Purification, Amino Acid Sequence, and cDNA Cloning of Trypsin Inhibitors from Onion (Allium cepa L.) Bulbs

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Three protease inhibitors (OTI-1–3) have been purified from onion (Allium cepa L.) bulbs. Molecular masses of these inhibitors were found to be 7,370.2, 7,472.2, and 7,642.6 Da by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), respectively. Based on amino acid composition and N-terminal sequence, OTI-1 and -2 are the N-terminal truncated proteins of OTI-3. All the inhibitors are stable to heat and extreme pH. OTI-3 inhibited trypsin, chymotrypsin, and plasmin with dissociation constants of $1.3 \times 10^{-9} \text{M}$, $2.3 \times 10^{-7} \text{M}$, and $3.1 \times 10^{-7} \text{M}$, respectively. The complete amino acid sequence of OTI-3 showed a significant homology to Bowman-Birk family inhibitors, and the first reactive site (P1) was found to be Arg17 by limited proteolysis by trypsin. The second reactive site (P1') was estimated to be Leu46, that may inhibit chymotrypsin. OTI-3 lacks an S-S bond near the second reactive site, resulting in a low affinity for the enzyme. The sequence of OTI-3 was also ascertained by the nucleotide sequence of a cDNA clone encoding a 101-residue precursor of the onion inhibitor.

Key words: amino acid sequence; cDNA cloning; onion bulbs; proteinase inhibitor; purification

Materials and Methods

Materials. Onion bulbs cultivated in Hokkaido were purchased commercially and stored at 4°C. Bovine trypsin, TLCK-treated bovine $\alpha$-chymotrypsin, alkaline protease, subtilisin BPN’, papain, porcine pepsin, and pronase E were obtained from Sigma Chem. Co. (St. Louis). Restriction enzymes and arginylendopeptidase were from Takara (Tokyo). The PCR primers were custom-synthesized by Hokkaido System Science. pBluescript II SK+ was from Stratagene. All the amino acid and peptide analogs were obtained from the Peptide Institute, Inc. (Osaka). All other chemicals were from Wako Chem. Co. (Osaka).
**Column chromatography.** Open column operations were done at 4°C. Anion-exchange chromatography was done on columns of DEAE-Toyopearl 650M (2.6 × 30 cm, Tosoh) and SuperQ-Toyopearl 650S (2.6 × 15 cm, Tosoh) equilibrated with 50 mM Tris-HCl (pH 8.0). A sample was put on, and eluted with a linear gradient of 0–0.3 M NaCl in the same buffer. Proteins were detected by the absorption at 280 or 220 nm on an Uber-30 spectrophotometer (Jasco). Reverse-phase HPLC was done on TSK-gel ODS-120T (0.46 × 25 cm, Tosoh) column with a linear gradient of acetonitrile from 0 to 40% in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min, and elution was monitored at 220 nm. Trypsin inhibitory activity was monitored as described previously.6

**Thermal and pH stability.** Thermal stability of inhibitors was tested in 50 mM Tris-HCl (pH 8.0). The inhibitor solution (50 µg/ml) was heated in a water bath at 80 to 100°C for 10–60 min, and cooled in ice. The residual trypsin inhibitory activities were then measured using 0.375 mM Bz-L-Arg-pNA in 10 mM CaCl₂-50 mM Tris-HCl (pH 8.0) at 25°C. Stability of inhibitors was examined in buffers of various pHs. Twenty-five µl of inhibitor solutions (500 µg/ml) were diluted with 100 µl of various buffers (20 mM each): KCl–HCl (pH 1 and 4), Tris-HCl (pH 8), and glycine-NaOH (pH 10 and 13) buffers. The mixtures were kept at 37°C for 1 h and neutralized with 125 µl of 1 mM CaCl₂-50 mM Tris-HCl (pH 8.0). The residual inhibitory activities were determined as described above.

