Polyacrylamide-Gel Electrophoresis of Reduced and Alkylated Soybean Trypsin Inhibitors[1]

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ABSTRACT

Polyacrylamide-gel electrophoresis of native and reduced-alkylated soybean trypsin inhibitors showed them to be different proteins rather than intramolecular disulfide forms or intermolecular disulfide polymers of a single protein. Reduction alone or reduction followed by alkylation caused one of the inhibitors to migrate much slower in 8*M* urea than did the native inhibitor. This decrease in mobility, which was accompanied by an increase in intrinsic viscosity, is attributed to an unfolding after cleavage of the disulfide cross-linkages.

Several soybean proteins have the ability to inhibit trypsin and so are referred to as soybean trypsin inhibitors (SBTI's). Previous reports from the Northern Laboratory describe isolation of four SBTI's which differ in their electrophoretic behavior as shown by moving-boundary (1) and by polyacrylamide-gel electrophoresis (2). The four SBTI's have different sedimentation coefficients (1), and one contains two disulfide bonds (3). Therefore, the different species of SBTI's could possibly be (a) various intramolecular disulfide structures of the same molecule (if two disulfides are present, there would be three possible intramolecular disulfide structures); (b) intermolecular disulfide polymers of a single protein (which would agree with the reported sedimentation coefficients); or (c) different proteins.

If only structures a or b (intra- and intermolecular disulfides) are present, then

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reduction and alkylation of the SBTI's and analysis by polyacrylamide-gel electrophoresis in 8M urea should reveal a single protein band. If structure c is present, then the electrophoretic pattern of the reduced-alkylated SBTI's should show different protein bands.

Our studies were undertaken, therefore, to compare the reduced-alkylated SBTI's by gel electrophoresis to determine if a single polypeptide chain is common to each of the SBTI's.

MATERIALS AND METHODS

Preparation of four SBTI's (SBTI-B1, -B2, -A1, -A2) has been described (1,4,5) and the samples in this study are a portion of those previously used (1,2). Commercial SBTI three-times crystallized (SBTI-3X, Gallard Schlesinger Chemical Manufacturing Company, Garden City, New York, Lot A4570) was used for viscometric analysis.

The SBTI's were reduced in potassium phosphate-sodium chloride buffer, pH 7.6, ionic strength 0.1, containing 8M urea. A molar ratio of 2-mercaptoethanol to disulfide content of 400:1 was selected to reduce the proteins, assuming 2 mol. of cystine per mol. (3) for all inhibitors. After 1-hr. reduction, acrylonitrile was added in a 2:1 molar ratio to 2-mercaptoethanol and the alkylation was allowed to proceed 30 min. while the pH was kept at 7.6. The solution was adjusted to pH 4.5 with hydrochloric acid; after the suspension had been dialyzed against water at 2°C. for 1 week, it was freeze-dried.

Quantitative amino acid analysis by the ion-exchange procedure of Moore et al. (6) at 50°C., after hydrolysis of the proteins for 24 hr. with 6N HCl, was used to determine to what extent the proteins were modified by reduction and alkylation. S-Carboxyethyl cysteine, the derivative of cysteine after alkylation and hydrolysis, elutes before glutamic acid (7). Thus, extent of alkylation can be determined during amino acid analysis. Addition of known S-carboxyethyl cysteine to the hydrolysates of native and reduced-alkylated SBTI before chromatography gave its elution position, as well as its color yield and recovery.

Polyacrylamide-gel electrophoresis was performed as previously reported by us (2), except that the patterns were destained by the procedure of Ferris et al. (8), which worked satisfactorily. The concentration of Cyanogum 41 was sometimes altered, but the amount of catalysts always remained the same.

Viscosity measurements were made at 25°C. with a Cannon-Fenske modified Ostwald viscometer, ASTM No. 100. Densities of the buffer and protein solutions were measured with a 5-ml. pycnometer. Intrinsic viscosities were evaluated by plotting $(\eta_{rel}-1)/c$ vs. c and extrapolating to zero concentration, where c is the concentration of protein in g. per 100 ml. of solution.

