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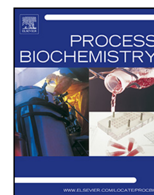

Exploiting the biological roles of the trypsin inhibitor from *Inga vera* seeds: A multifunctional Kunitz inhibitor

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ABSTRACT

Here, the purification, biochemical and biological properties of a trypsin inhibitor from *Inga vera* seeds (IVTI) are described. Partial amino acid sequence of IVTI showed that it belongs to the MEROPS I03 Kunitz inhibitor family. Moreover, it is composed of a single 20 kDa polypeptide chain with one disulfide bridge and was capable of inhibiting bovine trypsin at a 1:1 molar ratio with a 1.15 nM inhibition constant. IVTI was stable over a wide range of temperature, pH and concentration of a reducing agent. IVTI also inhibited the trypsin-like enzymes from the midgut of lepidopteran pests, such as *Anagasta kuehniella* (89%), *Spodoptera frugiperda* (83%), *Corcyra cephalonica* (80%), *Heliothis virescens* (70%) and *Helicoverpa zea* (64%). Furthermore, bioinsecticidal assays against *A. kuehniella* demonstrated that IVTI affected larval development by impairing weight gain and survival, as well as altering the duration of the larval cycle. IVTI was also fungicidal to *Candida buinensis* and bacteriostatic agent to *Escherichia coli*. Further assays revealed that IVTI is a chemopreventive agent against human epithelial colorectal adenocarcinoma cells (CACO-2), reducing cell viability by 70% at 200 $\mu g\,mL^{-1}$. In summary, these results demonstrate the multifaceted potential of IVTI as a biotechnological tool for agriculture and healthcare.

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1. Introduction

Plant peptidase inhibitors (PIs) are widely distributed in plant tissues, especially in seeds and tubers [1]. They play an essential role in plant development due to their modulation of peptidase activities that are normally involved in important physiological processes [2,3]. They might also be associated with plant defense

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mechanisms, by protecting them against insects and pathogens [1,4].

PIs exhibit a wide range of biological activities that has attracted much interest mainly because of their potential applications in agriculture and medicine [1,5]. As multifaceted molecules, they have been studied for improvement of plant resistance to pathogens and insects [4,6–8], antimicrobial agent [9,10] and anticancer agent [11–14].

In order to isolate and characterize PIs with distinct properties, many plant species, belonging to different botanical families, have been studied [15–17]. Among them, serinopeptidase inhibitors are the most explored class, which presenting different physicochemical characteristics, such as the disulfide bridge topology, molecular mass and inhibition specificity. These features allow classifying the serinopeptidase inhibitors into families known as Kunitz, Bowman–Birk, Potato I and II, squash, mustard and cystatins [18]. However, there has never been a comprehensive system of classification for these molecules. In view of this, Rawlings et al. [19] implemented a new method of classification in MEROPS database, which is based molecular structure and homology. This novel classification system intends to facilitate the exchange, storage and retrieval of information about PIs.

In this paper, we describe the purification and biochemical properties of a Kunitz inhibitor from *Inga vera* seeds, denominated IVTI. *I. vera* belongs to the Mimosoideae subfamily of Fabaceae, and it has a wide distribution from the North to the South of Brazil and it is mostly found in wetter areas [20]. In order to explore the potential of IVTI as a biotechnological tool, we investigated its biological activity against insect pests and pathogenic microorganisms, as well as the cytotoxicity effect to human normal and tumor cell lines.

2. Material and methods

2.1. Material

Inga vera seeds were obtained from our seed bank. Bovine pancreatic trypsin, bovine pancreatic α-chymotrypsin, bovine serum albumin (BSA), *N*-α-benzoyl-dl-arginine-*p*-nitroanilide (BApNA), *N*-α-benzoyl-dl-tyrosyl-*p*-nitroanilide (BTpNA), dithiothreitol (DTT) and electrophoresis reagents were purchased from Sigma (St. Louis, MO, USA). Chromatography supports were acquired from GE Healthcare. All other chemicals and reagents used were of analytical grade.

