Differential Expression of Kunitz and Bowman-Birk Soybean Proteinase Inhibitors in Plant and Callus Tissue

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ABSTRACT
Bowman-Birk soybean trypsin inhibitor (BBSTI) but not Kunitz soybean trypsin inhibitor (KSTI) was found in samples of undifferentiated and partially differentiated Amsoy 71 tissue culture callus. This suggests the differential metabolism of these two classes of proteinase inhibitors, whether the difference be in synthesis, in rates of degradation, or both. The differential metabolism of the proteinase inhibitors is also seen in the plant. Both BBSTI and KSTI were found in the hypocotyl, root, and epicotyl of the Amsoy 71 soybean seedling in addition to their expected presence in the cotyledons. Whereas the ratio of KSTI to BBSTI in the cotyledon was higher, the ratio of BBSTI to KSTI was higher in the extracotyledonary tissues of the seedling. The levels of both classes of proteinase inhibitors declined during seedling growth, except in the epicotyl and the proximal root. In both of these tissues, an increase in BBSTI, but not in KSTI content, expressed as milligrams inhibitor per plant part, occurred.

The KSTI and BBSTI in the soybean are small proteins which inhibit the proteolytic activities of mammalian pancreatic digestive proteinases. It has generally been thought that these proteinase inhibitors are confined to the developing seed in the maturing plant, to the dry seed, and to the cotyledon in the young seedling. Past attempts at detecting these proteinase inhibitors in soybean plant parts other than the cotyledon have yielded mixed results, some negative (4, 5), some positive (2, 11). Birk (2) reported that 1-week-old seedlings had all the trypsin inhibitor in the cotyledon, a little in the hypocotyl, and none in the leaves and roots. Hwang et al. (11) found Bowman-Birk inhibitor in 12-d cotyledons, epicotyl, hypocotyl, and root but concluded that only the cotyledons showed a significant amount of the proteinase inhibitors. In the mature plant, Goldberg reports evidence of proteinase inhibitor synthesis only in the cotyledon (5). The negative results appear to be correlated with plant maturity. All positive results were obtained with seedling material. The Kunitz and Bowman-Birk inhibitors are chemically distinct from those proteinase inhibitors found by Ryan and coworkers (6) in the vegetative tissue of potato, tomato, and other species.

Soybean seeds generally have more Kunitz than Bowman-Birk inhibitor (23); an exception being those strains which do not have Kunitz inhibitor at all (18). In this report, we demonstrate the presence of Kunitz and Bowman-Birk inhibitor in the vegetative tissues of the soybean seedling. Except for the cotyledon, the other plant tissues had more Bowman-Birk than Kunitz inhibitor. We also show that callus culture tissue originating from a Kunitz-inhibitor-bearing strain of soybean has only the Bowman-Birk inhibitor present.

MATERIALS AND METHODS
Plant Material. Seeds of soybean (Glycine max [L.] Merrill) strain Amsoy 71 (May Seed and Nursery Company, Shenandoah, IA) were germinated in a moist mixture of three parts of vermiculite to two parts of Jiffy Mix in a growth chamber in a 12-h day/12-h night cycle. The temperature during the day was 25°C, and at night was 20°C. The plants were harvested on specific days, and the separate tissues were frozen immediately at −78°C.

To obtain undifferentiated soy callus, Amsoy 71 was cultured in Murashige and Skoog (16) medium with 5 mg/l of 2,4-D. Growth conditions were 25°C and 12 h of light. The tissue used in these experiments was 10 weeks old since the time of last transfer and was selected for good growth, light green color, and absence of differentiation. Callus tissues from 15 bottles were pooled, homogenized for 30 s in ice water, and immediately frozen and lyophilized. Dry weight/fresh weight ratio was 3.8%.

To obtain partially differentiated soy callus, the callus grown on the medium described above was transferred to Murashige and Skoog medium containing 0.3 μM (average concentration) each of BA, ABA, and GAs. The tissues were pooled from a factorially designed experiment with each of the three growth regulators present at 0.1 or 0.5 μM in all possible combinations. The tissue used in these experiments was 21 weeks old, and tissue work-up was as described above. Dry weight/fresh weight ratio was 3.5%. The appearance of the tissue was markedly different from the first group: yellowish to light brown in color, and 60% of the surface, on the average, was covered with small, globular cell clusters of meristemoid cells, commonly called globular proembryoids.

