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Trypsin Inhibitor from *Lathyrus sativus* Seeds: Final Purification, Separation of Protein Components, Properties, and Characterization

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The trypsin inhibitor fraction LSTI-B″ from *Lathyrus sativus* seeds was purified to homogeneity as shown by gel filtration, sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis, and analytical ultracentrifugation, all showing that the molecular weight was 22 000. The sedimentation coefficient, $S_{20,w}$, was 2.0S and the isoelectric point was at pH 6.2 ± 0.2. No self-aggregation could be noticed by running column chromatography in Sephadex G-75 eluted with 4 M guanidine hydrochloride and also 8 M urea. The specific activity of LSTI-B″ was 1900 trypsin units inhibited/mg of protein with a 108-fold purification. The N-terminal amino acid of the protein was found to be glycine. The percent nitrogen in the protein was 15.38. When LSTI-B″ was subjected to gel electrophoresis in the absence of $NaDodSO_4$ in Tris–glycine buffer system at pH 8.3 five major protein bands could be demonstrated. The nonaggregation nature of the bands could be demonstrated by subjecting the fraction to gel electrophoresis in the absence of $NaDodSO_4$ with increasing gel concentration. The five proteins were taken to be "charge isomers"; they could be separated on a DEAE–cellulose column where a gradient was established between pH 9.0 and 6.0. The properties of these isomers are presented.

The presence of a trypsin inhibitor in *Lathyrus sativus* seeds was reported earlier by Roy and Rao (1971). Its partial purification, characterization, and biological properties were also reported in earlier communications (Roy and Rao, 1971; Roy, 1972a,b). The partially purified protein fraction IV(A) was shown to have five distinct protein components as demonstrated by differences in mobilities in polyacrylamide gel electrophoresis in the absence of sodium dodecyl sulfate ($NaDodSO_4$) (Roy, 1972a). This report deals with attempts to further purify and separate the active protein components, all having trypsin inhibitory property, in order to elucidate their physicochemical and biological properties.

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EXPERIMENTAL SECTION

Materials. Sephadex G-75 (for gel filtration), CM-Sephadex (C-50), and blue dextran 2000 were obtained from Pharmacia (Uppsala, Sweden), DEAE–cellulose chromedia (DE 11) medium fiber powder, nominal capacity 1.0 mequiv min^{-1}/g^{-1} obtained from Whatman Ltd. (England); trypsin, 213 units/mg (twice crystallized), and ovalbumin (twice crystallized) from Worthington Biochemical Corporation (Freehold, NJ); trypsin (about 20 000 fluid-gross unit/g), 1-fluoro-2,4-dinitrobenzene (FDNB), and glycine from E. Merck (Germany); vitamin-free casein from Nutritional Biochemicals Corporation (Cleveland, OH); ribonuclease (bovine, pancreas), myoglobin crystals, salt-free (lyophilized) from Schwarz/Mann (Orangeburg, NY); bovine serum albumin, β-mercaptoethanol (MCE), dansyl chloride, coomassie brilliant blue-R, hemoglobin,

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Table I. Purification Steps of the Trypsin Inhibitor from *Lathyrus sativus* Seeds[a]

purification step	active fractions	yield per 100 g of seeds, mL/mg	total protein, mg	total protein %	sp act., TUI/mg of protein	purificat., fold increase	recov. of total TUI activity, %
1	H_2SO_4 extract[c] (crude)	640	12210[b]		17.6	1	100
2	trichloroacetic acid extract[c] (fraction II)	354	425		242.5	13.7	47.09
3	lyophilized[c] acetone pptd (fraction III)	58	40.6	70	465.7	26.4	8.80[d]
4	DEAE-cellulose[c] column chromatography (fraction IV(A))	26.2	20.98	80	642.0	36.4	6.26[d]
5	Sephadex G-75 column chromatography (fraction LSTI-B″)	7.6	7.3[b]	96.2	1900.0	108.0	6.25

[a] Details of the purification methods and analysis are given in the text. [b] Total protein was calculated from nitrogen content determined by Kjeldahl method (N × 6.25). [c] Part of the values reported earlier (Roy and Rao, 1971; Roy, 1972a). These are reported for comparison. [d] The values given in earlier publication (Roy, 1972a) were recalculated and the corrected values are given here.

alcohol dehydrogenase, and cytochrome C from Sigma Chemical Co. (St. Louis, MO); acrylamide (for electrophoresis) and *N,N′*-methylene bisacrylamide from Eastman Kodak Co. (Rochester, NY); *N,N,N′,N′*-tetramethylenediamine (temed), and ammonium persulfate from E. C. Apparatus Corporation (Philadelphia, PA); sodium dodecyl sulfate ($NaDodSO_4$) and bromophenol blue from British Drug House Chemicals Ltd. (Poole, England); tris(hydroxymethyl)aminomethane (Tris) from Light & Co. Ltd. (Colnbrook, England); guanidine hydrochloride from Riedel-De Haën Ag (Germany). Other reagents used were chemically pure unless otherwise mentioned.

