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# An aminopeptidase (AP2) from sprouts of sweet potato (*Ipomoea batatas* (L.) Lam. cv. Tainong 64)

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**Abstract.** A neutral aminopeptidase (AP2) was purified 119-fold from sprouts of sweet potato (*Ipomoea batatas* L. Lam. cv. Tainong 64) using a 4-step procedure comprising extraction from sprouts, DEAE-cellulose column, Sephacryl S-300 column, and hydroxyapatite column chromatography. The molecular weight of AP2 was estimated to be about 63,000 by gel filtration of Sephacryl S-200, and the isoelectric point was 6.08. The pH optimum for L-alanine-4-nitroanilide was 7.6 in citrate-phosphate buffer, and the enzyme was most stable from pH 5.2 to 7.0 when the temperature was below 50°C. AP2 hydrolyzed $\beta$-naphthylamides and 4-nitroanilides of both L-alanine and L-leucine, some dipeptides and tripeptides. $K_m$ (mM) and $V_{max}$ ($\mu$mol/h • mg) toward Leu-4-nitroanilide, Ala-4-nitroanilide, Leu-Met, and Ala-Met were 0.076 and 1.29, 0.16 and 0.45, 0.74 and 3.67, and 1.18 and 4.41, respectively. AP2 was inhibited by the sulfhydryl group inhibitors *p*-chloromercuri-benzoate and 5,5-dithio-bis-(2-nitrobenzoic acid) and the metal chelators bathocuproine and 1,10-phenanthroline.

**Key words**: Aminopeptidase; *Ipomoea batatas*; Purification; Sprouts.

## Introduction

In a previous paper (Lin and Chan, 1992), we described the purification and characterization of an aminopeptidase of sweet potato sprout, designated as AP1. Here, we report a second aminopeptidase, designated as AP2, from the same source.

## Materials and Methods

Materials and methods were identical to those of the foregoing paper except that the substrate used in the activity staining after PAGE was Leu-NHNap instead of Ala-NHNap.

### *Enzyme Extraction and Purification*

All stages were carried out at 4°C. About 103 g frozen sprouts were homogenized with 8 vol (v/w) of 10 mM sodium phosphate buffer, pH 7.0 (buffer A) containing 1% (w/v) PVP-40 in a Polytron homogenizer (Luzern, Switzerland) at high speed for 1 min. 3 times at 4°C. The homogenate was filtered through 3 layers of cheesecloth and then centrifuged at 11,000 x g for 20 min. The supernatant, designated as crude extract, was dircetly applied to a buffer A pre-equilibrated DEAE-cellulose column (3 × 55 cm) and eluted with buffer A to wash off unbound proteins. Bound proteins were eluted stepwise using 0-0.15, 0.15-0.40 and 0.40-1.00 M NaCl in buffer A (total vol, 500 ml). Active fractions (5 ml per fraction) were pooled and concentrated by Diaflo ultrafiltration membranes PM10 and chromatographed on a Sephacryl S-300 column (3 × 90 cm) pre-equilibrated and eluted with buffer A. Active fractions were pooled and concentrated as described above and applied to a hydroxyapatite Bio-Gel HT column (3 × 40 cm) pre-equilibrated with buffer A and eluted with a linear gradient of 10-100 mM sodium phosphate buffer, pH 7.0 (total vol, 500 ml). Active fractions were pooled and concentrated by a PM10 membrane.

*Assays of Aminopeptidase Activity*

The hydrolysis of Ala-4-nitroanilide was measured spectrophotometrically as 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 citrate-phosphate buffer (pH 7.6, buffer B) and 0.2 ml of the enzyme solution, was incubated at 37°C for 6 h and terminated with 0.6 ml of 30% acetic acid. The absorbance of the reaction mixture was measured at 410 nm. One enzyme unit was defined as the amount of enzyme required to produce 1 $\mu$mol 4-nitroaniline/h under the assay conditions. This method was used during purification.