Preparation of Extracts. The frozen tissues from harvested plants were homogenized in a Waring Blendor using 6 ml cold 50 mM Tris-Cl (pH 8) buffer and 1 g of polyvinylpolypyrrolidone (wet weight, hydrated in the same buffer) per g tissue. The extracts were clarified by filtration through cheesecloth and by centrifugation at 27,000g for 40 min at 4°C. The extracts were dialyzed against 5 mM ammonium bicarbonate, lyophilized, and dissolved in 50 mM Tris-Cl (pH 8.0) to give an exact 10-fold concentration.

For extracts of the callus tissues, the lyophilized callus tissue powder was stirred in 50 mM Tris-Cl (pH 8) buffer at 4°C using 70 ml buffer for every g of powder. The extract was clarified by centrifugation, then concentrated down 5-fold by dialysis against water, lyophilized, and re-dissolution in an appropriate vol-

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2 Abbreviations: KSTI, Kunitz soybean trypsin inhibitor; BBSTI, Bowman-Birk soybean trypsin inhibitor.
Immunochemical Methods. Antibodies were obtained from rabbits which had been immunized with purified KSTI or glutaraldehyde-polymerized BBSTI (21). Qualitative detection of inhibitor was done by Ouchterlony analysis. The antibodies are specific for each class of inhibitor. Quantitative measurement of inhibitor protein was done by radial immunodiffusion (13) as modified by Tan-Wilson et al. (24). All extracts were tested against antisera from rabbits immunized with human hemoglobin to check for false positive results. To test for false negative or inaccurate results, known amounts of pure inhibitor were added to extracts and measured by radial immunodiffusion. All results were found to be accurate.

Immunoblotting was performed with electrophoresis run in the standard Davis system (3), with 15% w/v acrylamide gel and 4 M urea included in the gel and reservoir buffers, the latter to enhance the blot transfer. The proteins were transferred by electroblotting onto 0.2 μm pore size nitrocellulose membrane (Schleicher and Schuell) by cathodic transfer in 0.7% (w/v) acetic acid (25). The primary antibody used for staining was purified IgG from rabbit antisera. The purification was done by ammonium sulfate fractionation (8) followed by chromatography on DEAE-Affi-gel Blue (Bio-Rad) as per instructions of the supplier. The Bio-Rad immuno-blot assay using goat anti-rabbit IgG as secondary antibody and horse radish peroxidase conjugate was utilized to visualize the antigen-antibody complexes on the blot.

RESULTS

Presence of Bowman-Birk but Not of Kunitz Inhibitor in Soybean Callus Culture. Undifferentiated and partially differentiated soy callus were tested for the presence of Kunitz and Bowman-Birk proteinase inhibitors. The results of Ouchterlony analysis shown in Figure 1 show the presence of Bowman-Birk but not of Kunitz inhibitor, in both undifferentiated and partially differentiated soy callus tissue. In this figure, there are two immunoprecipitin lines between the BBSTI standard and the antibody well. This is most likely due to the tendency of BBSTI to form very tightly associated dimers and trimers (15) complicated by the necessity of using glutaraldehyde-polymerized BBSTI as antigen in the rabbit. Apart from this heterogeneity in aggregation state, the BBSTI used as standard antigen and as immunogen was purified to electrophoretic homogeneity. This standard BBSTI is the major but not the only Bowman-Birk inhibitor in Amsoy 71. The multiplicity of isoinhibitors found in the seed (22) could also be in the tissue culture and thus explain the diffuse character of the immunoprecipitin lines between tissue culture samples and antibody. The lack of KSTI and the presence of BBSTI was again confirmed in radial immunodiffusion. The lower limit of detection for KSTI in these experiments was 6 μg per g dry tissue. There was 0.16 mg BBSTI/g dry weight (0.006 mg/g fresh weight) of the undifferentiated callus tissue and 0.33 mg BBSTI/g dry weight (0.012 mg/g fresh weight) of the differentiated callus tissues. These amounts are fairly close considering the difference in the state of differentiation and the age of cultures (see "Materials and Methods").