Methods. Preparation of the partially purified trypsin inhibitor: The trypsin inhibitor was isolated and partially purified by the method described earlier (Roy and Rao, 1971; Roy, 1972a), and the fraction used was designated as fraction IV(A) (Table I). The final fraction was dialyzed aganist a large volume of distilled water at 4 °C for 24 h. The dialyzed material was lyophilized.

Column Chromatography. *Sephadex Column.* Chromatography was carried out using a column (81 × 2 cm) of Sephadex G-75, which was equilibrated with Tris-HCl buffer, pH 8.0, 0.01 M. Fraction IV(A) weighing 165.0 mg in 3-mL volume of the above buffer was fed to the column. Fractions (3.5 mL) were collected at a flow rate of 31 mL/h and analyzed for trypsin inhibitory activity. Fractions having antitryptic activity were dialyzed against distilled water in cold for 20–24 h and lyophilized (LSTI-B″). For further purification the chromatographic method was repeated. Other column chromatographic runs were made in 4 M guanidine hydrochloride buffer, pH 8.5; in 8 M urea, pH 7.75 solutions: in 0.1 M sodium phosphate buffer, pH 7.2; in Tris–glycine buffer, pH 8.3; and 0.05 M sodium acetate buffer, pH 5.0. In all these runs, the column dimension was kept almost constant.

In (80 × 2 cm) in Tris-HCl buffer system at pH 8.0, 0.01 M, the molelcular weight of the protein was determined using internal molecular weight standards, such as, bovine serum albumin, ovalbumin, myoglobin, ribonuclease, and cytochrome *c* as per the method described by Andrews (1964).

DEAE-Cellulose Column. In order to fractionate the purified protein into components, DEAE–cellulose column (82.5 × 2.0 cm) was used. Activated DEAE–cellulose was equilibrated in the column of the above dimensions with 0.05 M Tris-phosphate buffer, pH 9.0. The fraction LSTI-B″ weighing about 100 mg in the above buffer was fed to the column. A pH gradient was established between pH 9.0 and 6.0, both 0.05 M. Flow rate was maintained at 70 mL/h, and 3.5-mL fractions were collected and analyzed for trypsin inhibitory activity. The active fractions were pooled together for processing. DEAE–cellulose columns of the above dimensions equilibrated with sodium phosphate buffer, pH 7.0, 0.05 M, and another chromatographic run was conducted on DEAE–cellulose column preequilibrated with phosphate citrate buffer, pH 5.0, 0.07 M, and eluted by a pH gradient between pH 5.0 and 7.0, 0.07 M.

Assay of the Enzyme Activity. The inhibitory activity of the fractions was determined by the method described earlier (Roy and Rao, 1971), using 2% casein solution in sodium phosphate buffer, pH 7.6, 0.1 M, as substrate and trypsin as the enzyme. The test solutions were diluted suitably, so that the desired volume would show an inhibition in the range of 40 to 60%. In all these experiments, one trypsin unit (TU) was arbitrarily defined as an increase of 0.01 absorbance at 280 nm in 20 min for 10 mL of reaction mixture under the conditions mentioned and the trypsin inhibitory activity as the number of trypsin units inhibited (TUI). For the estimation of trypsin inhibitory activity of the fractions from the chromatographic columns, trypsin (20000 fluid gross units/g) was used, whereas for determining the stoichiometry, twice crystallized trypsin was used.

Protein Estimation. Protein was estimated by the method of Lowry et al. (1951), using bovine serum albumin (BSA) as standard unless mentioned otherwise. The Kjeldahl method was used in some cases for estimation of total nitrogen (Hawk's Physiological Chemistry, 1965).

Disc Gel Electrophoresis. Disc gel electrophoresis was carried out at pH 8.3 using 7.5% acrylamide system as described by Davis (1964). Also, disc gel electrophoresis with varying gel concentrations were carried out as described by Hedrick and Smith (1968). For $NaDodSO_4$ gel electrophoresis the method described by Weber and Osborn (1969) was followed. This was also followed for the determination of molecular weight of the protein, using proteins like bovine serum albumin, myoglobin, ovalbumin, alcohol dehydrogenase, and hemoglobin as standards. For staining the protein bands, either amido black or coomassie blue solution was used as described by the above-mentioned methods. In some cases either mercaptoethanol (MCE) or dithiothreitol (DTT) was used to provide a reduced condition to the electrophoretic systems.

