

Characterization of Seed Storage Proteins of Several Perennial Glycine Species

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ABSTRACT: Perennial *Glycine* species, distant relatives of soybean, have been recognized as a potential source of new genetic diversity for soybean improvement. The subgenus *Glycine* includes around 30 perennial species, which are well-adapted to drought conditions and possess resistance to a number of soybean pathogens. In spite of the potential of the perennial *Glycine* species for soybean improvement, very little is known about their storage proteins and their relationship with cultivated soybean seed proteins. We have examined the seed protein composition of nine perennial *Glycine* species by one- and two-dimensional (1-D and 2-D) gel electrophoresis. The relationship between cultivated soybean and perennial soybean seed proteins was examined by immunoblot analyses using antibodies raised against *G. max* β -conglycinin, glycinin A3 subunit, lipoxygenase, leginsulin, Kunitz trypsin inhibitor, and Bowman–Birk protease inhibitor. Additionally, we have measured the trypsin and chymotrypsin inhibitor activities from cultivated soybean and perennial *Glycine* species and have found marked differences between them. Our 2-D gel and immunoblot analyses demonstrate significant differences in the protein composition and size heterogeneities of the 7S and 11S seed storage proteins of soybean and perennial *Glycine* species. Perennial *Glycine* species accumulated a 45 kDa protein that was not detected in *G. max* and *G. soja*. This unique 45 kDa protein was immunologically related to the A3 glycinin subunit of *G. max*. The results of our studies suggest that even though the seed proteins of wild perennial *Glycine* species and *G. max* are immunologically related, their genes have diverged from each other during the course of evolution.

KEYWORDS: soybean, perennial *Glycine* species, glycinin, β -conglycinin, Kunitz trypsin inhibitor, Bowman–Birk inhibitor

INTRODUCTION

The genus *Glycine* is made up of two subgenera, namely, *Glycine* and *Soja*. The widely cultivated soybean (*Glycine max*) and its wild progenitor (*Glycine soja*) belong to the subgenus *Soja*. Both *G. max* and *G. soja* are native to northeastern Asia, are diploids ($2n = 40$), and are cross-compatible.¹ The subgenus *Glycine*, which encompasses about 30 wild perennial species, are indigenous to Australia and carry diploid ($2n = 40$), tetraploid ($2n = 80$), aneuploid ($2n = 38$) and aneutetraploid ($2n = 78$) chromosomes. The wild perennial *Glycine* species are well adapted to a wide range of climatic conditions and harbor many useful traits that can be exploited by breeders for soybean improvement. Earlier studies have shown that some perennial *Glycine* species carry resistance to soybean cyst nematode, rust, stem and root rot, brown spot, yellow mosaic virus, sclerotinia stem rot, and sudden death syndrome.² These examples highlight the potential of perennial *Glycine* species for increasing the disease resistance of cultivated soybeans by interspecific crosses.

North American soybean cultivars have a very narrow genetic base.^{3–5} Merely 17 ancestor soybean lines are responsible for the bulk of the genetic base of the North American soybean cultivars.⁴ This narrow genetic base possess a significant problem in case of an outbreak of pests or pathogens, in the absence of any resistant soybean germplasm. In this regard, the wild perennial *Glycine* species could serve as donor of valuable genes and could be exploited by breeders to widen the genetic base of the cultivated soybeans. Singh and associates were the

first to successfully generate intersubgeneric hybrids between *G. max* and *G. tomentella*.^{6,7} Numerous other attempts have been made to transfer useful genetic traits from wild perennial *Glycine* spp. to cultivated soybeans by intersubgeneric crosses.^{8–14}

The commercial and nutritive value of soybean is influenced by the seed protein concentration. North American soybean cultivars contain about 36–38% protein on a dry weight basis. An increase in protein concentration by a few percentage points would significantly improve the value of the crop. Breeders have exploited the genetic diversity found in *G. soja* germplasm to boost seed protein content and to increase the yield.^{15,16} A similar approach is also being undertaken using wild perennial *Glycine* spp. to improve the soybean seed composition. However, progress in this area has been limited because of reproductive barriers between *G. max* and *Glycine* spp. Refinement in methodology for intercrossing cytoplasmic and genetic diversity from *G. tomentella* into cultivated soybean has been recently developed.¹⁴ Some of the fertile lines derived from *G. tomentella* PI 441001 \times *G. max* cv. Dwight have been successfully grown in the field and are being evaluated for their agronomic performance.¹⁴ With advancement in the methodology it should be possible to exploit the genetic diversity of

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wild perennials *Glycine* spp. to improve the protein content of cultivated soybeans. To achieve this objective it is essential to first identify and characterize the abundant seed proteins of wild perennial *Glycine* spp. In contrast to the cultivated soybean, only limited information is available on the seed storage protein of wild perennial *Glycine* spp. Here, we have investigated the protein composition of several perennial *Glycine* species and demonstrate their immunological relatedness with cultivated soybean seed storage proteins.

MATERIALS AND METHODS

Plant Materials. Seeds of perennial *Glycine* species (*G. argyrea* Tind., PI 505151; *G. canescens* F.J. Herm. PI 509454; *G. clandestina* Wendl. PI 440958; *G. latifolia* (Benth.) Newell & Hymowitz B, PI 559303; *G. latrobeana* (Meissn.) Benth. PI 505187; *G. microphylla* Tind. PI 440956; *G. tabacina* (Labill.) Benth. PI 446978; *G. cyrtoloba* Tind. PI 440962; and *G. pescadrensis* Hayata PI 319697) and wild soybean (*G. soja* Sieb. & Zucc. PI 378683) were obtained from the USDA-ARS GRIN collection. Soybean (*G. max* L. Merr.) cultivars Williams 82 and Maverick seeds were from our laboratory collection. *Glycine* species were grown in a greenhouse under 16 h day length and 30/18 °C day–night temperatures. Plants were fertilized once a month with Miracle-Gro according to the manufacturer's (Scotts Miracle-Gro Company, OH) recommendations.

