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# Kunitz-Type Trypsin Inhibitor with High Stability from *Spinacia oleracea* L. Seeds

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**Abstract**—The trypsin inhibitor SOTI was isolated from *Spinacia oleracea* L. seeds through ammonium sulfate precipitation, Sepharose 4B-trypsin affinity chromatography, and Sephadex G-75 chromatography. This typical Kunitz inhibitor showed remarkable stability to heat, pH, and denaturant. It retained 80% of its activity against trypsin after boiling for 20 min, and more than 90% activity when treated with 6 M guanidine hydrochloride. The formation of stable SOTI–trypsin complex ($K_i = 2.3 \cdot 10^{-6}$ M) is consistent with significant inhibitory activity of SOTI against trypsin-like proteinases present in the larval midgut of *Pieris rapae*. Sequences of SOTI fragments showed homology with other inhibitors.

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*Key words*: *Spinacia oleracea* L., trypsin inhibitor, protein stability, Kunitz family, *Pieris rapae*

Proteinase inhibitors are proteins or peptides capable of inhibiting catalytic activities of proteolytic enzymes. They are roughly categorized as serine, cysteine, aspartic, and metalloproteinase inhibitors [1]. As a source of storage proteins and a mechanism of host defense, proteinase inhibitors are widely distributed in many types of plants. The two major families of serine proteinase inhibitors are the Kunitz and Bowman–Birk type inhibitors. They differ in their molecular weight, disulfide bond content, three-dimensional structure, and stability to heat and denaturing agents. In biological systems, proteinases are inactivated either by proteolytic degradation or by interaction with inhibitory pseudo-substrates with variable affinities to the catalytic sites of the enzymes [2, 3].

Proteinase inhibitors have been found as storage proteins [4] and endogenous regulators of proteolytic activity [5, 6]. They play important physiological roles in regulating diverse processes involving proteinases, such as intracellular protein breakdown, transcription, cell cycle progression, cell invasion, and apoptosis [6, 7]. It has been suggested that serine proteinase inhibitors regulate the activities of various proteinases involved in different physiological processes. However, their functions *in vivo* are not fully understood. Plant proteinase inhibitors have been shown to react as a protective mechanism against pests and epiphyte infection [8, 9]. In addition, the serine proteinase–inhibitor complex has been studied as a model of protein–protein recognition [3, 10-12].

Serine proteinase inhibitors have been studied in many plants such as Graminaceae, Leguminosae, and Solanaceae. However, in the common Chenopodiaceous plant *Spinacia oleracea* L., little attention has been drawn to its proteinase inhibitors. Spinach chloroplast dehydroascorbate reductase, one of a few proteinase inhibitors found in the Chenopodiaceae family, possesses an N-terminus whose amino acid sequence is homologous to Kunitz-type trypsin inhibitors in plants [13]. Preliminary studies carried out in our laboratory proved that *Spinacia oleracea* L. seed extract had anti-tryptic activity. In the present paper, we describe the purification, characterization, and *de novo* sequencing of *Spinacia oleracea* L. trypsin inhibitor (SOTI). Our research describes another study of proteinase inhibitors in Chenopodiaceae.

*Abbreviations*: BAPNA, $N_\alpha$-benzoyl-DL-arginine-*p*-nitroanilide; BBI, soybean Bowman–Birk trypsin inhibitor; MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; SOTI, *Spinacia oleracea* L. trypsin inhibitor; TNBS, trinitrobenzene sulfonate.

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## MATERIALS AND METHODS

**Purification of SOTI.** *Spinacia oleracea* L. seeds (100 g) were defatted in ether and homogenized in a tissue grinder. A crude extract was prepared as follows: the tissue homogenates were extracted in 800 ml of 0.02 M Tris-HCl (pH 8.0) at 4°C for 12 h and then centrifuged at 6000*g* for 20 min. The pellet was subjected to a second extraction in 400 ml of 0.02 M Tris-HCl (pH 8.0). To remove thermolabile proteins, the supernatant was incubated at 65°C for 15 min and then centrifuged at 6000*g* for 20 min. $(NH_4)_2SO_4$ was added to the supernatant, and the fraction precipitated between 35 and 75% saturation was collected. The collected precipitate was dialyzed against 0.02 M Tris-HCl (pH 8.0) at 4°C for 24 h and subjected to further purification.

