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Purification and properties of endopeptidases of sprouts of sweet potato (*Ipomoea batatas* L. Lam. cv. Tainong 64)

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Abstract. Five endopeptidase fractions (DCI, DCII, DCIII, DCIV, and DCV), assayed with hemoglobin at pH 3.25, were obtained from sprouts of sweet-potato (*Ipomoea batatas* L. Lam. cv. Tainong 64) through crude extract, ammonium sulphate precipitation, column chromatography on DEAE-cellulose. DCI, the major fraction, and DCII were further purified by column chromatography on hydroxyapatite, and Sephadex G-150 to obtain enzyme fractions GFI and GFII, respectively. GFI was further purified by discontinuous polyacrylamide gel electrophoresis (Disc-PAGE) to obtain enzyme fractions PPI1, PPI2, and PPI3. The mobility relative to bromophenol blue on 10% gel (Rm), molecular weight estimated by gel-filtration (Mn), molecular weight of subunits estimated by SDS-PAGE (Ms) of PPI1, PPI2, and PPI3 were 0.10, 0.12, and 0.16; 80K, 80K, and 80K; 27K, 80K, and 85K, respectively. PPI1 bound indigenous trypsin inhibitors strongly. GFII had Mn and Ms of 53K and 27K, respectively. Due to insufficient amounts, DCIII was further purified directly by gel-filtration to obtain enzyme fraction GFIII, which had Mn of 71K.

Key words: Endopeptidases; *Ipomoea batatas*; Properties; Purification; Sprouts.

Introduction

In plant tissues, most research on proteolysis has centered on the role of proteases in the mobilization of reserve proteins. However, considerable interest in the molecular basis of ontogenetical processes has led to the realization that proteases are intricately involved in plant metabolism.

Some examples of plant tissues in which endopeptidases have been studied are: (1) in monocots: immature ears of maize (Vodkin and Scandalios, 1980), germinating seeds of rice (Doi *et al.*, 1980), germinated seeds of sorghum (Garg and Virupaksila, 1970); (2) in dicots: sprouting potato tubers (Kitamura and Maruyama, 1986), the sarcocarp of snake-gourd fruit (Kaneda *et al.*, 1986), leaves of spinach (*Spinacia oleracea* L.) (Aducci *et al.*, 1986) and the latex of *Elaeophorbia drupifera* (Lynn and Clevette-Radford, 1985).

In sweet potato (2n=6x=90), we have reported the occurrence and some properties (Lin *et al.*, 1988), possible storage function (Lin and Chen, 1980; Lin, 1989) and drought responsive function (Lin, 1989) of trypsin inhibitors in roots and leaves. However, no information is available yet about whether trypsin inhibitors inhibit any indigenous proteases, or how themselves are metabolized. In order to answer these questions, knowledge of proteases in sweet potato tissues is essential. We have detected activities of endopeptidase (EC.3.4), aminopeptidase (EC.3.4.11, L-leucine-*p*-nitroanilide or L-alanine-β-naphthylamide as the substrate), and carboxypeptidase (EC. 3.16.17, hippuryl-L-lysine as the substrate) in sprouts and resting roots. We describe in this publication the purification and properties of five endopeptidase fractions, active at pH 3.25, from sprouts of Tainong 64 ('T64').

Materials and Methods

Chemicals

Acrylamide, ammonium persulphate, and bisacrylamide were products of Bio-Rad (Richmond, CA, USA). Calibration kits for electrophoresis and Sephadex gel-filtration, and Sephadex G-150 were obtained from Pharmacia (Uppsala, Sweden). Ammonium sulphate, DEAE-cellulose, monobasic sodium phosphate, dibasic sodium phosphate, trypsin (Art. 24581), acetic acid, isopropanol, and methanol were purchased from E. Merck (Darmstadt, FRG); N-acetyl-DL-phenylalanine-β-naphthylester (APNE), Coomassie blue, hemoglobin, dimethyl-formamide, L-leucine-p-nitroanilide (Leu-Np), tetrazotized o-dianisidine, and low molecular weight kit for electrophoresis were obtained from Sigma Chemical Company (St. Louis, MO, USA).

