technique is to be published later. It is observed that the naturally occurring carotenoids were not a source of appreciable error in the fluorometric methods.

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Further Purification, Fractionation, and Properties of Trypsin Inhibitor Isolated from *Lathyrus sativus*

Dwijendra N. Roy

The trypsin inhibitor isolated from *Lathyrus sativus* was further purified and fractionated by DEAE cellulose column into five proteins with high trypsin inhibitory activity and a sixth protein with negligible activity. The first five proteins could be eluted in one block at pH 7.0, while the sixth was eluted at pH 3.6. The five-protein fraction IV(A) on dialysis and lyophilization resulted in a yield of 26.2 mg per 100 g of original seed. This fraction, IV(A), showed five distinct protein bands by disc acrylamide gel electrophoresis.

Isolation and partial purification of a specific trypsin inhibitor from *Lathyrus sativus* (L.S.) and some of its properties were reported by Roy and Rao (1971). The partially purified trypsin inhibitor showed a growth depressing activity in rats (Roy, 1972).

The present report describes further purification and properties of this inhibitor.

EXPERIMENTAL

Preparation of Material. The trypsin inhibitor was isolated and partially purified by the method reported earlier (Roy and Rao, 1971) and was designated as Fraction III.

DEAE-Cellulose Chromatography. Columns of 75.5 × 1.5 cm were prepared with DEAE–cellulose (Whatman column chromedia, De 11, medium fibrous powder, normal capacity 1.0 mequiv/g), washed with distilled water before use. The column was first equilibrated for 48 hr with 0.05 *M* phosphate buffer, pH 7.0, at 25°C; 32.6 mg of partially purified inhibitor (Fraction III) dissolved in 5 ml of phosphate buffer, pH 7.0, was loaded on the column, eluted first with 0.05 *M* phosphate buffer of pH 7.0, and then subsequently with phosphate–citrate buffer of pH 3.6. The flow rate was maintained at 45.5 ml/hr and 6.2-ml fractions were collected. Fractionation on the DEAE–cellulose column was repeated at a higher load (62.4 mg) of the inhibitor and the reproducibility of the pattern of elution was confirmed.

Disc Gel Electrophoretic Studies. Disc gel electrophoresis was conducted at pH 8.3 using 7.5% acrylamide system as described by Davis (1964). The effect of urea on the preparation was studied by using 4.6 *M* and 8.0 *M* urea in all the solutions and buffers of pH 8.3. Electrophoresis was carried out by applying 165–200 μg of protein sample, at 5 mA per tube for 1.5 hr, using Bromophenol blue as the indicator for the moving front. After each run, the gels were stained with 1% Amido Black in 10% acetic acid, subsequently destained by repeated washings with 10% acetic acid until the clear protein bands were visible.

Protein Determination. Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Test for Carbohydrates. The purified fraction was subjected to hydrolysis by the method of Miyata *et al.* (1970). A 30-mg sample was hydrolyzed with 3 ml of 2 *N* H_2SO_4 by refluxing for 24 hr. The hydrolysate was neutralized with $Ba(OH)_2$ and then centrifuged. The clear supernatant was evaporated to a minimum volume. The presence of carbohydrates in this solution was examined by the Molisch test (Vogel, 1957).

Assay for Enzyme Activity. The inhibitory activity of the fractions was determined by the method described earlier (Kakade *et al.*, 1969; Roy and Rao, 1971). 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, about 20,000 Fuld-Gross units per gram) (5 mg/ml). The incubation mixture consisted of 0.5 ml of trypsin solution, 2 ml of 2% casein, 1.0 ml of phosphate buffer (pH 7.6, 0.1 *M*), 0.3 ml of HCl (0.001 *M*) solution and 0.2 ml test solution. The total volume was 4 ml in each case. Incubation was carried out at 37°C for 20 min, after which 6.0 ml of 5% TCA solution was added to stop the reaction. Corresponding blanks were run concurrently. In all these experiments, 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

National Institute of Nutrition, Indian Council of Medical Research, Jamai-Osmania, Hyderabad-7 (A.P.), India.