The crude extract was fractionated by ion-exchange chromatography on a DEAE-52 column (2.5 × 20 cm) equilibrated with 0.02 M Tris-HCl buffer (pH 8.0) and then eluted with 0.02 M Tris-HCl buffer containing a gradient of NaCl from 0 to 0.4 M. The inhibitory fraction was dialyzed against 0.1 M Tris-HCl (pH 8.0) containing 20 mM $CaCl_2$ and then subjected to a trypsin-Sepharose 4B affinity column (1 × 10 cm) equilibrated with the same buffer. The protein was further purified on a Sephadex G-75 column (1 × 80 cm) equilibrated with 0.15 M Tris-HCl (pH 8.0) containing 0.05 M NaCl. The elution profiles were monitored at 280 nm. The active fractions designated as SOTI were combined, dialyzed against distilled water, lyophilized, and subjected to SDS-PAGE to validate purity.

**Isolation of insect midgut extract.** *Pieris rapae* larvae were obtained from local *Brassica* crops and reared on fresh *Brassica* leaves at 25°C and 60% relative humidity. Midguts were removed from larvae (4th instars) on ice and placed into isotonic saline (0.15 M NaCl). The midgut tissues were then homogenized and centrifuged at 10,000*g* for 10 min at 4°C. The supernatants were freshly used as *P. rapae* proteinases.

**Determination of protein concentrations and purity.** Protein concentrations were determined by the Bradford method (Beyotime Bradford kit). Gelatin-polyacrylamide gel electrophoresis (gel-PAGE) was performed according to Felicioli et al. [14] using 15% separating gel (containing 0.5% (w/v) gelatin) and 5% stacking gel (without gelatin). After electrophoresis, the gel was washed with distilled water and incubated at 37°C for 30 min in 100 ml of 0.1 M Tris-HCl (pH 8.0) containing 5 mg trypsin. The bands of proteinase inhibitors, protected from proteolysis by trypsin, were stained with Coomassie blue R-250 after hydrolysis of the gelatin. SDS-PAGE (15% gel) was performed under non-reducing and reducing conditions as previously described [15]. The proteins used as molecular weight standards were phosphorylase (94 kD), bovine serum albumin (66 kD), ovalbumin (45 kD), carbonic anhydrase (30 kD), and α-lactalbumin (14 kD).

**Measurement of inhibitory activity of SOTI.** The inhibitory activity of SOTI and the proteolytic activities of trypsin and *P. rapae* proteinases were determined using $N_\alpha$-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) as the substrate [16]. One unit of proteolytic activity was defined as the amount of protein showing an increase of 0.1 absorbance unit at 410 nm, while one unit of inhibitory activity (UI) was defined as the amount of protein showing a decrease of 0.1 absorbance unit. The purified inhibitor samples (i.e. SOTI) were incubated with trypsin or *P. rapae* proteinases at 37°C for 10 min in 0.1 M Tris-HCl (pH 8.0) containing 0.02 M $CaCl_2$. BAPNA was then added to the mixture. After 10 min, the reaction was stopped by the addition of 33% (v/v) acetic acid, and the absorbance was measured at 410 nm.

**Determination of inhibition constant ($K_i$).** Proteinases were incubated with increasing concentrations of purified SOTI in 0.1 M Tris-HCl (pH 8.0) at 37°C, the residual proteolytic activity being measured with appropriate substrates. Apparent values of $K_i$ were determined by fitting the equation for slow-tight binding [17] to the experimental points using a nonlinear regression program integrated in EnzFitter.

**Isoelectric focusing.** Isoelectric focusing was done using a flatbed apparatus (LKB, USA). Ampholine solutions (40%, v/v) with the pH range of 3.5-10.0 were used with subsequent Coomassie brilliant blue G-250 staining. The inhibitors were detected using a negative staining technique according to Uriel and Berges [18].

**Formation of SOTI–trypsin complex.** Purified SOTI and trypsin were incubated at 37°C for 3 h in 0.1 M Tris-HCl (pH 8.0) containing 0.02 M $CaCl_2$. SOTI and trypsin were mixed at a molar ratio of 1 : 1. As a negative control, SOTI and trypsin were mixed in the same buffer without incubation. Laemmli sample buffer without β-mercaptoethanol was added to the samples 5 min before the SDS-PAGE run. The procedures for the electrophoresis and staining are as described by Felicioli [14].

