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Purification and characterization of an enzyme hydrolyzing L-methionine-4-nitroanilide from germinated sweet potato (*Ipomoea batatas* [L.] Lam. cv. Tainong 57) roots

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Abstract. Using L-methionine-4-nitroanilide substrate, a probable methionine aminopeptidase (MAP1) from germinated sweet potato (Tainong 57) roots was purified 121-fold with 14% yield by a five-step procedure comprising extraction, DEAE-Sephacel batch adsorption, and Sephacryl S-300 column, DEAE-Trisacryl-M column, and chromatofocusing column chromatography. The molecular weight of MAP1 was estimated by gel filtration on Sephacryl S-300 to be 163,300; the isoelectric point was 4.87. The molecular weight of the monomer was determined by SDS-polyacrylamide gel electrophoresis to be 54,800, suggesting that the holoenzyme contains three identical subunits. The enzyme was stable below 45 °C. Heat inactivation of MAP1 occurred in two steps and the corresponding thermodynamic parameters were determined. Among seventeen amino acid 4-nitroanilides tested, L-methionine-4-nitroanilide was a specific substrate. The enzyme retained 24% activity in the presence of 1 mM phenylmethane sulfonyl fluoride and 31% activity in the presence of 1 mM para-chloromercuribenzoate. Iodoacetic acid had no influence. One mM Mg^{2+} stimulated the enzyme 80%, while 2 μM Zn^{2+} inhibited 80% of the enzyme activity. MAP1 is quite different from the methionine aminopeptidases reported in literature.

Keywords: Heat inactivation; Inhibition; Methionine aminopeptidase; Subunits; Thermodynamic parameters; Zn^{2+}.

Abbreviations: EDTA, ethylene diaminetetraacetic acid; IAA, iodoacetic acid; MAP, methionine aminopeptidase; NHNan, 4-nitroanilide; pCMB, para-chloromercuribenzoate; PMSF, phenylmethane sulfonyl fluoride; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; 'T57', sweet potato variety Tainong 57.

Introduction

Methionine aminopeptidase (EC 3.4.11.x) catalyzes the removal of N-terminal methionine from proteins and is essential for *Escherichia coli* cell growth (Ben-Bassat et al., 1987; Chang et al., 1989; Roderick and Matthews, 1988). Polypeptides synthesized in the cytoplasm of eukaryotes are generally initiated with methionine, but N-terminal methionine is absent from most natural proteins. The removal of N-terminal methionine is also catalyzed by methionine aminopeptidase (MAP), one of the central enzymes in protein synthesis (Chang et al., 1990 and 1992; Moerschell et al., 1990; Tsunasawa et al., 1985). MAPs from various mammal tissues have been reported (Aoyagi et al., 1984; Aoyagi et al., 1985; Freitas et al., 1985; Kendall and Bradshaw, 1992; Matsuzawa et al., 1992; Termignoni et al., 1986). L-methionine-4-nitroanilide-hydrolyzing activity in sweet potato tissues has been reported (Lin and Tsai, 1991). Two sweet potato aminopeptidases, with molecular weights and isoelectric points of 115,000 and 5.53, and 63,000 and 6.08, respectively, and which hydrolyze 4-nitroanilides of both L-alanine and L-leucine, have also been reported (Lin and Chan, 1992). This paper describes an L-methionine-4-nitroanilide-hydrolyzing enzyme (MAP1) purified from germinated sweet potato (Tainong 57) roots, which may be a methionine aminopeptidase.

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Materials and Methods

Chemicals and Chromatographic Materials

Acrylamide, ammonium persulphate, and N, N'-methylene bisacrylamide were products of Bio-Rad (Richmond, CA, USA). Calibration kits for electrophoresis and Sephacryl S-300 gel-filtration, chromatofocusing kit (pH range 4 – 9), DEAE-Sephacel, DEAE-Trisacryl M, and Sephacryl S-300 were obtained from Pharmacia (Uppsala, Sweden). L-methionine-NHNan and PMSF were purchased from E. Merck (Darmstadt, Germany). Polyvinylpyrrolidone-40 (mol. wt. 40,000) was obtained from the Sigma Chemical Company (St. Louis, MO, USA). Diaflo ultrafiltration membranes PM10 (mol. wt. cutoff 10,000) were obtained from Amicon Inc. (Danvers, MA, USA).

