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TRYPSIN INHIBITOR OF *SOLANUM TUBEROSUM*: EXTRACTION, STABILITY, AND CHANGE OF ACTIVITY DURING STORAGE[1,2]

YAW-HUEI LIN and SHWU-JANE JENG

Institute of Botany, Academia Sinica, Taipei, Taiwan Republic of China

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Abstract

Three solvent systems for extraction of trypsin inhibitor of both potato tubers and chips were used. As far as specific per cent inhibition (per cent inhibition per mg protein) is concerned, extraction with acidic 70% ethanol is the best, followed by extraction with 0.05 M tris-HCl buffer (pH 7.4), and extraction with glass distilled water in this order. Sequence of mixing the reagents affected the extent of inhibition observed, a greater degree of proteolytic inhibition was observed in the order of (substrate-inhibitor+enzyme) than (enzyme-inhibitor+substrate). The trypsin inhibitor activity was quite stable towards heat and extreme low pH. Preincubation at 130°C for 30 min destroyed inhibitor activity completely. The specific per cent inhibition of both potato tuber and chip preparations showed a tendency to increase during storage. The reason for this unusual phenomenon is not known.

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 many of the *Leguminosae* (Vogel *et al.*, 1968). The presence of trypsin inhibitor in potato (table variety) was reported (Sohonie and Bhandarkar, 1954), however no detailed information about the extraction methods, change of inhibitor activity during the storage of potato tubers and potato chips, and stability of the inhibitor is available. This work was done to provide such information.

Materials and Methods

Materials

Potato (*Solanum tuberosum* Nong Lin No. 1) was purchased from local

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market near Nankang. Trypsin (10,000–13,000 BAEE units per mg) and DL-BAPA (DL-benzoyl arginine *p*-nitroanilide hydrochloride) were products of Sigma Co. (U.S.A.). Casein was a product of Wako (Japan). Other reagents are of enzyme grade.

Preparation of potato chips

Potato tubers were cut into small stripes with a knife and then dehydrated at 70°C in an oven overnight. Dried chips were assayed for trypsin inhibitor activity after being stored at room temperature for various times.

Extraction methods

Fresh potato tissues or dried potato chips were first cut into small pieces with a knife and then homogenized in a commercial fruit juicer with 5–10 volumes of various solvent systems and processed differently as described below.

Method 1. Distilled water was used. The homogenate was divided into two parts. One part was filtered through 4 layers of cheesecloth and then centrifuged at 9,000 rpm using a Sorvall SS-34 rotor for 15 min. The supernatant fluid was dialyzed twice against 100 volumes of distilled water for at least 4 hr and the dialyzed crude extract was assayed for trypsin inhibitor activity. The other part of homogenate was not filtered through cheesecloth. It was acidified to pH 4.2 and kept at 7°C overnight. Any precipitates formed were discarded by decantation. The clear solution was brought to 30% ammonium sulfate saturation. The supernatant fluid and the precipitates were dialyzed as with the former part. Dialyzed supernatant fluid was lyophilyzed and tested for inhibitor activity as the dialyzed precipitates.

Method 2. Tris-HCl, 0.05 M (pH 7.4), was used. The whole procedure was the same as the method 1.

Method 3. Seventy per cent ethanol containing 0.01 N HCl was used. The homogenate was filtered through 4 layers of cheesecloth. Part of the dialyzed filtrate (crude extract) was directly tested for inhibitor activity. The other was placed in a vacuumed desiccator with KOH to absorb HCl, ethanol and water. Dried samples were dissolved in distilled water to which solid NaCl was added to 15%. The solution was then kept at 7°C overnight. Any precipitates formed were isolated by filtration. Inhibitor assays were carried out with both precipitates and filtrate.

Trypsin activity assay with casein as substrate

This was done according to procedure reported by Kunitz (1946) and was the main method used throughout this work. Standard assays were run by

adding 1.0 ml trypsin solution (containing 20 μg trypsin) and 0.5 ml distilled water to tubes with 1.0 ml 1% casein (in large excess). Proteolytic reaction was allowed to proceed at 35°C for 20 min. The solutions were then poured into tubes containing 3.0 ml of 5% 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 solutions was determined by measuring the absorbance of the solutions at 280 nm. Control tests were run with crude inhibitor extract as enzyme source. Sample tests were done by preincubating crude inhibitor extract with 1.0 ml trypsin solution for 15 min in a total volume of 1.5 ml. The remainder of the procedure was the same as standard assays.

