Different Rates of Metabolism of Soybean Proteinase Inhibitors during Germination

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ABSTRACT

During germination, the content of the major Bowman-Birk proteinase inhibitor (BB-E) in the cotyledons of soybean (Glycine max [L.] Merrill cv. Fiskeby V) seeds decreases, becoming a minor form by the sixth day of germination. One of the three other minor species (BB-D) of this inhibitor in the dry seed increases to become the major form in six-day cotyledons. The other two minor species (BB-C and BB-F) also appear to decrease during germination, but at a slower rate compared to the original major inhibitor form BB-E. By 13 days of germination, no distinct Bowman-Birk inhibitor species can be discerned in the cotyledons. The content of the major Kunitz proteinase inhibitor (K-B) also decreases during germination, but much more slowly. One new form of the Kunitz inhibitor (K-A) becomes apparent by the sixth day. By the 13th day, the proportion of the main isoinhibitor to the late-appearing form is approximately two to one. This difference in the rates of disappearance and appearance of isoinhibitor species in the Bowman-Birk and Kunitz proteinase inhibitor classes suggests a differential metabolism of these two types of proteins and a possible difference in function in the soybean plant.

The seeds of legume species contain proteinase inhibitors active against serine proteinases. A number of possible functions for these inhibitors in plants have been proposed. These include the control of endogenous proteinases, particularly during dormancy of the seed, acting as a defense against the proteolytic enzymes of microbial, insect, avian, or mammalian predators, and serving as a storage depot, particularly of sulfur-containing amino acids (15, 16). The in vivo function of these proteinase inhibitors is not yet known. In some instances it seems likely that several of these functions may be served simultaneously. A possible storage function is implied by the observed decline in trypsin inhibitory activity during the germination of Vigna radiata (10), Phaseolus vulgaris (14, 18), and Pisum sativum (6). During the germination of the mung bean, V. radiata, the major proteinase inhibitor species present in the dry seed was found to be converted to three new active isoinhibitors (10). Phaseolus vulgaris proteinase inhibitors have also been shown to undergo limited proteolysis during the germination of the seed (19).

The inhibitors in V. radiata and P. angularis belong to the Bowman-Birk class (18). Soybeans, in general, have both the Bowman-Birk (BBSTI) and the Kunitz (KSTI) classes of proteinase inhibitor (8). That Kunitz inhibitors also show change during germination is suggested by the appearance of a new electrophoretically distinct protein in germinating beans (13), which is immunochemically cross-reactive with KSTI (5). In this paper, we demonstrate the disappearance of the original isoinhibitor forms of both BBSTI and KSTI and the appearance of new species during germination of the soybean. We found that the change in the Bowman-Birk isoinhibitor profile occurs much earlier and is more pronounced than the change in the relative proportions of the Kunitz inhibitors.

MATERIALS AND METHODS

Plant Materials and Other Reagents. Soybean seeds (Glycine max [L.] Merrill) cv. Fiskeby V, were obtained from Stokes Seeds (Buffalo, NY). Trypsin (bovine, twice crystallized), α-N-benzoyl-arginine ethyl ester (BAEE), and α-N-benzoyl-L-arginine-p-nitroanilide (BAPNA) were from Sigma.

Germination of Seeds and Collection of Plant Tissues. Seeds were hand-sorted for soundness and imbibed overnight in 50 g batches (approximately 300 seeds) in distilled H2O, then planted in sterile quartz sand. The plants were grown in an environmental chamber in a 16 h/8 h light/dark cycle with 24°C day and 18°C night temperature. The seedlings were harvested at the designated times. Germination time was reckoned relative to the beginning of imbibition. Those seeds showing no obvious growth were counted and discarded. From the sum of this count and that of the viable seeds, the fraction of the original dry weight (50 g) represented in the viable, germinated seeds was calculated. The cotyledons of seeds germinated for longer than 2 d were collected, rinsed with distilled H2O, blotted dry, and weighed. In the case of seeds germinated for 1 or 2 d, the tests were removed with the unexpanded embryonic axis remaining attached to the cotyledons. The cotyledons were stored at ~20°C until used.

