Change in Activity of Soybean Trypsin Inhibitor by Removal of C-terminal Amino Acid Residues during Seed Germination

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Abstract: Kunitz-type soybean trypsin inhibitor (KSTI) is a major factor lowering the nutritional quality of soybean proteins. KSTI might be degraded during protein processing. In the present work, we determined the effect of removal of C-terminal amino acid residues on the inhibitory activity of KSTI in the cotyledons during germination in Japanese soybean cultivars, Toyokomachi and Wasekogane. The type of KSTI in both cultivars was determined by analysis of the complete nucleotide sequences of PCR products amplified from genomic DNA and partial amino acid sequences of both cultivars. These KSTI were identical to $T^p$ in Toyokomachi and $T^p$ in Wasekogane. KSTI from cotyledons at 4 days after germination in both cultivars was separated clearly into two bands T1 and T2 in Toyokomachi and W1 and W2 in Wasekogane upon native-PAGE. Based on the C-terminal amino acid sequences, KSTIs with higher mobility ($T_1$, W1) were found to lack 13 amino acid residues at the C-terminus. The KSTI with lower mobility ($T_2$, W2) lacked 14 or 15 amino acid residues at the C-terminus. Further, KSTI with lower mobility showed high inhibitory activity compared with that of KSTI with higher mobility. Thus, the changes in the form of KSTI from T1 or W1 to T2 or W2 by removal of C-terminal amino acid residues during seed germination may change the structure at the active site and consequently increase the inhibitory activity of KSTI.

Key words: Cotyledons, C-terminal amino acids, Inhibitory activity, Kunitz-type soybean trypsin inhibitor.

Kunitz-type soybean trypsin inhibitor (KSTI) (Kunitz, 1947a, 1947b) accounts for about 5% of seed protein in soybean and is the major inhibitor of plant protein proteases. The KSTI with a molecular weight of approximately 20,100 inhibits the activity of bovine trypsin (Koide and Ikenaka, 1973a). KSTI has been hypothesized to serve as a storage protein, to supply sulphur-containing amino acids to the seedlings (Tan-Wilson et al., 1982; Wilson et al., 1988; McGrain et al., 1989), and to inhibit digestive proteolytic enzymes of mammals and insects (Green and Lyman, 1972). The real role of KSTI is still not fully understand. Nonetheless, it is clear that KSTI is a major factor lowering the nutritional quality of soybean proteins (Leiner, 1979).

Three different variants of KSTI, $T^i$, $T^r$ and $T^p$, have been demonstrated by polyacrylamide gel–electrophoresis (PAGE) (Singh et al., 1969; Hymowitz and Hadly, 1972) and these variants were designated as $T^a$, $T^p$ and $T^r$, respectively (Orf and Hymowitz, 1979).

The amino acid sequences of $T^p$ (Koide and Ikenaka, 1973a, 1973b, 1973c, Kim et al., 1985), $T^p$ (Kim et al., 1985) and $T^r$ (Kim et al., 1985), and amino acid composition of $T^a$, $T^p$ and $T^r$ (Kim et al., 1985) were analyzed. All three inhibitors consisted of 181 amino acid residues. The reactive site of these KSTIs was found at the same position (Arg–Lle–Glu) by Ozawa and Laskowski, Jr. (1966). Comparing the sequences of $T^p$ and $T^r$, there is only one substitution at position 55 (Gly → Glu) (Kim et al., 1985). While the amino acid residues at position 55 of $T^a$ and $T^p$ were identical and another eight or nine amino acid substitutions were found between the sequences of $T^a$ or $T^r$ and $T^p$ (Kim et al., 1985). These results were confirmed by complementary DNA sequences encoding $T^p$ and $T^p$ (Song et al., 1993). Further, the three forms are inherited as codominant alleles in a multiple allelic series at a single locus (Hymowitz, 1986).

$T^a$ and $T^r$ inhibited the activity of bovine trypsin at a ratio 1:1 when an enzyme concentration of $2.18 \times 10^{-8} M$ was used for the titration (Kim et al., 1985). By contrast $T^p$ inhibited the activity at a 10-fold higher concentration of the enzyme. That is, $T^a$ and $T^r$ showed higher affinity to trypsin than $T^p$. Further, Hartl et al. (1986) found that KSTI was initially cleaved by a limited specific pronase. In the soybean cv. Amsoy 71, which contains the $T^a$ variant of KSTI, this initial proteolysis removes the five carboxyl–terminal amino acid residues. Additionally, this proteolysis is catalyzed by cysteine protease (Wilson et al., 1988). From the results, Papasotis and Wilson (1991) suggested that the protease cleaves the native form of KSTI, $T^p$ yielding $T^a$ during germination.