Plant Material

Roots of sweet potato (*Ipomoea batatas* L. Lam. cv. Tainong 64) were purchased from local market. Roots were sprayed with 0.04% sodium azide and allowed to sprout in dark programmed at 32±1°C (12h) and 25±1°C (12h) with 75±2% relative humidity. Sprouts were used directly or frozen with liquid nitrogen and then kept at -17°C until use.

Enzyme Extraction and Purification

All stages were carried out at 4°C. Sprouts of 141 g were homogenized with 1 liter of 10 mM sodium phosphate buffer (pH 6.8, buffer A) in a Polytron (Luzern, Switzerland) homogenizer at 4°C. The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 10,500 x g for 40 min and the pellets were discarded. Fine ammonium sulphate (AS) was added to the supernatant fraction (i.e. crude extract). Precipitates from 0.2-0.8 saturated AS solution were collected, dissolved in buffer A, and dialyzed twice against 100 volumes of buffer A overnight. The dialyzed sample was loaded on a DEAE-cellulose column (3 x 72 cm) pre-equilibrated with buffer A. Unbound proteins were washed off with buffer A and bound proteins were eluted with a gradient of 0.3-0.6 M and then 1.0 M NaCl in buffer A at a flow rate of 24 ml/hr. Each fraction contained 5 ml. Five active fractions (DCI, DCII, DCIII, DCIV, and DCV) were obtained. DCI and DCII were separately dialyzed against 1 mM sodium phosphate buffer (pH 6.8) and then concentrated by ultrafiltration (Amicon, mol. wt. cutoff 10 K). The concentrated DCI was further purified successively by chromatography on a hydroxyapatite column (2.6 x 60 cm) and gel-filtration on a Sephadex G-150 column (2 x 65 cm) to obtain enzyme fractions HAI and GFI, respectively. DCII was further purified by the same process to obtain enzyme fractions HAII and GFII, respectively. Due to insufficient amounts, DCIII was further purified directly by gel-filtration on a Sephadex G-150 column (2 x 65 cm) to obtain enzyme fraction GFIII; DCIV and DCV were not purified further.

Polyacrylamide Gel Electrophoresis

A vertical system of discontinuous slab gel electrophoresis (177 x 105 x 1.5 mm, or 95 x 55 x 0.75 mm) was used for both identification and purification purposes. The gel system was prepared according to Davis (1964) with a stacking gel of 2.5% (w/v) acrylamide (pH 6.8), and a separating gel of 10% or 12.5% (pH 8.8). Electrophoresis was carried out at 125 volts for 30 min (stacking gel) and then at 150 volts for 5-8 hr unless otherwise specified. Proteins were detected by both Coomassie blue and activity staining. For purification purpose, gel portions of about 0.3 cm length containing endopeptidase activity were cut off with a razor blade. Gel portions were soaked in suitable buffers such as buffer A and concentrated by an ISCO Model 1750 concentrator (Lincoln, NE, USA). The molecular weight cutoff of cellulose membrane was 3,500. Concentrated samples were used for estimation of molecular weight in SDS-PAGE.

Assays of Endopeptidase Activity during Purification

This was based on the method of Doi *et al.* (1980). The reaction mixture contained 0.6 ml of 2% hemoglobin in 0.05 M glycine-HCl buffer (pH 3.25) and 0.2 ml enzyme solution. The enzyme reaction was carried out for 3 hr at 37°C. The reaction was stopped by the addition of 0.6 ml of 0.47 M trichloroacetic acid. After centrifugation of the reaction mixture, absorbance of the supernatant solution at 280 nm was measured. The amount of enzyme which gave an increase of absorbance 1.0 at 280 nm under the experimental conditions was defined as one enzyme unit. Although casein was used for endopeptidase assays

(Kunitz, 1946), we did not use it due to its insolubility at pH 3.25.

