

Purification and characterization of pathogenesis-related antifungal Beta-1, 3 glucanase from Basrai banana fruit

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Abstract: Pathogenesis-related proteins have been described as proteins that are encoded by the plant genome and that are induced specifically in response to infections by pathogens. These represent a collection of unrelated protein families which function as part of the plant defense system. Pathogenesis-related antifungal protein has been isolated from the pulp of ripe Basrai bananas and purified through ammonium sulphate precipitation, Sephadex G-75 gel filtration chromatography and electro-elution. The purified protein with acidic character (pI 6.81), has molecular weight of 34.5kDa, as determined by MALDI-TOF mass spectrometry. Mascot score obtained was 473 greater than 82, indicate extensive homology at a significant level (p.0.05) and the protein was identified as beta 1,3-glucanase with antifungal activity. It inhibited the growth of *Fusarium oxysporum* demonstrating the potential role of Basrai banana antifungal protein to control fungal diseases in plants, animals and human.

Keywords: Antifungal protein, banana, characterization.

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INTRODUCTION

Banana is an important fruit crop of Pakistan cultivated over an area of 33,000 hectares with annual production of 10, 3000 tones. Sindh contributes 70 to 80% in banana acreage and production Dwarf Cavendish variety (Basari) is grown over more than 98% of the area. The production declined drastically in early nineties due to wide spread of banana bunchy top diseases caused by banana bunchy top virus (BBTV), vector, black aphid and some other harmful diseases¹, Panama, now it is known as Fusarium wilt a highly destructive and the most notorious of all plant diseases which affects the vascular system of banana. The causal organism is a soil-borne fungus, *Fusarium oxysporum*².

Higher plants have developed various defense mechanisms against biotic and abiotic stresses, such as pathogen invasions, wounding, exposure to heavy metal, salinity, cold, and ultraviolet rays. These defense mechanisms include: physical strengthening of the cell wall through lignification, suberization, and callose deposition; production of phytoalexins which are secondary metabolites, toxic to bacteria and fungi; and synthesis of pathogenesis-related (PR) proteins such as β -1,3-glucanases, chitinases and thaumatin like proteins³.

PR proteins were first observed in tobacco plants infected with tobacco mosaic virus (TMV)⁴, and they were subsequently identified in many other plants species. Based on their primary structures, immunologic relationships, and enzymatic properties, PR proteins are currently grouped into seventeen families (PR-1 through 17)⁵⁻⁸. The PR-1 family consists of proteins with small size

(usually 14-17 kD) and antifungal activity. The PR-2 family consists of β -1,3-glucanases, which are able to hydrolyze β -1,3-glucans, a biopolymer found in fungal cell walls. The PR-3, -4, -8 and -11 families consist of chitinases belonging to various chitinase classes (I – VII). The substrate of chitinases, chitin, is also a major structural component of fungal cell walls.

Chitinases and β -1,3-glucanases could work synergistically to boost their antifungal activity both *in vitro* and *in vivo*. Therefore, the resistance against *Cercospora nicotianae* was greatly enhanced in tobacco which expressed both chitinase and glucanase⁹⁻¹¹.

In addition, some PR proteins, β -1,3-glucanases, chitinases and thaumatin-like proteins, have been implicated in regulating various developmental processes such as flower formation, fruit ripening, seed germination, and embryogenesis⁵.

The present study aims at investigating the isolation, purification and identification, of pathogenesis related antifungal protein from banana fruit. It further intends to highlight the vast scope of the use of this protein for the benefit of plant, animal and human diseases in economical manner.

MATERIALS AND METHODS

Protein extraction

Frozen tissue was grinded to a fine powder in a sterilized pestle and mortar. Protein was extracted by the addition of 2 mL of buffer (500mM Tris-Cl, pH 8.0, 5% (w/v) SDS, 10mM DTT and 10mM sodium diethyldithiocarbamate) to 1g of powdered tissue, incubated at 95°C for 5 minutes and then centrifuged at 12,000rpm for 5 minutes. Protein was concentrated

by precipitation with ice-cold TCA (Trichloroacetic acid) at a final concentration of 10% (w/v). After 15min incubation on ice the precipitated protein was collected by centrifugation at 12,000g for 15min, the resulting pellet was washed twice with ice-cold ethanol:ethyl acetate (2:1). Proteins were resuspended in 20mM Tris-C1, pH 8.0.

Protein purification

Ammonium sulphate precipitation

Protein was precipitated with 60, 80 and 90% of ammonium sulphate saturation and stirred overnight at 4°C. The pellet was obtained after centrifugation at 10,000rpm for 30 minutes at 4°C and extensively dialyzed at 4°C against 50mM phosphate buffer (pH 7.0) before subjected to gel filtration.

