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PARTIAL PURIFICATION AND SOME PROPERTIES OF TRYPSIN INHIBITORS OF SWEET POTATO (*IPOMOEA BATATAS* LAM.) ROOTS[1]

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Abstract

Trypsin inhibitors of water extract of sweet potato (var. Tainong No. 65) roots were purified successively by ammonium sulfate fractionation, gel-filtration, DEAE-cellulose column chromatography, CM-cellulose column chromatography. A 20-fold purification was obtained. Purified sample was shown by polyacrylamide gel electrophoresis to be not a single protein. Roots of Tainong 65 may contain 3-4 molecular species of trypsin inhibitor which were not easy to be separated by conventional protein purification techniques due to their similar physical and chemical properties.

Introduction

Natural inhibitors for proteolytic enzymes occur in micro-organisms, plants, and animals. Natural inhibitors of proteases of small and large molecular weights have been reported. Many inhibitors of the latter category are proteins. The first known plant protein inhibitor of protease is that from soybean, which was crystallized in 1946 by Kunitz. Other protein inhibitors were later found in wheat, barley and many of the *Leguminosae* (Vogel *et al.*, 1968). The presence of trypsin inhibitor in a non-leguminous plant, sweet potato, was first reported by Sohonie and Bhandarkar (1954). Their preparation was very thermolabile, although it was not completely pure. Isolation of trypsin inhibitor from sweet potatoes has been patented (Sugiura and Takeuchi, 1972). Three different trypsin inhibitors were found in a sweet potato, i.e. *Ipomoea batatas* LAM *var. edulis* Makino (Okinawa Kokei No. 14). The purification and some chemical and inhibitory properties of

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the trypsin inhibitors, named as inhibitors II and III, were described. Both inhibitors were fairly stable over a pH range from 2 to 11 at 37°C, and thermostable (Sugiura *et al.*, 1973). Modification of amino acid residues in inhibitor III has also been reported (Ogiso *et al.*, 1974). A brief report concerning the effect of varietal differences on the activity level and the heat stability of the trypsin inhibitor of sweet potatoes has been published (AVRDC Sweet Potato Report, 1975). A further survey on levels and heat stability of trypsin inhibitor activity among 53 sweet potato varieties was reported by our laboratory (Lin and Chen, 1980; Lin, 1982). Trypsin inhibitor activity of sweet potato roots showed seasonal variation and was related to changes of climatic factors such as cumulative temperature and cumulative rainfall (our unpublished data). Our unpublished data also showed that many agronomic characters of sweet potato were related to trypsin inhibitor activity. All these observations suggest that trypsin inhibitors of sweet potato roots may play important physiological roles. Purification and characterization of trypsin inhibitors of our local variety Tainong No. 65 which contains the highest trypsin inhibitor activity should give valuable information.

Materials and Methods

Materials

Roots of sweet potato HP-18 which was officially named as Tainong No. 65 were kindly provided by Chia-yi Agricultural Experimentation Station. Fresh roots were cut into chips immediately after being delivered to the laboratory. Chips were dried at 40–50°C and then frozen at −20°C or refrigerated at 7°C for later use. Trypsin (10,000–13,000 BAEE units per mg) was purchased from Sigma Co., U. S. A.; Casein and Folin phenol reagents for protein determinations were products of Wako Co., Japan; Ammonium sulfate (enzyme grade), CM-cellulose (0.6 meq/g, medium mesh), DEAE-cellulose (0.9 meq/g, medium mesh), and chemicals for polyacrylamide gel electrophoresis from E. Merck, Germany; Sephadex G-75 from Pharmacia Fine Chemicals, Sweden.