**Stability and chemical resistance of SOTI.** To measure pH stability, 0.2 ml of the inhibitor solution (0.5 mg/ml of SOTI in 0.05 M Tris-HCl containing 20 mM $Ca^{2+}$, pH 8.0) was incubated with 0.8 ml of 0.1 M pH buffers. These buffers were sodium citrate (pH 2.0-4.0), sodium acetate (pH 4.5-5.5), sodium phosphate (pH 6.0-7.0), Tris-HCl (pH 7.5-8.5), and sodium bicarbonate (pH 9.0-11.0). After a 16 h incubation at 4°C, the residual anti-tryptic activity of the solutions was measured in 50 mM Tris-HCl (pH 8.0) containing 20 mM $Ca^{2+}$.

To measure thermal stability, the inhibitor solution (0.1 mg/ml of SOTI in 0.05 M Tris-HCl buffer containing 20 mM $Ca^{2+}$, pH 8.0 at room temperature) was heated in a water bath at various times and temperatures. This solution was then cooled to room temperature before testing for residual inhibitory activity.

The protein inhibitor was modified with 20 mM trinitrobenzene sulfonate (TNBS) [19], cyclohexane-

dione, 4-nitrobenzenesulfonyl fluoride, or diethyl pyrocarbonate [20] for 12 h at pH 6.0, 25°C. To unfold SOTI and reduce the disulfide bond, 6 M guanidine hydrochloride and 20 mM dithiothreitol were used, respectively. After modification, residual inhibitory activity was measured.

**MS/MS (tandem mass spectrometry) and sequencing.** The purified SOTI was subjected under reducing conditions to SDS-PAGE on a 15% acrylamide gel. SOTI fragments were excised, trypsinized, and analyzed on a Micromass Q-TOF Premier (USA) mass spectrometer for sequencing. The MS/MS data were analyzed to deduce the amino acid sequence using online PEAKS software.

**Fluorescence and far-UV CD (circular dichroism) measurements.** Purified SOTI was dissolved in 50 mM Tris-HCl buffer (pH 8.0) to a final concentration of 20-25 μg/ml. The SOTI solution was excited at 280 nm, and the intrinsic fluorescence was measured at 300-400 nm with 5 nm slit widths on a Hitachi F-4500 (Japan) spectrofluorimeter at 25°C. Secondary structure of SOTI was estimated using an AVIA Model 400 CD spectropolarimeter. Purified SOTI was dissolved in 50 mM Tris-HCl buffer (pH 8.0) to a final concentration of 40 μg/ml. Each sample was scanned four times from 186 to 260 nm with a 0.2 cm pathlength.

## RESULTS AND DISCUSSION

**Purification of SOTI.** SOTI was extracted, precipitated by ammonium sulfate (35-75% saturation), and purified by ion-exchange chromatography on DEAE-52, affinity chromatography on trypsin-Sepharose 4B, and gel filtration on Sephadex G-75.

Only one peak from the ion-exchange chromatography showed anti-tryptic activity. Similarly, affinity chromatography and gel filtration also provided one active peak. Affinity chromatography was apparently very effective in isolating this inhibitor. During purification, however, the possibility of limited digestion of SOTI by the immobilized trypsin cannot be excluded. The inhibitor was purified by 63-fold with a yield of 14.9%.

SDS-PAGE showed that SOTI consists of a single polypeptide with a molecular mass of 23 kD (Fig. 1, a and b), which was also confirmed by gel filtration chromatography on Sephadex G-75. Gelatin-PAGE (Fig. 1c) showed that SOTI strongly inhibits trypsin. The procedure used to purify the protein was satisfactory since the purified protein exhibited a single band on SDS-PAGE under reducing conditions. The molecular mass of SOTI (23 kD) indicates its similarity to proteinase inhibitors of the Kunitz family, which generally have molecular masses of 18-24 kD and contain one or two polypeptides [21-23].

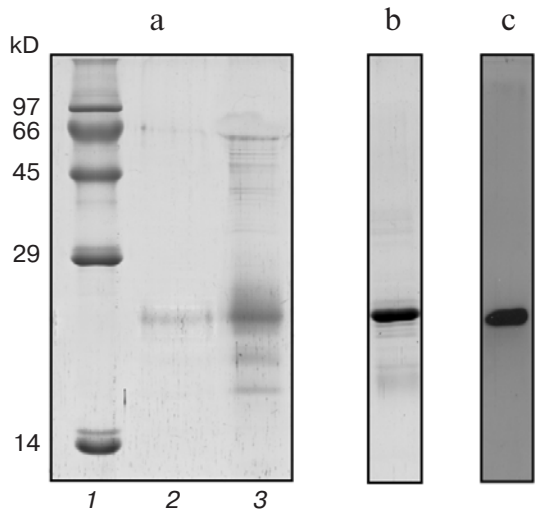


**Fig. 1.** a) SDS-PAGE. Lanes: *1*) markers; *2*) reduced sample after DEAE-52, Sepharose 4B-trypsin, and Sephadex G-75; *3*) reduced crude extract. b) SDS-PAGE: reduced sample after lyophilization. c) Gelatin-PAGE: unreduced sample after lyophilization.

