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The Purification and Characterization of a *Bacillus stearothermophilus* Methionine Aminopeptidase (MetAP)

Jae Min Chung, Il Yup Chung and Young Seek Lee*

Department of Biochemistry and Molecular Biology, Hanyang University, Sa-1-dong 1271, Ansan, Kyunggi-do, 425-791, Korea

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Methionine aminopeptidase (MetAP) catalyzes the removal of an amino-terminal methionine from a newly synthesized polypeptide. The enzyme was purified to homogeneity from *Bacillus stearothermophilus* (KCTC 1752) by a procedure that involves heat precipitation and four sequential chromatographs (including DEAE-Sepharose ion exchange, hydroxylapatite, Ultrogel AcA 54 gel filtration, and Reactive red 120 dye affinity chromatography). The apparent molecular masses of the enzyme were 81,300 Da and 41,000 Da, as determined by gel filtration chromatography and sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), respectively. This indicates that the enzyme is comprised of two identical subunits. The MetAP specifically hydrolyzed the N-terminal residue of Met-Ala-Ser that was used as a substrate, and exhibited a strong preference for Met-Ala-Ser over Leu-Gly-Gly, Leu-Ser-Phe, and Leu-Leu-Tyr. The enzyme has an optimal pH at 8.0, an optimal temperature at 80ºC, and pI at 4.1. The enzyme was heat-stable, as its activity remained unaltered when incubated at 80ºC for 45 min. The *Km* and *Vmax* values of the enzyme were 3.0 mM and 1.7 mmol/min/mg, respectively. The *B. stearothermophilus* MetAP was completely inactivated by EDTA and required Co^{2+} ion(s) for activation, suggesting the metal dependence of this enzyme.

Keywords: *Bacillus stearothermophilus*, Methionine aminopeptidase, Co^{2+} ion, Metalloenzyme

Introduction

Although the biosynthesis of polypeptides begins with methionine (formyl-methionine in bacteria), the majority of mature proteins do not have the amino acid at their N-termini (Flinta *et al.*, 1986). Methionine aminopeptidase (MetAP) is an enzyme that is responsible for removing the amino-terminal methionine (Ben-Bassat *et al.*, 1987; Bradshaw *et al.*, 1998). The hydrolysis of the methionine usually occurs only when the penultimate amino acid that is adjacent to the initiator methionine has a radius of gyration of 1.29 Å or less. These second amino acids are glycine, alanine, proline, serine, cysteine, threonine, and valine (Ben-Bassat *et al.*, 1987; Miller *et al.*, 1987; Chang *et al.*, 1990; Moerschell *et al.*, 1990; Kendall and Bradshaw, 1992). The protein with the removed methionine can be subjected to further modifications (Arfin and Bradshaw, 1988).

This enzyme seems to be ubiquitous and was found in a number of organisms that includes *Escherichia coli* (Ben-Bassat *et al.*, 1987), *Pyrococcus furious* (Tsunasawa *et al.*, 1997), *Salmonella typhimurium* (Movva *et al.*, 1990), *Saccharomyces cerevisae* (Chang *et al.*, 1990; Li and Chang, 1995), swine (Kendall and Bradshaw, 1992), and humans (Arfin *et al.*, 1995). Based on an amino acid sequence alignment, MetAP is divided into two major classes (type I and II) (Arfin *et al.*, 1995). An analysis of the X-ray structures demonstrates that both types of MetAP commonly possess a pseudo two-fold symmetry in a catalytic domain that consists of α-helices and β-strands, each coordinated with a single Co^{2+} ion (Bazan *et al.*, 1994; Lowther and Matthews, 2000). Type I and II primarily differ in the presence of a helical domain of approximately 60 residues that are inserted within the C-terminus of type II MetAP. The insert matches no known proteins in the sequence and structural motifs. The type I enzymes are found in bacteria, while the type II MetAPs are found in Archaea. Both types occur in yeast and humans (Chang *et al.*, 1990; Li and Chang, 1995).

EDTA treatment causes the loss of MetAP activity, indicating that MetAP is a metalloenzyme. Different metal ions are known to activate various MetAPs *in vitro* - Co^{2+} and Zn^{2+} for *E. coli* MetAP (Ben-Bassat *et al.*, 1987; Lowther *et al.*, 1999); Co^{2+}, Zn^{2+}, Mn^{2+}, and Ni^{2+} for yeast MetAP-Ib (Walker and Bradshaw, 1998): Co^{2+} for yeast MetAP-IIa (Chang *et al.*, 1992): and Co^{2+} and Mn^{2+} for human MetAP (Li and Chang, 1996). Co^{2+} is initially considered a primary

*To whom correspondence should be addressed.
Tel: 82-31-400-5512 Fax: 82-31-419-1760
E-mail: yslee@hanyang.ac.kr

cofactor for all MetAPs. However, Walker and Bradshaw recently demonstrated that the yeast MetAP I is dependent on Zn^{2+} rather than Co^{2+} in the presence of glutathione, where its reduced form abrogates Co^{2+}-dependency for the MetAP. This suggests that Zn^{2+} is likely a physiologically relevant metal (Walker and Bradshaw, 1998). In addition, Co^{2+} and Fe^{2+} strongly stimulate the activity of MetAP that is obtained from anaerobically cultured *E. coli* cells in the presence of reduced glutathione (Dsouza and Holz, 1999). Thus, the natural metals that are associated with this family of enzymes remains uncertain.

