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The Purification and Characterization of *Bacillus subtilis* Tripeptidase (PepT)

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A tripeptidase (PepT) was purified to homogeneity from *Bacillus subtilis* through four sequential chromatographies including DEAE-Sepharose ion exchange, hydroxylapatite, mono-Q FPLC ion exchange, and Superose-12 FPLC gel filtration. The apparent molecular mass of the enzyme was 49,200 Da and 51,400 Da as determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel filtration chromatography, respectively, and the enzyme exists in a monomeric form. The physico-chemical properties of the enzyme were as follows: optimum pH at 7.5, optimum temperature at 60°C, and pI at 4.9. The K_m and V_{max} values of the enzyme were 4.3 mM and 2.5 mmol/min/mg, respectively, with Met-Ala-Ser as substrate. The *B. subtilis* PepT requires Co^{2+} ion(s) for activation, while it is inactivated by EDTA and 1,10-phenanthroline, suggesting that it is a metalloprotein. The enzyme was not inhibited by any of serine protease, aspartic protease, or leucine amino-peptidase inhibitors. The enzyme showed comparable activities towards four different substrates including Met-Ala-Ser, Leu-Gly-Gly, Leu-Ser-Phe, and Leu-Leu-Tyr. The amino terminal sequence of PepT determined by Edman degradation was found to be MKEEIIERFTTYVXV and turned out to be identical to that of PepT deduced from a cloned *B. subtilis pepT*.

Keywords: *Bacillus subtilis*, Co^{2+} ion, Metalloenzyme, Tripeptidase (PepT).

Introduction

A tripeptidase (PepT) is known to be a component of the bacterial protease/peptidase system that exerts its functions of nutritional utilization of tripeptides (Miller and MacKinnon, 1974; Miller and Schwartz, 1978; Botbol and Scornik, 1991; Taylor, 1993). This enzyme catalyzes the removal of N-terminal amino acid residue from tripeptides with varying sequence but does not hydrolyze dipeptides, tetrapeptides, and N- or C-blocked tripeptides (Hermsdorf, 1978; Miller and Schwartz, 1978; Strauch *et al.*, 1983). The counterparts from *Escherichia coli* (Hermsdorf, 1978), *Salmonella typhimurium* (Strauch and Miller, 1983), *Lactococcus lactis* (Bosman *et al.*, 1990), and *Pediococcus pentosaceus* (Simitsopoulou *et al.*, 1997) have been purified and partially characterized. *E. coli* PepT has the molecular mass of 80,000 Da as determined by gel filtration chromatography (Hermsdorf, 1978) while *L. lactis* and *P. pentosaceus* PepTs have an M_r of approximately 100,000, consisting of two identical subunits each of 45,000–52,000 Da (Bosman *et al.*, 1990; Simitsopoulou *et al*, 1997). The PepTs are believed to be metalloproteins since these are inactivated by the addition of chelating agents and activated in the presence of metal ions. These enzymes, however, differ in requirement of metal ions as a cofactor. For instance, both *S. typhimurium* and *L. lactis* PepTs require Mn^{2+} or Zn^{2+} (Strauch and Miller, 1983; Bosman *et al.*, 1990), whereas *P. pentosaceus* PepT needs Mg^{2+}, Mn^{2+}, or Ca^{2+} for activation (Simitsopoulou *et al.*, 1997). *P. pentosaceus* PepT is insensitive to protease inhibitors such as iodoacetic acid, PMSF, and trypsin inhibitor (Simitsopoulou *et al.*, 1997). The molecular clonings of *pepT* genes have been reported from three bacterial species: *S. typhimurium* (Miller *et al.*, 1991) and *L. lactis* (Mierua *et al.*, 1994), and *Bacillus subtilis pepT* genes (Schrogel *et al.*, 1996) were recently cloned and a partial nucleotide sequence of *E. coli pepT* is also available (Lombardo *et al.*, 1993). The *pepT* genes encode polypeptides of about 410 amino acid residues in length, thus supporting the earlier estimates of the molecular mass of PepTs. The deduced amino acid

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sequences of the three PepTs are homologous to one another with approximately 50% identity (Miller *et al.*, 1991; Mierua *et al.*, 1994; Schrogel *et al.*, 1996). In addition, the analysis of the deduced amino acid sequences of PepTs reveals that there are several regions with strong sequence homology in PepTs and that at least one of these regions may be involved in metal binding (Mierua *et al.*, 1994; Schrogel *et al.*, 1996; Simitsopoulou *et al.*, 1997). These genes are shown to be dispensable for their growth (Miller *et al.*, 1991; Mierua *et al.*, 1994; Schrogel *et al.*, 1996), and expressed under an anaerobic condition (Miller *et al.*, 1991) or during stationary phase of growth (Schrogel *et al.*, 1996).

In this report, we have purified and characterized the enzyme that has a tripeptidase activity from *B. subtilis*, and determined its N-terminal sequence. The enzyme has a molecular mass of approximately 50,000 Da and possesses all common characteristics of PepT. We believe that this enzyme is a *B. subtilis* PepT.

