Characterization of Trypsin and Chymotrypsin Inhibitors in the Wild Perennial *Glycine* Species

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Eleven wild perennial species were used for characterizing the seed protease inhibitors in the genus *Glycine* Willd. subgenus *Glycine* to determine the presence and examine the variability and expression patterns. Seeds of all the species contained trypsin and chymotrypsin inhibitors. There were highly significant variations among the wild perennial species in the electrophoretic profiles of trypsin and chymotrypsin inhibitors, migration patterns of anti-KTI and anti-BBI (mAB 238) immunocrossreactive proteins, and trypsin and chymotrypsin inhibitor activities of seeds. These variations were greater in A genome species than those in B or C genome species. Seeds of the B genome species had the lowest trypsin and chymotrypsin inhibitor activities. Most of the trypsin inhibitors found in the wild perennial species had a weaker chymotrypsin inhibitor activity. All of the wild perennial species also contained DNA sequences that crosshybridized to the soybean BBI cDNA (pB38) and produced transcripts that were of the same size as but less abundant than those of soybean. The pattern of developmental expression of these transcripts in *Glycine clandestina* was the same as that found in the soybean seed. Variations in the trypsin and chymotrypsin inhibitor banding patterns are useful in the genetic studies of the protease inhibitors and biosystematic studies in the genus *Glycine*.

INTRODUCTION

Protease inhibitors are widely distributed among many species in several plant families, particularly among legumes (Liener and Kakade, 1980). These inhibitors have been proposed to function as storage proteins, regulators of endogenous proteases, and factors that protect plants from insect and pathogen attack (Liener and Kakade, 1980; Ryan, 1990).

Protease inhibitors in soybean seed are considered to be antinutritional in human and animal diets. After Osborne and Mendel (1917) first recorded that raw soybean meal (RSM) would not support normal growth in rats, several studies established that feeding RSM containing protease inhibitors to monogastric animals, such as chicks, rats, and quail, would lead to pancreatic hyperplasia, acinar adenoma, and overall growth reduction (Chernick et al., 1948; Madar et al., 1974; Gumbmann et al., 1986). Protease inhibitors in the RSM are conventionally inactivated by moist heat processing (DiPietro and Liener, 1989). However, the residual activity in soy protein is a cause of concern in various human foods, including infant formulas, flour, protein concentrates, soy sauces, textured soy fibers, tofu, etc. (Brandon et al., 1991).

Protease inhibitors in soybean seed are currently categorized into three classes: Kunitz trypsin inhibitors, Bowman–Birk inhibitors, and glycine-rich soybean trypsin inhibitors (GRSTI). The major and predominantly expressed form of Kunitz trypsin inhibitors is a 21.5-kDa protein (KTI) with two disulfide bonds and inhibition specificity toward trypsin (Liener and Kakade, 1980). At least 10 members with molecular weights ranging from 6000 to 10 000 have been described in the Bowman–Birk inhibitor class (Tan-Wilson et al., 1985). Of these, the major form, referred to as the Bowman–Birk inhibitor (BBI), is an 8-kDa protein and inhibits both trypsin and chymotrypsin simultaneously at independent reactive sites (Odani and Ikenaka, 1973; Birk, 1985). The GRSTI class contains two members with molecular weights of about 20 000. They inhibit trypsin and are relatively minor forms in soybean seed but are predominant in the vegetative tissue of soybean seedlings (Tan-Wilson et al., 1987).

Mies and Hymowitz (1973) reported the presence of variability in trypsin inhibitors among a few wild perennial *Glycine* species. However, there were no papers on the biochemical and molecular characterization of protease inhibitors in the wild perennial species of the genus *Glycine*. Exploration of variation among trypsin and chymotrypsin inhibitors in the genus *Glycine* will provide a basis for their use in genetic and biosystematic studies. Studying the BBI-related gene expression and regulation in the wild perennial species can aid in better understanding of these genes in soybean. Further study of protease inhibitors in the wild perennial *Glycine* may help us understand the nature and evolution of these inhibitors.

In this study, a general survey of a number of trypsin and chymotrypsin inhibitors in 11 perennial species of the genus *Glycine* using activity staining and immunoblotting methods was conducted. Molecular studies on the presence of BBI-related genes, their expression, and regulation are presented. These results were compared to those of soybean.

MATERIALS AND METHODS

i. Materials. The selected accessions of various wild perennial *Glycine* species (Table I) were grown in the greenhouse. Planting was done in 6-in. clay pots filled with clay loam soil/sand/peat moss (1:1:1), and the plants were fertilized once every other week. Mature dry seeds were used for extraction of proteins. Immature seeds and leaf material were used for isolating RNA and DNA, respectively.

The monoclonal antibodies to soybean BBI from mouse (mAB 238) and polyclonal antibodies to soybean KTI (anti-KTI) from rabbit were provided by Drs. D. L. Brandon and L. O. Vodkin, respectively. Purification and characterization of these antibodies have been described previously (Brandon et al., 1989; Vodkin, 1981). The cDNA clone of soybean BBI (pB38) was obtained from Dr. R. W. Hammond. Isolation and sequence of pB38 has been reported (Hammond et al., 1984).

ii. Extraction and Estimation of Seed Proteins. After the seed coat was removed, the seeds were crushed to a fine meal

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Table I. Species, Three-Letter Code, Accession Numbers, Diploid Chromosome Number (2n), Genomic Designation, and Presence or Absence of BBI of the Genus *Glycine* Used in the Study

species (code)	PI no.[a]	IL no.[b]	2n	genome[c]	BBI[d]
G. arenaria (ARE)	505204	689	40		+
G. argyrea (ARG)	505151	768	40	A_2A_2	+
G. canescens (CAN)	440932	434	40	AA	+
G. clandestina (CLA)	440958	490	40	A_1A_1	+
	440950	426	40	A_1A_1	+
G. microphylla (MIC)	440956	449	40	BB	-
	440972	503	40	BB	-
		1070	40	BB	-
G. latifolia (LAT)	253238	375	40	B_1B_1	-
	440980	515	40	B_1B_1	-
	446964	547	40	B_1B_1	-
G. tabacina (TAB)	373986	345	40	B_2B_2	-
	440985	422	80	AA, BB	+
	378704	348	80	BB, BB	-
G. cyrtoloba (CYR)	440962	480	40	CC	-
G. curvata (CUR)	505166	791	40	C_1C_1	-
G. tomentella (TOM)	441000	448	40	DD	+
	483223	576	40	DD	-
	505222	709	40	DD	-
	440998	398	38	EE	+
	373988	368	78	DD, EE	+
	441005	485	80	AA, DD	+
G. falcata (FAL)	233139	320	40	FF	+
G. max (Kunitz)			40	GG	+
(Williams 82)			40	GG	+

