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In vitro protease activities of four parts of germinated Tainong 57 sweet potato roots

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Abstract. Germinated 'Tainong 57' (T57) roots were divided into 4 parts: sprouts (P1); basal area of the sprout with 1 cubic centimeter each (P2); one third of the total root length containing sprouts (P3); and the rest of the total root containing no sprouts (P4). Protein contents (mg/g fr. wt.), trypsin-inhibitor activity (mg trypsin inhibited/g fr. wt., TIA), protease activities (unit/mg protein) using 51 synthetic substrates were measured with crude extracts of the four parts at pH 3.4, 7.0, and 8.9, respectively. Protein content increases from P4 and P3 via P2 toward P1; while TIA increases from P4 and P3 toward P2 and then decreases sharply in P1. Fourteen protease activities with relatively higher values are presented and grouped on two criteria, i.e. relative activities in the 4 parts and pH preference. Two and five groups were found based on the former and the latter, respectively. Good agreement between results of the two grouping procedures suggests that different roles are played by protease activities under study.

Key words: Enzyme extraction; *Ipomoea batatas*; Protein content; Protein metabolism; Trypsin inhibitor activity.

Introduction

We have reported the occurrence and 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 of sweet potato (*Ipomoea batatas* (L.) Lam.). However, no information is available yet about whether trypsin inhibitors inhibit any indigenous proteases, or how themselves and other storage proteins are metabolized. In order to answer these questions, knowledge of proteases in sweet potato tissues is essential. We have already detected activities of endopeptidase (Lin and Chu, 1988), aminopeptidase (Chen and Lin, 1989), and carboxypeptidase (EC. 3.16.17, hippuryl-L-lysine as the substrate; Lin, unpublished data) in sprouts and resting roots. We describe in this work a broad-range survey of *in vitro* protease activities, protein content and trypsin inhibitor activity (TIA) of the four parts of germinated Tainong 57 (T57) roots, patterns of which demonstrate changing of protein metabolism during germination. We also propose a sorting method for relating different protease activities.

Materials and Methods

Chemicals

All synthetic substrates of proteases were purchased from Sigma Chemical Company (St. Louis, MO. USA) except L-Ala-4-nitroanilide (abbreviated as Ala -NA), Benzyl L-Arg-NA (as Benz-Arg-NA), Nα-3 -(carboxy propionyl)-Phe-NA (as CP-Phe-NA), glutaryl Phe-NA (as G-Phe-NA), acetyl Tyr-NA (as Ace-Tyr-NA), Glu-l-NA, and Cystine-bis-NA which were products of E. Merck (Darmstadt, Germany). Benzylcarbonyl was abbreviated as Cbz and 2-naphthylamide as Nap. All other chemicals were also obtained from Sigma Chemical Company. For simplicity if no configuration (D or L) of an amino acid is in-

dicated it stands for L-form. For amide derivatives of aspartic or glutamic acid, that of COOH of position 1 is implied if not specified.

Plant Material

Sweet potato (*Ipomoea batatas* L. Lam. cv. Tainong 57) were purchased from local market. Roots were sprayed with 0.04% sodium azide and allowed to sprout for about one month in the dark programmed at 32±1°C (12 h) and 25±1°C (12 h) with 75±2% relative humidity. Only water was supplied during germinating period. Germinated roots were divided into four parts: sprouts (P1); basal area of sprout with 1 cubic centimeter (P2); one third of the total root length containing the sprouts (P3); and the rest of the total root not containing the sprouts (P4). Protease activities were assayed with crude extract of each part described above.

Enzyme Extraction

All stages of enzyme extraction were carried out at 4°C. Samples, except P3 and P4 which were peeled first, were homogenized in ca. 2.5 volumes of double-distilled water (v/w) with a commercial fruit-blender. After standing for 1 h the homogenate was centrifuged at 35000 X g for 60 min and the pellets were discarded. The floating materials of the supernatant were removed by filtering through Whatman filter paper #1 under suction and the clear solution was used as crude extract for various assays.

