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Purification of a lectin-like antifungal protein from the medicinal herb, *Withania somnifera*

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ABSTRACT

A 30 KDa monomeric acidic lectin-like protein was purified from the leaves of an important medicinal herb, *Withania somnifera* (L.) Dunal (Solanaceae), by a series of gel filtration and affinity chromatography methods. The inhibitory concentration of the protein ranged from 7 μg to 11 μg against major phytopathogens under *in vitro* conditions. The peptide sequence showed similarity to concanavalin A like lectin from *Canavalia ensiformis* and caused distinct cell wall adhesion of the protein treated hyphae under SEM. Further, the antifungal activity of the protein was compared with standard lectins like concanavalin A, phytohemagglutinin and wheat germ agglutinin.

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1. Introduction

Plants require a broad range of defence mechanisms to effectively combat invasion by microbial pathogens and possess both preformed and inducible mechanisms to resist pathogen invasion. Some of the responses are constitutive and pathogen non-specific, but a majority of them are induced after recognition of the pathogen. Elicitors produced and released by the pathogen induce defence response, comprising the reinforcement of cell walls, the production of phytoalexins and the synthesis of defence-related proteins [40]. Pathogenesis Related (PR) proteins in plants have been defined as proteins of a host that are induced only in response to attack by pathogens or by a related event [48]. They are induced locally in response to pathogen attack as well as systemically in both compatible and incompatible host/pathogen interactions. The recognized PR proteins have been extensively reviewed [7] and currently comprise 17 families of induced proteins [49]. These include one each of 1,3-glucanases [24,36], proteinase inhibitors [44], one specific peroxidase [23,17], PR-1 family with unknown biochemical properties [27], the thaumatin-like PR-5 family [46], the birch allergen Betv1-related PR-10 family [21], defensins [41,43], lipid-transfer proteins (LTPs) [16,34], thionins [4,30] and other proteins including 2S storage albumins [42,1] and ribosome inactivating proteins (RIPs) [28,38]. Important groups of antimicrobial proteins not induced by pathogen attack and hence not included under PR proteins are lectins and cysteine-rich peptides [7]. The role of PR proteins, their classification, mechanism of action, their role in defence mechanisms and generation of transgenics with increased resistance, has been extensively reviewed [47,37,13,11].

Withania somnifera, also known as Ashwagandha or Indian ginseng belongs to the family Solanaceae. In Ayurveda, it is considered as an adaptogen that works on a nonspecific basis to normalize physiological function. Fruits, leaves and seeds have been traditionally used in the Ayurvedic system as aphrodisiacs, diuretics and for treating memory loss. The present study was undertaken to identify constitutive, non race specific, broad spectrum antifungal protein from leaves of *W. somnifera* (L.) Dunal and characterize its effect on the pathogen hyphae.

2. Experimental

2.1. Plant material

Seeds of *W. somnifera* obtained from the Regional Forest Research Institute, Andhra Pradesh Forest Department,

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Rajahmundry, Andhra Pradesh, India were germinated and maintained in Polybags containing sand, red soil and celrich (commercial organic manure, SPIC India Ltd, India) in a ratio of 2:1:0.1.

2.2. Fungal culture

The fungal isolates of *Rhizoctonia solani* (*Phyllanthus* isolate), *Fusarium moniliforme* (rice isolate), *Macrophomina phaseolina* (blackgram isolate) and *Trichosporium vesiculosum* (*Casuarina* isolate) were grown and maintained in potato dextrose agar medium.