Determinations of Water Soluble Protein

The protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Trypsin Activity Assay with Casein as Substrate

This was done mainly according to procedure reported by Kunitz (1946). Standard assays were run by adding 0.5 ml double distilled water (DDW) and 1.0 ml trypsin solution (containing 20 μg trypsin in 0.25 mM HCl) to tubes containing

1.0 ml of 2% activated (35°C, 5 min) casein solution (in Na_2HPO_4–NaH_2PO_4 buffer, pH 7.6). Proteolytic reaction was allowed to proceed at 37°C for 20 min. The solution was then poured into tubes containing 3.0 ml of 10% trichloroacetic acid. The precipitates formed were centrifuged off after standing for 1 hr or longer at about 25°C. The concentration of split products in the supernatant solution was determined by measuring the absorbance of the solution at 280 nm. Control tests were run by preincubating 0.3 ml samples containing 0.3–0.45 mg protein and 0.2 ml DDW with 1.0 ml of 2% activated casein solution at 37°C for 15 min. Then 1.0 ml DDW was added and the mixture was allowed to stand at 37°C for another 20 min before being poured into 10% trichloroacetic acid. Sample tests were done by preincubating 0.3 ml sample and 0.2 ml DDW with 1.0 ml of 2% activated casein solution at 37°C for 15 min. Then 1.0 ml trypsin solution was added and proteolytic reaction was proceeded as standard assay.

Calculation of Trypsin Inhibitor Activity

The percentage of inhibition was calculated by a formula as 100% × ((A_{280} of standard + A_{280} of control) − A_{280} of sample)/(A_{280} of standard), and the specific % inhibition was defined as % inhibition per mg water soluble protein. Dilution of samples was made if % inhibition was close to 100% inhibition.

DDW Crude Extract

Six-hundred gms of sweet potato chips were blended with 1,800 ml DDW in a fruit juicer. The homogenate was filtered through 4 layers of cheesecloth twice and then centrifuged at 17,370×g for 10 min. The supernatant fluid was concentrated with a vacuum flash evaporator to about 246 ml. This was dialyzed twice against 100 volumes of distilled water for at least 4 h before processed to the next step. The dialyzed sample was called "crude extract".

Ammonium Sulfate (A.S.) Fractionation

Ground solid ammonium sulfate was added slowly in small amounts into crude extract (keeping pH at 7.0 by adding concentrated NaOH solution) to get a 20% A.S. saturation solution which was centrifuged as mentioned above after standing for 20 min. The precipitate showed little trypsin inhibitor activity and was discarded. To the supernatant solution ground solid A.S. was added as mentioned above to get a 30% A.S. saturation solution. As the previous step both precipitate and supernatant solution could be obtained. The precipitate was dissolve with a minimum amount of DDW and dialyzed as mentioned above. The dialyzed sample was called 20–30% A.S. fraction. The supernatant solution was processed further to get a 40% A.S. saturation solution and then a sample called 30-40% A.S. fraction could be obtained. This process was repeated to get 40–50%, 50–80%, >80%

A. S. fractions sequentially.

Gel Filtration

Sephadex G-75 was used. The column size was 2.6 cm (I. D.)×70 cm. The gel bed was 2.6 cm×60 cm and the flow rate was 20 ml/h. Elution solution was 10 mM phosphate buffer (pH 6.0). This step was used after A. S. fractionation.

DEAE-cellulose Column Chromatography

The column size was 2.5 cm×45 cm. The bed size was 2.5 cm×30 cm. The elution solution was a linear gradient with 10 mM phosphate buffer (pH 6.0) as starting buffer in the mixing chamber (300 ml) and 0.5 M NaCl in the same buffer as finishing solution in the reservoir (300 ml). The flow rate was 30 ml/h and each fraction contained 5 ml effluent. Sample used in this step was the only peak fraction obtained in Sephadex G-75 gel filtration as shown in Fig. 1.

CM-cellulose Column Chromatography

The combined peak fraction ($D_1+D_2+D_3$) obtained in the previous step was purified further by this method. The bed size was 2.5 cm×20 cm and the flow rate was 30 ml/h. The elution solution was a linear gradient of sodium acetate-acetic acid buffer from 10 mM (pH 4.0, 300 ml) to 30 mM (pH 5.5, 300 ml). Each fraction contained 5 ml effluent.

Polyacrylamide Gel Electrophoresis (PAGE)

Gel systems of both pH 8.3 (Burgess, 1968) and pH 3.2 (Panyin and Chalkley, 1969) were used to analyze protein bands of samples obtained at different purification steps which showed trypsin inhibitor activity.

