J. Microbiol. Biotechnol. (1998), **8**(6), 639–644

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Characterization of Alkaline Serine Proteases Secreted from the Coryneform Bacterium TU-19

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Received: August 20, 1998

Abstract Extracellular serine proteases were isolated from a soil bacterium, alkalophilic coryneform bacterium TU-19, which have been grown in a liquid medium optimized at 30°C and pH 10.0. Three different sizes, 120 kDa (protease I), 80 kDa (protease II), and 45 kDa (protease III), of serine proteases were purified using Sephadex G-150 and QAE-Sephadex chromatography (Kang *et al.* 1995. *Agric. Chem. Biotech.* **38:** 534–540). SDS-PAGE showed that the 120 kDa protease was degraded into the 80 kDa protease in 20 mM Tris-HCl (pH 8.0) buffer solution. This degradation was enhanced in the presence of 0.5 M NaCl and 5 mM EDTA, but was inhibited in the presence of 5 mM $CaCl_2$. These results indicated that the Ca^{2+} ion seems to stabilize the 120 kDa protease like other proteases derived from *Bacillus* species. The NH_2-terminal amino acid sequences of the 10 residues of both proteases were completely identical: Met-Asn-Thr-Gln-Asn-Ser-Phe-Leu-Ile-Lys. In contrast to this, the 80 kDa protease has 1.5 times higher specific activity than the 120 kDa protease does (Kang *et al.* 1995. *Agric. Chem. Biotech.* **38:** 534–540). Therefore the C-terminal of the 120 kDa protease seems to be autolyzed to the 80 kDa protease but this autolysis did not decrease the protease activity. Optimum pH and temperature of both 80 kDa and 120 kDa proteases were pH 10.5 and 45°C, respectively, and pH and thermal stability were almost identical. Several divalent ions except the Fe^{2+} ion showed similar effects on activities of both proteases, which are similarly resistant to three different detergents.

Key words: Autolysis, amino acid sequence analysis, C-terminal pro-sequences

Proteases secreted by alkalophilic microorganisms are of interest because they represent a major source of commercially produced proteolytic enzymes [7]. These enzymes are being used in the production of detergent, leather, and food and in pharmaceutical industries [4]. Therefore, we previously isolated an alkalophilic bacterium from soil and identified it as coryneform bacterium TU-19 by its morphological and physiological properties [1]. This bacterium produces at least three extracellular proteases (protease I, II, and III) and these proteases were classified as serine proteases based on inactivation of proteases by class-specific inhibitors [8].

Proteins destined for export are generally synthesized with a 15 to 25 amino acids long, hydrophobic N-terminal extension that somehow initiates the export process [3]. This so-called signal sequence is removed from the protein once export is under way through the action of an endoproteolytic signal peptidase. In addition to this signal sequence, many proteins contain N-terminal pro-sequences. These pre-proteins are processed into mature proteins by the function of peptidases in order to be functional proteins. In the case of bacteria, pro-sequences are indispensable for proper folding of the proteins, subtilisins from *Bacillus* species [19] and α-lytic protease from *Lysobacter enzymogens* [17].

On the other hand, C-terminal pro-sequences have been observed in several prokaryotic organisms as well as in melons, and their functions were estimated. IgA protease of *Neisseria gonorrhoeae* [16], serine protease of *Serratia marcescens* [15], and aqualysin I of *Thermus thermophilus* [9] have a long C-terminal pro-sequence for protein folding and translocation of proteases. The C-terminal pro-sequences of protease precursors from *Alteromonas* [22], *Achromobacter* [14], *Bacillus subtilis* [23], and *Cucumis melo* [23] are needed for secretion. However, the processing mechanism(s) of C-terminal pro-sequence to the mature proteins are not well described compared with that of the N-terminal pro-sequence.