Preparation of Plant Extracts. Cotyledons or dry seeds, the latter for the day zero determinations, were homogenized in a Waring Blendor for 2 min. The meal was then extracted by stirring overnight at 4°C in 0.05 M Tris-HCl buffer (pH 8) containing 0.3 mM phenylmethylsulfonylfluoride and 0.5 mM sodium iodoacetate, using a ratio of 10 ml buffer for every gram of cotyledons. The bries were filtered through cheesecloth, then centrifuged at 6,800 g at 4°C for 45 min. The supernatants were recentrifuged under the same conditions. Solid ammonium sulfate was then added at 0°C to reach 85% saturation and the material held overnight at 4°C. The ammonium sulfate precipitates were collected the next day by centrifugation at 6,000 g at 4°C for 1 h. The remaining pellets were reextracted with buffer, centrifuged, and added to the first extractions. These supernatants were acidified with HCl to a pH of 4.2, the precipitates removed by centrifugation at 6,800 g for 1 h and the pH of the supernatant adjusted to pH 8 with NaOH.

Proteinase Inhibitor Assays. Trypsin inhibitor activity was measured using α-N-benzoyl-arginine-ethyl ester as substrate (9). Assays of extracted gel slices were done using α-N-benzoyl-L-arginine-p-nitroanilide as substrate (4). One unit of inhibitor is defined as the amount that inhibits 1 mg active trypsin. For the

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enzyme preparation used in this study, the trypsin has been estimated by active site titration (1) to be 56% active.

Polyacrylamide Disc Gel Electrophoresis. Polyacrylamide disc gel electrophoresis was performed in 5-mm internal diameter glass tubes using the method of Davis (3) with 10% gels. Gels were sliced into 1.5-mm thick slices using a Yeda Scientific Instruments gel slicer. Individual slices were extracted with 200 μl 0.05 M Tris-HCl buffer (pH 8.0), by subjecting them to three freeze-thaw cycles over three days. Rf values were measured relative to the bromphenol blue tracking dye. When samples were to be compared visually, polyacrylamide disc gel electrophoresis was performed in the same system on 1.5-mm thick vertical gel slabs.

Column Chromatography. Gel filtration was performed with a 5 × 140 cm column of Sephadex G-75 (Pharmacia) equilibrated and eluted with 0.05 M Tris-HCl buffer (pH 8.0).

Ion-exchange chromatography was done with a 2.5 × 100 cm column of DEAE-cellulose (DE-52, Whatman) equilibrated with 0.05 M ammonium acetate (pH 6.5). The proteinase inhibitors were eluted with pH and concentration gradients of 2.7 liters with 0.05 M ammonium acetate (pH 6.5) as the initial buffer and 0.5 M ammonium acetate (pH 5) as the final buffer. The gradients were determined by measuring the pH and conductivity of column fractions.

Radial Immunodiffusion. Rabbit antisera specific to either KSTI or BBSTI were obtained as described previously (17). There was no cross-reaction between these two classes of inhibitor. The concentrations of Kunitz and Bowman-Birk inhibitors in gel slice extracts and in column fractions were determined by radial immunodiffusion (11). Known concentrations of the KSTI obtained commercially and BBSTI purified from the dry seed were used to construct standard curves. The concentrations of inhibitor standards were determined spectrophotometrically using an extinction coefficient E1%=10 of 10.01 and 4.6 for KSTI and BBSTI, respectively (8). Standard curves for the commercial KSTI and the two KSTI forms found in Fiskeby V are identical.

