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Soybean Inhibitors. I. Separation and Some Properties of Three Inhibitors from Commercial Crude Soybean Trypsin Inhibitor*

V. Frattali† and R. F. Steiner

ABSTRACT: Commercial crude soybean trypsin inhibitor (STI) has been separated into a number of components by diethylaminoethylcellulose chromatography. In addition to the well-known soybean trypsin inhibitor of Kunitz (Kunitz, M. (1947), *J. Gen. Physiol. 30*, 291) (referred to as "component F_2" in this paper when obtained in a chromatographically pure state), two other homogeneous protein inhibitors (F_1 and F_3) have been isolated and partially characterized. Unlike STI, neither F_1 nor F_3 is a stoichiometric inhibitor of tryptic activity. Also, F_1, like STI, is a moderate inhibitor of chymotrypsin, whereas F_3 has a marginal effect on this enzyme. F_1, F_2, and F_3 have different electrophoretic mobilities on acrylamide gel and different molecular weights within the range 18,000–24,000.

All three proteins contain tryptophan; however, F_3, unlike F_1 and F_2, is devoid of tyrosine. The pH profiles of the intensity of tryptophan fluorescence for F_1 and F_2 are similar, while that for F_3 differs from the other two in the pH range ~1–9. Of the estimated five tyrosine residues in F_1, four are readily iodinated under conditions where only two of the four residues in F_2 (STI) react to form diiodotyrosine.

A number of trypsin and chymotrypsin inhibitors have been isolated from soybean protein. The first to be crystallized and studied in detail was the classical soybean trypsin inhibitor of Kunitz (1945–1947), STI.[1] Although other inhibitors had been reported to be present in soybeans (Laskowski and Laskowski, 1954; Lipke *et al.*, 1954), only within the last few years have several of these been purified and characterized (Rackis *et al.*, 1959, 1962; Rackis and Anderson, 1964; Birk, 1961; Birk *et al.*, 1963; Yamamoto and Ikenaka, 1967).

In addition to the soybean inhibitors, a number of other trypsin and chymotrypsin inhibitors have been isolated in highly purified form from both plant and animal sources. Some examples include the four distinct lima bean inhibitors (Jones *et al.*, 1963), a pancreatic trypsin inhibitor (Kassell *et al.*, 1963), and chicken ovomucoid (Rhodes *et al.*, 1958). At least two chymotrypsin-specific inhibitors have been reported in the literature, namely, the crystalline *Ascaris* inhibitor which is specific for both α-chymotrypsin and chymotrypsin B (Peanasky and Laskowski, 1960) and an inhibitor isolated from potatoes (Ryan and Balls, 1962). This investigation reports the isolation of two additional inhibitors from commercial crude soybean trypsin inhibitor which, apparently, are quite distinct in properties from all other soy inhibitors.

Materials

All enzymes, enzyme substrates, and protein inhibitors were purchased from the Worthington Biochemical Corp., Freehold, N. J. Crude soybean trypsin inhibitor

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[1] Abbreviations used: STI, crystalline soybean trypsin inhibitor of Kunitz (1945–1947); crude STI, crude Kunitz inhibitor obtained from Worthington Biochemical Corp.; $SBTIA_1$ and $SBTIA_2$, soybean trypsin inhibitors A_1 and A_2 described by Rackis *et al.* (1962); TAME, *p*-toluenesulfonyl-L-arginine methyl ester; ATEE, *N*-acetyl-L-tyrosine ethyl ester.

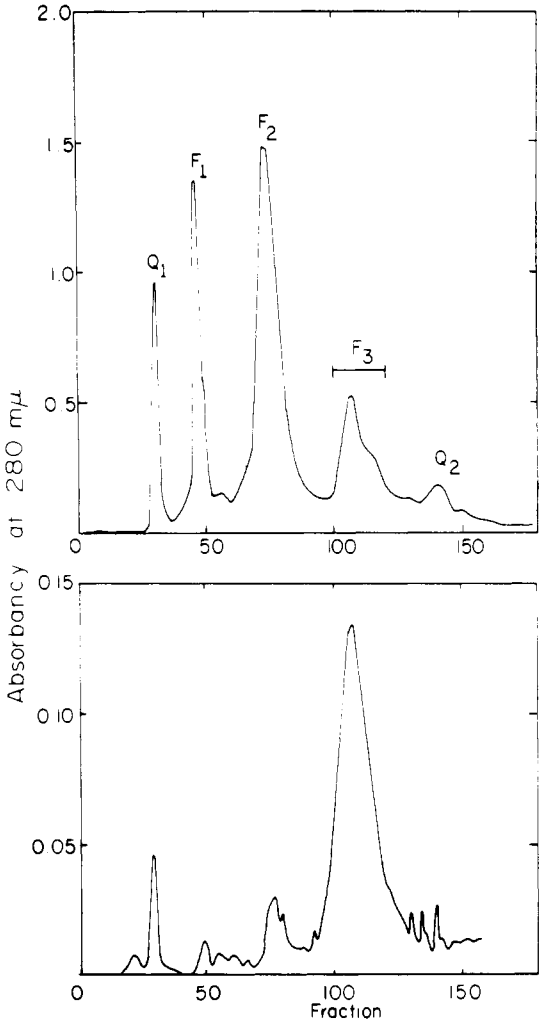

FIGURE 1: Upper: A representative gradient elution pattern of Worthington crude STI (lot 5494) from DEAE-cellulose. Initial and final buffers were, respectively, 0.05 M NH_4OAc (pH 6.5) and 0.5 M NH_4OAc (pH 5.0). Details are given in the text. Lower: Fractions 100–120 (indicated by the bracket in the upper graph) were pooled and dialyzed *vs.* 1.0 mM HCl. The retentate was concentrated to a volume of ~10 ml and applied to the DEAE-cellulose column. The buffer system and elution conditions were the same as those indicated in the Methods section for chromatography of crude STI.

