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NATURE OF PROTEINASE INHIBITORS RELEASED FROM SOYBEANS DURING IMBIBITION AND GERMINATION*

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Key Word Index—*Glycine max*; Leguminosae; soybean; Kunitz inhibitor; Bowman–Birk inhibitor; proteinase inhibitor; seed germination; seed imbibition; inhibitor release; inhibitor modification.

Abstract—Proteinase inhibitors are released to a smaller extent from soybeans which have been pre-equilibrated to an atmosphere of high relative humidity (r.h.) compared to those equilibrated to low r.h. Seeds pre-equilibrated to high r.h. also exhibited better germination. Regardless of the states of seed hydration, both Kunitz and Bowman–Birk soybean trypsin inhibitors are released in parallel with respect to each other and to other proteins at germination times up to 100 hr. After 48 hr of germination, new forms of trypsin inhibitor appear in the leachate which react with anti-Bowman–Birk trypsin inhibitor antibodies. No new forms of Kunitz trypsin inhibitor were observed immunochemically during the first 100 hr of germination.

INTRODUCTION

The leaching of intracellular substances from imbibing seeds has been reported. These released solutes range from very low MW electrolytes, amino acids and sugars [1–4] to high MW proteins [5]. Pre-equilibration of seeds to high r.h. reduces the release of these solutes and, at the same time, increases the fraction of germinating seeds vs non-germinating seeds. The loss of solutes has been attributed to a failure in membrane reorganization in the transition from the dehydrated to the hydrated state [6].

One class of proteins released from germinating soybean seeds is the proteinase inhibitor [5, 7]. Hwang and coworkers showed that the Bowman–Birk inhibitors were released within 24 hr of seed imbibition. They suggested that the release of these proteinase inhibitors may be beneficial to the plant, particularly with regard to defense of the plant against pathogens [5].

In this study, we show that proteinase inhibitors are released to a smaller extent from seeds which had been pre-equilibrated in an atmosphere of high r.h. These seeds also had better germination than seeds which were pre-equilibrated in atmospheres of lower r.h. Furthermore, we demonstrate that the release of the proteinase inhibitors from the seed appears to be non-selective. Kunitz, as well as Bowman–Birk type inhibitors, are released. In addition, modified forms of the Bowman–Birk type inhibitor are released during the later stages of germination, possibly merely reflecting modifications occurring within the seed itself. A preliminary report of this work was given at the 1981 meeting of the American Society of Biological Chemists.

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RESULTS AND DISCUSSION

Characterization of proteinase inhibitors in soybean Fiskeby V

Soybean Fiskeby V was found to have the Kunitz type inhibitor [8] and several iso-inhibitors of the Bowman–Birk type. The latter were separable by DEAE-cellulose chromatography. Unlike soybean cultivar Tracy [9] and the strain studied by Odani and Ikenaka [10], these iso-inhibitors all had the same amino acid composition (after acid hydrolysis) as that reported for the classical Bowman–Birk inhibitor [11]. The major classical Bowman–Birk inhibitor will be referred to as BBSTI and the minor species as AI, AII and AIII.

Characterization of antisera

Antibodies to Kunitz soybean trypsin inhibitor (KSTI) and to Bowman–Birk soybean trypsin inhibitor (BBSTI) were raised in rabbits. The antisera were specific in their reaction with the two classes of proteinase inhibitors. Antisera from rabbits immunized with KSTI reacted only with KSTI. Antisera from rabbits immunized with BBSTI reacted only with the Bowman–Birk class of inhibitors and not with KSTI. Anti-BBSTI antisera did not distinguish among the different forms of the Bowman–Birk type of inhibitors found in soybean Fiskeby V.

For quantitative studies, it was important to establish the quantitative equivalence of the reaction of anti-BBSTI antisera with BBSTI and the Bowman–Birk inhibitors AI, AII and AIII. On mixing various amounts of BBSTI, AI, AII and AIII together and testing in the radial immunodiffusion system, it was found that the reactions of the four inhibitor species with the anti-BBSTI antiserum used in the experiments reported here to be identical and additive. Thus, the values reported for BBSTI using the anti-

BBSTI antiserum are the sum of these four inhibitor species.