The aminopeptidase activity was also measured with Ala-$\beta$-naphthylamide as the substrate (Kitamura and Maruyama, 1985). The reaction was performed as when amino acid derivatives of 4-nitroanilide were used as the substrate. The enzymatic reaction was terminated by adding 0.6 ml of 2 % HCl in ethanol, and 0.6 ml of 0.06% dimethyl amino cinnamaldehyde in ethanol was added finally. The absorbance at 540 nm of the reaction mixture was determined. One enzyme unit was defined as the amount of enzyme required to produce 1 $\mu$mol naphthylamine/h under the assay conditions.

The enzymatic hydrolysis of peptide substrates was followed by estimation of the liberated amino acids with 2, 4, 6-trinitrobenzenesulfonic acid in the presence of cupric ions (Waters and Dalling, 1983) and using leucine as the standard.

*Assays of Other Proteolytic activities*

The endopeptidase activity was determined by measuring spectrophotometrically the increase in absorbance at 254 nm using N$\alpha$-Benzoyl-L-Arginine Ethyl Ester (BAEE) or N-Acetyl-L-Tyrosine Ethyl Ester (ATEE) as the substrate (Prescott and Wargner, 1976; Schwert and Takenaka, 1955). The reaction was performed and terminated as assay of aminopeptidase activity with amino acid derivatives of 4-nitroanilide as the substrate. The $A_{254}$ of the reaction mixture was measured.

The endopeptidase activity was also determined with casein as the substrate (Kunitz, 1946). The reaction mixture, which contained 0.2 ml of the enzyme solution and 0.6 ml of 2 % casein in buffer A, was incubated at 37°C for 6 h. The reaction was terminated by adding 0.6 ml of 0.44 M trichloroacetic acid. After standing at room temperature for 1 h, the reaction mixture was centrifuged, and $A_{280}$ of the supernatant was measured. Boiled instead of native enzyme solutions were used in controls for the above enzymatic reactions.

## Results and Discussion

*Purification of Sweet Potato AP2*

The crude extract showed AP activity. When it was applied to a DEAE-cellulose column, three AP peaks and six protein peaks were eluted with buffer A. The first AP peak emerged slightly ahead of the first protein peak; the second AP peak coincided with the second protein peak; and the third AP peak coincided with the fifth protein peak (see Fig. 1 in Lin and Chan, 1992). The second AP peak, which was eluted at ca. 0.25 M NaCl was further purified successively on Sephacryl S-300 and hydroxyapatite columns to obtain 119-fold purification and 3.7% yield (Table 1). Four protein peaks but only one AP peak, which coincided with the third protein peak, were found in a Sephacryl S-300 column. AP fractions were pooled and concentrated, and applied to a hydroxyapatite column. Two protein peaks and only one AP peak, which coincided with the second (major) protein peak and was named AP2, were

**Table 1.** *Purification of AP2 from sprouts of 'T64'*

The fresh weight of sprouts used was 103 g. One unit was defined as the amount of enzyme required to hydrolyze 1 $\mu$mol Ala-NHNan/h under the experimental conditions described in the text.

| Procedure | Activity (units) | Protein (mg) | Spec. Act. (units/mg) | Recovery (%) | Purification (fold) |
|---|---|---|---|---|---|
| Crude extract | 25.8 | 361 | 0.07 | 100 | 1 |
| DEAE-cellulose | 1.02 | 3.77 | 0.27 | 4.0 | 3.86 |
| Sephacryl S-300 | 0.68 | 0.51 | 1.33 | 2.6 | 19.0 |
| Hydroxyapatite | 0.96 | 0.12 | 8.36 | 3.7 | 119 |

found. The chromatographic behaviour, as well as the molecular size of the sweet potato AP2, were similar to those of other plant APs.

Our AP2 preparation was homogeneous as examined by 7.5% Disc-PAGE pH 8.8 (Fig. 1).

