Korean J. Chem. Eng., **28**(1), 203-208 (2011)
DOI: 10.1007/s11814-010-0347-8

Purification and properties of a milk-clotting enzyme produced by *Bacillus amyloliquefaciens* D4

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(*Received 6 April 2010 • accepted 6 June 2010*)

Abstract−The milk-clotting enzyme from *Bacillus amyloliquefaciens* D4 was purified to 17.2-fold with 20% recovery by precipitation in ammonium sulfate and ion-exchange chromatography. The molecular mass of the enzyme was 58.2 kDa as determined by SDS-PAGE, and it was proved to be a metalloprotease by EDTA inhibition. The enzyme was active in the pH range 5.5-7.0 and was inactivated completely by heating at 55 °C for 20 min. The highest level of enzyme activity was obtained at 65 °C, pH 5.5, in the presence of 25 mM $CaCl_2$. The milk-clotting activity was inhibited only slightly by Na^+ and K^+ and significantly by Cu^{2+}, Zn^{2+} and Sn^{2+}. The K_m value of this enzyme was 0.471 mg/mL. The high level of milk-clotting activity coupled with a low level of thermal stability suggested that the milk-clotting enzyme from *B. amyloliquefaciens* D4 should be considered as a potential substitute for calf rennet.

Key words: Milk-clotting Enzyme, Rennet, Purification, Enzyme Properties, *Bacillus amyloliquefaciens*

INTRODUCTION

The inclusion of a milk-clotting enzyme is very important in the production of cheese. The enzyme clots milk and has an important role in the process of cheese maturation [1]. Calf rennet has traditionally been used by the dairy industry for the manufacture of cheese with good flavor and texture. The worldwide increase of cheese production coupled with a reduced supply of calf rennet has prompted a search for calf rennet substitutes, including microbial and plant rennets [2]. However, most plant rennets have proved unsuitable because they impart a bitter taste to the cheese [3].

Microbial rennet appears to be more promising because its production is cheaper, biochemical diversity is greater, and genetic modification is easier [4]. Many species of microorganism are known to produce a milk-clotting enzyme that can potentially substitute for calf rennet, and *Rhizomucor pusillus*, *Rhizomucor miehei*, *Endothia parasitica* and *Irpex lactis* are used extensively as sources of milk-clotting enzymes in the manufacture of cheese [5].

The Tibetan Plateau in China, a region that embraces diverse geographic environments and experiences a considerable range of temperature, is home to a diversity of microbial species and plentiful microbial germplasm resources. We developed a procedure for the isolation of microorganisms with milk-clotting activity and isolated 52 bacterial strains from the yak grazing soil in the Tianzhu Tibetan autonomous county in the north-eastern Tibetan Plateau. We found that *Bacillus amyloliquefaciens* D4 has the highest milk-clotting activity of all these strains.

Bacillus amyloliquefaciens is Gram-positive, catalase-positive, aerobic, rod-shaped and motile. It is known for its catabolic properties and the ability to degrade complex macromolecules. It has been shown that *B. amyloliquefaciens* produces a number of enzymes, including a thermostable xylanase [6], an α-amylase [7], a subtilisin-like proteinase [8] and a cellulase [9], but no milk-clotting enzyme was reported for *B. amyloliquefaciens*. We isolated *B. amyloliquefaciens* D4 and found that it had a high level of milk-clotting activity. The purpose of the present work was to purify and characterize the milk-clotting enzyme produced by *B. amyloliquefaciens* D4.

MATERIALS AND METHODS

1. Microorganism and Fermentation Medium

B. amyloliquefaciens D4 was isolated from soil collected on the Tibetan Plateau of China. The strain has been deposited with the China General Microbiological Culture Collection Center; CGMCC NO.3290. The microorganism was maintained in the laboratory at 37 °C on LB agar slants (1.0% (w/v) peptone, 1.0% (w/v) beef extract, 0.5% (w/v) NaCl and 2.0% (w/v) agar, pH 7.2).

The fermentation medium (100 mL), containing 18% (w/v) wheat bran juice, 4% (w/v) sucrose, 2% (w/v) skim milk and 0.2% (w/v) $NaH_2PO_4 \cdot 2H_2O$, was sterilized (autoclaved at 121 °C for 15 min) in a 250 mL Erlenmeyer flask. The medium was inoculated with the microorganism and incubated at 37 °C for 2 days on an orbital shaker at 170 rpm.

