Isolation and amino-acid sequence of two inhibitors of serine proteinases, members of the squash inhibitor family, from *Echinocystis lobata* seeds*

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Two serine proteinase inhibitors (ELTI I and ELTI II) have been isolated from mature seeds of *Echinocystis lobata* by ammonium sulfate fractionation, methanol precipitation, ion exchange chromatography, affinity chromatography on immobilized anhydrotrypsin and HPLC. ELTI I and ELTI II consist of 33 and 29 amino-acid residues, respectively. The primary structures of these inhibitors are as follows:

**ELTI I** KEQRVCPRLMRCKRDSDCAQCTCQKQSGFCG
**ELTI II** RVCPRLMRCKRDSDCAQCTCQKQSGFCG

The inhibitors show sequence similarity with the squash inhibitor family. ELTI I differs from ELTI II only by the presence of the NH₂-terminal tetrapeptide Lys-Glu-Glu-Gln.

The association constants ($K_a$) of ELTI I and ELTI II with bovine-trypsin were determined to be $6.6 \times 10^{10} \text{ M}^{-1}$, and $3.1 \times 10^{11} \text{ M}^{-1}$, whereas the association constants of these inhibitors with cathepsin G were $1.2 \times 10^7 \text{ M}^{-1}$, and $1.1 \times 10^7 \text{ M}^{-1}$, respectively.

Among small protein inhibitors of serine proteinases the squash inhibitors, isolated exclusively from squash plants, are the smallest ones. They are built of 27 to 33 amino-acid residues and each is cross-linked by three disulfide bridges. They were discovered by the end of 1979 [1]. In spite of their small size, association constants ($K_a$) for the interaction of the squash inhibitors with β-trypsin are among the highest for trypsin inhibitors (in the range of $10^{11} - 10^{12} \text{ M}^{-1}$) [2]. Over 40 representatives of this well established family have been sequenced [2–13], structures of 4 inhibitors were studied by X-ray crystallography [14, 15] or multidimensional ¹H NMR [5, 16–18], more than 40 analogues with defined specificity and activity were chemically synthetized [5, 16, 19, 20] and 4 were obtained by genetic engineering [9, 19, 21, 22].

**MATERIAL AND METHODS**

**Material.** The seeds of *Echinocystis lobata* were obtained from Wrocław University Botanical Garden.

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Reagents. CM-Sephadex C-25 was from Pharmacia Fine Chemicals (Uppsala, Sweden); Bio-Gel P 2 was purchased from Bio-Rad Labs (Richmond, Calif. U.S.A.); α-N-benzoyl-DL-arginine p-nitroanilide (BAPNA), p-nitrophenyl p'-guanidinobenzoate (NPGB), Suc-Ala-Ala-Pro-Phe-pNa, Tos-Gly-Pro-Arg-pNa were obtained from Sigma Chem. Co. (St. Louis, MO, U.S.A.); bovine trypsin was prepared according to Wilimowska-Pelc & Mejbaum-Katzenellenbogen [23], bovine β-trypsin was isolated from the preparation obtained according to [23] as described by Liepniex & Light [24]; anhydrotyspin was prepared according to Ako et al. [25]

Methods. Trypsin concentration was determined by spectrophotometric titration with NPGB [26]. The standardized trypsin solution was used to titrate CMT I. Cathepsin G was in turn titrated with standardized CMTII. Trypsin and cathepsin G activities were assayed with BAPNA or Tos-Gly-Pro-Arg-pNa and Suc-Ala-Ala-Pro-Phe-pNa as substrates, respectively, according to Erlanger et al. [27]. One unit of the antitrypsin activity was defined as that amount of the inhibitor which reduced by half the activity of 2 mg of trypsin. Cathepsin G from human leukocytes was purified by affinity chromatography on immobilized CMTI [28]. The association equilibrium constants of the inhibitors with β-trypsin and cathepsin G were measured by the method developed in M. Laskowski’s laboratory [29] in conditions described by Ołtewski et al. [30]. Anhydrotyspin and CMTI were immobilized on Sepharose 4B with divinyl sulfone by the method of Pepper [31]. Amino-acid analysis were performed after hydrolysis of samples at 105°C for 24 h with 6.0 M HCl containing 0.1% phenol in sealed, evacuated tubes. After removal of HCl, the amino acids were derivatized with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate, and AQC-derivatives were identified by reverse-phase-HPLC [32]. The amino-acid sequences of inhibitors (30-100 pmole) were determined with a ProSequencer model 6600 (Milligen) and PTH-analyser using the program provided by the manufacturer. Sequences of all inhibitors were determined without fragmentation and in their disulfide bridges form, therefore no signal was detected at cysteine residues and assignment of this residue was based on homology with other squash inhibitors.