RESULTS

Proteinase Inhibitor Content of Cotyledons during Germination. The concentration of cross-reacting material assayed with anti-KSTI and anti-BBSTI antisera were determined by radial immunodiffusion of the initial crude extracts. The data (Fig. 1) are expressed relative to both the initial dry weight and the fresh weight at harvest of the cotyledons. There is a definite but gradual decline in the amount of inhibitor protein in the cotyledons during the first 13 d of germination. However, throughout this period the amount of inhibitor per gram fresh weight remains relatively constant for both types of inhibitor. Trypsin inhibitor activity was not assayed due to the inclusion of phenylmethylsulfonyl fluoride in the extraction buffer.

Appearance of New Bowman-Birk Proteinase Inhibitor Species during Plant Development. In addition to the gradual decline in the amount of BBSTI protein during soybean germination, the form of the anti-BBSTI cross-reacting material also changes significantly. This was demonstrated by disc gel electrophoresis and by ion exchange chromatography. Initial crude extracts were subjected to electrophoresis on cylindrical gels, with slicing and elution as described in "Materials and Methods." The material extracted from the slices was assayed for cross-reactivity to anti-BBSTI antiserum by radial immunodiffusion. The data, shown in Figure 2, are given as the fraction of cross-reacting material in each gel slice relative to the total recovered in all slices of that gel.

The Bowman-Birk soybean trypsin inhibitor in dry seed, i.e. at day 0, appears at an Rf of 0.6 in this gel system. This inhibitor form has the same amino acid composition and amino-terminal

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**Fig. 1.** Trypsin inhibitor content in the cotyledons of germinating soybeans. Kunitz trypsin inhibitor (O) and Bowman-Birk trypsin inhibitor (●) as mg inhibitor/g initial dry weight; Kunitz (□) and Bowman-Birk (■) inhibitors as mg inhibitor protein/g fresh weight. Each point is the average of three determinations.

**Fig. 2.** Analysis of polyacrylamide disc gel electrophoresis of soybean cotyledon extracts. Cotyledons were harvested from plants at the indicated stages of growth. Top to bottom, extracts from cotyledons 0, 4, 8, and 13 d old. Left column, immunologically cross-reactive protein. Extracted gel slices were analyzed for Kunitz (O) and Bowman-Birk (■) inhibitors. Right column (in same order), gel slices were assayed for trypsin inhibitory activity (—). The percentage of inhibitor activity or inhibitor protein in each slice relative to the total in the entire gel is plotted against the Rf value, measured relative to the bromphenol blue front.
sequence determined to reside 7 as an authentic sample of Bowman-Birk soybean trypsin inhibitor kindly supplied by Dr. S. Odani. At day 4, a significant amount of anti-BBSTI cross-reacting material appears at a lower Rf. This becomes unmistakably clear by day 6. By day 13, anti-BBSTI cross-reacting material appears in slices ranging from an Rf of 0.23 to 0.6. No single form of the inhibitor predominates at this late date.

The decrease in the content of the original Bowman-Birk soybean trypsin inhibitor and the appearance of new forms during plant development is confirmed by column chromatographic separation of these forms. The ammonium sulfate-precipitated proteins prepared from the cotyledons of seeds germinated 0, 4, 6, and 13 d were subjected to acid treatment to remove much of the soybean storage proteins. Essentially quantitative recovery of BBSTI (and KSTI) protein, as determined immunochemically, was achieved in the acid treatment step. Further removal of large mol wt proteins as well as separation of anti-KSTI and anti-BBSTI cross-reacting material was accomplished by gel filtration on Sephadex G-75.