2. Enzyme Purification

All enzyme purification steps were done at 4 °C.

The crude enzyme was harvested by centrifugation at 8,000 g for 10 min and the supernatant was used for the study.

The crude enzyme extract was precipitated with ammonium sulfate (30-80% saturation). The precipitate obtained after centrifugation at 12,000 g for 15 min was suspended in 50 mM sodium phosphate buffer (pH 7.0) and dialysed (7 kDa cutoff) overnight against several changes of distilled water to remove the salt.

Ten milliliters of the partially purified enzyme solution were sub-

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jected to ion-exchange chromatography on a DEAE-Sephadex A-25 column (30 cm×2.6 cm) pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.0). The column was eluted at a flow rate of 0.8 mL/min with an increasing linear gradient of NaCl from 0 to 0.5 M in 50 mM sodium phosphate buffer (pH 7.0) and 5 mL fractions were collected. The protein content of each fraction was determined by measuring the absorbance at 280 nm. The fractions with enzyme activity were pooled and dialysed overnight against distilled water and then lyophilized.

3. SDS-PAGE and Protein Quantification

The molecular mass of the purified enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described [10] using molecular mass markers of 14.4, 20.0, 26.0, 35.0, 45.0, 66.2 and 94.0 kDa. The total protein content was measured by the Bradford method [11] with bovine serum albumin (BSA) as the standard.

4. Milk-clotting Activity Determination

The milk-clotting activity was determined as described [12]. A 5 mL portion of the substrate (10% skim milk in 10 mM $CaCl_2$) was incubated for 5 min at 35 ºC and then 0.5 mL of enzyme extract was added. The length of time starting from the addition of the enzyme extract to the formation of the first particles was recorded, and the milk-clotting activity was calculated as:

$$SU=2400\times5\times D/T\times0.5 \quad (1)$$

Where T is milk-clotting time (s), and D is dilution of the enzyme. One Soxhlet unit (SU) of milk-clotting activity was defined as the amount of enzyme required to clot 1 mL of substrate within 40 min at 35 ºC.

5. Enzyme Properties

5-1. Effects of Temperature and pH on Enzyme Activity and Stability

The optimum temperature for the activity of the purified enzyme was determined by assaying the milk-clotting activity at intervals of 5 ºC from 30-85 ºC. The optimum pH for the activity of the purified enzyme was determined by assaying the milk-clotting activity in the pH range 5.5-8.5, by adjusting the pH of the substrate (skim milk) with 0.1 M HCl or 0.1 M NaOH as appropriate. The maximum milk-clotting activity obtained was taken to be 100%.

To determine the thermal stability, the purified enzyme was incubated at 5 ºC intervals from 35-85 ºC, and the length of the incubation was varied from 0 to 60 min. After incubation, the residual milk-clotting activity was determined and the activity obtained with an incubation time of 0 min was taken to be 100%.

To determine the pH stability, the enzyme was dispersed (1 : 1, v/v) in the following 0.1 M buffer solutions: glycine-HCl (pH 3.5-4.0), citrate/phosphate (pH 4.5-5.5), sodium phosphate (pH 6.0-8.5) and carbonate/bicarbonate (pH 9.0-11.0), and kept at room temperature for 24 h. The residual milk-clotting activity was determined and the maximum activity obtained was taken to be 100%.

5-2. Effect of Substrate Concentration (Skim Milk)

The effect of substrate concentration on the milk-clotting activity of the purified enzyme was determined by increasing the concentration of the skim milk from 25 to 200 g/L. The maximum activity obtained was taken to be 100%.

5-3. Effect of $CaCl_2$ Concentration

The effect of the concentration of $CaCl_2$ on the milk-clotting activity of the purified enzyme was determined by increasing the concentration of the calcium chloride solution at intervals of 5 mM from 0 to 35 mM. The maximum activity obtained was taken to be 100%.

5-4. Effect of Other Metal Ions

The effect of metal ions Li^+, Mn^{2+}, Zn^{2+}, Mg^{2+}, Cu^{2+}, K^+, Na^+ and Sn^{2+} on the milk-clotting activity was determined at metal ion concentrations of 0 mM, 1 mM, 5 mM and 10 mM. The milk-clotting enzyme was incubated at room temperature for 40 min with metal ions. The milk-clotting activity obtained without metal ions was taken to be 100%.