(lot SIC 5494) is an acetone powder of a bentonite-purified aqueous extract of defatted soybean meal.[2]

Chromatographically pure α-chymotrypsin (EC 3.4.4.5) (lot CDS 6606), free of autolysis products, is prepared by Worthington according to the method of Yapel *et al.* (1966). Trypsin (EC 3.4.4.4) (lot TRL 6261) was twice crystallized; soybean trypsin inhibitor prepared according to the method of Kunitz (1947) (lot SI 618) was thrice crystallized.

DEAE-cellulose was obtained from Bio-Rad Laboratories as their "Cellex-D" (lot B-2232) which had an exchange capacity of 0.9 mequiv/g. Polyacrylamide gels were prepared with "Acra Gel" (premixed acrylamide and *N,N'*-methylenebisacrylamide supplied as Model 4-570 by Arden Instruments Inc., Rockville, Md.). Inorganic salts were analytical grade and all solutions were prepared with glass-distilled water.

Methods

DEAE-cellulose Chromatography. DEAE-cellulose was freed from fines by washing the material in distilled water, allowing the bulk to settle, and then decanting the suspended fines. The collected residue was then alternately washed with 1-l. quantities of 0.01 M KOH and 0.01 M HCl and, finally, with several liters of distilled water. This material was then equilibrated with 0.05 M NH_4OAc (adjusted to pH 6.5 by the addition of NH_4OH). A 2 × 60 cm column of adsorbent was prepared by allowing 1 l. of starting buffer, 0.05 M NH_4OAc (pH 6.5), to pass through the column.

Crude STI was dissolved in starting buffer, approximately 300 mg/5–10 ml, and the pH was readjusted to 6.5 by the addition of a small volume of 0.1 M NH_4OH. Upon application of the sample, the column was eluted with a gradient of pH and ionic strength using the two buffers 0.05 M NH_4OAc (pH 6.5) and 0.5 M NH_4OAc (pH 5.0) (adjusted to the stated pH by the addition of HOAc). Gradients were produced with a rectangular Varigrad (Peterson and Sober, 1959; Peterson and Rowland, 1961) in which the following volumes of 0.5 M NH_4OAc (pH 5.0) were placed in compartments 1–9, respectively: 0, 20, 40, 60, 80, 100...100 ml. The first five compartments were then brought to 100 ml by the addition of 0.05 M NH_4OAc (pH 6.5). Elution was performed at 4 ± 1° with an initial flow rate of approximately 1 ml/min; 5.0-ml fractions were collected. Protein peaks emerging from the column were identified by their absorbancy at 280 mμ.

Enzyme Assays. TRYPSIN. Trypsin activity was determined by using TAME as substrate. The assay kit, supplied by Worthington as "Determatube TAME," was utilized. The rate of esterolysis of the substrate by the enzyme at 25.0° was followed from the absorbancy change at 247 mμ.

The fraction of inactive trypsin in the commercial product was calculated by determining the per cent inhibition produced at two different STI concentrations and extrapolating to 100% inhibition. (Chromatographically purified STI was used; this material is referred to as "component F_2" in this paper. Molecular weight and extinction coefficient values for chromatographically pure STI given by Rackis *et al.* (1962) and listed in Table III were assumed.) Since trypsin is stoichiometrically inhibited by STI, a positive deviation from unity in the molar ratio of enzyme to inhibitor at 100% inhibition is a measure of the amount of inactive trypsin

[2] Personal communication by Dr. A. L. Baker, Technical Director, Worthington Biochemical Corp.

