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Purification and Characterization of Co^{2+}-Activated Extracellular Metalloprotease from *Bacillus* sp. JH108

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Abstract An extracellular protease was purified to homogeneity from the culture supernatant of psychrotrophic bacteria *Bacillus* sp. JH 108 using procedures including ammonium sulfate fractionation, anion exchange chromatography, gel filtration chromatography, and cation exchange chromatography. The enzyme exhibited a molecular weight of 36 kDa, an optimum pH of 8 to 9, and optimum temperature of 60°C. The enzyme preferentially hydrolyzed leucine at the N-terminus of peptides and thus can be classified as an aminopeptidase. It was strongly inhibited by metal chelating agents such as EDTA and 1,10-phenanthroline. The activity lost by EDTA was restored with Zn^{2+} or Co^{2+}. These divalent cations also stimulated the native enzyme. This suggests that the enzyme is a metalloprotease acting as a leucine aminopeptidase.

Key words: Psychrotrophs, metalloprotease, leucine aminopeptidase, Co^{2+}-stimulated exopeptidase

Microbial proteases have certain unique characteristics, and are now widely used in the food, leather, detergent, and pharmaceutical industries. Accordingly, many studies on elucidating the function and characteristics of these proteases have been carried out. Microbial extracellular proteases are produced by various microorganisms including *Bacillus* [40], *Serratia* [41], *Vibrio* [8], and *Pseudomonas* [11]. These organisms produce several proteolytic enzymes, which seemingly generate small peptides from protein-based growth substances. Most extracelluar proteases that exhibit a maximal activity at a neutral pH are metalloproteases which require a metal ion such as zinc (Zn^{2+}) for their enzyme activity [3]. The *Bacillus* metalloprotease has an optimum pH around 7 and exhibits high hydrolytic activity toward the synthetic substrate furylacroylglycylleucine amide which is not easily hydrolyzed by most serine proteases [28]. Acidic metalloproteases exhibit their highest activity in a low pH region between 4-6, as in the case of the proteases from *Pseudomonas caseicolum* and *Penicillum roqueforti* [10]. Alkaline metalloproteases are also found widely in nature and have been shown to have an optimum pH range from 7 to 9 and a broad substrate specificity [17].

Aminopeptidases occur ubiquitously in organisms and are exopeptidases that catalyze sequential removal of amino acids from the unblocked N-termini of peptides and proteins. Aminopeptidases play a role in several important physiological processes including the catabolism of exogenously supplied peptides, and are necessary for the final steps of protein turnover [14]. Bacteria display several aminopeptidase activities which are either localized in the cytoplasm, on membranes, associated with the cell envelope or secreted into the extracellular media. Most aminopeptidases are metalloenzymes, although cysteine and serine aminopeptidases have also been identified [13]. The best studied aminopeptidases are large oligomeric metalloenzymes (bovine lens leucine aminopeptidase) that contain zinc, however, the role of the metal ion in the enzyme activity has not been completely elucidated [5].

In this study, the psychrotrophic *Bacillus* from trout intestines was isolated, and the purification and enzymological properties of a leucine aminopeptidase activated by Co^{2+} are described.

Materials and Methods

Chemicals

Unless specifically indicated, chemicals were obtained from Sigma. Water used in this study was prepared with a Millipore Milli-Q system and had resistance of 18 MΩ/cm^{-1}. The medium for the bacteria culture was purchased from Difco Co.

Bacteria Culture

The microorganism used in this study was *Bacillus* sp. JH108 isolated from rainbow trout whose habitat is in cold

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fresh-water (< 17°C). Cells were grown in a modified nutrient broth containing 2% skim milk for 24 h at 30°C while shaking at 120 rpm. The culture was centrifuged at 10,000 × g for 20 min (4°C).

Protease Assay

The enzyme activity was measured with azocasein. The standard mixture (0.5 ml) contained 50 mM Tris-HCl (pH 7.0), 0.1% azocasein, and the enzyme. The reaction mixture was incubated at 30°C for 60 min and then stopped by adding an equal volume of 10% TCA and standing the mixture on ice for 10 min. After removal of the precipitated azocasein by centrifugation (12,000 × g, 3 min), the absorbance was measured at 440 nm. The hydrolytic activity towards a synthetic substrate was estimated using amino acid *p*-nitroanilide. Unless otherwise indicated, the reaction mixture (0.1 ml) contained 0.2 mM amino acid p-nitroanilide and 50 mM MOPS (pH 7.0). The reaction was initiated by the addition of concentrated enzyme after preincubation of the reaction mixture at 30°C for 5 min, and then incubated at 30°C for the indicated times. The 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 for the production of 1 micromole of *p*-nitroanilide per minute [3].

