Property and Amino Acid Sequence of a Subtilisin Inhibitor from Seeds of Beach Canavalia (Canavalia lineata)

Hideki Katayama, Yasuhiro Soezima,* Satoshi Fujimura, Shigeyuki Terada, and Eiji Kimoto
Department of Chemistry, Faculty of Science, Fukuoka University, 8–19–1 Nanakuma, Jonan-ku, Fukuoka 814–01, Japan
*Laboratory of Biochemistry, Faculty of Engineering, Kyushu Sangyo University, Matsukadai, Higashi-ku, Fukuoka 813, Japan
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A subtilisin inhibitor was purified from the seeds of Canavalia lineata by ammonium sulfate precipitation, ultrafiltration on a YM-30 membrane, column chromatography on DEAE-Toyopearl and SP-Toyopearl, followed by reverse-phase HPLC. The inhibitor (CLSI-I) is a low molecular weight protein (M , about 6500) containing no half-cystine residue, and quite stable as to extreme heat and pH treatment. CLSI-I inhibited subtilisin-type serine proteases including S. griseus alkaline protease. The amino acids of CLSI-I were sequenced by manual Edman degradation after enzymatic digestion with Achromobacter lyticus lysyl endopeptidase and Staphylococcus aureus V8 protease. CLSI-I contains 65 amino acid residues and showed a high homology to potato inhibitor I family proteins.

Potato I family inhibitors occur in several plants.1) The first protein of this family is potato tuber chymotrypsin inhibitor, characterized by unusually high heat-stability, a low M (about 8000), and the presence of a single disulfide bond. Analogous inhibitors were found in tomato4) and pumpkin.5) Protease inhibitors isolated from barley,6,7) cowpea (Vigna unguiculata),8,9) broad bean (Vicia faba),10) and adzuki bean (Vigna angularis)11,12) showed similar properties to the potato tuber inhibitor but they have no disulfide bridge in the molecules. We have found three subtilisin inhibitors from the seeds of beach Canavalia (Canavalia lineata).13) Two of them (CLSI-II and III) have already been purified to homogeneity and sequenced.14) They belong to the Kunitz-type inhibitor family. We here describe the purification, characterization, and amino acid sequence of the third inhibitor (CLSI-I) from the same seeds. This inhibitor shares several physicochemical properties of potato I family inhibitors, and showed a high homology to subtilisin inhibitors from other leguminous seeds.

Materials and Methods

Materials. Defatted powder of C. lineata seeds was prepared as described previously.13) Achromobacter lyticus lysyl endopeptidase was purchased from Wako Chem. (Osaka). Thermolysin and Streptomyces griseus alkaline protease were purchased from Seikagaku Kogyo Co. (Tokyo). C. lineata asparagus specific endopeptidase and Agkistrodon halys metalloprotease H6 were purified in our laboratory. All other enzymes were obtained from Sigma Chem. Co. (St. Louis). Fluorogenic substrates (Abz-Gly-Phe-Arg-Leu-Leu-Nba and Abz-Ala-Leu-Asn-Ala-Dna)14) were kindly supplied by Dr. N. Nishino (Kyushu Institute of Technology). All other peptide and amino acid derivatives were purchased from Peptide Institute Inc. (Osaka).