*General Properties*

The molecular weight of AP2 was estimated to be 63,000 by gel-filtration on a Sephacryl S-200 column. AP2 showed an electrophoretic mobility of 0.031 relative to bromophenol blue on 7.5% PAGE at pH 8.8 and an isoelectric point (pI) of 6.08 as estimated by chromatofocusing.

*Effect of Temperature on Activity and Stability*

AP2 activity was assayed under the standard conditions (pH 7.6 for 6 h) at various temperatures. The enzyme activity was the highest at 30°C-37°C. The purified AP2 in 10 mM Na-phosphate buffer, pH 7.0, was incubated for 20 min. at various temperatures, and the residual activites were assayed under standard conditions after being cooled to 4°C. The AP2 preparation was stable up to 50°C, and then was quickly inactivated at higher temperatures.

*Effect of pH on Activity and Stability*

AP2 activity was assayed at 37°C for 6 h in glycine-HCl, citrate, citrate-phosphate, Tris-HCl, or glycine-NaOH buffer at various pHs. The pH-activity profile (Fig. 2) indicated that the pH optimum was 7.6 in citrate-phosphate buffer. In this respect, the present enzyme resembles the group of enzymes, i. e. neutral aminopeptidases, which are characterized by their ability to hydrolyze peptide bonds between the $\alpha$-carboxyl group of various amino acids and the amino group of some aromatic amines, for instance, $\beta$-naphthylamine, 4-nitroaniline, and 4-(phenylazo)-phenylamine, optimally at pH 7 to 8 (Elleman, 1974; Kolehmainen and Mikola, 1971; Ninomiya *et al.*, 1983; Vodkin and Scandalios, 1980; Waters and Dalling, 1984). AP2 was kept

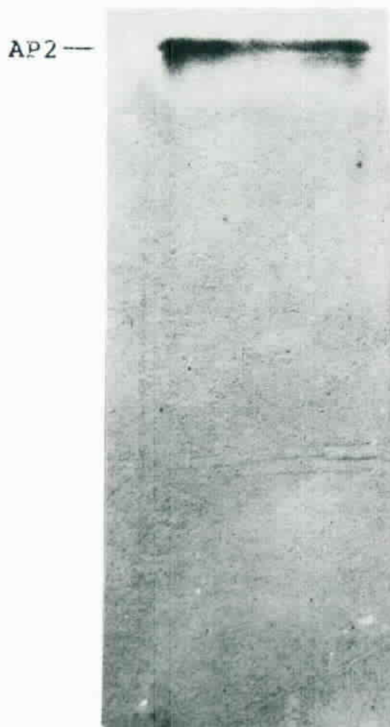


Fig. 1. The activity staining of AP2 on 7.5% polyacrylamide gel, pH 8.8. AP2 sample containing ca. 5 $\mu g$ protein was applied. The anode was at the bottom.