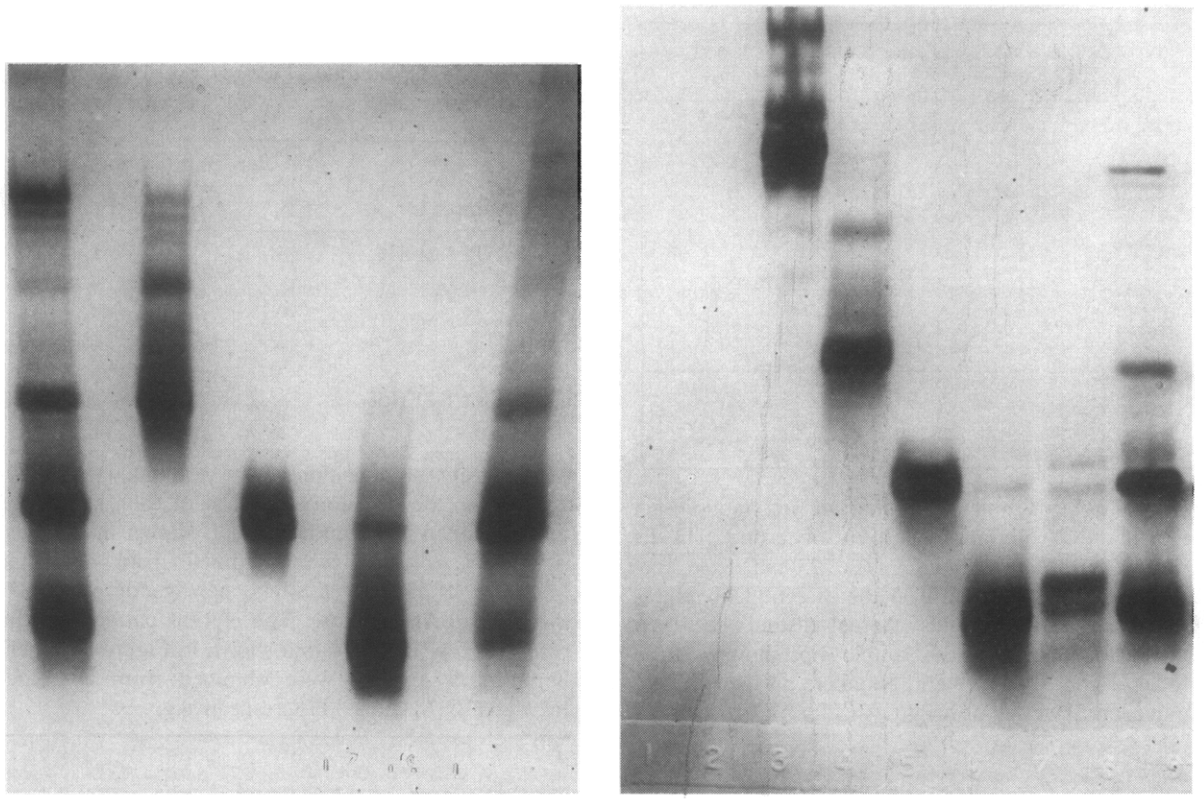

FIGURE 2: Acrylamide gel electrophoretic migration patterns. Left: For crude STI, F_1, F_2, F_3, and crystalline STI, Worthington lot 618 (from left to right). The direction of migration for all components is from the cathode (top of photo) to the anode. Experimental details are given in the Methods section. See Table I for the per cent composition of the various components in each sample. Right: For Q_1, F_1, F_2, F_3, Q_2, and crude STI (from left to right).

in the commercial enzyme. Accordingly, the crystalline enzyme used in this study was calculated to be 48% inactive.

An optical factor of 0.64 for trypsin (Green, 1953; Baines *et al.*, 1964) was used to convert absorbancy at 280 mμ to concentration (milligrams per milliliter). A molecular weight of 23,800 was assumed (Cunningham, 1954).

CHYMOTRYPSIN. Chymotryptic activity was determined spectrophotometrically at 237 mμ by following the time course of hydrolysis of ATEE at 25.0°. The assay kit supplied by the Worthington Corp. as "Determatube CHY" was utilized. The assumption was made that the particular lot of chymotrypsin used throughout this investigation was 100% active. The concentration of chymotrypsin (milligrams per milliliter) was computed from the absorbancy at 280 mμ; a conversion factor of 0.50 (Dixon and Neurath, 1957) was used. To convert to a molar concentration a molecular weight of 25,000 was assumed (Wilcox *et al.*, 1957).

Enzyme Inhibition. Unless otherwise specified, the same general format for determining chymotryptic and tryptic activity was observed. All microliter aliquots were delivered with certified Lang–Levy pipets; stock solutions of enzyme in 1.0 mM HCl were prepared fresh daily.

To 2.0 ml of an HOAc–KOAc buffer (pH 5.0, ionic strength 0.033) was added 200 μl of stock enzyme solution and an aliquot (from 25 to 100 μl) of a stock inhibitor solution. At known time intervals after the addition of inhibitor to the enzyme, a 100-μl aliquot of enzyme–inhibitor solution was mixed with 2.0 ml of substrate and the rate of the appropriate esterolytic reaction was determined spectrophotometrically.

Polyacrylamide Gel Electrophoresis. Gel electrophoresis was performed with the Arden 500 electrophoresis system (Arden Instruments Inc., Rockville, Md.). The cylindrical unit produces two gel slabs with dimensions 6 in. wide, 7 in. long, 1/8 in. thick. The buffer consists of 5.39 g of Tris/l., 0.463 g of disodium EDTA/l., and 2.75 g of boric acid/l. (pH 8.37). Monomer solutions of "Acra Gel," 5% w/v, were prepared with the above-mentioned buffer as solvent. Protein samples were dissolved in 30% sucrose–Tris–borate–EDTA buffer; 20 μl of ~2% protein solution was applied to preformed slots in the gel slab. Electrophoresis was conducted for 2 hr at 300 V and ~120 mA; during a run, cooling is provided by running tap water through an inner cylinder of the apparatus.

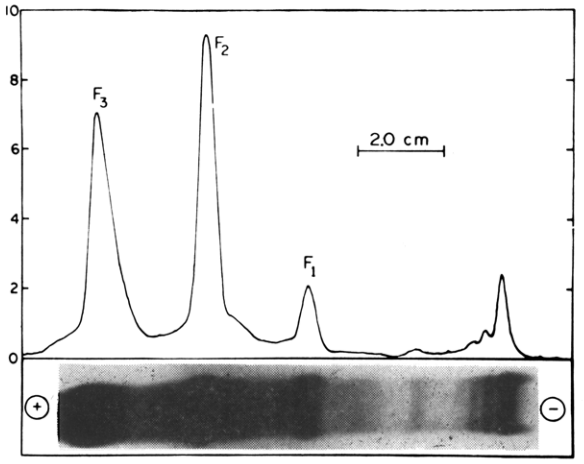

FIGURE 3: Densitometer tracing of acrylamide gel electrophoretic migration pattern of crude STI as portrayed in Figure 2 (left). The 2.0-cm scale corresponds to linear distance in the direction of migration of the various bands. Actual distance between point of application of the sample (not shown) to the middle of band F_3 = 13 cm. Numbers on the ordinate are in arbitrary units of height.