Materials and Methods

Reagents Bacto trypton, yeast extract, and bacto agar were obtained from Difco (Detroit, USA). 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). The molecular weight markers for gel filtration and isoelectric focusing were supplied by Bio-Rad (California, USA). Hydroxylapatite fast flow was purchased from Calbiochem (San Diego, USA), and Mono-Q and Superose-12 column were obtained from Pharmacia (Uppsala, Sweden). Metal ions were purchased from Fisher Scientific (Fair Lawn, USA).

Bacterial stain and culture conditions The bacterial strain used in this study was *Bacillus subtilis* KCTC 1027 obtained from Korean Cell Line Bank. Cells were grown in medium containing 24 g yeast extract, 12 g tryptone, 27.3 ml glycerol, 3 g K_2HPO_4, and 1 g KH_2PO_4 per liter, pH 7.0–7.2, for 36 h at 37°C. Forty ml of 40% (w/v) D(+)-glucose was added to the culture medium when growth reached early stationary phase. The cells were harvested by centrifugation at 3000 × *g* at 4°C for 40 min.

Enzyme purification All procedures were carried out under chilled condition (4°C), unless otherwise mentioned. The harvested cells (wet weight, 90 g) were suspended in 10 vol of buffer A (50 mM potassium phosphate, pH 7.0, and 0.1 mM PMSF), and were then disrupted by a bead beater (20 cycle, beating for 15 s and cooling for 45 s by immersing in ice/water). Cell debris were removed by centrifugation at 10,000 × *g* for 30 min and the supernatant were centrifuged at 100,000 × *g* for 1 h. The supernatant was treated with ammonium sulfate. A protein precipitate obtained at 40~70% ammonium sulfate saturation was dissolved in 20 ml of buffer B (20 mM potassium phosphate, pH 7.0, and 0.1 mM PMSF). After dialysis against buffer B, the protein solution was applied to a DEAE-Sepharose CL-6B column (2.5 × 20 cm) that had been equilibrated with the same buffer. After washing with buffer B, proteins were eluted with 500 ml of a linear gradient of 0–0.5 M KCl in buffer B at a flow rate of 60 ml/h. The active fractions (Nos. 58–63), 36 ml, were collected, dialyzed, and concentrated by ultrafiltration using YM10 membrane (Oh and Lee, 1998). The concentrated protein fraction, 10 ml, was dialyzed against buffer C (10 mM potassium phosphate, pH 7.0), and then applied to a hydroxylapatite column (2.5 × 20 cm) preequilibrated with buffer B. Unbound proteins were removed by washing with buffer C. The elution was carried out with 400 ml of a linear gradient of 10–500 mM potassium phosphate at a flow rate of 60 ml/h. Thirty ml of the active fractions (Nos. 23–27) were collected, dialyzed, and then concentrated to 0.2 ml by Centri-Prep. YM10 and Microcon 10. The concentrated solution was loaded onto a Mono-Q FPLC ion exchange chromatography (5 × 50 mm) prequilibrated with buffer D (20 mM potassium phosphate, pH 7.0, 0.1 mM PMSF, 50 mM NaCl). After the column was washed with the buffer D, the proteins were eluted with a linear gradient of KCl (0.1–0.5 M) in 40 ml buffer B, at a flow rate of 20 ml/h. The active fractions (Nos. 24–28) were collected, dialyzed against buffer E (50 mM potassium phosphate, pH 7.0, 50 mM NaCl), and concentrated to 0.2 ml by ultrafiltration with Centri-Prep. YM10 and Microcon 10. The concentrated protein fractions (5 ml) were applied to the Superose-12 gel filtration column (5 × 50 mm) preequilibrated with buffer E. Elution was carried out with 90 ml of the same buffer at a flow rate of 12 ml/h.

Determination of molecular mass The molecular mass of the enzyme was determined by sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and gel filtration. The molecular masses on gel were estimated with molecular mass standards including bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), glyceraldehyde-3-phosphate dehydrase (36,000 Da), trypsinogen (24,000 Da), trypsin inhibitor (20,100 Da), and α-lactoalbumin (14,200 Da). After electrophoresis, protein bands were visualized by staining the gel with Coomassie brilliant blue R250 (MERCK, Darmstadt, Germany). The purified enzyme was applied to Superose-12 gel filtration preequilibrated with buffer E and eluted with the same buffer at a flow rate of 12 ml/h. The chromatographic column was calibrated with the protein standards of thyroglobumin (670,000 Da), γ-globulin (158,000 Da), ovalbumin (44,000 Da), myoglobin (17,000 Da), and vitamin B-12 (1,350 Da).

The protein determination and enzyme assay The protein concentration was determined by the Lowry method (Lowry *et al.*, 1951). The activity of tripeptidase was determined by the modification of Ben-Bassat's method (Ben-Bassat *et al.*, 1987). The measurement of activity was carried out in a final 100 μl of reaction mixure containing 10 μl of enzyme and 90 μl of 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. The reaction was stopped by immersing in boiling water for 2 min, and then the mixture was added to 900 μl of a solution that contained 300 μl each of three color development solutions: solution A, solution B, and solution C containing 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 Tris·HCl, pH 7.4. The final reaction mixtures were incubated at 30°C for 30 min, and the absorbancies were measured at 440 nm. The specific

activities of PepT were expressed as μmol L-methionine/min/mg protein. One unit of activity is defined as 1 μmol of amino acids produced per min under the assay condition used.

