Characterization of Calcium-Activated Bifunctional Peptidase of the Psychrotrophic *Bacillus cereus*

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The protease purified from *Bacillus cereus* JH108 has the function of leucine specific endopeptidase. When measured by hydrolysis of synthetic substrate (*N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide), the enzyme activity exhibited optimal activity at pH 9.0, 60°C. The endopeptidase activity was stimulated by Ca^{++}, Co^{++}, Mn^{++}, Mg^{++}, and Ni^{++}, and was inhibited by metal chelating agents such as EDTA, 1,10-phenanthroline, and EGTA. Addition of serine protease inhibitor, PMSF, resulted in the elimination of the activity. The endopeptidase activity was fully recovered from the inhibition of EDTA by the addition of 1 mM Ca^{++}, and was partially restored by Co^{++} and Mn^{++}, indicating that the enzyme was stabilized and activated by divalent cations and has a serine residue at the active site. Addition of Ca^{++} increased the pH and heat stability of endopeptidase activity. These results show that endopeptidase requires calcium ions for activity and/or stability. A Lineweaver-Burk plot analysis indicated that the K_m value of endopeptidase is 0.315 mM and V_{max} is 0.222 μmol of *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide per min. Bestatin was shown to act as a competitive inhibitor to the endopeptidase activity.

Key words: *Bacillus cereus*, leucine endopeptidase, Ca^{++}-activated protease

Because of their broad biochemical diversity and their susceptibility to genetic manipulation, microorganisms represent an excellent source of enzymes. Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus Bacillus. Bacterial neutral proteases are active in a narrow pH range (pH 5 to 8) and have relatively low thermostability. Neutral bacterial proteases belong to the metalloprotease type and require divalent metal ions for their activity (Rao *et al.*, 1998). Bacterial alkaline proteases are characterized by their high activity at alkaline pH and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry.

Proteases are grossly subdivided into two major groups, exopeptidases and endopeptidases, depending on their site of action. Exopeptidases cleave the peptide bond promixal to the amino or carboxy termini of the substrate, while endopeptidases cleave peptide bonds which are distant from the termini of the substrate. Based on the functional group present at the prominent group, proteases are classified into serine protease, cysteine protease, aspartic protease, and metalloprotease (Beynon and Bond, 1989).

Metalloproteases are the most diverse category of the catalytic types of proteases (Barrett, 1995). They are characterized by the requirement of a divalent metal ion for their activity. About 30 families of metalloproteases have been identified, of which 17 contain only endopeptidases, 12 contain only exopeptidases, and 1 contains both endo- and exopeptidases (Rawlings and Barrett, 1993). Based on the specificity of their action, metalloproteases can be divided into four groups: neutral, alkaline, *Myxobacter* I, and *Myxobacter* II. The neutral proteases displayed specificity for hydrophobic amino acids, but the alkaline proteases exhibited to have a very broad specificity. *Myxobacter* protease I is specific for small amino acid residues on either side of the cleavage bond, whereas *Myxobacter* protease II is specific for lysine residue on the amino side of the peptide bond. Activities of metalloproteases depend on the presence of bound divalent cations. All of the metalloproteases are inhibited by chelating agents such as EDTA, 1,10-phenanthroline, and EGTA (Beynon and Bond, 1989).

In this study, the characteristics of the leucine endopeptidase, stimulated by Ca^{++}, from psychrotrophic *Bacillus cereus* were analyzed.

Materials and Methods

Chemicals

Unless otherwise stated, chemicals were obtained from

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Sigma. Water used in this study was prepared with a Millipore Milli-Q system. The medium for bacterial culture was purchased from Difco (USA).

Protease Purification

The protease was purified as described previously (Jung *et al.*, 1999). The enzyme activity was measured spectrophotometrically using azocasein as substrate during each purification step.