Protein Estimation

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

Determination of Trypsin Inhibitor Activity

Assay of trypsin inhibitor activity (TIA) followed Lin and Chen's modified procedure (1980) of Kunitz (1946). For each TIA determination, three assays (the standard, the control, and the sample) were undertaken. The standard assay was performed by adding 0.5 ml of double-distilled water and 1.0 ml trypsin solution containing 20 μg trypsin in 0.25 mM HCl to tubes containing 1.0 ml of 2% heated (100°C, 15 min) casein solution in 0.1 to 0.2 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.6. The proteolytic reaction was allowed to proceed at 37°C for 20 min and then was terminated with 3.0 ml of 10% trichloroacetic acid (TCA). The precipitate formed was centrifuged after standing for at least 1 h at about 25°C. The concentration of TCA-soluble peptides with aromatic amino acids in the supernatant was determined by measuring the absorbance of the solution at 280 nm (Blackburn, 1968). The control assay was performed by preincubating 0.3 ml of the sample and 0.2 ml of double-distilled water with 1.0 ml of 2% heated casein solution at 37°C for 15 min. Subsequently, 1.0 ml of double-distilled water was added and the mixture was allowed to stand at 37°C for a further 20 min before the reaction was terminated with 3 ml of 10% TCA. The sample assay was performed by preincubating 0.3 ml crude extract and 0.2 ml of double-distilled water with 1.0 ml of 2% previously heated casein solution at 37°C for 15 min. Finally, 1.0 ml trypsin solution was added and proteolytic reaction was carried out as for the standard assay. The percentage of inhibition (PI) was calculated from: $[(A_{280}$ of standard + A_{280} of control) − A_{280} of sample)]/A_{280} of standard × 100%, where A_{280} was the absorbance of the solution at 280 nm, and was converted to micrograms of trypsin inhibited. Samples were diluted to give values of PI around 50, hence PI>100 was possible for original crude extracts.

Assays of Protease Activities

Protease assays for each synthetic substrate (Erlanger *et al.*, 1961) were carried out at three pHs: 3.4 (glycine-HCl buffer), 7.0 (phosphate buffer), and 8.9 (Tris-HCl buffer). Each synthetic substrate was dissolved in N,N-dimethylformamide as stock solution and diluted with double-distilled water before use. Crude extracts of the four parts were diluted with appropriate amount of double-distilled water to ca. 0.25 mg protein/ml as enzyme sources. Each microplate contained 270 μl of the reaction mixture which consisted of 20 mM buffer, 2.5 mM substrate, and crude extract with ca. 30 μg protein. The enzyme reaction was started by adding 120 μl of the crude extract to a mixture of buffer and substrate solution and carried out at 37°C for 50, 100, and 150 min. The reaction mixture was monitored by measuring the absorbance at 405 nm at each time interval. Absorbance at zero time was used as the blank value for each corresponding assay. A Microplate EL 309 Autoreader manufactured by Bio-Tek Instruments (Winooski, Vermont, USA) was used. The amount of enzyme which gave an absor-

bance of 1.0 at 405 nm in 50 min was defined as one enzyme unit.

Results

Protein Contents and Trypsin Inhibitor Activity of The Four Parts

Table 1 shows protein contents and TIA of the four parts of germinated T57 roots. Sprouts (P1) contain the highest protein content, followed by basal area of sprout (P2), and both P3 and P4 are the lowest based on mg protein per g fresh weight. With an average of 3.415 mg protein per g fresh weight of dormant T57 roots, the increasing gradient of protein content from P4 and P3 to P2, and finally to P1 is obvious. In sharp contrast to protein content, P1 and P2, respectively, contain the lowest and the highest TIA. Because dormant T57 roots contain an average TIA of 1.210 mg trypsin inhibited per g fresh weight, it seems that trypsin inhibitors (TIs) are mobilized from root parts (P4 and P3) to P2 where TIs accumulate to some extent. Then TIs serve as one of amino acid sources for protein systhesis in sprouts.

Protease Activities of The Four Parts

In general, protease activities in dormant roots of sweet potato are very low or undetectable (data not shown) except a few enzymes such as tripeptidyl peptidase (Lin and Wang, 1990).

