BIOCHEMICAL CHARACTERIZATION OF AN IMMUNODOMINANT ALLERGEN/ANTIGEN OF *ASPERGILLUS FUMIGATUS*.

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ABSTRACT

An 18kDa protein was identified as a major immunodominant allergen/antigen secreted by a wild type isolate and various clinical isolates of *A.fumigatus*. The protein was purified to homogeneity and the N-terminal amino acid was found to be alanine. The N-terminal 20 amino acid sequence of 18kDa was found to be similar to restrictocin, a cytotoxin secreted by *Aspergillus restrictus*. Mass spectroscopic analysis of the purified allergen revealed a molecular size of 17.01 kDa. Immunoreactivity of the purified allergen with monoclonal antibodies and specific IgG and IgE antibodies of the patients of aspergillosis confirmed that this protein is Asp fl.

KEY WORDS : *Aspergillus fumigatus*, allergen, antigen, 18kDa antigen, RIA

INTRODUCTION

Fungi of the genus *Aspergillus* have been implicated in a diverse spectrum of human diseases ranging from allergic asthma, allergic bronchopulmonary aspergillosis (ABPA), aspergilloma to invasive aspergillosis. *A.fumigatus* is the major causative agent in *Aspergillus* induced diseases. The predominance of *A.fumigatus* over other *Aspergilli* in human diseases can be attributed to its ability to grow at human body temperature, its small spore size and complex antigenic structure.

The wide spectrum of disease states associated with the disease aspergillosis and the pathogen *A.fumigatus* to some extent is attributed to biologically active virulent factors of

the fungus. In view of these complexities proper definition of antigen(s) relevant to diagnosis and pathogenesis could not be explored fully. Currently the immunoassays to detect circulating antibodies to *A.fumigatus* are based on crude extracts or partially purified fractions of *A.fumigatus*. The array of *A.fumigatus* antigens identified for diagnosis depends on various factors like culture conditions, (culture filtrate versus mycelial intracellular extract), the strain used, the methods of extraction etc. Moreover the cross reactivities among different species of *Aspergillus* as well as between *Aspergilli* and other fungi and bacteria also affect the sensitivity of various serodiagnostic methods (1-4).

Attempts made by various researchers to isolate and purify diagnostically relevant antigens of *A.fumigatus* have clearly shown that antigenic preparations from a single strain of *A.fumigatus*

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were not sufficient to detect specific circulating antibodies in the sera of all patients (5). Kurup et. al. (3) while studying 11 strains of *A.fumigatus* and subsequently testing their culture filtrate antigens against sera from different patients of aspergillosis demonstrated that individual antigens reacted differently with the sera and detection of specific antibodies varied from 40-76%. Similar observations were noticed by other workers. In the light of all these variations in antigenic preparations, identification and characterisation of immunodominant antigens from different isolates would result in predictable reactivity that may enable comparison of multicentered results. These approaches may lead to designing of international standards of diagnostic antigens of *Aspergillus*.

In the current study, four strains of *A.fumigatus* isolated from ABPA and aspergilloma patients along with a wild type isolate were screened to study the antigenic profile in the culture filtrate of these isolates. After screening with sera of patients of aspergillosis, biochemical characterisation of an immunodominant antigen with a MW 18 kDa was carried out and the results are reported in the current paper.

MATERIALS AND METHODS

Isolation of strains : Clinical specimens originating from lower respiratory tract of patients referred to Medical Mycology laboratory at Vallabh Bhai Patel Chest Institute were cultured on Sabouraud dextrose agar (4) and incubated at 37°C for 1-2 weeks. The sputum and bronchial aspirate specimens were also examined by direct microscopy and serum samples were collected for detection of serum precipitins against etiologic *Aspergillus* species. Subsequently

strains isolated from ABPA and aspergilloma patients confirmed by clinical and laboratory findings were selected for the present study. Metabolic antigens from these strains along with a soil isolate 2605 obtained from Indian Type Culture Collection, Indian Agricultural Research Institute, Delhi, were prepared as standardized by Sandhu et. al. (4) and modified by Banerjee et. al. (6).

Sodium dodecyl sulphate poly-acrylamide gel electrophoresis (SDS-PAGE) : The antigens were electrophoresed on 10% polyacrylamide gels according to the method of Laemmli (7).

Immunological characterisation: The immunoblot method was carried out as described by Towbin et. al. (8).