RESULTS AND DISCUSSION

Effect of Reduction and Reduction-Alkylation

Figure 1 shows the results of polyacrylamide-gel electrophoresis of various trypsin inhibitors isolated from soybean whey (1) before and after reduction and alkylation. Figure 1 also shows the gel pattern of soybean whey proteins and a relative mobility scale (R_m) in which an R_m of 1.00 is assigned to native SBTI-A2 which corresponds to the major band of five-times crystallized SBTI (2).

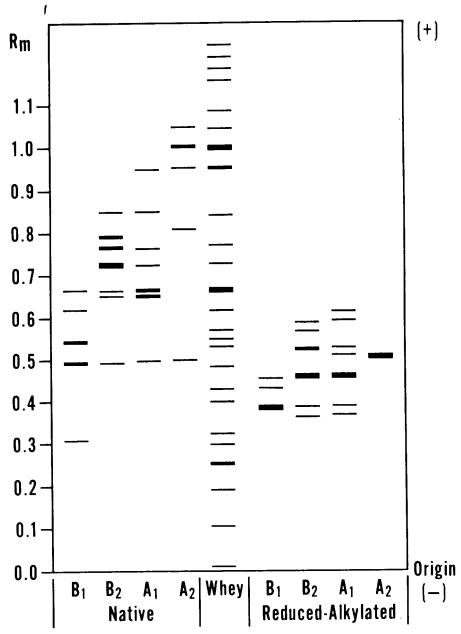


Fig. 1. Polyacrylamide-gel electrophoretic patterns of native and reduced-alkylated SBTI-B1, -B2, -A1, -A2. Electrophoresis buffer was 0.025M glycine, pH 9.2, containing 8M urea.

Mobilities of the reduced-alkylated SBTI's were lower than mobilities of the native proteins. SBTI-B2 and -A1 appeared similar in number and kinds of bands in both the native and the reduced-alkylated states, but there were differences in the relative amounts of the various bands. The two preparations may be mixtures of the same proteins but present in different ratios[2].

The largest decrease in mobility on reduction-alkylation occurs with SBTI-A2. This decrease is not due to changes in charge, because amino acid analyses of 24-hr. hydrolysates of modified and native proteins showed only minor changes in the molecule (Table I). The number of residues of each amino acid per 21,500 g. was identical for both proteins except for cystine, aspartic acid, and valine. The native protein contained two residues of cystine. In the reduced-alkylated protein there was only a trace of cystine (because of incomplete reduction-alkylation), whereas three residues of S-carboxyethyl cysteine were measured. The modified protein had 25 instead of 24 residues of aspartic acid and 11 residues of valine instead of 10. These differences are not significant. The modified and native proteins each contained 10 mol. of lysine per mol. of protein. The amino acid values agree well with those for crystalline SBTI reported by Wu and Scheraga (3) and included in Table I.

[2]This conclusion is inconsistent with an earlier report (1) which indicated that $s_{20,w}$ values for SBTI-B1 and -B2 differed markedly from the $s_{20,w}$ values for SBTI-A1 and -A2. However, recalculation of the original ultracentrifuge data, assuming a partial specific volume of 0.735, yielded respective $s_{20,w}$ values of 1.8, 1.7, 1.8, and 2.3S for SBTI-B1, -B2, -A1, and -A2 with protein concentrations of 0.5%. Our observations of gel-electrophoretic similarities and the small differences in $s_{20,w}$ values indicate that SBTI-B2 and -A1 may differ less than was originally believed. The recalculated $s_{20,w}$ values are more consistent with the specific activities reported by Rackis and Anderson (1). The smaller $s_{20,w}$ values for SBTI-B1 and -B2 probably mean smaller molecular weights, which account for the increases in their specific activities since activities were calculated on a weight basis.

