

DISTRIBUTION OF TRYPSIN INHIBITORS
IN PLANT FOODSTUFFS

by

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INTRODUCTION

After the isolation by Kunitz (1945) (1, 2, 3) of a substance in soybean seeds which is a specific inhibitor of trypsin, substances possessing similar properties were isolated from a number of plant and animal sources.

Trypsin inhibitors have attracted the attention of scientists investigating the nutritive value of proteins, studying the reaction of these inhibitors with enzymes or working on the unique pharmacological properties of these inhibitors for clinical application in the field of medicine. More recently, their roles in plant physiological functions have been studied.

One of the objectives of this study was to examine various plant tissues other than legume seeds to determine if trypsin inhibitors are of widespread occurrence. It was quickly established that sweet corn is a relatively rich source of a trypsin inhibitor, and the investigation was extended to include its isolation and characterization.

REVIEW OF LITERATURE

The first detection of a trypsin inhibitor was reported in soybeans by Bowman and by Ham and Sandstedt in 1944 (4, 5). Subsequently, it was isolated in crystalline form by Kunitz (1, 2, 3). Since then many investigations have been carried out on proteinase inhibitors. Most of the trypsin inhibitors have been found in seeds, and all the Leguminosae seeds contain trypsin inhibitors. However, trypsin inhibitor is not restricted to this part of the plant, as Honacar and Sohonie (6) reported the existence of trypsin inhibitor in both the tuber and leaves of sweet potatoes. Mitchell et al. (7, 8) found trypsin inhibitory activity in alfalfa leaves. Vogel et al. (9) isolated a trypsin inhibitor from beet and sugar beet. In cereal grains such as wheat,

oat, corn, barley and rye, inhibitory activities also have been found (10).

The inhibitor molecule has a random coil structure which is interrupted by an ordered helical structure. Soybean trypsin inhibitor is nonhelical in the native state, but it can be converted in part to the α -helical form when high concentrations of propanol are added (11).

The reactive sites of trypsin inhibitors were determined by cleavage upon incubation with trypsin. Part of the reactive site of many, or possibly all, naturally occurring trypsin inhibitors consists of a trypsin-accessible Arg-X or Lys-X bond (where X is an amino acid of the protein chain) contained within a disulfide loop (12). In some trypsin inhibitors (arginine inhibitors) such as soybean, Kazal bovine pancreatic, corn, wheat, rye, peanut and chicken ovomucoid inhibitors, modification of lysyl residues was without significant effect on inhibitory activity, in contrast to observations on lysine inhibitors such as lima bean, porcine pancreatic and Kunitz bovine pancreatic inhibitors (13).

The reaction of soybean (12, 13) or corn (15) inhibitor with trypsin resulted in a modified inhibitor. It was assumed that the native inhibitor consists of one protein chain, while the modified inhibitor consists of two chains joined by a disulfide bridge. In the first stage of interaction of trypsin and inhibitor, a specific cleavage of an essential bond, either arginine or lysine, is involved and it was concluded that arginine or lysine is located within a disulfide loop of the inhibitor. Proteolysis of the inhibitor by the enzyme is not essential for inhibition, and the rate-limiting step is most probably a conformational change (16). The trypsin-trypsin inhibitor complex subsequently may form an ester bond at the active site serine residue of the modified inhibitor (17). Trypsin combines with the inhibitor in a stoichiometric, reversible reaction (5). The existence of an equilibrium of the

reaction between virgin and modified inhibitor was demonstrated (18).

A low molecular weight growth inhibitor was prepared from soybean trypsin inhibitor by column chromatography which decreased weight gain in animal feeding experiments (19). In addition to the inhibition of body weight gains, chickens also exhibited a decrease in egg production when raw soybean meal was fed (20). The simplest explanation would be that the inhibitor inhibited intestinal proteolysis (21).

Pancreatic hypertrophy, together with excessive enzyme secretion, was observed in rats and chickens fed soybean flour or crystalline inhibitor (22, 23). Inhibition of proteolysis was more noticeable in the first week of life, but it later was counterbalanced by hypertrophy of the pancreas. Several observations had shown that trypsin inhibitor caused an excessive stimulation of the pancreas, resulting in loss from the body of a large number of essential amino acids required for growth of the animal. These losses of amino acids occurred because of inhibition of proteolytic activity in the intestinal tract by the trypsin inhibitor (24).

Trypsin inhibitor may cause metabolic disturbance of the utilization of methionine and other amino acids. In rats, feeding unheated soybeans did not selectively impair the availability or tissue utilization of methionine (25). Barnes and Kwong found an increase in conversion of ^{35}S -methionine to ^{35}S -cystine in rat pancreas by oral administration of crystalline soybean trypsin inhibitor (26). They suggested that there was a metabolic block in the utilization of cystine for protein synthesis.

At present, the physiological importance to plants of trypsin inhibitors is not known. Perhaps they exert some control on the proteolytic enzyme system of the plant. However, Ofelt (27) and Birk (28) indicated that the inhibitors of soybean did not function as inhibitors of plant proteolytic systems. The

observation by Ambe and Schonie (29) that inhibitor concentration was relatively high in young, growing tissue but low in older tissue suggested that these inhibitors may play an important role in regulation of protein metabolism.

Shain and Mayer (30, 31) found a trypsin inhibitor in lettuce seeds, but the inhibitor disappeared in the course of germination. Inhibitors in the seed or other natural storage organ may serve as inhibitors of the proteolytic enzyme of these tissues during the synthesis and storage of protein, prior to dormancy, when there is a need for rapid protein accumulation.

Since trypsin inhibitors occur widely in plants which are colonized by Azotobacter, Vogel et al. (9) believe their function in these plants is to prevent the plants from being overrun by symbiotic bacteria by protecting the plant tissues at the colonization site against the action of the bacterial proteinases.

Recently, Green and Ryan (32) found that wounding the leaves of potato or tomato plants by insects or by mechanical means induced a rapid accumulation of a powerful inhibitor of major intestinal proteinases of animals. This response to wounding of the leaves is possibly a defense mechanism.

The physiological functions of trypsin inhibitors in plants are still obscure and more detailed studies in this field should be undertaken in the future.

EXPERIMENTAL

Occurrence of Trypsin Inhibitors in Various Plant Tissues

Measurement of Inhibitor Activity

Trypsin inhibitor activities of various fresh plant tissues were determined by a modification of the methods of Beauchene et al. (33) and Ramirez

et al. (7).

A 3 percent solution of casein was prepared in 0.107 M phosphate buffer, pH 8.4, and the pH of the solution was readjusted to 8.4 with dilute sodium hydroxide. A trypsin solution containing 30 mg of trypsin (1:300) in 100 ml of 0.107 M phosphate buffer, pH 8.4, was prepared just before use. The assay mixture consisted of 5 ml of the casein solution, 2 ml of the trypsin solution, the inhibitor solution and sufficient distilled water to make a final volume of 15 ml. The final assay mixture was 0.05 M with respect to phosphate.

