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Purification and structural characterization of a novel milk-clotting enzyme from *Bacillus velezensis*

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This study isolated a high milk-clotting enzyme-producing strain DS-1 from Longnan Douchi, identified as *Bacillus velezensis* based on 16S rDNA. The enzyme was purified by ammonium sulfate precipitation and DEAE-Sephadex A-25 chromatography, yielding 56.1 kDa, 6.57-fold purification, and 68.57% recovery. It showed optimal activity at pH 5.5 and 60°C, stability at pH 5.5–9.5 and 35–45°C, and belonged to the aspartic protease family. Physicochemical property prediction indicated that the enzyme is an acid protease with an isoelectric point of 4.98 and an instability index of 36.93, suggesting good stability. Secondary structure prediction showed that the α-helix content was 31.75%, which is higher than that of milk-clotting enzymes from *Bos taurus*, *Camel*, and other sources. Sequence alignment revealed that the proportions of identical amino acid residues between this enzyme and those from *Bos taurus*, *Camel*, *Cryphonectria parasitica*, *Rhizomucor pusillus*, and *Bacillus subtilis* were all below 15%, indicating significant differences. This study provides a theoretical basis for the development and application of novel microbial milk-clotting enzymes.Structural prediction revealed a 422-amino-acid enzyme with more α-helices than β-sheets and a pocket-shaped active site.

Keywords: *Bacillus velezensis*, Milk-clotting enzyme, Purification, Structure prediction, Active site

INTRODUCTION

Rennet is a specialized protease that hydrolyzes specific peptide bonds in κ-casein, disrupting the stability of casein micelles and thereby leading to the formation of milk gel[1]. During cheese ripening, rennet continues to play a role, which is crucial for the development of cheese quality and flavor[2]. Traditional rennet is derived from the fourth stomach (abomasum) of calves or lambs; however, its limited availability cannot meet the growing demands of the cheese industry. Microorganisms offer advantages such as ease of cultivation, short growth cycles, and minimal constraints from climatic factors, making the production of rennet from microbial sources more cost-effective[3]. Consequently, the screening of excellent microorganisms for rennet production has remained a research hotspot. According to existing reports, microorganisms capable of producing rennet are widely distributed, though most are derived from dairy products, dairy processing environments, and soil[3-5]. Additionally, some studies have reported the isolation of microorganisms with good milk-clotting activity from traditional fermentation starters (Jiuqu)[6].

China has a wide variety of traditional soybean products, including douchi (fermented black soybeans), sufu (fermented tofu), soy sauce, and soybean paste, which harbor abundant microbial resources. Li *et al*.[7] isolated

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a strain of *Bacillus mojavensis* LY-06 from douchi that produces nattokinase, with an enzyme activity of 1434.64 U/mL. Yao *et al*.[8] isolated a fibrinolytic enzyme-producing strain, *Bacillus licheniformis* SFD-Y5, from douchi; after optimization, the enzyme activity reached 2434.45 ± 28.49 IU. Liu *et al*.[9] isolated a poly-γ-glutamic acid-producing strain, *Bacillus velezensis* CAU263, from douchi, which achieved a yield of 158.5 g/kg in shake flask cultivation. Bao *et al*.[10] screened eight protease-producing Bacillus strains from traditional soybean paste, among which strain BJ-15 exhibited a protease activity of 35.24 U/mL. Zhou *et al*.[11] isolated *Bacillus amyloliquefaciens* from douchi, which showed a protease activity of 185.6 U/mL. These studies indicate that traditional soybean products are rich in functional microorganisms.

Longnan Douchi is a traditionally fermented soybean product with regional characteristics from the Longnan area of Gansu Province, harboring various microorganisms including *Bacillus* species[12]. To date, studies on functional microorganisms in Longnan Douchi, particularly those producing rennet, have rarely been reported. This study aims to isolate and screen bacterial strains producing rennet from Longnan Douchi, and to carry out the purification and structural prediction of the rennet enzyme, thereby laying the foundation for the development of high-quality microbial rennet.

Materials and Methods

Materials

The culture media were as follows: Casein medium: peptone 2.5 g/L, glucose 10 g/L, yeast extract 1 g/L, casein 10 g/L, agar 20 g/L, skim milk 50 g/L, agar 20 g/L, pH 7.0. Slant medium: beef extract 3 g/L, peptone 10 g/L, NaCl 5 g/L, agar 20 g/L, pH 7.0. Seed medium: beef extract 3 g/L, peptone 10 g/L, NaCl 5 g/L, pH 7.0. Sterilization was performed at 121°C for 20 min. Fermentation medium for enzyme production: bran extract 18 g/100mL (18 g of bran boiled in 100 mL of tap water for 10 min, filtered, and then made up to 100 mL with tap water; natural pH), sucrose 40 g/L, skim milk powder 20 g/L, Na_2HPO_4 2 g/L, natural pH. Sterilization was carried out at 121°C for 20 min. DEAE-Sephadex A-25 was purchased from Beijing Ruida Henghui Technology Development Co., Ltd. Coomassie Brilliant Blue was purchased from Beijing Ruida Henghui Technology Development Co., Ltd. All other commonly used biochemical reagents were of analytical grade.

