A novel cysteine proteinase inhibitor from seeds of Enterolobium contortisiliquum and its effect on Callosobruchus maculatus larvae

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Abstract

This study focused on the characterization of a novel cysteine proteinase inhibitor from Enterolobium contortisiliquum seeds targeting the inhibition of the growth of Callosobruchus maculatus larvae, an important cosmopolitan pest of the cowpea Vigna unguiculata during storage. The inhibitor was isolated by ion-exchange besides of size exclusion chromatography. EcCI molecular mass is 19,757 Da, composed of two polypeptide chains. It strongly inhibits papain (Ki app 0.036 nM) and proteinases from the midguts of C. maculatus (80 μg mL⁻¹, 60% inhibition). The inhibitory activity is reduced by 40% after a heat treatment at 100 °C for 2 h. The protein displayed noxious activity at 0.5% and 1% (w/w) when incorporated in artificial seeds, reducing larval mass in 87% and 92%, respectively. Treatment of C. maculatus larvae with conjugated EcCI-FIT and subsequent biodistribution resulted in high fluorescence intensity in midguts and markedly low intensity in malpighian tubules and fat body. Small amounts of labeled proteins were detected in larvae feces. The detection of high fluorescence in larval midguts and low fluorescence in their feces indicate the retention of the FITC conjugated EcCI inhibitor in larval midguts. These results demonstrate the potential of the natural protein from E. contortisiliquum to inhibit the development of C. maculatus.

1. Introduction

Legumes have high levels of proteins, some of which are characterized as enzymes, storage proteins, and proteinase inhibitors [1–3]. Enterolobium contortisiliquum, a member of the Fabaceae family and Mimosoideae subfamily, are trees whose height exceeds 20 m and are commonly recognized as black ear due to the form of their fruits [4]. Its cotyledon has a high amount of proteins, several of which, including a serine proteinase inhibitor, have been characterized from seeds [5–10]. Inhibitors can act as highly definite substrates for target enzymes by forming a very stable enzyme-inhibitor complex that dissociates significantly more slowly than enzyme-substrate or enzyme-product complexes [11].

Proteins isolated from plants that block the activities of proteolytic enzymes are grouped into distinct families based on their primary sequences, the similarity of disulfide bond locations, and the position of reactive sites. These families are named Bowman-Birk, Potato I, Potato II, Kunitz, squash, barley, cystatin, and miscellaneous [1,11–13].

The Kunitz inhibitors family, extracted mainly from leguminous seeds, is the best characterized among other plant inhibitor families because of their abundance in seeds [1,14]. Kunitz inhibitors purified from Leguminosae seeds show molecular masses about 20 kDa (180 amino acid residues, with four cysteines in two disulfide bonds) [11,13]. Oliva et al. [1] regrouped the plant Kunitz members in subgroups according to the presence of cysteine residues from 1 to 4. The inhibitor of serine proteases found in E. contortisiliquum belongs to the Kunitz subgroup 4 with 2 S–S.

Proteinase inhibitors may play a role as reserve protein in plants [13,15] and may be implicated in plant defense mechanisms. These proteins may be produced during the normal development constitutively or induced in response to an injury [16,17]. The harmful action of these proteins on the growth and survival of insect larvae is well documented, parameters evaluated in experimental studies of insecticides [18,19].

Various insect orders contain chewing or sucking insects that are...
predators of legumes. Some, such as beetles or weevils from the Bruchidae family, Coleoptera order, have become skilled seed predators. The weevil Callistobruschus maculatus is a serious pest of several Vigna species, particularly Vigna unguiculata (L.) Walp. [20], attacking stored seeds and affecting crop quality and yield due to low seed germination rates. The reduction in potential seed germination in V. unguiculata is closely related to the degree of infestation by the weevil and can reach 100% in seeds with more than four holes, a situation which indicates the emergence of adult insects [21,22].

