Evidence, Isolation, Purification, and Some Properties of a Trypsin Inhibitor in *Lathyrus Sativus*

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Pulverized *Lathyrus Sativus* seeds were extracted with phosphate buffer (pH 7.5) and with distilled water. The extracts proved to have antiproteolytic activity when they were tested with trypsin. The heat labile nature of the inhibitor in the extracts could be observed. Subsequently, the trypsin inhibitory factor was isolated and partially purified from *L. Sativus* seeds. The heat lability and homogeneity of the isolated protein were also studied.

The presence of a trypsin inhibitor in soybeans and navybeans has been reported (Bowman, 1944; Ham and Sandstedt, 1944). The existence of a trypsin inhibitor in Indian pulses and vegetables has also been reported by different workers (Sohonie and Bhandarkar, 1954, 1955), in sweet potato (Sohonie and Honawar, 1956), and in field beans (Banerjee and Sohonie, 1969). The trypsin inhibitors have been isolated in pure crystalline forms from soybean (Kunitz, 1945, 1946, 1947) and from Indian field beans and double beans (Sohonie and Ambe, 1955). The preparation and the chemistry of the trypsin inhibitors have also been reviewed by Laskowski and Laskowski (1954) and Laskowski (1955).

The presence of a trypsin inhibitor in *Lathyrus Sativus* seed (L.S. seed) has so far not been reported, though Liener (1962) and Pusztai (1967) appear to have raised this question inconclusively. Sastry *et al.* (1963) reported that rats fed diets containing 50% or 100% L.S. powder showed significant reduction in growth rate, when compared with those of controls fed wheat- and maize-based diet. Rats fed 50% L.S. had a better growth rate than those fed on 100% L.S. diets. It has been observed (Esh and Som, 1952; Kuppuswamy *et al.*, 1958) also that autoclaving increases the digestibility of L.S. These observations suggest that L.S. may contain an antiproteolytic factor(s). Evidence for the presence of such an inhibitor in L.S. seeds is being reported in this paper.

EXPERIMENTAL

The Presence of Antiproteolytic Activity in Crude L.S. Extracts. The raw extracts from finely ground L.S. seeds were obtained by shaking 5 g of the pulse powder with 100 ml of 0.1 *M* phosphate buffer, at pH 7.5 [16 ml of NaH_2PO_4 (0.2 *M*), and 84 ml Na_2HPO_4 12 H_2O (0.2 *M*) diluted to 200 ml], or with 100 ml of distilled water for 3 hr using microid shaker at room temperature. The extracts were then centrifuged at 15° C and 700 × *g* for 20 min. The supernatants were diluted ten times and then used for the experiments; otherwise the inhibitions were extremely high.

Portions from the phosphate buffer extracts and water extracts were heated in boiling water bath for 10, 20, 30, 60, and 120 min and also autoclaved at 15 lb pressure for 15 and 30 min. The samples were cooled down to room temperature before being added to the reaction mixture for detecting the proteolytic activity.

Assay for Enzymatic Activity. The method developed by Kakade *et al.* (1969) was used for determining the trypsin inhibitory activity in the crude preparations (phosphate buffer and water extracts). A 2% casein solution in phosphate buffer (0.1 *M*, pH 7.6) was used as substrate, while the enzyme used was trypsin (E. Merck) (5 mg/ml). The incubation mixture consisted of 0.5 ml trypsin solution, 2 ml of 2% casein, phosphate buffer (pH 7.6, 0.1 *M*) of volumes 0.6 ml to 1.0 ml, HCl solution (0.001 *M*) of volumes 0.1 ml to 0.5 ml, L.S. extracts of phosphate buffer, and water extracts of volumes 0.2 ml and 0.4 ml. In all cases the total volume of incubation mixture were kept at 4 ml. Incubations were carried out at 37° C for 20 min after which 6.0 ml of 5% TCA solution was added to stop the reaction, and corresponding blanks were run concurrently. In this method one trypsin unit (TU) was arbitrarily defined as an increase of 0.01 absorbance unit at 280 mμ in 20 min for 10 ml of reaction mixture under the conditions described, and the trypsin inhibitory activity as the number of trypsin units inhibited (Kakade *et al.*, 1969).

