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DEGRADATION OF TRYPSIN INHIBITORS DURING SOYBEAN GERMINATION

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Abstract—The principal Bowman–Birk soybean trypsin inhibitor in the soybean seed, BBSTI-E, is converted to BBSTI-D by limited proteolytic removal of the last two amino acid residues at the carboxyl terminus. The enzyme that catalyses this activity, Protease B1, works at an optimum pH of 4, first appears at day one and peaks at day four. The data also suggest that a second enzyme, Protease B2, appears by day 9 and catalyses extensive proteolysis of both BBSTI-E and BBSTI-D, the latter at a much faster rate. Three separate activities, measured at pH 7 in the presence of sulphydryl reducing agents, with peaks at days one, three and eight, catalyse extensive proteolysis of BBSTI-D, BBSTI-E and of BBSTI-E and −D, respectively Immunostained Western blots show that other Bowman–Birk isoinhibitors are also digested during germination and early growth.

INTRODUCTION

In addition to bulk storage proteins, soybeans synthesize and accumulate proteinase inhibitors during seed development During germination and early growth, the proteins stored in the soybean are degraded in a series of proteolytic steps Of the three classes of trypsin inhibitors [1], the Kunitz (KSTI), the Bowman-Birk (BBSTI), and the glycine-rich (GRSTI) soybean trypsin inhibitors, only the first two undergo proteolysis during the first 12 days [2–4]. The principal Kunitz and Bowman–Birk soybean trypsin inhibitors in the strain studied are the Ti^a and the BBSTI-E forms. Both inhibitors are subjected to an initial limited specific proteolysis prior to their more extensive degradation. The initial proteolytic cleavage of Ti^a to the modified form, Ti^a_m, involves the hydrolysis of the Leu176-Asp177 bond near the carboxyl-terminus [5]; that of BBSTI-E to BBSTI-D involves the Lys69-Glu70 bond, again near the carboxyl-terminus [6]

In this work, we demonstrate the presence of enzymatic activities in the cotyledons of soybean seedling which catalyse the conversion of BBSTI-E to BBSTI-D, as well as other enzymes which catalyse the more extensive degradation of BBSTI-E and BBSTI-D The levels of the various enzyme activities on successive days after planting are correlated with the timing of the *in vivo* decline of BBSTI-E and the appearance and subsequent decline of BBSTI-D.

The seeds of the soybean cultivar we have studied also contain a number of inhibitors homologous to BBSTI-E As a group, these Bowman–Birk inhibitors have very high sulphur amino acid contents especially compared to the storage proteins, glycinin and β-conglycinin. The BBSTI-E form in subgroup I has 14 half-cystines per molecule of 71 residues The inhibitors in subgroup II have the same proportion of half-cystines while BBSTI-B in subgroup III has 10 half-cystines in a molecule of 68 residues. Isoinhibitors had been previously classified into subgroups according to inhibitor specificity and immunochemical cross-reactivity but primarily by their amino acid compositions and in some cases, published amino acid sequences. By these criteria, forms included in each subgroup are related by proteolysis occurring in the developing seed [1]. In this paper, we also followed changing levels of these other Bowman–Birk isoinhibitors during germination and early growth.

We have recently described three enzymatic activities in the soybean which degrade the Kunitz inhibitors [7]. The degradation of the Bowman–Birk trypsin inhibitors is compared to that of the Kunitz trypsin inhibitors.

RESULTS AND DISCUSSION

Temporal pattern of degradation of the Bowman–Birk isoinhibitors during germination and early growth

Soybeans were planted and the cotyledons harvested at designated days for the first 12 days after the initiation of imbibition. The cotyledon extracts were analysed for their isoinhibitor complements. Isoinhibitor species were separated by PAGE Several gels were run, using samples equivalent to 0 0006, 0.006, or 0.06 cotyledons in each lane The gels were stained for trypsin inhibitor activity Parallel gels were blotted and immunostained with BBSTI and Kunitz trypsin inhibitor specific antibodies. The results are summarized in Table 1. The level of BBSTI-E declines by day two. BBSTI-D appears in its place. The level of BBSTI-D then declines by day 10. Ti^a declines by day three to be replaced by Ti^a_m which then decreases by day 12 Although the patterns are similar, the two native inhibitors do not appear to undergo the same rate of degradation

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Table 1 Temporal pattern of trypsin isoinhibitor distribution in the cotyledons of the soybean in germination and early seedling growth*

