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Abstract An ammonium sulfate precipitation of fermentation broth produced by *Bacillus subtilis* FBL-1 resulted in 2.9-fold increase of specific protease activity. An eluted protein

fraction from the column chromatographies using DEAE-Cellulose and Sephadex G-75 had 94.2- and 94.9-fold higher specific protease activity, respectively. An SDS-PAGE revealed a band of purified protease at approximately 37.6 kDa. Although purified protease showed the highest activity at 45°C and pH 9.0, the activity remained stable in temperature range from 30°C to 50°C and pH range from 7.0 to 9.0. Protease activity was activated by metal ions such as Ca^{2+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Ca^{2+} and K^{+}, but 10 mM Fe^{3+} significantly inhibited enzyme activity (53%). Protease activity was inhibited by 2 mM EDTA as a metalloprotease inhibitor, but it showed good stability against surfactants and organic solvents. The preferred substrates for protease activity were found to be casein (100%) and soybean flour (71.6%).

Keywords: protease; *Bacillus*; metalloprotease; organic solvent; purification

1. Introduction

Proteases (E.C.3.4.21-24) catalyze the cleavage of peptide bond in protein molecules resulting in smaller fragments such as peptides and/or amino acids. They are distributed broadly in nature and a wide variety of microorganisms. Proteases are usually divided into

two groups, exopeptidases or endopeptidases, depending on their site of hydrolysis. Exopeptidases break the peptide bonds formed between the end amino acid and the rest of peptide chain, but endopeptidases hydrolyze the peptide bonds found within the polypeptide or protein. There was an attempt to classify proteases based on structural features of enzyme active center, rather than origin, specificity, or physiological action [1]. Proteases are then divided into four classes based on the type of functional group present at the active site and their mechanism of action: 1) serine protease, 2) aspartic protease, 3) cysteine/thiol protease, and 4) metalloprotease [1, 2].

Proteases are extensively used in a variety of industries, including detergent, leather, pharmaceuticals, food, textile, bakery, soy-processing, peptide synthesis, and X-ray film. The estimated value of worldwide sales of enzymes has been over 3 billion U.S. dollars, and the market for proteases accounts for approximately 60% of the total worldwide sale of enzymes [3-6]. Proteases have been isolated, purified, and identified in living organisms and bacteria. Microorganisms are good source of proteases due to a number of advantages; 1) the broad biochemical diversity, 2) the rapid growth, 3) the limited space required for cell cultivation, and 4) the ease at which the enzymes can be genetically manipulated to generate new enzymes for various applications [3]. Some bacteria, yeasts, and fungi are able to produce

proteases, but only those microorganisms that produce enough amounts of extracellular proteases are of industrial importance [7]. Microbial proteases are widely different not only in their functions but also in their properties.

Recently, most of the industrial processes are carried out at harsh conditions, where the enzymes are unstable under extremely high temperature, high or low pH, high concentration of organic solvents and detergents, but only a limited class of proteases is recognized as commercial resource. Alkaline serine proteases such as subtilisin Carlsberg, subtilisin BPN', and Savinase are the major application as detergent industrial source, and some metalloproteases are usually used in brewing and therapeutic industry [6].

We have successfully isolated and examined *B. subtilis* FBL-1 to produce potential protease [7, 8]. One of the possible objectives of purifying and characterizing a bacterial protease has been the production of enzymes for commercial purposes. Therefore, in the present study, a bacterial protease was produced by *B. subtilis* FBL-1, which was then purified and characterized by ammonium sulfate precipitation, column chromatographies, and SDS-PAGE. In addition, enzymatic properties of the purified protease were further investigated to characterize the effects of enzyme activity and stability on organic solvents, detergents, temperature, pH, oxidizing agents, and reducing agents.

2. Materials and Methods

2.1. Bacterial strain

Bacillus subtilis FBL-1 KCCM 43196 isolated from soil was procured by Food Bioengineering Laboratory in Yeungnam University, Daegu, South Korea [7, 8]. Stock cultures were preserved in 1.5 mL sample tubes containing 50% (v/v) glycerol at -70°C until use. In order to activate cultures, strains were inoculated into tryptic soy broth (TSB; BD, Sparks, MD, USA) and then grown at 37°C and 200 rpm for 15 h.