5-5. Effect of Inhibitors

Different protease inhibitors, including a serine-protease inhibitor (phenylmethylsulfonyl fluoride (PMSF) at 5 mM and 10 mM), a metalloprotease inhibitor (ethylene-diaminetetraacetic acid (EDTA) at 5 mM and 10 mM), an aspartic protease inhibitor (pepstatin A at 10 μM and 20 μM) and a cysteine-protease inhibitor (iodoacetamide at 2 mM and 4 mM) were added separately to the purified enzyme. The mixture was incubated at room temperature for 30 min and the residual milk-clotting activity was tested. The milk-clotting activity obtained without the inhibitors was taken to be 100%.

5-6. Michaelis-Menten Constant Determination

Solutions of casein at concentrations in the range 0.2-2% in 20 mM potassium phosphate buffer (pH 6.5) were used as the substrate and the proteolytic activity was determined as described [12]. The Michaelis-Menten constant K_m was calculated from the double reciprocal Lineweaver-Burk plot [13].

5-7. *N*-terminal Amino Acid Sequence

After SDS-PAGE, each purified enzyme band was transferred onto a polyvinylidene fluoride (PVDF) membrane. The enzyme band on the PVDF membrane was cut out and the N-terminal sequences of the purified enzyme were determined by automated Edman degradation with an ABI PROCISE^{TM} 492cLC protein sequencer.

6. Statistical Analysis

All experiments were done in triplicate and the results are expressed as mean±standard deviation. The data were analyzed by one-way ANOVA using SPSS version 17.0 and the level of statistical significance was set at $P<0.05$.

RESULTS AND DISCUSSION

1. Purification and Molecular Mass

Because the purity of an enzyme can impact the product quality, the milk-clotting enzyme of *B. amyloliquefaciens* D4 was purified to electrophoretic homogeneity. Partial purification of the enzyme, 1.5-fold purification and 58.13% recovery was achieved by precipitation in 30-80% saturated ammonium sulfate. Passage through a DEAE-Sephadex A-25 column eluted with a gradient of NaCl further purified the enzyme to 17.2-fold with 20% recovery. The results of the purification procedure are summarized in Table 1. The purified enzyme has a molecular mass of 58.2 kDa as determined by SDS-PAGE (Fig. 1(a)), which is higher than those in the literature (34-49 kDa) for other microbial milk-clotting enzymes [1,14-17].

The elution pattern of the milk-clotting enzyme after ion-exchange chromatography (DEAE-Sephadex A-25 column, elution with a linear gradient of 0-0.5 M NaCl) is shown in Fig. 1(b). The enzyme was eluted at 0.14-0.19 M NaCl, and a brown pigment associated with the enzyme was removed in this step. The partially purified enzyme was separated into two peaks but with only one having milk-

Table 1. Purification steps of milk-clotting enzyme from *B. amyloliquefaciens* D4

Purification steps	Milk-clotting activity (SU)	Protein content (mg/mL)	Specific activity (SU/mg)	Purification fold	Recovery (%)
Crude enzyme	4645.16	0.349	13309.91	-	100
$(NH_4)_2SO_4$ Fractionation (30-80%)	18000	0.939	19169.33	1.5	58.13
DEAE-SephadexA-25	800	0.0035	228571.43	17.2	20

