Kunitz Soybean Trypsin Inhibitor is Modified at its C-terminus by Novel Soybean Thiol Protease (Protease T1)

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Abstract: Kunitz soybean trypsin inhibitor (KSTI) is hydrolyzed during seed germination to yield amino acids needed to support initial seedling growth. The type of KSTI from Glycine max (L.) Merrill cv. Toyokomachi is KSTI-Ti. The KSTI-Ti from 4-day-old post-germination cotyledons (KSTI-Ti) has 3 or 4 amino acid residues cleaved off at the C-terminus. This KSTI modification is important to understand the mechanism of degradation in seed reserve proteins by proteases. Protease K1 also cleaves amino acid residues at the C-terminus of KSTI but it removes 5 amino acid residues. Therefore, we presumed the KSTI-Ti was produced by a protease other than protease K1. In this study, the protease T1 responsible for cleavage of KSTI-Ti at the C-terminus was purified. The enzyme was estimated to have a molecular mass of 33 kDa from its mobility on SDS-PAGE gels. The N-terminal amino acid sequence of the purified protease T1 corresponded to amino acids Phe-73 to Phe-92 of both thiol protease isoforms A and B from the soybean leaf, and shared 83% identity with the partial amino acid sequence of the membrane-associated cysteine protease from mung bean seedlings, a protease known to perform post-translational cleavage of C-terminal peptides of target proteins. Finally, this enzyme was shown to convert KSTI-Ti to KSTI-Ti.

Key words: C-terminus degradation, Inhibitory activity, Kunitz soybean trypsin inhibitor, Protease T1.

Wilson et al. (1988) found both the Kunitz-type soybean trypsin inhibitor (KSTI) and Bowman-Birk-type soybean trypsin inhibitor (BBSTI) in soybeans (Glycine max) and demonstrated that they are subjected to hydrolysis during germination and seedling growth (Tan-Wilson et al., 1982; Wilson and Chen, 1983). In the soybean cv Amsoy 71, which contains the Ti variant of KSTI, the five C-terminal residues of KSTI-Ti are removed by initial proteolysis to yield KSTI-Ti (Hartl et al., 1986). This proteolysis is catalyzed by protease K1 (Wilson et al., 1988), which is a thiol protease with a molecular mass of approximately 29,000 Da (Papastoitsis and Wilson, 1991), but its amino acid sequence has not yet been determined.

In our previous work (Momonoki et al., 2002), we determined changes in the inhibitory activity of KSTI by removal of C-terminal amino acid residues during seed germination in two Japanese soybean cultivars. KSTI from 4-day-old post-germination cotyledons was separated clearly into two bands on native-PAGE gels. Based on the C-terminal amino acid sequences, the lower mobility version of KSTI was missing 2 amino acid residues at the C-terminus, while the higher mobility version of KSTI lacked 3 or 4 amino acid residues at the C-terminus. The higher mobility KSTI had a higher inhibitory activity than the lower mobility KSTI, suggesting that these two versions differ conformationally so as to affect their active site. The reserve proteins of the legume seed are hydrolyzing during germination to yield amino acids needed to support initial seedling growth (Wilson et al., 1988). The KSTI modification is important to understand the mechanism of degradation of seed reserve proteins by proteases.

The protease K1, which was purified by Wilson’s research group, removes 5 amino acid residues off the C-terminus of KSTI, while our KSTI from 4-day-old post-germination cotyledons removed 3 or 4 amino acid residues from the C-terminus. This difference in proteolytic specificity suggests that we are working on a protease different from protease K1. In this study, we purified and identified this protease, which we call protease T1. It has a molecular mass of approximately 33,000 Da, is responsible for C-terminal modification of KSTI from Glycine max (L.) Merrill cv Toyokomachi, and was previously reported as type of KSTI-Ti (Momonoki et al., 2002). The purified protease T1 modified KSTI-Ti to KSTI-Ti. We found that this is a thiol protease, and determined its N-terminal amino acid sequence.
Materials and Methods

1. Plant materials

Soybean plants (Glycine max (L.) Merril cv. Toyokomachi) were grown in an incubator at 28°C under a 14-h photoperiod at 420 µmol m⁻² s⁻¹. Two seeds were placed in a vinyl pot (9 cm in diameter) filled with a mixture of compost and vermiculite (4 : 1), and the cotyledons harvested daily for 10 days after seeding. The plant materials were stored at −80°C until use.

