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Screening, purification, and characterization of proteinase from 3 *Lactobacillus delbrueckii* subsp. *bulgaricus*

Running title: Screening, purification, and characterization of LAB proteinase

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Abstract

Three strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* were selected for the proteinases property in order to improve milk gel firmness. Respective proteinases were purified by ultra-filtration, anion exchange, and hydrophobic interaction chromatographies. The 3 purified proteinases were determined to have molecular masses of about 39, 40, and 52 kDa. Optimal activities of the purified enzymes occurred at pH 6.0 and 40 °C. They are metallopeptidase, activated by Fe^{2+}, inhibited by Ba^{2+}, Zn^{2+}, Mn^{2+}, Xi^{2+}, Fe^{3+}, Cu^{2+}, and EDTA, and serine proteinase which are inhibited by PMSF.

Keywords Proteinase, purification, characterization, *Lactobacillus delbrueckii* subsp. *bulgaricus*, yogurt quality.

Introduction

In the past few decades, many studies have been performed on yogurt in order to improve its texture properties. The preliminary research work on yogurt has been concentrated on the technological character of lactic acid bacteria (LAB) and mainly directed at screening strains. However, little work has been done to achieve deep understanding of the underlying mechanism of gel formation by which fine yogurt texture might be produced. As yogurt is a complex system with so many interactions involved during gel formation, the properties of gels strongly depend on the interaction forces between milk proteins. The nature (strength, type, number, and duration or relaxation) of these interactions forming the gel network is highly dependent on the properties of the LAB culture. Yet, there is still much to be done about the physicochemical basis of the textural properties of yogurt gels plus the nature of the culture strains involved.

LAB proteinases are known to contribute positively to the texture formation of fermented dairy products [1, 2]. The proteolytic activity of LAB might exert an effect on the formation and stability of

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milk protein gels [3]. It has been reported that enzymatic hydrolysis of casein gave yogurts with different firmness, viscosity, degree of syneresis [4, 5], and the supplementation of the proteolytic strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* reduced the fermentation time in making and improved firmness [6]. Rheological research on milk gel demonstrated that limited proteolysis could lower the gel point and improve gelation [7-9]. The proteolytic activity of LAB has been correlated to their high acidification rates since proteinase negative mutants did not produce such high levels of acid [1, 10].

The cell-envelope proteinase is a key enzyme in the proteolytic system of LAB, as it initially degrades casein for the rapid growth of LAB in milk. In the past few years, cell-envelope proteinase from several LAB strains has been purified and characterized. Such strains included *Lactobacillus casei* subsp. *casei* IFPL731 [11], *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ397 [12], *Lactococcus lactis* subsp. *lactis* [13], and *Lactobacillus casei* DI-1 [14].

In our study, 12 isolates from Chinese fermented milk were chosen for the analysis of LAB proteinase and its contribution to yogurt texture quality. Amongst, 3 LAB strains were selected for further proteinase's purification and characterization. The purified proteinases of the latter 3 LAB selections are now under further research for the mechanism on how they improve yogurt quality.

Materials and methods

Microorganisms and propagation conditions

A total of 12 isolates were selected among the isolates from traditional Chinese fermented dairy products. These isolates were routinely propagated in Man-Rogosa-Sharpe (MRS) broth and kept frozen (at -80°C) in the same broth with 20% (v/v) glycerol. These isolated strains were preserved in the laboratory of Food Science and Engineering at Harbin Institute of Technology (HIT). The strains

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were subcultured twice successively in 12% reconstituted skim milk (Nestlé, China) at 37°C for 18 h before use.

Preparation of LAB proteinase

The strains were grown in MRS broth at 37 °C for 20 h, and the cells were harvested by centrifugation at 4000 g for 10 min at 4°C and washed 3 times with 50 mmol/L Tris-HCl buffer (pH 7.0). The washed cells were suspended in the same buffer containing 1 mg/mL lysozyme and incubated for 3 h at 37°C. Cell debrises were removed by centrifugation at 10,000 g for 15 min at 4°C. The clear supernatant was passed through the filter paper and a 0.22 μm filter membrane to remove contaminant bacteria. The filtered liquid was directly concentrated by ultra-filtration with a Millipore Lab-scale TFF System (Millipore, MA, USA) with a 10 kDa cut-off and then was freeze-dried to obtain crude enzyme powder.

