

A New Glycoprotein Allergen/Antigen with the Protease Activity from *Aspergillus fumigatus*

Shailly Nigam^a P.C. Ghosh^b P. Usha Sarma^a

^aMolecular Biochemistry and Diagnostics Division, Centre for Biochemical Technology, Delhi, and

^bDepartment of Biochemistry, University of Delhi South Campus, New Delhi, India

Key Words

Aspergillus fumigatus · Protein · Purification techniques · Immunoreactivity · Sequencing · Protease

Abstract

Background: *Aspergillus fumigatus* is an opportunistic fungus causing allergic and invasive aspergillosis in humans and animals. It secretes an array of complex biologically active glycoprotein antigens and allergens. It is important to identify and characterize probable potential virulent factors playing a major role in the pathogenesis of aspergillosis. **Methods:** Using protein purification techniques (lectin affinity chromatography, gel filtration, electroelution and high-pressure liquid chromatography), a major antigen/allergen with a molecular weight of 56 kD (gp56) from *A. fumigatus* was purified to homogeneity. The protein was characterized by immunoblot, ELISA and protease assays. The N-terminal amino acid sequencing was performed. **Results:** The gp56 protein showed a single band on silver staining and isoelectric focussing. The protein to carbohydrate ratio was 1.5:1 and gp56 gave a protein band at a molecular weight of 34 kD on enzymatic deglycosylation. It also exhibited IgG and IgE immunobinding with antibodies present in sera

of allergic bronchopulmonary aspergillosis patients. The gp56 exhibited protease activity and N-terminal seven-amino acid sequence showed homology with fungal serine proteases. **Conclusions:** The gp56 protein by virtue of its proteolytic activity could be one of the virulent factors of *A. fumigatus* involved in establishing infection in the host along with other factors.

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Introduction

Aspergillus fumigatus is a ubiquitous mould known to cause a number of human diseases, in particular allergic bronchopulmonary aspergillosis (ABPA), aspergilloma and invasive aspergillosis. These varied forms of the disease associated with single species of the fungus are attributed to secretion of biologically and immunologically active complex macromolecular proteins during colonization and establishment of infection. Understanding the immunodominant antigens/allergens and functionally important proteins of this medically important opportunistic fungus is necessary to unravel the mechanism of the pathogenesis in ABPA, invasive and other forms of aspergillosis.

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Correspondence to: Dr. P. Usha Sarma, Scientist G, Sr. Deputy Director
Molecular Biochemistry and Diagnostics Division
512, Center for Biochemical Technology
Mall Road, Delhi 110007 (India)
Tel. +91 11 7666158, Fax +91 11 7667471, E-Mail u_sarma@hotmail.com

Few antigens/allergens were identified with varied biological functions and have been implicated in the immunopathogenesis of *Aspergillus*-related diseases. Cytotoxic metabolites of *A. fumigatus* (gliotoxin, fumagillin and restrictocin) are thought to facilitate fungal growth by inhibiting macrophage functions and causing generalized immunosuppression [1]. *A. fumigatus* conidia secrete low-molecular-weight diffusates and inhibitory factors which inhibit chemotaxis and phagocytosis [2, 3]. Recent evidence supports the view that proteases may have a facilitating role for fungal adhesion and tissue penetration during the initiation of an infection [4]. However, *A. fumigatus* mutants lacking either alkaline protease or metalloprotease, or both were able to invade lung tissues causing mortality in murine models [5, 6]. This suggests the presence of other yet to be characterized virulent determinants. It is conceivable that proteolytic enzyme activities are one of a number of factors, each with a minor effect, that combine to facilitate disease progression.

Multifunctional glycoproteins with immunological and enzymatic activities (toxins, proteases, elastases, cat-alases, collagenases, ribonucleases and superoxide dismutases) are a few proposed virulent determinants. Molecular studies on glycoprotein antigens/allergens and their immunological and biological characterization would facilitate the understanding of the mechanism of immunopathogenesis and occurrence of different clinical forms. In addition, well-characterized *Aspergillus* antigens would aid in the development of sensitive and accurate diagnostic assays for aspergillosis. Such assays can easily be used in diagnostic laboratories.

