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Identification of virulence factors and diagnostic markers using immunosecretome of *Aspergillus fumigatus*[☆]

Awanit Kumar^a, Rizwan Ahmed^a, P.K. Singh^b, P.K. Shukla^{a,*}

^aMedical Mycology Lab, Division of Fermentation Technology, CSIR-Central Drug Research Institute, Lucknow-226 001, India

^bPlant Molecular Biology and Genetic Engineering Division, CSIR-National Botanical Research Institute, Lucknow-226 001, India

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ABSTRACT

Aspergillus fumigatus is a prime causative agent for various allergic and invasive aspergillosis. There has been a dramatic increase of such cases in last three decades yet the early diagnosis and virulence factor identification remains the challenge. In the present study secretome analysis of proteins isolated from the culture filtrate was done by 2D gel electrophoresis coupled with MS/MS and the immunosecretome analysis was carried out using immunoblotting of 2D transfer blots and probed with the sera of patients, immunized rabbit and mice. The identified proteins were analyzed further for homology with human proteins by BLAST search and for secretory signal by SignalP. A total of 65 protein spots from 2D gel resulted in identification of 24 different proteins along with their isoforms and out of which 15 proteins were identified as immunogenic in human. These findings may be helpful in the identification of virulence factors involved in aspergillosis and also useful as diagnostic markers.

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1. Introduction

Species of *Aspergillus* are found worldwide as a saprophyte and among these *A. fumigatus* is known to be the commonest associated with human mycoses. *A. fumigatus* has high sporulating capacity that results in the presence of high concentration of minute conidia in the air [1]. *A. fumigatus* is responsible for a range of diseases such as allergic diseases (asthma, allergic sinusitis, and alveolitis), invasive aspergillosis, allergic bronchopulmonary aspergillosis (ABPA) and aspergilloma caused due to inhalation of minute asexual spores present in the environment. In almost all the cases of aspergillosis immunocompromised condition is mandatory which may be due to AIDS, prolonged use of chemotherapy in cancer patients or the use of immunosuppressant in the organ transplant cases [2]. *A. fumigatus* conidia inhaled by normal immunocompetent human are efficiently cleared by the innate immune response whereas in immunocompromised

persons the conidia grow and release virulence factors. The factors developed in *A. fumigatus* for survival in the soil environment (natural habitat) become virulent when it accidentally infects humans and escapes from the innate immunity of hosts. There is a diversity of virulence factors in different forms of aspergillosis as they are under polygenic control, and depend on the stage specific protein biosynthesis [3].

The number of cases of aspergillosis has increased in past three decades but the prognosis of such infections remains a challenge to clinicians pending prompt diagnosis. The diagnosis of such infections is largely dependent on the conventional methods that require isolation of the causal agent from the site of infected tissue/organ which is not always feasible. Further this requires longer time period to confirm the diagnosis therefore the better and more reliable techniques for early diagnosis are needed. This demands the exhaustive knowledge of fungal antigens which may be detected in the

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* Corresponding author. Tel.: +91 522 2612411; fax: +91 522 2623405.

E-mail addresses: pk_shukla@cdri.res.in, p_kshukla@yahoo.com (P.K. Shukla).

body fluids of patients. Few antigens specific for the ABPA, aspergilloma, and invasive aspergillosis are known and being evaluated for the diagnosis [4]. The secretory proteins of *A. fumigatus* contain enzymes (such as proteases, peptidases and phospholipases), secreted toxins, adhesins, and other molecules responsible to invade host immune systems and cause pathogenesis [3,5]. *A. fumigatus* secretes exoproteases and other cytoplasmic fungal products that are capable of compromising mucociliary clearance, breaching the airway epithelial barrier, and activating the innate immune system of the lung, including production of several cytokines [6]. The virulence caused by *A. fumigatus* may be augmented by its numerous secondary metabolites, including fumagillin, gliotoxin, helvolic acid etc. [2]. Thus, the identification and study of secreted proteins may divulge unique infection mechanisms that could lead to new control measures for the aspergillosis.

