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Research Paper

Isolation and Identification of Allergens From *Aspergillus Fumigatus* Isolated From Sericulture Grinage Industry, Hospitals and Agricultural Soil.

Microbiology

KEYWORDS : A. fumigatus, allergic diseases, SDS-PAGE electrophoresis.

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ABSTRACT

In the present study we isolated A. fumigatus from sericulture grinage industry, hospitals and agricultural soil. A. fumigatus is a major contaminant in sericulture grinage industry, hospitals and also associated with allergic diseases in industrial workers. A. fumigatus grows repeatedly on Sabouraud's agar medium (SAM). Among the three sources, fungus isolated from sericulture grinage industry grew faster. Proteinaceous allergens were extracted from mycelia of A. fumigatus. High amount of protein was yielded in A. fumigatus isolated from sericulture grinage industry. SDS-PAGE analysis clearly revealed that A. fumigatus isolated from contaminated sericulture grinage industry constitute a heterogenous group of protein allergens by expressing 48 KDa and 37 KDa proteins bands which were bands completely absent from isolates of hospital and agricultural soil.

Introduction:

A. fumigatus is a filamentous fungus found worldwide. It is considered an airborne saprophytic fungus. Because of this, it naturally lives in the soil and it is a common mould found among compost and plant surfaces. It plays a key role in recycling carbon and nitrogen from deceased organisms. Its conidia are blown by wind and float through the air. It is estimated that there are approximately ten conidia found within every cubic meter of air (Gow Neil et al., 2005). A. fumigatus the most frequently isolated species, causes a broad spectrum of diseases in the human beings. A. fumigatus primarily affects the lungs, and causes allergic bronchopulmonary aspergillosis (ABPA), Aspergilloma, and invasive aspergillosis. A. fumigatus is now the second most common fungal infection found in hospitalized patients, after Candida albicans [Ellis et al., 2000]. A. fumigatus a leading fungal pathogen, and one of the most opportunistic fungi found in a range of other patient groups, including cystic fibrosis patients, HIV-positive patients and other immuno-compromised individuals [Cimon et al., 2001; Bakare et al., 2003; Singh et al., 2005]. The spores of this organism are found virtually everywhere. Hence anyone can be at risk, and just breathing can cause onset of the disease. Colonization is associated with a syndrome usually seen in people with asthma and cystic fibrosis (CF), called allergic bronchopulmonary aspergillosis (ABPA) (J. Agbetile et al., 2012). ABPA, one of the many forms of aspergillus disease, results from a hyper reactive immune response to A. fumigatus without tissue invasion (Karen et al., 2010). The immune response characteristic of this disease include elevated total serum IgE and antigen specific IgG levels, and antigen induced lymphocyte transformation (Kurup et al., 1991).

Materials and Methods:

Culturing of A. fumigatus:

A. fumigatus shows active growth on Sabouraud's agar medium (SAM) which is selective medium for human pathogenic fungi. One gram of soil sample from sericulture grinage industry, hospitals and agricultural soil were taken in three separate conical flasks containing 50 ml sterile distilled water and used as inoculum. This inoculum (0.1ml each) was transferred on to Sabouraud's agar medium and incubated at room temperature for one week. After incubation, the fungal cultures were identified. Conidial morphology was determined by ocular and stage micrometer.

Extraction of protein from A. fumigatus:

A. fumigatus from contaminated groundnut kernels was grown on cellophane laid on Sabouraud's agar medium (SA). Cellophane associated fungal mats were collected at different time intervals. Fungal mats were frozen for 24 hr and then thawed before mixing with lysis buffers (PBS pH 7.3 and 1 mM EDTA). Samples were extracted by using sterile acid washed sand with mortar and pestle. Complete breakage of cells was monitored by microscopic observation. Homogenized fungal extracts were centrifuged at 20000 rpm for 30 min. The clarified extracts were stored at -70°C (Segurado et al., 1999).

Estimation of protein from cell free extracts:

The total protein content was determined with Folin Ciocalteau's reagent according to the Lowry et al. (Lowry et al., 1951). For the estimation of protein, solution was treated with 0.5 ml of the alkaline solution. Further, 5 ml of alkaline copper sulphate solution was added and allowed to stand for 10 minutes at room temperature. Then 0.5 ml of Follin reagent was added, thoroughly mixed by vertexing with a cyclomixer and allowed to stand for 30 minutes for colour development. Absorbance was measured at 750 nm in a Spectronic 20-D Spectrophotometer. Bovine serum albumin was used as standard. Standard curve was prepared by plotting absorbance values on y-axis and concentration of standard protein on x-axis. Extracts containing less than 1 mg of protein per ml were concentrated by precipitation with 7 volumes of cold acetone at 70°C for 24 hr. Precipitated protein was pelleted by spinning for 20 min at 1200x g at 4°C in a vacuum evaporator. Clarified extracts were centrifuged at 3000x g 15 minutes.