We observed that the C-terminal of the alkaline serine protease from the coryneform bacterium has been degraded by itself and this autolysis enhanced protease activity in the specific activity [8]. However the degradation of the C-terminal of the protease did not affect its characteristics,

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pH and temperature optimum as well as pH and thermal stability. Effects of divalent metal ions and detergents on activities of both proteases were also similar. Therefore, we assumed that this autolysis of the C-terminal could be one of the possible processing mechanisms for the C-terminal pro-sequence to the mature serine protease in alkalophilic bacteria.

MATERIALS AND METHODS

Medium and Culture Conditions

The strain used in this study was isolated from soil collected in Kyungsan, South Korea, and has been identified as the alkalophilic coryneform bacterium TU-19 [1]. The isolates were cultivated in a medium containing 10 g of glucose, 10 g of bacto-trypton, 5 g of bacto-yeast extract, 1 g of potassium phosphate, and 1 g of magnesium bicarbonate per 1 liter of distilled water. The pH of the medium was adjusted to 10.0 by addition of an appropriate volume of sterile sodium bicarbonate solution (20%, w/v) because the optimum pH for growth of TU-19 is 10.0 [1].

Assay of Protease Activity

Protease activity of crude or purified proteins was assayed by a modified method of Yanagida *et al.* [24]. An enzyme solution (120 µl) was mixed with 600 µl of pre-warmed casein (0.6%, w/v) in sodium bicarbonate-NaOH buffer solution, and incubated for 30 min at 30°C. Enzyme reaction was stopped by adding 600 µl of stop solution containing 0.11 M trichloroacetic acid, 0.22 M sodium acetate, and 0.33 M acetic acid. After the reaction mixture was allowed to stand for 30 min at room temperature and centrifuged at 1,500×g for 5 min, the light absorbance of the supernatant was measured at 275 nm. The protein concentration was measured according to the published method of Lowry *et al.* [12].

Protein Purification and SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Purification and separation of three different proteases, protease I, II, and III, from coryneform bacterium TU-19 have been reported [8]; Ammonium sulfate fractionation, gel filtration using Sephadex G-150, and QAE-Sephadex column chromatography were carried out according to the published methods. The purified proteins were separated on polyacrylamide gel (10%, w/v) containing 10% sodium dodecyl sulfate (10%, w/v) according to the method of Laemmli [11].

Effects of the Ca^{2+} and Na^{+} Ions and EDTA on Stability of Protease I

In order to determine the effects of Ca^{2+}, Na^{+} and ethylene diamine tetraacetic acid (EDTA) on the stability of protease I, 5 mM of $CaCl_2$, 0.5 M of NaCl, and 5 mM of EDTA·2Na were added to the protease I which was in 20 mM Tris-HCl (pH 8.0) buffer solution and incubated at 30°C for 0~5 h. This was followed by separation of proteins on polyacrylamide gel (10%, w/v) containing SDS (10%, w/v).

N-Terminal Amino Acid Sequence Analysis of Proteases

N-terminal amino acid sequences of proteases I and II were analyzed according to the published method [13]. After polyacrylamide (8%, w/v) gel electrophoresis, the gel was incubated in 3-[cyclohexylamino-1-propanesulfonic acid] (CAPS) buffer solution (10 mM CAPS; pH 11.0, 10% methanol) for 15 min. Electro-blotting (20 mA, 3 h) was carried out using polyvinylidine fluoride (PVDF) membrane. The PVDF membrane was stained with Coomassie Blue R-250 (0.1%, w/v) for 5 min followed by a destaining with solution containing 50% methanol (v/v) and 10% acetic acid (v/v). The destained gel was washed with triple distilled water over night and bands for protein were removed. Then, peptide sequencing was carried out with an automatic peptide sequencer (Porton Instrument model 1090).

