PURIFICATION AND IDENTIFICATION OF ASPERGILLUS NIGER INDUCED NOVEL PROTEIN FROM GROUNDNUT (ARACHIS HYPOGEAE L.)

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Abstract: A 110-kDa protein was over-expressed in groundnut (Arachis hypogaea L.) seedlings due to infection of Aspergillus niger. Densitometric quantification revealed that the protein start inducing after 4hr post infection and maximum induction is seen after 42hr. When subjected to SDS page, protein showed three subunits. The purification of induced protein from maximum induced fraction was carried out using ammonium sulphate precipitation followed by size exclusion chromatography using shephadex G-100. Purified tri-peptide were confirmed by two-dimensional gel and subjected to tryptic digestion, followed by identification using matrix-assisted laser desorption/ionization–time of flight mass spectrometry. This sequence segment was searched for homologous sequences using MASCOT data base program and no similarity was found with other reported sequences of groundnut proteins. Even when the expected value parameters were increased, it yielded the same result. The searches were done against non redundant database. Further, no match to this sequence was found from a search on the Express Sequence Tag. This shows that the sequence may be unique. Thus, we assume that induced protein is a unique and novel protein which is not reported earlier in groundnut plants.

Key words: Groundnut, Aspergillus niger, Unique Protein

INTRODUCTION

Groundnut (Arachis hypogaea L.) is an important food legume. It is primarily grown as a seed crop and is a rich source of edible fats and proteins. It is the third most important source of plant protein contributing 11% of world’s protein supply. In India Groundnut is an important oilseed crop, cultivated on 7.5 million hectares of land, with an annual production of 8 million tons. More than nine million farmers depend on this crop for their livelihoods. Groundnut is grown as a single crop in year. The average productivity of groundnut in India is around 1300 kg ha⁻¹ which used to be around 1000 kg ha⁻¹ in the last decade [1]. The low productivity in India is mainly due to diseases of groundnut which reduce yield, affect quality and increase the cost of production. About 25 to 50 % of the crop loss is due to fungal diseases [2].

Among various fungal diseases, collar rot caused by Aspergillus niger has got economical importance as the fungus causes rot of both seed and seedling which drastically reduces the plant stand [3]. In moist soil, seeds may be attacked and killed due to rotting. Seeds removed from soil show black sooty cover. The infected areas of seedlings are covered with black fungal spores. Mature plants are also attacked. Symptoms include wilt of branches permanently, and or wilting of entire plant. The dead and dried branches are easily detached from the collar region. Infected
pods reveal patches of black sooty spores [4].

Investigation of natural defense mechanism in plants in recent years has given new dimension to disease control. Instead of targeting the pathogen by using fungicides and pesticides, plants’ defense mechanism can be activated to control the diseases. Plants possess both preformed and inducible mechanisms to resist pathogen invasion. Whether or not a plant turns out to be susceptible or resistant is likely determined by the speed and magnitude with which these mechanisms are activated and expressed and by their effectiveness against individual pathogens with different modes of attack [5].

For better crop productivity, various molecular researches are being carried out throughout the world. Molecular markers such as Restriction Fragment Length Polymorphism (RFLP), Random Amplification of Polymorphic DNA (RAPD), and Amplified Fragment Length Polymorphism (AFLP) techniques are widely used to analyze the complex traits and identifying a Quantitative Trait Loci (QTLs) [6]. However, little progress was made as these techniques have shown limited polymorphism in groundnut plant which exhibits narrow genetic base and shows low genetic variation [7]. Several up-regulated genes in response to drought and Aspergillus infestation were identified using the cDNA microarray [8]. Several ESTs from A. hypogaea are presently accessible in public databases, but the protein expression data remain extremely inadequate. Proteomic research involves investigation of synthesis, turnover and modification of many proteins in order to understand gene function and characteristics of various genotypes [9]. However, very little information is available about the proteome changes to biotic stress in peanut [10]. Proteomic analyses may, therefore, provide a powerful tool to address biochemical and physiological aspects of plant responses to biotic stresses. [11]. In present study, we have carried out proteome research in groundnut seedling challenged with A. niger in three groundnut cultivar. The overall objective of this research was to identify probable resistant protein in groundnut since only a limited coverage of peanut proteome is available till date.