2. Purification of KSTI

Frozen cotyledons were homogenized with 10 mM Tris-HCl buffer (pH 8.0) containing 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM pepstatin A, 10 mM leupeptin, 0.2 M NaCl and 20% glycerol using a mortar and pestle. The extracts were centrifuged at 15,000 × g for 10 min at 4°C, and resultant supernatants were used as crude extracts. The crude extract was subjected to ammonium sulfate fractionation and the 20-60% saturation fraction was dissolved in 20 mM Tris-HCl buffer (pH 8.0) and resolved on a Sephadex G-75 (Amersham Biosciences, Piscatway, NJ, U.S.A.) gel filtration column. The protease-containing fractions were applied onto Sephadex G-100 (Amersham Biosciences, Piscatway, NJ, U.S.A.) gel filtration column. Fractions with trypsin inhibitory activity were applied to a Poros HQ /M (Applied Biosystems, Foster City, CA, U.S.A.) anion exchange columns pre-equilibrated with 20 mM Tris-HCl buffer (pH 8.0), and the proteins were eluted with a linear gradient spanning 0 to 400 mM NaCl. The distinct fractions containing KSTI were collected by electrophoresis (Model 422 Electro-Eluter, Bio-Rad Laboratories, Hercules, CA, U.S.A.) (Davis, 1964).

3. Measurement of KSTI inhibitory activity

The inhibitory activity of KSTI was determined by the method described by Kakade et al. (1974). Sixteen milligrams of benzoyl-DL-arginine-p-nitroanilide hydrochloride in 400 µL of dimethylsulfoxide was diluted to 40 mL with 50 mM Tris-HCl (pH 8.2) containing 2 mM CaCl₂·2H₂O (the substrate solution). Four milligrams of porcine pancreatic trypsin (4907 USP units mg⁻¹, Wako Pure Chemical Industries, Osaka) was dissolved in 200 mL of 1 mM HCl (the trypsin solution). Two hundred microliters of the trypsin solution was added to 200 µL of KSTI solution and pre-incubated for 1 min at 37°C. Five hundred microliters of substrate solution was then added and incubated at 10 min at 37°C. After a 10-min incubation, the reaction was terminated by adding 100 µL of 30% acetic acid, and the absorbance of the total reaction solution was measured at 410 nm.

4. Identification of protease that removes the C-terminal end of KSTI

The protease that removed C-terminal amino acid residues from KSTI was identified from a mixture of crude proteases and purified KSTI. Two-day-old cotyledons were homogenized in 20 mM Tris-HCl buffer (pH 8.0) using a mortar and pestle. The homogenate was centrifuged at 15,000 × g for 30 min at 4°C and the supernatant was brought to 80% saturation with ammonium sulfate, mixed gently for 30 min, and left at 4°C for 1 hour. The resulting precipitate was collected by centrifugation at 20,000 × g for 30 min at 4°C and resuspended in 3 mL of 20 mM Tris-HCl buffer (pH 8.0), followed by dialysis against 20 mM sodium acetate buffer (pH 4.0). The precipitate formed from dialysis was removed by centrifugation at 20,000 × g for 30 min at 4°C, and the supernatant was applied onto Sephadex G-100 (Amersham Biosciences, Piscatway, NJ, U.S.A.) gel filtration column. The protease-containing fractions were applied onto the first of two Poros HS /M (Applied Biosystems, Foster City, CA, U.S.A.) cation exchange columns pre-equilibrated with 20 mM sodium acetate buffer (pH 4.0), and the proteins were eluted with a linear gradient of 0 to 1,000 mM NaCl. After dialysis against 20 mM sodium acetate buffer (pH 4.0), the samples were applied to the second Poros HS /M cation exchange column. The fractions containing a high protease peak was used as proteases extracts and incubated with purified KSTI. Forty microliters of the purified protease extract or 20 mM sodium acetate buffer (pH 4.0) control solution were added to 10 µL of reaction buffer (20 mM sodium acetate buffer (pH 4.0), 50 mM mercaptoethanol) and mixed with one of the following protease inhibitors, 0.2 µM L-trans-epoxysuccinyl-leucylamido-(4-guanidinobutane) (E-64), 8.3 µM antipain, 7 µM leupeptin, 1 mM PMSF, 4.4 µM pepstatin A, or 1 mM EDTA. Finally, 5 µL of purified KSTI was added, and the reaction incubated for 24 h at 30°C, followed by resolution on native-PAGE gels (Davis, 1964) and KSTI activity staining.