Protein Determination

The protein concentration was estimated according to the method of Bradford [4] or by using the BCA kit [30]. Bovine serum albumin was employed as the standard.

SDS-Polyacrylamide Gel Electrophoresis

Proteins were analyzed on SDS-PAGE using 10% acrylamide gels by the method of Laemmli [20]. After electrophoresis, the gel was stained with Coomassie Brilliant blue R-250 to visualize protein bands.

Substrate Gel Electrophoresis

The protease activity was detected in SDS-polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gel was soaked in a nonionic detergent solution (2.5% Triton X-100 in 50 mM Tris-HCl, pH 7.0) for 15 min with gently shaking and then incubated at 30°C for the indicated times in a substrate buffer containing 0.02% NaN_3 in 50 mM Tris-HCl (pH 7.0). Thereafter, the gel was stained with Coomassie Brilliant blue R-250.

Purification of Metalloprotease

The enzyme yield depended on the cultivation time. Maximal enzyme production was achieved by incubating the bacteria in a nutrient broth composed of 1/5 of the amount of the original and 2% skim milk for 24 h at 30°C. All purification procedures were performed at 4°C. The purification of the enzyme was initiated by concentrating an 8-l culture supernatant. The protease was then precipitated from the culture supernatant with ammonium sulfate (30–60% saturation), the precipitate was dissolved in 40 ml of 20 mM Tris-HCl (pH 7.0), and dialyzed against the same buffer. The dialysate was loaded onto a column of DEAE-Sephadex A 50 (2.5×20 cm) equilibrated with 20 mM Tris-HCl (pH 7.0). After being washed with the same buffer, the column was eluted with a gradient of 0.1 to 0.4 M NaCl in 20 mM Tris-HCl (pH 7.0) buffer. The activity was detected at 0.1 M NaCl fractions. The active fractions were combined and solid ammonium sulfate was added to produce a final concentration of 60% saturation. The pellet was resuspended in 8 ml of 20 mM Tris-HCl (pH 7.0). This solution was applied to a column (1.5×100 cm) of Sephacryl S-100 equilibrated with 20 mM Tris-HCl (pH 7.0) and the column was eluted at a flow rate of 0.4 ml/min. The active fractions collected from the Sephacryl S-100 column were applied to a column of CM-Sepharose CL 6B (1.5×8 cm) equilibrated with 20 mM Tris-HCl (pH 7.0). After the column was washed with the same buffer, the enzyme was eluted batch-wise with 0.2, 0.5, and 0.8 M NaCl prepared in 20 mM Tris-HCl (pH 7.0) at a flow rate of 1 ml/min. The enzyme was eluted at 0.2 M NaCl. A single peak showing protease activity was concentrated to 2 ml, and the purity was confirmed by SDS-PAGE.

Effect of Metal Ions and Inhibitors on Proteolytic Activity

Metal ions and inhibitors with indicated concentrations were added to the assay mixture during prewarming of the enzyme at 37°C, 10 min before the addition of the substrate. Phenylmethylsulfonyl fluoride and pepstatin stock solutions were prepared in ethanol before being diluted with the assay buffer. The activity was then expressed relative to controls which did not contain inhibitors, but with the same amounts of ethanol. The metals used were chloride salts.

RESULTS

An enzyme exhibiting the properties of a leucine metallo-aminopeptidase (see below) was purified 545-fold by conventional chromatographic techniques (Table 1). The effect of metal chelating agents was examined by substrate gel electrophoresis during the purification steps. When the proteolytic activities were assayed on gels containing 0.1% gelatin in either the presence or absence of EDTA, several activity bands indicated by clear regions were observed without EDTA, demonstrating that the bacteria isolated from the rainbow trout intestine secreted many extracellular proteases. In the presence of EDTA, however, several bands including the target enzyme had disappeared. Figure

Table 1. Purification of metalloprotease from *Bacillus* sp. JH108.

