Heterogeneity of Soybean Trypsin Inhibitor. I. Chromatographic Fractionation and Polyacrylamide-Gel Electrophoresis[1]

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ABSTRACT

A soybean trypsin inhibitor (TI) fraction which contained all trypsin and chymotrypsin inhibitor activities of water-extractable soybean protein was obtained by Sephadex G-75 gel filtration. Eleven electrophoretic protein bands were obtained by polyacrylamide-gel electrophoresis of the inhibitor fraction. Ten bands had TI activity. The soybean TI fraction was fractionated by DEAE-cellulose chromatography into eight fractions. Seven of these (I, I-1, II, III, IV-1, IV-2, and V) were TI-active. Fractions I, I-1, II, and III were classified as cystine-poor inhibitors (less than 2% half-cystine) and were electrophoretically heterogeneous. Fractions I, I-1, and II were trypsin inhibitors with essentially no chymotrypsin-inhibitor activity. Fraction III had the highest TI activity. Fractions IV-1 and IV-2 were cystine-rich inhibitors (more than 8% half-cystine) and had relatively high chymotrypsin-inhibitor activity. Fraction V was identified as the Kunitz inhibitor and SBTIA-2. The major components of III, IV-1, and IV-2, respectively, corresponded to SBTIB-1, SBTIB-2, and SBTIA-1 of Rackis and Anderson. The N-terminal amino acid of II, III, IV-1, IV-2, and V was aspartic acid.

The presence of a number of trypsin (TI) and chymotrypsin inhibitors (CTI) of soybeans has been reported. The first to be crystallized was the classic soybean trypsin inhibitor (TI) of Kunitz (1,2,3). Rackis et al. (4,5,6) isolated four inhibitors by DEAE-cellulose column chromatography and identified one of them as the Kunitz inhibitor. The isolation of additional soybean trypsin inhibitors has been reported by Birk and co-workers (7,8,9), Aso (10), Yamamoto and Ikenaka (11), Frattali (12), and Frattali and Steiner (13).

The previous paper (14) reported fractionation of whole water-extractable soybean proteins by Sephadex G-200 and identification of the TI-active fraction. This paper reports an improved method of preparing the inhibitor-active fraction by Sephadex G-75, chromatographic fractionation into six TI-active fractions, and identification of ten TI-active electrophoretic bands. The relations of the inhibitors obtained in this study with those isolated by other workers are discussed.

EXPERIMENTAL

Materials

Defatted soybean meal was prepared by low-temperature n-hexane extraction from Hawkeye soybean produced in the U.S. The water-extractable protein was prepared according to the method as described previously (14). A commercial crystalline soybean trypsin inhibitor (SBTI), which corresponds to the Kunitz inhibitor (2) and SBTIA-2 of Rackis and Anderson (4), was obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Samples of SBTIB-1, SBTIB-2, and SBTIA-1 of Rackis and Anderson (4) were kindly supplied by A. C. Eldridge, Northern Regional Research Laboratory, USDA, Peoria, Illinois.

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Gel Filtration

Freeze-dried, water-extracted soybean protein (400 mg.) was dissolved in 2.0 ml. of 0.01M potassium phosphate buffer, pH 7.6, containing 0.4M NaCl and 0.01M mercaptoethanol. The sample was applied to a column (3.0 by 100 cm.) of Sephadex G-75, which had been equilibrated with the same buffer, and eluted at a flow rate of 30 ml. per hr.; 5.0-ml. fractions were collected. Protein peaks emerging from the column were identified by their absorbance at 280 nm. Two partly overlapping peaks were combined and dialyzed against distilled water and freeze-dried.

DEAE-Cellulose Chromatography

Diethylaminoethyl (DEAE) cellulose (Brown Co.) equilibrated with 0.01M potassium phosphate buffer, pH 7.6, was packed in a column (3.0 by 50 cm.). Samples of 400 mg., or 50 mg. for rechromatography, were applied to the column and eluted with a linear NaCl gradient provided by two identical 1-liter bottles. The protein concentration was determined by the method of Lowry et al. (15).

Enzyme Inhibition

Trypsin and chymotrypsin activities were determined by the casein-digestion method of Kunitz (2). The substrate was 1% casein solution in 0.1M potassium phosphate buffer at pH 7.6. The reaction was performed at 35°C. for 20 min. and then 5% trichloroacetic acid was added, and absorbance at 280 nm. of the filtrate was determined. Trypsin (twice-crystallized and salt-free) and chymotrypsin (salt-free, alpha-rich) were obtained from Nutritional Biochemical Corp. Activity was expressed in units equal to γ of trypsin or chymotrypsin inhibited per γ of trypsin inhibitor. Activity of chromatographic effluents was expressed as mg. of enzyme inhibited per ml. of the effluents.