Isoelectric focusing Isoelectric focusing was peformed at room temperature using the method described by Righetti (1983). Sample (2 μl) was loaded on 25% acrylamide gel in mini-IEF cell (Bio-Rad) and sequentially developed for 15 min at 100 V, for 15 min at 200 V, and finally for 60 min at 450 V. The gel was fixed with a solution containing 4% sulforsalicylic acid, 12.5% trichloroacetic acid, and 30% methanol for 30 min, and then stained with 0.04% Coomassie brilliant blue R-250 for 2 h. The standard proteins used were phycocyanin (pI 4.65), β-lactoglobulin B (pI 5.1), bovine carbonic anhydrase (pI 6.0), human carbonic anhydrase (pI 6.5), equine myoglobin (pI 7.0), human hemoglobin A (pI 7.1), human hemoglobin c (pI 7.5), lentil lectin (pI 8.0), and cytochrome *c* (pI 9.6).

Thermal and pH effects To measure the optimum temperature of the enzyme, each enzyme solution was preincubated at temperatures ranging from 4°C to 100°C for 30 min, and then the residual activity was measured. The stability at the optimum temperature was measured by incubating the enzyme solution at 60°C for every 10 min. The optimum pH was measured in different buffers with each unique range of buffering capacity: 0.1 M citrate-NaOH buffer for pHs 5.0–6.5, 0.1 M potassium phosphate buffer for pHs 6.0–8.0, 0.1 M HEPES buffer for pHs 7.5–8.5, and 0.1 M borate-NaOH buffer for pHs 8.0–9.3.

The effects of protease inhibitors, chelating agents, and divalent cations on enzymatic activities Effects of protease inhibitors were examined that 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 (Lee *et al.*, 1995) Also, the effects of chelating agents and metal ions on enzymatic acivities were examined: 0.5–10 mM EDTA, 1–10 mM 1,10-phenanthroline, $HgCl_2$, $NiCl_2$, $MnCl_2$, $CoCl_2$, $MgCl_2$, $CaCl_2$, $ZnCl_2$, and $CuCl_2$ (Kang and Hwang, 1996). 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. There was no detectable effect of the chelating agents on the activities of oxidase and peroxidase.

Determination of kinetic parameters The K_m and V_{max} of the enzyme were determined for the substrate, Met-Ala-Ser. The hydrolysis of the tripeptide was determined for the substance from 0.25–12 mM Met-Ala-Ser at 55°C and pH 7.4 for 10 min followed by the methods mentioned above. The K_m and V_{max} values were determined using a Lineweaver-Burk plot.

Substrate specificities *B. subtilis* PepT activities were tested toward four different tripeptides: each of 4 mM Met-Ala-Ser, Leu-Gly-Gly, Leu-Ser-Phe, and Leu-Leu-Tyr in 100 mM potassium phosphate buffer, pH 7.4, containing 0.2 mM $CoCl_2$.

N-terminal microsequencing of tripeptidase The purified protein preparation was separated by electrophoresis on an SDS-PAGE (12.5%) and electroblotted onto polyvinylidene difluoride membrane as described by Choli *et al.* (1989). The N-terminal sequence of the purified enzyme was determined by Edman degradation in a Milligen 6600B equipped with a phenylthiohydantoin amino acid analyzer.

Results

Purification of *B. subtilis* PepT The enzyme was purified by four sequential chromatography procedures including DEAE-Sepharose CL-6B ion exchange (Fig. 1), hydroxylapatite (Fig. 2), Mono-Q FPLC ion exchange (Fig. 3), and Superose-12 FPLC gel filtration (Fig. 4). The pure enzyme (0.3 mg) was obtained from the starting material (90 g wet weight of cells) (Table 1). The relatively poor yield from the ammonium sulfate fractionation seems to be due to contaminating proteases present in the cytosolic fraction as observed in many cases of protease purification (Simpson *et al.*, 1966; Kendall and Bradshaw, 1992).

Determination of molecular mass The SDS-PAGE and gel filtration chromatography analyses of purified enzyme revealed a single band with the molecular mass of 49,200 Da and 51,400 Da (Figs. 5 and 6), respectively. The apparent molecular weight of the *B. subtilis* PepT is similar to those of the monomeric forms from *L. lactis* and *P. pentosaceus* PepTs (Bosman *et al.*, 1990; Simitsopoulou *et al.*, 1997).

