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PRIMARY STRUCTURE OF A KUNITZ-TYPE TRYPSIN INHIBITOR FROM *ENTEROLOBIUM CONTORTISILIQUUM* SEEDS

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Key Word Index—*Enterolobium contortisiliquum*; Leguminosae; Mimosoideae; seeds; serine proteinase inhibitor; amino acid sequence; blood clotting enzymes.

Abstract—A trypsin inhibitor was isolated from *Enterolobium contortisiliquum* seeds. Starting with a saline extract, ECTI (*E. contortisiliquum* trypsin inhibitor) was purified as a homogeneous protein by acetone precipitation, ion-exchange chromatography (DEAE-Sephadex A-50), gel filtration (Sephadex G-75 and Superose 12) and reversed phase HPLC (μ-Bondapak C-18). The amino acid sequence was determined by automatic degradation and by DABITC/PITC microsequence analysis of the reduced and carboxymethylated protein and also of purified peptides derived from the protein by cleavage with iodosobenzoic acid and by enzymic digestion with trypsin, chymotrypsin and *Staphylococcus aureus* V8 protease. ECTI contains 174 amino acid residues in two polypeptide chains, an α-chain consisting of 134 residues and a β-chain made up of 40 residues. The inhibitor displays a high degree of sequence identity with other Kunitz-type proteinase inhibitors isolated from the Mimosoideae subfamily. The reactive site was identified (by homology) as the arginine–isoleucine peptide bond at position 64–65. ECTI inhibits trypsin and chymotrypsin in the stoichiometric ratio of 1:1 and also Factor XIIa, plasma kallikrein and plasmin, but not thrombin and Factor Xa.

INTRODUCTION

Proteinases in biological systems are controlled by various mechanisms. They can be inactivated by proteolytic degradation [1] or blocked by inhibitors which are actually pseudosubstrates displaying variable degrees of affinity towards the catalytic site of enzymes [2]. Thus, proteinase inhibitors play an important role in biological systems, especially those which depend upon limited proteolysis to control their activity [3].

The high content of proteins in legume seeds has stimulated many studies towards their proteins in the germination physiology. Other properties may be also important for the plant in the defence of the seed against insects or other pathogenic organisms. As legume seeds are an important protein source for man's nutrition, the toxicity of compounds such as lectins or proteinase inhibitors are of considerable interest. Plant proteinase inhibitors can also be taken as model compounds for inhibition of proteolytic enzymes, especially those related to trypsin and chymotrypsin, i.e. enzymes which are commonly responsible for digestion in animals and microorganisms [4]. As the role of inhibitors found in plant seeds is not yet fully understood, potential physiological interactions between plant protease inhibitors and endogenous animal proteases should not be neglected when considering their functions [5].

Legume seeds contain various proteinase inhibitors, classified in several families such as Kunitz type and Bowman–Birk type, potato I, potato II, squash, cereal super family and thaumatin-like among others [6].

The plant Kunitz-type inhibitors, which are not related at all to the animal Kunitz-type inhibitors, have M_r of *ca* 20 000 and their four cysteine residues form two disulphide bridges. They are found in all Leguminosae subfamilies: Mimosoideae, Caesalpinoideae, Papilionoideae and also in the Solanaceae family. The Mimosoideae usually contain proteinase inhibitors formed by two polypeptide chains linked by a disulphide bridge, thus differing from the other Kunitz-type single-chain inhibitors from the Caesalpinoideae and Papilionoideae subfamilies [6–8].

Enterolobium contortisiliquum, belonging to the Mimosoideae subfamily and widely spread in Brazil, is a tree more than 20 m high and its black ear-shaped fruits are toxic for cattle [9]. Previously the isolation of an aminopeptidase [10], an endopeptidase [11] and a thiol

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proteinase inhibitor [12], and preliminary results on the serine proteinase inhibitor from *E. contortisiliquum* seeds, were described [13]. The present paper describes the purification, primary structure determination and action on blood clotting enzymes of the trypsin inhibitor isolated from *E. contortisiliquum* seeds.