About 51 synthetic substrates for proteases were used in this work. However only the results of 14 substrates with relatively higher specific activity (unit/mg protein) are presented. Each protease activity is linear with time within 150 min, but only results obtained at 50 min are shown for clarity. Each bar represents the average of at least three estimates with values of coefficient of variance (CV) between 5 to 7% (data not shown).

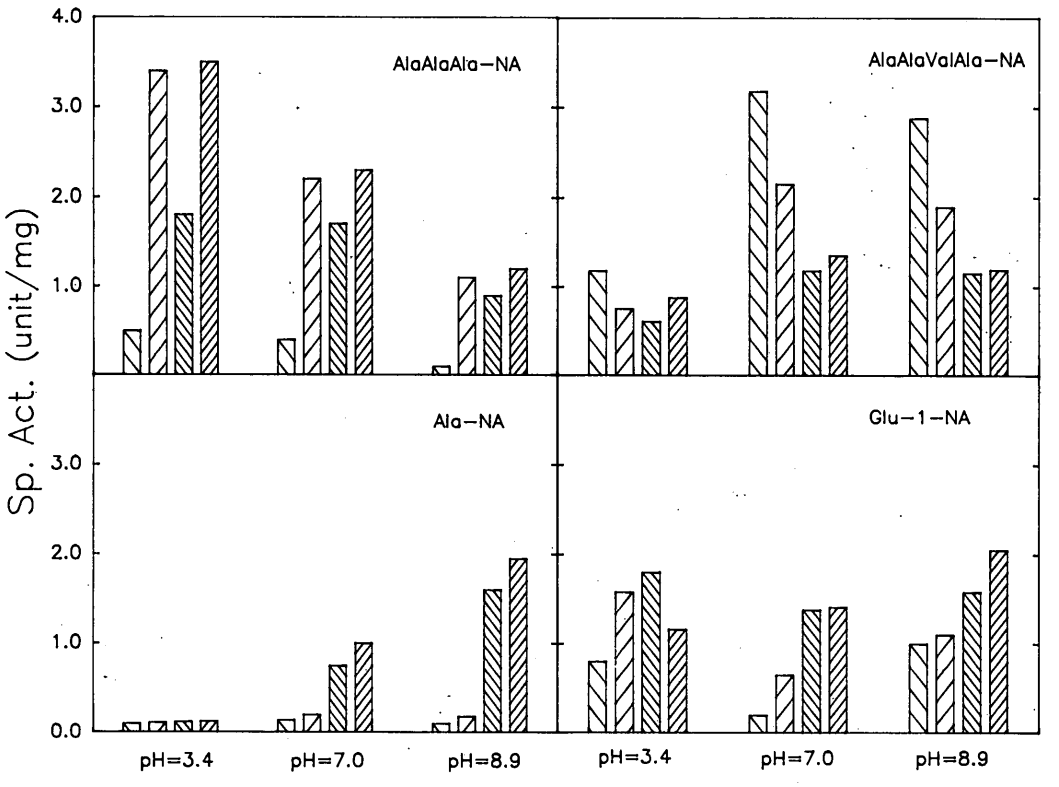

Fig. 1. Specific activities (unit/mg protein) of corresponding proteases that hydrolyze Ala-Ala-Ala-NA, Ala-Ala-Val-Ala-NA, Ala-NA and Glu-NA, respectively, of the four parts of germinated 'Tainong 57' (T57) sweet potato roots. ▨, sprouts (P1); ▨, base areas of sprouts with 1 cubic centimeter each (P2); ▨, one third of the total root length containing the sprouts (P3); ▨, two thirds of the total root length containing no sprouts (P4).

