Purification and Characterization of *Aeromonas caviae* Aminopeptidase Possessing Debittering Activity

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An aminopeptidase (EC 3.4.11.10) was purified to homogeneity from the culture filtrate of *Aeromonas caviae* T-64. The purified enzyme showed maximum activity at pH 8.5 and 50 °C and was inhibited by *o*-phenanthroline and EDTA. The M_r values estimated by MALDI-TOF mass spectrometry and SDS−PAGE were 29 500 and 31 000 Da, respectively. These results indicate that the enzyme is a monomeric metalloenzyme. The enzyme hydrolyzed the di- and tripeptides containing hydrophobic amino acid residues such as valine, isoleucine, leucine, tyrosine, and phenylalanine in the N-terminal and/or adjacent positions with high hydrolysis efficiency (K_{cat}/K_m). This substrate specificity was not restricted to the di- and tripeptides as the peptides longer than tripeptides were hydrolyzed at a high rate when hydrophobic amino acid residues were located in the N-terminal region.

Keywords: *Debittering; Aeromonas aminopeptidase; bitter peptide; substrate specificity; capillary electrophoresis*

INTRODUCTION

As previously reported, successful reduction of the bitter taste in protein hydrolysates can be achieved by using the aminopeptidase from *Aeromonas caviae* T-64 (Izawa *et al*., 1997). Peptides with two identical amino acids at the N terminus are not hydrolyzed at rates comparable with analogous amino acyl *p*-nitroanilides or amino acyl *β*-naphthylamides, which are often used for assaying aminopeptidase activity (Taylor, 1993). To further investigate the substrate specificity of the *Aeromonas* aminopeptidase, and thus give credence to the suggested cause of the debittering activity, we undertook to use peptides rather than peptide analogs for further characterization of this enzyme.

In this paper, the purification and characterization of the enzyme are reported. Using capillary electrophoresis, a rapidly evolving technique that affords peptide separation with high resolution, the substrate specificity of the enzyme is revealed to be in agreement with its debittering activity. On the basis of a detailed analysis of the kinetic parameters for the various peptides, the putative structure of the functional domain of the enzyme is also discussed.

MATERIALS AND METHODS

Microorganism and Cultivation. *A. caviae* T-64 used in this study was previously isolated from soil. The microorganism was cultivated aerobically on a rotary incubator at a speed of 200 rpm in a medium composed of tryptone 1% (w/v), glucose 0.1% (w/v), soytone 0.1% (w/v), yeast extract 0.1% (w/v), K_2HPO_4 0.1% (w/v), NaCl 0.1% (w/v), and $MgSO_4 \cdot 7H_2O$ 0.01% (w/v), pH 7.0. After 24 h of cultivation at 30 °C, the cells were removed by centrifugation.

Measurement of Enzyme Activities. Proteinase activity was assayed at 30 °C for 10 min in 100 mM phosphate buffer (pH 7.0) containing 2% casein as a substrate (Tokita and Hosono, 1972). Aminopeptidase activity was determined in 50 mM Tris-HCl buffer (pH 8.0) at 30 °C by using 2 mM Leu-pNA as a substrate. The reaction was initiated by adding enzyme to the preincubated substrate solution. The increase in absorbance at 405 nm was monitored at 10 s intervals for 5 min on a spectrophotometer (Beckman Model 600), and the initial rate of hydrolysis was calculated (Hayashi and Law, 1989). One unit of the aminopeptidase activity was defined as the amount of enzyme that produced 1 μmol of *p*-nitroaniline/min under the standard conditions.

Effect of pH and Temperature on the Enzyme Activity. Effect of pH on the enzyme activity was measured in 50 mM acetate (pH 4.0−5.8), 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 5.2−7.2), 50 mM phosphate (pH 6.5−8.2), 50 mM Tris-HCl (pH 7.0−9.0), 50 mM 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES) (pH 8.2−10.1), and 50 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) (pH 9.4−11.0) buffers containing 2 mM Leu-pNA as the substrate at 30 °C. Effect of temperature on the enzyme activity was measured in 50 mM Tris-HCl buffer (pH 8.0) containing 2 mM Leu-pNA as substrate at different temperatures from 0 to 80 °C.

