Soybean Bowman-Birk Trypsin Isoinhibitors: Classification and Report of a Glycine-Rich Trypsin Inhibitor Class[1]

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Ten soybean Bowman-Birk trypsin isoinhibitors have been purified from soybean seed, *Glycine max* (L.) Merr. In order of decreasing mobility in the Davis PAGE system, they are BBSTI-E′, BBSTI-E, BBSTI-D, BBSTI-C′, BBSTI-C, BBSTI-B′, BBSTI-B, BBSTI-A″, BBSTI-A′, and BBSTI-A. We assign the classical Bowman-Birk soybean trypsin inhibitor, BBSTI-E; its carboxyl-terminal proteolytic derivative, BBSTI-D; and a related BBSTI-E′ to subgroup I. Subgroup II consists of BBSTI-C′, -C, and -A″, which correspond to known homologous inhibitors in soybeans. BBSTI-B′ and BBSTI-B are in subgroup III. All inhibitors in these three subgroups have 70-80 residues with high half-cystine and low glycine contents. Subgroup IV inhibitors, BBSTI-A and BBSTI-A′, cross-react with anti-BBSTI-E antibodies. However, they are 3 times larger, have low half-cystine and high glycine contents, and do not appear to resemble the Bowman-Birk, the Kunitz, or the wound-induced proteinase inhibitors. We designate these to be in a separate class called glycine-rich soybean trypsin inhibitors. While the cystine-rich inhibitors are predominant in the cotyledon, the glycine-rich inhibitors are the major forms in the vegetative tissues of the seedling.

Dietary utilization of the rich protein resource in soybeans is limited by antinutritional components that, unless partially destroyed, result in a range of deleterious physiological effects in many animal species. Soybean trypsin inhibitors, which inhibit mammalian pancreatic serine proteinases, contribute to this antinutritional component (Rackis, 1965; Rackis and Gumbmann, 1981). The variation in nutritional quality of soybean cultivars stems partly from their very different levels of trypsin inhibitor and from varying proportions of trypsin inhibitors of the two classes: the Kunitz (Kunitz, 1947) and the Bowman-Birk (Birk et al., 1963) inhibitor classes [reviewed in Tan-Wilson and Wilson (1986)]. In the case of the better-studied Kunitz inhibitor, the nutritional quality of soybean strains also appeared to depend on the type of Kunitz isoinhibitor present in the seed (Bajjalieh et al., 1980). Quite ironically, the Bowman-Birk and Kunitz inhibitors contribute to the nutritional quality of soybeans by virtue of their relatively high half-cystine content. This supplements the low or negligible amounts of sulfur-containing amino acids in the storage proteins that comprise the bulk of the protein reserve in the seed.

A large number of Bowman-Birk trypsin inhibitors have been described in soybean cultivars since the first one isolated independently by Bowman and Birk. Odani and Ikenaka (1977a) purified and determined the amino acid sequence of inhibitor A, C-II, D-II, and E-I from the Japanese cultivar Sode-furi. Hwang et al. (1977) purified PI-I, PI-II, PI-III, PI-IV, and PI-V from the cultivar Tracy. Stahlhut and Hymowitz (1983) found five inhibitors—I, II, III, IV, and V—from cultivar Amsoy 71. Earlier we (Tan-Wilson et al., 1985a) studied the distribution of Bowman-Birk class inhibitors in eight soybean strains and found isoinhibitors that we designated as BBSTI-A, -A′, -A″, -B, -B′, -C, -C′, -D, -E, and -E′, in order of increasing mobility upon polyacrylamide gel electrophoresis in the Davis system. BBSTI-A, -A′, -C, and -E were found in all eight strains studied. The other forms were found in one to four strains. Each strain had four to eight different isoinhibitor forms.

Bowman-Birk isoinhibitor forms have been shown to differ in the extent of their interaction with trypsin and in their spectrum of inhibition of other pancreatic enzymes, chymotrypsin, and elastase (Odani and Ikenaka, 1977a; Wilson, 1981; Harry and Steiner, 1970; Turner et al., 1975). One would then expect part of the variation in nutritional quality of soybean cultivars to stem from the relative proportions of different Bowman-Birk isoinhibitor forms in the seed. It is important, therefore, to make some sense out of the proliferation of different Bowman-Birk inhibitor forms described by different laboratories, to determine which of the described inhibitors are equivalent, which are merely derived by proteolysis or deamidation of another, and which are truly different, i.e. coded for separately in the genome.

In this paper, we describe the purification of BBSTI-A, -A′, -A″, -B, -B′, -C, -C′, -D, -E, and -E′. We have determined their isoelectric points, amino acid compositions, and molecular weights, their abilities to inhibit bovine trypsin, chymotrypsin, and elastase, their ability to cross-react with anti-BBSTI-E antibodies, and their relative proportions in various parts of the soybean seedling. The determination of these characteristics enabled us to identify which inhibitors were equivalent to other isoinhibitor forms already described in the literature and to classify the inhibitor forms into separate groups. Each group consists of inhibitors appearing to be related by proteolysis. The number of such groupings would then give the minimum number of gene loci coding for the Bowman-Birk soybean trypsin inhibitors that have been studied.

EXPERIMENTAL SECTION

Materials. *Plant Materials.* Soybeans, *Glycine max* (L.) Merrill, cv. Amsoy 71, were from the May Seed and Nursery Co., Shenandoah, IA. Cultivars Altona and Panther were obtained from Johnny's Selected Seed Co., Albion, ME. Cultivar Fiskeby V was from Stokes Seed Co., Buffalo, NY.