Purification step	Total Volume (ml)	Total Protein (A) (mg)	Total Activity (B) (unit)	Specific Activity (unit/mg)	Purification fold	Recovery (%)
Crude Extract	4,000	20,700	12,740	0.615	1	100
Ammonium Sulfate 30-60%	50	255	1,428	5.6	9.1	11.2
DEAE-Sephadex A-50	35	3.4	1,013	298	485	8
Sephacryl S-100	20	1.5	494	329	535	3.9
CM-Sepharose CL-6B	6	0.55	184	335	545	1.4

One unit of the activity was defined as the increase of A_{440} by 0.001 per min at 30°C using azocasein as the substrate. (A), Determined by BCA assay; (B), One unit of activity was arbitrarily defined as the increase of the A_{440} by 0.001 per minute at 30°C.

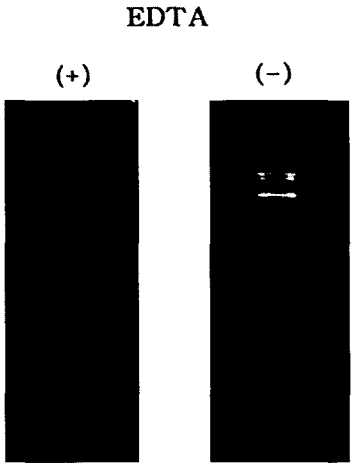

Fig. 1. Zymographic analysis of extracellular proteases using gelatin substrate gel.
Aliquots (20 µl) of the culture supernatant were electrophoresed in 10% SDS-polyacrylamide gels impregnated with 0.1% gelatin. Following electrophoresis, the gels were washed in 2.5% Triton X-100 and incubated at 30°C for 18 h in substrate buffer with or without 1 mM EDTA. Following incubation, the gels were stained in Coomassie Blue for 2 h and then destained. The enzyme-mediated gelatin lysis appears as the clear zones against the dark background (→).

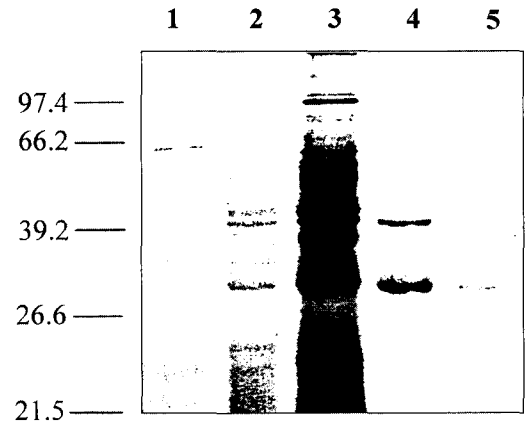

Fig. 2. 10% SDS-polyacrylamide gel electrophoresis of the metalloprotease during the purification steps.
The protease migrates as a single band with an apparent molecular mass of 36-kDa. Lane 1, crude extract; lane 2, ammonium sulfate 30-60% fractions; lane 3, DEAE Sephadex A-50 fractions; lane 4, Sephacryl S-100 fractions; lane 5, CM Sepharose CL-6B fractions. The bars on the left indicate the positions of the molecular weight markers.

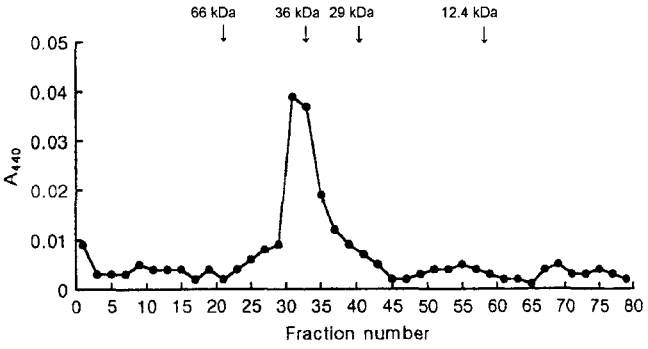

Fig. 3. Elution profiles of the purified protease from the gel filtration column.
The purified protease was chromatographed on a Sephacryl S-100 column (1.5×100 cm) as described in the text. Fractions of 1 ml were collected at a flow rate of 25 m/h. The proteolysis was assayed at 37°C using azocasein as the substrate, as described under Materials and Methods. The elutions of the molecular weight markers (BSA, 66 kDa; carbonic anhydrase, 29 kDa) are indicated by arrows at the top.