Amino Acid Analysis

Samples of TI fractions weighing approximately 2 mg. were hydrolyzed at 110°C. in 1 ml. of constant-boiling 6N HCl in sealed and evacuated glass ampules. The hydrolysates were dried under vacuum with gentle heat, and the residues dissolved in 1 ml. of buffer. Aliquots of 0.2 ml. were analyzed on a Technicon automatic amino acid analyzer with a 140- by 0.6-cm. column, Chromobeads type B resin, by a 22-hr. procedure. The N-terminal amino acid was determined by the method of Sanger (16).

Polyacrylamide Gel Electrophoresis

The method described by Yaguchi et al. (17) was used, but without urea and 2-mercaptoethanol. The 6% polyacrylamide gel was removed from the mold, washed with several changes of distilled water, and equilibrated against 0.076M tris-citrate buffer (pH 8.6). The discontinuous buffer system with bridge vessels containing 0.3M boric acid was used. A constant voltage of 150 was applied for 15 hr. at 5° to 10°C.

RESULTS

Preparation of Soybean Trypsin Inhibitor Fraction

Figure 1 shows a gel-filtration pattern of the water-extractable soybean protein

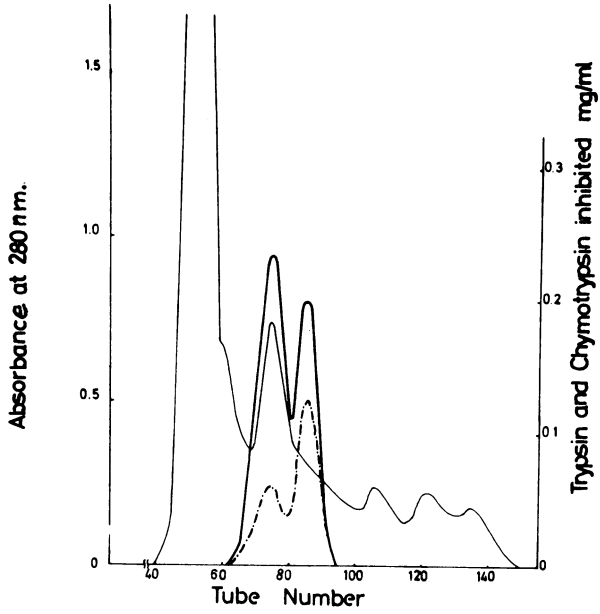

Fig. 1. Gel filtration of soybean protein on Sephadex G-75. Light line, absorbance at 280 nm.; heavy line, trypsin inhibition; dot-and-dash line, chymotrypsin inhibition.

on Sephadex G-75. The major portion of the soybean protein was eluted in the void volume and this fraction was devoid of TI and CTI activities. All TI and CTI activities of the soybean protein were recovered in a fraction which consisted of two partly overlapping peaks. The first TI peak of larger molecular size had relatively low, and the second peak rather strong, CTI activity. Both peaks were combined and subjected to DEAE-cellulose column chromatography. The inhibitor fraction obtained from Sephadex G-75 corresponded to inhibitor fraction D from Sephadex G-200 (14). The flow rate with Sephadex G-75 was two to three times as fast as that with Sephadex G-200 used previously.

DEAE Cellulose Chromatography

Figure 2 shows an elution pattern of the TI fraction on DEAE-cellulose column, and Table I shows some properties of the fractions. The proteins of the inhibitor fractions were separated into seven chromatographic fractions, but the last peak, VI, was devoid of both trypsin and chymotrypsin inhibitor activity. The first three fractions (I, I-1, and II) were trypsin inhibitors with no or little CTI activity. Fractions III and IV had high TI and CTI activity. Fraction V, the largest peak, was TI with minor CTI activity.

