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LEVEL AND HEAT STABILITY OF TRYPSIN INHIBITOR ACTIVITY AMONG SWEET POTATO (*IPOMOEA BATATAS* L.) VARIETIES[1,2]

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Abstract

Trypsin inhibitor activity (TIA) of water extract of roots of 53 sweet potato (*Ipomoea batatas* L.) varieties cultivated under similar conditions were determined. Before TIA test every water extract was treated in 4 ways, namely: with dialysis and heating, with dialysis but without heating, without dialysis but with heating, without dialysis or heating. TIA was expressed as either % inhibition or specific % inhibition (% inhibition/mg soluble protein). Stability of TIA to heat treatment (70°C, 10 min) varies among cultivars. Significant differences of TIA among cultivars were observed. As high as 99% inhibition (HP-18) and as low as 20% inhibition (Tainong-48) could be observed with other values in between. Analysis of variance of TIA expressed as % inhibition shows that cultivars and dialysis are significant ($P < 0.05$) sources of variance. Cultivars, dialysis, and heating are highly significant ($P < 0.01$) sources of variance when TIA was expressed as specific % inhibition. When water extract without either dialysis or heating was used, a significant positive correlation ($P < 0.05$) between TIA (% inhibition) and amount of crude total protein (calculated from total nitrogen) was found. A very significant positive correlation ($P < 0.01$) was found between TIA (% inhibition) and amount of water soluble protein.

Introduction

Natural inhibitors for proteolytic enzymes occur in plants and animals. The first known plant inhibitor was that from soybean, which was crystallized in 1946 by Kunitz. Other 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 in 1954 by Sohonie and Bhandarkar. Their preparation was very thermolabile, although it was not completely pure. Isolation of trypsin inhibitor from sweet potatoes

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has been patented (Sugiura and Takeuchi, 1972). Three different trypsin inhibitors were found in a sweet potato. 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 2–11 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). In order to utilize the trypsin inhibitors or to determine whether the trypsin inhibitor activity in sweet potato chips, as feed for swine in Taiwan, may cause adverse effect on pork production, detailed and systematic studies on the assay conditions of trypsin inhibitor activity and factors which may affect the activity level and the heat stability of the trypsin inhibitors are urgently needed. This report is the first one of the series to provide such information.

Materials and Methods

Materials

Sweet potato (*Ipomoea batatas* L.) varieties were planted under similar conditions in sandy loam at Chia-Yi Agricultural Experiment Station (Chia-Yi AES) in September 1976 and roots were harvested in February 1977. Forty kg of $(NH_4)_2SO_4$, 15 kg of $CaHPO_4$ or $Ca(H_2PO_4)_2$, and 40 kg of KCl per 0.1 hectare were applied before planting. Trypsin (10,000–13,000 BAEE units per mg) was purchased from Sigma Co. (U.S.A.). Casein was a product of Wako Co. (Japan).

Preparation of sweet potato chips

Fresh roots of different sweet potato varieties were cut into small stripes with a knife and then dehydrated at about 45°C in an oven overnight. Dried chips were used for trypsin inhibitor activity (TIA) assays.

Extraction method

Eight grams of sweet potato chips were first cut into small pieces with a knife and then homogenized with 5 volumes (v/w) of double distilled water (DDW) in a Polytron homogenizer (Switzerland) at 4°C. The homogenate was filtered through 4 layers of cheesecloth and then centrifuged at 9,000 rpm using a Sorvall SS-34 rotor (9,770 g) for 15 min. The supernatant fluid was divided into two parts. One part was used directly for trypsin inhibitor activity assays without dialysis. The other part of the homogenate was dialyz-

ed twice against 100 volumes of distilled water for at least 4 hr before TIA assays.