The targeted disruption of the MetAP gene resulted in a loss of cell viability or retarded growth. The inactivation of MetAP in *E. coli* and *S. typhimurium* had a lethal effect (Chang *et al*., 1989; Miller *et al.*, 1989). Similarly, the deletion of both MetAPI and MetAPII in yeast caused cell death, and the cell that lacked either one of the two exhibits had slow growth (Li and Chang, 1995). Thus, the presence of MetAP is necessary for cell viability. Although the physiological role of amino-terminal methionine removal is still unclear, there are several possible explanations. Amino acids, other than methionine at the N-terminus, are required for the activity of certain enzymes, as well as further post-translational modifications, such as acetylation (Taylor, 1993). Methionine, one of only two sulfur-containing amino acids, is recycled through such removal. The removal of amino-terminal methionine contributes to the regulation of protein stability (Bachmair *et al.*, 1985). In this study, we purified and characterized a MetAP from *Bacillus stearothermophilus*. This enzyme is a heat-stable, dimeric protein, and requires Co^{2+} as a cofactor.

Materials and Methods

Reagents L-amino acid oxidase, o-dianisidine, peroxidase, PMSF, $CoCl_2$, Met-Ala-Ser, Leu-Gly-Gly, Leu-Ser-Phe, Leu-Leu-Tyr, DEAE-Sepharose CL-6B, low molecular weight marker of protein, antifoam, and blue dextran were purchased from Sigma (St. Louis, USA). Molecular weight markers for gel filtration and isoelectric focusing were obtained from Bio-Rad (Hercules, USA). Hydroxylapatite fast flow was purchased from Calbiochem (San Diego, USA). Ultrogel AcA 54 was obtained from Sepracor-IBF (Marlborough, USA). Metal ions were purchased from Fisher Scientific (Fair Lawn, USA). Bacto trypton, yeast extract, and bacto agar were obtained from Difco (Detroit, USA).

Bacterial strain and culture conditions The bacterial strain that was used in this study was *Bacillus stearothemophilus* KCTC 1752, obtained from the Korean Cell Line Bank. The cells were grown for 36 h at 55°C in a 2.5 liter-fermentor with a medium that contained yeast extract 24 g, tryptone 12 g, K_2HPO_4 3 g, and KH_2PO_4 1 g per liter, pH 7.0-7.5. The agitation was maintained at 450 × rpm. A 40 ml of 40% (w/v) D(+)-glucose was added to a culture medium when the cell growth reached an early stationary phase. The cells were harvested by centrifugation at 3,000 × *g* at 4°C for 40 min. The average yield was 25 g (wet weight) per liter.

Enzyme purification All of the procedures were carried out at 4°C unless otherwise mentioned. The cells (wet weight, 80 g) were washed with ten volumes of buffer A (100 mM potassium phosphate, pH 7.0, 0.2 mM $CoCl_2$, and 0.1 mM PMSF), resuspended in three volumes of the same buffer, disrupted by ultrasonication at 80 watt (20 cycle, beating for 15 sec and cooling for 45 sec by immersing in ice/water), and centrifuged at 10,000 × *g* for 30 min. The supernatant was saved and subjected to ultracentrifugation at 100,000 × *g* for 2 h. The resulting supernatant was heat-treated by incubating at 70°C for 15 min, and was centrifuged to remove the precipitate. The supernatant was then treated with ammonium sulfate. A protein precipitate that was obtained at 40~80% ammonium sulfate saturation was dissolved in 20 ml of buffer B (20 mM potassium phosphate, pH 7.0, 0.2 mM $CoCl_2$, and 0.1 mM PMSF). After dialysis against buffer B, the protein solution was loaded onto a DEAE-Sepharose CL-6B column (2.5 × 20 cm) that had been pre-equilibrated with the same buffer. After washing with buffer B, the proteins were eluted with 500 ml of a linear gradient of 0-0.5 M KCl in buffer B at a flow rate of 30 ml/h. The active fractions (# 46-50), 30 ml, were pooled and concentrated to 10 ml by ultrafiltration with Centriprep YM10 (Millipore, Bedford, USA). The concentrated protein fraction was dialyzed against buffer C (20 mM potassium phosphate, pH 7.0), and then applied to a hydroxylapatite column (2.5 × 10 cm) that was pre-equilibrated with buffer C. Unbounded proteins were washed out extensively with buffer C. Elution was carried out with 400 ml of a linear gradient of 20-200 mM potassium phosphate at a flow rate of 30 ml/h. The active fractions (# 80-90), 33 ml, were pooled, concentrated to 3 ml by Centriprep YM10, and then dialyzed against buffer D (20 mM potassium phosphate, pH 7.0, 10% glycerol). The enzyme solution was loaded onto a Ultrogel AcA 54 gel filtration column (1.6 × 100 cm) that was pre-equilibrated with buffer D and the enzymes were eluted with buffer D at a flow rate of 10 ml/h. The active fractions (#57-65), 9 ml, were collected, dialyzed against buffer E (20 mM potassium phosphate, pH 7.0, 10% glycerol), and concentrated to 3 ml with Centriprep YM10 membrane. The concentrated protein fractions were applied to a Reactive red 120 CL agarose column (1 × 10 cm) that was pre-equilibrated with buffer E. Elution was carried out with 90 ml of the same buffer at a flow rate of 10 ml/h. Active fractions (#33-39) were pooled and concentrated to 1 ml.