RESULTS AND DISCUSSION

Extraction and fractionation of inhibitors

Extraction of inhibitors was carried out by stirring a suspension of E. loba flour (485 g) in 1.5 litres of 0.05 M acetate buffer, pH 4.5, for 1 h at room temperature. Insoluble materials were removed by centrifugation. The sediment was reextracted with 0.75 litres of the same buffer.

The supernatants obtained from two extractions were pooled, adjusted to 90% saturation with ammonium sulfate and centrifuged after 12 h to recover the precipitated proteins.

The salted out proteins were suspended in 250 ml of water, then methanol was added to a final concentration of 80% (v/v) and the mixture was stirred for 15 min. The precipitate formed contained mainly ammonium sulfate and protein insoluble in 80% methanol. After removing the precipitate by centrifugation, the supernatant containing methanol-soluble inhibitors, was evaporated in a stream of air in order to remove methanol.

Ion-exchange chromatography. The methanol-free solution was diluted with water to a conductivity corresponding to 0.05 M of NaCl, then 1 M acetate buffer, pH 4.7, was added to a final concentration of 0.05 M. The solution was applied onto a CM-Sephadex C-25 column (1.5 cm x 25 cm) equilibrated with 0.05 M acetate buffer, pH 4.7. Inhibitors, designated ELTI II and III, were eluted with a linear gradient of NaCl from 0 to 0.6 M (Fig. 1). The non-adsorbed protein still containing antitrypsin activity, was acidified to pH 3.0 with 1 M HCl and was applied to SP-Sephadex C-25 column (1.5 cm x 25 cm) equilibrated with 0.02 M citrate buffer, pH 3.0, and the inhibitor designated ELTI was eluted with the NaCl concentration gradient (0-0.6 M) (Fig. 2).

Affinity chromatography. The fractions showing trypsin inhibitory activity were combined, adjusted to pH 7.5 and applied onto a column (2 cm x 20 cm) filled with immobilized anhydrotyspin equilibrated with 0.1 M Tris/HCl buffer, pH 7.0. Inactive proteins were washed out with the same buffer and the bound inhibitors eluted with 0.02 M HCl (not shown) and lyophilized.
Reverse-phase and DEAE HPLC. The semi-preparative Delta PAK C18 column, equilibrated with 0.1% TFA in 20% acetonitrile, was used in the final step of purification of ELTI I and II. The proteins were eluted with an acetonitrile-water gradient in the presence of 0.1% TFA (Fig. 3). Upon rechromatography of the proteins showing antitrypsin activity the two inhibitors were purified to homogeneity (Fig. 4).

Fig. 1. Elution profile of Echinocystis lobata trypsin inhibitor II and III (ELTI II and ELTI III) from CM-Sephadex C-25. Proteins after ammonium sulfate and methanol fractionation, dissolved in 0.05 M Na-acetate, pH 4.7, were loaded onto a 1.5 cm \( \times \) 25 cm column equilibrated with the same buffer and eluted with a linear NaCl gradient of 0.0 to 0.6 M (- - -). The 10-mL fractions were collected and analyzed for \( A_{280} \) (- - -) and antitrypsin activity (- - -).

Fig. 2. Elution profile of Echinocystis lobata trypsin inhibitor I (ELTI I) from SP-Sephadex C-25. The non-adsorbed protein containing ELTI I eluted from CM-Sephadex C-25 column (Fig. 1), after acidification to pH 3.0 with 1 M HCl, was applied onto a 1.5 cm \( \times \) 25 cm SP-Sephadex C-25 column equilibrated with 0.01 M Na-citrate, pH 3.0. Protein were eluted with a linear NaCl gradient of 0.0 to 0.6 M (- - -). Fractions of 11 mL were collected and analyzed for \( A_{280} \) (- - -) and antitrypsin activity (- - -).

Fig. 3. Chromatography of ELTI I and ELTI II on a HPLC C18 column. The column (29 mm \( \times \) 300 mm, 15 \( \mu \)L) was equilibrated with 0.1% TFA in 20% acetonitrile (v/v) prior to loading, and eluted with a linear gradient of 20-60% acetonitrile in 0.1% TFA. The fractions with antitrypsin activity were rechromatographed. A and B denote ELTI I and ELTI II preparations, respectively, obtained after ion-exchange chromatography.