The use of proteinase inhibitors in the combating pests by targeting digestive enzymes of insects has received considerable attention. Bio-assays and experiments have demonstrated that feeding insects with rates. The reduction in potential seed germination in

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Inhibitor purification

The purification of the inhibitors from E. contortisiliquum seeds followed to the procedure shown in Fig. 1S, Supporting Information. The seeds (40 g) were homogenized in 0.02 M Tris/HCl, pH 8.0 (1:40 w/v) using a blender, centrifuged at 4000 × g, and the supernatant was fractionated with acetone at a final concentration of 80% v/v (80 mL of acetone and 20 mL of seed extract) at 4 °C. The precipitated protein was separated by centrifugation (30 min, 4 °C at 18,800 × g), and maintained at 25 °C until complete elimination of the residual acetone, solubilized in 0.02 M Tris/HCl buffer, pH 8.0, and applied to a DEAE-Sepharose column (2 × 25 cm) with 0.1 M Tris/HCl buffer, pH 8.0. The non-adsorbed material was removed by washing the column with an equilibration buffer and bound protein was eluted with NaCl (0.15 M and 0.3 M) in the buffer above.

Papain inhibition was measured by the inhibition of activity in the hydrolysis of 5 mM Z-Phe-Arg-p-Nan (Bachem, Bubendorf, Switzerland) as the substrate. A ÄKTA avant (GE Healthcare) was used in the process. Fractions from the DEAE-Sepharose column showing cysteine inhibitory activity (eluted with NaCl 0.15 M) were pooled and injected to a Superdex 75 column equilibrated with 0.05 M Tris/HCl buffer, pH 8.0. The fraction containing the inhibitor was then applied to a trespins-Sepharose column with 0.1 M Tris/HCl buffer, pH 8.0. The non-bounded fraction in the resin in which the cysteine inhibitor was detected was subsequently analyzed by C18 Vyda protein/peptide reverse phase (15/0.46 cm) eluted at a flow rate of 0.7 mL min⁻¹ with an acetonitrile gradient (0–100%) in trifluoroacetic acid (TFA) (0.1%, v/v).

Protein quantification in each purification step was determined by the Folin Ciocalteau assay [25] using serum albumin (BSA) at 0–500 μg mL⁻¹ as the standard curve.

2.1. Functional studies in the inhibitor

2.1.1. The inhibitory activity of EcCI on papain

The inhibition curve was prepared using 14 nM papain activated with 0.1 M NaH₂PO₄ pH 6.3, 0.01 M EDTA, 0.4 M NaCl, and 5 mM DTT at 40 °C for 10 min; this mixture was pre-incubated in the absence and presence of increasing concentrations of the inhibitor for 10 min at 40 °C, and subsequently added to the Z-Phe-Arg-p-Nan substrate (0.4 mM) (Calbiochem Ltda, Darmstadt, Germany); this mixture (final volume of 250 μL) was incubated for 30 min at 40 °C. The reaction was interrupted by adding 30 μL of 30% acetic acid (v/v). Hydrolysis of the substrate was checked by the absorbance of p-nitroaniline released at 405 nm in the Spectra max plus 384 (Molecular Devices). Inhibitory activity was determined by residual enzyme activity in the presence of EcCI compared to the control. The experiment was performed in duplicate.

2.2. Thermal stability of the inhibitor

Samples of EcCI in 10 mM PBA buffer (pH 7.0) were incubated at temperatures of 37 °C, 40 °C, 60 °C, 80 °C, and 100 °C for 30, 60, and 120 min. After heating, the samples were cool down to room temperature, and activity was measured and compared to the sample control that had been chilled on ice for 120 min. The inhibitory activity of papain was assayed as above.

2.2.2. Stability of EcCI at different pHs

EcCI was dialyzed against water and 500 μL aliquots were lyophilized. Lyophilized samples were reconstituted in 0.05 M sodium citrate buffer pH 3.0, 4.0, 5.0, and 6.0; 0.05 M sodium phosphate buffer pH 7.0; 0.05 M Tris/HCl buffer (pH 8.0 and pH 9.0); and 0.05 M sodium bicarbonate buffer pH 10.0.

After 3 h of incubation at 37 °C, the sample pH was fitted to 8.0, lyophilized, and reconstituted in 500 μL 0.05 M Tris/HCl, pH 8.0. The inhibitory activity of papain was subsequently determined.