Few glycoproteins isolated so far from *A. fumigatus* show specific IgG and IgE binding activities with the antibodies present in the sera of patients with aspergillosis and these allergens/antigens also exhibit lectin (concanavalin A, Con A)-binding property [7, 8]. It has been demonstrated that *A. fumigatus* antigens resolved into 18 bands and 12–14 of these were Con A-binding components, indicating the antigenic/allergenic nature of glycoproteins [9]. Few Con A-binding antigens/allergens of molecular weights of 18, 35, 45, 55, 66 and 70 kD have been identified, purified and characterized and have been shown to have biological activities [10–12]. The specific IgG and IgE immunoreactivity of these proteins was demonstrated with sera of patients with ABPA and aspergilloma. A 45-kD immunodominant glycoprotein antigen characterized in our laboratory showed protease and immunological activities [12]. More importantly, the Con A-bound fraction of *A. fumigatus* entrapped in liposomes could modulate an immune response in mice, leading to characteriza-

tion of functionally important allergens/antigens for understanding the host-pathogen interactions involved in the pathogenesis of *A. fumigatus* [13].

Most of the glycoprotein antigens of *A. fumigatus* remain poorly defined because of their complex nature. The present investigations were aimed at the identification of diagnostically relevant glycoprotein antigens/allergens of *A. fumigatus* in which 56 kD was characterized.

Materials and Methods

Antigen Preparation

The 3-week culture filtrate antigens (Af-3wcf) from the pathogenic strain *A. fumigatus*-285 were prepared [14]. Briefly, 150 ml glucose asparagine medium (*L*-asparagine 7 g, ammonium chloride 7 g, KH_2PO_4 1.31 g, sodium citrate 0.9 g, magnesium sulfate 1.5 g, ferric citrate 0.3 g, dextrose 10 g, glycerol 25 ml) was inoculated with the pure culture of *A. fumigatus* strain in a 500-ml conical flask. These were allowed to grow at 37°C as stationary culture. The culture filtrate proteins were precipitated by the addition of ammonium sulfate at 80% saturation. The precipitate obtained after centrifugation was dissolved in a minimum volume of distilled water followed by extensive dialysis against distilled water at 4°C. The protein sample was lyophilized and stored at –20°C. The *Aspergillus* allergens/antigens are usually prepared in liquid medium, which is the preferred medium as indicated by studies on various fungal allergens/antigens [15, 16].

Human Serum Samples

Serum samples from ABPA patients were obtained from VPCI, Delhi, India. ABPA is characterized by asthma, peripheral blood eosinophilia, immediate (type I) skin reactivity to *Aspergillus* antigens, precipitating serum antibodies against *Aspergillus* antigens, elevated total serum IgE concentrations, elevated serum IgG and IgE antibodies specific against *Aspergillus* species and a history of pulmonary infiltrates and bronchiectasis with normal tapering of the distal bronchi. Sera from healthy subjects without any indication of pulmonary disease were taken as controls.

Purification of 56-kD Antigen

Fifty milligrams of a protein-enriched fraction of *A. fumigatus* was applied to a column (1.5 × 20 cm) containing Con A Sepharose equilibrated with 10 mM Tris-HCl, pH 7.4 buffer containing 5 mM MgCl_2 and 5 mM CaCl_2 at 4°C. The unbound proteins were collected separately. The bound proteins were eluted using 0.2 M methyl- α -D-mannopyranoside in 10 mM Tris-HCl, pH 7.4 buffer containing 5 mM MgCl_2 and 5 mM CaCl_2 . The bound fraction was dialyzed against distilled water at 4°C. The Con A-bound protein fraction was lyophilized and stored at –20°C. One milligram of Con A-bound fraction was applied to prepacked Sephacryl S-100 HiPrep column 16/60 equilibrated with 0.05 M phosphate buffer, pH 7.2, containing 0.15 M NaCl. The sample was resolved using fast protein liquid chromatography. The protein fractions were collected at a flow rate of 0.5 ml/min and the absorbance measured at 280 nm. The fractions were concentrated, electrophoresed and electroblotted onto nitrocellulose membrane and probed for antigenicity with pooled sera from ABPA patients. The fractions enriched with the desired protein were

subjected to preparative SDS-PAGE (10%). The desired protein was electroeluted from the gel. The eluted protein was subjected to semi-preparative reverse-phase high-pressure liquid chromatography (HPLC) column (C18, 7.8 mm × 300 mm) on a Shimadzu HPLC apparatus. The elution of protein was performed using 10–70% gradient of acetonitrile in water containing 0.1% v/v trifluoroacetic acid at a flow rate of 1.0 ml/min and monitored at 280 nm. The major peak was collected. The purified protein was lyophilized and stored at –20 °C.