With publication of whole genome sequence of *A. fumigatus* [7] the study of pathobiology of this fungus became more feasible. The proteomic approach for better understanding of the host–pathogen interactions is the method of choice and the immunoproteomics is the tool for the discovery of biomarkers for early diagnosis of aspergillosis [4]. Secretome analysis of *A. fumigatus* and the immunogenic potential of the secretory proteins might help in understanding its virulence factors, drug targets, and targets for immunodiagnosis of the diseases. The present study is an attempt to analyze the secretome of *A. fumigatus* by 2D gel in combination with MS/MS analysis on MALDI-TOF-TOF platform and immunosecretome by western blotting of 2D gel transfer blot with pooled patient sera, immunized rabbit sera and immunized mice sera that may be useful for identification of target molecules for early diagnosis as well as in identification of virulence factors required to understand the manifestations caused by aspergillosis.

2. Materials and methods

2.1. Fungal strain and culture conditions

A. fumigatus strain used in this study is a patient isolate confirmed by ITS analysis [8]. *A. fumigatus* was grown on Sabouraud's dextrose agar (SDA) (BD Biosciences) slant for seven days and stored at 4 °C for further use. Conidia were harvested in physiological saline from 7 days old SDA slant cultures at 35 °C and the colony forming units (Cfu) determined by dilution and plating method. Erlenmeyer flasks containing 300 ml Sabouraud's dextrose broth (BD Biosciences) were inoculated with 1×10^6 conidia and kept on a rotary incubator shaker at 180 rpm at 35 °C for 14 days.

2.2. Isolation of secretory proteins

The mycelial growth was separated from 14 days old broth culture using Whatman filter paper No. 41 and the filtrate was sterilized through 0.22 μ m filter (Millipore, USA). The culture filtrate was cooled to 4 °C and chilled dry-acetone added to it in a ratio of 1:3, mixed vigorously and the mixture again kept overnight at 4 °C. Subsequently the precipitate thus formed

was separated by centrifugation at 10,000 *g* for 15 min at 4 °C. The pellet obtained was dissolved in phosphate buffer saline (PBS, pH 7.4) and the protein concentration was determined by Lowry's method and stored at –20 °C for further use. To check the consistency of secretory proteins from 14 days old *A. fumigatus* culture broth, the proteins were isolated in the similar manner on more than 3 occasions showing identical profiles.

2.3. Collection of patient sera

The sera of clinically suspected cases of aspergillosis visiting local Medical College hospital were obtained with informed consent and screened for antibody titers (IgG and IgE) by performing ELISA. A total of 22 patient sera were found to be positive for high IgG titers ($\geq 1:51,200$) against secreted antigens of *A. fumigatus* and were pooled for the present study. Out of these 22 patients, 11 cases were of ABPA (based on culture from sputum and elevated IgE and IgG level), 5 of aspergilloma (based on clinical symptoms and/or culture, chest X-ray and elevated IgG level) and 6 of IA (based on culture and elevated IgG level). Sera from healthy volunteers were used as negative control (control sera) (IgG $\leq 1:800$, IgE $\leq 1:400$). Due prior permission from local human ethics committee was also obtained prior to studies.

2.4. Immunization of experimental animals

One New Zealand bred albino rabbit and 10 BALB/c albino mice were immunized with secretory proteins of *A. fumigatus*. The secretory proteins (1 mg/ml) were mixed with Freund's complete adjuvant (1:1) and given subcutaneously in rabbit (500 μ g) and mice (50 μ g). Subsequently three booster doses of same quantity of the secretory proteins as above mixed with Freund's incomplete adjuvant (1:1) were given at an interval of 15 days. Serum from the immunized animals was collected at different time intervals and checked for optimum level of antibody titer by ELISA. Prior permission from local animal ethics committee was obtained.

