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SOME FACTORS AFFECTING LEVELS OF TRYPSIN INHIBITOR ACTIVITY OF SWEET POTATO (*IPOMOEA BATATAS* LAM.) ROOTS[1,2]

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Abstract

Trypsin inhibitor activity (TIA) of water extract of roots of 15 breeding lines or varieties of sweet potato (*Ipomoea batatas* Lam.) cultivated with random block design under similar conditions were determined. Before TIA test every water extract was treated in 2 ways, namely: with heating but without dialysis, without heating or dialysis. TIA was expressed as either % inhibition or specific % inhibition (% inhibition /mg soluble protein). Analysis of variance of TIA showed that varieties, heating, planting time, and blocks were very significant ($P<0.01$) sources of variance when TIA was expressed either as % inhibition or as specific % inhibition.

TIA level decreased from the stem end toward the distal end of T-57 roots. Variance analysis showed that individual plant, but not weight of the root, was a very significant ($P<0.01$) source of variance when TIA was expressed as % inhibition (data from unheated samples of T-65). TIA of T-65 root strips stored at 7°C for 2 years remained 52% of that obtained with fresh samples but the aged samples were more sensitive to heating than fresh ones. After being heated at 70°C for 10 min, TI of 3 other varieties (New 59-145, New-31, and Tainan-15) retained 72 to 86% of the original activity, which was comparable with samples of T-65. But after being heated at 100°C for 15 min, TI of the 3 varieties lost activity completely, while that of T-65 retained 51% activity after being heated at 100°C for 15 min.

Key words: Trypsin inhibitor activity; longitudinal distribution; heat stability; variance analysis; storage; sweet potato roots.

Introduction

Natural inhibitors for proteolytic enzymes occur in microorganisms, plants,

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[3] Abbreviations used: T-, Tainong-; TI, trypsin inhibitor; TIA, trypsin inhibitor activity.

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. Three different trypsin inhibitors were found in the sweet potato variety *Ipomoea batatas* Lam. *var. edulis* Makino (Kokei No. 14). The purification and some chemical and inhibitory properties of 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 difference 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). Differences in trypsin inhibitors of sweet potato roots of 4 American cultivars have also been reported (Dickey and Collins, 1984).

In this report, differences in trspsin inhibitor activity (TIA3) among sweet potato varieties were further examined. The effect of planting time and repeated blocks on the levels of TIA, the distribution of TIA in the roots, levels of TIA of roots with various weights from different individual plants, the effect of long term storage on the levels of TIA of sweet potato strips, and the effect of varietal difference on heat stability on TIA were investigated.

Materials and Methods

Materials

For variance analysis, sweet potato roots of 15 varieties or breeding lines: 65-HP-1, 65-HP-5, 64-HP-3, 64-HP-4, 64-HP-7, 65-HP-8, 64-HP-9, 65-HP-11, 64-HP-44, 64-HP-18, 64-HP-22, 64-HP-27, 65-HP-27, 64-HP-87, and T-57 were kindly provided by Chia-yi Agricultural Experimentation Station. Randomized block design with 4 repeated blocks was used for cultivation. The first batch was planted in the middle of August, 1977 and harvested in the middle of January, 1978. The second batch was planted in the middle of September, 1977 and harvested in the middle of February, 1978. Forty kg of $(NH_4)_2SO_4$, 15 kg of $CaHPO_4$ or $Ca(H_2PO_4)$, and 40 kg of KCl per 0.1 hectare were applied before planting. Fresh roots were cut into strips immediately after being delivered to the laboratory. Cutting process

could be carried out in the air or under the water which did not make any differences to TIA of the extract. Strips were dried at 40-50°C and then frozen at −20°C for later use.

For other experiments, roots of varieties T-65, 64, 62 and 57 were kindly provided by Chia-yi Agricultural Experimentation Station. Fresh samples without drying process were used for TIA assays.

Trypsin (10,000-13,000 BAEE units per mg), chymotrypsin (Type VII, TLCK treated), Na_2HPO_4, and NaH_2PO_4 were products of Sigma Co., USA. Trichloroacetic acid and HCl were purchased from E. Merck, West Germany. Casein and Folin phenol reagents were products of Wako Co., Japan.

