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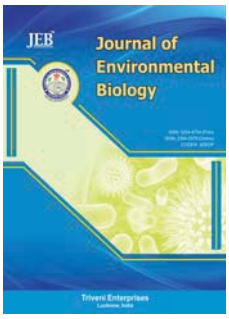

Purification and characterization of a novel thermostable antifungal protein with chitinase activity from mung bean

Authors Info

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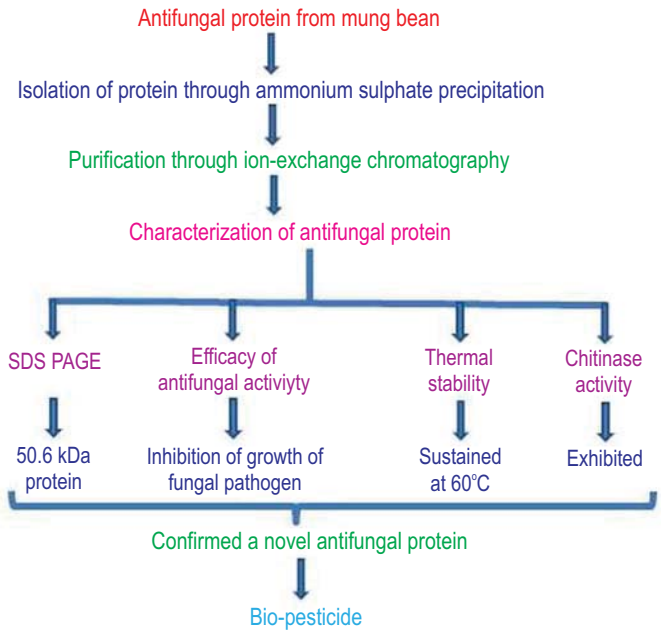
Abstract

Aim : Antifungal proteins are produced by many plant species and participate in defence mechanisms against number of fungal pathogens. The main objective of the study was to purify and characterize the thermo-stable antifungal compound with chitinase activity from mung bean *Vigna radiata* seeds to assess their antifungal potency.

Methodology : The antifungal protein was isolated through ammonium sulphate precipitation method followed by its purification through ion-exchange chromatography. The purified protein was characterized by evaluating its antifungal efficacy, thermal stability, chitinase activity and SDS PAGE profiling.

Results : Molecular mass of characterized antifungal protein was 50.6 kDa. Purified protein exhibited antifungal activity against pathogenic fungi *Macrophomina phaseolina* and *Magnaporthe grisea* and sustained its thermo-stability up to 60°C with chitinase activity.

Interpretation : The isolated antifungal proteins showed unique column chromatographic behaviour, molecular weight, specificity of chitinase activity and relatively high thermo-stability with potent antifungal activity. It can be used in different biomedical and pharmaceutical applications as bio-pesticides. Characterization of antifungal protein



Introduction

Mung bean [*Vigna radiata* (L.) Wilczek] is a valuable grain legume, widely cultivated in tropical and subtropical regions of Asia (Poehlman, 1991). Among legumes, it is the main source of amino acids, proteins, dietary fiber, unsaturated fatty acid and is used worldwide for human consumption, cattle feed and possess medicinal properties (Jo *et al*., 2006; Kulsum *et al*., 2007; Sharma and Mishra, 2009). It is used for the treatment of various ailments like hepatitis, gastritis, heart rash etc. (Huijie *et al*., 2003; Leung, 2007) due to its antihypertensive, antidiabetic and anticancer properties (Yang *et al*., 2008; Kumar and Singhal *et al*., 2009). The proximate composition of mung bean revealed that it posses high amount of antifungal proteins and their isolation may enhance its industrial applications.

Antifungal proteins are widely produced by many plant seeds used as food including legume seeds (Ye and Ng, 2000; Klomklao *et al*., 2011) and serve as a protective function against fungal invasion to the host (Kondori *et al*., 2010). These proteins participate in defence mechanisms against number of fungal pathogens (Ferrira *et al*., 2007). A number of antifungal proteins from legume seeds have also been purified and characterized by earlier researchers (Ajesh and Sreejith 2014; Kabir *et al*., 2016). During germination mung bean seeds secrete antifungal compounds that act as a potential protective role against fungal pathogens through proteolytic process, cell wall degradation, un-established plasma membrane, membrane pore formation, ribosomal destruction and participate in defence mechanisms, that antifungal compounds can be used as biofungicides (Ferrira *et al*., 2007; Klomklao *et al*., 2011).

