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QUARTERLY

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 KEEQRVCPRILMRCKRDSDCLAQCTCQQSGFCG

ELTI II RVCPRILMRCKRDSDCLAQCTCQQSGFCG

The inhibitors show sequence similarity with the squash inhibitor family. ELTI I differs from ELTI II only by the presence of the NH_2-terminal tetrapeptide Lys--Glu-Glu-Gln.

The association constants (K_a) of ELTI I and ELTI II with bovine-trypsin were determined to be 6.6×10^{10} M^{-1}, and 3.1×10^{11} M^{-1}, whereas the association constants of these inhibitors with cathepsin G were 1.2×10^{7} M^{-1}, and 1.1×10^{7} 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 crosss-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 $10^{11}-10^{12}$ 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 ^{1}H 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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Abbreviations: AQC, 6-amino-quinolyl-*N*-hydroxysucinimidyl carbamate; BApNa, α-*N*-benzoyl-DL-arginine *p*-nitroanilide; CMCTI, *Cucumis melo* var. *Conoman* trypsin inhibitor; CMTI, *Cucurbiata maxima* trypsin inhibitor; CPTI, *Cucurbita pepo* trypsin inhibitor; ELTI, *Echinocystis lobata* trypsin inhibitor; MCEI, *Momordica charantia* elastase inhibitor; MHTI, Hami melon trypsin inhibitor; NPGB, *p*-nitrophenyl-*p'*-guanidinobenzoate; TFA, trifluoroacetate.

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 Liepnieks & Light [24]; anhydrotrypsin 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 CMTI I. Cathepsin G was in turn titrated with standardized CMTI I. 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 I [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 Otlewski *et al.* [30]. Anhydrotrypsin 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 pmoles) 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. lobata* flour (485 g) in 1.5 litres of 0.05 M acetate buffer, pH 4.5, for 1 h at room temperature. Insoluble materials was 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 × 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 × 25 cm) equilibrated with 0.02 M citrate buffer, pH 3.0, and the inhibitor designated ELTI I 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 × 20 cm) filled with immobilized anhydrotrypsin 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 C_{18} 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).

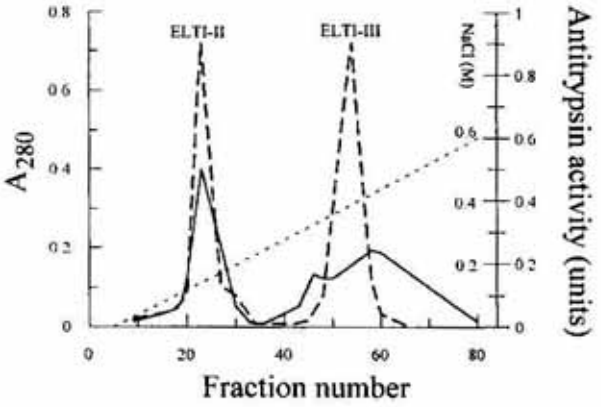

Fig. 1. Elution profile of Echinocystis lobata trypsin inhibitor II and III (ELTI II and ELTI III) from CM-Sephadex C-25.
Proteins after ammonium sulfate and methanol fractionation, dissolved in 0.05 M Na-acetate, pH 4.7, were loaded onto a 1.5 cm × 25 cm column equilibrated with the same buffer and 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 (– – –).

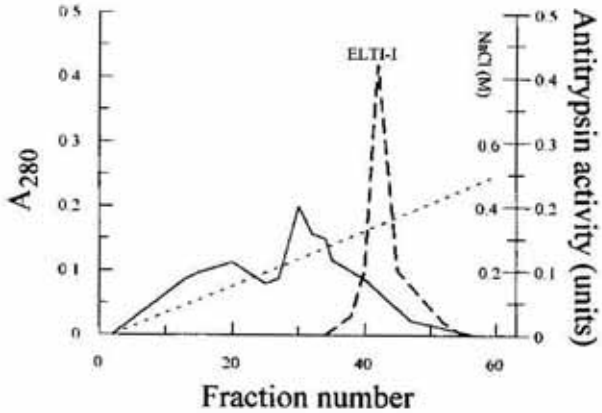

Fig. 2. Elution profile of Echinocystis lobata trypsin inhbitor I (ELTI I) from SP-Sephadex C-25.
The non-adsorbed protein containing ELTI I eluted from CM-Sephadex C-25 column (Fig. 1), after acidification to pH 3.0 with 1 M HCl, was applied onto a 1.5 cm × 25 cm SP-Sephadex C-25 column equilibrated 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 (– – –).