RESULTS

ECTI purification

ECTI was isolated in a homogenous form by the following procedure: *E. contortisiliquum* seeds were swollen in 0.15 M NaCl and homogenized in a blender. The proteins in the crude extract after centrifugation were precipitated by acetone (80% v/v) and the vacuum-dried powder was dissolved in 0.05 M Tris–HCl buffer at pH 8, at a final concentration of 10 mg ml^{-1}. This solution, 50 ml, was chromatographed on a DEAE-Sephadex A-50 column developed with a linear gradient from 0.05 to 0.5 M NaCl in the same buffer, and the active protein was eluted by 0.2 M NaCl. Fractions containing trypsin inhibitory activity were pooled, dialysed and lyophilized for the gel filtration step on a Sephadex G-75 column. The inhibitory active fractions were pooled, dialysed, lyophilized and then further chromatographed on a Superose 12 column (Fig. 1a).

The purified inhibitor exhibited a specific activity of 0.87 mg of trypsin inhibitor per mg of protein; the final yield in active material was 12%. The homogeneity of the preparation was assessed by reversed phase HPLC chromatography and SDS-PAGE (Fig. 1b). The single protein peak eluted from the μ-Bondapak C-18 column indicates that isoforms are absent in the final preparation.

Molecular mass

SDS-PAGE of the native inhibitor shows a homogeneous single polypeptide chain of M_r 20 000, and two polypeptide chains of M_r 16 000 and 6000 following reduction by dithiothreitol (Fig. 1c).

This value is in good agreement with other inhibitors from seeds of other species in the same family [7, 8]. The complete sequence shows that ECTI consists of 174 amino acid residues, corresponding to a calculated M_r of 19 630. Homology to other single-chain Kunitz-type inhibitors supports the hypothesis that the two chains in ECTI result from a proteolytic cleavage of a single-chain precursor [7].

Temperature and pH stability

The study of the effect of temperature (0–100°) and pH (2–12) on the trypsin inhibitory activity showed that the inhibitory activity was maintained at temperatures up to 60° for 10 min, but not at 100°. Pre-incubation of the inhibitor in the pH range 2.0–12.0 for 10 min did not affect trypsin inhibition.

Primary structure determination

The complete amino acid sequence was determined by automated Edman degradation on a 477A Applied Biosystem and manually by the DABITC/PITC method. The separation of the two polypeptide chains after the reduction and carboxymethylation process was performed by HPLC, using a μ-Bondpak C-18 column. The α-chain sequence was determined by submitting different samples to fragmentation by iodosobenzoic acid, and by enzyme digestion with trypsin, chymotrypsin and *Staphylococcus aureus* V8 protease. The peptide mixtures were resolved by an HPLC system. The β-chain sequence was determined automatically with 40 cycles of *N*-terminal sequencing. The complete amino acid sequence of ECTI consists of 174 amino acid residues, including four half-cystine residues forming in two disulphide bridges in the molecule (Fig. 2). Most of the sequence was deduced from the overlapping of peptides and fragments, but in those regions (residues 91–117) where there were no overlaps, the peptides were placed by their strong sequence identities with other Kunitz-type inhibitor sequence previously determined [7, 8]. The α-chain is a M_r 15 130 polypeptide with 134 amino acid residues, and the β-chain is a M_r 4500 polypeptide with 40 amino acid residues. The molecular mass for ECTI, calculated from the sequence of the two chains is 19 630, which corresponds to the estimation made by gel filtration and SDS-PAGE. The sequence homology suggests that the trypsin reactive site is the arginine–isoleucine polypeptide bond in position 64–65. ECTI shows significant homology (Fig. 3) to trypsin inhibitors from *Prosopis juliflora* (68%) and *Adenanthera pavonina* (57%), both belonging to the same Mimosae subfamily, and with SBTI which is a Papilionoideae [7, 8].

Homology is also observed with other Kunitz-type trypsin inhibitors, *Psophocarpus tetragonolobus* [14], *Erythrina latissima* [15], to a chymotrypsin inhibitor from *Schizolobium parayba* [16], to the bifunctional amylase–subtilisin inhibitor from the bran of rice [17], and even to an albumin from winged beans [18].