Isoelectric focusing of SOTI revealed p*I* value of 4.05. This result closely resembles that of CSTI [24], a Kunitz-type trypsin inhibitor (purified from *Calliandra selloi* Macbride seeds) that shows a p*I* of 4.0.

**$K_i$ of SOTI for trypsin.** The $K_i$ of SOTI against bovine trypsin was calculated as 2.3 μM using the equation for slow-tight binding inhibition [25]. This $K_i$ value indicates a high affinity between trypsin and SOTI, which is also shown for other plant trypsin inhibitors [26, 27].

**Formation of SOTI–trypsin complex.** The molar ratio of SOTI to trypsin was calculated using the tangent equation from the titration curve as 1.1 (Fig. 2a). SOTI incubated with trypsin in a 1 : 1 molar ratio produced a complex with a mass of approximately 46 kD, based on lane *4* of SDS-PAGE (Fig. 2b). This result was also confirmed when the incubation of the isolated complex with excess trypsin did not yield an additional complex with a mass of 70,000. This experiment suggested that a ternary complex was not formed and confirmed the presence of a single reactive site for trypsin, without hydrolysis products being detected. However, many trypsin inhibitors cannot form a stable complex with trypsin. COTI, another trypsin inhibitor, which we isolated from *Cassia obtusifolia* [28], can only form an inhibitor–trypsin complex in the absence of SDS and reducing agent. Therefore, it could only show a faint band on native PAGE. The stoichiometric ratio and the molecular mass of the SOTI–trypsin complex are similar to those for other Kunitz inhibitors [21-23, 26-30].

Far-UV CD spectra of SOTI, trypsin, and their complex are presented in Fig. 2c. The absence of negative peaks at 209 and 222 nm indicated a lack of helical structure in SOTI. Compared to the inhibitors CSTI and

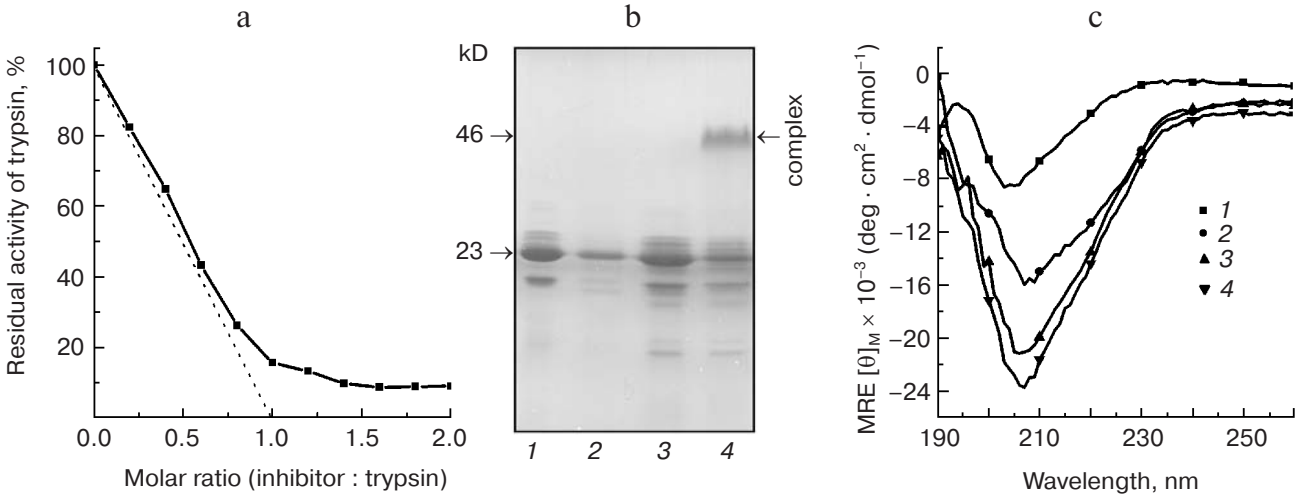


**Fig. 2.** a) Titration curve of trypsin inhibition by the purified inhibitor. The apparent inhibition constant ($K_i$) of inhibitor towards trypsin was determined by pre-incubating trypsin with increasing concentrations of the inhibitor, followed by measurement of the residual activity [33]. Full (100%) activity of trypsin corresponded to 225 U/ml. b) SDS-PAGE of SOTI–trypsin complex. Lanes: *1*) reduced trypsin; *2*) reduced SOTI; *3*) reduced trypsin and SOTI without incubation; *4*) reduced trypsin and SOTI with incubation at 37°C for 3 h. c) Far-UV CD spectra of SOTI, trypsin, and their complex: *1*) SOTI; *2*) trypsin; *3*) SOTI–trypsin complex; *4*) sum of SOTI and trypsin.