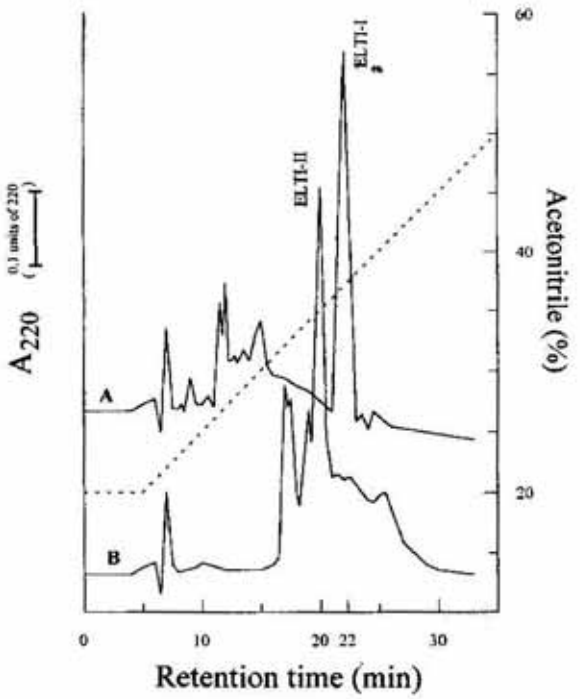

Fig. 3. Chromatography of ELTI I and ELTI II on a HPLC C_{18} column.
The column (29 mm × 300 mm, 15 µ) was equilibrated with 0.1% TFA in 20% acetonitrile (v/v) prior to loading, and eluted with a linear gradient of 20–60% acetonitrile in 0.1% TFA. The fractions with antitrypsin activity were rechromatographed. A and B denote ELTI I and ELTI II preparations, respectively, obtained after ion-exchange chromatography.

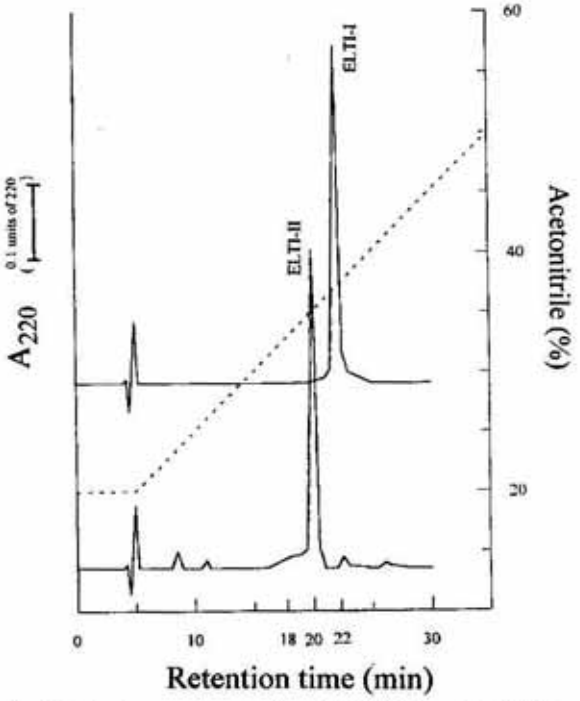

Fig. 4. Rechromatography of ELTI I and ELTI II on a HPLC C_{18} column.
The conditions were as described for Fig. 3. Peaks were pooled, evaporated and used for analysis and sequencing.