Optimum pH and pH Stability of Proteases

The following buffer solutions were prepared to learn the optimum pH of proteases; sodium acetate (pH 4.0-5.0), sodium phosphate (pH 6.0-6.5), Tris-HCl (pH 7.0-9.0), glycine-NaOH (pH 9.5-10.0), $NaHCO_3$-NaOH (pH 10.5), Na_2HPO_4-NaOH (pH 11.0-12.0), and KCl-NaOH (pH 12.5-13.0) at the concentration of 50 mM. Enzyme reaction was carried out in the presence of casein (0.6%, w/v) at 30°C for 30 min. pH stability was monitored by measuring the activities of proteases at the optimum pH after enzyme reactions were carried out at 30°C for 5 and 36 h at the respective pHs. Relative activities at the respective pHs were calculated corresponding to 100% of the highest activity at the optimum pH.

Optimum Temperature and Thermal Stability of Proteases

In order to measure the optimum temperature of proteases, enzyme reactions were carried out within the range of 10 to 80°C for 30 min in the presence of casein (0.6%, w/v) which had been adjusted to the optimum pH. The thermal stability of proteases was monitored by measuring the remaining activities of proteases after proteases were treated within the range of 30 to 70°C for 10 min. After thermal treatment of proteases which had been adjusted to the optimum pH, enzyme reactions were carried out by adding substrate at 30°C for 30 min.

Effects of Divalent Metal Ions on the Activities of Proteases

Several metallic salts, $CaCl_2 \cdot 2H_2O$, $CoCl_2 \cdot 2H_2O$, $ZnSO_4 \cdot 2H_2O$, $MnCl_2 \cdot 6H_2O$, $MgCl_2 \cdot 6H_2O$, $FeCl_2$, and $BaCl_2$,

were added to the enzyme solution to the final concentrations of 1 and 10 mM. Enzyme activities were measured by adding substrate to enzyme reactions which had been set at 30°C for 30 min in the presence of divalent ions.

Effects of Detergents on the Activities of Proteases

Three different detergents, SDS, triton X-100, and Tween 80, were added to enzyme solutions to a final concentration of 0.2%, 2.0%, and 2.0%, respectively. Enzyme activities were measured by adding substrate to enzyme reactions which had been set at 30°C for 30 min in the presence of detergents.

RESULTS AND DISCUSSION

Auto-Degradation of Protease I to Protease II

Among three different serine proteases isolated from coryneform bacterium TU-19 [8], 120 kDa protease (protease I) was degraded into 80 kDa protease (protease II) in 20 mM Tris-HCl (pH 8.0) buffer solution (Fig. 1). The degradative products started to appear after 3 h of incubation at 30°C. Degradation was enhanced by addition of NaCl (0.5 M of the final concentration) to the buffer solution (Lane 9 of Fig. 1). In the presence of NaCl, a single band corresponding the 80 kDa protease appeared on a gel after 2 h. However, the 40 kDa or smaller degradative products did not appear on gels, indicating that autolysis could occur by endo-protease activity of the 120 kDa protease. This kind of autolysis of protease in solution is a rare phenomenon, while an auto-proteolytic cleavage was observed in the alkaline protease purified from *Bacillus licheniformis* NS70 and kept at 4°C [10]. In order to search for the 40 kDa or smaller degradative products, expression of gene(s) encoding the 120 kDa protease and the 80 kDa protease may be examined. Furthermore, expressed proteases as well as purified proteases can be used to detect amounts of proteases present in cells using *in-situ* hybridization technique.

However, the degradative process was not observed when the 120 kDa protease was incubated in the presence of 5 mM $CaCl_2$ up to 5 h (Fig. 2). The effect of the Ca^{2+} ion on the delay of the 120 kDa protease degradation was also seen even in the presence of 0.5 M

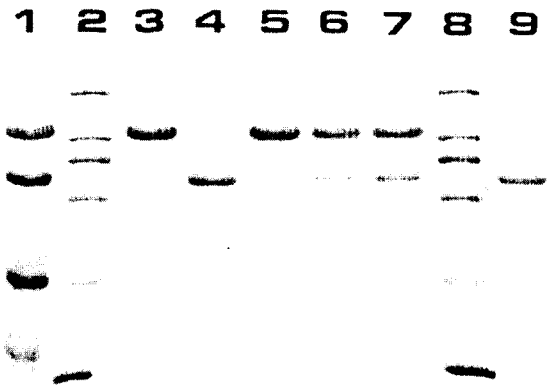

Fig. 1. Auto-degradation of protease I to protease II. SDS-PAGE (10%) was carried out at 10 mA.