Commercial Reagents. Bovine trypsin, α-chymotrypsin, and elastase, N^{α}-benzoylarginine ethyl ester, N^{α}-

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Table I. Final Steps in the Purification of the Bowman–Birk Soybean Trypsin Isoinhibitors

starting material	column[a]	elution	purify
pool containing BBSTI-A and -A′ from Figure 1, dry seed from cv. Panther	DE-52, 2.5 × 20 cm, 50 mM Tris-Cl, pH 8.5	15 mL/h, 150-mL linear gradient, 50 mM Tris-Cl (pH 8.5) as initial buffer, 50 mM Tris-Cl + 0.5 M NaCl as final buffer	BBSTI-A, BBSTI-A′
pool containing BBSTI-A″ from Figure 1	DE-52, 1.5 × 40 cm, 50 mM Tris-Cl, pH 8.5	60 mL/h, 1-L linear gradient, 50 mM Tris-Cl (pH 8.5) as initial buffer, 50 mM Tris-Cl + 0.3 M NaCl (pH 8.5) as final buffer	
pool containing BBSTI-A″ from column described above	DE-52, 1.5 × 40 cm, 50 mM Tris-Cl + 50 mM NaCl, pH 8.5	60 mL/h, 1-L linear gradient, 50 mM Tris-Cl + 50 mM NaCl (pH 8.5) as initial buffer, 50 mM Tris-Cl + 0.2 M NaCl (pH 8.5) as final buffer	BBSTI-A″
pool containing BBSTI-B and -B′ from Figure 1, dry seed from cv. Altona	DE-52, 2.5 × 60 cm, 50 mM Tris-Cl, pH 8	50 mL/h, 1-L linear gradient, 50 mM Tris-Cl (pH 8) as initial buffer, 50 mM Tris-Cl + 0.15 M NaCl (pH 8.0) as final buffer	BBSTI-B, BBSTI-B′
pool containing BBSTI-C from Figure 1		no further purification necessary	BBSTI-C
pool containing BBSTI-C′ from Figure 1	DE-52, 1.5 × 45 cm, 50 mM NH_4OAc, pH 6.5	60 mL/h, 1-L linear concn gradient, 50 mM NH_4OAc (pH 6.5) as initial buffer, 0.5 M NH_4OAc (pH 5) as final buffer	
pool containing BBSTI-C′ from column described above	DE-52, 1.5 × 70 cm, 50 mM Tris-Cl, pH 8.5	60 mL/h, 1-L linear gradient, 50 mM Tris-Cl (pH 8.5) as initial buffer, 50 mM Tris-Cl + 0.5 M NaCl as final buffer	BBSTI-C′
pool containing BBSTI-D from Figure 1 with Fiskeby V day 6 cotyledon as starting matl	DE-52, 1.5 × 17 cm, 50 mM Tris-Cl, pH 7.5	15 mL/h, 1-L linear gradient, 50 mM Tris-Cl (pH 7.5) as initial buffer, 50 mM Tris-Cl + 0.5 M NaCl (pH 7.5) as final buffer	BBSTI-D
pool containing BBSTI-E′ from Figure 1	DE-52, 1.5 × 80 cm, 0.2 M NH_4OAc, pH 5.4	50 mL/h, 1-L linear gradient, 0.2 M NH_4OAc (pH 5.4) as initital buffer 0.3 M as final buffer	
pool containing BBSTI-E′ from column described above	DE-52, 1.5 × 80 cm, 0.2 M NH_4OAc pH 5.4	50 mL/h, 0.2 M NH_4OAc (pH 5.4), no gradient	BBSTI-E′

[a] All columns were monitored as described for the column in Figure 1. In addition, when fractions were concentrated enough to show up on PAGE, they were monitored for electrophoretic mobility. Thus, fractions could be pooled conservatively with emphasis on purity rather than yield.

benzoyltyrosine ethyl ester, congo red elastin, *N,N′*-methylenebis(acrylamide), and *N,N,N′,N′*-tetramethylethylenediamine were from Sigma Chemical Co. Sephadex G-75 was from Pharmacia Fine Chemicals, while DEAE-cellulose (DE-52) was from Whatman. Servalyt Precote isoelectric focusing gels were purchased from Serva Biochemicals. Goat anti-rabbit IgG conjugated with alkaline phosphatase was from Bio-Rad Laboratories. Nitrocellulose membrane (BA-83, 0.2 μm) was from Schleicher and Schuell.

Immunochemical Reagents. Antibody specific for Bowman–Birk soybean trypsin inhibitor was raised in rabbits immunized with glutaraldehyde-polymerized BBSTI-E. The immunization protocol has been published in detail (Tan-Wilson and Wilson, 1982). Serum was used for radial immunodiffusion, but isolated IgG (Harboe and Ingild, 1973) was used for membrane ELISA.

Procedures. *Assays.* Trypsin inhibitor activity was determined with bovine trypsin with N^{α}-benzoylarginine ethyl ester as substrate. One unit of inhibitor was defined as the amount of inhibitor inhibiting 1 mg of active trypsin. The values reported here have been corrected for the amount of inactive trypsin in commercial preparations (Kassell, 1970). Chymotrypsin inhibitor activity was assayed with bovine α-chymotrypsin with N^{α}-benzoyltyrosine ethyl ester as the substrate (Hummel, 1959; Kress et al., 1968). Elastase inhibitor activity was assayed by using congo red elastin as the substrate (Naughton and Sanger, 1961). The amount of BBSTI protein in column fractions was determined by radial immunodiffusion (Mancini et al., 1965) with modifications (Tan-Wilson et al., 1983). BBSTI-E was used as standard. The concentration of BBSTI-E was determined spectrophotometrically at 280 nm with $E^{1\%}_{1cm} = 4.6$ (Kassell, 1970).