The reaction mixture was placed in a water bath maintained at 37°C. After 4 hours, 5 ml of 10 percent trichloroacetic acid were added to precipitate the trypsin and the undigested casein. The sample was filtered and the filtrate was adjusted to pH 7.0 with 40 percent sodium hydroxide. Ten ml of the filtrate were placed in a 15 ml graduated centrifuge tube and 5 ml of a suspension of copper phosphate in pH 9.1 borate buffer (33) were added. The content of the tube was mixed well, allowed to stand for 5-10 minutes and clarified by centrifugation. The absorbance of the supernatant solution was measured at 620 nm with a Beckman DU spectrophotometer.

Extraction of Plant Tissues

Extraction studies were performed by extracting 20-80 g of various fresh plant tissues with 200 ml of water in a blender for 1 minute at full speed. The residues were separated by centrifugation. The inhibitory activities of the crude extracts were measured by the casein digestion method mentioned above. The relative inhibitory activities of the plant tissues are shown in Plate I. and Table 1.

The data show marked differences in inhibitory activity of plants. Since legume seeds characteristically are high in trypsin inhibitor, it might be

EXPLANATION OF PLATE I

Relationship between percent inhibition and amount of plant tissue in various plants. The plant tissue concentration (mg/ml) showed was with respect to the final mixture.

- | | |
|---------------------|------------------|
| (A) Irish potato | (B) Sweet corn |
| (C) Brussel sprouts | (D) Sweet potato |
| (E) Spinach | (F) Broccoli |

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PLATE I

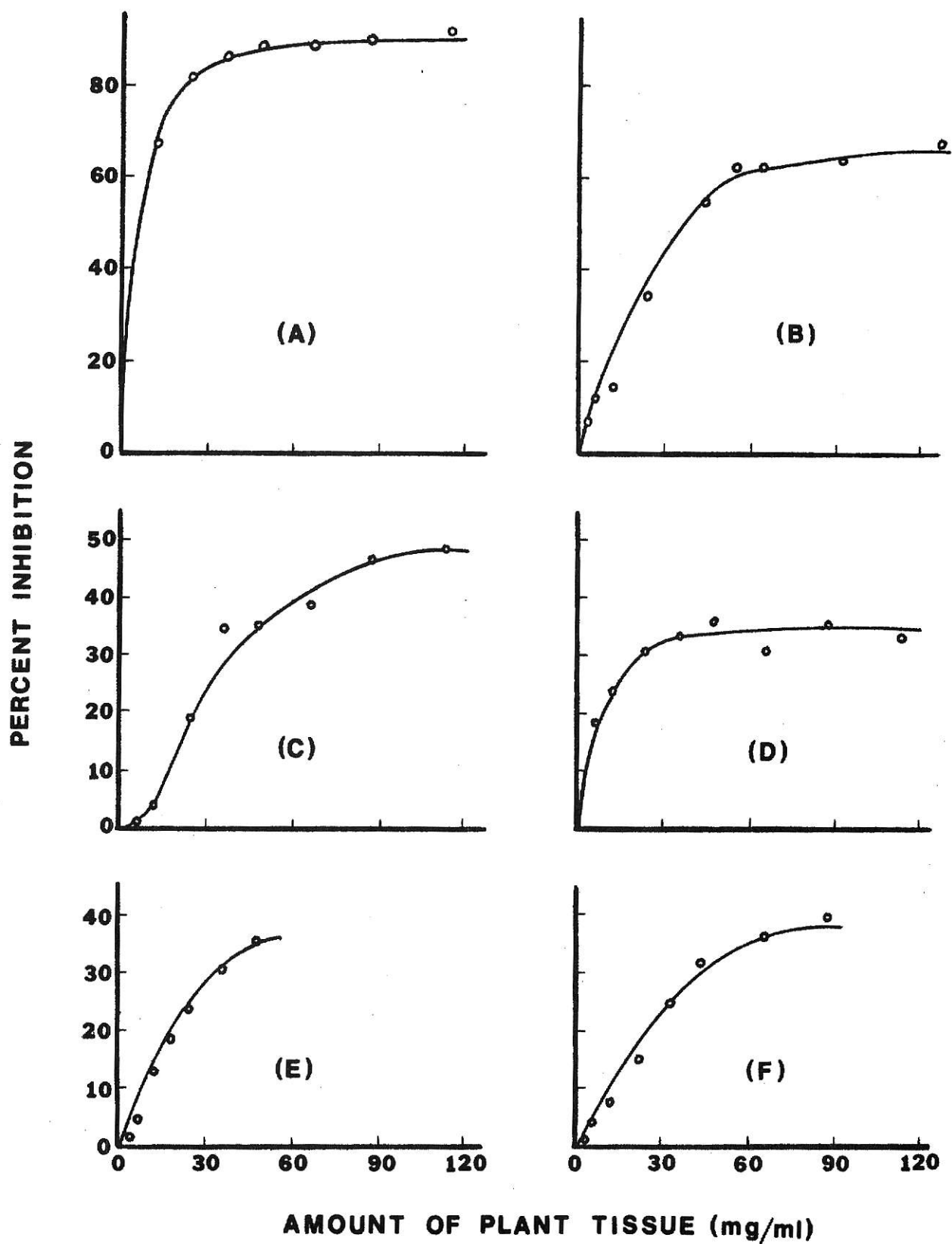


Table 1. Occurrence of trypsin inhibitors in various plant tissues.

Plant Tissue	Concentration (A) (mg/ml)	Percent inhibition (B)
Irish potato	48	88.7
sweet corn	66	62.3
sweet potato	48	36.0
spinach	48	35.5
broccoli	66	36.8
Brussels sprouts	88	46.7
cauliflower	48	8.5
cabbage	48	8.3
lettuce	48	5.5
white radish	48	26.0
red radish	48	9.6
carrot	36	6.3
cucumber	33	24.4
(C) asparagus, avocado, peach, nectarine and plum		-

(A) The concentration was based on the fresh tissue weight.

(B) Percent inhibitions were taken at the peaks of the trypsin inhibitor-concentration curve of plant tissues.

(C) Inhibition by these tissues was negligible and did not show a relationship with concentration.

expected that other storage organs would be high also. This was found to be true for Irish potato, sweet potato and sweet corn. However, fruits, which are largely storage tissues also, generally were low in activity, while other storage sites such as radish and cucumber were intermediate in activity. Leaf tissues such as spinach, broccoli and Brussels sprouts were high, but lettuce was low. Thus, there appears to be no consistent relationship between activity and storage function. Also, not all photosynthetic tissues were high. There is no apparent pattern of distribution which might suggest the metabolic function of trypsin inhibitors in plants. Their wide distribution does indicate that they have a physiological role and are not just unique substances produced by a few species of plants.