[a] A permanent plant introduction number assigned by the U.S. Department of Agriculture. [b] A temporary number given at the University of Illinois. [c] Genomic designations (Hymowitz et al., 1990; Singh et al., 1992). [d] Presence (+) or absence (-) of BBI based on enzyme-linked immunosorbent assay (Kollipara, 1992).

with flat-nosed hand-pliers, and 20-mg samples were weighed for protein extraction. A buffer solution containing 0.023 M $CaCl_2$ and 0.092 M Tris-HCl [tris (hydroxymethyl)aminomethane, pH adjusted with HCl], pH 8.1 (Tris–$CaCl_2$, pH 8.1 buffer) was added to a precise final concentration of 20 mg of seed meal/mL in a microcentrifuge tube. All of the seed samples were left in the refrigerator (4 °C) overnight before they were clarified by centrifugation at 10000*g* for 2 min. The supernatant of each sample was removed to a new tube and used immediately or stored at -20 °C for later use. Protein content of these samples was estimated by Bio-Rad microassay procedure (Bradford, 1976).

iii. Activity Assays. Both trypsin and chymotrypsin inhibitor activity assays were conducted according to Hummel's (1959) method using *p*-toluenesulfonyl-L-arginine methyl ester (TAME) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE), respectively, as substrates. The reagents were made as described by Friedman et al. (1991), Walsh (1970), and Walsh and Wilcox (1970) and filter-sterilized (0.45-μm nitrocellulose filters, Micron Separations Inc.).

a. Trypsin Assay. A total of 2.6 mL of assay buffer (10.34 mM $CaCl_2$, 41.38 mM Tris-HCl, pH 8.1) was added to a substrate (10 mM TAME, prepared fresh the same day by dissolving 37.9 g of TAME/10 mL of assay buffer) volume of 0.3 mL in a quartz cuvette (10-mm path length, 3.5-mL capacity). An aliquot of 0.1 mL of bovine trypsin (Sigma Chemical Co., catalog no. T-8253, dissolved and diluted to a final concentration of 20 μg/mL in 1 mM HCl) was added and mixed with the buffer and substrate immediately before the initiation of recording absorbance at 247-nm wavelength (A_{247}). Assay buffer (2.6 mL), substrate (0.3 mL), and 1 mM HCl without enzyme (0.1 mL) were used as a reference blank. The assays were monitored for 6 min in a UV–vis spectrophotometer (Hitachi Model U-2000) and confirmed the linear progression of the reaction curve. For inhibitor activity assays, 2.6 mL of assay buffer, 0.1 mL of the enzyme and 2–6 μL of the seed extract (i.e., corresponding to 40–120 μg of seed to yield 40–60% inhibition) were mixed in a cuvette and incubated for 6 min. Substrate (0.3 mL) was added after the incubation period, and recording at A_{247} was immediately initiated. The spectrophotometer was set to auto-zero with the assay tube against the reference blank just before the start of recording. Inhibitor activity assays were monitored for 3 min, and the slope of the reaction curve was recorded as change in A_{247} per minute. The trypsin inhibitor units per gram of seed was calculaed as

$$\text{TIU/g of seed} = [(T\Delta A_{247}/\text{min} - I\Delta A_{247}/\text{min}) \times 3 \times 1000]/(540 \times \text{gram seed weight})$$

where $T\Delta A_{247}$/min is the change in A_{247}/min in the absence of inhibitor (sample), $I\Delta A_{247}$/min is the change in ΔA_{247}/min in the presence of inhibitor, and g seed weight is the gram equivalent of seed extract used in the assay.

b. Chymotrypsin Assay. In these assays 1.4 mL of assay buffer (0.1 M $CaCl_2$, 0.1 M Tris-HCl, pH 7.8) was added to 1.5 mL of substrate solution. The substrate, 1 mM BTEE, was prepared fresh on the same day by dissolving 15.7 mg of BTEE in 50 mL of 50% (w/w) aqueous, spectral grade methanol (i.e., 3:2 methanol/H_2O by volume). To this assay solution was added 0.1 mL of bovine α-chymotrypsin (Sigma, catalog no. C-4129, dissolved and diluted to a final concentration of 20 μg/mL in 1 mM HCl) just before the assay was started. The progress of the reaction was monitored like that of trypsin (described above) for 6 min at wavelength 256 nm, and the linearity of the reaction curve was confirmed. The reference blank contained the same solutions as above except 0.1 mL of 1 mM HCl was used in place of enzyme solution. The inhibitor assay was conducted by incubating 7.5–15 μL of seed extract (i.e., a corresponding quantity of 150–300 μg of seed material to obtain 40–60% inhibition) with 0.1 mL of enzyme solution (i.e., 2 μg of α-chymotrypsin) and 1.4 mL of the assay buffer for 6 min. At the end of the incubation period, the reaction was started by adding 1.5 mL of 1 mM BTEE solution. The initial absorbance reading was adjusted to zero (auto-zero) against the blank, and immediately the reaction was monitored for 3 min. The slope of the linear curves, expressed as change in A_{256} per minute, was used to calculate the chymotrypsin

inhibitor units (CIU) as

$$\text{CIU/g of seed} = [(C\Delta A_{256}/\text{min} - I\Delta A_{256}/\text{min}) \times 3 \times 1000)]/(964 \times \text{gram seed weight})$$

where $C\Delta A_{256}$/min is the change in A_{256}/min in the absence of inhibitor (sample), $I\Delta A_{256}$/min is the change in A_{256}/min in the presence of inhibitor, and gram seed weight is the gram equivalent of seed extract used in the assay.