Sedimentation Velocity. These measurements were made with a Spinco Model E ultracentrifuge fitted with phase plate, Schlieren optics, and TRIC unit. The ex-

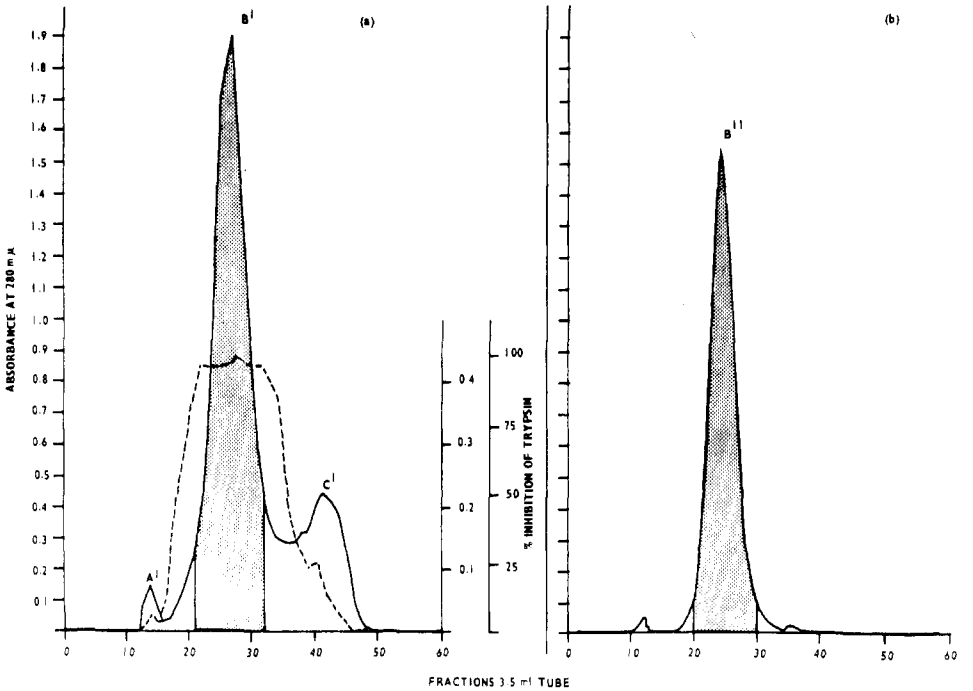

Figure 1. Chromatography of *Lathyrus sativus* trypsin inhibitor, fraction IV(A), on Sephadex G-75 column. Experimental conditions and assay method for anti-tryptic activity are described in the text. The solid line indicates the protein elution pattern and the dotted line indicates antitryptic activity. Fractions pooled together for rechromatography are shown in the shaded portion. The fractions designated as B′, Figure 1a are pooled together and the rechromatographed fractions are shown as B″, Figure 1b.

periment was conducted at only one protein concentration (1% solution in 0.01 M Tris-HCl buffer, pH 8.0). A standard 12-mm aluminum cell center-piece was used. Centrifugation was followed for 120 min at 59 780 rpm. Apparent sedimentation coefficient was finally corrected to standard conditions ($s_{20,w}$) (Schachman, 1959).

Molecular weight was determined by the Archibald method as described by Klainer and Kegeles (1955). The measurements were made at 25 ± 0.5°C. Photographs were taken 16, 32, and 48 min at bar angle 80°. Partial specific volume for calculation of molecular weight was assumed to be 0.750. For obtaining the concentration of the protein solution in terms of refractive index units, a double sector synthetic boundary cell (Spinco no. 6076-3) was used. Molecular weight reported here is the average of values calculated from three photographs.

Isoelectric Point. To determine the isoelectric point of the purified protein, the method described by Lampson and Tytell (1965) was followed. One milliliter of a 1% solution of LSTI-B″ in 0.1 M sodium phosphate buffer, pH 6.0, was applied to a 1.5 × 10 cm column of CM-Sephadex (C-50).

Stoichiometry. Pure protein solutions of the fractions were taken in water ranging from 0.0466 to 1.0 mg/mL, while purified trypsin (twice crystallized, Worthington Biochemical Corp.; 125 μg/mL) was used for the stoichiometric determinations. Incubation for activity was maintained in the conditions described under assay method.

