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PROTEOLYSIS OF KUNITZ SOYBEAN TRYPSIN INHIBITOR DURING GERMINATION

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Abstract—During the germination and seedling growth of soybeans (*Glycine max*) a new form of the Kunitz soybean trypsin inhibitor (KSTI) appears in the cotyledons distinct from the KSTI of the quiescent seed. This inhibitor has been purified from the germinated seeds of soybean cultivar Amsoy 71 and cultivar Fiskeby V. These cultivars contain the Ti^a and Ti^b variants of KSTI respectively in the dry seed. The inhibitors were characterized by sodium dodecylsulphate-polyacrylamide gel electrophoresis, amino acid analysis, and partial sequence determination. The newly appearing form in Amsoy 71 (Ti^a_m) was found to be identical to Ti^a except for the loss of the five residue sequence at the carboxyl-terminus of Ti^a. The Ti^b variant of KSTI was found to contain at least 199 amino acid residues (as opposed to 181 for Ti^a). The amino-terminal sequence of Ti^b was found to be identical to that of Ti^a for the first ten residues. The KSTI species appearing in Fiskeby V with germination, Ti^b_m, appears to be derived from Ti^b by the loss of the carboxyl-terminal decapeptide.

INTRODUCTION

During the process of seed germination and early seedling growth the reserves present in the seed, including the storage proteins, are mobilized via hydrolysis to support the growth of the seedling. The seeds of the Leguminosae are known to have high contents of proteins exhibiting inhibitory activity against mammalian and insect serine proteinase such as trypsin, chymotrypsin, and elastase [1, 2]. A number of physiological functions have been proposed for these inhibitors, including the inactivation of digestive enzymes from microorganisms and vertebrate and invertebrate herbivores, the regulation of endogenous seed proteinases, and serving as storage proteins in the seed, particularly as depots for sulphur-containing amino acids [2]. The latter possibility is especially attractive in view to the high half-cystine content of many of the legume proteinase inhibitors.

Both the Bowman–Birk type inhibitors and the Kunitz soybean trypsin inhibitor (KSTI) from soybeans (*Glycine max* L. Merrill) exhibit changes in their physical properties during seed germination [3]. In the case of the Bowman–Birk inhibitor, this has been demonstrated to be due at least initially to a limited specific proteolysis of the inhibitor [M. Madden, A. L. Tan-Wilson and K. A. Wilson, unpublished]. For KSTI, the cause has been less clear. After approximately four days of germination, a second electrophoretically less mobile form of the KSTI appears in the seed which is antigenically similar or identical to the form present in the quiescent seed. At ten days after the beginning of seed inhibition both forms of the KSTI have largely disappeared. This pattern of change has been noted in the soybean cultivars homozygous for each of the three KSTI genetic variants thus far described, as well as hybrids containing and simultaneously expressing two of the variants [4].

The KSTI species that appear during germination could arise as modifications of the KSTI present in the ungerminated seed. Alternatively, they could result from the expression of KSTI genes different from those expressed during seed development and maturation. Previously published results have been interpreted to support or reject both of the hypotheses [4, 5]. In this paper we report the isolation of the newly appearing KSTI species (Ti^a_m or Ti^b_m) found in the cotyledons of germinating soybeans containing either the Ti^a or Ti^b variant. Evidence is presented that these forms arise from the KSTI present in the ungerminated seed by limited specific proteolysis.

RESULTS AND DISCUSSION

Homogeneity of purified inhibitors

The purified Ti^a, Ti^a_m, Ti^b and Ti^b_m inhibitors were found to have R_f values of 0.50, 0.46, 0.46 and 0.42 on polyacrylamide disc gel electrophoresis (not shown). All were judged to be greater than 95% pure. The mobilities of the two native allelic variants (Ti^a and Ti^b) relative to each other and to the forms appearing upon germination (Ti^a_m and Ti^b_m) are consistent with the results previously reported by Orf and Hymowitz [6]. All chemical analyses were carried out on Ti^b and Ti^b_m solutions shortly after their purification, since prolonged storage (greater than 3 months, −20°, 1 mM HCl) gave rise to minor inhibitor forms, possibly due to deamidation. Freed [7], using a somewhat different isolation procedure, also noted modified forms of the inhibitor migrating slightly ahead of purified KSTI. No such problem was noted with Ti^a and Ti^a_m under similar conditions.