Trypsin activity assay with DL-BAPA as substrate

This followed the procedure of Erlanger *et al.* (1961). Standard assays, control tests, and sample tests were run under the same conditions described in the preceding section.

Protein determinations

Protein determinations were made by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Calculation of trypsin inhibitor activity

The percentage of inhibition was calculated by a formula as $\frac{(\text{standard}+\text{control})-\text{sample}}{\text{standard}}\times 100\%$, and the specific per cent inhibition was defined as per cent inhibition per mg protein.

Results

Comparison of three extraction methods

Table 1 shows that acidic ethanol extraction gives the highest specific per cent inhibition. This method is therefore the most suitable for purification of trypsin inhibitor from potato tissues. Water extraction method is simple and economic, and has oftenly been used although the results of assay revealed the lowest inhibition.

Sequence of mixing the reactants

Table 2 indicates that a change in the order of mixing the reactants exerted an influence on the extent of inhibition. A greater degree of proteolytic inhibition was observed in case of (substrate-inhibitor+enzyme) than (enzyme-inhibitor+substrate).

Table 1. *Comparison of three extraction methods for trypsin inhibitors from potato tubers*[1][2]

Extraction method	Fraction	Absorbance at 280 nm			% inhibition $\frac{(A+B)-C}{A}\times 100$	% inhibition per mg protein
		Standard (A)	Control (B)	Sample (C)		
Distilled water	Crude extract	0.221	0.140	0.174	84.6	165
	30% ammonium sulfate supernatant	0.206	0.054	0.258	none	none
	30% ammonium sulfate precipitate	0.175	0.281	0.287	96.5	71.4
Tris-HCl buffer	Crude extract	0.265	0.069	0.119	81.0	192
	30% ammonium sulfate supernatant	0.183	0.001	0.190	none	none
	30% ammonium sulfate precipitate	0.223	0.260	0.275	93.2	78.3
Acidic ethanol	Crude extract	0.186	0.051	0.066	91.9	287
	15% NaCl supernatant	0.196	0.094	0.107	93.4	259
	15% NaCl precipitate	0.196	0.024	0.111	55.6	59.1

(1) About 120 ml of crude extract was obtained with 100 gm fresh potato tubers by each method. The detailed extraction techniques and assay procedures were described in the text.

(2) The amount of casein used in each test was in large excess which could give an absorbance larger than 1.0 at 280 nm.

Table 2. *Effect of the order of mixing the reactants on the extent of inhibition*[1]

Order of mixing the reactants	Absorbance at 280 nm			% Inhibition $\frac{(A+B)-C}{A}\times 100$
	Standard (A)	Control (B)	Sample (C)	
Enzyme-inhibitor+Substrate	0.245	0.259	0.322	74
Substrate-inhibitor+Enzyme	0.245	0.259	0.283	90

(1) The inhibition of trypsin was studied under conditions under which (a) the substrate was added to a preincubated mixture of the enzyme and inhibitor (EI+S), and (b) the enzyme was added to a preincubated mixture of the substrate and inhibitor (SI+E). All other conditions were the same as described in the legend of Table 1.

Stability of trypsin inhibitor of crude extract toward extreme low pH and heat

The pH of the crude extract was adjusted to 0.8 by addition of 1 N HCl. The solution was kept 37°C for 30 min and the pH was adjusted back to neutral by addition of 1 N NaOH. This treatment did not affect inhibitor activity.

Table 3 indicates that trypsin inhibitor activity of crude potato extract is quite stable toward heat treatment. Preincubation of inhibitor solution between

100°C and 125°C for 30 min hardly decreases inhibitor activity. When pre-incubation period is 30 min, the minimum temperature required to destroy the inhibitor activity completely is 130°C.

Table 3. *Stability of trypsin inhibitor of crude potato extract toward heat*

Heat treatment	% Inhibition
Control	84.6
Preincubation at 100°C–125°C for 30 min	74.2
Preincubation at 130°C for 30 min	3.2

Change of trypsin inhibitor activity of potato during storage

Trypsin inhibitor is stable in fresh potato tubers during storage. This is shown in Fig. 1. Specific per cent inhibition increases even after storage for 4 months.

Trypsin inhibitor is also stable in dried stripes of potato tubers during storage. This is shown in Fig. 2. Specific per cent inhibition also shows a tendency to increase during storage.