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 DDW and 1.0 ml trypsin solution (containing 20 μg trypsin in 0.25 mM HCl) to tubes containing 1.0 ml of 1% 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 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 crude extract and 0.2 ml DDW with 1.0 ml of 1% 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 crude extract and 0.2 ml DDW with 1.0 ml of 1% activated casein solution at 37°C for 15 min. Then 1.0 ml trypsin solution was added and proteolytic reaction was proceeded as standard assays.

Determination of total nitrogen

This was done according to Horwity (1960). Crude total protein was calculated by multipling amounts of total nitrogen by 5.95.

Determination of water soluble protein

Protein determinations were made 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 $((A_{280}$ of standard + A_{280} of control) − A_{280} of sample)/(A_{280} of standard) × 100%, and the specific % inhibition was defined as % inhibition per mg water soluble protein.

Statistical analysis

Analysis of variance, and linear regression, least significant difference were done according to general statistical textbooks.

Results

The trypsin inhibitor activity of root extract of 53 sweet potato cultivars

Table 1 shows the TIA of 53 sweet potato cultivars. Crude extract of

Table 1. *Trypsin inhibitor activity of 53 sweet potato cultivars*[1,2]

Treatments / inhibitor activity / Varietal name	Dialysis				Without Dialysis			
	Heating[3]		Without Heating		Heating[3]		Without Heating	
	% inhibition	% inhibition/mg protein[4]	% inhibition	% inhibition/mg protein[4]	% inhibition	% inhibition/mg protein[4]	% inhibition	% inhibition/mg protein[4]
C57-6	70	121	71	123	70	84	80	96
Tainong-9	82	82	89	88	88	72	98	80
Tainong-16	47	46	61	60	65	56	76	66
HP-18	93	99	96	102	88	59	99	68
Tainong-59	43	41	52	50	44	57	51	66
Tainong-22	57	69	94	113	61	56	70	65
Tainong-10	60	43	61	44	63	51	72	59
Tainong-3	39	27	51	36	59	37	63	40
Tainong-41	60	59	64	63	62	37	75	45
Tainong-31	38	40	48	51	48	36	56	42
Tainong-23	68	38	73	41	72	34	82	38
Tainong-53	34	36	50	53	41	33	55	45
Tainong-20	12	11	77	67	55	32	70	40
Hon-Shin-Wey	4	6	16	23	25	32	33	42
Tainong-26	13	26	41	83	23	30	45	59
Tainong-46	44	59	57	76	36	28	48	37
Tainong-1	32	35	43	47	45	27	62	37
Tainong-40	47	146	61	188	31	25	62	50
Tainong-45	28	45	33	55	25	22	29	29
Tainong-42	30	30	40	40	36	21	49	29
Tainong-47	14	19	22	31	24	18	32	33
Tainong-43	16	27	19	32	22	19	46	40
Tainong-58	30	44	48	69	19	17	29	27
HP-4	35	111	59	193	18	17	29	27
Tainong-27	11	14	35	44	16	13	29	24
Tainong-34	21	34	30	47	8	8	29	30
Tainong-48	11	34	29	87	7	5	20	15
Chang-Hua	84	139	87	145	89	112	90	114
Tainong-50	67	99	82	120	75	68	81	73
C55-189	85	85	86	87	95	62	92	60
Tainong-61	88	100	91	104	91	61	93	62
Tainong-52	46	41	36	32	49	54	46	50
Tainong-25	64	48	52	39	80	53	78	52
Tainong-39	59	61	70	73	77	51	85	56
Porto Rico	95	66	95	66	92	45	95	46
Tainong-63	103	55	110	59	83	44	83	44
Tainong-19	60	47	61	48	80	43	82	44