Stability of the Aminopeptidase at Different Temperatures and pH. To estimate the thermal stability of the enzyme, the enzyme was preincubated in 50 mM Tris-HCl buffer (pH 8.0) for 30 min at different temperatures (from 0−80 °C), and the remaining activity was measured in the standard assay. To estimate the pH stability of the enzyme, the enzyme was preincubated with the same buffer condition described previously (from pH 4.0 to 11.0) for 30 min at 30 °C; the remaining activity was measured in the standard assay.

Effect of Inhibitors and Metal Ions. The enzyme was preincubated with inhibitors and metal salts for 30 min at 4 °C at various concentrations in 50 mM Tris-HCl buffer (pH 8.0; 50 μL). After incubation, aliquots (10 μL) of sample were mixed with the substrate solutions (990 μL) and the enzyme activities were assayed under the standard conditions. The effect of metal ions was measured in the same manner except that the enzyme was preincubated in the presence of 1 mM of $ZnCl_2$, $MgCl_2$, $CaCl_2$, $BaCl_2$, $NiCl_2$, $CoCl_2$, $MnCl_2$, and $CuCl_2$.

Purification of Aminopeptidase. Solid ammonium sulfate was added to the culture supernatant (12 L) to a final saturation of 90% at 4 °C. The precipitate was collected by centrifugation at 10000*g* for 60 min and dissolved in 170 mL of 10 mM Tris-HCl buffer (pH 8.0). The solution was then dialyzed against 10 L of the same buffer at 4 °C.

Dialyzed solution was applied to a DEAE-Toyopearl 650 (Tosoh Co., Tokyo, Japan) column (2.8 × 65 cm) equilibrated with 10 mM Tris-HCl buffer (pH 8.0). The column was eluted with a linear increasing gradient of NaCl from 0 to 0.4 M in the same buffer (1400 mL) at a flow rate of 22.5 mL/h.

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Fractions (18 mL) were collected and assayed for proteinase and aminopeptidase activity.

Aminopeptidase fractions were pooled and dialyzed against 20 mM Tris-HCl buffer (pH 8.0) containing 0.04 M NaCl. A fraction of dialyzed solution (about 100 mg of protein) was applied to a Mono-Q HR10/10 equilibrated with the same buffer containing 0.04 M NaCl. The column was eluted with a linear increasing gradient of NaCl from 0.04 to 0.2 M in the same buffer (280 mL) at a flow rate of 8 mL/min, and fractions (7 mL) containing aminopeptidase activity were pooled and stored at −20 °C.

A fraction of sample (about 1.1 mg of protein) was applied to a gel filtration column of Superose 12 HR15/60 previously equilibrated with 10 mM Tris-HCl buffer (pH 8.0) containing 0.2 M NaCl at a flow rate of 0.2 mL/min. The active fractions were pooled and used as purified aminopeptidase. The last two purification steps were repeated by applying a fraction of sample to each column.

Determination of Protein Content. Protein concentrations were determined spectrophotometrically at 280 nm using $E^{1\%}_{1cm} = 14.4$, which was calculated using the amino acid composition of *A. caviae* aminopeptidase (Izawa and Hayashi, 1996).

Molecular Mass Determination. SDS−PAGE was performed in 12.5% gel at pH 8.3 according to the method of Laemmli (1970). The gel was stained with Coomassie brilliant blue R-250.

The molecular mass measurement based on the MALDI-TOF mass spectrometry (Beavis, 1992; Karas *et al.*, 1991) was carried out by using Kompact MALDI II, Shimadzu, Kyoto, Japan. The calculated figures are the average of four measurements.

Determination of K_{cat} and K_m. Enzyme activity toward *p*-nitroanilide derivatives was measured under the standard conditions described above.