Gel filtration

The Sephadex G-75 (Sigma chemical, USA) was packed in column (1.6 x 70cm) and equilibrated 150 mM phosphate buffer (pH 7.0). The concentrated protein was subjected on a Sephadex G-75 column. Bound proteins were eluted with 150mM phosphate buffer (pH 7.0). Fractions containing (34-KDa protein) was concentrated at 4°C by ultrafiltration in an Amicon ultrafiltration cell fitted with 10,000 MW cut off membrane (PTGC 043 Millipore Company Co., USA) under 50 pounds/inch² nitrogen pressure. Protein concentration in each fraction was determined by dye-binding method¹². The protein fractions were analysed by SDS-PAGE¹³.

Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Homogeneity was checked by SDS-PAGE according to the method described by Laemmli, 1970. 12% resolving gel was prepared by mixing 7.5 ml of 2 M Tris-Cl pH 8.0, 8.4ml of 30% acrylamide/0.8% bis-acrylamide, 4ml distilled water, 10µl TEMED, 120µl (10%) freshly prepared ammonium persulphate and 200µl of 10% sodium dodesylsulphate (SDS).

The stacking gel was prepared by mixing 1.3ml of 0.5M Tris-Cl pH 6.8, 1.4ml of 30% acrylamide/0.8% bis-acrylamide, 7.2ml distilled water, 5µl TEMED, 100µl of 10% SDS and 100µl of (10%) freshly prepared ammonium sulphate.

Sample was prepared by mixing the sample with loading dye (0.5ml of 10% SDS, 0.5ml of 0.5M Tris-HCl pH 6.8, 0.5ml of glycerol containing 0.02% bromophenol blue, 9ml distilled water and 50µl β-mercaptoethanol). Protein size marker (Fermentas) was applied to determine the molecular weight of the protein.

Electro-elution

Required protein gel band was excised, cut to small pieces and transferred to dialyzing tube (boiled in ammonium bicarbonate solution) containing

elution buffer (Tris- acetate buffer (pH 7.4), 0.1% SDS and 100mM DTT (Fermentas, Cat# R0861). For electro-elution, horizontal agarose gel electrophoresis apparatus was filled with running buffer (50mM Tris-acetate buffer (pH 7.4), 0.1% SDS, and 0.5mM sod-thioglycolate). Dialysis tube containing gel pieces was aligned perpendicular to the current flow and run for 1 hour at 50 volts until the stained gel pieces were completely decolorized. After electrophoresis, protein extracts was pipetted out in separate sterile tube from dialysis tube and freeze dried. Resulting dried protein sample reconstituted with phosphate buffer saline (PBS, 137mM sodium chloride, 2.7mM potassium chloride, 4.3mM sodium hydrogen phosphate, 1.47mM potassium hydrogen phosphate, pH 7.4) and run on 15% SDS-gel. After staining and destaining, purified single protein band was obtained.

Protein sequence analysis

The protein band of interest was cut directly from gel with clean razor blade and analysed by MALDI-time of flight mass spectrometer from Saint Andrews University, Scotland, UK.

Antifungal activity

The antifungal activity assay at various stages of purification was conducted according to the method of Wang and Ng (2005). The assay for antifungal activity toward *F. oxysporum* was carried out in 100x15mm petri plates containing 10ml of malt extract agar. After the mycelia colony had developed, sterile blank paper disks (0.62cm in diameter) were placed at a distance of 0.5cm away from the rim of the mycelia colony. An aliquot of antifungal protein was added to a disk. The plates were incubated at 23°C for 72 hours until mycelia growth had covered the disks containing the control and produced crescents of inhibition around the disks containing samples with antifungal activity.

RESULTS AND DISCUSSION

Higher plants have a broad range of mechanisms to protect themselves against various threats like physical, chemical and biological stresses, such as wounding, exposure to drought, cold, air pollutants, ultraviolet rays and pathogen attacks, like fungi, bacteria and viruses¹⁵. These stresses can bring biochemical physiological changes in plants by producing phenolic compounds, phytoalexins and pathogenesis related (PR) proteins which consequently protect plants from various pathogen invasion³.

The protein was precipitated from the basrai banana extract by 60, 80 and 90% ammonium sulphate precipitation. Centrifugation was done to

obtain pellets. The pellets contained high salt content, which was further removed by dialysis. Dialysis was continued for 36 hours at 4°C. The dialysed fraction was centrifuged and supernatant was used for further purification. The dialysed fraction was analysed by SDS-PAGE as shown in Figure 1.