Plant Material

Fresh roots of sweet potato (*Ipomoea batatas* (L.) Lam. cv. Tainong 57) were purchased from a local market.

Enzyme Extraction and Purification

All stages were carried out at 4 °C. One-hundred grams of germinated 'T57' roots were homogenized three times with 8 volumes (v/w) of 10 mM sodium phosphate buffer, pH 7.0 (buffer A) containing 1% (w/v) PVP-40 in a Polytron homogenizer (Luzern, Swiss) at high speed for 1 min. The homogenate was filtered through three layers of cheesecloth and then centrifuged at 11,000 x g for 20 min. The supernatant liquid, designated as crude extract, was stirred with 20 g DEAE-Sephacel, which absorbed the enzyme. The solution was discarded and the DEAE-Sephacel was washed with 40 ml of buffer A. The enzyme was eluted from DEAE-Sephacel with 100 volumes of 300 mM NaCl in buffer A, concentrated and desalted by Diaflo ultrafiltration membrane PM 10, and chromatographed on a Sephacryl S-300 column (3 x 90 cm) which was pre-equilibrated and eluted with buffer A. Active fractions were pooled and applied to a DEAE-Trisacryl M column (3 x 40 cm) pre-equilibrated with buffer A and eluted with a linear gradient of 0.1 – 0.3 M NaCl in buffer A. Active fractions were concentrated and desalted as described above. The enzyme solution was finally purified by chromatofocusing (Lampson and Tytell, 1965). It was applied to a poly-buffer exchanger (PBE 94) column (1.2 x 29 cm), which was equilibrated with 25 mM imidazole-HCl buffer (pH 7.4). The enzyme was eluted with 200 ml of Polybuffer 74, which was adjusted to pH 4.0 by HCl, at a flow rate of 22 ml/h. Fractions were dialyzed before protein and enzyme assays.

Assay of Enzyme Activity

The hydrolysis of L-methionine-4-nitroanilide was measured spectrophotometrically at 405 nm, as previously reported (Erlanger et al., 1961, 1966). The reaction mixture, which contained 0.3 ml of 1 mM substrate solution, 0.2 ml of 0.15 M sodium phosphate buffer (pH 7.0, buffer B), 0.2 ml of the enzyme solution, and 0.1 ml of double-distilled water, was incubated at 37 °C for 30 min and terminated with 0.2 ml of 30% acetic acid. The absorbance of the reaction mixture was measured at 405 nm. One unit was defined as the amount of enzyme required to hydrolyze 100 μmole methionine-NHNan/min under the assay conditions.

When determining the influence of inhibitors, 0.1 ml of various concentrations of inhibitor solution were used instead of the double-distilled water in the above protocol.

Table 1. Purification of MAP 1 from germinated 'T57' roots.

One-hundred grams, fresh weight, of roots was used. One unit was defined as the amount of enzyme required to hydrolyze 100 μmole of methionine-NHNan/min under the experimental conditions described in the text.

Procedure	Activity (units)	Protein (mg)	Spec. Act. (units/mg)	Purification (fold)	Yield (%)
Crude extract	924	1106	0.84	1	100
DEAE-Sephacel	694	228	3.04	3.6	75
Sephacryl S-300	579	51.5	11.25	13.4	63
DEAE-Trisacryl M	497	10.5	47.38	56.4	54
Chromatofocusing	131	1.3	101.35	120.7	14

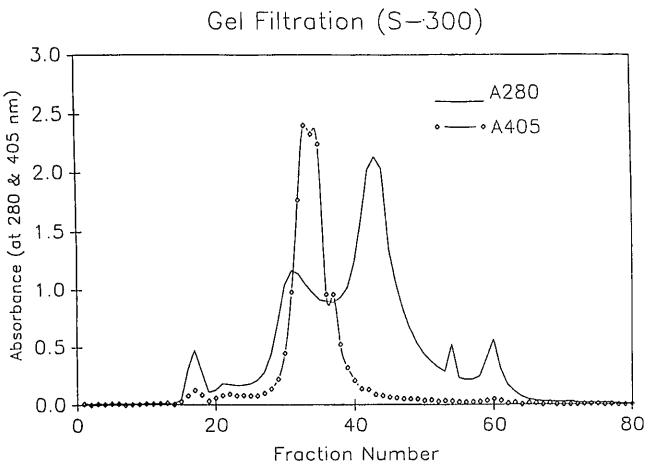

Fig. 1. Sephacryl S-300 column chromatography. Enzyme activity was determined by absorbance at 405 nm, protein concentration was determined by absorbance at 280 nm.

