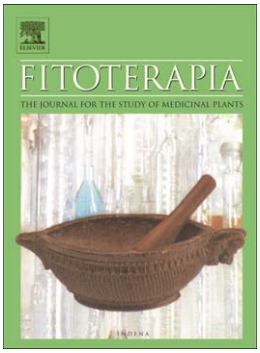

Purification of a lectin –like antifungal protein from the medicinal herb, *Withania somnifera*

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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.

Key words: Cell adhesion; Concanavalin A; Hyphal inhibition; Monomeric; phytopathogen

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 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 *Withania somnifera* (L.) Dunal and characterize its effect on the pathogen hyphae.

Experimental

Plant material

Seeds of *Withania somnifera* obtained from Regional Forest Research Institute, Andhra Pradesh Forest Department, Rajahmundry, Andhra Pradesh, India were germinated and maintained in Polybags containing sand, red soil and celrich (commercial organic manure, SPIC India Ltd, India) in ratio 2:1:0.1.

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.

Purification of Antifungal Protein

Thirty gram leaf tissues of *Withania somnifera* was homogenized in liquid nitrogen and extracted in three volumes of phosphate buffer, pH 7.0 (25mM Na_2PO_4; 250 mM NaCl; 10mM EDTA; 1mM PMSF; 1.5% PVPP; 0.2% activated charcoal and 100mM Ascorbic acid) and centrifuged at 9,000 rpm for 30 minutes at 4^0C. The supernatant was subjected to 60% ammonium sulfate precipitation. Subsequent to centrifugation, the protein pellet was suspended in 50mM sodium phosphate buffer, pH 7.0 and desalted by diafiltration using Microsep centrifugal device (Pall life sciences, Ann Arbor, MI, USA) with 3000Da cut off membrane. The extract was loaded to sepharose 6B column (Sigma Aldrich Ltd., USA) pre equilibrated with 50mM sodium phosphate buffer, pH 7.0. One ml. 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 50mM sodium phosphate buffer, pH 7.0, followed by protein injection and elution with the same buffer with flow rate of one ml./min. Pooled fractions were precipitated overnight with 60% ammonium sulfate at 4^0C. Subsequently, the precipitated protein was recovered by centrifugation and suspended in 25mM 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 – 0.5M) 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].

Determination of antifungal activity of purified fraction

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 35 .The pathogens tested were *T. vesiculosum, F. moniliforme, M. phaseolina and R. solani.* The plates were incubated for 48 hours and observations were made for the appearance of a crescent shaped inhibition zone.

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 minutes followed by loading of the purified protein (40ng/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.

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 10ml 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 100-2000 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.

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].

Scanning Electron Microscope study of protein treated hyphae

Hyphal mass of *T. vesiculosum* were suspended in 80µl of potato – dextrose broth containing 0.1% Triton X 100 and 8µg (in 20µl) of the purified protein. A control containing 80µl of hyphal suspension and 20 µl of sterile water was used and the microtitre plate was incubated at 30^0C for 72 hours. Subsequently, the hyphae were fixed in 4% glutaraldehyde in 0.2 M phosphate buffer pH. 6.0 for 1 hour. The hyphal mass was washed twice in sterile distilled water and dehydrated in alcohol series, air dried and subjected to SEM analysis.

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.

Comparison of antifungal activity of purified protein with standard lectins

One mg of standard lectins including concanavalin A, phytohemagglutinin and wheat germ agglutinin (Bangalore Genei Ltd., India) were dissolved in 50µl of sterile distilled water and tested for their toxicity against *T. vesiculosum* using *in vitro* hyphal extension inhibition bioassay as described earlier. 8 µg purified protein was also loaded to compare the efficacy of the *Withania* protein in comparison to the standard lectins.