Extraction Method

Eight grams of sweet potato strips (fresh or stored) were cut into small stripes with a knife. This procedure could be carried out in the air or under the water. Small pieces of sweet potato roots were then homogenized with 5 volumes (v/w) of double distilled water (DDW) in a Polytron homogenizer at 4°C. The homogenate was filtered through 4 layers of cheesecloth and then centrifuged in a Sorvall SS-34 rotor (9,770×g) for 15 min. The supernatant was used for TIA assays with or without dialysis, and with or without heating. Dialysis was done twice against 100 volumes of DDW. Heating was done at 70°C for 10 min or at 100°C for 15 min.

Trypsin Activity Assay with Casein as Substrate

This was done mainly according to procedure reported by Kunitz (1945, 1946). Standard assays were run by adding 0.5 ml 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 after standing for 1 h or longer at about 25°C were removed by centrifugation. The concentration of hydrolysate in the supernatant 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 carried out as standard assays.

The same process was used for chymotrypsin assays.

Determination of Water Soluble Protein

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

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/mg water soluble protein. Suitable dilution of samples was made to give % inhibition around 50. Percent inhibition and specific % inhibition were converted to μg trypsin inhibited per 0.3 ml extract and μg trypsin inhibited per mg protein, respectively, by multipling a coversion factor of 20.

Results

Inhibitory Activity of Crude Extract of Sweet Potato Roots to Trypsin

This is shown in Table 1 (Using T-57 roots). The inhibition was specific to trypsin. There was very slight inhibition to chymotrypsin. Roots of T-65, 64, and 62 gave similar results.

Table 1. *Inhibitory activity of crude extract of T-57 roots to trypsin*

Roots of sweet potato T-57 from 4 blocks were used separately for inhibitory activity tests. Each test consist of standard, control, and sample assays with triplicate determinations. So each figure shown is the mean and standard deviation of 12 determinations. Dilution factor of dialysis (defined as: volume before dialysis/volume after dialysis)=0.787±0.015 (n=3).

Protease	D		ND		D/ND	
	A	B	A	B	A	B
	Mean±S.D.					
Trypsin	68.0±15.6	36.8±2.0	109.4±26.4	34.6±3.2	0.62	1.06
Chymotrypsin	4.2± 1.4	2.4±1.2	4.0±0.9	1.3±0.6	1.05	1.85

1. D, dialyzed samples; ND, non-dialyzed samples; A, μg trypsin inhibited per 0.3 ml crude extract; B, μg trypsin inhibited per mg protein.

Variance Analysis of Trypsin Inhibitor Activity

Variance analysis of TIA expressed as % inhibition and specific % inhibition are shown in Tables 2 and 3, respectively. Varieties, heating, planting time, and blocks were very significant sources of variance in both cases.

Longitudinal Distribution of Trypsin Inhibitor Activity of T-57 Roots

This is shown in Table 4. TIA of the stem end (or the proximal end) was

Table 2. *Variance analysis of trypsin inhibitor activity expressed as percent inhibition*

Two batches of sweet potato roots were harvested in the middle of January, 1978 and in the middle of February, 1978 respectively. Growth period of both crops was 5 months.

Source of variance	Degree of freedom	Sum of square	Mean square	F-value
Variety (V)	14	223,974.91	15,998.21	1,083.89**
Heating (H)	1	6,876.82	6,876.82	465.91**
Planting time (T)	1	18,258.16	18,258.16	1,237.00**
Block (B)	3	4,979.35	1,659.78	112.45**
V×H	14	608.66	43.48	2.95**
V×T	14	15,449.61	1,103.54	74.77**
V×B	42	43,208.39	1,028.77	69.70**
H×T	1	0.04	0.04	0.03
H×B	3	464.61	154.87	10.49**
T×B	3	9,848.55	3,282.85	222.42**
V×H×T	14	592.44	42.31	2.87**
V×T×B	42	34,569.13	823.07	55.76**
H×T×B	3	112.59	37.53	2.54
V×H×B	42	2,956.98	70.40	4.77**
Error	282	4,162.95	14.76	
Total	479	366,063.12		

** significant at 1% level.

higher than that of the middle or the distal end no matter dialyzed or non-dialyzed samples were used. There seems to be a gradient of TIA levels from the stem end toward the distal end.

Comparison of Trypsin Inhibitor Activity of Sweet Potato Roots with Different Weights

This is shown in Table 5. Roots of two individual plants of T-65 were used. Roots with fresh weight over 200 grams and below 200 grams were assigned arbitrarily as "big" and "small", respectively. Analysis of variance using data of Table 5 was done. When data of unheated samples were used, individual plant but not weight of root was a very significant source of variance ($P<0.01$) as far as TIA (% inhibition, but not specific % inhibition) was concerned (Table 6). However, when data of heated (100°C, 15 min) samples were used, neither individual plant nor weight of root was a significant source of variance no matter TIA was expressed as % inhibition or specific % inhibition.