The protein determination and enzyme assay The protein concentration was determined by the Smith method (Smith *et al.*, 1985) using a BCA protein assay reagent (Pierce, Rockford, USA). The enzyme activity was determined by the modification of Ben-Bassat's method (Ben-Bassat *et al.*, 1987; Park *et al.*, 1999). The measurement of activity was carried out in a final 100 ml of the reaction mixture that contained 10 ml of an enzyme aliquot and 90 ml of a 100 mM potassium phosphate buffer (pH 7.4) with 4 mM Met-Ala-Ser and 0.2 mM $CoCl_2$. The reaction mixture was incubated at 55°C for 10 min. After boiling for 2 min, the reaction mixture was added to 900 ml of the mixture of three-color development solutions that contained 300 ml of each color development solution: solution A, solution B, and solution C that contained 6.6 mg of L-amino acid oxidase, 6.6 mg of o-dianisidine, and 2.1 mg of peroxidase, respectively, per 10 ml of 100 mM TrisHCl (pH 7.4). The final reaction mixtures were incubated at

30°C for 30 min, and the absorbencies were determined at 440 nm. The specific activities of enzyme were expressed as mmols L-methionine/min/mg protein. One unit of activity is defined as 1 mmol of amino acids produced per min under the assay condition used.

Confirmation of MetAP as an aminopeptidase To know whether the purified MetAP catalyzes the removal of the N-terminal residue of a substrate (Met-Ala-Ser), we attempted to fractionate the enzymatic product using HPLC. The enzymatic product (0.1 ml) was mixed with 0.1 ml of 10% sulfosalicyclic acid and centrifuged at 3,000 × *g* for 10 min. The denatured, aggregated enzyme in the mixture was removed by membrane filtration (pore size 0.22 μm). The supernatant was applied to a sodium cation ion exchange amino acid analyzer column that had been equilibrated with a Na328 buffer (Pickering Laboratories, USA). The amino acid and peptides were eluted with a pH gradient that was composed of Na328 and Na740 at 0.3 ml/min, 50°C for eluent, and at 0.3 ml/min at 130°C for trione. The eluted products were reacted with ninhydrin. Comparing the retention time of the reaction products and the substrate can recognize the cleavage of the N-terminal amino acid.

Determination of substrate specificity *B. stearothermophilus* MetAP activities were tested toward four different tripeptides as a substrate - 4 mM each of Met-Ala-Ser, Leu-Gly-Gly, Leu-Ser-Phe, and Leu-Leu-Tyr - in a 100 mM potassium phosphate buffer, pH 7.4, that contained 0.2 mM $CoCl_2$

Determination of molecular mass The molecular mass of the enzyme was determined by sodium dodecyl sulfate-polyacrylamide gel (12.5%) electrophoresis (SDS-PAGE) (Laemmli, 1970) and gel filtration. The molecular mass on the gel was estimated with a molecular mass standard that included bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), glyceraldehyde-3-phosphate dehydrase (36,000 Da), carbonic anhydrase (29,000 Da), trypsinogen (24,000 Da), trypsin inhibitor (20,100 Da), and -lactoalbulin (14,200 Da). After electrophoresis, the protein bands were visualized by staining the gel with Coomassie brilliant blue R250 (Merck, USA). For gel filtration, the purified enzyme was applied to a Sepharose CL-6B column (1.6 × 100 cm) and eluted with Buffer D at 10 ml/h. The chromatographic column was calibrated with standard proteins of thyroglobulin (690,000 Da), bovine serum albumin (66,000 Da), carbonic anhydrase (29,000 Da), and cytochrome c (12,400 Da).

Isoelectric focusing The isoelectric point was determined according to the manufacturers instructions using PhastGEL IEF gel (Pharmacia, USA). The standard proteins that were used were amyloglucosidase (pI 3.5), soy bean trysin inhibitor (pI 4.55), bovine carbonic anhydrase B (pI 5.85), human carbonic anhydrase (pI 6.55), horse myoglobin (pI 6.85), horse myoglobin (pI 7.35), lentil lectin (pI 8.15), lentil lectin (pI 8.45), and lentil lectin (pI 8.65).

