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Partial purification and characterization of a trypsin inhibitor isolated from *Adenanthera pavonina* L. seeds

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ABSTRACT

Trypsin inhibitors are important tools used by plants against parasites and once isolated may be directed to biotechnological applications. In this work, a trypsin inhibitor was extracted and isolated from the seeds of *Adenanthera pavonina* L. (Fabaceae). After, the biomolecule was purified and characterized in terms of its thermal stability and molecular weight. In addition its toxic effect against *Artemia salina* was investigated. The purification process was based in cetonic precipitation with acetone 70% (*v*/*v*) and anion-exchange chromatography on DEAE-Sephadex G-50. SDS–PAGE analysis, under reducing condition, showed that protein consists of a single polypeptide chain with molecular mass of approximately 14.1 kDa. The biomolecule is a competitive inhibitor with an inhibition stoichiometry of 1:2 for bovine trypsin. The inhibitor purified presented thermal stability in wide range of temperature (25–100 °C), and showed still effective inhibition (61%) until 100 °C during 30 min. Furthermore a volume of 0.16 mg/mL of the inhibitor was enough to present toxicity against the larvae of *Artermia salina* (100% of death rate after 72 h). The species *A. pavonina* is therefore source of trypsin inhibitors with high activity and could be used for obtaining the biomolecule in accord to its demand mostly in pharmaceutical and environment industry, medicine and others. As a likely source of potent trypsin inhibitors, the extract obtained from seeds of *A. pavonina* is discussed to be useful directly in the agro-industry, in the production of value-added bioproducts or in general biotechnological applications.

1. Introduction

Fabaceae (Leguminosae) is a largely distributed family of plants with high species diversity divided in three subfamilies: Caesalpinioideae, Mimosoideae, and Papilionoideae (Joly, 2002; Judd et al., 2009), which comprises leafy tropical forests, shrubs, herbs and vines (Joly, 2002).

Seeds from Fabaceae family present abundant protein content (Nikolić et al., 2012) and large amount of protease inhibitors (Negreiros et al., 1991; Chevreuil et al., 2014). Besides, in these plants, protease inhibitors were described in seeds, leaves and bulbs (Fürstenberg-Hägg et al., 2013) and play an essential role, such as protection against attack by herbivores and pathogens (Macedo et al., 2004) and enzymatic regulation (Migliolo et al., 2010; Ramos et al., 2012).

Trypsin inhibitors extracted from seeds of Fabaceae species have been purified and characterized with the aim of obtaining their practical use (Ramos et al., 2012), concerning their action on the plant protection against pathogenic agents (Valueva and Mosolov, 2004), in addition to providing the security of the protein stock, as well as in the regulation of the proteolytic cascade (Migliolo et al., 2010). The biotechnological importance of the trypsin inhibitors is mainly highlighted by their insecticidal function in the control of phytophagous insects (Silva et al., 2012; Cruz et al., 2013) and also by their antifungal activity (Kim et al., 2005).

In particular, the seeds of *Adenanthera pavonina* L. (subfamily Mimosoideae) have a peculiar feature related to their vibrant red color, for presenting dormancy and their extracts reported as producer of peptides with inhibitory action against the growth of insects and fungi as *Saccharomyces cerevisiae* and *Candida albicans* (Silva et al., 2012) in addition to their anti-inflammatory and analgesic properties (Olajide et al., 2004).

Artemia salina L. is a microcrustacean present in the fauna of marine ecosystems (Parra et al., 2001) and has been reported to provide preliminary assessment of general toxicity (Ferraz Filha et al., 2012). *A. salina* toxicity bioassay is considered a valuable test for toxicology and ecotoxicology for its rapidity, convenience and low cost (Costa et al., 2015; Rajabi et al., 2015).

The investigation of the characteristics of biomolecules extracted from *A. pavonina* L. seeds draws attention towards their likely biotechnological use and potential innovative applications, for example in the pharmaceutical and environmental industry. This fact justifies our proposal to purify the extract, to characterize it in terms of molecular

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weight and thermostability (25 °C–100 °C), to estimate its inhibitory potential by the ratio inhibitor:trypsin and also to evaluate its toxic effects against the microcrustacean *A. salina*.

2. Materials and methods

2.1. Collection of plant material

The seeds of *A. pavonina* L. were collected from three adult mother plants located at the campus of the Federal University of Pernambuco (Recife-PE-Brazil) during the period of March 2014 and registered in the herbarium UFP—Geraldo Muniz with number 77343, collected by Souza, D.D. 13/XI/2014.

2.2. Preparation of the protein extract

The cotyledons were collected from the seeds by pliers and subsequently ground to fine powder. For the extract preparation, 10% (*p*/*v*), three different buffers were tested (0.1 M sodium acetate buffer, pH 5.0; 0.050 M sodium phosphate buffer, pH 7.0 and 0.1 M Tris–HCl, pH 8.0) and the extractions were achieved under agitation (100 rpm) for 4 h at room temperature (25 °C). Next, the extracts were filtered, transferred to Falcons Tubes (50 mL) and finally centrifuged at 10.000 rpm (5 °C, 15 min). The supernatant was termed as crude extract (CE). An aliquot of 100 mL of CE was precipitated with acetone 70% and centrifuged at 3.500 rpm, 5 °C, for 20 min. After discarding the supernatant, the pellet was resuspended with 0.1 M Tris HCl buffer, pH 8.0, and submitted to the next purification step through the ion-exchange chromatography.