Measurement of proteinase activity

The assay for proteolytic activity was carried out as described by Christen [15]. It involved a combination of 0.3 mL of azocasein (Sigma-Aldrich, MO, USA) solution and 0.3 mL of enzyme solution (dissolved in 50 mmol/L phosphate buffer, pH 7.0). The contents of the tubes were mixed and incubated at 37 °C for 1 h. Respective reactions were stopped by the addition of 0.6 mL of 12% (w/v) trichloroacetic acid (TCA). The absorbance of the supernatant was read at 345 nm. Protein concentrations were estimated by the method using the Coomassie protein assay reagent [16] with bovine serum albumin as a standard.

Gel formation

Skim milk was reconstituted by dissolving low-heat skim milk powder (12%, w/w) in distilled water

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while gently stirring. To prevent the bacterial growth, 0.02% of sodium azide was added to 12 % reconstituted skim milk. Crude enzyme powder (with 20 % enzyme activity of the initial fermentation broth) was added prior to acidification with 1.75% glucono-δ-lactone (GDL). After the addition of GDL, the milk was gently stirred for 2 min and then incubated at 30 °C for 6 h when the final pH reached 4.5. Thereafter, the samples of the gel were transferred to a refrigerator and maintained at 4°C prior to texture analysis.

Texture analysis

The texture analyses of the gel samples were carried out after 24 h of refrigeration (at 4°C) by performing a penetration test with a Texture Analyzer (TA-TX2i, Stable Micro Systems, UK) equipped with a 40 mm cylindrical probe. The test speed was fixed at 1 mm/s, and the penetration depth was 20 mm. Force-time curves of the gel samples were obtained while operating at 4°C. Both, the maximum force values (gel firmness) and cohesiveness were calculated from the generated curves.

Identification of the LAB

All of the selected LAB strains were identified by sequencing of 16S rRNA gene and sugar fermentation reactions using the API 50 CHL system.

Purification of proteinase

The filtered liquid from the 0.22 μm filter membrane was directly concentrated by ultra-filtration with a Millipore Lab-scale TFF System (Millipore, MA, USA) with a 10 kDa cut-off. The concentrated crude enzyme of 20 mL were loaded on a DEAE 52 chromatography column (1.6×10 cm; GE, NJ,

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USA) previously equilibrated with 50 mmol/L of Tris-HCl (pH 7.0). The column was washed extensively with the same buffer and eluted with a linear gradient of 0 to 1.0 mol/L of NaCl in the same buffer at a flow rate of 3.0 mL/min. Fractions of 5 mL were collected and assayed for proteinase activity. The proteinase solution after DEAE chromatography was loaded onto a Hitrap Butyl FF column (1 mL; GE, NJ, USA) equilibrated with 1.0 mol/L $(NH_4)_2SO_4$ in 50 mmol/L phosphate buffer (pH 7.0) as a starting eluent. Non-interacting proteins were also eluted with the same starting eluent, and the column was eluted with a linear gradient of 1.0 to 0.0 mol/L of $(NH_4)_2SO_4$ by 50 mmol/L phosphate buffer (pH7.0, eluting buffer) at a flow rate of 1.0 mL/min. Fractions with proteinase activity were pooled, and then the active fractions were filtered at 4 °C in a bag filter with a 10 kDa cut-off. The purification system used was an ÄKTA purifier 100, equipped with a P-900 series pump, UV-900 monitor, pH/C-900 monitor, M-925 mixer, a complete set of motor valves, Frac-950 fraction collector, A-900 auto sampler, and AD-900 analog/digital converter connecting a 10A refractive index detector (GE, NJ, USA). All the steps were performed at 4°C, unless otherwise stated.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out for the determination of purity and the molecular mass of the enzyme as described by Laemmli [17]. A 5% acrylamide stacking gel and 12% acrylamide running gel were used. Proteins in the gels were stained with Coomassie Blue R250. The molecular mass of the enzyme was marked against a calibration kit of its molecular mass.

Characterization of the enzymes

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The effect of temperature (20, 30, 37, 42, 50 and 60°C) on the enzyme activity was measured in 50 mmol/L Tris-HCl buffer (pH 7.0) with azocasein as the substrate. To assess the thermal stability of the enzyme, the purified enzyme solutions were incubated for 1 h at temperatures ranging from 20°C to 60°C. The residual activity was then subsequently measured after incubation at 37 °C for 1 h with azocasein as the substrate.