The thermostable antifungal proteins with chitinase activity have not been studied in mung bean, therefore the present study was undertaken to purify and characterize thermo-stable antifungal compound with chitinase activity from mung bean seeds grown in arid and semi arid regions of Rajasthan, India for their antifungal potency.

Materials and Methods

Isolation of protein : Mung bean seeds were obtained from Agriculture University, Mandore, Jodhpur. Twenty-five gram seeds were soaked in distilled water for 24 hrs at room temperature and homogenized in 100 ml of 20 mM Tris–HCl buffer (pH 7.2). The homogenate was centrifuged to obtain crude extract. The crude extract was subjected to 0-30%, 30-60% and 60-90% ammonium sulphate fractionation following the method of Klomklao *et al*. (2011) as M1, M2 and M3, respectively. These residues were collected through centrifugation in different centrifuge tube and were dissolved in 20 mM Tris–HCl buffer (pH 7.2) and dialysed against distilled water in dialysis tubing (Sigma Aldrich). Each fraction was subjected to preliminary check for protein content and their antifungal activity and the ammonium sulphate fraction showing antifungal activity was further purified (M2 Fraction) while remaining fractions were discarded (M1 and M3). M2 fraction was further purified by the ion exchange chromatography on di-ethylamino ethyl (DEAE) cellulose (Hi Media, Mumbai, India) column (1× 15 cm) in 20 mM Tris–HCl (pH 7.2). Flow rate was maintained at 1 ml min^{-1} and each fraction of 2 ml was collected. The un-adsorbed proteins were collected separately and adsorbed proteins were eluted with buffer containing 1 M NaCl. Absorbance of each eluted fraction was analyzed for protein content with a UV-spectrophotometer at 280 mm.

Protein estimation : Protein concentration was estimated by dye binding method using bovine serum albumin (BSA) as standard (Bradford, 1976).

Characterization of antifungal protein by SDS PAGE : To determine purity of protein and its molecular mass, sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out. SDS-PAGE was performed according to the method described by Laemmli (1970) with 12% polyacrylamide gel. Electrophoresis gel was stained with Coomassie brilliant blue dye. The molecular mass of purified protein was determined with the comparison of its electrophoretic mobility with those of molecular mass marker proteins (Thermofisher Scientific, USA).

Assay of antifungal activity : Crude protein, ammonium sulphate fractions (M1, M2 and M3), ion exchange column fractions (M2P1 and M2P2) and heat treated proteins (M2P1) were subjected to assess their antifungal efficacy against pathogenic fungi *Macrophomina phaseolina* and *Magnaporthe grisea*, obtained from ICRISAT, Hyderabad. The young growing fungal mycelium inoculums were placed in the center of sterile Petri plates containing 15 ml potato dextrose agar. After the mycelium colony established on agar, sterile circular paper disks (0.5 cm in diameter) were placed one cm away from young growing fungal mycelium colony rim 10 µl aliquot (23.4 µg protein) of isolated protein was poured on to the disks and then incubated at 25ºC until the colony mycelia growth from the central disc had enveloped peripheral disks or formed crescents of inhibition around disks. Only 10 µl Tris–HCl buffer poured disc without protein was used as control.

Thermal stability : The purified antifungal protein (M2P1) was incubated at different temperature (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100°C) for 10 min to check its thermo-stability according to the method described by Sawasdipuska *et al.* (2011). After incubation, samples were cooled down at room temperature and then antifungal efficacy was re-confirmed.

Agar plate assay for chitinase activity : Colloidal chitin was prepared from chitin flakes following the method of Hsu and Lockwood (1975) with some modification. Colloidal chitin (1%) agar plates were prepared containing 15 ml of colloidal chitin agar media. After solidification of media, three sterile paper discs were

placed on chitin agar medium then heat treated 10 µl (23.4 µg protein) of ammonium sulphate precipitate (M2 fraction), M2P1 ion exchange column fraction and extraction buffer (as control) were poured on to discs. Plates were then incubated at 25°C for 48 hrs and then stained with 2% iodine solution. The development of clear zone around the discs were observed.