Fig. 4. Rechromatography of ELTI I and ELTI II on a HPLC C18 column. The conditions were as described for Fig. 3. Peaks were pooled, evaporated and used for analysis and sequencing.
ELTI II after this step of purification was still heterogeneous, therefore it was further purified on DEAE-5PW column equilibrated with 0.05 M bicarbonate buffer, pH 10.0, and eluted with a linear NaCl gradient. Finally, the active fractions after ion chromatography were purified on PAK C₁₈ column to yield pure inhibitor (not shown). Since its amino-acid sequence was identical to ELTI I, therefore the inhibitor ELTI III was not a subject of our further interest. The results of purification of the inhibitors are presented in Table 1.

### Amino-acid sequence of inhibitors

The amino-acid sequences of ELTI I and ELTI II are presented in Fig. 5 in comparison with the other published sequences of serine proteinase inhibitors of the squash family. The inhibitors from *E. lobata* are closely similar to the other squash inhibitor family. Both inhibitors studied...
Table 1
Purification of inhibitors from Echinocystis lobata seeds (485 g)

<table>
<thead>
<tr>
<th>Step</th>
<th>Weight (mg)</th>
<th>Activity (units)</th>
<th>Specific activity</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction with 0.05 M acetate buffer</td>
<td>13000</td>
<td>375</td>
<td>0.03</td>
<td>100.0</td>
</tr>
<tr>
<td>Ammonium sulfate precipitation and extraction with 80% methanol</td>
<td>206</td>
<td>184</td>
<td>0.9</td>
<td>49.0</td>
</tr>
<tr>
<td>CM-Sepahdex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ELTI I</td>
<td>29</td>
<td>39</td>
<td>1.3</td>
<td>10.4</td>
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<tr>
<td>ELTI II</td>
<td>15</td>
<td>43</td>
<td>2.8</td>
<td>11.5</td>
</tr>
<tr>
<td>SP-Sepahdex</td>
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<td></td>
<td></td>
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<tr>
<td>ELTI I</td>
<td>50</td>
<td>15</td>
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<tr>
<td>Anhydratrypsin</td>
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</tr>
<tr>
<td>ELTI I</td>
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<td>9.8</td>
<td>3.9</td>
<td>2.6</td>
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<tr>
<td>ELTI II</td>
<td>10</td>
<td>28.5</td>
<td>2.9</td>
<td>7.6</td>
</tr>
<tr>
<td>ELTI III</td>
<td>9</td>
<td>21.0</td>
<td>2.3</td>
<td>5.6</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ELTI I</td>
<td>0.7</td>
<td>5.0</td>
<td>7.1</td>
<td>1.3</td>
</tr>
<tr>
<td>ELTI II</td>
<td>2.7</td>
<td>19.6</td>
<td>7.3</td>
<td>5.2</td>
</tr>
<tr>
<td>ELTI III</td>
<td>3.2</td>
<td>10.1</td>
<td>3.1</td>
<td>2.7</td>
</tr>
<tr>
<td>DEAE-HPLC</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ELTI III</td>
<td>1.1</td>
<td>6.2</td>
<td>5.6</td>
<td>1.7</td>
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<tr>
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</tr>
<tr>
<td>ELTI III</td>
<td>0.5</td>
<td>3.6</td>
<td>7.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

are products of the same structural gene. ELTI I differs from ELTI II only by the presence of the NH₂-terminal tetrapeptide Lys-Glu-Glu-Gln, and probably this shorter form arose from the longer one by limited proteolysis. The same differences were observed between the inhibitors CM II III and IV, CPT II and III, MCE II, III and IV, LLTI I, II and III, MHT I and III, CMCT II and III (Fig. 5).

The association constants (Kₐ) of ELTI I and ELTI II with bovine β-trypsin were determined to be 6.6 × 10⁻¹⁰ M⁻¹ and 3.1 × 10⁻¹¹ M⁻¹, respectively. As in the case of CM II III and IV or CPT II and III the inhibitor with longer N-terminal extention is a weaker inhibitor of trypsin. The inhibitors from E. lobata, like inhibitors from Cucurbita maxima (CM II I) and Cucurbita pepo (CPT II II), inhibit cathespin G with the association constants of 1.2 × 10⁻⁷ M⁻¹ and 1.1 × 10⁻⁷ M⁻¹ for ELTI I and ELTI II, respectively.

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