Day†	0	1	2	3	4	6	8	10	12
Fresh weight (mg)	198	280	320	320	320	370	410	500	490
Colour									
Isoinhibitor	Y	Y	Y	Y	Y–G	G	G	G	G
KSTI-Ti[a]	+ + +	+ + +	+ + +	+ +	+ +	+ +	+ +	+ +	+
KSTI-Ti[a]$_m$					+ +	+ + +	+ + +	+ + +	+ +
BBSTI-E	+ + +	+ + +	+ +	+ +	+ +	+ +	+ +	+	+
BBSTI-D		+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +
BBSTI-E′	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+ +	+	+
BBSTI-C′	+ + +	+ + +	+ + +	+ +	−	+			
BBSTI-C	+ + +	+ + +	+ + +	+ +	+ +	+			
BBSTI-A″	+ + +	+ + +	+ + +	+ +	+	+			
BBSTI-B	+ + +	+ + +	+ + +	+ + +	+ +	+ +			
BBSTI-G ‡						+ +	+ +	+ + +	+ +
BBSTI-H ‡						+ +	+ +	+ + +	+ +
BBSTI-I ‡						+ + +	+ + +	+ +	+ +
BBSTI-J ‡					+ +	+ + +	+ + +	+ +	+ +
GRSTI-1	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
GRSTI-2	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +

+ + +Soybean cultivar Amsoy 71 Presence of isoinhibitor species is indicated by + + +, + +, or + to indicate decreasing immunostaining intensities and decreasing trypsin inhibitor negative stain

†From time of planting

‡Tentatively assigned to Bowman–Birk class because of reaction with anti-BBSTI-E antibodies (and not anti-KSTI antibodies), but could be GRSTI

Trypsin inhibitor bands corresponding to BBSTI-E′ in subgroup I, BBSTI-C′, BBSTI-C, and BBSTI-A″ in subgroup II, and BBSTI-B in subgroup III, all show a visible decrease in staining intensity within days three and six New bands exhibiting trypsin inhibitor staining, and immunostaining with anti-BBSTI-E-specific antibodies, designated BBSTI-G, -H, -I and -J in Table 1, begin to appear at days four to six, only to decline again at days 10 to 12 These inhibitors may represent limited proteolytic cleavage products of the subgroup II and III Bowman–Birk isoinhibitors

Conditions for BBSTI-E and BBSTI-D directed proteolytic activities

Day three cotyledons were selected as a source of possible BBSTI-E to BBSTI-D converting activity in assays described in the Experimental Section Incubation of BBSTI-E with day three cotyledon extracts resulted in a decrease in BBSTI-E as measured by scanning densitometry of Amido Black stained gels The pH conditions that gave the highest activity were pH 4 and pH 6 75. Only the activity at pH 4 resulted in the appearance of BBSTI-D (Fig 1A) The highest levels of proteolysis with BBSTI-D as substrate were found at pH 7 Activity was also detected at pH 3 5, 5.5 and 6.5 (Fig 1B) The activities operating at pH 4 were enhanced by the addition of 0.2 mM dithiothreitol, the activities at or near pH 7 had an absolute requirement for the thiol reagent

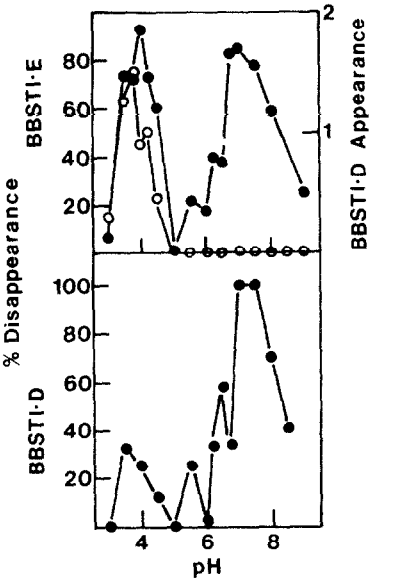

Fig 1 pH optima of proteolysis of BBSTI-E and BBSTI-D in extracts of day three cotyledons. Top BBSTI-E incubated for 4 hr with cotyledon extract equivalent to 0 35 mg fresh tissue –●–, % disappearance of BBSTI-E, –○–, appearance of BBSTI-D in terms of area under the peak of densitometer tracings, expressed in square inches Bottom BBSTI-D incubated for 2 hr with cotyledon extract equivalent to 0 35 mg fresh tissue –●– % , disappearance of BBSTI-D