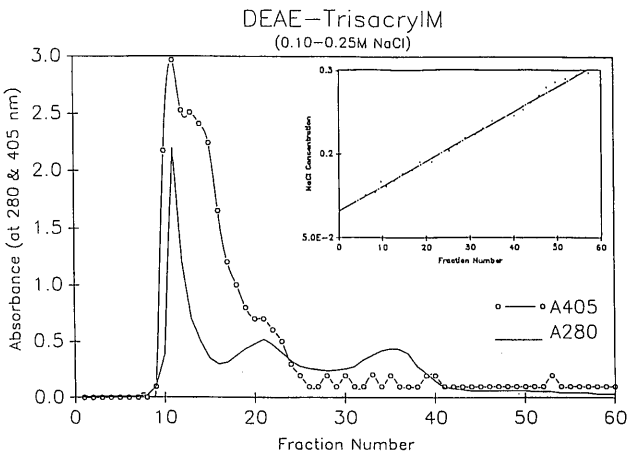

Fig. 2. DEAE-Trisacryl-M column chromatography. Enzyme activity was determined by absorbance at 405 nm, protein concentration was determined by absorbance at 280 nm.

Protein Estimation

The protein content of enzyme preparations was determined by the modified Bradford procedure (Compton and Jones, 1985). The assay consisted of the addition of 5 ml of the standardized Coomassie blue G-250 dye solution to 0.1 ml of protein solution, immediate mixing, and determination of A_{595}. Bovine serum albumin was used as the standard. During chromatography, protein content was estimated from A_{280}.

Estimation of Molecular Weight

The molecular weight of the enzyme was estimated by gel-filtration (Andrews, 1964) on a Sephacryl S-300 column (3 x 90 cm) with bovine serum albumin (67,000), aldolase (158,000), catalase (250,000), ferritin (440,000), and thyroglobulin (669,000) as standards. The molecular weight of the enzyme subunits was estimated by SDS-PAGE (Weber and Osborn, 1969) with α-lacalbumin (14,400), soybean trypsin inhibitor (20,100), carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (67,000), and phosphorylase b (94,000) as standards.

Thermal Inactivation of the Enzyme

Small quantities of enzyme solution (0.2 ml) with 0.2 ml of 0.15 M sodium phosphate buffer (pH 7.0, buffer B), and 0.1 ml of double-distilled water were preincubated at 25, 37, 45, 55, 65, and 75 °C for 10 – 70 min, and then removed and cooled in an ice bath for 20 min. Finally each residual enzyme activity was assayed in triplicate by adding 0.3 ml of 1 mM substrate solution and comparing with an identically prepared but unheated control sample. First order rate constants for inactivation (k) were obtained from the slopes of least square fits of ln (fractional activity remaining) versus time, $\ln E_t/E_o = -kt$, where E_t and E_o represent the residual enzyme activity at time t and the initial enzyme activity, respectively. Energy of activation to inactive form (E_a) can be obtained from the slope of Arrhenius plot ln k vs. 1/T according to equation

$$\ln k = -(E_a R^{-1} T^{-1}) + \text{constant}.$$

Calculation of Thermodynamic Parameters for MAP1 Inactivation

Thermodynamic parameters were calculated according to Eyring's transition state theory (Eyring, 1935).

$$k = k_b T h^{-1} e^{-\Delta F^*/RT} \quad (a)$$

$$E_a = \Delta H^* + RT \quad (b)$$

$$\Delta F^* = \Delta H^* - T\Delta S^* \quad (c)$$

Where ΔF^*, ΔH^*, ΔS^*, and E_a represent the free energy change, enthalpy change, entropy change, and energy of activation, respectively, for enzyme denaturation; k_b is Boltzmann's constant (1.3811×10^{-23} J K^{-1}), h is Plank's constant (6.6262×10^{-34} J s), T is the absolute temperature (K). Since inactivation rate constant k was obtained from the experiment described above, ΔF^* was readily calculated from equation (a). E_a was obtained as described above, therefore ΔH^* was calculated from equation (b). Finally, ΔS^* was calculated from equation (c).