SBTI (β-proteins), it was also lacking helixes, rich in β-sheets, and had more random coils in its secondary structure [24, 31]. The CD curve also indicated a change in the conformation during SOTI–trypsin complex formation. Analysis performed on the SOTI CD spectrum by CDPro revealed decreased content of β-sheets and increased content of α-helices, turns and unordered structure in the complex with trypsin (Table 1). On the other hand, X-ray crystallography has shown that complexes formed by Kunitz-type soybean trypsin inhibitor and porcine trypsin have more β-sheet, less α-helix, and less random coil than the SOTI–bovine trypsin complex [32].

**pH stability of SOTI.** SOTI displayed high stability at pH values ranging from 3.0 to 11.0 at 4°C (Fig. 3a). In fact, the residual anti-tryptic activity was still higher than 80% despite an incubation period of 12 h in buffer of any tested pH value.

The high stability of SOTI against pH changes seems to be a common feature for inhibitors from the Kunitz and Bowman–Birk families. The reported pH stable inhibitors included those isolated from *Canavalia lineata* [35], the thermostable trypsin and chymotrypsin inhibitor from *Vigna unguiculata* [36, 37], the chymotrypsin inhibitor isolated from *Schizolobium parahyba* seeds [38], the nine Kunitz and Bowman–Birk protease inhibitors isolated from wild soya (*Glycine soja*) seeds [9], the Kunitz-type inhibitor purified from *Peltophorum dubium* seeds [39], and the Bowman–Birk inhibitor from *Phaseolus aureus* [40].

**Thermostability of SOTI.** As thermostable proteins, many trypsin inhibitors can retain most of their activities against trypsin at 50-60°C. We measured the activity of SOTI at 25°C after incubation at 40-100°C for 20 min and found little loss of activity. Moreover, the analysis of the CD spectrum of SOTI at different temperatures has shown

**Table 1.** Secondary structure of SOTI, trypsin, and their complex

| Structure, % | SOTI | Trypsin | Complex | Calculation |
|---|---|---|---|---|
| α-Helix, % | 16.4 | 59.7 | 13.0 | 15.0 |
| β-Sheet, % | 60.6 | 17.2 | 10.4 | 11.8 |
| Turns, % | 21.0 | 24.8 | 19.7 | 36.7 |
| Unordered, % | 56.7 | 21.9 | 10.4 | 11.8 |

Note: Percentage of each secondary structure was estimated using the CDPro software with reference data set 9 (50 proteins) [34]. The values in the column "Calculation" were computed from the superposition of curves of SOTI and trypsin by CDPro.