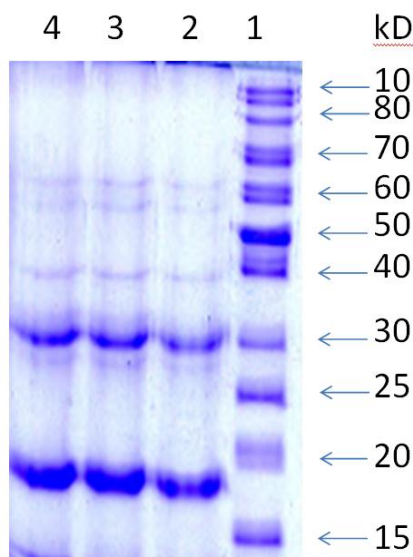


Figure 1: SDS-PAGE of ammonium sulphate precipitated fractions of Basari banana. From left to right, lane 1: protein size markers (Fermentas), lane 2: 60%, lane 3: 80% , lane 4: after 90% ammonium sulphate precipitation

Gel filtration on Sephadex G- 75 gel filtration medium, was used as a purification technique. The supernatant was lyophilized and loaded on a column (1.6X70cm) which was previously equilibrated with 150mM phosphate buffer (pH 7.0). Elution was done with same buffer and 0.5 ml fractions were collected in a flow rate of 0.5ml/min. The elution profile is shown in Figure 2. Fractions (0.5ml) were analyzed for protein contents by SDS-PAGE using 15% gel (Figure 3).

Partially purified protein was run on 15% SDS gel and required protein gel band was excised and subjected to electro elution. The eluted protein sample was analysed by SDS-PAGE. After staining and destaining, purified single protein band was obtained. Purified protein gel band was cut with sterilized razor and analysed by MALDI-TOF mass spectrometry. The protein identified by MALDI-TOF was beta 1,3-glucanase and results are depicted in Figure 4. Mascot score obtained for the protein was greater than 82 and the score >82 indicates identity or extensive homology at a significant level ($p < 0.05$). The results of Mascot score and sequence coverage of antifungal protein are shown in Figure 5 and Table 1.

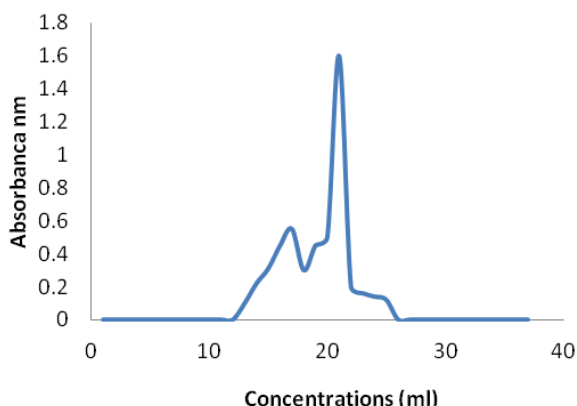


Figure 2: Gel filtration chromatography of ammonium sulphate precipitated fraction of Basari banana on a Sephadex G-75 (1.6 x 70 cm) column preequilibrated with 150 mM phosphate buffer (pH 7.0)

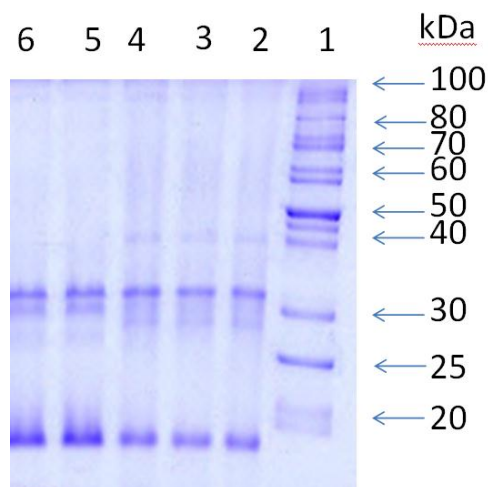


Figure 3: SDS-PAGE after gel filtration chromatography on Sephadex G-75 column. From left to right, lane 1: protein size markers (Fermentas); lane 2, 3,4,5 and 6: gel filtration fractions

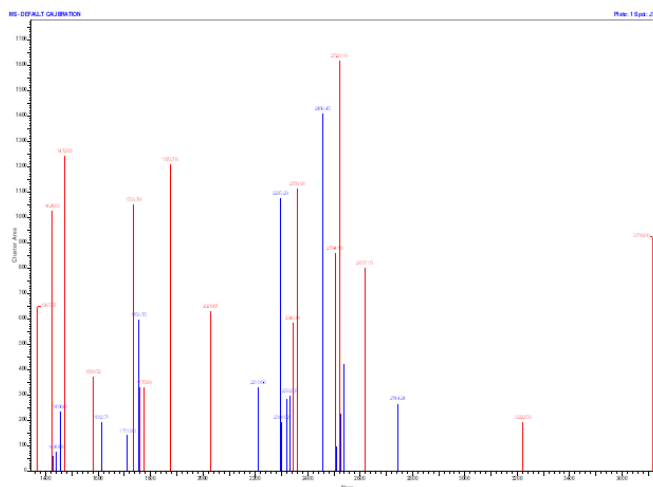
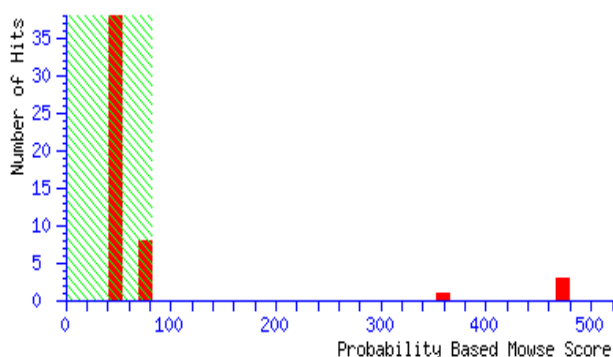


