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Properties of Chromatographically Purified Trypsin Inhibitors from Lima Beans

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The trypsin inhibitor fraction prepared from lima beans by the procedure of Fraenkel-Conrat *et al.* (1952) has been resolved by chromatography on DEAE-cellulose into four similar, chromatographically homogeneous components. The same substances may be obtained by direct extraction of the beans. Amino acid analyses of each component give integral molar ratios of residues. Assuming a molecular weight of about 10,000, as shown by Fraenkel-Conrat *et al.*, the substances contain by analysis 76, 77, 86, and 93 residues. The corresponding calculated molecular weights are 8291, 8408, 9423, and 9892. The inhibitors lack methionine and tryptophan, but are characterized by an unusually high content of cystine, ranging from 17.1 to 20.2 g of cystine per 100 g of protein. Cleavage of the disulfide bonds by oxidation with performic acid or by reduction with mercaptoethanol abolishes inhibitor activity. The oxidized and the reduced-carboxamidomethylated derivatives are still very resistant to the action of trypsin, however; an understanding of the basis for this resistance will depend upon further structural investigation of the inhibitors.

After the discovery by Kunitz (1947) of the presence in soybeans of a highly specific inhibitor of trypsin, substances possessing similar inhibitory properties were isolated from a number of sources. The material first obtained from lima beans by Fraenkel-Conrat *et al.* (1952) forms the subject of the present communication. The most active preparations, which Fraenkel-Conrat *et al.* were careful to point out might not be pure, were shown to have molecular weights of about 10,000 and to retain activity after exposure to extremes of *p*H and temperature or after treatment with proteolytic enzymes such as pepsin or papain. Amino acid analyses showed that these inhibitor preparations contained 16% cystine and were thus similar to keratin in degree of —S—S— cross-linking. Jirgensons *et al.* (1960) have examined such preparations chromatographically and reported further data on their amino acid composition and physical properties.

The present report describes the isolation and some of the properties of four similar inhibitors which may be prepared chromatographically either by direct extraction of lima beans or from the inhibitor fraction obtained by the procedure of Fraenkel-Conrat *et al.*

Materials and Methods

The lima beans (var. Fordhook, certified seed) were purchased from Burnett Bros., New York. The commerically prepared samples of lima bean trypsin inhibitor were obtained from Worthington Biochemical Corporation. Two lots, No. 539/40 and No. 541, were examined.

DEAE-Cellulose (exchange capacity 0.96 meq/g), Selectacel Type 40, Lot No. 1305, was obtained from Carl Schleicher and Schuell Co. The cross-linked dextrans, Sephadex G-75 and Sephadex G-25, were purchased from AB Pharmacia, Uppsala, Sweden.

The crystalline proteolytic enzymes were obtained from Worthington Biochemical Corporation: trypsin Lot No. TL 550 and Lot No. SF 926 and pepsin Lot No. P 636.

Extraction of Inhibitor from Lima Beans.—Four aqueous extractants were tested—water, 0.05 N HCl, 0.1 M ammonium formate at *p*H 3.20, and 0.25 N H_2SO_4. The extracts had *p*H values of 6.20, 4.25, 3.50, and 1.55, respectively, when measured at 25°.

All operations were performed in a cold room at 4–5°. The standard procedure was as follows: Finely ground beans (100 g) were extracted for 30 minutes with 80% ethanol (500 ml). The mixture was filtered at the water pump. The semidry meal was then stirred with 500 ml of the appropriate extractant for 1 hour and the mixture was centrifuged for 30 minutes at 1200 × *g*. The supernatant solution was removed, and the sediment was extracted for 1 hour with another 500-ml portion of extractant.

The extracts were adjusted to *p*H 5.0–5.1 with 5 N NH_4OH and combined, and solid ammonium sulfate was added with stirring to 50% saturation. After 2 hours, the precipitated protein was removed by centrifugation and taken up in 50 ml of 0.1 M ammonium formate–formic acid (*p*H 3.20). After removal of insoluble matter, the solution, which contained about

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90% of the inhibitory activity of the extract, was frozen until required for further study. From the specific activity of the purified preparations, it can be estimated that the beans contain about 0.5 g of inhibitors per 100 g.

