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Biocontrol of *Fusarium* species by a novel lectin with low ecotoxicity isolated from *Sebastiania jacobinensis*

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ABSTRACT

A lectin from *Sebastiania jacobinensis* bark was isolated using a combination of acetone precipitation, ammonium sulphate fractionation, ion exchange and gel filtration chromatographies. The lectin purified, with a molecular mass of 52.0 kDa and composed of two subunits of 24 kDa, is a glycoprotein with a neutral carbohydrate content of 6.94%. The lectin shows maximum activity over the pH range 4.0–7.5 and heat stability up to 70 °C. Our results show that the lectin is an incompetitive inhibitor for trypsin, with a *Ki* of 0.39 ± 0.02 μM. Fluorescence spectroscopy indicated the existence of a hydrophobic surface. The percentages of secondary structure are 75% α-helix, 10% β-sheet, 5% β-turn and 10% unordered. Lectin inhibits the mycelial growth of *Fusarium moniliforme* and *Fusarium oxysporum* with an IC_{50} value of 123 ± 0.5 and 303 ± 0.9 μg, respectively. *Artemia salina* Leach and embryos of *Biomphalaria glabrata* are not affected by the lectin, indicating low environmental toxicity. Alternative viewpoints are presented that might hopefully help in future efforts to develop safer and more effective microbial control agents.

1. Introduction

Increasing awareness of the potential impacts of crop-protection agents or pesticide use has led to the development of research with natural products to ensure that risks to man and the environment are limited. Thus, the regulation of natural products as crop-protection agents should have to undergo the same procedure as for a conventional chemical product (Neale, 2000). Antimicrobial agents are provided for control of certain diseases of wheat and other cereals caused by *Fusarium* species, including *Fusarium* head blight of wheat and other cereals. These agents can also improve yield of wheat plants and cereals. Plant lectins are a heterogeneous group of proteins or glycoproteins that share the capacity to identify a specific carbohydrate. Their widespread distributions in the plant kingdom suggest a physiologically important function (Sharon, 2007). They have attracted great interest because of their various biological activities, such as antiproliferative, antitumour, antifungal and antiviral properties (Peumans & Van Damme, 1998). Seeds, especially of leguminous species, are common sources of lectins, but they are also present in latex and bark of different species (Branco et al., 2004; Wititsuwannakul, Rukseree, Kanokwiroon, & Wititsuwannakul, 2008). The proteins provide an opportunity for discovery and a starting point for optimising complex cellular processes and molecular mechanisms. Providing rigorous and comprehensive characterisations for these proteins is invaluable to researchers and frees them to confidently pursue creative experimentation. Circular dichroism (CD) spectroscopy can be a valuable method for determining the secondary structures of proteins (Johnson, 1999). Intrinsic fluorescence and 4.4′-Bis-1-anilinonaphthalene-8-sulphonate (Bis-ANS) have been used in folding, stability studies and as evidence of conformational change in proteins, by assessing hydrophobic regions (Hawe, Sutter, & Jiskoot, 2008).

The salt-water crustacean, *Artemia salina* Leach, is used as food for fish. *A. salina* is sensitive to the effect of active substances and its mortality is used to monitor toxicity because it is highly sensitive to many chemical substances (Almeida, Silva, & Echevarria, 2002). *Biomphalaria glabrata* – Say, 1818 – is a snail of the Planorbidae family with a wide distribution in Brazil. The ease of observing antibiotic effects of drugs on embryonic development of *B. glabrata* and mortality of *A. saline* has urged their use as a way to monitor the environmental impact and selectivity of microbiological control agents (Mclaughlin & Rogers, 1998; Münzinger,

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1987). Antifungal proteins have been isolated from a large number of plants and have been separated into many types comprising thaumatin-like proteins, chitinases, β-1.3-glucanases, thionins, plant defensins, ribosome-inactivating proteins, protease inhibitor-like proteins and lectins, which might hopefully help in future efforts to develop safer and valuable microbiological control agents for genetically modified plants.

Sebastiania jacobinensis Müll. Arg. (Euphorbiaceae family) is a common tree found in tropical regions of Brazil. The bark of this plant is popularly used against infections and hypersensitivity processes. In view of the benefits that microbiologically controlled agents with low environmental toxicity provide to vegetable biotechnology, we describe the isolation and partial characterisation of an antifungal lectin from the bark of *S. jacobinensis* and its ecotoxicological profile.

2. Materials and methods

2.1. Chemicals

Reference samples of 4.4′-Bis-1-anilinonaphthalene-8-sulphonate (Bis-ANS) were purchased from Molecular Probes Inc., USA. The broad-range standard marker proteins, sugar, glycoproteins, *N*-benzoyl-L-arginine-4-nitroanilide (L-BAPNA) and phenylmethyl sulfonyl fluoride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cercobin 700 WP (Thiophanate methyl) was purchased from Iharabras SA. (Sorocaba, SP, BR). All the solvents and other chemicals used were of analytical grade from Merck (Darmstadt, Germany). All solutions were made with water purified by the Milli-Q system.