Extinction Coefficient. Protein solution of the components of the purified protein (LSTI-B″) was made in sodium phosphate buffer, pH 7.0, 0.05 M. The protein concentrations were in the range of 0.5 to 1.0 mg/mL.

Determination of N-Terminal Amino Acid. *I. Dinitrophenyl (DNP) Method.* N-terminal amino acid was determined by the method of Fraenkel-Conrat et al. (1955). For this, 4.4 mg of purified fraction (LSTI-B″) was used.

II. Dansyl (DNS) Method. The method used for dansylation was similar to that of Gray (1967) and Blackburn (1970). For this, 2 mg of LSTI-B″ was used.

Amino Acid Analysis. The separated protein components from LSTI-B″ were lyophilized and dried finally over P_2O_5 and were used for amino acid analysis. Each 1 mg in 1 mL of constant boiling HCl (5.7 N) was heated in evacuated sealed tubes for 24, 48, and 96 h at 110 °C. The hydrolysates were evaporated to dryness in vacuo and analyzed in an automatic amino acid analyzer employing conditions similar to those described by Spackman et al. (1958), but using Beckman special resins PA 25 and PA 38. Cystine was determined as cysteic acid after performic acid oxidation (Moore, 1963). Tryptophan content was estimated spectrophotometrically (Edelhoch, 1967).

RESULTS

The purification steps of the trypsin inhibitor from *Lathyrus sativus* seeds are summarized in Table I. There was a 108-fold increase in activity following its purification on Sephadex G-75 column. Chromatographic elution profile of the proteins are shown in Figure 1. The partially purified fraction IV(A) could be resolved into three peaks, shown as A′, B′, and C′. Of these protein peaks, only one major peak, B′, had antitryptic activity (Figure 1a). On rechromatography, the fraction B′ continued to show a single peak designated as B″ (LSTI-B″) (Figure 1b). The homogeneity of B″ was maintained when eluted with different buffer systems described earlier. As shown in Figure 2, the protein peak is coincidental with the peak of antitryptic activity obtained on final chromatography on Sephadex G-75 columns and the activity was constant across the peak. Rechromatographing LSTI-B″ on the Sephadex G-75 column in the presence of internal molecular weight standards yielded an apparent maximum molecular weight for LSTI-B″ of 22 000.

LSTI-B″ obtained in this fashion (Table I), when subjected to polyacrylamide gel electrophoresis with NaDodSO$_4$ only, showed the presence of a single band (Figure 3), indicating that the protein was pure as judged by this criterion as well. On the basis of mobility, the molecular

Table II. Disc Gel Electrophoretic Patterns of LSTI-B″ in Different Experimental Conditions[a]

pH	in buffer system	staining dye used	MCE[b]	DTT	$NaDodSO_4$	pattern of separations
8.3	Tris-glycine	amido black	−	−	−	5 bands
8.3	Tris-glycine	amido black	+	−	−	5 bands
7.2	sodium phosphate	coomassie blue	+	−	+	1 band
8.3	Tris-glycine	amido black	−	+	−	5 bands
7.2	sodium phosphate	amido black	−	+	+	1 band

[a] Details of the methods are given in the text. [b] (+) with; (−) without.

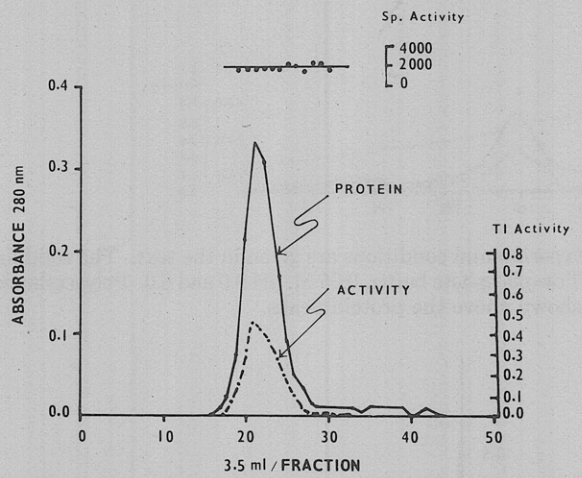

Figure 2. Rechromatography of fraction B″ on Sephadex G-75 column. Experimental procedure and assay method are described in the text. The solid line and dotted lines indicate the protein elution and activity, respectively. The specific activities of the fractions are also plotted.