Gel Filtration on Sephadex G-75.—The Sephadex G-75 (50–80 mesh) was prepared for use essentially as described by Crestfield *et al.* (1962) except that the final decantations and deaeration were performed on a suspension in 0.1 M ammonium formate buffer at *p*H 3.20. This suspension was used to pour a column 150 cm × 2 cm. The column was operated at 4–5° at flow rates of 16–20 ml/hour with the 0.1 M ammonium formate buffer as the eluting agent. Amounts of 50–400 mg of the commercial inhibitor preparations in sample volumes of 2–10 ml have been applied to this column without noticeable difference in the degree of fractionation obtained. Protein was detected in the effluent fractions (4 ml) by measurement of absorbancy at 280 mμ; trypsin inhibitor was detected in the manner described below.

Fractions containing inhibitor were pooled and either lyophilized or used immediately for chromatography.

Chromatography on DEAE-Cellulose.—The adsorbent was passed through acid and base cycles before use and was then equilibrated with sodium phosphate buffer (*p*H 7.60 ± 0.02; 0.01 M phosphate). The columns were poured and packed as described by Peterson and Sober (1962); an air pressure of 5 p.s.i. was used to compress the column. Finally the column was washed overnight with the 0.01 M phosphate which was employed as the starting buffer.

Protein samples were equilibrated with the initial buffer by passage through a column (40 × 4 cm) of Sephadex G-25 (270 mesh) previously equilibrated with the buffer. This column was successfully used with sample volumes up to 100 ml. It was operated in the cold (4–5°) at flow rates of 30–40 ml/hour; 10-ml fractions were collected. The fractions containing proteins were pooled.

The DEAE-cellulose columns were operated at 25°. Preparative columns had bed dimensions of 55 × 2 cm and were used with up to 120 mg of the material which had been obtained from a commercial inhibitor preparation by gel filtration on Sephadex G-75. After addition of the sample to the column, an exponential gradient of salt was applied through a 1-liter mixing chamber, the limiting buffer containing 0.01 M sodium phosphate and 0.4 M NaCl (*p*H 7.60 ± 0.02). Fractions (5.5 ml) were collected at flow rates of 20 to 25 ml/hour produced by an air pressure of 2 p.s.i.

When smaller columns (35 × 0.9 cm) were used with a load of about 25 mg of protein, the salt gradient was established through a 100-ml mixing vessel with an influent buffer containing 0.01 M phosphate and 0.3 M NaCl (*p*H 7.60 ± 0.02). These columns were eluted at flow rates of 5–6 ml/hour; 2-ml fractions were collected.

Protein was located in the effluent fractions by measurement of the ninhydrin color after alkaline hydrolysis of 100-μl aliquots according to the general procedure of Hirs *et al.* (1956), as modified by Crestfield *et al.* (1962). Trypsin inhibitor activity was measured by the procedure described below.

For rechromatography, pooled fractions were lyophilized, the resulting powder was dissolved in 10–20 ml of water, and the solution was equilibrated with the appropriate buffer with the aid of a Sephadex G-25 column. After rechromatography, protein was recovered from pooled fractions by lyophilization of the solution. The product was dissolved in 10–20 ml of 0.1 M acetic acid and the solution was passed through a column of Sephadex G-25 (35 × 2 cm) equilibrated with 0.1 M acetic acid. The fractions containing protein were combined and lyophilized.

Freshly prepared DEAE-cellulose columns were used for each chromatogram in the initial experiments. When it became apparent that all of the protein in the sample was eluted from the column by the buffer system employed, this practice was discontinued. The column was prepared for a new experiment by passing the starting buffer through it until no chloride could be detected in the effluent.