Studies on the Isolation of Antitryptic Activity. Five hundred grams of L.S. seed powder were extracted with ether until the washings were clear and colorless. The washed powder was air dried and then treated with 4 l. of 0.25 *M* sulfuric acid stirred for 1–2 hr, centrifuged at 15° C and 700 × *g* for 20 min, and the supernatant (fraction I) was assayed for antitryptic activity. In the second step, fraction I was saturated with $MgSO_4 \cdot 7H_2O$ and was allowed to stand overnight for complete precipitation. It was filtered, the residue was taken up into 2 l. of 2.5% TCA solution, heated on waterbath for 5 min, and filtered. The filtrate (fraction II) was again saturated with $MgSO_4 \cdot 7H_2O$ and kept overnight for complete precipitation. The precipitate which was separated by centrifugation was taken in 100 ml water, and dialyzed for 18–20 hr against running cold water at 5° C. It was centrifuged at 15° C and 700 × *g* for 20 min. The supernatant was treated with 6 volumes of cold acetone and was kept in cold overnight. This was centrifuged at 15° C and 700 × *g* for 20 min, and the residue was taken in minimum quantity of water and lyophilized. The dry substance was dissolved in minimum quantity of water and was reprecipitated with 6 volumes of cold acetone and the residue was again lyophilized (fraction III).

In order to check the purity and also to see the heat lability of the final fraction, the method of Kakade *et al.* (1969) was used. In these experiments, the method was essentially the same as described earlier. However, the incubation mixture consisted of 0.5 ml trypsin solution, 2 ml of 2% casein, 1.0 ml phsophate buffer, (0.1 *M*, pH 7.6), 0.1 ml trypsin inhibitor solution, and 0.4 ml HCl, (0.001 *M*). Trypsin inhibitory units (TUI) were calculated as described earlier in the case of crude extracts.

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Table I. Antitryptic Activities in Both Phosphate Buffer and Water Extracts Along with the Activities on Heat Treatment of the Extracts

Nature of extract	Heat treatment min/nature	Control[a]	Trypsin used: 0.2 ml extract Enzymatic activity[b] (TU) 48.5	Trypsin used: 0.4 ml extract Enzymatic activity[b] (TU) 48.5
Phosphate buffer extract (pH 7.5)	0	WB	20.4 (57.9)	7.1 (85.4)
	10	WB	29.6 (39.0)	16.8 (63.3)
	20	WB	25.9 (26.0)	30.5 (37.1)
	30	WB	38.7 (20.1)	34.0 (29.9)
	60	WB	47.5 (2.1)	42.5 (1.02)
	120	WB	48.5 (0.0)	48.0 (1.0)
	15	A	38.5 (20.6)	36.5 (24.7)
	30	A	46.1 (4.9)	44.2 (8.7)
Water extract	0	WB	18.1 (62.7)	7.4 (84.7)
	10	WB	18.7 (59.4)	7.6 (82.3)
	20	WB	21.8 (55.0)	10.8 (77.7)
	30	WB	22.0 (54.6)	11.5 (76.3)
	60	WB	26.0 (46.4)	12.3 (74.6)
	120	WB	44.6 (8.0)	37.7 (20.2)
	15	A	33.0 (31.9)	33.6 (30.7)
	30	A	35.5 (26.8)	38.7 (20.2)

[a] Not heated. WB Stands for heated in boiling water bath; A stands for autoclaved at 15 lb pressure. [b] Enzymatic activity expressed as trypsin unit (TU). The values in parentheses refer to percent inhibition. Incubation mixture: 2 ml of 2% casein solution (pH 7.6), 0.5 ml of trypsin solution (5 mg/ml), phosphate buffer (pH 7.6, 0.1 *M*) 0.6 ml to 1.0 ml, HCl solution (0.001 *M*) 0.1 ml to 0.5 ml, and *L. Sativus* phosphate buffer and water extracts of volumes as shown in the table. Incubation was carried out at 37° C for 20 min.