Released amino acids was quantitatively analyzed to determine the aminopeptidase activity for the various peptides. The reactions were monitored by capillary electrophoresis (Gordon *et al.*, 1988; Bergman *et al.*, 1991; Schwartz *et al.*, 1994) on a P/ACE System 2000 with a laser-induced fluorescence detector (Beckman). The enzyme reactions were carried out in 50 mM phosphate buffer for 20 min and terminated by heating in boiling water for 1 min. The reaction products were then derivatized with 7-fluoro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-F) as follows. Aliquots (50 μL) of the reaction mixtures were mixed with 25 μL of 100 mM/50 mM borate/phosphate buffer (pH 8.0) and 50 μL of 10 mM NBD-F ethanol solution and then incubated for 20 min at 60 °C. In the case of the substrate solutions containing >5 mM peptide, the reaction mixtures were diluted to 5 mM peptides with 50 mM phosphate buffer (pH 8.0) prior to derivatization. A fused silica capillary with an inner diameter of 75 μm and a total length 57 cm (50 cm to the detector) was used. Borate/phosphate buffer (50 mM/25 mM), pH 8.0−8.5, was employed as the electrolyte. Runs were carried out at 20 kV and at 23 °C.

Values for K_{cat} and K_m were calculated from two individual experiments. Each experiment was carried out at five different substrate concentrations. The substrate concentrations [S (mM)] were designed so that $1/S$ (mM) ranged from 20% to 200% of the $1/K_m$ (mM). The substrates Phe-pNA, Gly-pNA, Ser-Phe, and Leu-Asp were examined from 0.18 to 0.30 mM, from 1.0 to 2.0 mM, from 8.0 to 25 mM, and from 2.5 to 12.5 mM, respectively, owing to their low solubility and/or high K_m values. Calculation of the kinetic constants was carried out by using a computer program (GraFit, Erithacus Software, Staines, U.K.)

Chemicals. Diisopropyl fluorophosphate (DFP) was obtained from Wako Chemical (Tokyo, Japan), and *o*-phenanthroline was purchased from Sigma Chemical (St. Louis, MO). Other inhibitors were purchased from Boehringer Mannheim. Nva-Phe and Ile-Phe were purchased from Kokusan Chemical Co. Ltd. (Tokyo, Japan). Arg-Arg-Pro-Phe-Phe and Val-Tyr-Pro-Phe were synthesized by Sawady Technology Co. Ltd. (Tokyo, Japan). Other substrates used were obtained from Sigma. Tryptone, soytone, and yeast extract were obtained from Difco. All other chemicals were obtained as analytical grade.

Figure 1. Homogeneity of the purified aminopeptidase on SDS−PAGE.

Table 1. Purification of the Extracellular Aminopeptidase from *A. caviae* T-64

step	total activity (units)	total protein (mg)	specific activity (units/mg)	yield (%)	purifn fold
culture filtrate	39600	99000	0.4	100	1
$(NH_4)SO_4$ precipitate	27918	12690	2.2	70.5	6
DEAE-Toyopearl	6534	594	11.0	16.5	28
Mono-Q	3255	93	35.0	8.2	109
Superose 12	1980	36	55.0	5.0	138

RESULTS

Purification of Aminopeptidase. The culture of *A. caviae* T-64 showed proteinase and aminopeptidase activities, both of which were higher in the culture filtrate than in the intracellular fraction. In the DEAE chromatographic step, these active peaks were eluted together. Separation of proteinase from peptidase activity was achieved by column chromatography on Mono-Q HR10/10. The enzyme was purified 138-fold from the culture filtrate with a recovery of 5.0%, giving a specific activity of 55 units/mg as summarized in Table 1. The purified enzyme obtained after gel filtration was found to be homogeneous by SDS−PAGE (Figure 1).