Specificity

The inhibitory activity of ECTI specificity measurements assessed with different proteinases revealed a slow-binding tight (indicating tight binding of these enzymes by ECTI); the complexes with mechanism in a molar 1:1 stoichiometry, as was shown for plasma kallikrein (Fig. 4) when the inhibition curve was adjusted by a computer program using the indicated experimental values. The inhibition curves for other enzymes follow the same model, and the dissociation constants of the enzyme–inhibitor complexes calculated for each enzyme are compiled in Table 1. The dissociation constants (K_i) for plasma kallikrein and trypsin are in the range of 10^{-9} M and for plasmin 10^{-8} M; factor XIIa and chymotrypsin have lower dissociation constants (10^{-7} M). Factor Xa, tissue kallikrein and thrombin are not inhibited by ECTI.

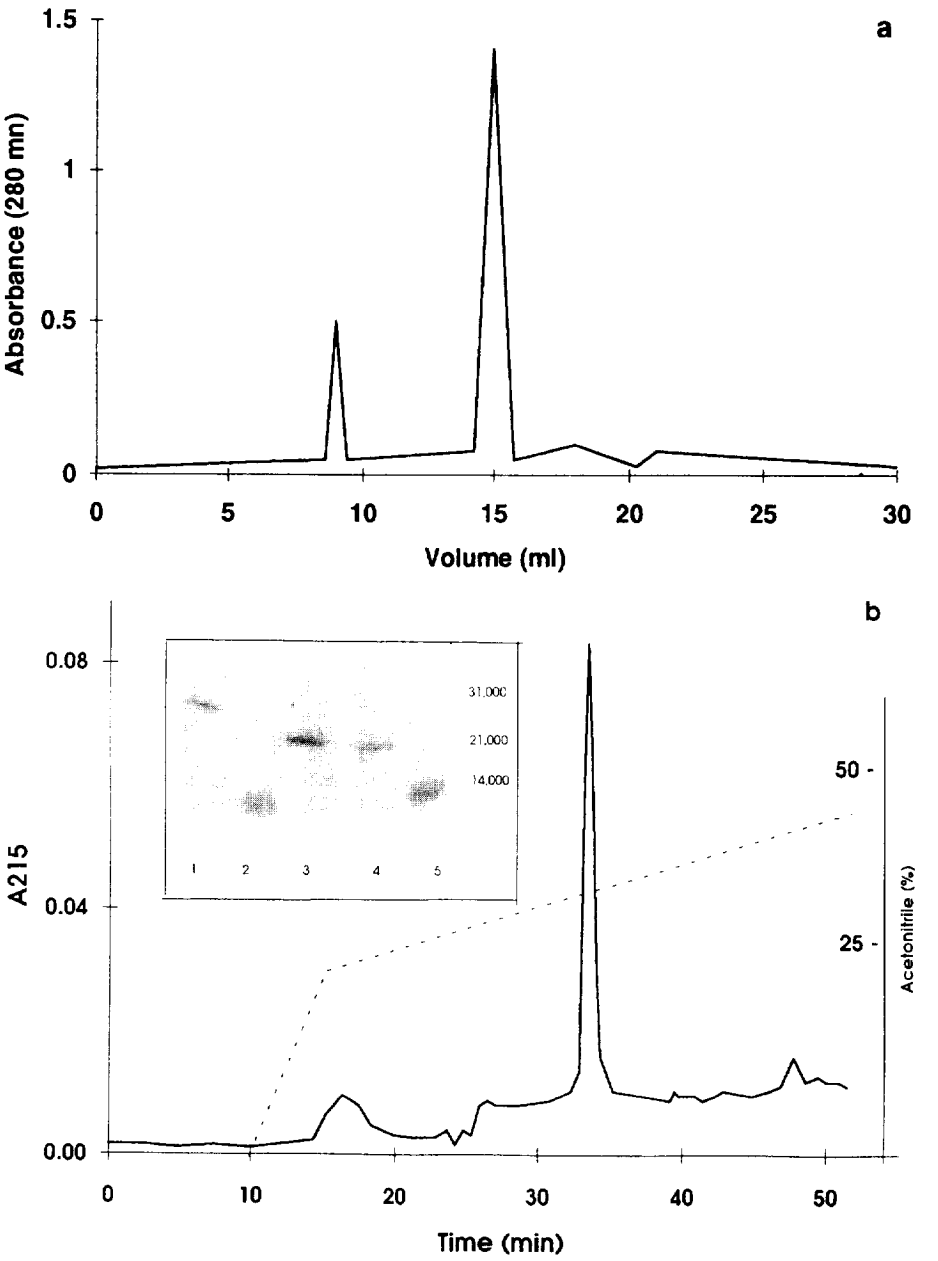

Fig. 1. Purification of ECTI. (a) Superose 12 (FPLC) chromatography of ECTI on DEAE-Sephadex column. Buffer: 50 mM Tris–HCl pH 8, 0.1 M NaCl; sample: 50 μg of protein; flow rate: 0.5 ml min^{-1}. (b) μ-Bondapak C-18 reversed phase chromatography (HPLC). Elution: acetonitrile gradient (0–100%) in 0.1% TFA; flow rate: 1 ml min^{-1}. Inset: SDS-polyacrylamide gel electrophoresis of the trypsin inhibitor from *Enterolobium contortisiliquum* seeds (ECTI). Gradient: 20–25%. 1, ECTI; 2, isolated β-chain; 3, isolated α-chain; 4, isolated β-chain (reduced); 5, isolated α-chain (reduced).