Temporal pattern of pH 4 proteolytic activities

Cotyledons from day 0 to day 14 seedlings were analysed for proteolytic activity at pH 4 with BBSTI-E and BBSTI-D as substrates. The results are presented in Fig. 2

The earliest activity appearing was directed primarily against BBSTI-D No discrete products were detected. This activity appears soon after imbibition, peaks at day one, and then declines rapidly (Fig. 2B). The major proteolytic activity from days three to eight as shown in Fig 2A catalysed the conversion of BBSTI-E to BBSTI-D. The enzyme responsible for this activity may have been present earlier than day three, but BBSTI-D appearance would be undetected due to destruction of the product BBSTI-D by the early activity degrading this isoinhibitor This interpretation of the data would be consistent with the *in vivo* detection of BBSTI-D as early as day one. Similarly, the percentage disappearance of BBSTI-E beyond day eight (Fig. 2A) can not be accounted for completely by the appearance of BBSTI-D. The data are consistent with the idea that there are two major BBSTI-E proteolytic enzymes active at pH 4 (to be referred to as the pH 4 enzymes) One which we shall designate as protease B1, converts BBSTI-E to BBSTI-D and another, designated protease B2, produces more extensive proteolysis of BBSTI-E without the production of obvious products. The two peaks of enzyme activity degrading BBSTI-D between days two and five and between days nine and 13 (Fig. 2B) may reflect second activities of protease B1 and protease B2, respectively Accounting for differences in assay conditions, the rate of proteolysis of BBSTI-D on day 12 is four times higher than that for BBSTI-E.

There are many parallels in the degradation of the Bowman–Birk and the Kunitz trypsin inhibitors. For both inhibitors, the proteolytic activities carried out *in vitro* at pH 4 can account *in vivo* for the entire temporal pattern of change from native to modified inhibitor and the total decrease in inhibitor protein. Limited proteolytic cleavage occurs near the carboxyl-termini for both native inhibitors and involves cleavage of a peptide bond with an acidic amino acid at the amino-terminal side [5, 6]. The temporal pattern of the activities converting BBSTI-E to BBSTI-D and of the native (T_I^a) to modified ($T_{I_m}^a$) Kunitz inhibitor appear to coincide at the same stage of germination and early growth (Table 1). For both systems, the data suggest that the enzyme catalysing the extensive proteolysis of the modified inhibitor may also digest the native inhibitor, although at a slower rate A similar process was reported previously for the proteolysis of pumpkin seed globulin [8] It is in contrast to the degradation of vetch seed protein in which the modification by limited proteolytic cleavage was needed for extensive proteolysis to occur [9] or to the degradation of the Bowman–Birk trypsin inhibitor in the mung bean which undergoes a series of limited proteolytic cleavages before being subjected to extensive proteolysis [10].

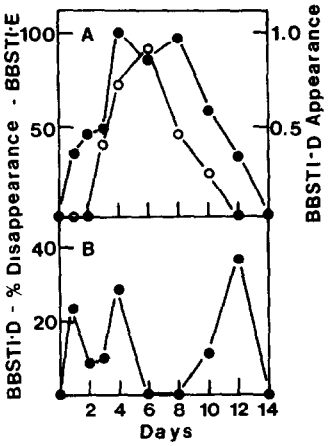

Fig 2 Temporal pattern of proteolysis of BBSTI-E and BBSTI-D at pH 4 Top BBSTI-E (4 4 μg) incubated for 2 hr with extracts made from the equivalent of 0.35 mg of cotyledons –●–, % disappearance of BBSTI-E, –○–, appearance of BBSTI-D in terms of area under the peak of densitometer tracings, expressed in square inches Bottom BBSTI-D (15 5 μg) incubated for 2 hr with extracts made from the equivalent of 0 35 mg of cotyledons –●–, % disappearance of BBSTI-D

The bulk storage proteins of the seed are sequestered in membrane-bound vesicles called protein bodies [11]. The larger proportion of both the Kunitz and the Bowman–Birk inhibitors have also been shown by immunocytochemical techniques to be in the protein bodies [12, 13] Many proteolytic enzymes responsible for digesting protein reserves in other legume seeds are cysteine proteinases working at acidic pH optima and these have been localized in the protein bodies [14, 15] It is thus reasonable to hypothesize that in soybeans, the pH 4 enzymes are together with at least the major part of the BBSTI-E in the protein bodies