Characterization of Purified Protein (gp56)

The purified protein was electrophoresed on 10% polyacrylamide gel with 0.1% sodium dodecyl sulfate. Isoelectric focussing of the purified antigen (3 µg) was performed on a polyacrylamide gel with an ampholine gradient of pH 3–9 (Pharmacia, Sweden) using the Phast system followed by silver staining.

Carbohydrate Analysis

The amount of carbohydrate in purified protein was estimated by the standard phenol-sulfuric acid method [13]. Purified gp56 (20 µg) was incubated with 1 U of N-glycanase F and 1 U of O-glycanase in a volume of 100 µl of sodium phosphate buffer, 0.1 M, pH 7.2, containing 0.25 mM EDTA. The gp56 was also mixed with the same buffer without N-glycanase and O-glycanase. The enzyme-treated sample was analyzed by 10% SDS-PAGE. Buffer containing the enzyme was used as the control.

Immunological Characterization

Indirect enzyme-linked immunosorbent assay (ELISA) was carried out for determining specific IgG/IgE immunobinding of gp56 [11]. The IgG/IgE immunoblot analysis was also performed. The specific IgG/IgE immunoreactivity of gp56 was determined in ELISA with the sera of 12 individual ABPA patients (1:100 v/v dilution). The IgG and IgE immunobinding was analyzed by immunoblotting with sera samples of 8 ABPA patients (1:100 v/v dilution).

Sodium Metaperiodate and Pronase Treatments

The gp56 protein (20 µg) was electrotransferred onto nitrocellulose membrane and subjected to periodate oxidation using 0.2 M sodium metaperiodate and the reaction was terminated by the addition of ethylene glycol. This was followed by IgG/IgE immunoblot analysis using pooled sera from ABPA patients. The untreated protein was taken as the control. The gp56 (15 µg) was incubated with 5 µg of pronase in 0.01 M Tris-HCl buffer, pH 7.4 at 37 °C for 24 h followed by immunoblot analysis for determining the antigenicity and allergenicity.

Protease Assay

Protease activity of gp56 antigen (10 µg) was analyzed on agarose gels copolymerized with either gelatin or casein hydrolysate as a substrate.

N-Terminal Sequencing and Homology Search

gp56 was subjected to electrophoreses (10% SDS-PAGE) followed by electroblotting onto polyvinylidene difluoride membrane. The protein band was cut and N-terminal amino acid sequencing was done on Protein Sequencer Prosize 491 (Perkin Elmer). The homology of sequence with known protein sequences was established using basic local alignment search tool.

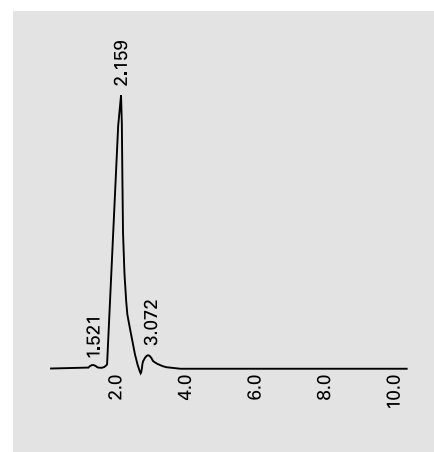


Fig. 1. Reverse-phase HPLC chromatogram of gp56. The protein was eluted at a flow rate of 1.0 ml/min with the buffer system 10–70% gradient of acetonitrile in water.