Names of those substrates with lower or non-detecable protease activity are listed below for reference: Ala-Ala-Phe-4-nitroanilide (as Ala-Ala-Phe-NA), Ace-Ala-NA, Ace-Leu-NA, Val-NA, Benz-DL-Arg-NA, Benz-Arg-NA, CBZ-Phe-NA, CP-Phe-NA, Ace-DL-Phe-NA, Glutaryl-Phe-NA, γ-Glu-NA, pyroglutamic acid-β-naphthylamide (as Pyro Glu-Nap), Glu-1-(4-methyl)-Nap, DL-Met-Nap, Formyl-Met-Nap, Cysteine-β-naphthylamide (as Cys-Nap), Benz-Cys-Nap, His-Nap, Ile-Nap, CBZ-Pro-Nap, Try-Nap, Tyr-Nap, Asn-Nap, Asp-Nap, Benz-Arg-(4-methyl)-Nap, Ala-(4-methyl)-Nap, Phe-Nap, Benz-DL-Phe-Nap, CBZ-Phe-Nap, succinly-Phe-Gly-Leu-Nap, hippuryl-Lys (as Hip-Lys), Hip-Arg, Hip-Gly-Lys, Hip-His-Leu, Hip-Phe, Benz-Gly-Phe, Hip-Gly-Gly.

Fig. 1 shows specific activities of corresponding proteases that hydrolyze Ala-Ala-Ala-NA, Ala-Ala-Val-Ala-NA, Ala-NA and Glu-NA, respectively, at pH 3.4, 7.0 or 8.9. All substrates except Ala-Ala-Val-Ala-NA were hydrolyzed more rapidly by crude extracts of P2, P3 or P4 than P1. Ala-Ala-Val-Ala-NA was hydrolyzed more rapidly by crude extract of P1 than P2, P3 or P4. This suggests that the enzyme(s) which degrades Ala-Ala-Val-Ala-NA may play a different role from that of the other 3 enzyme activities. The pH preferences of the 4 enzyme activities are different.

Fig. 2 shows specific activities of corresponding proteases that hydrolyze Leu-NA, Gly-NA, Pro-NA and Met-NA, respectively. That the estimates of P4 and P3 are higher than those of P2 and P1 with or without the other estimates equal to P2 and P1 is common to all 4 substrates at all 3 pHs. This suggests a common role of the 4 enzyme activities in degrading reserved proteins during germination. The pH preferences of enzyme activities hydrolyzing Leu-NA, Pro-NA, Met-NA are similar, namely pH 8.9>pH 7.0>pH 3.4. Slight change occurs for Gly-NA in which the order of pH 7.0 and 3.4 is reversed.

Fig. 3 shows specific activities of corresponding proteases that hydrolyze Lys-NA, Arg-NA, Ala-Ala-NA and Cystine-bis-NA, respectively. Specific activities hydrolyzing Lys-NA, Arg-NA and Cystine-bis