In addition, synthetic substrate for endopeptidases was used simultaneously in spectrophotometric analyses. Although N-acetyl-DL-phenylalanine-β-naphthylester was used for activity staining of endopeptidases on polyacrylamide gels (Chan and deLumex, 1982), we did not use it. Because the product released by the enzyme, β-naphthylamine, had weak absorbance at 340 nm and formed with tetrazotized o -dianisidine a color complex which had poor solubility in aqueous solution. Benzoyl-arginine-p-nitroanilide was not useful either. We finally used leucine-p -nitroanilide, originally used as a synthetic substrate for aminopeptidases (Erlanger *et al.*, 1961), as a substrate for endopeptidases according to the report that some endopeptidases can hydrolyze substrates of exopeptidases (Storey and Wagner, 1986). The reaction mixture contained 0.3 ml of 1.5 mM Leu-Np, 0.15 ml of 50 mM glycine-HCl buffer, pH 3.25, and 0.2 ml of the enzyme solution. The enzyme reaction was carried out for 3 hr at 37°C. The reaction was stopped by the addition of 0.6 ml of 30% acetic acid. After centrifugation of the reaction mixture, absorbance of the supernatant solution at 405 nm was measured. The amount of enzyme which gave an increase of absorbance 1.0 at 405 nm under the experimental conditions was defined as one enzyme unit.

Activity Staining of Endopeptidases after Polyacrylamide Gel Electrophoresis

The procedure of Chan and deLumex (1982) for activity staining of trypsin inhibitor was modified for endopeptidase staining. Gels were incubated at 37°C for 20-50 min with 160 ml of the substrate-dye solution immediately prepared before use, which consisted of 40 mg N-acetyl-DL-phenylalanine-β-naphthyl ester in 16 ml of dimethylformamide brought to 160 ml with 144 ml of 0.05 M glycine buffer (pH 3.25) or 0.05 M phosphate buffer (pH 7.0) in which 80 mg tetrazotized o -dianisidine were dissolved. Gels were destained with 2% acetic acid for 30 min and stored in a solution containing methanol: acetic acid: water = 5:1:5 (by vol) or dried overnight in hood at room temperature and ready for photographing. Trypsin instead of samples was used as a positive control. As negative controls, gels were stained as above except that substrate (APNE) was omitted.

When SDS-PAGE was finished, gels were immersed and shaked in 25% (v/v) isopropanol in 10 mM phosphate buffer, pH 7.2 (buffer B) for 10 min twice and finally in buffer B. The following procedure for staining was the same as described above.

Estimation of Molecular Weight

The molecular weights of proteases were estimated by Sephadex gel-filtration (Andrews, 1964) with ribonuclease A (mol. wt. 13.7K), chymotrypsinogen A (25K), ovalbumin (43K), bovine serum albumin (67K), and aldolase (158K) as standards. While the molecualr weights of subunits of proteases were estimated by SDS-PAGE (Weber and Osborn, 1969) calibrated with low molecular weight kit of Pharmacia. The kit contained α-lactalbumin (mol. wt. 14.4K), soybean trypsin inhibitor (20.1K), carbonic anhydrase (30K), ovalbumin (43K), bovine serum albumin (67K), and phosphorylase b (94K).

Protein Estimation

The protein contents of endopeptidase preparation were determined by A_{280} and with the folin phenol reagent (Lowry *et al.*, 1951) using crystalline bovine serum albumin as the standard.

Recovering-reexamination Process

Gel fractions with 0.3 mm length were cut from the top of the separating gel and each recovered sample was reexamined by PAGE and SDS-PAGE.

Results and Discussion

Purification of Endopeptidases

Five active fractions i.e. DCI, DCII, DCIII, DCIV, and DCV from sprouts of 'T64" were obtained from DEAE-cellulose column (Table 1 and Fig. 1). DCI represented the major active fraction and it was studied most extensively in this work. GFI (I of Sephadex G-150 column, Table 1) was obtained, after 5-step procedure, with 27.1-fold purification and 7.8% yield (Table 1). GFI was purified further to obtain enzyme fractions GFI1, GFI2, and GFI3, the purification data of which are not presented in Table 1 due to insufficient amounts.

Endopeptidases have been purified with fold of purification and yield of 5 and 3.2% (Kaneda *et al.*, 1986), 26 and 13% (Garg and Virupaksila, 1970), 200

Table 1. *Purification of endopeptidases of sprouts of sweet popato 'T64'*

Fresh sprouts of 141 g were used. Endopeptidases were assayed with hemoglobin and checked with Leu-Np, both at pH 3.25. One unit of enzyme activity was defined as the amount of enzyme required to increase absorbance 1.0 at 280 nm (hemoglobin) or at 405 nm (Leu-Np) under experimental conditions.

