Isolation of *Andrographis paniculata* Leaf Protein with Antifungal Property

MODHUMITA GHOSH*[1], D. THANGAMANI[1],
MANISHA THAPLIYAL[2], R. YASODHA[1] and K. GURUMURTHI[3]

[1]Division of Plant Biotechnology, Institute of Forest Genetics and Tree Breeding,
Forest Campus, R. S. Puram, Coimbatore- 641 002 India
[2]Forest Tree Seed Laboratory, Silviculture Division,
Forest Research Institute, Dehradun, India
[3]Institute of Forest Genetics and Tree Breeding, Forest Campus,
R. S. Puram, Coimbatore- 641 002 India

The crude protein extract from the leaves of Andrographis paniculata was found to inhibit the spore germination of two major pathogens *Aspergillus flavus* and *Macrophomina phaseolina.* The antifungal protein component was further purified from the crude extract and the molecular mass of toxic protein was estimated to be 39.5 KDa.

Keywords: plant defense mechanism, PR proteins, spore inhibition, systemic acquired resistance.

Plants have several inducible defense mechanisms that act to limit pathogen infection, like increased lignification, phytoalexin production and hypersensitive reaction (Hammond-Kosack and Jones, 1996). In addition, various novel proteins are induced which are collectively referred to as "pathogenesis-related" (PR) proteins. These proteins accumulate locally in the infected region, but are also induced systemically, associated with the development of systemic acquired resistance (SAR) against further infection. Induction of PRs has been found in many plant species belonging to various families (Van Loon, 1999) suggesting their role in adaptation to biotic stress condition. Some of the reports on purification of PR proteins are from tobacco (Van Loon, 1982), tomato (Vera and Conejero, 1988), barley (García-Olmedo et al., 1995; Christensen et al., 2002), *Arabidopsis* (Epple et al., 1995), maize (Malehorn et al., 1994), sunflower (Jung et al., 1993) and radish (Terras et al., 1992).

In an attempt to identify a new source of antifungal protein, *Andrographis paniculata* was studied and the activity of the antifungal protein component purified was tested against two potent pathogens, *Macrophomina phaseolina* and *Aspergillus flavus.* These are two major pathogens in the tropical countries with a broad host range, the former infecting about 500 plant species and the latter a common colonizer and better known for the production of a mycotoxin called aflatoxin (Sinclair, 1982; Malaguti, 1990; IARC, 1993).

*Corresponding author: E-mail: ghosm@ifgtb.res.in; fax: 91-0422-2430549

0238–1249/$ 20.00 © 2004 Akadémiai Kiadó, Budapest

Materials and Methods

Young leaves of *A. paniculata* were collected from the germplasm at the botanical garden of Institute of Forest Genetics and Tree Breeding, Coimbatore, India. Twenty-five grams of young leaves were extracted in 75 ml of ice-cold buffer containing 50 mM sodium acetate (pH 5.2), 1 mM ascorbic acid, 0.5 mM PMSF and 0.5% polyvinyl polypyrrolidone. Subsequent to centrifugation at 10,000 rpm for 15 minutes at 40 °C, the supernatant was precipitated with 30% ammonium sulphate. The pellet was discarded and the supernatant was desalted using sephadex G50 column (Amersham Biosciences Ltd., Sweden). The desalted protein was further purified using the Carboxy methyl cellulose (CMC) column. CMC was prepared by suspending ten grams of carboxy methyl cellulose (Bangalore Genei Ltd., India) in 15 volumes of 500 mM NaOH with continuous stirring and allowed settling for 30 minutes. The upper liquid phase was decanted and the swollen sorbent was suspended in distilled water. The sorbent was washed in distilled water till the pH became 8.0. This was followed by suspension in 15 volumes of 500 mM HCl and then washed till the pH was neutral. The sorbent was finally equilibrated with 50 mM ammonium acetate, pH 3.7.

The swollen sorbent was subsequently packed into column (55 cm × 1 cm) and was washed with ten bed volumes of 50 mM ammonium acetate, pH 3.7. The crude protein was loaded on the packed column and again washed with 500 ml of 50 mM ammonium acetate, pH 3.7. The bound proteins were eluted in a gradient of 50 mM to 500 mM ammonium acetate, pH 3.7. Three ml fractions were collected and the absorbance was read at 280 nm. The peak giving fractions were pooled and freeze- dried. The freeze-dried samples were suspended in 500 μl of sterile distilled water. The samples were further desalted and concentrated by passing through the microcon (Millipore) column with membrane nominal molecular weight limit [NMWL] of 3000 Daltons. The samples obtained were stored in –20 °C until further use. The fractions were subsequently assayed for their antifungal property.

The protein concentration was determined using Lowry's method. (Lowry et al., 1951). The molecular mass of the purified protein showing inhibition was determined in 12% native polyacrylamide gel using standard molecular weight markers (Bangalore Genei Ltd., India) and was stained with coomassie brilliant blue R250 (Hi Media Laboratories Pvt. Ltd., India). The molecular mass of the protein was determined using the Kodak 1D version 3.5 software.

The fractions separated after cation exchange chromatography was tested for their in vitro antifungal activity against *Macrophomina phaseolina* and *Aspergillus flavus.* Both the fungi were grown and maintained in potato dextrose agar. Fungal spores (1.2×10^3) were suspended in 80 μl of potato – dextrose broth containing 0.1% Triton × 100 in microtitre plate (12 × 8 wells). Serial dilutions were made individually for all the samples collected (1 μl, 5 μl, 10 μl, 15 μl and 20 μl of all protein fractions were made up to 20 μl with sterile water) and 20 μl of each fractionated protein at various dilutions were added to the spore suspension. A control containing 80 μl of spore suspension and 20 μl of sterile water was used and the plate was incubated at 32 °C. Observations were made for spore germination in both treated and untreated wells after 24, 48 and 72 hours and microphotographs were taken using Nikon UFX-DX.