Results

The gp56 antigen of *A. fumigatus* was purified to homogeneity by a combination of affinity chromatography, gel filtration and electroelution. The purified protein was then subjected to reverse-phase HPLC which gave a single peak. Figure 1 shows the reverse-phase HPLC profile of the electroeluted protein. The purified protein was homogenous on isoelectric focussing and gave a single band on silver staining at pI 6.8 (fig. 2a). The protein to carbohydrate ratio was 1.5:1 as determined by phenol sulfuric acid estimation. The gp56 on enzymatic deglycosylation by N-glycanase and O-glycanase resulted in a protein of a molecular weight of 34 kD as determined on silver staining (fig. 2b). The antigen was treated with N-glycanase to cleave N-linked oligosaccharides between N-acetylglucosamine and asparagine residues or O-glycanase to hydrolyze O-glycosidic bonds between N-acetylgalactosamine and serine residues. The presence of protease activity was observed using gelatin and casein hydrolysate as substrates in the agarose gel assay. The substrates (0.1% w/v) were copolymerized in 1% agarose. The clear zones around the wells indicated the presence of proteolytic activity of purified gp56 (fig. 3). The purified antigen, gp56, was subjected to electrophoresis followed by electroblotting for N-terminal amino acid sequencing. Efforts at N-terminal sequencing led to the identification of seven amino acids (A N/Q V V Q/P S N) only due to heavy glycosylation of gp56. Homology search of this protein sequence with other sequences available in the databank

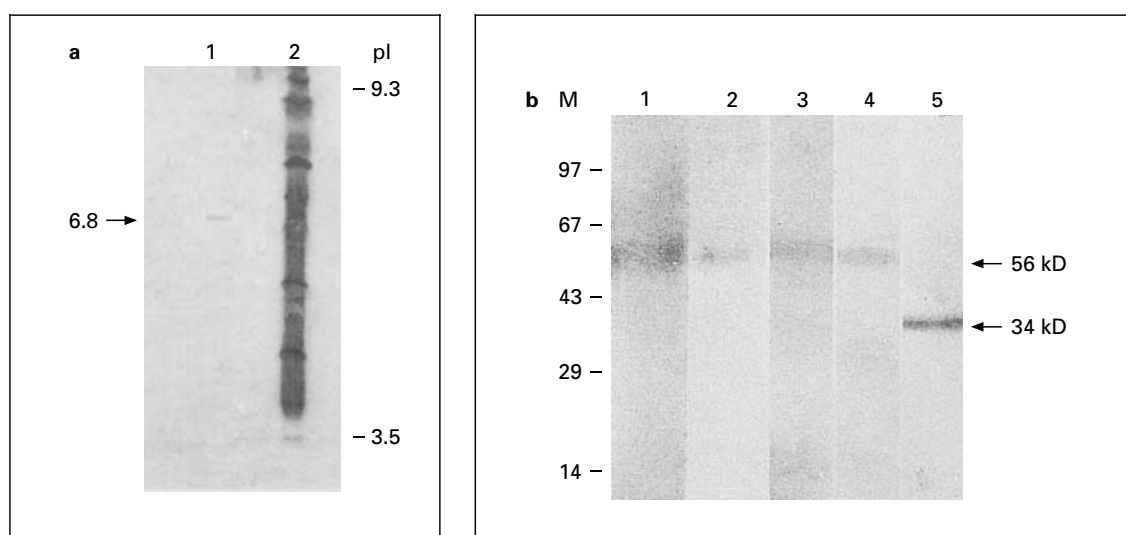


Fig. 2. **a** Isoelectric focussing of gp56. Lane 1: Purified gp56; lane 2: pI (3–9) marker. The arrow indicates the pI of gp56. **b** Protein profile and immunoreactivity of gp56. Lane 1: Silver-stained 10% SDS-PAGE gel of purified gp56; lanes 2 and 3: IgE and IgG reactivity with pooled sera of ABPA patients, respectively; lane 4: IgG binding with rabbit hyperimmune sera raised against Af-3wcf; lane 5: silver-stained deglycosylated gp56. M = Molecular weight markers in kilodaltons.