Protein Isolation for One-Dimensional Electrophoresis. Mature dry seeds (including the endocarp) were ground to a fine powder with a mortar and pestle. To isolate total protein, 10 mg of finely ground seed powder was transferred to a 2 mL microcentrifuge tube containing 1 mL of sodium dodecyl sulfate (SDS)-sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS (w/v), 10% glycerol (v/v), and 5% 2-mercaptoethanol (v/v)). The contents of the tube were vigorously vortexed for 10 min at room temperature followed by boiling for 5 min. Insoluble materials were removed by centrifugation at 15 800g for 5 min. The clear supernatant was designated as total seed protein fraction. Protein fraction enriched in legumin and Bowman–Birk protease inhibitor was isolated from 30 mg seed powder by extracting with 1 mL of 50% isopropanol as previously described.¹⁸ Kunitz trypsin inhibitor enriched fraction was isolated following the depletion of the abundant seed storage proteins by calcium precipitation.¹⁹ Briefly, 30 mg of seed powder was extracted with 1 mL of 50 mM Tris-HCl, pH 7.2, 1 mM EDTA by vigorously mixing on a vortexer for 10 min at room temperature. The slurry was subjected to centrifugation at 15 800g for 5 min. To the resulting clear supernatant, CaCl₂ was added to a final concentration of 10 mM, and the solutions was left standing at room temperature for 5 min. After incubation for 5 min at room temperature the solution was centrifuged as before, and the clear supernatant was treated as Kunitz trypsin inhibitor enriched fraction. Seed proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels using a Hoeffer SE 250 mini-Vertical electrophoresis apparatus (GE Healthcare, Pittsburgh, PA). Separation of proteins was achieved with a constant 20 mA/gel until the tracking dye reached the bottom of the gel. Following this, the gels were removed from the cassette and stained with Coomassie Blue R-250 solution.

Immunoblot Analysis. Seed proteins from *Glycine max*, *G. soja*, and wild perennial *Glycine* species were first resolved on SDS-PAGE gels. Resolved proteins were electrophoretically transferred to nitrocellulose membranes. The membrane was incubated with TBS (10 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 5% nonfat dry milk for 1 h at room temperature. Following this step, the nitrocellulose membranes were incubated overnight with antibodies that had been diluted 1:5000 in TBST (TBS with 3% nonfat dry milk containing 0.2% Tween 20). Subsequently, the membrane was washed three times with TBST and incubated with goat anti-rabbit IgG-horseradish peroxidase conjugate that had been diluted 1:5000 in TBST. Proteins reacting specifically with the antibodies were detected using the SuperSignal West Pico kit (Pierce, Rockford, IL).

Polyclonal Antibodies. Acrylamide gel purified glycinin acidic subunit A3 (40 kDa) and commercially purchased soybean trypsin-chymotrypsin inhibitor (Sigma) were used to raise antibodies in female New Zealand rabbits. The rabbits were injected with 200 µg of purified protein three times at 3 week intervals. Glycinin A3 and trypsin-chymotrypsin inhibitor antibodies were purified by 40% ammonium sulfate precipitation and DEAE-cellulose chromatography. The preparation of antibodies against β-conglycinin,²⁰ Kunitz trypsin inhibitor,²¹ Bowman–Birk inhibitor,²² and legumin¹⁷ has been previously described. Soybean lipoxygenase antibody was obtained from Joseph Polacco (Dept. of Biochemistry, University of Missouri, Columbia, MO).

Two-Dimensional Electrophoresis. Sample preparation for two-dimensional (2-D) electrophoretic analysis was carried out as previously described.¹⁹ For each perennial soybean species, seeds were ground into a fine powder and 250 mg was placed in a cold mortar/pestle. To this were added 50 µL of Plant ProteaseArrest (G-Biosciences) and 5 mL of extraction buffer (0.1 M Tris-Cl, pH 8.8, 0.9 M sucrose and 0.4% 2-mercaptoethanol). This slurry was again ground for several minutes to a liquid consistency and then placed into a 15 mL tube where 5 mL of Tris-equilibrated phenol was added for a 30 min extraction. Phase separation was achieved using 5000g 20 min centrifugation in a swing bucket rotor. The upper phenolic phase was removed and added to ten volumes of freshly prepared 100% methanol with 0.1 M ammonium acetate. Protein precipitation progressed for 4 h at –80 °C and was followed by centrifugation at 12000g for 20 min at 4 °C. The supernatant was discarded, and the protein pellet was resuspended vigorously in freshly prepared 100% methanol with 0.1 M ammonium acetate and 10 mM DTT (chilled to –20 °C). Washing of the insoluble proteins was repeated three times with the same solution and twice with freshly prepared 100% acetone containing 10 mM DTT. Incubation at –20 °C for 20 min was followed by centrifugation at 12000g for 10 min at 4 °C between each wash.

For IEF, 300 µg of the protein sample was loaded per strip using in-gel rehydration and linear gradient, 13 cm IPG strips (GE Healthcare, Pittsburgh, PA). Strips were brought to a rehydration volume of 250 µL with 7 M urea, 2 M thiourea, 1% CHAPS, 2% C7BzO, 5% glycerol, and 2.2% 2-HED, containing protein sample. IEF strips were equilibrated (post IEF) with 5% SDS in a urea-based solution (0.05 M Tris-Cl pH 8.8, 6 M urea, 30% glycerol, and 0.1% bromophenol blue) containing 2% DTT for 10 min and again but with 2.5% iodoacetamide for 10 min. Focused strips were placed onto a medium format 15% vertical second dimension and secured into place with a warm 1% agarose SDS-PAGE running buffer solution (0.2% SDS). Gels were run at an initial 10 mA/gel for 1 h and followed by 25 mA/gel for 3 h. After electrophoresis, the gels were immediately removed and fixed in 5:4:1 (methanol:water:acetic acid) for 30 min, followed by two brief rinses in water, and stained in a Coomassie G-250 solution for overnight.

Image Acquisition and Analysis. One- and two-dimensional electrophoresis (1DE and 2DE) Coomassie stained gels were destained with multiple changes of ultrapure H₂O to remove background. All gels were scanned separately using an Epson V700 Perfection scanner controlled through Adobe Photoshop. Images (1DE) were analyzed using Phoretix-Quant (TotalLab, Newcastle upon Tyne, U.K.) for band identification, location, and R_f.

Trypsin and Chymotrypsin Inhibitor Activity Assays. Dry seed powder (50 mg) was extracted with 1 mL of 50 mM Tris-HCl, pH 8.0 by vigorously mixing on a vortexer for 10 min at room temperature. The slurry was subjected to centrifugation at 15 800g for 5 min. The clear supernatant was collected and used to measure the trypsin or chymotrypsin inhibitory activity following the established protocol.²³ For the measurement of trypsin inhibitor activity, known amounts to seed protein extracts was added to 2 mL eppendroff tubes that contained the assay buffer (Tris-HCl, 20 mM CaCl₂, pH 8.2). Trypsin (20 µg) was added to the assay mixture, and the mixture was incubated for 15 min at 37 °C. Following this, 1 mM BAPNA was added and the mixture was incubated for an additional 10 min. The reaction was terminated by the addition of 30% acetic acid, and the absorbance at 410 nm was recorded. One trypsin inhibitor unit (TIU)