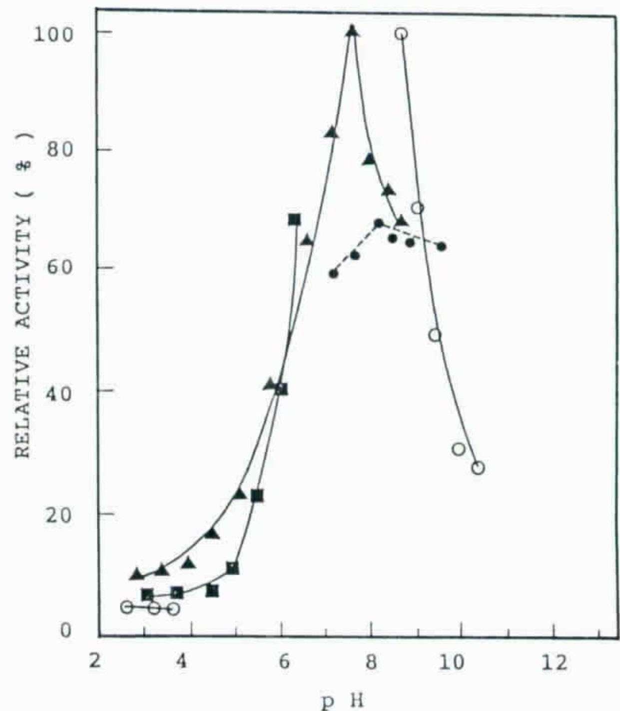


Fig. 2. The effect of pH on AP2 activity. The enzyme activity of AP2 containing 8.4 $\mu g$ protein was assayed as described in the text in: (○) 50 mM glycine-HCl (pH 2.8-3.4); (■) 14.3 mM citrate (pH 3.0-6.2); (▲) 28.6 mM citrate-57.2 mM phosphate (pH 2.8-8.2); (●) 14.3 mM Tris-HCl (pH 7.2-9.6); or (○) 14.3 mM glycine-NaOH buffer (pH 8.6-10.6). The maximal activity toward Ala-NHNan was 2.81 $\mu$mol/h · mg.

for 3 h at 37°C in glycine-HCl, citrate-phosphate, sodium phosphate, or glycine-NaOH buffer at various pHs. The residual activity was assayed under standard conditions. AP2 was stable between pH 5.2 and 7.0 (Fig. 3).

*Substrate Specificity*

(a) Synthetic substrates. AP2 *in vitro* effectively hydrolyzed Ala-NHNap, Leu-NHNap, Leu-NHNan, and Ala-NHNan, but had no detectable activity on substrates for carboxypeptidase or endopeptidase (Table 2). AP2 prefers $\beta$-naphthylamides with a hydrophobic side chain in the amino terminal residue (Ala-NHNap and Leu-NHNap), and has very low activity toward one with an acidic side chain in the amino terminal residue (Glu-NHNan). Leu-NHNan is a better substrate than Ala-NHNan. With regard to its narrow specificity on L-aminoacyl-NHNap or -NHNan, the present enzyme resembles one AP from barley grains (Sopanen and Mikola, 1975) and AP1 from pea seeds (Elleman, 1974). However, the enzyme prefers Ala-NHNap to Leu-NHNap while the reverse is true for the two APs mentioned above.

We started to purify the enzyme using Ala-NHNan instead of Ala-NHNap as the substrate because we did not know in the beginning that Ala-NHNap is a better substrate.

(b) Peptide substrates. AP2 hydrolyzed both dipeptides with Ala or Leu at the N-terminus and oligopeptides of alanine (Table 3). The rate of hydrolysis against Ala-Ser, Ala-Met, Leu-Ser, and Ala-Ala was 91, 86, 71 and 67%, respectively, of that against Leu-Met. The enzyme also hydrolyzed less effectively Leu-Leu, Leu-Ala, Ala-Leu and Leu-Phe. It had low activity toward DL-Ala-L-Asp, D-Leu-L-Leu, Leu-Gly, and Ala-Gly. AP2 had very low or no activity toward Leu-$\beta$-Ala, $\beta$-Ala-Gly, Leu-Asn, $\beta$-Ala-Ala, Ala-Asp, Ala-Lys, Ala-Pro, and Leu-Pro.

The activity of AP2 toward oligopeptides of Ala decreased with an increasing number of Ala residues.

