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TRYPSIN INHIBITORS IN PLANTS*

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Key Word Index—*Solanum tuberosum*; Solanaceae; *Zea mays*; Gramineae; trypsin inhibitors; trypsin inhibitor isolation.

Abstract—Trypsin inhibitors were found in several food plants. Potato and sweet corn were the most inhibitory, while fruits had negligible activity. Intermediate in activity were sweet potato, spinach, broccoli, Brussels sprouts and cucumber. The trypsin inhibitor of sweet corn was isolated by extraction in dilute salt solution, ammonium sulfate fractionation, chromatography with Sephadex G75 and CM-cellulose and lyophilization. Two components were demonstrated by disc gel electrophoresis. The inhibitor was heat stable. It had little inhibitory activity against papain but was moderately active against chymotrypsin.

INTRODUCTION

TRYPSIN inhibitors are distributed widely in legume seeds, and have been investigated extensively because of possible adverse effects on protein digestion when ingested by animals. More recent research has shown that they are present also in other plant tissues, such as sweet potato, beet, sugar beet, alfalfa leaves, lettuce seeds and cereal grains.[1] Although their functions in plants are obscure, several roles have been proposed for them, including control of protein hydrolysis and resistance to bacteria and insects.[1,2] It is important that the extent of their distribution in plants is examined further if their physiological roles are to be established. We have extended our studies to include plant tissues common in human diets because their inhibitors may affect man. It was established that sweet corn possesses considerable trypsin inhibitor activity, and the investigation was extended to include its isolation and characterization.

RESULTS

Inhibitory activities of aqueous extracts of various plant tissues are shown in Table 1. Potato and sweet corn were the most inhibitory, while fruits had negligible activity. Sweet potato was moderately active, but spinach, broccoli, Brussels sprouts and cucumber were appreciably inhibitory only at higher concentrations of the crude extracts. Although legume seeds generally are potent sources of trypsin inhibitors,[1] not all storage tissues in this study were inhibitory. Likewise, activity was not associated consistently with chlorophyll-containing tissues. There was no apparent pattern of distribution which might indicate the metabolic function of trypsin inhibitors in plants. However, their wide distribution indicates that they may have a physiological role.

Sweet corn inhibitor was extracted with 0·2 M NaCl and was precipitated by adding ammonium sulfate to 40% saturation at 4°. When the precipitate was dissolved in triethanolamine–NaCl buffer, pH 7·0, and the solution dialyzed against the buffer, some inactive precipitate formed. Passage of the supernatant through a Sephadex G75 column resolved

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[1] R. VOGEL, I. TRAUTSCHOLD and E. WERLE, *Natural Proteinase Inhibitors*, Academic Press, New York (1969).

[2] T. R. GREEN and C. A. RYAN, *Science* **175**, 776 (1972).

the isolate into three protein components with most of the inhibitory activity in the center band. When the component in the center band was chromatographed on a CM-cellulose column, one major protein band possessing most of the activity was obtained. This fraction was lyophilized and the residue was used for characterization studies. Table 2 shows the purification achieved with this procedure.

TABLE 1. TRYPSIN INHIBITION ACTIVITY OF CERTAIN FOOD PLANT TISSUES. PERCENTAGE REDUCTION IN TRYPSIN ACTIVITY INDUCED BY EXTRACTS

Plant tissue	mg fr. tissue per ml of assay mixture			
Potato (*Solanum tuberosum*)	67	82	89	90
Sweet corn (*Zea mays*, var. *saccharata*)	13	32	61	65
Sweet potato (*Ipomoea batatas*)	19	30	36	35
Spinach (*Spinacia oleracea*)	5	24	36	—
Broccoli (*Brassica oleracea*, var. *italica*)	2	15	34	40
Brussels sprouts (*Brassica oleracea*, var. *gemmifera*)	1	19	35	46
Cucumber (*Cucumis sativus*)	5	25	29	29
White radish (*Raphanus sativus*)	6	8	26	23
Red radish (*Raphanus sativus*)	7	8	10	8
Cabbage (*Brassica oleracea*, var. *capitata*)	—	4	8	—
Lettuce (*Lactuca sativa*)	2	4	6	—
Cauliflower (*Brassica oleracea*, var. *botrytis*)	3	6	9	—
Carrot (*Daucus carota*, var. *sativa*)	3	7	7	—
Asparagus (*Asparagus officinalis*, var. *altilis*)	3	—	—	10
Peach (*Prunus persica*)	8	—	—	7
Plum (*Prunus domestica*)	6	—	—	5
Anocado (*Persea americana*)	0	—	—	0

Disc gel electrophoresis of the isolated material showed the presence of a major component that possessed most of the inhibitory activity and a minor component that also was active. Repeated purification did not eliminate the minor component, and it was concluded that sweet corn contains two trypsin inhibitors. A similar result was found for soybean trypsin inhibitor.[3]

TABLE 2. PURIFICATION OF TRYPSIN INHIBITOR (ABSORBANCE AT 280 nm REPRESENTS PROTEIN CONCENTRATION. UNITS OF INHIBITOR REFLECTS DEPRESSION OF BAPA HYDROLYSIS BY TRYPSIN)

Purification stage	Absorbance at 280 nm	Units of inhibitor per assay	Specific activity	Relative purification
Crude extract	1·45	0·26	0·18	1·0
$(NH_4)_2SO_4$ precipitate	0·89	2·08	2·33	12·9
Sephadex G75	0·54	2·38	4·41	24·5
CM-Cellulose	0·41	2·21	5·40	30·0
CM-Cellulose, rechromatographed	0·50	2·62	5·24	29·1

A qualitative carbohydrate test showed the presence of carbohydrate, presumably as part of the inhibitor molecule because the purification procedure should have eliminated free carbohydrates. The inhibitor was heat stable. Assays with other proteolytic enzymes

[3] T. HYMOWITZ and H. H. HADLEY, *Crop Sci.* **12**, 197 (1972).

showed it to have little inhibitory effect against papain and moderate activity against chymotrypsin (Table 3). It acts as a non-competitive inhibitor of trypsin when examined by the double reciprocal method of Lineweaver–Burk.