iv. Polyacrylamide Gel Electrophoresis (PAGE). The electrophoresis was based on the Davis (1964) system as adapted by Tan-Wilson et al. (1985) with some modifications. The separating (resolving) gel contained 15% acrylamide, 0.8% *N,N'*-methylenebis(acrylamide) (bis), 0.1 M Tris-HCl, pH 8.8, and 4 M urea. The acrylamide was polymerized by adding *N,N,N',N'*-tetramethylethylenediamine (TEMED) and ammonium persulfate to a final concentration of 0.15% and 0.07%, respectively. The stacking gels were made up of 2.5% acrylamide, 1.25% bis, 0.059 M Tris base, 3.2% 1 M H_3PO_4, 1.33 μM riboflavin, 2.5% sucrose, and 1 M urea (pH not adjusted). The stacking gel was polymerized by warming the gel solution (after pouring into the gel mold) using fluorescent (100 W) lamps. The gels turned white when polymerized. The reservoir buffer contained 0.384 M glycine, 0.05 M Tris base, and 0.4 M urea (pH not adjusted). Sample buffer was made up of the reservoir buffer containing 40% sucrose and 0.01% bromophenol blue.

Samples were prepared by mixing seed extracts with the sample buffer in a 7:3 ratio and incubated at 50 °C for 20 min just before they were loaded into the sample wells of the gel. The gels were run at a constant current of 15 mA/gel (0.75 × 160 × 160 mm) for 4–5 h (Protean II xi Cell, Bio-Rad Laboratories). At the end of the electrophoresis, the stacking gels were removed and the separating gels were stained for trypsin or chymotrypsin inhibitor activities or used to transfer proteins to a nitrocellulose membrane for immunostaining (immunoblot analysis).

v. Trypsin and Chymotrypsin Inhibitor Activity Gels. After the proteins were resolved, the gels were stained for trypsin and chymotrypsin inhibitor activities based on Uriel and Berges (1968) with slight modifications. The gels were first washed in 0.1 M sodium phosphate buffer (pH 7.5) in a glass tray on an orbital shaker for three 5-min washes to remove the urea and equilibrate the gel with phosphate buffer. The gels were then incubated in phosphate buffer containing 15 mg/mL bovine trypsin or α-chymotrypsin for 15–20 min on the shaker at room temperature. At the end of incubation period, gels were rinsed three times for 2 min each rinse in distilled water before incubation in staining solution. The staining solution was prepared less than 15 min before use by dissolving 20 mg of *N*-acetyl-DL-phenylalanine β-naphthyl ester (APNE) in 8 mL of *N,N*-dimethylformamide and 40 mg of *o*-dianisidine, tetrazoitized (zinc chloride complex) in 80 mL of distilled water separately. These solutions were mixed immediately before they were poured on the gel. The gels were left in the stain for 4 h to overnight without shaking. The stained gels were rinsed in distilled water and stored in 7.5% acetic acid. All of the chemicals were obtained from Sigma, St. Louis, MO. The presence of trypsin or chymotrypsin inhibitors was visualized as clear bands with a dark violet or pink background.

vi. Immunoblotting. Alternatively, the fractionated proteins in the gels were transferred to a nitrocellulose membrane using 0.048 M Tris base and 0.039 M glycine (approximately pH 9.0) buffer with a semidry electroblotting system (Nova Blot, Pharmacia LKB Biotechnology Inc.). The transfer was done for 45 min–1 h at a constant current of 0.08 mA/cm². The immunostaining procedure was based on that of Vodkin and Raikhel (1986).

vii. Northern Blot Analysis. Total RNA was isolated from the seeds according to a scaled-down version of the protocol by McCarty (1986). The analytical procedure of RNA blotting was based on the paper by Alwine et al. (1977). The RNA was denatured with glyoxal and dimethyl sulfoxide (DMSO) according to the method of McMaster and Carmichael (1977) and resolved on 1.4% agarose gels as described by Sambrook et al. (1989) using 10 mM phosphate buffer (pH 7.0). Two to five micrograms of total RNA was used per sample. The gels were then used to transfer RNA to a nitrocellulose membrane (Magnagraph Nylon from Micron Separations Inc.) by capillary blotting procedure (Sambrook et al., 1989) using 10× SSC buffer (1X = 0.15 M NaCl + 0.015 M sodium citrate, pH 7.0). The RNA was then immobilized by baking the membranes at 80 °C for 30 min under vacuum, followed by UV cross-linking (200 μJ).

a. Hybridization Conditions. The membranes were placed in prehybridization solution (about 1 mL/cm²) containing 50% formamide, 5× SSC, 200 μg/mL sonicated and denatured salmon sperm DNA, 50 mM sodium phosphate buffer (pH 6.8), 1% SDS, 5× Denhardt's solution [1× = 50 mg of Ficoll + 50 mg of poly(vinylpyrrolidone) + 50 mg of bovine serum albumin per 1000 mL of H_2O], and 2.5% (w/v) dextran sulfate (MW 500 000) in sealed plastic pouches and incubated in a shaking water bath overnight at 42 °C. These membranes were then hybridized to ^{32}P-labeled (Feinberg and Vogelstein, 1983, 1984) BBI cDNA clone in a solution (about 1 mL/cm²) with the same composition as that of the prehybridization solution, except containing 20 mM phosphate buffer (pH 6.8), 1× Denhardt's solution, and 5% dextran sulfate. The hybridization conditions were the same as described for the prehybridization procedure. The probes were oligolabeled with a Random Primed DNA labeling kit from United States Biochemical Corp.

b. Washing Conditions. Following hybridization, the membranes were sequentially washed in 2× SSPE (1× = 0.15 M NaCl + 0.01 M NaH_2PO_4 + 0.0014 M Na_2EDTA, pH 7.4) and 0.5% SDS at 42 °C for 15 min, in 2× SSPE and 0.5% SDS at 65 °C for 30 min, and then in 0.2× SSPE and 0.2% SDS at 65 °C for 30 min–1 h. After the final wash, the membranes were exposed to X-ray film to visualize the hybridizing RNA bands.