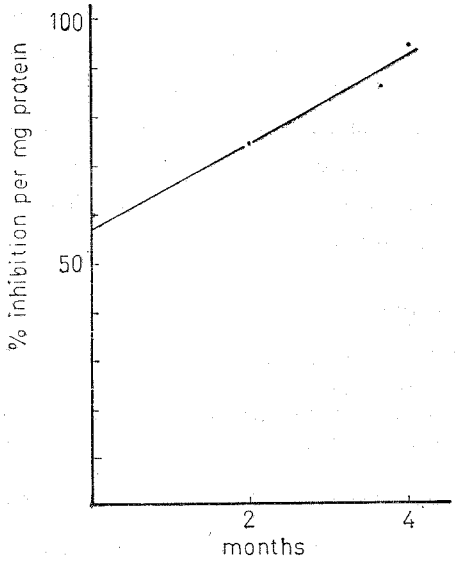

Fig. 1. Change of trypsin inhibitor activity of potato tubers during storage. Fresh potato tubers were stored at 7°C for the times shown and trypsin inhibitor activity of their crude extracts at each time was determined as described in legend of Table 1.

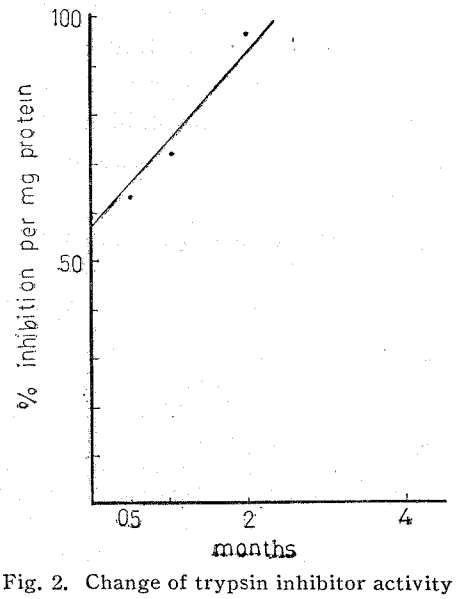

Fig. 2. Change of trypsin inhibitor activity of potato chips during storage. Dried potato chips were stored at room temperature for the times shown and trypsin inhibitor activity of their crude extracts at each time was determined as described in legend of Table 1.

Discussion

Among three extraction methods used, acidic ethanol extraction gives samples with the highest specific per cent inhibition. Therefore this method is most suitable for purification of trypsin inhibitor from potato tissues. Although water extraction gives samples with the lowest specific per cent inhibition, it is worth considering when simplicity and economy are the major concerns.

The inhibition of trypsin by various trypsin inhibitors has been studied by others under conditions similar to those described in legend of Table 2. A greater degree of proteolytic inhibition was observed in the latter case (SI+E) with the soy bean inhibitor, Kazal's pancreatic inhibitors and ovomucoid (Viswanatha and Liener, 1954). The results of Table 2 confirm to these general observations.

When DL-BAPA was used as substrate for trypsin activity assay no detecable inhibition was observed with both potato tuber and chip preparations. This is in agreement with observations made by Ryan and Balls (1962) when small molecule substrate of trypsin was used.

The specific per cent inhibition of both potato tuber and chip preparations shows a tendency to increase during storage. The reason for this is not completely understood. However, three suggestions may be made: (1) some proteins of potato tubers or chips are degraded substantially during storage while inhibitor is quite stable; (2) only small amounts of proteins are degraded, of which some are activators of trypsin; (3) there is a structural or conformational change of inhibitor molecules during storage. Further studies are required before this observation could be explained.

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馬鈴薯 (*Solanum tuberosum*) 之 Trypsin 抑制成分：抽取，穩定性及貯藏期間的活性變化

林 耀 輝　鄭 淑 楨

中央研究院植物研究所

乾馬鈴薯簽和新鮮的馬鈴薯塊根之 trypsin 抑制成分的抽取效果係以三種溶劑系統來比較。如果以比百分抑制（每一毫克蛋白質所產生的百分抑制）的大小爲評鑑標準，那麼抽取效果的好壞依次爲：酸性的百分之七十之乙醇，0.05 M 之 tris-HCl 緩衝液（pH 7.4），蒸餾水。抑制成分，trypsin 及基質（牛乳蛋白）這三者的混合次序會影響所產生的抑制程度。抑制成分和基質預先保溫然後再加 trypsin 所產生的抑制程度比抑制成分和 trypsin 預先保溫然後再加基質的大。Trypsin 抑制成分無論是對熱或者低 pH 都很穩定。攝氏130度以上加熱30分鐘才能完全破壞其活性。乾的馬鈴薯簽和新鮮的馬鈴薯塊根所含的抑制成分之比百分抑制都隨着儲藏時間之增加而增大，其中原因還不甚瞭解。