SUPPLEMENTARY MATERIAL

Identification and characterization of serine protease inhibitors from *Araucaria angustifolia* seeds

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Abstract

Araucaria angustifolia seeds are characterized by a relatively high content of starch and protein. This study aimed to verify the presence of α -amylase inhibitors in the seeds and to characterize a trypsin inhibitor found in the embryo tissues. Inhibitor purification was carried out by saline extraction of proteins, acetone precipitation, and affinity chromatography. Two protein bands of molecular weight estimated by SDS-PAGE at about 35 kDa were further examined by HPLC coupled to a mass spectrometer and were shown to be 36.955 Da (AaTI-1) and 35.450 Da (AaTI-2). The sequence of the N-terminal region shows that AaTI-1 and AaTI-2 are structurally similar to plant inhibitors of the serpin family. A mixture of AaTI-1 and AaTI-2, identified as AaTI, shows selectivity for inhibition of trypsin (K_{iapp} 85 nM) and plasmin (K_{iapp} 7.0 μ M), but it does not interfere with the chymotrypsin, human plasma kallikrein, porcine kallikrein, or other coagulation enzymes activity.

Keywords: *Araucaria angustifolia*, serpin, trypsin inhibitor, protein purification, chromatography, human plasmin inhibitor.

Experimental

Extraction of the inhibitors

Endosperm and embryo were ground individually in a blender with 0.15 M NaCl solution for 30 minutes and centrifuged at 3300 $\times g$ at 4°C for 15 minutes. The aqueous phases were fractionated with cold acetone, in the ratio 1:4 (v/v), and centrifuged at 7400 $\times g$ at 4°C for 15 minutes. The precipitates obtained were spread on a Petri plate and kept at room temperature until complete evaporation of acetone. The dry materials were solubilized in 0.05 M Tris-HCl, pH 8 and

applied onto trypsin-Sepharose affinity chromatography column previously equilibrated with the same buffer. The adsorbed materials were eluted with 0.5 M KCl-HCl, pH 2.

Inhibitory activities assays

Inhibitory activities of bovine trypsin and human plasmin were carried out by kinetic assays using p-nitroanilide-derivatives as substrates. The equilibrium dissociation constant (K_{iapp}) for trypsin was determined through pre-incubation of the enzyme with increasing concentrations of the inhibitor at 37°C in 50 mM Tris-HCl buffer, pH 8.0 containing CaCl_2 0.02% (v/v), in a final volume of 250 μL . BAPA substrate (10 mM) was added and the solutions were incubated at 37°C for 20 minutes. The reaction was stopped by adding 50 μL of acetic acid 40% (v/v), and the absorbance was read at 405 nm. For plasmin, 25 μL of the enzyme (28 μM) were pre-incubated at 37°C in 0.1 M Tris-HCl buffer, pH 7.7 containing 0.2 M NaCl, in a final volume of 250 μL with increasing concentrations of the inhibitor. After 10 minutes 20 μL of the substrate HD-Val-Leu-Lys-pNan (9 mM) were added. The reaction was stopped by adding 50 μL of acetic acid 40% (v/v), and the absorbance was read at 405 nm. The inhibitory activity of α -amylase was determined by a colorimetric reaction model described by in (Wood et al. 2012). Each experimental condition was performed in triplicate using the Morrison model (Oliva et al. 1999).

Fibrinolysis assay

A solution of 5 mL bovine fibrinogen (2.0 mg/mL) was prepared with 0.05M Tris-HCl, pH 7.5 and 0.15 M NaCl, 0.02 M CaCl_2 , 0.032 M MgCl_2 and sodium azide 0.02% (w/v). Another solution with 5.0mL agarose 1% (w/v) was prepared in the same buffer. The solutions were kept separately in a water bath at 40°C for 10 minutes. To the molten agarose solution was added 1 μL human thrombin (20 U/mL), then mixed into the fibrinogen solution, and homogenized in a Petri plate. After gel polymerization, three holes were made with a punch and 10 μL of inhibitor solution (6nM and 3nM), pre-incubated with plasmin (0.028 μM) at 37°C, was added to each of them. As a control, inhibitor solution was replaced by saline. The inhibitory activity was measured by the loss of opacity, showing that the presence of fibrinolysis.

Effects of pH and temperature

The effects of pH on AaTI activity were evaluated by a kinetic assay after incubation (45 min at 25°C) in selected buffers (10mM citrate phosphate buffer, pH 4.0-6.0, 10mM sodium phosphate, pH 7.0, 10mM Tris-HCl, pH 8.0-9.0). The temperature effects were evaluated after heating (30 min) at 30, 40, 50, 60, 70, 80, 90, and 100°C. The statistical significance was evaluated

using one-way ANOVA, followed by Tukey's test. A $p < 0.0001$ was considered to indicate significance (***). Data represent means $\pm$ SD of three independent experiments. Each experimental condition was performed in triplicate.