5. Activity staining for KSTI

The inhibitory activity of KSTI resolved on native-PAGE gels was detected using the staining method of Uriel and Berges (1968). Following electrophoresis, the gel was equilibrated in 50 mM sodium phosphate buffer (pH 7.2) for 3 min. Ten milligrams of porcine pancreatic trypsin (WAKO, Osaka, Japan) in 10 mL of 100 mM sodium phosphate buffer (pH 7.4) was added and incubated for an additional 10 min. The gel was then washed with 100 mM sodium phosphate buffer (pH 7.4) and soaked in 25 mg of N-acetyl-L-phenylalanine β-naphthyl ester in 10 mL of N,N-dimethylformamide for 1 min. Inhibitory activity was detected by staining the gel with 35 µL of tetrazoitiizd o-dianisidine in 10 mL of 100 mM sodium phosphate...
buffer (pH 7.4) for a few minutes. After staining, the gel was washed with distilled water, and trypsin inhibitory activity visualized as clear bands against a reddish-purple background.

6. Purification of thiol protease

The thiol protease that removes residues from the C-terminal end of KSTI was purified from 2-day-old cotyledons. Plant samples were homogenized in a 5-fold volume (v/w) of 20 mM Tris-HCl buffer (pH 8.0) containing 1 mM dithiothreitol (DTT) and 1 mM EDTA in a mortar with a pestle. The homogenate was centrifuged at 15,000 × g for 30 min and the supernatant was fractionated by addition of solid ammonium sulfate (35-70% saturation). After centrifugation at 20,000 × g for 30 min, the precipitate was suspended in 3 mL of 20 mM Tris-HCl buffer (pH 8.0), and dialyzed against 20 mM sodium acetate buffer (pH 4.0). The precipitate that formed during dialysis was removed by centrifugation at 20,000 × g for 30 min and the supernatant was resolved on a Sephadex G-100 (Amersham Biosciences, Piscatway, NJ, U.S.A.) gel filtration column.

Fractions containing thiol protease activity were applied to the first of two Poros HS/M (Applied Biosystems, Foster City, CA, U.S.A.) cation exchange column pre-equilibrated with 20 mM sodium acetate buffer (pH 4.0), and eluted with a linear gradient of 0 to 1,500 mM NaCl. The active fractions were then applied to thiopropyl Sepharose 6B column (covalent chromatography) (Applied Biosystems, Foster City, CA, U.S.A.) in batches and incubated for 24 h at 4º C. These flow-through fractions were collected and dialyzed against 20 mM sodium acetate buffer (pH 4.0), and the precipitate that formed during dialysis was removed by centrifugation at 20,000 × g for 30 min and applied to the second Poros HS/M cation exchange column by the method described above. The eluates from the Poros column were incubated with the trypsin inhibitor. Fractions containing active thiol protease were evaporated (centrifugal vaporizer CVE-100, EYELA, Tokyo, Japan), and subjected to SDS-PAGE analysis and N-terminal amino acid sequencing (Procise 492HT, Applied Biosystems, Foster City, CA, U.S.A.). SDS-PAGE was performed as described by Laemmli (1970) using 12.5% gel. The molecular mass of the purified thiol protease was estimated 33 kDa from comparison to the electrophoretic mobility of size standards: Myosin (200.0 kDa), β-galactosidase (116.2 kDa), albumin (66.2 kDa), aldolase (42.4 kDa), carbonic anhydrase (30.0 kDa) and myoglobin (17.2 kDa).