Isolation of Inhibitor from Sweet Corn

In assays of various plant tissues, it was found that sweet corn had a relatively high trypsin inhibitory activity. Therefore, it was used as isolation material for further studies.

Trypsin Assay

Method II of Erlanger et al. (34), based on hydrolysis of benzoyl-DL-arginine-p-nitroanilide (BAPA) to p-nitroaniline, was used to follow the progress of isolation and purification of the inhibitor.

Substrate Stock Solution (10^{-3} M) : 43.5 mg DL-BAPA was dissolved completely in 1 ml of dimethylsulfoxide and the solution was brought to 100 ml with 0.05 M Tris buffer, pH 8.2, containing 0.02 M calcium chloride. The temperature of the stock solution was never allowed to fall below 25°C.

Enzyme Solution : A concentration of 6.0 mg of trypsin (1:300) per ml

of 0.001 M hydrochloric acid was used.

For the assay, 0.9 ml of water was added to 5 ml of substrate stock solution, and the mixture was allowed to equilibrate in a thermostatically controlled water bath at 25°C for 5 minutes. At zero time, 0.1 ml of enzyme stock solution was added, and the reaction was allowed to run for 10 minutes. A suitable control without enzyme solution also was set up. Addition of 1.0 ml of 30 percent acetic acid terminated the reaction, and the quantity of p-nitroaniline produced by the enzymic reaction was estimated spectrophotometrically by measuring absorbance at 410 nm with a Beckman DU spectrophotometer. A standard curve was prepared by dilution of a stock solution of p-nitroaniline (Fig. 1). Rate of hydrolysis of DL-BAPA as a function of enzyme concentration is shown in Fig. 2.

One unit of enzyme was defined as the amount of enzyme which liberates one micromole of p-nitroaniline per minute under the specified conditions. Since the assay reaction was run for 10 minutes, 10 micromole of p-nitroaniline therefore would be produced by one unit of enzyme solution. From the standard curve, Fig. 1, 10 micromoles of p-nitroaniline produced an absorbance of 0.092 at 410 nm.

Trypsin Inhibitor Assay

The inhibitor solution was diluted with 0.05 M sodium acetate buffer, pH 5.4, containing 0.02 M calcium chloride. One unit of enzyme solution was prepared with a concentration of 0.5 mg trypsin per ml of 0.001 M hydrochloric acid. One ml of inhibitor solution was added to 1 ml (1 unit) of trypsin solution. The mixture was incubated at 25°C for 5 minutes, and 0.5 ml was used for trypsin assay (35).

One unit of trypsin inhibitor was defined as the amount of inhibitor

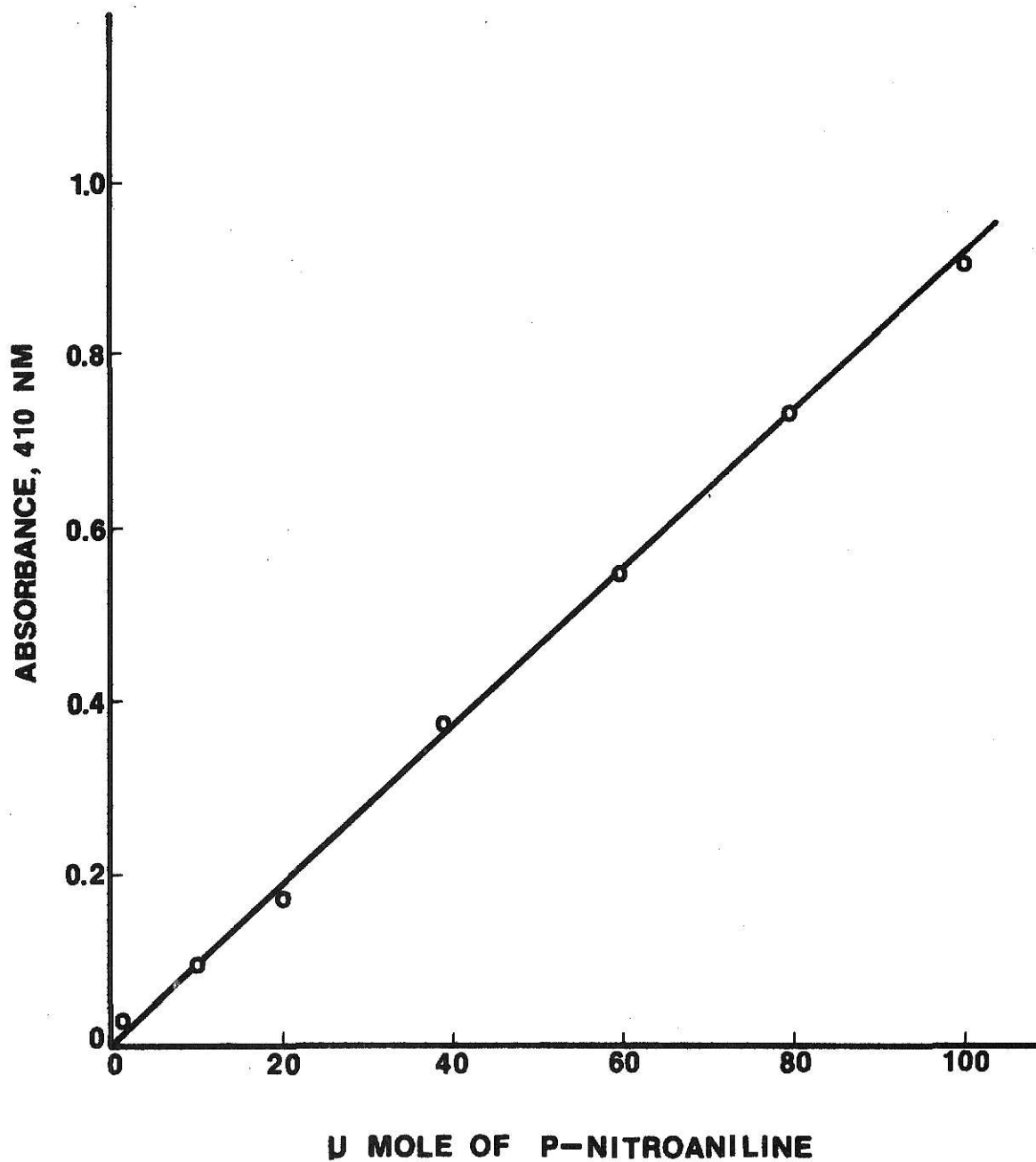


Fig. 1. Standard curve for p-nitroaniline.