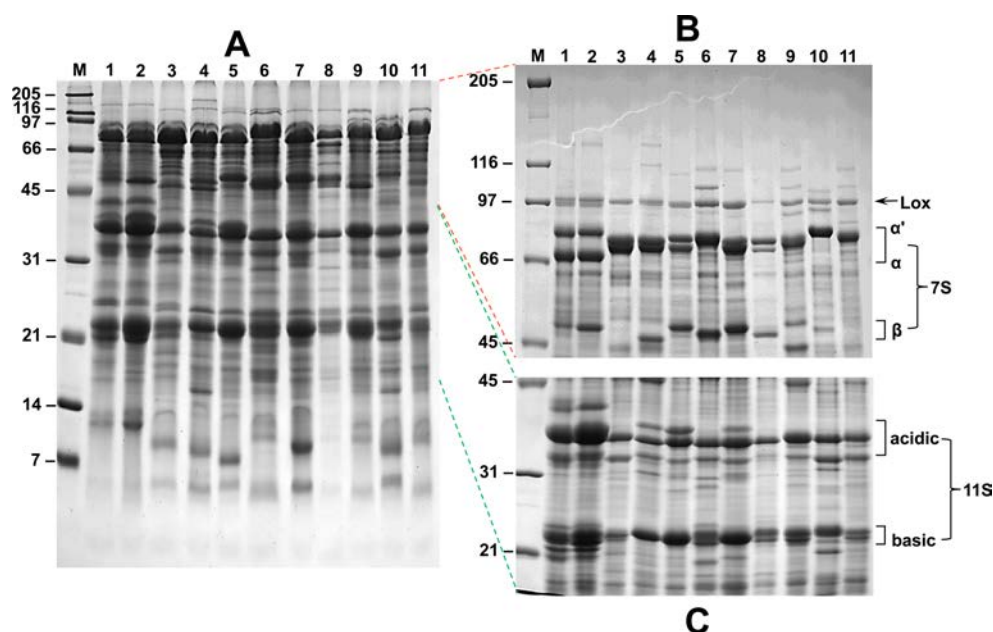


Figure 1. SDS-PAGE analysis of total seed proteins. Total seed proteins extracted from *G. max* cv. Williams 82 (lane 1), *G. soja* (lane 2), *G. argyrea* (lane 3), *G. canescens* (lane 4), *G. clandestina* (lane 5), *G. latifolia* (lane 6), *G. latrobeana* (lane 7), *G. microphylla* (lane 8), *G. tabacina* (lane 9), *G. cyrtoloba* (lane 10), and *G. pescadrensis* (lane 11) were analyzed by SDS-PAGE. Resolved proteins were detected by staining the gel with Coomassie Blue. Seed proteins were separated by SDS-PAGE with 15% (A), 8% (B), and 12% (C) resolving gels. Lane M contains the molecular weight markers whose sizes in kilodaltons are shown on the left side of the figure. Lox, lipoxygenase; 7S, β -conglycinin; 11S, glycinin.

was defined as the amount of inhibitor that reduces the absorbance of the noninhibited reaction by 0.01. Chymotrypsin inhibitor activity was measured by incubating known amounts to seed protein extracts with chymotrypsin (80 μ g) in the assay buffer (Tris-HCl, 20 mM CaCl₂, pH 7.8) at 37 °C for 15 min. *N*-Glutaryl-L-phenylalanine-4-nitroanilide (GLUPHEPA, 1 mM) was added, and the assay mixture was left for an additional 45 min at 37 °C. Reactions were terminated by the addition of 30% acetic acid, and absorbance at 410 nm was recorded. One chymotrypsin inhibitor unit (TIU) was defined as the amount of inhibitor that decreases the absorbance of the noninhibited reaction by 0.01.

RESULTS

Wild Perennial *Glycine* Species Accumulate Abundant Seed Proteins That Differ in Number and Size among Species. Total proteins from dry seeds of nine wild perennial *Glycine* species were analyzed by SDS-gel electrophoresis. *G. soja* and *G. max* cv Williams 82 (cultivar used to produce the reference genome sequence) were included in this analysis for comparison. The seed total protein profile of *G. soja* and soybean cultivar Williams 82 was strikingly similar (Figure 1A–C). The abundant seed storage proteins of soybean and *G. soja* (7S and 11S globulins) had identical protein profiles. In both, the 7S globulin was made up of three subunits (α' , α , and β) with apparent molecular weights of 76, 72, and 53 kDa, respectively (Figure 1). The 11S glycinin was represented by two abundant groups of proteins with molecular weights of 37–44 kDa and 17–22 kDa. These proteins are the products of the five-glycinin genes (*gy1*, *gy2*, *gy3*, *gy4*, and *gy5*). In contrast, the seed storage proteins of the nine wild perennial *Glycine* species showed considerable variability in their size and number (Figure 1). Seed proteins with relative mobilities similar to that of the 7S and 11S seed storage proteins of *G. max* and *G. soja* were seen in the perennials (Figure 1). However, the number, abundance, and sizes of the seed proteins differed among the nine wild perennial *Glycine* species (Figure 1). Seed proteins

with identical molecular weights representing the three subunits of 7S globulin (76, 72, and 53 kDa) of *G. max* and *G. soja* were not detected in the wild perennial *Glycine* species with the exception of *G. cyrtoloba*, which retained the 76 kDa protein (Figure 1). A 42 kDa glycinin A3 subunit and a 12 kDa glycinin A5 subunit polypeptides, which accumulates in *G. max* and *G. soja*, were not detected in any of the wild perennial *Glycine* species. A comparison of the protein profile also reveals that the wild perennial *Glycine* species contain protein bands that were either unique or much more abundant than in *G. max* and *G. soja* (Figure 1). Interestingly, a high molecular protein (100 kDa) was present in *G. max*, *G. soja*, and all wild perennial *Glycine* species examined in this study (Figure 1).

Seed Proteins of the Wild Perennial *Glycine* Species Are Immunologically Related to *G. max* Proteins. The immunological relationship among the seed storage proteins of wild perennial *Glycine* species and *G. max* seed proteins was evaluated by Western blot analyses. An antibody raised against the β -subunit of β -conglycinin was used to detect the presence of similar proteins in the wild perennial *Glycine* species (Figure 2). We have previously shown that this antibody recognizes not only the β -subunit but also the α' and α subunits as well.²⁴ As expected, the antibody recognized all three subunits of the 7S globulin in *G. max* and *G. soja* (Figure 2A). The three subunits of the β -conglycinin of *G. max* and *G. soja* had similar molecular weights. In contrast, the beta subunit of β -conglycinin antibody reacted strongly against variable size protein migrating closer to the to *G. max* and *G. soja* β -subunit (Figure 2B). In addition, the antibody also reacted against few other high molecular weight proteins corresponding to the *G. max* α' and α subunits of β -conglycinin. Interestingly, β -conglycinin antibody failed to react with any proteins from *G. cyrtoloba* seed extracts (Figure 2A).