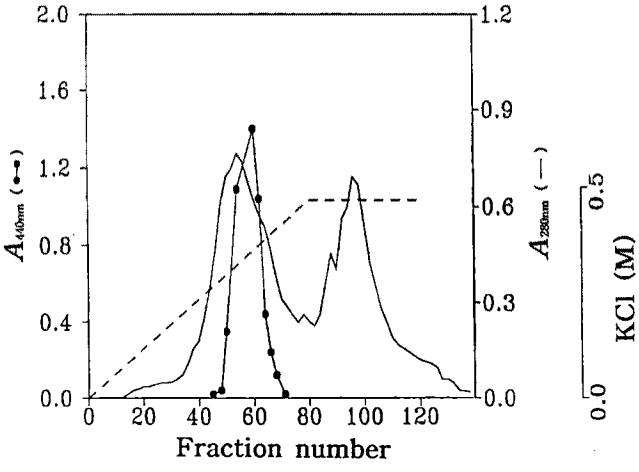

Fig. 1. DEAE-Sepharose CL-6B ion exchange chromatography of *B. subtilis* PepT. Ammonium sulfate saturated fraction (40–70%) was dialyzed against buffer B and loaded onto a DEAE-Sepharose CL-6B column (2.5 × 20 cm) that had been equilibriated with the same buffer. The enzyme was eluted with a 500 ml of linear gradient from 0.0 to 0.5 M KCl at a flow rate of 60 ml/h. The active fractions from 58 to 63 were pooled (36 ml), concentrated to 10 ml by ultrafiltration, and dialyzed against buffer C for the next chromatography. The solid and dashed lines represent relative protein concentrations (A_{280}) and KCl gradient, respectively. The solid line with closed circles refers to PepT activity measured at 440 nm.

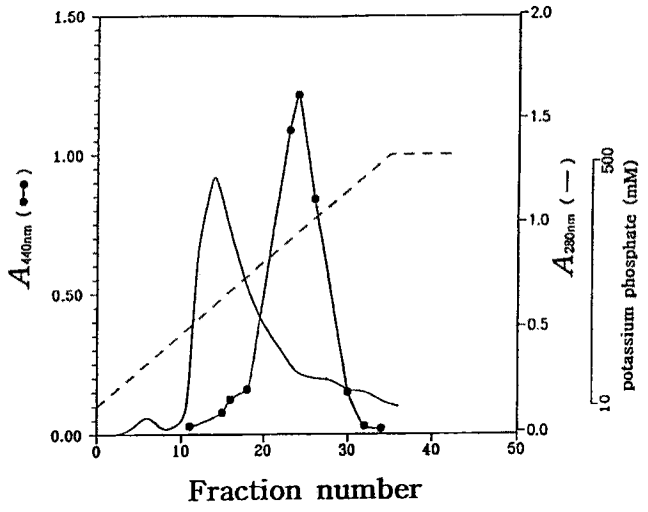

Fig. 2. Hydroxylapatite chromatography of *B. subtilis* PepT. The active fraction (10 ml) from the DEAE-Sepharose CL-6B ion exchange chromatography (Fig. 1) was applied to a hydroxylapatite column (2.5 × 10 cm) preequilibriated with buffer C. The enzyme was eluted with a linear gradient of 10–500 mM potassium phosphate buffer (400 ml), pH 7.0, at a flow rate of 60 ml/h. The active fractions from 23 to 27 were pooled (30 ml), concentrated to 0.2 ml by ultrafiltration, and dialyzed for the subsequent chromatography. The solid and dashed lines represent relative protein concentration (A_{280}) and potassium phosphate gradient, respectively. The solid line with closed circles refers to PepT activity measured at 440 nm.

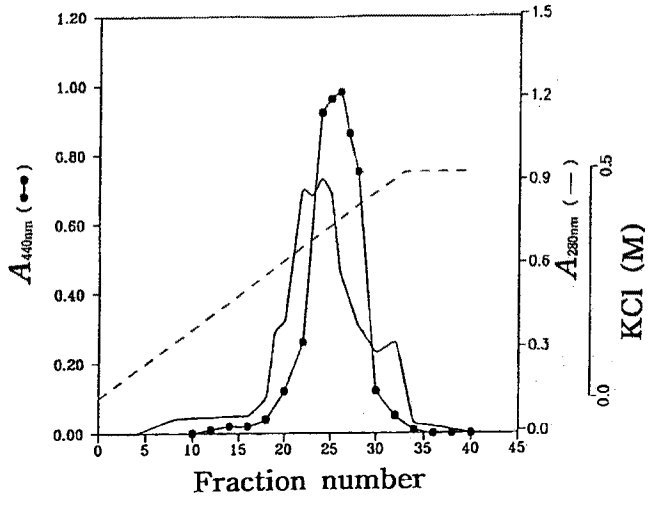

Fig. 3. Mono-Q FPLC ion exchange chromatography of *B. subtilis* PepT. The concentrated, active fraction (0.2 ml) from the hydroxylapatite chromatography (Fig. 2) was loaded onto a Mono-Q column (5 × 50 mM) preequilibriated with buffer D. The active fractions (Nos. 24–28) were eluted with a linear gradient of KCl (0.1–0.5 M) in buffer B at a flow rate of 20 ml/h, pooled (5 ml), concentrated to 0.2 ml by ultrafiltration, and dialyzed against buffer E for the subsequent chromatography. The solid and dashed lines represent relative protein concentrations (A_{280}) and KCl gradient, respectively. The solid line with closed circles refers to PepT activity measured at 440 nm.