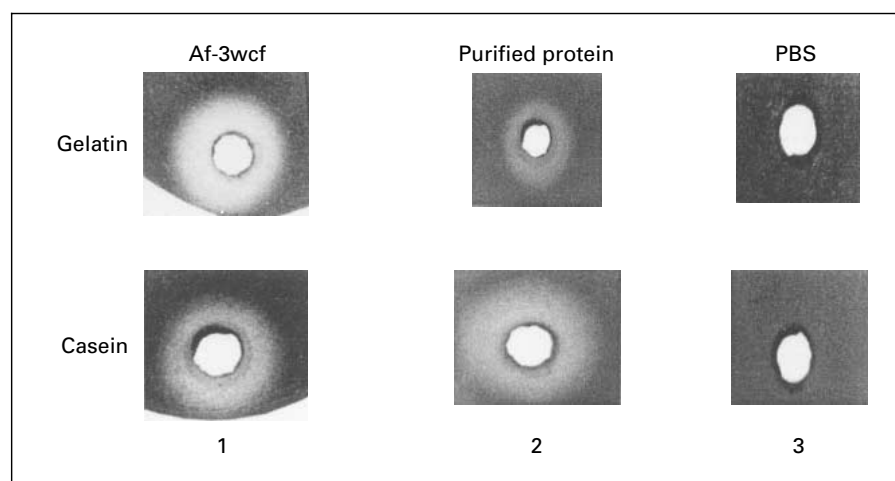


Fig. 3. Protease activity of gp56. Protease activity with gelatin and casein is shown. PBS was used as a negative control. The substrates (0.1% w/v) were copolymerized in 1% agarose w/v in PBS. The clear zones around the wells indicate the proteolytic activity.

revealed a sequence similarity with a family of serine proteases. It exhibited 71 and 29% identities with serine proteases from *Penicillium notatum* (Pen n 13) and *A. fumigatus* (Asp f 13) [17, 18]. Trypsin digestion of gp56 produced eight peptides. Immunoblot analysis of these peptides using sera of ABPA patients demonstrated three IgG- and IgE-binding peptides with molecular weights of 38, 17 and 10 kD (fig. 4).

The gp56 protein showed IgG immunobinding with rabbit hyperimmune sera raised against Af-3wcf. It also reacted with IgG and IgE antibodies present in pooled sera of ABPA patients on immunoblot (fig. 2b). The indirect ELISA established the specific IgG and IgE immunoreactivity of gp56 (table 1) with a serum pool of ABPA patients and with the sera of 12 individual patients (fig. 5). The gp56 antigen exhibited 65.9% IgG- and

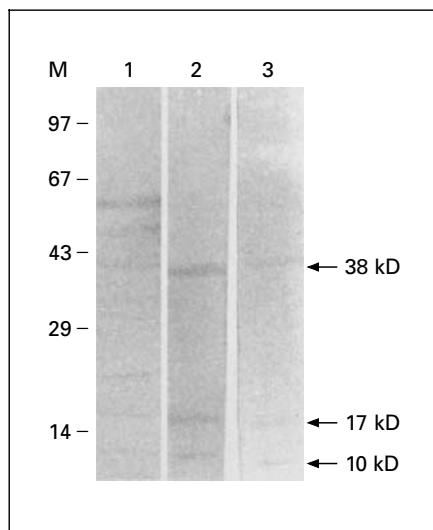


Fig. 4. Trypsin digestion of gp56. Lane 1: Coomassie blue-stained profile of trypsin-digested gp56; lanes 2 and 3: IgG and IgE immunoblot of trypsin-digested gp56 with pooled sera of ABPA patients, respectively. M = Molecular weight standards in kilodaltons. The arrows indicate three immunoreactive peptides with their molecular weights.

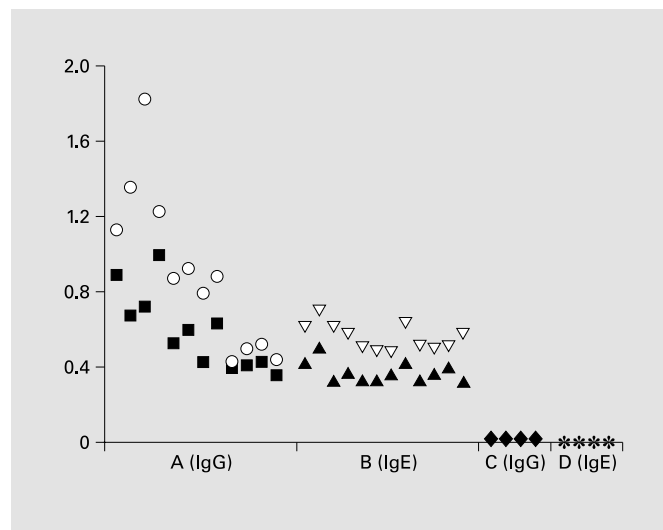


Fig. 5. Specific IgG and IgE binding of gp56 and Af-3wcf with sera of 12 ABPA patients in ELISA. Four control sera samples were also taken. Section A (IgG): ○ and ■ represent IgG binding with Af-3wcf and gp56, respectively; section B (IgE): ▽ and ▲ represent IgE binding with Af-3wcf and gp56, respectively; section C (IgG) and D (IgE) show IgG and IgE binding, respectively, for four control serum samples with Af-3wcf and gp56. All readings are mean values representing three readings for each sample.