viii. Isolation of DNA. Genomic DNA from the leaves was isolated as described by Dellaporta et al. (1983) with some modifications. Two to five grams of fresh or frozen (−80 °C) tissue was finely ground in liquid nitrogen using mortar and pestle, and 25 mL of extraction buffer [500 mM NaCl, 50 mM Na_2EDTA, 100 mM Tris-HCl, pH 8.0, 10 mM *o*-phenanthroline, 1% SDS, 0.07% β-mercaptoethanol (v/v), and 0.002% ethidium bromide] was added with continued grinding. The ground slurry of tissue was transferred to a sterile 250-mL centrifuge bottle, and the remaining tissue material in the mortar was washed with an additional 25 mL of the extraction buffer. The samples were incubated at 65 °C for 10 min and then were added with 17 mL of 5 M potassium acetate and incubated on ice for 15–30 min. After centrifugation at 12000*g* for 10 min, the supernatants were filtered through a sterile Miracloth filter into another sterile 250-mL centrifuge bottle containing 33 mL of 2-propanol. The samples were incubated at −20 °C for 30 min and centrifuged at 12000*g* for 10 min, the supernatant was discarded, and the pellets were resuspended in the TE buffer (10 mM EDTA and 25 mM Tris-HCl, pH 8.0). The resuspended samples were subjected to cesium chloride density gradient centrifugation using the standard procedure (Sambrook et al., 1989).

ix. Southern Blot Analysis. This analysis was based on the procedure by Southern (1975). Ten-microgram DNA samples were digested with *Eco*RI and resolved on 0.8% agarose gels using TAE (40 mM Tris base, 2 mM Na_2EDTA, 20 mM sodium acetate, 29.6 mM glacial acetic acid, pH 7.8) electrophoresis buffer. After electrophoresis, the gels were depurinated with 0.25 M HCl for 10 min, denatured in 0.5 M NaOH and 1.5 M NaCl for 20 min, and neutralized with 1.5 M NaCl and 1 M Tris-HCl, pH 8.0, for 20 min. The DNA from these gels was transferred to a nitrocellulose membrane, immobilized, prehybridized, hybridized, and visualized the hybridizing bands exactly as described above for northern blot analysis.

RESULTS

i. Trypsin and Chymotrypsin Inhibitors in the Wild Perennial Species in *Glycine*. The trypsin and chymotrypsin inhibitor activity gels revealed the presence of several inhibitors with varying band intensities in all of the species examined. Furthermore, a tremendous amount of variation in the migration patterns of these inhibitors was also observed among the species used for analysis (Figures 1 and 2). A great majority of trypsin inhibitors were also found to be chymotrypsin inhibitors.

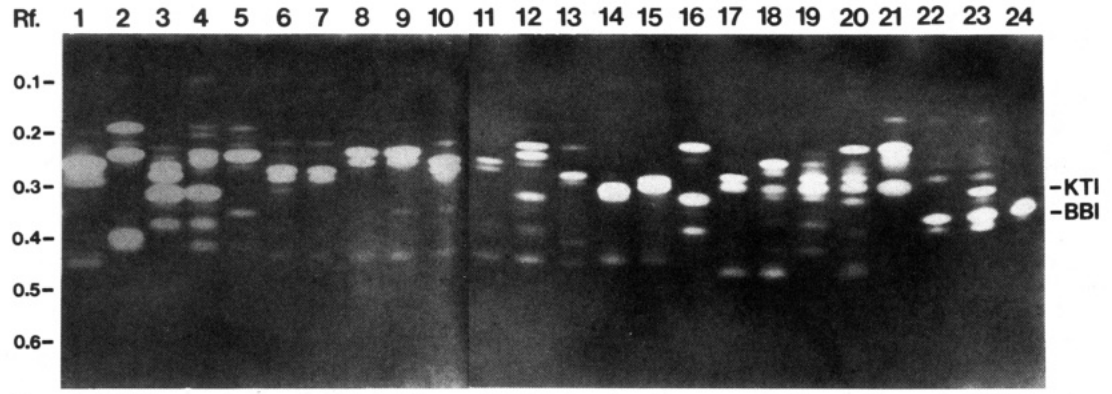

Figure 1. Trypsin inhibitor activity gel (15%, 4 M urea PAGE) showing the presence of several trypsin inhibitors in each species with variation in the intensities of bands in the genus *Glycine*. The samples [species (2*n*) IL no. or soybean cultivar] in the lanes are as follows: 1, ARE 689; 2, ARG 768; 3, CAN 434; 4, CLA 490; 5, CLA 426; 6, MIC 449; 7, MIC 503; 8, LAT 375; 9, LAT 515; 10, LAT 547; 11, TAB(40) 345; 12, TAB(80) 422; 13, TAB(80) 348; 14, CYR 480; 15, CUR 791; 16, TOM(40) 448; 17, TOM(40) 576; 18, TOM(38) 398; 19, TOM(78) 368; 20, TOM(80) 485; 21, FAL 320; 22, Kunitz; 23, Williams 82; 24, purified BBI standard.

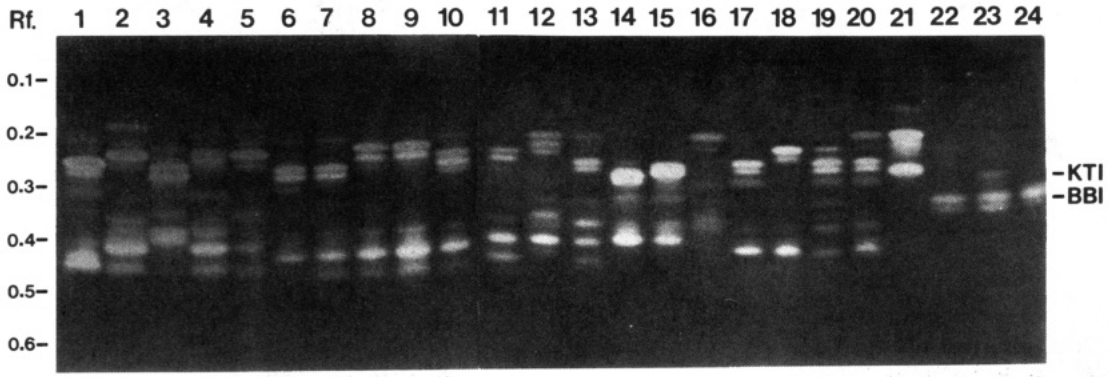

Figure 2. Chymotrypsin inhibitor activity gel (15%, 4 M urea PAGE) showing the presence of several chymotrypsin inhibitors in each species with variation in the intensity of bands in the genus *Glycine*. The sequence of samples is same as in Figure 1.