To understand the roles of KSTI during seed germination, we determined changes in inhibitory activity of KSTI by removal of C-terminal amino acid residues during seed germination in two soybean cultivars, Toyokomachi and Wasekogane. The type of KSTI in the cultivars was determined from the complete nucleotide


Abbreviation: KSTI, Kunitz-type soybean trypsin inhibitor.
Fig. 1. Banding profiles of extract from Toyokomachi and Wasekogane cotyledons on native–PAGE. Top panel shows the proteins extracted from cotyledons of Toyokomachi, and bottom panel shows those of Wasekogane. The number on each lane indicate the day after germination. The bands of KSTI (T1, T2, W1 and W2) are indicated by arrows. Lane M, thyroglobulin (669,000), ferritin (443,000), lactate dehydrogenase (139,850), albumin (66,267) and trypsin inhibitor (20,100) were used for molecular mass standard.

sequences of PCR products amplified from genomic DNA and partial amino acid sequences of both cultivars.

Materials and Methods

1. Plant materials

Young seedlings of *Glycine max* (L.) Merr. cv. Toyokomachi and Wasekogane were used for the experiments. Each vinyl pot (dia 9 cm) filled with compost: vermiculite (4:1) was seeded with 2 seeds and kept in an incubator at 28°C, under a 14 h photoperiod at 14,000 lux. The cotyledons were collected daily for 10 days to determine the changes of KSTI during germination. The cotyledons of the 4 d old soybean plants were used for analysis of partial amino acid sequence and determination of inhibitory activity of KSTI. The plant materials was homogenized with 10 mM Tris–HCl buffer (pH 8.0) containing, 0.2 mM EDTA, 1 mM PMSF, 5 mM peptatin A, 10 mM leupeptin, 0.2 M NaCl and 20% glycerol. The amount of buffer added was 4 ml for a cotyledon and 1 ml for a seed. The extracts were centrifuged at 14,000 g for 10 min and the supernatant was used for native–PAGE (Davis, 1964).

2. Purification of KSTI from germinating soybeans

In preliminary experiments, the proteins were extracted from 0 day (dry seeds) to 10 days after germination using cotyledons of two soybean cultivars, Toyokomachi and Wasekogane. The patterns of proteins in native–PAGE (Fig.1) confirmed the results of Papastoisitis and Wilson (1991). The protein from dry seeds (0 day) showed one band of KSTI in native–PAGE and 2 bands after germination. Thus, these bands from Toyokomachi cultivar were defined as T1 and T2 and these from the Wasekogane cultivar as W1 and W2, respectively (Fig. 1). The proteins containing KSTI were collected by electrophoresis (Davis, 1964) from these bands and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (Laemmli, 1970). The molecular weight of these proteins was approximately 20,000 (Fig. 2) and these proteins showed trypsin inhibitory activity. Further, the 20 amino acid residues at the N-terminus of these proteins confirmed the results of Kim et al. (1985). Consequently, these proteins were extracted as KSTI.

For analysis of amino acid sequences and determination of inhibitory activity, the KSTI was purified from cotyledons of the 4 d old soybean plants by electrophoresis using the Davis method (Davis, 1964). The protein extracts from cotyledons of the 4 d old soybean plants
was separated by 12.375% polyacrylamide gel (160 mm × 160 mm, 1 mm thick). Both sides of the gel after electrophoresis were cut 10 mm off and stained with 0.25% Coomassie Brilliant Blue R–250 and destained to detect KSTI bands. The pieces of gel were put back to the same position and fitted together with non-stained gel. Then, the strip gel containing KSTI bands was cut off from the non-stained gel and repeated electrophoresis for the strip gel. The KSTI in the gel was eluted with 5 mM Tris buffer containing 192 mM glycine using electro-eluter (Bio–Rad 422, USA). Elution was conducted at 4°C, at 8 mA for 4 h and about 200 μL of the eluate containing KSTI was collected. The purity of the KSTI was confirmed by SDS-PAGE (Fig. 2), and the purified KSTI was used for analysis of the amino acid sequence and determination of inhibitory activity.