A previous study investigated the glycinin (11S) composition of *G. canescens*, *G. tabacina*, and *G. clandestina*.²⁵ Soybeans

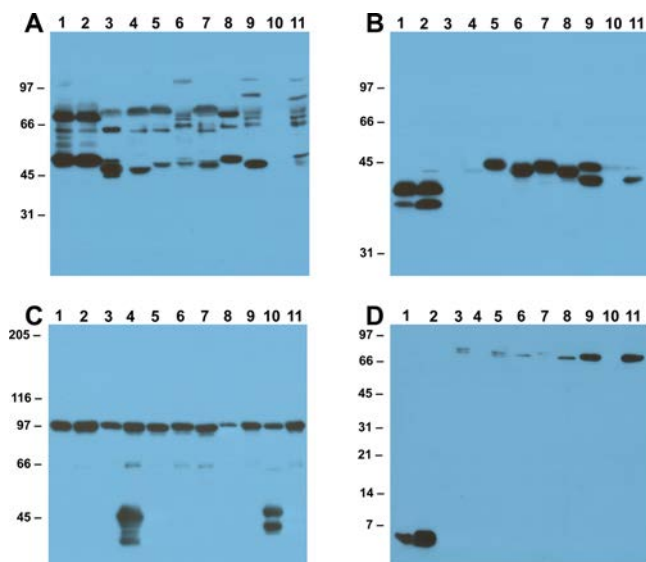


Figure 2. Detection of immunologically related soybean seed proteins in wild perennial species by Western blot analysis. Seed proteins extracted from *G. max* cv. Williams 82 (lane 1), *G. soja* (lane 2), *G. argyrea* (lane 3), *G. canescens* (lane 4), *G. clandestina* (lane 5), *G. latifolia* (lane 6), *G. latrobeana* (lane 7), *G. microphylla* (lane 8), *G. tabacina* (lane 9), *G. cyrtoloba* (lane 10), and *G. pescadrensis* (lane 11) were separated by SDS-PAGE. Resolved proteins were electrophoretically transferred to nitrocellulose membranes and probed with β -conglycinin (A), A3 glycinin (B), lipoygenase (C), and leginsulin (D) specific antibodies. Cross-reacting proteins were identified using anti-rabbit IgG-horseradish peroxidase conjugate followed by chemiluminescent detection. The numbers on the left side of the figure indicate the sizes of protein standards in kilodaltons.

contain two distinct groups of glycinin. Group I glycinins consists of $A_{1a}B_2$, $A_{1a}B_{1b}$, and A_2B_{1a} , while group II contains A_3A_4 , and $A_5A_4B_3$. It was reported that the perennial *G. tomentella* accumulated proteins homologous with both groups of glycinins but lacked subunit diversity.²⁵ Additionally, it was reported the A3 glycinin (42 kDa) component was lacking in *G. canescens*, *G. tabacina*, and *G. clandestina*. We also observed that a protein band corresponding to A3 glycinin was absent in all the wild perennial *Glycine* species examined in this study. To confirm this observation, we performed Western blot analysis using an antibody raised against *G. max* A3 glycinin. This antibody reacted strongly against a 42 kDa and 40 kDa polypeptide in *G. max* and *G. soja* protein extracts (Figure 2B). In contrast, the antibody recognized slightly higher molecular weight (44–47 kDa) proteins from seed extracts of *G. canescens*, *G. clandestina*, *G. latifolia*, *G. latrobeana*, *G. microphylla*, *G. tabacina*, and *G. pescadrensis* (Figure 2B). However, only a faint reaction with a 47 kDa protein was detected in *G. argyrea* and *G. cyrtoloba*, while in the case of *G. canescens* no reaction was detected (Figure 2B).

Soybean contains three distinct isoforms of lipoxygenase (LOXs) that can be distinguished by their differences in optimum pH and substrate specificity.²⁶ Lipoxygenases are monomeric proteins (94–97 kDa) and are often associated with the “beany” flavor of soybean.²⁷ The occurrence of lipoxygenase in the wild perennial *Glycine* species was examined by Western blot analysis utilizing *G. max* LOX specific antibodies. The LOX antibody specifically recognized a high molecular weight (94–97 kDa) protein from seed extracts of *G. max* and *G. soja* as well as all the perennial *Glycine* species

(Figure 2C). Lower exposure of the X-ray films indicate that the immunoreactive band may be composed of more than a single protein band, indicating that these bands may represent the different LOX isozymes. In the case *G. canescens* and *G. cyrtoloba*, the LOX antibody also reacted strongly against 42 and 46 kDa proteins (Figure 2C). We also examined the accumulation of leginsulin, a cysteine-rich soybean peptide, in perennial *Glycine* species. The leginsulin-specific antibodies recognized a 4 kDa protein from the seed extracts of *G. max* and *G. soja*, but no reaction against a similar size protein was detected in any of the perennial *Glycine* species (Figure 2D). However, a positive reaction against a high molecular weight protein was seen in most of the perennial *Glycine* species (Figure 2D).

Detection and Activity Measurement of Kunitz and Bowman–Birk Protease Inhibitors in Perennial *Glycine* Species. Soybean seed contains two types of protease inhibitors, namely, Kunitz trypsin inhibitors (KTI) and Bowman–Birk inhibitors (BBI). These proteins are considered to be antinutritional compounds because they inactivate digestive proteases and interfere with optimal animal growth and development. KTI is a 21 kDa protein, whereas BBI is made up of a group of cysteine-rich small molecular weight proteins. We utilized antibodies raised against soybean KTI to detect the presence of homologous proteins in wild perennial *Glycine* species (Figure 3). Western blot analysis detected the presence

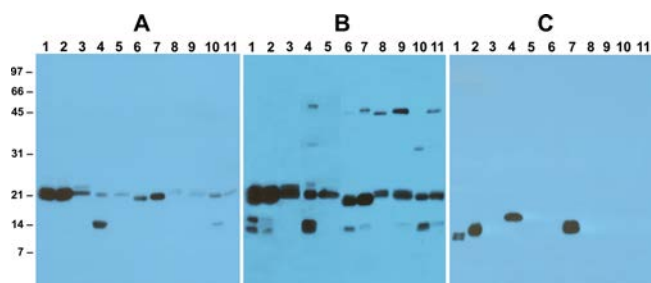


Figure 3. Detection of protease inhibitors in wild perennial species by Western blot analysis. Seed proteins extracted from *G. max* cv. Williams 82 (lane 1), *G. soja* (lane 2), *G. argyrea* (lane 3), *G. canescens* (lane 4), *G. clandestina* (lane 5), *G. latifolia* (lane 6), *G. latrobeana* (lane 7), *G. microphylla* (lane 8), *G. tabacina* (lane 9), *G. cyrtoloba* (lane 10) and *G. pescadrensis* (lane 11) were separated by SDS-PAGE. Resolved proteins were electrophoretically transferred to nitrocellulose membranes and probed with Kunitz trypsin inhibitor (KTI) (A), chymotrypsin inhibitor (B), and Bowman–Birk protease inhibitor (BBI) (C) specific antibodies. Immunologically related proteins were identified using anti-rabbit IgG-horseradish peroxidase conjugate followed by chemiluminescent detection. The numbers on the left side of the figure indicate the sizes of protein standards in kilodaltons.