2.2. Production of protease

The cells from stock cultures were inoculated to sterile 100 mL growth medium (TSB) and dispensed into 250 mL Erlenmeyer flasks, followed by incubation at 37ºC for 15 h. This was then inoculated aseptically at 1.5% (v/v) into 250 mL Erlenmeyer flask containing 100 mL production medium, which were incubated on a shaking incubator (VS-8480SF; Vision

Scientific Co., Daejeon, Korea) at 37ºC and 200 rpm. The production medium was composed of 32.4 g/L, yeast extract 15.0 g/L, KH_2PO_4 1.0 g/L, and $MgSO_4 \cdot 7H_2O$ 0.6 g/L. After 36 h of cultivation, cells were centrifuged at 13,000 × *g* and 4°C for 15 min with a high-speed refrigerated centrifuge (Supra 21K; Hanil Scientific Inc., Gimpo, Korea). The supernatant was collected and used as a crude enzyme preparation.

2.3. Protease activity assay

Protease activity was measured using casein as a substrate by the modified Folin & Ciocalteu's method [9]. A 20 µL of the enzyme was mixed with 500 µL of 0.5 M glycine-NaOH buffer (pH 9.0) containing 1% (w/v) casein and incubated at 40°C for 10 min with control. The enzyme reaction was stopped by addition of 2 mL of 10% (w/v) trichloroacetic acid. The mixture was incubated at room temperature for 15 min, followed by centrifuged at 16,000 × *g* for 15 min. The supernatant was mixed with 2.5 mL of 0.5 M Na_2CO_3 and then 500 µL of 20% (v/v) 2 N Folin & Ciocalteu's reagent was added. The mixture was incubated at 40°C for 10 min then the absorbance of mixture at 660 nm was measured by a spectrophotometer (UV-1600; Shimadzu Co., Kyoto, Japan). One unit of enzyme activity was

defined as the amount of enzyme required to liberate 1 µg of tyrosine per minute under the standard assay conditions.

2.4. Measurement of protein concentration

Protein concentration was determined by the BCA (bicinchonic acid) method using bovine serum albumin as a standard [10]. During chromatographic purification steps, the protein content of each fraction was monitored by measuring the absorbance at 280 nm.

2.5. Enzyme purification

Culture supernatant was subjected to ammonium sulfate precipitation for purification of protease. Ammonium sulfate fractions of 30-80% were collected by centrifugation at 13,000 × *g* and 4°C for 60 min, and the pellet was dissolved in a minimum amount of 0.1 M Tris-HCl buffer (pH 7.0). The protein was dialyzed against the same buffer to remove the residual salt at 4°C overnight with changing buffer solution. The dialysate was loaded onto a DEAE-Cellulose column (15 × 300 mm), which was equilibrated with 0.1 M Tris-HCl buffer (pH

7.0). Proteins were eluted with a linear gradient of NaCl (0-0.5 M) dissolved in the same buffer, and each fraction of 2.0 mL was collected. The column was washed with the same buffer until the absorbance of effluent at 280 nm reached zero. Enzyme activity and protein concentration of each fraction were measured. After then, the resultant fractions showing protease activity were loaded onto a Sephadex G-75 column (15 × 300 mm), which was equilibrated with 100 mM Tris-HCl buffer (pH 7.0). The fractions of 1 mL each were collected, and enzyme activity and protein concentration were measured. All purification procedures were carried out at 4°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 5% staking gel and a 12% resolving gel was used to determine the purity and molecular weight of the enzyme by the method of Laemmli [11]. Protein bands were visualized by silver staining method. Molecular weight of the purified enzyme was estimated by comparing the relative mobility of standard molecular weight marker protein (Bio-rad, Hercules, CA, USA).

2.6. Effect of pH on protease activity and stability

The effect of pH on protease activity was measured at different pH values. The pH of the

reaction mixture was adjusted to the desired values by using 0.1 M of buffers containing 1% (w/v) casein as a substrate as follows; citric acid buffer (pH 3.0 to 5.0), phosphate-citrate buffer (pH 5.0 to 7.0), Tris-HCl buffer (pH 7.0 to 9.0), and glycine-NaOH buffer (pH 9.0 to 12.0). The pH stability of protease was determined by pre-incubation in the above mentioned buffers at room temperature for 30 min and 60 min. The relative activity of enzyme was quantified under the standard assay conditions.