In all these cases the protein concentrations were obtained by the method of Lowry *et al.* (1951). To check the purity and homogeneity of the final purified fraction which had the trypsin inhibitory property, paper electrophoretic separation was used. Different buffer systems having pH ranging from 2.2 to 9.0 were used and in each case, the voltage was maintained at 250 V and the running time was 3 hr. The staining solutions were bromophenol blue (0.1%) in methanol, and ninhydrin (0.1%) in acetone.

RESULTS

Studies with Crude L.S. Seed Extracts. Effect of Heating Buffer and Water Extracts of L.S. on Proteolytic Activity of Trypsin. Table I shows the results where trypsin was incubated with casein as substrate with or without L.S. seed phosphate buffer and water extracts raw (unheated), and after heat treatment for 10, 20, 30, 60, and 120 min, and autoclaving for 15 and 30 min. For each sample two levels of each extract were used. Enzyme activity was expressed in trypsin units. Original activity of the enzyme without the addition of the L.S. extract in the incubation mixture was treated as control value. Percent inhibitions were calculated after comparing with control values, and the data are given in Table I. It is seen that the raw (unheated) samples of phosphate buffer extract and the water extract have pronounced antiproteolytic activity. And the heated or autoclaved samples lose antiproteolytic activity progressively with increasing heating periods from 10 to 120 min and autoclaving at 15 and 30 min. The inhibition of the enzyme activity in the raw sample and the loss of antiproteolytic activity on heating or autoclaving appear to show the heat labile nature of trypsin inhibitor. All 0.4 ml extracts have more proteolytic inhibitory effect than all 0.2 ml levels.

Studies with Purified Fractions. Table II shows the stepwise fractionation of the 0.25 *M* sulfuric acid extract of L.S. seeds. Total trypsin inhibitory activity, fold increase of activity per mg of protein, and total volumes of fractions at the time of purification are shown in this table. The yield of the purified protein (fraction III) having maximum trypsin inhibitory activity was 58 mg per 100 g of L.S. seeds. The final product was found to be a buff colored, slightly lustrous, and light powdery substance. A 26.4 times purification was observed when a comparison of the trypsin inhibitory units per mg of protein (TUI/mg protein) of the fractions is made.

To test the thermolability of the final purified protein (fraction III), a solution of the fraction (1 mg/ml) in water was prepared. Portions from this solution were heated in water-bath for different timings up to 60 min. The effect of heat treatment with time and the percentage inhibition obtained are depicted in Figure 1. This shows that the fraction III from L.S. seeds has a high trypsin inhibitory activity and is thermolabile. The trypsin-inhibitory property of the above fraction is reduced gradually with increase of heating time.

When fraction III was subjected to paper electrophoresis at 250 V for 3 hr, with different buffers of pH ranging from 2.2 to 9.0, only one single protein spot could be found which could be detected by bromophenol blue. In case of bromophenol blue reaction, a green spot in acetic acid was obtained which, on exposure to ammonia vapor, turned blue. With ninhydrin, the spot gave a very faint ninhydrin positive reaction. The mobilities of the protein are shown in Table III.

The nitrogen content of the protein (fraction III) was found to be 12.09% on dry weight basis (Micro-Kjeldahl Method).

DISCUSSION

The presence of antitryptic activity in L.S. has been demonstrated for the first time. The antiproteolytic activity was demonstrable in water extracts and extracts of phosphate

Table II. Isolation and Purification Steps for Trypsin-Inhibitor from L.S. Seeds

Fractions	Total volume (ml)	Protein (mg/ml)	TUI[a] per 0.1 ml	Total TUI	TUI/mg protein	Fold increase
I (H_2SO_4 extract)	3300	18.5	32.6	1,075,800	17.62	1
II (TCA extract)	1170	1.2	29.1	340,000	242.50	13.7
III Lyophilized acetone precipitated	290 mg	70%	32.6	94,540	465.71	26.4

[a] TUI stands for trypsin inhibitory unit. For TUI activity, incubation mixture: 0.5 ml trypsin solution (5 mg/ml), 2 ml of 2% casein, 1.0 ml phosphate buffer, 0.1 ml of the fraction [concentration for fraction III solution (1 mg/ml)] and 0.4 ml of HCl (0.001 *M*) solution. Incubation was carried out at 37° C for 20 min.