Lane 1; After ammonium sulfate fractionation (55%~80%) of extracellular proteins of TU-19. Lanes 2 and 8; Molecular weight standards in kDa (top to bottom): myosine, 205 kDa; β-galactosidase, 116 kDa; phosphorylase b, 97.4 kDa; BSA, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa. Lane 3, purified protease I; Lane 4, purified protease II; Lanes 5, 6, and 7, protease I incubated in 20 mM Tris-HCl (pH 8.0) buffer solution at 30°C for 1 h, 3 h, and 5 h, respectively; Lane 9, protease I incubated in 20 mM Tris-HCl (pH 8.0) buffer solution containing 0.5 M NaCl at 30°C for 2 h.

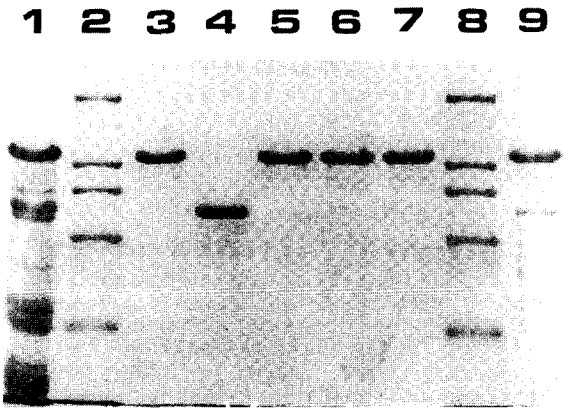

Fig. 2. The protective effect of Ca^{++} ion on the auto-degradation of protease I.

Lanes 1~4 and 8, same as described in Fig. 1; Lanes 5, 6, and 7, protease I incubated in the 20 mM Tris-HCl (pH 8.0) buffer solution containing 5 mM $CaCl_2$ at 30°C for 1 h, 3 h, and 5 h, respectively; Lane 9, Protease I incubated in 20 mM Tris-HCl (pH 8.0) buffer solution containing 0.5 M NaCl and 5 mM $CaCl_2$ at 30°C for 2 h.

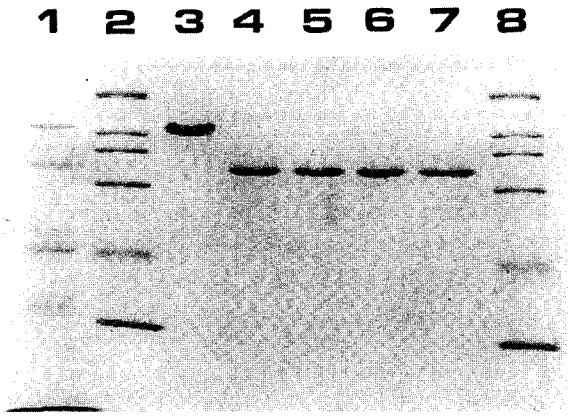

Fig. 3. The effect of EDTA on the auto-degradation of protease I.

Lanes 1~4 and 8, same as described in Fig. 1; Lanes 5, 6, and 7, protease I incubated in 20 mM Tris-HCl (pH 8.0) buffer solution (pH 10.0) containing 5 mM EDTA at 30°C for 1 h, 3 h, and 5 h, respectively.

NaCl. The addition of $CaCl_2$ to the solution containing NaCl or the addition of NaCl to the solution containing $CaCl_2$ had the same effect on the delay of the 120 kDa protease degradation. Addition of EDTA, a chelate compound of the Ca^{2+} ions, to the solution containing the 120 kDa protease did enhance the degradation of the 120 kDa protease to 80 kDa (Fig. 3). In Fig. 3, the band for the 80 kDa protease showed up on the gel when the 120 kDa protease was incubated in the presence of the 5 mM EDTA for 1 h. These results indicated that the Ca^{2+} ion stabilized the 120 kDa protease like other proteases derived from *Bacillus* species [2, 5].