High performance liquid chromatography experiments

High performance liquid chromatography was carried out on a Vydac C₁₈ column previously equilibrated with solvent A. Solvent A consisted of 0.1% TFA in water and solvent B in 0.1% solvent A in acetonitrile. Sample was injected in solvent A and, after 2 min begun the elution by solvent B under the following steps gradient condition: 5% solvent B for 5 minutes, from 5 to 70% B during 22 minutes, from 70 to 80% B during 33 minutes and from 80 to 100% B during 5 minutes. The flow rate was 2mL/min and the eluted peaks were monitored at 280 nm.

Primary sequencing and amino acid determination

The primary structure was obtained with an automated sequencer, Shimadzu PPSQ-23, according to the manufacturer's instructions, using the Edman degradation technique.

The amino acid analysis was performed with an amino acid analyzer Biochrom 20 plus.

SDS-PAGE

Homogeneity and the apparent molecular weight were obtained by SDS-PAGE, under non-reducing conditions, on 15% polyacrylamide gels in the Mini-Protean system (Bio-Rad, Life Science Research, USA). Protein bands were visualized by Coomassie brilliant blue staining (Sigma Aldrich, USA).

Liquid-chromatography electrospray-ionization mass spectrometry (LC/ESI-MS)

The purified peptides were characterized by liquid-chromatography electrospray-ionization mass spectrometry (LC/ESI-MS). LC/ESI-MS data were obtained on a Waters instrument (model 3100) coupled to a Waters Alliance system (model 2695) using a Phenomenex Gemini C18 column (2.0 x 150 mm, 3.0 μ m particle size, 110 Å pore size). Solvent A was 0.1% TFA in water, and solvent B was 60% ACN in solvent A. The gradient was 5–95% B for 30 min, and the peptides were detected at 220 nm. Mass measurements were performed in a negative mode with the following conditions: mass range between 200 and 2000 m/z, nitrogen gas flow rate of 4.1 L.h⁻¹, capillary voltage of 2.3 kV, cone voltage of 32 V, extractor voltage of 8 V, source heater set at 100 °C, solvent heater set at 400 °C, ion voltage of 1.0 V, and a multiplier voltage of 800 V.

Figure S1. Affinity Chromatography of the crude extract from the embryo

Figure S2. Reverse phase high performance liquid chromatography of the Fraction B (AaTI) from the affinity chromatography.

Figure S3. Polyacrylamide Gel Electrophoresis of the Fraction B from the affinity chromatography. 1 - Saline extract (100 µg); 2 – Propanone precipitated (100 µg); 3 – Protein standard (14.3; 18.4; 29; 43; 68; 97.4 and 200 kDa); 4 - trypsin-Sepharose eluate (30 µg); 5 - trypsin-Sepharose eluate reduced by dithiothreitol (30 µg)

Figure S4. Mass spectrum of the peak eluted in the retention time of 27.6 minutes from the reverse phase liquid chromatography.

Figure S7. Functional stability at different pH values. AaTI was subjected to many pH values for 15 min before inhibitory activity against trypsin be measured. AaTI was stable at experimental conditions, showed no significance changes in activity. $p\text{-value} < 0.001$ was considered to indicate significance (***)).

Figure S8. Inhibitory activity assay for bovine trypsin. The equilibrium dissociation constant (K_{iapp}) was determined through pre-incubation of the enzyme with increasing concentrations of the inhibitor at 37°C in 50 mM Tris-HCl buffer, pH 8.0 containing CaCl_2 0,02% (v/v), in a final volume of 250 μL . BAPA substrate (10 mM) was added and the solutions were incubated at 37°C for 20 minutes. The reaction was stopped by adding 50 μL of acetic acid 40% (v/v), and the absorbance was read at 405 nm.

Figure S9. Inhibitory activity assay for human plasmin. In the assay, 25 μL of plasmin (28 μM) were pre-incubated, at 37°C in 0.1 M Tris-HCl buffer, pH 7.7 containing 0.2 M NaCl, in a final volume of 250 μL , with increasing concentrations of AaTI. After 10 minutes 20 μL of the substrate HD-Val-Leu-Lys-pNan (9 mM) were added. The reaction was stopped by adding 50 μL of acetic acid 40% (v/v), and the absorbance was read at 405 nm.

Figure S10. A picture of the fibrin plate after 18 hours of incubation with plasmin (A) 10 μL of plasmin (2.8 μM), (B) plasmin preincubated with AaTI (6.0 μM) (C) Plasmin with AaTI (3.0 μM).

Table S1. Amino acid composition of the fraction eluted at 27.6 minutes in reverse-phase high performance liquid chromatography of AaTI. The values are expressed in percentage (%) of amino acids.

Amino acids	(%)
Ala	6
Arg	12
Asp	14
Glu	6
Gly	7
His	6
Ile	7
Leu	9
Lys	2
Phe	4
Pro	4
Ser	6
Thr	4
Val	9

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