2.5. SDS-PAGE and immunoblotting

Secretory proteins of *A. fumigatus* were resolved on denatured polyacrylamide gel (12%) by the conventional method of Laemmli in a vertical mini gel system [9] using 80 to 100 μ g protein per lane and stained with Coomassie brilliant blue R250 (CBB). For immunoblotting the secretory proteins of *A. fumigatus* thus resolved were electro-transferred to nitrocellulose membrane. Transblotted proteins on the membrane were checked with Ponceau S stain and the membrane was cut into strips (proteins per lane), washed with 20 mM Tris, 0.89% NaCl, pH 7.4 (TBS) containing Tween-20 and blocked with 2% fat free milk solution in TBS for 1 h at 37 °C. The strips were washed once again with TBS containing 0.05% tween-20 (TBST) to remove excess blocking agent. Then the membrane strips were incubated with primary antibody (pooled patient sera, rabbit sera, and pooled mouse sera, 1:100 dilutions in each case) against secretory proteins, diluted in TBS and incubated for 2–3 h at 37 °C with constant gentle shaking. The membrane was washed with TBST as above and incubated with HRP-labeled

secondary anti-human, anti-rabbit and anti-mouse respectively for 90 min. After washing the immunodominant proteins were visualized using 3,3'-di-amino benzidine (DAB) as substrate, catalyzed by H₂O₂.

2.6. Two-dimensional gel electrophoresis and immunoblotting

Isoelectric focusing was carried out using pre-cast 13 cm Immobilized pH Gradient (IPG) strips with a range of 4–7 (GE Healthcare, USA). One milligram of secretory proteins were precipitated with 10% TCA containing acetone and kept at –20 °C for at least 1 h. The precipitated proteins were pelleted at 10,000 g for 15 min at 4 °C, the pellet was washed thrice with chilled acetone, air dried and dissolved in 100 µl of sample preparation solution containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) IPG buffer (pH 4–7), 40 mM DTT and kept for 1 h at room temperature with vortex mixing. Then 150 µl of rehydration solution [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (v/v) IPG buffer (pH 4–7), 0.002% (w/v) bromophenol blue] was added to it, kept on vortex mixer for 15–30 min and centrifuged at 10,000 g for 15 min at 4 °C to remove any residual particles. The IPG strip was placed in the resultant solution in a rehydration tray for 16–18 h at room temperature. Isoelectric focusing was carried out over Ettan IPGphor 3 (GE Healthcare, USA) for a total of 80,000–100,000 Vh (volt-hours) with gradual increase in the voltage according to the program, step and hold at 150 V for 2 h, step and hold at 500 V for 2 h, gradient 1000 V for 1 h, gradient 8000 V for 3 h and final step and hold 8000 V up to 80,000 Vh. The strip was removed and equilibrated with SDS equilibration buffer containing 75 mM Tris-Cl pH 8.8, 6 M Urea, 30% (v/v) Glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue. Equilibration was done twice, first with DTT (10 mg/ml) then with iodoacetamide (25 mg/ml) for 15 min each under shaking conditions. The proteins in equilibrated strips were resolved in isocratic gel (10%) at a constant current of 15 mA. After completion of electrophoresis either CBB staining was performed to visualize the proteins or the gel was used for transfer the proteins to the nitrocellulose paper for immunoblot experiments using patient sera, rabbit sera and mice sera separately as mentioned earlier.