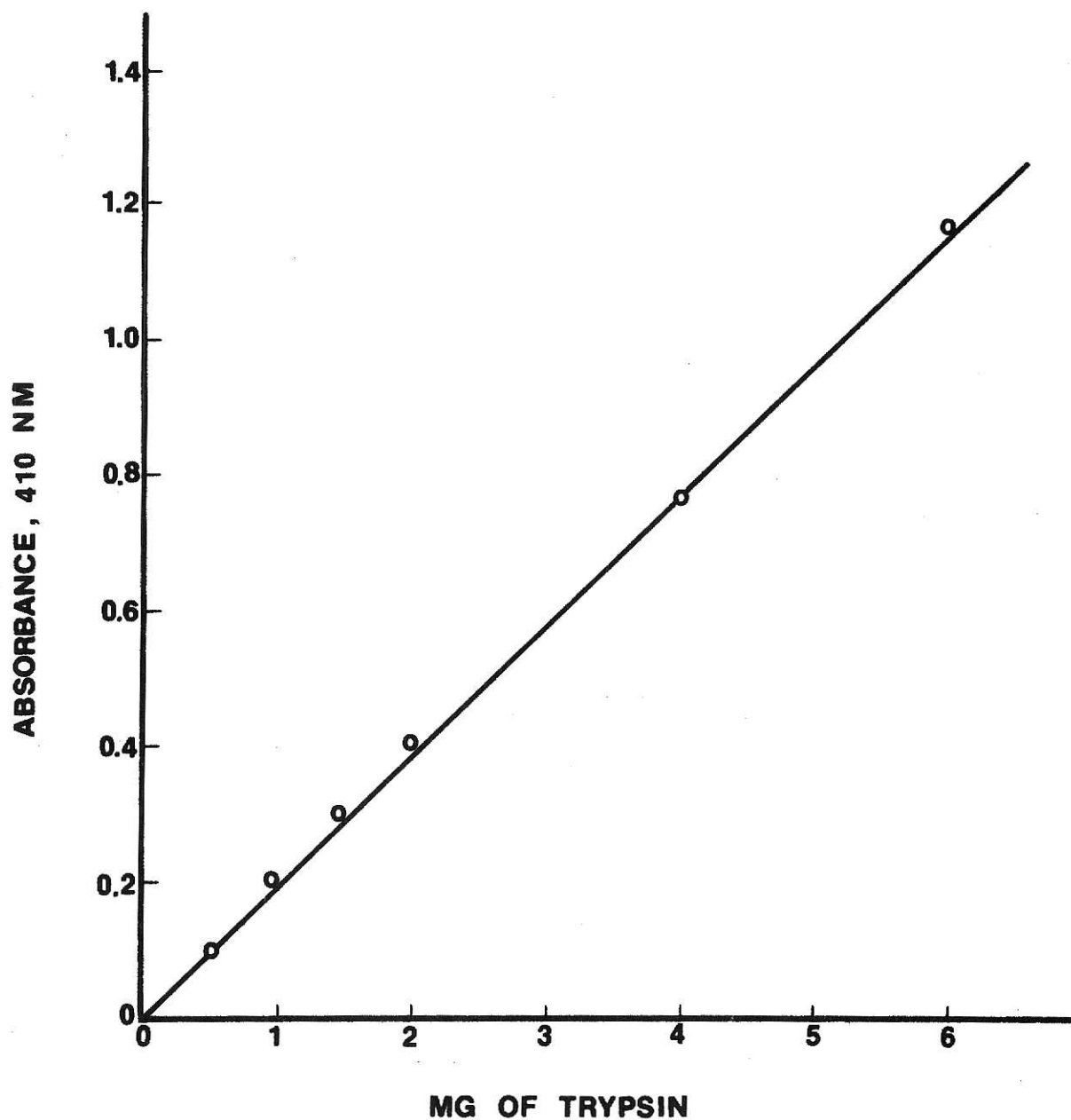


Fig. 2. Rate of hydrolysis of DL-BAPA as a function of enzyme concentration.

which decreases the reaction rate by one trypsin unit, or decreases the absorbance at 410 nm by 0.092 (Fig. 1).

Protein Determination

In the initial stages of purification, protein concentrations were estimated spectrophotometrically at 280 nm with a Beckman DU spectrophotometer.

Preparation of Trypsin Inhibitor from Sweet Corn

All the operations were carried out at room temperature except where otherwise stated. The protein solutions were stored at 4°C.

Four hundred fifty grams of sweet corn, variety Royal Gold, were homogenized with two volumes of acetone in a blender for 1 minute at full speed and the homogenate was filtered under suction. The residue was washed with acetone on the Buchner funnel until the yellow color was removed. The residue then was rapidly air-dried.

Step I. Preparation of Crude Extract

The sweet corn powder was agitated for 12 hours at 4°C with 1 l of 0.2 M sodium chloride. The extract was adjusted to pH 8.0 by adding sodium hydroxide and was centrifuged at 5,000 g for 20 minutes. The residue was re-extracted with 1 l of the sodium chloride solution. The two supernatants were combined.

Step II. Ammonium Sulfate Fractionation

Solid ammonium sulfate was added gradually to the supernatant from step I until it was 40 percent saturated. The suspension was stirred for 1 hour at 4°C, after which it was kept overnight at 4°C. The active precipitate was collected by centrifugation at 12,000 g for 30 minutes, and was suspended in 150 ml (3/40 of the original volume of extract) of 0.05 M triethanolamine in 0.1 M sodium chloride buffer, pH 7.0. The suspension was stirred for 30

minutes. The precipitate was separated by centrifugation and washed again with 50 ml (1/40 of the original volume) of the triethanolamine-sodium chloride solution. The two active supernatants were combined, and the solution was extensively dialyzed against 0.05 M triethanolamine in 0.1 M sodium chloride buffer, pH 7.0, at 4°C for 48 hours. When a precipitate formed during the dialysis, it was removed by centrifugation. The dialyzed solution was concentrated by evaporation by hanging the dialysis tubing in front of an electric fan. The concentrated extract was used for further fractionation by column chromatography.

Step III. Sephadex G-75 Chromatography

To obtain satisfactory flow rates and good separations, the gel must be prepared carefully. The gel should be allowed to swell in excess solvent and allowed to stand for a period of time. A minimum of 24 hours is required for Sephadex G-75 to swell at room temperature, but the process of swelling can be accelerated by putting the gel slurry in a boiling water bath. Air bubbles may be trapped in the gel slurry, causing uneven flow when the bed is packed. This can be avoided by swelling the slurry in a boiling water bath also (36, 37). The bed volume of dry Sephadex G-75 is 12-15 ml per g. Therefore, 35 g of dry gel were swelled in 600 ml of water on a boiling water bath for 3 hours. The swelled gel was packed into a 2.5 x 110 cm column and equilibrated with 0.05 M triethanolamine in 0.1 M sodium chloride, pH 7.0. The inhibitor solution was placed on the column and elution was accomplished with the same buffer at a flow rate of 16 cm/hr at room temperature. The eluate was collected in 5 ml fractions. Absorbance of each fraction was measured with a Beckman DU spectrophotometer at 280 nm and trypsin inhibitor activity was assayed by the procedure described earlier.