Measurement of inhibitory activity. The enzyme activity of A. oryzae protease, protease K, pronase E, subtilisin BN, subtilisin Carlsberg, and subtilisin E was measured in 1 mM CaCl₂-50 mM Tris-HCl (pH 8.0) using Z-Ala-Ala-Leu-pNA (Z-AAA-pNA) as a substrate. Z-Gly-Gly-Leu-pNA and 50 mM Borax-NaOH (pH 11.0) were used for S. griseus alkaline protease. Bz-Tyr-pNA, Suc-Ala-Ala-Ala-pNA, and Phe-Arg-Leu-pNA were used for z-chymotrypsin, elastase, and papain, respectively. Inhibitory activity toward several enzymes was measured as described previously.13) In brief, sample solutions (100 μl) were incubated with 2.5 ml of enzyme solution in appropriate buffer at 37°C for 2 min. Then, 100 μl of substrate in dimethylsulfoxide were added. The liberation of p-nitroaniline was monitored at 410 nm on a Ubest-30 spectrophotometer (JASCO). Fluorogenic substrates were used for serratoppleidase. S. caeciptius protease, B. polymyxa protease, thermolysin, A. halys protease H6,13) trypsin, and asparagine-specific endopeptidase. The increase of fluorescence was monitored on a FP-550A spectrofluorometer (JASCO) at 440 nm with the excitation at 380 nm. A. lyticus lysyl endopeptidase activity was measured spectrophotometrically using Boc-Val-Leu-lys-MCA as the substrate. α-Amylase activity was measured as described.17)

SDS-PAGE and amino acid analysis. SDS-PAGE was done using a 16.5% polyacrylamide-3% cross-linked gel and 0.1% SDS-0.1 M Tris-tricine buffer system.18) Proteins were detected by staining the gel with a 0.1% Coomassie brilliant blue R-250. Amino acid composition was analyzed by an automated amino acid analyzer (JASCO) using o-phthalaldehyde and sodium hypochlorite after samples were hydrolyzed with 0.25% phenol-2% thioglycolic acid-6 M HCl at 110°C for 20 h in evacuated, sealed tubes.

Kinetic measurements. Dissociation constant (Kₐ) of enzyme-inhibitor complex was calculated by Henderson’s method.19) The Kₐ of Z-Ala-Ala-Leu-pNA was 0.073 mM for subtilisin BN and 0.100 mM for subtilisin Carlsberg, and the Kₐ of Z-Gly-Gly-Leu-pNA was 0.034 mM for S. griseus alkaline protease.

Thermal and pH stability. The inhibitor solution (50 μg/ml) in 50 mM TES-NaOH buffer (pH 7.0) was heated in a water bath at 50 to 100°C, and cooled in ice for 10 min. The residual subtilisin inhibitory activities were then measured as described above. Stability of CLSI-I was also examined in various buffers (20 mM each): KCl-HCl (pH 1 to 2), acetate (pH 1 to 5), phosphate (pH 6), Tris-HCl (pH 8), and glycine-NaOH buffers (pH 10 to 13). Twenty-five μl of inhibitor solutions (500 μg/ml) were diluted with 100 μl of buffers. The mixtures were kept at 37-70°C for 10-60 min and diluted with 125 μl of 1 mM CaCl₂-50 mM Tris-HCl (pH 8.0). The residual inhibitory activity was measured immediately.

Chromatography. Anion-exchange chromatography was done on a DEAE-Toyopearl 650M column (2.6 × 40 cm, Tosoh) equilibrated with

Abbreviations: Abz, 2-aminobenzoyle; CLSI, Canavalia lineata subtilisin inhibitor; Dna, 2,4-dinitroanilinoethylamide; pNA, 4-nitroaniline; Nba, 4-nitrobenzamide.; TFA, trifluoroacetic acid; Z, carbenzoxyl; Pyr, pyrogallitic acid; Suc, succinyl; Bz, benzoyl; Boc, t-butoxycarbonyl; MCA, 4-methylcoumaryl-7-amine; TES, Tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid. Amino acid symbols except Gly denote the t-configuration.
50 mM Tris-HCl (pH 9.0). Elution was done with a linear gradient of 0.08-0.12 M NaCl in the same buffer. Fractions of 5 ml were collected. Cation-exchange chromatography was done on a SP-Toyopearl 650M column (2.6 x 40 cm; Tosoh) equilibrated with 50 mM citrate buffer (pH 3.2). Proteins were eluted with a linear gradient from the same buffer to 0.2 M NaCl-50 mM citrate buffer (pH 4.5). Fractions of 5 ml were collected. Reverse-phase HPLC was done on a μBondasphere C4 column (7.6 x 300 mm; Waters) with a linear gradient of 0 to 60% acetonitrile in 0.1% TFA for 60 min at a flow rate of 1 ml/min.