Purification of Soybean Bowman–Birk Trypsin Isoinhibitors. The pool of soybean BBSTI was purified from Amsoy 71 seeds by ammonium sulfate fractionation, acid precipitation, and Sephadex gel filtration as described previously (Tan-Wilson et al., 1985a). The BBSTI pool was then fractionated on a DEAE-cellulose column (DE-52) in 0.05 M ammonium acetate, pH 6.5, buffer. The inhibitors were eluted with a linear gradient of 0.05 M ammonium acetate, pH 6.5, as the initial buffer and 0.5 M ammonium acetate, pH 5.0, as the final buffer. Fractions were monitored for absorbance at 280 nm, trypsin inhibitor activity, and cross-reaction to anti-BBSTI-E antibody by radial immunodiffusion (Tan-Wilson et al., 1983). Fractions were pooled, dialyzed against water, and lyophilized. These were then subjected to polyacrylamide gel electrophoresis in the Davis (1964) system in 10% gels and in the Davis system with 4 M urea in 15% gels. The chromatogram, indicating where Bowman–Birk trypsin inhibitors BBSTI-A, -A′, -A″, -B, -B′, -C, -C′, -D, -E, and -E′ elute, is shown in Figure 1.

Table I lists further chromatographic procedures required for the final purification of the isoinhibitors other than BBSTI-E. BBSTI-E, the classical inhibitor, was purified previously by a modification (Tan-Wilson and Wilson, 1982) of a procedure established by Odani and Ikenaka (1977a). A single band on polyacrylamide gel electrophoresis in both systems and close to integer values for the numbers of amino acid residues per molecule were criteria for purity.

Amino Acid Analysis. Samples were hydrolyzed in vacuo with 5.7 N HCl at 110 °C for 20 and 48 h. Analysis was performed on a Glenco MM-70 amino acid analyzer equipped with a ninhydrin detection system. BBSTI-A and BBSTI-A′ were also hydrolyzed in vacuo in methanesulfonic acid + 3-(2-aminoethyl)indole (Simpson et al., 1976). To analyze for half-cystine as cysteic acid, the samples were subjected to performic acid oxidation (Hirs, 1967).

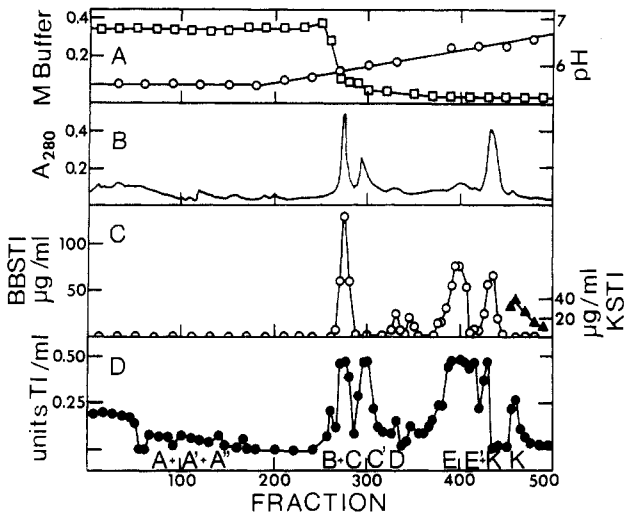

Figure 1. Initial separation of Bowman–Birk isoinhibitors by ion-exchange chromatography. The BBSTI pool from Amsoy 71 was fractionated on a DEAE-cellulose (DE-52, Whatman) column (2.5 × 150 cm) at room temperature, equilibrated in 50 mM ammonium acetate, pH 6.5. The column was eluted at 100 mL/h with a 5.4-L linear buffer concentration gradient using 0.5 M ammonium acetate (pH 5) as the final buffer. The pH and concentration gradients are shown in panel A. Column fractions were monitored for absorbance at 280 nm (panel B), cross-reaction to anti-BBSTI-E antibodies in radial immunodiffusion tests (panel C), and trypsin inhibitor activity (panel D). Pools were dialyzed vs. water and then lyophilized. Electrophoresis was done to determine which of the BBSTI isoinhibitors were present in each pool and are marked on this figure by the initials A, A′, A″, B, C, C′, D, E, and E′. K represents some Kunitz trypsin inhibitor still remaining in the BBSTI pool.

Molecular Weight Determination. Molecular weights were calculated from amino acid compositions of the inhibitors. For BBSTI-A and BBSTI-A′, analysis was also done by the determination of electrophoretic mobility on different pore size polyacrylamide gels relative to known molecular weight standards by the method of Hedrick and Smith (1968).

Isoelectric Point Determination. Servalyt Precotes (Serva Biochemicals) in the range pH 4–7, anode fluid pH 3, and cathode fluid pH 7 were used. The actual pH gradient in the gel was determined with a surface pH electrode. Protein bands were detected by staining with Serva Blue W.