TABLE 3. EFFECT OF SWEET CORN INHIBITOR ON ACTIVITY OF TRYPSIN, CHYMOTRYPSIN AND PAPAIN (CASEIN WAS USED AS THE SUBSTRATE)

Enzyme	Inhibitor concn. (mg/ml)	Absorbance at 280 nm	Absorbance decrease	Percentage inhibition
Trypsin	0	0·663	—	—
0·1 mg/ml	0·1	0·263	0·400	60
	0·2	0·220	0·443	67
Chymotrypsin	0	0·337	—	—
25 μg/ml	0·1	0·275	0·062	18
	0·2	0·202	0·132	39
Papain	0	0·158	—	—
2 mg/ml	0·1	0·166	—	—
	0·2	0·145	0·013	8

EXPERIMENTAL

Survey method. 20–60 g of fresh tissue were macerated with 200 ml H_2O for 1 min. Inhibitory activities of the extracts were measured by the method of Ramirez *et al.*[4] by determining the extent of hydrolysis of casein by trypsin in the presence and in the absence of the plant extracts. Hydrolysis was measured by adding a copper phosphate suspension to deproteinized hydrolysates and measuring absorbance of the resulting blue solution at 620 nm.

Isolation and characterization. 450 g of fresh sweet corn were macerated with 1 l. acetone for 1 min. The mixture was filtered through a Buchner funnel and the residue was washed with acetone until the yellow color was removed. The powder was suspended in 1 l. of 0·2 M NaCl and the mixture was agitated for 12 h at 4°. The extract was adjusted to pH 8 with NaOH and centrifuged at 5000 *g* for 20 min. The residue was re-extracted with 1 l. of 0·2 M NaCl and the two supernatants were combined. $(NH_4)_2SO_4$ was added to 40% saturation and, after 18 hr at 4°, was collected by centrifuging at 12 000 *g* for 30 min. The ppt. was suspended in 150 ml of 0·05 M triethanolamine-0·1 M NaCl buffer, pH 7·0. The suspension was stirred for 30 min and centrifuged. The residue was extracted again with 50 ml of the buffer and the two supernatants combined, and dialysed against the buffer for 48 hr at 4°. The dialyzed solution was centrifuged to remove inactive ppt. and the supernatant concentrated in a dialysis bag suspended in front of an electric fan. A 2·5 × 110 cm column of Sephadex G75 was equilibrated with 0·05 M triethanolamine–0·1 M NaCl buffer, pH 7. Elution of the inhibitor was accomplished at room temp. with the same buffer at a flow rate of 16 cm/hr. The eluate was collected in 5 ml fractions and the absorbance of each was determined at 280 nm. Inhibitory activity of each fraction was measured by method II of Erlanger *et al.*[5] using benzoyl-DL-arginine-*p*-nitroanilide (BAPA) as enzyme substrate. One trypsin unit was the amount of enzyme which liberated 1 μmol of *p*-nitroanaline per min from BAPA. Under the conditions of the assay, one unit was equivalent to 0·092 absorbance at 410 nm. One inhibitor unit was defined as the amount of inhibitor which depressed the reaction by one trypsin unit, or depressed absorbance at 410 nm by 0·092. Fractions with inhibitory activity were combined, dialyzed against H_2O for 24 hr and concentrated. The solution was placed on a CM-cellulose column prepared according to the method of Peterson and Sober.[6] Gradient elution was performed using 300 ml of the starting buffer (0·03 M phosphate, pH 6) in the mixing chamber and 300 ml of 0·3 M phosphate buffer, pH 8·5 in the reservoir. The active fractions were combined and re-chromatographed on the same CM-cellulose column. The active fractions were combined, dialyzed and lyophilized. Homogeneity of the isolate was studied by disc electrophoresis on 7% polyacrylamide gels by the method of Davis.[7] Carbo-

[4] J. S. RAMIREZ and H. L. MITCHELL, *J. Agric. Food Chem.* **8**, 393 (1960).
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[6] E. A. PETERSON and H. A. SOBER, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. 5, p. 3, Academic Press, New York (1962).
[7] B. J. DAVIS, *Ann. New York Acad. Sci.* **121**, 404 (1964).

hydrate content was examined by the method of Dubois *et al.*[8] Thermostability was established by heating a solution containing 0·5 mg of the isolate per ml H_2O at 100° under reflux. Samples were withdrawn after 0·5, 1, 2, 4 and 6 hr, and inhibitory activities were determined on cooling. Even after 6 hr at 100° 82% of the inhibitory activity remained. Chymotrypsin and papain inhibition were determined by the assay procedures of Kunitz,[9] using casein as a substrate and conducting the assays both with and without the isolated inhibitor.

[8] M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS and F. SMITH, *Analyt. Chem.* **28,** 350 (1956).
[9] M. KUNITZ, *J. Gen. Physiol.* **30,** 291 (1947).