Determination of Trypsin Activity.—The casein digestion method of Kunitz (1947) was used to determine trypsin activity. Portions of the inhibitor solution and the trypsin solution (100 μl of a solution containing 400 μg of trypsin TL550 per ml of 5 mM HCl) were made up to 1 ml with Tris-HCl buffer (0.1 M; *p*H 7.60 ± 0.02) and maintained at 25° for 5 minutes. Then 1 ml of casein solution (1 g "Hammarsten" casein in 100 ml of the same Tris-HCl buffer) was added. Digestion was allowed to proceed at 25° for 15 minutes, and stopped by the addition of 3 ml of trichloroacetic acid (5% w/v). After 1 hour, the mixture was filtered and the optical density of the filtrate at 280 mμ was determined in a 1-cm cell in the Zeiss PMQ II spectrophotometer.

Tryptic activity was determined by reference to a standard curve. The inhibitor assay was found to be most reliable in the range of 40–60% inhibition; when accurate results were required, the inhibitor concentration was chosen to give values in this range. Control digests containing no trypsin and digests corresponding to zero time were included with each set of assays. Assays were performed in duplicate except when a series of chromatographic fractions was being tested.

It was estimated from observations of the rate of hydrolysis of benzoyl-L-arginine ethyl ester that trypsin TL550 contained 60% active trypsin. Tryspin inhibitor activities reported in this paper have been corrected so that activities are expressed as mg of active trypsin inhibited per mg of inhibitor preparation.

Amino Acid Analysis.—Protein samples were hydrolyzed at 110° in 6 N HCl in evacuated sealed tubes for 22 hours and 72 hours (*cf.* Moore and Stein, 1962). The amino acid compositions of hydrolysates were determined by ion-exchange chromatography with use of automatic procedures (Spackman *et al.*, 1958). Cystine plus cysteine was determined as cysteic acid by a modification (Moore, 1963) of the procedure of Schram *et al.* (1954). Oxidation was allowed to proceed for 16 hours.

Performic Acid Oxidation, Reduction, and Carboxamidomethylation.—Samples purified by gel-filtration were oxidized with performic acid under the conditions used by Hirs (1956) with ribonuclease. The approximate completeness of oxidation was checked by analysis for cysteic acid. Reduction with mercaptoethanol was performed along the lines described by White (1960) and Anfinsen and Haber (1961) under the conditions which Crestfield *et al.* (1963) used with ribonuclease. Reduced-carboxamidomethylated material was prepared by substituting iodoacetamide for iodoacetic acid in the alkylation step following reduction. The completeness of the reduction and alkylation was determined by analysis for carboxymethylcysteine in a hydrolysate of the derivative.

Results

Gel Filtration.—The material used in our first studies was a commercial preparation of inhibitor (Lot No.

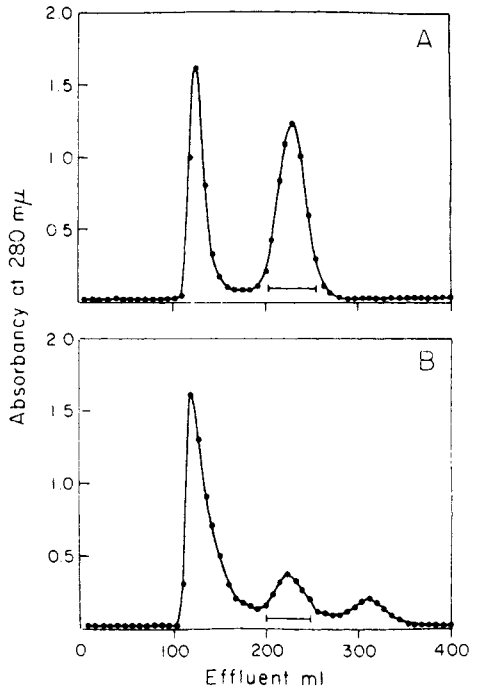

FIG. 1.—Gel filtration of lima bean trypsin inhibitors on columns of Sephadex G-75 (150 × 2 cm) in 0.1 M ammonium formate-formic acid (*p*H 3.20). *A*, 300 mg Worthington Lot No. 539 40. *B*, 10 ml of solution obtained from lima beans extracted with 0.25 N H_2SO_4.