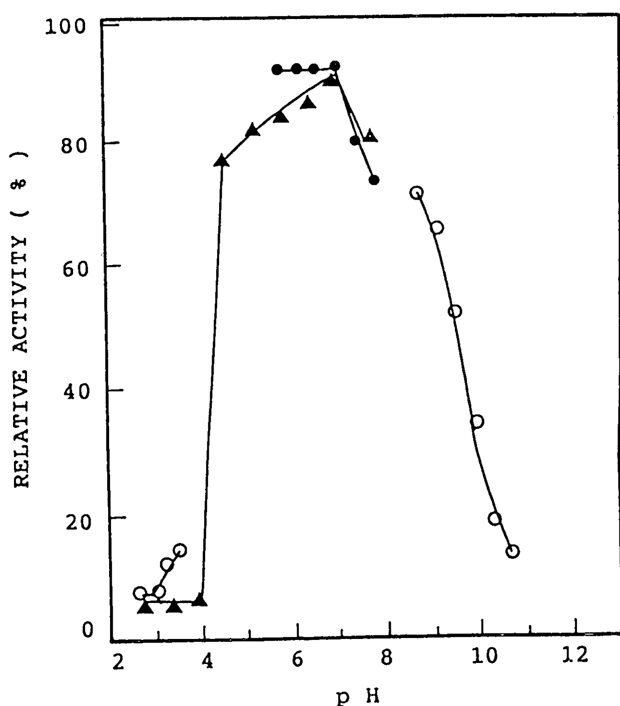


Fig. 3. The effect of pH on the stability of AP2. AP2 containing 8.4 $\mu$g protein was preincubated for 3 h at 37°C in: (○) 25 mM glycine-HCl (pH 2.8-3.4); (▲) 50 mM citrate-100 mM phosphate (pH 2.8-7.6); (●) 50 mM sodium phosphate (pH 5.8-7.8); or (○) 25 mM glycine-NaOH (pH 8.6-10.6). The residual activity was then assayed as described in the text. The maximal activity toward Ala-NHNan was 2.81 $\mu$mol/h • mg.

**Table 2.** *AP2 activity toward synthetic substrates*

In each assay, 8.4 $\mu$g of the purified AP2 was used. The final concentration of synthetic substrates and casein was 0.429 mM and 0.429%, respectively. Activity toward Leu-NHNan, Ala-NHNap, and Leu-NHNap were 2.09, 3.25, and 3.04 $\mu$mol/h • mg protein, respectively. No activity toward S-Bz-Cys-NHNap and Bz-Phe-NHNap was detected.

| Substrate | Activity (%) |
|---|---|
| Leu-NHNan | 100 |
| Ala-NHNan | 54 |
| Glu-NHNan | 15 |
| Su-Phe-NHNan | 13 |
| Cystine-bis-NHNan | 19 |
| Hippuryl-Lys | 0 |
| Hippuryl-Arg | 0 |
| Hippuryl-Phe | 0 |
| Bz-Cys-NHNan | 6 |
| Ac-Tyr-NHNan | 4 |
| Glutaryl-Phe-NHNan | 3 |
| Bz-Arg-NHNan | 2 |
| Ac-Tyr-EE | 0 |
| Bz-Arg-EE | 0 |
| Casein | 0 |

**Table 3.** *AP2 activity toward peptide substrates*
The substrate concentration was 0.5 mM. In each assay, 0.89 $\mu$g of the purified AP2 was used. The details were the same as those given in the text except that the reaction was carried out for 12 h. Activity toward Leu-Met was 2.5 $\mu$mol/h • mg protein. A standard curve was prepared using L-leucine.

| Peptide substrate | Activity (%) |
|---|---|
| Leu-Met | 100 |
| Ala-Ser | 91 |
| Ala-Met | 86 |
| Leu-Ser | 71 |
| Ala-Ala | 67 |
| Leu-Leu | 57 |
| Leu-Ala | 57 |
| Ala-Leu | 52 |
| Leu-Phe | 48 |
| Ala-Phe | 38 |
| DL-Ala-L-Asp | 29 |
| D-Leu-L-Leu | 24 |
| Leu-Gly | 19 |
| Ala-Gly | 14 |
| Leu-$\beta$-Ala | 10 |
| $\beta$-Ala-Gly | 5 |
| Leu-Asn | 5 |
| $\beta$-Ala-Ala | 0 |
| Ala-Asp | 0 |
| Ala-Lys | 0 |
| Ala-Pro | 0 |
| Leu-Pro | 0 |
| Ala-Ala-Ala | 48 |
| Ala-Ala-Ala-Ala | 16 |
| Ala-Ala-Ala-Ala-Ala | 23 |
| Ala-Ala-Ala-Ala-Ala-Ala | 4 |