The BBSTI pools from the Sephadex G-75 columns were subjected to column chromatography on DEAE-cellulose as described in “Materials and Methods.” The column fractions were monitored for absorbance at 280 nm, trypsin inhibitor activity and anti-BBSTI cross-reacting material. The column elution profiles are shown in Figure 3. Six peaks of activity were obtained on the column charged with material from d 0 seeds and are marked A to F in Figure 4. These shall be referred to in the text as BB-A, BB-B, BB-C, BB-D, BB-E, and BB-F. BB-A is the breakthrough peak which has trypsin inhibitory activity but does not precipitate with either anti-BBSTI or anti-KSTI on Ouchterlony plates. BB-

B appears in all four columns and coincides with the sharp drop in pH. Polyacrylamide gel electrophoresis of this pool shows that BB-B is a mixture of small amounts of the subsequently eluted forms of BBSTI (BB-C, BB-D, BB-E and BB-F) and thus probably represents in large part an artifact resulting from the rapid pH drop. Pools BB-C, BB-D, BB-E and BB-F are of most interest. The progressive change in the proportions of the BBSTI forms from days 0 to 6 can be seen by comparing the four elution profiles in Figure 3 and in the summary provided in Figure 4. Inhibitor BB-E is the principal Bowman-Birk species in the dry seed. Upon germination, both trypsin inhibitory activity and amount of BBSTI protein chromatographing at this position decreases steadily. Inhibitor BB-D quite clearly increases during the first 6 d, both in terms of trypsin inhibitor activity and in amount of BBSTI protein, to become the major form. Isoinhibitor BB-F remains a minor form throughout the first 6 d of germination. The ratio of BB-F to BB-D decreases as BB-D gains prominence, whereas the ratio of BB-F to BB-E increases as BB-E is gradually lost. Although the individual fractions in pool BB-C showed little or no cross-reactivity with an early anti-BBSTI antiserum, the pool, tested with a later antiserum of higher titer and wider specificity for antigen, did show a small amount of immunoprecipitate formation. The changes in the amount of isoinhibitor BB-C during germination are similar to the changes found in BB-F. By day 13, all the isoinhibitor forms, including BB-D, have virtually become indistinguishable from a background of various cross-reacting forms of the Bowman-Birk inhibitor eluting in almost every fraction collected from the DEAE-cellulose column.
amide gel electrophoresis, BB-C, BB-D, BB-E, and BB-F exhibited Rs values of 0.35, 0.46, 0.49, and 0.47.

Appearance of New Kunitz Proteinase Inhibitor Species during Plant Development. The KSTI pools from the gel filtration columns were chromatographed on DEAE-cellulose as described above. The elution profiles for the crude KSTI preparations from days 0, 6, and 13 are shown in Figure 5. At day 0, only one major peak with trypsin inhibitor activity reacting with anti-KSTI antiserum is present. The other inhibitor peaks are BBSTI inhibitors inasmuch as they precipitate with anti-BBSTI antiserum. These early peaks represent inclusion of some BBSTI in the KSTI pools from the Sephadex G-75 column. The main KSTI inhibitor form, designated K-B, diminishes by day 6, in relation to another form, designated K-A. By day 13, the ratio of species K-B to species K-A is about 2:1. This is unlike the situation with BBSTI in which no distinctly separable forms are left by day 13. The iso-inhibitor form K-A migrates somewhat slower than the form K-B (Rs 0.43 versus 0.47) on polyacrylamide gel electrophoresis.

The progressive change in KSTI species during soybean germination and development is summarized in Figure 6. Inasmuch as there are only two forms of KSTI, the pattern is very simple. As the initial forms of KSTI in the seed decreases, the other form of KSTI increases. The proportions of the two KSTI forms were calculated on the basis of trypsin inhibitory activity and on the amount of KSTI protein as determined by radial immunodiffusion.

DISCUSSION

We have demonstrated that the decrease during seed germination in the content of the major Bowman-Birk inhibitor species is more rapid than that of the major Kunitz inhibitor species. New forms of BBSTI appear within the first 4 d of seed germination. No major distinct species can be discerned by the 13th d. This is in contrast to the slower appearance of a new KSTI species and the persistence of both KSTI forms even after 13 d of germination and growth. These results were obtained both by electrophoretic analysis of the initial crude extracts and by column chromatographic analysis of extracts after acid precipitation of the storage proteins. Better resolution was obtained by the later method, but the first was necessary to show that the appearance of new inhibitor species was not caused by the purification steps prior to column chromatography. Similar results were obtained when the experiment was first done a year before with a different lot of beans. Furthermore, in a previous report (17), we analyzed the proteinase inhibitors leached from growing seeds into the surrounding medium and observed the same rapid change in BBSTI in the leachate in comparison to the KSTI form of inhibitor.