Release of inhibitors from seeds pre-equilibrated at various r.h.s

Pre-equilibration of soybean seeds to different r.h.s had a significant effect on the extent of inhibitor release occurring in the first 24 hr of germination. Pre-treatment at high r.h. (80%, seed water content 9.7% by wt) reduced the release of inhibitor and protein measured after 24 hr of imbibition. Equilibration to lower (20%) r.h. increased both protein and inhibitor release, and reduced the water content in the seed to 5% by wt. The differences between the amount of protein and inhibitor in the leachate of seeds pre-equilibrated to the two different conditions were found to be significant at the $P < 0.01$ level (Student's t-test). The data shown in Fig. 1 and in Table 1 show that the proteinase inhibitors were released in parallel with total protein release. Additionally, as the table indicates, pre-equilibration at the higher r.h. increased the fraction of seeds germinating after 24 hr of imbibition.

These data suggest that the release of protein into leachates, and perhaps the release of proteinase inhibitor into leachates, is not advantageous, but rather detrimental to the seed with respect to success in germination. It has been suggested [6] that such protein release could result from the failure of dehydrated cellular membranes to reorganize fully to their normal selectively permeable, lamellar conformation before the cellular contents are fully hydrated. Exposure of the dry seed to high r.h.s would allow partial membrane reorganization prior to exposure of the seed to liquid water.

Release of inhibitors during the first 100 hr of germination

The proteinase inhibitor activity, anti-KSTI and anti-BBSTI-cross-reacting material and protein content were determined in leachates of soybean seeds germinated up to 100 hr. Seeds pre-equilibrated to 50% r.h. were used. The results are shown in Fig. 2 and Table 2. Hwang *et al.* [5] reported a rapid rise and approximately parallel release of the different forms of Bowman–Birk inhibitors in the leachate up to 24 hr of germination. Our results show the same rapid release of the Bowman–Birk inhibitor during the first 24 hr, continuing at a lower rate up to 50 hr, then levelling off. The Bowman–Birk type of inhibitors accounted for *ca* 50% of the trypsin inhibitory activity and 5% of the protein in the leachate at 24, 48, 76 and 100 hr of germination. This number was calculated from the amount of this class of inhibitor as measured by radial immunodiffusion, and the stoichiometry of interaction of these inhibitors with bovine trypsin. The Kunitz inhibitors accounted for *ca* 10% of the trypsin inhibitory activity and 3% of the protein in the leachate from 24 to 100 hr. Thus, the data show very clearly that the Kunitz and Bowman–Birk type of inhibitors are released in parallel. This complements the report of Hwang *et al.* [5] that the different iso-inhibitors of the Bowman–Birk class found in soybean cultivar Tracy were released in parallel. Our data also show that collectively, the proteinase inhibitors appear to be released in parallel with the other released proteins.

To examine the nature of the released proteinase inhibitors more closely, we analysed concentrated samples of the leachates on PAGE. The gels were sliced, each slice eluted as described in Experimental, and the eluate analysed for trypsin inhibitory activity, for KSTI and for BBSTI, the latter two by radial immunodiffusion. To more clearly represent changes in distributions, the data are given as the fraction of activity, KSTI or BBSTI in each slice relative to the total recovered in all slices of the particular gel.

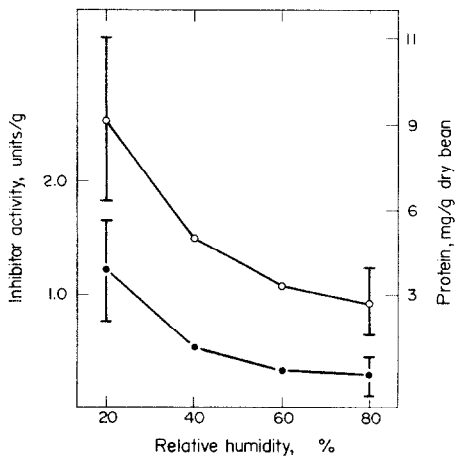

Fig. 1. The effect of seed pre-equilibration to different r.h.s on proteinase inhibitor release. ●—●, Trypsin inhibitor activity; ○—○, protein. Points at 20% and 80% r.h. represent the average of six growth experiments; those at 40% and 60% r.h., the average of two growth experiments. Vertical lines indicate ± s.d. for the indicated data.