**Kinetic measurements.** For the kinetic measurement, Bz-L-Arg-pNA, Suc-Leu-Leu-Val-Tyr-pNA, and Boc-Glu-Lys-Lys-MCA were used as substrates for trypsin (3.5 × 10⁻⁸ M), chymotrypsin (2.7 × 10⁻⁸ M), and plasmin (3.5 × 10⁻⁸ M), respectively. For MCA substrates, the increase in fluorescence was measured with a FP-550A spectrofluorometer (Jasco) at 440 nm with excitation at 360 nm. The dissociation constant, Kᵅ, for the inhibitor-enzyme complex was calculated by the methods of Green and Work,8 or Henderson.9

**Electrophoresis.** SDS-PAGE was done in the presence of 2-mercaptoethanol using a 10% polyacrylamide slab gel (3% cross-linking) and 0.1% SDS-0.1 M Tris-0.1 M tricine (pH 8.25) buffer system as described by Schagger and von Jagow.10 Proteins were detected by staining the gel with 0.1% Coomassie brilliant blue G-250 in 5% perchloric acid. The isoelectric point (pI) was calculated from a calibration curve obtained by relative mobilities of several marker proteins (pI 4.75–6.45).

**Molecular mass analysis.** Mass spectrum was measured on a Voyager DE-STR MALDI-TOF-mass spectrometer (PerSeptive Biosystems). Approximately 50–80 pmol of inhibitor was dissolved in 10 µl of 0.1% trifluoroacetic acid-50% acetonitrile containing α-cyano-4-hydroxycinnamic acid (10 mg/ml) as the matrix, and 4-µl samples were analyzed. The spectrum was calibrated by the molecular mass of apomyoglobin.

**Sequence analysis.** S-Pyridylethylthiolation was done according to Friedman et al.12 Pe-inhibitor was digested with *Achromobacter* protease I (E/S = 1:50) at 37°C for 6 h in 20 mM Tris-HCl (pH 9.0), or with BrCN (100 equivalents) at room temperature for 24 h in 70% formic acid. A large fragment obtained from BrCN-cleavage was further digested by arginylen-dopeptide (E/S = 1:50) at 37°C for 6 h in 20 mM Tris-HCl (pH 9.0). The digests were lyophilized, dissolved in 0.1% TFA, and then fractionated by reverse-phase HPLC on a TSK-gel ODS-120T column (0.46 × 25 cm, Tosoh) in 0.1% TFA with an appropriate gradient of acetonitrile. The amino acid sequences of the peptides were analyzed by automatic protein sequencing system (PPSQ 21, Shimadzu).

**Limited proteolysis.** OTI-3 (500 µg) was incubated with trypsin (78 µg) in 130 µl of 50 mM citrate buffer (pH 3.0) containing 10 mM CaCl₂ at 37°C for 4 h. The modified inhibitor was purified by reverse-phase HPLC on a Cosmosil 5C₈-A R-300 column (0.46 × 15 cm, naicalai tesque).

**cDNA cloning.** Onion bulbs were homogenized and the RNA was extracted with an RNeasy Plant Mini kit (Qiagen). Total RNA was reverse-transcribed into cDNA using an adaptor-linked oligo (dT) primer (5’-GGCCACGCGTCACTAGTCGACTAGTA-3’). The partial nucleotide sequence of OTI cDNA was amplified from onion bulb cDNA by PCR using an adaptor primer (5’-GGCCACGCGTCACTAGTCGACTAGTA-3’) and a degenerate primer 5’-TGTYGGAGYWSNTGNYGNWNTGNYGAY-3’ which corresponds to the amino acid sequence of Cys₅-Asp₁₆ in OTI-3. The 0.4-kb fragments obtained were used as a probe for standard plaque hybridization. An *Allium cepa* cDNA library was constructed using a SMART cDNA library construction kit (Clontech). After λTriplEx2 phage clones containing full-length OTI cDNAs were purified, they were converted to plasmids by cre-lox recombination.10 Finally, full-
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length cDNAs subcloned into pTriplEx2 were isolated. Nucleotide sequences of randomly selected clones were analyzed with an ABI Prism 377 DNA sequencer (Applied Biosystems).

Results

Puriﬁcation of inhibitors

Onion bulbs (3.5 kg) were homogenized in 0.05% L-ascorbic acid-50 mM Tris-HCl (pH 7.6) and the extract was centrifuged at 14,000 × g for 20 min. The supernatant was brought to 80% saturation with (NH₄)₂SO₄. The precipitate was collected and dialyzed against 50 mM Tris-HCl (pH 7.6). The solution was put on a DEAE-Toyopearl 650M column. Trypsin-inhibitory activity was eluted in the second peak (Fig. 1A). This was then put through HPLC on a TSK-gel ODS-120T column (Fig. 1B). Since the product gave three major and some minor bands on isoelectric focusing, it was further puriﬁed by anion-exchange chromatography on a SuperQ-Toyopearl 650S column, giving three inhibitors (OTI-1–3, Fig. 1C). Yields of OTI-1, 2, and 3 were 0.6, 1.2, and 1.1 mg from 3.5 kg of onion bulbs, respectively.