1 indicates that the enzyme has a small molecular weight. An examination of the enzyme by SDS-PAGE revealed a highly purified preparation containing only a single protein band (Fig. 2). SDS-PAGE gave a molecular mass of 36 kDa, and the native enzyme exhibited a monomeric form of 36 kDa as determined by gel filtration chromatography on a calibrated Sephacryl S-100 column (Fig. 3).

Effect of pH and Temperature

The enzyme activity was measured using L-Leucine p-nitroanilide as the substrate over a pH range from 4.0 to 11.0. The enzyme was active over pHs 6 to 11 with an optimum at pH 9.0 (Fig. 4). A variation in the buffers had a slight effect on the enzyme activity: at pH 8, the Tris buffer yielded 98% of the activity compared with pH 9.0, and the activity in the HEPES buffer was 93.5% of that in the Tris buffer at the same pH and concentration. When the proteolytic activities were examined with a protein substrate (Azocasein), the pH profile was somewhat different as

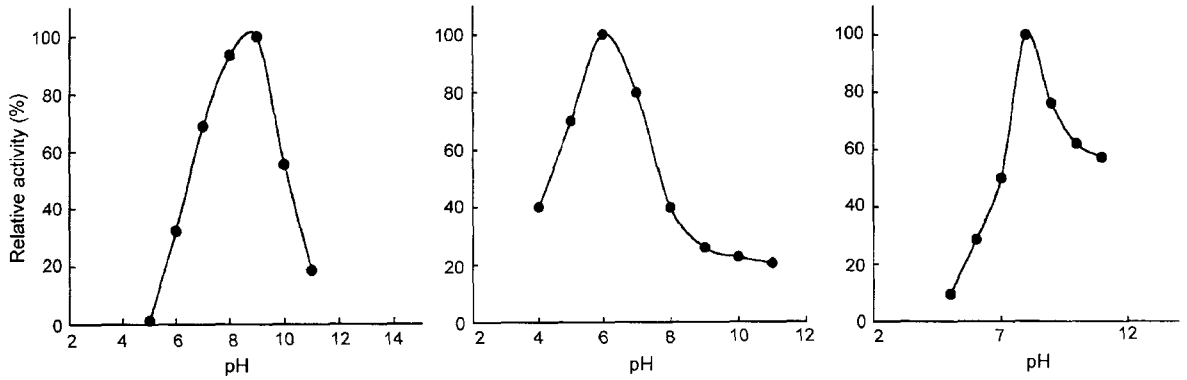

Fig. 4. Effect of pH on the activity of Leucine aminopeptidase.
Panel A, the hydrolysis of Azocasein was measured at 37°C in 50 mM buffers. Azocasein (0.1%) was incubated for 120 min with enzyme (2.6 μM). The activities were determined as described in Materials and Methods. Panel B, the hydrolysis of L-Leucine *p*-nitroanilide was measured at 37°C in 50 mM buffers. The enzyme activity is given in the relative amount of liberated p-NA for 30 min. Panel C, relative activities in 50 mM buffers. The hydrolysis of L-Leucine *p*-nitroanilide was measured at 37°C in buffers containing 1mM $CoCl_2$. The buffers used were MES-NaOH for pHs 5, 6, and 7, MOPS-NaOH for pHs 6, 7, and 8, Tris-HCl and HEPES-NaOH for pHs 7, 8, and 9, and CAPS-NaOH for pHs 9, 10, and 11. The variations of the activities in the different buffers at the same pHs were normalized to the values obtained with the Tris buffer.

shown in Fig. 4B. The maximum proteolysis occurred at pH 6. Furthermore, in contrast to the synthetic substrate (Leu-p-NA) (Fig. 4A), a significant decrease of enzyme activity was observed with the protein substrate at an alkaline pH above 8. This could be partly due to destabilization of the protein substrate. When 1 mM Co^{++} was included in the reaction mixture (Fig. 4C), a high level of hydrolysis of L-Leucine *p*-nitroanilide was observed at alkaline pHs and the optimum pH was around 8. This may be due to stimulation of the enzyme activity by Co^{++} at higher pHs. The effect of temperature on the catalytic activity is shown in Fig. 5. The enzyme activities with L-Leucine *p*-nitroanilide as the substrate were measured at various temperatures for 10, 30, 60, and 120 min. Judging from the initial velocity, the enzyme exhibited its maximal activity at around 60°C. This activity was immediately lost at above 70°C because of thermal inactivation (Fig. 5A). At 10°C, the enzyme had over 30% of its activity at optimum temperature. Between 60 and 120 min, there was a greater increase in the protease activity at 37°C than at above 50°C (not shown), presumably indicating more stability at lower temperatures. The hydrolysis of azocasein was also influenced by temperature (Fig. 5B). The protein substrate was hydrolyzed at the highest rate at 45°C, pH 7.