Thermal and pH effect The enzyme solutions were preincubated at temperatures ranging from 4°C to 100°C for 30 min, then enzymatic activities were measured. The enzyme stability was measured by incubating the enzyme at 80°C every 10 min for 45

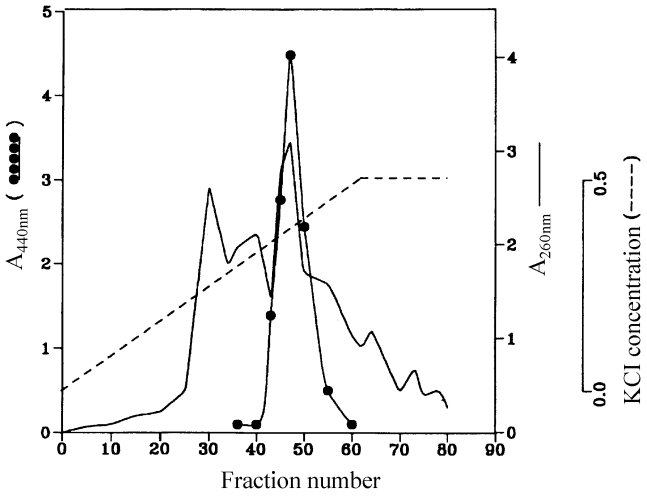

Fig. 1. DEAE-Sepharose CL-6B ion exchange chromatography of *B. stearothermophilus* MetAP. Ammonium sulfate saturated fraction (40-80%) was dialyzed against buffer B and loaded onto a DEAE-Sepharose CL-6B column (2.5 × 20 cm) that had been equilibrated with the same buffer. The enzyme was eluted with a 500 ml of a linear gradient from 0.0 to 0.5 M KCl in Buffer B at a flow rate of 30 ml/h. The active fractions from 46 to 50 were pooled (30 ml), concentrated to 10 ml, and dialyzed against buffer C for the next chromatography. The solid and dashed lines represent relative protein concentrations (O.D. at 280 nm) and KCl gradient, respectively. The closed circles refer to MetAP activity (O.D. at 440 nm).

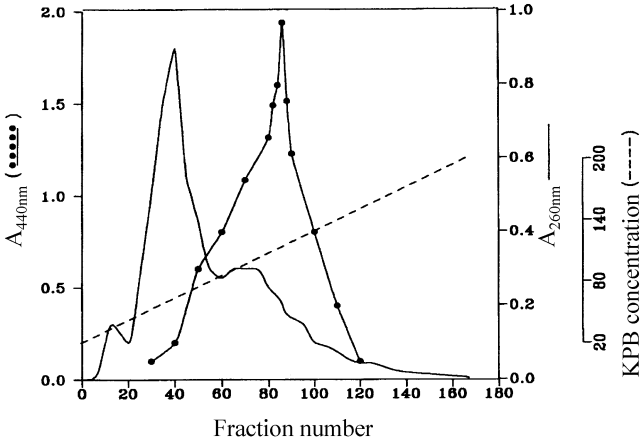

Fig. 2. Hydroxylapatite chromatography of *B. stearothermophilus* MetAP. The active fraction (10 ml) from the previous chromatography (Fig. 1) was applied to a hydroxylapatite column (2.5 × 10 cm) that was pre-equilibrated with buffer C. The enzyme was eluted with a linear gradient of 400 ml of 20-200 mM potassium phosphate buffer, pH 7.0, at a flow rate of 30 ml/h. The active fractions from 80 to 90 were pooled (33 ml), concentrated to 3 ml, and dialyzed for the subsequent chromatography. The solid and dashed lines represent protein concentration (O.D. at 280 nm) and potassium phosphate gradient, respectively. The solid line with closed circles refers to MetAP activity measured at 440 nm.

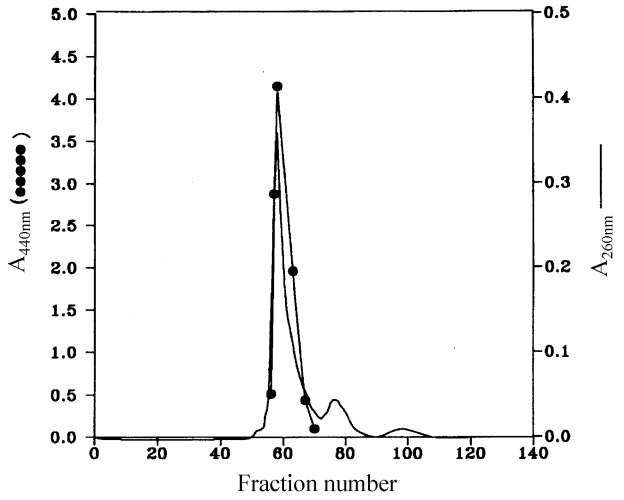

Fig. 3. Ultrogel AcA 54 gel filtration chromatography of *B. stearothermophilus* MetAP. The concentrated, active fraction (3 ml) from the hydroxylapatite chromatography (Fig. 2) was loaded onto a Ultrogel AcA 54 column (1.6 × 100 cm) that was pre-equilibrated with buffer D. The enzyme was eluted with buffer D at a flow rate of 10 ml/h. The active fractions (from 57 to 65) were pooled (9 ml), concentrated to 3 ml, and dialyzed against buffer E for the subsequent chromatography. The solid lines with or without the closed circles represent MetAP activity (O.D. at 440 nm) and protein concentration (O.D. at 280 nm), respectively.