The purity and molecular mass of these preparations were examined by SDS-PAGE. Each inhibitor gave a major band of 18 kDa and a faint band of 8 kDa in the absence of 2-mercaptoethanol (Fig. 1D). On the other hand, only 8-kDa bands were observed in the presence of a reducing agent. Molecular masses of OTI-1–3 measured by MALDI-TOF-MS were 7,370.2, 7,472.2, and 7,642.6 Da, respectively. Isoelectric points were estimated to be 5.8, 5.2, and 4.6 for OTI-1, 2, and 3, respectively.

Thermal and pH stability

Thermal stability was examined at pH 8.0. Trypsin inhibitory activities of three OTIs were retained even after the solution was heated at 100°C for 10 min (data not shown). On the other hand, the activities of OTI-1 and 2 decreased upon prolonged incubation at 80–100°C for 60 min (Fig. 2A).

All the inhibitors were quite stable in several extreme pH solutions. After standing at 37°C for 1 h,
the three inhibitors retained the full trypsin inhibitory activity (Fig. 2B). These observations agree with the fact that most of the BBI type inhibitors are quite stable to pH and heat.5,6,15)

**Inhibitory property**

All the inhibitors strongly inhibited trypsin (Fig. 3A). The stoichiometry of OTI-3 and trypsin was 1:1. α-Chymotrypsin and plasmin were also inhibited, but weakly (Fig. 3A and B). We examined the effects of onion inhibitors on elastase, subtilisin BPN’, bacterial alkaline protease, *Achromobacter* protease I, arginylendopeptidase, thrombin, papain, pepsin, thermolysin, and pronase E. However, none of these enzymes was inhibited by the inhibitors (data not shown).

The approximate dissociation constants ($K_d$) of OTI-3-enzyme complexes were $1.3 \times 10^{-9}$ M, $2.3 \times 10^{-7}$ M, and $3.1 \times 10^{-7}$ M for trypsin, chymotrypsin, and plasmin, respectively. The $K_d$ value for trypsin was not influenced by the presence of a 4-molar excess of chymotrypsin (data not shown). This indicates that the anti-tryptic and anti-chymotryptic sites are independent of each other and these enzymes bind different regions of OTI.

**Amino acid sequences of onion inhibitors**

Amino acid analysis of OTIs showed similar compositions to one other (Table 1). High contents of
half-Cys residues, characteristic of Bowman-Birk family inhibitors, were observed. Three inhibitors were S-pyridylethylated (Pe) and the N-terminal sequences were analyzed by direct Edman degradation. They showed a similar sequence except the length of the N-terminal three residues (Fig. 4A).

The complete amino acid sequence of OTI-3 was analyzed. Pe-OTI-3 was cleaved chemically by BrCN or digested enzymatically by Achromobacter protease I (K), BrCN (M), and arginylendopeptidase (R) are shown below the sequence. (C) Sequences of Pe-OTI-2-derived peptides by BrCN cleavage.

Identification of anti-tryptic site

Limited tryptic hydrolysis of OTI-3 at pH 3 gave a modified inhibitor where the peptide bond at the trypsin reactive site is cleaved. The modified inhibitor was directly put through amino acid sequence analysis, yielding a sequence of RAP-DLARXE—together with the N-terminal sequence of GEEEGGX-XD. Thus, we could identify the trypsin reactive site to be Arg17–Arg18. A chymotrypsin reactive site could not be identified in a similar way. This may reflect the lower affinity of OTI for this enzyme.