Results and Discussion

The crude protein extract from mung bean seeds was fractionated by ammonium sulphate precipitation to obtain three fractions M1 (0-30%), M2 (30-60%) and M3 (60-90%). Each fraction was evaluated for the antifungal potential against two phytopathogenic fungi (*M. phaseolina* and *M. grisea*). Protein fraction M2 exhibited the maximum antifungal activity as compared to M3 fraction, while M1 fraction did not exhibit any antifungal activity against tested phytopathogenic fungi, and consequently M1 and M3 fraction were discarded. M2 fraction was further purified by ion exchange chromatography on DEAE-cellulose resulted into two major UV absorbing peaks (unbound fraction M2P1 and bound fraction M2P2 protein) (Fig. 1). Both proteins were further tested for their antifungal potential and found that M2P1 peak from DEAE cellulose exhibited a crescent shape zone of inhibition against pathogen *M. phaseolina* and *M. grisea* (Fig. 2a, b) on PDA culture medium, while M2P2 peak could not check the growth of both pathogenic fungal mycelium and finally discarded. M2P1 fraction was further characterized by SDS PAGE and its molecular mass of 50.6 kDa with single band (Fig. 3), representing the purified antifungal protein from mung bean seeds. The purified yield of M2P1 protein was processed for its thermo-stability from 10-100°C and incubated for 10 min (Fig. 4). Moreover, this M2P1 protein fraction retained significant antifungal activity up to 60°C for 10 min and subsequently decrease in antifungal activity was observed form 70 to 100°C. Purified protein M2P1 was also investigated for its chitinase activity on colloidal chitin agar plates and found that it showed clear zone surrounding paper disc when iodine solution was used which indicated good chitinase activity of purified protein M2P1 (Fig. 5). Chitinase activity was also observed after heat treatment of purified protein. The protein yield of different fractions of mung bean seeds at purification stage revealed that the protein in 30-60% ammonium sulphate precipitation fraction (M2) and DEAE cellulose fraction (M2P1) of seed was 6.5 and 0.1872 mg g^{-1}, respectively. Ammonium sulphate precipitation method was used for protein isolation and purification by ion exchange chromatography on DEAE cellulose in the present study. Many researchers have also used this method for isolating seed protein (Shahid *et al*., 2008; Sawasdipuksa *et al*., 2011; Ajesh and Sreejith, 2014).

Crude proteins were purified by ion exchange method and two un-adsorbed peaks were found from fraction M2P1 and NaCl treated adsorbed fraction M2P2. This may be due to the fact that the higher salt concentration increased the solubility of protein, leading to the higher concentration of protein in the extract and denaturation of mung bean crude protein at high salt concentration. Benjakul *et al*. (2000) reported increased solubilisation of proteins isolated from cow pea and pigeon pea when an alkaline solution was used.

Good quantity of antifungal protein (M2P1) with the yield of 0.187 mg g^{-1} was purified on DEAE cellulose ion exchange column chromatography with molecular mass of 50.6 kDa in the present study, which was within the range of previously reported

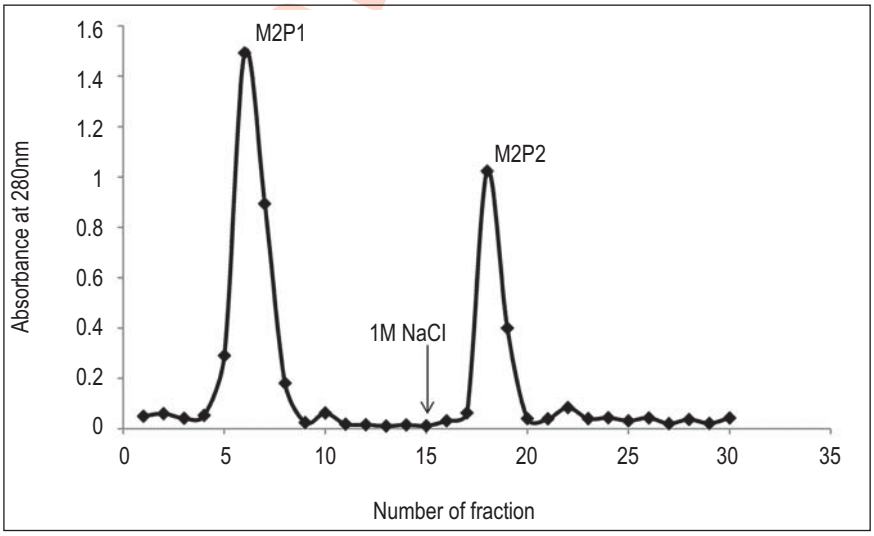


Fig. 1 : Ion exchange chromatography of ammonium sulphate precipitate (30-60%) or isolated M2 fraction of mung bean on DEAE cellulose column (1× 15 cm)