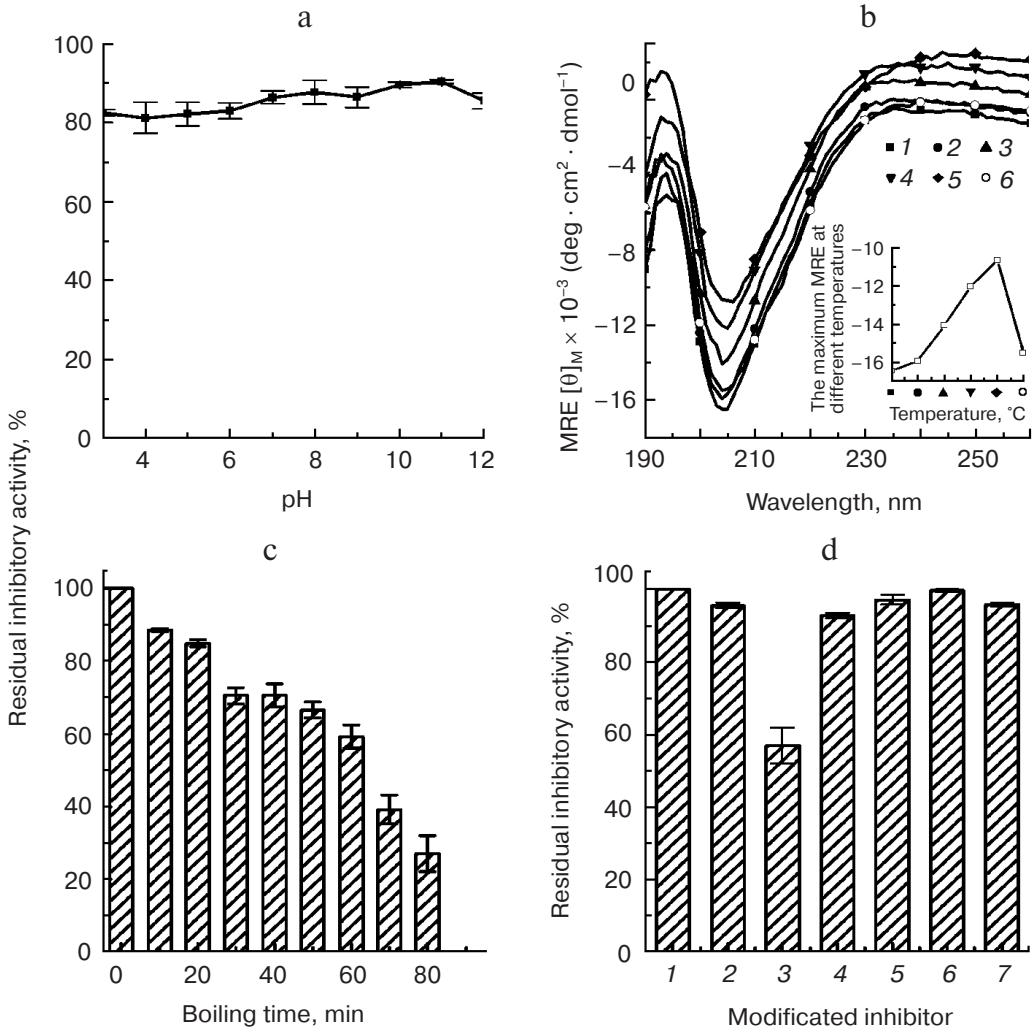


**Fig. 3.** a) pH stability of SOTI. b) Far-UV CD spectra of SOTI at different temperatures (°C): *1*) 25; *2*) 20; *3*) 40; *4*) 60; *5*) 80; *6*) after return to 25°C. c) Thermal stability of SOTI. d) Effect of chemical modification and denaturants on inhibitory activity of SOTI: *1*) native SOTI; *2*) arginine modified; *3*) lysine modified; *4*) tyrosine modified; *5*) histidine modified; *6*) GuHCl denaturant; *7*) reduced by dithiothreitol.

that the amount of β-sheet and turn decreased and the amount of α-helix and unordered structure increased gradually with increasing temperature (Table 2). However, it was found that the CD curve of heated SOTI resembled the curve of native SOTI when the temperature was returned to 25°C (Fig. 3b). Interestingly, $\lambda_{min}$ (wavelengths with minimum mean residue ellipticity) had no shift when the temperature was decreased from 80 to 25°C, and the minimum mean residue ellipticity was near the value of unheated SOTI. This result indicated that the changes of inhibitor conformation upon heating were reversible. Further experiments have shown that the inhibitor retained more than 80% of its activity when incubated at 100°C for 20 min, but there was a 40% loss of activity in 60 min and an 80% loss in 80 min (Fig. 3c). Therefore, all of these results suggest that SOTI has high thermostability.

In the past, inhibitors of Bowman–Birk family were believed to be more stable to heat than those belonging to Kunitz family. It was reported that the former retained their full inhibitory activity even after being heated at 90°C for 60 min [40, 41]. A possible explanation for the difference in heat stability between the two inhibitor families is their cysteine content. In fact, while the Kunitz inhibitors typically contain two disulfide bonds, those belonging to Bowman–Birk family are stabilized by up to eight disulfide bonds [22]. But SOTI, which is thought to be a Kunitz inhibitor, showed higher heat stability than those inhibitors belonging to the Bowman–Birk family reported before. To explain this, more experiments (such as X-ray crystallography) concerning the conformation and structure of SOTI needs to be carried out.