Screening and identification of rennet-producing strains

Douchi specimens obtained from Kang County (Longnan City, Gansu Province, China) were kept at 4 °C and immediately transported back to the laboratory. After sieving, 1 g of each specimen was suspended in 99 mL of sterile physiological saline, followed by serial dilution with sterile physiological saline to concentrations ranging from 10^{-3} to 10^{-7}. Transfer 1 mL of the diluted sample and spread it onto casein plate medium. Incubate at 37 °C for 48 h. Select colonies with a large ratio of the precipitation zone to the hydrolysis zone, further streak-purify them on plates, and after obtaining pure cultures, assign numbers to them and transfer to slant medium. Inoculate the screened strains into fermentation medium for enzyme production, and incubate at 37 °C with shaking at 170 r/min. After cultivation, measure the rennet activity. Suspend the bacterial cells in 10 μL of sterile water, denature at 99 °C for 10 min, centrifuge, and use the supernatant as the template. Amplify the 16S rDNA using universal primers and perform sequencing. Compare the obtained sequences with the nucleic acid sequence database in GenBank using BLAST at NCBI. After alignment using ClustalX 1.8, construct a phylogenetic tree using MEGA4.0 software[13])

Extraction and separation/purification of rennet

The screened strain was inoculated into seed culture medium and incubated at 37 °C with shaking at 170 rpm for 24 h to obtain liquid seed culture. The liquid seed was then inoculated into fermentation medium for enzyme production at an inoculum size of 4% (v/v) and incubated at 37 °C with shaking for 48 h. After fermentation, the culture broth was centrifuged at 8,000 r/min for 10 min to obtain the crude enzyme solution[14].

A precise volume of the crude enzyme solution was placed in an ice-water bath. While slowly stirring, finely ground and dried ammonium sulfate powder was gradually added to reach 30% saturation. After 30 min, the mixture was centrifuged at 12,000 × g and 4 °C for 10 min. A small amount of the supernatant was taken for enzyme activity and protein content assays, and the precipitate was collected for further use. A precise volume of the supernatant was taken, and the above procedure was repeated to achieve ammonium sulfate saturations of 40%, 50%, 60%, 70%, and 80%, respectively. The rennet activity of the supernatant at each saturation level was measured. The salt-precipitated crude enzyme solution was placed into a dialysis bag and dialyzed slowly against distilled water at 4 °C for 24 h. Complete removal of ammonium sulfate was checked using 1% $BaCl_2$ solution. Ion exchange chromatography was performed using DEAE-Sephadex A-25 anion exchanger packed in a 30 cm × 2.6 cm column. Linear gradient elution was applied with a sample load of 30 mL. Fractions with high enzyme activity were collected, desalted by dialysis against distilled water overnight at 4 °C, and then freeze-dried for further use[15]. Purity identification and molecular weight determination of the enzyme were conducted by SDS-PAGE[16].

Determination of the N-terminal 15-amino-acid sequence

After SDS-PAGE of the purified rennet, the resolved pure protein was transferred onto a PVDF (Polyvinylidene Fluoride) membrane using an electroblotting apparatus. The N-terminal amino acid sequence was then analyzed by Edman degradation, which was commissioned to Shanghai GeneCore Bio-Technologies Co., Ltd.[17].

Determination of rennet activity

According to the method of Arima *et al*.[18], 5 mL of 100 g/L skim milk (prepared with 0.01 mol/L $CaCl_2$ solution) was incubated at 35 °C for 5 min. Then, 0.5 mL of crude enzyme solution was added, and the mixture was rapidly blended. The time (in seconds) from enzyme addition to milk coagulation was accurately recorded. The amount of enzyme required to coagulate 1 mL of 100 g/L skim milk in 40 min was defined as one Soxhlet Unit (SU). The rennet activity was calculated using the following formula:

MCA (SU/mL) = (2400 / t) x 10

where, MCA (milk clotting activity) represents the rennet clotting activity, and t is the clotting time in seconds.

Primary structure analysis of rennet

The sequences of rennet from different sources—Bos taurus (NP_851337.1), Camelus dromedarius (CAC19554.1), *Cryphonectria parasitica* (pdb|4ER1|E Chain E), *Rhizomucor pusillus* (BAA76606.1), and *Bacillus subtilis* (WP_069149540.1)—were retrieved from the NCBI database and compared with rennet from *Bacillus velezensis*. Multiple sequence alignment of their amino acid sequences and primary structures was performed using the MUSCLE tool (https://npsa.lyon.inserm.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_muscle.html) on the NPS@ server.

