Initiation of the Degradation of the Soybean Kunitz and Bowman-Birk Trypsin Inhibitors by a Cysteine Protease

Gregory Papastoius and Karl A. Wilson

Department of Biological Sciences, State University of New York at Binghamton, Binghamton, New York 13902–6000

ABSTRACT

Protease K1 activity initiates the degradation of the Kunitz soybean trypsin inhibitor (KSTI) during germination and early seedling growth. This enzyme was purified nearly 1300-fold from the cotyledons of 4-day-old soybean (Glycine max [L.] Merrill) seedlings. Protease K1 is a cysteine protease with a molecular weight of approximately 28,000. It cleaves the native form of KSTI, Ti*, to Tiâ′, the same modified form observed in vivo. In addition to attacking KSTI, protease K1 is also active toward the major Bowman-Birk soybean trypsin inhibitor, as well as the α, α′, and β subunits of soybean β-conglycinin. The properties and temporal variation of protease K1 during germination indicate that it is responsible for initiating the degradation of both KSTI and Bowman-Birk soybean trypsin inhibitor in the soybean cotyledon.

During germination the reserve proteins of the legume seed are hydrolyzed to yield amino acids needed by the seedling until it becomes established as a photosynthetic autotroph. We previously demonstrated that the Kunitz and Bowman-Birk-type trypsin inhibitors of legume seeds are also subjected to hydrolysis during germination and seedling growth (17, 20, 21). The soybean Glycine max (L.) Merrill contains both KSTI and BBSTI, each of which is initially cleaved by a limited specific proteolysis. In the soybean cv Amsoy 71, which contains the Ti* variant of KSTI, this initial proteolysis removes the five carboxyl-terminal residues (Asp-Lys-Glu-Ser-Leu) of KSTI-Ti* to produce KSTI-Tiâ′ (9). This proteolysis is catalyzed by protease K1. Protease K1 is essentially absent from dry soybeans but increases greatly during germination, peaking at day 4 of growth (22). The degradation of the major soybean Bowman-Birk-type inhibitor, BBSTI-E, similarly involves an initial truncation at the carboxyl terminus to produce BBSTI-D (12). This reaction is catalyzed by protease B1, which exhibits a temperature variation in activity similar to that of protease K1. Both of these inhibitor-degrading activities are distinct from another activity in the seedling cotyledon, protease G1, which specifically degrades the acidic chains of the storage globulin glycinin (22).

Preliminary studies of the K1 proteolytic activity in crude extracts of germinated soybean seeds suggest that protease K1 is a cysteine protease (22). Here we describe the purification and further characterization of protease K1. These results indicate that this protein also accounts for much, if not all, of the protease B1 activity noted in the cotyledon.

MATERIALS AND METHODS

Plant Materials, Substrates, and Reagents

Soybeans (Glycine max [L.] Merrill), cv Amsoy 71, were purchased from May Seed and Nursery Co., Shenedoah, IA. Germination and growth were carried out as previously described (22) for 4 d. Cotyledons were harvested and stored at −20°C until needed.

The native form of KSTI (as found in Amsoy 71), the Ti* variant, was purchased from Worthington Biochemical Corp., and the modified form, Tiâ′, was purified as previously described (9). BBSTI-E, the major form of Bowman-Birk-type inhibitor in the soybean, and BBSTI-D were purified by the method of Tan-Wilson et al. (18) and Madden et al. (12), respectively. Glycinin and β-conglycinin were prepared by the method of Wilson et al. (23). Concentrations of these proteins were determined spectrophotometrically assuming the A280 of a 1 mg/mL solution of KSTI (Ti* and Tiâ′), BBSTI (E and D), glycinin, and β-conglycinin of 1.00, 2.27, 1.23, and 1.82, respectively.