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Analysis of KSTI inhibitors from cultivar Amsoy 71

As expected from the sequence data of Koide and Ikenaka [8], Ti^a exhibited a single band on sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with a molecular weight of approximately 21 500 (Table 1). Ti^a_m similarly appears as a single species on SDS-PAGE but with a slightly lower M_r of 21 000.

The amino acid composition of Ti^a_m shows extensive similarity to the composition of Ti^a (Table 1). Ti^a_m appears to be a somewhat smaller protein, composed of 175 amino acid residues, compared to the 181 of Ti^a. From the compositional data it would appear that Ti^a_m is derived from Ti^a through the loss of single residues of aspartate, serine, glutamate, lysine, leucine and isoleucine. With the exception of isoleucine, these amino acids are located at the carboxyl-terminus of Ti^a [8]. Temporarily, ignoring the isoleucine discrepency, these data, in addition to the molecular weight data above, suggest that Ti^a_m arises from Ti^a by limited specific proteolysis at the carboxyl-terminus.

This hypothesis is confirmed by sequence analysis of the termini of the two inhibitors. Edman-dansyl degradation indicate that the amino-terminal sequence of Ti^a_m is identical to that of Ti^a for at least the first five residues (Table 2). Digestion of performic acid oxidized Ti^a_m with a combination of carboxypeptidase A and B resulted in the release of leucine, lysine, glutamine and phenylalanine in that order (Table 3). Digestion of the oxidized inhibitor with carboxypeptidase A alone resulted in the release of leucine only, while treatment with carboxypeptidase B alone was ineffective in releasing any amino acid (data not shown). The carboxyl-terminal sequence of Ti^a_m is thus -Phe-Gln-Lys-Leu, i.e. that of Ti^a up to Leu 176. The data therefore support the identification of Ti^a_m as a proteolytic degradation product of Ti^a resulting from the loss of the carboxyl-terminal residues Asp 177—Leu 181. We believe the apparent loss of an isoleucyl residue is an artifact resulting from the incomplete hydrolysis of Ile 57—Ile 58 and Ile 19—Leu 20 peptide bonds during amino analysis. No other data are consistent with a loss of this isoleucyl residue.

Analysis of KSTI inhibitors from Fiskeby V

SDS-PAGE indicates that both Ti^b and Ti^b_m are composed of single polypeptide chains with apparent

Table 1. Comparison of the amino acid composition of the KSTI proteins

	Residues/molecule*					
Residue	Ti^a	Ti^a_m	Ti^a-Ti^a_m	Ti^b	Ti^b_m	Ti^b-Ti^b_m
Asx	26	(25.1) 25	1	(29.8) 30	(29.7) 30	—
Thr	7	(6.7) 7	—	(8.8) 9	(8.4) 8	1
Ser	11	(10.2) 10	1	(12.7) 13	(11.0) 11	2
Glx	18	(17.2) 17	1	(20.9) 21	(17.7) 18	3
Pro	10	(9.9) 10	—	(9.5) 10	(9.8) 10	—
Gly	16	(16.0) 16	—	(17.9) 19	(17.8) 18	—
Ala	8	(8.0) 8	—	(9.0) 9	(9.3) 9	—
1/2 Cys†	4	(3.8) 4	—	(4.1) 4	(3.8) 4	—
Val	14	(13.8) 14	—	(14.9) 15	(15.0) 15	—
Met	2	(1.6) 2	—	(1.0) 1	(0.8) 1	—
Ile	14	(13.0) 13	1	(15.0) 15	(14.6) 15	—
Leu	15	(13.9) 14	1	(14.0) 14	(13.0) 13	1
Tyr	4	(3.8) 4	—	(3.2) 3	(3.2) 3	—
Phe	9	(8.7) 9	—	(10.6) 11	(10.2) 10	1
Lys	10	(9.4) 9	1	(11.7) 12	(10.6) 11	1
His	2	(1.8) 2	—	(1.1) 1	(0.9) 1	—
Arg	9	(8.7) 9	—	(10.7) 11	(10.2) 10	1
Trp	2	(1.7) 2	—	ND‡	ND	—
Total	181	175	6	197	187	10
Amino-terminus§	Asx	Asx		Asx	Asx	
M_r‖	21 500	21 000		22 000	21 500	

*Values for Ti^a were calculated from the amino acid sequence [8]. Values for Ti^a_m, Ti^b, and Ti^b_m were calculated based upon 8 residues of alanine, 14 residues of leucine and 13 residues of leucine respectively. Values in parentheses are the best values based upon the average of the 20, 48 and 72 hr hydrolysate values, except for Thr and Ser which were found by extrapolation to zero time values. Unenclosed values are the best integer values.