3. Analysis of the amino acid sequence

Purified KSTI was separated by SDS-PAGE. SDS-PAGE was performed as described by Laemmlı (1970) using 12.5% polyacrylamide gel. Protein samples containing 10 mM Tris–HCl (pH 6.8), 1% SDS, 1% 2-mercaptoethanol and 20% glycerol were boiled for 3 min. Electrophoresis was performed with a RAPIDAS mini slab gel electrophoresis apparatus (ATTO, Tokyo). After electrophoresis, separated peptides were electroblotted onto a polyvinylidene difluoride (PVDF) membrane with transfer buffer in a semidyb blotting apparatus (Bio–Rad, USA). Finally, the blotted peptides were sequenced with a gas-phase protein sequence from the N-terminus (477A/120A, Applied Biosystems, Foster City, USA) and C-terminus (Procise C494, Applied Biosystems).

To confirm the type of KSTI, we analyzed the internal regions of protein sequences. The KSTI proteins separated by SDS-PAGE were digested with Staphylococcus aureus V8 protease following the method of Cleveland et al. (1977). Separated peptides were loaded onto SDS-PAGE gels and electroblotted onto PVDF membrane. The peptides on the blotted membranes were sequenced. On the other hand, for in situ digestion, the PVDF membranes were cut into small pieces after thorough washing with a digestion buffer containing acetonitrile. In situ digestion with Chromobacter protease I (lysylendopeptidase) was performed in 20 mM Tris–HCl (pH 9.0) containing 10% acetonitrile at 37°C for 15 h at enzyme-to-substrate ratio of 1:100 (mol/mol). Peptides were separated by a high performance liquid chromatography (HPLC, Model 172 HPLC Separation System, Applied Biosystems, USA) using a C18 column (220 × 2.1 mm), and eluted with a linear gradient of 0–70% Solvent B for 60 min at a flow rate of 0.2 mL/min using the following solutions. Solvent A: 0.08% trifluoroacetic acid (TFA) in water, and Solvent B: 0.08% TFA in 70% acetonitrile. Digestion solution was diluted with water to decrease the acetonitrile concentration before injection. The fractionated peptides were manually collected by monitoring the absorbance at 214 nm. Amino acid residues were determined in each peak.

4. Complete nucleotide sequences of the KSTI gene

Total genomic DNA was extracted from the fine meal of soybean dry seed (100 mg) by the combined procedure of the benzyl chloride method using ISOLPLANT DNA extraction kit (Nippon Gene, Osaka, Japan) and cetyltrimethylammonium bromide (CTAB, Sigma, St. Louis, USA) method. The DNA was further purified following the procedure of Marmur (1961). The primer pair to amplify the region containing the KSTI gene was designed based on the mRNA nucleotide sequence of Kunitz trypsin inhibitor K7P (Accession No. S45092; Johfuku and Goldberg, 1989) from the GenBank database. The sequences of primers were KT3–1: 5′-aactaagaaagcttctcatgc-3′ (location 390–411) for the forward and K7P–4: 5′-aattgctgctaaacaagac-3′ (location 1100–1122) for the reverse, and K7P–1/4 primer pair yielded a 733-bp amplified product. For further sequence determination additional primers for sequencing were designed based on determined sequence: K7P–2 (for both cultivars); 5′-gacacctgtggattac-3′ for the reverse, K7P–3 (for Wasekogane); 5′-cggagccctcttttttcag-3′ for the forward and K7P–5 (for Toyokomachi); 5′-ccggaagccctctttttcag-3′ for the forward.

A polymerase chain reaction (PCR) was performed in a 50 μL reaction mixture containing 10 ng of template DNA, 0.5 μM of each primer, 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 1.5 mM MgCl2, 0.2 mM of each dNTP, and 1.25 units of Taq DNA Polymerase. The PCR was carried out for 35 cycles consisting of denaturation for 1 min at 94°C, annealing for 1 min at 55°C and extension for 1 min at 72°C using a Gene–Amp automated thermal cycler (Model 9700, PE Applied Biosystems, Foster City, USA).

The PCR products were extracted from agarose gels using a DNA extraction kit (Easy Trap ver. 2, TaKaRa Shuzo, Ohtsu, Japan). The purified DNA was sequenced in both strands with an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems) using an automated DNA sequencer (Model 310, PE Applied Biosystem) according to the manufacturer's instructions. The primers designed for PCR were used for the cycle sequencing reaction. MacVector nucleotide and protein analysis software (Oxford Molecule, Ltd., Oxford, England) were used for analyzing nucleotide sequence and protein translation.