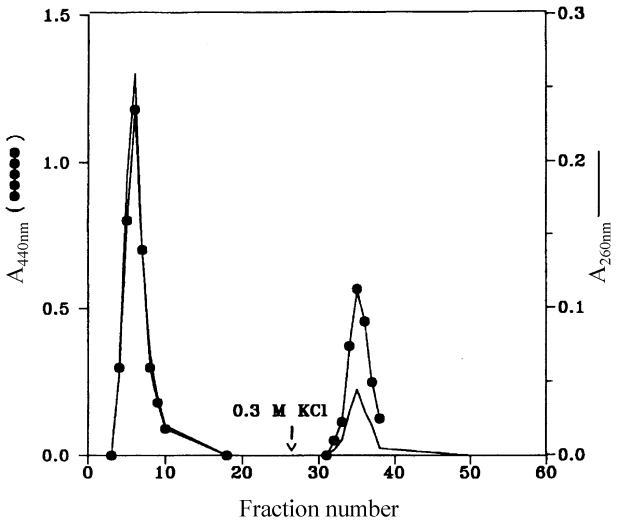

Fig. 4. Reactive red 120 agarose chromatography of *B. stearothermophilus* MetAP. The concentrated, active fraction (3 ml) (Fig. 3) was applied to the Reactive red 120 agarose column (1.0 × 10 cm) that was pre-equilibrated with buffer E. The MetAP eluted 90 ml of the same buffer at a flow rate of 10 ml/h with 0.3 M KCl in buffer E. The active fractions from 33 to 39 were pooled (3.5 ml) and concentrated to 1 ml for the subsequent analysis. The solid line with or without the closed circles represent MetAP activity (O.D. at 440 nm) and protein concentration (O.D. at 280 nm), respectively.

min. The pH optimum was measured in different buffers, each with a unique range of buffering capacity: 0.1 M citrate-NaOH buffer for pH 5.0-6.5, 0.1 M potassium phosphate buffer for pH 6.0-8.0, 0.1 M HEPES buffer for pH 7.5-8.5, and 0.1 M borate-NaOH buffer for pH 8.0-9.3.

The effects of protease inhibitors, chelating agents, and divalent cations on enzymatic activities The effects of protease inhibitors were examined. They included 0.1 mM TLCK, 0.1 mM leupeptin, 0.1 mM chymostatin, 0.1 mM PMSF, 0.01 mM pepstatin, 0.01 mM bestatin, and 1 μM aprotinin. Also, the effects of the chelating agents and metal ions on enzymatic activities were examined. They were 0.5-10 mM EDTA, 1-10 mM 1, 10-phenanthroline, 1-5 mM of $CaCl_2$, $CoCl_2$, $CuCl_2$, $MgCl_2$, $HgCl_2$, $FeCl_2$, $MnCl_2$, $NiCl_2$, and $ZnCl_2$. To avoid any possible influence of metal ions on L-amino acid oxidase and peroxidase in color development solutions, the metal ions were removed by adding 4 mM EDTA prior to the color reaction and by incubation for 10 min.

Determination of kinetic parameters The *Km* and *Vmax* of the enzyme were determined by a Lineweaver-Burk plot for the substrate concentration of 0.063, 0.13, 0.25 0.5 1.0 2.0, and 4.0 mM Met-Ala-Ser.

Results and Discussion

Purification of *B. stearothermophilus* MetAP The enzyme was purified by four sequential chromatography procedures. These included DEAE-Separose CL-6B ion exchange (Fig.

Table 1. Purification of MetAP from *B. stearothemophilus*

Purification Step	Total Protein (mg)	Total Activity (units)[a]	Specific Activity (units/mg)	Purification Folds	Yield (%)
Crude extract	1237	20709	16.7	1	100
Heat Precipitation	275	12425	45.2	2.7	59.9
$(NH_4)_2SO_4$ 40%-80%	116.9	10147	86.8	5.2	48.9
DEAE-Sepharose	33.1	6627	200.4	12	32.0
Hydroxylapatite	3.1	1522	491	29.4	7.3
Ultro gel AcA54	0.46	787	1710.9	102.5	3.8
Reactive red 120 agarose	0.11	567	5145.5	308.7	2.74

[a]One unit of the enzyme activity was defined as that catalyzing the formation of 1 mmol of free L-Methionine a minute.

1), hydroxylapatite (Fig. 2), gel filtration (Fig. 3), and Reactive red 20 agarose columns (Fig. 4) following heat inactivation and ammonium sulfate precipitation. The purification yield and fold were 2.7% and 309, respectively (Table 1). The specific activity of the final enzyme was 5,145 mmol/min/mg of protein. The significant loss of activity (40%) during heat inactivation at 70°C is likely due to the co-precipitation of MetAP with other denatured proteins. $CoCl_2$ was excluded in buffers from the step of hydroxylapatite column chromatography since the metal ions bound the column to prevent protein adsorption. The exclusion of $CoCl_2$, therefore, seemed to affect a marked decrease in the enzyme yield (from 32 to 7.3%) during the step of hydroxylapatite column chromatography. The column chromatography also likely removed other contaminated aminopeptidases including leucine aminopeptidases (LAPs), which are known to be able to hydrolyze Met-Ala-Ser that is used as a substrate. It could also contribute to the reduction of the enzyme yield, as evidenced in previous studies (Simpson *et al.*, 1976; Kendall and Bradshaw, 1992). A substantial amount of the enzyme was eluted with a wash buffer of reactive red 120 agarose column chromatography. This indicates that the enzyme is not efficient in binding to this column. Hence, we repeatedly recovered the enzyme from the eluent. *Km* and *Vmax* that was obtained by a Lineweaver-Burk plot were 3.0 mM and 1.7 mmol/min/mg, respectively, using Met-Ala-Ser as a substrate.