Peptidase Assay

The enzyme activity was measured with a synthetic substrate. Hydrolytic activity toward the synthetic substrate was estimated with *p*-nitroanilide of amino acids. Unless otherwise indicated, the reaction mixture (0.1 ml) contained 0.2 mM amino acid p-nitroanilide, 50 mM MOPS (pH 7.0). The reaction was initiated by the addition of the concentrated enzyme after 5 min preincubation of the reaction mixture at 30°C, and incubation at 30°C for the indicated times. Activity was determined by measuring the release of *p*-nitroanilide at 405 nm with an extinction coefficient of 9,620 $M^{-1}cm^{-1}$. One unit of hydrolytic activity was defined as the amount of enzyme required to produce 1 μmole of *p*-nitroanilide per min (Beynon and Bond, 1989).

Determination of Kinetic Parameters

The kinetic parameters for the hydrolysis of aminoacyl *p*-nitroanilides were measured. Purified enzyme was incubated in 50 mM Tris-HCl (pH 8.0) at 37°C for 10 min. Aminoacyl *p*-nitroanilides were then added to achieve final concentrations of 0.1 - 1 mM. The initial velocity of substrate hydrolysis was determined by measuring the amount of *p*-nitroanilide liberated. The K_m and k_{cat} values were calculated from a Lineweaver-Burk reciprocal plot.

Effect of Metal ions and Inhibitors on the Proteolytic Activity

Metal ions and inhibitors were added to the assay mixture 10 min before the addition of substrates. Phenylmethylsulfonyl fluoride and pepstatin stock solutions were prepared in ethanol before being diluted with assay buffer. The activity was then expressed relative to the control reactions containing equal amounts of ethanol without an inhibitor. Metals were used as chloride salts.

Heat Stability of the Protease

The purified enzyme in an assay mixture was treated at 60°C or 70°C for varying amounts of time. Afterward, the reaction was initiated by the addition of substrate at 37°C for 30 min. The remaining activity was compared with the enzyme activity without heat treatment.

Results

In the previous study, the extracellular protease studied

Table 1. Substrate specificity of the protease

Substrate	Initial Velocity (μM/min)
N-succinyl-Ala-Ala-Pro-Leu *p*-nitroanilide	0.216
N-acetyl-L-Alanine *p*-nitroanilide	0.002
N-succinyl-Ala-Ala-Val *p*-nitroanilide	0.000
N -benzoyl -DL-Arginine *p*-nitroanilide	0.002
N-p-tosyl-Gly-Pro-Lys *p*-nitroanilide	0.000
N-glutaryl L-Phenylalanine *p*-nitroanilide	0.002
N-benzoyl L-Tyrosine *p*-nitroanilide	0.000
N-succinyl-Ala-Ala-Pro-Asp *p*-nitroanilide	0.000

Enzyme assay was carried out at 37°C in the reaction mixture containing 50 mM Tris-HCl (pH 8.0) and 0.2 mM of each synthetic substrate. Changes in the absorbance at 405 nm were monitored continuously for 60 min.

here was purified and characterized. The enzyme preferentially hydrolyzed leucine at the N-termini of peptides and can thus be classified as an aminopeptidase (Jung *et al.*, 1999). Amino acid residues other than leucine and alanine at the N-termini of peptides were not hydrolyzed by this enzyme. When the peptide hydrolyzing activity was assayed with synthetic compounds acting as substrates for endopeptidase, the enzyme had the capacity to hydrolyze the substrates for endopeptidase. Thus, this enzyme exhibited the activities with exopeptidase, as well as with endopeptidase. However, the extracellular protease from *Bacillus amyloliquefaciens* demonstrated activity only with endopeptidase (Son and Kim, 2003). Enzymological properties of the enzyme, previously characterized as leucine aminopeptidase, were analyzed in this study.