2.2.4. Inhibitory activity of EcCI on cysteine proteinases present in the intestine of C. maculatus larvae

Larvae of C. maculatus (twenty-eighteen days old) were removed from infested seeds, placed on a stereomicroscope, and dissected with tweezers to isolate midguts. The intestines were macerated manually in 0.15 M NaCl solution under constant agitation for 1 h at 8 °C. The extract was centrifuged at 4000 g and 4 °C for 10 min. The collected supernatant was frozen at –20 °C.

This supernatant was 15-fold diluted in 0.1 M NaH₂PO₄ pH 6.3 buffer; 0.01 M EDTA; 0.4 M NaCl; and 5 mM DTT and incubated at 37 °C for 10 min to activate enzymes; 20 μL aliquots were incubated in the absence and presence of increasing concentrations of EcCI for 10 min at 37 °C. After this period, 20 μL of the Z-Phe-Arg-p-Nan substrate (5 mM) was added, and the final reaction volume was adjusted to 250 μL. Hydrolysis was monitored for 60 min, the reaction was stopped with 30 μL of acetic acid 40% (v/v), and absorbance was measured at 405 nm.

2.3. Structural characterization of EcCI

2.3.1. The N-terminal sequence identification

EcCI was denatured with 200 μL of 50 mM Tris/HCl buffer pH 8.5 containing 6.0 M guanidinium HCL, 1.0 mM EDTA, and 5.0 mM and reduced by dithiothreitol for 3 h at 37 °C. The S-pyridyl ethylation of cysteines was achieved by the addition of 5 μL 4-vinylpyridimidine for 3 h at 37 °C with incubation under a nitrogen atmosphere in the dark. After the incubate was applied onto a C18 and subsequently, the N-terminal sequences of the separated chains were determined by Edman degradation (PPSQ-23 Sequencer, Shimadzu, Tokyo, Japan) [26].

2.3.2. Mass spectrometry

The EcCI inhibitor molecular weight was determined by Liquid Chromatography coupled Electrospray Ionization Mass Spectrometric using the Waters 3100 apparatus attached to a Waters e 2695 separation module and a 2489 detector. A column (C18 2.1/150 mm, 60 Å, 3.5 μM/ Waters Nova-Park) was equilibrated with 0.1% trifluoroacetic (TFA) in Milli-Q water. The protein was eluted with 90% acetonitrile containing 0.1% TFA in Milli-Q water in a linear gradient (5–95%) for 30 min (wavelength 214 and 220 nm) at the flow rate of 0.4 mL min⁻¹ in positive mode (ES+), and under the following conditions: mass range between 200 and 2000 m/z; nitrogen gas flow of 6.0 L/h; 4.0 kV capillary; 40 V-cone voltage; 3.0 V extractor; source heater at 120 °C, solvent heater at 400 °C, 1.0 V ion energy; and 500.85 V multiplier [27].

2.3.3. SDS – PAGE

Electrophoresis non-reducing, and reducing (dithiothreitol, 200 mg mL⁻¹) conditions followed to the procedure described by Laemmli [28].
2.4. Spectroscopic measurements

Conformational studies of EcCI (0.1 mg mL$^{-1}$) were carried out in a Jasco J-810 circular dichroism spectropolimeter at 25 °C in a 1 mm path length cuvette. The CD spectrum was recorded in the 190–250 nm range as an average of eight scans. Data were expressed as the mean residue ellipticity ($\theta$) [29]. The percentage of secondary structure was estimated by deconvoluting the CD spectrum using the CDPro software package, which contains three CD analysis programs: CONTINLL, SELCON3, and CDSSTR with a protein reference set of 37 proteins [30, 31].

To analyze the pH effect on the secondary structure of EcCI, the protein (0.1 mg mL$^{-1}$) was incubated in phosphate-borate-acetate buffer (PBA) at 10 mM during 3 h and pH values of 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0. CD measurements were recorded as above [29].

