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Purification and biochemical properties of a Kunitz-type trypsin inhibitor from *Entada acaciifolia* (Benth.) seeds

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ABSTRACT

A new trypsin inhibitor (EATI) was isolated from *Entada acaciifolia* (Benth.) seeds. EATI is a competitive inhibitor with a molecular mass of 20 kDa and an inhibition stoichiometry of 1:1 for bovine trypsin. The dissociation constant (K_i) calculated was 1.75 nmol/L, displaying a high affinity between enzyme and inhibitor. Both Native PAGE and RP-HPLC revealed that EATI is composed of four isoinhibitors that share the amino acid composition and the amino-terminal sequence homolog to Kunitz-type inhibitors. EATI is stable to denaturation by heat (up to 70 °C), pH (2–10), urea (8 mol/L) and its inhibitory activity was unaltered in different concentrations of DTT (up to 100 mmol/L). CD analysis revealed that EATI in reduced form underwent structural modifications associated with a decrease in thermal and pH stabilities, suggesting that their disulfide bonds are not involved in the structuring of its reactive site, but are important for maintenance of its conformational stability. This behavior makes EATI one of the few inhibitors described in the literature with high DTT resistance.

1. Introduction

Protein protease inhibitors (PIs) are proteins able of inhibiting the catalytic activity of proteolytic enzymes. These inhibitors are found throughout all life forms, but plants are recognized as a potential source for most of the naturally occurring PIs which have been isolated and well characterized [1]. The most described plant inhibitors are among the families of Fabaceae, Poaceae and Solanaceae, occurring in reproductive and storage organs and also in vegetative tissues [2], being synthesized constitutively during normal development or induced in response to herbivory, corroborating the evolution of PIs as one of the natural defensive strategies against insect pests and pathogens [3,4].

Kunitz and Bowman-Birk are the most studied inhibitor families and are found in abundance in various leguminous plants [3,5]; Kunitz family is usually 18–24 kDa heterogeneous proteins consisting of a number of isoinhibitors, with two disulfide linkages, a low cysteine content, and a single trypsin reactive site in one of the protein loops, whereas Bowman-Birk family shows smaller proteins (8–10 kDa), with seven disulfide linkages and present two independent reactive sites for trypsin and chymotrypsin [5,6].

The use of PIs for crop protection has been studied extensively [4,7]. PIs inhibit insect gut proteases by binding tightly to the active site, leading to essentially inactive complex formation. The inability to utilize ingested protein and to recycle proteolytic enzymes results in a critical amino acid deficiency, which affects the growth, development and survival of the insects [8]. For this reason, the incorporation of genes encoding PIs into transgenic plants has been proposed as a method for preventing seed damage by insect attacks, while presenting few or no side effects in vertebrates [9]. The possibility of using PIs as candidates for protein miniaturization has been increasing considerably. Rational design of models with properties similar to the reactive site is a valuable tool for a better comprehension of structure–function relationships, providing additional information for the development of better inhibitors for multidomain PIs [10,11].

Although PIs have been detected in the seeds of many plant species, there are many taxa where they have not yet been studied and thus, these could represent a source of novel information on PIs structures and properties [12,13], contributing to drug design for the control of diseases and pathologic processes [14,15]. *Entada acaciifolia* (Benth.) belongs to the subfamily

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Mimosoideae of the Fabaceae and presents a wide distribution in Brazilian southeast and center west. The present paper reports the purification, biochemical characterization, and amino-terminal sequence of EATI, a trypsin inhibitor isolated from *E. acaciifolia* seeds.

2. Materials and methods

2.1. Materials

E. acaciifolia seeds were collected locally (Campinas, SP, Brazil) and identified by the Laboratory of Plant Taxonomy, University of Campinas. The seeds were washed and air-dried before use. Bovine pancreatic trypsin, bovine serum albumin (BSA), soybean trypsin inhibitor (STI), 5,5′-dithio-bis(2-nitrobenzoic acid) (DNTB), L-cysteine and Nα-benzoyl-D,L-arginine 4-nitroanilide hydrochloride (BAPNA) were purchased from Sigma (St. Louis, MO, USA), as were sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) molar mass markers, acrylamide, bis-acrylamide, dithiothreitol (DTT) and other electrophoresis reagents. Chromatographic supports were from GE Healthcare. All other chemicals and reagents used were of analytical grade.