Tainong-30	20	65	31	98	31	41	34	45
Okinawa-100	41	39	51	50	57	40	60	42
Tainong-44	42	46	47	51	56	37	58	38
Tainong-60	33	32	42	40	56	33	55	32
Tainong-56	55	49	57	52	54	32	57	34
Tainong-17	30	18	53	32	60	30	63	32
Tainong-57	82	75	85	77	95	65	95	62
Tainong-51	28	31	31	36	52	42	55	43
Centenial	87	82	89	84	93	70	96	71
Tainong-55	80	111	82	115	86	70	92	74
Tainong-24	50	51	32	33	80	50	64	40
Tainong-37	56	114	20	40	59	71	37	45
Tainang-15	29	35	13	16	56	41	46	33
Tainong-49	44	37	52	43	56	34	44	27
Tainong-62	31	63	−4	−8	42	37	21	19
Tainong-54	28	59	−41	−86	98	61	62	37
Least Significant difference								
LSD 5%	11	25	8	18	8	8	8	8
LSD 1%	15	34	11	25	11	10	11	11

(1) 0.3 ml of crude extract corresponding to 99.5 ± 13.4 mg (one standard deviation) chip was used without dilution for TIA tests.
(2) Relative error of 10% trichloroacetic acid precipitation method was about 10%. Each figure was the average of duplicate trials.
(3) Crude extract was heated at 70°C for 10 min before TIA tests.
(4) Water soluble protein determined with Lowry's method.

each varieties was treated differently as shown before TIA tests. The data of crude extract without dialysis are more suitable for reference when feeding experiments are to be considered. While it is better to use the data of crude extract with dialysis when purification and nature of trypsin inhibitor are the major concern.

Stability of TIA to heat treatment (70°C, 10 min) varies among cultivars. Since relative error of trichloroacetic acid precipitation method is about 10%, we may use this value as a basis for judging whether TIA of crude extract of a particular sweet potato cultivar changes significantly after heating. Based on data obtained with undialyzed crude extract and average value ± 0.1 average value as judging range, we may roughly divide sweet potato cultivars into three classes according to stability of TIA (both % inhibition and specific % inhibition) of their root extract. This is also shown in Table 1. Class I consists of 27 cultivars from C57-6 on top to Tainong-48 in the middle of Table 1. Class II consists of 20 cultivars from Chang-Hua to Tainong-55. Class III consists the remaining 6 cultivars: Tainong-24, 37, 49, 62, 54 and

Tainang-15. TIA of root extract of class I cultivars falls outside lower limit of judging range, namely average value—0.1 average value, after heating. TIA of root extract of class II cultivars keeps within judging range, namely average value ± 0.1 average value, after heating. While that of class III cultivars falls outside upper limit of judging range, namely average value +0.1 average value.

For example, % inhibition of samples of Tainong-40 without heating is 62, while after heating % inhibition is 31 which is much smaller than 62–6.2 = 55.8, so this cultivar was assigned as class I. For Chang-Hua cultivar, % inhibition after heating is 89 which keeps well within 90 ± 9, so it was assigned as class II. Per cent inhibition after heating of Tainong-24 is 80 which is larger than 64 + 6.4 = 70.4, therefore this cultivar was assigned as class III. It should be mentioned that although Tainong-9, HP-18, and Tainong-45 were assigned as class I, their % inhibition after heating and lower limit of judging range were 88, 88.2; 88, 89.1; 25, 26.1; respectively. So these 3 cultivars are on the borderline between class I and II.

It is clear from Table 1 that significant differences of TIA exist between cultivars. As high as 99% inhibition (HP-18) and as low as 20% inhibition (Tainong-48) could be observed with other values in between. As we will discuss later, the actual TIA range among cultivars might be broader than what we present here if diluted crude extract of each cultivar was used.

Amount of soluble protein and crude total protein of roots of 53 sweet potato cultivars

Table 2 shows various amounts of soluble protein and crude total protein of roots of 53 sweet potato cultivars. These data are useful for calculation of relationship between TIA and protein content which will be presented later.

Variance analysis of TIA expressed as % inhibition

Table 3 shows variance analysis of TIA expressed as % inhibition. From data of Table 3, it is clear that variation of TIA in different cultivars, effect of dialysis on TIA, and interaction between heating and dialysis are significant ($P < 0.05$).