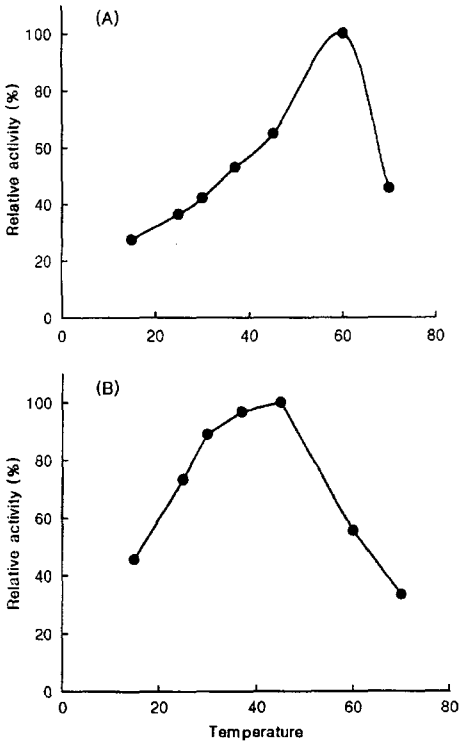

Fig. 5. Effect of temperature on the activity of Leucine aminopeptidase.
Panel A, the enzyme activities towards L-Leucine *p*-nitroanilide were measured at different temperatures. L-Leucine *p*-nitroanilide (0.2 mM) was incubated with 2.6 μM enzyme for 30 min at pH 8. Panel B, the enzyme activity was determined using Azocasein (0.1%) as the substrate in the reaction buffer (pH 7) for 120 min.

Substrate Specificity

The specificity of the enzyme was explored using a series of amino acid p-nitroanilde substituents (Table 2). The enzyme rapidly hydrolyzed L-Leucine *p*-nitroanilide more than other aminoacyl p-nitroanilides. There was no measurable hydrolysis of Glu-pNA, Lys-pNA, Arg-pNA, *N*-benzoyl Arg-pNA, and *N*-benzoyl Tyr-pNA. However, the enzyme was able to release *p*-nitroanilide at a lower level (5%) from Phe-pNA containing a modified moiety at its free NH_2 group (*N*-glutaryl-Phe-pNA). Alanyl and methionyl p-nitroanilide were also cleaved at 30–50% of the rate of Leu-pNA. The kinetic parameters for the hydrolysis of the hydrolyzable substrates by the enzyme were then estimated. For the Leu-pNA hydrolysis, the Michaelis constant (K_m) and

maximum initial velocity (V_{max}) determined by a Lineweaver-Burk plot were 0.61 mM and 0.125 μmol of the pNA released/min, respectively (Fig. 6A). These values are comparable with other aminopeptidases, which range between 0.1 and 2 mM [13, 15]. The K_m and V_{max} values for the hydrolysis of Ala-pNA were estimated to be 0.3 mM and 0.023 μM/min, and 0.46 mM and 0.014 μM/min for Met-pNA, respectively.

Table 2. Substrate specificity of the metalloprotease.

Substrate	Initial Velocity (μM/min)
L-Leucine *p*-nitroanilide	0.041
L-Alanine *p*-nitroanilide	0.022
L-Methionine *p*-nitroanilide	0.015
L-Lysine *p*-nitroanilide	0
L-Glutamic acid γ-(*p*-nitroanilide)	0
N-Acetyl-L-Alanine *p*-nitroanilide	0.001
N_α-Benzoyl-DL-Arginine *p*-nitroanilide	0.001
N-p-Tosyl-Gly-Pro-Lys *p*-nitroanilide	0
N-Glutaryl L-Phenylalanine *p*-nitroanilide	0.001
N-Benzoyl L-Tyrosine *p*-nitroanilide	0

Each assay was carried out at 37°C in 50 mM Tris-HCl (pH 8.0) and the change in absorbance at 405 nm was monitored for each substrate.

