Subclassification of Soybean Bowman-Birk Isoinhibitors

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Trypsin inhibitors are proteins in the soybean which inhibit vertebrate pancreatic proteinases. Multiple forms of the two inhibitor classes, the Kunitz and the Bowman-Birk inhibitors, are found in the soybean. The three Kunitz isoinhibitors are well characterized with regard to amino acid sequence, genetic inheritance and affinity for trypsin. Enough Bowman-Birk trypsin inhibitors have been purified and characterized that identities can be established and inhibitors classified into subgroups. The first subgroup includes the classical double-headed inhibitor of bovine trypsin and chymotrypsin, BBSTI-E; its proteolytic derivative, BBSTI-D, and BBSTI-E′. Subgroup II has the double-headed weak trypsin inhibitors, BBSTI-C′, C and A″, related by proteolysis at the amino terminus. Subgroup III has the even weaker trypsin inhibitor BBSTI-B′ and its apparent proteolytic derivative, BBSTI-B. Subgroup I, II and III inhibitors have 70–80 residues with high half-cystine and low glycine content. Subgroup IV consists of the strong trypsin inhibitor BBSTI-A and its apparent proteolytic derivative BBSTI-A′. These have about 200 residues, only two half-cystine residues per molecule and 25 residue percent glycine. They cross-react better than do Subgroup III inhibitors with anti-BBSTI-E antibodies. While the cystine-rich BBSTI-E and BBSTI-C are predominant in the cotyledon, the storage organ of the plant, the glycine-rich trypsin inhibitors are predominant in the vegetative tissues of the seedling.

Proteinase inhibitors in the soybean limit an animal's maximum use of the rich protein resource in the seed. These protein proteinase inhibitors form complexes with vertebrate pancreatic proteinases and inhibit their enzymatic activity. The Kunitz soybean trypsin inhibitors (KSTI) and the Bowman-Birk soybean trypsin inhibitors (BBSTI) are the two major classes (1–4). Multiple forms of both inhibitor classes are found in the soybean. The three Kunitz isoinhibitors are well characterized with regard to amino acid sequence and genetic inheritance. The affinity of different KSTI forms for bovine trypsin can differ by as much as a thousand-fold (5,6). As summarized in a recent review (7), there is almost as much variation in the binding of bovine trypsin to the few BBSTI forms that have been studied. However, the number and variation of BBSTI forms are not yet clearly defined. A listing of all the names given to multiple forms of this class of inhibitor (4,8-13) would number at least 50, when in fact the same isoinhibitor may be designated with as many as five different names.

Although the seed contains two or three times as much KSTI as BBSTI by weight (14), the BBSTI are just as significant for the following reasons. The molecular weight of KSTIs is 22,000; that of BBSTIs is 8000 (15). Thus, the molar concentration of BBSTI in the seed is at least equivalent to that of KSTI. BBSTIs are double-headed. Each molecule can inhibit two molecules of proteinases simultaneously (16). Depending on the isoinhibitor, the proteinases may be two trypsin molecules or one molecule of trypsin and one molecule of either chymotrypsin or elastase (8). BBSTIs are not readily inactivated by digestion in the stomach nor by heat processing of soy meal as are KSTIs (17,18). Therefore, BBSTI is considered to be a significant antinutritional component in the soybean.

BBSTIs are also important in a beneficial sense. At least one form of BBSTI is stored in the protein body, the same membrane-bound compartment in which the storage proteins of the seed are sequestered (19). This BBSTI is digested during the early stages of germination (20) and thus functions like a storage protein which complements the low sulfur-containing amino acid content of the storage proteins. BBSTIs also have been shown to inhibit digestive enzymes of insect and

TABLE 1

Concordance of Multiple Forms of Bowman-Birk Inhibitors

Ref.[a]	(4)	(8)	(9)	(12)	(13)
				V	
					BBSTI-E′
	AA	A	PI-V	III	BBSTI-E
					BBSTI-D
		D-II	PI-IV		BBSTI-C′
			PI-III		BBSTI-C
		E-I	PI-II		BBSTI-A″
		C-II			
					BBSTI-B′
					BBSTI-B
			PI-I		
					BBSTI-A′ = GRSTI-2
					BBSTI-A = GRSTI-1

[a]Literature references to the Bowman-Birk trypsin inhibitor variants listed in each column.