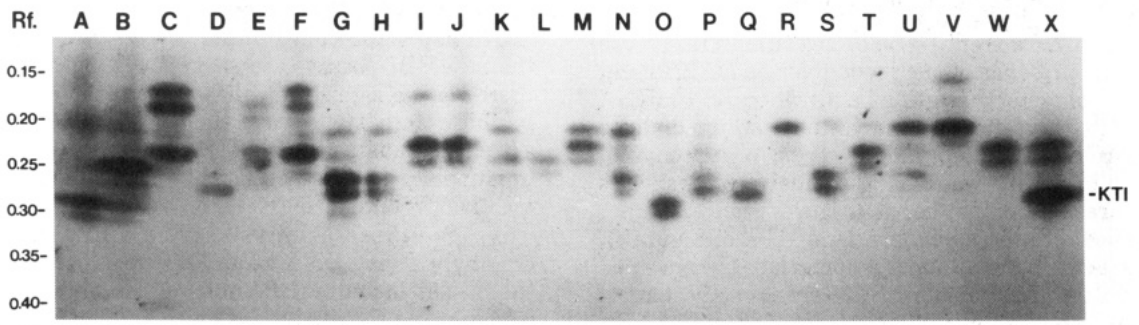

Figure 3. Immunoblot analysis of anti-soybean KTI crossreactive proteins in the selected accessions of various species of the genus *Glycine*. The samples [species (2*n*) IL no. or soybean cultivar] in the lanes are as follows: A, purified KTI; B, ARE 689; C, ARG 768; D, CAN 434; E, CLA 490; F, CLA 426; G, MIC 449; H, MIC 503; I, LAT 375; J, LAT 515; K, LAT 547; L, TAB(40) 345; M, TAB(80) 422; N, TAB(80) 348; O, CYR 480; P, CUR 791; Q, TOM(40) 448; R, TOM(40) 576; S, TOM(38) 398; T, TOM(78) 368; U, TOM(80) 485; V, FAL 320; W, Kunitz; X, Williams 82.

Similarly, several of the chymotrypsin inhibitors were also trypsin inhibitors with a majority showing weaker inhibition. The latter type of inhibitors migrated with R_f values ranging approximately between 0.35 and 0.5 (Figures 1 and 2). There were a greater number of inhibitors and also more variability in A genome species (lanes 1–5) than in the B genome species (lanes 6–11). The tetraploid *Glycine tomentella* accessions, $2n = 78$ and 80 (DDEE and AADD genomes, respectively) also had a large number of bands with inhibitory activities (lanes 19 and 20). There was also more uniformity in the banding pattern within the B genome species (lanes 6–11) and within C genome species (lanes 14 and 15 of Figures 1 and 2). The cv. Kunitz, a null for KTI, did not contain a KTI band with inhibitory activity; cv. Williams 82, on the other hand, had a KTI band with strong trypsin inhibitor activity (lanes 22 and 23, respectively, of Figure 1) and also with very weak chymotrypsin inhibitor activity (lane 23 of Figure 2). Both cultivars, however had a BBI band present. The fainter band migrating slightly faster than the BBI may be a proteolytic derivative [e.g., BBSTI-D of Tan-Wilson et al. (1978)] of the BBI.

ii. Immunoblot Analysis with Anti-KTI and mAB 238 Antibodies. Several anti-KTI immunocrossreactive bands were observed in most of the accessions examined (Figure 3). There were also two distinct anti-KTI immunostainable bands that migrated more slowly than the KTI band in both cv. Kunitz and cv. Williams 82 (lanes W and X). This might be due to the fact that the KTI antiserum used was polyclonal (against nondenatured KTI) and crossreacted with the other isoinhibitors. The faint crossreactive band that migrated slightly faster than and KTI, seen in both Williams (lane X) and purified KTI standard (lane A), may be the specific derivative of

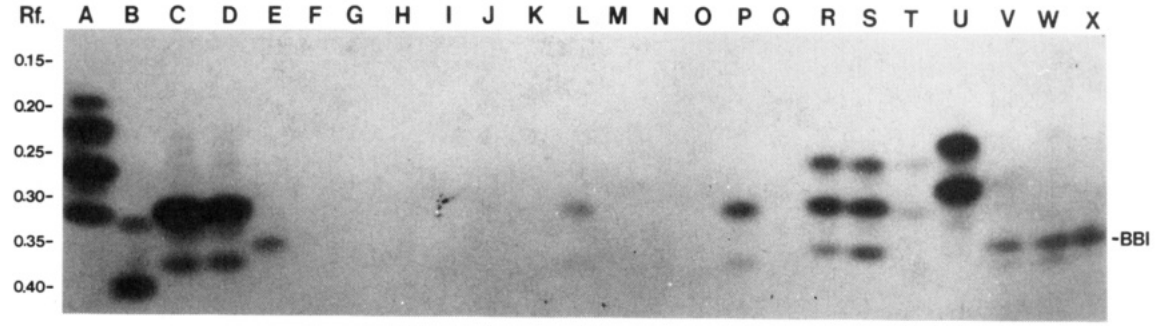

Figure 4. Monoclonal antibody 238 crossreactive proteins in the selected accessions of various species of the genus *Glycine*. The samples [species ($2n$) and IL no. or soybean cultivar] in the lanes are as follows: A, ARE 689; B, ARG 768; C, CAN 434; D, CLA 490; E, CLA 426; F, MIC 449; G, MIC 503; H, LAT 375; I, LAT 515; J, LAT 547; K, TAB(40) 345; L, TAB(80) 422; M, TAB(80) 348; N, CYR 480; O, CUR 791; P, TOM(40) 448; Q, TOM(40) 576; R, TOM(38) 398; S, TOM(78) 368; T, TOM(80) 485; U, FAL 320; V, Kunitz; W, Williams 82; X, purified BBI standard.