SDS-Polyacrylamide gel electrophoresis (SDS PAGE) :

Separation of fungal allergic immune-dominant antigens was demonstrated by PAGE technique performed by method of Laemmli et al., 1979. After estimation of protein concentration from fungal allergens, PAGE was carried out using 12.5% separating gel and 5% stacking gel. The gels were stained with silver nitrate. The separating gel consisted of 10% (W/V) acrylamide, N,N-methylene bis acrylamide (Sigma, USA) at a concentration such that the ratio of monomer to bis was 30:0.8, 0.375 M, Tris-HCL (pH 8.8) and 0.1% SDS. It was chemically polymerized with 0.05% (W/V) ammonium persulphate (Sigma, USA) and 0.05% (W/V) TEMED (Merck, FRG). The solution was cast into slabs and overlayed with n- butanol to exclude contact with air. The stacking gel containing 4% (W/V) acrylamide 0.12 M, Tris-HCl (pH 6.8), 0.1% SDS, 0.05% (W/V) ammonium persulphate

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0.05%(V/V)TEMED. Samples, 50-200 µgs were digested with an equal volume of sample buffer (0.0625 M Tris-HCl, pH 6.8) 10% (V/V) glycerol, 5% ß-Mercaptoethanol, 2% SDS and 0.02% bromophenol blue by heating in a boiling water bath for 3 minutes. After cooling, the samples along with protein markers (Sigma, USA) were loaded into the slots. The samples were stacked and run at 120 V for about 6 hr using 0.025 M Tris, 0.192M glycine buffer (pH 8.3) containing 0.1% SDS as electrode buffer. After electrophoresis, gels were fixed in PBS buffer for 1hr, Then the gels were treated with 50% ethanol for 10 minutes each time and with Na2S2O3 for one minute. Gels were washed with distilled water and soaked in silver nitrate (AgNO3) solution for ½ hr. Further gels were washed with distilled water.

Results:

Growth of A. fumigatus on Sabouraud's agar medum:

Filamentous growth is an important morphological characteristic of A. fumigatus. It grows rapidly on the selective Sabouraud's agar medium and forms white cotton colonies, which later turn to green. After formation of conidia the colony of A. fumigatus become dark green and powdery. It produces a typical and smooth walled conidiophores whose tip widens gradually into a dome shaped vesicle of 20-30µm in diameter. Phialides are found on the upper half of 2/3 parts of the vesicle with 5-10 × 2-3µm diameter which produces dark green and spherical conidia in chains.

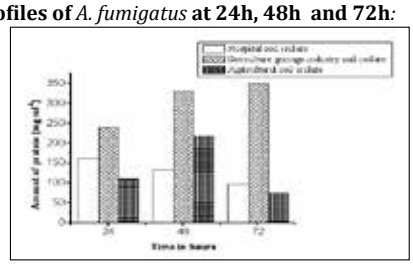
Growth and pigment characterization:

A. fumigatus isolated from various sources was repeatedly grown on Sabouraud's agar medium. Among the three sources, fungus isolated from sericulture grinage industry grew faster than hospitals and agricultural soil. However, pigmentation is very thick in the fungus isolated from hospitals. Pigment production is the predominant character during immunopathogenesis and phagocytic activity of human phagocytic cells.

Protein profiles of A.fumigatus:

Proteinaceous allergens were extracted from mycelia of *A. fumigatus* grown on SA medium. High amount of protein was obtained with *A. fumigatus* isolated from sericulture grinage industry up to 72 hr (fig.1). Low concentration of protein was yielded from agricultural soil isolate. Interestingly, the protein concentration was initially increased (24 hrs) in the isolate from hospital, which gradually decreased by 72 hr. The total protein content from sericulture grinage industry was high (357µg/ml) when compared with the lowest amount in agricultural soil isolate (85µg/ml). Amount of protein present in cell free extracts (CFE) of *A. fumigatus* isolated from three different sources was estimated. Protein concentration increased in the order of SGI>H>AS during 24hrs of growth, SGI>AS>H at 48 hrs and SGI>H>AS at 72 hr incubation (SGI-Sericulture grinage industry, H-Hospital, AS-Agricultural soil). Protein concentration increased from 24-48 hr in hospital and agricultural soil fungal isolates whereas in Sericulture grinage industry isolate protein concentration continuously increased from 24-72 hr. However, the isolate from sericulture grinage industry showed maximum amount of protein concentration. Production and expression of proteins from isolates of *A. fumigatus* having strong allergenic activity were reported (12). The concentration of the proteins reflects the pigmentation, pathogenicity and phagocytic functions. *A. fumigatus* from hospitals exhibited light pigmentation and poor protein concentration. The protein concentration of Sericulture grinage industry is very high with thick pigmentation. These results strongly suggest that pigment production is always associated with allergenicity .