Table 1. ELISA absorbance values for IgG and IgE immunobinding of gp56 and Af-3wcf

Serum used	With gp56		With Af-3wcf	
	IgG	IgE	IgG	IgE
Pooled sera from ABPA patients	0.624 ± 0.002	0.395 ± 0.004	0.947 ± 0.010	0.523 ± 0.004
Control sera (NHS)	0.034 ± 0.001	0.012 ± 0.008	0.043 ± 0.001	0.014 ± 0.009
Af-RHS	0.723 ± 0.007	–	1.212 ± 0.005	–
Rabbit preimmune sera	0.094 ± 0.009	–	0.078 ± 0.003	–

The gp56 and Af-3wcf were coated at a concentration of 1 µg/100 µl/well. The sera of the patients were used at a dilution of 1:100 v/v for IgG and 1:50 v/v for IgE. The rabbit serum raised against *A. fumigatus* (Af-RHS) was used at a dilution of 1:500 v/v. All the readings are mean values representing three readings for each sample. NHS = Normal human serum.

75.5% IgE-immunobinding activities on comparison with the well-characterized diagnostic *A. fumigatus* antigens used for the diagnosis with an indirect ELISA. Four control serum samples from normal subjects were also taken as controls. The immunoreactivity of the protein in immunoblot was demonstrated with sera of 8 individual

patients (fig. 6). The role of carbohydrate moieties of gp56 was studied after periodate oxidation. The immunological activity of gp56 was retained after periodate oxidation; however, a decrease in the activity was observed. A decrease in IgG and IgE activities on Western blot was observed after pronase treatment.

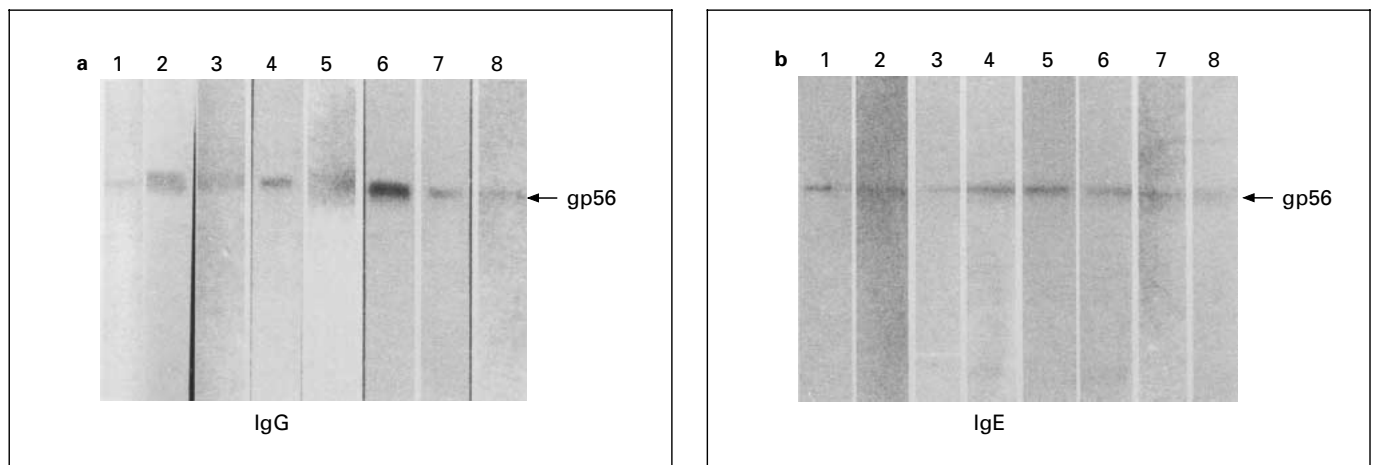


Fig. 6. Immunoblots showing IgG (**a**) and IgE (**b**) binding of gp56 with serum samples from 8 ABPA patients. The serum dilution was 1:100 v/v and 1:50 v/v for IgG and IgE, respectively.