Similar to some plant aminopeptidases (Kolehmainen and Mikola, 1971; Ninomiya *et al.*, 1981; Pallavieini *et al.*, 1981), the ability of AP2 of 'T64' sprouts to hydrolyze peptide substrates with lower rates than the corresponding aminoacyl-$\beta$-naphthylamides or aminoacyl-4-nitroanilides is in contrast to other plant aminopeptidases which hydrolyze peptide substrates at much higher rates than aminoacyl-$\beta$-naphthylamide substrates (Waters and Dalling, 1983; 1984).

AP2 is different from AP1 found in the same source (Lin and Chan, 1992) in several aspects: AP2 retains 95% of activity after being preincubated at 55°C for 15 min, while AP1 retains only 15% of activity; the mol. wt., estimated by gel-filtration, and isoelectric point of the former are 63,000 and 6.08 while those of the latter are 115,000 and 5.53, respectively; AP2 prefers Leu-NHNan to Ala-NHNan while the reverse is true for AP1.

*Kinetic Properties*

AP2 activity was assayed under standard conditions at pH 7.6 with various amounts of enzyme preparation, ranging from 4.2 to 16.9 $\mu$g, and with a final concentration of 0.429 mM Ala-NHNan for different reaction times up to 16 h. There was a linear relationship between reaction velocity and enzyme concentration in the range used for reaction time up to 6 h. The effect of substrate concentration on the rate of hydrolysis of Leu-NHNan in citrate-phosphate buffer, pH 7.6, was investigated at substrate concentrations from 0.05 to 0.45 mM when 8.4 $\mu$g of AP2 preparation was used. The apparent Michaelis-Menten constant ($K_m$) and maximum velocity ($V_{max}$) for the enzyme were calculated from the double-reciprocal plot of Lineweaver-Burk to be 0.076 mM and 1.29 $\mu$mol/h/mg, respectively. No significant inhibition was observed at high substrate concentrations.

$K_m$ and $V_{max}$ values of some representative substrates are summarized in Table 4. The highest ratio of $V_{max}$ to $K_m$ was found for Leu-NHNan.

*Effects of Protease Inhibitors*

AP2 was strongly inhibited by *p*-CMB, PMSF, leupeptin, 5,5-dithio-bis-(2-nitrobenzoic acid) and $ZnSO_4$; moderately by bathocuproine and 1,10-phenanthroline; and slightly by $CuSO_4$. But it was not significantly affected by thiol compounds, iodoacetamide, iodoacetic acid, various trypsin inhibitors, EDTA, or pepstatin (Table 5).

The observation that AP2 was inhibited by sulfhy-

**Table 4.** *$K_m$ and $V_{max}$ values for the hydrolysis of some representative substrates by AP2*

| Substrate | $K_m$ (mM) | $V_{max}$ ($\mu$mol/h • mg) | $V_{max}/K_m$ ($\mu$mol/h • mg • mM) |
|---|---|---|---|
| Leu-NHNan | 0.076 | 1,29 | 18.4 |
| Ala-NHNan | 0.16 | 0.45 | 2.81 |
| Leu-Met | 0.74 | 3.67 | 4.96 |
| Ala-Met | 1.18 | 4.41 | 3.74 |

**Table 5.** *Effects of some reagents on AP2 activity*
In each assay, 8.4 $\mu$g of the purified AP2 was used, and the final concentration of Leu-NHNan was 0.429 mM. The control activity was 1.15 $\mu$mol/h · mg.