Procedure	Hemoglobin as substrate					Leu-Np as substrate				
	Activity (units)	Protein (mg)	Spec. Act. (units/mg)	Recovery (%)	Purification (fold)	Activity (units)	Protein (mg)	Spec. Act. (units/mg)	Recovery (%)	Purification (fold)
Crude extract	1196.0	1120.00	1.07	100.00	1.00	690.0	1120.00	0.62	100.0	1.00
0.2-0.8 ammonium sulfate	650.0	560.00	1.16	54.00	1.08	538.0	560.00	0.96	78.0	1.50
DEAE-cellulose										
I	133.0	35.60	3.74	11.00	3.50	51.0	35.60	1.43	7.0	2.31
II	38.0	12.40	3.06	3.00	2.80	15.0	12.40	1.21	2.0	1.95
III	27.0	33.80	0.80	2.00	0.75	43.0	33.80	1.27	6.0	2.05
IV	21.0	26.40	0.80	1.75	0.75	24.0	26.40	0.91	3.0	1.47
V	41.0	105.00	0.39	3.00	0.37	2.6	105.00	0.02	0.3	0.03
Hydroxyapatite										
I	99.0	7.60	13.00	8.00	12.10	97.0	7.60	12.80	14.0	20.60
II	—[a]	—	—	—	—	—	—	—	—	—
Sephadex G-150										
I	94.0	3.23	29.00	7.80	27.10	24.0	3.23	7.43	3.5	12.00
II	5.9	4.90	1.20	0.50	1.10	6.4	4.90	1.31	1.0	2.10
Preparative PAGE										
I 1	—	—	—	—	—	—	—	—	—	—
I 2	—	—	—	—	—	—	—	—	—	—
I 3	—	—	—	—	—	—	—	—	—	—

[a]Data not available.

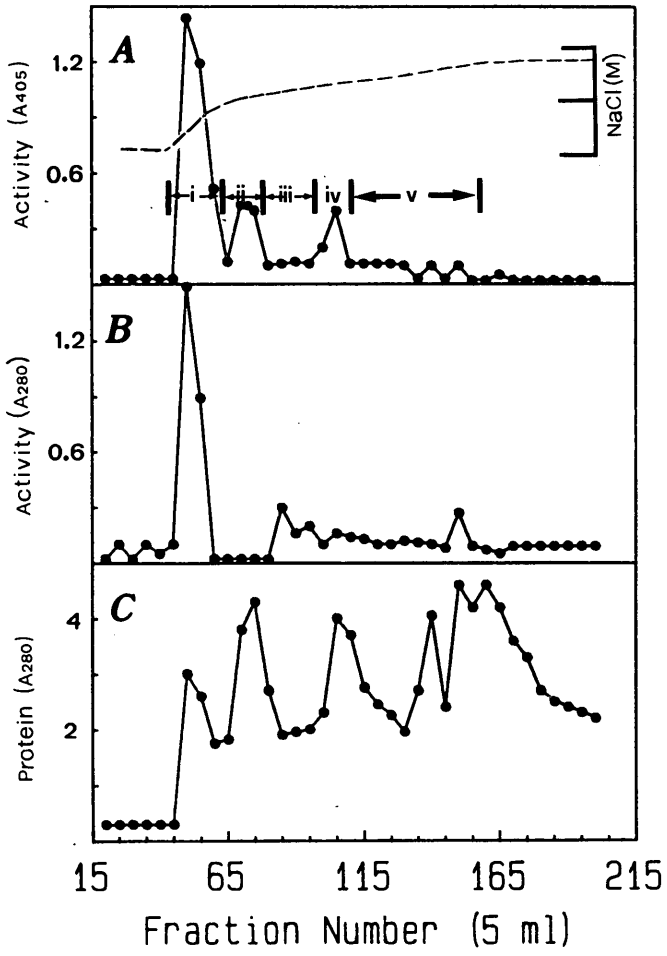

Fig. 1. Chromatography of endopeptidases from sprouts of 'T 64' on DEAE-cellulose column. The dialyzed preparation (56 ml containing 690 mg protein) was loaded on a column (3 x 72 cm) which was previously equilibrated with 10 mM sodium phosphate buffer, pH 6.8. Unbound proteins were washed off the column with the same buffer and bound proteins were eluted with a gradient of 0.3-0.6 M NaCl and then with 1.0 M NaCl in the same buffer at a rate of 24 ml/hr. Each fraction contained 5 ml. Enzyme activity was assayed using Leu-Np in glycine buffer, pH 3.25 (A); and hemoglobin in glycine buffer, pH 3.25 (B). Amount of protein was estimated by absorbance at 280 nm (C). i: fraction number 43-65; ii: No.66-78; iii: No.79-97; iv: No. 98-110; v: No.111-157.