Purification: The third week culture filtrate protein was loaded onto a 0.9x30 cm phenyl sepharose column equilibrated with 25% saturated $(\text{NH}_4)_2\text{SO}_4$ solution in 0.01M sodium phosphate buffer, pH-6.8. The adsorbed proteins were then eluted out with a gradient of decreasing $(\text{NH}_4)_2\text{SO}_4$ concentration (20%, 15%, 10% and 5%) and simultaneously increasing ethylene glycol concentration (10%, 20%, 30%, and 40%). Finally the column was washed with 50% ethylene glycol. Fraction III contained the maximum concentration of 18 kDa protein as revealed by SDS-PAGE and competitive radio immunoassay. This fraction was loaded onto a preparative SDS-PAGE and 18 kDa protein was electroeluted (9). The eluted protein was then loaded onto a reverse phase preparative column (C 18) (Waters High Performance Liquid Chromatography (HPLC) system) equilibrated with acetonitrile containing 0.1% trifluoroacetic acid (TFA) and eluted with water-acetonitrile (containing 0.1% TFA) at a flow rate of 1 ml/min. Peak 3

collected from preparative HPLC was subjected to analytical HPLC system using the reverse phase column.

Densitometric scanning : The Coomassie brilliant blue stained gels containing 18 kDa protein in different day culture filtrates as well as the purified protein were scanned at 650 nm using CS 9000 Dual Wavelength Flying Spot Scanner (Shimadzu).

Monoclonal antibodies against Asp fl, an 18 kDa allergen designated as Asp fl : Monoclonal antibodies (Mab4A6B5) raised against Asp fl was a gift from Dr. Karla Arruda, Division of Allergy and Clinical Immunology, University of Virginia, USA (10).

Radioimmunoassay (RIA) : In a direct binding assay, the radiolabelled antigen (200 µl; 4500-5000 counts per 10s) and the serial dilution of antibody (hyperimmune rabbit serum, patient sera, monoclonal antibody starting from 1:100- 1:50,000; 200 µl) were mixed and the volume was made to 500 µl with phosphate buffered saline (0.01M, pH 7.4, 0.9% NaCl). Tubes were incubated overnight at room temperature. The antigen-antibody complex was precipitated by the addition of 200µl of anti-rabbit γ-globulin. The tubes were allowed to stand for 24 hr at 4°C and then centrifuged at 4000 rpm for 15 min at 4°C. The radioactivity in the pellet was estimated in a γ-counter. The results of the direct binding assay were expressed as B/ Tx100 where B is the number of counts bound to the antibody and T is the total counts that were added.

Competitive RIA : Varying concentrations of competitors (100µl of unlabelled 18 kDa allergen, different fractions obtained during purification of the

allergen, culture filtrates at different stages of growth) were added to the reaction tubes prior to the addition of iodinated 18 kDa allergen/antigen. (The dilution of the competitors was also made in RIA buffer). The competitive assay results were expressed as B/B₀ x100 where B is the number of counts bound to the antibody in the presence of cold competitor and B₀ is the total counts in the absence of competitor.

N-terminal amino acid determination of Asp fl : This was carried out by 4 N'-N-Dimethyl-aminoazobenzeneisothiocyanate (11) and Dansyl chloride methods. 100 µg of purified 18 kDa protein was dissolved in 50µl water and 100 µl of DABITC solution (10nmol/ml of DABITC in pyridine, freshly prepared) was added, heated at 70°C for 75 min. and then cooled. 600 µl of extraction solvent (heptane-ethylacetate 2:1 v/v) was added for extracting excess reagents and by products. This was then vortexed and discarded. This step was repeated thrice. The aqueous phase was vacuum dried, followed by heating at 55°C for 60 min. after addition of 150 µl of 50% TFA and again vacuum dried. 50 µl of water was added followed by addition of 200 µl butylacetate. Organic layer was separated followed by extraction with 200 µl of butylacetate. The pooled butylacetate was vacuum dried. The modified N-terminal amino acid was dissolved in 10 µl of 80% ethanol and further confirmed by thin layer chromatography (TLC) analysis. In dansylation method also, 100 µg of lyophilized protein was taken to which 10µl of sodium bicarbonate and 10µl dansyl chloride was added and incubated at 50°C for one hour. This was vacuum dried followed by addition of 50 µl 6N HCl and kept overnight at 110°C for hydrolysis. The sample was vacuum dried and dissolved in 5µl of ethanol and subjected for confirmation by TLC analysis (9).

Sequencing of the purified 18 kDa protein

The sequencing of the purified protein was performed using a gas phase sequencer (Applied Biosystems, Inc.) in NIH, USA.

Mass spectral analysis for Asp fl : This was carried out in Kartos Kompact Maldi 3V4 mass spectrophotometer in NIH, USA.

RESULTS

Antigenic profile of *A.fumigatus* isolates

SDS-PAGE profile of the culture filtrate proteins from different isolates showed proteins with MW ranging from 12 to 80 kDa. Fig. 1. shows SDS-PAGE pattern of 3 WCF proteins of *A.fumigatus*, both soil and clinical isolates. Immunoblot analysis with pooled sera of ABPA patients showed both IgG and IgE reactivity predominantly with 70kDa, 55kDa, 45kDa, 34kDa and 18kDa. Although the staining pattern of these proteins differed slightly, the 18kDa was found to be one of the major and common antigen in all the isolates.