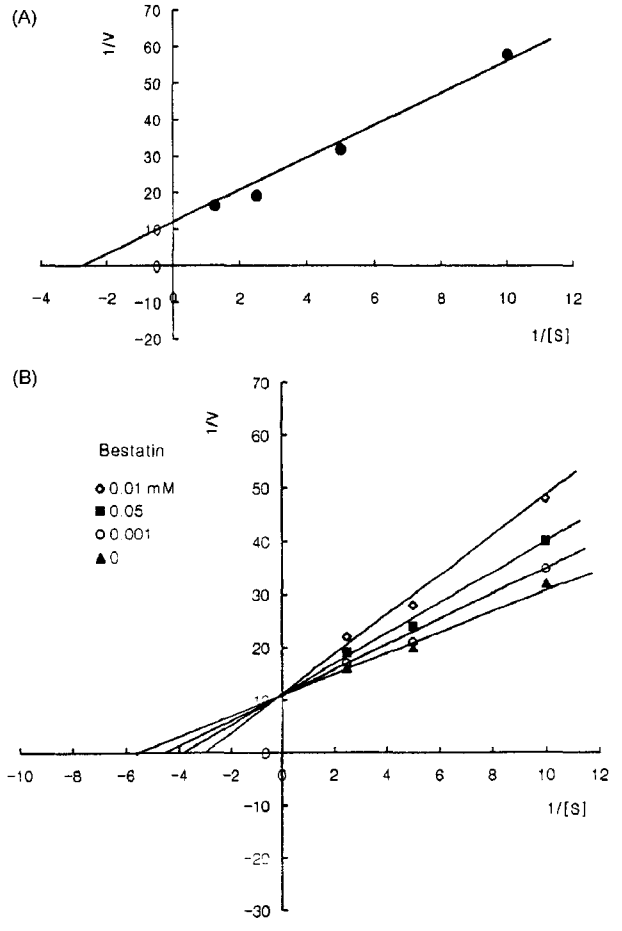

Fig. 6. Analysis of kinetic parameters for the hydrolysis of Leu-p-NA.

Kinetic parameters of the enzyme using Lineweaver-Burk plot of 1/v against 1/s. The leucine aminopeptidase activity was measured in the reaction buffer containing the substrate within a range of 0.1-2 mM at 37°C and pH 8 (A). Bestatin, at indicated concentrations, was added to each of the reactions (B).

Effect of Inhibitors

The effect of a series of protease inhibitors on the enzyme activity was studied (Table 3). In these experiments, each compound at an indicated concentration was tested after a 10 min preincubation with the enzyme. The effect of the inhibitors on the enzyme activity against leucine-pNA was examined. Iodoacetamide had no significant inhibitory effect on the activity. The serine-type protease (leupeptin, phenylmethylsulfonyl fluoride) and aspartic protease (pepstatin) inhibitors also had no effect on the enzyme

Table 3. Effect of protease inhibitors on enzyme activity.

Inhibitor	Concentration (mM)	Remaining activity (%)
No addition		100
EDTA	0.001	94.1
	0.01	29.4
	0.05	11.8
	0.1	0
1,10-Phenanthroline	0.01	99.9
	0.05	24.9
	0.1	0
Bestatin	0.001	93.5
	0.01	76
	0.05	57
	0.1	23
Leupeptin	0.01	95.9
	0.05	89.1
	0.1	85.9
	1	65.6
PMSF	0.002	102
	0.01	96.8
	0.1	81.1
	1	74
Pepstatin A	0.001	96.3
	0.01	88.9
	0.05	77.8
	1	69.3
Iodoacetamide	0.002	100
	0.01	99.2
	0.1	88.1
	1	81.5

The enzyme (0.26 μM) was preincubated with each metal ion in 50 mM. MOPS (pH 7) at 37°C for 10 min and the activities were determined at 37°C for 120 min with 0.2 mM L-Leucine *p*-nitroanilide as substrate.

activity. In contrast, bestatin, a specific inhibitor of leucine aminopeptidase, showed an inhibitory effect similar to that of EDTA. Bestatin behaved as a competitive inhibitor of the enzyme activity. The K_i value for the hydrolysis of leucine *p*-nitroanilide was determined to be 5.8 μM by a Dixon plot (Fig. 6B). Furthermore, the enzyme activity was strongly inhibited by metal chelating agents. When 0.1 mM EDTA or 1 mM 1,10-phenanthroline were added, no activity was detected. The slightly stronger effect of EDTA over 1,10-phenanthroline on enzyme activity is a common feature of neutral metalloproteases of the *Bacillus* species [31].