7. Thiol protease assay

Thiol protease activity was determined by the modified method of Asano et al. (1999). One hundred and-fifty micro litters of sample solution was mixed with 37.5 µL of reaction buffer (20 mM sodium acetate buffer, 50 mM mercaptoethanol and 1 M NaCl, pH 4.0) and pre-incubated for 2 min at 30ºC. Then, after addition of 62.5 µL of the 40 µM Z-Phe-Arg-MCA substrate solution (PEPTIDE INSTITUTE, INC., Osaka), it was incubated for 5 min. The reaction was stopped by addition of 375 µL of 7 µM leupeptin and spectrophotometric reading taken at 370 nm (Ex) and 460 nm (Em).

8. Analysis of N-terminal amino acid sequences of thiol protease

The purified thiol protease separated by SDS-PAGE was electroblotted onto a polyvinylidene difluoride (PVDF) membrane using a semidy blotting apparatus (Bio-Rad, Hercules, CA, U.S.A.). After the PVDF membrane was stained with Coomassie brilliant blue R-250, visible bands were excised and their N-terminal amino acid sequences determined using a pulsed-liquid phase protein sequencer.

Results

1. Changes in KSTI banding patterns during germination

To determine the changes in the inhibitory activity of KSTI during germination, we collected the crude extracts of KSTI cotyledons every day from day 0 (dry seed) to day 10 cotyledons. The crude extracts of KSTI were analyzed by native-PAGE, which showed one band at 0 day and 2 bands after 2 days of germination (Fig. 1). These results were first reported by Papastoitioitis and Wilson (1991), and we reported that the type of KSTI from Japanese cultivar “Toyokomachi” was Ti b.
Sugawara et al. — Identification of Soybean Protease T1 Modifying C-terminus of KSTI

(Momonoki et al., 2002). In the present study, the lower band was identified KSTI-\(T1^b\) and the upper band was designated as KSTI-\(T1^{b'}\), which lacked 3 or 4 amino acid residues at C-terminus (Momonoki et al., 2002).

2. Purification of KSTI

KSTI was purified from dry seeds. The crude seed extracts were combined and applied to a Sephadex G-75 gel filtration column (Fig. 2A). All active fractions from this gel filtration column were applied to a Poros HQ/20, anion-exchange column (Fig. 2B), where the major peak contained the active fractions. These active fractions were applied to a native-PAGE gel, and the protein band containing KSTI excised for purification. The KSTI was further separated on SDS-PAGE gel, and the purified protein of approximately 20,000 Da was isolated (Fig. 2C).
3. Changes in inhibitory activity of KSTI-\( T_i^b \) and KSTI-\( T_i^b' \) during germination

KSTI-\( T_i^b \) and KSTI-\( T_i^b' \) were purified from day 0 (dry seed) to day 10 cotyledons as described above. As shown in Fig. 3, the inhibitory activity of KSTI-\( T_i^b \) increased slightly until 3 days after seedling and decreased thereafter. KSTI-\( T_i^b' \) first appeared in 2-day-old post-germination cotyledons. The inhibitory activity of KSTI-\( T_i^b' \) increased rapidly until 5 days after seedling, then rapidly declined, so that the activity at day 7 was nearly the same level as that recorded at 2 days.

4. Characterization of the protease responsible for C-terminal modification of KSTI

To characterize the protease responsible for the C-terminal modification of KSTI, we treated the crude protease samples with various protease inhibitors known to affect specific protease classes. The reaction mixture containing protease inhibitor(s), crude protease sample and KSTI-\( T_i^b \) and/or KSTI-\( T_i^b' \) were determined by their mobility on native PAGE gels and trypsin inhibitory active staining. As shown in lane 7 of Fig. 4, the reaction of the crude protease with KSTI-\( T_i^b \) preparation did not yield a band corresponding to the KSTI-\( T_i^b' \) on the native-PAGE, while it gave a band corresponding to the KSTI-\( T_i^b' \), suggesting that this crude protease sample contained a protease that may be responsible for the KSTI modifications. Use of thiol protease inhibitors E-64, antipain and leupeptin prevented the generation of KSTI-\( T_i^b' \) from KSTI-\( T_i^b \); indicating that the protease responsible for the C-terminus modification of KSTI is thiol protease. Reactions in the presence of PMSF and pepstatin A yielded two bands corresponding to KSTI-\( T_i^b \) and KSTI-\( T_i^b' \) implying that these inhibitors partially inhibited the protease activity. In contrast, the reaction in the presence of EDTA displayed only one band corresponding to KSTI-\( T_i^b' \), showing that this inhibitor did not interfere with the proteolytic reaction.