TABLE I. AMINO ACID COMPOSITION OF NATIVE AND REDUCED-ALKYLATED SOYBEAN TRYPSIN INHIBITOR-A2 AND CRYSTALLINE INHIBITOR[a]

Amino Acid	Reduced-Alkylated	Native	Crystalline Inhibitor[b]
Lysine	10	10	11
Histidine	2	2	2
Arginine	8	8	9
Aspartic acid	25	24	29
Threonine	6	6	8
Serine	9	9	13
Proline	10	10	10
Glutamic acid	17	17	21
Glycine	15	15	18
Alanine	8	8	9
N recovered, %	95.61	99.13	...
Valine	11	10	12
Cystine	Tr	2	2
Methionine	2	2	3
Isoleucine	13	13	14
Leucine	14	14	16
Tyrosine	4	4	4
Phenylalanine	8	8	9
S-carboxyethyl cysteine	3	...	...
Tryptophan	...	...	2
N, as NH_3, %	7.07	7.54	...

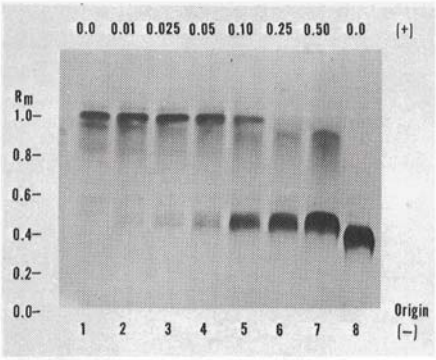
[a]Residues per 21,500 g. protein.

[b]See ref. 3.

The polyacrylamide-gel patterns of the reduced-alkylated proteins suggest that the four SBTI's are not intra- or intermolecular disulfide structures of a single polypeptide chain, since the treated proteins do not migrate as one common band.

The slower mobility of reduced-alkylated SBTI's implies that the various inhibitors all contain disulfide bonds, since cystine is the major amino acid affected under the conditions used. Reduction and alkylation of disulfides apparently allow the proteins to assume a more unfolded conformation in 8M urea which, in turn, either increases resistance to electrophoretic migration or causes retardation because of sieving effects of the gel, or both. These possibilities were studied in further experiments.

The first experiment was to determine the effect of reducing the disulfide bonds of crystalline SBTI-3X with mercaptoethanol on gel electrophoresis (Fig. 2). Unmodified and reduced-alkylated crystalline SBTI-3X were dissolved in gel buffer. 2-Mercaptoethanol was added to portions of the solution of commercial SBTI to give concentrations from 0.01 to 0.5M of the reducing agent. After being allowed to stand 0.5 hr., the samples were inserted in the polyacrylamide gel (5% Cyanogum 41 gel with pH 9.2 glycine buffer containing 8M urea). The reduced-alkylated inhibitor migrated ~0.5 as fast as the native inhibitor. Concentrations of 2-mercaptoethanol of 0.025M and less did not affect the migration of SBTI; however, 0.25M and higher concentrations of the mercaptan completely converted the inhibitor (the band at R_m = 1.0) to a slower-moving form (R_m ~0.5). Presumably the slow species is a reduced form of the molecule, since its mobility is about the same as that of the reduced-alkylated protein. At 0.1 and 0.05M, 2-mercaptoethanol reduction appears incomplete, since both slow- and fast-moving components are present. The presence of only two major bands at these lower concentrations of 2-mercaptoethanol indicates that if the inhibitor with only one



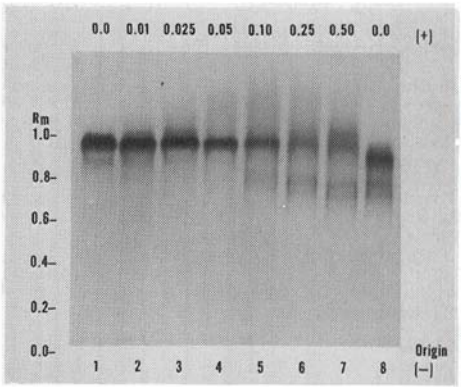


Fig. 2(left). Electrophoretic patterns of SBTI on 5% polyacrylamide with 0.025M glycine, pH 9.2, buffer containing 8M urea. Sample 1 is SBTI without reducing agent. Samples 2 to 7 are SBTI containing 0.01, 0.025, 0.05, 0.10, 0.25, and 0.50M 2-mercaptoethanol, respectively. Sample 8 is reduced-alkylated SBTI. Top abscissa scale represents concentrations of 2-mercaptoethanol in moles per liter.