M_r Value. The M_r of the purified enzyme was estimated to be 29 500 Da by MALDI-TOF mass spectrometry. A single protein band corresponding to a M_r value of 31 000 Da was observed via the SDS−PAGE. These results indicated that the enzyme exists as a monomer.

Effect of Temperature and pH on the Activity and Stability of the Enzyme. The enzyme showed maximum activity at 50 °C (Figure 2). The relative activities at 20 and 30 °C were 11% and 47%, respectively, of the optimal activity. The optimal pH for the enzyme activity was nearly pH 8.5 (Figure 3).

The effects of temparature and pH on the enzyme stability are shown in Figures 4 and 5. The enzyme was

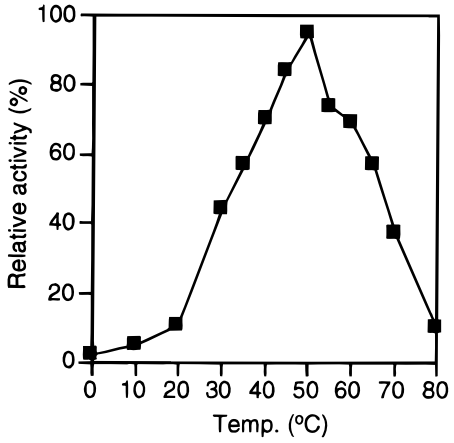

Figure 2. Effect of temperature on enzyme activity.

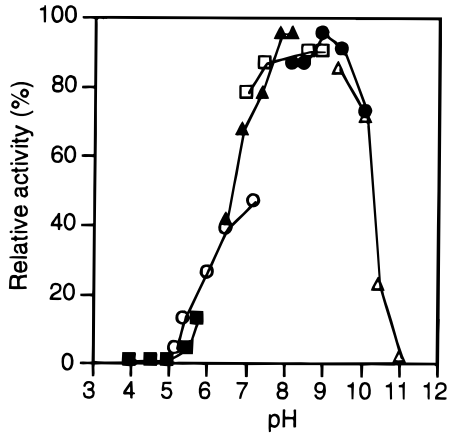

Figure 3. Effect of pH on enzyme activity. Buffers used: ■, acetate buffer; ○, MES buffer; ▲, phosphate buffer; □, Tris-HCl buffer; ●, CHES buffer; △, CAPS buffer.

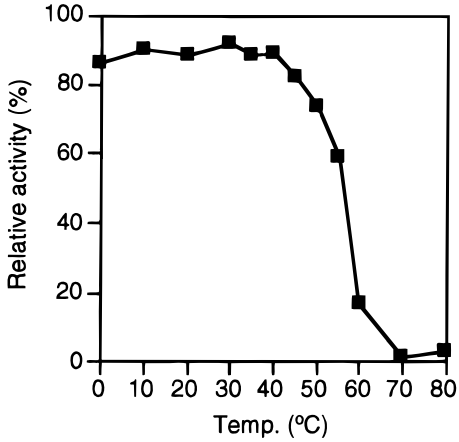

Figure 4. Effect of temperature on stability of the enzyme.

stable below 40 °C for 30 min. The enzyme was stable between pH 8.0 and pH 9.0 for 30 min.

Effect of Inhibitors and Metal Ions. The influence of inhibitors on the activity of the aminopeptidase is summarized in Table 2. The enzyme activity was not affected by inhibitors commonly used for serine, cysteine, and aspartic acid proteases. The enzyme was strongly inhibited by metal chelating agents such EDTA (10 mM) or *o*-phenanthroline (0.1 mM). It was observed that the inhibition by EDTA was pH dependent and more effective at pH 7.2 than at pH 8.0. Besides chelating agents, the aminopeptidase inhibitor bestatin (Suda *et al.*, 1976) strongly inhibited enzyme activity. None of the tested metal ions (Zn^{2+}, Mg^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, and Cu^{2+}) enhanced activity of the enzyme, while Co^{2+}, Mn^{2+}, and Cu^{2+} inhibited the activity.