5. Purification and identification of thiol protease

The crude extracts from 2-day-old post-germination cotyledons were purified by chromatography using Sephadex G-100, two successive Poros HS/M, and thiopropyl Sepharose 6B (covalent chromatography) columns. The active fractions (Fig. 5A) from the Sephadex G-100 gel filtration column were collected and applied to the first Poros HS/M column equilibrated with 20 mM sodium acetate buffer (pH 4.0), and the adsorbed proteins eluted with a linear NaCl gradient ranging from 0 to 1.5 M, as shown in Fig. 5B. The active fractions were eluted as double peaks, and the specific activity responsible to C-terminal modification of KSTI-\( T_i^b \) was found in the 1st peak (data not shown). All active fractions from the first Poros HS/M column were applied to a thiopropyl Sepharose 6B covalent binding column in batches and incubated for 24 h at 4°C. The thiol protease activity responsible for C-terminal modification of KSTI-\( T_i^b \) was found in the flow-through fraction, it was applied to the second Poros HS/M (Fig. 5C-a). The active fractions were eluted from the major peak, in fractions 32 to 40 (Fig. 5C-b). After this series of purification procedures, the yield of active thiol protease from 80 g of soybean cotyledons was about 1.12 µg of protein. The molecular mass of the purified thiol protease was estimated as 33 kDa by its mobility on an SDS-PAGE gel (Fig. 6A). To determine the N-terminal
Identification of Soybean Protease T1 Modifying C-terminus of KSTI

Sugawara et al.

Fig. 6. Purification of 33 kDa protease T1 by SDS-PAGE.
A, silver stained SDS-PAGE profile of active protease T1 fractions (32 to 40) after the second Poros HS/M column. The 33 kDa protease T1 band is indicated by arrows. Line M, molecular mass standards: phosphorylase b (97,400 Da), bovine serum albumin (66,267 Da), aldolase (42,400 Da), carbonic anhydrase (30,000 Da), soybean trypsin inhibitor (20,100 Da) and lysozyme (14,400 Da); B, SDS-PAGE gel stained with Coomassie Brilliant Blue (CBB) showing purified 33 kDa protease T1 indicated by arrow. Lane M, myosin (200,000 Da), β-galactosidase (116,248 Da), albumin (66,267 Da), aldolase (42,400 Da), carbonic anhydrase (30,000 Da) and myoglobin (17,201 Da).