The addition of phenylmethylsulfonylfluoride and of iodoacetate to the extract buffer ensured that new iso-inhibitor forms did not arise by the in vitro action of serine or sulfhydryl endopeptidases or of plant carboxypeptidases after the cotyledons were extracted. The new inhibitor species could be newly synthesized proteins, expressing genes other than those which code for the main KSTI and BBSTI species found in the dry seed, or the iso-inhibitors which appear during germination could be the result of modification of the existing inhibitor species, as had been seen in P. vulgaris (14) and in V. radiata (10). It is also possible that both types of events occur. This will be resolved after purification and structural characterization of the soybean inhibitors which appear during germination.

We had previously characterized this soybean strain by subjecting the ethanol extract of soybean meal to a succession of CM-cellulose and DEAE-cellulose chromatography as described by Odani and Ikenaka (12). Whereas their soybean strain yielded five iso-inhibitors with distinctly different amino acid compositions, we found four with virtually identical amino acid compositions. The major Bowman-Birk inhibitor chromatographs with the BB-E described in this paper. The three minor peaks elute

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**Fig. 5.** Elution profile of DEAE-cellulose chromatography of the Kunitz inhibitor pools from the gel filtration of soybean cotyledon inhibitor preparations. Cotyledons were from plants harvested at the indicated stages of growth. From top to bottom: days 0, 6, 13. Fractions were monitored for trypsin inhibitor activity (---), and Kunitz inhibitor protein (●). The pH (○) and buffer concentration (- - -) gradients shown for day 0 are the same for days 6 and 13.

**Fig. 6.** Contribution to total KSTI inhibitor activity (top) and KSTI cross-reacting protein (bottom) by K-A (●) and K-B (●) during soybean germination.
SOYBEAN PROTEINASE INHIBITORS

Jefferson strain has been characterized as having a slower mobility through polyacrylamide electrophoresis system (3). The Kunitz inhibitor (K-B) in this soybean strain, Fiskeby V, migrates slower than does commercial KSTI in the Davis (3) polyacrylamide disc gel system, suggesting that KSTI is the product of the Ti allele. This was confirmed by the similar electrophoretic mobility of K-B and KSTI from soybean of the variety Jefferson kindly provided by Dr. T. Hymowitz. The Jefferson strain has been shown to have the Ti allele product (2). Although still cross-reacting to KSTI, the new band which appears during germination migrates even more slowly and thus does not correspond to any of the three known allelic forms of KSTI found in dry seeds.

Whereas the Bowman-Birk class of proteinase inhibitors occurs in a broad range of legume species, the Kunitz inhibitor type has been described thus far in only a limited number of species. In fact, in soybean, where the Kunitz type inhibitor was initially described, varieties lacking the Kunitz inhibitor have been found. This suggests that these two classes of inhibitors might have different, though possibly overlapping functions in the legume. The difference in the rates of change in the pattern of iso-inhibitor species for these two classes supports this idea. For instance, proteolysis or deamidation of the inhibitor would logically be a necessary occurrence if the proteinase inhibitors were to function as storage for amino acids or amino groups, respectively. Inhibitors of endogenous seed proteinases which digest reserve seed proteins would also be expected to lose inhibitor activity as the storage proteins are mobilized in the germination process. However, it would be undesirable for proteinase inhibitors which function solely as allelochemicals against microbial, insect, avian, and mammalian predators to lose inhibitor activity. The rates at which the different classes of proteinase inhibitors are degraded during germination may thus in part reflect their relative contributions to these different functions.

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LITERATURE CITED