Fig. 3(right). Electrophoretic pattern of SBTI on 5% polyacrylamide with 0.025M glycine, pH 9.2, buffer without urea. Sample 1 is SBTI without reducing agent. Samples 2 to 7 are SBTI containing 0.01, 0.025, 0.05, 0.10, 0.25, and 0.50M 2-mercaptoethanol, respectively. Sample 8 is reduced-alkylated SBTI. Top abscissa scale represents concentrations of 2-mercaptoethanol in moles per liter.

disulfide bond reduced is present as an intermediate, it has a mobility identical to that of either the native or the completely reduced form; i.e., R_m = 1.0 or 0.5. It should be emphasized that concentrations of the mercaptan in the proten samples during electrophoresis are unknown, because the protein and 2-mercaptoethanol probably have different mobilities and therefore they would separate during analysis. Attempts to prepare gels by adding various amounts of 2-mercaptoethanol to the gel buffer were unsuccessful[3].

Figure 3 shows the pattern from a 5% polyacrylamide gel prepared with pH 9.2 glycine buffer containing no urea. The protein samples used for Fig. 2 (in pH 9.2 glycine containing 8M urea plus various concentrations of 2-mercaptoethanol) were inserted in the electrophoresis gel. After electrophoresis for 2 hr., staining, and destaining, all samples lacked the band at R_m ~0.5 which was observed when the proteins were analyzed in 8M urea (Fig. 2). Nearly all samples migrated at R_m 0.8 to 1.0. This migration probably suggests refolding of the reduced protein back to its original shape. In samples that had the higher concentrations of 2-mercaptoethanol, streaking of the bands was observed. This streaking may be caused either by disulfide polymer formation or the formation of mixed disulfides. Apparently SBTI can be reduced or reduced-alkylated and still remain folded, like the native protein with the absence of urea. However, if the polyacrylamide gel contains 8M urea, all the proteins except native SBTI are unfolded, and the two forms can be differentiated. Since reduced-alkylated crystalline SBTI has a slower mobility than native SBTI, i.e. 0.5, this difference in migration may afford a means for studying

[3]In our usual procedure for preparing polyacrylamide gels, 0.01M 2-mercaptoethanol was used in the buffer. However, after polymerization, the reducing agent could not be detected in the gel by the nitroprusside test. Apparently the ammonium persulfate in the polymerization mixture oxidized the 2-mercaptoethanol at this concentration (0.01M), but at higher concentrations (0.20 to 0.25M) gelation did not occur, as noted earlier (2).

equilibria between native and reduced forms of SBTI if the reduced form is alkylated to "freeze" it in the reduced-alkylated state.

Varying the concentration of Cyanogum 41 altered the relative rate of migration. In gels containing 4 to 16% Cyanogum 41 and 8M urea, the modified protein always traveled more slowly than the native protein. In a 16% polyacrylamide gel, the R_m of reduced-alkylated SBTI was only 0.35 as compared to 0.5 in a gel containing 4% polyacrylamide.

It is well known (3,9,10) that SBTI-3X has a unique structure. Steiner (11) has reported that reduction of SBTI causes loss of trypsin inhibitor activity. Upon reoxidation of the sulfhydryl groups, the protein becomes active and again inhibits trypsin. We have confirmed loss of activity upon reduction and have found that the reduced-alkylated SBTI is also inactive. The biologically active structure is therefore intimately related to integrity of its disulfide bonds.