Cloning of cDNA of onion inhibitor

To confirm the complete sequence of OTI-3, we have cloned the cDNA encoding the inhibitor. A degenerate oligonucleotide was designed according to the N-terminal part of three OTIs, and a partial fragment of OTI cDNA was obtained by a 3'-RACE reaction with onion bulb mRNA. The amplified DNA fragment was confirmed to encode OTI and used for screening of a cDNA library constructed from the same mRNA source. More than 2,000 positive clones were detected out of 2 × 10^6 clones screened, and five clones, named cOTI-1 to -5, were used for sequence analysis. The nucleotide sequences of these clones were completely identical to each other except for two nucleotides adjacent to the polyadenylation site. The nucleotide sequence of cOTI-1 is shown in Fig. 5. The open reading frame encodes 101 amino acids, and the N-terminal 31 residues are the propeptide. Its deduced amino acid sequence is consistent with the sequences of purified inhibitors. OTI-1, 2, and 3 start from Glu35, Glu34, and Gly32 of the precursor protein (indicated by arrowheads in Fig. 5), respectively.

Discussion

Here we present the first report on the purification of BBI-family inhibitors from a Liliaceae plant. Three trypsin inhibitors (OTI-1–3) have been purified to homogeneity. SDS-PAGE analysis of these inhibi-
Fig. 6. Alignment of Amino Acid Sequences of Bowman-Birk Inhibitors.

(a) soybean C-II, (b) soybean D-II, (c) Canavalia lineata II, (d) Phaseolus angularis I, (e) soybean BBI, (f) Vicia angustifolia, (g) peanut A-II, (h) Job’s tears, (i) Foxtail millet II, (j) Common wheat. Two reactive sites are boxed. Half-cystine residues are shaded.

The amino acid composition (Table 1) and N-terminal residues (Fig. 4A) have suggested that three OTIs have almost the same amino acid sequences. However, the N-terminal sequence of OTI-1 and 2 are different from OTI-3 due to limited proteolysis at the N-terminals. Isoinhibitors with different N-terminal lengths have also be reported in many BBIs. Because the truncated form of OTI-1 and 2 were less stable at high temperature (Fig. 2A), the N-terminal two residues (Gly-Asp) have a role in stabilizing the protein. Nevertheless, we could not detect any difference in their inhibitory activity on trypsin.

In Fig. 6, the amino acid sequences of several BBIs from various plants are compared. OTI-3 showed a significant sequence homology with other inhibitors (25–33% identity). The locations of 14 Cys residues in Leguminosae inhibitors are completely preserved (a–g in Fig. 6). On the other hand, only 10 or 12 Cys residues in inhibitors from Gramineae plants (h–j) as well as onion are preserved, and two Cys residues around the second reactive site are missing. This seems to be a common feature for BBIs from monocotyledonous plants such as Gramineae and Liliaceae.

From the first and second reactive sites of other inhibitors, the reactive sites (P) for trypsin and chymotrypsin of OTI was estimated to be Arg and Leu, respectively. The anti-trypsic site was directly confirmed by a limited proteolysis of OTI-3 with trypsin. However, we could not determine the second reactive site in a similar manner. This may be due to the low affinity of this site for chymotrypsin, and an equilibrium between virgin and modified inhibitors is not directed toward the limited proteolysis. The lack of an S-S linkage near the second reactive site and the unique insertion on Asp residue just before the second reactive site are not in favor of tight binding of OTI to the active site of chymotrypsin. Having no convincing data about the plasmin inhibition site, we can assume that the first reactive site is also an inhibitory site for plasmin with respect to the substrate specificity.

The amino acid sequence of OTI was further confirmed by a cDNA cloning experiment (Fig. 5). The open reading frame encoded 101 amino acids with a 31-residue pro-sequence. Three onion iso inhibitors seemed to be derived from a single OTI gene. Although Southern blot analysis was done to find the copy number of OTI gene in the onion genome, we could not obtain clear results, presumably due to the largeness of the onion genome; it is generally believed that the genome sizes of Liliaceae plants are very large, as the genome of the fritillary is estimated to be approximately 40 times larger than that of a human. Because we could not isolate any other clones that encode different OTI or OTI-like sequences during the screening experiment, we concluded that onion contains only one BBI gene. OTIs were thus derived by the cleavage at different sites from a common precursor.

The physiological advantage of the production of multiple iso inhibitors to this plant is not clear. OTIs are unique in the respect that they were purified from the bulb, a tissue for nutrition storage lying underground, although their primary structures show a classical aspect of BBI-family protein. Therefore, OTIs may play a protective role against feeding by nematodes or soil-living larvae, in addition to the roles proposed previously, including the regulation of endogenous proteinases and the storage of sulfur amino acids.

References

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