Blood clotting

The ability of ECTI to affect blood clotting, such as thrombin time (TT), prothrombin time (PT) and activated partial thromboplastin time (APTT) was assayed using normal human citrated plasma. The increase in APTT indicates that ECTI blocks the contact phase of blood clotting ($n = 6$; $p < 0.01$), but not TT and PT, which are not significantly altered in the presence of ECTI.

ECTI strongly inhibits bovine trypsin and chymotrypsin and also some serine proteinases involved in the blood clotting cascade and fibrinogen proteolysis: human plasma kallikrein, factor XIIa and plasmin. ECTI showed no inhibitory activity on factor Xa, thrombin or tissue kallikrein or as on cysteine proteinases such as

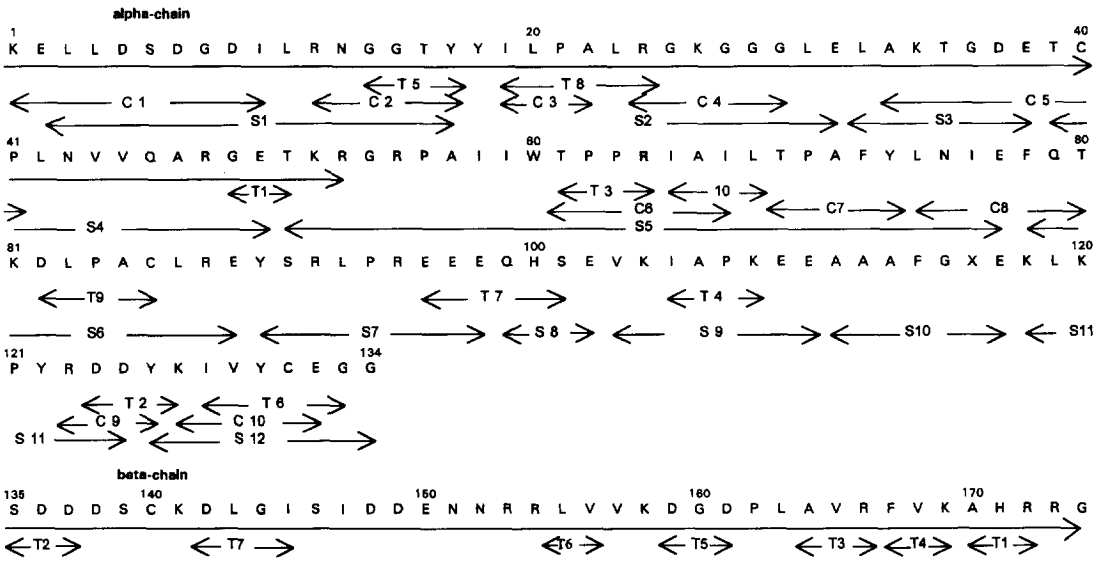

Fig. 2. Amino acid sequence of ECTI (*E. contortisiliquum* trypsin inhibitor), a double-chain Kunitz-type inhibitor. T, tryptic peptides; C, chymotryptic peptides; S, peptides from digestion with protease from *S. aureus*. ###, Amino acid residues identified by automated sequencing.

papain and bromelain. ECTI does not affect TT or PT, but APTT increases in the presence of ECTI inhibitor. This pattern of inhibition may reflect the conformation of the reactive site of the enzymes. Some proteinases such as thrombin, tissue kallikrein and factor Xa may have a conformation in the reactive site area that blocks the accessibility of ECTI to the catalytically active serine.