†Determined as cysteic acid after performic acid oxidation [22].

‡ND, Not determined.

§Determined by the Edman-dansyl technique [20].

‖Determined by SDS-PAGE [16].

Table 2. Terminal sequence analysis of KSTI species*

Ti^a	Asp-Phe-Val-Leu-Asp-Asn-Gln-Gly-Asn-Pro-Leu . . . . . Gln-Phe-Gln-Lys-Leu-Asp-Lys-Glu-Ser-Leu
Ti^a_m	Asx-Phe-Val-Leu-Asx . . . Phe-Gln-Lys-Leu
Ti^b	Asx-Phe-Val-Leu-Asx-Asx-Glx-Gly-Asx-Pro . . . Ser
Ti^b_m	Asx-Phe-Val-Leu-Asx-Asx-Glx-Gly-Asx-Pro . . . Lys

*Residues were determined by →, Edman-dansyl degradation; ←, carboxypeptidase digestion (A, B, or A + B); ⇐, hydrazinolysis.

Table 3. Release of amino acids from performic acid oxidized KSTI-Ti by carboxypeptidases A + B*

Residue	mole released/mole protein 1 hr	4 hr
Leu	0.33	0.76
Lys	0.35	0.72
Gln	0.12	0.42
Phe	0.10	0.36

*Digestion with carboxypeptidases A + B was carried out as described in ref. [21]. Released amino acids were identified directly on the amino acid analyser.

molecular weights of approximately 22000 and 21500 respectively. Ti^b_m is thus slightly smaller than Ti^b, analogous to the Ti^a_m/Ti^a pair. Amino acid compositional data indicates extensive similarity between Ti^b and Ti^b_m (Table 1). Inhibitor Ti^b_m appears to be derived from Ti^b by the loss of three glutamyl, two seryl, and single threonyl, phenylalanyl, leucyl, lysyl and arginyl residues. The above data suggest a relationship between these two inhibitors similar to that established between the KSTI species of Amsoy 71; i.e. that Ti^b_m arises via limited proteolysis of Ti^b.

Examination of the amino-termini of Ti^b and Ti^b_m by the Edman-dansyl degradation revealed both to have the same amino-terminal sequence as Ti^a and Ti^a_m (Table 2). Digestion of the performic acid oxidized inhibitors with combined carboxypeptidases A and B yielded ambiguous results, as did the digestion of oxidized Ti^b with carboxypeptidase A alone. Digestion of native Ti^b with carboxypeptidase B alone resulted in no released amino acids. Similar treatment of native Ti^b_m resulted in the release of 0.84 moles of lysine per mole of inhibitor during an 8 hr incubation, while treatment with carboxypeptidase A alone released no amino acids. The carboxyl-terminus of Ti^b_m was thus established as lysine (Table 2). The carboxyl-terminus of Ti^b was unambiguously identified as serine by hydrazinolysis (0.73 mole serine/mole inhibitor, no other termini detected).

The analyses of the Kunitz inhibitors from both soybean cultivars therefore supports a general model of proteolysis at the carboxyl-terminus of the Kunitz inhibitor to produce the inhibitor observed in the cotyledons of the young seedling. Previous reports [9] of immunological identity between the original and post-germination forms of the Kunitz inhibitor are readily understandable in light of this precursor–product relationship.

The appearance of new inhibitor species during germination derived from the inhibitors present in the quiescent seed has been observed in the adzuki bean (*Vigna angularis*) [10] and the mung bean (*Vigna radiata*) [11]. In the mung bean the major dry seed inhibitor, MBTI-F, is converted in a sequential manner tot the electrophoretically distinct inhibitors MBTI-E and then MBTI-C [11]. Sequence determination of these inhibitors shows that inhibitor E is derived from inhibitor F by proteolysis at the carboxyl-terminus [12]. Recent work in our laboratory with the soybean indicates that the major Bowman–Birk inhibitor of the quiescent seed, BBSTI-E, is converted to BBSTI-D [3], through the loss of two amino acid residues, again at the carboxyl-terminus [M. Madden, A. L. Tan-Wilson and K. A. Wilson, in press]. BBSTI-D however appears earlier in seedling development than does the modified KSTI [3], possibly indicating the involvement of different modifying proteinases for the two types of inhibitors.