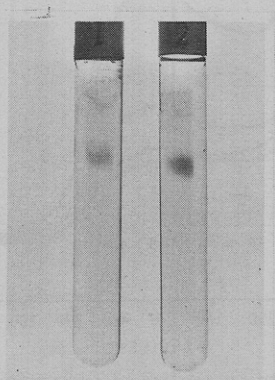

Figure 3. $NaDodSO_4$ gel electrophoresis of LSTI-B″. Separation pattern of 20- and 50-μg quantities of LSTI-B″ in 1 and 2 are shown. Methods of separation and condition of staining are described in the text.

weight was determined using internal standards which yielded a molecular weight of LSTI-B″ as 22 000.

When LSTI-B″ was subjected to analytical ultracentrifugation for 120 min under conditions described earlier, a single symmetrical peak (Figure 4) was obtained, showing again the homogeneous nature of the fraction. The sedimentation coefficient ($s_{20,w}$) was found to be 2.0S. The weight average molecular weight obtained by the Archibald method was found to be 22 400 ± 1500.

LSTI-B″ which showed its homogeneous nature by the above three methods, when subjected to gel electrophoresis under the conditions described earlier in Tris–glycine buffer system at pH 8.3 (Roy, 1972a), displayed five protein bands (Figure 5). The bands as eluted from the gels separately as described by Weber and Osborn (1975) were found to be active against trypsin. The mobility of each band at each acrylamide concentration relative to the dye front was determined (R_f), and when log R_f values were

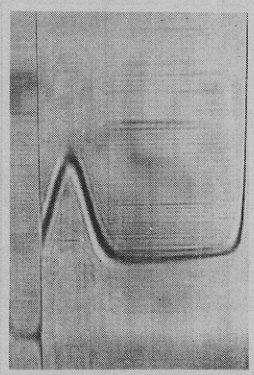

Figure 4. Sedimentation velocity pattern of LSTI-B″. The analytical procedure is described in the text. Sedimentation proceeds from left to right; the photograph was taken 65 min after reaching maximum speed at bar angle 45°.

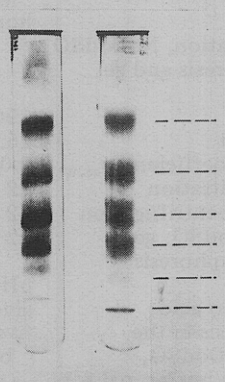

Figure 5. Polyacrylamide gel electrophoretic pattern of LSTI-B″. Experimental methods are described in the text. About 50- and 70-μg quantities of protein were administered as shown in 1 and 2, respectively.

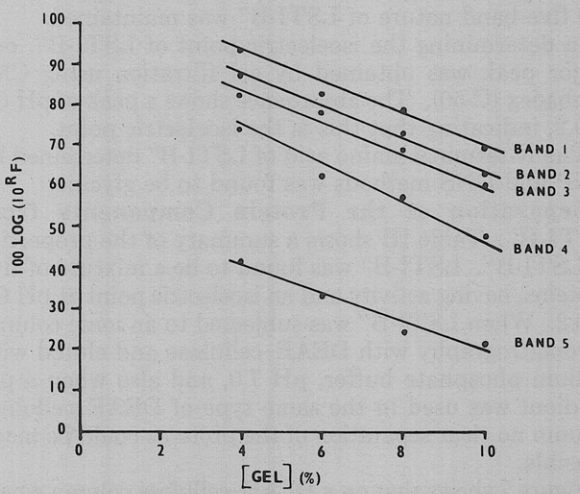

Figure 6. Polyacrylamide gel electrophoresis with varying acrylamide concentrations (4, 6, 8, and 10%). The log R_f of the bands are plotted against increasing gel concentrations.

plotted against acrylamide concentrations the mobilities of the respective proteins were seen parallel to one another