Membrane ELISA. Several dilutions of different BBSTI isoinhibitors were applied onto nitrocellulose membrane in a 96-well pattern set by a Bethesda Research Laboratories (Gaithersberg, MD) dot-blot apparatus. The blots were developed by (1) incubation with 3% (w/v) gelatin in 0.05 M Tris-Cl + 0.5 M NaCl, pH 7.5 (TBS), (2) incubation with anti-BBSTI-E antibody diluted in 1% gelatin in TBS, (3) washing with TBS, (4) incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase diluted in 1% gelatin in TBS, and (5) washing with TBS. The blots were then stained with alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate in the presence of nitro-blue tetrazolium (Blake et al., 1984). Color densities of the resulting dots were measured by reflectance densitometry with a Hoeffer GS300 scanning densitometer. The relative peak heights of the standards, as measured on the densitometer recording, were plotted vs. the amount of the known BBSTI solutions to yield standard curves. Molar concentrations of BBSTI standard solutions were determined by the absorbance at 280 nm and extinction coefficients calculated from amino acid analysis data.

Identification of Isoinhibitors in Vegetative Tissues. Soybean seedlings were grown for varying lengths of time, harvested, and separated into cotyledons, epicotyls, hypocotyls, and roots. Extracts were made, and the BBSTI fractions were isolated by gel filtration on 2.5 × 140 cm Sephadex G-75 column equilibrated with 0.05 M Tris-Cl, pH 8.0. The isoinhibitors were separated by polyacrylamide gel electrophoresis in the Davis (1964) system with a 4 M urea included. The isoinhibitors were visualized after blotting, staining with rabbit anti-BBSTI-E IgG as the primary antibody and goat anti-rabbit IgG conjugated to horseradish peroxidase as the second antibody. Details are given in Tan-Wilson et al. (1985b).

RESULTS

The Bowman–Birk isoinhibitors BBSTI-A, -A′, -A″, -B, -B′, -C, -C′, -D, -E, and -E′ were purified as described in the Experimental Section. The isoinhibitors were then characterized as to their amino acid compositions, molecular weights, isoelectric points, and extent of cross-reaction to anti-BBSTI-E antibodies, their abilities to inhibit bovine trypsin, bovine chymotrypsin, and bovine elastase, and their presence in various plant tissues in the soybean seedling.

Physical Properties. Amino acid compositions of the purified Bowman–Birk inhibitors are given in Table II. BBSTI-A″, -B, -B′, -C, -C′, -D, -E, and -E′ were all generally similar in compostion to BBSTI-E, whose amino acid sequence was determined by Odani et al. (1972). BBSTI-A and -A′ were very different in relative amino acid percentages so the calculation of the number of residues per molecule was based on a separate determination of molecular weight. The molecular weights of BBSTI-A and BBSTI-A′ were estimated by the procedure of Hedrick and Smith (1968) to be 24 600. The number of amino acid residues per molecule given in Table II were then calculated to give MW 20 751 and 19 720 for BBSTI-A and for BBSTI-A′, respectively. Because the amino acid compositions for these two isoinhibitors were so unusual for Bowman–Birk inhibitors, samples were also hydrolyzed by the method of Simpson et al. (1976) for amino acid analysis. These hydrolyses yielded the same results as hydrolysis in hydrochloric acid.

The amino acid compositions of our isoinhibitors were compared to those published for other soybean Bowman–Birk type inhibitors. BBSTI-E has a composition exactly like the classical BBSTI, which has also been referred to as inhibitor A by Odani and Ikenaka (1977a), PI-V by Hwang et al. (1977), and inhibitor III by Stahlhut and Hymowitz (1983). BBSTI-C′ corresponds to inhibitor D-II described by Odani and Ikenaka (1978) and PI-IV described by Hwang et al. (1977). BBSTI-C corresponds to PI-III described by Hwang et al. (1977). BBSTI-A″ has the same amino acid composition as inhibitor E-I described by Odani and Ikenaka (1978) and as inhibitor PI-II described by Hwang et al. (1977). The identity of PI-IV to D-II as well as that of E-I to PI-II was quoted by Foard et al. (1982).

We know that certain isoinhibitors are related to others by proteolysis, and we can make hypotheses for other such relationships on the basis of their amino acid compositions. BBSTI-D has been shown to be generated by removal of two amino acid residues from the carboxyl terminus of BBSTI-E (Madden et al., 1985). This proteolysis occurs primarily in the cotyledons during germination (Tan-Wilson et al., 1982). BBSTI-E′ has the same amino acid composition as BBSTI-E but with an additional seven residues, suggesting that BBSTI-E is a cleavage product of BBSTI-E′. Foard et al. (1982) have raised the possibility

Table II. Amino Acid Compositions of the Bowman–Birk Isoinhibitors

	residues/molecule[a]									
residue	BB-E′	BB-E	BB-D	BB-C′	BB-C	BB-A″	BB-B′	BB-B	BB-A	BB-A′
Asx	11	11	10	12	11	10	8	9	15	16
Thr	3	2	2	3	3	3	3	3	9	9
Ser	11	9	9	11	8	8	6	9	42	40
Glx	7	7	6	6	7	5	9	7	30	31
Pro	7	6	6	5	4	5	4	4	8	8
Gly	1	0	0	2	2	1	7	4	45	42
Ala	5	4	4	0	0	0	4	3	14	13
$^1/_2$ Cys[b]	14	14	14	14	14	14	10	10	2	2
Val	1	1	1	0	0	0	4	1	8	8
Met	1	1	1	2	2	2	1	1	1	1
Ile	2	2	2	1	1	1	4	2	6	5
Leu	2	2	2	3	3	3	5	3	8	7
Tyr	2	2	2	2–3	2	2	2	2	5	4
Phe	2	2	2	1	1	1	3	2	5	4
Lys	5	5	5	4	4	4	4	4	6	6
His	1	1	1	1	1	1	1	1	3	3
Arg	3	2	2	5	4	5	3	3	5	4
total	78	71	69	72–73	68	65	78	68	212	203
MW[c]	8522	7865	7621	8084	7584	7407	8453	7432	20751	19886
MW[d]									24600	24600

[a] All values are the average of the 20- and 48-h hydrolysis data, except Ser and Thr, which were determined by extrapolation of the data to zero time. [b] Determined as cysteic acid. [c] Calculated from the amino acid composition data. [d] Determined by the method of Hedrick and Smith (1968).