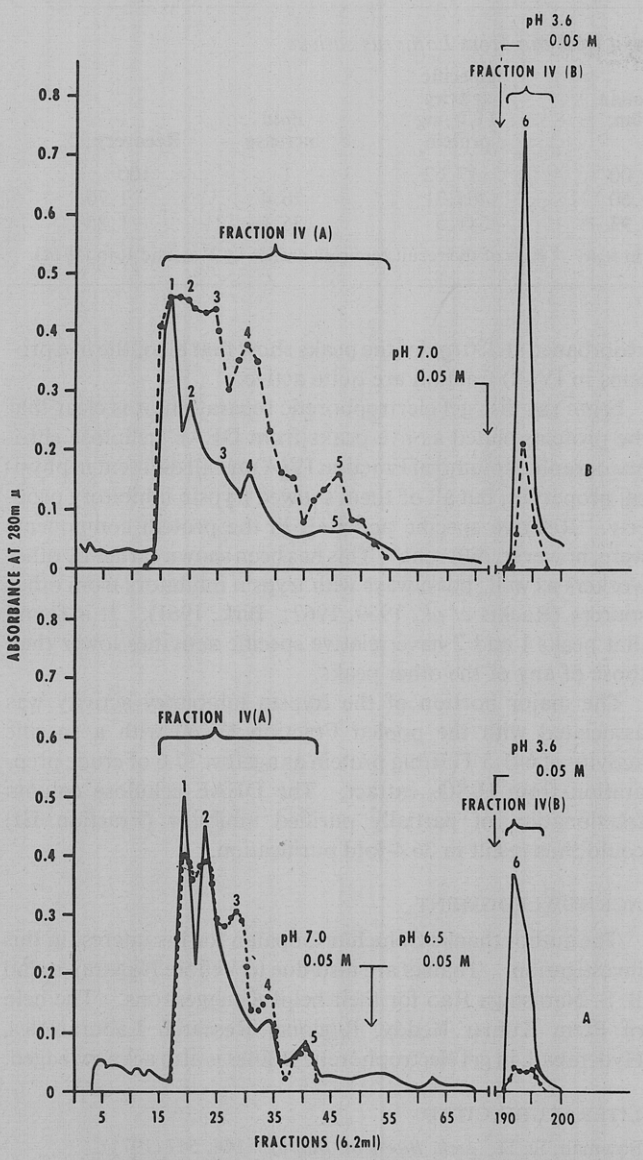

Figure 1. Chromatography of trypsin inhibitor (Fraction III) on a DEAE–cellulose column. Solid line indicates the protein content in the effluents, and dotted lines represent trypsin inhibitory activity. A: 32.6 mg of Fraction III in 5 ml of solution was applied. B: 62.4 mg of Fraction III in 5 ml of solution was applied

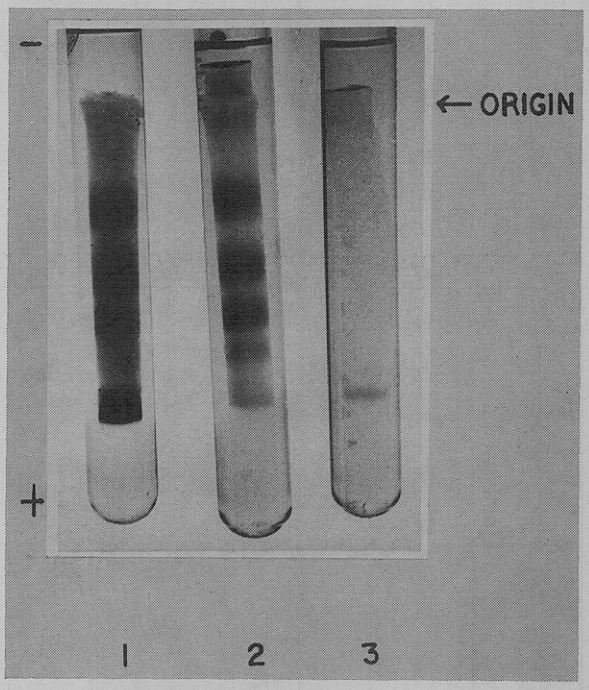

Figure 2. Polyacrylamide gel electrophoretic patterns of fractions of trypsin inhibitor before and after purification and fractionation through DEAE–cellulose column. 1: Fraction III before chromatographic separation. 2: Fraction IV(A), containing five active peaks. 3: Fraction IV(B), containing a single peak with low activity