2.7. Effect of temperature on protease activity and stability

The effect of temperature on protease activity was carried out by incubation of reaction mixture at different temperatures ranged between 30°C and 60°C in 0.5 M glycine-NaOH buffer (pH 9.0) containing 1% (w/v) casein as a substrate. Thermal stability of the protease was determined by pre-incubation of protease at 30-70°C for 30 min and 60 min. The relative activities were quantified under the standard assay conditions.

2.8. Substrate specificity

Substrate specificity of the protease was determined using different substrates. The reaction mixtures were prepared by adding 1% (w/v) of casein, bovine serum albumin (BSA), soybean flour, and gelatin in 0.5 M glycine-NaOH buffer (pH 9.0). The enzyme activity was determined as described above.

2.9. Effect of metal ions on protease activity

The effect of metal ions on enzyme activity was investigated by incubating the reaction mixture with NH_4Cl, $CaCl_2 \cdot 2H_2O$, $FeCl_3 \cdot 6H_2O$, $FeSO_4 \cdot 7H_2O$, $MgSO_4 \cdot H_2O$, $MnCl_2 \cdot 4H_2O$, KCl, $CaCO_3$, and $ZnCl_2$ at concentrations of 1 mM and 5 mM for 30 min and 60 min at room temperature, respectively. The enzyme activity measured under the absence of metal ions was considered as 100%.

2.10. Effect of detergents, oxidants, and reductants on protease activity

The enzyme solution was incubated at room temperature for 30 min in 0.5 M glycine-NaOH buffer (pH 9.0), containing 1% (v/v) Tween 20, 1% (v/v) Tween 80, 1% (v/v) Triton X-100, 1

mM and 10 mM sodium dodecyl sulfate, 2% (v/v) H_2O_2, and 2% (v/v) 2-mercaptoethanol. Esterase activity was assayed by the spectrophotometric method and compared with the activity of the enzyme in the absence of detergent. Protease activity was measured and compared with the proteolytic activity of the enzyme in the absence of surfactants, oxidants, and reductants.

2.11. Effect of organic solvents on protease activity

The enzyme solution was incubated at room temperature for 30 min in in 0.5 M glycine-NaOH buffer (pH 9.0), containing 25 or 50% (v/v) of 1-butanol, benzene, *n*-hexane, 2-propanol, dimethyl sulfoxide (DMSO), and ethyl alcohol. Tween 80 was used as an emulsifier for water-immiscible solvents. Protease activity was measured and compared with the proteolytic activity of the enzyme in the absence of organic solvents.

2.12. Effect of inhibitors on protease activity

The effect of several inhibitors on protease activity was investigated by incubating the

enzyme solution with ethylenediaminetetraacetic acid (EDTA, 2 mM and 10 mM), phenylmethylsulfonyl fluoride (PMSF, 1 mM), and diisopropyl fluorophosphates (DIFP, 0.1 mM). The purified enzyme was pre-incubated with each inhibitor at room temperature for 30 min and then the residual activity was measured under the standard assay condition.

3. Results and Discussion

3.1. Purification of protease

B. subtilis FBL-2 was cultivated in optimized medium for 36 h. The crude enzyme preparation was subjected to 30-80% ammonium sulfate precipitation, followed by dialyzed against 0.1 M Tris-HCl buffer (pH 7.0) at 4°C overnight by changing the fresh buffer every 4 h. The dialyzate was loaded onto DEAE-Cellulose column at a flow rate of 2 mL/min. As shown in Fig. 1A, the fractions showing high protease activity (Fractions 5 to13) were pooled, desalted, concentrated, and loaded again onto Sephadex G-75 column. Fractions 15 to 38 showed high protease activity (Fig. 1B). Purification factors and recoveries at each step are shown in Table 1. The enzyme was purified 94.89-fold with a yield of 2.3% from the crude

extract, and the specific activity was increased to 3378.1 U/mg-protein.