The effect of temperature on the secondary structure of EcCI was also analyzed. Protein samples in 10 mM Tris/HCl buffer pH 8.0 (0.1 mg mL$^{-1}$) were heated at 25, 40, 60, 80, and 100 °C for 2 h. The CD spectrum in the 195–200 nm UV range was recorded as described above.

2.5. Bioassay

2.5.1. Insect

The C. maculatus colony was kept at the Proteins Chemistry and Function Laboratory of the Department of Biochemistry at the Federal University of São Paulo, São Paulo, SP, Brazil. Bruchids were raised on V. unguiculata host seeds (cv. Fradinho), acquired at city supermarkets, and maintained in glass bottles at 28 °C and the relative humidity was maintained at around 60–80% inside a B.O.D. incubator.

2.5.2. Artificial seeds

Artificial seeds (400 mg) prepared using a cylindrical brass mold filling up with fine flour of V. unguiculata cotyledons containing different concentrations (w/w) of EcCI were offered over 24 h to three C. maculatus females (2 days old) in the same conditions described in section 2.6.1. After 24 h, the females and excess eggs were removed to keep only four eggs on each seed. Artificial seeds containing only V. unguiculata flour were used as controls. Seeds were opened after 18 days of incubation, (28 °C and 60–80% relative humidity) larvae were weighed, and emergence numbers determined [29].

2.5.3. FITC (isothiocyanate fluorescein) conjugated to EcCI

FITC (50 mg mL$^{-1}$ in DMSO) was diluted with 0.75 M bicarbonate buffer pH 9.5 and added to EcCI in a 1:1 (w/w) ratio. The tube was wrapped in aluminum foil and incubated at room temperature with rotation for 1 h, dialyzed against Milli-Q water for the elimination of the unconjugated FITC, and lyophilized for further incorporation into artificial seeds [29].

2.5.4. Artificial seeds containing EcCI coupled to FITC

FITC-EcCI (1.0% w/w) was mixed with cowpea flour compacted and inserted into gelatin capsules. Three C. maculatus larvae (fourth instar) present in the seeds of V. unguiculata were transferred to gelatin capsules to allow feeding movements. After a feeding period of 48 h, were dissected for analysis by confocal microscopy (Leica TCS SP8) of the midguts, Malpighian tubules, and fat bodies [29, 32, 33].

3. Results

3.1. Purification of E. contortisiliquum cysteine proteinase inhibitor (EcCI)

The saline extract of EcCI from E. contortisiliquum seeds was fractionated by acetone (80% v/v) and purified by ion-exchange chromatography over a DEAE-Sepharose column (Fig. 1A). The fraction exhibiting inhibitory activity toward the papain enzyme was pooled, lyophilized and onto to Superdex 75 column (Fig. 1B). The fraction containing the cysteine proteinase inhibitor was chromatographed on trypsin-Sepharose to reduce the contamination of the serine proteinase inhibitor (data not shown). The purification phases are in Table 1. Conformational studies of EcCI were carried out in a Jasco J-810 circular dichroism spectropolarimeter at 25 °C in a 1 mm path length cuvette. The CD spectrum was recorded in the 190–250 nm range as an average of eight scans. Data were expressed as the mean residue ellipticity ($\theta$) [29]. The percentage of secondary structure was estimated by deconvoluting the CD spectrum using the CDPro software package, which contains three CD analysis programs: CONTINLL, SELCON3, and CDSSTR with a protein reference set of 37 proteins [30, 31].

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The effect of temperature on the secondary structure of EcCI was also analyzed. Protein samples in 10 mM Tris/HCl buffer pH 8.0 (0.1 mg mL$^{-1}$) were heated at 25, 40, 60, 80, and 100 °C for 2 h. The CD spectrum in the 195–200 nm UV range was recorded as described above.