Azocasein, leupeptin, pepstatin A, Na iodoacetate, N-ethylmaleimide, PMSF, BAPA, leucine p-nitroanilide, carbobenzyloxy phenylalanyl-alanine, and the oxidized A chain of bovine insulin were from Sigma Chemical Co. Twice-crystallized acrylamide, N,N’- methylendisacrylamide, SDS, and Serva Blue G were obtained from Serva Fine Biochemicals. CM- and DEAE-Trisacryl M were purchased from IBF Biotechnics (Columbia, MD), and Sephacryl S-200 was from Pharmacia Fine Chemicals. All other chemicals were reagent grade or better, and twice-distilled water was used throughout. All pH adjustments were performed at room temperature (21 ± 1°C).

Purification of Protease K1

Crude extracts were prepared by homogenizing the partially thawed cotyledons (approximately 100 g per preparation) in 50 mm Tris·Cl + 10 mm 2-mercaptoethanol, pH 8 (5 mL of buffer per g of tissue). This and all subsequent operations were carried out at 0 to 4°C. The homogenate was filtered.

1 Supported by National Science Foundation grant PCM 8301202.
2 Present address: Department of Arthritis, Boston University Medical School, Boston University, Boston, MA.
3 Abbreviations: KSTI, Kunitz soybean trypsin inhibitor; BBSTI, Bowman-Birk soybean trypsin inhibitor; BAPA, N-benzoyl-L-arginine p-nitroanilide.
through cheesecloth and centrifuged at 19,000g for 90 min. The resulting clear supernatant was applied to a 5 × 42 cm column of DEAE-Trisacryl M equilibrated to 50 mM Tris-Cl + 2 mM 2-mercaptoethanol, pH 8.0. The column was eluted at 160 mL/h with 300 mM of the same buffer, followed by a 3.2-L linear NaCl gradient, 0 to 0.6 M, in the same buffer. Fractions of 10 mL were collected.

The fractions containing the majority of the protease K1 activity were pooled, dialyzed against 50 mM Na acetate + 5 mM 2-mercaptoethanol, pH 5.0, and applied to a CM-Trisacryl M column (2.5 × 48 cm) equilibrated to the same buffer. The column was eluted at 60 mL/h with a linear NaCl gradient, 0 to 0.6 M, in the buffer (total gradient volume 1.6 L). Fractions of 5 mL were collected. The fractions containing protease K1 activity were pooled, concentrated by ultrafiltration on an Amicon YM-5 membrane, and applied to a 2.5 × 91 cm Sephacryl S-200 column equilibrated to 50 mM Tris-Cl + 2 mM 2-mercaptoethanol, pH 8.0. The column was eluted with the same buffer at 40 mL/h, with fractions of 5 mL collected. The single peak of protease K1 was pooled and rechromatographed on a 2.5 × 26 cm DEAE-Trisacryl M column in 50 mM Tris-Cl + 5 mM 2-mercaptoethanol, pH 8.0. Elution was carried out at 25 mL/h, with a 0 to 0.6 M NaCl gradient (800 mL total). The protease peak fractions were pooled to yield the final protease K1 preparation.

Protease Assays

Assays for protease K1 were carried out as described previously (22). The assay reaction consisted of 20 µL of the desired sample and 40 µL of substrate mixture. The latter contained 7.5 µg KSTI-Ti, 3.5 mM 2-mercaptoethanol, 0.3 µg amphotericin B, 60 µg kanamycin, and Mcllvaine's pH 4.0 citrate/phosphate buffer (14). For each sample two identical reactions were set up. One was immediately frozen at −20°C to serve as a zero-time control, and the other was incubated for 24 h at 30°C. For each set of assays two substrate standards (zero time and +24 h) were prepared in a similar manner except that the corresponding buffer was substituted for the enzyme sample. After incubation, all of the reactions were examined for conversion of Tp to Tpₐ using PAGE (6) followed by staining with Coomassie brilliant blue G and quantitation by scanning densitometry (22). No conversion of Tp to Tpₐ was observed in the assay in the absence of added protease K1.