TABLE I: Per Cent Composition of Various Components in Crude STI, Crystalline STI, and Chromatographically Isolated F_1, F_2, and F_3.

Sample	Per Cent Composition[a]			
	F_1	F_2	F_3	Other
Crude STI (lot SIC 5494)	8	41	41	10
F_1[b]	89	0	0	11
F_2[b]	0	~100	0	0
F_3[b]	0	5	95	0
Crystalline STI (lot SI 618)	6	77	11	6

[a] Per cent compositions were estimated from the area under the densitometer trace of each sample on the developed acrylamide gel slab shown in Figure 2 (left). [b] Sample F_1 was obtained from combined fractions 48–55 after a single passage of crude STI through DEAE-cellulose. The elution pattern for this run was very similar to that shown in Figure 1 (upper). Samples F_2 and F_3 were obtained from combined fractions 70–78 and 99–118, respectively.

Protein bands were identified with an Amido Black stain.

Amino Acid Analyses. Analyses conducted at the Naval Medical Research Institute were made with a Spinco automatic amino acid analyzer, Model 120B. Aqueous protein solutions were acidified with an equal volume of ~12 N HCl and hydrolyzed at 110° for 22 hr in sealed, evacuated ampoules. One analysis performed by Analytica Corporation, Long Island, N. Y., was conducted in a similar fashion. All protein samples were desalted prior to hydrolysis by passage through a Sephadex G-100 column equilibrated with 1 mM HCl.

Ultracentrifugal Analyses. With one exception, molecular weight estimates were made by the sedimentation equilibrium method of Van Holde and Baldwin (1958) using interference optics and a 3.0-mm column. Molecular weights were calculated from the slope of a plot of ln J *vs.* r^2. The molecular weight of chromatographically purified STI (F_2) was determined by the Yphantis (1964) meniscus depletion method.

Extinction Coefficients. Extinction coefficients were calculated with protein solutions of known absorbancy at 280 mμ using Charlwood's (1957) method to determine absolute concentration.

Iodination. Protein solutions were prepared in 0.1 M Tris buffer (pH 9.0) at 25°. The procedure outlined below was used for the two runs mentioned in this paper. To each of a series of test tubes were added 0.50 ml of 0.1 M Tris (pH 9.0) and 0.10 ml of a given stock protein solution. All samples were cooled to 4° and an aliquot (from 5 to 200 μl) of a precooled 0.05 M I_2–0.2 M KI solution was added to each tube. Five minutes after the addition of the iodine solution, the reaction was quenched by the addition of 0.40 ml of a 0.1 M $Na_2S_2O_3$–0.5 M lysine solution of pH 9.0. Solutions were warmed to room temperature and the number of moles of diiodotyrosine formed was computed from the absorbancy at 325 mμ, assuming a molar extinction coefficient of 5.0×10^3 for diiodotyrosine (Edelhoch, 1962).

Ultraviolet Fluorescence. The intensity of emission of fluorescent radiation was measured with an Aminco–Bowman spectrofluorometer, equipped with an Osram light source. The activation wavelength was 290 mμ; emission was observed at 350 mμ. With the use of a hollow cell holder, through which water from a constant temperature bath was circulated, solution temperature in any given cuvet was regulated at 25.0 ± 0.1°. Titrations were performed by the addition of small volumes of dilute HCl or KOH, 0.1–3.0 M, with a 0.20-ml pipet. For this determination, a control solution was maintained at a fixed pH and alternate readings of emitted fluorescence were recorded for the control and the solution being titrated. The observed intensity of the variable solution was then corrected for dilution and normalized with respect to the control.

Spectrophotometric Data. All ultraviolet absorbancies were obtained with a Beckman Model DU spectrophotometer which is equipped with a pair of thermospacers for temperature control and a photomultiplier attachment.

Results

DEAE-cellulose Chromatography. Figure 1 (upper

TABLE II: Amino Acid Composition.

	F_1 Residues/18,300 g		F_3 Residues/23,400 g		Best Estimates		
					F_1	F_3	STI[a]
Lys	10.4	12.4	21.8	21.0	11 ± 1	21	11
His	2.3	2.7	4.8	4.9	3	5	2
NH_3[b]	13.7	12.7	29.4	24.4	13	27 ± 2	16
Arg	8.4	8.8	8.1	7.7	9	8	9
Asp	21.8	20.8	29.1	30.7	21	30	29
Thr[c]	6.5	5.4	4.7	4.8	6	5	8
Ser[c]	14.6	15.6	11.1	11.1	15	11	13
Glu	22.3	24.7	46.9	51.0	23 ± 1	49 ± 2	21
Pro	7.6	8.4	6.8	5.9	8.0	6	10
Gly	10.8	10.0	11.1	10.2	10	10	18
Ala	6.1	4.4	5.5	4.5	5	5	9
Cys-$^1/_2$[c]	14.3	13.3	12.1	12.8	14	12	4
Val	5.0	3.5	3.0	1.5	4	2	12
Met	5.5	5.6	9.6	10.5	6	10	3
Ile	6.7	6.6	8.2	7.5	7	7	14
Leu	10.9	10.6	13.8	12.5	11	13	16
Tyr[c]	4.8	4.2	Trace	<0.1	5	0	4
Phe	4.0	3.1	2.6	1.4	4	2	9
Trp					1[d]	3[d]	2
Tyr[d]	5.0		0.5				
Trp[d]	1.3		2.5				