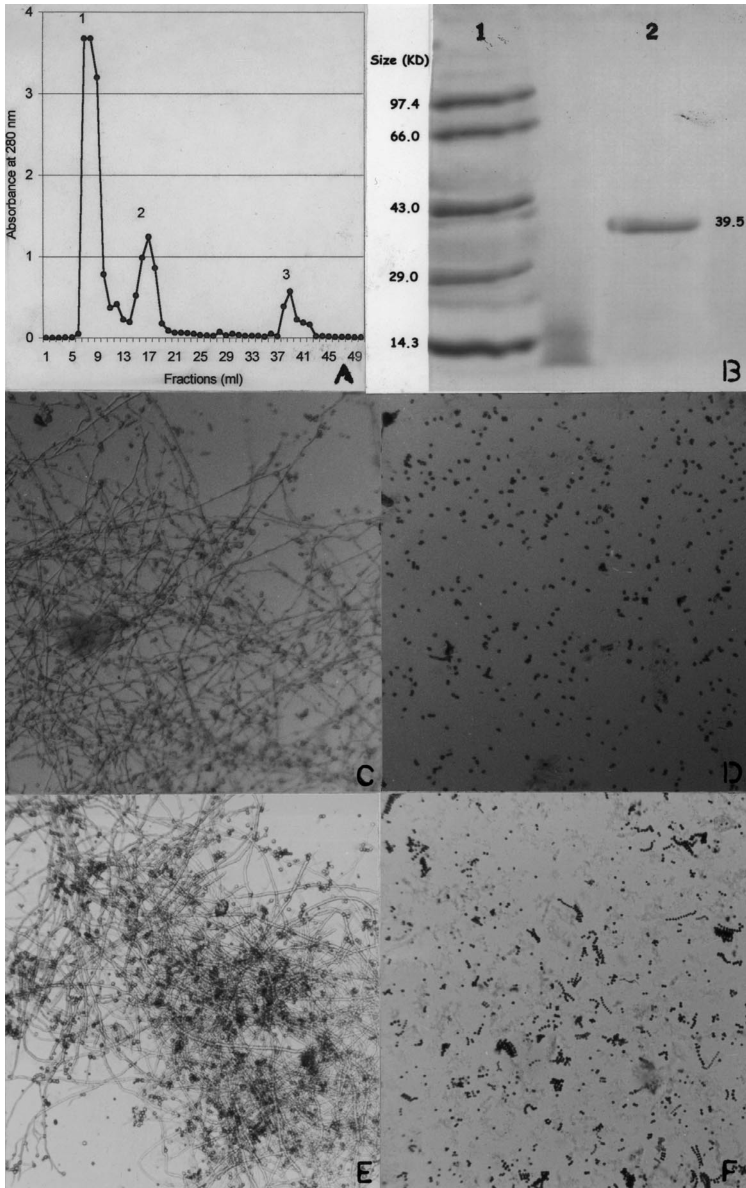

Fig. 1. A. Cation exchange chromatogram of the crude leaf protein of *Andrographis paniculata*
B. Native polyacrylamide Gel Electrophoresis of the purified protein.
Lane 1: Standard molecular weight markers; Lane 2: 39.5 KD Antifungal protein
C. Spore germination of *A. flavus* in control well (after 48 hrs)
D. Inhibition of *A. flavus* spore germination in protein treated well (after 48 hrs)
E. Spore germination of *M. phaseolina* in control well (after 48 hrs)
F. Inhibition of *M. phaseolina* spore germination in protein treated well (after 48 hrs)

Results and Discussion

The crude protein was purified through CMC column and three peaks were collected *(Fig. 1A).* All the three freeze dried desalted fractions were tested for their antifungal activity against *A. flavus* and *M. phaseolina.* The first fraction completely inhibited the spore germination of both the pathogens even after 72 hours *(Fig. 1C–F).* The optimal concentration of the protein component in the fraction 1 showing total inhibition to spore germination was found to be approximately 15 μg ml^{-1} in M. phaseolina and 24 μg ml^{-1} in *A. flavus.* In the control wells, the spores germinated by 24 hours and by 72 hours profuse hyphal extension were observed for both pathogens. The other two fractions (2 and 3) showed no antifungal activity and the growth was comparable to the control wells (not shown). The molecular mass of the protein component in the first fraction was determined to be approximately 39.5 KD *(Fig. 1B).*

Several reports are available on antifungal proteins showing toxicity to *A. flavus* and *M. phaseolina.* Chen et al. (1998) purified a 14 KD protein from maize kernels and reported their association with the resistance of the kernel against *A. flavus.* In another study, maize trypsin inhibitor was identified to have toxic property against *A. flavus* that inhibited the alpha amylase activity of the pathogen (Chen et al., 1999). Uncharacterized low molecular weight antifungal protein showing toxicity against *A. flavus* was identified from seeds of *Sorghum bicolor* (Ghosh and Ulaganathan, 1996) and from leaves of *Moringa oleifera* (Umar Dahot, 1998) while a class I chitinase from cotton was also shown to have antifungal activity against *A. flavus* (Chlan et al., 2001). Ghosh et al. (2002) reported identification and purification of an uncharacterized 20 KD antifungal proteins from *Plumbago capensis* against *M. phaseolina.* The present study also reports the toxicity of an uncharacterized protein from leaves of *Andrographis paniculata* against *A. flavus* and *M. phaseolina.* These proteins are usually single gene encoded and non- race specific and hence can be a promising candidate gene in developing transgenics for disease resistance.

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