For the routine assay of protease K1 activity, as in monitoring of chromatography columns, activity was reported as the percentage of Tpₐ present at zero time converted to Tpₐ after 24 h. In this assay, the percentage of Tp converted to Tpₐ is directly proportional to the amount of protease K1 activity present up to approximately 60 to 70% conversion. However, this relationship is only linear to approximately 35% conversion. For more accurate quantitation of the protease (e.g. to calculate recovery of activity and in the inhibition and subcellular localization studies), the sample was assayed over a range of different dilutions. Only those assays occurring within the linear portion of the activity/concentration relationship were used to calculate protease K1 activity. One unit of protease K1 activity under these conditions was defined as converting 1 mg of Tp to Tpₐ in 24 h.

Protease G1 activity was carried out at pH 4 as previously described (22). Activities in column fractions were reported as the percentage of the glycinin acidic chains present at time zero converted to the degraded form A' (22) after 24 h. Assays for β-cglycinin degradation were carried out in a similar manner substituting β-cglycinin for glycinin in the reaction mixture. Protease B1 activity, which catalyzes the conversion of BBST1-E to BBST1-D, was assayed by the method of McGrain et al. (13). For the comparison of the activity of purified protease K1 against BBST1-E, β-cglycinin, and glycinin, assays were performed over a range of enzyme dilutions to ensure that the assays were linear with respect to enzyme activity.

General endopeptidase activity was measured with azocasein as substrate by the method of Wilson et al. (22) with the exception that 2-mercaptoethanol (2 mM) was substituted for DTT (1 mM). Carboxypeptidase was assayed using carboxy-enzol-oxy-phenylalanyl-alanine, and leucine aminopeptidase was assayed with leucine p-nitroanilide (22). Activity hydrolyzing BAPA was assayed by the method of Catsimpoolis et al. (5).

Electrophoresis

PAGE of the purified protease preparation was performed in 7% (w/v) gels by the method of Davis (6) and stained either with Coomassie brilliant blue G (Serva Blue G) or with the silver-staining method of Wray et al. (25). SDS-PAGE was performed according to the method of Laemmli (11).

Mol Wt Determination

The mol wt of the purified protease K1 was estimated by gel filtration on a 2.5 × 91 cm column of Sephacryl S-200 equilibrated to 50 mM Tris-Cl + 10 mM 2-mercaptoethanol, pH 8.0. The column was calibrated with BSA, chicken ovalbumin, KSTI, myoglobin, RNase, and cyt c. Mol wts of 66,200, 45,000, 21,500, 17,600, 13,700, and 12,300 were assumed for these standards. The apparent mol wt for protease K1 was also determined by SDS-PAGE, using BSA, ovalbumin, carbonic anhydrase (mol wt 29,000), KSTI, and lysozyme (mol wt 14,000) as standards.

Reaction of Protease K1 with Insulin A Chain

Oxidized A chain of bovine insulin (200 µg) was incubated with 3.6 milliunits of purified protease K1 in a total volume of 60 µL of 83 mM ammonium formate + 1.7 mM 2-mercaptoethanol, pH 4.5, for 20 h at 30°C. Controls in which either the insulin polypeptide or the enzyme were omitted were also run. After incubation the reactions were examined by reverse phase HPLC in a manner similar to that described by Wilson et al. (24).

Purification of Protein Bodies

Soybeans were germinated and grown in continuous darkness using a 12-h, 25°C/12-h, 20°C cycle, and the cotyledons were harvested on day 4. These cotyledons were minced with a razor blade and ground in a chilled mortar and pestle with 50 mM Tris-Cl + 1 mM Na2EDTA + 0.1 mM MgCl₂ + 14%
RESULTS

Purification of Protease K1

Crude extracts of soybean cotyledons from day 4 of growth contain a number of proteolytic activities including proteases K1, B1, and G1 and general endopeptidase. A number of peptidases such as carboxypeptidase, BAPAase, and leucine aminopeptidase are also present (13, 22). Application of crude extract to DEAE-Trisacryl M at pH 8 resulted in the retention of all of these activities on the column. However, the proteases and peptidases, including proteases K1 and G1 and general endopeptidase, eluted from the column near the beginning of the salt gradient associated with a single major protein peak containing both glycycin and β-conglycinin (Fig. 1). Minor peaks of proteases K1 and G1 were occasionally observed eluting later in the gradient (as in Fig. 1) but were sometimes absent. These results suggest that under these conditions protease K1 and the other proteolytic activities are strongly associated with the two major storage globulins.