[a] Obtained from Wu and Scheraga (1962). [b] Corrected for decomposition of serine and threonine (Rees, 1946). [c] The following corrections, according to Moore and Stein (1963), were made for the estimated decomposition after 20-hr acid hydrolysis: threonine, cystine, and tyrosine 5%; serine 10%. [d] Determined spectrophotometrically according to the method of Edelhoch (1967). Corresponding values for the tyrosine and tryptophan content of F_2 (residues/21,600 g) were calculated to be 4.8 and 2.7, respectively.

illustration) illustrates a typical elution pattern of crude STI from a DEAE-cellulose column. Protein fractions that yielded essentially homogeneous components when subjected to gel electrophoresis are labeled F_1, F_2, and F_3.[3] Component F_2 is the classical inhibitor, STI, isolated and characterized by Kunitz (1945–1947). Peaks Q_1 and Q_2 contain multiple protein fractions, as will be demonstrated later, which are not further resolved with the present chromatographic system. Figure 1 (lower) illustrates the elution pattern for component F_3 obtained from pooled fractions 100–120. It can be seen that the principal contaminant, ~5% F_2, is well separated from component F_3. Component F_3, free of F_2, was used in the enzyme inhibition studies. Occasionally, upon reuse of the column with other samples from the same lot of crude STI, F_1 eluted as a split peak. This occurrence, however, is not a reflection of heterogeneity but may well be the result of minor changes in ionic strength of the solvent used to dissolve crude STI.

Polyacrylamide Gel Electrophoresis of Chromatographically Purified Components. The migration patterns of the three major components obtained by a single passage of crude STI through a DEAE-cellulose column along with those for crude STI and commercial crystalline STI are given in Figure 2 (left). Estimates of the per cent composition of the major components in any given sample, obtained from the area under the densitometer trace of each of the five samples seen in Figure 2 (left), are given in Table I. A reproduction of a trace of crude STI is given in Figure 3. With the Tris–borate–EDTA buffer, all bands migrate toward the anode; the actual distance travelled by F_3 in Figure 3, as measured from point of application of the sample to the middle of the band F_3, is 13 cm. Figure 2 (right) demonstrates the heterogeneous character of the protein eluting as peaks Q_1 and Q_2.

Amino Acid Analysis. Results of amino acid analyses

[3] These combined letter and numerical designations, along with Q_1 and Q_2, are used exclusively in this paper. The terms F_1, F_2, and F_3 are used to represent the *first*, *second*, and *third* major protein *fractions* that elute from the DEAE-cellulose column and are, apparently, homogeneous. To restate a point, the protein component, F_2, is a highly purified form of soybean trypsin inhibitor crystallized and characterized by Kunitz (1945–1947). Hence, the authors prefer that in any future reference to "F_2" some designation as "chromatographically pure soybean trypsin inhibitor of Kunitz" be used.

TABLE III: Comparison of Molecular Weights and Specific Extinction Coefficients of Soybean Inhibitors.

Inhibitor	Mol Wt	ϵ_{280}[a]	Reference
F_1	18,300	0.716	This paper
F_2[b]	22,000	1.04	This paper
$SBTIA_2$[b]	21,600	0.994	Rackis *et al.* (1962)
STI[c]	22,700	0.900	This paper
STI[c]	21,500 ± 800	0.944	Wu and Scheraga (1962)
F_3	23,400	0.634	This paper

[a] Extinction coefficient for 1 mg/ml at 280 mμ, 1.0-cm cell. [b] Chromatographically pure STI. [c] Commercial, five-times crystallized.

of F_1 and F_3 are given in Table II; best estimate values for these two components are compared with similar data for STI (Wu and Scheraga, 1962). From inspection of the data, it is seen that all three are acidic in character, F_3 more so than the other two. One of the more striking differences between chromatographically pure STI and both F_1 and F_3 is the high sulfur content of the latter pair. Since all half-cystine residues presumably participate in S–S bonds in the protein molecules, the only other trypsin inhibitors that show a greater degree of cross-linkage are the 1.9S soybean inhibitor of Yamamoto and Ikenaka (1967) and the lima bean inhibitor components isolated by Jones *et al.* (1963).

Of particular interest is the presence of tryptophan and absence of tyrosine in component F_3. This peculiar combination has been reported in one other proteinase inhibitor (Peanasky, 1963). The reverse of this combination of chromophores is known to occur in another soy inhibitor (Birk *et al.*, 1963), the four lima bean trypsin inhibitors (Jones *et al.*, 1963), a pancreatic trypsin inhibitor (Kassell *et al.*, 1963), and a number of avian ovomucoids (Stevens and Feeney, 1963).