The pH optimum was determined at a fixed assay temperature of 37°C at various pH ranging from 5.0 to 8.0 using 50 mmol/L citrate-Tris-borate buffer. The pH stability of the purified proteinase was conducted in citrate-phosphate-borate buffer (pH 4.0-9.0) at 37°C for 30 min. The pH of the reaction mixture was adjusted with 50 mM citrate buffer (pH 3.7-6.0), 50 mM Tris-HCl buffer (pH 7.0-9.0), and 50 mM borate buffer (pH 10.0).

The enzymes were pre-incubated in the presence of various metals (Na^{+}, Ba^{2+}, $K^{+,}$ Ca^{2+}, Mn^{2+}, Mg^{2+}, Li^{2+}, Fe^{2+}, Zn^{2+} or Cu^{2+}), phenylmethylsulphonyl fluoride (PMSF) and ethylene diamine tetraacetic acid (EDTA) in 50 mmol/L Tris-HCl buffer (pH 7.0) at a final concentration of 1.0 and 10.0 mmol/L, respectively. The enzymatic activity was measured after incubation at 37°C for 1 h with azocasein as the substrate.

Statistical analysis

All trials were carried out in triplicate and repeated 3 times. The experimental data are presented as the mean±SD. One-way ANOVA was applied to the results of technological properties, using Duncan's test for comparison of the means (P<0.05). The SPSS software package (version 16.0, SPSS, IL) was employed for the statistical analysis.

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Results and discussion

Screening and identification for LAB with proteinase that improves GDL gel quality

Texture analysis is a valid way to determine the quality of milk curd. While the enzyme properties of the strains were investigated by GDL gel texture analysis, 12 strains were selected according to the texture analysis. As shown in Table 1, the gel firmness was increased by the crude proteinase of the LAB strains. The results confirmed that the cell-envelope proteinase were involved in the development of texture characteristics of the milk gel as the report of [18]. The differences in increasing gel firmness between the crude proteinase of the selected strains are clearly demonstrated in Table 1. Proteinase of *Lactobacillus* SB5, SB25, CH9-3 4.0, and SH3 4.3 appeared to have a positive effect on gel quality. Interestingly, SB25 was eliminated for its instability between the passages.

The 3 LAB strains (SB5, CH9-3 4.0, and SH3 4.3) were identified as *Lactobacillus delbrueckii* by sequencing of 16S rDNA gene and the accession numbers of these strains in GenBank were provided in our previous work [19] . Moreover, based on sugar fermentation reactions using the API 50 CHL system, they were identified as *Lactobacillus delbrueckii* subsp. *bulgaricus*.

Purification of proteinase

The cell-free extract was concentrated to 20% of the original volume by ultra-filtration with a 10 kDa cut-off, and there were many kinds of protein with a large variety of molecular weight in the crude enzyme (Fig. 3). The DEAE-52 chromatograph led to the separation of two major fractions and the first fraction showed the highest enzyme activity (Fig. 1). Anion exchange chromatography (DEAE-52) was used first to exclude the impurities, and proteinase was extracted (Fig. 1). During hydrophobic interaction

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(Hitrap Butyl FF) chromatography, proteinase activity was detected as a symmetrical peak (Fig. 2). The enzyme obtained from the final Hitrap Butyl FF chromatographic step showed a protein band through an SDS-PAGE as shown in Fig. 4. The specific enzyme activity observed with azocasein as the substrate after each purification step is summarized in Table 2. As shown in Table 2, the enzymes from *Lactobacillus delbrueckii* subsp. *bulgaricus* CH9-3 4.0, SH3 4.3 and SB5 were purified about 43, 33, and 32-fold with specific activities of 54.4, 80.2, and 61.1 U/mg from the cell-free extract by ultra-filtration and the two different column chromatography steps. The recovery of proteinase activity was about 46.6%, 39.3%, and 40.4%, respectively. The purified enzymes were homogenous on SDS-PAGE and their molecular masses were estimated to be 39, 40, and 52 kDa, in that order. In this study, the proteinases from the cell-free extract was purified, and the enzyme could be released from the cell wall by digestion with lysozyme. It was suggested that the release of the enzyme from the cell wall by lysozyme treatment may be a result of changes in the interaction between the enzyme and those cell wall components in close association with it.