The elution pattern is shown in Fig. 3. Fractions possessing trypsin

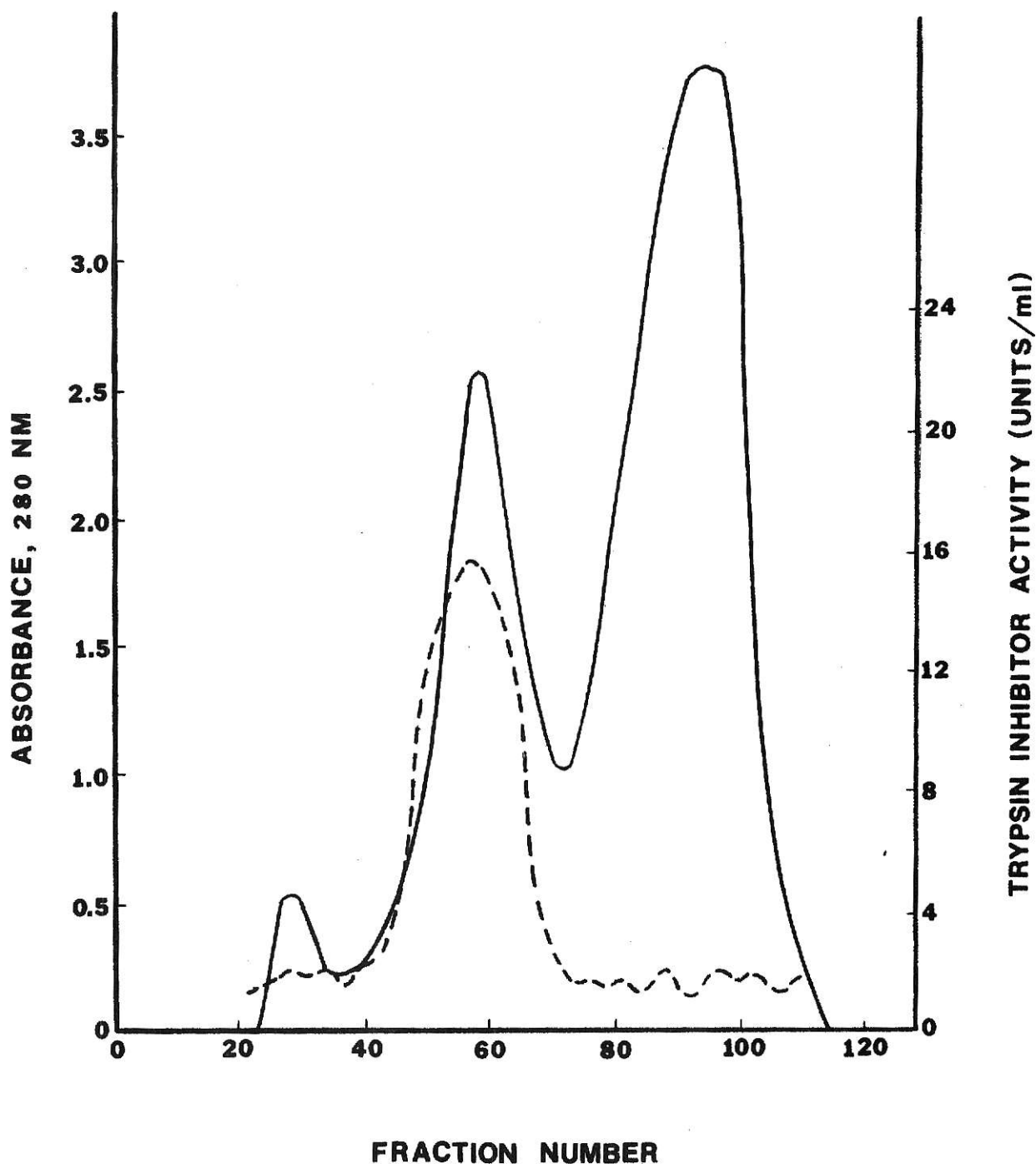


Fig. 3. Elution and activity patterns obtained with Sephadex G-75 chromatography. Solutions with absorbances at 280 nm (—) greater than 2.0 were diluted 1:5 with the buffer prior to measurement. Trypsin inhibitor activity (---) was measured by hydrolysis of DL-BAPA.

inhibitor activity were pooled, dialyzed against water for 24 hours and concentrated by evaporation.

Step IV. CM-cellulose Chromatography

A CM-cellulose column was prepared by the method of Peterson and Sober (38). Twenty g of dry CM-cellulose were suspended in distilled water and the fine particles were decanted off. Then the adsorbent was allowed to sink into a solution of 0.5 M sodium chloride and 0.5 M sodium hydroxide, the suspension was filtered and the residue was washed with a similar solution until no more color was removed. This was followed by the addition of sufficient 1 M hydrochloric acid to make a strongly acid suspension, which was filtered immediately and washed free of acid with water. The filtered cake again was suspended in 0.03 M pH 6.0 phosphate starting buffer and packed into a 2.5 x 30 cm column, the bottom of which was covered with glass wool, and was allowed to settle under gravity. The equilibrated sample was applied to the column, and the column was washed with 50 ml of the starting buffer. Thereafter, gradient elution was performed using 300 ml of the starting buffer in the mixing chamber and 300 ml of 0.3 M pH 8.5 phosphate buffer in the reservoir. The eluate was collected in 5 ml fractions. Protein in the eluate was determined spectrophotometrically by measuring the absorbance with a Beckman DU spectrophotometer at 280 nm, and the activity was measured by standard assay. The elution and activity patterns are shown in Fig. 4. The active fractions were pooled and the resulting solution was dialyzed, concentrated and rechromatographed through the same column, following the procedure described above.

Step V. Lyophilization

The rechromatographed CM-cellulose fractions possessing trypsin inhibitor activity were pooled and the solution was dialyzed and concentrated. The concentrated sample of about 30 ml was placed in a beaker and frozen. The