| Addition | Concentration (mM) | Activity (%) |
|---|---|---|
| Control | | 100 |
| *p*-CMB | 0.2 | 0 |
| PMSF | 2.0 | 11 |
| | 1.0 | 35 |
| Leupeptin | 1.0 | 27 |
| | 0.1 | 51 |
| 5, 5-Dithio-bis-(2-nitrobenzoic acid | 1.0 | 36 |
| | 0.1 | 68 |
| 1, 10-Phenanthroline | 2.0 | 34 |
| | 1.0 | 51 |
| $ZnSO_4$ | 1.0 | 35 |
| $CuSO_4$ | 1.0 | 68 |
| Bathocuproine | 1.0 | 36 |
| | 0.5 | 37 |
| 2-mercaptoethaonl | 5.0 | 90 |
| | 1.0 | 106 |
| DL-dithiothreitol | 1.0 | 92 |
| Iodoacetamide | 1.0 | 100 |
| Iodoacetic acid | 1.0 | 100 |
| Aprotinin | 0.86 | 113 |
| Soybean TI | 1.0[a] | 100 |
| TI from turkey egg white | 1.0[a] | 100 |
| TI from roots of sweet potato | 0.1[a] | 100 |
| EDTA | 2.0 | 110 |
| | 1.0 | 106 |
| Pepstatin | 5.0 | 100 |

[a]mg/ml.

dryl group inhibitors and metal chelators is similar to that for AP1 from the primary leaf of wheat (Waters and Dalling, 1984).

In spite of the strong inhibiton by *p*-CMB, AP2 was not activated by thiol compounds. No simple explanation for this relationship can be offered at present, but similar observations were reported on aminopeptidases from pea seeds (AP1) (Elleman, 1974), pig reproductive fluids (Basha *et al.,* 1978), and Japanese apricot (Ninomiya *et al.,* 1981). In this respect, it is different from the APs of barley grain (Sopanen and Mikola, 1975) and pea seeds (AP1) (Elleman, 1974) just mentioned.

Bachmair *et al.* (1986) proposed that the *in vivo* half-life of a yeast protein is related to the property of its amino-terminal residue. Thus AP2, which has rather specific substrate requirements, may play a key role in cellular regulation if their proposal can be extended from yeast to higher plants, and if removal of the amino-terminal residue of some proteins by aminopeptidases speeds up later hydrolysis by other proteases.

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# 臺農 64 號甘藷芽之一種胺肽酶 (AP2)

**林耀輝　陳慶源**

中央研究院　植物研究所

經過粗抽處理、DEAE-cellulose、Sephacryl S-300, 及 hydroxyapatite 管柱層析共四步驟從臺農 64 號甘藷芽可得一胺肽酶(AP2)，其純化倍數爲 119。AP2 分子量以 Sephacryl S-200 膠體滲透法測定爲 63,000；而等電點爲 6.08。以 L-alanine-4-nitroanilide 爲基質在 citrate-phosphate 緩衝液測得之最適 pH 爲 7.6, AP2 在 pH 5.2 和 7.0 之間且在 50°C以下時是穩定的。AP2 水解 L-alanine-4-nitroanilide, L-leucine-4-nitroanilide, L-alanine-$\beta$-naphthylamide, L-leucine-$\beta$-naphthylamide, 和某些雙肽及參肽。AP2 對基質 Leu-4-nitroanilide, Ala-4-nitroanilide, Leu-Met, 及 Ala-Met 之 $K_m$ (mM) 和 $V_{max}$ ($\mu$mol/h・mg) 分別爲 0.076 和 1,29, 0,16 和 0.45, 0.74 和 3.67, 1.18 和 4.41。AP2 受-SH 抑制劑4-chloromercuri-benzoate, 5,5-dithio-bis-(-2-nitrobenzoic acid); 以及金屬螯合劑bathocuproine及1,10-phenanthroline 所抑制。