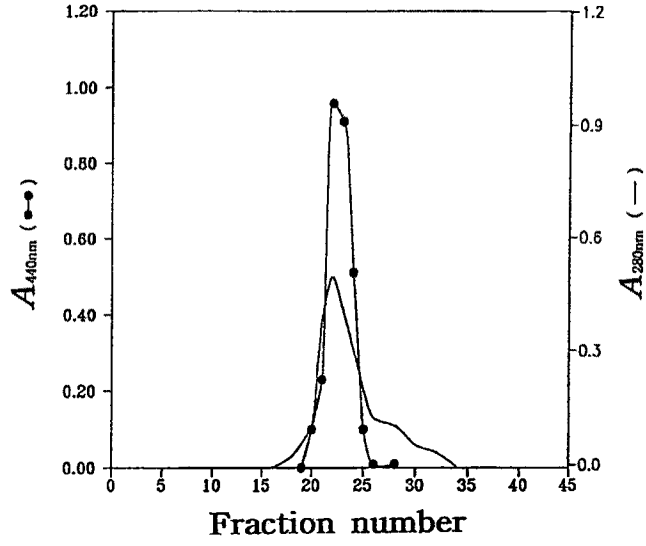

Fig. 4. Superose-12 gel filtration chromatography of *B. subtilis* PepT. The concentrated, active fraction (0.2 ml) from mono-Q ion exchange chromatography (Fig. 3) was applied to Superose-12 column (1.0 × 10 cm) preequilibriated with buffer E. The PepT was eluted with 90 ml of the same buffer at a flow rate of 12 ml/h. The solid lines with or without the closed circles represent PepT activity (A_{440}) and relative protein concentration (A_{280}), respectively.

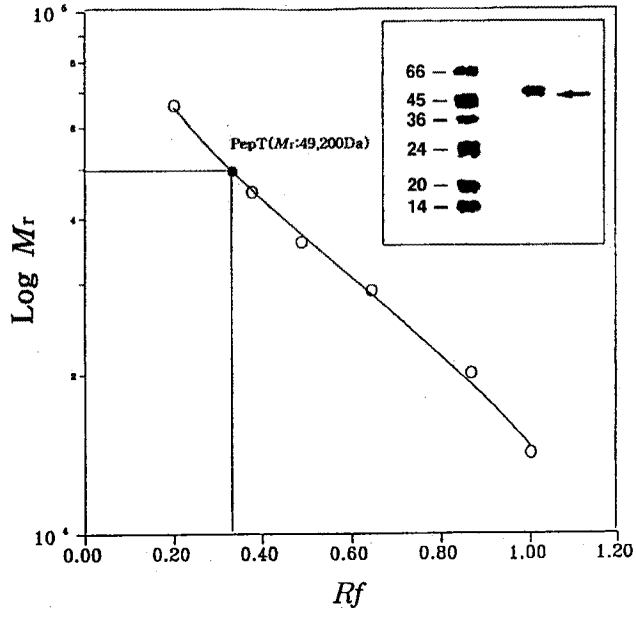

Fig. 5. SDS-PAGE of *B. subtilis* PepT. The electrophoresis was carried out according to the method of Laemmli using a 12.5% polyacrylamide gel. The purified enzyme, 3 μg, was subject to SDS-PAGE. The arrow in the inset indicates *B. subtilis* PepT. Molecular weight standards are (top to bottom): bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), glyceraldehyde-3-phosphate dehydrase (36,000 Da), trypsinogen (24,000 Da), trypsin inhibitor (20,100 Da), and α-lactoalbumin (14,200 Da).

Table 1. Purification of PepT from *B. subtilis*.

Purification Step	Total Protein (mg)	Total activity (μmol/min)	Specific activity (units/mg/min)	Purification (fold)	Yield (%)
Crude extract	2251	12406	5.5	1	100
Ammonium sulfate (40–70%)	645	8062	12.5	2.3	64
DEAE-Sepharose CL-6B ion exchange	89	2335	26.1	4.7	18
Hydroxylapatite	6.4	849	132.6	24.9	6.8
Mono-Q FPLC ion exchange	1.1	411	373.6	67.2	3.3
Superose-12 FPLC gel filtration	0.3	286	1021.5	182	2.3

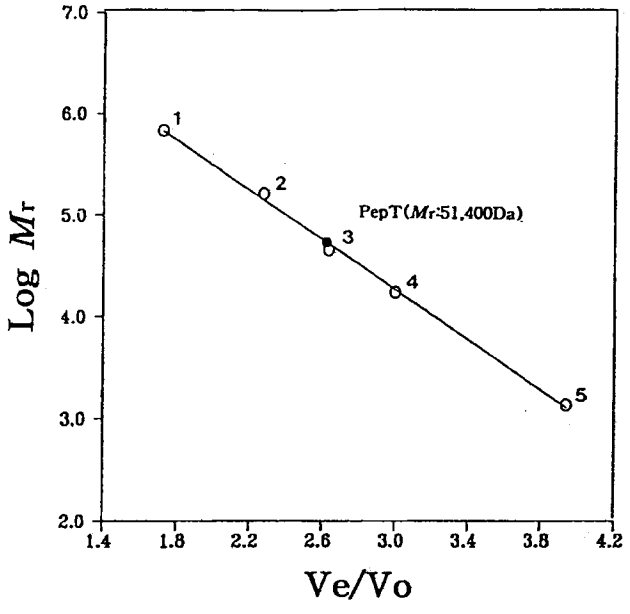

Fig. 6. Determination of a molecular mass of *B. subtilis* PepT. The molecular mass of the purified enzyme, 0.3 μg, was determined by a Superose-12 FPLC gel filtration chromatography. Molecular weight standards are: thyroglobulin (670,000 Da), γ-globulin (158,000 Da), ovalbumin (44,000 Da), myoglobin (17,000 Da), and vitamin B-12 (1350 Da).