2.7. Mass spectrometric analysis

The gel piece containing corresponding protein spots was excised from the 2D gel stained with CBB and de-stained with 100 mM ammonium bicarbonate (ABC) in 50% acetonitrile, dehydrated with 100% acetonitrile (ACN) and dried in a speed vac for 1 h. In gel digestion of proteins was carried out overnight with 25 µl trypsin (Sigma Chemical Co.) stock solution (20 µg/ml) as per instructions of the manufacturer. The reaction was stopped with 1% trifluoroacetic acid (TFA) and the supernatant collected by centrifugation. Peptides were re-extracted from the gel pieces with 100 µl of 0.1% TFA in 50% ACN solution by vortex mixing for 30 min followed by brief centrifugation and pooled the supernatants. The pooled supernatant was concentrated on a speed vac and 0.5 µl of the concentrated peptide solution was spotted over the MALDI plate followed by 0.5 µl of matrix (alpha-cyano-4-hydroxycinnamic acid) solution (1 mg/ml) and cal-mix solu-

tion was spotted at the corresponding position. Prior to MS/MS analysis the instrument (MALDI-TOF-TOF, model 4800, ABSciex, USA) was calibrated with the cal-mix standard (Sigma Chemical Co.). The mass spectrometric data were generated using the reflector positive mode. The peptides with higher signal intensity were selected for MS/MS analysis. The peptide mass fingerprinting and peptide fragment ion data obtained were analyzed for the search of proteins in the all entries of the NCBI database with the help of Mascot software (Matrix Science) online. The search parameters were-peptide tolerance of 100 ppm, one missed cleavage, carbamidomethylation of cysteine as fixed modification and methionine oxidation as variable modification, precursor ion mass tolerance of 20 ppm, fragment ion mass tolerance of 0.05 Da. The individual ions scores >59 indicate identity or extensive homology ($p < 0.05$) and considered as significant. The spectra of common contaminants were removed by searching against contaminant database, and the remaining data were used for the identification of proteins.

2.8. Bioinformatics analysis

Identified proteins were analyzed by the use of SignalP software version 3.0 available online (<http://www.cbs.dtu.dk/services/SignalP/>) for the prediction of the signal peptide and the probability. Hidden Markov Model (HMM) was used for the prediction of signal peptide and cleavage site in the proteins. Homology search was performed to find out sequence similarity of identified proteins with the human proteins by alignments. The NCBI BLASTp (protein-protein BLAST) algorithm tool was used for the sequence alignment of the identified proteins with non-redundant protein sequence database of human (taxid: 9606).

3. Results and discussion

Fungal infections in general pose two major challenges to treat them; the first is the prompt and reliable diagnosis and the second, limited availability of broad spectrum antifungal agents. Many times it is extremely difficult to diagnose a case of mycoses at an early stage due to basically lack of awareness and suitable method of diagnosis which at present is largely dependent on the isolation of causal fungus from the site of infection. This requires the infected material which is many times out of reach or not sufficient and not always possible to get and then its growth on multiple media. The fungi may take longer periods to grow, may be from few days to 3 weeks. To improve upon the diagnosis people have tried for the detection of antibodies against fungi but the detection of antigen is a better option as in the former case, false positive cases may result because of the well known fact that once the antibodies are produced they remain elevated for a longer period even after the infection is over. There are a few diagnostic tests available for the detection of antigens of *A. fumigatus* and these are reliable to some extent. The situation is further complicated due to the development of resistance against the available limited antifungal armamentarium. To improve upon the situation it becomes important to analyze and know about the metabolic or secreted proteins of a fungus

at various time intervals. The study of secretory proteins seems to be of value in the discovery of circulating biomarkers that may play an important role in prompt diagnosis as well as targeted therapeutic developments. The secretory proteins are released during the germination of conidia and growth of hyphae, which facilitate the fungus to escape from the innate immunity of the host and create the hostile environment to their survival. In the present study an effort was made to study the secretome of *A. fumigatus* which is the most common cause of aspergillosis in human beings. The secretory proteins of *A. fumigatus* were extracted from 2 to 22 days old culture filtrate on alternate day and found that 14 days old culture filtrate contains the maximum number of proteins in comparison to others (data not shown). Hence for this study the 14 days culture filtrate proteins were used in subsequent study.