Figure 4: MALDI-Spectra of beta1,3-glucanase

Mascot Search Results

Search title : SampleSetID: 6882, AnalysisID: 13183,
 MaldiWellID: 180947, SpectrumID: 78646, Path=\\30 July
 Service Plate\\22Sept09\\Yasmeen Banana 2 Comb Uni
 Database : UniProt combined_090409 (7966092 sequences;
 2613551657 residues)
 Timestamp : 22 Sep 2009 at 08:57:27 GMT
 Warning : A Peptide summary report will usually give a
 much clearer picture of MS/MS search results.
 Top Score : 473 for Q9SQL1, Beta-1,3-glucanase
 (Fragment) OS=Musa acuminata PE=1 SV=1
 Probability Based Mowse Score

Ions score is $-10 \cdot \log(P)$, where P is the probability that the
 observed match is a random event.
 Protein scores greater than 82 are significant ($p < 0.05$).
 Protein scores are derived from ions scores as a non-probabilistic
 basis for ranking protein hits.



Fixed modifications: Carbamidomethyl (C)

Variable modifications: Oxidation (M)

Cleavage by Trypsin: cuts C-term side of KR unless next residue
 is P

Sequence Coverage: 54

Matched peptides shown in **Bold Red**

1 LVAVPTRVQS IGVCYGMGLGN NLPPPSEVVS
 LYKSNNIAR **RLYDPNQAAAL**
 51 **QALRNSNIQV LLDVPRSDVQ SLASNPAAAG**
DWIRRNVAAY WPSVSFRYIA
 101 **VGNELIPGSD LAQYILPAMR NIYNALSSAG**
LQNQIKVSTA VDTGVLGTSY
 151 PPSAGAFSSA AQAYLSPIVQ FLASNGAPLL
 VNVYPYFSYT GNPGQISLPY
 201 ALFTASGVVV QDGRFSYQNL FDAIVDAVFA
 ALER **VGGANV AVVSESQWP**
 251 **SAGGGAEAST SNAQTYNQNL IRHVGGGTPR**
RPGKEIEAYI FEMFNENQKA
 301 **GGIEQNFGLF YPNKQPVYQI SF**

Figure 5: The Mascot search results of beta 1,3-glucanase.

Table 1: Protein identification by MS/MS.

Sample	Banana
Mascot protein hit (Uniprot accession)	Q9SQL1
Protein	beta-1,3-glucanase
Mascot score	473
Theoretical molecular mass (kDa)	34.511
pI	6.81
Sequence coverage	54%

Antifungal protein was identified as beta-1,3-glucanase by MALDI-TOF mass spectrometry with molecular weight of 34.5kDa and its maximum sequence homology is correlated with previously reported beta-1,3-glucanase of *Musa acuminata*¹⁶. Several reports described beta-1,3-glucanases having molecular mass in the range of 33 to 44 kDa^{17,18}.

Beta-1,3-glucanases are abundant proteins widely distributed in plants and were one of the first Pathogenesis-related protein families known to enzymatic activity¹⁹. Beta-1,3-glucanases have direct role in defense against pathogens because a substrate of these enzymes is a major component of the cell walls of many fungi²⁰. Combinations of beta-1,3-glucanase and chitinase have antifungal activity against several fungi in vitro²¹. Purified beta-1,3-glucanase from Basrai banana exhibited antifungal activity against *F. oxysporum* (Figure 6). However, independently glucanase inhibited only one of these fungi, *Fusarium solani*. Combined effect of chitinase and glucanase may enhance antifungal activity against various fungi²¹.



Figure 6: Antifungal activity of Basrai banana antifungal protein toward *F. oxysporum*.

In the present study, beta-1,3-glucanase antifungal protein isolated from basrai banana inhibits destructive fungal contamination. This gives a new potential for banana improvement.

Basrai banana antifungal protein can be used to control fungal diseases in plants, animals and human.

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