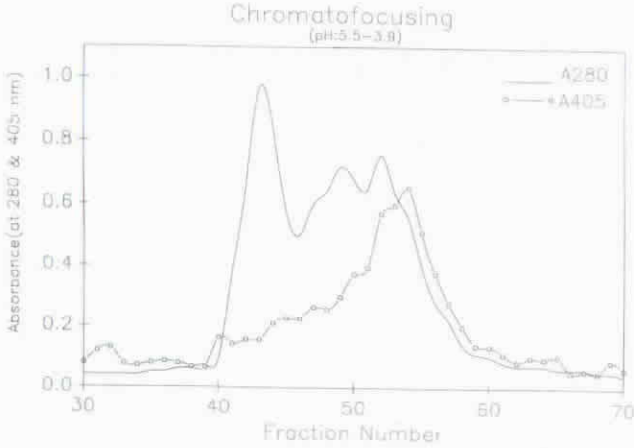

Fig. 3. Chromatofocusing column chromatography. Enzyme activity was determined by absorbance at 405 nm, protein concentration was determined by absorbance at 280 nm.

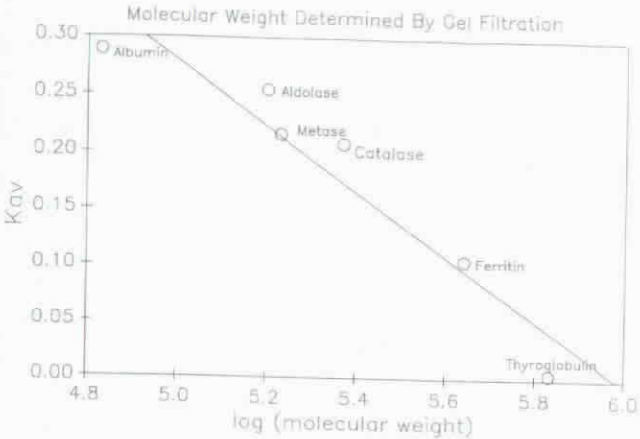

Fig. 4. Estimation of molecular weight of native MAP1 from germinated 'T57' roots by gel-filtration on Sephacryl S-300 column.

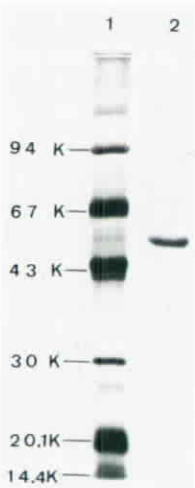

Fig. 5. Estimation of molecular weight of monomer of MAP1 from germinated 'T57' roots by 7.5% SDS-PAGE, pH 8.8. Lane 1, low molecular weight kit; lane 2.5 μg purified MAP1.

Results

Purification of the Enzyme

The purification results are summarized in Table 1. Starting with crude extract, a homogeneous enzyme preparation was obtained with 121-fold purification and 14% yield. Figs. 1–3 show details of Sephacryl S-300, DEAE-Trisacryl-M, and chromatofocusing column chromatography. The DEAE-Sephacel batch treatment resulted in a 3.6-fold purification. When the enzyme was eluted from DEAE-Sephacel, concentrated, desalted, and chromatographed on a Sephacryl S-300 column, six protein peaks, two enzyme peaks, and one enzyme shoulder were found (Fig. 1). Only the main enzyme-peak fractions were combined and applied to a DEAE-Trisacryl M column. We found three protein peaks and

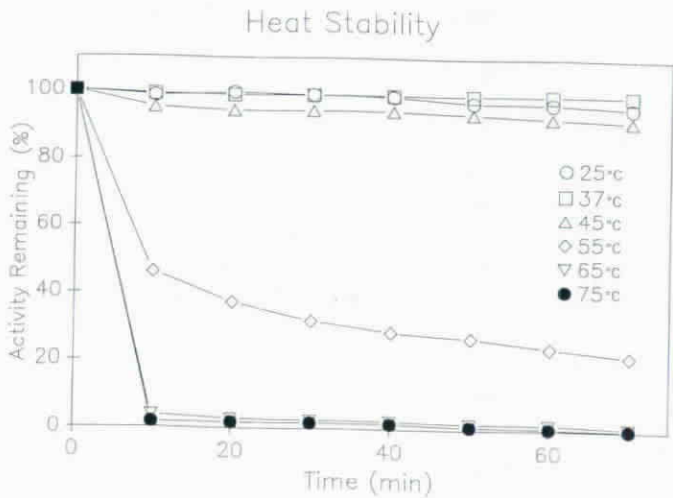

Fig. 6. Heat stability of native MAP1 from germinated 'T57' roots.

