 ml) was heated for 10 minutes at 35 °C, then a 0.1 ml enzyme solution containing 10 mM of Me^{2+} was added. The duration of coagulation was determined in seconds. Milk-clotting activity was expressed as 1 / duration of coagulation. For 100 % milk coagulation activity were taken the value of 1 / duration of coagulation of the control samples of recombinant chymosin (Me^{2+} not added).

Statistical processing of the obtained data was carried out in the computing environment of the Excel spreadsheet processor (Microsoft Corporation, USA). For quantitative variables, the results are presented as an arithmetic mean (M) with an indication of the standard deviation (±SD).

Results and discussion

The results of studying the effect of various Me^{2+} on the MA of the engineering variant of recombinant reindeer chymosin and commercial recombinant cow chymosin and recombinant camel chymosin are presented in Table 1.

Table 1 Effect of divalent metal cations on MA of recombinant chymosins

Me2+, added to the recombinant chymosin	Milk-clotting activity, % (± σ)		
	rChn-Rta-S198R	rChn-Bos	rChn-Cam
Control (-Me^{2+})	100	100	100
Cu^{2+}	54.3±1,0	50.0±0,3	44.9±0,3
Mg^{2+}	111.7±1,2	111.1±1,1	107.7±1,0
Ni^{2+}	14.2±0,2	13.2±0,1	12.1±0,2
Co^{2+}	73.4±0,5	72.4±0,5	70.6±1,2
Ba^{2+}	96.4±1,2	114.9±1,1	110.0±0,6
Mn^{2+}	131.3±0,8	131.5±0,9	120.1±1,5
Ca^{2+}	107.2±0,5	115.6±0,7	107.7±1,0

According to the effect of divalent metal cations on the MA of recombinant chymosin, all studied Me^{2+} can be divided into two groups: stimulating coagulation ability (containing Mg^{2+}, Mn^{2+}, Ca^{2+}) and inhibiting it (Cu^{2+}, Ni^{2+}, Co^{2+}). The greatest stimulating effect was registered for manganese ions in the presence of which the MA of enzymes increased by 1.2-1.3 times. The addition of Mg^{2+} and Ca^{2+} to recombinant chymosin solutions led to increase milk-clotting activity of rChn-Rta-S198R, rChn-Bos and rChn-Cam by 1.1-1.2 times.

The maximum inhibitory effect was possessed by Ni ions, which caused a decrease in MA rChn-Rta-S198R and comparison enzymes by 7.1-8.3 times. The introduction of copper and cobalt salts into recombinant chymosin preparations led to decrease MA by 1.4-2.2 times.

It is worth highlighting the Ba ions, which demonstrated a multidirectional effect: MA rChn-Rta-S198R in their presence decreased by 3.4%, and MA rChn-Bos and rChn-Cam increased by 14.9 and 10.0 %, respectively. Perhaps the insignificant depressing effect of Ba^{2+} on the engineering variant of recombinant chymosin of reindeer is the result of the S198R substitution introduced into its amino acid sequence.

In general, our data on the effect of Me^{2+} on the MA of recombinant chymosin are in good agreement with the results of other authors obtained for recombinant chymosin of the cow, recombinant chymosin of the double-humped camel and recombinant chymosin of the Altai maral (*Cervus elaphus sibiricus*).[14-16] The mechanism of effect of Me^{2+} on MA of recombinant chymosin in the above works is not discussed.

It is possible that the nature of the effect of divalent metal ions on the milk-clotting activity of chymosins is associated with the electronegativity of individual ions and with the peculiarities of the distribution of surface charges in enzyme molecules.

As an attempt to know the mechanism of Me^{2+} effect on the MA of enzymes, we compared the atomic masses, ionic radii and electronegativity of metal ions on the Pauling scale (Table 2). The general dependence of the Me^{2+} effect on the MA of recombinant chymosin on the mass of the atom or its ionic radius was not revealed.

Table 2 Some physicochemical properties of divalent metal ions

Metal	Parameter		
	Atomic masse (Da)	Ionic radii (pm)	Electronegativity (Pauling scale)
Cu^{2+}	63.5	73	1.9
Mg^{2+}	24.3	66	1.31
Ni^{2+}	58.7	69	1.91
Co^{2+}	58.9	72	1.88
Ba^{2+}	137.3	134	0.89
Mn^{2+}	54.9	80	1.55
Ca^{2+}	40.1	99	1

As follows from the data presented in Table 2, the nature of the effect of Me^{2+} on MA of recombinant chymosin may be associated with electronegativity. Electronegativity is a fundamental chemical property that characterizes the ability of an atom in a molecule to shift common electron pairs to itself (in other words, to attract electrons of other atoms to itself). Comparing the charge characteristics of the studied divalent ions, we drew attention to the following dependence: the electronegativity of cations inhibiting MA of recombinant chymosin lies in the range of 1.88-1.91, whereas for Me^{2+} stimulating enzymes this indicator is lower (0.89-1.55). The fact that Ba ions (electronegativity = 0.89) do not stimulate, but slightly suppress MA rChn-Rta-S198R is probably the result of a point amino acid substitution of S198→R, which, as noted above, leads to an increase in the total positive charge of the enzyme surface.

It can also be assumed that the differences we observed in the degree of suppression or stimulation by the same metals of the milk-clotting activity of different recombinant chymosin are related to the peculiarities of the distribution of surface charges in the molecules of enzymes.

Conclusion

Divalent metal cations act differently on the milk-clotting activity of recombinant chymosines of various origins: Mg^{2+}, Mn^{2+}, Ca^{2+} stimulate the milk-clotting activity of the studied enzymes, whereas Cu^{2+}, Ni^{2+}, Co^{2+} suppresses it. Ba ions inhibit the activity of rChn-Rta-S198R, but stimulated the coagulation ability of recombinant chymosin of the cow and recombinant chymosin of the camel. It is possible that the nature of the effect of divalent metal ions on the milk-clotting activity of chymosines is associated with the electronegativity of individual ions and with the peculiarities of the distribution of surface charges in enzyme molecules.

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Conflicts of interests

The authors declare that there are no conflicts of interest.

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