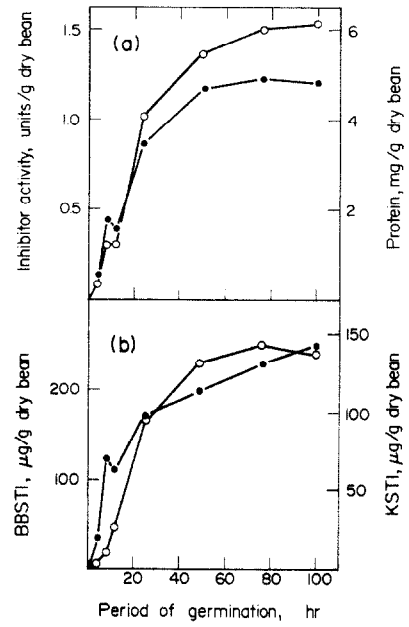

Fig. 2. Release of trypsin inhibitors from germinating soybeans. (a) ○—○, Trypsin inhibitor activity; ●—●, protein. (b) ●—●, KSTI; ○—○, BBSTI. KSTI and BBSTI were measured by radial immunodiffusion. All determinations were performed in triplicate. The data shown are from a single experiment, representative of several experiments with similar results.

Table 1. Proteinase inhibitor release from seeds pre-equilibrated to high and low r.h.s

	r.h.	
	20%	80%
Percent germination*	19	56
Proteinase inhibitor released† (units/g dry bean)	1.22 ± 0.47	0.30 ± 0.16
KSTI released (μg/g dry bean)	58 ± 6	7 ± 4
BBSTI released (μg/g dry bean)	33 ± 9	6 ± 5
Protein released (mg/g dry bean)	8.69 ± 2.42	2.94 ± 1.00

*After imbibing for 24 hr, seeds were germinated on moist filter paper for 3 days at 21°.

†All amounts in this table were measured in leachates after 24 hr imbibition. Average ± s.d. from four trials at each pre-treatment. Based on weight of seeds prior to pre-equilibration.

Table 2. Soybean proteinase inhibitors released during germination

	Period of germination (hr)			
	24	48	76	100
Protein released (mg/g dry bean)	3.47	4.65	4.90	4.81
Proteinase inhibitor released (units/g dry bean)	1.02	1.36	1.50	1.56
KSTI released (mg/g dry bean)	0.096	0.112	0.129	0.140
(% total protein released)	2.77	2.41	2.47	2.91
(% total inhibitor released)*	11.3	9.8	10.3	10.8
BBSTI released (mg/g dry bean)	0.161	0.229	0.250	0.241
(% total protein released)	4.64	4.92	5.10	5.01
(% total inhibitor released)†	47.4	50.5	50.1	46.3

*Calculated from the amount of released inhibitor, assuming a 1:1 stoichiometry of inhibitor combining with trypsin, and M_R values of 20 000 and 24 000 for KSTI and trypsin, respectively.

†Calculated as described in (previous footnote), but assuming inhibitor MW of 8000.

Figure 3 shows the data for ungerminated seed extract and for leachates at 24, 48, 76 and 100 hr of germination in succession from top to bottom. These data show that, within the limits of resolution of this method, only one species of Kunitz inhibitor was released throughout the time examined. In fact, the Kunitz inhibitor in the seed, and in the 24 hr and the 100 hr leachates all showed immunological identity (Ouchterlony analysis).

The situation is quite different with regard to the Bowman–Birk inhibitor. The Bowman–Birk type inhibitors from ungerminated seeds, BBSTI, AI, AII and AIII, all co-migrated and were seen as a single band at R_f 0.65. Starting at 48 hr of germination time, a new species of inhibitor, cross-reacting with anti-BBSTI antiserum but banding at R_f 0.55, appeared in the leachate. This modification was not detectable by Ouchterlony analysis, either due to the limitation of this method of analysis, or due to the modification occurring at a site on the inhibitor not bound by antibody.