2.2. Purification of I. vera trypsin inhibitor

I. vera seeds were dried at room temperature and stored at −20 °C until use. The seeds were then ground and defatted using hexane. A crude extract was obtained by extraction of this meal (100 g) with 100 mM potassium phosphate pH 7.6 (1:10, w/v) for 2 h at room temperature, with subsequent centrifugation at 7.500*g* for 30 min. The supernatant was dialyzed against water at 4 °C and lyophilized before the inhibitory activity against trypsin was assayed. For IVTI purification, the crude extract (150 mg) was dissolved in a 50 mM Tris-HCl pH 8.0, and loaded onto a DEAE-Sepharose column (2 × 20 cm) equilibrated in the same buffer. Proteins were eluted with a 0–1 M NaCl linear gradient in 50 mM Tris-HCl pH 8.0 at a flow of 40 mL h^{-1}. The fractions containing inhibitory activity were pooled, dialyzed and lyophilized. For the next purification step, the peak containing inhibitory activity was loaded onto a trypsin-Sepharose column (2 × 10 cm) equilibrated with 100 mM sodium phosphate pH 7.6 containing 100 mM NaCl. The elution of protein bound to the column was performed with 100 mM HCl at a flow rate of 30 mL h^{-1}. Furthermore, IVTI was subjected to a reverse phase high performance liquid chromatography (RP-HPLC) for analytic analyze and amino-terminal sequencing. Therefore, it was used a Discovery® BIO Wide Pore C8 column (25 × 4.6 mm, 10 μm) previously equilibrated with 0.1% (v/v) of trifluoroacetic acid (solvent A), followed by a linear gradient from 0 to 100% (v/v) of 66% (v/v) acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid (solvent B). In all purification steps, the proteins were monitored at 280 nm.

2.3. Protein estimation

Protein concentration was measured by the Bradford [21] method, and 1 mg mL^{-1} of bovine serum albumin (BSA) was used as the standard.

2.4. Inhibitory activity assays

The inhibitory activity assays were performed according Erlanger et al. [22] by measuring the residual hydrolytic activity of bovine trypsin and chymotrypsin against the substrates BApNA and BTpNA, respectively. For trypsin inhibitory activity, aliquots of 50 μL of trypsin (0.125 mg mL^{-1} in 1 mM HCl) were incubated with different concentrations of IVTI in 50 mM Tris-HCl pH 8.0. Following incubation at 37 °C for 15 min, 500 μL of 1 mM BApNA were added to the reaction. After 30 min, the reaction was stopped by adding 250 μL of 30% (v/v) acetic acid (1 mL final assay volume). To determine chymotrypsin inhibitory activity, aliquots of 75 μL of chymotrypsin (2 mg mL^{-1} in 1 mM HCl) were incubated with different concentrations of IVTI in 50 mM Tris-HCl pH 8.0. Following incubation at 37 °C for 15 min, 25 μL of 5 mM BTpNA were added, and the reaction was carried out for 5 min. The reaction was stopped by adding 250 μL of 30% (v/v) acetic acid (500 μL final assay volume). The substrate hydrolysis was monitored at 410 nm. One trypsin inhibitory activity unit (TIU) or one chymotrypsin inhibitory activity unit (CIU) was defined as a decrease of 0.01 units of absorbance at 410 nm in relation to control samples (without inhibitor).

2.5. Polyacrylamide gel electrophoresis

SDS-PAGE (12.5% (w/v)) was performed as described by Laemmli [23] using low range molecular weight standards (14.4–97.4 kDa). The proteins separated on the gels were subsequently detected by staining the gel with 0.1% (w/v) Coomassie Brilliant Blue R-250 and destaining with a solution of distilled water, methanol and acetic acid (5:4:1–v/v/v).

2.6. Mass spectrometry analysis and amino acid sequencing

The amino-terminal sequence of IVTI was determined by direct sequencing using automated Edman degradation with a PROCISE amino acid sequencer (Applied Biosystems). The phenylthiohydantoin (PTH) amino acids were identified in a model 140C microgradient PTH amino acid analyzer (Waters system) based on their retention times.

UltrafleXtreme MALDI-TOF/TOF equipment (BrukerDaltonics, Bremen, Germany) was used for the analyses. The matrix applied was α-cyano-4-hydroxycinnamic acid (CHCA) at saturated solution. The matrix and samples were prepared in acetonitrile (ACN) and water with 0.1% trifluoroacetic acid (30:70, v/v). The sample solutions and the matrix (1:1 v/v) were mixed in equal amounts and, spotted onto a ground stainless steel MALDI target (1 μL). The analyses of peptides from tryptic difestion and protein intact were performed by reflector (600–4000 Da) and linear (15–50 kDa) mode, both using positive ion mode. The experimental conditions used for MS analyses in reflector mode were: pulsed ion extraction of 110 ns, laser frequency of 1000 Hz and voltages of ion source 1

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