and 2.5% (Kitamura and Maruyama, 1986), 281 and 16% (Aducci *et al.*, 1986), 293 and 16% (Vodkin and Scandalios, 1980). Vodkin and Scandalios (1980) indicated: "Assigning a specific role to a particular enzyme is difficult because such metabolic processes as intracellular protein breakdown, abnormal fragment degradation, and peptide transport and hydrolysis are probably the cumulative effects of a number of proteases and peptidases which can have broad and overlapping substrate specificities."

One should notice the possibility of such concerted action of endopeptidases against protein substrates such as hemoglobin. This may account for the low overall fold of purification listed in literature (Garg and Virupaksila, 1970; Kaneda *et al.*, 1986) and Table 1. This may also account for the low fold of purification of DCIII, IV, and V. The activity ratio of endopeptidase to aminopeptidase of the crude extract, 1196/690=1.73, is high relative to that, 94/24=3.92, of GFI which has been purified 27.1 fold. When crude extract was purified one step further by ammonium sulfate precipitation enzyme activity lost 46% activity. This may be due to the instability and/or concerted action of endopeptidases described above.

One should also notice the separation of indigenous inhibitors from the corresponding proteases. The apparent total activity against Leu-Np of the more purified HAI (I of hydroxyapatite column, Table 1) is larger than the less purified DCI (97 and 51 units, respectively) may be due to the removal of inhibitors of aminopeptidases.

Purification and General Properties of Endopeptidase Fractions PPI1, PPI2 and PPI3

When DCI was subjected to chromatography on hydroxyapatite column, only one endopeptidase peak (HAI), coincided with the protein peak, was found (Fig. 2). HAI did not match the active peak assayed with Leu-NP, suggesting that the later may be an aminopeptidase active in pH 3.25.

When HAI was subjected to chromatography on Sephadex G-150 column, only one endopeptidase peak (GFI) was found (Fig. 3). GFI separated from the main protein peak indicating the effective removal of other proteins from GFI at this step.

When GFI was subjected to non-denaturing Disc-PAGE, three endopeptidase bands (PPI1, PPI2 and PPI3) with Rm values of 0.10, 0.12, and 0.16, respectively, were found (figure not shown). GFI and PPI1 were found to contain trypsin inhibitors, (Fig. 4B and Fig. 5). Since trypsin inhibitors have mol. wt. ca. 20 K to 40K (Lin *et al.*, 1988) and since GFI has gone