**Chemical modifier and denaturant stability of SOTI.** Chemical modification was carried out to determine essential groups for the inhibitory activity of SOTI. The inhibitory activity against trypsin by SOTI treated by

**Table 2.** Secondary structure of SOTI at different temperatures

| Temperature, °C | α-Helix, % | β-Sheet, % | Turns, % | Unordered, % |
|---|---|---|---|---|
| 25 | 16.4 | 59.7 | 13.0 | 15.0 |
| 20 | 18.1 | 58.6 | 11.2 | 12.5 |
| 40 | 20.9 | 54.0 | 13.0 | 12.8 |
| 60 | 28.4 | 49.5 | 10.9 | 11.3 |
| 80 | 20.9 | 47.3 | 10.7 | 15.9 |
| Return to 25 | 17.3 | 52.3 | 15.0 | 15.6 |

TNBS (to modify lysine) was reduced by 43% (Fig. 3d). Other chemical modifications of SOTI and 6 M guanidine hydrochloride had little effect on its activity. Consistent with this result, many trypsin inhibitors that we found with NCBI Protein Blast search program contain lysine in their active sites [42-44].

The intramolecular disulfide bridges have been believed to be responsible for the functional stability of Kunitz-type inhibitors. However, dithiothreitol had little effect on the activity or stability of SOTI, in contrast to the inhibitor from *Erythrina caffra* [45] and COTI from *Cassia obtusifolia* [28]. Hence, the stability of SOTI may not be due to the presence of disulfide bridges.

**Anti-insect activity of SOTI.** The inhibitory activities of SOTI to midgut extract from *P. rapae* larvae were determined by *in vitro* enzyme assays. The results have shown that SOTI inhibited 50% of the proteolytic activity of midgut extract at a concentration of 20 μg/ml. In comparison, BBI (soybean Bowman–Birk trypsin inhibitor) inhibited 30% of the activity at 200 μg/ml (Fig. 4). However, considering its 8 kD molecular weight, BBI should have a higher inhibitory activity against trypsin than SOTI if they were at the same molar concentration. Since SOTI displays more inhibitory activity towards proteolytic enzymes in midgut extract of *P. rapae* larvae, it shows more potential as a bio-pesticide.

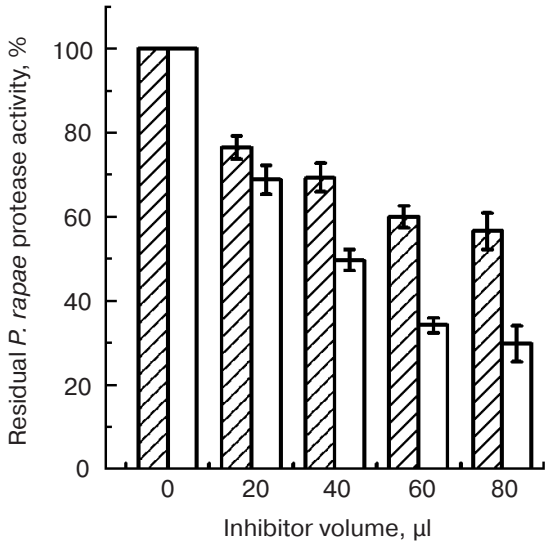


**Fig. 4.** Inhibitory activities of SOTI and BBI towards proteolytic enzymes of midgut extract from *P. rapae* larvae. Hatched columns, SOTI (20 μg/ml). Open columns, BBI (200 μg/ml). Full (100%) activity of *P. rapae* proteases corresponded to 170 U/ml.

**MS/MS and partial sequencing.** Sequences of some SOTI fragments were determined from MS/MS data (Fig. 5a). Given the sequences of several candidate fragments, the NCBI Protein Blast search program showed similarity between SOTI and other inhibitors. Multiple sequence alignments of SOTI and its related sequences were carried out using the DNAMAN program with the default parameters. The multiple alignments were manually adjusted if necessary. One of the SOTI peptides, with *m/z* 445.7997 z 2 (TAVVCNSPK), could be found in a Kunitz-type proteinase inhibitor from human serum and urine [46]. Another protein sequence found in a trypsin inhibitor from winged bean seeds (*Psophocarpus tetragonolobus* L.) was similar to the peptide *m/z* 587.3015 z 2 (VGLDSDAELVR) (Fig. 5b) [47].

In summary, the results presented in the present study demonstrate that SOTI can form a stable complex with trypsin. This protein also displays remarkable stability to heating over a wide pH range, chemical modifications, and denaturants. The inhibitor shows high stability under alkaline conditions, which resembles the pH environment in midgut of many insects, suggesting that SOTI could be used for pest control. However, since inhibitors to proteases can prevent the digestion and absorption of proteins by animals, SOTI is considered as a kind of antinutrition agent. Moreover, SOTI is difficult to denature even after it is boiled or cooked because of its remarkable thermal stability. Therefore, when considering transferring SOTI genes to plants as a pest control method, one must be cautioned that SOTI is only suitable for non-food crops (such as cotton).