Results

Ammonium Sulfate Fractionation

Table 1 shows that although the distribution of trypsin inhibitors of Tainong No. 65 roots spreads over various A. S. saturation fractions, the main activity resided at 20–30% and 30–40% A. S. saturation fractions.

Results of Purification

Table 2 shows results of five steps' purification. Samples obtained at each step were subjected to both gel systems of polyacrylamide gel electrophoresis to analyze their protein patterns.

Table 1. *Ammonium sulfate fractionation of trypsin inhibitors from Tainong-65*

Ammonium Sulfate Fraction (%)	Volume (ml)	Total Protein (mg)	Total % Inhibition	Specific % Inhibition $\left(\frac{\text{Total \% Inhibition}}{\text{mg protein}}\right)$	Purification (fold)	Recovery (%)
crude extract	246	7,872	820,000	104.2	1	100
20-30	56	2,121	371,300	175.1	1.68	45.28
30-40	26	1,404	167,000	119.0	1.14	20.37
40-50	19	540	39,900	73.94	0.71	4.87
50-80	20	244	10,000	40.98	0.39	0.012
>80	20	4	268	60.06	0.03	0.0003

Table 2. *Summary of purification of trypsin inhibitors from Tainong-65*

Fraction	Volume (ml)	Total Protein (mg)	Total % Inhibition	Specific % Inhibition $\left(\frac{\text{Total \% Inhibition}}{\text{mg protein}}\right)$	Purification (fold)	Recovery (%)
Crude Extract	246	7,872	820,000	104.2	1	100
Ammonium Sulfate (30-40%)	26	1,404	167,000	119.0	1.142	20.4
Sephadex G-75 Peak	76	83.6	20,310	242.9	2.331	2.477
DEAE-cellulose Peak						
1		37.66	17,990	477.6	4.583	2.194
2		14.72	4,544	308.7	2.963	0.554
3		64.77	19,300	298.0	2.860	2.354
CM-cellulose Peak						
1	4	0.471	394	2,000.0	19.19	0.048
2	9	1.601	3,224	2,013.0	19.32	0.393
3	7	14.38	7,338	510.3	4.897	0.895
4	8	4.602	3,719	808.1	7.755	0.454

Gel Filtration

Fig. 1 shows results of Sephadex G-75 gel filtration. Samples applied to the column were from 30–40% A. S. saturation fraction. Those used in Fig. 2 and Fig. 3 were from 20–30% and 40–50% A. S. saturation fractions, respectively. Obviously the protein peak and trypsin inhibitor activity peak do not separate well in Sephadex G-75 gel. Void volume fraction which did not show inhibitor activity was not shown in all the figures. Sample of 20–30% A. S. saturation fraction was used up, so 30–40% A. S. saturation fraction (Fig. 1) was used for the following experiments.

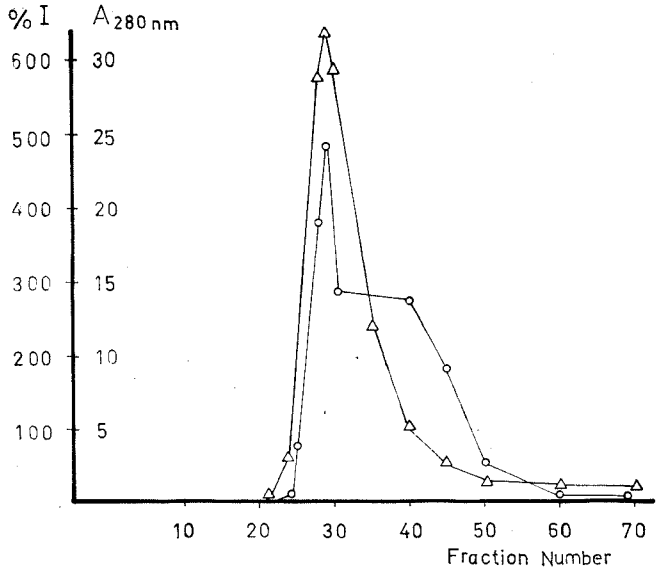

Fig. 1. Gel filtration of 30%-40% A.S. saturation fraction on Sephadex G-75 column. Sephadex G-75 was equilibrated with elution solution: 10 mM phosphate buffer (pH 6.0) and flow rate was 20 ml/hr. Every 5.5 ml (100 drops) was collected as a fraction. ○, inhibition activity; △, A_{280nm}.