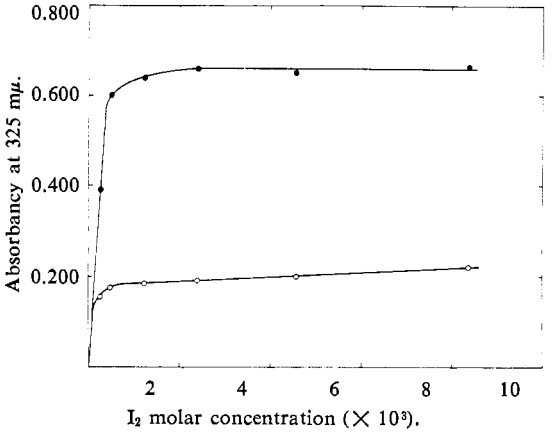

FIGURE 4: Iodination of the tyrosine residues in components F_1 (●) and F_2 (○) as followed by the absorbancy increase at 325 mμ. Concentration of F_1 = 0.598 mg/ml, that of F_2 = 0.481 mg/ml. The solvent is 0.1 M Tris (pH 9.0) at 25°. The calculated number of moles of diiodotyrosine formed per mole of protein at an I_2 concentration of 4.5×10^{-3} M are 4.0 for F_1 and 1.8 for F_2.

Molecular Weights and Extinction Coefficients. In Table III are tabulated the molecular weights and extinction coefficients for F_1, F_2, and F_3, along with similar values for STI. Partial specific volume values ($\bar{v}$) for F_1 (0.710 ml/g) and F_3 (0.708 ml/g) were calculated from amino acid analyses for these proteins according to the method of Cohn and Edsall (1943); that for F_2, $\bar{v}$ = 0.735 ml/g, was obtained from Rackis *et al.* (1962).

Inhibiting Capacity. Table IV is a compilation of results of inhibition of tryptic and chymotryptic activity by the various components. Since inhibition of trypsin by F_2 is stoichiometric, *i.e.*, a given quantity of inhibitor completely abolishes the activity of an equimolar quantity of enzyme, the corresponding data have not been included in the table. Inspection of the data shows that neither F_1 nor F_3 is a stoichiometric inhibitor[4] for either enzyme. It is seen that, ranked according to potency of inhibition of tryptic and chymotryptic activity, the following order persists: $F_2 > F_1 > F_3$. The inhibiting effect of F_2 on chymotrypsin using ATEE as substrate is in fair agreement with data of Wu and Laskowski (1955) who investigated the same enzyme–inhibitor system using casein as substrate. They observed 50% inhibition of chymotryptic activity at a constant STI concentration of 20 μg/ml and two different enzyme concentrations, 10 and 25 μg/ml (inhibition ratios 10:20 = 0.50, 25:20 = 1.25). By interpolation of the data in Table IV, 50% inhibition of the esterolytic activity of chymotrypsin with ATEE as substrate is produced at enzyme and inhibitor concentrations of 1.11 and 1.52 μg/ml, respectively (inhibition ratio, 1.11:1.52 = 0.73).

As noted previously, inhibition of trypsin by F_1 is nonstoichiometric. Additionally, it was found that

[4] It is assumed throughout this text that "nonstoichiometric inhibition" arises from the fact that the enzyme–inhibitor complex has a high dissociation constant.

TABLE IV: Inhibiting Capacity of Components F_1, F_2, and F_3 for Tryptic and Chymotryptic Activity.[a]

I/E[b]	E (μg/ml)	Exposure Time of I to E (min)	Act. (%)[c]	Comments
		Trypsin-F_1		
1.3	0.56	2	49	
1.3	0.56	10	70	
1.3	0.56	20	78	
3.7	0.56	2	37	
3.7	0.56	10	62	
3.7	0.56	20	84	
3.7	0.56	30, 45, 60	87	
7.5	0.56	2	25	
7.5	0.56	20	61	
7.5	0.56	30, 45	78	
1.4	0.56	2	31	*d*
1.4	0.56	10	35	*d*
1.4	0.56	20	41	*d*
1.4	0.56	2	29	*e*
1.4	0.56	10	35	*e*
1.4	0.56	20	46	*e*
4.0	0.53	2	26	*f*
4.0	0.53	10	43	*f*
4.0	0.53	20	72	*f*
1.3	0.59	~2	47	*g*
1.3	0.59	~2	47	*h*
		Trypsin-F_3		
2.3	0.59	~2	95	
11	0.58	~2	73	
22	0.58	~2	45	
		Chymotrypsin-F_1		
0.82	0.95	2, 10, 20	97	
1.6	0.95	2, 10, 20	90	
3.4	1.2	2, 10, 20	64	
6.9	1.2	2, 10, 20	45	
14	1.2	2, 20	27	
		Chymotrypsin-F_2		
0.52	1.1	2, 10, 20	79	
1.1	1.1	2, 10, 20	64	
2.1	1.1	2, 10, 20	44	
		Chymotrypsin-F_3		
2.4	1.2	~2	97	
7.1	1.2	~2	97	
14	1.2	~2	94	

[a] The procedure for determining enzyme inhibition as outlined in the text was followed unless otherwise stated. [b] Molar ratio of inhibitor to enzyme. All data involving trypsin were corrected for 48% inactive material in the commercial enzyme. The assumption that inactive enzyme does not bind inhibitor was made. Chymotrypsin was assumed to be fully active. Molar concentrations of F_1 and F_3 were calculated with molecular weight and extinction coefficient data given in Table III. The molarity of F_2 was calculated using the data of Rackis *et al.* (1962) (*cf.* $SBTIA_2$ in Table III). [c] Residual enzymic activity expressed in per cent. [d] Inhibitor exposed to enzyme in 0.045 M Tris–8.9×10^{-3} M $CaCl_2$ (pH 8.1) for the specified time prior to assay. [e] Inhibitor exposed to enzyme in 0.045 M Tris (pH 8.1) for the specified time prior to assay. [f] Inhibitor exposed to enzyme in 8.9×10^{-3} M $CaCl_2$, HOAc–KOAc (pH 5.0, ionic strength 0.015) for the specified time prior to assay. [g] Inhibitor was 60% iodinated. [h] Inhibitor was fully iodinated.