MATERIALS AND METHODS

Plant material and seed germination: Three varieties of groundnut seeds (Arachis hypogaea L.), GG 11, GG 20 and GG 24, were obtained from Junagadh Agricultural University, Junagadh, Gujarat, India. Seeds were surface-sterilized with 0.1% HgCl₂, rinsed carefully with sterile water and put for germination in sterilized petri dishes with sterile filter paper damped either with distilled water (control) or spore suspension of A. niger. Similar seed germination was also carried out in pots containing sterile soil. Infection by spore suspension was always made in seeds grown for 48 hrs.

Isolation and inoculation of fungal culture: A. niger was isolated on potato dextrose agar from infected groundnut plant obtained from Agricultural University Junagadh, Gujarat, India, which was confirmed by spore structure. It was maintained on PDA plate. Inoculum was prepared by flooding sterilized water on sporulated plate and spores were collected and counted using Neubauer chamber. A suspension containing 10⁵ spores /ml was prepared and used for infection.

Disease severity: Disease incident was calculated as follows. A total of 200 seeds of each variety were incubated with A. niger spore suspension, the infected seeds were counted and the percentage of disease incident was calculated.

Sample collection: Treated and untreated plant material was powdered using liquid nitrogen (Ln₂) and stored at -4°c for further analysis. Each treatment was replicated three times. For all the analysis the samples were collected in triplicates. The experiment was repeated twice.

Protein extraction and electrophoresis: Total protein was extracted according to Roulin and Buchala [12]. Plant material was frozen in Ln₂ and grounded to fine powder with a pre-chilled mortar and pestle and extracted by adding 100mM Tris buffer (ph 7.2) containing 1mM phenyl methane sulphonyl fluoride (PMSF) and 1% polyvinylpyrrolidone (PVP). Extracted protein was quantified by method of Lowry et al. [13]. Protein profiling was carried according to Liang et al. [14]. Total protein from all three varieties were profiled using SDS/Native PAGE (10% separating gel with 4% stacking gel) according to the method of Laemmli [15], with the Mini-PROTEIN Dual Slab Cell System (BIO-RAD). Total proteins (120µg) from each sample were loaded onto SDS-PAGE gels. Low-range protein markers (Sigma) were used as molecular mass
Fig. 1: Native PAGE of showing induced protein and purified protein. Well no. M = molecular weight marker, 1 = GG 11 control, 2 = GG 11 infected with A. niger, 3 = GG 20 control, 4 = GG 20 infected with A. niger, 5 = GG 24 control, 6 = GG 24 infected with A. niger.

Fig. 2: SDS PAGE showing Purified tri-peptide. Well no. M = molecular weight marker, 1= Purified tri-peptide.

Fig. 3: Bioassay of partially purified protein
The crude protein fraction of 42 hr post infection was precipitated out using different levels of ammonium sulfate saturation from 0% to 80%. The pellets were collected after each saturation interval by centrifugation at 12,000 g for 20 min at 4°C, and dissolved in 0.01 M Tris HCl buffer pH 7.2, and checked by running Native PAGE for maximum precipitation. From these, dissolved pellets were loaded onto Sephadex G-100 (1.5 cm × 10 cm) using 0.01 M sodium phosphate buffer, pH 7.2 as eluant. Further purification was carried out by gel filtration using Sephadex G-75 (1.5 cm × 15.0 cm). Peak fractions of G-75 gel filtration were collected and reloaded onto a Sephadex G-75 equilibrated with 0.01 M sodium phosphate buffer, pH 7.2. The column was washed with the same buffer. The entire purification procedure was monitored by UV spectrophotometry, and Native PAGE. The eluants containing band of interest was further subjected for sequencing.

Protein digestion and sequencing: Protein bands of interest were cut from polyacrylamide gels and digested overnight using trypsin (Sigma) as described elsewhere [17]. The cleaved peptides were eluted, concentrated by vacuum centrifugation and separated by RP nano-LC (LC1100 series, Agilent Technologies, Palo Alto, California; column: Zorbax 300SB-C18, 3.5 μm, 150 mm × 0.075 mm; eluate: 0.1% formic acid in 0–60% acetonitrile). The peptides were analyzed by on-line MS/MS (LC/MSD TRAP XCT mass spectrometer, Agilent Technologies) [18]. Thereafter, a database search was conducted using the MS/MS ion search (MASCOT, http://www.matrixscience.com) against all plants entries of NCBInr (GenBank; http://www.ncbi.nlm.nih.gov/index.html) with subsequent parameters: trypsin digestion, up to one missed cleavage site, fixed modifications: carbamidomethyl (C), variable modifications: oxidation (M), peptide total: ± 1.2 Da, MS/MS total: ± 0.6 Da, peptide charge: +1, +2 and +3.