2.3. Purification of antifungal protein

Thirty gram leaf tissues of *W. somnifera* was homogenized in liquid nitrogen and extracted in three volumes of phosphate buffer, pH 7.0 (25 mM Na_2PO_4; 250 mM NaCl; 10 mM EDTA; 1 mM PMSF; 1.5% PVPP; 0.2% activated charcoal and 100 mM Ascorbic acid) and centrifuged at 9000 rpm for 30 mins at 4 °C. The supernatant was subjected to 60% ammonium sulfate precipitation. Subsequent to centrifugation, the protein pellet was suspended in 50 mM sodium phosphate buffer, pH 7.0 and desalted by diafiltration using Microsep centrifugal device (Pall life sciences, Ann Arbor, MI, USA) with 3000 Da cut off membrane. The extract was loaded to sepharose 6B column (Sigma Aldrich Ltd., USA) pre equilibrated with 50 mM sodium phosphate buffer, pH 7.0. One milliliter fractions were collected and the protein in the pooled fractions was recovered by ammonium sulfate precipitation with 60% relative saturation. This sample was further fractionated through Superose 12 10/300 GL column using FPLC system (GE Healthcare, Piscataway, NJ, USA). The column was washed with 50 mM sodium phosphate buffer, pH 7.0, followed by protein injection and elution with the same buffer with flow rate of 1 ml/min. Pooled fractions were precipitated overnight with 60% ammonium sulfate at 4 °C. Subsequently, the precipitated protein was recovered by centrifugation and suspended in 25 mM phosphate buffer, pH 7.0. The protein sample was desalted by diafiltration and loaded on a concanavalin A sepharose column (Sigma Aldrich Ltd., USA) and eluted with a gradient of methyl-D-mannopyranoside (0–0.5 M) in binding buffer. The eluted fractions were pooled, concentrated and desalted by diafiltration. The protein concentration of each fraction was determined using Bradford's reagent (Sigma Aldrich Ltd., USA) [5].

2.4. Determination of antifungal activity of purified fraction

2.4.1. Hyphal extension inhibition assay

The protein fractions at different concentrations were tested for their antifungal activity using *in vitro* hyphal extension assay as described in Ref. [35]. The pathogens tested were *T. vesiculosum, F. moniliforme, M. phaseolina and R. solani.* The plates were incubated for 48 h and observations were made for the appearance of a crescent shaped inhibition zone.

2.5. Determination of molecular weight and pI of the protein

The inhibiting protein fraction purified through concanavalin A sepharose column was resolved in gradient 8–25 SDS–PAGE precast gel in Phast automated electrophoretic system (GE Healthcare, Piscataway, NJ, USA) along with standard molecular weight markers (Bangalore Genei Ltd, India) and silver stained as described by the manufacturer. The pI value of the protein was determined by isoelectric focusing in IEF 3–9 precast gel using the Phast automated electrophoretic system (GE Healthcare, Piscataway, NJ, USA). Initially the gel was prefocused for 10 min followed by loading of the purified protein (40 ng/μl) along with broad range (pH 3–10) IEF marker (GE Healthcare, Piscataway, NJ, USA.). After completion of the run, the gel was stained with silver nitrate as described by the manufacturer.

2.6. In gel tryptic digestion and peptide sequencing

The purified protein was separated on a 12% SDS–PAGE and stained with Ezee Blue gel stainer (Bangalore Genei Ltd, India). Gel plugs containing protein spots were subjected to automated tryptic digestion on a ProGest Workstation (Genomic Solutions, Ann Arbor, MI) using the standard ProGest long trypsin protocol. Following digestion, the peptide extracts were lyophilized in a vacuum concentrator, re-suspended in 10 ml 0.1% formic acid and used for MS–MS analyses.

Mass spectroscopic peptide separation and sequencing was carried out on Applied Biosystems QSTAR PULSARi™ quadrupole time of flight mass spectrometer coupled to an Amersham Ettan™ MDLC nano HPLC workstation (Applied Biosystems, CA, USA). TOFms spectra were collected between the mass range 100 and 2000 amu throughout the gradient elution and precursor ion selection and product ion spectra were generated using Applied Biosystems BioAnalyst™ software's fully automated switching and acquisition procedures. Only multiple charged precursor ion species were selected for fragmentation and peptide sequencing.

2.7. Database searches and protein identification

For protein identification, all MS–MS product ion spectra generated from sample were used in a MASCOT (www.matrixscience.com) database search of the NCNInr database of all available Viridiplantae sequences [32].

2.8. Scanning electron microscope study of protein treated hyphae

Hyphal mass of *T. vesiculosum* were suspended in 80 μl of potato–dextrose broth containing 0.1% Triton X 100 and 8 μg (in 20 μl) of the purified protein. A control containing 80 μl of hyphal suspension and 20 μl of sterile water was used and the microtitre plate was incubated at 30 °C for 72 h. Subsequently, the hyphae were fixed in 4% glutaraldehyde in 0.2 M phosphate buffer pH. 6.0 for 1 h. The hyphal mass was washed twice in sterile distilled water and dehydrated in alcohol series, air dried and subjected to SEM analysis.

2.9. Hemagglutinating activity

Agglutination assays were carried out in glass tubes in a final volume of 1 ml consisting of 400 μl of protein solution and 600 μl of 1% suspended human red blood cells. Agglutination was inspected visually, 1 h after the addition of the erythrocyte suspension.

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