Results and Discussion

Total leaf proteins and the subsequently precipitated proteins were initially tested for their antifungal activity by hyphal extension inhibition assay using *T. vesiculosum* as the test pathogen. The precipitated protein showed a distinct inhibition zone. Further, this was purified through gel filtration and four distinct peaks were recorded. All the peak giving fractions were pooled, precipitated and tested for their antifungal activity. Peak one inhibited the hyphal extension of the pathogen and this fraction was further purified through Superose 12 10/300 GL column using FPLC system. Two distinct fractions were documented and the peak giving fractions were pooled, precipitated and tested for antifungal activity. The second fraction showing antifungal activity was further purified through affinity chromatography and the protein fractions were pooled and precipitated. The fraction showed a distinct zone of inhibition at a concentration of 7µg. This fraction also inhibited *F. moniliforme* and *M. phaseolina* at a concentration of 9µg and *R. solani* at 11µg concentration.

The purified protein was separated on a precast SDS PAGE and presence of a single band was observed at approximately 30 KDa revealing the monomeric nature of the protein. The pI of the purified protein was determined to be approximately 4.0. The protein was further characterized by peptide mass fingerprinting using LC MSMS. The digest generated four unique peptide fragments with sequence coverage of 21% and 20% with concanavalin A lectin from *Canavalia ensiformis* with PDB accession number 1DQ4B and 1DQ2A respectively (Figure 1).

Plant lectins are proteins with sugar-binding properties, whose biological role in the plant is ill-defined [8;18]. Recent findings have indicated that lectins play a crucial role in symbiotic nitrogen fixation in legumes [15] and contribute to plant defense against fungal and insect attack [33]. Only a few lectins with antifungal properties are reported to have a role in first line defense. *Urtica dioica* agglutinin (UDA) from the stinging nettle and hevein from the *Hevea brasiliensis*, are low molecular weight, monomeric chitin-binding lectins that possess antifungal properties *in vitro* [6; 50; 12]. An antifungal lectin devoid of chitinase activity was reported from potato tubers [20]. Similarly, a monocot mannose-binding lectin, gastrodianin purified from the terminal corm of the orchid *Gastrodia elata* inhibited the growth of both ascomycete and basidiomycete plant pathogens *in vitro* including *Valsa ambiens, Gibberella zeae, Botrytis cinerea, Armillaria mellea, Rhizoctonia solani,* and *Ganoderma lucidum* [22; 52]. It also imparted increased disease resistance against fungi in transgenic plant systems [52; 51; 12]. A homodimeric lectin with molecular weight of 67 KDa was isolated from seeds of red kidney beans, *Phaseolus vulgaris*. It exerted a suppressive effect on growth of the fungal species *Fusarium oxysporum, Coprinus comatus,* and *Rhizoctonia solani* [54]. Similarly, a novel lectin (AMML) was isolated from roots of a Chinese herb, *Astragalus mongholicus* showing antifungal activity against *Botrytis cincerea, Fusarium oxysporum, Colletorichum* sp., and *Drechslera turcia* but not against *Rhizoctonia solani* and *Mycosphaerella arachidicola* [53]. A 50 KDa lectin (PSL) purified from the seeds of *Pisum sativum* showed antifungal activity against *Aspergillus flavus, Trichoderma viride* and *Fusarium oxysporum* [39].

In *W. somnifera* the report on identification of antifungal protein is limited to the purification of a monomeric acidic glycoprotein (WSG) with molecular mass of 28 KDa from the root tubers.

The protein was identified as a trypsin-chymotrypsin protease inhibitor and demonstrated a fungistastic effect by inhibiting the spore germination and hyphal growth of *Aspergillus flavus, Fusarium oxysporum, F. verticilloides* and showed antibacterial activity against *Clavibacter michiganensis subsp. michiganensis* [19]. In the present report, the 30 kDa monomeric lectin purified from leaves of *W. somnifera* also revealed broad spectrum antifungal activity.

Plant lectins have been reported to interfere with normal cell wall deposition and assembly of fungal cell wall components. Several quantifiable growth disruptions during fungal spore germination including sensitivity to osmotic lysis, adventitious branching of the spore germ tubes and inhibition of germ tube elongation was reported [3]. The binding of lectins like wheat germ agglutinin, soybean agglutinin and peanut agglutinin on the cell wall and vesicles of *Aspergillus* and *Penicillium* were demonstrated using fluorescein conjugated lectins. The three lectins inhibited incorporation of [3H] acetate, N-acetyl-D-[3H] glucosamine and D-[14C] galactose into young hyphae of *Aspergillus ochraceus*, indicating interference with fungal growth [2]. Similarly antifungal lectins with specificity to chitin were reported from *Urtica dioica* (UDA) and hevein from rubber tree [6; 50; 12].