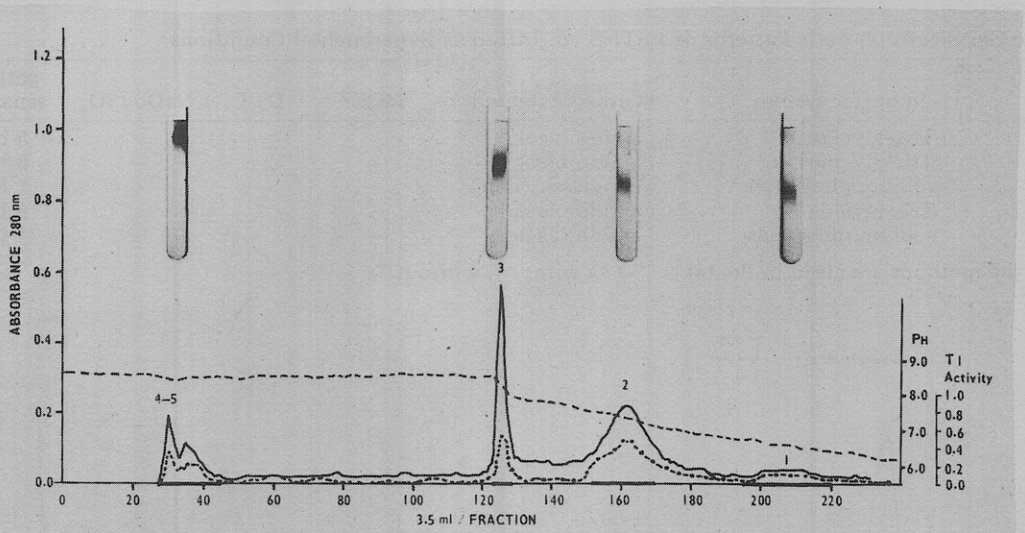

Figure 7. Chromatography of LSTI-B″ on a DEAE–cellulose column. Experimental conditions are given in the text. The solid line and the dotted line indicate protein and activity curves. Eluting solvent was Tris–phosphate buffer, 0.05 M, pH 9.0 and 6.0. Polyacrylamide gel electrophoretic pattern at 8% gel concentration of the proteins are shown above the protein peaks.

Table III. Summary of the Properties of LSTI-B″

solubility	highly soluble in water and salt solutions
thermolability	thermolabile with little resistance (Roy and Rao, 1971).
behavior under ultracentrifugation, $NaDodSO_4$ gel electrophoresis and gel filtration	homogeneous
sp act.	1900 TUI/mg of protein
nitrogen content	15.38%
sedimentation coefficient, $s_{20,w}$	2.0S
mol wt by gel filtration	22 000
by ultracentrifugation	22 400 ± 1500
by $NaDodSO_4$ gel electrophoresis	22 000
isoelectric point	pH 6.2 ± 0.2
N-terminal amino acid	glycine
gel electrophoresis in the absence of $NaDodSO_4$ in Tris–glycine buffer, pH 8.3 at different gel concentrations	five separate protein bands "charge isomers"

(Figure 6). Table II shows that under different conditions of disc gel electrophoresis without $NaDodSO_4$ treatment the five-band nature of LSTI-B″ was maintained.

In determining the isoelectric point of LSTI-B″, one major peak was obtained by gel filtration using CM-Sephadex (C-50). The absorbance shows a peak of pH 6.2 ± 0.2, indicating that this is the isoelectric point.

The N-terminal amino acid of LSTI-B″ determined by DNP and DNS methods was found to be glycine.

Separation of the Protein Components from LSTI-B″. Table III shows a summary of the properties of LSTI-B″. LSTI-B″ was found to be a mixture of five proteins, having activity and an isoelectric point at pH 6.2 ± 0.2. When LSTI-B″ was subjected to an ionic column chromatrography with DEAE–cellulose and eluted with sodium phosphate buffer, pH 7.0, and also when a pH gradient was used in the same type of DEAE–cellulose column no clear separation of the proteins could be made possible.

Figure 7 shows that on a DEAE–cellulose column where a pH gradient was established between pH 9.0 and 6.0, the elution profile was markedly different, being a clear separation of the five proteins. The first peak designated as 4–5 shows two slightly overlapping proteins, while peaks 3, 2, and 1 are well-separated proteins. The individual

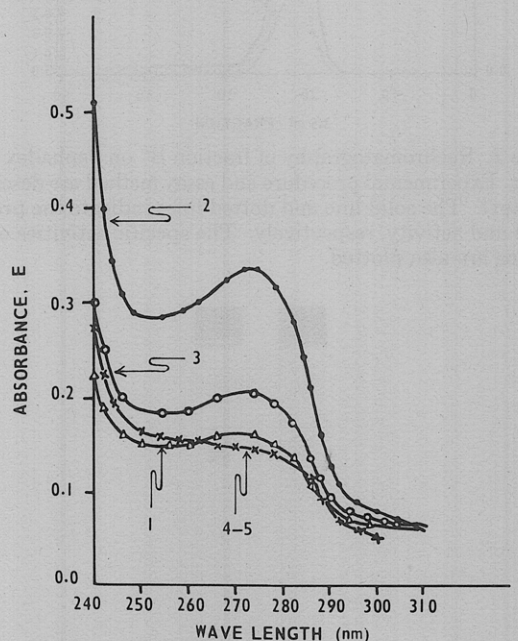

Figure 8. Absorption spectra of the protein components of LSTI-B″.

pooled fractions were dialyzed and lyophilized and were subjected to disc gel electrophoresis at pH 8.3 with a gel concentration of 8.0%; distinct protein bands could be seen (Figure 7). The proportions of the components of LSTI-B″ as 1, 2, 3, and 4–5 were 13, 30, 12, and 19% of the total having specific activities as 1278, 2200, 1340, and 1171, respectively.

