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PROTEOLYSIS OF SOYBEAN BOWMAN–BIRK TRYPSIN INHIBITOR DURING GERMINATION*

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Key Word Index—*Glycine max*; Leguminosae; soybean germination; trypsin inhibitor; proteolysis.

Abstract—The Bowman–Birk type trypsin inhibitor, BBSTI-D, which appears in the cotyledons of germinated soybeans (*Glycine max*), was isolated in homogeneous form. BBSTI-D has an amino acid composition identical to the native Bowman–Birk soybean trypsin inhibitor (BBSTI-E) except for the loss of one glutamyl/glutaminyl residue and one aspartyl/asparaginyl residue. The amino-terminal sequence of BBSTI-D was identical to that of BBSTI-E. These data, as well as the compositions of the tryptic peptides from reduced carboxymethylated BBSTI-D, indicate that BBSTI-D is derived from BBSTI-E by the loss of the carboxyl-terminal residues Glu^{70}–Asn^{71}.

INTRODUCTION

The dicot seed generally contains large quantities of food reserves, typically (but not exclusively) in the cotyledons. During germination and early seedling growth these reserves are mobilized to supply the developing plant with its energy and metabolic precursor needs. Legume seeds are notable for their large content of proteinase inhibitors which are themselves proteins [1, 2]. A number of functions have been suggested for these inhibitors, including serving as storage depots, particularly for sulfur containing amino acids [2]. We have recently demonstrated that the trypsin inhibitor of the mung bean [*Vigna radiata* (L.) Wilczek] undergoes extensive, though specific, proteolysis during the early stages of germination and seedling growth [3, 4]. We have also reported the disappearance of the Bowman–Birk soybean trypsin inhibitor BBSTI-E and several other Bowman–Birk type trypsin inhibitors from the soybean [*Glycine max* (L.) Merrill] during seedling growth [5]. Concomitant with the disappearance of the trypsin inhibitor characteristic of the ungerminated seed there is the appearance of a new Bowman–Birk type inhibitor, BBSTI-D. It was not apparent from our initial results whether the newly appearing BBSTI-D arises as a breakdown product of a pre-existing Bowman–Birk type inhibitor (e.g. BBSTI-E), or via *de novo* synthesis.

In this paper we present evidence indicating that the soybean trypsin inhibitor BBSTI-D is the product of the limited specific proteolysis of BBSTI-E during seedling growth. This proteolysis results in the removal of the carboxyl-terminus of the inhibitor molecule, and thus resembles the initial step in the degradation of the mung bean trypsin inhibitor during seedling growth.

RESULTS

Gel filtration of the crude inhibitor preparation on Sephadex G-75 at pH 8 resulted in a single peak of material cross-reacting with anti-BBSTI-E antibody (not shown). This was well separated from the bulk of the eluting protein, as well as the majority of the Kunitz trypsin inhibitor. Ion exchange chromatography of the BBSTI-E cross-reactive material on DEAE-cellulose yielded four major peaks of trypsin inhibitor, all of which also reacted, to varying extents, with anti-BBSTI-E antiserum (Fig. 1). On the basis of their elution positions the last two peaks were identified as BBSTI-D and BBSTI-E [5]. The BBSTI-D recovered from this column was further purified by rechromatography on DEAE-cellulose at pH 7.5 (Fig. 2). A homogeneous preparation of BBSTI-D was obtained by pooling the center fractions of the inhibitor peak.

The purified BBSTI-D migrated as a single band of R_f 0.47 in the 4 M urea polyacrylamide gel electrophoresis system (not shown), compared to an R_f of 0.54 for BBSTI-E. The amino acid composition of BBSTI-D is identical to BBSTI-E, less one aspartyl/asparaginyl residue and one glutamyl/glutaminyl residue (Table 1). Edman-dansyl sequence analysis demonstrated that the first five residues of the amino-terminus of BBSTI-D are the same as those of BBSTI-E (Table 2). These data suggest that BBSTI-D is identical to BBSTI-E except for the loss of the carboxyl-terminal residues Glu^{70}–Asp^{71} [6].