Characterization of the milk-clotting enzyme

Effects of temperature on enzyme activity

The temperature dependence of the enzyme's activity was evaluated by measuring its milk-clotting ability across a range of 30–75 °C, at 5 °C intervals.

Effects of temperature on enzyme stability

The thermal stability of the enzyme was assessed by incubating it at various temperatures (35, 45, 55, and 65 °C) for durations ranging from 0 to 60 min. After each incubation, the remaining milk-clotting activity was determined, and the value obtained without any incubation (0 min) was set as the reference (100%).

Effects of pH on enzyme activity

We determined the enzyme's optimal pH by assaying milk-clotting activity at pH 5.5–8.5. The skim milk substrate was adjusted to each pH using 0.1 M HCl or 0.1 M NaOH. The maximum activity recorded was set to 100%.

Effects of pH on enzyme stability

pH stability was evaluated by incubating the enzyme with a 1:1 (v/v) ratio in a series of 0.1 M buffers, including 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). After 24 h of incubation at room temperature, the remaining milk-clotting activity was measured. The maximum activity observed under these conditions was taken as the reference (100%).

Effect of inhibitors

The effect of various protease inhibitors on enzyme activity was examined by separately supplementing the purified enzyme with a serine-protease inhibitor (10 mM PMSF), a metalloprotease inhibitor (10 mM EDTA), an aspartic protease inhibitor (20 μM pepstatin A), and a cysteine-protease inhibitor (2 mM iodoacetamide). Following a 30 min incubation at room temperature, the remaining milk-clotting activity was assessed. Enzyme activity measured without any inhibitor served as the reference (100%).

Prediction of physicochemical properties of rennet

The physicochemical properties of the enzyme, including isoelectric point (pI), net charge, and water solubility, were determined using the peptide property calculation tool from Innovagen AB (https://pepcalc.com)[19]. The instability index of the enzyme was predicted using the ProtParam tool on Expasy (https://web.expasy.org/cgi-bin/protparam/protparam)[20]. The proportion of hydrophobic amino acids in the enzyme was determined using the peptide hydrophobicity analysis tool from Peptide 2.0 Inc. (https://www.peptide2.com).

Prediction of the secondary structure of rennet

The secondary structure of rennet from *Bacillus velezensis* was predicted using PRABINPS@: SOPMA (https://npsa.lyon.inserm.fr/) and compared with the secondary structures of rennet from Bos taurus (NP_851337.1), Camelus dromedarius (CAC19554.1), *Cryphonectria parasitica* (pdb|4ER1|E Chain E), *Rhizomucor pusillus* (BAA76606.1), and *Bacillus subtilis* (WP_069149540.1)[21].

Prediction of the tertiary structure of rennet

The tertiary structure and B-factor of the enzyme were predicted using the I-TASSER On-line Server (http://zhanglab.ccmb.med.umich.edu/I-TASSER)[22]. The active site of the enzyme was predicted using the COACH server in conjunction with the predicted tertiary structure, with TM-score and Cov serving as confidence evaluation metrics[23,31].

Results and Discussion

Isolation, screening, and identification of rennet-producing strains

The bacterial diversity in traditional fermented soybean products was analyzed, and preliminary isolation of rennet-producing microorganisms was conducted. Fig. 1a shows the colonies and milk-clotting zones of the strains on casein plates. It can be observed that the microorganisms formed distinct milk-clotting zones on the casein plates, indicating their potential for rennet production. Table 1 presents the enzyme production of the preliminarily screened rennet-producing microorganisms from douchi samples. Among them, strain DS-1 exhibited the highest rennet activity, reaching 1983.8 SU/mL after 48 h of cultivation in bran extract medium. The 16S rDNA sequence of the strain was amplified by PCR and sequenced, with a length of 1401 bp. It was consistent with the 16S rDNA fragment size of *Bacillus velezensis* BMB205 within the genus *Bacillus*, showing 99.99% similarity, indicating that this strain is a strain of *Bacillus velezensis*, designated as *Bacillus velezensis* DS-1. Fig. 1b shows the phylogenetic tree of strain DS-1. This strain is evolutionarily close to *Bacillus velezensis* ZB42 and *Bacillus velezensis* BMB205.

