Serine Proteinase Inhibitor from Wax Gourd
(Benincasa hispida [Thunb] Cogn.) Seeds

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Received October 3, 2005; Accepted November 23, 2005

Two squash family protease inhibitors were obtained from wax gourd (Benincasa hispida [Thunb] Cogn.). Even though they were distinctly separated by reversed-phase chromatography, the amino acid sequences of two inhibitors were identical. Both inhibitors were converted into each other, perhaps due to cis-trans isomerization of characteristic Pro in the C-terminal region.

Key words: serine protease inhibitor; Benincasa hispida; wax gourd; squash

The squash family inhibitors consist of 28–30 amino acid residues, including three disulfide bridges.1) Inhibition of serine proteinases in this family follows the canonical standard mechanism. Since squash family inhibitors inhibit biologically important molecules such as trypsin, activated Hageman factor,2) human leucocyte elastase, and cathepsin G,3) they appear to be a helpful tool for studying the interaction between proteinases and inhibitors. They should also make possible a variety of applications because of their low molecular weight. The three-dimensional structure of the complex of trypsin and Cucurbita maxima trypsin inhibitor I (CMTI-I)4) and the solution structure of CMTI-I5) have been investigated by X-ray crystallography and NMR respectively. Chemical syntheses of squash family inhibitors6,7) have created powerful tools for the investigation of their function-structure relationship.

Benincasa hispida (Thunb) Cogn., belonging to the family Cucurbitaceae, is a vegetable used widely in Thailand, India, and other semi-tropical countries. In Ayurveda, wax gourd is recommended for management of peptic ulcer, hemorrhages from internal organs, and epilepsy and other nervous disorders.8) Since these diseases are related to serine proteinases, the inhibitors might play important roles in controlling diseases.9–11) Here we report the structures and inhibitory activities of serine protease inhibitors from wax gourd seeds planted locally in Thailand.

Serine protease inhibitors were extracted from dried seeds of wax gourd purchased at Khonkaen Province, Thailand. After removal of seed coats, seeds were homogenized in acetone and washed twice with five volumes (w/v) of acetone to remove fat. Defatted seed powder (9.65 g) was extracted with 100 ml of 5 mM Tris-HCl buffer, pH 8, for 2 h at room temperature with mechanical stirring. The supernatant was recovered by centrifugation (6,000 × g, 20 min). The sediment was re-extracted by the same procedure. The crude extract was heated at 100°C for 10 min to remove heat-labile protein as precipitate. The supernatant collected by centrifugation was adjusted pH to 5 with HCl, then re-centrifuged. The extract obtained was applied on a SP-Toyopearl column (1.5 × 22 cm; Tosoh, Tokyo, Japan) equilibrated with 10 mM sodium acetate buffer, pH 5.0, and then eluted with a linear gradient of 0–2 M NaCl in the same buffer. Thus one trypsin inhibitor fraction was obtained.

The trypsin inhibitor fraction was further separated into two active fractions, BHTI-I and BHTI-II (Benincasa hispida trypsin inhibitor-I and II), by reversed-phase HPLC (column, Protein and Peptide C18; 4.6 × 250 mm; Grace Vydac, Columbia, MD, USA), as shown in Fig. 1. The yields of BHTI-I and II were 33.6 nmol and 26.5 nmol respectively.

Using a gas-phase peptide sequencer (PPSQ-10 Shimadzu, Kyoto, Japan), the amino-terminal sequences of BHTI-I and II were determined up to the 20th amino acid. Both inhibitors were digested with lysyl endopeptidase (Takara Bio, Shiga, Japan), after they were reduced and S-alkylated. The amino acid sequences of N-terminal and C-terminal peptides of BHTI-I and II were determined as follows: N-terminal peptides of BHTI-I and II, RRCPRIYMECK; and C-terminal peptides of BHTI-I and II, HDSDCLADCVCLPGICG. As shown in Table 1, the amino acid sequences of BHTI-I and BHTI-II were identical. The mass numbers measured by TOF mass spectrometry (REFLEX-III, Bruker Daltonics Inc., Billericia, MA, USA) were 3280.03 for BHTI-I and 3280.06 for BHTI-II, which coincided with the expected one, 3279.91, from amino acid sequences.

By re-chromatographies on the same reversed-phase column of each inhibitor, BHTI-I was separated into BHTI-I and BHTI-II (Fig. 2A), and BHTI-II was also separated into BHTI-I and BHTI-II (Fig. 2B).
suggests that BHTI-I and II are converted into each other and exist as a mixture in the equilibrium state, even though the two inhibitors have identical amino acid sequences. No such exchange between two conformers has been reported in other squash family inhibitors. It has been reported that some peptides containing proline take two exchangeable conformers due to cis-trans conversion of proline, and that the two conformers can be separated by reversed phase chromatography.12–14) Therefore, the conversion between BHTI-I and II might be caused by cis-trans isomerization of Pro(24), which is characteristic of BHTI-I and II.