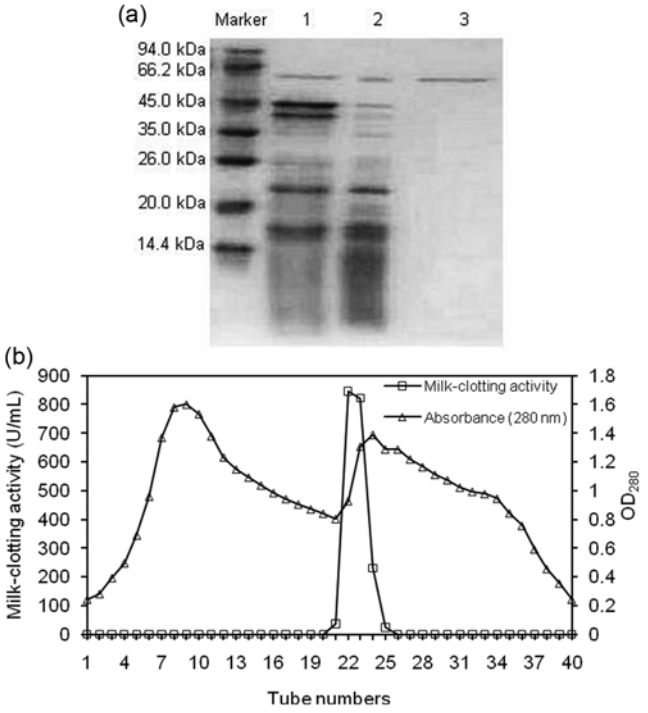

Fig. 1. Purification and molecular mass of the milk-clotting enzyme produced by *B. amyloliquefaciens* D4. (a) SDS-PAGE electrophoretogram of the *B. amyloliquefaciens* D4 milk-clotting enzyme after various purification steps. Marker lane, standard molecular mass markers; lane 1, crude milk-clotting enzyme; lane 2, milk-clotting enzyme purified by precipitation in $(NH_4)_2SO_4$; lane 3, milk-clotting enzyme purified by ion-exchange chromatography through DEAE-Sephadex A-25. (b) Ion-exchange chromatography through DEAE-Sephadex A-25 of the crude enzyme of *B. amyloliquefaciens* D4.

clotting activity. Earlier studies reported that the milk-clotting enzymes from different sources have different chromatographic/activity results. Our result was in accord with the milk-clotting enzyme from *Bacillus sphaericus* [18]. However, this result was different from the milk-clotting enzyme from *Mucor bacilliformis* [19], where the ion-exchange chromatography elution pattern showed two peaks of proteins but both fractions had milk-clotting activity.

2. Effects of Temperature and pH on Enzyme Activity and Stability

The milk-clotting activity increased with increased temperature in the temperature range 30-60 °C and the optimum temperature for the purified enzyme was 65 °C. (Fig. 2(a)). Different enzymes have different optimum temperatures, mainly depending on the enzymes' structure. This result was in accord with the milk-clotting enzyme from *Penicillium oxalicum* [20] and *Bacillus pumilus* TYO-67 [21], but different from calf rennet, which has an optimum temperature in the range 40-42 °C. This substantial difference in optimum temperature between the microbial milk-clotting enzyme and calf rennet suggests strongly that they are suitable for use under different conditions.

The maximum milk-clotting activity was at pH 5.5 for the purified enzyme, and the activity decreased with increasing pH (Fig. 2(b)). This result was similar to what is reported for the milk-clotting enzymes from *Mucor miehei* [22], *Rhizopus oryzae* [1] and glutinous rice wine mash liquor [23]. Like calf rennet, the purified enzyme from *B. amyloliquefaciens* D4 had a higher level of milk-clotting activity in the acidic range. This is attributed, in part, to the solubilization of the colloidal calcium phosphate (CCP) that is an integral part of casein micelles under acidic conditions.

The heat stability of the purified enzyme is shown in Fig. 2(c). It was fully active after 60 min of incubation at 35 °C but inactivated after 20 min at 55 °C. Compared with the purified enzyme from *B. sphaericus* [18], which completely lost its activity after 20 min at 70 °C, and *Mucor pusillus* [14] and *Rhizomucor miehei* [15], which lost their activity after 30 min at 65 °C, the *B. amyloliquefaciens* D4 enzyme has a lower level of thermostability. After the milk has been clotted, most of the milk-clotting enzyme that was added to the milk is included in the whey during cheese production [24]. A residue of rennet is unwanted because it will continue working and will hydrolyze the whey proteins. The low level of thermostability of the *B. amyloliquefaciens* D4 enzyme made it easy to inactivate, which allows the whey to be reused.

The stability of the purified enzyme at different pH values is shown in Fig. 2(d). The enzyme was stable in a relatively wide range of pH 5.5-7.0, with maximum stability at pH 6.5. Outside either end of this range, the activity of the enzyme decreased drastically, which showed that the *B. amyloliquefaciens* D4 enzyme was stable in the acidic to neutral range. The low pH sensitivity is very important for the coagulant because the use of highly pH-sensitive rennet can lead to reduced yields and defective cheese due to a soft coagulum at cutting [25]. The low pH-sensitivity of the *B. amyloliquefaciens* D4 enzyme is useful for cheese making.