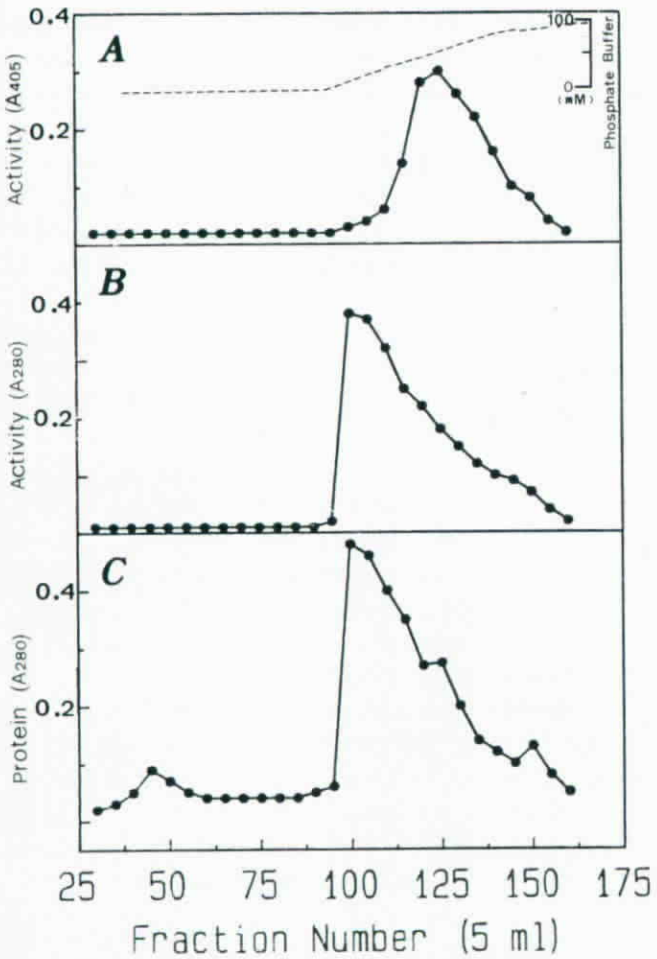

Fig. 2. Chromatography of DCI from sprouts of 'T 64' on hydroxyapatite column. DCI containing 35.6 mg protein was applied on a hydroxyapatite column (2.6 x 60 cm) pre-equilibrated with 1.0 mM sodium phosphate buffer, pH 6.8. Unbound proteins were washed off with the same buffer. Bound proteins were eluted with a gradient of 1-100 mM sodium phosphate buffer, pH 6.8, with a flow rate of 18 ml/hr. Each fraction contained 5 ml and was assayed as described in Fig. 1.

through 5 purification steps each with different separating principles, this finding is quite significant. It indicates that 1). Trypsin inhibitors bind very strongly to GFI and PPI1; 2). The smearing activity band of trypsin inhibitors suggests that this is a mixture of trypsin inhibitors, possibly containing hydrolyzed molecules.

Observations under light microscope (125X) after activity staining of trypsin inhibitor, aminopeptidase, or endopeptidase of free-hand sections of frozen sprout tissues reveal that active protease spots coincide with

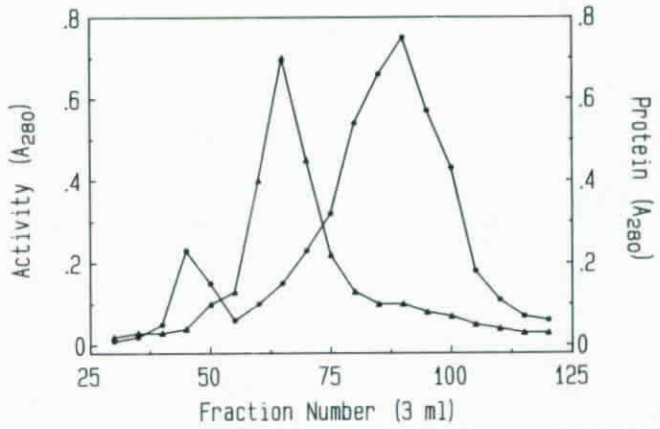

Fig. 3. Chromatography of HAI from sprouts of 'T 64' on Sephadex G-150 column. HAI containing 7.6 mg protein was applied on a Sephadex G-150 column (2 x 65 cm) pre-equilibrated with buffer A containing 0.25 M NaCl. Proteins were eluted with the same solution at a flow rate of 12 ml/hr. Each fraction contained 3 ml and was assayed as described in Fig. 1. No enzyme activity was detected when Leu-Np was used as the substrate. The mol. wt. of the active fraction was estimated to be 80 K. ▲, activity; ●, protein.

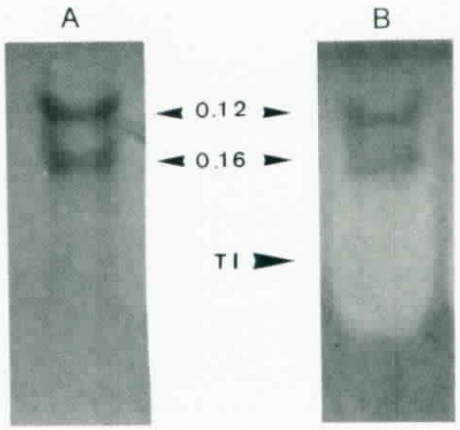

Fig. 4. Activity staining of endopeptidases or endopeptidases and trypsin inhibitors of GFI of 'T 64' sprouts in Disc-PAGE. Anodic runs at pH 8.8 in 10% w/v gel. A, activity staining of endopeptidases; B, activity staining of endopeptidases and trypsin inhibitor. GF 1 of 0.45 milli units was used in each case. Endopeptidase with Rm 0.10 was not shown in this figure. TI, trypsin inhibitor.

trypsin inhibitors (Lin, unpublished data). GFI, especially PPI1, may be a good candidate to start with in the work searching for indigenous proteases which are responsible for the degradation of trypsin inhibitors of sweet-potatoes.