To explore this possibility further, the tryptic peptides from reduced, carboxymethylated BBSTI-D were examined. Gel filtration of the tryptic digest on Sephadex G-25 yielded three peptide fractions, I, II, and III (Fig. 3). These were further fractionated by paper chromatography (Fig. 4). Tryptic peptides IA, IIE, IIIA, and IIIB were obtained in homogeneous form. Their amino acid com-

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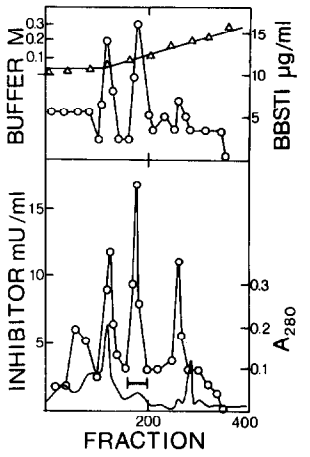

Fig. 1. Ion exchange chromatography of the Bowman–Birk type inhibitor pool from Sephadex G-75 gel filtration. The inhibitor pools from three gel filtration runs were pooled, dialyzed against 50 mM NH_4OAc, pH 6.5, and applied to a 2.5 × 100 cm column of DEAE-cellulose equilibrated to the same buffer. The column was eluted with a linear NH_4OAc concentration gradient as described in the text. Fraction size was 7.5 ml. Top frame: —○—, Bowman–Birk type inhibitor protein, mg/ml; —△—, molar concentration of NH_4OAc. Bottom frame (same column run): ——, A_{280}; —○—, trypsin inhibitory activity (units/ml). The fractions containing BBSTI-D were pooled as indicated.

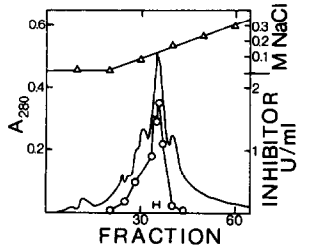

Fig. 2. Ion exchange chromatography of BBSTI-D on DEAE-cellulose at pH 7.5. The inhibitor recovered from the ion exchange chromatography in Fig. 1 was applied to a 1.5 × 17 cm column of DEAE-cellulose equilibrated to 50 mM Tris–HCl, pH 7.5. The column was eluted with a linear NaCl concentration gradient (see Experimental). ——, A_{280}; —○—, Bowman–Birk type inhibitor protein (by radial immunodiffusion), mg/ml; —△—, M NaCl. The purified BBSTI-D was pooled as indicated.

positions (Table 3) indicate their identity to residues 1–16, 38–43, 17–23, and 24–28, respectively, of BBSTI-E. Peptide IIC was identified as an approximately equimolar mixture of residues 17–23 and 29–37. Peptides isolated from a tryptic digest of reduced, carboxymethylated BBSTI-E were identical to these BB-D peptides on the basis of amino acid composition and mobility on PC and TLC (data not shown). Identical peptides have also been described by Odani *et al.* [7].

Peptide IC exhibits an amino acid composition consistent with its identity to residues 38–69 of BBSTI-E. A

Table 1. Composition of BBSTI-D

Amino acid	Residues/molecule* Hydrolysis time 20 hr	48 hr	72 hr	Best	Integer	BBSTI-E†
Asx	10	10	10	10	10	11
Thr‡	2.3	2.3	2.3	2.3‡	2	2
Ser‡	8.3	7.6	6.9	8.9‡	9	9
Glx	6.0	6.5	6.4	6.3	6	7
Pro	5.5	5.5	6.0	5.7	6	6
Gly	0	0	0	0	0	0
Ala	4.0	4.3	4.3	4.2	4	4
1/2 Cys§	13.6	ND	ND	13.6	14	14
Val	1.0	1.4	1.3	1.2	1	1
Met	0.9	0.8	0.8	0.8	1	1
Ile	1.8	2.0	1.9	1.9	2	2
Leu	2.2	2.5	2.3	2.3	2	2
Tyr	1.8	1.8	1.7	1.8	2	2
Phe	1.9	1.9	2.0	1.9	2	2
Lys	4.5	4.7	4.7	4.6	5	5
His	0.9	0.9	0.9	0.9	1	1
Arg	2.0	2.2	2.1	2.1	2	2
Total					69	71

*Calculated assuming 10 residues of Asx per molecule of BBSTI-D.

†Calculated from amino acid sequence [6].

‡Values from extrapolation of 20, 48 and 72 hr hydrolysis data to zero time.

§Determined as cysteic acid; ND, not determined.

Table 2. Determination of amino-terminal sequence of BBSTI-D

Residue No.	Dansyl amino acid recovered Major	Minor*
1	Asp	Glu Ser
2	Asp	Glu Ile Leu
3	Glu	Asp Ile Leu
4	Ser	Glu Asp
5	Ser	Asp Glu

BB-D amino-terminal sequence:
Asx–Asx–Glx–Ser–Ser–
BB-E amino-terminal sequence:
Asp–Asp–Glu–Ser–Ser–†

*Minor DNS amino acids listed in descending order of predominance.