EXPERIMENTAL

Plant materials and reagents. Soybean seeds [*Glycine max* (L.) Merrill] varieties Amsoy 71 and Fiskeby V wee obtained from May Seed Company (Shenandoah, IA), and Stokes Seeds (Buffalo, NY) respectively. Sephadex G-75 was from Pharmacia Fine Chemicals, while DEAE-cellulose (DE-52) was from Whatman, Ltd. Sequencing reagents, including phenylisothiocyanate and trifluoroacetic acid were from Pierce Chemical Co. Hydrazine (95%) was from Eastman Kodak Co. Carboxypeptidase A and B, treated with diisopropylfluorophosphate, and commercial KSTI were obtained from Sigma Chemical Co. Protein standards for SDS-PAGE were from Bio-Rad Laboratories. F1700 micropolyamide TLC plates were products of Schleicher and Shuell Co. Reagent grade pyridine was refluxed with ninhydrin and redistilled prior to use as an electrophoretic or chromatographic solvent. All other chemicals were reagent grade or better. All pH measurements were made at room temp. (21 ± 1°) unless otherwise indicated. Double distilled water was used throughout.

Germination of seeds and collection of plant tissue. Seeds were hand selected for soundness, rinsed in H_2O, and then planted in moist vermiculite. Plants were grown on a 12 hr day (25°)/12 hr night (20°) cycle. Time of growth was reckoned from the time of planting. After the appropriate period of growth, cotyledons were harvested, rinsed in H_2O and blotted dry. Cotyledons exhibiting microbial infection were discarded. The remaining cotyledons were weighed and stored at −80° until used.

Purification of inhibitors. The Ti^b form of KSTI was purified from ungerminated cultivar Fiskeby V soybeans. Seeds were ground to a coarse powder in a Waring blender. The meal was defatted at room temp. by stirring with 3 ml of Me_2CO per g of ground seed for 40 min, followed by filtration and air drying for 24 hr. Extraction of the inhibitor was achieved by stirring the defatted meal overnight at 4° in 10 ml of 50 mM Tris–HCl + 0.5 mM sodium iodoacetate + 0.33 mM phenylmethylsulphonyl fluoride, pH 8.0 for every g of meal. The homogenate was filtered through cheesecloth and centrifuged at 12000 *g* for 1 hr at 4°. The supernatant was then adjusted to 85% saturation in $(NH_4)_2SO_4$ at 0° and allowed to stand overnight at 4°. The precipitate was collected by centrifugation, and the supernatant acidified to pH 4.2 at 0° with HCl. The copious ppt was removed by centrifugation and the pH of the supernatant adjusted to 8.0 with NaOH.

This soln was chromatographed on a column of Sephadex G-75 (5 × 140 cm) as previously described [3]. Fractions were monitored for A_{280} and for reactivity with anti-BBSTI and anti-KSTI antibodies by radial immunodiffusion. Fractions showing KSTI cross-reactivity were pooled and dialysed against 50 mM NH_4OAc, pH 6.5. The equilibrated sample was applied to a DEAE-cellulose column (2.5 × 100 cm) equilibrated with the same buffer. The inhibitor was eluted with a linear gradient of 0.05–0.5 M NH_4OAc, the latter buffer at pH 5, as previously described [3]. The resulting KSTI peak was pooled and rechromatographed under the same conditions to obtain the final preparation of Ti^b.

Ti^b_m was purified from the cotyledons of Fiskeby V germinated for either 6 or 13 days. The crude inhibitor fraction was prepared as described above, except that the initial defatting step was omitted. Gel filtration of this preparation was as above. Subsequent chromatography on DEAE-cellulose as above resulted in good resolution of the two KSTI species, Ti^b_m, eluting at 0.26 M buffer, and Ti^b, eluting at 0.28 M. Each KSTI inhibitor peak was rechromatographed and thus purified to homogeneity.

The Ti^a_m inhibitor was prepared from Amsoy 71 cotyledons germinated for 8 days. Extraction, chromatography and sample treatment was as described for Ti^b_m. Ti^a_m was found to elute at 0.24 M buffer, and Ti^a at 0.27 M buffer, on the DEAE-cellulose column.

Radial immunodiffusion. Rabbit antisera to KSTI were prepared as previously described [3, 13]. Radial immunodiffusion was performed as described in ref. [14]. Standards were prepared from commercially obtained KSTI (the Ti^a variant). Concns of these standards were determined spectrophotometrically using an extinction coefficient $E^{1\%}_{1cm}$ of 10.01 for KSTI at 280 nm. There was no difference in standard curves obtained with commercially available KSTI (Ti^a) and the KSTI species (Ti^b and Ti^b_m) purified from Fiskeby V [14].