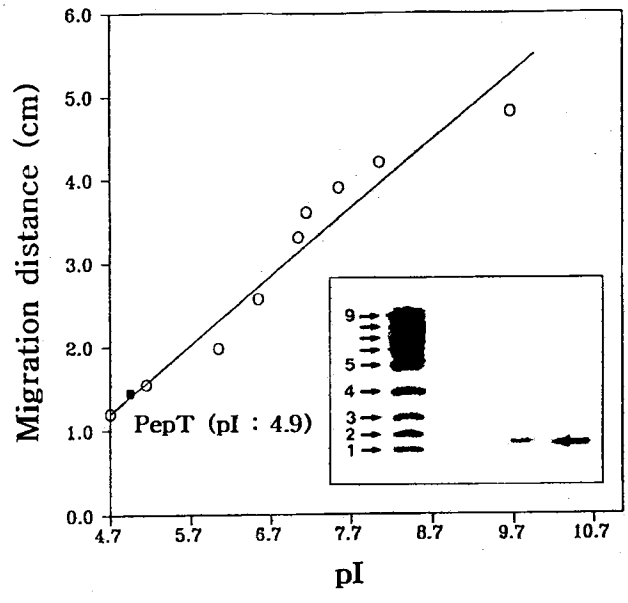

Fig. 7. Determination of pI of *B. subtilis* PepT by isoelectric focusing. Isoelectric focusing was carried out as described in Materials and Methods. The arrows in the inset indicate pI standards from bottom to top: phycocyanin (pI 4.65), β-lactoglobulin B (pI 5.1), bovine carbonic anhydrase (pI 6.0), human carbonic anhydrrase (pI 6.5), equine myoglobin (pI 7.0), human myoglobin (pI 7.1), human hemoglobin C (pI 7.5), lentil lectin (pI 8.0), and cytochrome *c* (pI 9.6).

The enzymatic and physicochemical properties The pI of this enzyme was estimated to be 4.9 (Fig. 7), indicating that the enzyme is a slightly acidic protein. The K_m and V_{max} towards Met-Ala-Ser were 4.3 mM and 2.5 mmol/min/mg, respectively (Fig. 8). The apparent K_m of 4.3 mM is similar to 5.3 mM of *E. coli* PepT (Hermsdorf, 1978). The optimum temperature of the purified enzyme was 60°C (Fig. 9a), yet the enzymatic activity was declined rapidly after 10 min of incubation at 55°C (data not shown). This enzyme had an optimum pH at 7.5 (Fig. 9b). As shown in Table 2, the purified enzyme was not affected by the addition of any of protease inhibitors including serine protease inhibitors (0.1 mM TLCK, 0.1 mM leupeptin, 0.1 mM chymostatin, 1 μM aprotinin, and 0.1 mM PMSF), aspartic protease inhibitor (0.01 mM pepstatin), and leucine aminopeptidase (LAP) inhibitor (0.01 mM bestatin). These results suggest that *B. subtilis* PepT does not belong to any of a family of serine protease, aspartic protease, or LAP. On the other hand, this enzymatic activity was inhibited in the presence

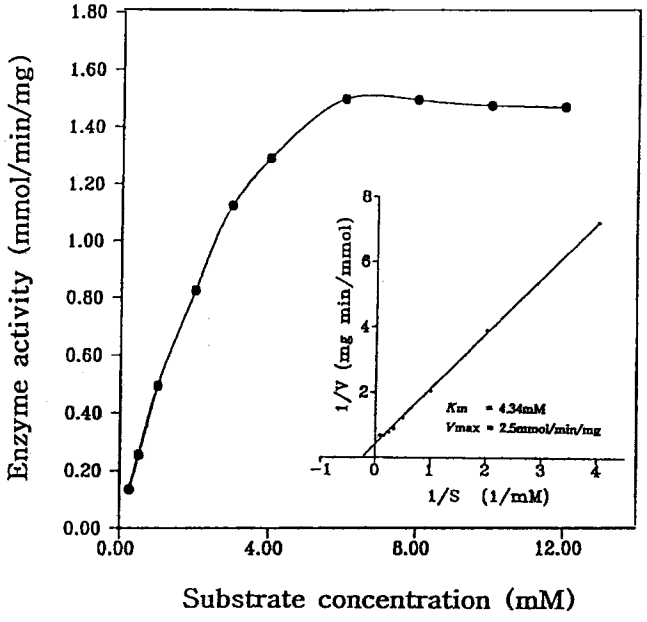

Fig. 8. Effect of substrate concentration on *B. subtilis* PepT activity. The PepT activity was assayed in the presence of 0.3 μg of purified enzyme with various concentrations of Met-Ala-Ser. The inset indicates a Lineweaver-Burk plot.

Table 2. Effect of protease inhibitors on *B. subtilis* PepT.

Protease inhibitors	Relative activity (%)
None	100
Aprotinin[a]	94
Chymostatin[b]	100
Leupeptin[b]	97
PMSF[b]	100
TLCK[b]	100
Pepstatin[c]	97
Bestatin[c]	100

Different concentrations were used: [a] 0.1 μM, [b] 0.1 mM, and [c] 0.01 mM.