Expression of 18kDa protein in different days culture filtrates (day 1, 3, 5, 7, 14 and 21) was analyzed by comparing its level with mycelial growth, changes in pH and concentration of total protein in the culture filtrate extract. Comparison with area of known quantity of BSA on gel scan with the areas corresponding to the 18kDa protein in culture filtrates on different days, showed that day 1, 2, 3, 7, 14 and 21 culture filtrates comprised 14.1%, 20.6%, 27%, 12%, 16% and 17.8% of the total protein loaded respectively. Increase in concentration of

Fig. 1. SDS-PAGE pattern of 3 WCF proteins of *A. fumigatus* (both soil and clinical isolates)

Lane 1: Clinical isolate 1 (isolates from sputum of ABPA patient), Lane 2: Clinical isolate 2 (isolates from sputum of ABPA patient), Lane 3: Clinical isolate 3 (isolates from sputum of ABPA patient, strain no. 285), Lane 4: Clinical isolate 3 (isolates from sputum of aspergilloma patient), Lane 5: Soil strain, Lane 6: Molecular weight markers.

the 18 kDa protein correlated with mycelial growth and weight of the fungus and a fall in the pH of the culture medium (4.9-5.2). After day 3-4, the level of 18 kDa protein was observed to drop and then again found to increase after day 7 coinciding with the fall in the mycelial yield and rise in the pH, that is, during the autolytic phase. The presence of 18 kDa protein in culture media at a high concentration both during early phase of growth and lytic phase indicates its secretory as well as somatic nature. This 18-kDa protein which appeared to be a predominant antigen/allergen was chosen for purification and further characterisation.

Purification, biochemical and immunological characterisation

The culture filtrate proteins after 100% ammonium sulfate saturation were loaded onto phenyl-Sepharose CL-4B column. On elution, four protein peaks designated as fractions I, II, III and IV were obtained. Fig.2 shows SDS-PAGE (10%) profile of the fractions obtained during phenyl-Sepharose CL-4B chromatography. Of the total protein loaded 92% bound to the phenyl-Sepharose column. By competitive RIA, fraction III showed the highest inhibition when compared with third week culture filtrate preparation in Mab based RIA. Fig. 3 shows results of a competitive RIA using ^{125}I -18 kDa antigen and monoclonal antibody specific to the 18 kDa antigen in contrast to the other three fractions. Further the phenyl

Fig. 2. SDS-PAGE (10%) analysis of fractions obtained during phenyl-Sepharose CL4B chromatography

Lane 1 & 5 : Molecular weight marker., Lane 2 : Flowthrough with 25% $(\text{NH}_4)_2\text{SO}_4$, Lane 3 : Fraction I obtained with 20% $(\text{NH}_4)_2\text{SO}_4$ and 10% ethylene glycol., Lane 4 : Fraction II obtained with 15% $(\text{NH}_4)_2\text{SO}_4$ and 20% ethylene glycol., Lane 6 : Fraction IIIa obtained with 10% $(\text{NH}_4)_2\text{SO}_4$ and 30% ethylene glycol., Lane 7 : Fraction IIIb obtained with 10% $(\text{NH}_4)_2\text{SO}_4$ and 30% ethylene glycol.

sepharose fractions when analyzed by SDS-PAGE, fraction III obtained with 10 % of ammonium sulphate and 30% ethylene glycol was found to be rich in 18 kDa protein, therefore it was subjected to preparative SDS-PAGE and the protein corresponding to the MW 18 kDa was eluted by electroelution. The eluted protein was loaded onto a preparative HPLC column. The HPLC profile showed three peaks. Peak 3 with a retention time of 28 min. was found to be the major

Fig 3. Results of a competitive RIA using ^{125}I -18kDa antigen and monoclonal antibody specific to the 18kDa antigen. Fraction 1,2,3,4 and 3WCF preparation were used as competitors.

peak containing the 18 kDa protein. The peak 3 also eluted as a symmetrical single peak on analytical HPLC with the retention time of 28 min. Fig. 4 shows the rerun of the protein recovered from peak 3 obtained on preparative HPLC. SDS-PAGE of the protein of this peak indicated a molecular weight of 18 kDa. Fig. 5 shows the SDS-PAGE (10%) pattern of the purified 18 kDa protein. Densitometric scan of the gel also revealed a single sharp peak covering 100 % area at the position of the 18 kDa protein marker.

Immunoreactivity of the purified protein

The 18 kDa allergen/antigen showed reactivity with both IgG and IgE antibodies in the

Fig. 4. Rerun of the protein recovered from peak 3 obtained on preparative HPLC.