Absorption spectra of the protein components of LSTI-B″ as 1, 2, 3, and 4–5 are shown in Figure 8. The extinction coefficient values were 3.20, 3.38, 3.45, and 1.83 of the fractions 1, 2, 3, and 4–5, respectively.

Figure 9 shows the inhibitory curves of the protein components on pure trypsin (23 000). The molar ratios of the inhibitors to trypsin were found to be 1:3, 1:4, 1:3, and 1:2 for the components 1, 2, 3, and 4–5, respectively.

Table IV presents the amino acid composition of the protein components 1, 2, 3, and 4–5. These are the averages of values obtained from hydrolysates of three different periods, i.e., 24, 48, and 96 h. But in the case of serine and threonine the three values are extrapolated to zero times. No methionine could be found in any of the

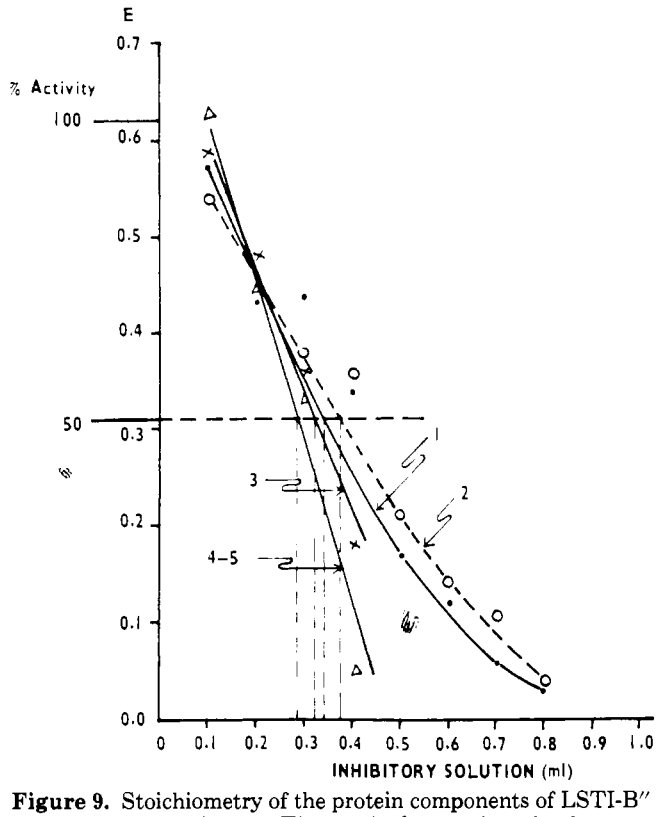

Figure 9. Stoichiometry of the protein components of LSTI-B″ with trypsin are shown. The methods are given in the text. Calculations were made on 50% inhibition only.

samples, even when higher concentrations of the hydrolysates were analyzed.

DISCUSSION

The trypsin inhibitor, LSTI-B″, was found to be homogeneous on the criteria of gel filtration on Sephadex G-75 column, analytical ultracentrifugation, and NaDodSO$_4$ gel electrophoresis, and thereby the molecular weight of the protein was found to be 22000. Other supporting evidences for purity and homogeneity are (1) the protein maintained its homogeneous characteristics when eluted from Sephadex G-75 column with 4 M guanidine hydrochloride, with 8 M urea solutions, and with various other buffer solutions of pH and molarity, (2) the N-terminal amino acid was only glycine, (3) the isoelectric point was at pH 6.2 ± 0.2, and (4) the specific activities over the protein peak eluted from Sephadex G-75 column were constant, and the average was 2000 TUI/mg of protein, which was in agreement with the isolated product, LSTI-B″, estimated separately (1900 TUI/mg of protein).

When LSTI-B″ was subjected to disc gel electrophoresis in Tris-glycine buffer at pH 8.3, it displayed five distinct protein bands (Figure 5). This characteristic could be demonstrated in some other gel electrophoretic conditions, but only when no NaDodSO$_4$ was used. This could be clarified further on electrophoresis as different gel concentrations when the separate entities of the proteins with different mobilities could be observed (Figure 6). If the protein species were the result of oligomerization of one protein, the lines would then have different slopes and would converge to a single point (cf., Koontz and Shiman, 1976). However, if the protein species represented by the bands were of similar size, but of different charges, the lines would be parallel, i.e., have identical slopes (i.e., Hedrick and Smith, 1968). The protein bands in this case did show similar types of parallelism in mobilities (Figure 6), providing evidence that they were charge isomers.