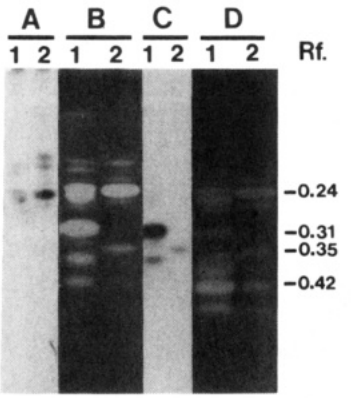

Figure 5. Comparison of various trypsin and chymotrypsin inhibitors visualized by (A) immunostaining with anti-soybean KTI antiserum, (B) trypsin inhibitor activity, (C) immunostaining with mAB 238, and (D) chymotrypsin inhibitor activity analyses in (1) CLA 490 and (2) CLA 426.

KTI [Ti^a_m of Wilson et al. (1988)]. Vodkin (1981) also recorded the presence of such a doublet band (KTI and the additional faster migrating band) when KTI mRNA were in vitro translated and analyzed by sodium dodecyl sulfate/PAGE and fluorography. Trypsin and chymotrypsin inhibitors in several wild perennial *Glycine* species also appeared as doublet bands (Figures 1 and 2).

All species with the A genome (lanes A–D of Figure 4), E genome (lanes R and S), and F genome (lane U) contained two or more darkly stained mAB 238 crossreactive bands, while the tetraploid species *Glycine tabacina* (with AABB genome) and *G. tomentella* (with AADD genome) showed only two faint bands (lanes L and T, respectively). The electrophoretic variant CLA, IL 426 (lane E), showed a single band with a different R_f value compared to either of the two darker bands in the standard CLA, IL490 (lane D), as seen in Figure 4. The B genome (lanes F–K and M) and C genome (lane N and O) species did not have any immunostainable bands and are therefore referred to as BBI-nulls. *G. tomentella* ($2n$ = 40) with D genome contained accessions that were BBI-positive (lane P) and BBI-null (lane Q). The A genome species ARE, CAN, and CLA and the F genome FAL contained the darkest bands, indicating the higher content of BBI.

A closer comparison between the inhibitor activity bands and immunostainable bands in two accessions of *Glycine clandestina*, IL490 (standard) and IL426 (a variant), was done by resolving the seed extract proteins in the same gel simultaneously, followed by analyzing the sections of the gel for inhibitor activities and crossreactivities to the antibodies. Figure 5 shows a composite of such an analysis. There were several inhibitor activity bands that did not crossreact with the antibodies. Almost all of the trypsin inhibitors (lanes in B) were also chymotrypsin inhibitors. Some are obviously weak inhibitors, e.g., the band with R_f 0.31 of IL490 was a strong inhibitor of trypsin (lane B.1) and a weak inhibitor of chymotrypsin (lane D.1) as indicated by the clearness of the band.

iii. Protein Content and Trypsin and Chymotrypsin Inhibitor Activities. There were highly significant differences in the soluble protein content and also in both trypsin and chymotrypsin inhibitor activities among the species tested (Table II). The protein content of most of the wild perennial species was significantly lower than that of soybean. Both trypsin and chymotrypsin inhibitor activities were significantly lower in the B genome species compared to the A genome species. *Glycine falcata* had the highest CIU/g seed weight of any genotype tested, including soybean (Table II). The C genome species also were lower in trypsin inhibitor activity but close to A genome species in their chymotrypsin inhibitor activity. In the case of TOM 40 (D genome) the BBI-null accession IL576 had significantly lower trypsin inhibitor activity than the BBI-positive accession IL448, but interestingly, there was no significant difference in the chymotrypsin inhibitor activity between them. Overall, the inhibitor activity estimates (Table II) agree fairly well with the immunoblot analyses, especially with the mAB 238 crossreactive proteins (Figure 4).

iv. Southern Analysis. The BBI cDNA (pB38) crosshybridizing DNA sequences were present in all of the species, including BBI-nulls, of the genus *Glycine* that were used in the analysis (Figure 6). Overall, there was a high degree of polymorphism in the BBI gene for *Eco*RI sites among the species. The *Eco*RI restriction fragment lengths were also more polymorphic in the A genome species (lanes A–E) than those in the B genome (lanes F–K and M) and C genome (lane N and O) species. No polymorphisms were observed between the accessions within the species *G. clandestina* (lanes F and G), *Glycine latifolia* (lanes H–J), and *G. tomentella* $2n$ = 40 (lanes P and Q).

v. Northern Analysis and Developmental Expression of BBI. The pattern of developmental expression of BBI transcripts in the seed of *G. clandestina* (IL490) correlated with that of soybean cv. Williams 82 (Figure 7). However, the transcripts in CLA, IL490, were at least 1 order of magnitude less abundant than those in soybean at early– and midmaturation stages. At late-maturation stage the level of message decreased more dramatically in soybean than in CLA, IL490 (Figure 7, lanes 4 and 8). Early to midmaturation stage embryos had the highest level of BBI mRNA.