Discussion

Glycoprotein antigens/allergens of *A. fumigatus* are highly complex in nature and not many of them have been isolated and characterized. The lack of information on well-characterized antigens has resulted in there being no WHO reference standards or international commercial standards for the immunodiagnosis of aspergillosis. However, an increase in the incidence of aspergillosis necessitated efforts towards characterization of antigens/allergens for diagnosis.

The diagnostic potential of Con A-bound glycoprotein antigens/allergens is well documented. Further, liposomal formulations of glycoprotein antigens/allergens have been observed to lead to immunomodulation resulting in a Th1-type immune response in mice [13]. Hence the characterization of glycoprotein antigens is anticipated to be useful for basic and applied studies. With this in view, the current work on a 56-kD Con A-binding gp antigen of *A. fumigatus* was undertaken.

Few Con A-bound antigens with molecular weights of 88, 55, 58 and 45 kD have been reported to be proteins with a varying sugar content of 10, 23, 46 and 40%, respectively [10, 11, 19, 20]. The antigen, gp56, reported here showed a sugar content of 38.5%. The IgG/IgE immunoreactivity of gp56 decreased with sodium meta-periodate and pronase treatments. This indicates that both the protein and carbohydrate part of the antigen may contribute to antigenic and allergenic epitopes. The amount and nature of carbohydrate moieties may play a role in the immunoreactivity of *A. fumigatus* glycoprotein

antigens/allergens [21]. In view of its specific immunoreactivity with IgE and IgG antibodies gp56 may find a use in developing a diagnostic immunoassay for *A. fumigatus*-induced allergic disorders. Recent functional studies on glycoprotein antigens and allergens of *A. fumigatus* and human lung surfactant proteins A and D clearly demonstrated the inhibition of histamine and the inhibition of the binding of specific IgE and IgG antibodies induced by *A. fumigatus* glycoprotein antigens and allergens [22–25].

The N-terminal sequence of gp56 showed homology to fungal serine proteases [17, 18] and not with the reported 55-kD antigen from *A. fumigatus*, which indicates that these proteins are different [10]. In addition, reported gp55 was shown not to possess any protease activity when fluorogenic peptide substrates were used. However, the antigen reported here displayed protease activity with gelatin and casein substrates. Biological activities like elastinolytic and proteolytic activities are known to be retained even after protein purification steps [11, 26, 27]. The gp45 antigen has also been reported to exhibit protease activity with gelatin substrate [11]. Another glycoprotein protease of 32 kD has been reported with N-linked high mannose groups [28].

Studies on the cross-reactivity among *Aspergillus* allergens/antigens indicated the presence of common epitopes in various antigens/allergens of *A. fumigatus*. Monoclonal antibodies raised against 58 kD reacted with the 45-kD antigen as well [12]. Extensive enzymatic and chemical mapping as well as immunological cross-reactivity studies on *A. fumigatus* culture filtrate antigens of a molecular weight of 60, 40 and 37 kD suggested the possibility of a

common peptide core in the structure [7, 8]. It has been thought that differences in molecular weights and antibody binding profiles of glycoproteins could be due to a varied sugar and phosphate content. These glycoprotein antigens may be derivatives of a multifunctional macromolecular glycoprotein complex. But extensive work on the characterization of these antigens/allergens, their epitopic sequences and their homologies needs to be carried out to identify any possible occurrence of intrinsic properties in these allergenic proteins.

Proteases isolated, purified and characterized from *A. fumigatus* and *Aspergillus flavus* have been proposed as potential virulence factors [4]. Studies on various mutants deficient in alkaline protease/metalloprotease have established that more than one protease is involved in virulence [6, 28–30]. So there is a need to study proteases to establish the colonization potential of *A. fumigatus* in humans. The gp56 protein identified herein exhibited homology to serine proteases from *P. notatum* and

A. fumigatus [26, 27]. Alkaline and/or serine proteases are major allergens prevalent in airborne *Penicillium* and *Aspergillus* species possessing a similar protein structure and strong antigenic/allergenic cross-reactivity [31–36].

Detailed investigations of 56 kD, with respect to a structure-function relationship, epitope mapping, and identification of gene encoding, are required to investigate its role in immunopathogenesis. A new allergen of *A. fumigatus* with a molecular weight of 56 kD, pI 6.8 and protease activity reported in the current work can be considered as one of the diagnostic antigens.

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