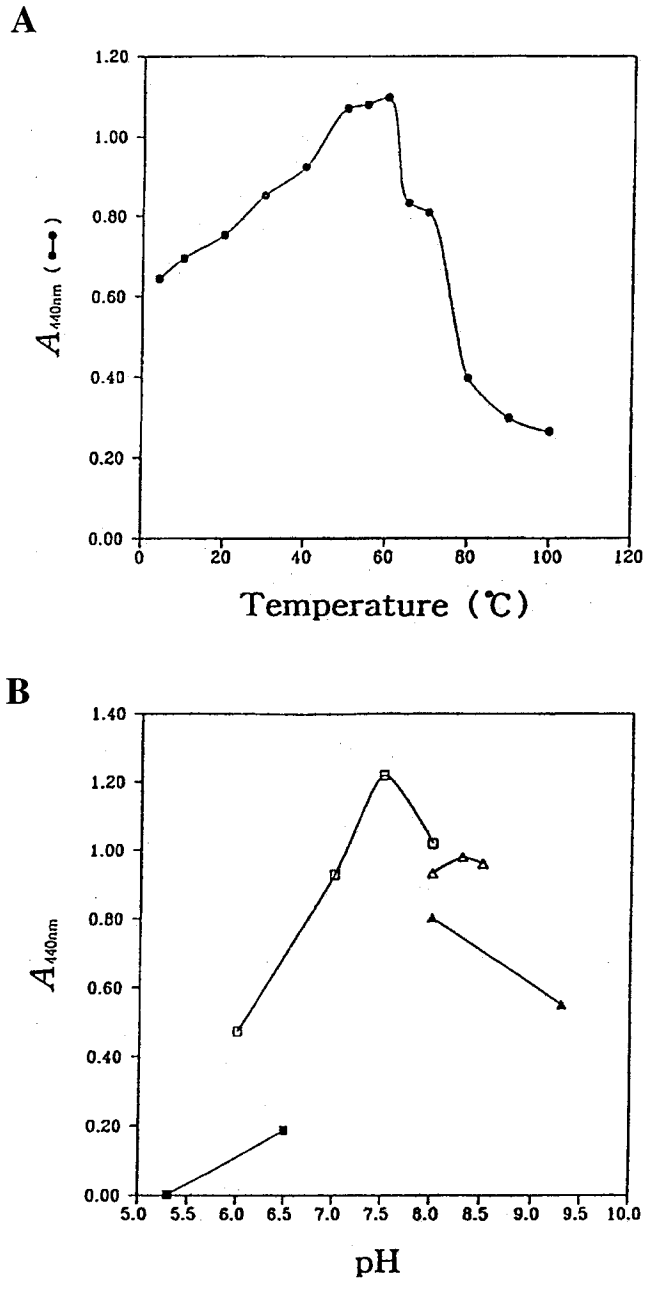

Fig. 9. Effects of temperature and pH on PepT activity. The relative enzyme activities of *B. subtilis* PepT for Met-Ala-Ser were determined at different temperatures (A) and pHs (B). ■–■, pH range 5.0–6.5 (0.1 M Citrate-NaOH); □–□, pH range 6.0–8.0 (0.1 M potassium phosphate); ▲–▲, pH range 7.5–8.5 (0.1 M HEPES); △–△, pH range 8.0–9.3 (0.1 M Borate-NaOH).

of chelating agents such as EDTA and 1,10-phenanthroline which blocked the tripeptidase activities by 70% and 80%, respectively, at concentrations as low as 0.5–1.0 mM (Fig. 10). This result prompted us to examine whether divalent cation(s) affect the enzymatic activity. Only Co^{2+} ion enhanced the acivity by 3 fold, whereas the others had little effect (Zn^{2+}, Cu^{2+}, Mg^{2+}, and Mn^{2+}) or significantly decreased the activity (Ca^{2+}, Ni^{2+}, Fe^{2+}, and Hg^{2+}) (Table 3), suggesting that this enzyme is a metallopeptidase that requires Co^{2+} ion(s) as a cofactor. 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 4, this enzyme had comparable activities on any of the substrates used and did not have a preference for any of them like most other tripeptidases reported (Hermsdorf, 1978; Strauch and Miller, 1983; Bosman *et al.*, 1990; Simitsopoulou *et al.*, 1997).

N-terminal sequence of the enzyme N-terminal sequence of purified enzyme was determined by Edman degradation. The N-terminal sequence of purified enzyme

Table 3. Effects of divalent cations on activity of *B. subtilis* PepT.

	Relative activity (%)[a]			
	Concentrations (mM)			
Ions	1.0	2.0	4.0	10.0
Cu^{2+}	116	103	100	97
Mg^{2+}	65	61	63	42
Mn^{2+}	113	73	81	84
Zn^{2+}	81	68	67	65
Co^{2+}	310	268	255	250
Ni^{2+}	51	36	36	31
Ca^{2+}	13	3	6	13
Fe^{2+}	10	0	0	0
Hg^{2+}	0	0	0	0

[a] 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 incubating with 4 mM EDTA prior to the color reaction for 10 min.