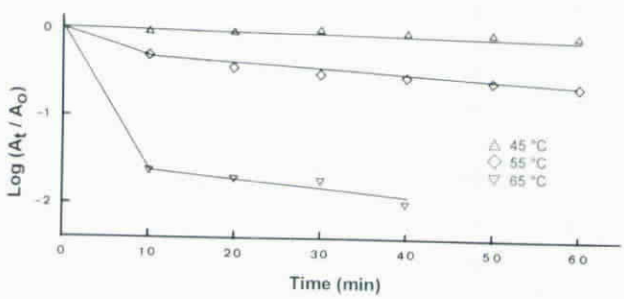

Fig. 7. Thermal inactivation of MAP1 of germinated 'T57' roots.

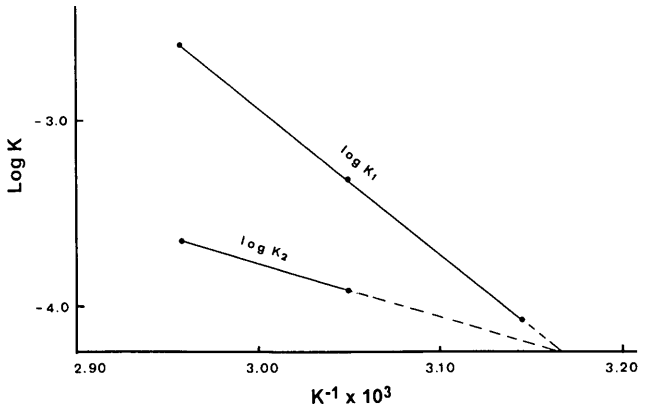

Fig. 8. Arrhenius plots for two-step thermal inactivation rate constants of MAP1 from germinated 'T57' roots.

one major and several minor enzyme peaks with two shoulders (Fig. 2). Fractions 8 – 17 were combined, desalted, and subjected to chromatofocusing (Fig. 3). There were three protein peaks with pI 4.44 (fraction no. 43), 4.69 (frac. no. 49), and 4.79 (frac. no. 52), respectively, while only one major enzyme peak with pI 4.87 (frac. no. 54) and one enzyme shoulder with pI 4.79 (frac. no. 52) were found. Fractions 53 – 56 were combined and designated as purified enzyme; the homogeneity was confirmed by Sephacryl S-300 gel-filtration (Fig. 4) and SDS-PAGE (Fig. 5).

General Properties

The molecular weights of the enzyme and subunit of the enzyme were estimated to be 163,300 by gel-filtration (Fig. 4) and 54,800 by SDS-PAGE (Fig. 5), respectively.

Influence of Temperature on Stability

The enzyme is quite stable below 45 °C. After heating at 45, 55, and 65 °C for 10 min, the remaining activity was 95%, 46%, and 2.5%, respectively. The enzyme completely loses its activity after being heated at 75 °C for 10 min (Fig. 6). Comparison of the data from heating at 45, 55, and 65 °C suggest that denaturation of the enzyme may occur in two steps: the holoenzyme dissociates into monomers in step one and the monomers denaturate in step two (Fig. 7). By Arrhenius plots, E_a of step one (E_{a1}) was calculated to be 185.9 kJ and E_a of step two (E_{a2}) was calculated to be 55.3 kJ (Fig. 8). Other thermodynamic parameters of the denaturation of the enzyme are shown in Table 2.

Table 2. Thermodynamic parameters for the denaturation of 'T57' MAP 1 at 65 °C

Step	k (s^{-1})	ΔF^* (kJ)	E_a (kJ)	ΔH^* (kJ)	ΔS^* (J/K)
1	2.67×10^{-3}	44.35	185.9	183.1	410.6
2	2.22×10^{-4}	47.38	55.3	52.5	15.3

Influence of pH on Activity

The influence of pH on the purified MAP1 is similar to that on crude enzyme (Lin and Tsai, 1991). The enzyme is slightly more active at pH 8.9 than at pH 7.0, while at pH 3.4 it exhibits only 40% of the activity it does at pH 7.0.