Variance analysis of TIA expressed as specific % inhibition

Table 4 shows variance analysis of TIA expressed as specific % inhibition. The effect of all sources of variance examined on TIA is highly significant ($P < 0.01$).

Table 2. *Amount of soluble protein and crude total protein of roots of 53 sweet potato cultivars*

Amount of protein / Varietal name	Soluble protein (mg)/0.3 ml crude extract		Total crude protein (mg)[1]
	Dialysis	Without dialysis	
C57-6	0.576	0.828	2.529
Tainong-9	1.008	1.224	3.645
Tainong-16	1.008	1.152	2.277
HP-18	0.954	1.476	3.711
Tainong-59	1.044	0.780	4.398
Tainong-22	0.828	1.080	6.384
Tainong-10	1.392	1.224	5.178
Tainong-3	1.425	1.584	3.537
Tainong-41	1.008	1.662	4.158
Tainong-31	0.960	1.332	2.871
Tainong-23	1.812	2.157	4.938
Tainong-53	0.948	1.225	4.530
Tainong-20	1.152	1.728	7.971
Honshinwey	0.684	0.792	2.610
Tainong-26	0.492	0.756	2.976
Tainong-46	0.744	1.272	5.193
Tainong-1	0.912	1.692	5.364
Tainong-40	0.324	1.224	5.211
Tainong-45	0.612	1.152	2.340
Tainong-42	1.008	1.692	4.809
Tainong-47	0.720	1.368	4.449
Tainong-43	0.594	1.152	2.820
Tainong-58	0.696	1.104	5.043
HP-4	0.312	1.080	5.802
Tainong-27	0.792	1.200	4.203
Tainong-34	0.624	0.960	4.326
Tainong-48	0.336	1.464	3.165
Changhua	0.612	0.792	2.727
Tainong-50	0.684	1.098	3.780
C55-189	0.885	1.446	5.055
Tainong-61	0.897	1.509	5.034
Tainong-52	1.128	0.912	2.931
Tainong-25	1.320	1.524	4.356
Tainong-39	0.960	1.512	4.389
Porto Rico	1.440	2.070	7.215
Tainong-63	1.872	1.896	9.612
Tainong-19	1.260	1.872	3.168
Tainong-30	0.312	0.756	3.507

Okinawa-100	1.032	1.440	7.332
Tainong-44	0.912	1.509	3.039
Tainong-60	1.020	1.692	5.694
Tainong-56	1.116	1.674	3.723
Tainong-17	1.680	1.998	6.126
Tainong-57	1.029	1.548	5.169
Tainong-51	0.876	1.260	5.514
Centenial	1.059	1.410	6.525
Tainong-55	0.729	1.320	6.363
Tainong-24	0.972	1.602	3.426
Tainong-37	0.486	0.828	3.495
Tainang-15	0.846	1.368	4.200
Tainong-49	1.200	1.656	4.887
Tainong-62	0.492	1.116	5.340
Tainong-54	0.480	1.704	6.924

(1) Total crude protein (mg) contained in that amount of sweet potato chips giving 0.3 ml crude extract. This was obtained by multipling total nitrogen (mg) by a factor of 5.95.

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

Source of variance	Degree of freedom	Sum of square	Mean square	F-value
Replication	1	727.91	727.91	0.049
Cultivars (C)	52	1,253,402.64	24,103.89	1.63*
Heating (H)	1	27,701.31	27,701.31	1.87
C×H (interaction)	52	796,847.44	15,323.98	1.03
Dialysis (D)	1	93,807.40	93,807.40	6.32*
C×D	52	765,480.52	14,720.77	0.99
H×D	1	59,812.71	59,812.71	4.03*
C×H×D	52	770,932.02	14,825.61	458.42**
Error	211	6,824.17	32.34	
Total	423	3,775,536'19		

* significant ($P < 0.05$)
** Very significant ($P < 0.01$)

The covariation relationship between TIA and protein content

From data of Table 1 and Table 2, we can plot figures showing correlation between TIA (expressed as % inhibition or specific % inhibition) and protein content (as total crude proteins or soluble proteins) under various treatment combinations. These are presented in Fig.1 (A and B).