Table III. Classification of BBSTI into Subgroups

			residue % amino acid					inhibn of bovine enzymes		
subgroup	BBSTI	p*I*	$^1/_2$ Cys	Gly	Asx	Glx	Ser	trypsin	chymotrypsin	elastase
I	E′	3.42	18	1	14	9	14	+	+	–
	E	3.95	20	0	16	10	13	++	+	–
	D	3.95	20	0	15	9	13	+	+	–
II	C′	4.76	19	3	16	8	15	+	+/–	–
	C	4.94	21	3	16	10	12	+	–	–
	A″	5.03	22	2	15	8	12	+	+/–	–
III	B′	4.79	13	9	10	12	8	+/–	–	–
	B	4.79	15	6	13	10	10	+/–	–	–
IV	A	5.28	1	21	7	14	20	++	–	–
	A′	5.28	1	21	8	15	20	++	–	–

that the in vitro translation product of Bowman–Birk trypsin inhibitor mRNA may be longer than the classical BBSTI at the amino terminal end, supporting this idea.

BBSTI-C′ and BBSTI-A″ are also related by proteolysis. Odani and Ikenaka (1978) showed that inhibitor E-I (our BBSTI-A″) was like D-II (our BBSTI-C′), only lacking nine amino acid residues at the amino terminus. The amino acid composition of BBSTI-C is between those of BBSTI-C′ and BBSTI-A″. BBSTI-C might be an intermediate in the BBSTI-C′ to BBSTI-A″ conversion, or an alternative cleavage product of BBSTI-C′.

The amino acid compositions of BBSTI-B′ and BBSTI-B suggest another proteolytic derivation, BBSTI-B′ being the larger one by 10–12 residues. Likewise, BBSTI-A has the same amino acid composition as BBSTI-A′ except that BBSTI-A has 8–10 additional amino acid residues.

Table III summarizes the characteristic amino acid residue percentages. The isoelectric points are also given in Table III. BBSTI-E′, -E, -D, -C′, -C, and -A″ have the high half-cystine and low glycine contents and higher aspartate + asparagine content than glutamate + glutamine content characteristic of the classical Bowman–Birk inhibitor. BBSTI-B and BBSTI-B′ have lower half-cystine and higher glycine contents and nearly equivalent amounts of aspartate + asparagine and glutamate + glutamine residues. These inhibitors have very low serine contents. BBSTI-A and BBSTI-A′ have very low half-cystine and very high glycine contents and more glutamate + glutamine than aspartate + asparagine (the opposite to that found for the classical Bowman–Birk inhibitor). The BBSTI-A and BBSTI-A′ forms have very high serine contents.

Spectrum of Enzyme Inhibitor Activity. The purified isoinhibitors were tested for their abilities to inhibit bovine trypsin, bovine chymotrypsin, and bovine elastase. These results are given in Table III. None of these inhibitors inhibit bovine elastase. BBSTI-E′, -E, and -D, as is characteristic of the classical Bowman–Birk inhibitor, are double-headed, inhibiting both trypsin and chymotrypsin simultaneously (Seidl and Liener, 1972). BBSTI-C′, -C, and -A″, as previously noted for inhibitors D-II and E-I, inhibit bovine trypsin weakly. At high molar ratios of inhibitor to enzyme, we could detect weak inhibition of chymotrypsin. BBSTI-B and -B′ exhibit the weakest inhibition of trypsin and do not inhibit bovine chymotrypsin. BBSTI-A and -A′ are strong inhibitors of bovine trypsin. They do not inhibit chymotrypsin.

Immunochemical Cross-Reactivity to Anti-BBSTI-E Antibodies. Since only BBSTI-E′, -E, and -D give good immunoprecipitin rings upon radial immunodiffusion with anti-BBSTI-E antibodies, we developed a membrane ELISA (enzyme-linked immunoadsorbent assay) dependent only upon primary binding of antigen to antibody. This assay was based on the dot immunobinding assay of Hawkes et al. (1982). We used reagents developed for the immunodetection of protein blots on nitrocellulose membrane and incorporated reflectance scanning desitometry for quantitation of color intensities. Details are given in the Experimental Section. Results comparing BBSTI-B′, BBSTI-A″, and a sample containing BBSTI-A

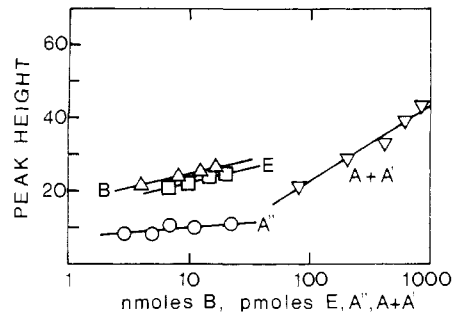

Figure 2. Immunological cross-reactivity of Bowman–Birk isoinhibitors. The extent of immunological cross-reaction of one isoinhibitor from each subgroup to anti-BBSTI-E antibodies is compared here by membrane ELISA. The color intensities are represented here as peak height, measured as the number of squares on the recording chart. Symbols: □, for BBSTI-E; ○, for BBSTI-A''; △, for BBSTI-B; ▽, for BBSTI-A + A'.