Sera from a number of clinically diagnosed cases of aspergillosis were collected and antibody titer determined. A total of 22 patient sera were found positive for aspergillosis by performing ELISA (titer 1:51,200) using secretory proteins of *A. fumigatus* while the ELISA titer with immunized rabbit and mice serum were found to be more than 1:51,200. The single dimensional SDS-PAGE transfer blots were primed separately with polyclonal antibodies of pooled patient sera (22 positive cases), rabbit sera, mice sera and normal human sera as well as pooled pre-immune sera of mice and rabbit (Fig. 1). Multiple bands of immunogenic proteins were observed after developing the blots in each case except in the case of normal human sera.

The secretory proteins of *A. fumigatus* were resolved in 2D gel electrophoresis (pH 4–7, length 13 cm) where CBB staining revealed the presence of more than 100 protein spots (Fig. 2A).

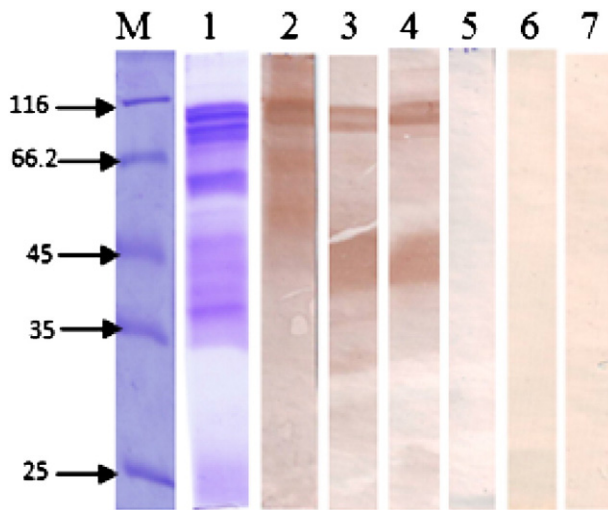


Fig. 1 – SDS-PAGE profile and immunoblotting analysis of *A. fumigatus* secretory proteins. Lane M, molecular weight marker; Lane 1, *A. fumigatus* secretory proteins (the gel was stained with Coomassie brilliant blue R250); Lane 2, immunoblotting with pooled patient sera; Lane 3, immunoblotting with pooled mice sera; Lane 4, immunoblotting with rabbit sera; Lane 5, immunoblotting with pooled sera of normal person; Lane 6, immunoblotting with pre-immune sera of rabbit; Lane 7, immunoblotting with pooled pre-immune sera of mice.

Most of these protein spots were in the mass range of 35–100 kDa in the acidic range of pI 4–6. Many of the protein spots appeared as multiple spots in a row and few of the representative spots from these rows were selected arbitrarily for MS/MS analysis and turned out to be same proteins in most of the cases. These protein spots found at different pI positions turned out to be isoforms of the same protein where variation in their positions may be due to glycosylation or other post-translational modifications. This way 65 selected protein spots were identified as 24 different proteins (including 3 hypothetical proteins) with significant score and sequence coverage (Table 1). All the proteins were matched with the *A. fumigatus* (Af 293) proteins at NCBI database. It was observed that most of the proteins identified are of the enzymatic nature and involved in the metabolism of carbohydrate, peptide or amino acids and lipids. Some of these are required to degrade the polymeric substrate to get nutrition and others may be involved in the morphogenesis and adhesives [10–14]. Many of these proteins have been reported for other fungi and microorganisms but not well studied in the case of *A. fumigatus*.

Studies on immunogenic nature of these secretory proteins are of particular importance to understand the pathogenic nature and virulence factor of the diseases associated with *A. fumigatus*. The pooled patient sera of aspergillosis cases as well as the sera from laboratory immunized animals were used to identify the immunodominant proteins. Out of 24 identified proteins 15 turned out to be immunogenic in nature as revealed by the identification of proteins from the immunoblots (Fig. 2B) using pooled patient sera. Other proteins which did not exhibit immunogenicity might be due to non-immunogenic nature or due to presence of fewer antibodies against them. Whereas in case of immunoblots with laboratory immunized rabbit and mice sera, most of the 2D resolved spots in the said range were detected as the immunodominant proteins except the alkaline proteases, aspartyl aminopeptidases which were detected in pooled patient sera and the hypothetical proteins (Fig. 2B, C and D). The proteins which did not show immunogenicity in case of patient sera (because of unknown reason) did have the capability to evoke the immune system as revealed by the study with experimental animals sera. The immunodominant nature of the proteins might be useful for elucidation of the diagnostic markers as well as the vaccine candidate.