2.2. Purification of E. acaciifolia trypsin inhibitor

Defatted *E. acaciifolia* seed powder was extracted with 10 volumes of 0.1 mol/L sodium phosphate buffer, pH 7.6, overnight at 4 °C, and centrifuged at 5000 × *g* for 30 min. The supernatant (crude extract) was dialyzed against water at 4 °C, lyophilized and assayed for antitrypsin activity. This extract, dissolved in 0.1 mol/L sodium phosphate buffer, pH 7.6, containing 0.1 mol/L NaCl, was applied to a Sephadex G-75 column (80 cm × 3 cm) equilibrated with the same buffer at a flow rate of 12 mL/h. The fractions containing inhibitory activity were pooled, dialyzed, lyophilized and loaded to a DEAE-Sepharose (ion exchange) column (12 cm × 3 cm), pre-equilibrated with 50 mmol/L Tris–HCl buffer, pH 8.0, at a flow rate of 40 mL/h. The elution of proteins was made with a linear gradient of NaCl (0–1 mol/L). The peak that showed inhibitory activity was applied to a trypsin-Sepharose column (12 cm × 3 cm) equilibrated with 0.1 mol/L sodium phosphate buffer, pH 7.6, containing 0.1 mol/L NaCl at a flow rate of 30 mL/h. The protein bound to the column was eluted with 0.05 mol/L HCl, dialyzed and lyophilized.

Additionally, EATI was subject to a reverse phase high performance liquid chromatography (RP-HPLC) C4 column (4.6 mm × 150 mm) previously equilibrated with 0.1% (v/v) of TFA (solvent A), followed by a linear gradient from 0 to 100% (v/v) of 66% (v/v) of acetonitrile in 0.1% (v/v) of aqueous trifluoroacetic acid (solvent B) at a flow of 1 mL/min. The elution profile was monitored at 280 nm.

2.3. Protein quantification

Protein concentrations were determined by Coomassie blue staining using the Bradford method [16]. BSA (1 mg/mL) was used as standard.

2.4. Polyacrylamide gel electrophoresis

SDS–PAGE (12.5%) in the absence and presence of DTT (0.1 mol/L) was carried out as described by Laemmli [17]. Low range molecular weight standards (14.4–97.4 kDa) were used. The proteins were detected by staining with 0.1% of Coomassie brilliant blue R-250. For Native PAGE (15%) [17], 2 μg of purified EATI was used and the gel was submitted to APNE staining. The gel was fixed in a TCA 12.5% solution, incubated by 30 min in 0.1 mol/L sodium phosphate buffer, pH 7.4 containing 0.1 mg/mL trypsin and then transferred for a solution containing APNE (2.5 mg/mL in dimethylformamide) and *o*-dianisidine tetrazotized (0.55 mg/mL in 0.1 mol/L sodium phosphate buffer, pH 7.4) until the appearance of transparent bands against a pink background.

2.5. Amino acid analysis

The amino acid analysis of EATI was performed in a Pico-Tag amino acid analyzer (Water System), as described by Henrison and Meredith [18]. The sample was hydrolyzed in 6 mol/L HCl and 1% of phenol at 106 °C for 24 h. The hydrolysates were reacted with 20 μL of fresh derivatization solution (methanol/triethylamine/water/phenyl isothiocyanate, 7:1:1:1, v/v) for 1 h at room temperature. After pre-column derivatization, phenylthiocarbamyl (PTC)-amino acids were identified by comparing their RP-HPLC retention times to those of standard PTC-amino acids. The identification of proteins with similar amino acid composition was made with AACompIdent in ExPASy proteomics server [19].

For quantitation of total sulfhydryl groups, Ellman's reagent (DNTB) was used. The quantification of equimolar solutions of EATI and STI (10 mg/mL) were determined and the concentration of total sulfhydryl groups compared with a calibration curve obtained with L-cysteine (1000 μM).