†From ref. [6]; also confirmed by our own determination.

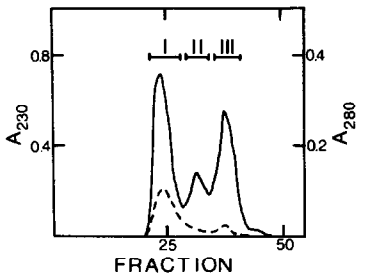

Fig. 3. Fractionation of tryptic peptides from reduced carboxymethylated BBSTI-D. The digest derived from *ca* 5 mg of the inhibitor was chromatographed on a 1.5 × 90 cm column of Sephadex G-25 as described in the text. Three peptide fractions, I, II, and III, were pooled as indicated. ——, A_{230}; – – –, A_{280}.

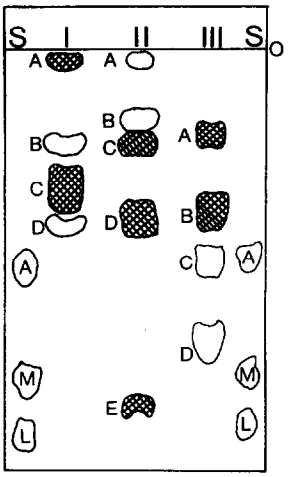

Fig. 4. Fractionation of tryptic peptides by descending paper chromatography. The solvent used was *n*-BuOH–HOAc–H_2O–pyridine (90:18:72:60). S, standard containing alanine (A), methionine (M) and leucine (L). I, II, III, pools from gel filtration (Fig. 3). O is the origin. Peptides strongly stained by ninhydrin are cross hatched. Weakly stained peptides are outlined only.

similar peptide was obtained from BBSTI-E in this study, as well as by Odani *et al.* [7], but representing residues 38–71. No evidence of a dipeptide corresponding to Glu^{70}–Asn^{71} was found in the tryptic digest of reduced carboxymethylated BBSTI-D, again indicating the absense of Glu^{70}–Asn^{71} from the BBSTI-D molecule.

DISCUSSION

The compositional, amino-terminal sequence and peptide compositional data are all consistent with BBSTI-D being identical in sequence to BBSTI-E except for the hydrolysis of the Lys^{69}–Glu^{70} peptide bond resulting in the loss of the carboxyl-terminal dipeptide Glu^{70}–Asn^{71}. While the possibility that this inhibitor species could arise via *de novo* synthesis as a distinct gene product separate

Table 3. Composition of tryptic peptides from reduced carboxymethylated BBSTI-D

	Residues/molecule*											
Amino acid	IA Found	IA Expected	IC Found	IC Expected	IIC Found	IIC Expected§	IIE Found	IIE Expected	IIIA Found	IIIA Expected	IIIB Found	IIIB Expected
Asx	3.0	3	4.3	4	1.5	2	0	0	0.7	1	1.0	1
Thr†	0.8	1	1.0	1	0	0	0	0	0.2	0	0	0
Ser†	1.7	2	2.3	3	2.6	3	0.8	1	1.0	1	1.0	1
Glx	1.6	2	3.0	3	1.0	1	0	0	1.0	1	0	0
Pro	0.5	1	2.9	3	1.8	2	0	0	1.6	2	0	0
Gly	0	0	0	0	0	0	0	0	0	0	0	0
Ala	1.2	1	1.6	2	1.1	1	1.2	1	0.2	0	0	0
1/2 Cys†‡	3.1	4	4.5	6	2.2	3	1.8	2	0	1	0.9	1
Val	0	0	1.0	1	0	0	0	0	0	0	0	0
Met	0	0	0	0	0	0	0	0	0	0	0.5	1
Ile	0	0	1.3	2	0	0	0.7	1	0	0	0	0
Leu	0.1	0	0.7	1	1.0	1	1.0	1	0.1	0	0	0
Tyr	0	0	1.5	2	0	0	0	0	0	0	0	0
Phe	0	0	1.7	2	0	0	0	0	0	0	0	0
Lys	1.7	2	1.9	2	1.2	1	0	0	0.1	0	0	0
His	0	0	0	0	1.0	1	0	0	0.1	0	0	0
Arg	0	0	0	0	0.8	1	0	0	0.6	1	0.6	1
Total		16		32		16		6		7		5
Sequence location	1–16		38–69		17–23 29–37		38–43		17–23		24–28	

*All values are from 20 hr hydrolyses. Expected values are calculated from the sequence of BBSTI-E [6].