+ BBSTI-A' to BBSTI-E are shown in Figure 2. Color-staining intensities on a molar basis for BBSTI-A'' were just less than half of those for corresponding amounts of BBSTI-E. Color intensities for a sample containing a mixture of BBSTI-A and BBSTI-A' fall in the same range as those for BBSTI-E but only when amounts of BBSTI-A + BBSTI-A' were 10 times higher. BBSTI-B' showed the weakest cross-reaction, the amounts having to be 10^3 times higher than the amount of BBSTI-E needed to give color intensities in the same range.

Isoinhibitor Distribution in Various Times of the Soybean Seedling. In the dry seed and in the cotyledon and hypocotyl 1 day after the beginning of imbibition, BBSTI-E and BBSTI-C are present in proportionally greater concentrations than BBSTI-A and -A', as determined by electrophoresis, blotting, and immunostaining. The same situation prevails in the day 8 cotyledon. In the day 8 hypocotyl, root, and epicotyl, as well as in all the plant parts of the day 12 seedling, the BBSTI-A band stains as dark or darker than those of BBSTI-C and BBSTI-E. Considering that BBSTI-A is weak in cross-reaction to anti-BBSTI-E antibodies compared to BBSTI-E itself, we can conclude at least qualitatively that the predominance of BBSTI-E and BBSTI-C in the cotyledon, the storage organ of the seed, is replaced by the predominace of BBSTI-A in the vegetative tissues of the seedling.

DISCUSSION

Classification of Bowman–Birk Isoinhibitors into Subgroups. The Bowman–Birk soybean trypsin isoinhibitors can be classified into four subgroups by virtue of their distinctive amino acid compositions, molecular weights, spectrum of enzyme inhibitor activity, and immunochemical cross-reactivity.

We designate BBSTI-E', BBSTI-E, and BBSTI-D as belonging to subgroup I. These are the inhibitors identical to or very similar to the classical Bowman–Birk inhibitor. Isoinhibitors in this group have 14 half-cystine residues. BBSTI-E has been shown to have seven disulfide bridges in a molecule with only 71 amino acid residues. These inhibitors have only one or no glycine residues per molecule and have more aspartate + asparagine residues than glutamate + glutamine residues. BBSTI-E has been shown to have two separate inhibitory reactive sites, one for trypsin and one for chymotrypsin (Seidl and Liener, 1972). BBSTI-E' and BBSTI-D also inhibit both trypsin and chymotrypsin.

BBSTI-C', BBSTI-C, and BBSTI-A'' belong to another subgroup designated as subgroup II. Their amino acid compositions are similar to those of the inhibitors in subgroup I. The amino acid sequences of inhibitors E-I and D-II (Odani and Ikenaka, 1978) also show considerable homology to the sequence of BBSTI-E. Moreover, the sequence at the two inhibitory reactive sites show that inhibitors E-I and D-II are double-headed, but having two inhibitory sites for trypsin. Thus, they should be classified into a separate subgroup from that of BBSTI-E. In the immunological comparison shown in Figure 2, BBSTI-A'', a member of this subgroup, was closest of the three isoinhibitors that were checked for immunochemical relatedness to BBSTI-E. The inhibitors in this subgroup are poor inhibitors of bovine trypsin and even poorer, perhaps nonspecific, for chymotrypsin.

BBSTI-B and BBSTI-B' can be grouped together in subgroup III characterized by a somewhat lower apparent half-cystine content (10 instead of 14 half-cystine residues per molecule) and a decidedly higher glycine content (four to seven instead of zero to two per molecule) than that characteristic of subgroups I and II: These are the poorest trypsin inhibitors. BBSTI-B' was also the farthest from BBSTI-E in immunological relatedness in the membrane ELISA.

There are at least three more soybean Bowman–Birk inhibitors that have been well characterized and for which we have not found exact correspondence to any of the 10 that we have purified. One is inhibitor C-II purified and sequenced by Odani and Ikenaka (1977b) from the strain Sode-furi. This inhibitor is double-headed, inhibiting both trypsin and elastase. It is structurally similar to their inhibitors D-II and E-I, which are the same as inhibitors we have classified in subgroup II. Another inhibitor is PI-I purified by Hwang et al. (1977) from cultivar Tracy. The amino acid composition of this inhibitor most closely resembles that of BBSTI-B and BBSTI-B', which would put it in subgroup III. However, Foard et al. (1982) state that PI-I is a cleavage product of PI-IV. In this case, PI-I would belong to subgroup II as we have defined it. A third inhibitor is inhibitor V described by Stahlhut and Hymowitz (1983). This inhibitor migrates even faster than BBSTI-E', when we use their electrophoretic system.

Individual soybean strains may also lack one or another representative of the three Bowman–Birk inhibitor subgroups. Two of eight soybean strains we studied (Tan-Wilson et al., 1985a) do not have either BBSTI-B or BBSTI-B'. Stahlhut and Hymowitz (1983) found 3% of 470 strains of soybean not to contain inhibitor III, the classical Bowman–Birk inhibitor, which we call BBSTI-E.

Norioka and Ikenaka (1983) have classified the Bowman–Birk type inhibitors from various legume species according to homologies of their amino acid sequences, particularly around their inhibitory reactive sites. Our isoinhibitors BBSTI-C', -C, and -A'' fall in their group I classification, together with the mung bean F, garden bean II', and adzuki bean IA inhibitors. Our BBSTI-E falls under their group II, together with lima bean IV, adzuki bean II, and the *Macrotyloma axillare* DE-3 and DE-4 inhibitors. Norioka and Ikenaka have designated two other groupings, III and IV, represented by the *Vicia angustifolia* and peanut inhibitors A-II and B-II, respectively. Our BBSTI-B and -B' with their lower half-cystine and higher glycine contents do not appear to fit into either of these groupings.