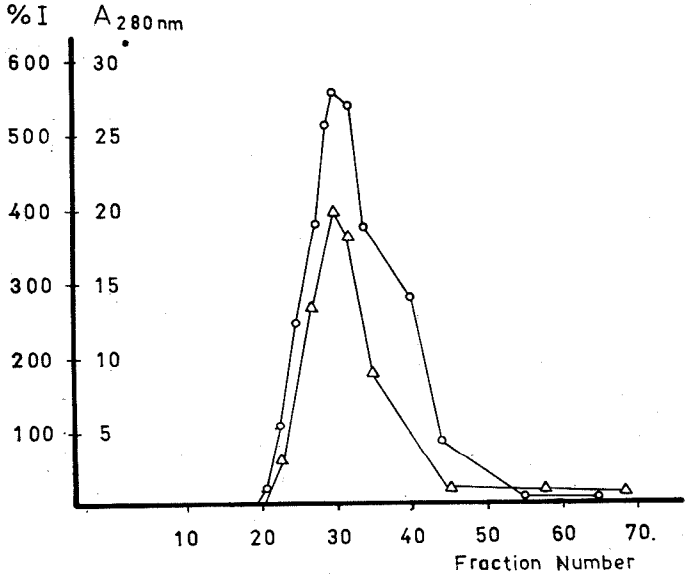

Fig. 2. Gel filtration of 20%-30% A.S. saturation fraction on Sephadex G-75 column. Conditions used were the same as Fig. 1. ○, inhibition activity; △, A_{280nm}.

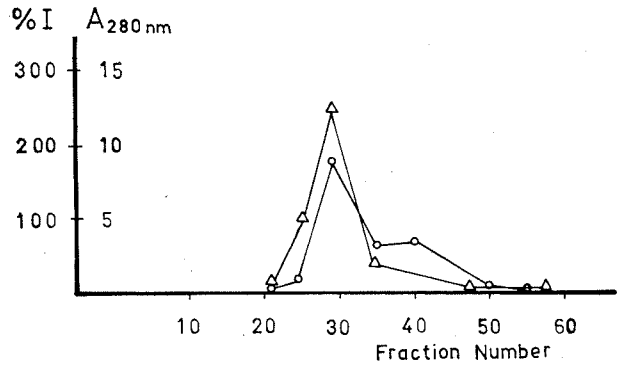

Fig. 3. Gel filtration of 40%-50% A.S. saturation fraction on Sephadex G-75 column. Conditions used were the same as Fig. 1 and Fig. 2, ○, inhibition activity; △, A_{280nm}.

Elution Pattern of DEAE-cellulose Column Chromatography

Fig. 4 shows that trypsin inhibitor activity peaks and the main protein peak already have tendency to separate. Three activity peaks are assigned D_1 (fraction No. 56-69), D_2 (fraction No. 71-74), and D_3 (fraction No. 76-102) from left to right. But patterns of polyacrylamide gel electrophoresis show that D_1, D_2, and D_3 have little difference. In order to get enough active fractions for further experiments, D_1, D_2, and D_3 were pooled together to proceed the next step.

Elution Pattern of CM-cellulose Column Chromatography

Fig. 5 shows that trypsin inhibitor activity spreads over a broad range. Based on Fig. 5, the degree of purification of peaks P_1, P_2, P_3, and P_4 can be found in Table 2. The highest degree of purification is about 20 folds.

Electrophoresis Pattern of P_1, P_2, P_3, and P_4 from CM-cellulose Column Chromatography

Fig. 6 shows protein patterns of pH 8.3 PAGE (containing 8 M urea) system. Proteins were stained with Coomassie brilliant blue. The patterns of all four fractions were quite similar.