Molecular mass of *B. stearothermophilus* MetAP The purified enzyme migrated approximately as a 41 kDa protein, as shown in SDS-PAGE (Fig. 5A). A gel filtration chromatography analysis of the purified enzyme revealed a single band with an apparent molecular mass of 81,300 Da (Fig. 5B). These results suggest that *B. stearothermiphilus* MetAP is composed of two identical subunits. MetAPs from other species have a broad range of molecular mass; *E. coli*, *S. typhimurium*, yeast, and porcine MetAPs are 29,300, 29,300, 43,200, and 67,000 Da, respectively (Ben-Bassat *et al.*, 1987; Miller *et al.*, 1987; Chang *et al.*, 1990; Kendall and Bradshaw, 1992). However, all of the MetAPs that have been purified are shown to be active in a monomeric form. In contrast, the dimeric form of the *B. stearothermiphilus* MetAP appears unique to the thermophilic bacteria.

The substrate specificity and kinetic parameters of the MetAP The reaction products of the purified MetAP were examined to see if they *per s*e catalyzed the removal of the N-terminal residue of the substrate (Met-Ala-Ser). A HPLC analysis of the reaction products revealed three retention times. Compared to the peaks of free amino acids that were used as a standard, one peak was for the substrate itself and the second peak was for free methionine. There was as yet no peak that corresponded to either Ser or Ala. Hence, the third peak must correspond to the dipeptide, Ala-Ser, indicating that the enzyme belongs to a family of aminopeptidase that specifically hydrolyzes the N-terminal residue of substrate.

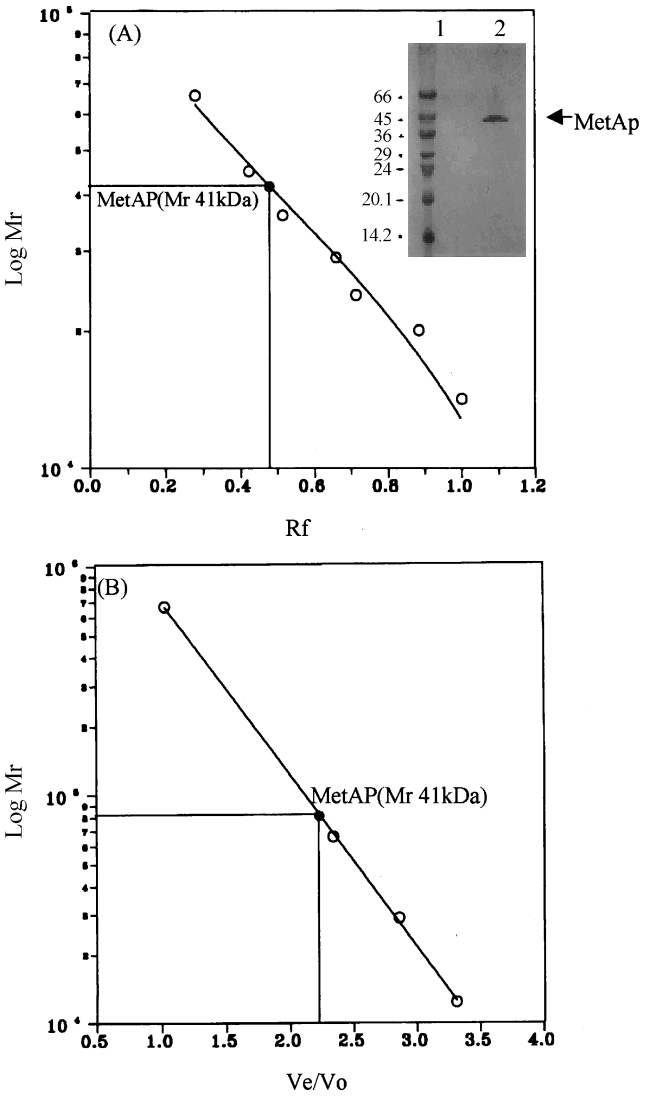

Fig. 5. Determination of a molecular mass of *B. stearothermophilus* MetAP. (A). SDS-PAGE (12.5%) was performed. Lane 2 in the inset indicates the purified *B. stearothermophilus* MetAP. A molecular weight standard (top to bottom) was shown in lane 1: bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), 3. glyceraldehyde-3-phosphate dehydrase (36,000 Da), trypsinogen (24,000 Da), trypsin inhibitor (20,100 Da), and -lactoalbumin (14,200 Da). (B). Determination of molecular weight of *B. stearothermophilus* MetAP by Sepharose-CL-6B gel filtration chromatography. The standard proteins were thyroglobulin (670,000 Da), bovine serum albumin (66,000 Da), cationic anhydrase (29,000 Da), and cytochrome c (12,400 Da).