Extraction and separation/purification of rennet from *Bacillus velezensis*

Since enzyme purity affects product quality, the rennet from *Bacillus velezensis* DS-1 was purified using ammonium sulfate precipitation and DEAE-Sephadex A-25 column chromatography[24,25]. The results of the purification process are summarized in Table 2. The enzyme was partially purified by 30–80% saturated ammonium sulfate precipitation, achieving a purification fold of 1.15 and a recovery yield of 95.05%. Further purification was carried out by DEAE-Sephadex A-25 column chromatography with NaCl gradient elution, resulting in a purification fold of 6.57 and a recovery yield of 68.57%. Fig. 2b shows the elution profile of rennet after ion exchange chromatography (DEAE-Sephadex A-25 column eluted with a linear gradient of 0–0.5 M NaCl). As shown in the figure, the rennet activity was mainly concentrated in fractions 22–25, while almost no activity was detected in the other fractions. The molecular weight of the enzyme was determined to be 55.1 kDa by SDS-PAGE (Fig. 2a). Which is close to milk-clotting enzymes from *B. amyloliquefaciens* D4[26] and higher than others (34–49 kDa)[27-30].

Sequence identification of the milk-clotting protease from *Bacillus velezensis*

The N-terminal 15-amino-acid sequence of the rennet protease from *Bacillus velezensis* was determined to be MTAVNQTISKVVNGK. After BLAST alignment in the NCBI database, the amino acid sequence of the enzyme is shown in Fig. 3 and Table 3. The enzyme consists of a total of 422 amino acid residues, among which hydrophobic amino acids account for 41.23%, acidic amino acids for 15.64%, basic amino acids for 13.03%, and neutral amino acids for 30.09%. Multiple sequence alignment using the MUSCLE tool revealed that the amino acid sequence of this enzyme differs considerably from those of the other five rennets. The proportion of different amino acid residues exceeded 60%, while the proportions of identical and similar amino acid residues were below 15% and below 14%, respectively.

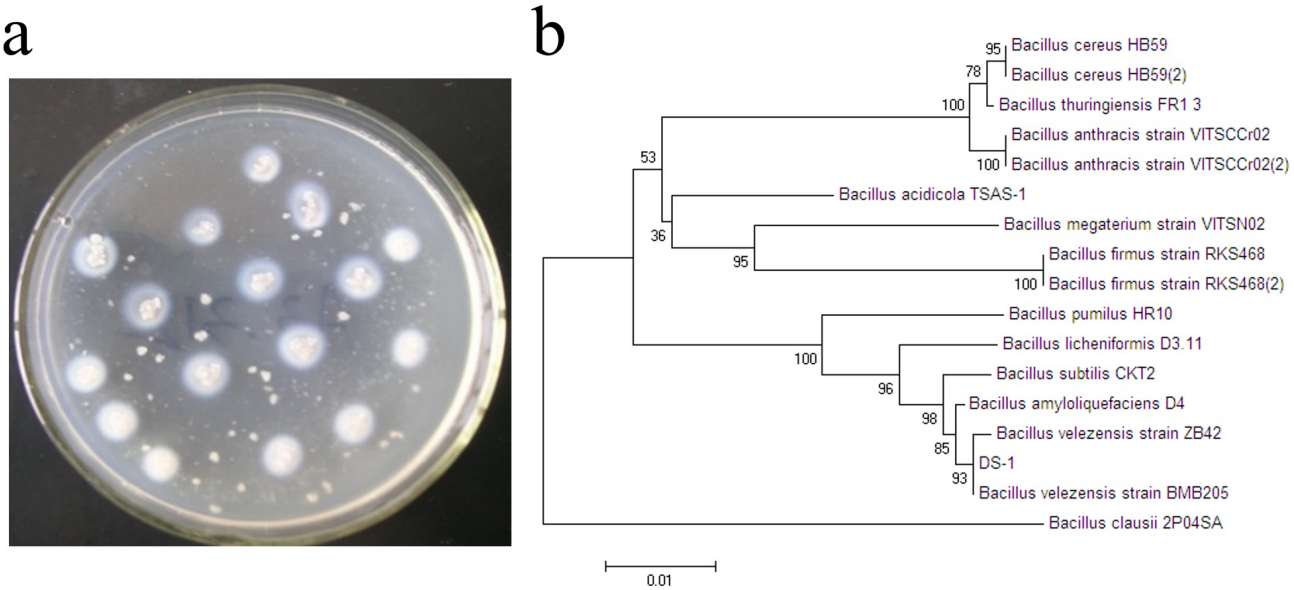

Figure 1. Colonies and milk-clotting circles of strains on casein plates (a) and phylogenetic tree of strain DS-1(b)

Table 1. Colony diameter, precipitation zone diameter, and milk-clotting activity of different strains on casein plates

