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Isolation and Purification of Cuticle Degrading Extra Cellular Proteases from Entomopathogenic Fungal Species of *Beauveria bassiana*

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ABSTRACT

The entomotoxic protein of Beauveria bassiana (Bals.) (Ascomycota:Hypocreales) was precipitated with 90% saturated $(NH_4)2SO_4$ and the precipitate was eluted in Sephadex G-25 column. The protein content was higher (0.026 µg/ml) supported by UV spectroscopy absorbance. The vibration frequency in FT-IR appeared between the ranges of 1650– 1580cm-^{1} due to N-H bond formation for amines. Bioassays of Beauveria bassiana reveal that caused significantly higher insecticidal activity.Amylase, Protease and Lipase (digestive enzymes), Esterase, Glutathione S-Tranferase and Lactate dehydrogenase level/activity in insects. Hence, the Beauveria bassiana was subjected to sodium dedosyl sulphate–poly-acrylamide gel electrophoresis (SDS-PAGE)

Key word: *Beauveria bassiana, Protein Extraction, FT-IR, SDS-PAGE*

INTRODUCTION

Crop protection plays a paramount and integral role in modern day agricultural production. Indiscriminate use of synthetic pesticides results in drastic problems such as environmental pollution, health hazards etc in humans. One of the proven technically and commercially sustainable options for crop protection proved is the use of biocontrol agents. *Beauveria bassiana* (Bals.) Vuillemin is one of the well-understood and widely used entomopathogenic fungi infecting wide range of economic pests mainly belonging to Lepidoptera, Coleoptera and Hymenoptera. It is a soil born pathogen of many insect pests, and its potential as a microbial insecticide has heightened interest in its ecology in soils. As there are ample evidences that the commercial insecticides can inhibit growth of fungi in in vitro and in vivo conditions, the problem of understanding compatibility of the latter with chemical pesticides remain pressing. Moorhouse[13] studied the effect of Dichlorvos and Triazophos on the growth of *B.bassiana* and observed complete inhibition in growth at ten times the recommended rate. According to Reddy[16] in vitro growth and conidial germination was not effected by nematicide in entomopathogenic fungi.

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The differences in pathogenecity as well as to other abiotic stress are signs of naturally occurring genetic variation during co-evolution of the fungus and its host and might be relevant if the fungus is to be developed as a mycopesticide. Analyzing total soluble protein patterns of the isolates may give insight regarding differences between tolerant and less tolerant isolates of *B. bassiana* at biochemical level. In the present study isolates of *B. bassiana* were evaluated for compatibility with four commonly used organophosphorous pesticide compounds with an objective to understand the threshold concentrations of test chemical permitting survival of biocontrol agent. Further, SDS-PAGE was performed to study total soluble mycelial protein profiles among the two categories in order to understand differences if any at biochemical level.

MATERIALS AND METHODS

Fungal cultures

B. bassiana isolates used in the present study were isolated from National Fungal Cultural Collection of INDIA (NFCCI) from PUNE, India. Pure cultures were established and maintained on PDA (Potato Dextrose Agar) slants.

Isolation of culture filtrate

The *Beauveria bassiana* was grown on the PDB (Potato Dextrose Broth) And PDYB (Potato Dextrose Yeast Broth) Media and keep in Growth room for 20 days after inoculation.

Biochemical Analysis

Preparation of protein sample

After 20 days of growth on PDB (Potato Dextrose Broth) and PDYB (Potato Dextrose Yeast Broth) medium, semi-liquid culture was filtered by suction through no. 1 filter paper (Whatman Ltd. Kent, England). The filtrate was passed through 0.45 µm pore size filter (Millipore Corp), fungal matt was separated and freeze dried at -4^0 C (LG, Japan). This was used to produce entomotoxin and subsequently used to assay the virulence of the toxin. Protein from the dried fungal matt was ground with 90% ice cold saturated $(NH_4)2SO_4$ solution (681gL^{-1}) for protein precipitation. The precipitated protein was collected by centrifugation at 8000 rpm for 30 minutes. The crude protein was subjected to a gel filtration through a Sephadex G-25 (Sigma) column (1 x 10 cm) in 50 mM tris/HCl buffer at PH 8.

UV–spectroscopy analysis

The fungal protein fractions collected through Sephadex G-25 column were analyzed using UV spectrometer (Schumadzu, Japan) to determine protein content. The samples were prepared by mixing 100 µl of aliquots with 2 ml of distilled water. The pre-run Tris HCl buffer through Sephadex G-25 column sample was used as the blank. The base line was corrected with the blank solution between 600 nm and 100 nm. The absorbance peak reading of the each fraction was recorded categorized in it.

Fourier Transform Infrared Spectroscopy (FT-IR) analysis

FT-IR spectra of *Beauveria bassiana* was determined on a FTIR spectrometer in the range of 4000–400cm^{-1} with a resolution of 4 cm^{-1}.The FT-IR was carried out for each sample separately for the identification of possible resonance vibration of chemical bonds.