Effect of Structure on Migration

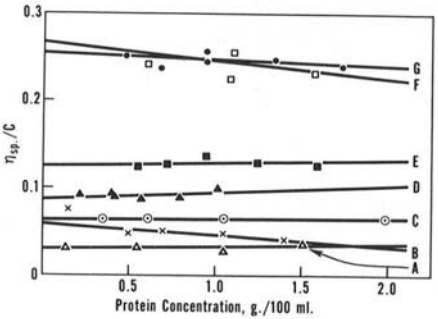
Rupture of the two disulfide bonds of SBTI causes gross changes in the protein structure in urea, as exhibited by the marked changes in intrinsic viscosity shown in Fig. 4. The intrinsic viscosity of SBTI-3X in 0.025M glycine, pH 9.2, was 0.03 (curve A). The intrinsic viscosity of native SBTI in 0.025M glycine, pH 9.2, containing 8M urea, was 0.06 (curve B). This value compares favorably with the viscosity of SBTI-3X in 9M urea reported by Edelhoch and Steiner (10) as shown in curve C.

When SBTI-3X was dissolved in 0.025M glycine, pH 9.2, containing 0.5M 2-mercaptoethanol, the intrinsic viscosity increased to 0.09 (curve D). Reduced-alkylated SBTI in 0.025M glycine had an intrinsic viscosity of about 0.13 (curve E).

A drastic increase in the intrinsic viscosity to 0.27 was observed when SBTI-3X was dissolved in 0.025M glycine, pH 9.2, containing 8M urea and 0.5M 2-mercaptoethanol (curve F). Likewise, the viscosity of reduced-alkylated SBTI was 0.26 when the protein was dissolved in 0.025M glycine, pH 9.2, containing 8M urea (curve G).

Viscosity measurements (Fig. 4) suggested that SBTI existed in four states in our study; i.e., a compact structure in glycine buffer (curve A); an expanded structure in glycine buffer and 8M urea (curve B); a structure where the protein is either reduced or reduced-alkylated but remains partially folded in absence of urea (curves D and E); and a structure where the protein is reduced or reduced-alkylated and more unfolded in 8M urea (curves F and G).

Resolution of the folded and unfolded forms of SBTI in Figs. 1 and 2 and lack of resolution in pattern of Fig. 3 may depend on size of the protein particle in relation to the average pore size of the acrylamide gel. Zweig and Whitaker (12) report that the average pore size of a 5% polyacrylamide gel is 63 A. They also calculated that a protein of 20,000 MW would have the following dimensions as a function of shape: for a sphere, the diameter equals 36 A; for an ellipsoid with major and minor axes of a= 2b, 57 by 29 A; and for an ellipsoid with a=4b, 91 by 22 A. Therefore, if the native and the reduced-alkylated SBTI's (in the absence of 8M urea) have respective dimensions similar to those of the first two examples, we should observe no marked difference in migration due to sieving. However, if reduced-alkylated SBTI in 8M urea has a molecular dimension on the order of 91 by 22 A, particle sieving by the polyacrylamide (13,14) may become significant.



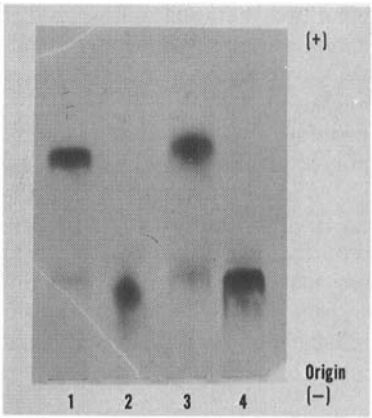


Fig. 4(left). Reduced viscosities of native and modified commercial SBTI as a function of protein concentrations. A, native SBTI in 0.025M glycine, pH 9.2; B, native SBTI in 0.025M glycine, pH 9.2, 8M urea; C, native SBTI in pH 7.0 buffer 9M urea from Edelhoch and Steiner (10); D, native SBTI in 0.025M glycine, pH 9.2, 0.5M RSH; E, reduced-alkylated SBTI in 0.025M glycine, pH 9.2; F, native SBTI in 0.025M glycine, 8M urea, and 0.5M RSH; G, reduced-alkylated SBTI in 0.025M glycine, pH 9.2, and 8M urea.