N-Terminal Amino Acid Sequence Analysis of Proteases

In order to find out a direction of the degradation of the 120 kDa protease, N-terminal amino acid sequencing analysis was carried out on both 120 kDa and 80 kDa proteases. The N-terminal amino acid sequences of the 10 residues of both proteases were Met-Asn-Thr-Gln-Asn-Ser-Phe-Leu-Ile-Lys indicating that the C-terminal region of the 120 kDa protease was degraded. The C-terminal degradation did not decrease the protease activity; the 80 kDa protease contains 1.5 times higher specific activity than that of the 120 kDa protease [8]. Therefore, we concluded that the C-terminal of the 120 kDa protease was autolyzed to the 80 kDa protease.

The N-terminal amino acid sequences of proteases I and II of alkalophilic coryneform bacterium TU-19 are completely different from those of other alkalophilic serine proteases from several other bacteria (Fig. 4). However, other serine proteases share high levels of amino acid identity in the N-terminal regions.

Characteristics of Proteases I and II

Both proteases I and II have the same optimum pH 10.5, and contain above 80% enzyme activity in the range of pH 9.5 to pH 11.0 (Fig. 5A). At pH 12.0, the enzyme activities of protease I and protease II were 22% and 33% of those at pH 10.5, respectively. The pH stability of protease I was also similar to that of protease II; the remaining activities of both proteases were above 80% in the range of pH 7.5 to 12.0 after 5 h incubation of the enzyme solution at 30°C and the remaining activities of protease I and protease II were 80% and 82% when they were incubated at pH 10.0 for 36 h (Fig. 5B).

Optimum temperature and thermal stability of protease I were also almost identical to those of protease II (Fig. 6A). Both proteases showed the highest activity at 45°C and the remaining activities of the protease I and protease II at 55°C were 41% and 36%, respectively. However both lost their activity above 80°C. Thermal stability was monitored by measuring activities of proteases in the

Protease I	Met-Asn-Thr-Gln-Asn-Ser-Phe-Leu-Ile-Lys
Protease II	Met-Asn-thr-Gln-asn-Ser-Phe-Leu-Ile-Lys
Subtilisin Carlsberg	Ala-Gln-thr-Val-Pro-Tyr-Gly-Ile-Pro-Leu
Subtilisin BPN'	Ala-Gln-Ser-Val-Pro-Trp-Gly-Val-Ser-Arg
Bacillus 221	Ala-Gln-Ser-Val-Pro-Trp-Gly-Ile-Ser-Arg
HS 682	Ala-Gln-Ser-Val-Pro-Trp-Gly-val-Ser-Gln

Fig. 4. Comparison of the N-terminal amino acid sequences of proteases I and II of the alkalophilic coryneform bacterium TU-19 with those of other serine proteases of several alkalophilic bacteria.

Subtilisin carlsberg is from *Bacillus licheniformis* [18], Subtilisin BPN' is from *Bacillus amyloliquefaciens* [13]. Bacillus 221 is from *Bacillus* sp. No. 221 [6]. HS 682 is from *Thermoactinomyces* sp. HS 682 [21].