Delineation of a New Class of Soybean Trypsin Inhibitor. BBSTI-A and BBSTI-A' are structurally different from the isoinhibitors in the other three Bowman–Birk inhibitor subgroups. These molecules are 3 times larger than the classical Bowman–Birk inhibitor. They have only two half-cystine residues per molecule of

more than 200 residues. At most, these inhibitors could only have one disulfide bridge. This very fact would disqualify them from classification as members of the Bowman–Birk class of inhibitors (Laskowski and Kato, 1980), in spite of the relatively strong immunological reaction we find in these inhibitors with anti-BBSTI-E antibodies. BBSTI-A and BBSTI-A′ have very high glycine and serine contents as well as more glutamate + glutamine than aspartate + asparagine residues per molecule. They are very good inhibitors of bovine trypsin so, functionally, they are closer to the classical BBSTI than the isoinhibitors in subgroups II and III. Kunitz soybean trypsin inhibitors are also good inhibitors of bovine trypsin and have molecular weights of approximately 20 000. However, BBSTI-A and BBSTI-A′ do not in any way resemble the Kunitz trypsin inhibitors in their amino acid compositions, electrophoretic mobility, and isoelectric points. The amino acid compositions of BBSTI-A and -A′ do not resemble those inhibitors in the family represented by potato tuber and tomato wound-induced inhibitors (Graham et al., 1985) either.

The one characteristic that makes these inhibitors distinctly different from the other described plant proteinase inhibitor families is their very large percentage of glycine residues. We therefore consider BBSTI-A and BBSTI-A′ not as Bowman–Birk inhibitors but as a separate class of glycine-rich soybean trypsin inhibitors (GRSTI) and therefore assign them the names GRSTI-1 and GRSTI-2 in place of BBSTI-A and BBSTI-A′, respectively.

Proteolytic Derivation of Some Isoinhibitors from Others. Within each of the three Bowman–Birk inhibitor subgroups and our newly designated glycine-rich inhibitor class, there are a number of isoinhibitors that appear to be related by proteolysis. The BBSTI-E to BBSTI-D and the BBSTI-C′ to BBSTI-A″ relationships are supported not just by amino acid composition but also by amino acid sequence data (Madden et al., 1985; Odani and Ikenaka, 1978). The other possible relationships, BBSTI-C′ → BBSTI-C, BBSTI-C → BBSTI-A″, BBSTI-B′ → BBSTI-B, and GRSTI-1 → GRSTI-2 are only suggested by the amino acid compositions. The multiplicity of inhibitors could just as well be the result of multiple genes coding for isoinhibitors.

The proteolysis of BBSTI-E → BBSTI-D should be considered quite distinct from the others that we have hypothesized. There is only a trace of BBSTI-D in the dry seed. BBSTI-D arises during germination, with cleavage occurring near the carboxyl terminus. This type of proteolysis has also been described for the mung bean (*Vigna radiata*) Bowman–Birk type inhibitor (Lorensen et al., 1981; Wilson and Chen, 1983).

The other proteolytic events are hypothesized in order to explain the presence of isoinhibitor forms present in the dry seed. Presumably these proteolytic events occur during seed maturation. In many cases, the larger forms are the ones that we had found previously to be in the seed of only a few strains of soybean. When present, these isoinhibitors are only found in small quantities. They may thus represent proteolysis that has not been completed prior to desiccation in just a few strains, or even in just a limited number of seeds of a strain. For example, BBSTI-E′ was found only in two out of eight strains and only in small quantities compared to the predominant BBSTI-E found in all eight strains. The distribution of BBSTI-C′ relative to BBSTI-C and of BBSTI-B′ relative to BBSTI-B follows the same pattern.

The apparent loss of significant numbers of amino-terminal residues during posttranslational modification of plant proteinase inhibitors has been noted in peanut (Norioka and Ikenaka, 1983), garden bean (Wilson and Laskowski, 1975), adzuki bean (Yoshikawa et al., 1979; Kiyohara et al., 1981), mung bean (Zhang et al., 1982; Wilson and Chen, 1983), and lima bean (Stevens et al., 1974), as well as the soybean (Odani and Ikenaka, 1978). It seems likely that at least some of the proteolysis may result from exposure of the inhibitors to proteinases involved in the posttranslational processing of the major storage proteins of the seed. BBSTI-E′ has one Thr, two Ser, one Pro, one Gly, one Ala, and one Arg residue more than BBSTI-E. BBSTI-C′ has the same sequence as BBSTI-A″ plus the additional sequence Ser-Asp-Gln-Ser-Ser-Ser-Tyr-Asp-Asp at the amino terminus. The predominance of hydrophilic residues, especially the acidic and hydroxyl-containing amino acids, at the amino-terminal portion of the sequence seems characteristic of most of the Bowman–Birk type inhibitors so far sequenced. We note however that BBSTI-B′ contains, relative to BBSTI-B, additional hydrophobic as well as hydrophilic amino acid residues. The same observation can also be made for GRSTI-1 relative to GRSTI-2.