Temperature effects on the enzyme activity and its stability

The enzyme from *Lactobacillus delbrueckii* subsp. *bulgaricus* (CH9-4 4.0, SH3 4.3, and SB5) showed high activity at temperatures ranging from 37 °C to 50 °C, with the maximum activity at 42 °C (Fig. 5), and retained 31.5%, 12.6% and 10.1% of their activity when they were pre-incubated for 30 min at 60 °C, respectively (Fig. 6). These data indicated that the enzymes from the three selected strains were more stable when heated with the substrate, and the optimum temperature of the proteinases were consistent with the optimum growth temperature of the *Lactobacillus delbrueckii*. The proteinase that has been purified from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ397 [12] presented the

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maximum activity at 42 °C. Proteinase purified from *Lactococcus lactis* subsp. *lactis* [13] exhibited the maximum activity at 40°C, and proteinase obtained from *Lactobacillus casei* DI-1 [14] had a maximum activity at 37°C. All of these results indicated that the proteinases from different sources have the maximum activity at different temperatures.

Effects of pH on the proteinase activity and its stability

The enzyme showed high activity in the pH range between 5.5 and 7.0 with optimum activity at pH 6.0 (Fig. 7). Less than 70 % of the maximum activity was measured at acidic pH of 5.0 and alkalinic pH of 8.0, respectively. These data indicated that the enzymes from the three strains have been more stable when incubated for 30 min over the pH range 5-8 (Fig. 8). These data indicated that the optimum pH of the enzymes from the three selected strains were consistent with the optimum growth pH of the *Lactobacillus delbrueckii*. Our optimum pH conditions were similar to the proteinase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ397 [12]. However, the optimum pH were apparently different from some other proteinases obtained from other LAB, such as *Lactococcus lactis* ssp. *lactis* [13] or *Lactobacillus helveticus* L89 [20], the optimum pH of which were in the range from 7.0 to 7.5.

Effects of metal ions and inhibitors on enzyme activity

The effects of various compounds on the enzyme activity are summarized in Table 3. Interestingly, the proteinase from the three selections was not significantly influenced by Na^{+}, K^{+}, Li^{2+}, Ca^{2+}, and Mg^{2+} at a concentration of 1 mmol/L and 10 mmol/L. The enzymes were strongly activated by Fe^{2+} and strongly inhibited by Ba^{2+}, Zn^{2+}, Mn^{2+}, Xi^{2+}, Fe^{3+}, and PMSF. Most activity was lost in the presence of Cu^{2+} and EDTA, which indicated that the proteinases were somehow metallopeptidase. Because their

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activity were inhibited by the serine proteinase inhibitor PMSF, the proteinases were hypothesized to be serine proteinase. Under the same conditions, our findings were different from those of Guo [13], whom reported that the proteinase from *Lactococcus lactis* ssp. *lactis* was strongly inhibited by Ni^{2+}, EDTA, and PMSF, while being activated by Mn^{2+}, Mg^{2+}, and Ca^{2+}.

Conclusions

In the present study, proteinase produced by three strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* (CH9-4 4.0, SH3 4.3, and SB5) were purified and characterized. The purification to homogeneity of the enzyme was achieved by ultra-filtration, ion exchange chromatography (on DEAE-52), and hydrophobic interaction chromatography (on Hitrap Butyl FF column). When the final purification step ended, the enzymes from the selections were purified 43, 33, and 32-folds with specific activities of 54.4, 80.2, and 61.1 U/mg and 46.6%, 39.3%, and 40.4% recovery. The purified enzymes were homogenous on SDS-PAGE and their molecular masses were estimated to be 39, 40, and 52 kDa, in that order. The enzymes showed optimum temperature at 42°C and optimum pH of 6.0. The high proteolytic activity of proteinases was achieved at the pH values from 5.5 to 7.0 and at moderate temperature which all suggested specific good stability. The purified proteinases are now under further investigation to explore the mechanism of improving yogurt quality during curding.

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Table 1. Curd with 20 % crude enzyme rheology evaluated using the TA-XT2i Texture Analyser