Substrate specificity was measured by comparing the relative activities toward four different tripeptides: Met-Ala-Ser, Leu-Gly-Gly, Leu-Ser-Phe, and Leu-Leu-Tyr. As shown in Table 2, this enzyme had a strong preference for Met-Ala-Ser (100%) over the others (more or less 30%). This data suggest that this enzyme is neither a leucine aminopeptidase that is specific for the N-terminal leucine residue, nor a tripeptidase that shows

Table 2. Substrate specificity of *B. stearothermophilus* MetAP

Substrate[a]	Relative activity (%)
Met-Ala-Ser	100
Leu-Gly-Gly	34
Leu-Ser-Phe	30
Leu-Leu-Tyr	32

[a]Each substrate was used at a concentration of 4 mM in 100 mM potassium phosphate buffer, pH 7.4, containing 0.2 mM $CoCl_2$.

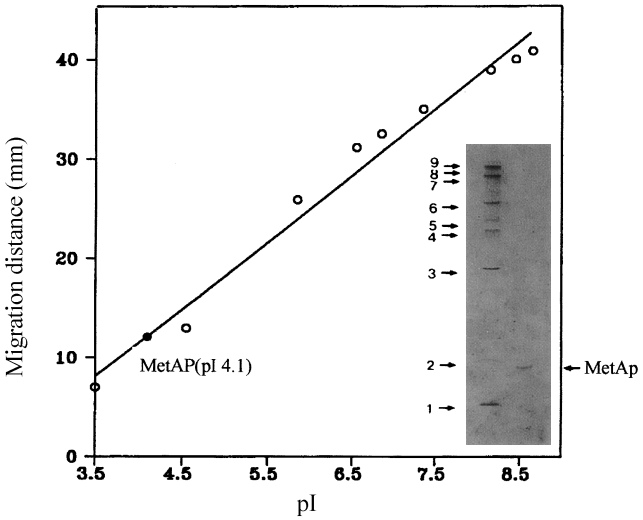

Fig. 6. Determination of pI of the purified enzyme by isoelectric focusing. The arrows in the inset indicate the pI standards from top to bottom: lentil lectin (pI 8.65), lentil lectin (pI 8.45), lentil lectin (pI 8.15), horse myoglobin (pI 7.35), horse myoglobin (pI 6.85), human carbonic anhydrase (pI 6.55), bovine carbonic anhydrase (pI 5.85), soybean trypsin inhibitor (pI 4.55), and amyloglucosidase (pI 3.5).

no preference for any of these substrates (Simitsopoulou *et al.*, 1997; Park *et al.*, 1999). However, more peptide substrates with a variety of residues, both at their amino-termini and at the next (penultimate) residue (other than the tripeptides used in this study), are necessary in order to confirm the identity of this enzyme.

Physicochemical properties of the enzyme This enzyme had a pI at 4.1 (Fig. 6); therefore, it is more acidic than the MetAPs from other species (5.4 and 7.8 for *S. typhimurium* (Miller *et al.*, 1987) and yeast (Chang *et al.*, 1990), respectively). The optimum temperature of the enzyme activity was 85°C (Fig. 7A), which is much higher than temperature (55°C) at which the optimal growth occurs. Since the enzymatic activity did not decline after 45 min of incubation at 80°C (Fig. 7B), thermostability is supported. This enzyme had a pH optimum at 8.0 (Fig. 7C), which is also higher than the enzymes from yeast and porcine (Chang *et al.*, 1990; Kendall and Bradshaw, 1992).