Isoelectric Focusing

Samples of crude extract; 0-20%, 20-40%, 40-60%, 60-80% A.S. saturation fractions; peak of Sephadex G-75 gel filtration were analyzed by isoelectric focusing (Ampholine PAG plate with a pH range of 3-11 was kindly provided by LKB Ltd.).

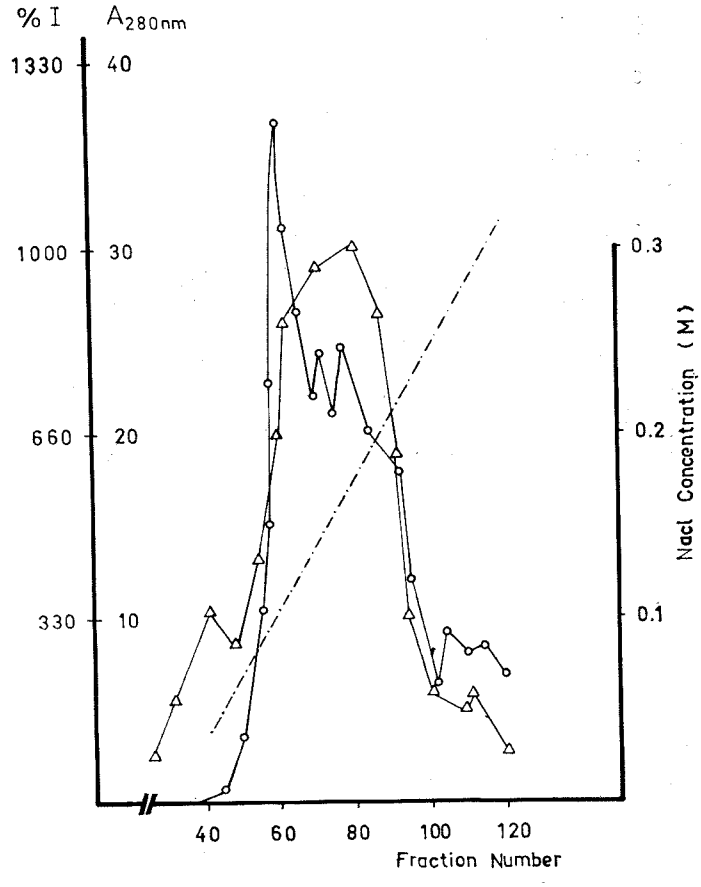

Fig. 4. Elution profile of DEAE-cellulose column chromatography. Column size was 2.5 (ID) × 45 cm, bed size 2.5 × 30 cm. Ten-ml sample obtained from Sephadex G-75 gel filtration (Fig. 1) was applied to the column and was eluted with a linear gradient of NaCl (0-0.5 M) in 10 mM phosphate buffer (pH 6.0). Flow rate was 30 ml/hr and every 5 ml was collected as a fraction. ○, inhibition activity; △, A_{280nm}; ·-·-, NaCl concentration.

Results showed that all patterns were quite similar with proteins distributed between pH 3.5–4.3. So the majority of the proteins are acidic proteins.

Discussion

Trypsin inhibitors of roots of sweet potato *Tainong* No. 65 may have many molecular species with similar physical and chemical properties such as molecular weights (Fig. 1-Fig. 3); pI (results of isoelectric focusing). Although DEAE-cellulose and CM-cellulose column chromatographies can separate samples into 3–4