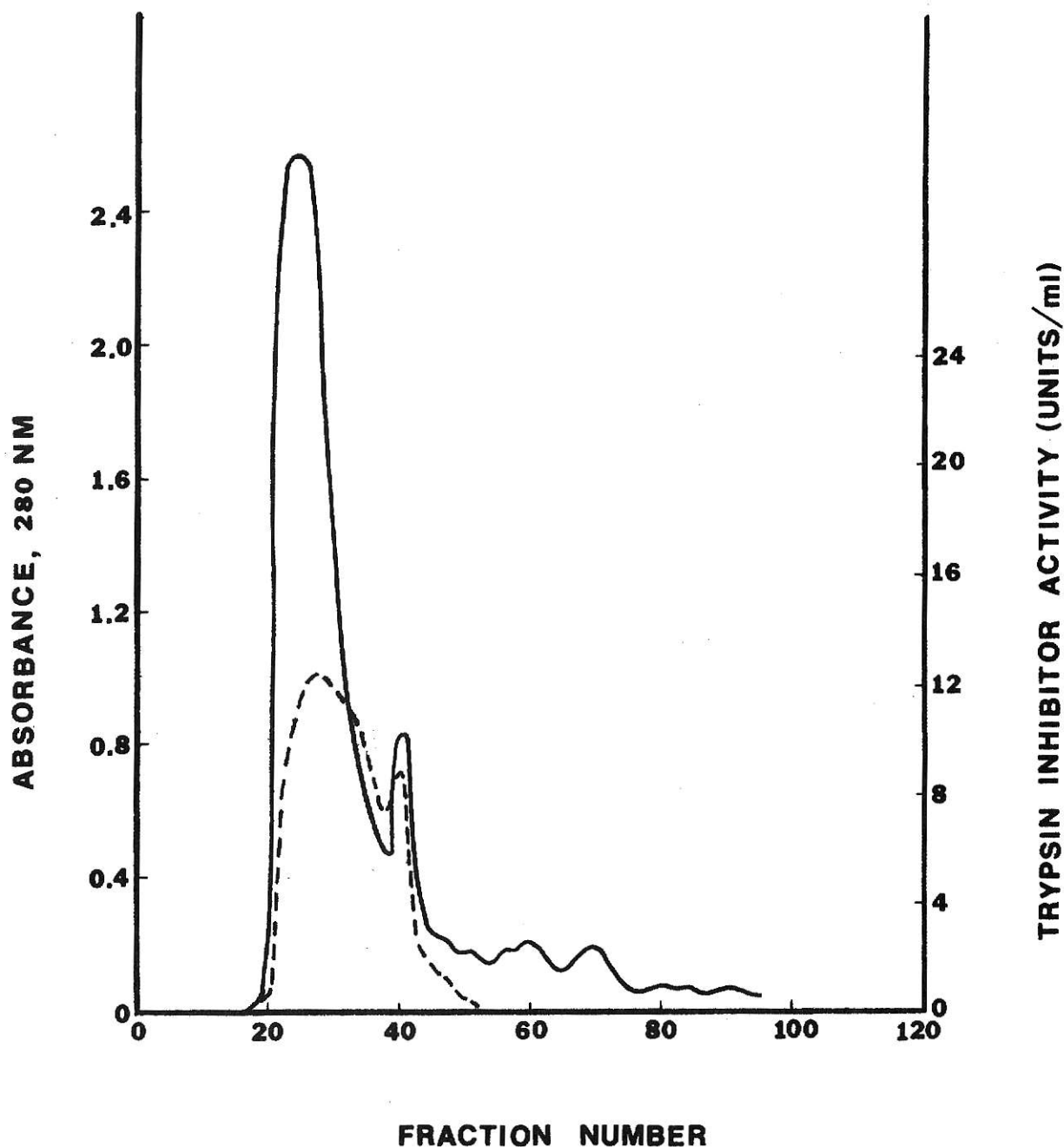


Fig. 4. Elution activity patterns obtained with CM-cellulose chromatography. Initial and final buffer were 0.03 M pH 6.0 phosphate and 0.3 M pH 8.5 phosphate. Elution with absorbance at 280 nm (—) greater than 2.0 were diluted 1:5 prior to measurement. Trypsin inhibitor activity (----) was measured by hydrolysis of DL-BAPA.

beaker was transferred to a desiccator containing anhydrous calcium chloride and the desiccator was evacuated with a vacuum pump. After three hours, the stopcock of the desiccator was closed and the desiccator was stored at 4°C overnight. The inhibitor activity was determined by dissolving the lyophilized sample in 0.05 M sodium acetate buffer, pH 5.4, containing 0.02 M calcium chloride.

The results of purification of sweet corn trypsin inhibitor are shown in Table 2.

The trypsin inhibitor was gradient eluted from the CM-cellulose column with the pH changing from acidic to basic. When rechromatographed on CM-cellulose with the same buffer, the inhibitory substances were eluted from the column at the same pH and same buffer salt concentrations as before. A single inhibitory protein peak was obtained by rechromatography and showed no increase in the inhibitory activity over the rechromatographed starting material.

Properties of the Purified Inhibitor

Homogeneity

Disc electrophoresis in 7 percent polyacrylamide gels was conducted according to the procedure of Davis (39). A RDS disc reagent kit from Canalco Co. was used for this purpose. Persulfate was used to catalyze the polymerization of the gels. Tris-glycine buffer, pH 9.5, served as the electrode tray buffer and bromophenol blue as the tracking dye. The inhibitor in concentrated sucrose and gel solution was layered on the spacer gel under the buffer. Two hundred ug of inhibitor per tube was used. A current of 4 mA per tube was applied for 1.5 hours. The gel was removed from the tube and the protein bands were stained with Coomassie blue as described by Chrambach et al. (40). There were two protein bands on the electrophoresis gel, as shown in Plate II. Six

Table 2. Purification of trypsin inhibitor.

Step	Absorbance at 410 nm (A)	Decrease of A_{410} (B)	Units of inhibitor per tube (C)	Absorbance at 280 nm (D)	Specific activity (units of I/ A_{280}) (E)	Relative purification (F)
Crude extract	0.232	0.024	0.26	1.45	0.18	1.0
(NH_4) ₂ SO ₄ precipitate	0.065	0.191	2.08	0.89	2.33	12.9
Sephadex G-75	0.037	0.219	2.38	0.54	4.41	24.5
CM-cellulose	0.053	0.203	2.21	0.41	5.40	30.0
CM-cellulose rechromat.	0.015	0.241	2.62	0.50	5.24	29.1

(A) Absorbance of trypsin-substrate reaction without inhibitor was 0.256 (blank).

(B) The decrease of absorbance at 410 nm compared to the blank (A).

(C) Since one unit of inhibitor was defined as the amount of inhibitor which decreases trypsin activity by one unit or decreases the absorbance at 410 nm by 0.092, the units of inhibitor were calculated as: (decrease of absorbance at 410 nm) / (0.092).

(D) The absorbance of inhibitor sample at 280 nm.

(E) Specific activity was defined as units of inhibitor per unit of protein content (absorbance at 280 nm). It was calculated as (units of inhibitor) / (absorbance at 280), or (C) / (D).

(F) The relative purification of crude extract was assumed as 1. The relative purification of inhibitor samples from various steps was obtained by comparing the specific activity to crude extract's; for example, the relative purification of (NH_4)₂SO₄ precipitate was calculated as follows: $2.33/0.18 = 12.9$.