Chromatography of the protease pool from DEAE-Trisacryl on CM-Trisacryl M at pH 5.0 is shown in Figure 2. The majority of the applied protein and carboxypeptidase activity, and all of the leucine aminopeptidase and BAPAase, did not bind to the column, eluting in fractions 40 to 200 (data not shown). Protease K1 activity eluted as a peak at approximately 200 mm NaCl, whereas endopeptidase activity eluted earlier at 130 mm NaCl. Protease G1 activity eluted as two peaks, one corresponding to the endopeptidase peak and the second overlapping the first half of the protease K1 peak.

The fractions containing protease K1 were pooled, concentrated, and subjected to gel filtration on Sephacryl S-200 at pH 8.0 (Fig. 3). Protease K1 and protease G1 activity were separated from the bulk of the higher mol wt-containing proteins. The protease K1 pool was finally rechromatographed on DEAE-Trisacryl M at pH 8. The activity eluted as a single peak at approximately 60 mm NaCl (Fig. 4). This peak was pooled and used as the final protease K1 preparation.

Protease K1 was purified with an overall recovery of 8% and a nearly 1300-fold increase in specific activity (Table I).

Purity and Mol Wt of the Protease K1 Preparation

Electrophoresis of the final protease K1 preparation in the Davis PAGE system followed by staining with Coomassie blue revealed a single major protein band of Rf 0.20 which...
expressed as by pooled a x 2.5- between however, treated.

Figure 3. Purification of protease K1 by gel filtration. The pool from Figure 2 was concentrated by ultrafiltration and applied to a column of Sephacryl S-200 equilibrated to 50 mM Tris-Cl + 2 mM 2-mercaptoethanol, pH 8.0. — , Aprotinin; ○, protease K1. Activities of proteases K1 and G1 are expressed as the percentage of substrate converted to product in 24 h by 12 and 20 μL, respectively, of the indicated column fractions.

accounted for 90% of the total stained material. Silver staining of an identical gel (Fig. 5A) revealed two minor species of Rf 0.43 and 0.09. On the basis of the silver staining the major (Rf 0.20) protein is at least 86% of the total.

SDS-PAGE with silver staining (Fig. 5B) revealed only two polypeptides of similar mobility corresponding to mol wt s of 29,000, and 30,200. The lower mol wt species accounted for approximately 60% of the staining in the preparation illustrated. However, the exact ratio between the two forms varied between preparations, with the larger form dominating in preparations that were carried out rapidly without freezing of the protease between stages of the purification. The multiple forms may thus, in part, represent partial proteolysis of protease K1, either by other proteases in the crude extract or autolysis by protease K1 itself.

Analytical gel filtration on Sephacryl S-200 at pH 8 yielded an apparent mol wt for protease K1 of 29,000 (Fig. 6), in good agreement with the results of SDS-PAGE.

**pH Optimum and Mechanistic Class of Protease K1**

Our initial studies (22) indicated a pH optimum of approximately 4.0 for protease K1 activity in crude extracts. The purified enzyme exhibited a sharp pH optimum at pH 3.5 to 4.0 with KSTI-Ti as substrate. No activity was observed at or below pH 3.0 or at or above pH 4.5 (data not shown).