Fraction IV was rechromatographed on DEAE-cellulose with milder NaCl gradient, and yielded three fractions (Fig. 3). The first fraction was essentially identical with fraction III, judged from chromatographic position, polyacrylamide-gel electrophoresis, and inhibitory activities. The other two fractions (IV-1, IV-2) had high TI and CTI activities. The specific activities were

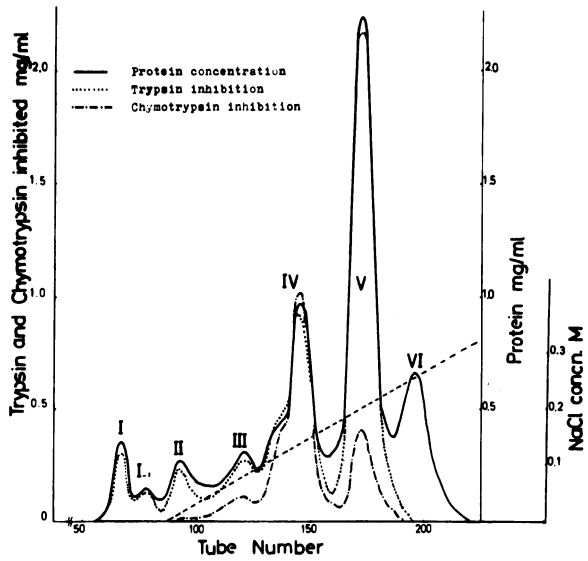

Fig. 2. Chromatographic pattern of trypsin inhibitor (TI) fraction on DEAE-cellulose.

TABLE I. SOME PROPERTIES OF TRYPSIN INHIBITOR FRACTIONS

	Fraction						
	I	I-1	II	III	IV-1	IV-2	V
Salt conc. for elution, M	0	0	0.005	0.07	0.11	0.14	0.21
Trypsin inhibition, specific activity[a]	0.87	0.87	0.90	1.88	1.67	0.80	0.97
Chymotrypsin inhibition, specific activity[a]	0	0	0.15	1.63	1.63	1.02	0.24
N-terminal amino acid			asp.	asp.	asp.	asp.	asp.

[a]Enzyme inhibited, γ per γ inhibitor.

higher in IV-1 and IV-2. The inhibitor activities of IV-2 did not coincide with the protein peak IV-2, suggesting that IV-2 contained more than one protein component. Fraction V of Fig. 2 was rechromatographed on a DEAE-cellulose column and the results were as shown in Fig. 4. A single peak was obtained, suggesting its homogeneity.

Amino Acid Composition

Table II shows the amino acid composition of six chromatographic fractions of Figs. 2 and 3. Methionine, tyrosine, and histidine contents were quite similar among these fractions and the differences were less than 1%. These fractions, however, differed greatly in the amounts of cystine, alanine, glycine, and proline. The

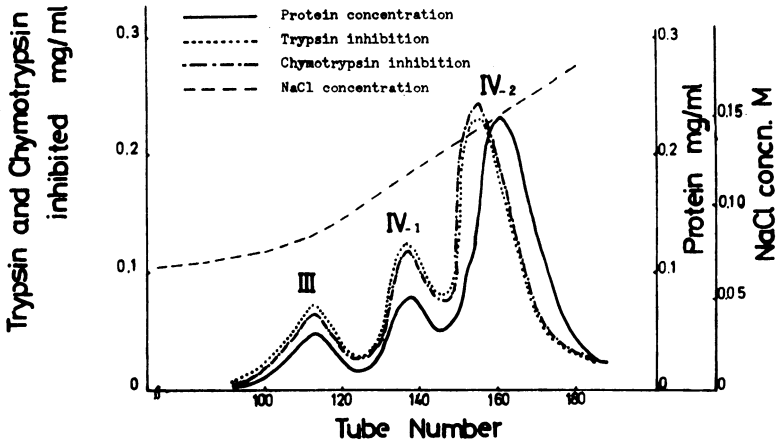

Fig. 3. Rechromatography of fraction IV of Fig. 2 on DEAE-cellulose.

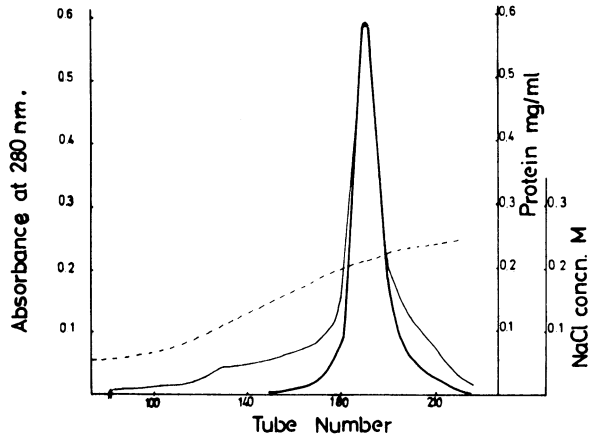

Fig. 4. Rechromatography of fraction V of Fig. 2 on DEAE-cellulose. Light line, absorbance at 280 nm.; heavy line, protein concentration; dashed line, NaCl concentration.