Strain number	Colony diameter (mm)	Precipitation zone diameter (mm)	Milk-clotting activity (SU/mL)
DS-1	2.0±0.1	11.0±0.2	1983.6±3.0
DS-2	3.1±0.2	8.3±0.1	21.1±4.2
DS-3	3.5±0.2	7.3±0.2	11.3±1.9
DS-4	2.6±0.2	7.8±0.2	26.4±3.1
DS-5	3.3±0.2	7.5±0.2	16.1±0.6
DS-6	5.2±0.2	8.5±0.1	81.2±2.1
DS-8	2.5±0.2	9.0±0.2	111.6±5.0
DS-9	3.1±0.1	7.2±0.2	126.7±4.2
HB-1	3.6±0.1	8.1±0.1	18.3±1.9
HB-2	2.2±0.2	4.5±0.1	22.4±3.5
HB-3	3.3±0.1	7.5±0.2	16.5±0.9

Table 2. Purification procedure of milk-clotting protease from strain DS-1

Purified step	Milk-clotting activity (U)	Total protein/ mg	Specific activity/ $U.mg^{-1}$	Purification fold	Recovery rate %
Fermentation broth	95981.8	981.3	97.8	1	100.00
$(NH_4)_2SO_4$	91235.3	809.2	112.7	1.15	95.05
DEAE-SephadexA-25	65815.5	102.3	643.3	6.57	68.57

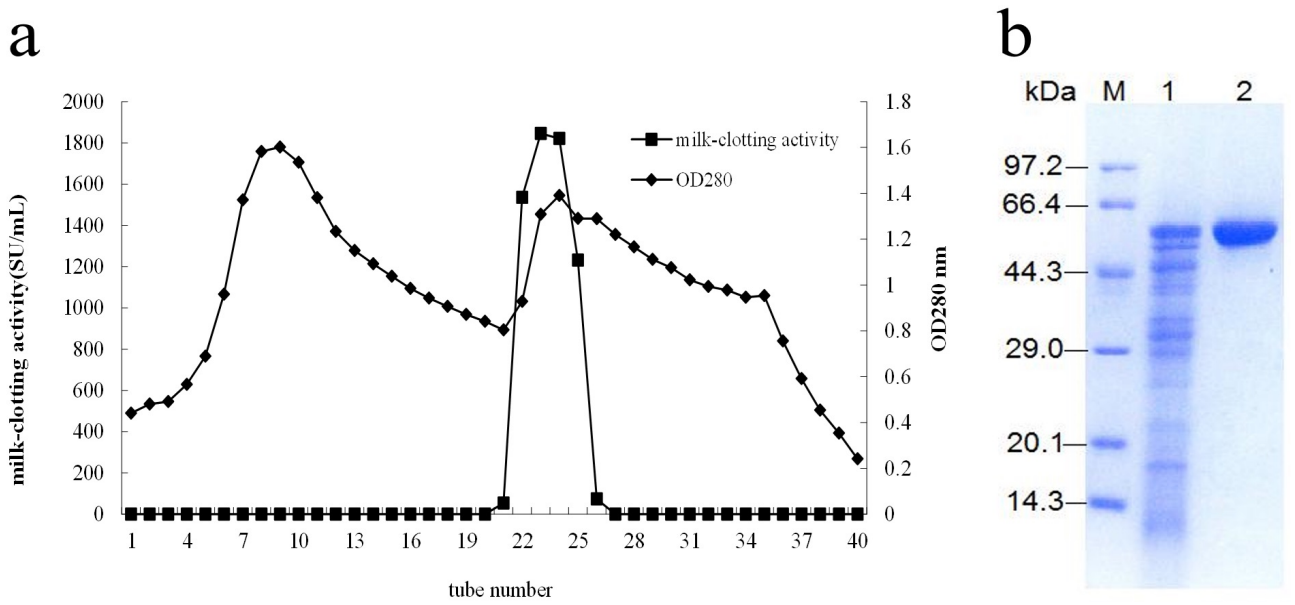

Figure 2. Column chromatography purification (a) and SDS-PAGE of the milk-clotting enzyme (b)

Characterization of the DS-1 enzyme

pH is an important parameter affecting both milk-clotting enzyme activity and the degree of cheese ripening (Bansal *et al*., 2025). The optimal pH of DS-1 enzyme is 5.5, and it exhibits good stability within the pH range of 5.5–9.5. The optimal temperature is 60 °C, and it shows favorable thermal stability between 35 °C and 45 °C (with residual activities of 75%–52% after 60 min), but its thermal stability decreases significantly above 55 °C (retaining only 36% activity after 10 min) (Fig. 4). The pH characteristics of this enzyme are highly compatible with the cheese ripening environment (pH 5.0–6.0), and its temperature properties are suitable for the medium-temperature processing commonly used in cheese production. In summary, DS-1

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        10         20         30         40         50         60
MTAVNQTISK VVNGKRVITK KPELLAPAGN LEKLKIAVHY GADAVFIGGQ EYGLRSNADN

        70         80         90        100        110        120
FSIEEIAEGV EFAKKYGAKI YVTTNIFAHN ENMDGLEEYL KALGDAKVAG IIVADPLIIE