3.2. Functional studies of the EcCI inhibitor

3.2.1. Inhibitory activity of EcCI on papain

EcCI inhibits the cysteine proteinase papain with an apparent dissociation constant (K$\text{app}$) of 0.036 nM (Fig. 2A). The inhibitor loses 40% of its activity after exposure to 100 °C for 120 min in 10 mM PBA buffer, pH 7.0 (Fig. 2B); its functionality is best maintained within the pH range of 8.0 and 9.0 (Fig. 2C).
3.2.2. Inhibitory activity of EcCI on cysteine proteinases from intestinal extracts of C. maculatus larvae

The inhibitory effect of EcCI was tested on enzymes extracted from the C. maculatus larvae midgut. EcCI (80 μg mL⁻¹) inhibited 60% of proteolytic activity present in the larval extract (Fig. 2 D).

3.3. Structural characterization of the EcCI inhibitor

SDS-PAGE electrophoresis of EcCI showed an apparent molecular mass of approximately 18.0 kDa and reducing conditions showed two polypeptide chains (Fig. 3 A). The exact EcCI molecular mass is 19,757 Da, as determined by mass spectrometry (Fig. 3 B). The N-terminal sequence of the isolated alpha chain SNLLLTDGNNLGYYILPAHSGKGGG determined by the Edman degradation as well some amino acids residues from the isolated B chain (Fig. 4 A and B) compared to other sequences deposited in the protein database using the Blast protein program available at http://blast.ncbi.nlm.nih.gov/ have similarity to Kunitz inhibitors, such as the inhibitors purified from Prosopis juliflora [34], Adenatera pavonina [35], Copaifera langsdorffii [36], E. contortisiliquum [7] and Bauhinia bauhinioides [37] among others.

3.4. Spectroscopic measurements

CD spectroscopy was used to characterize EcCI secondary structure. The spectrum showed a negative band at 201.5 nm. Based on CD, the EcCI secondary structure was estimated to be composed of 19% α-helices, 26% β-sheets, 24% β-turns, and 31% unordered structure (Fig. 5 A). Three programs (CONTIN, SELCON3, and CDSSTR) were used to improve the reliability of the protein CD analysis, which resulted in less than 2.0% deviation.

EcCI thermal stability was also analyzed by CD. The EcCI CD spectrum showed approximately 46% decrease in the intensity of ellipticity at 201.5 nm with an associated displacement of the band at 208.5 nm in the temperature range from 25 to 100 °C demonstrating that the EcCI conformation is stable in the temperature range from 25 °C to 40 °C (Fig. 5 B).

In contrast, extreme pHs have little effects on the EcCI conformation because no change to its secondary structure was observed after exposure to different pHs (Fig. 5 C).

3.5. EcCI inhibitor effects on C. maculatus larval development

The EcCI inhibitor was toxic to C. maculatus larvae. Fig. 6 A shows that the inhibitor reduces larvae body mass by 87% and 92% at

### Table 1

| Steps                  | Volume (mL) | Protein (mg/mL) | Total protein (mg) | UI/mL | UI/total | EA | Purif. Yield (%) |
|------------------------|-------------|-----------------|--------------------|-------|----------|----|------------------|
| Precipitation at 80% acetone | 400         | 2.9             | 1160               | 50    | 20,000   | 17.24 | 1 100            |
| DEAE-Sepharose          | 132         | 1.6             | 211.2              | 37.5  | 4950     | 23.44 | 1.36 24.75       |
| Superdex 75             | 120         | 0.04            | 4.8                | 10    | 1,200    | 250  | 14.5 6           |

*a* Inhibition Unit per mL.  
*b* Total Inhibition Unit (UI/mL x total volume).  
*c* Specific activity.  
*d* Fold purification.
concentrations of 0.5% and 1.0% (w/w), respectively when compared to control larvae.

3.6. Effects of conjugated EcCI-FIT on C. maculatus larvae

Treatment of C. maculatus larvae with conjugated EcCI-FIT and subsequent biodistribution resulted in high fluorescence intensity in the midgut of larvae (Fig. 6B a-b), and markedly lower intensity in the Malpighian tubules (Fig. 6B c-d) and fat body (Fig. 6B e-f). Few labeled proteins were detected in larvae feces (Fig. 6B g-h).