Substrate Specificity

The catalytic activity of enzymes towards various synthetic aminoacyl p-nitroanilides, acting as substrates for endopeptidase, was measured (Table 1). The enzyme hydrolyzed *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide more rapidly than other aminoacyl *p*-nitroanilides. There was no apparent hydrolytic activity when N-succinyl-Ala-Ala-Val-*p*-nitroanilide, *N*-p-tosyl-Gly-Pro-Lys-*p*-nitroanilide, *N*-succinyl-Ala-Ala-Pro-Asp-*p*-nitroanilide, *N*-acetyl-L-Ala-*p*-nitroanilide, *N*-benzoyl-Arg-*p*-nitroanilide, *N*-glutaryl-Phe-*p*-nitroanilide, and *N*-benzoyl-L-tyrosine *p*-nitroanilide were used as substrates.

Effect of Temperature and pH

Using the following buffers, MES-NaOH (pH 5 - 7), MOPS-NaOH (pH 6 - 8), Tris-HCl (pH 7 - 9), HEPES-NaOH (pH 7 - 9) and CAPS (pH 9 - 11), the enzyme activity was estimated with *N*-succinyl Ala-Ala-Pro-Leu *p*-nitroanilide as a substrate over a pH range from 4.0 to 11.0. The enzyme displayed a broad pH activity profile in the neutral to basic range with optimum pH at 9.0 (Fig. 1).
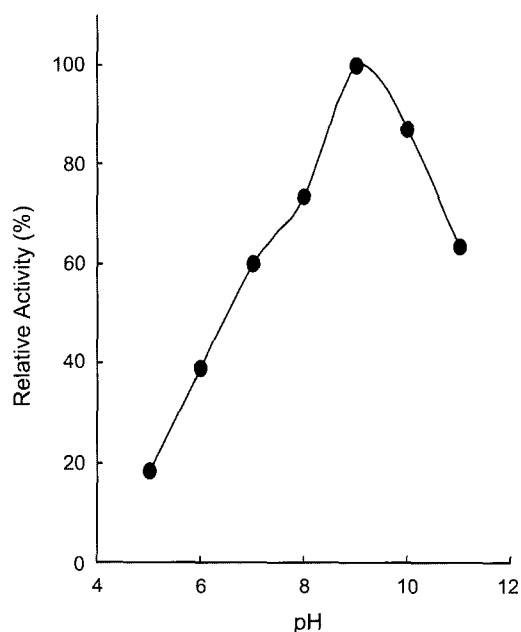
Fig. 1. Effect of pH on endopeptidase activity. The enzyme activity was measured using N-succinyl-Ala-Ala-Pro-Leu-p-nitroanilide as substrate in the 50 mM buffers of indicated pH at 37°C.
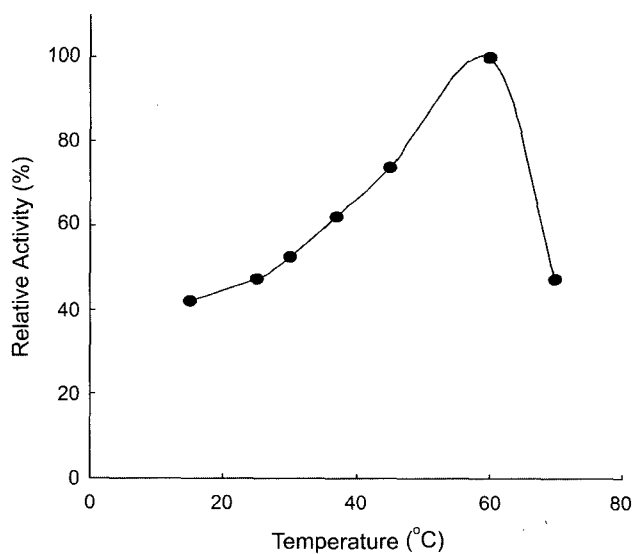

Fig. 2. Effect of temperature on endopeptidase activity. The protease activity towards N-succinyl-Ala-Ala-Pro-Leu-p-nitroanilide was measured at pH 8.0 at indicated temperatures.

Fig. 3. Analysis of kinetic parameters for the hydrolysis of N-succinyl-Ala-Ala-Pro-Leu-p-nitroanilide. Kinetic parameters of the enzyme using Lineweaver-Burk plot of 1/V against 1/[S]. Endopeptidase activity was measured in the reaction buffer containing the substrate within a range of 0.1 - 2 mM at 37°C and pH 8.0.