SignalP analysis was used to determine the presence of signal peptide sequence to ascertain the secretory nature of these proteins and prediction of cleavage site of signal peptide. Most of the identified proteins were confirmed as secretory protein with the presence of signal sequences with good prediction value and cleavage site position as mentioned in Table 2. Few of these proteins such as aspartyl aminopeptidase, amidase, and 2 hypothetical proteins though present in the secretory proteins of *A. fumigatus* and identified by MS/MS analysis did not contain any signal sequence. This may either be due to disintegration of fungal cells during incubation period where these proteins got passage to secretory proteins or signalP could not recognize their secretion property because of their different secretion mechanism [15,16].

Further, the identified proteins when searched for homology by sequence similarity with human proteins using NCBI

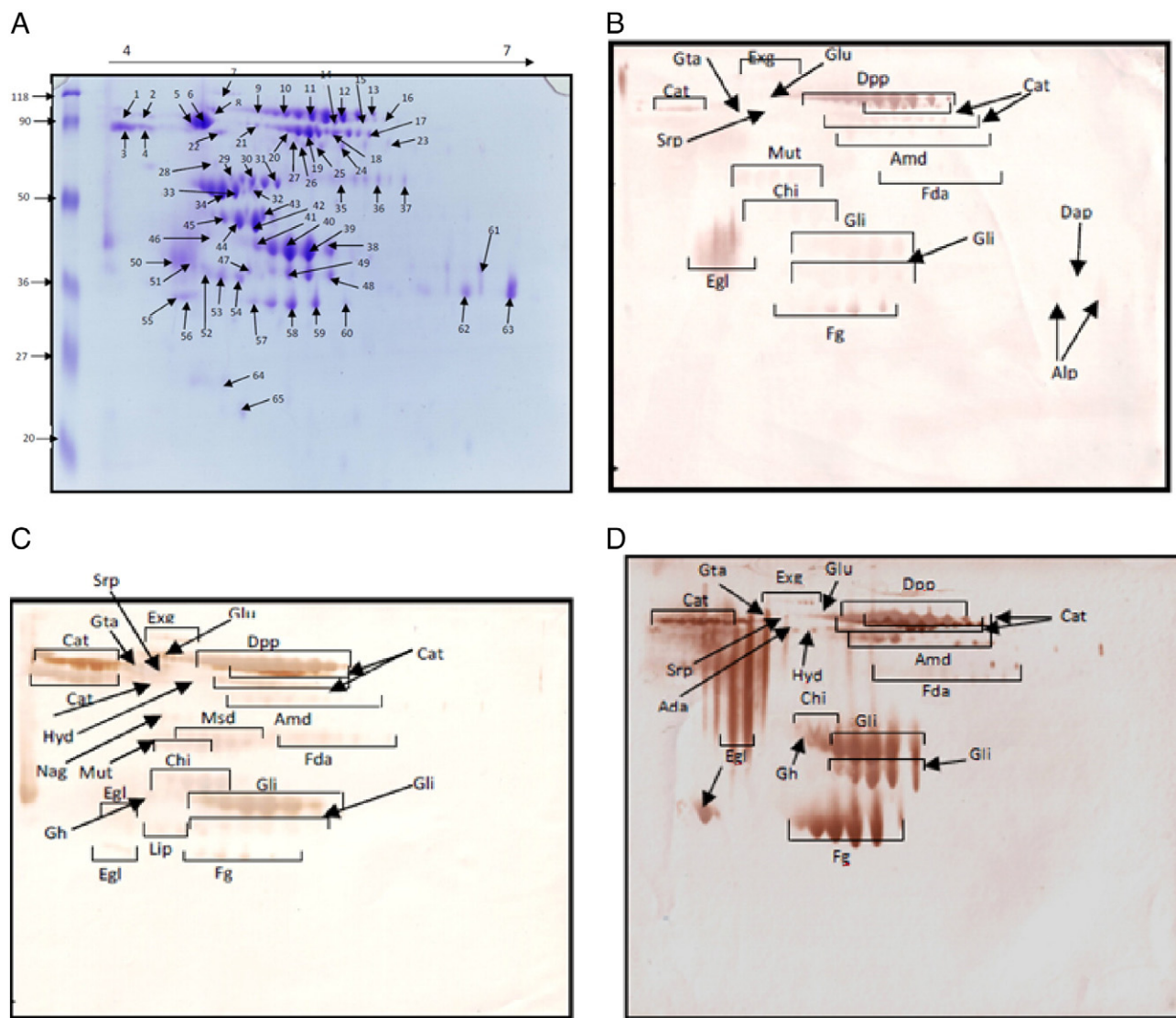


Fig. 2 – 2D gel electrophoresis profile of *A. fumigatus* secretory proteins and immunoblotting. One mg of protein was used for 2D sample preparation and loaded on to 13 cm immobilized pH gradient strip (4–7) and isoelectric focusing carried out followed by 2nd dimension SDS PAGE to resolve the proteins on the basis of their charge and molecular weight. After electrophoresis the 2D gel was either stained with CBB to visualize the protein spots and used for MS/MS analysis or transfer the proteins to nitrocellulose paper for immunoblot experiments. **A**, CBB stained 2D gel electrophoresis profile of secretory proteins (pI 4–7). The protein spots that were identified by MS/MS are numbered and listed in [Table 1](#); **B**, immunoblotting with pooled patient sera; **C**, immunoblotting with rabbit sera; **D**, immunoblotting with pooled mice sera; Note: letters coded with the arc represents the corresponding proteins mentioned in [Table 2](#).