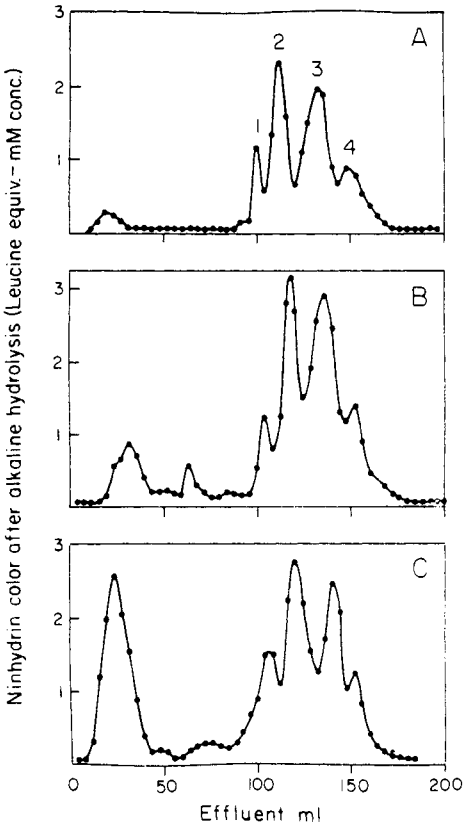

FIG. 2.—Chromatography of lima bean trypsin inhibitors on DEAE-cellulose. Column: 0.9 × 35 cm. Eluants: 0.01 M sodium phosphate, *p*H 7.6, 100 ml mixing chamber, with influent buffer 0.01 M phosphate + 0.3 M NaCl, *p*H 7.6. *A*, Worthington Lot No. 541 (20 mg). *B*, inhibitor fraction from beans extracted with 0.25 N H_2SO_4. *C*, inhibitor fraction from beans extracted with 0.1 M ammonium formate-formic acid (*p*H 3.20).

539 40) which is described by the manufacturers as being Fraction III obtained by the procedure of Fraenkel-Conrat *et al.* (1952). The authors stated that the material at this stage of purification had about 70% of the highest specific activity reported. They removed inactive material by fractional precipitation with ammonium sulfate at *p*H 6. We find that similar purification can be achieved by gel-filtration on columns of Sephadex G-75. The results of a typical experiment are shown in Figure 1*A*. All of the detectable trypsin inhibitor activity was associated with the second peak, emerging at about 60% of the total column volume. The specific activity of material in the pooled fractions was 2.0–2.1 mg trypsin mg, in good agreement with the highest values reported by Fraenkel-Conrat *et al.* The recovery of inhibitor in the pooled fractions was 80–90%.

In Figure 1*B* is shown the result of a typical experiment in which 10 ml of the solution obtained from lima beans by direct extraction with 0.25 N H_2SO_4 was passed through the Sephadex G-75 column. The second peak again contained all the detectable inhibitor activity. The somewhat lower specific activity (1.6–1.8 mg mg inhibitor) indicated that this material still contained some inactive protein. Material obtained by extraction with the other aqueous solutions gave results very similar to those shown in Figure 1*B*.

Amino acid analysis of the inhibitor obtained from the commercial preparation by gel filtration revealed that, although many amino acids were present in amounts corresponding very closely to whole numbers of residues, methionine, histidine, glycine, tyrosine, and phenylalanine were exceptions; methionine in particular was always present in small amount (0.05–0.10 residues per molecule of 10,000 m.w.). These results suggested that the inhibitor still was not pure, and experiments were therefore performed to determine the optimum conditions for chromatography of the inhibitor.

Chromatography.—On DEAE-cellulose the purified inhibitor was readily resolved into a small inactive fraction emerging virtually unretarded and four major fractions which gave a series of sharp but overlapping peaks as shown in Figure 2*A*. Comparison of the inhibitor activities of the fractions on the basis of the ninhydrin color yield after alkaline hydrolysis indicated that the materials in the four peaks had almost identical specific activities. The results of the chromatography of freshly prepared extracts of lima beans are given in Figure 2, *B* and *C*. The active components of all three preparations have similar chromatographic properties.