Strains	Firmness (g)	Consistency (g·sec)	Cohesiveness (g)	Index of Viscosity (g·sec)
control	125.8±3.6^{a}	2098.1±78.6^{abc}	-65.8±3.0^{abc}	-84.0±2.6^{abc}
SB3	146.6±16.7^{def}	2316.0±301.3^{def}	-72.8±8.9^{e}	-85.8±10.4^{bcd}
SB5	167.2±12.1^{ef}	2712.0±115.1^{ef}	-73.6±6.9^{cde}	-97.8±6.2^{cd}
SB7	150.8±5.1^{bcde}	2466.8±78.9^{cde}	-79.0±2.3^{de}	-102.4±5.6^{d}
SB25	164.0±5.2^{def}	2681.5±67.4^{ef}	-79.7±2.7^{de}	-105.7±4.7^{d}
CH9-3 4.0	160.0±2.1^{cdef}	2543.1±17.6^{def}	-68.3±0.4^{bcd}	-91.8±1.1^{bcd}
SH3 4.3	178.3±5.0^{f}	2900.8±67.0^{f}	-81.6±2.1^{e}	-103.7±4.6^{d}
3 4.5	125.5±3.6^{a}	1999.1±2.3^{a}	-72.7±0.9^{bcde}	-86.4±1.3^{abc}
SP1.1	141.9±11.9^{abcd}	2395.6±209.2^{bcde}	-61.9±6.1^{abc}	-84.7±7.4^{abc}
MOIA	129.4±2.0^{ab}	2081.5±8.5^{ab}	-54.3±2.2^{a}	-74.2±1.5^{a}
MYC	148.5±13.8^{bcde}	2361.8±177.5^{abcde}	-65.0±4.8^{abc}	-84.8±7.3^{abc}
LBH	139.7±5.0^{abc}	2239.2±36.6^{abcd}	-60.7±0.6^{ab}	-82.8±1.9^{ab}
Q10L	149.8±12.6^{bcde}	2452.9±228.4^{bcde}	-68.1±8.0^{bcd}	-88.3±7.7^{abc}

$^{a-g}$ Means values (mean±SD) are significantly different from each other ($P<0.05$) with different letters in the same column.

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Table 2. Summary of the purification of LAB proteinase

	Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
CH9-3 4.0	Cell-free extract	8.10	10.30	1.27	100.00	1.00
	ultra-filtration	0.77	10.20	13.25	99.03	10.43
	DEAE-52	0.24	7.50	30.93	72.82	24.35
	Butyl FF	0.09	4.80	54.39	46.60	42.82
SH3 4.3	Cell-free extract	4.40	10.70	2.46	100.00	1.00
	ultra-filtration	0.32	10.60	38.10	99.07	15.49
	DEAE-52	0.17	9.40	54.48	87.85	22.15
	Butyl FF	0.05	4.20	80.18	39.25	32.59
SB5	Cell-free extract	7.10	13.60	1.91	100.00	1.00
	ultra-filtration	0.65	13.50	21.46	99.26	11.24
	DEAE-52	0.23	12.10	52.63	88.97	27.55
	Butyl FF	0.09	5.50	61.11	40.44	31.94

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Table 3. Effect of the metal ions plus inhibitors on enzyme activity

Compound	CH9-3 4.0 Relative activity (%)		SH3 4.3 Relative activity (%)		SB5 Relative activity (%)	
	1 mmol/L	10 mmol/L	1 mmol/L	10 mmol/L	1 mmol/L	10 mmol/L
None	100.0±2.8^{ab}	100.0±2.8^{ab}	100.0±2.0^{abc}	100.0±2.0^{a}	100.0±1.0^{ab}	100.0±1.1^{ab}
Na^{+}	103.6±2.3^{b}	109.6±2.3^{b}	105.8±0.5^{bc}	108.7±0.5^{b}	100.3±2.2^{ab}	102.5±0.9^{b}
K^{+}	104.3±5.0^{b}	105.3±0.9^{ab}	105.5±1.4^{bc}	108.3±1.1^{b}	100.6±2.6^{ab}	104.4±2.6^{b}
Li^{2+}	98.0±1.9^{ab}	101.7±0.5^{a}	106.4±1.4^{c}	108.3±1.0^{b}	97.8±1.3^{a}	102.2±2.2^{b}
Mg^{2+}	99.7±0.4^{ab}	101.4±3.6^{a}	105.1±1.4^{bc}	108.0±1.4^{b}	99.7±1.3^{ab}	96. 9±0.9^{a}
Ca^{2+}	100.9±0.4^{ab}	99.7±2.0^{a}	98.7±0.2^{abd}	78.2±1.0^{d}	100.6±1.8^{ab}	87.6± 0.9^{d}
Ba^{2+}	96.3±3.7^{d}	65.8±1.9^{e}	103.5±1.4^{bcd}	75.3±6.7^{de}	103.4±1.3^{b}	87.6± 2.6^{d}
Fe^{2+}	100.8±0.5^{ab}	124.4±3.2^{c}	96.5±0.5^{ad}	167.7±6.4^{c}	93.17±1.8^{c}	130.4±2.6^{c}
Fe^{3+}	81.8±3.2^{d}	13.5±0.6^{h}	93.6±1.8^{d}	0.09±0.6^{i}	86.6± 1.3^{d}	3.4±0.4^{j}
Zn^{2+}	80.5±3.3^{d}	67.8±0.8^{e}	63.5±2.7^{f}	32.4±0.5^{f}	61.2± 1.4^{e}	37.3±0.9^{f}
Mn^{2+}	86.2±0.8^{cd}	54.3±2.0^{f}	105.8±1.8^{cd}	68.9±1.4^{e}	99.1±2.2^{ab}	73.0±1.3^{e}
Xi^{2+}	54.0±1.6^{e}	17.8±2.4^{h}	96.5±2.3^{ad}	15.7±2.3^{h}	90.7±2.6^{cd}	18.9±0.4^{i}
Cu^{2+}	27.7±0.7^{f}	15.5±0.9^{g}	39.4±0.5^{f}	23.7±1.8^{g}	33.2±1.3^{f}	13.4± 0.5^{h}
EDTA	28.4±1.3^{f}	15.4±2.1^{h}	30.1±3.6^{h}	26.3±1.8^{fg}	29.5±0.4^{f}	28.3± 0.6^{g}
PMSF	89.2±2.6^{c}	78.0±4.6^{d}	86.0±5.9^{e}	76.0±5.8^{de}	86.5±5.2^{d}	76.5±5.1^{e}