Effect of Storage on Trypsin Inhibitor Activity of Sweet Potato Strips of T-65 Roots

This is shown in Table 7. It is quite amazing that about half of the original

Table 3. *Variance analysis of trypsin inhibitor activity expressed as specific percent inhibition*

Two batches of sweet potato roots were harvested in the middle of January, 1978 and in the middle of February, 1978 respectively. Growth period of both crops was 5 months. Specific % inhibition was % inhibition per mg soluble protein.

Source of variance	Degree of freedom	Sum of square	Mean square	F-value
Variety (V)	14	383,285.69	27,377.55	404.05**
Heating (H)	1	16,856.98	16,856.98	248.78**
Planting time (T)	1	77,418.98	77,418.98	1,142.58**
Block (B)	3	117,945.61	39,315.20	580.23**
V×H	14	9,437.06	674.08	9.95**
V×T	14	194,747.25	13,910.52	205.30**
V×B	42	514,821.75	12,257.66	180.90**
H×T	1	8.52	8.52	0.13
H×B	3	1,711.66	570.55	8.42**
T×B	3	2,114.33	704.87	10.40**
V×H×T	14	905.41	64.67	0.95
V×H×B	42	16,993.34	404.60	5.97**
V×T×B	42	321,804.25	7,662.01	113.08**
H×T×B	3	698.18	232.73	3.43
Error	282	19,107.77	67.76	
Total	479	1,677,856.70		

** significant at 1% level.

Table 4. *Longitudinal distribution of trypsin inhibitor aciivity of T-57 roots*

Each set of experiment (I or II) consisted of standard, control, and sample assays with triplicate determinations for each assay.

Part	D		ND		D/ND	
	A	B	A	B	A	B
	Mean±S.D.					
The stem end						
I	72.0±1.6	35.6±0.8	122.8±0.8	33.6±0.2	0.59	1.06
II	70.8±1.0	35.0±0.6	122.6±0.6	33.6±0.2	0.58	1.06
The middle						
I	64.4±0.8	36.8±0.4	94.4±0.6	29.8±0.2	0.68	1.23
II	65.8±0.6	37.4±0.4	91.4±0.6	28.8±0.2	0.72	1.30
The distal end						
I	63.8±0.5	42.2±1.6	80.2±0.4	29.2±0.2	0.80	1.45
II	65.2±0.6	43.2±0.8	84.4±0.4	30.8±0.2	0.77	1.40

1. D, ND, A, and B are the same as Table 1.

Table 5. *Comparison of trypsin inhibitor activity of sweet potato roots with different weights*

Ten times diluted, non-dialyzed crude extract of T-65 roots was used. Roots with fresh weight over 200 grams were arbitrarily designated as "big" and those below 200 grams as "small".

Plant	Weight of Root (g)	H				NH			
		A		B		A		B	
I	669	328	334	88	90	650	644	174	173
	513	322	341	76	80	656	662	154	156
	338	297	285	89	86	570	551	172	166
	90	341	328	87	84	601	607	153	155
	67	377	358	106	101	585	566	164	159
	63	327	333	80	80	685	697	165	168
II	603	378	372	101	100	598	591	160	158
	451	199	193	78	75	393	368	154	144
	189	360	354	106	104	566	530	167	156
	102	162	168	57	55	312	349	105	118

H, crude extract was heated at 100°C for 15 min; NH, unheated samples; A, and B are the same as Table 1.

Table 6. *Variance analysis of trypsin inhibitor activity expressed as % inhibition*

Data of samples without heating or dialysis were used (Table 5).

Soruce of variance	Degree of freedom	Sum of square	Mean square	F-value
Replication (R)	1	12,432.25	12,432.25	1.93
Plant (P)	1	192,282.25	192,282.25	29.81**
Size of root (S)	1	7,921.00	7,921.00	1.23
P×S	1	56.25	56.25	0.009
P×R	1	5,329.00	5,329.00	0.83
S×R	1	756.25	756.25	0.12
P×S×R	1	121.00	121.00	0.019
Error	8	51,604.00	6,450.50	
Total	15	270,502.00	18,033.47	

** significant at 1% level.

TIA remained after strips had been stored at 7°C for 2 years. In average, heating at 70°C for 10 min and 100°C for 15 min reduced TIA to 69% and 29% of untreated samples, respectively. This suggests that TIA of stored strips was more sensitive

to heat treatments than fresh ones (88% versus 69%, 51% versus 29%). But the stability of TIA of T-65 roots toward heat treatment is quite unique if we compare with data obtained using samples of other varieties or breeding lines under the same heat treatments (Table 8). Table 8 shows that TIA of root samples of New 31, Tainan 15, and New 59-145 were as stable as that of T-65 to heating at 70°C for 10 min. However heating at 100°C for 15 min destroyed almost completely TIA of crude extract of New 31, Tainan 15, and New 59-145, a sharp contrast to that of T-65.