EcCI protein was visualized on the cellular surface of the midgut (Fig. 6 C a-b) using microscopic analysis at 63× magnification (Fig. 6C); however increase in EcCI protein in malpighian tubules (Fig. 6C c-d) and fat bodies (Fig. 6C e-f) was observed around to the cells, indicating that EcCI was not internalized.

4. Discussion

A large number of inhibitors have been purified from several families of legumes such as Mimosoideae [7,38], Caesalpinioideae [39,40], and Papilionoideae [41,42]. Two inhibitors have been described in E. contortisiliquum seeds, the serine proteinase inhibitor EcTI [7] and a...
such as the canecystatin recombinant inhibitor [46], cystatin extracted from apples [16], the P. juliflora inhibitor [47], and the ApTI inhibitor from A. pavonina [19] with Ki’s of 3.3 nM, 0.21 nM, 0.59 nM, and 1.0 μM, respectively.

The EcCI amino-terminal region is not similar to phytocystatins, the archetype of plant cysteine protease inhibitors [48,49], but is similar to BbCI, fitting the classical plant Kunitz inhibitor family, mainly characterized from seeds of Fabaceae, and the Mimosoideae, Papilionoideae, and Caesalpinioideae taxonomic subfamilies. The EcCI N-terminal region shows similarity with purified proteins from the Caesalpinioideae subfamily such as Bauhinia bauhinoides [37], Copaifera langsdorffii [36], and Caesalpinia echinata [50], and the following Mimosoideae subfamily members: Adenathera pavonina [35], Enterolobium contortisiliquum [7], Leucaena leucocephala [51], Acacia confused [52] and Prosopis juliflora [34]. These groups include proteins with a molecular weight of approximately 20–22 kDa, consisting of one or two polypeptide chains and generally four cysteine residues that form two disulfide bonds (CyS39-CyS83 and CyS36-CyS145, in comparison of the soybean trypsin inhibitor) [11,13]. However, the BbCI inhibitor is devoid of cysteine residue [37].

The EcCI molecular mass of 19,757 kDa is comparable with other Kunitz inhibitors [1] but differs from EcTI (19,851 kDa) [10]. It should be noted that inhibitors isolated from species in the Mimosoideae subfamily, including EcCI, are constituted by two polypeptide chains with a long chain (designated A or α) of approximately 13–16 kDa, an intra-chain disulfide bridge, and a small chain (designated B or β) of around 5–6 kDa that binds alpha chain by a disulfide bridge.

Although no structural similarity was detected between EcCI and classical inhibitors of cysteine proteases, the amino-terminal region showed similarity to proteins such as BbCI [37], ApTI [19,53], and the inhibitor extracted from Prosopis juliflora [47], which are described as inhibitors of this class of enzymes. Secondary structure analysis shows a high proportion of β-structures and disordered structures indicating that EcCI belongs to the class of β proteins and confirming its similarity to the Kunitz family of inhibitors whose structural feature is the high percentage of betas and disordered structures [52,54,55].

EcCI, unlike EcTI, is thermostable: its inhibitory activity against papain decreases by only 40% after 2 h exposure to 100 °C, while EcTI is less heat resistant and shows total decreased functionality after 10 min of exposure to heat [7]. The loss of functionality of proteins in even short treatment periods at extreme pH conditions is common. However, EcCI
Fig. 6. Bioassay. (A) EcCI inhibitory effect on the development of larvae. Effect of EcCI on hatched larvae mass. The inhibitor was incorporated into artificial seeds of *V. ungiculata* at concentrations of 0.5% and 1.0% (w/w). *N* = 20 larvae. *Statistical significance compared to the control. The data represent means ± SEM (p-value < 0.05 by One-way ANOVA). (B) EcCI-FITC conjugate in *C. maculatus* larvae, visualization with 10× magnification. a-b midgut, c-d Malphigian tubules, e-f fat body, g-h feces. Bar = 173 μm. (C) EcCI-FITC conjugate in *C. maculatus* larvae, visualization at 63× magnification. a-b midgut, c-d Malphigian tubules, e-f fat body. Bar = 32.3 μm.
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Declaration of competing interest

The authors declare that they have no competing interests.

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