Effect of Metal Ions

The effect of different metal ions on the enzyme activity was investigated by adding the indicated concentrations of metals to the reaction mixture. As shown in Table 4, the addition of Cu^{2+}, Ni^{2+}, or Zn^{2+} resulted in decrease of the enzyme activity. Mg^{2+}, Ca^{2+}, and Fe^{2+} had no effect on the enzyme activity. However, the addition of 1 mM Co^{2+} increased the proteolytic activity two-fold over the control. The activity eliminated by EDTA was fully restored by the addition of Co^{2+}. A less efficient reactivation (50%) occurred with Mn^{2+}, Mg^{2+}, and Zn^{2+}. The other metal ions had no effect on the restoration of the enzyme activity. The metal contents of the enzyme were determined by ICP-MS. This analysis indicated that the enzyme contained Zn^{2+} (1.2±0.2 atoms per molecule of enzyme), but no Co^{2+}.

Table 4. Effect of metal ions on the activity of Leucine aminopeptidase.

Cation	Concentration (mM)	Relative activity (%)
No addition		100
Mn^{2+}	0.1	100
	1	57
Co^{2+}	0.1	116
	1	204
Ni^{2+}	0.1	80
	1	27
Mg^{2+}	0.1	112
	1	119
Ca^{2+}	0.1	94
	1	105
Fe^{2+}	0.1	91
	1	77
Zn^{2+}	0.1	91
	1	53
Cu^{2+}	0.1	54
	1	12
EDTA (0.1 mM)		0
$+Zn^{2+}$	1	40
$+Co^{2+}$	1	112
$+Mn^{2+}$	1	66
$+Mg^{2+}$	1	66
$+Ca^{2+}$	1	0
$+Ni^{2+}$	1	0
$+Fe^{2+}$	1	0

The enzyme (0.26 μM) was preincubated with each reagent in 50 mM MOPS (pH 8.0) at 37°C for 10 min. To assay the reactivation effect of metal ions on EDTA-treated Leucine aminopeptidase, the enzyme was first incubated with 0.01 mM EDTA (100% inhibition) in the reaction mixture at 37°C.

DISCUSSION

This study describes the purification of an aminopeptidase from psychrotophic bacteria and the characterization of some of its properties. The substrate and inhibitor specificity results support the conclusion that the enzyme can be classified as a metalloprotease and leucine aminopeptidase.

Aminopeptidases are widely distributed in nature and are generally classified in terms of their substrate specificity, i.e. preference for neutral, acidic, or basic amino acid. These enzymes can also be classified based on their catalytic mechanism: metallo-, cysteine-, serine-, and aspartyl-aminopeptidase, the former type being predominant in bacteria [13]. The activity of this enzyme was characterized by an activation effect involving either Mn^{2+} or Mg^{2+}, and the maximum peptidase activity was achieved using substrates containing a leucine residue at the N-terminus. The second type of aminopeptidase activity was characterized by requirements for Co^{2+}, Zn^{2+}, or Mn^{2+}, and the maximum activity was achieved using alanine-β-naphthylamide as the substrate [22]. This enzyme was subsequently named alanine aminopeptidase. The third type of aminopeptidase seemed to be similar to the second one except that it lacked the requirement for metal ion. This type of enzyme together with alanine aminopeptidase were inhibited by puromycin whereas leucine aminopeptidase was not [15]. Aspartyl (or glutamyl) aminopeptidase exhibited a preference for N-terminal aspartyl and glutamyl residues [39].

The results in this study revealed that the enzyme was optimally active at an alkaine pH of 8-9 and at 60°C. The molecular mass of the enzyme estimated by Sephacryl S-100 gel filtration was 35,600 and by SDS-PAGE, 36,000, indicating that the native enzyme was active in a monomeric form. Cobalt (Co^{2+}) was identified as the activator of the enzyme. Other divalent metal ions such as Mg^{2+} and Ca^{2+} did not affect the enzyme activity and inhibitory effects with Cu^{2+}, Ni^{2+}, and Zn^{2+} were also found. The enzyme activity was inhibited by both EDTA and 1,10-phenanthroline. A metal determination using an ICP-

MS analysis showed that the protease contained zinc ions and no other ions. Therefore, these results indicate that the protease is a metalloenzyme. The enzyme activity eliminated by EDTA was reactivated by Co^{2+} and Zn^{2+}. The effect of the metal chelators was thus reversible; the most potent metal ion that reactivated the EDTA-treated enzyme was Co^{2+}.