Table 4. *Variance analysis of trypsin inhibitor activity expressed as specific % inhibition*

Source of variance	Degree of freedom	Sum of square	Mean square	F-value
Replication	1	222.27	222.27	1.28
Cultivars (C)	52	199,687.18	3,840.13	22.16**
Heating (H)	1	4,255.21	4,255.21	24.56**
C×H (interaction)	52	22,066.27	424.35	2.45**
Dialysis (D)	1	21,654.92	21,654.92	124.99**
C×D	52	57,405.18	1,103.94	6.37**
H×D	1	488.03	488.03	2.81**
C×H×D	52	9,008.72	173.24	3.89**
Error	211	9,382.06	44.46	
Total	423	324,169.90		

* significant ($P > 0.05$)
** very significant ($P < 0.01$)

Discussion

We standardize the extraction procedure of sweet potato roots, however the final volume of crude extract of different cultivars varies. For crude extracts of 53 cultivars used in this experiment, 24.11 ml ± 3.24 ml (one standard deviation) is the distribution of final volumes. Although water content and texture of chips of various cultivars might be different, experimental errors might be introduced during extraction procedure or dialysis, we are not sure which one is the major source causing variation in final volume of crude extract. So we did not make correction for volume difference of crude extract when calculating % inhibition shown in Table 1. As specific % inhibition was obtained by dividing % inhibition of 0.3 ml extract by mg of soluble protein in 0.3 ml extract, TIA expressed with this definition automatically correct any volume differences of crude extract of various cultivars. Based on pure logical reasoning we may predict that specific % inhibition is better than % inhibition as a way to express TIA when calculating variance analysis or correlation coefficient. This reasoning is supported by data of Table 3 and 4 and Fig. 1.

All samples used in this experiment was not diluted. This makes the detection of sweet potato cultivars with low TIA easy. However, this also puts a ceiling on those cultivars such as Tainong-9, HP-18, Changhua, C55-189 etc. which show very high TIA. For those cultivars our data might be an under estimate of their TIA. The range of TIA of 53 cultivars tested might be much broader if diluted samples are used.