2.2. Purification of *S. jacobinensis* bark lectin (SejaBL)

S. jacobinensis bark was collected from trees in the semi-arid region, state of Pernambuco, Brazil. *S. jacobinensis* bark powder was homogenised overnight at 4 °C in 10 mM Tris–HCl buffer (pH 8.5) with 0.2% (v/v) Triton X-100 detergent. The homogenate was centrifuged at 5000 g for 20 min (crude extract) followed by lyophilisation. A solution of acetone/water (4:1) was prepared and stored at −20 °C. In brief, two volumes of cold acetone were added to the crude extract (2:1), mixed well and kept on ice for 10 min. The solution was centrifuged at 10,000 g for 20 min at 4 °C. The supernatant was concentrated under vacuum until the total evaporation of acetone was achieved. The residual aqueous solution obtained was precipitated with $(NH_4)_2SO_4$ 60% of saturation. The precipitate was dialysed against the Tris–HCl buffer; this fraction was applied to a CM-Cellulose column (2 × 20 cm) previously equilibrated with the Tris–HCl buffer containing 150 mM NaCl. Following removal of unadsorbed proteins, the column was eluted with 1 M acetic acid. UV absorbance was used to monitor the elution and fractions (up to 0.100 abs) eluted with acetic acid were pooled, dialysed, concentrated by ultrafiltration (Amicon ultra-15, Mr 10,000 cut-off) and applied to a Sephadex G-100 column (2 × 70 cm) equilibrated with 150 mM NaCl. The column was eluted with the same solution at a flow rate of 0.5 ml min^{-1}. The first fraction eluted (SejaBL), after concentration by ultrafiltration in 10 mM phosphate buffer (pH 7.0), was applied to a Superdex 75 HR 10/30 column coupled to an ÄKTA purifier system (GE). The column was preequilibrated and eluted with 300 mM NaCl, at a flow rate of 0.5 ml min^{-1}, monitored by absorbance at 215 nm. The lectin was submitted to reverse-phase chromatography on a C-18 column performed on an HPLC system (Shimadzu) monitored at 215 nm, as described below. The total protein content of the crude extract and the purified lectin were determined by Lowry, Rosebrough, Farr, and Randall (1951), using the BSA standard curve, at a range of 0–500 μg ml^{-1}.

2.3. Hemagglutination activity and sugar specificity

Rabbit and human glutaraldehyde erythrocytes were obtained as described by Bing, Weyand, and Stavinsky (1967). Hemagglutinating activity (HA) defined as the lowest sample dilution showing hemagglutination was evaluated as described by Correia and Coelho (1995). Specific hemagglutinating activity (SHA) corresponded to the ratio between HA and protein concentration. The carbohydrate binding specificity of lectin was determined by HA inhibition using several sugars (D-glucose, *N*-acetyl-D-glucosamine, D-arabinose, D-mannose, L-fucose, L-raphinose, D-galactose, L-threalose, D-xylose, D-sucrose, L-rhamnose, L-celobiose, D-lactose, methyl-α-D-mannopyranoside and methyl-α-D-glucopyranoside) and glycoproteins (bovine serum albumin, casein, thyroglobulin, ovalbumin, fetuin and asialofetuin).

2.4. Effect of pH, temperature and metal ions

The effect of pH on HA was evaluated by incubating the lectin (0.5 mg ml^{-1}) at different pH values for 1 h at room temperature in selected buffers (10 mM citrate phosphate buffer, pH 4–7 and 10 mM tris–hydrochloric acid buffer, pH 8–11) and remaining SHA was determined in pH 7.0 at 25 °C. Heat stability was determined by incubation of lectin solution at different temperatures (30–90 °C for 30 min and 100 °C, 30–90 min) and remaining SHA was determined in pH 7.0 at 25 °C. The effects of Mg^{2+}, Zn^{2+} and Ca^{2+} were determined by incubation at the same volume of any metal ion (5, 10 and 20 mM) in 150 mM NaCl and lectin. An aliquot (50 μl) of the mixture was distributed in microtitre plate wells and the HA was analysed as described for the carbohydrate inhibition assay.

2.5. Molecular weight determination

Polypeptide chain analyses were performed after disulfid bridge reduction and alkylation. Previously lyophilised samples were reduced by Friedman reaction (Friedman, Krull, & Cavins, 1970) with some modifications as follows: the lectin (1.5 mg) was dissolved in 250 μl, 50 mM Tris–HCl, pH 8.6, 6 M urea, 10 mM EDTA, 179 mM DTT and incubated for 3 h at 37 °C, in the dark, before N_2 purging. The free sulphydryl groups were exposed to 100 μl iodoacetate and the reaction was continued for another 2 h under the same initial conditions. The iodoacetate derivative chains were desalted and separated on a C-18 column (Vydac-protein peptide ultrasphere) performed on a HPLC system (Shimadzu LC-10AD-kyto, Japan) and monitored at 215 nm. The column was equilibrated with solvent A (0.1% TFA in H_2O) and eluted using solvent B (90% acetonitrile: 10% H_2O: 0.1% TFA) in a non-linear gradient, where B = 5% at t = 5 min; B = 70% at t = 27 min; B = 80% at t = 60 min and B = 100% at t = 69 min. SDS–PAGE was carried out according to Laemmli (1970) and stained with a silver kit (Bio-Rad). Acid gel electrophoresis for native proteins was performed with Davis' system (1964). SDS–PAGE on reducing conditions was made after the Friedman reaction. Glycoproteins were detected by periodic acid-schiff (PAS) and estimation of carbohydrate content of the protein samples was done by the phenol sulphuric acid method, with a curve of D-mannose as a standard.

2.6. Fluorescence spectroscopy

Intrinsic fluorescence emission of the lectin solution (0.2 mg ml^{-1} in 10 mM phosphate buffer, pH 7.0) was measured at 25 °C using a spectrofluorimeter (JASCO FP-6300, Tokyo, Japan) in a cuvette (1-cm pathlength rectangular quartz). The excitation wavelength was 295 and 280 nm; emission spectra were recorded at a range of 305–450 nm and band passes were 5 nm. The