Sequencing: CLSI-I (270 μg) was digested with lysyl endopeptidase (5 μg) in 0.2 M Tris-HCl (pH 9.0) at 37°C for 6 h. Another 100 μg of the inhibitor was digested with V8 proteinase (5 μg) in 0.1 M NH4HCO3 at 37°C for 6 h. Peptides in the digests were purified by reverse-phase HPLC on a Biofine RPC-SC18 column (4.6 x 250 mm, JASCO). The amino acid sequences of peptides were analyzed by manual Edman degradation using 4-N,N-dimethylaminoazobenzene-4'-isothiocyanate/phenylisothiocyanate double coupling method.26

Results
Purification of inhibitor

The defatted powder was stirred in water at 4°C, and the extract was centrifuged. The supernatant was heated at 80°C for 15 min and centrifuged. The supernatant was ultra-filtered on a YM-30 membrane (Amicon). The filtrate was lyophilized and dialyzed against 50 mM Tris-HCl (pH 9.0) using Spectra/Per MWCO 3500 dialysis tube (Spectrum Medical Inc.). After removal of insoluble materials the supernatant was chromatographed on a DEAE-Toyopearl 650M column. As reported previously,13 subtilisin inhibitory activity was separated into three peaks (CLSI-I, -II, and -III) (Fig. 1). Fractions (No. 32-42) containing CLSI-I were pooled and lyophilized. The crude preparation was dialyzed against 50 mM citrate buffer (pH 3.2) and put on an SP-Toyopearl 650M column. The inhibitor was eluted in the second peak as indicated by a bar in Fig. 2. The fractions (No. 77-87) were pooled, lyophilized, and dissolved in 0.1% TFA. The solution was separated by reverse-phase HPLC on a μBondasphere C4 column. As shown in Fig. 3, CLSI-I was eluted as a sharp peak at the retention time of 47 min. Finally pure CLSI-I (0.8 mg) was obtained from 750 g of the defatted powder.

Purity and amino acid composition

Figure 4 shows an electrophoregram of CLSI-I on SDS-PAGE. The inhibitor was homogeneous and the molecular mass was estimated to be 6500 Da. From the amino acid analysis (Table), CLSI-I contained no half-cystine residue.

![Fig. 1. Separation of C. lineata Subtilisin Inhibitors by Anion-exchange Chromatography.](image1)

Crude inhibitor preparation from 200 g of the defatted meal of C. lineata seed was put on a DEAE-Toyopearl 650M column (2.6 x 40 cm) equilibrated with 50 mM Tris-HCl (pH 9.0). The proteins were eluted with a linear gradient of 0.08-0.12 M NaCl in the same buffer. Fractions of 5 ml were collected.

![Fig. 2. Cation-exchange Chromatography of CLSI-I on SP-Toyopearl 650M Column.](image2)

The fraction I in Fig. 1 was put on the column (2.6 x 40 cm) equilibrated with 50 mM citrate buffer (pH 3.2). The proteins were eluted with a linear gradient of 0-0.2 M NaCl in the same buffer. Fractions of 5 ml were collected.

![Fig. 3. Purification of CLSI-I by Reverse-phase HPLC.](image3)

The inhibitor was chromatographed on μBondasphere C4 column (7.6 x 300 mm) with a linear gradient of acetonitrile in 0.1% TFA at a flow rate of 1 ml/min.

![Fig. 4. SDS-PAGE Profile of Purified CLSI-I.](image4)

Lane 1: marker proteins, ovalbumin (46 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.5 kDa), aprotinin (6.5 kDa), and insulin B-chain (3.4 kDa). Lane 2: purified CLSI-I.
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H. KATAYAMA et al.