Material with trypsin inhibitory activity but not cross-reacting with either anti-KSTI or anti-BBSTI antiserum appeared at lower R_f values. This material, by our estimation, constituted *ca* 40% of the trypsin inhibitory activity in the leachates between 24 and 100 hr of germination time (Table 2). Based on our own purification studies, this material does not appear to correspond to any of the well-characterized Bowman–Birk type iso-inhibitors reported in other strains of soybeans [9, 10]. However, besides KSTI and BBSTI, a number of other, less-well-characterized inhibitors have been reported in soybeans (e.g. inhibitors F_1 and F_3, see ref. [12] for a review of

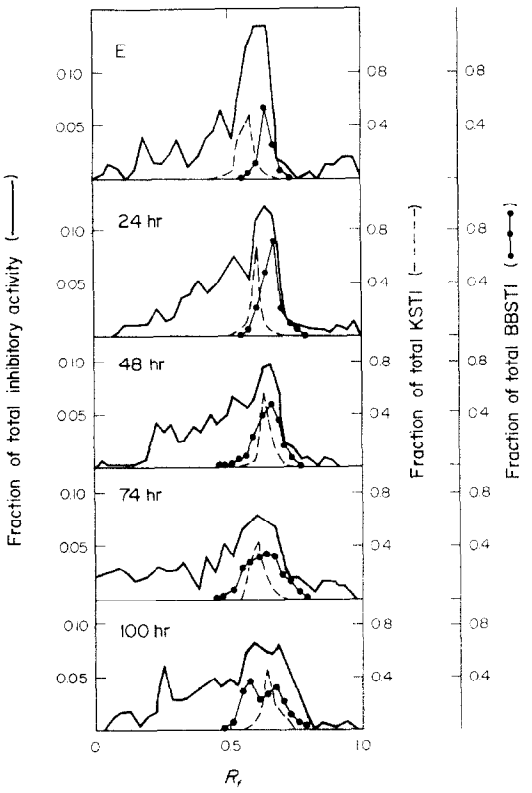

Fig. 3. Analysis of protease inhibitors in leachates by PAGE and radial immunodiffusion of gel slices. Approx 2.2 A_{280} units of material from leachate was applied to each gel. Gel slices were analysed for trypsin inhibitory activity (——), KSTI (----) and BBSTI (●—●). R_f, distance measured relative to the bromophenol blue dye front. From top to bottom: extract of ungerminated seeds, leachates at 24, 48, 76 and 100 hr of germination.

soybean trypsin inhibitor nomenclature). Such inhibitors may account in part or in whole for this activity.

The inhibitor proteins initially released from the seed appear to be identical to the inhibitor present in the ungerminated seed on the basis of their electrophoretic and immunochemical properties. However, at later germination times, a new form of BBSTI appears distinct from that of the ungerminated seed. If BBSTI of all forms in the seed are released non-selectively, the release of this modified BBSTI form in the leachate could be an indication of modification of BBSTI occurring in the seed during germination. This would not be unusual considering previous reports of conversion of the mung bean (*Vigna radiata*) and adzuki bean (*Phaseolus angularis*) BBSTI-type inhibitors, by limited proteolysis, to new forms of inhibitors [13, 14].

EXPERIMENTAL

Plant materials and other supplies. Soybean [*Glycine max* (L.) Merrill var. Fiskeby V] seeds were obtained from Stokes Seeds (Buffalo, NY). Trypsin (bovine, twice crystallized), soybean trypsin inhibitor (Kunitz), α-*N*-benzoyl-L-arginine ethyl ester and glutaraldehyde were purchased from Sigma.

Preparation of soybean inhibitors. Commercially available Kunitz soybean trypsin inhibitor (KSTI) was found to contain a small amount of contaminant when examined by PAGE. The inhibitor was purified to homogeneity by ion-exchange chromatography on DEAE-cellulose in 50 mM Tris–HCl buffer, pH 7.5. The column was eluted with a linear gradient from 0 to 1 M NaCl.