       130        140        150        160        170        180
TCRRVAPDVE IHLSTQQSLS NWKAVQFWKE EGLDRVVLAR ETSGLEIKEM KEKVDIEIET

       190        200        210        220        230        240
FIHGAMCIAY SGRCVLSNHM TARDSNRGGC CQSCRWDYDL YQTDGANAVA LYDEEDAPFA

       250        260        270        280        290        300
MSPKDLKLIE SIPQMIEMGI DSLKIEGRMK SIHYVATVVS VYRKVIDAYC ADPENFVIQK

       310        320        330        340        350        360
EWLDELNKCA NRDTAPAFFE GTPGYEEQMF GEHGKKTTFD FAGLVLAYNE DTQMVTLQQR

       370        380        390        400        410        420
NFFKQGDEVE FFGPEIDNFT FTIGTIWDED GNELDAARHP LQIVTFKVDK KIYPSNMMRK

GK
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Figure 3. The amino acid sequence of milk-clotting protease from *Bacillus velezensis*

Table 3. Amino acid sequence alignment of milk-clotting protease from *Bacillus velezensis* with other enzymes

Sources of rennet	Identical %	Strongly similar %	Weakly similar %	Different %	Proportion of hydrophobic amino acids/%
Bos taurus	13.40	12.04	9.13	65.44	39.90
Camelus dromedarius	12.84	13.45	8.58	65.11	40.16
Cryphonectria parasitica	12.21	12.21	11.35	64.24	38.48
Rhizomucor pusillus	14.15	12.62	10.52	62.72	39.34
Bacillus subtilis	14.26	12.11	7.79	65.84	36.28

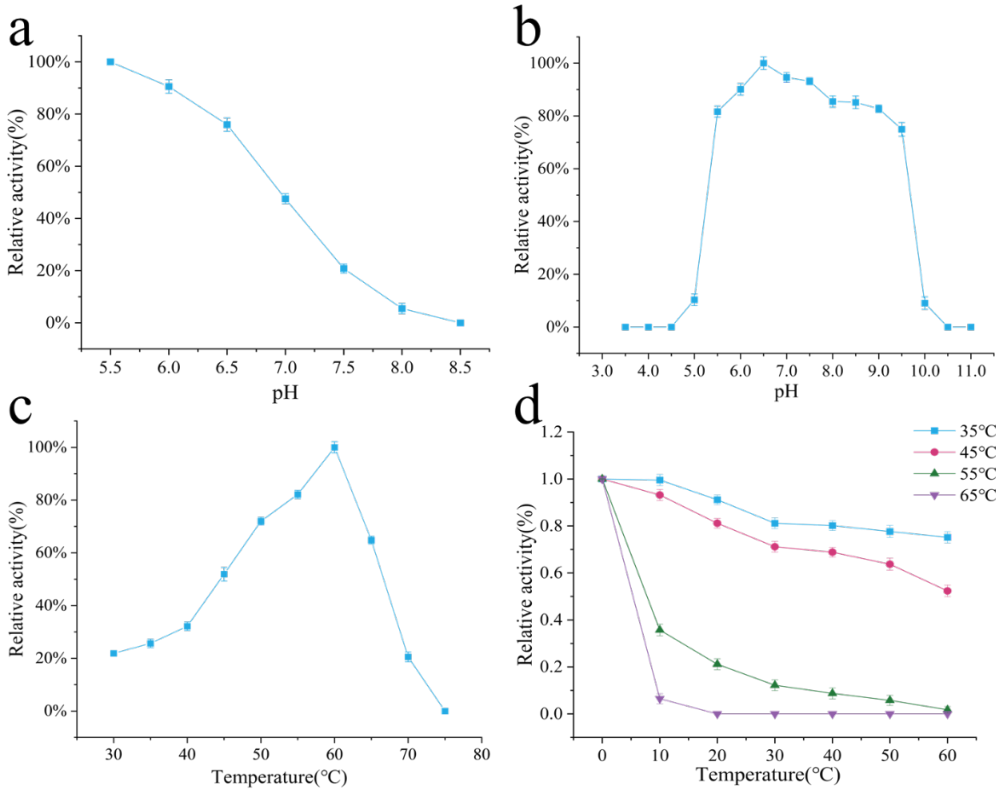

Figure 4. Enzymatic properties of milk-clotting enzyme produced by strain DS-1. (a) Influence of different pH on enzyme activity. (b) pH stability of milk-clotting enzyme. (c) Effect of temperature on milk-clotting activity. (d)Thermo-stability of milk-clotting enzyme

is an acidic, medium-temperature protease with a strong enzymatic foundation for application in cheese ripening.