The distinctive binding capacity of ECTI for serine proteases may perhaps reflect a potential selectivity for inhibition of enzymes from herbivorous insects, as well as from other plant or animal sources.

EXPERIMENTAL

Seeds. Mature seeds were manually harvested from wild trees, in the Pantanal region of Brazil (Corumbá, MS), and seeds were manually peeled to expose the cotyledons.

Inhibitor purification. In a typical experiment, 30 g of 0.15 M NaCl swollen cotyledons were homogenized in a blender with 300 ml 0.15 M NaCl, centrifuged at 3000 *g*, and the supernatant was taken as the starting material (crude extract). The proteins in the crude extract were pptd by 80% (v/v) Me_2CO at 4°. The sediment sepd by centrifugation was dried *in vacuo*, and dissolved in 50 mM Tris–HCl buffer, pH 8.

Chromatography on DEAE-Sephadex. The above soluble fr. was applied to a DEAE-Sephadex column (2 × 25 cm) equilibrated with 50 mM Tris–HCl buffer, pH 8. After extensive washing with the equilibrium buffer, the inhibitor was eluted with 0.2 M NaCl in a linear gradient 0.05–0.5 M NaCl (250 ml of each soln). Protein elution was followed by measuring A 280 nm, and the inhibitory activity was followed by trypsin inhibition using BAPNA as substrate. The frs containing trypsin inhibitory activity were pooled, dialysed and lyophilized.

Gel filtration. The inhibitor from the previous step was submitted to gel filtration on a Sephadex G-75 column (1.4 × 85 cm) equilibrated with 0.1 M Tris–HCl buffer, pH 8, 0.1 M NaCl. The protein and inhibitory activity thus obtained were measured as described above, and ECTI was further chromatographed on a Superose 12 column in a FPLC system, in 50 mM Tris–HCl, pH 8, 0.1 M NaCl, with 0.5 ml min^{-1}.

Reversed phase chromatography. The gel filtered native protein as well as reduced and carboxymethylated protein and peptides obtained after chemical cleavage or enzymic digestions of the inhibitor were sepd on a μ-Bondapak C-18 reversed phase column in an HPLC system. The sepn was achieved by an MeCN gradient (0–100%) in 0.1% TFA (v/v) during 75 min, at 1 ml min^{-1}, at room temp.

SDS-electrophoresis. The homogeneity and the M_r was assessed by SDS-PAGE using a 10–20% acrylamide gradient gel as described by in ref. [19] and stained by Coomassie Blue.

Structure determination. For determination of ECTI structure, the native inhibitor was reduced and carboxymethylated [20]. The component α- and β-chains were sepd by reversed phase HPLC on μ-Bondapak C-18. These chains were hydrolysed by iodosobenzoic acid [21] and by enzyme digestion with trypsin [22], chymotrypsin [23] and *S. aureus* V8 protease [24]. Automatic sequencing was carried out on an Applied Biosystems Model 477A sequencer, and manual sequencing determined by the DABITC/PITC method [25].