Chromatographic separations on a larger scale were carried out with another commercial sample, Lot No. 539 40. The results are shown in Figure 3*A*. The pattern obtained with this sample, whether it was chromatographed on an analytical or a preparative scale, was noticeably different from that shown in Figure 2. The relative amounts of protein eluted in the peaks numbered 3 and 4 are different, probably as a result of fractionation during the isolation process, but subsequent analysis showed the components to have the same amino acid composition as components 3 and 4 from Lot No. 541. The result of rechromatography of the material in the four peaks is shown in Figure 3, *B*, *C*, *D*, and *E*. The chromatographic system gives thoroughly reproducible results.

Amino Acid Compositions.—The analyses were performed on samples from the main peaks obtained in the rechromatography experiments. The yield of com-

ponent 3 was higher from Lot No. 541 (Fig. 2A) than from Lot No. 539/40 (Fig. 3A), and the cut from the center of the peak was nearly homogeneous by chromatography. The data are summarized in Table I.[1]

Each component contains several amino acids that are present in amounts corresponding to one residue per molecule of a protein of molecular weight of about 10,000 (Fraenkel-Conrat *et al.*, 1952). The analytical data for all of the amino acids in each fraction are in good agreement with integral molar ratios. The nearly complete absence of glycine in component 2 demonstrates the efficiency of the chromatographic separation from its neighboring components. The absence of methionine and tryptophan in all of the components is evidence that the inhibitors have been well separated from the major proteins of the lima bean.

On the basis of the amino acid analyses, components 1, 2, and 4, which contain 77, 76, and 86 residues, respectively, could all be derived from component 3, which contains 93 residues, were it not that there appear to be 13 residues of aspartic acid in component 3 and 14 in component 2. Without further study it is not possible to be certain whether the experimental values for aspartic acid, which are 13.1 and 13.7 residues in this instance, are actually significantly different.

The data confirm the earlier observations that these proteins are rich in cystine. Component 2, for example, contains 20.2 g of cystine per 100 g of protein—a value higher than that for human hair (18 g per 100 g; Schram *et al.*, 1954). The half-cystine residues presumably all participate in —S—S— bonds in the protein molecules, since there are no —SH groups detectable by exposure of the inhibitors to iodoacetate in 8 M urea followed by analysis for carboxymethylcysteine.

The values for half-cystine are those obtained by analysis for cysteic acid after performic acid oxidation. The degree of decomposition of cystine *per se* during acid hydrolysis of the unoxidized proteins varies from sample to sample, as can be seen from the data in Table I.

Activity.—Components 1 to 4 had the following activities in terms of mg trypsin inhibited by 1 mg of protein: 2.69, 2.77, 2.28, and 2.44, respectively. When these results are expressed in molar terms, on the basis of a molecular weight of 23,800 for trypsin (Cunningham, 1954) and the calculated molecular weights for the components (Table I), the moles of trypsin inhibited by one mole of inhibitor are 0.95, 0.99, 0.95, and 0.97, respectively. Thus, all four samples have essentially the same activity on a molar basis, and each combines with the enzyme in equimolar amount.

Properties of Oxidized and Reduced Inhibitors.—Oxidation by performic acid or reduction with mercaptoethanol in 8 M urea abolishes the inhibitor activity of all of the components. The ratio of trypsin to inhibitor in these experiments was about 1:2, so that 1% residual inhibitor activity would have been detected. In a preliminary experiment, the reduced inhibitor was found to regain full activity on reoxidation in the presence of air according to the procedure of White (1960).

[1] The effluent curves show the presence of a small unidentified component emerging between *allo*-isoleucine and isoleucine. In some 24-hour hydrolysates the size of this peak approaches that from glycine or valine, which are present to the extent of 1 residue per molecule of inhibitor. The quantity of this unidentified component decreases with time of hydrolysis; at 72 hours the amount is about half that at 24 hours. The ratio of color produced with ninhydrin at 440 mμ and 570 mμ is normal for an amino acid. The nature of this constituent of the acid hydrolysates has not been determined.