5. Measurement of inhibitory activity of KSTI

The inhibitory activity of KSTI to trypsin was measured by the method of Kakade et al. (1974). Forty milligrams of benzyl-α-L-arginine p-nitroanilide (BAPA) hydrochloride was dissolved in 400 μL dimethylsulfoxide and diluted to 40 mL with 50 mM Tris–HCl
(pH 8.2) containing 2 mM CaCl₂ 2H₂O and incubated at 37°C. Four milligrams of porcine pancreas trypsin (WAKO) was dissolved in 200 mL 1 mM HCl. KSTI was diluted with 10 mM Tris–HCl (pH 8.0) to 0, 1.6, 3.2, 4.8, 6.4 and 8.0 µg per 200 µL using test tubes. After dilution, 200 µL of trypsin solution (20 ng/µL) was added to each tube and incubated for 1 min at 37°C. After 1 min, 500 µL of BAPA was added to each tube and incubated for 10 min at 37°C. After 10 min, the reaction was terminated by adding 100 µL of 3% acetic acid to each tube and then the absorbance was detected at 410 nm.

Results

1. Changes of KSTI on native-PAGE after germination

The KSTI was extracted from 0 day (dry seeds) to 10 days after germination, and separated by native-PAGE (Fig. 1). There are differences in the mobility of KSTIs from cotyledons between two cultivars, Toyokomachi and Wasekogane (Fig. 1). The KSTI from dry seeds (0 day) in both cultivars showed one band in native-PAGE. Then the KSTI was separated into two bands at the 2nd or 3rd day in Toyokomachi and at the 2nd day in Wasekogane after germination. These bands from Toyokomachi were defined as T1 and T2 and those from Wasekogane as W1 and W2, respectively. The KSTI in cotyledons disappeared at the 6th day in Wasekogane and at the 8th day in Toyokomachi after germination.

2. Determination of amino acid sequences at N and C-termini of KSTI

The samples of KSTI in both cultivars were obtained from cotyledons at the 4th day after germination. At this time the KSTI was separated into two bands by native-PAGE in both cultivars. The partial amino acid sequences of the KSTI (T1, T2 or W1, W2) were analyzed. Figure 3 shows the results. In T1, T2, W1 and W2, the N-terminal amino acid sequences uniformly showed that the mature protein has Asp²⁶ at the N-terminal.

The C-terminal amino acid sequences of these KSTI were also analyzed. Of the two bands obtained from Toyokomachi, the C-terminal amino acid sequence was determined as V-D-K-E corresponding to Val²⁰¹-Lys³⁰³ and Q-K-V-D corresponding to Gln¹⁹⁹-Asp²⁰². In the Wasekogane KSTI, the C-terminal amino acid sequence of the higher mobility band (W1) was determined as L-D-K-E corresponding to Leu²⁰¹-Glu²⁰⁴, and the lower mobility band as a mixture of two kinds of proteins with C-terminal sequences of K-L-D-K corresponding to Lys²⁰⁰-Lys³⁰³ and Q-K-L-D corresponding to Gln¹⁹⁹-Asp³⁰³.

3. Determination of Type of KSTI

The types of KSTI in both cultivars were determined by analyzing the complete nucleotide sequences and partial amino sequences (Fig. 4 and 5). The 675-bp DNA determined contained the entire KSTI gene without any introns. The nucleotide sequences determined for Toyokomachi was exactly identical to Tp from the complete nucleotide sequences by Song et al. (1993), while the sequence for Wasekogane was identical to Tp (Song et al., 1993). Further, from the partial amino acid
sequences, Wasekogane KSTI was completely identical to $T^{a}$ (Kim et al., 1985). However, Toyokomachi KSTI was not identical to $T^{a}$ analyzed by the partial amino acid sequences (Kim et al., 1985) and the amino acid composition (Kim et al., 1985). There are differences at amino acid residues 37 and 38 from the N-terminus.

### 4. Inhibitory activity of KSTI caused by removal of C-terminal amino acid residues

The inhibitory activities of KSTI to trypsin in both cultivars are shown in Fig. 6. The KSTI with lower mobility (T2 or W2) in both cultivars, which lacked 14 or 15 amino acid residues from the C-terminus, showed significantly higher inhibitory activity than that with higher mobility (T1 or W1), which lacked 13 amino acid residues from the C-terminus. Further, there is a difference in the inhibitory activity between the two cultivars.

The inhibitory activities of KSTI in Wasekogane (W1 and W2) were higher than that of Toyokomachi (T1 and T2).