Most microbial neutral metalloproteases contain 1 atom of zinc per molecule [7]. The *Streptomyces griseus* aminopeptidase is a calcium-activated zinc metalloenzyme characterized by a high enzymic activity [2]. The aminopeptidase from *Lactobacillus casei* subsp. *rhamnosus* hydrolyzes a wide range of substrates and its enzyme activity is inhibited by EDTA. Cobalt (Co^{2+}) was the only metal ion which dould stimulate the aminopeptidase activity [1]. The metalloprotease from *Pseudomonas fluorescens* is a Zn-containing metalloenzyme, which has been shown to be strongly inhibited by EDTA. No activation by Co^{2+} has been observed with this enzyme [19]. The fact that enzyme activity can be stimulated by replacement of a prosthetic ion by Co^{++} has already been observed in the Zn-containing metalloprotease of *Bacillus cereus* [9]. Similar results were also reported by Van Wart and Lin [35] for porcine kidney leucine aminopeptidase. This enzyme was shown to be a zinc metalloenzyme which is activated by Mn^{2+} and Mg^{2+}, and inhibited by Ni^{2+}, Cu^{2+}. These results can be explained by supposing that these ions interact with a metal binding site that is different from that originally occupied by Zn^{2+}. The presence of metal binding sites for both Zn^{2+} and Co^{2+} has also been suggested by Garner and Behal (1974) [12]. The presence of a secondary metal-binding site was clearly demonstrated for leucine aminopeptidase isolated from bovine lens [32]. In this case, enzyme activation with Mn^{2+} or Mg^{2+} results from the binding of these ions to a secondary site, named a regulatory site, without displacement of the Zn^{2+}. The inhibition and activation of an enzyme can both result from the binding of metal ions to a regulatory site. The aminopeptidase from porcine liver exhibits a broad specificity, an optimum pH between 6.5 and 7.0, and is activated by Co^{2+}, which is pH-dependent. However, cobalt ion has an inhibitory effect on the enzymic activity at above pH 7.5 [18].

In this study, PMSF, leupeptin, iodoacetamide, and p-chloromercuribenzoic acid had no inhibitory effects on the enzyme activity against leucine *p*-nitroanilide. The SH-residues were not significantly involved in the functional groups of the enzyme. Like leucine aminopeptidase, proteasein cleaved L-leucine-*p*-nitroanilide more rapidly than other aminoacyl-*p*-nitroanildes. The highest enzymatic velocity and hydrolytic coefficient (k_{cat}/K_m) were obtained with L-leucine-*p*-nitroanilide. Accordingly, the protease under study is a leucine aminopeptidase. In support of the above contention, the enzyme was markedly inhibited by bestatin, a recognized leucine aminopeptidase inhibitor.

The native enzyme molecular mass was lower than those of other aminopeptidases such as the aminopeptidase from *Staphylococcus chromogenes* (450 kDa) [42], human tissues (liver, pancreas; 235 kDa, intestine; 206 kDa) [29], chicken skeletal muscle (185 kDa) [25], porcine intestinal mucosa (130 kDa), rat brain (102 kDa) [37], porcine liver (96 kDa) [18], carp ordinary muscle (84 kDa) [6], *Streptomyces rimosus* (83 kDa) [35], human serum (58 kDa) [32], and human melanoma cell (52 kDa) [34]. The zinc-binding leucine aminopeptidase from *Aeromonas proteolytica* is active as a monomer with an M_r of 30,000 that binds two Zn^{2+} atoms [26].

The purified aminopeptidase from psychrotrophic *Bacillus* sp. JH108 had an optimal activity temperature at 50°C and maintained a relatively high enzyme activity at lower temperatures (below 20°C). Similar results have been previously reported, where low optimum temperatures have been observed with other proteases from psychrophiles and psychrotrophs. An alkaline protease from *Escherichia freundii* had an optimum temperature of 25°C at pH 10 [24]. However, a yeast, isolated in Antarctica, *Candida humicola*, produced a protease with an optimum temperature of 37°C [27] and the psychrophile *Xanthomonas maltophila* produced a protease with an optimum temperature of 50°C [21].

Psychrotrophic microorganisms have been isolated not only from permanently cold environments but also from seasonally cold environments because of their ability to tolerate higher temperatures [23]. Because of their relatively high activities at low temperatures, psychrotrophs are of great interest for their potential biotechnological and industrial applications.

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