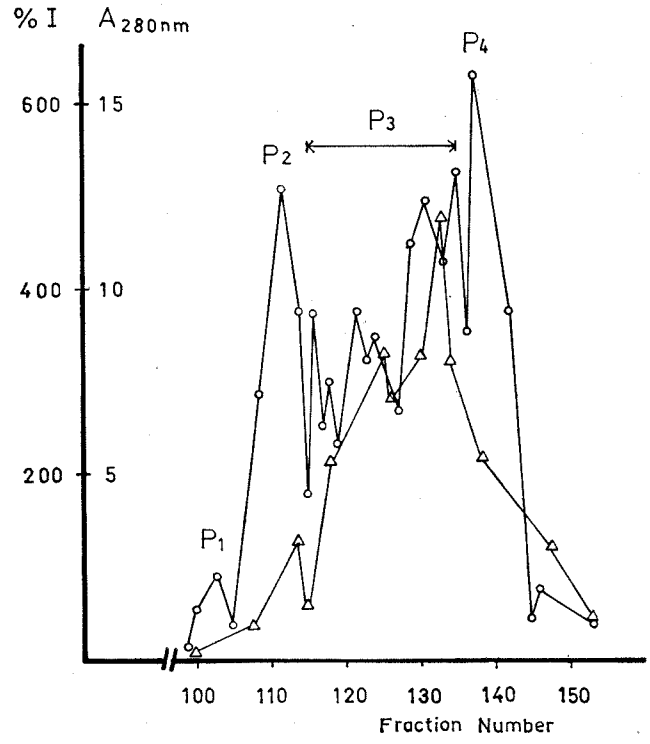

Fig. 5. Elution profile of CM-cellulose column chromatography. The bed size was 2.5×20 cm. Six-ml concentrated DEAE-peak fractions was applied to the column and eluted with a linear gradient of NaOAc-HOAc buffer from 10 mM (pH 4.0, 300 ml) to 30 mM (pH 5.5, 300 ml). The flow rate was 30 ml/hr and every 5 ml was collected as a fraction. ○, inhibition activity; △, A_{280nm}.

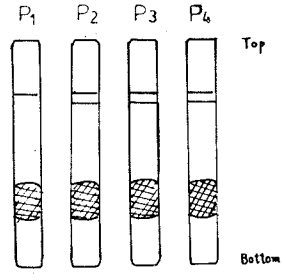

Fig. 6. Disc gel electrophoresis of peak fractions P_1, P_2, P_3, and P_4 from CM-cellulose column chromatography. PAGE system of pH 8.3 with 8 M urea (see METHODS) was used. A current of 3 mA per tube was applied for 3 hrs. P_1, fraction No. 98-104 of CM-cellulose column chromatography; P_2, fraction No. 106-114; P_3, fraction No. 116-136; P_4, fraction No. 138-150. Cathode, top; anode, bottom.

peaks, however results of PAGE showed that protein components of these peaks overlapped to some extent. Although we don't know the exact reason, the results are reproducible. It is quite striking that our results were quite different from those of Sugiura *et al.* (1973) using roots from different sweet potato varieties (Tainong No. 65 vs. Okinawa Kokei No. 14). They got 3 well separated molecular species. So far we have obtained data which showed that trypsin inhibitor activities of various sweet potato varieties changed with seasons, however we don't know yet whether relative amounts of different molecular species of trypsin inhibitor of the same variety also change with seasons.

We have tried to prepare trypsin covalently bound to Sepharose 4B and used this as affinity column to separate trypsin inhibitors of roots of Tainong No. 65. However trypsin inhipitors failed to bind to the column. We keep trying.

Acknowledgement

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Addendum

A batch of Sepharose 4B covalently bound to trypsin was kindly provided recently by Prof. T. H. Hseu, Institute of Molecular Biology, National Tsing Hua University. Sample of 30-40% A. S. saturation fraction was purified further by this affinity column. The purified trypsin inhibitor fraction was subjected to PAGE and was shown to contain 3 protein bands.

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甘藷胰蛋白酶抑制劑之純化和性質

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臺農65號甘藷塊根以二次蒸餾水淬取，硫酸銨沈澱分劃，膠體滲透，DEAE-cellulose 柱體層次分析，CM-cellulose 柱體層次分析共五個步驟可得胰蛋白酶抑制劑約20倍之純化。以膠體電泳分析純化後的試樣顯示未能得到單一蛋白質。臺農65號甘藷塊根可能含有 3-4 種胰蛋白酶抑制劑。它們之間的理化性質很接近（有部份實驗數據支持這一看法），故不易以傳統的蛋白質分離技術分離純化。