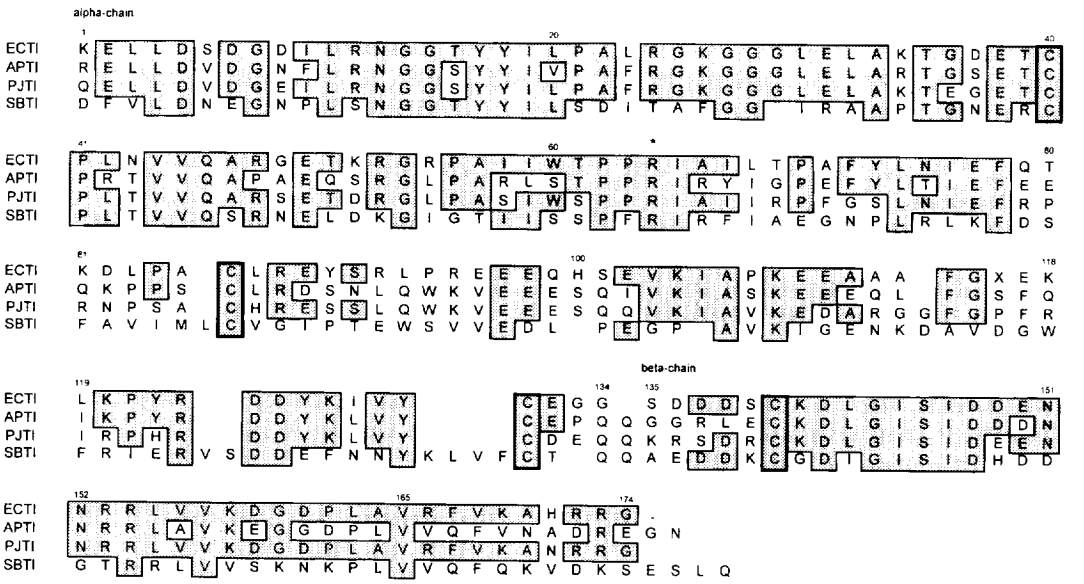

Fig. 3. Homology to other plant Kunitz-type inhibitors. 1, ECTI (*E. contortisiliquum* trypsin inhibitor); 2, APTI (*Adenanthera pavonina* trypsin inhibitor) [7]; 3, PJTI (*Prosopis juliflora* trypsin inhibitor) [6]; 4, SBTI (soybean trypsin inhibitor) [6]. *Indicates the reactive site.

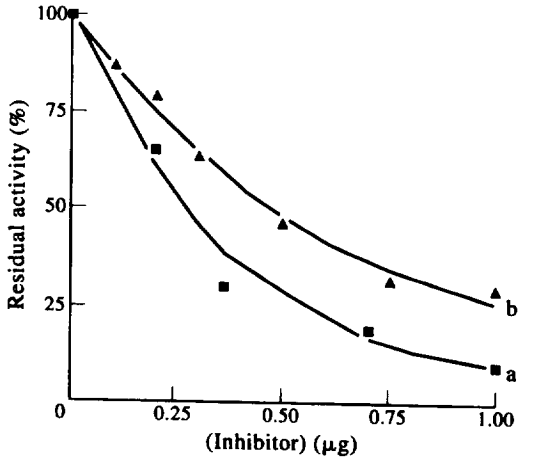

Fig. 4. Trypsin and HuPK inhibition. (a) Trypsin (10 $\mu g\,ml^{-1}$) was pre-incubated with increasing concentrations of ECTI in 50 mM Tris–HCl buffer, pH 8, 37 °C for 15 min. Residual activity was measured with 1 mM Bz–Arg–pNa. (b) Human plasma kallikrein (5 $\mu g\,ml^{-1}$) was incubated under the same conditions, but the residual activity was assessed using 1 mM Ac–Phe–Arg–pNa.

Table 1. Dissociation constants (K_i) for proteinases

Enzyme	K_i (10^{-9} M)
Trypsin	1.56
Chymotrypsin	120
HuPK	5.0
Factor XIIa	150
Plasmin	18
Papain	NI
Factor Xa	NI

K_i was determined by assuming a slow-tight binding, and the experimental points of curves, as those in Fig. 4, for each enzyme and suitable chromogenic substrates, were fitted into Morrison's equation [26].

Kinetic constant determination. The dissociation constants of the enzyme–inhibitor complexes were measured following Morrison's procedure [26]. The enzymes were pre-incubated with ECTI, and the remaining enzyme activity was measured with synthetic substrates. The inhibition constants were determined by the Enzifitter Program.

Clotting time. TT, PT and APTT were measured by standard procedures [27]. All incubations were performed in the presence of ECTI to check the inhibition activity on clotting time, physiological saline was used in the control.

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