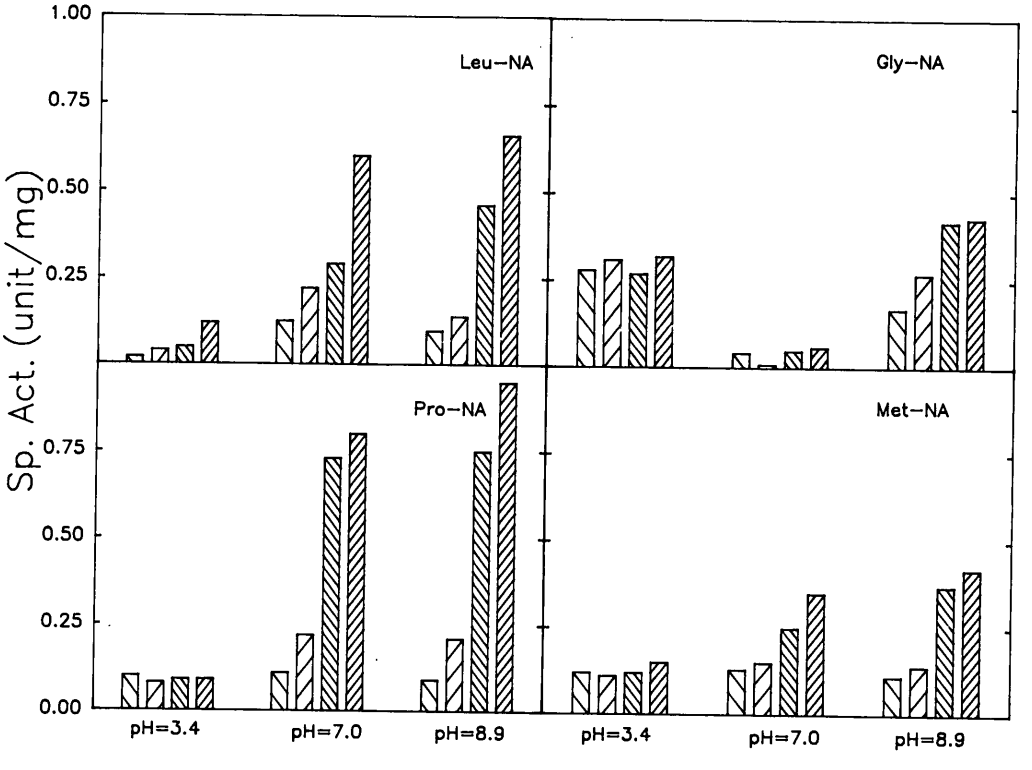

Fig. 2. Specific activities of corresponding proteases that hydrolyze Leu-NA, Gly-NA, Pro-NA and Met-NA, respectively, of the four parts of T57 germinated roots. Symbol assignments are the same as in Fig. 1.

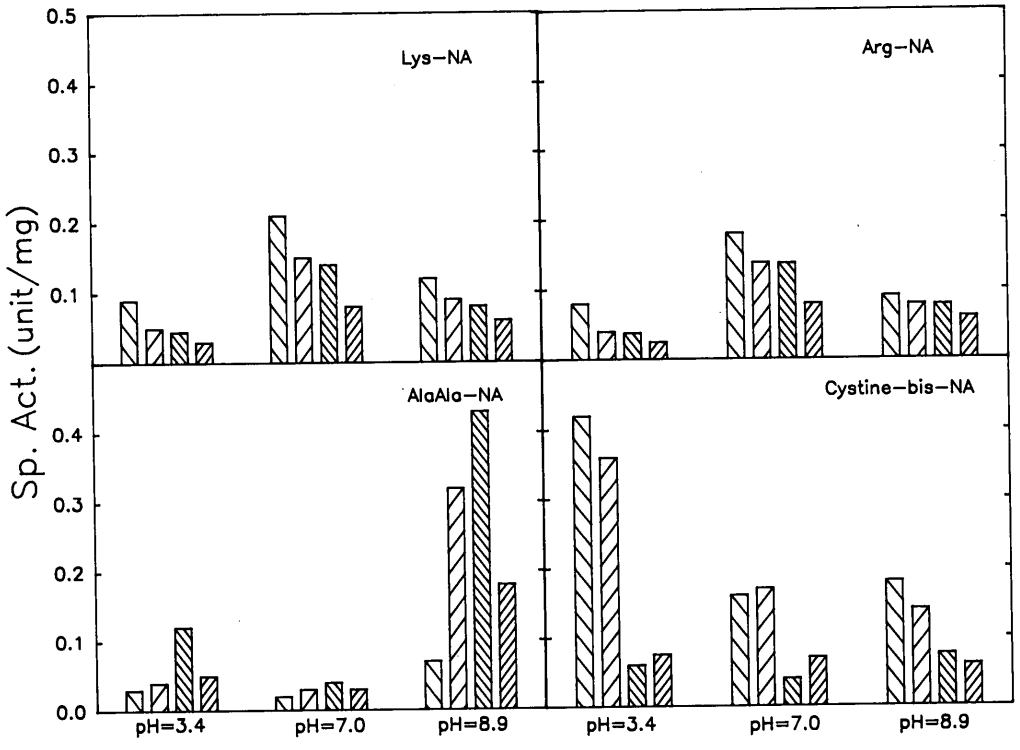

Fig. 3. Specific activities of corresponding proteases that hydrolyze Lys-NA, Arg-NA, Ala-Ala-NA and Cystine-bis-NA, respectively, of the four parts of T57 germinated roots. Symbol assignments are the same as in Fig. 1.