SDS-PAGE

Tricine-SDS-PAGE was used to determine number and relative molecular weight of polypeptides/proteins found in the *B. bassiana* entomotoxins. All solutions and reagents were prepared according to [17]. A 16% separating gel (pH 8.2) was prepared from N, N'-methylene Bisacrylamidestock solution (49.5% T, 3% C). Similarly gel buffer stock solution (3.0M Tris and 0.3% SDS, pH 8.2); anode electrophoresis buffer (2M Tris pH 8.9) and cathode electrophoresis buffer (0.1M Tris, 0.1M Tricine, and 0.1% SDS pH 8.2) were prepared. Acrylamide stock solution **[**Acrylamide (49.5%), methylene bis acrylamide (3%C) and keep at 4^{0}C]; gel buffer (pH 8.4) [3.0M Tris and 0.3% SDS kept at room temperature], Glycerol 70%, cathode buffer (Upper buffer) pH 8.2 (1M Tris, 1M Tricine, 1% SDS and keep in RT), anode buffer (lower buffer) pH 8.9 (2.1M Tris keep in RT), Ammonium per sulphate (APS) [APS 10% kept at 4^{0}C] and TEMED were prepared and used for this study.

Equal amount of 20 uL entomotoxin was denatured into the buffer solution of 100% Glycerol, 2-Mercaptoethanol, 20% SDS and 1M Tris at pH 6.8, and then loaded to gels. A

constant voltage of 30 V was maintained until the samples concentrated on the stacking gel, before adjusting the voltage to 80 V for the remaining separation time.

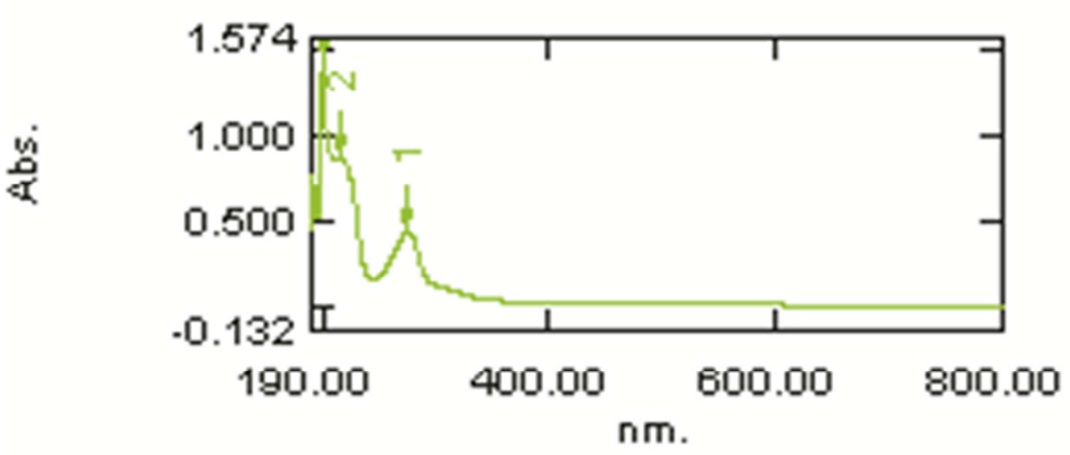
Electrophoretic separations were maintained for 7-8 hrs with 25 mA/gel stable current. The gel was stained overnight in 0.1% Coomassie Blue R-250 (in 30% methanol, 10% acetic acid) and then destained. After destaining, the gel was placed in 7.5% acetic acid for preservation and imaged with Olympus camera attached to Gel documentation unit

(Biotech, Yerkard, India) with D-Gel Das software.

RESULTS

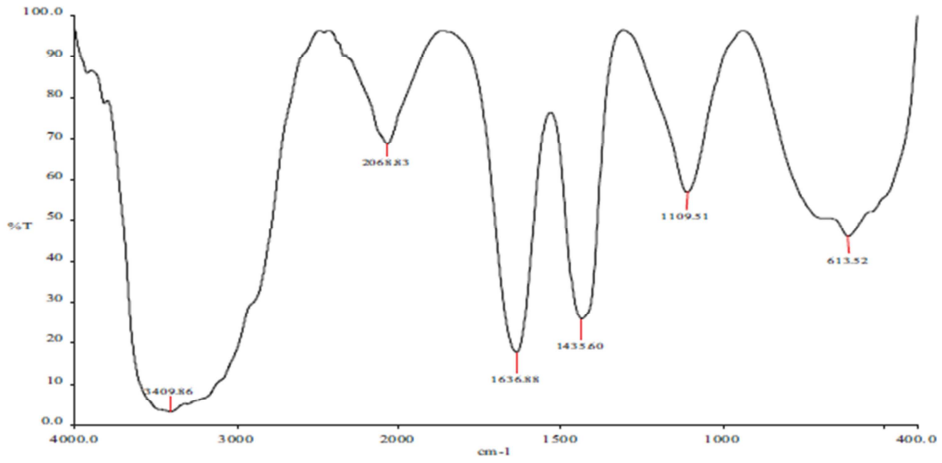
Purification of entomotoxin

The toxic protein of *B. bassiana* was precipitated with 90% $(NH_4)2SO_4$ and the precipitate was eluted from Sephadex G-25 column. Totally 12mL elutes (each 1 mL) were collected, separated primarily and analyzed by UV - visible spectroscopy. It was observed that the total protein surface plasmon resonance band occurs initially at 210 nm. The proteins surface plasmon band recorded between 210-280 nm in an aqueous medium. Based on the UV absorption the eluents.