Substrate Specificity

The hydrolytic activity of MAP1 against synthetic substrates is shown in Table 3. The enzyme strongly prefers Met-NHNan. Of seventeen substrates tested, the enzyme cannot hydrolyze eight and hydrolyzes four at very low rates. Gly-NHNan, the second most readily

Table 3. Hydrolytic activity of MAP1 from germinated 'T57' roots against synthetic substrates.

In each assay, 0.5 µg of MAP1 was used. The final concentration of synthetic substrates was 0.375 mM.

Substrate	Activity (%)
Met-NHNan	100
Gly-NHNan	26
Ala-NHNan	24
Pro-NHNan	22
Ala-Ala-Ala-NHNan	17
Ala-Ala-Val-Ala-NHNan	6
CBZ-Phe-Ala-NHNan	6
Val-NHNan	1
Ace-Ala-NHNan	1
Ala-Ala-NHNan	0
Arg-NHNan	0
Cystine-bis-NHNan	0
Glu-NHNan	0
γ-Glu-NHNan	0
N-CP-Phe-Ala-NHNan	0
CP-Phe-NHNan	0
Glutaryl Phe-NHNan	0

Abbreviations: **Ace,** acetyl; **CBZ,** carbobenzoxy; **CP,** 3-carboxy-propionyl.

Table 4. Influence of some reagents on 'T57' MAP1 activity.
The mixtures each containing 0.5 μg MAP1 in 0.2 ml solution of germinated 'T57' roots, 0.2 ml buffer B, and 0.1 ml of various reagents with the indicated final concentrations, were incubated for 30 min at 25 °C, and the levels of MAP1 activity of the mixtures were then assayed by adding 0.3 ml substrate under standard conditions.

Addition	Concentration mM	Activity %	Type[a]
Control		100	
EDTA	1	89	M
	2	73	
	5	70	
pCMB	1	31	C or S
IAA	1	104	C
1,10-Phenanthroline	0.4	87	M, C, or S
	0.6	81	
	1	81	
PMSF	1	24	S or C

[a]Mechanistic set of peptidases: **C** for cysteine type; **M** for metallo type; **S** for serine type (Storey and Wagner, 1986).

Table 5. Influence of metal ions on 'T57' MAP1 activity.
The details are the same as in Table 3 for control assays. For others, metal ions were included, with the final concentrations shown.

Addition	Concentration mM	Activity %
Control		100
$MgCl_2$	0.5	156
	1.0	179
$CaCl_2$	0.5	117
	1.0	127
$MnCl_2$	0.5	90
	1.0	101
$NiCl_2$	0.5	83
	1.0	81
	4.0	35
$CoCl_2$	0.2	46
	0.5	55
$CuCl_2$	0.5	20
	1.0	6
$FeCl_3$	0.2	27
	0.5	8
$ZnCl_2$	0.5	1

Table 6. Influence of Zn^{2+} on 'T57' MAP1 activity.
The details are the same as in Table 5, with Cl^- as the counter ion.

Addition	Concentration μM	Activity %
Control		100
Zn	50	1.0
	40	1.2
	30	2.4
	20	3.5
	18	3.6
	16	3.8
	14	4.4
	12	5.1
	10	5.8
	8	7.0
	6	9.1
	4	13.1
	2	21.2

hydrolyzed substrate, is hydrolyzed at only 26% of the rate of Met-NHNan hydrolysis.

Influence of Inhibitors

The influence of some reagents on MAP1 activity are shown in Table 4. The catalytic mechanism of MAP1 from germinated 'T57' roots is serine type.

Influence of Metal Ions

The influence of metal ions on MAP1 activity is shown in Table 5. Mg^{2+} activates MAP1 of germinated 'T57' roots; while Zn^{2+}, Fe^{3+}, Cu^{2+}, Co^{2+}, and Ni^{2+} inhibit the enzyme activity. The influence of Zn^{2+}, which exhibits the strongest inhibition (80% inhibition by 2 μM Zn^{2+}), was further studied and is summarized in Table 6.