Means values (mean±SD) are significantly different from each other (P<0.05) with different letters in the same column.

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Figure captions

Fig. 1 Proteinase of screened strains separated by DEAE Cellulose DE-52 chromatography

a-c: Elution curves of DEAE-chromatography for proteinase from strains CH9-3 4.0, SH3 4.3 and SB5

↓ The arrow indicates the active fraction containing proteinase activity

Fig. 2 Proteinase of screened strains separated by HiTrap Butyl FF chromatography

a-c: Elution curves of HIC chromatography for proteinase from strains CH9-3 4.0, SH3 4.3 and SB5

↓ The arrow indicates the active fraction containing proteinase activity

Fig. 3 SDS–PAGE of proteinase after ultrafiltration

M, standard marker; Lane CH9-3 4.0; Lane 2, SH3 4.3; Lane 3, SB5

Fig. 4 SDS–PAGE of proteinase during purification steps

M, standard marker; Lane 1-3, proteinase of CH9-3 4.0, SH3 4.3 and SB5 after DEAE chromatography; Lane 4-6, proteinase of CH9-3 4.0, SH3 4.3 and SB5 after HIC chromatography

Fig. 5 Effect of temperature on activity of purified proteinase

Fig. 6 Residual activity of purified proteinase after 30 min incubation at temperatures ranging from 20 to 90°C

Fig. 7 Effect of pH on the activity of purified proteinase

Fig. 8 Residual activity of purified proteinase after 30 min incubation at pH ranging from 3.0 to 10.0