Protease K1 activity was previously shown to require the presence of reduced thiol for activity, suggesting it to be a cysteine protease (22). The strong inhibition of the purified enzyme by iodoacetate and N-ethylmaleimide (Table II) confirms this assignment of mechanistic class. Protease K1 is also

![Figure 4](image_url) **Figure 4.** Rechromatography of protease K1 on DEAE-Trisacryl M. The pool from Sephacryl S-200 chromatography was applied to a 2.5- x 26-cm column of DEAE-Trisacryl M equilibrated to 50 mM Tris-Cl + 5 mM 2-mercaptoethanol, pH 8.0. The column was eluted with a linear gradient of NaCl as described in the text. — , Aprotinin; ○, protease K1 activity; C, M NaCl. The final preparation of protease K1 was pooled (bar) and dialyzed against 50 mM Tris-Cl + 10 mM 2-mercaptoethanol, pH 8.0. Activities of proteases K1 and G1 are expressed as the percentage of substrate converted to product in 24 h by 12 and 20 μL, respectively, of the indicated column fractions.

![Figure 5](image_url) **Figure 5.** PAGE and SDS-PAGE of the final protease K1 preparation. A, PAGE in the nondenaturating system of Davis. B, SDS-PAGE in the system of Laemmli (M, standards, in thousands, are indicated on right). Both gels were silver stained. Lanes 1 and 2 contain 3.1 and 0.8 milli-A280 units of enzyme, respectively; top, origin.

<table>
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* Starting material was 130 g of day 4 cotyledons. * One A280 unit of protein is defined as that amount which, when dissolved in 1 mL of buffer, yields an absorbance of 1.000 at 280 nm with a 1-cm light path. * One unit of protease K1 activity is defined as converting 1 mg of TP to TPm in 24 h under the conditions of the assay.
significantly inhibited by leupeptin, which was also found to inhibit the activity in crude extracts (22). Pepstatin A was found to increase protease K1 activity above the control. The mechanism of this apparent activation is not known.

**Substrate Specificity**

Purified protease K1 was found to be active against a number of soybean seed proteins in addition to KSTI-Ti* (Table III). The major soybean Bowman-Birk protease inhibitor, BBSTI-E, was cleaved to produce BBSTI-D (as identified by the mobility of the product on SDSPAGE) at pH 4.0. Thus, protease K1 also possesses protease B1 activity (13). In fact, on a molar basis, protease K1 cleaves BBSTI-E more rapidly (eightfold higher rate) than KSTI-Ti*. The enzyme was, in contrast, inactive at pH 4 toward both KSTI-Ti* and BBSTI-D, indicating that the enzyme is specific for the native protease inhibitors.

Protease K1 is also very active in degrading the subunits of β-conglycinin at pH 4.0. The α', α, and β subunits were also degraded at rates 1.5- to twofold higher than that observed with KSTI (Table III). Unlike the degradation of the protease inhibitors, the degradation of β-conglycinin by protease K1 does not produce any discrete products observable on SDS-PAGE. Protease K1 was found to have no activity toward glycinin (pH 4.0, 5.0, or 6.0), azocasein (pH 5.7), or the oxidized A chain of bovine insulin (pH 4.5).

**Subcellular Localization of Protease K1**

The friability of the protein bodies in germinated soybean cotyledons (as well as the large amount of lipid present) resulted in a low recovery of intact protein bodies from the sucrose gradient. Under the conditions used here the intact protein bodies from day 4 cotyledons sedimented through the gradient and formed a pellet at the bottom of the tube. Microscopic examination revealed no contamination with starch grains. Typically 3 to 4% of the total protein bodies were recovered intact in this fraction based on the recovery of glycinin and KSTI in the pellet.

The protein body fraction was judged to be free of mitochondria, peroxisomes, and cytosolic proteins, as evidenced by the absence of markers for these compartments, fumarase, catalase, and leucine aminopeptidase, respectively (Table IV). In contrast, marker proteins for the soybean protein bodies, KSTI and α-mannosidase, are both enriched in the protein body fraction compared to the original homogenate. Protease K1 was likewise found to be present at high specific activity in the protein body compared to the homogenate. The low recovery of intact protein bodies complicates the interpretation of these results. However, it is apparent that, at least in the population of protein bodies recovered by this method, both KSTI and protease K1 appear together in the same subcellular compartment.