2.3. Protein concentration

The total protein concentration was estimated following the method described by Bradford (1976). A curve was plotted to correlate the optical density (spectrophotometer GE, Ultrospec 7000, UK) using serum bovine albumin (BSA) concentrations (from 100 to 1000 μg/mL) as standard.

2.4. Ion exchange chromatography

The adsorptive character of the pre-purified extract was tested through two columns (80 mm × 10 mm) with different resins: CM cellulose (cationic exchanger) equilibrated with 0.05 mM sodium acetate buffer, pH 5.0, and by DEAE—Sephadex G-50 (anionic exchanger) equilibrated with 0.1 M Tris–HCl buffer, pH 8. For both procedures, the adsorbed material was eluted with 0.3 M KCl solution.

2.5. Inhibitor characterization

2.5.1. Determination of the inhibitory activity

The assays were carried out as described by Kakade et al. (1969), following a method in microplates. For the tests were used: 110 μL of 0.1 M Tris–HCl buffer, pH 8.0; 30 μL of a commercial trypsin (Sigma-Aldrich, St. Louis, USA) at 0.36 mg/mL of concentration and 30 μL of the purified sample. After 15 min of incubation, 30 μL of the substrate [Cloridrate of N-Alfa-Benzoil-DL-Arginina-P-Nitroanilida (BapNa)], dissolved in 1.5 mM dimethyl sulfoxide (DMSO), was added. The mixture was incubated one more time at 37 °C for 15 min and subsequently it had its optic density measured (at 415 nm) with a Microplate reader (iMark™). For estimating the inhibition stoichiometry, the ratio inhibitor:trypsin about kinetic studies was achieved as the similar method described before and conducted with modifications in concentrations of the inhibitor (0.16 to 0.0012 mg/mL).

2.5.2. Thermal stability

The experiment was conducted according to Chaudhary et al. (2008). Aliquots of 300 μL of inhibitor solution (0.16 mg/mL) were submitted to different temperatures (25 °C, 30 °C, 37 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C, 90 °C and 100 °C) for 30 min, followed by determination of inhibitory activity.

2.5.3. SDS-PAGE method

After the process of purification in column, the samples were solubilized in the buffer (Urea 7 mol/L; Thiourea 2 mol/L; DDD 85 mmol/L;Triton X-100, 2.5% *v*/*v*; Pharmalyte™, 0.5% *v*/*v*; Isopropanol, 10% *v*/*v*) and submitted to electrophoresis (SDS-PAGE method, 12%) following the

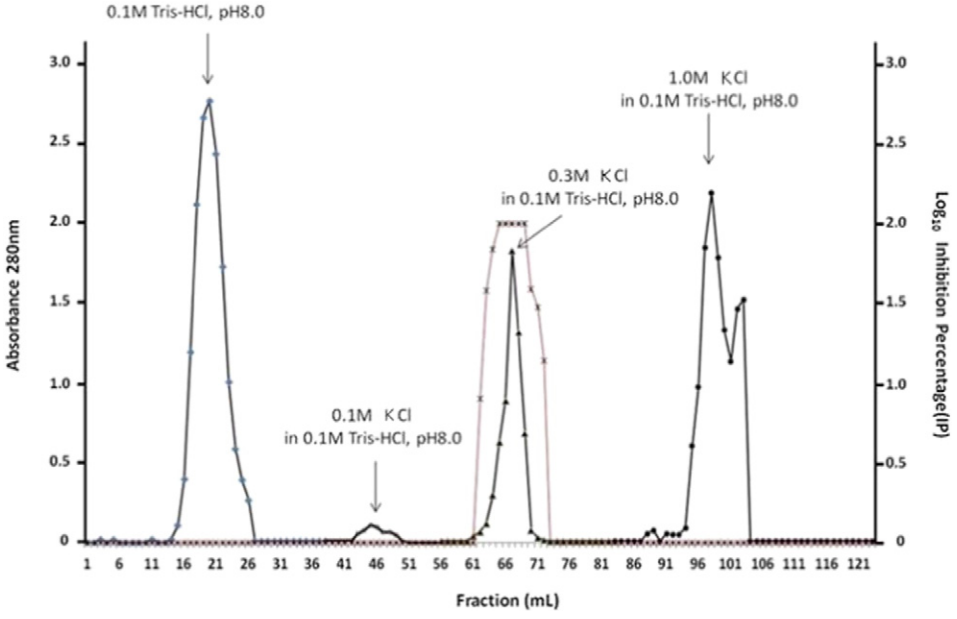

Fig. 1. Chromatogram of the acetone-precipitate (70%) obtained from cotyledons of *A. pavonina* seeds. DEAE-Sephadex G50 column (80 mm × 10 mm) was used and adjusted with Tris–HCl buffer 0.1 M. The protein content (1.8 mg) was chromatographed and eluted in a constant flow rate of 1 mL/3 min with ionic strength gradient by different KCl concentrations (0–1.0 M) and monitored at 280 nm.