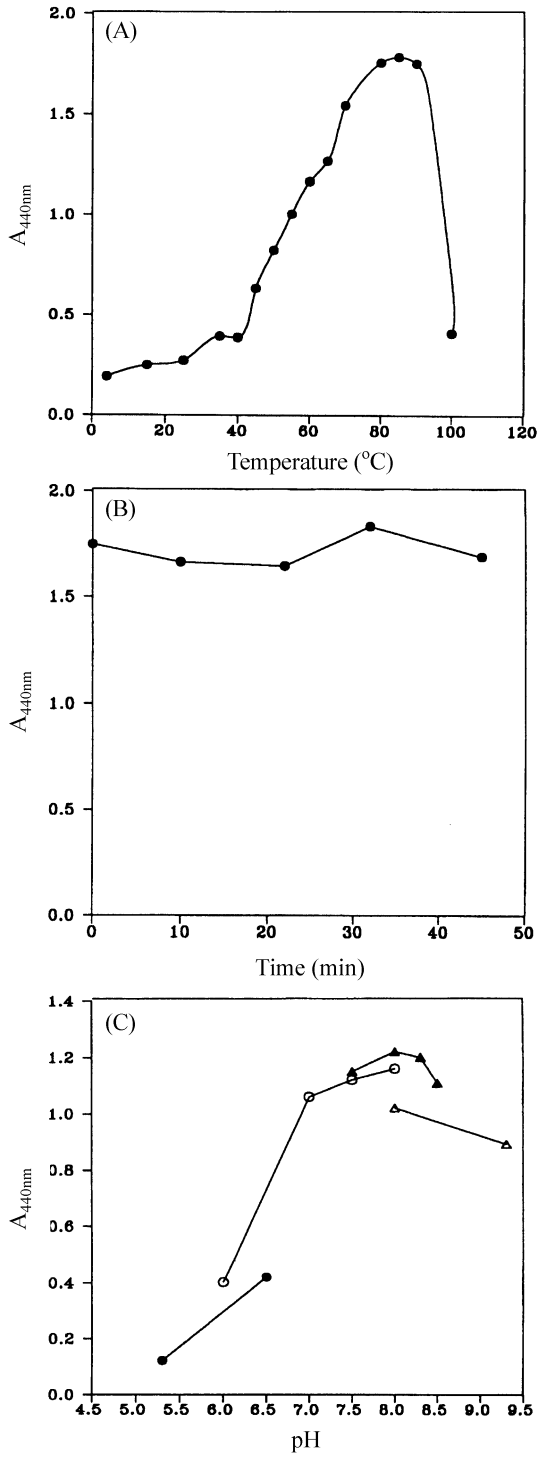

Fig. 7. Effects of temperature and pH on activity of *B. stearothermophilus* MetAP. (A). The enzyme has an optimum temperature at 85°C, exhibiting a typical bell shape in response to temperature. (B). The enzyme activity remains unchanged for 45 min at 80°C. (C). The pH optimum of this enzyme is 8.0. Different buffers were used for this purpose as follows: pH range 5.0-6.5 (0.1 M Citrate-NaOH), ● - ● ; pH 6-8 (0.1 M potassium phosphate), ○ - ○ ; pH 7.5-8.5 (0.1 M HEPES), ▲-▲; pH 8-9.3 (0.1 M Borate-NaOH), △ - △ .

Table 3. Effect of protease inhibitors on *B.stearothermophilus* MetAP

Protease inhibitors	Relative activity (%)
None	100
Aprotinin	100
Chymostatin	97
Leupeptin	95
PMSF	100
TLCK	94
Pepstatin	91
Bestatin	95

Effect of protease inhibitors on enzymatic activity As shown in Table 3, the purified enzyme was unaffected by the addition of any of the protease inhibitors. These include serine protease inhibitors (0.1 mM TLCK, 0.1 mM PMSF, 0.1 mM leupeptin, 0.1 mM chymostatin, 1 mM aprotinin), an aspartic protease inhibitor (0.01 mM pepstatin), and an inhibitor of leucine aminopeptidase (0.01 mM bestatin). These results suggest that the purified enzyme does not belong to any of the family of serine protease, aspartic protease, or leucine aminopeptidase.

Effects of divalent cations and chelating agents on enzymatic activities This enzymatic activity was nearly completely inhibited by 1 mM EDTA and 1 mM 1, 10-phenanthroline. Among a number of metal ions, only Co^{2+} ion enhanced the activity; 0.1 mM Co^{2+} was high enough for activating the enzyme by two-fold. In contrast, other divalent cations had little effect (Mg^{2+}), significantly decreased (Cu^{2+}, Ca^{2+} and Zn^{2+} at 1-5 mM), or abrogated the activity (Mn^{2+}, Ni^{2+}, Fe^{2+} and Hg^{2+} at 1 mM) (Table 4). This collectively suggests that this enzyme is a Co^{2+}dependent metallopeptidase. The X-ray structures of the three MetAPs from *E. coli*, *P. furious*, and humans have been resolved. These enzymes commonly have a pseudo two-fold symmetry that has two metal ions per catalytic subunit (Roderick and Matthews, 1993; Liu *et al.*, 1998; Tahirov *et al.*, 1998). However, recent experiments determined that *S. cerevisae* MetAP I (containing Zn^{2+}) has a substantially higher activity than the Co^{2+}-coordinated enzyme under *in vivo* conditions (Walker and Bradshaw, 1998). For instance, the Co^{2+}-enzyme is inactivated in the environment where the yeast cell contains a high concentration of reduced glutathione in the cytosol, unlike the Zn^{2+} enzyme. This supports the view that yeast MetAP I is a Zn^{2+}-metalloprotease *in vivo*. A similar finding comes from the study of tripeptidases (PepTs), which are also traditionally considered Co^{2+}-dependent metalloenzymes. When a recombinant PepT from *Bacillus subtilis* is prepared in depletion of any metal ions by extensive dialysis in the presence of EDTA, Zn^{2+} (650 nM) stimulates the enzyme activity at much less of a concentration than Co^{2+} (1700 nM). In addition, the binding of Zn^{2+} to the *B. subtilis* PepT is shown to promote more stability upon exposure to denaturants than Co^{2+} does (Cha *et al.*, 2000). Although Zn^{2+} strongly inhibits the enzyme activity at relatively high, nonphysiological concentrations (>2 mM), a similar result is also observed in the yeast MetAP I (Walker and Bradshaw, 1998). Collectively, these facts leave a question as to what metal ion is a naturally authentic ligand. In a future study, an atomic absorption spectroscopy analysis is required to reveal the identity of metal ion(s) that are associated with the *B. stearothermophilus* MetAP.

Table 4. Effect of divalent cations on *B.stearothermophilus* MetAP

Metal ions	Concentration (mM)	Relative activity (%)
Co^{2+}	0.1	207
	1	201
	2	198
	4	185
Mg^{2+}	0.1	110
	1	97
	2	109
	4	85
Cu^{2+}	0.1	97
	1	73
	2	48
	4	12
Ca^{2+}	0.1	97
	1	67
	2	50
	4	48
Zn^{2+}	2.5	62
	5	29
Hg^{2+}	1	0
Fe^{2+}	1	0
Mn^{2+}	1	0
Ni^{2+}	1	0

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