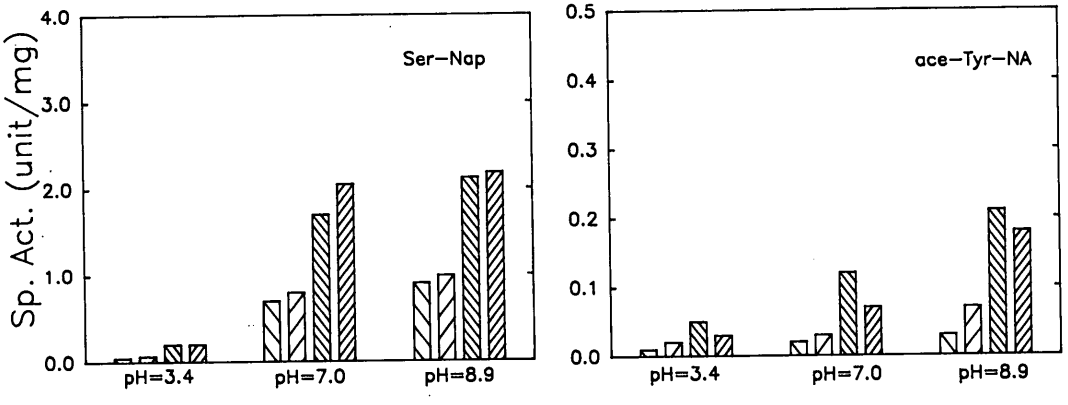

Fig. 4. Specific activities of corresponding proteases that hydrolyzing Ser-Nap and Ace-Tyr-NA, respectively, of the four parts of T57 germinated roots. Symbol assignments are the same as in Fig. 1.

-NA, respectively, of P1 or P2 are higher than those of P3 or P4, a similar situation to that of Ala-Ala-Val -Ala-NA in Fig. 1. While P3 is the highest followed by P2 in case of Ala-Ala-NA. The pH preference for Lys -NA and Arg-NA is 7.0>8.9 or 3.4. For Ala-Ala-NA and Cystine-bis-NA the order is 8.9>3.4>7.0 and 3.4>7.0 or 8.9, respectively.

Fig. 4 shows specific activities hydrolyzing Ser -Nap and Ace-Tyr-NA, respectively, of P4 or P3 are higher than those of P2 or P1. The pH preference of Ace-Tyr-NA and Ser-Nap is the same: 8.9>7.0>3.4.

Table 1. *Protein contents and trypsin inhibitor activity of the four parts of germinated 'Tainong 57' (T57) roots*
The average protein content of resting T57 roots was 3.415 mg per g fresh weight and the average TIA was 1.210 mg trypsin inhibited per g fresh weight.

	Part[a]			
	1	2	3	4
Protein content (mg/g fr. wt.)	5.519	4.751	2.915	3.206
Trypsin inhibitor activity (mg trypsin inhibited/g fr. wt.)	0.360	1.410	1.037	1.040

[a] Part 1, sprouts; part 2, basal areas of sprouts with one cubic centimeter each; part 3, one third of the total root length containing the sprouts; part 4, the rest of the total root containing no sprouts.

Discussion

Results of Table 1 indicate an increasing gradient of protein content from P4 and P3 to P2 and finally to P1 based on mg protein per g fresh weight. Since plant leaves in general (Rost *et al.*, 1979) and leaves of sweet potato in particular (Lin and Ho, 1986) contain much higher water content than roots, the observed gradient of protein content will be increased if based on mg protein per g dry matter. Since no nutrients were supplied, the observed gradient clearly shows the direction of protein mobilization during germination of T57 roots.