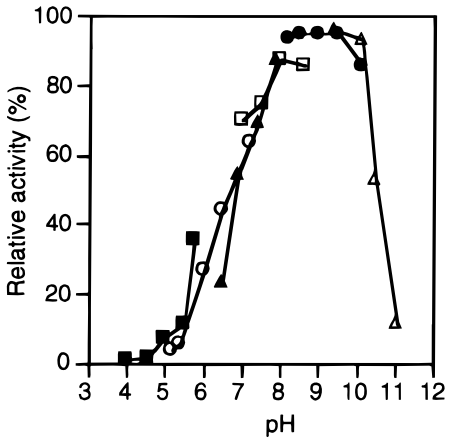

Figure 5. Effect of pH on stability of the enzyme. Buffer conditions were the same as in Figure 3.

Table 2. Effect of Inhibitors

inhibitor	concn (mM)	rel activity
control (no inhibitor)		100
EDTA[a] (pH 8.0)	10	59
	1	90
EDTA[a] (pH 7.2)	10	7
	1	44
o-phenanthroline	1	1
	0.1	11
bestatin	0.5	0
	0.05	1

[a] Preincubated for 3 h at 30 °C. No inhibition was observed in the presence of the following chemicals: 0.1 mM antipain, 1 mM chymostatin, 0.1 mM leupeptin, 30 mM AEBSF, 1 mM PMSF, 0.03 mM aprotinin, 1 mM DFP, 1 mM TPCK, 1 mM TLCK, 1 mM PCMB, 0.2 mM E-64, 1 mM pepstatin, and 1 mM phosphoramidon.

The metal ion requirement for enzyme activity was tested by adding metal ions (described above) to the EDTA-inactivated enzyme. The purified enzyme was dialyzed against 50 mM Tris-HCl buffer (pH 7.2) containing 10 mM EDTA at 5 °C for 24 h, and then excess EDTA was removed by using Sephadex column chromatography. The column was previously equilibrated with 50 mM Tris-HCl buffer containing 0.01 mM EDTA. After incubation of the EDTA-inactivated enzyme for 30 min at 4 °C at pH 8.0 in the presence of 1 mM metal ions, the activity was measured according to the standard assay. Incubation with Zn^{2+} resulted in the enzyme activity being restored to 40% of its pre-EDTA-treatment activity. However, incubation with Mg^{2+}, Ca^{2+}, Ba^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, and Cu^{2+} did not restore any enzyme activity. The EDTA-inactivated enzyme was found to be unstable since after storage for 24 h at 4 °C it was not reactivated by Zn^{2+}.

Influence of N-Terminal Amino Acid Residue. To investigate the effect of the N-terminal amino acid on the enzyme activity, kinetic parameters for amino acid pNAs and a series of dipeptides with L-phenylalanine at the C terminus were measured (Table 3). The enzyme liberated all amino acids except the acidic amino acids from the tested substrates. Proline iminopeptidase activity was confirmed since Pro-pNA and Pro-Phe were hydrolyzed. The enzyme readily hydrolyzed substrates possessing hydrophobic amino acids at the N-terminal position.

A methyl group as a branch on the side chain of the amino acid reduced the K_m value as shown by the