Structural study of the rennet protease from *Bacillus velezensis*

Physicochemical properties of the rennet protease from *Bacillus velezensis*

The physicochemical properties of the rennet protease from *Bacillus velezensis* and several proteases with similar sequences were predicted, and the results are shown in Table 4. The molecular weight of the *B. velezensis* rennet protease was relatively smaller than that of the other proteases listed in the table. However, its instability index was below 40, indicating that the enzyme is stable. The net charge, predicted under neutral pH conditions, represents the overall surface electrostatic charge of the rennet. All values were negative. The net charge of the *B. velezensis* rennet protease was very close to that of Bos taurus chymosin but much lower than that of Camelus dromedarius rennet. The number of negatively charged residues differed only slightly among the three enzymes, whereas the *B. velezensis* rennet protease had fewer positively charged residues than Camelus dromedarius rennet. The isoelectric point (pI) of this enzyme was similar to those of the two known rennets listed in the table. All pI values of the tested enzymes were below 7, indicating that they are all acidic proteases.

The secondary structure of a protein refers to the local spatial arrangement and conformation of the main-chain atoms in the polypeptide chain, primarily including α-helices, β-sheets, and random coils. Among these, α-helices play a major role in stabilizing the secondary structure. The secondary structure of the rennet from *Bacillus velezensis* was predicted using its complete amino acid sequence. The results showed that the proportion of α-helices was higher than that of β-sheets (Fig. 5). Moreover, the proportion of α-helices was also higher than that of the other five rennets from different sources, indicating that the rennet protease from *Bacillus velezensis* may possess a more stable secondary structure (Table 5).

Tertiary structure of the rennet protease from *Bacillus velezensis*

The tertiary structure of the rennet protease from *Bacillus velezensis* is shown in Fig 6a, where different colors represent different structural elements. The prediction model yielded a C-score of -2.50, an estimated TM-score of 0.42 ± 0.14, and an estimated RMSD of 13.0 ± 4.2 Å. This structure differs considerably from that of Bos taurus chymosin[32], which may be attributed to species differences, and also suggests that this is a novel rennet protease. Using the COACH server, seven amino acid residues in the active site of the *Bacillus velezensis* rennet protease were predicted: 172 E, 173 K, 251 S, 252 I, 253 P, 254 Q, and 256 I (Fig. 6b). The prediction results showed a TM-score of 0.503 and a Cov value of 0.678. As observed from the predicted tertiary structure, the active site of the enzyme is located in the lower left part, with a small number of α-helices and some random coils on the outer layer. Some amino acid residues, such as 173 K and 254 Q, are located on the molecular surface, facilitating substrate entry and binding. Fig. 6c reveals that the active site resembles a pocket. Two amino acid residues (172 E and 173 K) are located on one side of the pocket, while five residues (251 S, 252 I, 253 P, 254 Q, and 256 I) are located on the opposite side, with the pocket in the middle. The substrate can enter the pocket and undergo catalytic reactions.

Conclusions

In this study, *Bacillus velezensis* DS-1, screened from Longnan Douchi, exhibited high rennet-producing capacity. After purification, the specific activity of the enzyme reached 643.3 SU/mg. The rennet is an acid protease with a molecular weight of 55.1 kDa and good structural stability. The purified enzyme exhibits distinct enzymatic properties, including an optimal pH of 5.5 and optimal temperature of 60 °C, with broad pH stability and moderate thermal stability. Its α-helix content is higher than that of several known rennets, suggesting a relatively stable spatial conformation. The tertiary structure model revealed that the active center of the enzyme adopts a

Table 4. Comparison of physicochemical properties of milk-clotting protease from strain DS-1 and other similar proteases

Sources of rennet	Number of residues	iso-Electric point	Net charge at pH 7	Instability index	Water solubility	Properties
Bacillus velezensis	422	4.98	-18.7	36.93	Good	Acidic
Bos taurus	381	4.97	-10.9	37.77	Poor	Acidic
Camelus dromedarius	381	5.90	-3.9	35.10	Poor	Neutral
Cryphonectria parasitica	330	4.14	-14	23.21	Poor	Acidic
Rhizomucor pusillus	427	4.48	-19	31.65	Poor	Acidic
Bacillus subtilis	521	6.39	-2	29.56	Good	Neutral

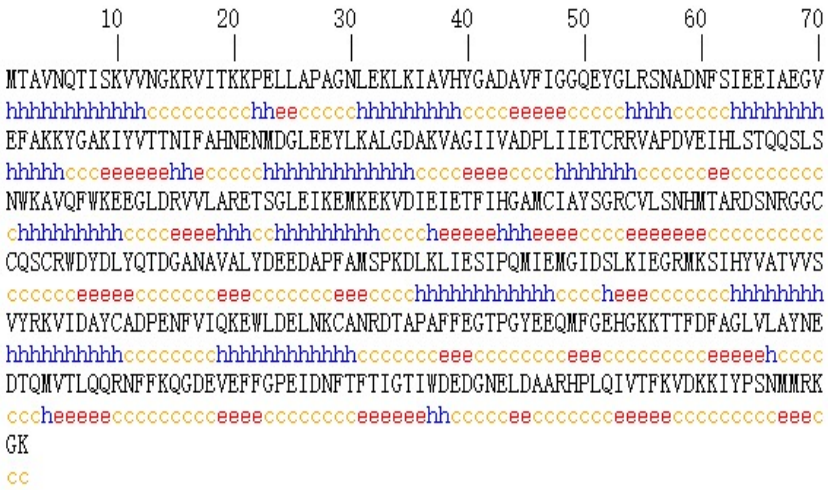

Figure 5. The secondary structure prediction of the milk-clotting protease from *Bacillus velezensis*