Temporal pattern of proteolytic activities at neutral pH

The pattern of proteolysis of BBSTI-E and BBSTI-D at pH 7 was studied in a manner similar to that used for the acidic activities The results are shown in Fig. 3 All activities at pH 7 result in rapid, extensive proteolysis of the native or the modified inhibitor, without observable intermediates The earliest proteolytic activity is seen in the dry seed and declines after day two. This is active primarily towards BBSTI-D However, any actual role for this enzyme in the proteolysis of the Bowman–Birk inhibitors depends upon subcellular localization of enzyme and BBSTI-D in the same compartment, considering that the only activity we have found to produce BBSTI-D is one that functions at pH 4.

The activity hydrolysing BBSTI-E peaks at day three. The profile in the succeeding days could be interpreted as the result of a third activity directed toward both BBSTI-E and BBSTI-D. Accounting for differences in assay conditions, we find that at day eight, the rate of proteolysis of BBSTI-D is 12 times that of the proteolysis of BBSTI-E

Assay conditions for BBSTI-D proteolysis at pH 4 and at pH 7 are equivalent, except for pH, so the two late appearing activities are of comparable magnitude. However, their appearance on different days and the pH profiles (Fig. 1) show that the pH 7 proteolytic enzymes are distinct from the pH 4 enzymes. This poses a question with regards to the subcellular localization and function of the enzymes with near neutral pH optima especially since no such extensive proteolytic system of enzymes working at neutral pH exists for the Kunitz inhibitor We suggest four possibilities (i) The pH 7 proteolytic en-

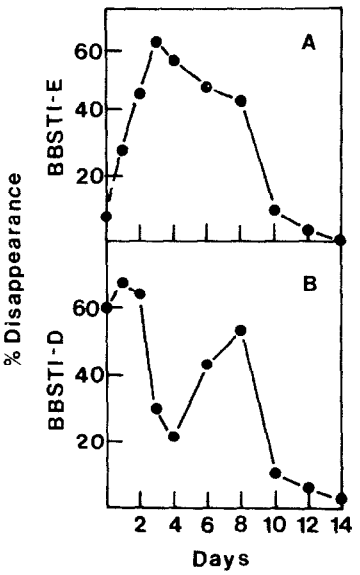

Fig 3 Temporal pattern of proteolysis of BBSTI-E and BBSTI-D at or near neutral pH Top BBSTI-E (4 4 μg) incubated for 3 hr at pH 6 75 with extracts made from the equivalent of 0 7 mg of cotyledons –●–, % disappearance of BBSTI-E Bottom BBSTI-D (15 5 μg) incubated for 2 hr at pH 7 with extracts made from the equivalent of 0 35 mg of cotyledons –●–, % disappearance of BBSTI-D

zymes may have a totally unrelated function We may be witnessing an artifact of an *in vitro* assay where substrate and enzyme which may never come in contact in the cotyledon are brought together (ii) Some BBSTI-E and its derived BBSTI-D may be in the cytosol either to begin with or as a result of the rupture of protein bodies Separate proteolytic enzymes may exist to catalyse their degradation (iii) BBSTI-E may be in the protein bodies but the other isoinhibitor forms may be in the cytosol. The pH 7 enzymes may be in the cytosol principally to catalyse the degradation of the subgroup II and III Bowman–Birk inhibitors (iv) The protein bodies may not be able to maintain an acidic pH optimum when the protein bodies are depleted of protein or phytate reserves. The pH 7 enzymes may be there to catalyse the proteolysis of any remaining trypsin inhibitor in the vesicles

Overall scheme of proteolysis

The degradation of all the Bowman–Birk soybean trypsin inhibitors in the cotyledons of the soybean seedling at a time when the storage proteins are being rapidly mobilized indicates that all these proteins serve a storage function in the seed. The existence of several proteolytic enzyme activities directed towards BBSTI-E and BBSTI-D indicates that the storage function which these proteins serve is significant to the needs of the plant If all these activities were to actually work *in vivo*, we see an overall scheme that is not a simple linear pathway, but one that exhibits several possible alternative routes to the ultimate degradation of BBSTI-E First, there is the pathway in which BBSTI-E is modified by limited proteolysis with accompanying enzyme activities catalysing extensive proteolysis of both BBSTI-E and BBSTI-D The latter could occur throughout the first 13 days of growth but would be due to separate enzymes peaking at day one, day four and day 12 The pH 7 system consisting of at least three separate activities peaking at day one, day three and day eight may represent another alternative route to degradation of those Bowman–Birk inhibitors that through rupture of protein bodies or through intrinsic cellular location, may be in an environment of near neutral pH Instead of the appearance and sustained presence of proteolytic activity until substrate has been depleted, we observe a series of pulses of enzyme activity active at different stages of germination and early growth The effect would thus be a slow, regulated release of amino acids rather than a large burst as a result of the digestion of all the protein reserves