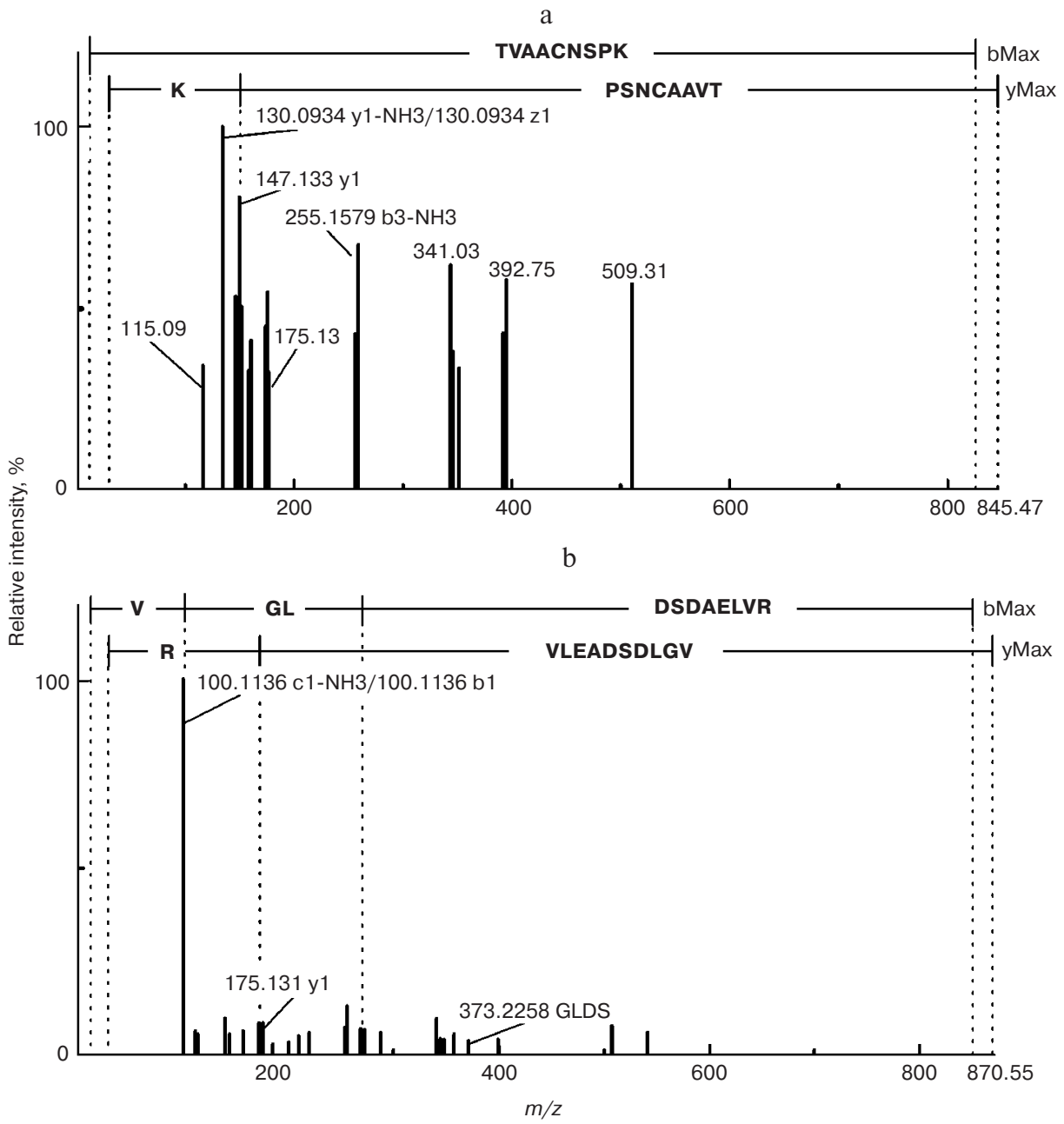


**Fig. 5.** The *de novo* sequences of SOTI fragments calculated by PEAKS. a) Sequence TAVVCNSPK of the ion *m/z* 445.7997 z 2. b) Sequence VGLDSDAELVR of the ion *m/z* 587.3015 z 2.

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