### Discussion

Storage proteins in seeds are hydrolyzed to yield amino acid during germination. The KSTI is also subjected to hydrolysis during germination and seedling growth (Tan-Wilson et al., 1982; Wilson and Chen, 1983; Wilson et al., 1988). Soybean cotyledons 4 d after germination contain the $T^{a}$ variant of KSTI. Initial proteolysis, which is catalyzed by protease K1, removes the five carboxyl-terminal residues (Asp-Lys-Glu-Ser-Leu) of KSTI-$T^{a}$ to produce KSTI-$T^{a}_{18}$ (Hartl et al., 1986). Further, protease K1 produces in vitro the same product, KSTI-$T^{a}_{18}$, which is observed during the in vivo degradation of KSTI-$T^{a}$ (Hartl et al., 1986). Papainotisis and
Wilson (1991) proposed that the native storage protein is first cleaved specially, at 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. Therefore, the inhibitory activity of KSTI can be changed during germination. In this study, we determined the changes in the inhibitory activity of KSTI by removal of C-terminal amino acid residues during seed germination using two Japanese soybean cultivars, Toyokomachi and Wasekogane.

The type of KSTI in the cultivars was determined by analyzing the complete nucleotide sequences of PCR products amplified from genomic DNA and the partial amino acid sequences of Toyokomachi and Wasekogane. The present study showed that the 675-bp DNA contained the entire KSTI gene without any introns. Previously, the three variants of KSTI, $T^a$, $T^b$ and $T^c$, were analyzed for the amino acid composition (Kim et al., 1985), the partial amino acid sequences (Kim et al., 1985) and the complete nucleotide sequences except $T^c$ (Song et al., 1993). The complete nucleotide sequence determined in this study was identical to $T^a$ (Song et al., 1993) for Toyokomachi and $T^b$ (Song et al., 1993) for Wasekogane. Further, the partial amino acid sequence of Wasekogane KSTI was identical to that of $T^a$ (Kim et al., 1985). However, the KSTI from Toyokomachi was not identical to $T^b$ determined by amino acid composition (Kim et al., 1985) and the partial amino sequence (Kim et al., 1985). In this study we determined the complete nucleotide sequence of KSTI from genomic DNA of both cultivars. A 675-bp DNA was determined to contain the entire KSTI gene without any introns. Further, the complete nucleotide sequence of KSTI from Toyokomachi was exactly identical to $T^b$ (Song et al., 1993). Song et al. (1993) analyzed the complete nucleotide sequence of the KSTI gene from cDNA. Thus, we determined the type of KSTI as $T^a$ for Wasekogane and $T^b$ for Toyokomachi.

To determine the effect of changes in primary structure on the inhibitory activity of KSTI, we analyzed the partial amino acid sequences at the N-terminal and C-terminal and inhibitory activity of KSTI obtained from cotyledons of both cultivars at the 4th day after germination. The KSTI was separated clearly into two bands with higher mobility (T1, W1) and lower mobility (T2, W2) upon native–PAGE.

The N-terminal amino acid sequences in both cultivars showed that the mature protein has Asp at the N-terminal. Song et al. (1993) presumed that the 25 amino acid residues in N-terminal region of KSTI could be the signal peptide. Further, in this study, the N-terminal 25 residues were predicted to be the signal peptide, based on SignalP program (http://www.cbs.dtu.dk/services/SignalP) (Nielsen et al., 1997). Thus, we concluded that the N-terminal region of KSTI protein, that is, the N-terminal 25 residues, were removed during maturation.

Based on C-terminal amino acid sequences of these KSTI, the KSTI with higher mobility in both cultivars (T1, W1) lacked 13 amino acid residues (Ser$^{205}$–Glu$^{217}$) from the C-terminus. Further, the KSTI with lower mobility in both cultivars (T2, W2) lacked 14 amino acid residue (Glu$^{204}$–Glu$^{217}$) or 15 amino acid residues (Lys$^{203}$–Glu$^{217}$) from the C-terminus of KSTI. On the other hand, Koide et al. (1973c) reported that the C-terminal residue of KSTI was Leu$^{206}$. These authors, however, analyzed the C-terminal sequence of KSTI obtained from dry seeds in that KSTI did not yet undergo sequential degradation.

Differences in the inhibitory activity were found between KSTI with the higher and lower mobilities and...
between the cultivars. KSTI (T2, W2) with lower mobility showed high inhibitory activity than KSTI (T1, W1) with higher mobility. This means that the inhibitory activity of KSTI is related to the removal of C-terminal amino residues. Further, the inhibitory activity of Wasekogane KSTI (W1 or W2) was higher than that of Toyokomachi KSTI (T1 or T2). The difference between KSTI (T1, W1) with higher mobility and KSTI (T2, W2) with lower mobility in native-PAGE can be due to the conformational change of the protein molecule caused by the removal of C-terminal amino acid residues. The changes in the structure from the KSTI (T1 or W1) to the KSTI (T2 or W2) by the removal of C-terminal amino acid residues during germination may change the structure involving the active site and consequently increase the inhibitory activity of KSTI.

References