Molecular weight of the purified protease was verified by SDS-PAGE and silver staining method. As shown in Table 1, after ammonium sulfate precipitation step, the recovery ratio of protein and enzyme were as low as 2.5% and 7.1%, respectively. In addition, ammonium sulfate precipitation resulted in more concentrated proteins other than protease. This low amount of recovery for a target enzyme and low selectivity of ammonium sulfate precipitation might result in denser protease band of crude extract than that of ammonium sulfate fraction (Fig. 2). However, the protease was dramatically purified in the next chromatographic separation steps, which was visualized in Fig. 2. By comparing the relative mobility of standard marker proteins, molecular weight of the purified protease was estimated to be approximately 37.6 kDa, which was similar to molecular weight of protease derived from *B. subtilis* RKY3 (38 kDa) [12].

3.2. Effect of pH on protease activity and stability

The effect of pH on protease activity and stability was examined over a pH range from pH 3.0 to 12.0. As shown in Fig. 3A, enzyme showed the highest activity at pH 9.0 (glycine-

NaOH buffer), but it was declined rapidly beyond pH 9.0. As shown in Fig. 3B, the purified enzyme was stable between pH 7.0 and 9.0 for 30 and 60 min, respectively. In addition, the enzyme activity could be retained approximately 80% of its initial activity at pH 6.0 to 9.0 after incubation for 30 min. Similar results of pH effect on protease activity have been reported, and the proteases produced by *B. subtilis* Y-108 [13], *B. tequilensis* P15 [14], *B. cereus* SV1 [15], and *B. cereus* AK1871 [16] showed their optimum pH at 7.5 to 8.0 and high pH stability at 7.0 and 9.0.

3.3. Effect of temperature on protease activity and stability

The effect of temperature on protease activity was investigated. As shown in Fig. 4A, the protease activity was highest at 45°C, and the enzyme activity at 50°C was sustained with 98.5% of maximum activity. However, the protease activity was rapidly declined beyond 50°C. As shown in Fig. 4B, the purified protease could retain 100% relative activity at 30 to 50°C for 30 min and 60 min, respectively. Similar effects of temperature on protease activity have been reported. For example, the protease produced by *B. subtilis* Y-108 showed its optimum temperature at 50°C and thermal stability at 25 to 50°C [13]. The protease from *B.*

subtilis RKY3 had its optimum temperature at 60°C, and its thermal stability was rapidly declined above 40°C [12].

3.4. Substrate specificity

The purified protease was reacted with different substrates such as casein, BSA, soybean flour, or gelatin. As shown in Table 2, casein was found to show the highest substrate specificity (100%) to the purified enzyme, followed by soybean flour (71.6%) and BSA (22.3%). However, the enzyme could not assimilate gelatin as a substrate. McConn *et al.* [17] previously reported that a neutral protease derived from *B. subtilis* was active in hydrolyzing casein but its ability to hydrolyze gelatin and egg albumin was only limited. This result suggests that the purified protease form *B. subtilis* FBL-1 show similar aspect of *B. subtilis* reported by McConn *et al.*.

3.5. Effect of metal ions on protease activity

The effect of metal ions on protease activity was shown in Table 3. The relative activity of

protease in the presence of 10 mM Mg^{2+}, Ca^{2+}, and Mn^{2+} were 41%, 30%, and 23%, respectively. Though the enzyme was significantly inhibited by 10 mM Fe^{3+}, Fe^{2+} led to activation of protease activity. Similar effect of metal ions on protease activity has been reported. Proteases produced by *B. tequilensis* P15 [14], *B. cereus* SV1 [15], *B. cereus* AK1871 [16], *Bacillus* sp. B001 [4], *B. mojavensis* A21 [18] and *Bacillus sp.* AK.1 [19] were activated by presence of Ca^{2+}. Some earlier reports have also showed that thermal stability of protease was improved in the presence of Ca^{2+} [20]. It may be explained by strengthening the interactions inside protein molecules and by combining Ca^{2+} to autolysis site to prevent autolysis and thermal unfolding [19, 21].