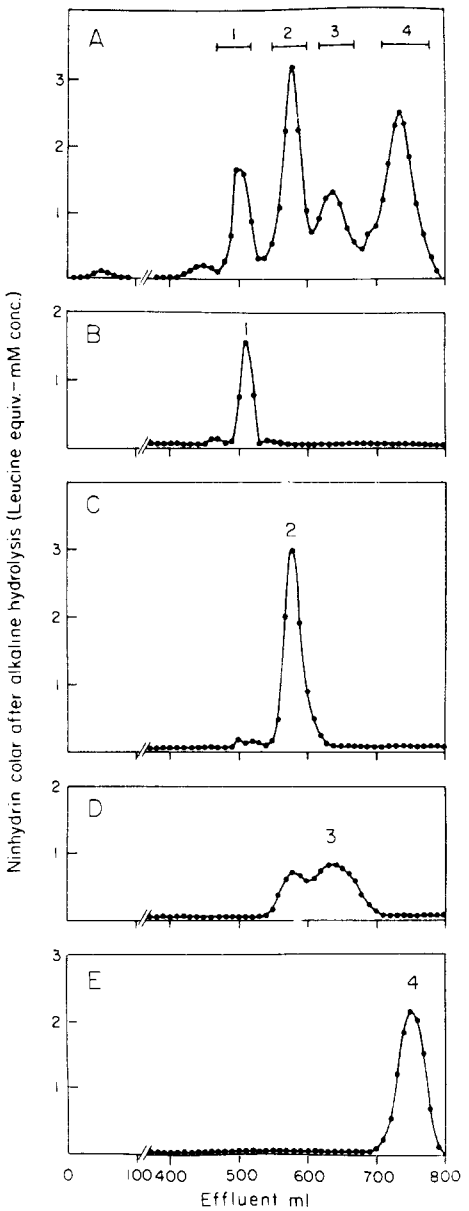

FIG. 3.—Chromatography of lima bean trypsin inhibitors on DEAE-cellulose. Column: 55 × 2 cm. Eluants: Same as for Fig. 2, with a 1-liter mixing chamber. *A*, 120 mg purified inhibitor from Worthington Lot No. 539/40. *B*, *C*, *D*, and *E*, rechromatography of components 1–4 from *A*. Worthington Lot No. 541 gave a higher yield of component 3; a nearly chromatographically homogeneous sample of this component was isolated from a cut taken from the center of the main peak in the rechromatography experiment with this lot.

Both the oxidized and the reduced-carboxamidomethylated inhibitors are resistant to the action of trypsin. They were tested under the conditions which Hirs *et al.* (1956) found gave 70 to 95% hydrolysis of the susceptible bonds at lysine and arginine residues in oxidized ribonuclease (substrate concentration 0.5%, enzyme concentration 0.02%, temperature 25°). The ninhydrin method was used to monitor the degree of hydrolysis of the modified inhibitors by trypsin. After 4 hours at 25°, there was an increase of only 4% in the ninhydrin color when the oxidized proteins were treated with the enzyme, and there was no detectable increase when the reduced-carboxamidomethylated derivatives were treated in the same way.

Each of the four inhibitors present in the fraction

TABLE I

AMINO ACID COMPOSITIONS OF TRYPSIN INHIBITORS FROM LIMA BEANS

Determined by ion exchange chromatography after 22 and 72 hours of acid hydrolysis. Results expressed as numbers of amino acid residues per molecule.[a]