PPI1, PPI2, and PPI3 have Rm values of 0.10, 0.12, and 0.16; mol. wt., estimated by gel-filtration, of 80K, 80K, and 80K; mol. wt. of subunits, estimated by SDS-PAGE, of 27K (Fig. 5), 80K, and 85K, respectively. Although PPI3 was active at pH 3.25, it is not known at present whether its optimal pH is in acidic range. Hence, PPI3 may be the same endopeptidase as the one with Rm value of 0.161 reported in another manuscript (Lin and Chu, 1989).

Purification and General Properties of Endopeptidase Fraction GFII

When DCII was subjected to chromatography on hydroxyapatite column, 3 protein peaks (one major and two minors) but no active enzyme fractions were found using both Leu-Np and hemoglobin as substrates (figure not shown). When the major protein peak was subjected to chromatography on Sephadex G-150 column, two enzyme peaks, using hemoglobin as the substrate, were found (Fig. 6). Peak 1 corresponded to GFI. The mol. wt. of peak 2 (GFII) was 53K estimated by gel-filtration. When GFII was subjected to SDS-PAGE, one polypeptide band with mol. wt. of 27 K was found, suggesting that GFII consists of two identical subunits.

Purification of Endopeptidase Fraction GFIII

When DCIII was directly subjected to chromatography on Sephadex G-150 column, two protein peaks and only one endopeptidase peak were found (Fig. 7). The enzyme peak separated well from the protein peaks indicating the removal of most contaminating proteins at this step.

General Properties of Endopeptidases

General properties of PPI1, PPI2, PPI3, GFII and GFIII are summaried in Table 2. Each molecule of PPI1 consists of 3 identical polypeptide subunits with a total mol. wt. of 81K. Each molecule of PPI2 and PPI3 is a polypeptide with a mol. wt. of 80K and 85K, respectively. Each molecule of GFII consists of 2 identical polypeptide subunits with a total mol. wt. of 54 K.

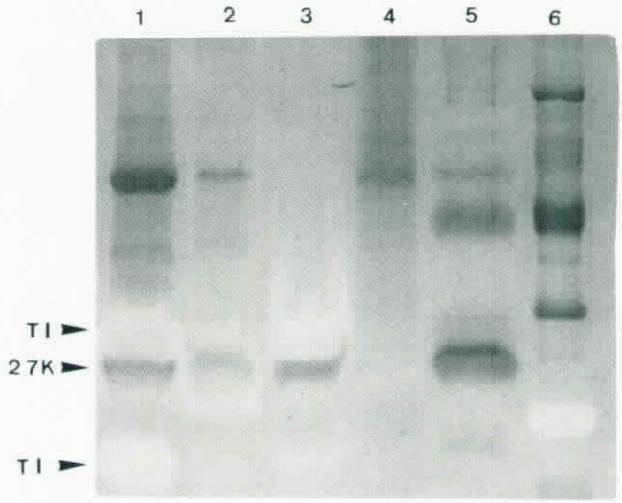

Fig. 5. Activity staining of endopeptidases and trypsin inhibitors of various endopeptidase preparations of 'T 64' sprouts in SDS-PAGE (10% gel). Lane 1, 2, 3, 4, 5, and 6: DCI, DCII, PPI 1, 0.2-0.8 ammonium sulfate fraction, low mol. wt. kit of Sigma, low mol. wt. kit of Pharmacia, respectively. Samples containing about 15 μg protein were applied.