Table 3. Influence of N-Terminal Amino Acid

substrate	$K_{cat} \pm$ SE (s^{-1})	$K_m \pm$ SE (mM)	K_{cat}/K_m ($mM^{-1}\ s^{-1}$)
Leu-pNA	38 ± 3.4	0.10 ± 0.018	380
Met-pNA	8.2 ± 0.34	0.96 ± 0.080	8.5
Phe-pNA	6.3 ± 2.1	0.52 ± 0.26	12
Val-pNA	0.26 ± 0.0082	0.19 ± 0.014	1.4
Ala-pNA	0.26 ± 0.026	5.9 ± 1.2	0.044
Lys-pNA	0.077 ± 0.0025	1.9 ± 0.16	0.041
Arg-pNA	0.028 ± 0.00040	2.0 ± 0.080	0.014
Gly-pNA	0.012 ± 0.0047	7.2 ± 3.4	0.002
Pro-pNA	0.0037 ± 0.00010	1.6 ± 0.11	0.002
Glu-pNA	<0.001[a]		
Leu-Phe	41 ± 1.5	0.050 ± 0.0048	820
Ile-Phe	3.4 ± 0.079	0.030 ± 0.0026	113
Nva-Phe	10 ± 0.41	0.45 ± 0.058	22
Met-Phe	22 ± 0.91	1.1 ± 0.10	20
Phe-Phe	158 ± 12	2.1 ± 0.27	75
Val-Phe	1.3 ± 0.094	0.070 ± 0.0093	19
Ala-Phe	1.2 ± 0.069	14 ± 1.2	0.090
Lys-Phe	4.2 ± 0.46	5.0 ± 1.2	0.84
Arg-Phe	6.0 ± 0.38	4.6 ± 0.54	1.3
Gly-Phe	0.013 ± 0.00070	33 ± 2.8	<0.001
Pro-Phe	0.41 ± 0.18	31 ± 15	0.013
Ser-Phe	3.3 ± 2.2	173 ± 126	0.020
Tyr-Phe	11 ± 0.85	6.6 ± 1.1	1.7
Glu-Phe	<0.001[a]		

[a] Initial rate of hydrolysis measured at 5 mM substrate concentration.

results for Leu-Phe (0.050 mM), Ile-Phe (0.030 mM), and Val-Phe (0.070 mM), which were less than one-sixth of that for the nonbranched substrate Nva-Phe (0.45 mM). In contrast to the similar low K_m values for these branched substrates, K_{cat} values of Ile-Phe and Val-Phe were $^1/_{12}$ and $^1/_{30}$, respectively, of that of Leu-Phe, indicating that the methyl group at more distal position enhanced the catalytic process and a minimum length of side chain is required for effective hydrolysis of the substrates. Of the dipeptides having a N-terminal aliphatic amino acid, Ala-Phe showed the lowest K_{cat}/K_m ratio (0.090 $mM^{-1}\ s^{-1}$). Gly-Phe showed the lowest K_{cat}/K_m ratio (<0.001 $mM^{-1}\ s^{-1}$) among the tested dipeptides.

In contrast to the effect of a methyl group, a hydroxyl group on the side chain had a negative influence on the enzyme activity, as the K_{cat}/K_m ratio for Tyr-Phe was $^1/_{40}$ of that of Phe-Phe. Similarly, Ser-Phe gave a lower K_{cat}/K_m ratio (0.020 $mM^{-1}\ s^{-1}$) than Ala-Phe (0.090 $mM^{-1}\ s^{-1}$).

Phenylalanine was liberated from Phe-Phe at an extremely high rate of hydrolysis, but due to its relatively high K_m value, the K_{cat}/K_m ratio for Phe-Phe was lower than those of Leu-Phe and Ile-Phe.

Influence of the Adjacent Amino Acid Residue. Kinetic parameters for leucyl dipeptides and other peptides are summarized in Table 4. These data revealed that the nature of the amino acid residue in the adjacent position to the N-terminal amino acid also significantly affects the enzyme activity.

Leucine at the C terminus reduced the K_m value, as Leu-Leu gave the lowest K_m (0.0070 mM). An acidic residue affected the enzyme activity even in the adjacent position, as the K_m for Leu-Asp was 20-fold greater than that of any other leucine dipeptide. The highest K_{cat} was observed when Leu-Phe was used as a substrate, and the substrates containing bulky residues such as methionine, arginine, and tyrosine tended to give high K_{cat} values. The only exception to this tendency was observed when Leu-Leu was used as a substrate.

Comparison of the kinetic values for Leu-Leu, Leu-Phe, Phe-Leu, and Phe-Phe indicated that Phe at the C terminus enhances K_{cat} and K_m values.