	Component 1			Component 2			Component 3			Component 4		
Amino Acid	22 hr.	72 hr.	Integer[b]	22 hr.	72 hr.	Integer[b]	22 hr.	72 hr.	Integer[b]	22 hr.	72 hr.	Integer[b]
Aspartic acid	12.1	12.0	12	13.7	13.7	14	13.1	13.0	13	13.3	13.1	13
Glutamic acid	5.94	5.88	6	5.29	5.30	5	7.10	7.10	7	6.99	7.06	7
Glycine	0.92	0.98	1	0.02	0.05	0	0.92	0.97	1	1.01	1.00	1
Alanine	3.05	3.02	3	3.15	3.10	3	3.68	3.75	4	2.64	2.63	3
Valine	0.95	1.00	1	1.00	0.96	1	1.05	1.12	1	0.98	1.05	1
Leucine	2.88	2.77	3	3.03	3.05	3	3.00	3.05	3	3.14	3.06	3
Isoleucine	3.83	3.92	4	3.92	4.02	4	4.64	4.78	5	4.12	4.14	4
Serine	10.8	9.00	12	10.5	8.60	12	13.3	11.0	15	11.9	10.3	13
Threonine	4.01	3.24	4	3.02	2.58	3	4.95	4.80	5	4.82	4.46	5
Half-cystine	10.6	9.30	12[c]	14.2	13.1	14[c]	15.8	15.0	16[c]	11.1	13.3	14[c]
Proline	5.75	5.80	6	5.97	5.94	6	6.99	7.10	7	7.05	6.54	7
Phenylalanine	1.02	1.02	1	1.01	1.03	1	1.84	1.72	2	2.10	1.87	2
Tyrosine	0.93	0.87	1	0.90	0.83	1	1.85	1.74	2	0.97	0.71	1
Histidine	5.12	5.14	5	2.97	2.97	3	5.93	5.92	6	6.00	5.82	6
Lysine	4.12	4.15	4	4.06	4.06	4	4.26	4.10	4	3.82	3.70	4
Arginine	1.88	1.93	2	1.97	1.94	2	2.10	2.15	2	2.04	2.03	2
Amide-NH_3[d]	5.64		(4)[e]	6.28		(5)[e]	6.80		(5)[e]	6.36		(5)[e]
Total			77			76			93			86
Molecular[f] weight		8408			8291			9892			9423	

[a] Tryptophan and methionine were absent (<0.02 residues/molecule). [b] The values for serine, threonine, and tyrosine were obtained by extrapolation to zero time (*cf.* Moore and Stein, 1962). [c] Determined as cysteic acid in the performic acid oxidized protein; the experimental values were 12.2, 14.0, 15.9, and 14.3, respectively. [d] Approximate values for amide-NH_3 were obtained by correcting the total NH_3 content of 22-hr. hydrolysates for the NH_3 formed in the decomposition of serine and threonine. [e] Not included in the total number of residues. [f] Calculated from the amino acid analyses. An unknown peak between *allo*-isoleucine and leucine has not been taken into consideration (see text).

used for these experiments contained four lysine and two arginine residues. Were each lysyl and arginyl bond to be hydrolyzed, an increase in ninhydrin color of over 100% would be anticipated. With the oxidized inhibitor, the action of chymotrypsin gave ten times as much of an increase in ninhydrin color as did the action of trypsin.

DISCUSSION

Homogeneity of the Components.—From the chromatographic results and the amino acid analyses, it seems likely that homogeneous preparations of inhibitors 1, 2, 3, and 4 have been obtained. In the experiments of Jirgensons *et al.* (1960), rechromatography of a given zone obtained from the DEAE-cellulose column showed a series of peaks. In the present experiment, the sample to be chromatographed has been carefully equilibrated with a buffer of exactly the same *p*H and ionic strength as the buffer with which the column is equilibrated. This precaution has been found to be essential to avoid multiple zoning in the initial chromatography as well as in the rechromatography.

The Significance of Several Active Components.—The appearance of several components with the same activity suggests the possibility that they are artifacts formed during isolation. The effluent curves in Figure 2 show that aqueous extracts of the beans prepared at 4° with ammonium formate buffer at *p*H 3.2 or with 0.25 N H_2SO_4 (*p*H 1.5) contain the same four fractions that are present in Fraction III isolated by the procedure of Fraenkel-Conrat *et al.*, a method which includes adsorption on and elution from bentonite. A different lot of Fraction III (Fig. 3) does show different relative amounts of the components, however, which may indicate that some fractionation has occurred during the adsorption step. The yield of inhibitor in the direct extractions was about 80% of the extractable inhibitor activity of the beans, whereas the yield of Fraction III was 40%. The lower recovery is compatible with the supposition that fractionation of the components had occurred.