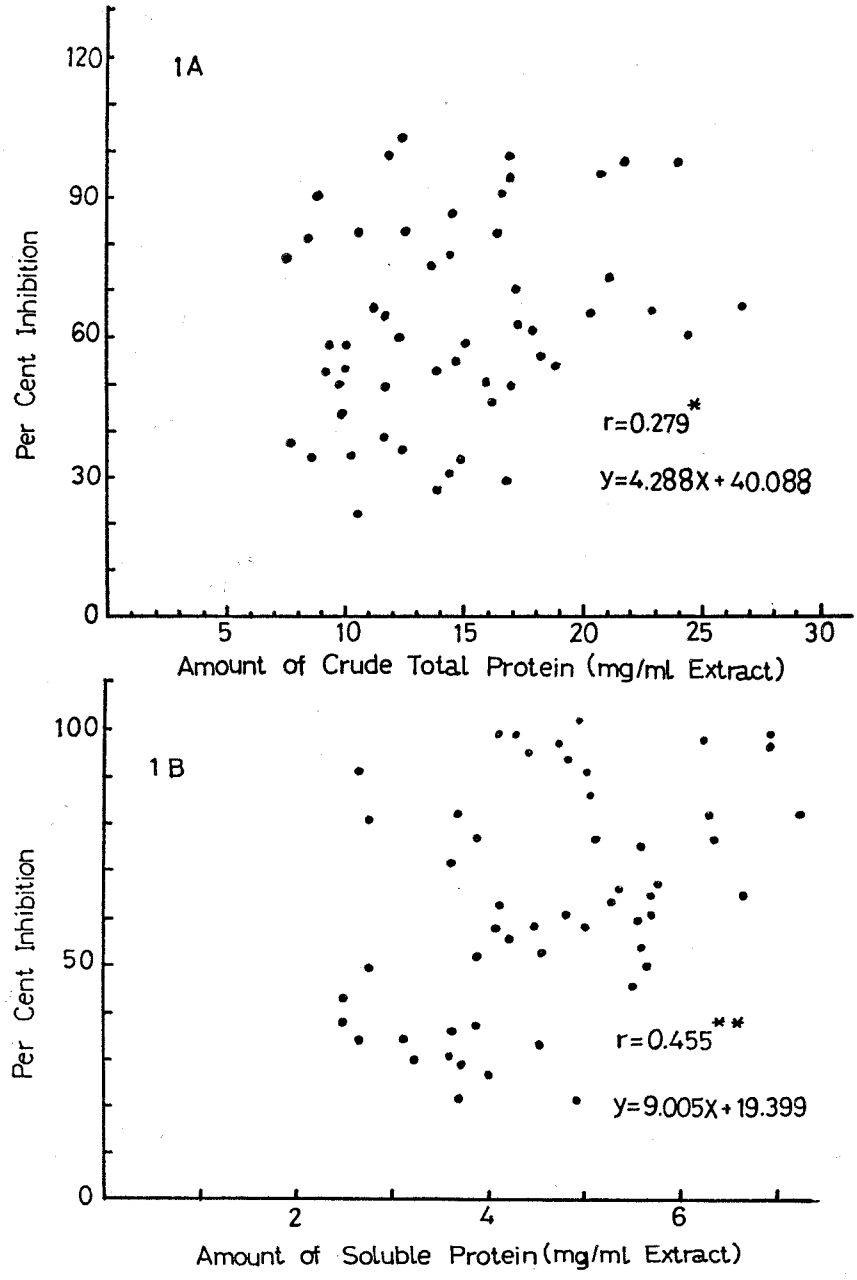

Fig. 1. Correlation between per cent inhibition and protein content

Samples with neither dialysis nor heating were used. Linear regression was used to calculate correlation coefficient (r). Significance test of r was done by checking whether $\sqrt{N-2} \cdot \frac{|r|}{\sqrt{1-r^2}} > t_{N-2}(\alpha)$, where $N = 52$ and α stands for level of significance.

We have mentioned that Tainong-9, HP-18, and Tainong-45 fall on the borderline between class I and class II (Table 1). Heating at a higher temperature, say 100°C, 20 min, should be helpful for clearing this ambiguity.

Increasing of TIA after heating (class III) is quite interesting. Since we have detected trypsin activators in old leaves of some sweet potato cultivars (manuscript in preparation), we can make an possible explanation. Namely, if we assume trypsin activators locate in roots of some sweet potato cultivars, then TIA expressed by crude extract of a particular cultivar will depend on the net equivalents of trypsin inhibitors and activators. Heating samples of class III at 70°C, 10 min destroys mainly the activity of trypsin activators, so net TIA increases. For convenience of discussion, we assigned arbitrarily cultivars with % inhibition below 40, between 40 and 70 and above 70 as low, median and high TIA cultivars respectively. Data of Table 1 may provide various kinds of information. How to use these data depends on interests of researchers. For example, if we are searching for suitable sweet potato cultivars with low TIA, then Tainong-48, 34, 27, 58, 47, 45, 62, Honshinwey, and HP-4, etc, are among the best candidates. These cultivars have low TIA as both % inhibition and specific % inhibition are concerned. If what we need are median TIA cultivars with high soluble proteins, then we should pick up Tainong-3, 20, 1, 42, 41, 44, 60, and 17. For those who are interested in chemical and biochemical studies of trypsin inhibitors of sweet potato cultivars, Tainong-9, 22, 61, 63, HP-18, Changhua, C55-189, Porto Rico, and Centennial are recommended ones.