of a 21–22 kDa protein in all nine perennial *Glycine* species that reacted with different affinity to the soybean KTI antibody (Figure 3A). Interestingly this reactivity was much stronger when chymotrypsin inhibitor antibody was employed (Figure 3B). In some cases, the CTi antibody also reacted against a couple of lower molecular weight (12–14 kDa) proteins. However, the CTi related proteins were not detected in all of the wild perennial *Glycine* species. The accumulation of the low molecular weight protein was strikingly higher in *G. canescens* (Figure 3B). We also employed a synthetic BBI peptide antibody in our analysis. Using this peptide antibody, we were able to detect their accumulation only in *G. max*, *G. soja*, *G. canescens*, and *G. latrobeana* (Figure 3C).

We also determined the trypsin and chymotrypsin inhibitor activity from the seeds of the wild perennial *Glycine* species and compared it to that found in *G. max* and *G. soja*. Significant differences in the specific activities of KTi and BBI were observed between species belonging to the subgenus “Soja” and subgenus “Glycine”. *G. max* cultivar W82 and *G. soja* exhibited significantly higher KTi activities when compared with the wild perennial *Glycine* species (Figure 4A). In contrast, the BBI

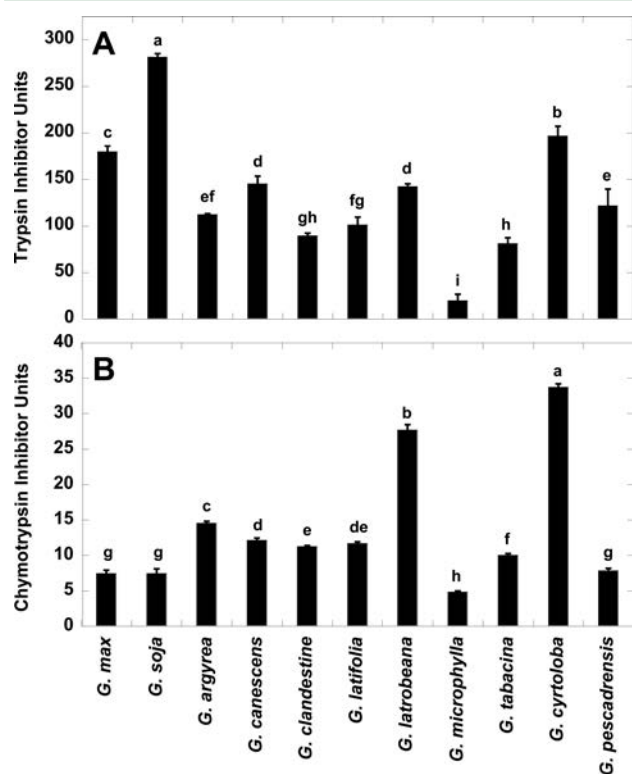


Figure 4. Trypsin and chymotrypsin inhibitor activity measurement in seed extracts. *N*-benzoyl-L-arginine ethyl ester and *N*-glutaryl-L-phenylalanine-4-nitroanilide were used as substrates for the measurement of trypsin and chymotrypsin inhibitor activity, respectively. Inhibitor units were defined as the amount of inhibitor that decreases the absorbance of the noninhibited reaction by 0.1. Letters above columns indicate results of one-way analysis of variance ($p < 0.05$) and Tukey's honest significant difference test ($\alpha = 0.005$). Different letters on the top of the columns indicate significant differences between mean.

activity was significantly higher in wild perennial *Glycine* species except *G. microphylla* and *G. pescadrensis* when compared to that of *G. max* and *G. soja* (Figure 4B). In particular, *G. cyrtoloba* and *G. latrobeana* showed the highest BBI activity among the nine wild perennial *Glycine* species, and the same two *Glycine* species also had the highest KTi activity among all the wild perennial *Glycine* species examined in this study (Figure 4A).

2-D Gel Analyses of Wild Perennial *Glycine* Species Reveal Size and Charge Heterogeneity of Glycinin and β -Conglycinin Subunits. Analyses of soybean seed proteins by high-resolution 2-D gel electrophoresis and subsequent identification of various protein spots by mass spectrometry have been reported by several groups.^{19,28–30} We have previously identified a total of 36 protein spots as representing the different isoelectric forms of glycinin and β -conglycinin.^{31,32} 2-D gel analyses revealed that these protein spots were prominent in both *G. max* cultivar Williams 82 and *G. soja*,

members of the subgenus “Soja” (Figure 5). Matrix-assisted laser desorption/ionization time-of-flight analysis identified protein spots 1–7 as components of α' -, α -, and β -subunits of the β -conglycinin, while protein spots 8–12 correspond to the glycinin acidic and basic subunits (Figure 5). Even though these protein spots were common for both *G. max* and *G. soja* and shared similar molecular weights, the relative intensity of the protein spots differed slightly (Figure 5). Protein spots 13 and 14 represent the Kunitz trypsin inhibitor and Bowman–Birk protease inhibitors, respectively. The two abundant proteins (spots 1 and 2) correspond to the β -conglycinin α' and α subunits with apparent molecular weights of 70 and 66 kDa. The isoelectric point of these proteins was 5.4 and 4.8, respectively. The β -subunit of β -conglycinin (50–52 kDa) is represented by an isoelectric series (pI 5.8 to 6.3) of spots (Figure 5). The 11S globulin of soybean was composed of two groups of proteins. One group of proteins was made up of a series of protein spots of approximately 36–40 kDa with acidic pI values, while the other group was represented by protein spots migrating around 21 kDa with basic pI values (Figure 5). Prominent protein spots corresponding to other glycinin subunits (spots 8, 9, 11) lipoxygenase, sucrose binding protein, basic 7S globulin, KTi, and BBI were also identified (Figure 5).