Comparison of the sequences of BHTI-I and II with those of known squash trypsin inhibitors available in the BLAST database revealed the highest homology score, 92% identity with LLTIs (trypsin inhibitors extracted from Lagenaria leucantha Rusby, var. Gourda Makino seeds) and LLDTIs (trypsin inhibitors extracted from Lagenaria leucantha Rusby, var. Depressa Makino seeds) (Table 1).

Table 1. Amino Acid Sequences, \( K_i \) Values of Trypsin Inhibitor from Wax Gourd Seeds, and Amino Acid Sequence Homology of Squash Family Inhibitors

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Amino Acid Sequence</th>
<th>( K_i ) (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BHTI-I</td>
<td>RRCPRIYMECKHDSCLADVCVCLPDGECG</td>
<td>7.8 \times 10^{-10}</td>
</tr>
<tr>
<td>BHTI-II</td>
<td>RRCPRIYMECKHDSCLADVCVCLPDGECG</td>
<td>9.6 \times 10^{-10}</td>
</tr>
<tr>
<td>LLTI-II(10)</td>
<td>RRCPRIYMECKHDSCLADVCVCLPDGECG</td>
<td>6.5 \times 10^{-11}</td>
</tr>
<tr>
<td>LLTI-III(10)</td>
<td>RRCPRIYMECKHDSCLADVCVCLPDGECG</td>
<td>3.0 \times 10^{-11}</td>
</tr>
<tr>
<td>LLDTI-I(11)</td>
<td>RRCPRIYMECKHDSCLADVCVCLPDGECG</td>
<td>2.4 \times 10^{-10}</td>
</tr>
<tr>
<td>CMTI-I(4)</td>
<td>RVCPRILMECKHDSCLADVCVCLPDGECG</td>
<td>* in this study; -E, pyroglutamic acid; arrow, reactive site.</td>
</tr>
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Fig. 1. Reversed-Phase Chromatography of Inhibitor Fraction Obtained by Cation-Exchange Chromatography.
Inhibitor fraction obtained by cation-exchange chromatography was applied on reversed-phase column (Proteins and Peptides C18, 4.6 x 250 mm, Grace Vydac, Columbia, MD, USA) equilibrated with 0.1% trifluoroacetic acid. Adsorbed proteins were eluted by a 40-min linear gradient of acetonitrile from 0% to 40% in equilibrating solution at 1 ml/min flow rate. Chromatography was performed using HPLC (model L-7100, Hitachi, Tokyo, Japan), and proteins were detected by absorbance at 230 nm. Two inhibitor fractions, BHTI-I and BHTI-II, were obtained.

Fig. 2. Reversed-Phase Chromatographies of BHTI-I and II.
BHTI-I (A) and II (B) were re-chromatographed under the conditions described in Fig. 1. Inhibitors were detected by absorbance at 215 nm.
The trypsin inhibitory activities of BHTI-I and II were titrated in 50 mM Tris buffer, pH 8.0, using N-alpha-benzoyl-l-arginine ethyl ester (Peptide Institute, Osaka, Japan) as a substrate. Briefly, bovine pancreas trypsin, of which the activity was titrated preliminarily with a known amount of purified BHTI, was incubated with various amounts of BHTI in 2.0 ml of reaction buffer for 4 min at 30 °C to form the enzyme-inhibitor complex. The reaction was started by adding of 100 µl of 10 mM substrate. Degradation of the substrate was monitored by measuring absorbance at 253 nm using a spectrophotometer (U-3300 Hitachi, Tokyo, Japan). Trypsin inhibitory activities were expressed in terms of \( K_i \) calculated according to Green and Work.10 The \( K_i \) values of BHTI-I and II were 7.8 × 10^{-10} M and 9.6 × 10^{-10} M, respectively. These values are almost the same as expected, since BHTI-I and II exist as a mixture in the equilibrium state, perhaps due to conversion between the two conformers. But the inhibitory activity of BHTI-I was slightly stronger than that of BHTI-II.

The trypsin inhibitory activities of BHTIs were compared with those of other squash family inhibitors of which the \( K_i \) values were determined under the same conditions in our laboratory. As shown in Table 1, the trypsin inhibitory activity of BHTI-I was lower than that of LLTI-II. The \( K_i \) values of BHTI-I and II were about 12-fold and 15-fold larger than that of LLTI-II, even though the amino acid sequences of BHTI-I and LLTI-II differ in only two amino acids, at the 24th and 25th positions. The crystal structure of the complex of CMTI-I and trypsin indicated that the primary binding segment of CMTI-I to trypsin is Arg(1) to Glu(9), and the second His(25) to Gly(29). The amino acid sequence of CMTI-I is shown in Table 1. Since two amino acids, Pro(24) and Gln(25), are located near the secondary binding segment, both amino acids, especially Pro(24), might affect the inhibitory activity of BHTI.

References