Table I. Relative Specific Activities of the Peaks Eluted from DEAE–Cellulose Column

Fractions	Peak	Relative specific activity
19	1	90.0
23	2	98.2
28	3	225.8
34	4	238.5
40	5	191.4
192	6	12.5

relative specific activity of peak = trypsin units inhibitor (TUI)/ absorbance at 280 mμ
(Mikola and Suolinna, 1969)
Trypsin units inhibited: for assay of trypsin inhibition, incubation mixture was the same as described in text.

mixture under the conditions mentioned and the trypsin inhibitory activity as the number of trypsin units inhibited (TUI).

RESULTS

DEAE–Cellulose Column Chromatography. Figure 1 shows the elution profile of the protein components in the partially purified fraction (Fraction III). The elution pattern was reproducible when two levels of the protein were loaded, as shown by A and B in the figure. As measured by absorbance at 280 mμ, maximum protein was eluted out in Fraction IV(A) when 0.05 *M* phosphate buffer of pH 7.0 was used as an eluent. A comparatively small amount of protein in Fraction IV(B) was eluted out when the eluent was 0.05 *M* phosphate citrate buffer, pH 3.6. Fraction IV(A) consisted of five protein peaks, which, however, was not completely resolved. Fraction IV(B) consisted of only one protein peak. The relative specific activities of the five protein fractions in Fraction IV(A) and the single protein in Fraction IV(B) are shown in Table I. Almost all the trypsin inhibitory activity is located in five protein components in Fraction IV(A). The trypsin inhibitory activity in Fraction IV(B) is negligibly small.

The other properties are shown in Table II.

Disc Gel Electrophoresis. On acrylamide gel electrophoresis, Fraction IV(A) was seen to resolve into five distinct protein bands, while Fraction IV(B) gave rise to a single protein band (Figure 2). The resolution of Fractions IV(A) and IV(B) is shown in numbers 2 and 3, respectively, while No. 1 indicates the separation of partially purified trypsin inhibitor (Fraction III). The pattern of protein bands observed on gel electrophoresis was closely similar to protein fractions eluted from DEAE–cellulose column. When electrophoresis was conducted in 8 *M* and 4.6 *M* urea, the same pattern of protein bands was observed in Fractions IV(A) and IV(B).

Enzymatic Activity. Figure 3 shows the pattern of trypsin inhibition with the increase in concentration of the trypsin inhibitor, IV(A) in the medium. It was observed that 6.6 μg of Fraction IV(A) could result in 50% inhibition of 1250 μg of trypsin. This worked out that 1 μg of Fraction IV(A) could cause 50% inhibition of 189 μg of trypsin, or 3.07 TU. For

Table II. Purification Steps of the Trypsin Inhibitor from *Lathyrus sativus*

Active fractions	Yield per 100 g of L.S. seeds, mg	Protein, %	Total protein in fraction, mg	Specific activity TUI[a]/mg protein	Fold increase	Recovery, %
H_2SO_4 extract[b]	...	...	61,050.00	17.62	1	100
Fraction III[b]	58.00	70	40.60	465.71	26.4	1.76
Fraction IV(A)	26.17	80	20.93	641.5	36.4	1.25

[a] TUI stands for trypsin units inhibited. Assay method same as described in text. [b] Part of the result reported earlier by Roy and Rao (1971).