3.6. Effect of surfactants, oxidants, and reductants on protease activity

The effect of various chemicals such as surfactants, oxidants, and reductants on protease activity was investigated. As shown in Table 4, the enzyme was stable in the presence of 1% (v/v) nonionic surfactants like Tween 20, Tween 80, and Triton X-100. However, the enzyme was inhibited by the presence of 1 mM SDS as an anionic surfactant and 69% of the enzyme activity was inhibited by addition of 10 mM SDS. In addition, hydrogen peroxide (H_2O_2) and

2-mercaptoethanol inhibited the protease activity by 12% and 36%, respectively. Similar effects of surfactants, oxidants, and reductants on protease activity have been reported. Protease from *B. tequilensis* P15 was stable in the presence of nonionic surfactants such as Tween 20, Tween 80, and Triton X-100, but it was inhibited in the presence of anionic surfactant such as SDS by 47.4% [14]. However, the protease produced by *B. mojavensis* was stable in the presence of high concentration SDS up to 1% (w/v) [21].

3.7. Effect of organic solvents on protease stability

The relative activity of protease after exposure to organic solvents is shown in Table 5. Enzymes are generally inactivated in the presence of organic solvents such as 1-butanol, benzene, *n*-hexane, 2-pronpanol, dimethyl sulfoxide, or ethyl alcohol. However, the protease from *B. subtilis* FBL-1 was rarely inhibited by water-immiscible solvent such as *n*-hexane even at 25% and 50%. In addition, the enzyme was quite stable in the presence of 1-butanol and DMSO at 25% and 50%. Benzene and 2-propanol at 50% significantly lowered the enzyme activity to 69.4% and 42.8%, respectively. According to the previous studies, the protease from *Aeromonas veronii* PG01 [22] was inhibited in the presence of DMSO at 50%,

the enzyme from *B. pumilus* 115B [23] was stable in the presence of hexane and benzene, the protease from *B. tequilensis* P15 [14] was unstable in the presence of hexane, and the enzyme from *B. cereus* AK187 [16] was significantly unstable in the presence of butanol.

3.8. Effect of inhibitors on protease activity

The purified protease derived from *B. subtilis* FBL-1 was completely inhibited by the EDTA as a metalloprotease inhibitor (Table 6). The enzyme activity was almost reduced to 11% and 3.67% in the presence of 2 mM and 10 mM EDTA, respectively, but it was nearly not inhibited in the presence of PMSF and DIFP. Serine residue in the active site of the proteinases is irreversibly acylated by PMSF or DIFP, which results in inactivation of the enzymes. Therefore, the reagents such as PMSF or DIPF are serine protease inhibitors [24]. Cysteine proteases are generally inactivated with oxidative agents, metal ions, or alkylating agents. However, inhibition of metalloproteases is achieved with chelating agents (EDTA) or sodium dodecyl sulfate [25]. Therefore, the results obtained here suggest that the purified protease derived from *B. subtilis* FBL-1 should be considered to be a metalloprotease.

4. Conclusion

The protease from *B. subtilis* FBL-1 was purified and characterization for industrial application. The enzyme showed pH stability from 7.0 to 9.0 and thermostability from 30°C to 50°C. The protease activity was strongly activated by divalent metal ions. EDTA as a metal chelator almost inhibited protease activity, but no inhibition was observed when DIFP was added, suggesting that the protease from *B. subtilis* FBL-1 might be classified into a neutral metalloprotease. In addition, the enzyme activity could be highly stable even in the presence of nonionic surfactants, reducing agents, or organic solvents. It's stability against various chemicals makes this enzyme a potential biocatalyst for industrial applications. These study efforts need to get more knowledge on metalloproteases in *B. subtilis* for potential industrial applications such as brewing and grain starch isolation industries.

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Figure Legends

Fig. 1. Chromatograms obtained by (A) DEAE-Cellulose ion-exchange chromatography and (B) Sephadex G-75 gel filtration chromatography. Both columns were equilibrated with 100 mM Tris-HCl buffer (pH 7.0).

Fig. 2. SDS-PAGE of the purified protease produced by *B. subtilis* FBL-1. Lane 1, molecular weight marker proteins; lane 2, crude extract; lane 3, ammonium sulfate fraction; lane 4, DEAE-Cellulose fraction; lane 5, Sephadex G-75 fraction.

Fig. 3. Effect of pH on (A) protease activity and (B) stability. The buffer systems used were as follows: 0.1 M citric acid buffer for pH 3.0-5.0, 0.1 M phosphate-citrate buffer for pH 5.0-7.0, 0.1 M Tris-HCl buffer pH 7.0-9.0, and 0.1 M glycine-NaOH buffer for pH 9.0-12.0. The highest enzyme activity was considered as 100%, and error bars showed standard deviations of triplicate samples.