When sweet potato organs of the same kind, such as roots or leaves, were compared, TIA correlated positively with total water-soluble protein (Lin and Chen, 1980; Lin and Ho, 1986; Lin, 1989). Since now we are comparing different kinds of organs, namely sprouts vs. germinated roots, we must consider that the ratios of amount of TIs to total water-soluble protein are different in different organs. Knowing that TI is one of the storage proteins of sweet potato roots (Lin and Chen, 1980; Lin, 1989), results of Table 1 suggest that reserved proteins are mobilized in yet unknown mechanisms from root parts (P4 and P3) to basal area of sprout (P2) as reflected by change of TIA. P2 serves as a relocation area from where reserved proteins serve as a source of amino acids for protein synthesis in sprouts. By unknown mechanisms, TIs are converted into other proteins.

Vodkin and Scandalios (1980) have indicated the possible "cumulative effects of a number of proteases and peptidases which can have broad and overlapping substrate specificities." In order to find out the possible relationship among different protease and peptidase activities of the four parts, those described in this work may be sorted into various groups and subgroups depending on the decreasing order of specific enzymatic activities or pH preference. Thus two groups are found according to the first criterion. Group 1 contains those with estimates in P4 or P3 larger than P2 or P1 (with or without other estimates equal to P2 or P1): Ala-Ala-Ala-NA, Ala-Ala-NA, Ala-NA, Glu-NA, Leu-NA, Gly-NA, Pro-NA, Met-NA, Ser-Nap and Ace-Tyr-NA. Group 2 contains those with estimates in P1 or P2 larger than P3 or P4 (with or without other estimates equal to P3 or P4): Ala-Ala-Val-Ala-NA, Lys-NA, Arg-NA and Cystine-bis-NA.

Five groups may be established according to the second criterion. Group 1 contains those with estimates at pH 8.9>7.0>3.4: Leu-NA, Ala-NA, Met-NA, Pro-NA, Ser-Nap and Ace-Tyr-NA; subgroup 1 contains Gly-NA and Ala-Ala-Na (pH 8.9>3.4>7.0). Group 2 contains Ala-Ala-Ala-NA (pH 3.4>7.0>8.9) with one subgroup of Cystine-bis-NA (pH 3.4>7.0 or 8.9). Group 3 contains Lys-NA and Arg-NA (pH 7.0>8.9 or 3.4). Group 4 and 5 contain Ala-Ala-Val-Ala-NA (pH 8.9 or 7.0>3.4) and Glu-NA (no obvious pH preference), respectively.

The proposed sorting methods seem quite useful according to the fact that 8 out of 10 members in Group 1 based on the first criterion are sorted into the same group, namely Group 1, based on the second criterion. Thus application to other proteases in the future is promising. At the same time, good agreement of the results between two grouping procedures suggests that different roles are played by protease activities belonging to different groups.

Lack of a particular protease activity does not necessarily mean that the enzyme does not exist in the tissue examined. There are several possible reasons, such as: the existence of particular inhibitors, the assay conditions are not appropriate, etc.

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台農 57 號甘藷塊根發芽後四個部份的粗抽液之各種蛋白酶活性

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已發芽之台農 57 號甘藷塊根分成四個部份：芽(P1)；芽之基部邊長各 1 公分之方塊(P2)；含芽佔總塊根長度 1/3 之部位(P3)；及其餘佔總塊根 2/3 且不含芽之部位(P4)。各部份之粗抽液用來測定蛋白質含量(每克鮮重所含之蛋白質以毫克表示)，胰蛋白酶抑制因子活性(每克鮮重所抑制之胰蛋白酶以毫克表示，簡稱爲 TIA)，各種蛋白酶活性(使用 51 種合成基質，每種分別在 pH 3.4、7.0 及 8.9 下測定)。蛋白質含量從 P4 及 P3 經 P2 向 P1 遞增；而 TIA 從 P4 和 P3 向 P2 增加却在 P1 迅速地下降。只有活性較高之 14 種基質的結果在本篇報告中詳述且依據兩種標準(即在四個部份之相對之比活性大小和對 pH 之喜好情形)加以歸類。分別得到 2 種和 5 種類別。由上述兩種不同標準而歸類所得之結果頗爲一致，因此肯定了歸類法之有用性，且指出分屬不同類之蛋白酶可能扮演不同之生化、生理功能。