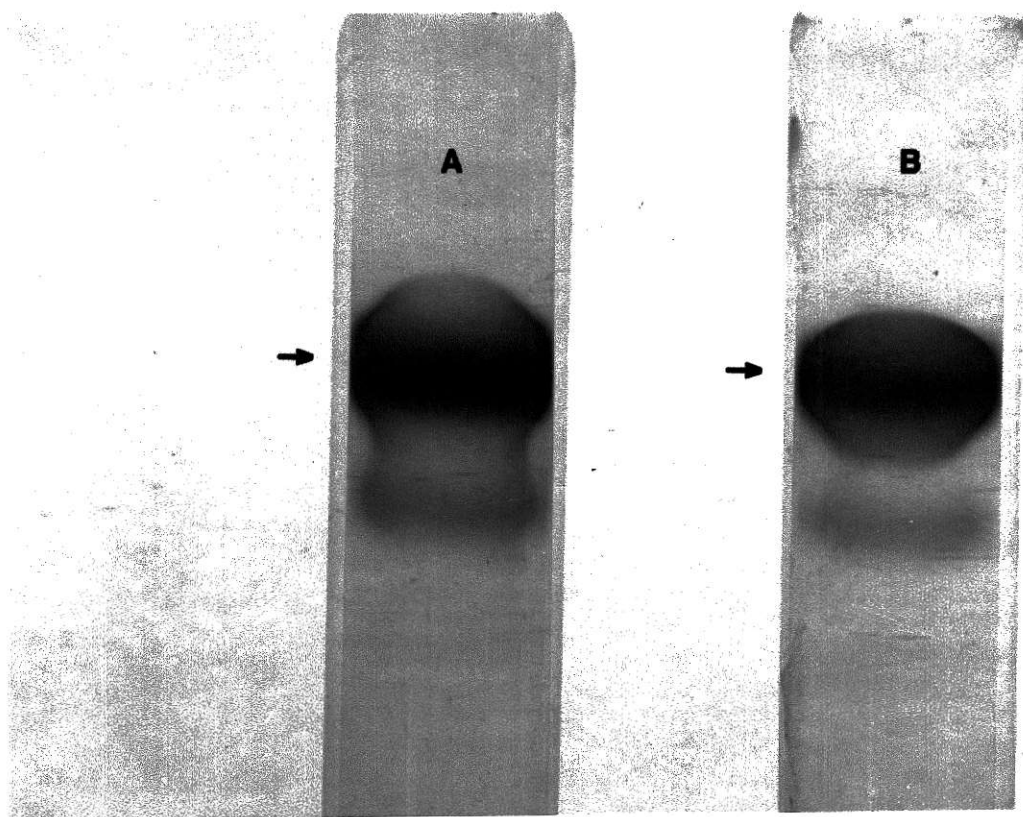
EXPLANATION OF PLATE II

Disc electrophoresis of isolated sweet corn trypsin inhibitor.
Higher trypsin inhibitory activity was found in upper bands (see arrows)
(A) 200 ug of inhibitor. (B) 100 ug of inhibitor.

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PLATE II

more tubes were prepared and carried through the procedure described above, but only one gel was stained after removing from the tube. The gels in the remaining five tubes were removed and the two protein bands were separated manually, using the stained sample as a guide, and corresponding bands were combined. The gel fractions were soaked overnight in 0.05 M sodium acetate buffer, pH 5.4, containing 0.02 M calcium chloride, during which proteins in the gel were extracted into buffer. The inhibitory activity of the lower band was very low. It was concluded that the isolated inhibitor was slightly contaminated.

Carbohydrate Content

The presence of carbohydrate in the purified protein preparations was determined by the method of Dubois et al. (41). One ml of various concentrations of trypsin inhibitor solution were pipetted into test tubes, and 1 ml of 5 percent phenol in water was added to each tube. Then 5 ml of concentrated sulfuric acid was rapidly added with the stream of acid being directed against the liquid surface rather than against the side of the tube in order to obtain good mixing. The tubes were allowed to stand for 10 minutes, then were shaken and placed in a water bath for 20 minutes at 25°C. The absorbance was measured at 490 nm. Carbohydrates with free or potentially free reducing groups give an orange-yellow color when treated with phenol and concentrated sulfuric acid.

Little orange-yellow color developed in the phenol-sulfuric acid reaction with the purified preparation. The amount of carbohydrate in purified inhibitor was negligible even when the concentration of trypsin inhibitor was brought up to 0.5 mg per ml, the absorbance at 490 nm then being 0.103. These data indicate that the isolated inhibitor contains some carbohydrate. It may be present as part of the inhibitor molecule, or as a contaminant.

Effect of Heat

An inhibitor solution was prepared which contained 0.5 mg per ml of water. The solution was placed in a flask and heated in a boiling water bath. A water-cooled condenser was attached to the flask to avoid evaporation. Portions of the solution were removed after 0.5, 1, 2, 4 and 6 hours. The inhibitory activity of each portion was determined by standard assay after the solution was cooled to room temperature. The results (Table 3) show that the purified inhibitor is heat stable.

Inhibition of Other Proteolytic Enzymes Compared to Trypsin

The proteolytic assays of Kunitz (42) were used for trypsin, chymotrypsin and papain by using a casein substrate. A suitable concentration of enzymes and inhibitor were incubated with 1 percent casein in borate buffer at 35°C, and the reaction products were estimated at 280 nm. The data are shown in Table 4. The data show that the isolated inhibitor had high inhibitory activity to trypsin, and very low activity to papain. The isolated inhibitor inhibited chymotrypsin at half efficiency compared to trypsin.

Kinetic Studies

The inhibition was studied by the double reciprocal method of Lineweaver and Birk to determine the type of inhibition. In this method a trypsin progress curve was obtained by holding the enzyme concentration constant and varying the substrate concentration and time. The substrate was DL-BAPA. A constant concentration of inhibitor solution was added and the inhibitory effect was measured for the same concentrations of substrate and reaction time as used for the trypsin progress curve.

The experimental data are shown in Table 5. From these values a double reciprocal plot was prepared as shown in Fig. 5. The plot shows that the isolated inhibitor is a non-competitive inhibitor and has a K_i value of about 0.95×10^{-3} M.

Table 3. Effect of heat on activity of inhibitor.

Treatment	Heating time (hr.)	Absorbance at 410 nm	Decrease of absorbance	Percent inhibition
Inhibitor absent	-	0.247	-	-
Inhibitor present	0	0.014	0.233	94
	0.5	0.018	0.229	93
	1	0.017	0.230	93
	2	0.027	0.220	89
	4	0.036	0.211	85
	6	0.045	0.202	82

0.5 mg/ml of isolated trypsin inhibitor was used in the experiment.

Table 4. Inhibition of other proteolytic enzymes compared to trypsin, using casein as substrate.

	Concentration of inhibitor (mg/ml)	Absorbance at 280 nm	Decrease of absorbance	Percent inhibition
Trypsin	0	0.663	-	-
	0.1	0.263	0.400	60
	0.2	0.220	0.443	67
Chymotrypsin	0	0.337	-	-
	0.1	0.275	0.062	18
	0.2	0.205	0.132	39
Papain	0	0.158	-	-
	0.1	0.166	-	-
	0.2	0.145	0.013	8

The concentrations of trypsin, chymotrypsin and papain were 0.1 mg/ml, 25 ug/ml and 2 mg/ml respectively.