half-cystine contents of fractions I, II, and III were less than 2%; that of fractions IV-1 and IV-2, more than 8%. Alanine was highest in III and lowest in V. Glycine of IV-1 and IV-2 was much lower than that of other fractions, whereas their proline content was higher than that of other fractions. The amino acid composition of fraction V was quite similar to that of Kunitz's crystalline TI (11). The N-terminal amino acid of five fractions (Table I) was aspartic acid. Aspartic acid was also reported for the N-terminal amino acid of SBTIA-1 and A-2 by Rackis and Anderson (4) and of the 1.9S inhibitor of Yamamoto and Ikenaka (11).

Polyacrylamide Gel Electrophoresis

Figures 5, 6, and 7 show polyacrylamide-gel electrophoretic patterns of the

TABLE II. AMINO ACID COMPOSITION OF TRYPSIN INHIBITOR FRACTIONS EXPRESSED AS MOLES PER 100 MOLES OF AMINO ACID RESIDUES RECOVERED

	Fraction					
	I	II	III	IV-1	IV-2	V
Aspartic acid	9.25	10.39	9.41	11.95	11.15	14.37
Threonine	5.50	5.71	5.28	4.57	4.05	3.85
Serine	7.41	6.46	6.51	9.34	8.04	5.94
Glutamic acid	11.48	10.28	11.46	9.81	11.04	10.53
Proline	6.73	5.25	4.84	7.34	8.11	6.70
Glycine	9.00	8.90	10.10	4.93	5.69	9.28
Alanine	10.40	10.73	12.72	8.00	6.69	4.85
Valine	5.72	7.08	6.36	3.84	5.25	6.41
Cystine/2	1.51	0.74	1.48	9.89	8.70	2.94
Methionine	2.16	1.69	1.89	1.82	1.78	1.18
Isoleucine	4.20	5.17	4.43	3.65	4.48	7.28
Leucine	8.52	7.87	7.41	5.33	6.88	8.06
Tyrosine	1.96	2.47	2.18	2.72	2.62	2.21
Phenylalanine	4.03	4.07	3.25	3.22	3.81	4.85
Lysine	5.91	7.30	5.89	6.88	6.85	5.77
Histidine	1.76	1.99	2.11	2.37	1.60	1.46
Arginine	4.46	3.90	4.68	4.34	3.26	4.32

original TI fraction from Sephadex G-75, and chromatographic fractions of Figs. 2, 3, and 4. SBTIB-1, SBTIB-2, SBTIA-1 of Rackis and Anderson (4), and a commercial crystalline TI (SBTI) which corresponds to the Kunitz inhibitor and SBTIA-2 of Rackis and Anderson (4) were used as standards.

The original TI fraction (Fig. 7, "TIF") was highly heterogeneous and consisted of at least 11 electrophoretic components. The proteins were eluted from the unstained gel with the phosphate buffer, and TI activity was detected in ten bands which moved to the anode, whereas the component which moved toward the cathode was free of activity. The TI-active electrophoretic bands were named from band 1 near the slot to band 10 which was closest to the cathode (Fig. 7).

As shown in Fig. 5, fraction I consisted of bands 1 and 2, and a TI-inactive component which moved toward the cathode. Fraction I-1 contained bands 4 and 5. Fractions II and III were highly heterogeneous and contained different amounts of bands 2, 3, 4, 5, 6, and 7. Band 6 was identified as SBTIB-1 of Rackis and Anderson (4).

Fractions IV-1 and IV-2 (Fig. 6) have similar electrophoretic behavior with SBTIB-2 and SBTIA-1 respectively, and it appeared that the major component of IV-1 was band 9 and that of IV-2, band 8. Since Eldridge et al. (18) reported that the electrophoretic mobility of SBTIB-2 in polyacrylamide gel was faster than that of SBTIA-1, and Rackis and Anderson (4) reported that SBTIB-2 was eluted earlier than SBTIA-1 from the DEAE-cellulose column, it was concluded that IV-1 corresponded to SBTIB-2 and IV-2 corresponded to SBTIA-1.

Fraction V (Fig. 7) was homogeneous and contained only band 10 which corresponded to the major component of a commercial crystalline TI. Thus fraction V was identical with the Kunitz inhibitor and SBTIA-2 of Rackis and Anderson (4).