Discussion
Soybean seeds contain about 40% protein, 5% of the amino acid sequence of the 33 kDa thiol protease, we combined all of these bands and resolved it on an SDS-PAGE gel (Fig. 6B) and sequenced. As shown in Fig. 7, the N-terminal amino acid sequence of 33 kDa thiol protease corresponded to the amino acid sequence from Phe-73 to Phe-92 of both thiol protease isoforms A (accession no. U71380) and B (accession no. U71379) from the leaf of Glycine max. However, KSTI was contained only in the cotyledon but not soybean leaf, and the functions of thiol protease isoforms A and B are unknown. Additionally, the N-terminal amino acid sequence of our 33 kDa thiol protease shared 83% identity to the partial amino acid sequence of membrane-associated cysteine protease (MCP) (accession no. AB038598) (Okamoto et al., 2001) from mung bean (Vigna mungo) seedlings. Thus, the thiol protease purified from 2-day-old post-germination cotyledons is different from other proteases which cleave KSTI, and we designate this new species as protease T1.
which are trypsin inhibitors of the KSTI and BBSTI classes. The reserve proteins of these legume seed are hydrolyzed during seed germination to yield amino acids needed to support seedling growth until establishing photosynthetic autotrophism (Wilson et al., 1988). Papastoitsis and Wilson (1991) previously found that KSTI and BBSTI in legume seed are hydrolyzed during seed germination and seedling growth (Tan-Wilson et al., 1982; Wilson and Chen, 1983). Wilson’s group purified protease K1, which removes five C-terminal residues (Asp-Lys-Glu-Ser-Leu) from KSTI-Ti to produce KSTI-Ti-m (Wilson et al., 1988). However, KSTI-Ti-b from Japanese soybean cultivar “Toyokomachi” had only 3 (Glu-Ser-Leu) or 4 (Lys-Glu-Ser-Leu) amino acid residues removed from the C-terminus (Momonoki et al., 2002). Thus, the removal of C-terminal residues from KSTI-Ti was also considered to be mediated by a protease different from protease K1. In this study, we purified a protease T1, which was distinct from protease K1, and produced KSTI-Ti-m by cleavage of KSTI-Ti at the C-terminus. Protease T1 had a molecular mass of approximately 33 kDa, in contrast to protease K1 whose molecular mass is approximately 29 kDa. Papastoitsis and Wilson showed that only KSTI-Ti-m was detected on native-PAGE after reacting with pepstatin A suggesting that protease K1 was not inhibited by pepstatin A. However, we found both KSTI-Ti-b and KSTI-Ti-s in reaction mixtures of purified KSTI and our protease extracts after pepstatin A treatment suggesting that our 33 kDa protease T1 was partially inhibited by pepstatin A. These results showed that the modification of KSTI-Ti-b to KSTI-Ti-s was dependent not only on protease K1 but also on other thiol proteases. For this reason, we purified the 33 kDa protease T1 responsible for removal of amino acid residues from the C-terminus of KSTI-Ti, and confirmed that protease T1 converted KSTI-Ti-b to KSTI-Ti-s. During chromatographic purification, protease T1 did not couple with thiopropyl Sepharose 6B, which normally reacts with proteins containing thiol groups to form mixed disulfides. This indicates a defect in the structure between the resin linker arm and the catalytic thiol group of thiol protease T1. Indeed, no covalent coupling was detected when activated thiol Sepharose 4B was subjected to covalent chromatography of Lactobacillus casei thymidylate synthase (Bradshaw and Dunlap, 1993). Therefore, the failure to immobilize the thiol protease T1 in resin probably reflected the inability of the spacer arm of the thiopropyl Sepharose 6B matrix to enter the catalytic site of this protease, possibly due to its size and/or its distribution of charges.

The N-terminal amino acid sequence of protease T1 corresponds to amino acids Phe-73 to Phe-92 of thiol protease isoforms A (accession no. U71380) and B (accession no. U71379) from soybean leaf, although the physiological significance of this region has yet to be determined. Interestingly, the N-terminal amino acid sequence of protease T1 shares 83% identity with the partial amino acid sequence of membrane-associated cysteine protease (MCP) (accession no. AB038598) (Okamoto et al., 2001) from germinating mung bean (Vigna mungo) cotyledons. These protease homologues are members of the papain family of thiol proteases, such as peptidase C1 (Rawrings and Barrett, 1994). The N-terminal polypeptides of mature papain-type thiol proteases were comparatively longer, and possessed the inhibitor 129 domain, which inhibits catalytic sites (Rawrings and Barrett, 1994). MCP and thiol protease isoform B possess an inhibitor 129 domain at the N-terminal polypeptide region. Therefore, protease T1 may also possess this domain, which may be activated by proteolytic cleavage as occurs in other papain family proteases including MCP. MCP is localized in the protein storage vacuole (PSV) and endoplasmic reticulum (ER), and is involved in post-translational cleavage of the C-terminal propeptide including the KEDL-tail of SH-EP, a known thiol protease, which plays a major role in the degradation of seed storage protein (Okamoto et al., 2001). Thus, protease T1 may also modify KSTI-Ti-m at its C-terminal end in the PSV or ER, and consequently the activated KSTI-Ti-m may regulate the degradation of storage proteins during germination. Other trypsin inhibitors might also be modified at their C- or N-termini by papain type proteases similar to protease T1, in turn regulating the degradation of...
storage proteins during germination by changing the levels of inhibitory activity.

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