3. Substrate Concentration and K_m

The effect of the concentration of skim milk on the activity of the purified enzyme is shown in Fig. 3. The milk-clotting activity reached a maximum when the skim milk concentration was increased to 50 g/L. Moreover, its K_m was 0.471 mg/mL when casein was the substrate. The K_m is the concentration of substrate required to produce 50% of the maximum velocity value [26]. Each enzyme has a characteristic K_m for a given substrate. The K_m value provides information about the affinity of the enzyme and substrate. A high K_m indicates a low affinity, and vice versa. The K_m value obtained in this study revealed that this milk-clotting enzyme has a lower affinity towards the casein substrate when compared with other mi-

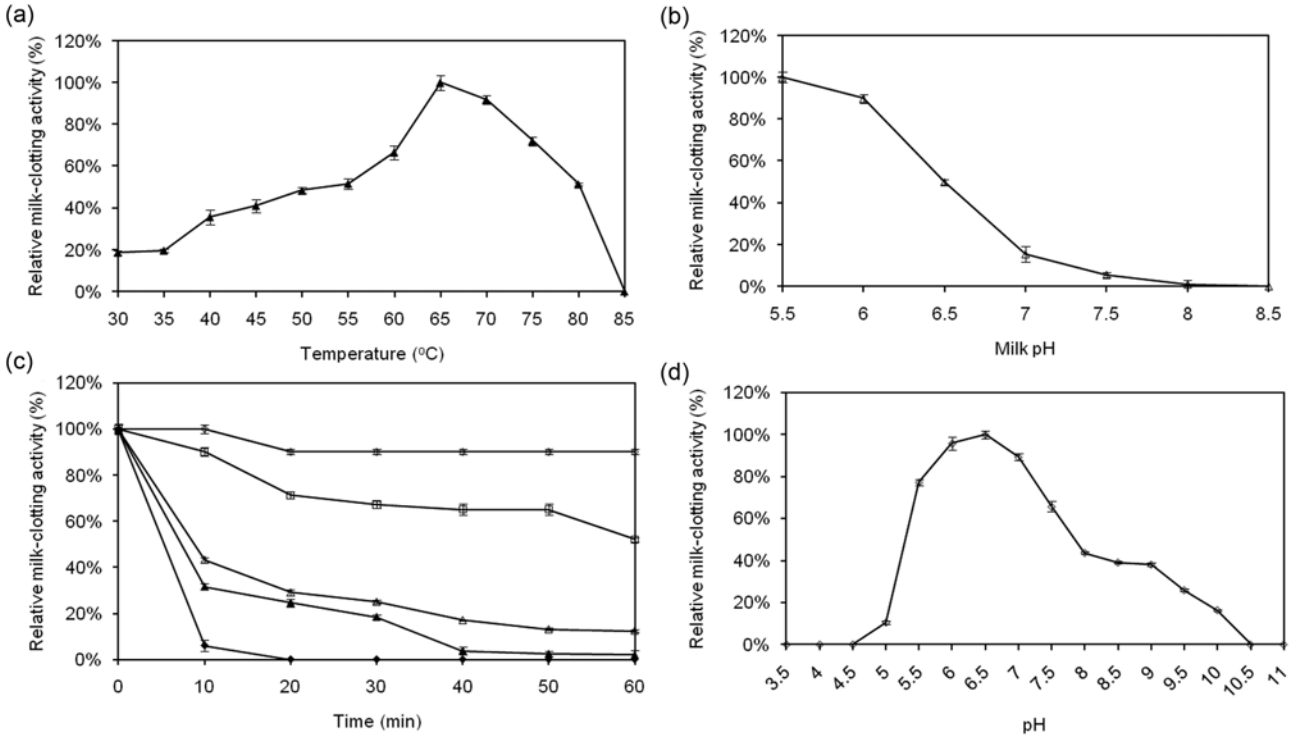

Fig. 2. Effects of temperature and pH on enzyme activity and stability. (a) Effect of temperature on the milk-clotting activity of the purified *B. amyloliquefaciens* D4 enzyme. Error bars represent the standard deviation of triplicate experiments. (c) Heat stability of the purified *B. amyloliquefaciens* D4 enzyme at 35 °C (◇), 40 °C (□), 45 °C (△), 50 °C (▲), and 55 °C (◆). Error bars represent the standard deviation of triplicate experiments. (b) Effect of milk pH on milk-clotting activity of the purified *B. amyloliquefaciens* D4 enzyme. Error bars represent the standard deviation of triplicate experiments. (d) pH stability of the purified *B. amyloliquefaciens* D4 enzyme. Error bars represent the standard deviation of triplicate experiments.