F_1 became less potent as the length of time of exposure to trypsin increased when both were incubated in 0.1 M HOAc (pH 5.0) prior to assay (Table IV). Substitution of the acetate buffer with 0.05 M Tris–0.01 M $CaCl_2$ (pH 8.1) was found to enhance the potency of F_1 and to decrease the rate of decay of its inhibiting capacity. Neither effect can be ascribed to a loss of activity by trypsin at this slightly alkaline pH for in a control experiment tryptic activity remained constant over a 20-min period. Repetition of this experiment with calcium ion absent produced essentially the same results; the trypsin control did, however, demonstrate a loss of activity (~15%) in 20 min.

Reactivity of Tyrosine Groups. When F_1 is iodinated at 3°, it is found that four of the estimated five tyrosine groups react to form diiodotyrosine (Figure 4). The same reaction with the companion inhibitor (F_2) results in iodination of two of the four tyrosine residues. This latter observation has been reported previously (Steiner, 1966); it has been repeated and is presented here for comparative purposes. The fact that most of the tyrosine residues in F_1 are readily accessible for reaction with iodine suggests that these residues are not deeply buried in the interior of the molecule.

Tyrosine Ionization. Figure 5 shows the pH profile of tyrosine ionization for F_1, as monitored by the increase in absorbancy at 295 mμ.

Tryptophan Fluorescence. Ultraviolet emission by tryptophan-containing proteins has been shown to be due almost entirely to this chromophore, the contribution of tyrosine being largely suppressed (Teale, 1960). Since F_3 is devoid of tyrosine, the fluorescent display at 350 mμ can only arise from the indole group of tryptophan. The dependence of the intensity of

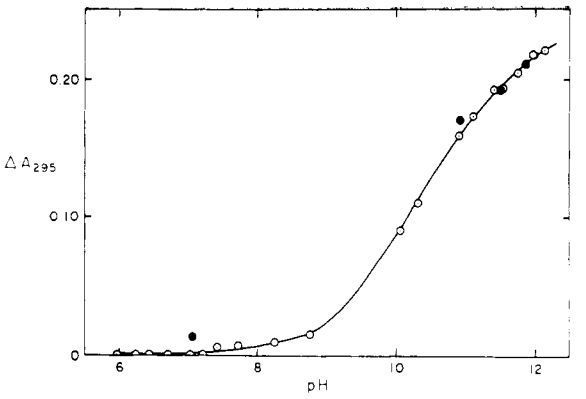

FIGURE 5: Effect of alkaline pH on the absorbancy of F_1 (0.53 mg/ml) in 0.1 M phosphate buffer at 295 mμ. The protein blank was maintained at pH 6.0. Open circles represent the forward titration from pH 6.0; closed circles are for the reverse titration from pH 12.2.

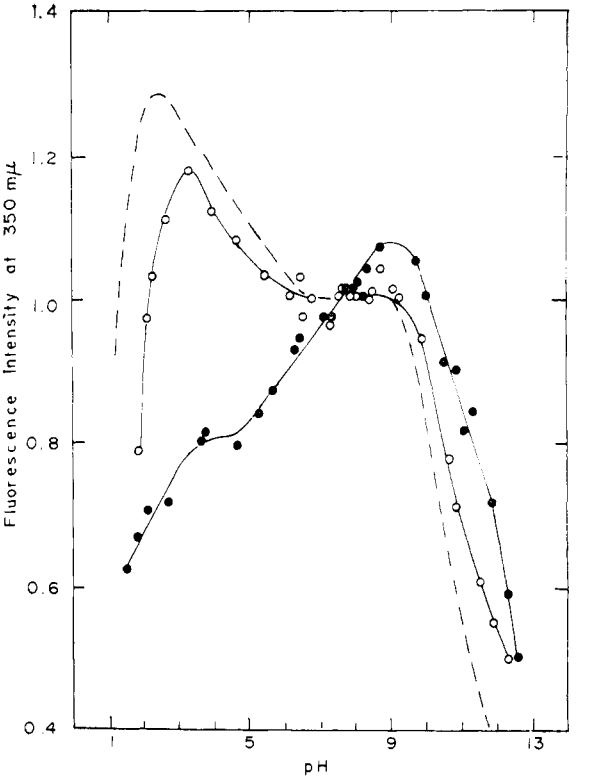

FIGURE 6: Effect of pH on the intensity of fluorescence of F_1 (○) and F_3 (●) in 0.1 M KCl–0.01 M Tris. Protein concentration ≅0.02%. The dashed curve is a representation of similar data for crystalline STI in 0.3 M KCl from Steiner and Edelhoch (1963).

fluorescence on pH for F_3 is quite different from that obtained with F_1 and STI (Figure 6). The latter two inhibitors, although dissimilar in several respects, exhibit closely corresponding fluorescence intensities over a wide pH range. On the other hand, the profile for F_3 from pH ~1 to 9 is altogether different. While F_3 demonstrates an almost uniform increase in intensity in this range, F_1 and STI exhibit maxima at pH 2–3 with plateau regions from pH 6 to 9. All three, however, undergo alkaline quenching beyond pH 9.