Table Amino Acid Compositions of Intact CLSI-I and Peptides Derived from Lysyl Endopeptidase and V8 Protease Digestion of CLSI-I*

<table>
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<th>Amino acid</th>
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<th>K3</th>
<th>K4</th>
<th>K5</th>
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<tr>
<td>Arg</td>
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<td>1.2 (2)</td>
<td>—</td>
<td>0.8 (1)</td>
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Total 65 4 2 8 13 16 21 11 9 25

* Values from the sequence are cited in parentheses. K1–K6 and V1–V3 denote the peptides obtained from lysyl endopeptidase and V8 protease digests of CLSI-I, respectively.

Fig. 5. Stability of Inhibitor.
(a) Thermal stability of CLSI-I. The inhibitor solution was heated in 50 mM TES-NaOH (pH 7.0) for 10 min (○), 30 min (△), and 60 min (□) at the indicated temperatures and the residual subtilisin inhibitory activities were assayed using Z-AAL-pNA as substrate in 50 mM Tris-HCl (pH 8.0) containing 1 mM CaCl2. (b) Stability of CLSI-I as a function of pH. Subtilisin inhibitory activities were assayed using samples of inhibitor solutions after the treatment in buffers of indicated pH for 1 h at 37°C.

Stability as to heat and pH treatment
CLSI-I was heated at pH 7.0 and 50–100°C for 10–60 min, and the residual inhibitory activity against subtilisin BPN' was measured in 1 mM CaCl2–50 mM Tris–HCl (pH 8.0). The inhibitor was extremely stable against heat treatment (Fig. 5a). A great reduction of inhibitory activity occurred only after prolonged treatment at 100°C.

CLSI-I was treated at 37°C for 60 min in buffer solutions from pH 1 to pH 13 and the remaining inhibitory activity was measured. The activity was retained almost completely at 37°C even in extreme pH solutions (Fig. 5b). However, the inhibition activity was significantly reduced to about 50% at 60°C for 10 min at pH 1 and 13 and was lost almost completely at 70°C for 1 h at both pHs.

Inhibitory specificity spectrum of CLSI-I
Inhibitory activity of CLSI-I was tested against several...
enzymes. CLSI-I dose-dependently inhibited some microbial serine proteases such as proteinase K, subtilisin BPN', subtilisin Carlsberg, subtilisin E, A. oryzae protease, and S. griseus alkaline protease (Fig. 6). A weak and partial inhibition was also observed toward pronase E. Other serine proteases such as trypsin and chymotrypsin were not inhibited. Inhibition against cysteine proteases, aspartic proteases, and α-amylase was not observed.

The dissociation constants ($K_i$) of enzyme–inhibitor complexes at 37°C were calculated: $9.6 \times 10^{-8}$ m for subtilisin BPN', $7.3 \times 10^{-9}$ m for subtilisin Carlsberg, and $1.7 \times 10^{-7}$ m for S. griseus alkaline protease.

**Amino acid sequence**

Since CLSI-I contains no cysteine residue, native inhibitor was digested directly with lysyl endopeptidase and V8 protease. After enzymatic digestion, the resulting peptides were purified by reverse-phase HPLC (data not shown). Six peptides (K1–K6) and three peptides (V1–V3) were obtained from lysyl endopeptidase and V8 protease digests, respectively. The amino acid compositions of these peptides are cited in the Table. These peptides were sequenced by a manual Edman degradation. The results of sequencing are shown in Fig. 7 together with the complete amino acid sequence of CLSI-I. The position of peptide K2 was estimated from the comparison with the sequences of analogous inhibitors (VSI and ASI). The order of peptides K6 and K4 was identified from the partially sequenced amino acid composition of peptide V3. As cited in the Table, the Val contents of peptides K6 and V3 as well as of the intact inhibitor are lower than those expected from the sequence. This may be due to the presence of an acid-resistant bond, Val-Val, in these peptides.