2-D gel analysis of seed proteins of wild perennial *Glycine* species revealed both striking similarity and differences in the number of protein spots and their migration relative to that of cultivated soybean (Figure 5). An examination of the seed protein 2-D gel profiles of the wild perennial *Glycine* species reveal a shift in the isoelectric points of 36–40 kDa glycinin protein spots from 4.8–5.4 to 5.6–6.8 (Figure 5). This shift is very pronounced in *G. latifolia*, *G. tabacina*, *G. cyrtoloba*, and *G. pescadrensis* (Figures 5 and 6). In these perennial *Glycine* species the acidic glycinin subunits were resolved into 6–10 protein spots in contrast to *G. max* where it was separated into about 6 spots (Figure 5). Another striking feature of perennials is the presence of an abundant 45 kDa protein spot with an isoelectric value of 5.2 (Figure 5 and 6). With the exception of *G. argyrea*, this spot was prominent in all other perennial species investigated in this study (Figure 5). Interestingly, all the wild perennial *Glycine* species, with the exception of *G. canescens*, lacked prominent protein spots corresponding to the glycinin A4 subunit (Figure 5). Similarly, a protein spot corresponding to the low molecular weight A5 glycinin was also absent in all the wild perennial *Glycine* species. All the perennial *Glycine* species displayed prominent protein spots of approximately similar sizes and isoelectric points corresponding to the *G. max* KTi and BBI (Figures 5–7).

DISCUSSION

The genus *Glycine* comprises two subgenera, *Soja* and *Glycine*. It is estimated that these two subgenera diverged about 5 million years ago.³³ The subgenus *Glycine* includes around 30 wild perennial species. The members of the subgenus *Soja* (*G. max* and *G. soja*) are diploid ($2n = 40$), while members of subgenus *Glycine* include diploid ($2n = 40$), tetraploid ($2n = 80$), aneuploid ($2n = 38$), and aneutetraploid ($2n = 78$) species.^{34,35} On the basis of the ability to produce fertile hybrids and meiotic chromosome pairing, the wild perennial *Glycine* species have been grouped into seven “genome groups”, A–G.^{2,36} On the basis of isozyme and DNA phylogenetic analyses, two additional genomic groups H and I have been added to this list.^{37,38} In this study we have examined the protein composition of *Glycine* species belonging to the genomic

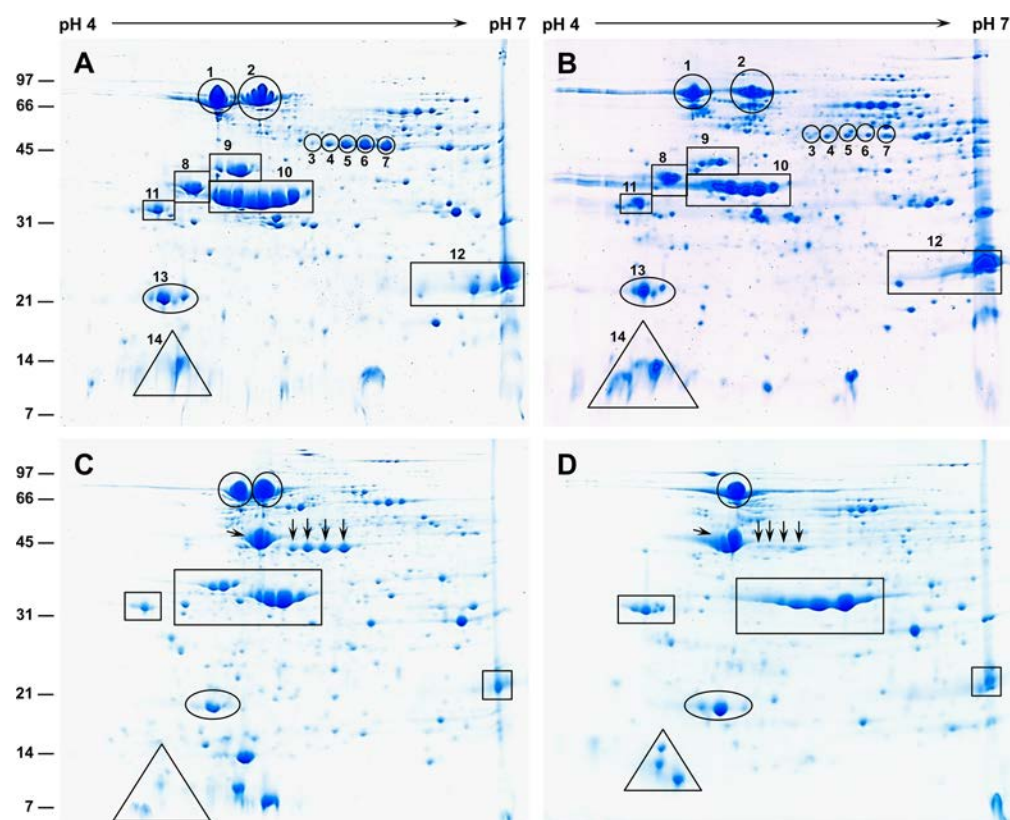


Figure 5. Two-dimensional gel electrophoresis comparison of seed proteins. Seed proteins (300 μg) were first separated by isoelectric focusing on pH 4–7 strips followed by separation by SDS-PAGE on 16% gels. The gels were stained with colloidal Coomassie blue G-250: (A) *G. max* cv Williams 82, (B) *G. soja*, (C) *G. canescens*, and (D) *G. latifolia*. Proteins corresponding to the 7S globulins are circled; 11S proteins are enclosed in boxes; Kunitz trypsin inhibitors are enclosed by oval shaped circles; the Bowman–Birk protease inhibitors are enclosed in triangles. Arrows in panels C and D point to proteins that appear to be unique to the perennials. The position and sizes of the molecular weight markers in kilodaltons are shown on the left side of the figure.

groups A (*G. argyrea*, *G. canescens*, *G. clandestina*), B (*G. latifolia*, *G. microphylla*), A/B' (*G. pescadrensis*), B/B' (*G. tabacina*), C (*G. cyrtoloba*), and I (*G. latrobeana*). A comparison of the seed protein profiles of members of the two subgenera, *Soja* and *Glycine*, clearly reveals significant differences among them. Marked differences in their molecular weights of the α' - and α subunit of the β -conglycinin were observed in the wild perennials. In an earlier study, the accumulation of the β -subunit of the β -conglycinin was not detected in the perennials.²⁵ However, our Western blot analysis clearly demonstrates the accumulation of the β -subunit of β -conglycinin in all the perennials species examined in this study with the exception of *G. cyrtoloba*. This discrepancy may be due to the fact that the accumulation of the β -subunit of β -conglycinin is highly variable, and its accumulation has been shown to be influenced by nitrogen availability.^{39,40} Interestingly, in some of the perennial *Glycine* species (*G. latifolia*, *G. latrobeana*, *G. tabacina*, *G. cyrtoloba*, and *G. pescadrensis*) proteins corresponding to the α' -subunit of the β -conglycinin were not detected. Our observation suggests that the genes encoding the 7S β -conglycinin of cultivated soybean and the wild perennial *Glycine* species may have diverged during the course of evolution. Cloning and sequence elucidation of the 7S β -conglycinin genes of the wild perennial *Glycine* species should shed light on the extent of their genetic diversity.