Table III. The Electrophoretic Mobility of Trypsin Inhibitor (Fraction III) at Different pH

Buffer system	pH	Mobility cm/250 V/3 hr	Polarity
(1) Pyridine:acetic acid:water 5:0.5:94.5%	3.6	2.9	towards cathode
(2) Formic acid:pyridine: water 4:0.3:95.7%	2.2	2.0	-do-
(3) Pyridine, glacial acetic acid and water (Smith, 1960)	5.3	1.4	-do-
(4) Pyridine, glacial acetic acid, and water (Smith, 1960)	6.5	0.9	-do-
(5) Sodium borbital, and HCl buffer (Colowick and Kaplan, 1955)	9.0	1.2	towards anode

The trypsin inhibitor moves as a single entity under these experimental conditions.

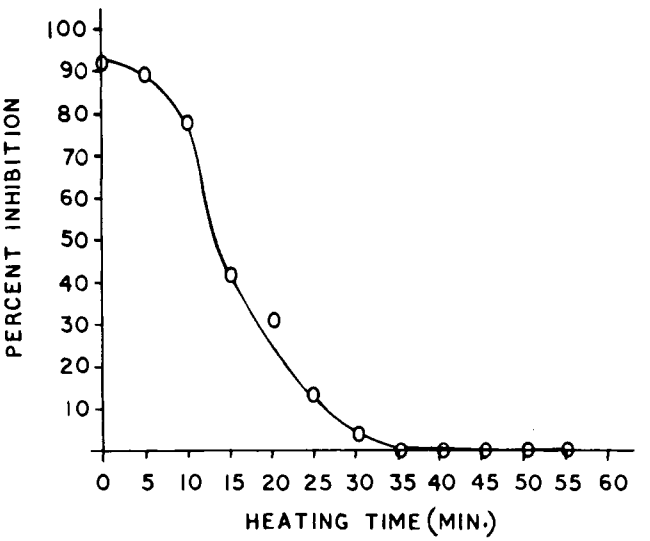

Figure 1. Effect of heat on tryptic inhibitory property of purified fraction III. For the assay of trypsin inhibition, incubation mixture: Same as described in Table II. Incubation was carried out at 37° C for 20 min

buffer when incubated with trypsin under standardized conditions. The inhibitory activity could be destroyed almost completely by heating up to 120 min. Autoclaving up to 30 min brought about destruction of the inhibitory activity to a major extent. This was evident in the case of both the water extracts and extracts of phosphate buffer. It was also shown that in all cases 0.4 ml extract had more inhibitory property than that with 0.2 ml extract.

Purification of the isolated trypsin inhibitor yielded a buff colored lustrous powder which, on paper electrophoresis at different pH's, showed it to be a single protein component, with a nitrogen content of 12.09%, on dry weight basis, and which value is close to the values of other proteins having trypsin inhibitory activity described by Liener and Kakade (1969). The purified inhibitor was thermolabile and was found to be four times more susceptible to heat treatment than the crude preparations.

The excessive consumption of L.S. is known to be responsible for the causation of the crippling paralysis Lathyrism, which is prevalent in parts of central India (Ganapathy and Dwivedi, 1961; Nagarajan, 1969; Sarma and Padmanaban, 1969). The presence of an unusual amino acid, *N*-oxalyl-α, β-diamino propionic acid with pronounced neurotoxic properties when administered to experimental animals (Nagarajan *et al.*, 1963; Adiga *et al.*, 1963), could be a major factor responsible for human lathyrism. The role of other factors present in L.S. in the causation of the disease in human subjects is still not completely understood. The presence of a trypsin inhibitor in L.S. could perhaps assume some significance since the population ingesting this legume as a staple consume it as unleavened bread. The heat treatment in the preparation of such unleavened bread may not be adequate to result in the complete destruction of the inhibitor (unpublished data indicate the destruction is only up to 48%).

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