Discussion

Homogeneity. All three major components (F_1, F_2, and F_3) are obtained in a relatively pure state directly from the DEAE-cellulose column. This point is verified by polyacrylamide gel electrophoresis of selected, pooled fractions from the column. Since component F_2 is obtained in a highly purified state using crude STI as the starting material, this may well be a method of choice, as a one-step operation, for preparing rather homogeneous preparations of STI. Eldridge *et al.* (1966) have amply demonstrated that nine commercial samples of crystalline soybean trypsin inhibitor separate into six or more bands when analyzed electrophoretically on polyacrylamide gel in an 8 M urea medium.

Inhibiting Capacity. Most trypsin inhibitors reported to date (Kunitz, 1947; Birk *et al.*, 1963; Rackis *et al.*, 1962; Yamamoto and Ikenaka, 1967) react stoichiometrically with trypsin to abolish the enzyme's proteolytic and/or esterolytic activity. Neither F_1 nor F_3 has been found to be this potent. In the case of F_1, solution composition appears to be a critical factor and the best conditions found to date are given in Table IV. It remains to be seen if the trypsin–F_1 complex is more stable at pH 8 rather than at pH 5 as a result of the change in pH or the change in specific ion on going from an acetate to a Tris buffer. It should be mentioned that the gradual loss of inhibiting capacity of F_1 as a function of time of exposure of this inhibitor to trypsin may permit the classification of F_1 as a "temporary inhibitor" of trypsin. Laskowski and Wu (1953) suggested the term "temporary inhibition" to describe the case in which an inhibitor is also a substrate for an enzyme. Accordingly, at least two reported cases fall in this category, Kazal's inhibitor–trypsin (Laskowski and Wu, 1953), and ovomucoid–trypsin (Gorini and Audrain, 1952, 1953). However, since the results of an experiment designed to determine if F_1 is digested by trypsin were inconclusive,[5] classification of F_1 as a temporary inhibitor of trypsin cannot be made without reservation at the present time.

Inhibition of chymotryptic activity is best accomplished with component F_2; F_1 has some effect, F_3 possesses the least inhibiting capability. On the other hand, two soybean inhibitors, purified inhibitor AA (Birk *et al.*, 1963) and the 1.9S inhibitor (Yamamoto and Ikenaka, 1967), are fairly potent chymotrypsin inhibitors. Reportedly, purified inhibitor AA is 13 times more efficient than crystalline STI, and the 1.9S inhibitor combines with chymotrypsin in an equimolar amount to retard the activity of the enzyme. Additionally, it has been determined that $SBTIA_1$ (Rackis

[5] Unpublished data of V. Frattali and R. F. Steiner.

et al., 1962; Rackis and Anderson, 1964) counteracts the esterolytic activity of chymotrypsin on the substrate ATEE. In a preliminary experiment, about 50% inhibition of chymotrypsin (1.2 μg/ml) was observed at an approximate molar ratio of 0.45 (inhibitor/enzyme). When first obtained, F_1 was thought to be $SBTIA_1$ because both have very similar electrophoretic mobilities on acrylamide gel. However, discrepancies in inhibiting capacity and molecular weight eventually indicated their dissimilarity.

In summary, the order of inhibition of trypsin by soy fractions characterized to date is: STI (F_2), $SBTIA_2$ = $SBTIA_1$ = purified inhibitor AA (Birk *et al.*, 1963) = 1.9S inhibitor (Yamamoto and Ikenaka, 1967) > F_1 > F_3. The approximate order for chymotrypsin is: 1.9S inhibitor $\geqslant$ purified inhibitor AA $\geqslant$ $SBTIA_1$ > STI (F_2) > $F_1 \gg F_3$.

Composition and Structure. Apart from the conspicuous absence of tyrosine and presence of tryptophan in component F_3 and the large number of S–S bonds in F_1 and F_3, little can be said of the composition of the three components. It remains to be seen if the disulfides in F_1 and F_3 are intrachain bridges, as is the case of the two S–S bonds in STI (Wu and Scheraga, 1962; Steiner, 1965), or are interchain bridges.

The fact that four of the five tyrosine residues in F_1 are readily iodinated suggests at least two possibilities. (a) These aromatic residues normally exist at or near the surface of the molecule and are readily accessible to a reactive molecule in solution or (b) under the conditions of the iodination reaction, the structure of F_1 is so perturbed as to expose these residues which ordinarily prefer to exist in the interior of the macromolecule. A decision on this matter cannot be reached without further experimental evidence.

Significance. All components are distinct proteins which exist in the commercial preparation of crude STI and some commercial preparations of crystalline STI. No new component is formed during the separation and isolation of the constituents of crude STI. On the basis of the substantial differences that are observed in the amino acid analyses, it seems unlikely that all three components (F_1, F_2, and F_3) arise from a common parent molecule. It is possible that the production of multiple inhibitors, including the inhibitors of Rackis and Anderson (1964), Birk *et al.* (1963), and Yamamoto and Ikenaka (1967), is a result of genetic heterogeneity; however, this point must be regarded as pure conjecture at present.

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