The SEM studies conducted in the present study revealed a distinct cell wall adhesion in protein treated hyphae, when compared to water treated hyphae, suggesting a probable binding of the *Withania* lectin to the cell wall components of the pathogen. Morphological changes in the fungal vesicles were also observed in the treated hyphae.

The antifungal nature of the purified lectin from *Withania* was compared with standard lectins through *in vitro* assay. Standard lectins like concanavalin A from jack bean, phytohemagglutinin

from *Phaseolus vulgaris* and wheat germ agglutinin from *Triticum vulgaris* did not show any antifungal activity at 1mg concentration, while a clear zone of inhibition was observed with the purified protein from *Withania*. Concanavalin A is not reported to have antifungal activity [31] while there are no reports on the toxicity of phytohemagglutinin on microbes. The present investigation also confirmed their non toxic nature on hyphal extension. However, wheat germ agglutinin, a chitin specific lectin purified from wheat germ was reported as antifungal against *F. graminearum* and *F. oxysporum* in earlier reports [9, 26]. However, the present study did not reveal a significant effect of the lectin against the hyphal growth of *T. vesiculosum*.

The hemagglutinating activity of lectins have been widely documented. However, a mannose binding tetrameric lectin purified from the bulbous tissues of *Lycoris aurea* showed non agglutination of human erythrocytes while a strong agglutination was observed with rabbit erythrocytes [25]. Hevein, a chitin binding antifungal lectin, failed to agglutinate untreated or trypsin treated erythrocytes from rabbit, pigeon or human origin [29]. In elderberry (*Sambucus nigra*), a hevein – like protein showing chitin specificity showed no agglutinating activity [45]. Similarly, in the present study, the purified protein from *Withania* did not reveal a hemagglutinating activity against the human erythrocytes.

Lectins distinguish themselves from all other plant proteins by their specific carbohydrate-binding activity. Molecular, biochemical, cellular, physiological and evolutionary arguments indicate that lectins have a role in plant defense [33]. The chitin-binding plant lectins recognize a carbohydrate that is a typical constituent of the cell wall of fungi and the exoskeleton of invertebrates, revealing its role in first line defense against pathogens. The antifungal lectin

purified from the leaves of *W. somnifera* suggests a similar role in plant defense against pathogens.

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Figure 1 Similarity of protein sequence from *W. somnifera* (WSP) with Concanavalin A lectin from *Canavalia ensiformis*

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1DQ4B     1 ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQNGKVGTAHIIYNSVDKR 60

1DQ2A     1 ADTIVAVELDTYPNTDIGDPSYPHIGIDIKSVRSKKTAKWNMQNGKVGTAHIIYNSVDKR 60

WSP       ------------------------------------------------------------ VGTAHIIYNSVDKR

IDQ4B     61 LSAVVSYPNADSATVSYDVDLDNVLPEWVRVGLSASTGLYKETNTILSWSFTSKLKSNST 120

IDQ2A     61 LSAVVSYPNADSATVSYDVDLDNVLPEWVRVGLSASTGLYKETNTILSWSFTSKLKSNST 120

WSP       ----------------------------------------VGLSASTGLYKETNTILSWSFTSK----------

IDQ4B     121 HETNALHFMFNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGRALFYAP 178

IDQ2A     121 HETNALHFMFNQFSKDQKDLILQGDATTGTDGNLELTRVSSNGSPQGSSVGRALFYAP 178

WSP       ------------------------------------------------------------------------------------------

IDQ4B     179 VHIWESSAVVASFEATFTFLIKSPDSHPADGIAFFISNIDSSIPSGSTGRLLGLFPDAN 237

IDQ2A     179 VHI WESSAVVASFEATFTFLIKSPDSHPADGIAFFISNIDSSIPSGSTGRLLGLFPDAN 237

WSP       ----------------------------------------------------------------------LLGLFPDAN
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