Bioassay of partially purified protein: Anti-fungal activity of partially purified protein against A. niger was carried out by the agar cup diffusion method [19]. Potato dextrose agar plate was prepared by dissolving a weighed ingredient in water at 7.6 pH. Two cups were made by punching agar surface from same distance to center with a sterile cork borer and punched part of the agar media was removed by scooping. In the center of PDA plate 48 h old A. Niger inoculated. Each cup was added 200 µL partially purified protein (20 µg/mL) carefully. Plates were then incubated at 35°C for 24–48 h to observe growth. Bioassay of this partially purified protein was also performed to check its activity against A. Niger infection to germinating seed of groundnut. Seeds of groundnut were put for germination for 24 in two sterile petri plate with filter paper soaked with water. After 20h in one plate partial purified protein applied on germinating seeds. Both the plates were treated with same size of inoculum of A. niger after 24h and incubated 35°C for 24 h.

RESULTS AND DISCUSSION

Germinating seeds of the three groundnut varieties GG 11, GG 20 and GG 24 infected with A. niger showed 76, 56 and 70% of disease severity, respectively, which means GG 20 was found more tolerant compared to other two varieties. The survival of about 50% of seeds of GG 20 variety in spite of exposure to A. niger in soil generated our interest in investigating natural resistance mechanism in this variety. Native PAGE profiling of all three varieties post infection with A. niger was carried out which showed one induced protein bands in GG20 variety (Fig. 1). Molecular mass of induced protein found to be 110 kd by molecular size chromatography. Densitometric quantification of induced protein was carried out which showed maximum induction found three fold post 42 h of pathogen infection. Purification
of protein of interest was carried out using ammonium sulphate precipitation which showed maximum precipitation at 40% ammonium sulphate concentration further purification of induced protein carried out using size exclusion chromatography (Fig. 1). Purified protein subjected to SDS-PAGE for subunit confirmation showed that this protein contains three subunit of about 41 kDa, 39 kDa and 30 kDa (Fig. 2). Sequencing of all three peptide was carried out by LC–ESI–MS/MS. This sequence segment was searched for homologous sequences using MASCOT data base program in which 41 kDa peptide [MLVESEGR] shown maximum score 44 for hypothetical protein (Q1T5F9_MEDTR) *Medicago truncatula*, 39 kDa peptide [AAFLNN-DYTK] shown maximum score 96 for p27SJ (gi|57868106) *Hypericum perforatum*. As far as protein identity concern more than 43 MASCOT score would be identical for protein identification [20] but in sequenced peptides were not found significant score for any reported protein sequences of groundnut or other plant defense related proteins.

Even when the expected value parameters were increased, it yielded the same result. The searches were done against non redundant database. Further, no match to this sequence was found from a search on the Express Sequence Tag of groundnut, shows that the sequence may be unique. Mittra et al. [21] reported similarly novel cadmium induced protein in wheat. Varietal differences of protein profiles in mature groundnut seeds were investigated and identified 20 proteins by Kottapalli et al. [22]. More than 250 groundnut leaves protein identified by Katam et al. [23]. Although numerous PR proteins reported by different authors in plants, but very less emphasis was given on the identification of defense related proteins in groundnut. Direct antifungal activity assay of partially purified protein revealed that this protein do not have any growth inhibiting activity on *A. niger*; however, to our surprise this protein showed decrease infection severity of *A. niger* and increased rate of germination when applied on germinated seedling prior to 4 hrs of infection (Fig. 3). Thus, we assumed that this induced protein is a unique and novel protein which is not reported earlier in groundnut. The complete sequence of induced protein and its functional role against *Aspergillus niger* however, await future investigation.

In summary, we report for the first time, a 110-kDa *Aspergillus niger* induced protein. Using bioassay result we assumed that this protein have role in defense response against *Aspergillus niger* in groundnut. This 110-kDa trimeric protein seems to be a new protein, as search for the occurrence of other such biotic stress proteins in plants do not show homology to this induced protein. The exact role and nature of this protein however, awaits further investigations.

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**REFERENCES**