Using least significant difference listed in Table 1, we may draw a conclusion that TIA varies significantly among cultivars. Since all 53 cultivars we used were planted at the same time under same conditions, the variation among cultivars must have genetic basis. Manipulation of TIA level may be achieved by breeding programs.

The amount of water soluble protein of Tainon-59, Tainon-10 and Tainon-52 increases after dialysis. There is no good explanation except experimental errors.

From Table 3 and Table 4, it is clear that for variance analysis specific % inhibition is more sensitive than % inhibition. Dialysis affects TIA of crude extract significantly (Table 3). This suggests that crude extract of sweet potato roots consists of dialyzable and nondialyzable factors which inhibit proteolytic activity of trypsin. This suggestion is supported by the finding that interaction between heating and dialysis is significant (Table 3).

Data of Table 4 support the conclusion drawn from Table 3. Besides, they also provide additional information. For example, heating as well as dialysis affects TIA of crude extract of sweet potato roots very significantly.

It is understandable that heating causes very significant change of TIA if nondialyzable components of trypsin inhibitors of sweet potato roots are of protein nature. The extent of effect caused by heating varies from cultivar to cultivar (C × H is very significant). This is in agreement with the possible classification of sweet potato into three classes according to heat stability of TIA judged by a different criterion (Table 1).

The experimental conditions of Fig. 1A (samples without either dialysis or heating) is comparable to those of AVRDC'S report. A similar conclusion can be drawn from our results. Namely, there is a significant positive correlation between TIA (% inhibition) and amount of crude total protein calculated from total nitrogen. But the positive correlation we found is not as strong as what they got. More close correlation was found when amount of water soluble protein was used instead of crude total protein (Fig. 1B). For most sweet potato cultivars examined breeders will face a dilemma: when they try to increase the protein content of the roots they increase TIA at the same time. However the situation is not completely hopeless. It is still possible to breed a cultivar with high protein content and low TIA if enough capital, time and patience are invested.

When correlation between specific % inhibition and amount of water soluble protein was tested, values of r were calculated to be 0.242, 0.243, 0.085, and 0.191 for samples with both dialysis and heating; samples with dialysis, without heating; samples without dialysis, with heating; and samples with neither dialysis nor heating respectively. No correlation could be found.

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不同甘藷品種之胰蛋白酶抑制因子——活性的高低及熱穩定度

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本實驗使用在相同栽培條件下生長的53個品種(或品系)的甘藷塊根的水抽液分析其所含的胰蛋白酶抑制因子的活性（TIA)。在分析 TIA 之前，每一水抽液都有四種不同處理，就是：透析之後又經加熱；只透析不加熱；不透析但經加熱；既不經透析又不經加熱。TIA有兩種表示法：抑制百分率（% inhibition）和比抑制百分率(抑制百分率/可溶蛋白質之毫克數）。TIA 對加熱處理（70°C 10分鐘）的穩定性隨品種不同而有顯著的差異。最高的可達99%(如 HP-18) 最低的爲20%（臺農48號）其他的品種則介於兩者之間。TIA 以抑制百分率表示時變方分析（ANOVA）的結果顯示品種和透析是顯著的變異來源（$P < 0.05$)。如果 TIA 以比抑制百分率表示時，則品種、透析、加熱都是極顯著的變異來源。以不經透析也不經加熱的水抽液做實驗，發現 TIA（抑制百分率）和粗蛋白質的含量（從總氮量換算過來）兩者之間有顯著的正相關（$P < 0.05$)；TIA（抑制百分率）和水溶蛋白質的含量兩者之間有極顯著的正相關（$P < 0.01$)。