Western blot analysis using serum samples of ABPA and aspergilloma patients. Fig. 6 & 7 shows the immunoblots of the 18 kDa protein for specific

Fig. 5. SDS- PAGE (10%) of the purified 18kDa protein

Lane 1 & 2: Different quantities of 18 kDa protein loaded, Lane 3: Molecular weight markers

IgG with sera of aspergilloma and ABPA patients, rabbit antiserum and monoclonal antibody and specific IgE with sera of ABPA patients. Immunological activity of the purified protein was further confirmed by using polyclonal rabbit sera raised against *A. fumigatus* and a Mab (clone 4A6) raised by Arruda et. al. (10) in Western blot analysis.

N-terminal amino acid determination of Asp fl:

The N-terminal residue of the protein by DABITC and Dansyl chloride methods was observed to be alanine.

Mass spectral data also showed that the N-terminal sequence of this protein is:

ATWTCINQQLNPKTNKWEDK

Fig. 6. Immunoblots of the 18kDa protein for specific IgG with sera of aspergilloma and ABPA patients, rabbit antiserum and monoclonal antibody.

Lane 1: Serum of ABPA patient., Lane 2: Serum of aspergilloma patient, Lane 3: Rabbit antiserum and Lane 4: Monoclonal antibody.

Fig. 7. Immunoblots of the 18 kDa protein for specific IgE with sera of ABPA patients.

Lane 1 & 2 : Sera of ABPA patients

and a molecular weight of 17.01kDa. This sequence is similar to the sequence of the 18 kDa allergen/antigen reported by Arruda et. al. (12, 13).

DISCUSSION

A.fumigatus secretes an array of antigens allergens, which are multifunctional in nature. These include proteins with enzymatic activities such as chymotrypsin, proteinase, elastase, ribonuclease, catalase and superoxide dismutase. The role of these antigens in the immunopathogenesis of *Aspergillus* related diseases is not clear. Establishment of the biological function of *Aspergillus* proteins with allergenicity and antigenicity facilitates a better understanding of the mechanism of immunopathogenesis of this clinically complex fungal disease.

Arruda et. al. (10) reported an 18 kDa immunodominant allergen/antigen which binds to

IgE and IgG from allergic and IgG from aspergilloma patients. Moreover it has also been reported to be one of the major antigens secreted *in vivo*, in urine of patients of invasive aspergillosis and in regions of necrosis surrounding fungal colonies in the kidneys of mice infected with *A.fumigatus* (14-16).

Present work on isolation of a major antigen/allergen in the isolates of clinical origin of *A.fumigatus* led to the characterisation of a protein of molecular weight 18 kDa which reacted with the IgG and IgE antibodies in patients of ABPA and aspergilloma. This 18 kDa protein was not only immunodominant but also abundant in the third week culture extract comprising approximately 17.8% of the total protein. As this protein was found to be present both during the early phase of the growth and lytic phase, third week culture filtrate was taken for purification as it contains both the somatic as well as the metabolic antigens. The purified antigen retained its immunological and biological activity and was homogeneous on SDS-PAGE. The N-terminal amino acid was found to be alanine. The N-terminal 20 amino acid sequence was observed to be similar to restrictocin. However, the mass spectral analysis showed a MW of 17.01 kDa. The differences in MW reports of this antigen may be due to glycosylation as majority of antigens of *A.fumigatus* are highly glycosylated.

Ribonuclease and cytotoxic activities (17, 18) of this protein may have a role in the pathogenesis of aspergillosis. Asp fl was found to be a major allergenic cytotoxin with an IC_{50} of 4.16 nm (18) which is significantly higher than other fungal toxins (200 times restrictocin and 2 times alpha sarcin) (19, 20).

The sequencing data of the purified 18 kDa protein exhibited 100% homology with that of the deduced sequence of Aspfl (12, 13). This sequence homology of this protein with that of alpha-sarcin, produced by *A.giganteus*, mitogillin and restrictocin produced by *A.restrictus* (21-23) is as reported.

The deduced amino acid sequence of Asp fl from ABPA patient showed the presence of four cysteines with two disulphide bridges and histidine residues (13, 24) reported to be important for ribonuclease activity of fungal ribotoxins (20). Identification and elimination/modification of immunologically active domains of the protein might result in synthesis of functional peptides devoid of allergenicity for applications.

Further, identification of antigenic determinants would facilitate a better

understanding on its possible role in pathogenesis. Epitope mapping of Asp fl would offer advancement in the development of diagnostic technologies for aspergillosis based on synthetic epitopes. Biochemical and immuno-logical characterisation of 18 kDa allergen/ribonuclease from an Indian clinical isolate in the current study is anticipated to lead to development of reference standards.

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