Table IV. Amino Acid Composition of Fractions 1, 2, 3, and 4-5[a]

amino acids	nearest integer, mol/22 000 of protein			
	1	2	3	4-5
Ala	14	15	14	14
Arg	2	3	7	7
Asp	24	26	26	24
$^1/_2$-Cys	19	24	23	26
Glu	16	18	14	12
Gly	8	7	8	5
His	9	6	6	6
Ile	4	4	5	10
Leu	6	6	5	4
Lys	22	17	15	23
Met	0	0	0	0
Phe	6	6	6	6
Pro	16	12	16	12
Ser	20	20	19	18
Thr	19	18	20	20
Trp	8	5	3	2
Tyr	4	6	6	2
Val	8	19	12	12

[a] Average values from hydrolysates at 24, 48, and 96 h at 110 °C under vacuum are presented. Conditions are given in the text. Serine and threonine values were obtained by linear extrapolation to zero hydrolysis time. Half-cystine determined as cysteic acid as separate samples after oxidation with performic acid, hydrolyzed for 96 h at 110 °C under vacuum. Tryptophan was analyzed as per the method by Edelhoch (1967).

Since LSTI-B″ has its isoelectric point at pH 6.2 ± 0.2, the charge isomeric protein components could not be separated on a DEAE-cellulose ionic column by eluting with buffer of pH 5.0 or pH 7.0, whereas when a gradient was established on a similar type of DEAE-cellulose column between pH 9.0 and 6.0, separation of the components could be achieved.

The separated protein components from LSTI-B″ designated as 1, 2, 3, and 4-5 had different mobilities on polyacrylamide gel electrophoretic field in the absence of NaDodSO$_4$ (Figure 7). Their other properties such as specific activities, binding capacity with trypsin, absorption coefficient are different from one another. The amino acid compositions of the components were also different (Table IV).

Considering these data, it seems to be clear that the five protein components have identical molecular weights of 22000, with glycine as the N-terminal amino acid, and have identical isoelectric points. But because of a difference of amino acid residues on the protein molecules, there are variations in their overall residual charges on the molecules, giving evidence of "charge isomers" or "isoinhibitors" in this case.

There are reports that trypsin inhibitors isolated and purified from many plant sources are generally one single protein component. For example, kidney bean (Pusztai, 1966), navy bean (Wagner and Riehm, 1967), and black eyed peas (Ventura and Filho, 1967) are reported to have single protein trypsin inhibitors. But increasing evidence appears to be forthcoming to show that there is more than one protein component in trypsin inhibitors from many sources.

Extensive work has been done in various components of trypsin inhibitors from soybean which has been reviewed (Steiner and Frattali, 1969; Kassell, 1970), and it has been observed that soybean seems to have at least five and possibly six protease inhibitors (Liener and Kakade, 1969). Similarly, lima bean has six components (Fraenkel-Conrat et al., 1952; Tauber et al., 1949; Jones et al., 1963; Haynes

and Feeney, 1967), and navy bean has five protein components with antitryptic properties (Bowman, 1971). If a comparison is made in the light of physicochemical properties of the LSTI-B″ along with its individual protein components with some of the reported values of the plant protease inhibitors (Liener and Kakade, 1969), it seems that all of them are different in their own way; but still, if an attempt is made to draw some type of similarities, the properties like isoelectric point, extinction coefficient, molecular weight, stoichiometry, and amino acid composition may be comparable between LSTI-B″ and its components, with Kunitz inhibitors, Bowman–Birk inhibitors, 1.9S inhibitors from soybean, and also with those of navy beans. In the amino acid analysis of the purified inhibitors it is observed that the amino acids like aspartic acid, half-cystine, glutamic acid, lysine, and serine values are relatively higher than that of other amino acid components. Absence of methionine is found in LSTI-B″, which was also observed in lima and black eyed peas. Of all the reported values (Liener and Kakade, 1969), in the majority of the cases, the N-terminal amino acid is aspartic acid, whereas in the case of mung bean it is serine. LSTI-B″ has only glycine as the N-terminal amino acid which may be an exception from others reported so far. Considering all the comparisons and findings it may be concluded that *Lathyrus sativus* is the first of its kind in naturally occurring plant sources of trypsin inhibitors to have active components behaving as isoinhibitors. This aspect needs further investigation and elucidation.

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