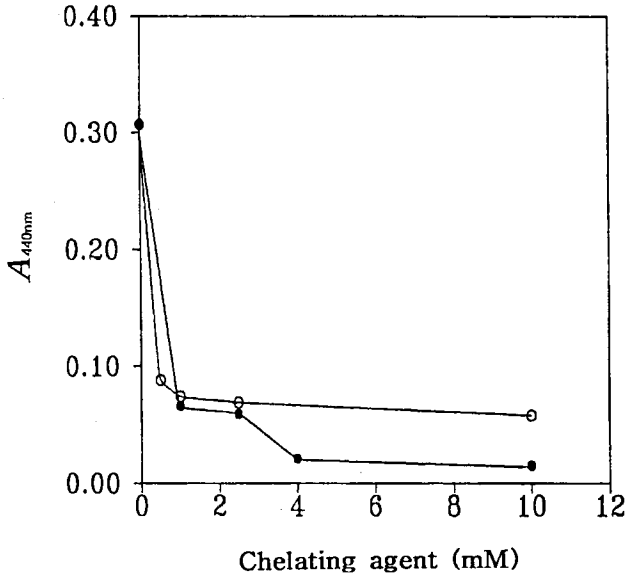

Fig. 10. Effects of EDTA and 1,10-phenanthroline on *B. subtilis* PepT activity. PepT activities were measured in the presence of various concentrations of EDTA (○–○) and 1,10-phenanthroline (●–●).

Table 4. Substrate specificity of *B. subtilis* PepT.

Substrate[a]	Relative activity (%)
Met-Ala-Ser	100
Leu-Gly-Gly	97
Leu-Ser-Phe	95
Leu-Leu-Tyr	88

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

was found to be MKEEIIERFTTYVXV where X denotes an unambiguous amino acid residue. This sequence turned out to be identical to that expected from a cloned *B. subtilis pepT* gene (Schrogel *et al.*, 1996).

Discussion

We have purified and characterized a PepT from *B. subtilis*. We conclude that the purified enzyme is the PepT on the basis of following criterions. First, the molecular mass of the purified enzyme, approximately 50,000 Da, was similar either to those of the monomeric form of the counterparts that have been purified from *L. lactis* (Bosman *et al.*, 1990) and *P. pentosaceus* (Simitsopoulou *et al.*, 1997) or to those of gene products that are expected from cloned *pepTs* from *S. typhimurium* (Miller *et al.*, 1991), *L. lactis* (Mierua *et al.*, 1994), *and B. subtilis* (Schrogel *et al.*, 1996). Second, the enzyme is a metallopeptidase like tripeptidases isolated from *S. thyphimurium*, *L. lactis*, and *P. pentosaceus*, although they require different metal ions for their activation (Hermsdorf, 1978; Miller and Schwartz, 1978; Strauch and Miller, 1983): Mn^{2+} and Zn^{2+} stimulate *S. typhimurium* and *L. lactis* PepTs while Mg^{2+}, Mn^{2+}, or Ca^{2+} are needed for *P. pentosaceus* PepT activity. The activity of *B. subtilis* PepT is reversibly inhibited by EDTA and can only be restored by Co^{2+} ion(s), yet we do not know how many Co^{2+} ions are required. Third, PepTs isolated from *E. coli*, *S. typhimurium*, *L. lactis*, and *P. pentosaceus* are specific for tripeptides with varying sequences, yet little or no activities are detected against dipeptides, tetrapeptides, and N- or C-blocked tripeptides (Hermsdorf, 1978; Bosman *et al.*, 1990; Simitsopoulou *et al.*, 1997). In our experiment, *B. subtilis* PepT was able to hydrolyze four different tripeptides with similar efficiency. However, whether this enzyme has the activity for substrates other than tripeptides remains to be clarified. Finally, the N-terminal sequence of the purified enzyme has a homology of 50% to the regions of putative *S. typhimurium* (Miller *et al.*, 1991) and *L. lactis pepT* products (Mierua *et al.*, 1994), and furthermore is identical to that of the deduced *B. subtilis pepT* product (Schrogel *et al.*, 1996). In addition, the enzyme was not affected by protease inhibitors such as serine, leucine, and aspartic protease inhibitors. Taken together, these results suggest that the purified enzyme from *B. subtilis* is the PepT with common characetristics of tripeptidases.

L. lactis and *P. pentosaceus* PepTs are composed of two identical subunits forming a dimer which is its active form (Bosman *et al.*, 1990; Simitsopoulou *et al.*, 1997). On the other hand, *E. coli* PepT has a molecular mass of 80,000 Da as determined by gel filtration chromatography (Hermsdorf, 1978), yet it is not known whether *E. coli* PepT is present in a monomeric or dimeric form. Our data suggest that *B. subtilis* PepT may be active in a monomeric

form since gel filtration chromatography demonstrated that a molecular mass with approximately 50,000 Da had an enzymatic activity. In addition, β-mercaptoethanol or DTT did not inhibit the enzymatic activity (data not shown).

In this study we demonstrated that *B. subtilis* PepT is a metalloenzyme because its activity is strictly dependent upon the presence of Co^{2+} ion(s). A gene encoding *B. subtilis* PepT was also available as previously reported (Schrogel *et al.*, 1996). These facts would facilitate, in further study, to investigate the catalytic function of PepT at a molecular level including the role of Co^{2+} ion(s) in the mechanism of enzymatic reaction and involvement of Co^{2+} ion(s) at the active site.

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