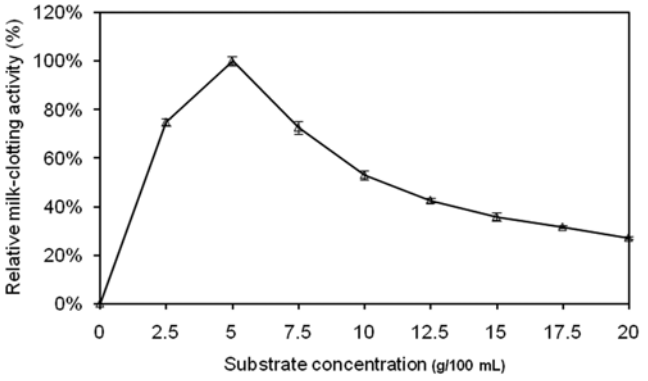

Fig. 3. Effect of substrate concentration on the milk-clotting activity of the purified *B. amyloliquefaciens* D4 enzyme. Error bars represent the standard deviation of triplicate experiments.

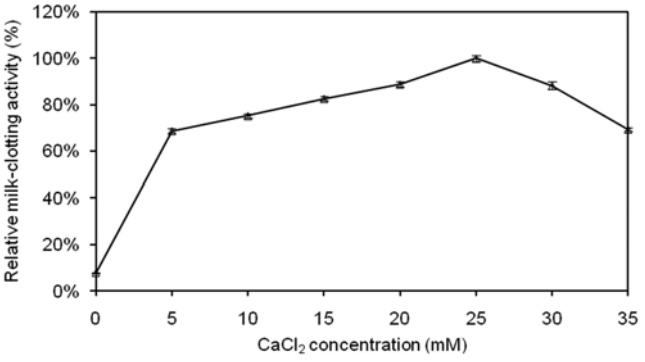

Fig. 4. Effect of $CaCl_2$ concentration on the milk-clotting activity of the purified *B. amyloliquefaciens* D4 enzyme. Error bars represent the standard deviation of triplicate experiments.

crobial milk-clotting enzymes, whose K_m values for casein were 0.388±0.002 g % [19] and 0.371±0.067 g % [27].

4. Effect of the Concentration of $CaCl_2$

Calcium had a positive effect on the activity of the milk-clotting enzyme. Calcium has been described as important in milk clot formation, when its concentration is high enough [28]. Fig. 4 shows that the milk-clotting activity was highest at 25 mM $CaCl_2$. In the range 0-20 mM $CaCl_2$, the coagulation rate increased with increasing concentration of Ca^{2+}. Milk-clotting activity decreased at concentrations of $CaCl_2$ higher than 25 mM. This positive effect of Ca^{2+} at lower concentrations has been attributed to the action of Ca^{2+} on the second step of coagulation caused by the neutralization of casein micelles' negative residues (phosphoserine and carboxylic groups) by Ca^{2+} and calcium-phosphate complexes [29]. The lower level of activity at higher concentrations of Ca^{2+} results from a progressive saturation of negative residues of the micelles with increasing concentration of Ca^{2+} in the medium [30].

5. Effect of Other Metal Ions

Fig. 5 shows that different metal ions had different effects on milk-

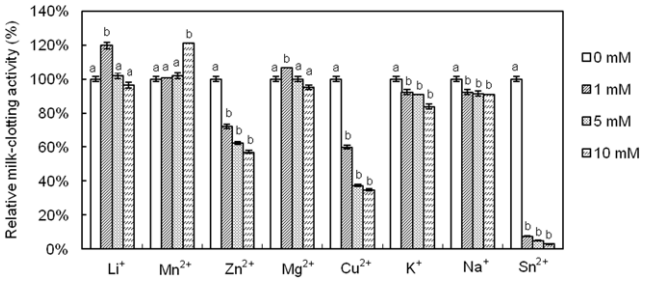

Fig. 5. Effect of metal ions on the milk-clotting activity of the purified *B. amyloliquefaciens* D4 enzyme. Error bars represent the standard deviation of triplicate experiments.

clotting activity. Na^+ and K^+ had only a slight inhibitory effect, whereas Cu^{2+}, Zn^{2+} and Sn^{2+} inhibited the milk-clotting activity significantly. In contrast, Mn^{2+} had a significant stimulatory effect on milk-clotting activity, whereas 1 mM Li^+ and 1 mM Mg^{2+} promoted the activity slightly, but had no effect when present at concentrations of only 5 mM or 10 mM. These results were similar to those reported by Wang et al. [23], who reported that Na^+ and K^+ inhibited the enzyme activity in glutinous rice wine mash liquor. In contrast, Na^+ and K^+ had no effect on the activity of milk-clotting enzyme from *Bacillus polymyxa* B-17 [31].