Polyacrylamide disc gel electrophoresis. PAGE was performed by the method of ref. [15] in 1.5 mm thick 10% (w/v) acrylamide slab gels and stained with Coomassie blue R (0.25% in 45.4% MeOH + 9.2% HOAc). The gels were destained by diffusion in 12.5% (w/v) isopropanol + 10% HOAc. R_f values were calculated relative to the bromphenol blue tracking dye. SDS-PAGE was performed in 12.5% (w/v) acrylamide slab gels according to the method of ref. [16]. Staining was as above.

Sesquencing procedure. Samples for amino acid analysis were hydrolysed *in vacuo* with 5.7 N HCl at 110° for 20, 48 and 72 hr. Analysis of hydrolysed samples was performed on a Glenco MM-70 amino acid analyser equipped with a ninhydrin detection system. For the determination of tryptophan, samples were hydrolysed as described in ref. [17].

The amino-terminal residue of each of the KSTI inhibitors was detected using the dansylation method described in ref. [18]. Dansyl-amino acids were chromatographed and identified on micropolyamide TLC sheets according to the method of ref. [19].

Inhibitor amino-termini were manually sequenced using the Edman-dansyl technique of ref. [20] with the following modification. After the coupling and drying steps, reaction by-products were removed by addition of 100 μl of H_2O followed by extraction with three 400 μl aliquots of butyl acetate. Dansyl amino acids were identified as described above.

Carboxyl-terminal residues were identified by carboxypeptidases A plus B digestion according to the method of ref. [21]. Performic acid-oxidized inhibitors were prepared by the method of ref. [22]. Liberated amino acids were identified by amino acid analysis. Alternatively, the hydrazinolysis procedure of ref. [23] was used.

During all phases of analysis of the experimental samples (Ti^b, Ti^b_m, and Ti^a_m), identical analysis of Ti^a, whose sequence is known [8], was carried out as a procedural control and reference.

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REFERENCES

1. Richardson, M. (1977) *Phytochemistry* **16**, 159.
2. Ryan, C. A. (1973) *Plant Physiol.* **24**, 173.
3. Tan-Wilson, A. L., Rightmire, B. R. and Wilson, K. A. (1982) *Plant Physiol.* **70**, 493.
4. Orf, J. H., Miles, D. W. and Hymowitz, T. (1977) *Bot. Gaz.* **138**, 255.
5. Freed, R. C. and Ryan, D. S. (1978) *Cereal Chem.* **55**, 534.
6. Orf, J. H. and Hymowitz, T. (1979) *J. Am. Oil. Chem. Soc.* **56**, 722.
7. Freed, R. C. (1981) Ph.D. Thesis, Univ. of Wisconsin.
8. Koide, T. and Ikenaka, T. (1973) *Eur. J. Biochem.* **32**, 417.
9. Freed, R. C. and Ryan, D. S. (1978) *J. Food Sci.* **43**, 1316.
10. Yoshikawa, M., Kiyohara, T., Iwasaki, T. and Yoshida, I. (1979) *Agric. Biol. Chem.* **9**, 1989.
11. Lorensen, E. L., Prevosto, R. and Wilson, K. A. (1981) *Plant Physiol.* **68**, 88.
12. Wilson, K. A. and Chen, J. C. (1983) *Plant Physiol.* **71**, 341.
13. Tan-Wilson, A. L. and Wilson, K. A. (1982) *Phytochemistry* **21**, 1547.
14. Tan-Wilson, A. L., Rightmire, B. R. and Wilson, K. A. (1983) *J. Immunol. Methods* **61**, 99.
15. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404.
16. Laemmli, U. K. (1970) *Nature* **227**, 680.
17. Simpson, R. J., Neuberger, M. R. and Liu, T.-Y. (1976) *J. Biol. Chem.* **251**, 1936.
18. Gray, W. R. (1972) *Methods Enzymol.* **25**, 121.
19. Woods, K. R. and Wang, K. T. (1967) *Biochim. Biophys. Acta* **133**, 369.
20. Gray, W. R. (1972) *Methods Enzymol.* **25**, 333.
21. Wilson, K. A. and Laskowski, M. Sr. (1975) *J. Biol. Chem.* **250**, 4261.
22. Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 199.
23. Schroeder, W. A. (1972) *Methods Enzymol.* **25B**, 138.