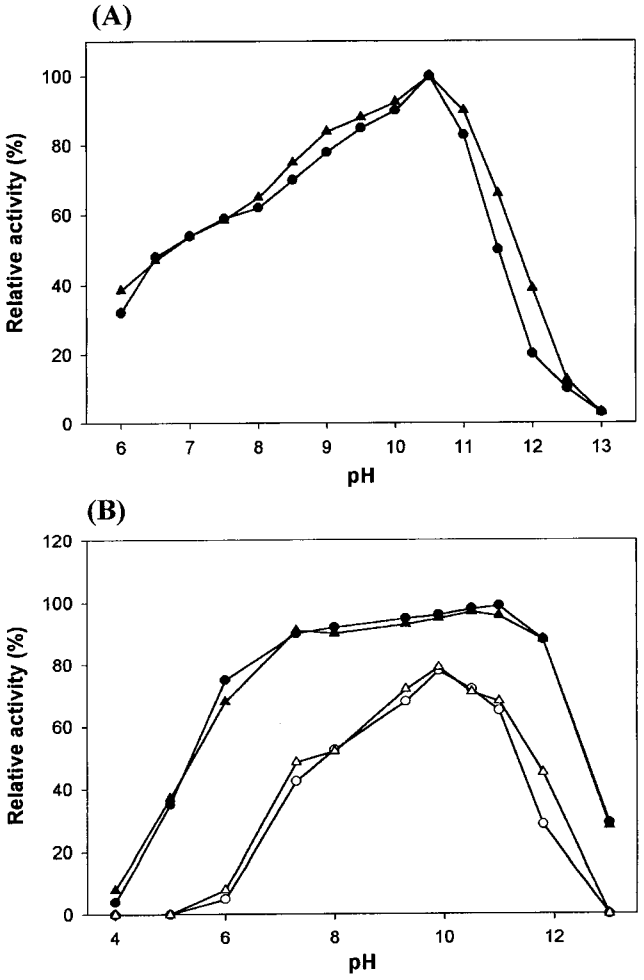

Fig. 5. Effect of pH on the activities of proteases I and II.

(A) Optimum pH of protease I (●-●) and protease II (▲-▲). The enzyme activities of enzyme solutions were measured at the indicated pH. The following buffer systems were utilized; sodium acetate (pH 4.0-5.0), sodium phosphate (pH 6.0-6.5), Tris-HCl (pH 7.0-9.0), glycine-NaOH (pH 9.5-10.0), $NaHCO_3$-NaOH (pH 10.5), Na_2HPO_4-NaOH (pH 11.0-12.0), and KCl-NaOH (pH 12.5-13.0) at the concentration of 50 mM. The activity at pH 10.5 was taken as 100% activity. (B) pH stability of protease I (●-● and ○-○) and protease II (▲-▲ and △-△). The enzyme solutions were pre-incubated at 30°C for 5 h (●-● and ▲-▲) and 36 h (○-○ and △-△) in the buffer systems as indicated in (A). The residual activities were measured at 30°C.

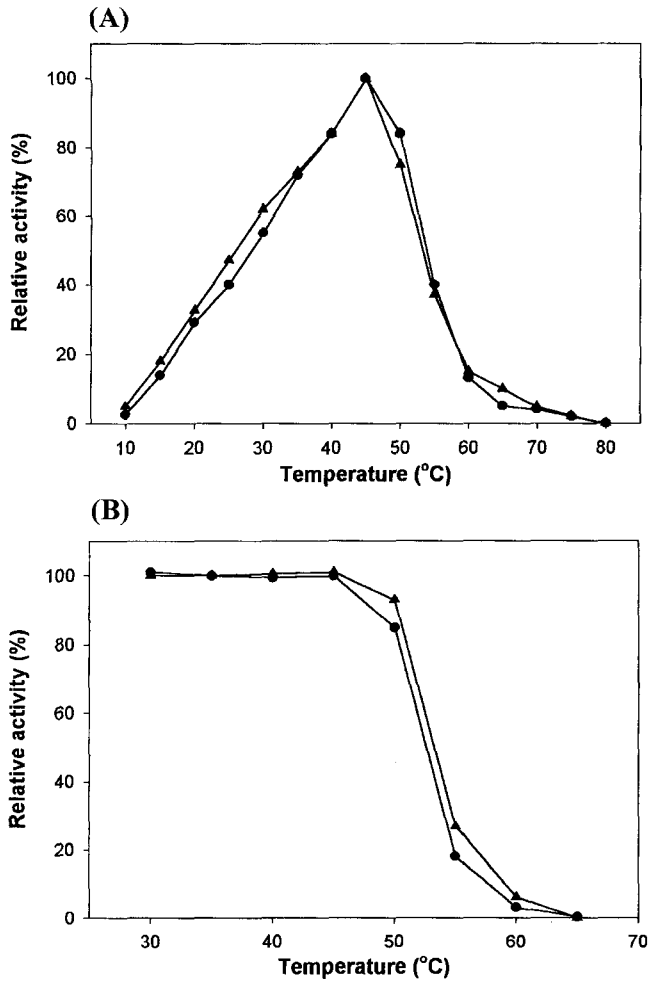

Fig. 6. Effect of temperature on the activities of proteases I and II.