Proteinase Inhibitors in Plant Tissues during Germination. The presence of both classes of proteinase inhibitors was tested and confirmed by Ouchterlony analysis of extracts of vegetative tissue from 18-d-old soybean plants. We measured the amounts of KSTI and BBSTI in leaf, epicotyl, cotyledon, hypocotyl, and root tissue in soybean seedlings harvested at designated times during the first 14 d of growth. The roots were divided at time of harvest into two portions, that portion proximal to the stem and having lateral roots and that portion distal to the stem, whenever such a distinction could be made. The amounts of the inhibitors were determined by radial immunodifussion.

For these measurements, pure Kunitz inhibitor of the Ti' form (15) and Bowman-Birk inhibitor of the BBSTI-E form (23), the latter being the classical inhibitor first sequenced by Odani and Ikenaka (17), were used as standards for the quantitation of mg inhibitor per ml extract. The measurement of Kunitz inhibitor quantities are accurate since radial immunodiffusion standard curves using our antisera have been shown to be identical for the two forms of Kunitz inhibitor that were subsequently found in this work to be in these plant tissues (24). Our antibody to the Bowman-Birk inhibitor cross-reacts most strongly with BBSTI-E, the eliciting immunogen and the major form of BBSTI in Amsoy 71 dry seed (unpublished results). It also cross-reacts, though weakly in comparison to BBSTI-E, with all other Bowman-Birk isoinhibitors found in Amsoy 71 seed. Thus, measurements of BBSTI would be under- rather than over-estimates. Unless the proportions of strong and weak cross-reacting inhibitors were to change significantly, the trends depicted should be quite accurate. The results are expressed in mg inhibitor per g fresh tissue weight in Figure 2. Plots of mg inhibitor per mg protein (not shown) show the same patterns.

As expected, there is twice as much Kunitz as Bowman-Birk inhibitor in the dry seed and cotyledon in the very early stages of germination. However, the amounts of Bowman-Birk inhibitor in the other tissues of the seedling were found to be higher by weight per g tissue compared to the amounts of Kunitz inhibitor. Epicotyl from 8 to 14-d plants had twice as much BBSTI compared to KSTI. Hypocotyl from 4 to 8-d plants had 1.5 to 2 times as much BBSTI as KSTI. The same ratios are observed in the roots. Since the mol wt of BBSTI is 2.5 times that of BBSTI, the ratios in terms of moles BBSTI to KSTI are that much larger. The same relative levels of BBSTI to KSTI in the extracotyledonary tissues are also found in the embryonic axis after 1 d of imbibition where we obtain 8.7 and 3.9 mg of BBSTI and of KSTI, respectively, per g fresh tissue.

The pattern of distribution in the various plant parts of the two classes of inhibitor is similar. Through the first 2 weeks of seedling growth, cotyledons have 10 to 30 times more BBSTI and KSTI, respectively, compared to stem and 100 to 150 times more compared to root. The hypocotyl portion of the stem had more inhibitor compared to the epicotyl portion. Virtually all the root inhibitor was found proximal rather than distal to the
stem. No proteinase inhibitor was found in the leaves, although quantities of inhibitor added to leaf extract could be accurately determined by our techniques. The levels of inhibitor expressed as mg inhibitor per g tissue declined in all parts, leveling off after 10 to 12 d.

The distribution pattern appears somewhat different for KSTI and BBSTI when comparing plots of mg inhibitor per plant part as in Figure 3. Specifically, while the amounts of KSTI in the epicotyl and roots show a decline, the amounts of BBSTI in these plant parts increase during germination.

Epicotyl and root tissues have levels of BBSTI in the same range as those found in the callus tissues, when comparing mg BBSTI/g dry or fresh weight.