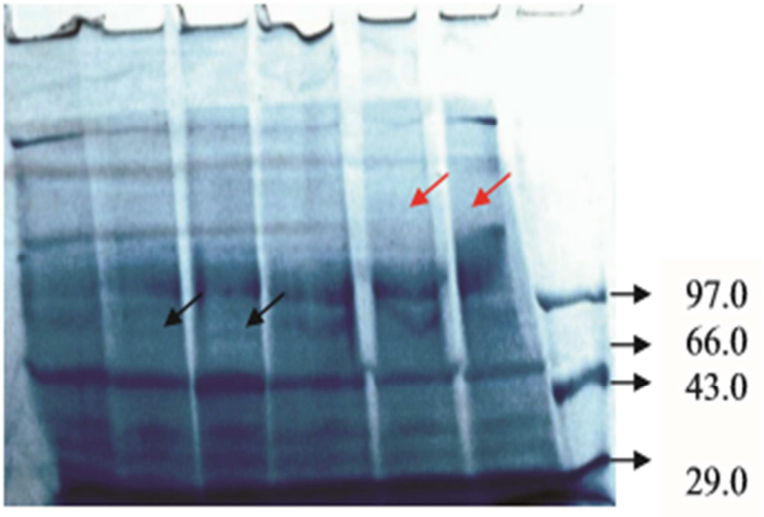


Fourier Transform Infrared Spectroscopy (FT-IR) analysis

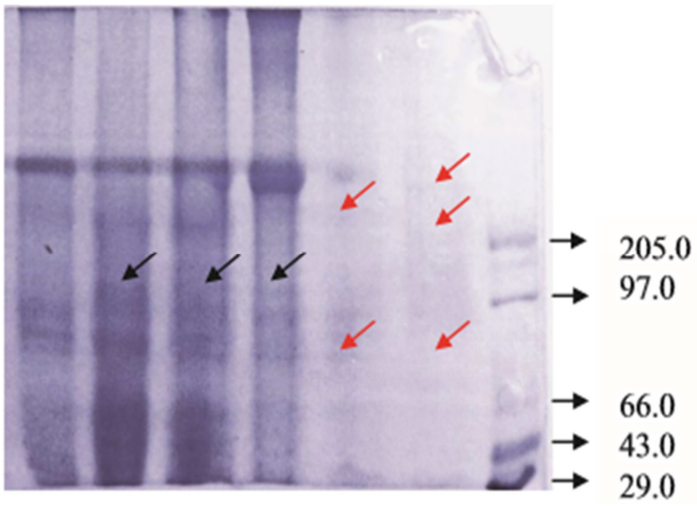
FTIR analysis reveals that the *Beauveria bassiana* show characteristic vibration between 1637 - 1639 cm^{-1}(N-H for amines) and also between 1435 - 1440 cm^{-1} for (C-H for methyl group)



SDS-PAGE - Entomotoxin



The SDS-PAGE analyses of FI, FII and FIII revealed that the fractions contain different polypeptides having low molecular polypeptides, isolated through Sephadex G-25 Column of *Beauveria bassiana* contains a single band (M.W. -10656)



DISCUSSION

Naturally occurring entomopathogenic fungi are important regulatory factors in insect populations. Many species are employed as biological control agents of insect pestsunder field and glasshouse crops, orchards, ornamentals, range, and lawn, stored products, and forestry and also used in vector insects of veterinary and medical importance. Protein absorbs strongly at 280 nm due to a number of its constituent amino acids. The peptide bonds found in the amino acids also absorb at 205 nm. In the present study the Sephadex column fraction was analyzed by UV-visible spectroscopy. The result confirms that the fractions showed the absorption of proteins. In another analysis, the samples were subjected to FT-IR spectra. When the purification/specificity of the polypeptide increased, the quantity of protein content decreased. The same findings were proved in the present study. The entomotoxin of *B. bassiana* showed high of

proteins content. The spectrum results showed the presence of proteins as secondary amines. Previous reports showed that the filamentous fungi like *B. bassiana* had been shown to contain multiple types of enzymes presence of protein in the metabolic mixture of *Fusarium oxysporm* which was identified using FT-IR spectra at the absorption range of 1500–1600 cm^{-1}. Present results also confirm the presence of protein in the range of 1400-1600 cm^{-1} in *B. bassiana* toxic fractions in the form of secondary amines/amides. FT-IR spectroscopy is a sensitive method to analyses the presence of protein in the fungal metabolites. In the present fractions, the peaks represent not only secondary amines, but also the presence of alcohols (3300-3400 cm^{-1}), ketons (1100-1200 cm^{-1}) and aldehydes (600-700 cm^{-1}) with respective ranges.

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