Fig. 2a : Antifungal activity of purified protein (fraction M2P1) against *M. phaseolina*. (C) Control; 10 µl of 20 mM Tris–HCl buffer. (A); 23.4 µg purified protein in 10 µl of 20 mM Tris–HCl buffer and (B); 46.8 µg purified protein in 20 µl of 20 mM Tris–HCl buffer

Fig. 2b : Antifungal activity of purified protein (M2P1) against *M. grisea*. (C) Control; 10 µl of 20 mM Tris–HCl buffer. (A); 23.4 µg purified protein in 10 µl of 20 mM Tris–HCl buffer and (B); 46.8 µg purified protein in 20 µl of 20 mM Tris–HCl buffer

antifungal proteins and higher from the earlier reported antifungal protein from mung bean seeds. Ye *et al*. (1999) characterized thaumatin like antifungal protein from French beans (*P. vulgaris* cv kentucky wonder) having molecular mass 20 kDa. Ye and Ng (2000) designated Mungin protein (18 kDa), isolated from *P. mungo* seed. A 67 kDA homodimeric lectin protein has been reported from *P. vulgaris* by Ye *et al*. (2001). Proteins Phasein A and Phasein B were isolated form *P. vulgaris* cv. Pinto and characterized by Ye and Ng (2002) with their molecular weight of 28 and 32 kDa, respectively.

In the present study, 10 µl aliquots of purified protein (23.4 µg) were used against pathogenic fungi to check antifungal assay. Similarly, 0.5 to 25 mg ml^{-1} concentration of proteins was ascribed by Osborn *et al*. (1995) and Almeidia *et al*. (2000). Praxedes *et al*. (2011) used concentration ranging from 12.4 to 28.2 mg ml^{-1}. In their experiment, protein isolated and purified from mung bean seeds showed strong antifungal activity against phytopathogens *i.e., M. phaseolina* and *M. grisea*. Correspondingly, Ye and Ng (2000) isolated Mungin protein from mung bean seed which exhibited an inhibitory action *against Coprinus comatus, Rhizoctonia solani* and *Botrytis cinerea*. Wang *et al*. (2005) reported mung bean chitinase which inhibited the growth of *Fusarium solani*, *Fusarium oxysporum*, *Mycoshaerella arachidicola, Pythium aphanidermatum* and *Sclerotium rolfsii*.

In the present study, purified antifungal protein having chitinase activity remained unadsorbed (unbound) in nature on ion exchange chromatography on DEAE-cellulose, in contrast to other studied antifungal chitinase proteins. However, the chromatographic behavior of antifungal chitinase proteins are distinct in general. Most of the chitinases were adsorbed on anion exchangers eg. *Aeromonass chubertii* chitinases (Liu *et al*., 2009). Some are adsorbed on cation exchangers, eg. emperor banana chitinase (Ho and Ng, 2007). Some are adsorbed on both kinds of ion exchangers, eg. *Bacillus cereus* chitinase (Wang et al. 2009). In some cases chitinase was purified by affinity chromatography eg. *Tamarindus indica* chitinase (Rao and Gowda, 2008).

The molecular weight (50.6 kDa) and chitinase activity of the novel antifungal protein purified and characterized under present study is unique protein as compared to previously reported antifungal proteins having chitinase activities due to different molecular weights. The molecular masses of most of the chtinase proteins reported so far ranges between 30kDa to 60KDa. However, chitinase with different molecular masses have been reported earlier. The molecular mass of small chitinase eg. *Panax ginseng* chitinase (Lam and Ng, 2001) is as low as 15 kDa. The molecular mass of some chitinase are higher eg. *Oreochrom isniloticus* chitinase (Molinari *et al*., 2007) and *Bombex mori* chitinase (Kabir *et al*., 2006) are 75