Bowman–Birk soybean trypsin inhibitor (BBSTI) was purified from the Fiskeby V seeds by the method of ref. [10] with the exception that CM-Sephadex was used in place of CM-cellulose. The purified BBSTI was found to be identical in electrophoretic mobility, amino acid composition and amino terminal sequence analysis to a sample of BBSTI kindly supplied by Dr Odani. No evidence of the Bowman–Birk-type inhibitors CII, DII and EI found by Odani and Ikenaka [10] or of the Bowman–Birk-type inhibitors PI–PIV found by Hwang *et al.* [5] in cultivar Tracy was found in Fiskeby V. Instead, using the procedures of Odani and Ikenaka, we found three other minor Bowman–Birk inhibitor species, all with the same amino acid compositions as BBSTI upon acid hydrolysis. Collectively, these minor forms eluted as a single peak before the Bowman–Birk soybean trypsin inhibitor on the CM-Sephadex column. These were resolved into three separate peaks on further chromatography on DEAE-cellulose. Those iso-inhibitors migrate with BBSTI on the PAGE system of ref. [15] using 10% gels.

Germination of seeds. Seeds were surface sterilized and germinated in H_2O at room temp. in the dark. The leachates were harvested as described in ref. [7]. Only seeds with intact testa and free of any obvious damage were used.

Seeds were pre-equilibrated to different r.h.s by holding aliquots of seeds for 3 weeks at 21 ± 1° in sealed containers over aq. H_2SO_4 solns. Solns of 28, 40, 50 and 60% (v/v) H_2SO_4 were used to obtain r.h.s of *ca* 80, 60, 40 and 20%, respectively. The H_2O content of unimbibed seeds was determined from weights before and after drying at 110° for 48 hr.

PAGE. This was performed using the method of ref. [15] with 10% gels. Electrophoresis was at 5 mA/gel for *ca* 90 min. Gels were stained in 1% (w/v) amido Schwartz in 7.5% (v/v) HOAc, and destained by leaching in 7.5% HOAc. Alternatively, gels were sliced into 1.5 mm-thick slices using a Yeda Scientific Instruments gel slicer (Rehovot, Israel). Individual slices were extracted with 200 μl 50 mT Tris–HCl buffer, pH 8.0, by subjecting them to four freeze–thaw cycles over 2 days. R_f values were measured relative to the bromophenol blue tracking dye.

Trypsin inhibitor assays. This was measured as described in ref. [16] using α-*N*-benzoyl-L-arginine ethyl ester as substrate. One unit of inhibitor was defined as inhibiting 1 mg of active trypsin. The values reported have been corrected for the amount of inactive trypsin protein. For the enzyme preparation used in this study, the trypsin has been estimated by active site titration [17] to be 56% active.

Protein content determination. The protein content of leachates was determined by the method of ref. [18].

Preparation of antisera. New Zealand white rabbits were immunized with purified KSTI according to the following schedule: 0.5 mg in Freund's complete adjuvant, subcutaneous, day 1; 0.5 mg in Freund's incomplete adjuvant, subcutaneous, day 14; 0.5 mg in Freund's incomplete adjuvant, intramuscular, 4 additional times at 2-week intervals. Rabbits bled 1 week after the last injection showed a

positive immune response. Booster injections of 0.5 mg KSTI in Freund's incomplete adjuvant administered intramuscularly and followed by bleeding 1 week later were given at 1-month intervals.

BBSTI was first polymerized with glutaraldehyde [19] to enhance its immunogenicity. Rabbits were injected with polymerized BBSTI according to the following schedule: 1 mg in Freund's complete adjuvant, subcutaneous, on day 1; 1 mg in incomplete adjuvant, intramuscular, days 10 and 20. Rabbits bled 1 week later showed a strong response to the polymerized BBSTI and a weak response to the unpolymerized inhibitor. Upon further boosting at monthly intervals with 1 mg polymerized inhibitor in incomplete Freund's adjuvant administered intramuscularly, the response to unpolymerized inhibitor became significantly stronger.

Quantitation of inhibitor by radial immunodiffusion. The amounts of KSTI and of BBSTI in unknown samples was determined by radial immunodiffusion [20]. Known concentrations of the purified inhibitors were used to construct standard curves. Concentrations of inhibitor were determined spectrophotometrically using $E^{1\%}_{1cm}$ 10.01 and 4.6 for KSTI and for BBSTI, respectively [21].

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