**Discussion**

The seeds of *C. lineata* contain at least three subtilisin inhibitors (CLSI-I, II, and III). We have already reported the purification and amino acid sequences of CLSI-II and -III, which are Kunitz-type inhibitors. Isolation of the third inhibitor (CLSI-I) has been done by reverse-phase HPLC (Fig. 3). The inhibitory spectra of two inhibitors (CLSI-II and

![Fig. 7. Summary of the Sequencing of CLSI-I.](image)

Peptides derived from CLSI-I by digestion with lysyl endopeptidase (K) and *S. aureus* V8 protease (V) are shown below the whole sequence. Residues not identified are indicated by small letters. The whole inhibitor sequence of CLSI-I was presumed from comparing with subtilisin inhibitors from leguminous seeds. N-Terminal part of the inhibitor was directly sequenced from CLSI-I and is shown by an underline.

![Fig. 8. Comparison of the Amino Acid Sequences of Potato I Family Inhibitors.](image)

VSI, broad bean subtilisin inhibitor; ASI-II, adzuki bean subtilisin inhibitor; PI-L, potato tuber inhibitor; TI-I, tomato leaf inhibitor; DI-I, tomato fruit inhibitor; CMTI-V, pumpkin trypsin inhibitor V; CI-Ic and CI-II, barley chymotrypsin inhibitors; LIE, leech inhibitor Eglun. N-Terminal part of VSI was not yet analyzed. One letter codes in italics indicate positions where polymorphisms have been identified. The arrow indicates the putative of CLSI-I which is estimated from those of ASI and VSI. Residues identical to CLSI-I are enclosed in solid boxes.
-III) have already been reported.\textsuperscript{13} CLSI-I showed a wider inhibitory spectrum than CLSI-II and -III, and inhibited several microbial serine proteases including \textit{S. griseus} alkaline protease, \textit{A. oryzae} protease, proteinase K, subtilisin BPN', subtilisin Carlsberg, and subtilisin E. Among three inhibitors, only CLSI-I suppressed the activity of \textit{S. griseus} alkaline protease. Lorenzo \textit{et al.}\textsuperscript{21} isolated three iso-inhibitors from jack bean (\textit{C. ensiformis}) seeds. The iso-inhibitors are heat-stable and inhibit several microbial proteases. They have an identical \( M_r \) of 8000 with different isoelectric points of 6.6, 6.3, and 6.0. Though the properties of these inhibitors are quite similar to CLSI-I, we could not detect any iso-inhibitors of CLSI-I in the seeds of \textit{C. lineata}.

Judging from several properties, CLSI-I is a member of the potato inhibitor I family.\textsuperscript{23} Comparison of the amino acid sequences of potato I family inhibitors are cited in Fig. 8. CLSI-I showed the highest sequence homology (79\%) to subtilisin inhibitors from broad bean (VSI)\textsuperscript{10} and adzuki bean (ASI).\textsuperscript{12} Other inhibitors from cereals\textsuperscript{4-7,23} and leech\textsuperscript{24} gave much lower sequence homology. \( P_1 \) sites of these inhibitors are according to the substrate specificity of target enzyme.

A disulfide bridge is present near the reactive site of many protease inhibitors to maintain an adequate conformation for the inhibitory activity.\textsuperscript{25} However, some potato I-type inhibitors as well as CLSI-I contain no disulfide bridge. An Ala-Asp peptide bond was identified as the reactive site of VSI\textsuperscript{10} and ASI.\textsuperscript{26} We could estimate from the sequence homology that the reactive site of CLSI-I might be Ala\textsuperscript{41}-Asp\textsuperscript{42}. This explains why the inhibitor does not affect the activity of trypsin and chymotrypsin, because the amino acid residues near the reactive site of CLSI-I are well conserved in these legumous subtilisin inhibitors.

References