Fig. 5(right). Polyacrylamide-gel electrophoretic patterns of samples: 1, native SBTI; 2, SBTI in phosphate buffer treated with 0.5M RSH, then acrylonitrile; 3, SBTI in phosphate buffer, 8M urea, then treated with acrylonitrile; 4, reduced-alkylated SBTI. Gel contained 5% polyacrylamide, 0.025M glycine, pH 9.2, and 8M urea.

This phenomenon then could explain the electrophoretic patterns of Figs. 1,2, and 3. If the protein is native, reduced, or reduced-alkylated and in glycine buffer with no urea, the mobility will be affected only slightly and the R_m will be near 1.00 (Fig. 3). If the same three protein forms are in 8M urea and buffer, only the native protein will move with an R_m of 1.00 on electrophoresis; the reduced and reduced-alkylated proteins will migrate much more slowly (R_m ~0.5) because of unfolding of the molecules as implied by their higher intrinsic viscosities. SBTI in 0.025M glycine, pH 9.2, containing 0.5M 2-mercaptoethanol (curve D, Fig. 4) has a higher intrinsic viscosity than SBTI in 0.025M glycine and 8M urea (curve B, Fig. 4). This higher intrinsic viscosity suggests that the sample may indeed be reduced and partially unfolded in the absence of 8M urea. To test this possibility, commercial SBTI-3X (Gallard Schlesinger Lot A4570) was dispersed in phosphate buffer, pH 7.6, μ= 0.1, containing 0.5M 2-mercaptoethanol. After the mixture was held 1 hr., acrylonitrile (2:1 molar ratio of acrylonitrile:mercaptan) was added and the reaction allowed to proceed for 45 min. After the pH was lowered to 4.5 with HCl, the sample was dialyzed against water and freeze-dried. Analysis of this sample and of the necessary controls is recorded in Fig. 5.

Sample 1 is the native starting material. Sample 2 is the sample treated with 0.5M 2-mercaptoethanol and acrylonitrile (as described above). Sample 3 is a control sample consisting of SBTI in buffer containing 8M urea (no 2-mercaptoethanol) which was treated with acrylonitrile. Sample 4 was reduced-alkylated SBTI dispersed in phosphate buffer containing 8M urea. As noted in the figure, sample 2, which was reduced in absence of urea, migrates at about the same rate as the completely reduced-alkylated SBTI, sample 4. The slower mobility of sample 2 confirms that SBTI in phosphate buffer, pH 7.6, μ = 0.1, containing 0.5M 2-mercaptoethanol, is indeed in a reduced state.

Relation of Isolated SBTI's

Yamamoto and Ikenaka (15) recently isolated and characterized two soybean trypsin inhibitors, one of which appeared to be identical with the Kunitz inhibitor (16) and with SBTI-A2. The second inhibitor had a sedimentation coefficient of 1.9S, had a MW of 16,400, and contained more than 19% cystine. This cystine content corresponds to 13 disulfide bonds per mol. as compared to two disulfide bonds per mol. of Kunitz inhibitor or SBTI-A2. The relation of this 1.9S inhibitor to the inhibitors described here is unclear. The results of Yamamoto and Ikenaka, however, support our conclusions that the various inhibitors differ in their primary structures. Because of its high disulfide content, the 1.9S inhibitor cannot be a degradation product of the Kunitz inhibitor. Nevertheless, the possibility that SBTI-B1, -B2, and -A1 are proteolytic degradation products of SBTI-A2 cannot be ruled out yet. Ozawa and Laskowski (17) suggest that the 60 to 70 amino acid residues from the carboxyl terminal end of the Kunitz inhibitor may be unessential for activity. Another possibility for the multiplicity of trypsin inhibitors in the soybean is genetic heterogeneity (2, 18). At the present time the latter possibility is conjecture. Clearly, further studies are needed to determine the relationship among the various inhibitors that have been isolated. Gel electrophoresis appears to be a useful tool for this purpose.

Acknowledgments

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