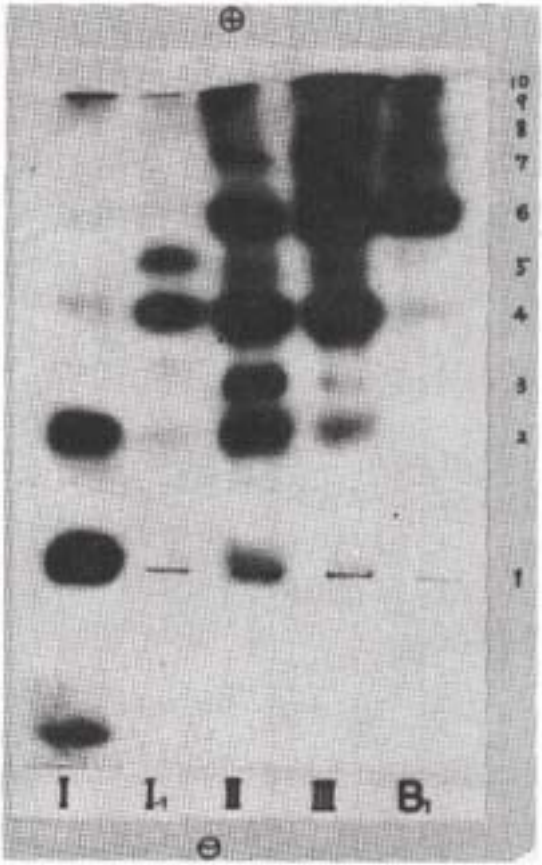

Fig. 5. Polyacrylamide-gel electrophoretic pattern of DEAE fractions (I, I-1, II, III) of Fig. 2 and SBTIB-1 (B-1).

DISCUSSION

The preparation of soybean TI fraction by means of Sephadex G-75 is simple and effective. All of the TI's and CTI's in soybean proteins extracted with water are confined in the small effluent fraction and separated from other soybean proteins. The present method with Sephadex G-75 reduces the time required for the gel filtration by one-half or one-third compared to the previous method with Sephadex G-200 (14). The gel-filtration behavior of the soybean inhibitors suggests that the components are relatively small proteins and have similar molecular sizes. The molecular weights (MW) reported (4,6,7,8,9,11,12,13) for various soybean trypsin inhibitors range from 8,000 (12) to 24,000 (9).

The present results indicate that the soybean TI is highly heterogeneous and consists of at least ten components. The amino acid composition, electrophoretic behavior, relative position in DEAE-cellulose chromatography, and inhibitory

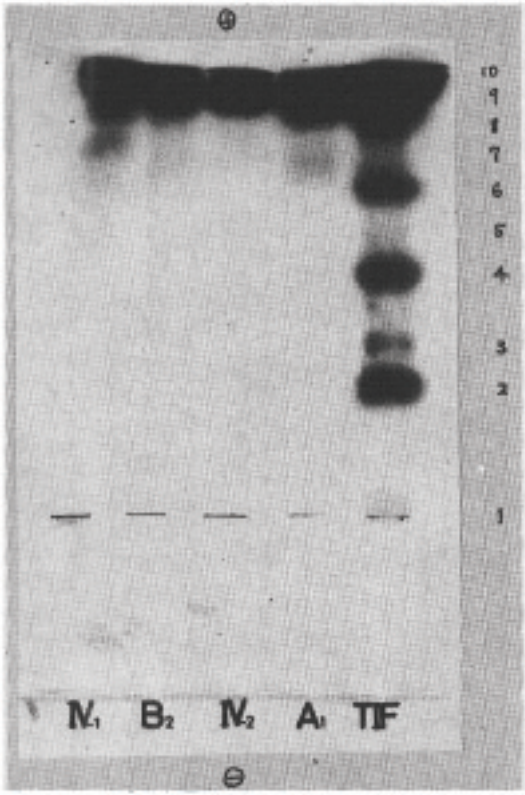

Fig. 6. Polyacrylamide-gel electrophoretic pattern of DEAE fractions (IV-1, IV-2) of Fig. 3, SBTIB-2 (B-2), SBTIA-1 (A-1), and TI fraction.

activities of fraction V clearly indicate that it is homogeneous and identical with the Kunitz inhibitor (1,2) and SBTIA-2 of Rackis and Anderson (4). Eldridge et al. (18) showed that most commercial trypsin inhibitors tested contained the Kunitz inhibitor as a major component. Other TI fractions obtained in this study can be classified into cystine-rich (IV-1, IV-2) or cystine-poor (I, I-1, II, and III) trypsin inhibitor fractions, depending on whether the cystine contents are higher or lower than that of the Kunitz inhibitor.