The pH activity curve was quite similiar to the pH profile with L-leucine p-nitroanilide as a substrate (Jung *et al.*, 1999). To establish optimum temperature, the hydrolysis of N-succinyl-Ala-Ala-Pro-Leu-p-nitroanilide was estimated in the range of 15 to 70°C. The effect of temperature on the enzyme activity is shown in Fig. 2. This enzyme activity was maintained within a broad temperature range. The enzyme displayed an apparently optimum activity at 60°C. The effect of temperature on endopepti-
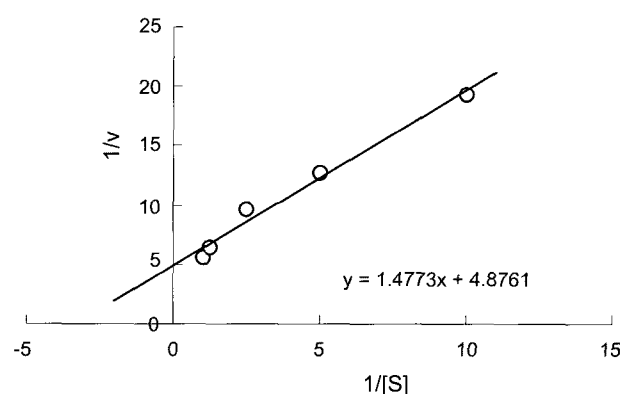
dase activity is very similar to the effect of temperature on the activity of leucine aminopeptidase (exopeptidase), activity of the enzyme (Jung *et al.*, 1999). This enzyme contained more than 30% of its relative activity to optimum temperature at 10°C, and also retained 30% of activity at 70°C.
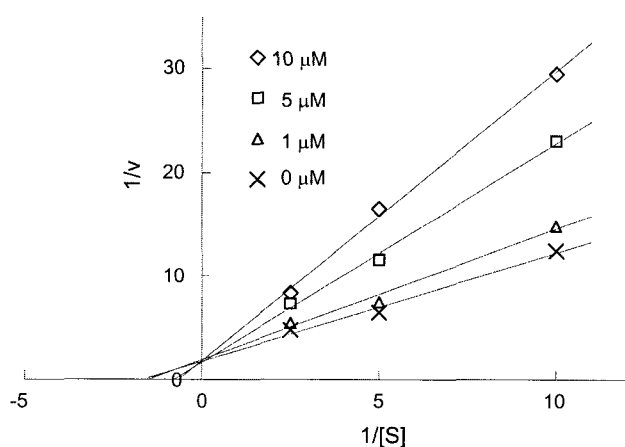
Determination of Kinetic Parameters

The kinetic parameters of the endopeptidase activity were measured by assaying the enzyme in the presence of a range of substrate concentrations. A Lineweaver-Burk plot of the data indicated that the K_m value of endopeptidase is 0.315 mM and V_{max} is 0.222 μmol of p-nitroanilide released/min (Fig. 3). These values are comparable with other peptidases, which range between 0.1 mM and 2 mM, and between 0.014 μM /min and 0.025 μM/min, respectively.

Effect of Inhibitors

The effect of protease inhibitors on hydrolytic activity of protease toward N-succinyl-Ala-Ala-Pro-Leu-p-nitroanilide as a substrate was measured (Table 2). The activity of protease was inhibited by EDTA, 1,10-phenanthroline, and EGTA, known as metalloprotease inhibitors. The partial inhibition by EGTA may indicate that Ca^{++} is required for full activity. EDTA and 1,10-phenanthroline significantly decreased the enzyme activity. This result indicated that divalent metal ions might be necessary for enzyme activity, acting as cofactors. The activity of peptidase was completely inhibited by PMSF, which acts as an inhibitor of serine protease. However, the serine residue in the endopeptidase did not respond to TLCK or TPCK, which typically inhibit the serine protease member of chymotrypsin and trypsin, respectively. It could be concluded from these data that this endopeptidase is a metal-activated enzyme with a loose interaction with metal, and seems to