BLAST revealed that out of 24 only 3 proteins viz. beta-1,3-endoglucanase, thioredoxin reductase, and FAD-dependent oxygenase did not have any match with human proteins and therefore may be used as diagnostic markers. Role of beta-1,3-endoglucanase (EglC) in cell wall morphogenesis and during sexual development in *Aspergillus nidulans* has been discussed [17] and recently Hartl et. al characterized the GPI-anchored endo beta-1,3-glucanase (Eng2) of *A. fumigatus* with a similar role in morphogenesis with open question remaining [11]. Thioredoxin reductase (GliT) a component of the gliotoxin biosynthetic cluster, provides the self protection to *A. fumigatus* against gliotoxin [18] which is an important virulent factor that has a major role in pathogenesis [19] and therefore, GliT

may be potentially exploited as a target molecule against aspergillosis. Some other proteins such as glutaminase, exo-beta-1,3-glucanase, and extracellular serine rich protein had less than 10% homology. Glutaminase A has a role in the amino acid metabolism. Glutamine is converted to glutamic acid by the action of glutaminase, indicating its utilization in nitrogen rich substrate as the glutamic acid is a preferred amino acid for metabolism. The primary role of exo- β -1,3-glucanase is to release glucose units from the non-reducing end of the β -1,3-glucan chain present in the polysaccharides. Cell wall associated exo- β -1,3-glucanase has been studied in *A. fumigatus* where it has probably a role in hyphal morphogenesis [12]. The third protein with less than 10% homology

Table 1 – MS/MS analysis and identified proteins spots of Fig. 2A.