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Fig.1

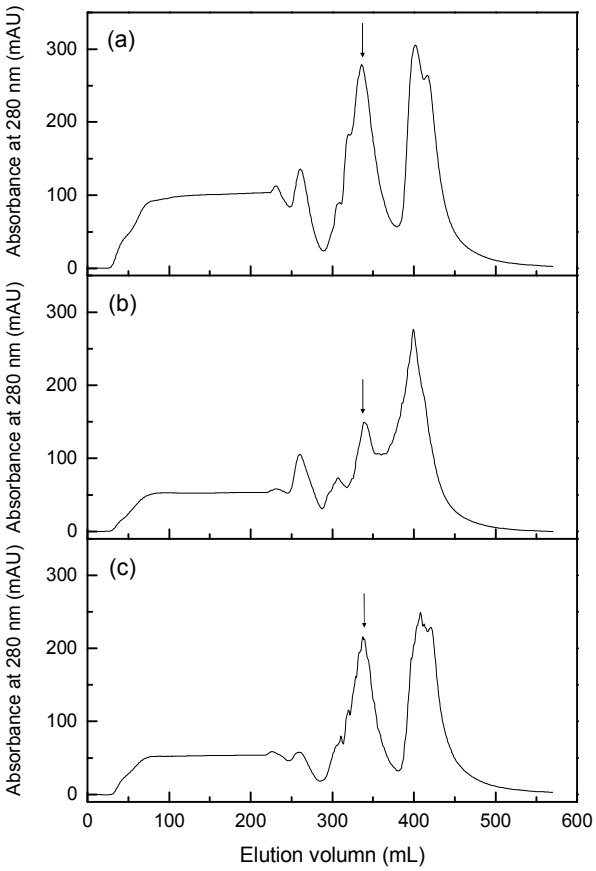

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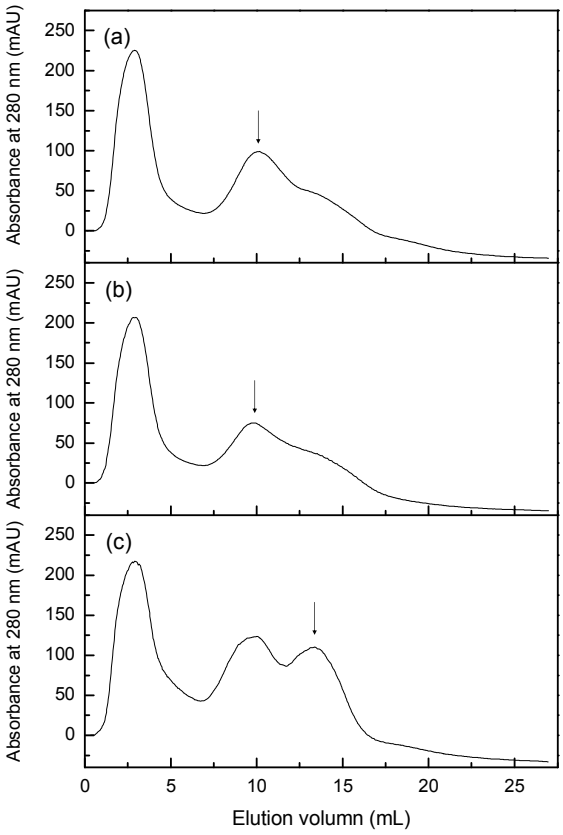
Fig. 2



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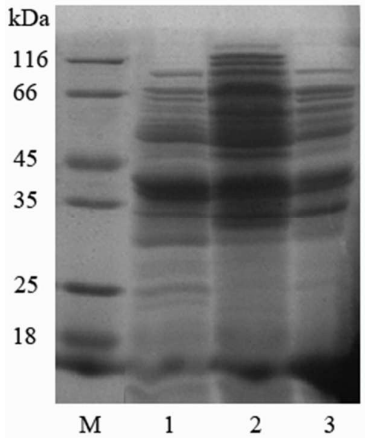