Table 2. Effect of protease inhibitors on endopeptidase activity

Inhibitor	Concentration (mM)	Activity (%)
No addition		100
EDTA	0.01	55
	0.10	36
	0.50	16
1,10-phenanthroline	0.01	80
	0.10	45
	0.50	36
EGTA	0.01	83
	0.10	66
	0.50	66
Bestatin	0.001	94
	0.005	61
	0.010	46
	0.100	43
PMSF	0.01	33
	0.10	0
	0.50	0
TPCK	0.01	100
	0.10	85
	0.50	60
TLCK	0.01	80
	0.10	80
	0.50	80
Iodoacetamide	0.01	93
	0.10	93
	0.50	93

Table 3. Effect of metal ions on endopeptidase activity

Metal Ion	Concentration(mM)	Activity (%)
No addition		100
Ca^{++}	0.1	111
	1.0	186
Co^{++}	0.1	100
	1.0	125
Mg^{++}	0.1	100
	1.0	158
Mn^{++}	0.1	100
	1.0	139
Ni^{++}	0.1	103
	1.0	113
Zn^{++}	0.1	84
	1.0	88
Cu^{++}	0.1	67
	1.0	13
Fe^{++}	0.1	0
	1.0	0
EDTA(1 mM)[a]		0
+ Ca^{++} (1 mM)		111
+ Co^{++} (1 mM)		70
+ Mn^{++} (1 mM)		70
+ Mg^{++} (1 mM)		0
+ Zn^{++} (1 mM)		0
+ Ni^{++} (1 mM)		0
+ Fe^{++} (1 mM)		0

[a]To assay the reactivation effect of metal ion on EDTA-treated endopeptidase, the enzyme was first incubated with EDTA to eliminate the metals. The enzyme protein was recovered by spun column and the restored activity was assayed by adding metal ions back into the reaction mixture

Fig. 4. Analysis of kinetic parameters for the hydrolysis of *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide in the presence of Bestatin. Bestatin was added to each reaction at indicated concentrations. Bestatin is shown to be a competitive inhibitor to leucine endopeptidase activity. The K_i value for the hydrolysis of *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide was determined to be 6.7 μM.

have a serine residue at the active site. It was also inhibited by bestatin, which acts as a substrate analogue of *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide. Bestatin acts as a competitive inhibitor on the enzyme activity (Fig. 4). The K_i value for the hydrolysis of *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide was determined to be 6.7 μM, as determined by an analysis of the Dixon plot.

Effect of Metal ions
The effect of various metal ions on enzyme activity is shown in Table 3. Addition of Ca^{++}, Mn^{++}, and Mg^{++} stimulated the activity of the enzyme, while the addition of Cu^{++} and Fe^{++} inhibited activity. Co^{++}, Ni^{++}, and Zn^{++} did not affect enzyme activity. Notably, the addition of 1 mM Ca^{++} increased the activity to double that of the control.
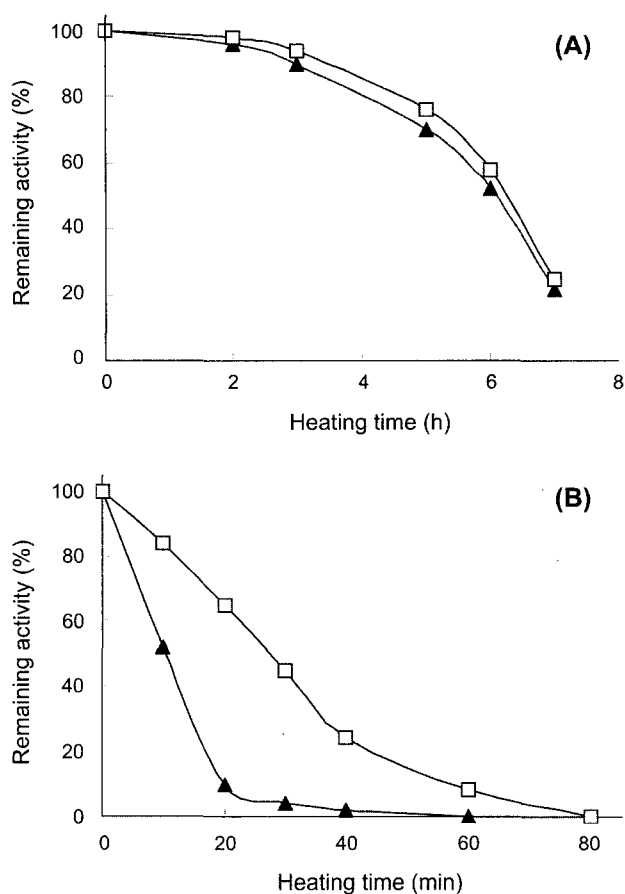
Fig. 5. Heat-stability of the endopeptidase. The enzyme was heat-treated at 60°C (A) or 70°C (B) for the indicated times. The remaining activity of the enzyme towards N-succinyl-Ala-Ala-Pro-Leu-p-nitroanilide was measured at 37°C and pH 8.0 in the absence (▲) or presence of 1 mM Ca^{++}(□).