†Not corrected for destruction.

‡As *S*-carboxymethylcysteine.

§Assuming an equimolar mixture of residues 17–23 and 29–37.

from BBSTI-E cannot be disproven by the data presented here, it seems unlikely. It is more likely that BBSTI-D is derived from BBSTI-E by a specific proteolysis during early seedling growth. We have demonstrated that the initial event in the degradation of the mung bean (*Vigna radiata*) trypsin inhibitor during germination is the removal of the carboxyl-terminal tetrapeptide Lys^{77}–Asp–Asp–Asp^{80} [4]. Degradation then proceeds though a number of specific cleavages including an internal cleavage and the removal of the eight residues of the amino-terminus. A similar modification by limited proteolysis during germination has also been noted in the adzuki bean (*Vigna angularis*) [8]. Proteolysis during seed maturation or storage presumably accounts for the presence of some apparently truncated inhibitors found in the ungerminated seeds of the garden bean (*Phaseolus vulgaris*) [9] and the soybean [10].

We have no information as to the nature of the proteolytic enzymes involved in the production of BBSTI-D from BBSTI-E. It is of note, however, that we have found that a similar limited proteolysis is inflicted upon the Kunitz trypsin inhibitors of soybean cultivars Fiskebey V and Amsoy 71 during germination. In the latter cultivar, which contains the Ti^a variant [11] of the Kunitz inhibitor, the cleavage occurs between Leu^{176}–Asp^{177}, resulting in the loss of the carboxyl-terminal pentapeptide sequence Asp^{177}–Leu^{181} [Hartl, P. and Wilson, K. A., unpublished results]. Thus, with both types of proteinase inhibitors the initial proteolysis occurs to the amino-terminal side of an acidic residue, i.e. at Lys–Glu in BBSTI-E and Leu–Asp of Kunitz soybean trypsin inhibitor Ti^a variant.

While the initial proteolysis of the soybean and mung bean Bowman–Birk type trypsin inhibitors at the carboxyl-terminus is remarkably similar, there is a distinct difference in the rates at which these modifications occur. The conversion of the native mung bean trypsin inhibitor (MBTI-F) is essentially complete by 48 hr after imbibition of the seed [3]. In contrast, BBSTI-E is present in large quantities in the soybean until at least 6 days after the beginning of imbibition. In part this may be explained by the somewhat slower germination and growth of the soybeans. However, it is possible that this difference in kinetics may also be due at least in part to the different growth habits of the mung bean and soybean seedlings. The cotyledons of the mung bean serve primarily only as storage organs. During germination and seedling growth they rapidly undergo senescence and abscission. In contrast, the cotyledons of the soybean become photosynthetic organs for the young plant in addition to serving as storage organs. The cotyledons of the soybean persist much longer on the plant. Under these two different circumstances it is not surprising that the degradation of a cytosolic protein such as the Bowman–Birk type trypsin inhibitors should proceed at a different pace. In spite of these differences, the proteolysis of the Bowman–Birk type proteinase inhibitors during seed germination and early seedling growth would appear to be a general phenomenon in the legumes.

EXPERIMENTAL

Plant materials and reagents. Soybean seeds, *Glycine max* (L.) Merrill, cv. Fiskeby V, were obtained from Stokes Seeds, Buffalo, NY. The seeds were equilibrated to an atmosphere of 80% relative humidity at room temp. for 1 week prior to use. The seeds were then imbibed for *ca* 1 hr in H_2O, followed by planting in moist vermiculite. The plants were grown at 23°, with a 16 hr light/8 hr dark cycle. Six days after planting the seedlings were harvested, the cotyledons dissected free of the axes, rinsed and blotted dry. The cotyledons were stored at −20° until needed.

L-1-Tosylamide-2-phenylethyl chloromethyl ketone-treated bovine trypsin was prepared by the method of ref. [12]. Reagent grade pyridine was refluxed with ninhydrin and redistilled prior to use in chromatographic solvents and sequence reactions. Constant boiling (5.7 M), HCl, phenylisothiocyanate and trifluoroacetic acid were from Pierce. All other chemicals were reagent grade or better, and ×2 distilled H_2O was used throughout. All pH adjustments were made at room temp. (21 ± 1°).