Fig. 4. Effect of temperature on (A) protease activity and (B) stability. The highest enzyme

activity was considered as 100%, and error bars showed standard deviations of triplicate samples.

Table 1. Summary of the purification step for the proteolytic enzyme from *Bacillus subtilis* FBL-1

Purification steps	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Crude extract	7,073.0	251,794.5	35.6	100	1
$(NH_4)_2SO_4$ (30-80%)	175.4	17,795.3	101.5	7.1	2.85
DEAE-Cellulose	3.9	12,937.1	3,352.4	5.1	94.17
Sephadex G-75	1.7	5,669.9	3,378.1	2.3	94.89

Table 2. Substrate specificity of the purified protease from *Bacillus subtilis* FBL-1

Substrates (1%, w/v)	Relative activity (%)
Casein	100 ± 7.4
Bovine serum albumin	22.3 ± 1.3
Soybean flour	71.6 ± 4.3
Gelatin	0

Table 3. Effect of metal ions on protease activity

Metal ions	Relative activity (%)	
	1 mM	10 mM
None	100 ± 1.7	100 ± 1.6
Mg^{2+} ($MgSO_4$)	118 ± 12.9	141 ± 4.4
Fe^{2+} ($FeSO_4$)	119 ± 8.1	110 ± 3.6
Fe^{3+} ($FeCl_3$)	125 ± 4.7	47 ± 3.5
Mn^{2+} ($MnCl_2$)	123 ± 3.3	123 ± 6.5
NH^{4+} (NH_4Cl)	105 ± 2.9	108 ± 4.6
Ca^{2+} ($CaCl_2$)	116 ± 0.8	130 ± 3.3
Ca^{2+} ($CaCO_3$)	106 ± 5.8	112 ± 2.1
K^{+} (KCl)	107 ± 3.2	116 ± 3.0
Zn^{+} (ZnCl)	104 ± 1.7	93 ± 4.2

Table 4. Effect of surfactants, oxidants, and reductants on protease activity

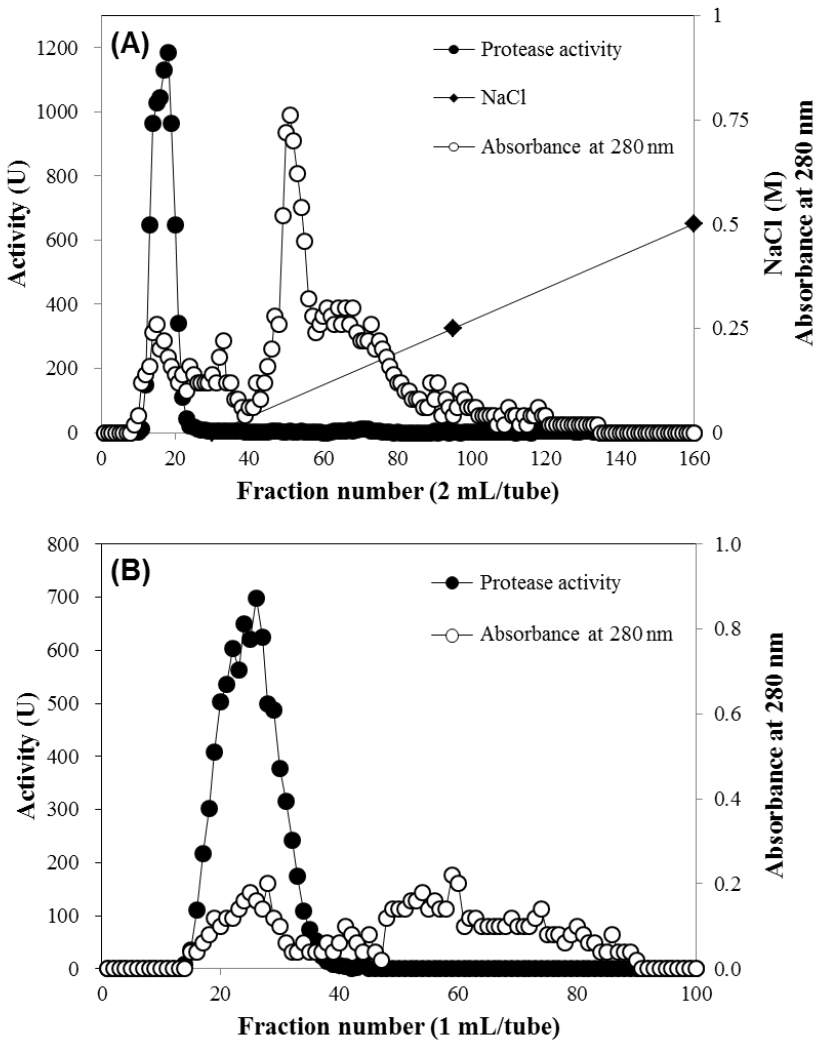
Surfactants, oxidants, or reductants	Concentration	Relative activity (%)
None	-	100 ± 4.6
Tween 20	1% (v/v)	77 ± 1.2
Tween 80	1% (v/v)	88 ± 1.0
Triton X-100	1% (v/v)	77 ± 2.9
Sodium dodecyl sulfate (SDS)	1 mM	86 ± 2.1
	10 mM	31 ± 0.7
H_2O_2	2% (v/v)	88 ± 2.9
2-Mercaptoethanol	2% (v/v)	64 ± 3.7