The activity of EDTA-treated enzyme could be completely restored by the addition of Ca^{++} and partially restored by the addition of Co^{++} and Mn^{++}. The other metal ions had no effect on the restoration of enzyme activity. This result may suggest that Ca^{++} ions are required for the stimulation of endopeptidase activity, and Co^{++} and Mn^{++} ions affect endopeptidase activity.

Heat Stability

Thermal stability of the endopeptidase was measured at 60°C for 3 - 7 h. The activity was very stable to the heat treatment for 3 h. A significant decrease in the activity was not observed. After 5 h of incubation at 60°C, 70% of activity was maintained (Fig. 5A). However, protease was unstable at 70°C, and heat treatment at 70°C for 30 min resulted in a complete loss of activity (Fig. 5B). After heat treatment for 20 min, the enzyme retained 10% of its activity. However, the enzyme was stabilized by the addition of Ca^{++} ions. More than 80% of the activity was maintained by heat treatment at 70°C for 10 min when 1 mM Ca^{++} was added to the reaction mixture (Fig 5B). The

enzyme retained approximately 20% of its activity after heat treatment for 40 min at 70°C. These results suggest that Ca^{++} ion stabilizes the enzyme.

Discussion

This study describes the characterization of an endopeptidase from the psychrotrophic strain of *Bacillus cereus*. The results of the study revealed that the enzyme was optimally active at an alkaline pH of 8 - 9 and at 60°C. On the basis of its optimum pH, this protease would be grouped into the alkaline protease category. The enzyme retained higher levels of activity at low temperature. At temperatures below 20°C, the enzyme maintained over 40% of its activity at the optimum temperature. This protease retained higher hydrolytic activity towards synthetic substrates than protein substrates, especially at low temperatures. These data suggest that the enzyme may have increased structural flexibility at low temperatures. This prominent feature is common to cold-active enzymes, which exhibit high levels of activity at low temperatures. Cold-adapted microorganisms, which include psychrophiles and psychrotrophs, are known to produce cold-active enzymes. Homologous counterparts of the cold-active enzymes are produced by mesophilic or thermophilic microorganisms, but are less active at low temperatures (Kulakova *et al.*, 1999). In order to obtain high catalytic efficiency, cold-active enzymes have probably evolved to possess high conformational flexibility, although stability has been sacrificed (Davail *et al.*, 1994). Therefore, thermal instability of cold-adapted enzymes is regarded as a consequence of their conformational flexibility. When compared to their mesophilic counterparts, cold-active enzymes display a high thermosensitivity and a higher catalytic efficiency over a temperature range of roughly 0 - 30°C. However, the protease studied here was shown to be somehow thermotolerant.