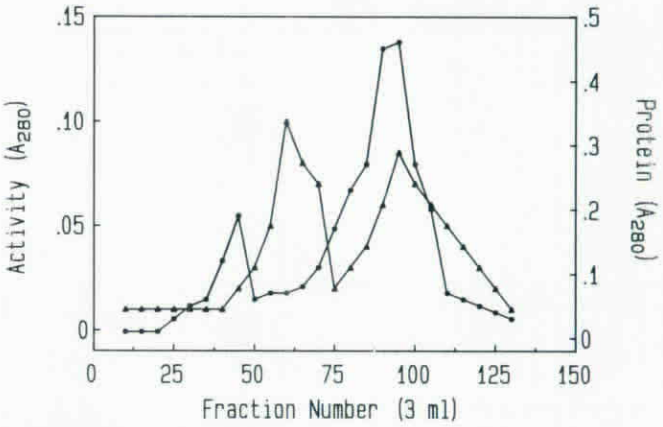

Fig. 6. Chromatography of HAII from sprouts of 'T 64' on Sephadex G-150 column. HAII containing 4.9 mg protein was applied on the same Sephadex G-150 column as Fig. 3. Active fractions were eluted and assayed as described in Fig. 1. No enzyme activity was detected when Leu-Np was used as the substrate; while 2 active fractions were found when hemoglobin was used as the substrate. The mol. wt. of the 2 fractions were estimated to be 80 K and 53 K, respectively. Hence the first fraction seems to be the same as GFI and the second fraction was designated as GFII. ▲, activity; ●, protein.

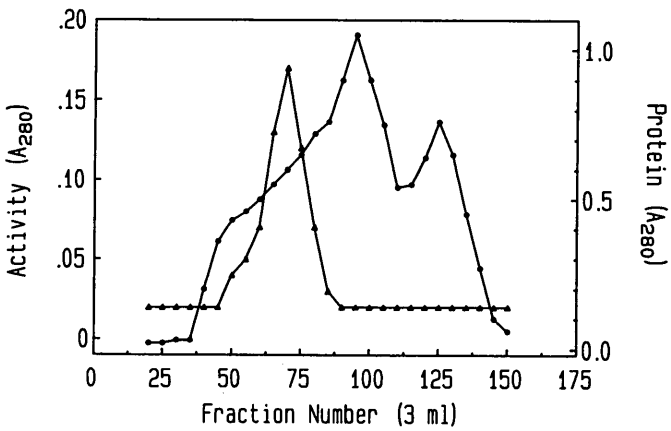

Fig. 7. Chromatography of DCIII preparation from sprouts of 'T 64' on Sephadex G-150 column. DCIII containing 11 mg protein was loaded on the same Sephadex G-150 column as Fig. 3. Fractions were eluted and assayed as described in Fig. 1. When hemoglobin was used as the substrate, only one activity peak (GFIII) was detected. The mol. wt. of GFIII was estimated to be 71 K. ▲, activity; ●, protein.

Table 2. *General properties of endopeptidases from sprouts of sweet potato 'T64'*

Endopeptidase	Rm (10% PAGE)	mol. wt. (Sephadex G-150)	mol. wt. (10% SDS-PAGE)	Subunits
PPI 1	0.10	80 K	27 K	3
PPI 2	0.12	80 K	80 K	1
PPI 3	0.16	80 K	85 K	1
GFII	-[a]	53 K	27 K	2
GFIII	-	71 K	-	-

[a]Data not available.

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台農64號甘藷芽之蛋白質內切酶之純化及性質

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經過粗抽液、硫酸銨沉澱，及 DEAE-cellulose 管柱層析共三步驟可得到5個蛋白質內切酶之活性部份：DCI、DCII、DCIII、DCIV及DCV；此乃以台農64號甘藷芽爲材料而以 hemoglobin 在 pH3.25所分析獲得者。DCI (最主要之活性部份)及DCII再連續以 hydroxyapatite 及 Sephadex G-150管柱層析純化而分別獲得 GFI及GFII。GFI再以不連續聚丙烯醯胺凝膠電泳法純化而得PPI1，PPI2及PPI3。這三者之相對泳動率(R_m，在10%凝膠上相對於 bromophenol blue者)，由膠體滲析估計之分子量(Mn)，及由 SDS-聚丙烯醯胺凝膠電泳所估計之次單元之分子量(Ms)分別爲：0.10，0.12，及0.16；80K，80K，及80K；27K，80K，及85K。PP I 1和內在之胰蛋白酶抑制因子結合得很緊。GFII之 Mn 及 Ms分別爲53K及27K。因爲不足量之故，DCIII直接以膠體滲析法純化而得 Mn 爲71K 之 GFIII。