![Figure 6](image-url)
DISCUSSION

The investigations of a number of workers (8, 16), including our own (19, 20), have suggested a general pattern for storage globulin mobilization in the dicot cotyledon. The native storage protein is first cleaved specifically, and in a limited number of sites on the polypeptide(s), by an initiating protease. The modified storage protein then becomes susceptible to more extensive degradation by other proteases and possibly also the initiating protease. This pattern is not only seen in the legumin- and vicilin-type storage globulins but also with other seed proteins such as the Kunitz and Bowman-Birk-type trypsin inhibitors.

Two types of initiating proteases have thus far been described in the dicot seed. The first is presently known through only one example, proteinase F (24) in the mung bean (Vigna radiata), a serine protease that initiates the degradation of the Bowman-Birk-type trypsin inhibitor MBTI-F. Proteinase F has been demonstrated to be present in the dry seed in the same subcellular compartment (the protein body) as its substrate, MBTI-F. It apparently becomes active in the degradation of MBTI-F at or soon after the hydration of the seed in imbibition.

The second group of initiating proteases has been termed the "protease A" family of enzymes by Shutov and Vaintraub (16). These are cysteine endopeptidases that are absent in the dry seed and only appear during germination. The soybean protease K1 described here clearly belongs to the protease A family in terms of its mechanistic class and timing of appearance (22). Its mol wt (29,000) is similar to those of the cysteine endopeptidases isolated from Phaseolus vulgaris (4), Vigna mungo (15), Vigna radiata (1), and protease A of Vicia sativa (16).

Protease K1 meets the criteria required of the initiating protease for KSTI degradation in the soybean (20). Protease K1 produces in vitro the same product, KSTI-T1\textsubscript{m}, that is observed during the in vivo degradation of KSTI-T1\textsuperscript{a} (9). The time course of the appearance of protease K1 activity after germination correlates well with the time course of KSTI-T1\textsuperscript{a} disappearance and KSTI-T1\textsubscript{m} appearance (22). Finally, both KSTI and protease K1 are localized in the same subcellular compartment, the protein bodies. Similar observations support the contention that protease K1 is responsible, at least in part, for the initiation of the degradation of the major Bowman-Birk-type inhibitor, BBSTI-E, in the soybean. Like KSTI, BBSTI-E is compartmentalized in the protein bodies (7). Thus, all of the necessary criteria, the production of the same product (BBSTI-D) in vivo (12) and in vitro, correlation between the time courses of conversion of BBSTI-E to BBSTI-D (17) and the appearance of this protease, and the localization of both the protease and its putative substrate in the same compartment are again met.

The involvement of protease K1 in the in vivo degradation of \( \beta \)-conglycinin is less certain. The time course of protease K1 appearance and the degradation of the \( \alpha \) and \( \alpha' \) subunits of \( \beta \)-conglycinin are approximately comparable. However, whereas the \( \alpha \) and \( \alpha' \) subunits are initially degraded in vivo to a product of mol wt 51,000, "band 4" (23), no such product is observed in in vitro using purified protease K1 at any pH value between 4.0 and 6.0. Furthermore, degradation of the \( \beta \) subunit of \( \beta \)-conglycinin is not observed in vivo until after day 6 of growth (23) but was observed in vitro during incubation with protease K1. Protease K1 activity has by this time already begun to decline to values less than one-half of the peak level on day 4 (22).

These results demonstrate that one cannot necessarily generalize the degradation pathway of a particular protein (or family of proteins) as observed in one species of legume to the degradation of a homologous protein in another legume species. Whereas the degradation of the Bowman-Birk-type trypsin inhibitor of the mung bean is initiated by a serine protease (proteinase F), the degradation of the major BBSTI is initiated by a cysteine protease (protease K1). The two legume species likewise differ in when these initiating proteases are synthesized in the cotyledons. Thus, although some generalizations between species are possible (e.g. a specific initiating cleavage, followed by more generalized proteolysis), considerable variation occurs in the enzymatic apparatus used to carry out this process.

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LITERATURE CITED

nin, α-conglycinin, KSTI and BBSTI (Abstr 785). Plant Physiol 92: 133a