Evidence of Proteolytically Modified Inhibitor in Extracotyledonary Tissues. Kunitz proteinase inhibitors are proteolytically modified (unpublished results) during germination. This is demonstrated by the appearance of a new isoinhibitor form in the cotyledons of germinating soybeans (19, 23). This form migrates slower than the original Ti₄ form in PAGE in the Davis system. We examined the Kunitz isoinhibitor pattern in extracts by gel electrophoresis, blotting onto nitrocellulose and visualizing only the KSTI by immunostaining (Fig. 4). As expected, there was a single band of native Ti₄ Kunitz inhibitor in day 1 cotyledon and two bands corresponding to Ti₄ and its proteolytically modified form Ti₄ₐ in day 8 cotyledon. Ti₄ was also present in extracts of hypocotyl and root, showing that the same native isoinhibitor found in the seed and cotyledons is present in the other tissues of the seedling. We also detected traces of Ti₄ₐ in extracts of day 10 hypocotyl and day 6 root, suggesting either the occurrence of proteolysis in these tissues or the transport of proteolytically modified inhibitor from the cotyledon to these tissues. Corresponding work with BBSTI has to await the identification and characterization of the eight BBSTI forms which we find in the Amsoy 71 dry seed (unpublished results).

DISCUSSION

The difference in the expression of the Kunitz and Bowman-Birk inhibitors is most clearly demonstrated in the experiments on soy callus tissues. In both undifferentiated and partially...
differentiated tissues, we have found Bowman-Birk but not Kunitz inhibitors. Unlike the presence of inhibitors in the differentiated plant structures, the presence of these inhibitors must be due to synthesis. The results suggest that the Bowman-Birk inhibitors are synthesized whereas the Kunitz inhibitors are not, or are synthesized at levels well below our limits of detection. However, there is also the possibility that the Kunitz inhibitors are synthesized and then rapidly degraded. In this regard, it is worth noting that KSTI could hardly be found in the 14-d-old seedling, whereas BBSTI or its degradation products were still detectable. Wong et al. (28, 29) found that the Proteinase Inhibitor I from tobacco tumor, variegated or etiolated leaf tissues was the same, but different from that of either normal callus or crown gall callus tissues. We do not know yet whether the BBSTI in the callus tissues will show the same iso-inhibitor pattern as the BBSTI in the seed or whether we will find evidence of post-translational modification.

The possibility that BBSTI is synthesized while KSTI is not is particularly intriguing since this is not the case in the developing seed except for those few strains with the recessive ti gene. Hammond et al. (7) found the same timing and extent of expression of the BBSTI as that of the Kunitz inhibitor and the 1S storage protein by comparing their mRNA in developing seeds. These mRNA simultaneously accumulate early during the mid-maturation stage of seed development, reach a steady state, then decline toward maturity. The level of regulation for the BBSTI in the seed has not been established, but it is known that the expression of the storage proteins and of the Kunitz inhibitor in the seed is controlled by the mRNA levels (5). There is some evidence in another legume species to suggest that the BBSTI gene may be regulated separately. Working with the garden bean, Phaseolus vulgaris, Wilson (26) found Bowman-Birk type inhibitors to appear quite late in the development of the seed compared to the majority of the other extractable proteins. In an evolutionary sense, it is interesting to note that while all common legume crop species studied have homologous Bowman-Birk inhibitors, only the soybean has the Kunitz inhibitor (26).

The presence of the proteinase inhibitors in the epicotyl, hypocotyl, and roots of the seedling might be due to one or more of the following: (a) transport from the cotyledon; (b) synthesis in the plant part; or (c) expansion of the embryonic axis. The origins of the inhibitors might even be different for different plant tissues and for the two inhibitor classes. Note the increase in BBSTI but decrease in KSTI per plant part in epicotyl and root. This can be further complicated by variable rates of degradation of the same class of inhibitor in different plant parts or by variable rates of degradation of the two classes of inhibitor in the same plant part. The latter was demonstrated in the more rapid proteolysis of the Bowman-Birk BBSTI-E form compared to the Kunitz TiE form in the cotyledons of cultivar Fiskev K during germination (23). In Amsoy 71, which bears the TiE form of the Kunitz inhibitor, the overall cotyledon content of KSTI falls more rapidly than does the BBSTI (Fig. 2). Whether the difference is found in the distribution between the cotyledon and the axis of the seed, synthesis, transport, or rates of degradation, the data on the relative levels of KSTI and BBSTI show that the metabolism of KSTI and BBSTI in the cotyledon is different from that in extracotyledonal tissues.