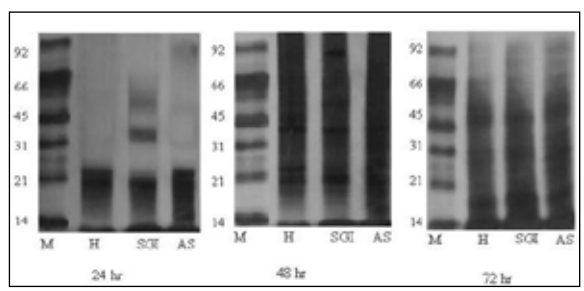
Fig.1: Protein profiles of *A. fumigatus* at 24h, 48h and 72h:



Characterization of allergens by using SDS-PAGE:

A. fumigatus was cultured on SA medium at 37°C has been observed to yield more consistent protein allergens. Present results clearly suggest that the most reliable mycelial associated allergens can be obtained from 2-4 day stationary cultures of the fungus. The SDS- PAGE gel electrophoresis clearly demonstrated the variability in protein profiles of different A. fumigatus strains. We have identified several fractions of A. fumigatus associated allergens comparing with standard molecular markers (fig.2). Some of these fractions were evaluated and found to be specific to Aspergillus. These findings suggest that A .fumigatus mycelial extracts constitute a very heterogenous group of antigens. PAGE analysis clearly revealed that A.fumigatus isolated from sericulture grinage industry constitutes a heterogenous group of protein allergens by expressing 48 KDa and 37 KDa protein bands. These bands are completely absent from hospital, and agricultural soil. Surprisingly protein expression is similar during 24 hr. This homogenous expression of proteins disturbed during 48 and 72 hrs of growth. Appearance of low molecular weight proteins in sericulture grinage industry, hospital fungal isolates is due to their high pathogenicity which is according to reports of (Arruda et al,.1992).

Fig.2: Characterization of allergens by using SDS-PAGE:



Discussion:

In this study we isolated A. fumigatus from different sources such as sericulture grinage industry, hospital areas and agricultural soil were repeatedly grown on SA medium. Fungus isolated from sericulture grinage industry grew faster than hospital and agricultural soil. Conidial morphology of these fungal isolates (Sericulture grinage industry, hospital areas and agricultural soil) were evaluated and found to vary. Conidia from hospital isolate were smooth walled whereas from sericulture grinage industry and agricultural soil isolates have spiny conidia. Proteinaceous allergens were extracted from mycelia of A. fumigatus grown on SA medium. High amount of protein was yielded in A. fumigatus isolated from sericulture grinage industry up to 72 hr, and low concentration of protein was yielded from agricultural soil isolate. SDS-PAGE analysis clearly reveals that heterogeneous group of protein allergens from A. fumiagatus isolated from sericulture grinage industry constitute, which are completely absent from isolates of hospital areas and agricultural soil. Heterogeneous expression of proteins was similar in all isolates during 24hr but were disturbed during 48 and 72hr. Different antigenic patterns are produced when the organism is cultured in Czapek-Dox medium and in a protein hydrolysate medium such as Sabouraud's medium. Moreover, the presence of high concentrations of hexose in both media induces an acidic pH during growth which greatly influences the pattern of antigens produced (Little et al., 1993; Moutaouakil et al., 1993; Latge et al., 1994; Latge eta al., 1995). The chemical nature of the antigens of the fungus is protein, polysaccharide and glycoprotein (Kurup et al., 1977; Hearn et al., 1992). SDS-PAGE electrophoresis pattern clearly demonstrates the variability in proteins of different A. fumigatus strains. Out of 22 antigens recognized from Aspergillus species, 13 antigens showed various enzyme activities and reacted with sera of ABPA patients (Tran-van et al., 1969). A. fumigatus antigens induce a strong eosinophillic inflammation in the lungs that persists over several days. Pathologic lesions affect the surface of the airways the interstitial tissue of the lungs and the alveoli. Antigen antibody complexes of A. fumigatus enhance the T-cell response in lungs. A. fumigatus antigens develop not only lung eosinophilia but also blood

and bone-morrow eosinophilia (Murail et al., 1992).

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