2.6. Amino-terminal sequencing

The peaks separated through of RP-HPLC had its amino-terminal sequence determined in a Shimadzu PPSQ-10 Automated Protein Sequencer performing Edman degradation. Sequences were determined from samples blotted onto polyvinylidene fluoride membranes after SDS-PAGE. Phenylthiohydantoin-amino acids were detected at 269 nm after separation on a RP-HPLC C18 column (4.6 mm × 250 mm) under isocratic conditions, according to the manufacturer's instructions. Percentage sequence identity with trypsin inhibitors was searched for automatic alignment, performed using the NCBI-BLAST search system [20]. Multiple alignments were performed using CLUSTAL W (2.0.12) [21].

2.7. Assay of inhibitory activity

Trypsin inhibitory activity was determined by measuring the residual hydrolytic activity of bovine trypsin towards the substrate BAPNA [22]. Aliquots of 4 μL of trypsin (0.25 mg/mL) were incubated with different concentrations of EATI in 50 mmol/L Tris–HCl buffer containing 20 mmol/L Ca^{2+}, pH 8.0. After 15 min incubation at 37 °C, 200 μL of 1 mmol/L BAPNA was added (final 270 μL assay volume). The total time of assays was 30 min. The changes in absorbance at 410 nm, after subtracting the blank solution, were recorded on a VersaMax Microplate Reader (Molecular Devices). One trypsin inhibitory activity unit (TIU) was defined as the decrease in 0.01 units of absorbance per 30 min at 37 °C.

2.8. Kinetic analyses

For determination of kinetic parameters of dissociation constant (K_i) and the mode of EATI inhibition, Dixon plot and Eadie–Hofstee Plot were used, respectively. The stoichiometric ratio between EATI/trypsin also was determined. Increasing concentrations of EATI were added to a fixed concentration of enzyme and the residual enzymatic activity determined. Each result was represented by the mean of three independent assays.

2.9. Stability of inhibitory activity against bovine trypsin

For stability studies, an EATI solution (1 mg/mL in 50 mmol/L Tris–HCl buffer, pH 8.0) was heated for 30 min at different temperatures (37–100 °C), and then cooled to 0 °C before testing for residual inhibitory activity. To measure pH stability, the inhibitor solution was prepared with an equal volume of diverse buffers (0.1 mol/L final concentration): sodium citrate (pH 2–4), sodium acetate (pH 5), sodium phosphate (pH 6–7), Tris–HCl (pH 8) and sodium bicarbonate (pH 9–10). After incubation in each buffer for 1 h at 37 °C, the remaining inhibitory activity was measured as previously described. All experiments were carried out in six replicates and the results are the means of three assays ± SD.

EATI was incubated with DTT at final concentrations of 1, 10 and 100 mmol/L for 15–120 min at 37 °C. The reaction was terminated by adding iodoacetamide at twice the amount of each DTT concentration, and the residual inhibitory activity on trypsin was then determined. A positive control with soybean trypsin inhibitor-STI (1 mg/mL) was performed.

2.10. Stability of reduced EATI

In order to evaluate the thermal and pH stability of reduced inhibitor, EATI was incubated with 100 mmol/L DTT for 2 h at 37 °C. The reaction was terminated by adding iodoacetamide at twice the amount of each DTT concentration and the inhibitor was immediately submitted to the assays of thermal and pH stability, as previously described.

2.11. Circular dichroism of EATI

Far-UV CD spectra (190–250 nm) were collected on a JASCO J-715 using a 1 mm path length quartz cell. A protein concentration of 0.1 mg/mL dissolved in 10 mmol/L sodium phosphate buffer, pH 7.6, was utilized, and each spectrum obtained is the result of 32 accumulations. Measurements were taken for native EATI, EATI treated with 8 mol/L urea and 1 mmol/L DTT for 2 h. The molar ellipticity was obtained and the percent secondary structure was predicted using the DICHROWEB server [23] through the CONTIN algorithm [24].

2.12. Statistical analysis

The data were examined using one-way analysis of variance (ANOVA) with a *p*-value of >0.05 considered significant. The analysis was performed with the GraphPad InStat program (GraphPad software).