The addition of sodium chloride to cheese serves a number of purposes. Sodium chloride improves the flavor, texture and color of cheese, it kills off the starter cultures used in the cheese-making process, preventing further growth and acid development, and it helps to adjust the moisture content of cheese by forcing water out [32]. The lower level of sensitivity to sodium chloride of the *B. amyloliquefaciens* D4 enzyme makes it beneficial in cheese making.

6. Effect of Inhibitors

Protease inhibitors were used to identify the group at the active site of the enzyme (Table 2). Inhibition studies showed the sensitivity of the purified enzyme to a serine protease inhibitor (PMSF), a cysteine protease inhibitor (iodoacetamide), a metalloprotease inhibitor (EDTA) and an aspartic protease inhibitor (pepstatin A). The fact that PMSF, pepstatin A and iodoacetamide did not inhibit the enzyme activity showed that the purified *B. amyloliquefaciens* D4 enzyme was not a serine protease, aspartic protease or cysteine protease. The strong inhibition, 97.83% at 5 mM EDTA and 98.05% at 10 mM EDTA, showed that the enzyme belongs to the metallo-

Table 2. Effect of inhibitors on the milk-clotting activity of the purified enzyme from *B. amyloliquefaciens* D4

Inhibitor	Concentration (mM)	Residual activity (%)
Control	0	100±0.23
EDTA	5	2.17±0.22
	10	1.95±0.15
Iodoacetamide	2	95.76±0.93
	4	94.93±0.95
PMSF	5	98.24±0.91
	10	96.99±1.0
Pepstatin A	0.01	105.13±0.97
	0.02	94.38±0.93

	1	5	10	15
Bacillus amyloliquefaciens D4	V	N	G T L M Q Y F E W Y T P N	
Rhizomucor pusillus	V	N	N T L L G G D I Q Y T D V	
Rhizomucor miehei	V	N	N T I I G G D I A Y T D V	
Bacillus amyloliquefaciens	V	N	K A E Q I Y Y R A I T V Y	

Fig. 6. Comparison of the *N*-terminal amino acid sequences of the purified *B. amyloliquefaciens* D4 enzyme with those of several microbial proteases and calf chymosin.

protease group.

7. *N*-terminal Amino Acid Sequence

The first 15 amino acid residues of *N*-terminal sequence of the enzyme were VNGTLMQYFEWYTPN (Fig. 6). When compared to the National Center for Biotechnology Information (NCBI) protein database by BLAST search, the sequences of the purified enzyme did not show significant homology to calf chymosin. However, the sequences of the purified enzyme showed some homology with the sequences of milk-clotting aspartic proteinase (40%) from *Rhizomucor pusillus* [33], Mucor rennin (33.3%) from *Rhizomucor miehei* [34] and neutral protease (26.7%) from *Bacillus amyloliquefaciens* [35]. The differences in those basic amino acids among proteases may result from their differences in their primary structures.

CONCLUSIONS

In this study, the purification and properties of the milk-clotting enzyme from *B. amyloliquefaciens* D4 were investigated. The purified enzyme is a metalloprotease with a molecular mass of 58.2 kDa and it has high specific milk-clotting activity (228,571.43 SU/mg), low thermostability and low sensitivity to pH. Moreover, the medium used for enzyme production is wheat bran, which is cheap. These results suggest that *B. amyloliquefaciens* D4 is a possible commercial source of milk-clotting enzyme for cheese making. An evaluation of its potential for use in cheese-making will be the subject of future work.

ACKNOWLEDGEMENTS

The authors are grateful to the Agricultural Biotechnology Project of Gansu Province, China (GNSW-2006-14) and Beijing Science and Technology Project (D10110504600000) for financial support of this work.

REFERENCES

1. S. Kumar, N. S. Sharma, M. R. Saharan and R. Singh, *Process. Biochem*., **40**, 1701 (2005).
2. M. T. H. Cavalcanti, M. F. S. Teixeira, J. L. Lima Filho and A. L. F. Porto, *Bioresour. Technol*., **93**, 29 (2004).
3. S. Raposo and A. Domingos, *Process. Biochem*., **43**, 139 (2008).
4. J. M. Ageitos, J. A. Vallejo, A. B. F. Sestelo, M. Poza and T. G. Villa, *J. Appl. Microbiol*., **103**, 2205 (2007).
5. S. Neelakantan, A. K. Mohanty and J. K. Kaushik, *Curr. Sci*., **77**, 143 (1999).
6. J. D. Breccia, F. Siñeriz, M. D. Baigori, G. R. Castro and R. Hatti-