TABLE 2

Classification of BBSTI into Subgroups

Subgroup	BBSTI	Residue percent[a] 1/2 Cys	Gly	Number residues	Cross-reactivity[b]	Inhibition[c] Tr	Ch
I	E′	18	1	78		+	+
	E	20	0	71	1	++	+
	D	20	0	69		+	+
II	C′	19	3	72		+	+/-
	C	21	3	68		+	-
	A″	22	2	65	0.5	+	+/-
III	B′	13	9	78		+/-	-
	B	15	6	68	0.001	+/-	-
IV	A	1	21	212		++	-
	A′	1	21	203	0.1	++	-

[a]Number of particular amino acid residue/total number of residues.

[b]Reciprocal of molar ratio of isoinhibitor to BBSTI-E to achieve the same binding as BBSTI-E to anti-BBSTI-E IgG.

[c]Tr, bovine trypsin; Ch, bovine chymotrypsin.

microbial pests (21,22), and proteins homologous to the BBSTIs are induced in alfalfa leaves upon wounding (23). Thus the BBSTIs also are thought to serve as a defense mechanism in the seed and in the plant. BBSTIs also may prove to be beneficial to animals. One form has been shown to prevent X-ray induced malignant transformation of cells in culture (24).

It is important, therefore, to organize the information on the different Bowman-Birk inhibitor forms described by different laboratories to determine which of the described inhibitors are equivalent, which are derived by proteolysis or deamidation of another and which are truly different, i.e., coded for separately in the genome.

SUBCLASSIFICATION OF BOWMAN-BIRK INHIBITORS

Enough BBSTI forms have been purified to homogeneity and amino acid compositions and sequences available (8,9,12,25) so that identities can be established. The information is presented in Table 1.

Highly similar amino acid sequences allow for a subclassification of all but three of these proteins. For example, amino acid sequence data shows that BBSTI-D has the same sequence as BBSTI-E minus two carboxyl-terminal amino acid residues (26). Inhibitor E-I has the same sequence as inhibitor D-II minus nine amino acid residues at the amino-terminal end (27). Other assignments of isoinhibitors to subgroups as presented in Table 2 are based on amino acid compositions. The amino acid composition for a representative member from each subgroup is shown in Table 3. Other proteins assigned to each group have nearly the same composition with minor differences which could arise through proteolysis. For subgroups I and II, all such extra residues are hydrophilic. For subgroups III and IV, the extra residues are hydrophilic and hydrophobic. Table 2 further shows how the subgroups differ in the extent of immunochemical cross-reactivity and the spectrum of enzyme inhibition (25).

Subgroup I includes the classical Bowman-Birk inhibitor which has 14 half-cystine residues bonded in seven disulfide bridges in a molecule with only 71 amino acid residues (29). The proteins in this subgroup have only one or no glycine residues per molecule. BBSTI-E has been shown to have two inhibitor reactive sites, one for trypsin and one for chymotrypsin (16,29). BBSTI-E′ and BBSTI-D also inhibit both bovine trypsin and chymotrypsin (25).

Subgroup II inhibitors are closest in homology to subgroup I inhibitors. This is evident in the homology of amino acid sequences as well as in immunochemical cross-reactivity to antibodies elicited in rabbits by BBSTI-E. In enzyme-linked immunoadsorbent assays done on nitrocellulose membrane, the staining intensity for an isoinhibitor from subgroup II is half that for the same concentration of BBSTI-E. Inhibition for trypsin is weak. There is some inhibition for chymotrypsin but only at very high inhibitor to enzyme ratios (25).

BBSTI-B and BBSTI-B′ are in subgroup III. A thousand times more BBSTI-B is needed to reach the same staining density as BBSTI-E in membrane ELISA. Of all the BBSTIs in Table 2, these are the weakest in trypsin inhibition (25).

All inhibitors in these three subgroups have 70–80 residues with high half-cystine and low glycine contents. They are homologous to Bowman-Birk inhibitors found in other legume species. These inhibitors have been classified by Norioka and Ikenaka (30). Our subgroup II inhibitors fall in the same subgrouping as the mung bean F, garden bean II′ and adzuki bean IA inhibitors. Our subgroup I inhibitors are classified to be most homologous to lima bean IV, adzuki bean II and the *Macrotyloma axillare* DE-3 and DE-4 inhibitors. Our subgroup III inhibitors do not coincide with any of their other groupings.