Ultrastructural studies using colloidal gold-labeled antibodies specific for the classical Bowman–Birk inhibitor indicate this protein to be localized, at least in part, in the protein bodies of the cotyledonary parenchyma (Horisberger and Tacchini-Vonlanthen, 1983). The amino-terminal processing might be a step in targeting the inhibitors after synthesis to their ultimate intracellular location(s).

We can arrive at an estimate of the minimum number of gene loci that are needed to account for the trypsin inhibitors in the soybean strains that we have studied. For the Kunitz trypsin inhibitor, there is one gene locus coding for three allelic forms, Ti^a, Ti^b, and Ti^c, as well as the recessive ti allele in strains that do not have a functional Kunitz inhibitor (Singh et al., 1969; Clark et al., 1970; Orf and Hymowitz, 1979). For the Bowman–Birk class, there must be at least three gene loci, one coding for each of the three subgroups. Foard et al. (1982) have shown that there are mRNAs in the developing soybean seed coding for the classical Bowman–Birk inhibitor and for the inhibitor they designate as PI-IV, equivalent to our BBSTI-C′ and therefore belonging to subgroup II. This indirectly demonstrates the existence of at least those two separate loci for a subroup I and a subgroup II isoinhibitor (Hammond et al., 1984). Finally, there should be at least one gene coding for the new glycine-rich soybean trypsin inhibitor we have described in this report.

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Dry-Matter Accumulation and Carbohydrate Composition in Developing Normal- and High-Lysine Sorghum Grain[1]

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Eight sorghum [*Sorghum bicolor* (L.) Moench] varieties (four high lysine and four normal) were evaluated 21, 31, and 61 days after flowering (DAF) for changes in total dry-matter accumulation and carbohydrate composition. The chemically induced high-lysine mutant P-721 opaque was not significantly different from P-721 normal in whole-kernel weight, endosperm weight, germ weight, germ percent, and moisture percent as well as in carbohydrate composition. High-lysine natural mutants from Ethiopia (IS 11758, IS 11167, YM-3) showed lower kernel weight, lower endosperm weight, higher percent germ, and higher percent moisture than normal sorghum varieties at all stages of grain development. More significantly, distinct differences were found in the overall carbohydrate profile and the rate of starch synthesis, as well as the level of total sugars at the various stages of grain development in the Ethiopian high-lysine and Ethiopian normal varieties. Between 21 and 31 DAF, starch synthesis was delayed and a concomitant accumulation of sugars occurred in the high-lysine sorghums. The level of sugars was the highest at the late-dough stage (31 DAF), providing a nutritional basis for the Ethiopian tradition of consuming the high-lysine varieties at the late-dough stage.

Cereals provide most of the carbohydrates and about 50% of the annual human dietary protein requirement. This estimate would be even higher if an assessment was made of how much of the diverse cereals are consumed at their respectively perferred immature stages. In Eastern Africa and specifically in Ethiopia, almost every cereal crop is consumed (green-fresh, roast, or boiled etc.) sometime before maturity. Ear roasts of corn (*Zea mays* L.) or sorghum are special delicacies of their respective seasons. In the United States vegetable corn types are widely used before maturity.

Research on protein and carbohydrate improvements of vegetable corn (Barbosa, 1971; Glover and Crane, 1972) utilizing the opaque-2, sugary-2 and other endosperm mutants indicate that double-mutant combinations such as su_1, o_2; sh_2, o_2; and bt_2, o_2 can improve the nutritional quality of maize at immature stages of development. Tossello (1974) evaluated some endosperm mutants and their double-mutant combinations with opaque-2 for their protein and carbohydrate quality at the immature stages of 21 and 42 days after pollination. He reported that the overall carbohydrate profile and protein yield and quality of the mutants and their double-mutant combinations were superior to the normals both at 21 and 42 days after pollination. The data demonstrated that several of the double-mutant combinations may be of interest for their food value as a fresh product (fresh boiled or roasting ear corn) and possibly as a snack food or in dry cereal breakfast food products.

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While similar genes in sorghum have essentially the same pattern of effects, work on vegetable sorghum types is lacking. Sorghum grain has a wide range of variation in endosperm composition. Waxy, floury, corneous, sugary, and high-lysine types exist, as they do in corn. Several workers (Quinby and Martin, 1954; Creech, 1965; Webster, 1965; Gorbet and Weibel, 1972) have reported on the many carbohydrate mutants affecting starch content and the relative properties of amylose and amylopectin in the starch. Singh and Axtell (1973) reported the protein and carbohydrate composition of mature whole-kernel samples of two high-lysine sorghum lines from Ethiopia. Shannon (1968) suggested that all carbohydrates in maize, whether starch, phytoglycogen, or sucrose, begin to accumulate at about the same physiological age. This age was dependent upon environmental conditions during the first 2 weeks following pollination.

At the present time genes in two naturally occuring high-lysine lines in sorghum, IS-11167 and IS-11758 (Singh and Axtell, 1973), and one induced mutant, P-721 opaque (Mohan and Axtell, 1975), are known to change drastically the relative proportion of certain storage proteins in the sorghum endosperm. The chemical composition and nutritional value of these mutants were shown to be superior to any known sorghum lines. However, the effects of these mutations on absolute and relative changes in protein quality and carbohydrate profile in the endosperm during grain development have never been investigated. Farmers in Wollo, Ethiopia (where the two high-lysine lines originated), indicated that they consumed the grain of these lines at the dough stage as head-roast. They also claimed to use them this way because of the sweet flavor of these lines but make no mention of their nutritional quality.

The overall purpose of this study was to evaluate the nutritional value and carbohydrate profile of high-lysine and normal varieties of sorghum at the milk-dough stage

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