Table 5. Effect of organic solvents on protease activity

Solvents	Relative activity (%)	
	25% (v/v)	50% (v/v)
None	100 ± 2.4	100 ± 2.4
1-Butanol	77.6 ± 3.1	77.4 ± 5.8
n-Hexane	102.3 ± 3.5	100 ± 8.1
Benzene	66.1 ± 3.5	30.6 ± 1.0
2-Propanol	75.8 ± 4.1	57.2 ± 2.4
Dimethyl sulfoxide (DMSO)	79.3 ± 3.2	73.1 ± 1.6
Ethyl alcohol	87.6 ± 3.8	61.2 ± 3.6

Table 6. Effect of inhibitors on protease activity

Inhibitors	Concentration (mM)	Relative activity (%)
None	-	100 ± 2.94
Ethylene-diaminetetraacetic acid (EDTA)	2	11 ± 0.47
	10	3.67 ± 0.47
Diisoprophyl fluorophoshpahte (DIFP)	0.1	86.33 ± 3.68
Phenylmethly sulfonyl fluoride (PMSF)	1	84.67 ± 6.13



426

427

428 Fig. 1

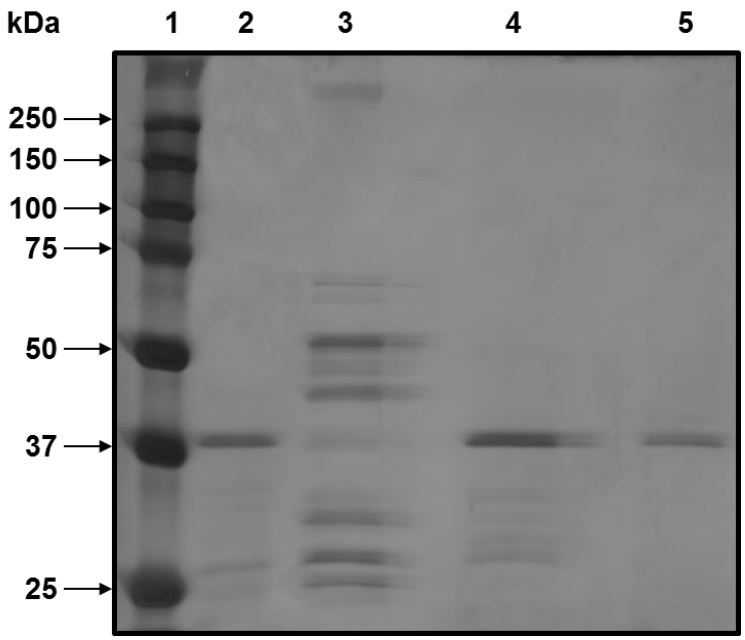


Fig. 2

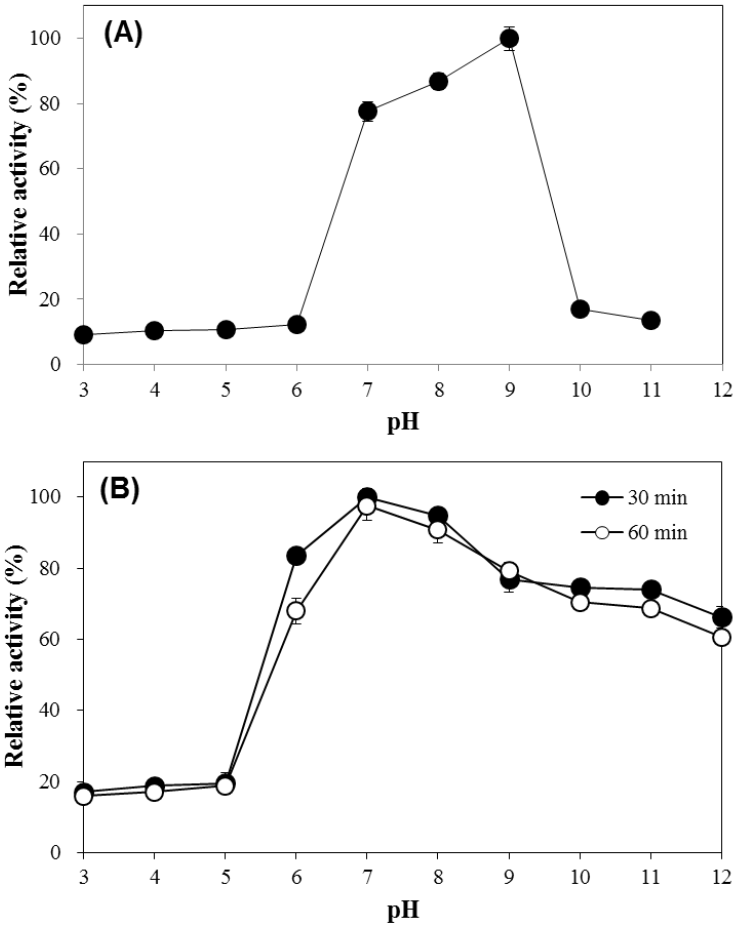


Fig. 3

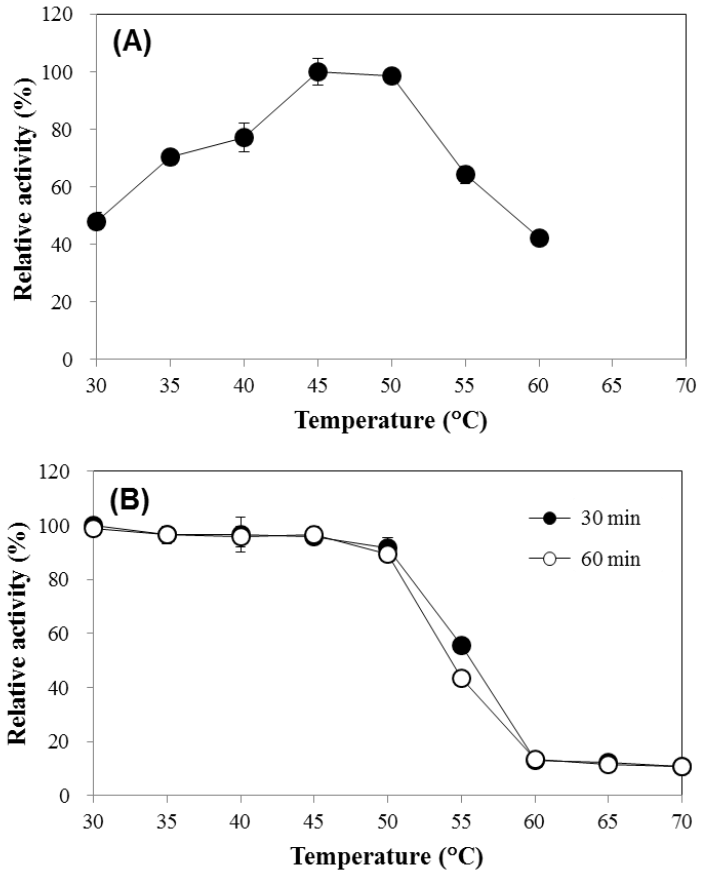

(A)
Relative activity (%)
Temperature (°C)
(B)
30 min
60 min
Relative activity (%)
Temperature (°C)

Fig. 4