The reaction time for trypsin and chymotrypsin was 20 minutes, and for papain it was 2 hours.

Table 5. Effects of inhibitor and substrate concentrations on hydrolysis of DL-BAPA by trypsin.

Inhibitor concentration (ug/ml)	Substrate concentration(S) (10^{-3} M)	Velocity(V) (u mole p-nitroaniline/min)	1/S	1/V
0	0.83	3.00	1.2	0.33
	0.50	2.20	2.0	0.45
	0.17	1.01	6.6	0.99
	0.08	0.50	12.0	1.78
1.04	0.83	1.20	1.2	0.83
	0.50	0.93	2.0	1.06
	0.17	0.37	6.6	2.70
	0.08	0.24	12.0	4.55

0.5 mg/ml of trypsin solution was used in the experiment, and the final mixture was 0.021 mg/ml with respect to the trypsin concentration.

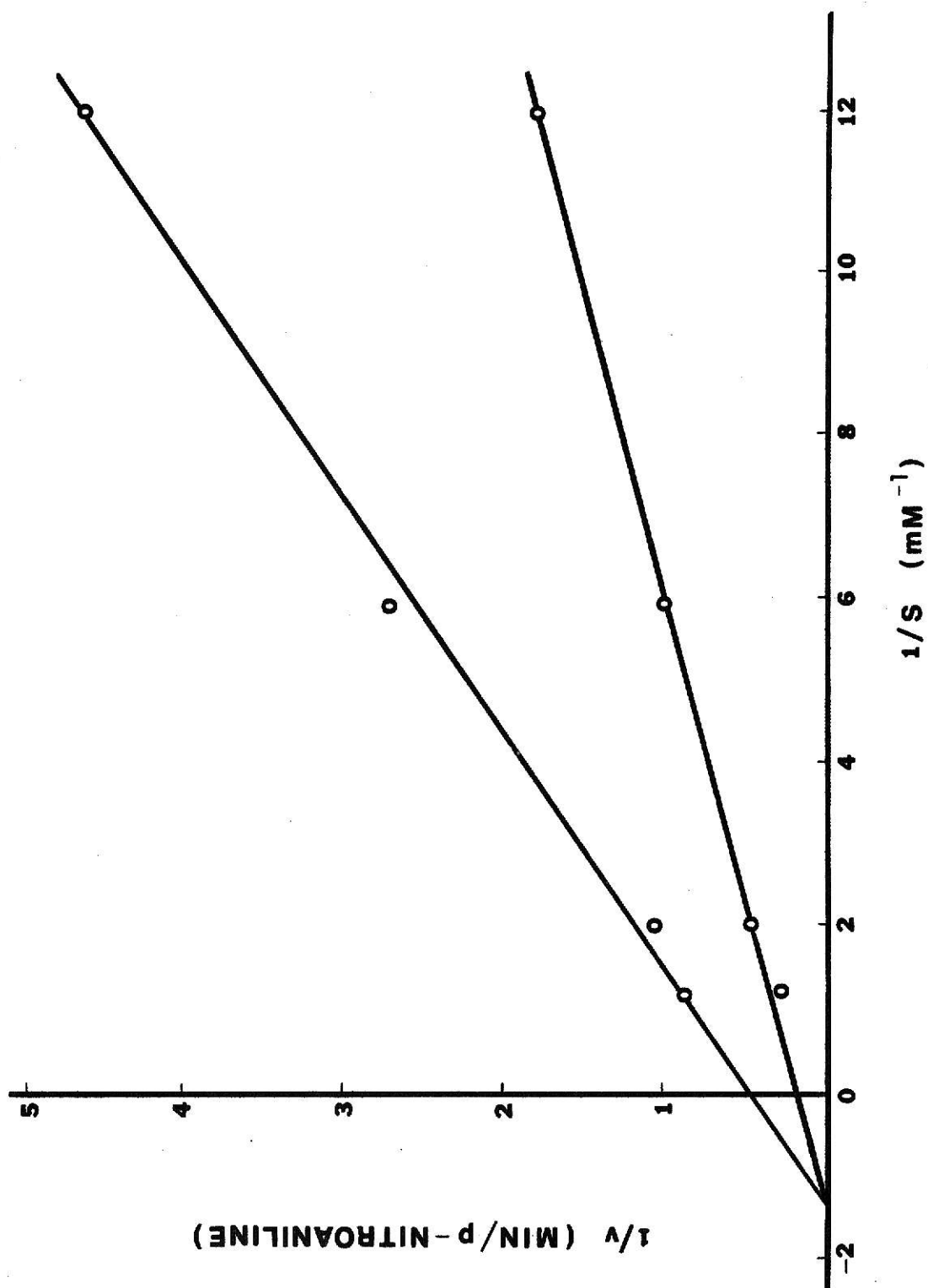


Fig. 5. Double reciprocal plot of sweet corn inhibitor on hydrolysis of DL-BAPA by trypsin.

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DISTRIBUTION OF TRYPSIN INHIBITORS
IN PLANT FOODSTUFFS

by

YING CHEN

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AN ABSTRACT OF A MASTER'S THESIS

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Most research on trypsin inhibitors of plant origin has been done on legumes. This investigation has shown that trypsin inhibitor is distributed widely among plants and in various tissues. The inhibitory activity was found to be high in some storage organs such as Irish potato, sweet corn and sweet potato but low in fruits. Among the leaf tissues, spinach, broccoli and Brussels sprouts were high in trypsin inhibitor, but cabbage and lettuce were low. There appears to be no consistent relationship between inhibitory activity and plant physiological function.

A trypsin inhibitor in sweet corn was isolated for further investigation of inhibitor properties. Isolation studies were carried out by sodium chloride extraction, ammonium sulfate precipitation and Sephadex and CM-cellulose column chromatography. Inhibitory activity of the isolated material was determined by the amount of decrease in trypsin activity on hydrolysis of benzoyl-DL-arginine-p-nitroanilide. A yellow solution of p-nitroaniline was produced during hydrolysis and was measured spectrophotometrically at 410 nm.

The isolated inhibitor is heat stable, and is a non-competitive inhibitor with a K_i of about 0.95 mM, as measured by trypsin hydrolysis of benzoyl-DL-arginine-p-nitroanilide. The isolated inhibitor inhibited chymotrypsin at half efficiency compared to trypsin, but had very low inhibitory activity on papain. Two protein bands were found by disc electrophoresis in isolated trypsin inhibitor, but the lower band had very low inhibitory activity. A negligible amount of carbohydrate was found by phenol-sulfuric acid test. This suggests that the isolated inhibitor may be slightly contaminated or it may be a glycoprotein.