When the total RNA from the midmaturation stage seeds of various *Glycine* species were probed with pB38,

Table II. Protein Content, Trypsin Inhibitor Units (TIU), and α-Chymotrypsin Inhibitor Units (CIU) of the Selected Accessions and Cultivars of Various Species in the Genus *Glycine*

species (2n) (IL no./cultivar)	% protein[a]	TIU/g seed[b]	TIU/g protein[c]	CIU/g seed[d]	CIU/g protein[c]
G. arenaria (40) (IL689)	11.91[e] ± 0.08	8519 ± 107	71 528	218 ± 0	1830
G. argyrea (40) (IL768)	13.24 ± 0.01	7160 ± 223	54 078	280 ± 0	2115
G. canescens (40) (IL434)	10.69 ± 0.05	8642 ± 123	80 842	280 ± 0	2619
G. clandestina (40) (IL490)	10.43 ± 0.05	6296 ± 0	60 364	270 ± 5	2589
G. clandestina (40) (IL426)	9.81 ± 0.10	2222 ± 0	22 650	52 ± 5	530
G. microphylla (40) (IL449)	7.70 ± 0.05	1420 ± 62	18 442	41 ± 5	532
G. microphylla (40) (IL503)	6.21 ± 0.12	988 ± 62	15 910	52 ± 52	837
G. latifolia (40) (IL375)	7.87 ± 0.02	1049 ± 62	13 329	73 ± 5	928
G. latifolia (40) (IL515)	11.48 ± 0.09	2901 ± 62	25 270	166 ± 5	1446
G. latifolia (40) (IL547)	9.06 ± 0.03	1790 ± 123	19 757	88 ± 5	971
G. tabacina (40) (IL345)	8.74 ± 0.10	617 ± 62	7 059	124 ± 0	1419
G. tabacina (80) (IL422)	9.03 ± 0.16	2469 ± 123	27 342	171 ± 0	1894
G. tabacina (80) (IL348)	9.65 ± 0.08	1111 ± 107	11 513	104 ± 5	1078
G. cyrtoloba (40) (IL480)	9.91 ± 0.06	3457 ± 62	34 884	233 ± 0	2351
G. curvata (40) (IL791)	11.06 ± 0.03	3086 ± 62	27 902	187 ± 0	1691
G. tomentella (40) (IL448)	9.27 ± 0.014	3395 ± 123	36 624	171 ± 0	1845
G. tomentella (40) (IL576)	8.63 ± 0.09	2778 ± 0	32 190	166 ± 5	1924
G. tomentella (38) (IL398)	9.38 ± 0.07	3457 ± 62	36 855	114 ± 5	1215
G. tomentella (78) (IL368)	9.53 ± 0.08	3951 ± 62	41 459	135 ± 5	1417
G. tomentella (80) (IL485)	9.26 ± 0.08	3704 ± 0	40 000	156 ± 0	1685
G. falcata (40) (IL320)	13.18 ± 0.19	8086 ± 62	61 351	358 ± 0	2716
G. max (40) (Kunitz)	13.71 ± 0.18	7593 ± 107	55 383	290 ± 5	2115
G. max (40) (Williams 82)	14.14 ± 0.15	11728 ± 163	81 388	332 ± 5	2304

[a] Content of proteins soluble in Tris–$CaCl_2$ buffer (pH 8.1), least significant difference (LSD at $\alpha = 0.05$) = 0.285. [b] LSD ($\alpha = 0.05$) = 271.7. [c] TIU/g of Tris–$CaCl_2$ buffer soluble seed protein. [d] LSD ($\alpha = 0.05$) = 11.1. [e] Based on three samples.

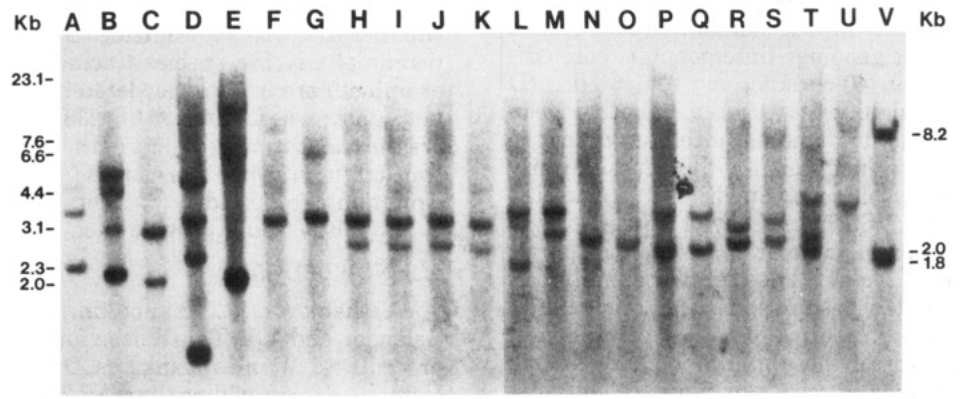

Figure 6. Southern analysis of the wild perennial *Glycine* species showing the presence of polymorphisms in *Eco*RI fragment lengths when probed with ^{32}P-labeled pB38. The samples [species (2n) and IL no. or soybean cultivar] in the lanes are as follows: A, ARE 689; B, ARG 768; C, CAN 434; D, CLA 490; E, CLA 426; F, MIC 449; G, MIC 503; H, LAT 375; I, LAT 515; J, LAT 547; K, TAB(40) 345; L, TAB(80) 422; M, TAB(80) 422; M, TAB(80) 348; N, CYR 480; O, CUR 791; P, TOM(40) 448; Q, TOM(40) 576; R, TOM(38) 398; S, TOM(78) 368; T, TOM(80) 485; U, FAL 320; V, Williams 82.

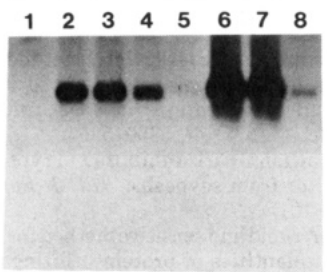

Figure 7. Developmental expression of the BBI in *G. clandestina*. Total RNA from the seeds at different stages of development were probed with ^{32}P-labeled pB38 (BBI). The samples in the lanes are as follows: (1) immature, (2) early-maturation, (3) midmaturation, and (4) late-maturation stage seed RNA (10 μg/lane) from CLA 490; (5) immature, (6) early-maturation, (7) midmaturation, and (8) late-maturation stage seed RNA (5 μg/lane) from soybean cv. Williams 82.