Table 4. Influence of Adjacent Amino Acid

substrate	$K_{cat} \pm$ SE (s^{-1})	$K_m \pm$ SE (mM)	K_{cat}/K_m ($mM^{-1}\ s^{-1}$)
Leu-Leu	4.0 ± 0.15	0.0070 ± 0.0011	571
Leu-Met	34 ± 2.6	0.021 ± 0.0037	1619
Leu-Phe	41 ± 1.5	0.050 ± 0.0048	820
Leu-Val	7.7 ± 0.35	0.11 ± 0.011	70
Leu-Ala	4.1 ± 0.27	0.073 ± 0.012	56
Leu-Arg	22 ± 1.1	0.067 ± 0.0093	328
Leu-Gly	15 ± 0.31	0.98 ± 0.048	15
Leu-Pro	<0.001[a]		
Leu-Asp	2.9 ± 0.31	24 ± 3.4	0.12
Leu-Ser	14 ± 1.4	1.1 ± 0.23	13
Leu-Tyr	17 ± 1.5	0.057 ± 0.015	298
Phe-Leu	7.9 ± 0.60	0.23 ± 0.036	34
D-Leu-L-Leu	<0.001[a]		
L-Leu-D-Leu	<0.001[a]		
Phe-Phe-Phe	172 ± 9.1	0.099 ± 0.018	1737
Leu-Leu-Leu	36 ± 5.7	0.039 ± 0.011	923
Leu-Leu-Tyr	22 ± 2.0	0.018 ± 0.0039	1222

[a] Initial rate of hydrolysis measured at 5 mM substrate concentration.

Table 5. Enzyme Activity on the Peptides Longer than Tripeptides

substrate	rel activity (μmol/s·μmol)
Ala-Ala-Ala-Ala	15.5[a]
Phe-Phe-Phe-Phe	226[b]
Val-Tyr-Pro-Phe	189[b]
Arg-Arg-Pro-Phe-Phe	26.9[b]
Ala-Ala-Ala-Ala-Ala-Ala	8.1[a]

[a] Enzyme activity measured at 5 mM substrate concentrations. [b] Enzyme activity measured at 0.5 mM substrate concentrations.

The enzyme does not possess X-proline aminopeptidase activity and its specificity is enantioselective as Leu-Pro, L-Leu-D-Leu, and D-Leu-L-Leu were not hydrolyzed.

The enzyme activity was affected by the presence of a third amino acid in the substrate. The values of K_{cat}/K_m for Phe-Phe-Phe, Leu-Leu-Leu, and Leu-Leu-Tyr were 2−23-fold greater than that of the corresponding dipeptides Phe-Phe and Leu-Leu. The increase in K_{cat}/K_m for Leu-Leu-Leu and Leu-Leu-Tyr were largely caused by the increase in K_{cat}, while that for Phe-Phe-Phe was by decrease in K_m.

Enzyme Activities on Peptides Longer than Tripeptides. The enzyme activities on the peptides longer than tripeptides are shown in Table 5, in which Val-Tyr-Pro-Phe and Arg-Arg-Pro-Phe-Phe have been reported to exhibit bitterness 3.3 and 50 times greater than caffeine, respectively (Ishibashi *et al.*, 1987). Hydrophobicity in the N-terminal regions of these peptides also enhanced the enzyme activity as Phe-Phe-Phe-Phe and Val-Tyr-Pro-Phe-Phe were hydrolyzed at a high rate in comparison to another peptides.

DISCUSSION

The enzyme was strongly inhibited by metal chelating agents such as EDTA (10 mM) or *o*-phenanthroline (0.1 mM). In particular, the enzyme was very sensitive to *o*-phenanthroline, which is known to be a specific inhibitor for Zn^{2+} metalloproteases. The addition of Zn^{2+} to the EDTA-inactivated enzyme restored up to 40% of the native enzyme activity. These results indicate that the enzyme is a Zn^{2+} metalloenzyme. The metal ion contained in the enzyme seems to bind tightly

to the enzyme, since complete inactivation of the enzyme requires long incubation such as 24 h at 4 °C in the presence of 10 mM EDTA.