Fig. 3

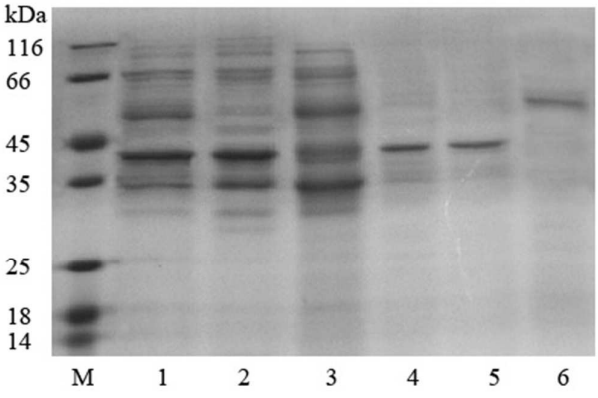

Fig. 4

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Fig. 5

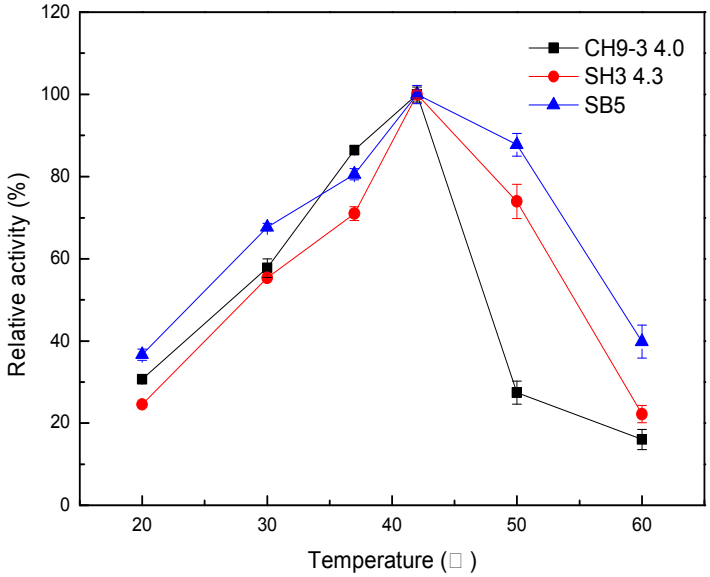

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Fig. 6

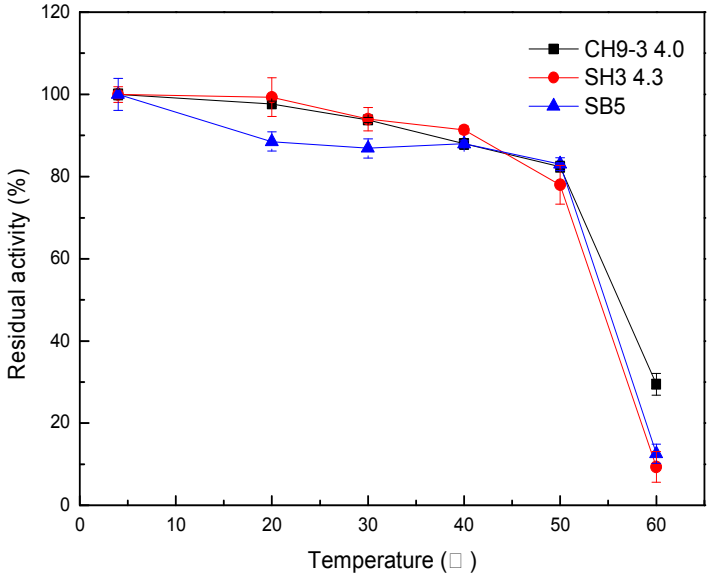

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Fig. 7

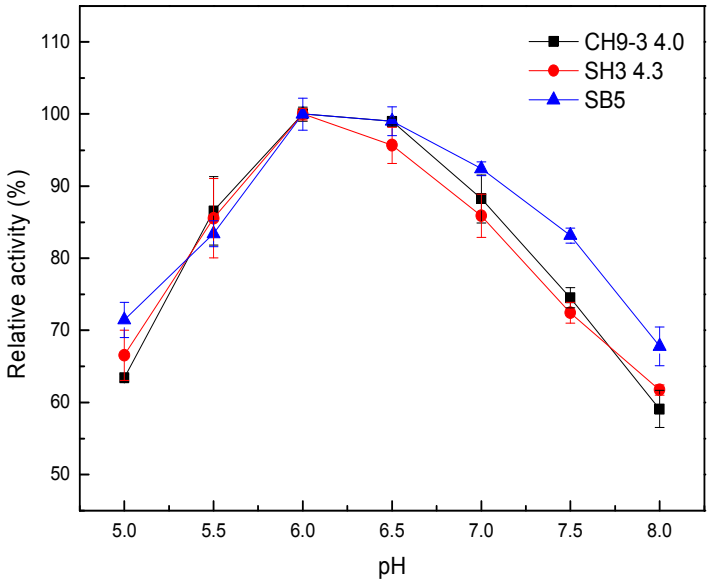

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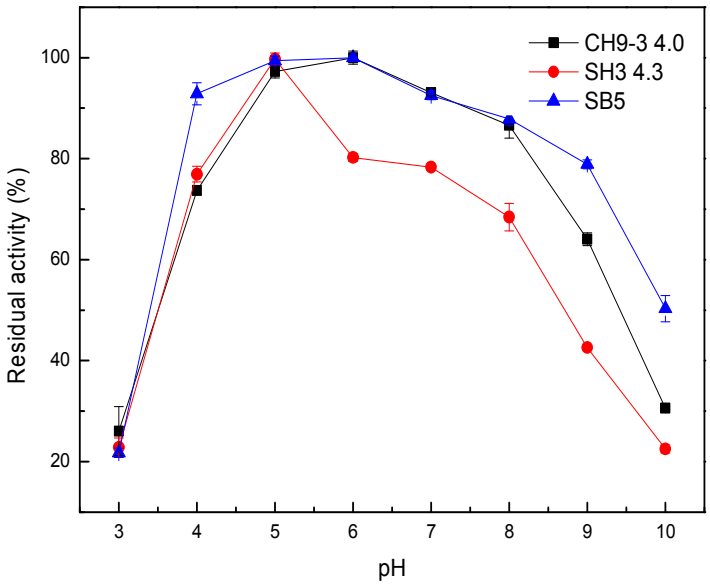
Fig. 8



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