(A) Optimum temperatures of protease I (●-●) and protease II (▲-▲). The enzyme activities of enzyme solutions in 50 mM $NaHCO_3$-NaOH (pH 10.5) were measured at the indicated temperatures. The activity at 30°C was taken as 100% activity. (B) Thermal stability of protease I (●-●) and protease II (▲-▲). The enzyme solutions in 50 mM $NaHCO_3$-NaOH (pH 10.5) were pre-incubated at the indicated temperatures for 10 min and the residual activities were measured at 30°C.

range of 30°C to 70°C (Fig. 6B). Enzyme solutions adjusted at the optimum pH were set at the respective temperature for 10 min and cooled. Then enzyme reactions were carried out at 30°C for 30 min and the enzyme activities were measured. Protease I and protease II showed 100% activities up to 45°C but lost their activities at 65°C.

Several divalent metal ions, Ca^{2+}, Co^{2+}, Mn^{2+}, Mg^{2+}, and Ba^{2+}, did not affect the activities of the two proteases, but Fe^{2+} and Zn^{2+} did (Table 1). In the case of the protease I, the remaining activities were 35% and 37% in the presence of 10 mM Fe^{2+} and 10 mM Zn^{2+}, respectively. Similar results were obtained in the case of protease II; The remaining activities were 28% and 32% in the presence of 10 mM Fe^{2+} and 10 mM Zn^{2+}, respectively. However,

Table 1. Effects of divalent ions on the activities of protease I and protease II.

Metal ions	Concentration (mM)	Relative activity*	
		Protease I	Protease II
Ca^{2+}	1	96	100
	10	105	100
Co^{2+}	1	97	100
	10	102	104
Mn^{2+}	1	98	103
	10	95	97
Mg^{2+}	1	92	100
	10	102	110
Fe^{2+}	1	86	35
	10	35	28
Zn^{2+}	1	83	85
	10	37	32
Ba^{2+}	1	90	104
	10	97	101

*The activity of the proteases without metal ions corresponds to 100% activity.

the remaining activity of the protease II was 35% in the presence of 1 mM Fe^{2+}. Inhibitory effects of Fe^{2+} and Zn^{2+} on enzyme activities were also found in other alkalophilic proteases [2, 5].

Both proteases seem to be resistant to detergents; The remaining activities of protease I and protease II were 75%~88% in the presence of 0.1% SDS, 2.0% Triton X-100, and 2.0% Tween 80 (Table 2). This resistance to detergents is also found in other alkaline protease from *Bacillus* species [10, 20].

Based on the above results, we concluded that the C-terminal of the 120 kDa protease was autolyzed to the 80 kDa protease, and this autolysis did not decrease the protease activity. In addition, the degradation of the C-terminal of the protease did not affect its characteristics, pH and temperature optimum, as well as pH and thermal stability. Effects of divalent metal ions and detergents on activities of both proteases were also similar. Therefore, autolysis of the C-terminal could be one of the possible processing mechanisms for the C-terminal pro-sequence to the mature serine protease in alkalophilic bacteria.

Table 2. Effect of detergents on the enzymatic activities of protease I and protease II.

Detergents	Concentration (w/v)	Relative activity* (%)	
		Protease I	Protease II
SDS	0.2%	75	86
Triton X-100	2.0%	84	80
Tween 80	2.0%	75	88

*The activity of the proteases without detergents corresponds to 100% activity.

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