From the kinetic parameters for the peptide substrates, it was found that both the substrate binding and the catalytic action of the enzyme are influenced by not only the N-terminal amino acid residue but also by the residue in the adjacent position. It is also apparent that the hydrophobicity of the amino acid side chain correlates to the affinity of the enzyme for the substrates. In addition, a higher K_{cat}/K_m ratio was observed for the tripeptides than for the dipeptides. These observations show that the binding site of the enzyme could fit peptides containing several hydrophobic amino acid residues.

In general, K_{cat} values observed for the dipeptides that had phenylalanine at the C-terminal position were higher than those observed for the corresponding amino acid pNAs, probably because of the K_{cat}-enhancing effect that phenylalanine has when it is present in the position adjacent to the N-terminal amino acid. The binding of the enzyme to a bulky amino acid residue may provide a structure for the enzyme−substrate complex that is advantageous to the catalytic process.

It can be pointed out that the kinetic properties of the enzyme described here are similar to those of *Aeromonas proteolytica* aminopeptidase (Prescott *et al*., 1971): (1) Both enzymes are strongly inhibited by *o*-phenanthroline, but for both enzymes inhibition by EDTA is less effective and also pH dependent (Prescott and Wilkes, 1966). (2) They are both monomeric metalloenzymes with M_r values of 29 500 (Prescott *et al*., 1971). (3) Unlike mammalian or plant leucine aminopeptidase (Frederik *et al*., 1973; Kohno *et al*., 1986; Mikkonen, 1992), the addition of Mn^{2+} or Mg^{2+} to the native enzymes does not affect the activity. (4) They have the highest K_{cat}/K_m ratio for N-terminal leucine and give a low K_m with a branched substrate (Wagner *et al*., 1972). (5) They require an N-terminal residue more bulky than valine for effective hydrolysis of the substrate. (6) Both show strong specificity not only for the N-terminal residue but also for the adjacent residue, and the dipeptides Leu-Met, Leu-Phe, and Leu-Arg were hydrolyzed at high rates (high K_{cat}), while Leu-Leu gives the lowest K_{cat} (Wagner *et al*., 1972). The first observation is not restricted to the two enzymes, as it has been commonly observed in other aminopeptidases, regardless of their source (Kohno *et al*., 1986; Mars and Monnet, 1995; Itoh and Nagamatsu, 1995; Sharma and Ortwerth, 1986). However, the purified enzyme markedly differs from *A. proteolytica* aminopeptidase in thermostability, which is stable at pH 8.0 and 70 °C for 1 h (Prescott *et al*., 1966, 1971).

We have previously shown that there is high homology (64% identity) between these two enzymes in their mature domains (Izawa and Hayashi, 1996). The similarity in the amino acid sequences and the kinetics properties of the two enzymes strongly suggests that they have a similar conformation in their functional domains. On the basis of these observations, the enzyme should be classified as *Aeromonas* aminopeptidase (EC 3.4.11.10).

As previously reported, the bitter taste of peptide solutions prepared by hydrolyzing casein and soy protein is effectively reduced by treatment with the enzyme, and the hydrophobic amino acids having Δf values of >1500 cal/mol, such as valine, isoleucine, leucine, tyrosine, and phenylalanine, accounted for >76% of the free amino acids released by the enzyme (Izawa *et al*., 1997). The purified enzyme hydrolyzed the di- and tripeptides containing these amino acid residues in the N-terminal and/or adjacent positions with high hydrolysis efficiency (K_{cat}/K_m). This substrate specificity was not restricted to di- and tripeptides as the peptides longer than tripeptides were hydrolyzed at a high rate when hydrophobic amino acids residues were located in N-terminal region. The data described here give credence to the debittering ability of *A. caviae* aminopeptidase.

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LITERATURE CITED

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