In contrast to the 7S β -conglycinin, the 11S glycinin subunits appear to be more conserved between cultivated soybean and the wild perennial *Glycine* species. Even though the glycinin

subunits of *G. max* and the wild perennial *Glycine* species reveal similar relative mobility on SDS-PAGE gels, differences in the isoelectric points are evident as demonstrated by 2-D gel analysis. Staswick and associates²⁵ examined the glycinin composition of *G. canescens*, *G. tomentella*, *G. tabacina*, and *G. clandestina* and compared it to that of *G. max*. Additionally, these authors also purified the 11S glycinins from *G. tomentella* by DEAE-Sephadex chromatography and determined their NH₂-terminal acid sequences. On the basis of these analyses, it was concluded that glycinin subunits homologous to G1 (A₁B₂), G2 (A₂B₁), and G4 (A₃A₄B₃) were absent in *G. tomentella*.²⁵ However, a comparison of the partial genomic sequence and their deduced amino acid sequences of Gy1 glycinin subunit of *G. latifolia* to their counterparts in *G. max* and *G. soja* indicated that Gy1 genes are highly conserved among the two subgenera, *Soja* and *Glycine*.⁴¹ Earlier, we cloned and elucidated the nucleotide and their deduced amino acid sequence of the Gy4 gene from *G. microphylla*.⁴² A comparison of Gy4 sequences of *G. max* and *G. microphylla* revealed significant differences within intron 3 of Gy4, suggesting that the Gy4 genes of wild perennial *Glycine* species and *G. max* may have diverged during the course of evolution.^{41,42} This possibility is strengthened by a recent comparative mapping study between *G. latifolia* and soybean that found extensive chromosome rearrangements in the genus *Glycine*.⁴³

Previous studies have indicated that glycinin G5 subunit (A₃B₄) was absent in wild perennial *Glycine* species.^{25,41} Our

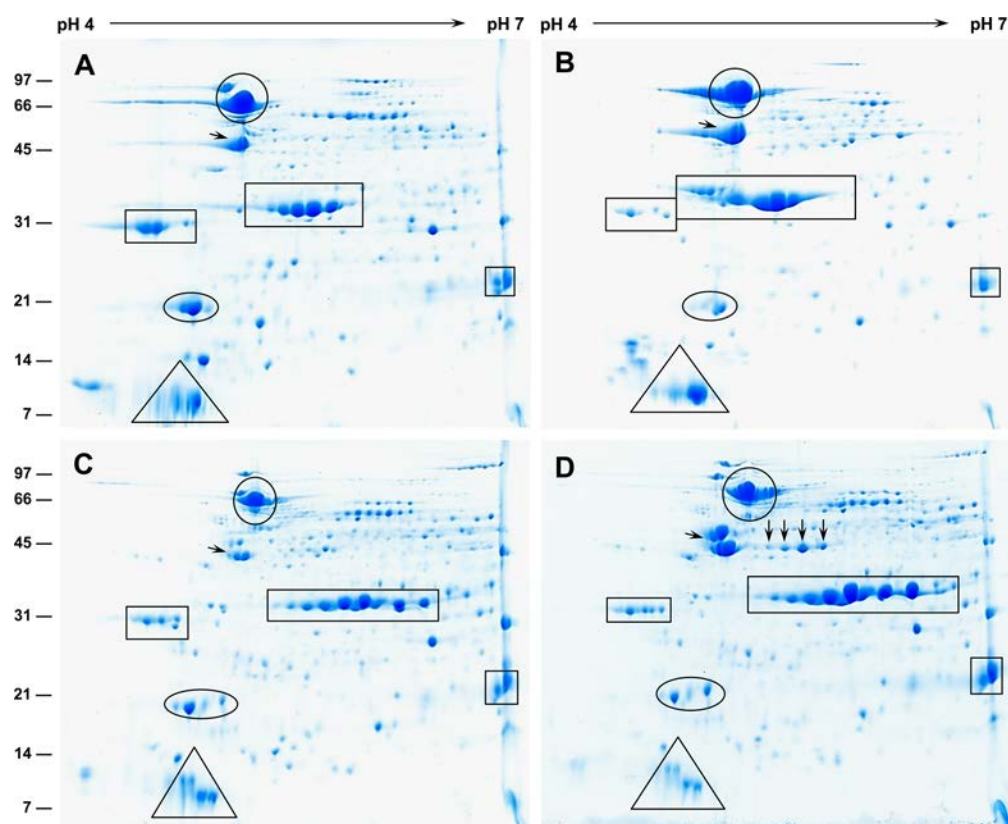


Figure 6. Two-dimensional gel electrophoresis comparison of seed proteins. Seed proteins (300 μg) were first separated by isoelectric focusing on pH 4–7 strips followed by separation by SDS-PAGE on 16% gels. The gels were stained with colloidal Coomassie blue G-250: (A) *G. cyrtoloba*, (B) *G. latrobeana*, (C) *G. pescadrensis*, and (D) *G. tabacina*. Proteins corresponding to the 7S globulins are circled; 11S proteins are enclosed in boxes; Kunitz trypsin inhibitors are enclosed by ovals; and the Bowman–Birk protease inhibitors are enclosed in triangles. Arrows in panel D point to proteins that appear to be unique to the perennials. The position and sizes of the molecular weight markers in kilodaltons are shown on the left side of the figure.

SDS-PAGE analysis of the seed proteins also indicated a 42 kDa protein corresponding to the *G. max* glycinin A3 subunit was absent in all the wild perennial *Glycine* species examined in this study. However, we observed that most of the perennial *Glycine* species accumulated a unique 50 kDa polypeptide that was conspicuously absent in both *G. max* and *G. soja*. Interestingly, antibodies raised against the purified A3 glycinin subunit of *G. max* reacted specifically against the 44–47 kDa protein, suggesting that protein homologous to A3 is present in the perennial *Glycine* species. In cultivated soybean, the glycinins are synthesized as a precursor protein that is processed into acidic and basic subunits that are linked by a single disulfide bond. A specific protease is responsible for the cleavage of the acidic and basic subunits, and the cleavage site is highly conserved.⁴⁴ It is likely that the *Gy5* gene in the wild perennial *Glycine* species has an altered cleavage site leading to the failure of the protease to process the precursor protein into its corresponding subunits. Cloning and the nucleotide sequence determination of the wild perennial *Glycine* *Gy5* gene should clarify this possibility.