Crude inhibitor preparation. Cotyledons (100 g) were homogenized with 1 l. of 50 mM Tris–HCl, pH 8, containing 0.3 mM phenylmethylsulphonyl fluoride and 50 mM Na iodoacetate. This and all subsequent steps were carried out at 5° unless otherwise noted. After stirring 15 hr, the brie was filtered through 3 layers of cheesecloth, and centrifuged at 10000 *g* for 45 min. The supernatant was collected, and solid $(NH_4)_2SO_4$ added to reach 85% satn. The mixture was held 15 hr, followed by collecting the precipitated protein by centrifugation as above. The resulting pellet was redissolved in 50 mM Tris–HCl, pH 8, and then adjusted to pH 4.2 with 1 M HCl. The ppt produced was removed by centrifugation, and the supernatant adjusted to pH 8 with NaOH.

Gel filtration. The crude inhibitor preparation derived from 100 g of tissue was applied to a 5 × 140 cm column of Sephadex G-75 equilibrated to 50 mM Tris–HCl, pH 8. The column was eluted at room temp. with the same buffer at 100 ml/hr with fractions of 11.5 ml collected.

Ion exchange chromatography. The Bowman–Birk trypsin inhibitor containing fractions from 2 or 3 gel filtration runs were pooled and extensively dialysed against 50 mM NH_4OAc, pH 6.5. This material was then applied at room temp. to a 2.5 × 100 cm column of DEAE-cellulose (Whatman DE-52) equilibrated to the same buffer. The column was subsequently eluted with a linear concn gradient generated with 2.7 l. of 50 mM NH_4OAc, pH 6.5, as the initial buffer and 2.7 l. of 0.5 M NH_4OAc, pH 5 as the final buffer. Elution was at 65 ml/hr, with 7.5 ml fractions collected. The gradient was monitored by conductivity and pH.

The BBSTI-D [5] pool from ion exchange chromatography was dialysed against 5 mM NH_4HCO_3 and lyophilized. To further purify the inhibitor, the lyophilized powder was dissolved in 50 mM Tris–HCl, pH 7.5, and applied to a 1.5 × 17 cm column of DEAE-cellulose equilibrated at room temp. to the same buffer. The column was eluted with a linear gradient, 0–0.5 M NaCl, in the buffer, with a total gradient vol. of 150 ml. Fractions of 2 ml each were collected, at a flow rate of 15 ml/hr.

Assay of trypsin inhibitor activity and protein. Trypsin inhibitor activity was measured using bovine trypsin and α-*N*-benzoyl-L-arginine ethyl ester as substrate [13]. Kunitz and Bowman–Birk type trypsin inhibitor proteins were quantitated by radial immunodiffusion as previously described [5]. While the anti-Bowman–Birk antiserum used was raised by the immunization of rabbits with pure BBSTI-E, it also cross-reacted with BBSTI-D.

Polyacrylamide slab gel electrophoresis. This was performed by a modification of the method of ref. [14] with a 1.5 mm thick 15% (w/v) acrylamide gels. Urea, 4 M, was included in the separating and stacking gels, as well as the reservoir buffer, to

eliminate self-association of the inhibitor. The gels were stained for 15 hr with 1.5% w/v Amido Schwartz in 7.5% HOAc and destained by diffusion in 7.5% HOAc.

Amino acid analysis. Samples for amino acid analysis were hydrolyzed *in vacuo* at 110° in 5.7 M HCl for 20, 48 and 72 hr. Amino acid analyses were carried out using a Glenco MM-70 amino acid analyser equipped with a ninhydrin detection system. Performic acid oxidation was performed as described in ref. [15] for the determination of half-cystine as cysteic acid.

Tryptic peptides of BB-D. The methods used for the reduction, carboxymethylation and tryptic digestion of BBSTI-D and BBSTI-E were as described in ref. [4]. For the preparative isolation of peptides, gel filtration was initially utilized. A sample of the tryptic digest of reduced, carboxymethylated BBSTI-D equivalent to *ca* 5 mg of inhibitor was applied to a 1.5 × 90 cm column of Sephadex G-25 equilibrated to and eluted with 10 mM NH_4HCO_3. Fractions of 3.1 ml were collected at a flow rate of 40 ml/hr. Peptide fractions from gel filtration were further fractionated by descending PC on Whatman 3 MM paper. The solvent used was *n*-BuOH–HOAc–H_2O–pyridine (90:18:72:60). Peptides were detected by spraying with 0.05% ninhydrin in EtOH and developing for 15 hr at room temp. The purity of the final peptide preparations was ascertained by TLC as described above.

Amino-terminal sequence determination. Amino-terminal sequencing was carried out using the manual Edman-dansyl technique of ref. [16]. Dansyl amino acids were identified by TLC on polyamide sheets.

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