Discussion

Crude extracts of roots of T-57, 65, 64, and 62 exhibited high inhibitory activity toward trypsin while negligible inhibitory activity toward chymotrypsin (Table 1).

Using 53 sweet potato varieties, Lin and Chen (1980) have shown that dialysis and varieties are significant ($P<0.05$) source of variation of TIA expressed as % inhibition while dialysis, heating, and varieties are very significant ($P<0.01$) source of variation of TIA expressed as specific % inhibition. Dickey (1983) also

Table 7. *Trypsin inhibitor activity of sweet potato root strips stored at 7°C for 2 years*

Stored strips (500 g) of T-65 were ground and extracted with 2,500 ml DDW. For experiment I, 2,104 ml extract was obtained. Supernatant of 1,950 ml was obtained after centrifugation at 9,770× g for 15 min. For experiment II, 2,145 ml and 1,965 ml were obtained for extract and supernatant, respectively. Ten times diluted supernatant solution without dialysis was used for inhibition assays. For comparison, data of fresh samples were listed in experiment III.

Exp.	Treatment	A	B	RIA
		Mean±S.D. (n=4)		
	no heating	39.8±1.0	20.4±0.6	1.00
I	70°C, 10 min	25.8±1.2	13.2±0.6	0.65
	100°C, 15 min	10.8±1.6	5.6±0.8	0.27
		Mean±S.D. (n=3)		
	no heating	45.8±0.4	25.2±0.2	1.00
II	70°C, 10 min	32.8±0.4	18.0±0.2	0.72
	100°C, 15 min	14.2±0.8	7.8±0.4	0,31
		Mean±S.D. (n=3)		
	no heating	82.3±1.4	41.7±0.7	1.00
III	70°C, 10 min	72.4±1.0	36.7±0.5	0.88
	100°C, 15 min	42.0±0.6	21.3±0.3	0.51

1. A and B are the same as Table 1. RIA, residual inhibitory activity = TIA of heated sample/TIA of unheated sample.

Table 8. *Effect of heat treatments on trypsin inhibitor activity of three sweet potato varieties*

Variety	Treatment	D[1]			ND		
		A	B	RIA[2]	A	B	RIA
		Mean±S.D. (n=6)			Mean±S.D. (n=4)		
New 59-145	without heating	17.4±0.2	17.2±0.6	1.00	34.6±0.6	15.6±0.2	1.00
	70°C, 10 min	17.0±0.6	17.2±0.6	0.98	24.8±0.2	11.2±0.1	0.72
	100°C, 15 min	0.4±0.01	0.4±0,01	0.02	2.8±0.04	1.20±.02	0.08
New 31	without heating	2.6±0.01	11.4±0.04	1.00	1.0±0.003	1.0±0.003	1.00
	70°C, 10 min	1.8±0.0	7.8±0,08	0.69	−(1.2±0.006)	−(1.2±0.006)	—
	100°C, 15 min	0.3±0.003	1.4±0.02	0.12	−(4.8±0.06)	−(4.8±0.06)	—
Tainan 15	without heating	14.2±0.4	18.8±0.6	1.00	28.6±0.2	18.4±0.12	1.00
	70°C, 10 min	15.2±0.4	20.2±0.6	1.07	24.6±0.2	15.8±0.12	0.86
	100°C, 15 min	0.2±0.002	0.2±0.002	0.01	−(4.2±0.02)	−(2.6±0.01)	—

[1] D, ND, A, and B are the same as Table 1.
[2] RIA, residual inhibitory activity=TIA of heated sample/TIA of unheated sample.

found significant variability in TIA levels in 10 American high protein selections. In this report, using 15 breeding lines or varieties, we found that varieties, heating, time of planting (month), and blocks are very significant sources of variation of TIA expressed as % inhibiton or specific % inhibition (Tables 2 and 3).