Glycine soja, the wild relative of *G. max*, is commonly found in China, Taiwan, Korea, Japan, and Russia.⁴⁵ Tetraploid species including members of the *G. tomentella* polyploid complex, *G. pescadrensis*, *G. dolichocarpa*, and *G. tabacina*, all originated in Australia. Interestingly, *G. tomentella* and *G. tabacina* also occur in Taiwan, Philippines, Ryukyu islands, and South Pacific islands. In an attempt to define the systematic relationship among *Glycine* species, Hsieh and associates

analyzed the seed proteins of *G. soja*, *G. tomentella*, and *G. tabacina* by SDS-PAGE and Western blotting.⁴⁶ The authors observed considerable differences in heat soluble protein profiles and seed maturation proteins and concluded that antibodies specific to seed maturation proteins (GmPM1, GmPM2, and GmPM8) could be exploited to distinguish between and within other *Glycine* species. Similarly, protease inhibitors have also been used to study the genomic diversity among *Glycine* species.⁴⁷ Soybean accumulates two types of protease inhibitors, Kunitz trypsin inhibitor (KTI) and Bowman–Birk protease inhibitor (BBI), in the seeds. Three KTI genes (KTI-1, KTI-2, and KTI-3) have been shown to express specifically in seeds, with KTI-3 being the most abundant.⁴⁸ They encode proteins of about 20–22 kDa and specifically inhibit trypsin. BBI is encoded by a multigene family and encodes proteins of variable sizes (6–14 kDa) that can inhibit both trypsin and chymotrypsin. The migration pattern of BBI has been used to study the genomic diversity within *G. tomentella* accessions. A monoclonal antibody against soybean BBI (MAB 238) was used as a genetic marker to study the genomic relationship in the genus *Glycine*.⁴⁷ This immunological investigation led to the identification of BBI-nulls in several wild perennial *Glycine* species belonging to the B-genome (*G. latifolia*, *G. microphylla*, and *G. tabacina*) and C-genome (*G. curvata* and *G. cyrtoloba*). Our immunoblot analysis employing a BBI peptide antibody confirmed the absence of BBI in the members of the B-genome. However, in contrast to the earlier report,⁴⁷ we were unable to detect the presence of

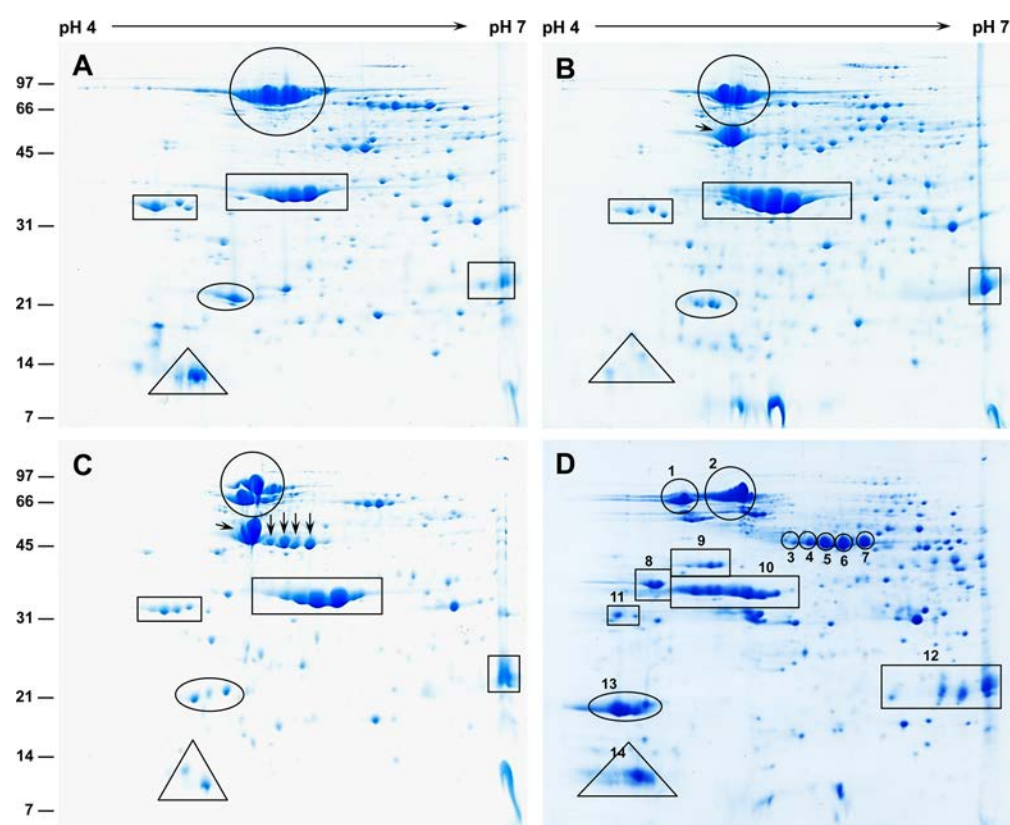


Figure 7. Two-dimensional gel electrophoresis comparison of seed proteins. Seed proteins (300 μg) were first separated by isoelectric focusing on pH 4–7 strips followed by separation by SDS-PAGE on 16% gels. The gels were stained with colloidal Coomassie blue G-250: (A) *G. argyrea*, (B) *G. clandestina*, (C) *G. microphylla*, and (D) *G. max* cv Maverick. Proteins corresponding to the 7S globulins are circled; 11S proteins are enclosed in boxes; Kunitz trypsin inhibitors are enclosed by ovals; the Bowman–Birk protease inhibitors are enclosed in triangles. Arrows in panel C point to proteins that appear to be unique to the perennials. The position and sizes of the molecular weight markers in kilodaltons are shown on the left side of the figure.

BBi in *G. argyrea* and *G. clandestina*, members of the A-genome. It is interesting to note that antibodies raised against soybean chymotrypsin inhibitor, unlike the BBi peptide antibody, were able to detect the presence of BBi related proteins in several wild perennial *Glycine* species. This discrepancy may be due to the fact that the different BBi antibodies may be recognizing different epitopes of the BBi. Even though the immunoblot analysis indicates the absence of BBi in some perennial *Glycine* species, enzyme assays clearly establish chymotrypsin inhibitor activity in all the perennial *Glycine* species examined in this study. It has been estimated that soybean contains at least 10 BBi isoforms with different specific inhibitory activities on trypsin, chymotrypsin, and/or elastase. BBi activity detected in seed extracts of wild perennial *Glycine* species is most likely due to the presence of numerous BBi isoforms.

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