Kaul, *Enzyme Microb. Technol*., **22**, 42 (1998).
7. H. Chung and F. Friedberg, *Biochem. J*., **180**, 387 (1985).
8. N. P. Balaban, L. A. Malikova, A. M. Mardanova, G. N. Rudenskaya and M. R. Sharipova, *Biochemistry* (Moscow), **4**, 459 (2007).
9. Y.-J. Lee, B.-K. Kim, B.-H. Lee, K.-I. Jo, N.-K. Lee, C.-H. Chung, Y.-C. Lee and J.-W. Lee, *Bioresour. Technol*., **99**, 378 (2008).
10. U. Laemmli, *Nature*, **227**, 680 (1970).
11. M. M. Bradford, *Anal. Biochem*., **72**, 248 (1976).
12. K. Arima, J. Yu and S. Iwasaki, *Methods. Enzymol*., **19**, 446 (1970).
13. H. Lineweaver and D. Burk, *J. Am. Chem. Soc*., **56**, 658 (1934).
14. A. Nouani, N. Belhamiche, R. Slamani, S. Belbraouet, F. Fazouane and M. M. Bellal, *Int. J. Dairy. Technol*., 112 (2009).
15. S. Preetha and R. Boopathy, *World. J. Microbiol. Biotechnol*., **13**, 573 (1997).
16. J. L. Sardinas, *Appl. Microbiol*., **16**, 248 (1968).
17. A. G. Vishwanatha, Rao. Appu and S. A. Singh, *Appl. Microbiol. Biotechnol*., **1**, 1 (2009).
18. A. Magda, EI-Bendary, E. Maysa, H. Moharam and H. A. Thanaa, *J. Appl. Sci. Res*., **3**, 695 (2007).
19. L. B. Areces, M. B. D. J. Bonino, M. A. A. Parry, E. R. Fraile, H. M. Fernández and O. Cascone, *Appl. Biochem. Biotechnol*., **37**, 283 (1992).
20. A. M. Hashem, *Bioresour. Technol*., **75**, 219 (2000).
21. M. Yasuda, M. Aoyama, M. Sakaguchi, K. Nakachi and N. Kobamoto, *Appl. Microbiol. Biotechnol*., **51**, 474 (1999).
22. M. Z. Sternberg, *J. Dairy. Sci*., **54**, 159 (1971).
23. Y. Wang, Q. Cheng, Z. Ahmed, X. Jiang and X. Bai, *Korean. J. Chem. Eng*., **1**, 1 (2009).
24. M. J. Sousa, Y. Ardo and P. L. H. McSweeney, *Int. Dairy. J*., **11**, 327 (2001).
25. M. Harboe and P. Budtz, Technology of Cheesemaking. In: Law, Barry A. Ed., New York, Academic Press (1999).
26. H. S. Kim, M. D. Legoy and D. Thomas, *Korean J. Chem. Eng*., **1**, 35 (1989).
27. H. M. Fernandez-Lahore, R. M. Auday, E. R. Fraile, M. Biscoglio de Jimenez Bonino, L. Pirpignani, C. Machalinski and O. Cascone, *J. Peptide Res*., **53**, 599 (1999).
28. S. G. Anema, S. Kim Lee and H. Klostermeyer., LWT-*Food Sci. Technol*., **40**, 99 (2007).
29. C. Merheb-Dini, E. Gomes, M. Boscolo and R. da Silva, *Food Chem*., **120**, 87 (2010).
30. M. S. Pires, G. A. Orellana and C. A. Gatti, *Food Hydrocolloids*, **13**, 235 (1999).
31. H. Matta and V. Punj, *Int. J. Food Microbiol*., **42**, 139 (1998).
32. M. R. Payne and K. R. Morison, *Int. Dairy. J*., **9**, 887 (1999).
33. H. Yamazaki, Y. Ohnishi and S. Horinouchi. Published Only in Database of NCBI (1999).
34. G. L. Gray, K. Hayenga, D. Cullen, L. J. Wilson and S. Norton, *Gene*., **48**, 41 (1986).
35. H. Shimada, M. Honjo, I. Mita, A. Nakayama, A. Akaoka, K. Manabe and Y. Furutani, *J. Biotechnol*., **2**, 75 (1985).