Since dilution factor of dialysis 0.787 is larger than 0.62 (ratio D/ND in Table 1), dialysis must remove some small TIs together with other small molecules including small peptides. Dilution and removal of small TIs could explain why % inhibition drops to 0.62 of the original value while specific % inhibition remains the same (Table 1). Table 4 shows gradient distribution of TIA from the stem end toward the distal end. In both the middle and the distal end most dialyzable components are probably small non-TI peptides. Therefore % inhibition of dialyzed samples did not drop to the same extent as the stem end and specific % inhibition became larger than 1.00.

Both thermolabile TI and thermostable TI have been reported (Sohonie and Bhandarkar, 1954; Sugiura *et al.*, 1973). Stability of TIA toward heating may be studied by heating aqueous samples at 70°C for 10 min or 100°C for 15 min. Thus, TIA of roots of New 59-145, New 31, and Tainan-15 retained more than 69% of the value of unheated samples after being heated at 70°C for 10 min which was comparable with T-65. However, heating at 100°C for 15 min completely destroyed TIA of root samples of the 3 varieties while destroyed only half TIA of T-65 (Tables 7 and 8). Residual inhibitor activity reflects heat stability of TIA of a particular variety.

Time of planting (month) and block were newly found factors which affect TIA levels (Table 3). The former indicates that climatic factors such as rainfall, temperature *etc.* may have tremendous effects on TIA levels of sweet potato roots. The latter suggests that soil environments of the plant may also affect TIA levels. Both factors may function via differential controls over metabolism of TI molecules and non-TI molecules especially proteins and small polypeptides. Dickey and Collins (1984) investigated the longitudinal distribution of TIA in 4 American sweet potato varieties (the high TI "Centennial" and "Pope" and the low TI "Jewel" and "Caromex"). TIA of the stem end was the highest in all cultivars. TIA of the distal end was the lowest in case of Centennial and Jewel. In Pope, TIA of the distal end was slight higher than the middle. In Caromex, TIA of the distal end was the same as the stem end and was higher than the middle. Table 4 shows that the order of TIA levels in roots of T-57 was the stem end>the middle>the distal end, which was in agreement with that of Centennial and Jewel. The concentration of soluble protein decreased from the stem end to the distal end. However, the stem end of T-57 roots had not only the highest TIA but also the highest specific TIA. It suggests that both concentration of TIs and concentration ratio of TI polypeptides/non-TI polypeptides decrease from the stem end toward the distal end (data of samples without dialysis). Data of dialyzed samples show that from the stem end toward the distal end relatively more and more non-TI small polypeptides were moved out of dialysis bag leaving samples with enriched TI polypeptides in a relative sense. So higher specific TIA were observed at the distal end.

Tables 5 and 6 show that individual plant but not size of the roots is a very significant source of variation ($P<0.01$).

Comparison of results of Tables 7 and 8 indicate the unusual stability of TIs of T-65 roots both to long-time storage and heat treatments. This property deserves further investigations.

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甘藷塊根之胰蛋白酶抑制活性的若干決定性因子

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以逢機區集設計在相同栽培條件下收穫之15種甘藷育種品系或品種的塊根爲材料，研究其水抽液的胰蛋白酶抑制活性（以下簡稱抑制活性）。水抽液之預先處理有兩種方式：經加熱處理但不經透析；和不經加熱處理也不經透析。抑制活性也有兩種表示方式：一種是百分抑制活性；另一種爲比百分抑制活性（即百分抑制活性除以樣品液中可溶性蛋白質之毫克數）。不論抑制活性是以那種方式表示，變方分析顯示品種、加熱處理、栽種時間和栽種區域都是極顯著（$P<0.01$）之變異來源。

臺農57塊根之抑制活性的縱向分佈乃從接近蔓這端經中間部位，然後向較遠端遞減。當抑制活性以百分抑制表示時，植株個體是極顯著之變異來源，但塊根大小却不是（以臺農65號塊根之未加熱水抽液爲材料）。臺農65號甘藷簽在攝氏7度儲藏兩年後，殘留抑制活性對熱處理比新鮮材料較敏感；但是殘留抑制活性高達原來之52%，相當特別。另外三種品種（新 59-145，新 31，和臺南15號）之塊根粗抽液經攝氏70度加熱10分鐘後保有原來的72至86%的抑制活性，這一點可以和臺農65號相提併論。但是經攝氏 100 度加熱15分鐘後，前三品種材料之抑制活性幾乎全消失而臺農65號材料竟仍殘留一半的抑制活性。