Compared with thermolysin, this protease appeared to have a strong specificity for hydrolyzing peptides. This protease hydrolyzed the synthetic-peptide substrates bearing leucine at the P1 site, but could not hydrolyze those bearing tyrosine, arginine, lysine, valine, alanine, aspartic acid, and glutamic acid. This high substrate specificity may suggest that this protease would not play a significant nutritional role for the hydrolysis of protein substrates in *Bacillus* sp. It seems that enzymes with broader substrate specificity are more important for nutritional demand.

Metal chelators exerted a strong inhibitory effect on protease activity, suggesting that metal could be required for maximum activity of the endopeptidase to take place. The inhibition of EDTA and 1,10-phenanthroline on enzyme activity is a common feature of metalloprotease of *Bacillus* spp. (Takii *et al.*, 1987) and *Bacillus subtilis* (Matsubara and Feder, 1971). Ca^{++} has been demonstrated as the stimulator of the enzyme. Other divalent metal ions,

such as Co^{++}, Mg^{++}, Mn^{++}, and Ni^{++}, slightly enhanced the enzyme activity; Zn^{++}, Cu^{++}, and Fe^{++} inhibited the enzyme activity. Stimulation by Ca^{++} has also been demonstrated for enzymes from *Helicobacter pylori* (Windle and Kelleher, 1997), *Streptomyces exfoliatus* (Kim *et al.*, 1998), and *Vibrio anguillarum* (Farrell and Crosa, 1991). From the analysis of ICP-MS, the metal determination exhibited that a present protease contained zinc ions, with no other ions present (Jung *et al.*, 1999). These results also indicated that this protease is a metalloenzyme. Zn^{++} ions are actually necessary for the full activity of other microbial metalloproteases, whereas Ca^{++} ions are essential for their stability because they maintain the structure of the enzymes (Matsubara and Feder, 1971; Silder *et al.*, 1986). Based on data from this study, this may also be true for this protease. Calcium appears to have a protective effect of Ca^{++} upon heat denaturation (Croux *et al.*, 1990). This protective effect was also observed in our study. When Ca^{++} ions were added, the thermostability of enzyme was significantly increased. This indicates that calcium ions might be involved in the stabilization of the structure of enzymes when exposed to heat treatment. These properties are consistent with the pH stability (Watanabe *et al.*, 1993) and heat stability (Tajima *et al.*, 1976) of bacterial metalloproteases. The stabilizing effect by Co^{++} and Ca^{++} on heat denaturation is a well-known phenomenon of microbial neutral metalloproteases (Silder *et al.*, 1986).

The enzyme activity against *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide was also inhibited by PMSF, a serine protease inhibitor, but was not inhibited by other serine protease inhibitors such as TLCK, TPCK or iodoacetamide. The inhibitory effect of PMSF on the protease does not appear to be a general feature of the microbial metalloproteases. However, it was reported that DFP inhibited a neutral metalloprotease from *Bacillus cereus* (Sierecka, 1998). Our results suggest that this protease is the metal-activated endopeptidase with a serine residue at the active site, a protease which is essential for the catalytic reaction.

Most importantly, this protease exhibited both aminopeptidase and endopeptidase activities. The activity of aminopeptidase by the enzyme was described previously (Jung *et al.*, 1999). The k_{cat}/K_m value for *N*-succinyl-Ala-Ala-Pro-Leu-*p*-nitroanilide was higher than that for L-leucine-*p*-nitroanilide. Taken together, data in this study suggest that this enzyme has a dual function as a leucine-specific endopeptidase and a leucine aminopeptidase with higher specificity towards peptidyl leucine than free leucine on the N-terminus. This is the first detailed report of an extracellular protease with dual-function and leucine-specific endopeptidase activity, as well as leucine aminopeptidase. According to Rawlings and Barrett (1993), main groups of microbial metalloproteases may be distinguished. The protease in this study seems to be similar

to those of the M3 group, which possess activity of both endo- and exopeptidases. This protease seems to have an unusual feature; the leucine-specific endopeptidase activity is activated by Ca^{++}, while leucine aminopeptidase activity is activated by Co^{++}.

Acknowledgement

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