EXPERIMENTAL

Plant materials and extracts Seeds of *Glycine max* (L) Merrill cv Amsoy 71 (Lincoln Seed Company) were grown on Jiffy Mix in a growth chamber in a 12 hr 12 hr light/dark cycle Temperatures were set at 25° in the light and 20° in the dark Cotyledons were harvested at designated days for a period of 12 days of growth Cotyledons were stored frozen at −20° For determination of the trypsin isoinhibitor forms cotyledons were homogenized in 7 ml cold 50 mM Tris–HCl 0 3 mM phenylmethylsulphonyl fluoride (PMSF)–0 5 mM Na iodoacetate (NaIAA) 0 5 mM EDTA, pH 8 0, per g fr wt of tissue The PMSF, NaIAA and EDTA serve to inhibit serine, cysteine and metallo-proteinase activity during homogenization and sample handling For assays of proteolytic activity these compounds were omitted Cotyledons were extracted with cold 50 mM Na-Pi–2 mM dithiothreitol (DTT), pH 7 0 using 7 ml/g fr wt of tissue Extracts were clarified by centrifugation

Enzyme assays Substrate (4 4 μg BBSTI-E or 15 5 μg BBSTI-D) was incubated with 5 μl of cotyledon extract in buffer The buffers were citrate–phosphate formulated according to McIlvaine [16] with DTT added to reach a concentration of 0 2 mM in the final incubation mixture (total vol of 50 μl) Incubation mixtures were prepared in duplicate One was frozen immediately at −70° and the other incubated at 37° for the designated time period, then frozen as above Samples were subjected to PAGE in the Davis [17] system, using 15% acrylamide for the BBSTI-E proteolytic assays and 17% acrylamide for the BBSTI-D assays The gels were fixed in 10% sulphosalicylic acid, stained in 1 5% Amido Black dissolved in 7 5% HOAc then destained in 7 5% HOAc The band intensities were determined by scanning densitometry with a Hoefer densitometer The peak areas were determined by planimetry. The percentage of disappearance of substrate was calculated as [(area under the peak corresponding to the substrate band at time 0 − the corresponding area at time t)/ (the area at time 0)] × 100 BBSTI-E and BBSTI-D stain with different intensities and the change in stain intensity per concentration of trypsin inhibitor is different so that the % of BBSTI-E that is converted to BBSTI-D cannot be readily calculated

Preliminary experiments had established the range of BBSTI-E and BBSTI-D concentrations in which band intensities would be proportional to inhibitor concentration and amount The addition of antibiotics, 4 8 μg/ml amphotericin B and 95 μg/ml kanamycin did not change the results The amount of extract used and the incubation time were varied to reach % disappearance of substrate between 10 and 90%, whenever possible Assays were done under different conditions to ensure that the temporal patterns of activities would reflect the enzyme concentrations on different days in the extracts

Determination of trypsin isoinhibitor forms PAGE gels in the Davis system with 4 M urea added were run to analyse extracts of equivalent numbers of cotyledons that had been harvested at different days of growth The gels were stained for trypsin inhibitor activity [18] Parallel gels were blotted onto PVDF (polyvinylidene difluoride by Millipore) and immunostained The primary antibodies were IgG from sera of rabbits immunized with either pure Kunitz soybean trypsin inhibitor of the T_1^a form or glutaraldehyde-polymerized BBSTI-E [19] The secondary antibodies were goat anti-rabbit IgG conjugated to alkaline phosphatase. The secondary antibodies, as well as the 5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium substrates for the colour reaction were obtained from BioRad In this detection system, the anti-Kunitz antibodies were specific for the Kunitz trypsin inhibitors The anti-BBSTI-E antibodies could be used to detect all Bowman–Birk isoinhibitor forms and glycine-rich trypsin inhibitors The identities of the different forms were based on comparison with known purified inhibitors and from the mobilities of the bands relative to the mobility of BBSTI-E

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