DEAE fractions IV-1 and IV-2 belong to the cystine-rich inhibitor group which possess as high CTI activity relative to TI activity and eluted from the DEAE cellulose column with 0.10 to 0.15M NaCl. As judged from the electrophoretic and chromatographic behaviors of SBTIB-2 and SBTIA-1 of Rackis and Anderson (4) with fractions IV-1 and IV-2, it is reasonable to assume that IV-1 corresponds to SBTIB-2, and SBTIA-1 is one of the components of IV-2, the 1.9S TI of Yamamoto and Ikenaka (11). Component F-1 of Frattali and Steiner (13) and the low-MW inhibitor of Frattali (12) are high in cystine. Their chromatographic and inhibitory characteristics also suggest that they are probably components of this group. Because of relatively high CTI activity and chromatographic behavior, the

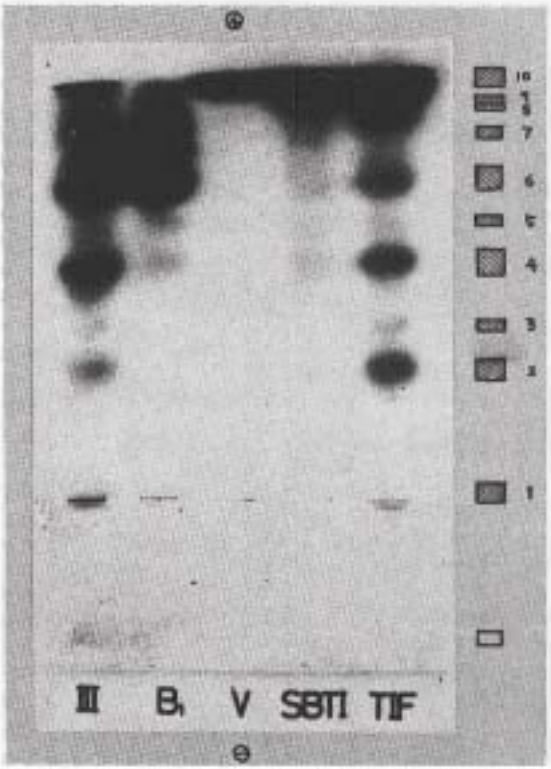

Fig. 7. Polyacrylamide-gel electrophoretic pattern of fraction III of Fig. 3, SBTIB-1 (B-1), fraction V of Fig. 4, commercial SBTIA-2 (SBTI), and TI fraction. Cross-hatched: TI activity positive; blank: TI activity negative.

The acetone-insoluble inhibitors of Birk et al. (7,8,9) and Aso (10) are also cystine-rich, and have relatively high CTI activity and similar chromatographic behavior. Recently, Frattali (12) has shown that 1.9S inhibitor, commercial inhibitor AA of Birk (7,9), and the low-MW inhibitor of Frattali (12) have similar electrophoretic behavior on polyacrylamide-gel, and that the amino acid composition of 1.9S inhibitor and the low-MW inhibitor is nearly identical. It is quite possible that both IV-1 and IV-2 obtained in this study are still heterogeneous and that further fractionation and characterization are necessary to establish relations among various cystine-rich inhibitors.

DEAE fractions I, I-1, II, and III belong to the cystine-poor inhibitor group which eluted from the DEAE cellulose column with less than 0.1M NaCl. These individual fractions were still heterogeneous and contained two or more TI-active electrophoretic components (bands 1 to 7). The major component (band 6) of fraction III corresponded to SBTIB-1 of Rackis and Anderson (4). The components (bands 1 to 5) of fractions I, I-1, and II appeared free of chymotrypsin inhibitor activity, and the minor chymotrypsin inhibitor activity of fraction II may be attributed to the contaminating components (bands 6 and 7) of fraction III. The

presence of CTI-free and cystine-poor TI components in soybean which correspond to the electrophoretic bands 1 to 5 has not been reported in the literature.

Component F-3 isolated by Frattali and Steiner (13) is devoid of tyrosine, and was eluted from the DEAE-cellulose column after the Kunitz inhibitor was eluted. It appears that component F-3 does not correspond to any of the inhibitor fractions obtained in this study.

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