Three possible functions have been ascribed to the proteinase inhibitors in the seed. These are: (a) defense against the digestive enzymes of microbial, avian, or mammalian predators; (b) storage of sulfur-containing amino acids in the seed; and (c) inhibition of endogenous proteinase in the seed (26). The presence of KSTI and of BBSTI in the hypocotyl, epicotyl, and root of the seedling, the general decline in the amounts of inhibitor in these parts during germination, and the presence of a proteolytically modified form of KSTI in hypocotyl and root support the idea that these inhibitors serve as storage proteins. Their relatively higher sulfur amino acid content, especially the BBSTI with 14 half-cystines per molecule complement the low sulfur content of glycine and conglycinin.

In the mung bean and pea, there is evidence of a cytosolic, low mol wt protein in the hypocotyl with an amino acid composition similar to that of the storage proteins. This is rapidly degraded during germination, before the mobilization of the protein-body-bound reserves of the cotyledons or the hypocotyl itself (14). Other studies show reduced proteolytic activation of Bowman-Birk inhibitors, also cytosolic (1), before degradation of the bulk storage protein of the cotyledon (12).

In the soybean, the Bowman-Birk inhibitors have been localized in different subcellular structures of the cotyledon and the embryonic axis. In the cotyledon, the BBSTI were found in the protein bodies, cytosol, and intercellular space. The embryonic axis exhibited strong marking intensity for BBSTI mostly in the cytosol and the cell walls (10). Consistent with our results, marking intensity for KSTI was much weaker in the embryonic axis compared to the cotyledon. In both parts of the seed, KSTI was found in the protein bodies, cytosol, and the cell walls (9).

It is useful to imagine the provision of amino acids to the growing plant as consisting of two phases. In the first phase, that of early germination and seedling growth, amino acid requirements are met by cytosolic storage proteins found both in the cotyledon and the embryonic axis, but mostly in the latter. These storage proteins would include the proteinase inhibitors with the higher ratio of sulfur-rich BBSTI to KSTI, and perhaps a low mol wt protein with amino acid composition similar to that of the main storage protein as was found in mung bean and pea. The latter is not a necessary feature of our hypothesis since KSTI, found in soybean but not in mung bean and pea, could fulfill this function. The proteolysis of these cytosolic storage proteins would provide the amino acids needed by the emerging plant structures which arise by expansion of the embryonic axis. This would explain why we find higher ratios of BBSTI to KSTI in the extracotyledonal tissues of the plant. This phase would encompass 0 to 3 d after germination of Amsoy 71 under our growth conditions. After this time, we see the mobilization of the bulk storage protein in the protein bodies of the cotyledon. These storage proteins would consist of glycinein, conglycinin, and the proteinase inhibitors with the higher KSTI to BBSTI ratio. During the second phase, the amino acid requirements of the plant are met in most part by the proteolysis of these storage proteins, the proteolysis occurring in the cotyledon itself, the amino acids being transported out to the growing plant structures. The mixture of amino acids supplied to the growing plant at this time would have a lower relative content of sulfur-containing amino acids. However, by this time, the plant would presumably be able to utilize inorganic sulfur, absorbed through the rapidly expanding root system, for the synthesis of the required sulfur-containing amino acids.

LITERATURE CITED

1. BAUMGARTNER, B. MJ CHRISSPELS 1976 Partial characterization of a proteinase inhibitor which inhibits the major endopeptidase present in the cotyledons of mung bean. Plant Physiol 58: 14-16

2. BIRK, Y 1967 Chemistry and nutritional significance of proteinase inhibitors from plant sources. Ann NY Acad Sci 146: 388-399


7. HAMMOND RW, DE FOARD, BA LARKINS 1984 Molecular cloning and analysis