Table 5. The secondary structure prediction of the milk-clotting protease from strain DS-1

Origin	Alpha helix	Extended strand	Random coil
Bacillus velezensis	31.75%	21.33%	46.92%
Bos taurus	18.56%	27.84%	53.60%
Camelus dromedarius	24.81%	22.18%	53.01%
Cryphonectria parasitica	10.31%	32.42%	57.27%
Rhizomucor pusillus	12.18%	26.46%	61.36%
Bacillus subtilis	30.90%	18.43%	50.67%

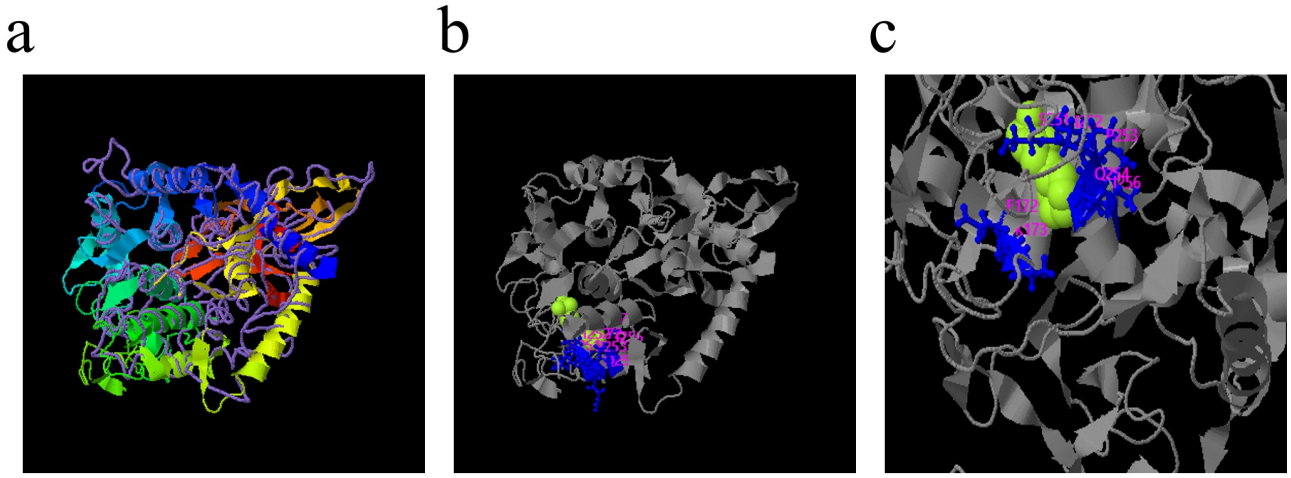

Figure 6. The prediction of tertiary structure, active sites and normalized B-factor of milk-clotting protease from *Bacillus velezensis*. (a) Tertiary structure of the rennet protease from *Bacillus velezensis*; (b) Active site of the rennet protease from *Bacillus velezensis*; (c) Enlarged view of the active site of the rennet protease from *Bacillus velezensis*. Pink represents α-helices, yellow represents β-sheets, blue-and-white indicates random coils, and purple represents the active site

typical pocket-like conformation, with seven key amino acid residues collectively forming the catalytic site. Comparative analyses of sequences and structures with rennets from various sources imply that this enzyme is evolutionarily relatively independent, potentially indicating a unique rennet variant based on computational predictions. These findings lay an important foundation for further elucidating the interaction mechanism between this enzyme and κ-casein, conducting enzyme molecular modification, and evaluating its application potential in cheese processing.

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CRediT authorship contribution statement

Wendi Yang: Conceptualization, Methodology, Data curation, Visualization, Writing – original draft. Yafeng Zhang: Formal analysis, Supervision, Writing – review & editing, Validation. Weibing Zhang, Haijun Qiao: Project administration, Funding acquisition, Writing – review & editing. Zhongming Zhang: Funding acquisition, Project administration, Resources, Writing – review & editing.

Declaration of competing interest

All authors ensure the absence of known conflicts of interest or personal relationships that could bias the research work presented in this paper.

Data availability

Data are available within the article.

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