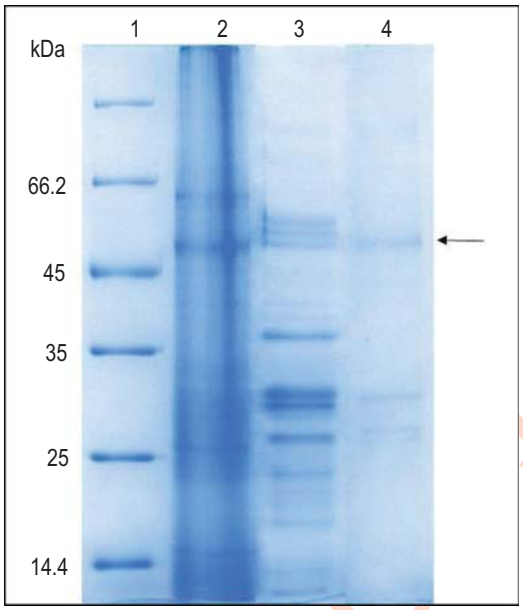


Fig. 3 : Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of isolated fraction M2P1. From left to right; lane 1: molecular marker, lane 2 : Crude Protein, lane 3: 30-60% ammonium sulphate fraction, Lane 4 : Ion exchange fraction

Fig. 4 : Thermostability test of purified protein (M2P1) against *M. phaseolina* with 10°C to 100°C (10°C interval for 10 min.) with 23.4 µg in 10µl buffer and control (C: only buffer)

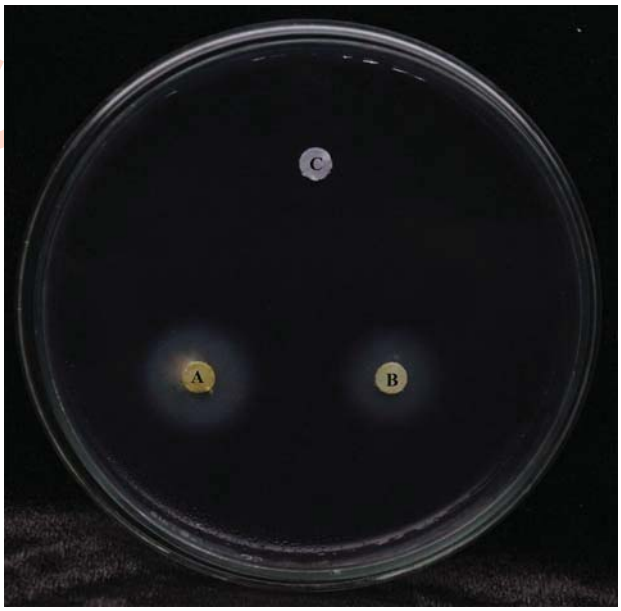


Fig. 5 : Chitinase activity on colloidal chitine agar media. (C) : Control; (A) Ammonium sulphate precipitate fraction (M2); (B) Ion exchange column fraction (M2P1)

kDa. Lam and Ng (2010) characterized Acaconin, a chitinase like antifungal protein, from *Acacia confuse* seeds having molecular mass of 32 kDA. Ye *et al*. (2001) characterized antifungal lectin exhibiting sequence similarity to chitinase has been isolated from *P. vulgaris* seeds having molecular mass of 67 kDa. Wang *et al*. (2008) purified and characterized chitinase from peanut (*Arachis hypogaea*) and demonstrated that chitinase protein exhibited a molecular mass of 34.4 kDa.

The heat treatment of purified antifungal protein (M2P1) was carried out from 10°C to 100°C for 10 min. and it was found that the purified protein remained thermostable up to 60°C and exerted inhibitory effect on pathogengic fungal growth, but it was rapidly inactivated when incubated at temperature above 60°C. Plants defend themselves against pathogens by producing secondary metabolites and proteins with antifungal activity. Yan *et al*. (2015) reviewed plant antifungal proteins and their applications in agriculture emphasizing thermostability and activity over a wide range of pH as desirable traits of such proteins. Ramos *et al.* (2015) purified thermostable osmotin/thaumatin- like antifungal protein from the latex of *Calotropis procera* that retained its antifungal activity over a wide pH range.

This antifungal protein from mung bean seed can be used as an alternative eco-friendly bio-fungicides and can play a promising role in different biomedical and pharmaceutical applications.

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