TABLE 3

Amino Acid Compositions of Representative Bowman-Birk Isoinhibitors

Residue	Residues/Molecule[a]			
	BB-E	BB-C	BB-B	BB-A
Asx	11	11	9	15
Thr	2	3	3	9
Ser	9	8	9	42
Glx	7	7	7	30
Pro	6	4	4	8
Gly	0	2	4	45
Ala	4	0	3	14
1/2 Cys[b]	14	14	10	2
Val	1	0	1	8
Met	1	2	1	1
Ile	2	1	2	6
Leu	2	3	3	8
Tyr	2	2	2	5
Phe	2	1	2	5
Lys	5	4	4	6
His	1	1	1	3
Arg	2	4	3	5
Total	71	68	68	212
MW[c]	7865	7584	7432	20751
MW[d]				24600

[a]All values are the average of the 20- and 48-hr 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 (28).

GLYCINE-RICH SOYBEAN TRYPSIN INHIBITOR CLASS

Subgroup IV inhibitors are good inhibitors of bovine trypsin and require only 10 times higher molar concentration of inhibitor to reach the same binding to anti-BBSTI-E antibodies. However, they are three times larger, have low half-cystine and very high glycine contents. At most, these inhibitors could have only one disulfide bridge, in contrast to the seven disulfide bonds in the much smaller BBSTI-E, the one most distinguishing structural characteristic of the Bowman-Birk inhibitors. BBSTI-A and BBSTI-A′ do not resemble the Kunitz soybean trypsin inhibitor or the wound-induced inhibitors of tomato and potato, (31). Their strongest distinguishable characteristic is the very large percentage of glycine residues. Recently, we have begun to consider these inhibitors as belonging to a separate class of glycine-rich soybean trypsin inhibitors and have assigned the names of GRSTI-1 and GRSTI-2 in place of BBSTI-A and BBSTI-A′, respectively (25).

The multiple forms of proteinase inhibitors which cross-react with anti-Bowman-Birk inhibitor antibodies thus arise from the presence of multiple gene loci coding for at least one protein in each of the three Bowman-Birk subgroups and at least one glycine-rich trypsin inhibitor. Further variation results from proteolytic cleavage.

DIFFERENTIAL METABOLISM OF THE DIFFERENT PROTEINASE INHIBITOR CLASSES

Proteinase isoinhibitors can be identified on Western blots of cotyledon extracts. When following the time course of first appearance of isoinhibitor during seed development, we have found isoinhibitors belonging to the three Bowman-Birk subgroups I, II and III appearing very closely after the Kunitz proteinase inhibitors (unpublished data). It is also at this time that most of the storage proteins are being synthesized (32). However, the glycine-rich inhibitors are not evident until the seeds have reached the green mature stage, when seed weights reach their maximum.

In the seed of the Amsoy 71 cultivar, the Bowman-Birk inhibitors BBSTI-E and BBSTI-C are present in greater concentrations than the glycine-rich isoinhibitors. As germination proceeds, the Bowman-Birk inhibitors are degraded. The glycine-rich trypsin inhibitors remain and are also found in the epicotyl, hypocotyl and root of the seedling (25). Thus, the predominance of the Kunitz and Bowman-Birk inhibitors in the cotyledon, the storage organ of the seed, is replaced by the predominance of the glycine-rich inhibitors in the vegetative tissues of the seedling. These differences suggest that the glycine-rich inhibitors serve a different function from that served by the Bowman-Birk and Kunitz inhibitors. The difference may not be a difference in kind. The GRSTI may be functioning for storage or defense or both. However, the time and location within the plant in which these different inhibitor classes function are clearly different.

SIGNIFICANCE IN COMPARISON OF SOYBEAN STRAINS

There are soybean strains that are known to lack the Kunitz trypsin inhibitor (33). Of 470 strains studied, Stahlhut and Hymowitz (12) found 3% not to contain the classical Bowman-Birk inhibitor. Two of eight strains that we have looked at lack representatives of the Bowman-Birk inhibitor subgroup III (13). Possibly, the lack of one proteinase inhibitor is compensated for by the presence of other inhibitors in the same class or even by inhibitors belonging to another class exercising similar functions in the plant, storage and/or defense. Variation in the amino acids that are deposited and in the types of proteinases that the total collection of inhibitors of the seed can inhibit would be advantageous to the plant. The very large difference in the association constant for binding to bovine trypsin among the Kunitz and Bowman-Birk isoinhibitors may reflect wide proteinase inhibitor specificities. If this is indeed the case, then the strategy of selecting strains that contain the least nutritionally deleterious isoinhibitors for a particular animal species is compatible with maintaining the viability of the plant when other inhibitors contributing to the same plant function are left in the seed.

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