Discussion

The inactivation of purified MAP1 from germinated 'T57' roots may occur in two steps: the holoenzyme dissociates into monomers in step one and the monomer denatures in step two (Figs. 7 and 8, Table 2). At 65 °C step one is twelve times faster than step two (Table 2), due mainly to much larger inactivation entropy change (S_1^*/S_2^* = 26.9) which outweighs ΔH^*, ($\Delta H_1^*/\Delta H_2^*$ = 3.49). The ratio E_{a1}/E_{a2}, (185.9/55.3 = 3.36) suggests that interaction between subunits gives the holoenzyme some protection against heat inactivation. Since the Arrhenius plots for the inactivation rate constants of 'T57' MAP1

Table 7. A brief comparison of MAPs from various sources

Source	Mol. wt. Holoenzyme	Mol. wt. Monomer	Monomers per holoenzyme	pI of holoenzyme	Reference
Escherichia coli	29,333	29,333	1	—	Ben-Bassat et al., 1987
Saccharomyces cerevisiae	36,000	34,000	1	7.8	Chang et al., 1990
Salmonella typhimurium	29,140	29,292	1	5.55 - 5.25	Wingfield et al., 1989
Porcine liver	67,000	70,000	1	—	Kendall & Bradshaw, 1992
Germinated 'T57' roots	163,300	54,800	3	4.87	This paper

(Fig. 8) intersect at 42.7 °C, below 42.7 °C step one is slower than step two.

The enzymes that are involved in N-terminal processing are extremely specific for N-terminal methionine and also for the second amino acid in the peptide. Since we do not have data to clarify the specificity for the second amino acid, we could not exclude the possibility that the activity we observed is that of one of many broad specificity aminopeptidases that is able to attack N-terminal methionine peptides. On the other hand, both the apparent molecular weight and isoelectric point of MAP1 are different from those of sweet potato aminopeptidases 1 and 2 (Lin and Chan, 1992) and so it may be a methionine aminopeptidase.

The porcine-liver enzyme is similar to methionine aminopeptidases isolated from *Escherichia coli, Salmonella typhimurium*, and yeast in that it also is stimulated by Co^{2+}. It is, however, much larger than these enzymes and differs somewhat in specificity, particularly with that of the yeast enzyme (Kendall and Bradshaw, 1992). Our enzyme is unique in several ways: (1) it contains three subunits; (2) pI of the holoenzyme is 4.87; (3) Mg^{2+} at 1 mM increases activity 80% more than does the control; (4) PMSF, Zn^{2+}, Fe^{3+}, and Cu^{2+} inhibit the activity very strongly. A brief comparison of MAPs from various sources is shown in Table 7.

In contrast to the structure of the prokaryotic enzyme, the yeast methionine aminopeptidase consists of two functional domains: a unique N-terminal domain containing two motifs resembling zinc fingers, which may allow the protein to interact with ribosomes, and a catalytic COOH-terminal domain resembling those of other prokaryotic methionine aminopeptidases. Furthermore, unlike with the prokaryotic gene, the deletion of the yeast MAP1 gene is not lethal, suggesting for the first time that an alternate NH-terminal processing pathway(s) exists for cleaving methionine from nascent polypeptide chains in eukaryotic cells (Chang et al., 1992).

The exact biological function of sweet potato MAP1 requires further study.

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臺農 57 號甘薯發芽塊根之一種胺基端甲硫胺酸水解酶之純化及性質

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經過粗抽液、DEAE-Sephacel 批次吸附、Sephacryl S-300, DEAE-Trisacryl-M 及 chromatofocusing 管柱層析共五步驟從臺農 57 號甘薯發芽塊根可得一胺基端甲硫胺酸水解酶 (MAP1)，其純化倍數爲 121，產率爲 14%。原態 MAP1 之分子量以 Sephacryl S-300 膠體滲透法測定爲 163,300；而等電點爲 4.87。其單元體之分子量以 SDS-PAGE 測定爲 54,800；所以原態 MAP1 應含三個相同的單元體。MAP1 在45°C以下時是穩定的。MAP1 受熱失活時明顯地經歷兩個步驟：步驟一乃三個單元體分散開，步驟二乃單元體之多肽鏈的散開；其相關之熱力學參數已分別得到。在測試之 17 種基質中 MAP1 對 L-methionine-4-nitroanilide有極高之特異性。在 1 mM 的phenylmethane sulfonyl fluoride 及 para-chloromercuribenzoate 存在下 MAP1 只分別剩下 24% 及 31% 之活性；iodoacetic acid 並無影響。One-millimolar Mg^{2+}增加 MAP1 80% 活性；而 2 μM Zn^{2+}就可抑制 80%的活性。綜合上述，本實驗得自臺農 57 號甘薯發芽塊根之 MAP1 很多方面均不同於文獻已報告者。

關鍵詞：熱變性；抑制；次單元；熱力學參數；鋅離子。