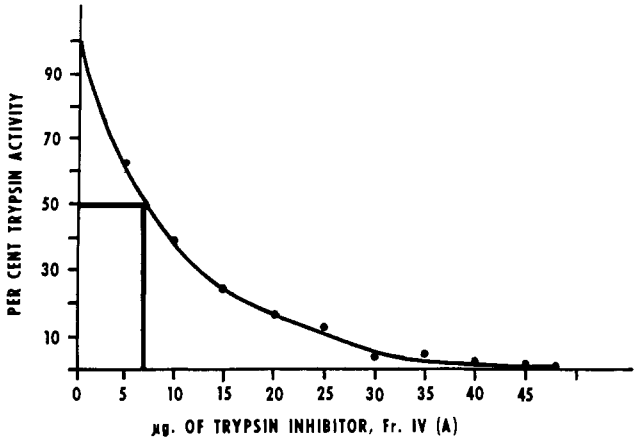

Figure 3. Activity curve of Fraction IV(A). Method described in text. Incubation mixture: 0.5 ml of trypsin solution (2.5 mg/ml); 0.5 ml of phosphate buffer, pH 7.6, 0.1 *M*; 2.0 ml of casein solution (2%); trypsin inhibitory solution, Fraction IV(A) (50 μg/ml) of volumes ranging from 0 to 0.9 ml. Total volumes maintained at 4.0 ml

complete inhibition, 1 μg of Fraction IV(A) was equivalent to 27.7 μg of trypsin. Therefore, for complete inhibition, the Fraction IV(A):trypsin was 1:28. The trypsin that was used in these experiments had 20,000 TU/g (or 20 TU/mg of the enzyme), while the trypsin inhibitor IV(A) was found to have a specific activity (TUI/mg protein) of 641.5. Considering 80% protein of the Fraction IV(A), the TUI/mg of fraction was found to be 513.2. It shows that 1 unit of trypsin to 1 unit of trypsin inhibitor IV(A) had a 26 to 1 ratio.

Carbohydrate Content. No carbohydrate could be detected in Fraction IV(A).

DISCUSSION

Extensive work has been done on trypsin inhibitors from soybeans (Kunitz, 1945, 1946, 1947, 1948; Bowman, 1946; Birk, 1961; Birk *et al.*, 1963; Rackis *et al.*, 1959, 1962; Rackis and Anderson, 1964; Yamamoto and Ikenaka, 1967; Fretalli and Steiner, 1968). It is evident that there are at least five and possibly six proteinase inhibitors in soybean (Liener and Kakade, 1969), six components in lima beans (Fraenkel-Conrat *et al.*, 1952; Tauber *et al.*, 1949; Jones *et al.*, 1963; Haynes and Feeney, 1967), and five components of trypsin inhibitors in navy beans (Bowman, 1971). Increasing evidence appears to be forthcoming to show that there is more than one protein component in tryspin inhibitors from other sources as well.

The partially purified protein having significant trypsin inhibitory activity from L.S. seeds has been reported earlier, and herein it was shown to be single protein on the basis of paper electrophoretic studies (Roy and Rao, 1971). However, it could be further resolved into six clear protein bands in gel electrophoretic separation. The ratios of the activity to absorbance at 280 mμ of the peaks show that all of the five proteins in IV(A) fraction are quite active.

From the disc gel electrophoretic separation, it is clear that the proteins eluted as five peaks from DEAE-cellulose chromatographic column in Fraction IV(A) are all different in physical properties, but all of them showed trypsin inhibitory property. Relative specific activities in the protein components were, however, different. This has been shown earlier by other workers as well, but always with trypsin inhibitors from other sources (Rackis *et al.*, 1959, 1962; Birk, 1961). It is found that peaks 1 and 2 have relative specific activities lower than those of any of the other peaks.

The major portion of the trypsin inhibitory activity was associated with the pooled Fraction IV(A) with a specific activity of 641.5 TUI/mg protein as against 17.6 of crude preparation from H_2SO_4 extract. The DEAE-cellulose column fractionation of partially purified inhibitor (Fraction III) could thus result in 36.4-fold purification.

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