Since the same results are obtained when the extract is prepared at either *p*H 3.2 or *p*H 1.5, there is no indication that exposure of a single active protein to acidic conditions is responsible for the formation of the several components. The stability of component 3 was checked by allowing it to stand at *p*H 1.5 for 24 hours at room temperature; there was no increase in ninhydrin color value and, hence, probably no cleavage of peptide bonds.

It is possible that the several active components arise from a parent molecule by enzymatic action in the beans. Although further exploration of this subject is needed, the general resistance of the inhibitors to enzymatic action makes this unlikely. Fraenkel-Conrat *et al.* showed the activity of Fraction III to be resistant to pepsin and papain. In the present experiments, component 3 (1 mg per ml) was exposed to pepsin (200 μg per ml) for 24 hours at *p*H 1.5 without detectable loss of activity. These experiments, in which the survival of activity was measured, do not exclude the possibility that there may have been some cleavage at nonessential bonds in the molecules.

We conclude that the proteins in our chromatographic fractions are probably constituents of the mature dry seed. It is possible that changes taking place during development of the seed or during subsequent drying of the seed, or genetic heterogeneity, may be responsible for the production of the multiplicity of inhibitors; decision on this point must await further experimental work.

Specific Properties of the Inhibitors.—The experiments with the oxidized and reduced inhibitors focus attention upon several of the special features of the molecules. Since neither the completely oxidized nor the reduced proteins are active as inhibitors, at least

some of the disulfide bonds must remain intact to maintain the structure that combines with trypsin. The observation that the extended chain, devoid of disulfide bonds, remains very resistant to tryptic hydrolysis raises several questions for further study. Since the oxidized derivative is hydrolyzed appreciably by chymotrypsin, the resistance to trypsin appears to be fairly specific.

Resistance to trypsin might arise in several ways. For example, the lysine or arginine residues might be of the D-configuration. Fraenkel-Conrat *et al.* reported microbiological assays for lysine and arginine which are in reasonable agreement with the values found in our chromatographic analyses; this result would suggest that these amino acids are of the L-configuration. Hydrolysis at lysine residues could be inhibited by unexpected linkages involving the ϵ-NH_2 groups; Fraenkel-Conrat *et al.* showed, however, that these NH_2 groups can be acetylated, which is evidence that they are free.

Resistance to trypsin might be explained by the presence of lysylprolyl (and, by analogy, arginylprolyl) linkages, which are not split by trypsin (Bell, 1954; *cf.* Hirs *et al.*, 1956). The inhibitors contain 4 lysine and 2 arginine residues and 6 or 7 proline residues per molecule. It is thus possible that each residue of lysine and arginine could be followed by a residue of proline. An answer to this question will come from studies on the sequence of amino acid residues in the chain.

Comparison with Other Inhibitors.—The amino acid analyses recorded in Table I, when compared with published values for pancreatic trypsin inhibitor (Green and Work, 1954), soybean trypsin inhibitor (Scheraga and Wu, 1962), and ovomucoid (Lewis *et al.*, 1950), emphasize the very different natures of these substances. If the manner of interaction with trypsin is the same for each of these proteins, then only relatively short segments of the peptide chains of both trypsin and inhibitor can be involved in the interaction.

The presence of a number of components with inhibitor activity is not unique. Rackis *et al.* (1959) and Birk (1962) each found two trypsin inhibitors in soybeans by chromatography on DEAE-cellulose. Other crystalline preparations of trypsin inhibitors from plant sources have been obtained by Sohonie and co-workers (1958) from Indian double bean (Dolichos Loblob) and field beans (*V. faba*), but the low specific activities reported for the crystalline products suggest that these preparations may not be homogeneous.

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