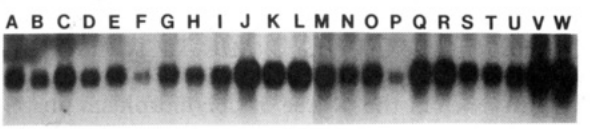

Figure 8. Northern analysis of total RNA from midmaturation stage seed from various species of the genus *Glycine* probed with ^{32}P-labeled pB38 (BBI). The samples [species (2n) and IL no. or soybean cultivar] in the lanes are as follows: A, ARG 768; B, CAN 434; C, CLA 490; D, CLA 426; E, MIC 449; F, MIC 503; G, LAT 375; H, LAT 515; I, LAT 547; J, TAB(40) 345; K, TAB(80) 422; L, TAB(80) 348; M, MIC 1070; N, CYR 480; O, CUR 791; P, TOM(40) 448; Q, TOM(40) 709; R, TOM(38) 398; S, TOM(78) 368; T, TOM(80) 485; U, FAL 320; V., Kunitz; W, Williams, 82.

all of the accessions contained the same size crosshybridizing RNA as soybean (Figure 8). There was significant variation in the abundance of these transcripts among various species. However, the level of RNA did not correlate with the inhibitor activities of various species. For example, the B genome species *G. tabacina*, nulls for BBI (lanes K and M of Figure 4) with lower levels of trypsin and chymotrypsin inhibitor activities (Table II), showed higher levels of BBI transcripts (lanes J and L of Figure 8). *Glycine arenaria* could not be included in the northern analysis since it did not produce pods in the greenhouse for isolation of RNA. The signal intensities were stronger in the soybean (lanes V and W) compared to those in the

wild species (lanes A–U) depsite a loading of only 2 μg/lane in the case of soybeans as opposed to 5 μg/lane in the wild species (Figure 8).

DISCUSSION

The trypsin and chymotrypsin inhibitors varied in both number and migration patterns among various wild perennial species. There was also variation in the intensity of these bands (Figures 1 and 2). This could be due to the lower concentration of an inhibitor fraction or the lower affinity (weaker inhibition) for the bovine trypsin or α-chymotrypsin. The trypsin inhibitor band with R_f 0.31 in CLA, IL490, is a good example of the latter, where it inhibited trypsin very strongly but has a very weak reaction with α-chymotrypsin (Figure 5). Most of the slower migrating inhibitors in all of the species were specific to both trypsin and chymotrypsin, while the faster migrating inhibitors, especially in the B genome species, were α-chymotrypsin-specific (Figures 1 and 2). Although there were no crossreactive proteins common to both mAB 238 and anti-KTI antibodies in the two accessions used in the comparison (Figure 5), it may be necessary to compare the other species also in the same way.

The variation in the inhibitor banding pattern was also observed among the accessions within a species (lanes 8–10 of Figures 1 and 2). However, the variation was much less dramatic. The variation increased from species to species within the same genome (intragenomic comparisons) to species with different genomes (intergenomic comparisons). By contrast, in 40-chromosome tomentellas (D genome) there was a significant variation in both trypsin and chymotrypsin inhibitor banding patterns dividing the accessions into four distinct groups with high uniformity within each group (Kollipara, Singh, and Hymowitz, unpublished results). Such a behavior renders these protease inhibitors excellent markers for phylogenetic studies in the genus *Glycine*. These protease inhibitors, in fact, have been widely used for biosystematic studies, especially in legumes (Weder, 1985). The variations in the migration patterns of these inhibitor bands among the accessions within a species provide excellent biochemical phenotypic markers to study the genetics and inheritance of Bowman–Birk inhibitors. Such study was not possible since there was no variation in the BBI banding pattern in soybean (Kollipara, 1992).

The A genome species CAN, CLA, and ARG and the closely related ARE are morphologically and geographically more diverse than the B (MIC, LAT, and TAB 40) and C genome (CYR and CUR) species (Doyle et al., 1990). The difference in the degree of polymorphism observed in the BBI-related genes between A genome and B and C genome species (Figure 6) and also in the trypsin and chymotrypsin inhibitor patterns (Figures 1 and 2) agrees with such data.

The presence of anti-KTI and mAB 238 crossreactive proteins in the wild perennial species suggests that these inhibitors are highly conserved during evolution. A definite pattern of the presence of mAB 238 crossreative proteins in various wild perennial relatives of soybean (Figure 4) clearly reflected the intergenomic relationships that have been established through morphology, cytology, biochemistry, and molecular biology (Singh and Hymowitz, 1985; Singh et al., 1988, 1989, 1992; Doyle et al., 1990). The large number and the enormous variability of the protease inhibitors present in the wild perennial *Glycine* species also indicate that the BBI family of inhibitors may contain several more members than were previously thought. Although the presence of such a large number of isoinhibitors may complicate the problem of characterizing the BBI gene family, it will provide an excellent opportunity to understand the evolution of these genes.

There were several chymotrypsin inhibitors present in the B genome species (Figure 2) but none were crossreactive with mAB 238 (Figure 4). However, the CIU/g seed values of the B genome species were significantly lower than those of the A and D–G genome species. This could be partly due to the low affinities of these inhibitors for bovine α-chymotrypsin.

The relative abundance of pB38 crosshybridizing transcripts (BBI-related) at various developmental stages was parallel in both the wild perennial species *G. clandestina* and soybean (Figure 7). This suggests that the molecular basis for the developmental regulation of the BBI-related genes in the wild perennial species may be very similar. This pattern of expression during soybean embryogenesis has been well documented in storage proteins (glycinins and conglycinins), lectin, and Kunitz trypsin inhibitor (Goldberg et al., 1989). It is interesting to note that the BBI-related genes in the wild species were conserved enough to hybridize with the BBI cDNA from soybean under high stringency conditions (see Materials and Methods). The B genome species, in particular, did not have proteins that would crossreact with mAB 238 but had transcripts which crosshybridized to the cDNA of soybean BBI (Figure 8). These transcripts were also about the same size as those of the soybean BBI. It is not known whether the crosshybridizing transcripts in the wild perennial *Glycine* species (including all of the species examined) are in fact translated into proteins. It is also not known whether the mAB 238 crossreacting proteins are the translation products of the pB38 crosshybridizing transcripts. In vitro translation followed by immunoprecipitation, as reported by Foard et al. (1982) and Hammond et al. (1984), would provide such information.

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