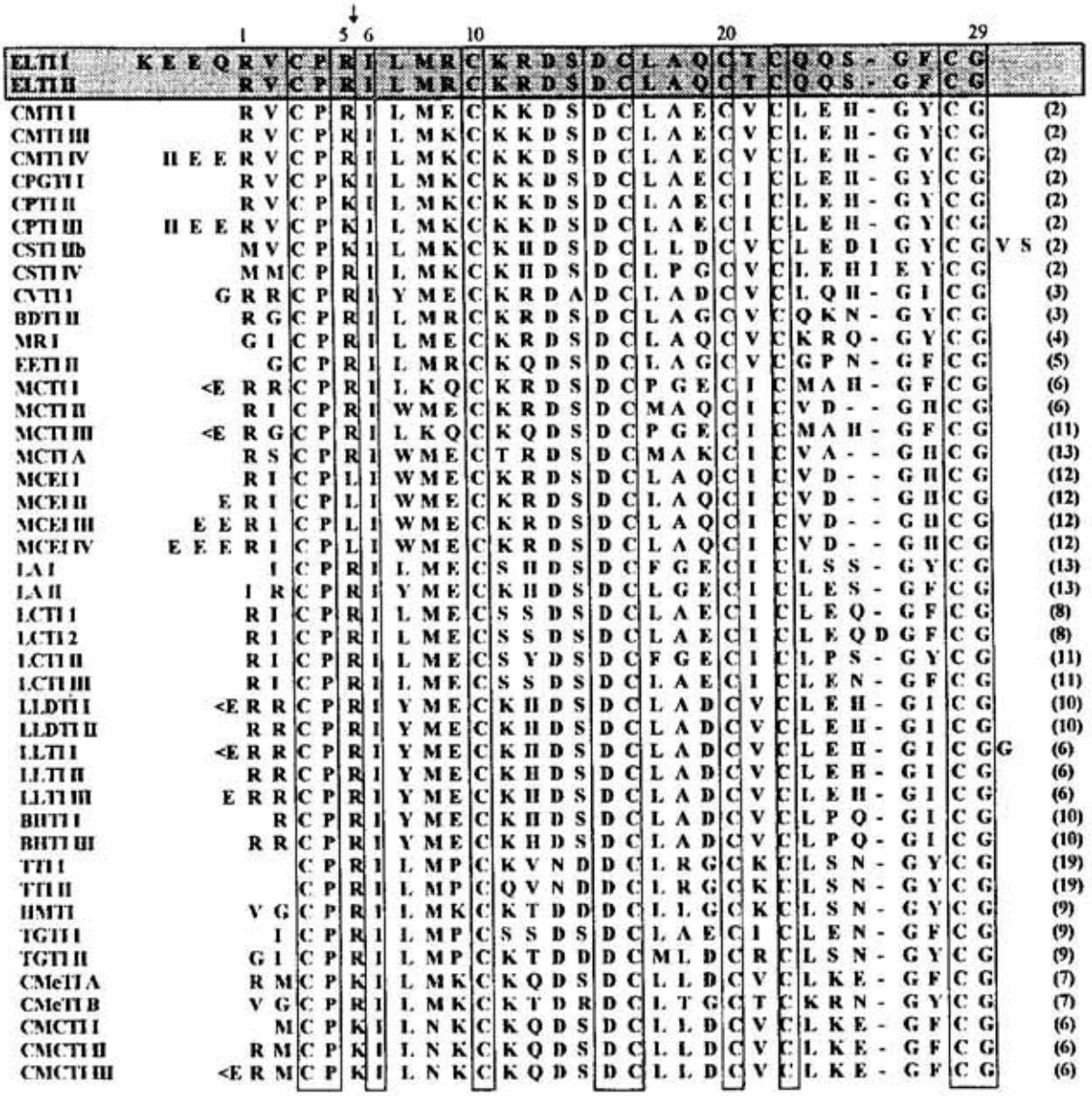

Fig. 5. Alignment of amino-acid sequences of ELTI I and ELTI II with those of 41 other low molecular inhibitors belonging to the squash inhibitor family.

TI, trypsin inhibitor; EI, elastase inhibitor; EL, *Echinocystis lobata;* CM, *Cucurbita maxima;* CPG, *Cucurbita pepo* var. *Giromontia;* CP, *Cucurbita pepo;* CS, *Cucumis sativus;* CV, *Citrulus vulgaris;* BD, *Bryonia dioica;* MR, *Momordica repens;* EE, *Ecballium elaterium;* MC, *Momordica charantia;* LC, *Luffa cylindrica;* LLD, *Lagenaria leucantha* var. *Depressa;* LL, *Lagenaria leucantha;* BH, *Benincasa hispida;* T, *Trichosanthes;* HM, Hami melon; TG, towel gourd; CMe, *Cucumis melo;* CMC, *Cucumis melo* var. *Conomon;* LA, *Luffa acutangula;* ↓ indicates the reactive site peptide bond.

ELTI III 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 C_{18} 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)

Step	Weight (mg)	Activity (units)	Specifiic activity	Yield (%)
Extraction with 0.05 M acetate buffer	13000	375	0.03	100.0
Ammonium sulfate precipitation and extraction with 80% methanol	206	184	0.9	49.0
CM-Sephadex				
ELTI II	29	39	1.3	10.4
ELTI III	15	43	2.8	11.5
SP-Sephadex				
ELTI I	50	15	0.3	4.0
Anhydrotrypsin				
ELTI I	2.5	9.8	3.9	2.6
ELTI II	10	28.5	2.9	7.6
ELTI III	9	21.0	2.3	5.6
RP-HPLC				
ELTI I	0.7	5.0	7.1	1.3
ELTI II	2.7	19.6	7.3	5.2
ELTI III	3.2	10.1	3.1	2.7
DEAE-HPLC				
ELTI III	1.1	6.2	5.6	1.7
RP-HPLC				
ELTI III	0.5	3.6	7.2	1.0

are products of the same structural gene. ELTI I differs from ELTI II only by the presence of the NH_2-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 CMTI III and IV, CPTI II and III, MCEI II, III and IV, LLTI I, II and III, MHTI I and III, CMCTI II and III (Fig. 5).

The association constants (K_a) of ELTI I and ELTI II with bovine β-trypsin were determined to be $6.6 \times 10^{10}\ M^{-1}$ and $3.1 \times 10^{11}\ M^{-1}$, respectively. As in the case of CMTI III and IV or CPTI 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* (CMTI I) and *Cucurbita pepo* (CPTI II), inhibit cathepsin G with the association constants of $1.2 \times 10^7\ M^{-1}$ and $1.1 \times 10^7\ M^{-1}$ for ELTI I and ELTI II, respectively.

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