Spot no.	Identified protein	NCBI ID (gi)	Theoretical MW/PI	Observed (approx.) MW/PI	Score	No. of peptides matched	Coverage (%)
1	Catalase	1857716	79.924/5.65	92/4.2	466	9	16
2	Catalase	2431866	79.986/5.50	92/4.3	855	12	19
3	Catalase	2431866	79.986/5.50	86/4.2	1296	18	27
4	Catalase	2431866	79.986/5.50	86/4.3	1318	18	27
5	Glutaminase A (GtaA)	71000415	76.214/4.71	90/4.6	1240	16	28
6	Extracellular serine rich protein	1463233345	86.001/4.82	90/4.7	174	3	5
7	Exo- β -1,3-glucanase (ExgO)	70996120	101.357/5.19	110/4.8	469	8	12
8	β -glucosidase	70990956	95.093/5.01	95/4.7	542	9	16
9	Secreted dipeptidyl peptidase (DppV)	71001112	79.753/5.59	95/5.0	504	13	23
10	Secreted dipeptidyl peptidase (DppV)	71001112	79.753/5.59	93/5.2	553	14	20
11	Secreted dipeptidyl peptidase (DppV)	2340046	79.753/5.59	90/5.4	122	4	7
12	Secreted dipeptidyl peptidase (DppV)	2340046	79.753/5.59	90/5.6	91	3	4
13	Secreted dipeptidyl peptidase (DppV)	2340046	79.753/5.59	92/5.7	367	9	13
14	Catalase	2431866	79.986/5.50	90/5.7	891	15	24
15	Catalase	1857716	79.924/5.65	90/5.5	139	5	9
16	Catalase	2431866	79.986/5.50	90/5.6	162	7	14
17	Catalase	2431866	79.986/5.50	85/5.7	912	15	23
18	Catalase	2431866	79.986/5.50	85/5.5	1141	13	22
19	Catalase	1857716	79.924/5.56	85/5.4	92	3	5
20	Catalase	2431866	79.986/5.50	85/5.2	418	9	17
21	Hydrolase	146323010	72.427/5.38	90/5.0	171	5	13
22	Adenosine deaminase family protein	146323525	66.433/4.86	85/4.7	802	14	23
23	Amidase	70997715	65.283/6.48	80/5.8	343	6	12
24	Amidase	70997715	65.283/6.48	80/5.6	783	10	21
25	Amidase	70997715	65.283/6.48	80/5.4	868	10	21
26	Amidase	70997715	65.283/6.48	80/5.3	907	10	21
27	Amidase	70997715	65.283/6.48	80/5.2	333	6	13
28	β -N-acetyl hexosaminidase (NagA)	70983560	67.781/5.58	68/4.8	281	5	16
29	Mannosidase I	45826518	58.582/5.14	60/4.9	827	12	35
30	Mannosidase I	45826518	58.582/5.14	58/5.1	545	10	30
31	Mannosidase I	45826518	58.582/5.14	58/5.2	804	14	35
32	Mannosidase I	45826518	58.582/5.14	55/5.1	129	3	9
33	α -1,3-glucanase/mutanase	70989361	54.330/4.98	55/4.9	921	12	42
34	α -1,3-glucanase/mutanase	70989361	54.330/4.98	55/4.8	219	5	16
35	FAD-dependant oxygenase	70985799	55.277/6.52	60/5.4	565	9	25
36	FAD-dependant oxygenase	70985799	55.277/6.52	60/5.7	504	8	23
37	FAD-dependant oxygenase	70985799	55.277/6.52	60/6.0	526	5	15
38	Thioredoxin reductase (GltT)	70992029	36.209/5.44	40/5.5	652	7	29
39	Thioredoxin reductase (GltT)	70992029	36.209/5.44	40/5.4	781	9	35
40	Thioredoxin reductase (GltT)	70992029	36.209/5.44	40/5.2	806	8	35
41	Thioredoxin reductase (GltT)	70992029	36.209/5.44	40/5.1	155	3	12
42	Chitinase class V	146323424	49.350/5.24	48/5.2	782	9	34
43	Chitinase	30385694	47.708/5.10	45/5.1	306	6	26
44	Chitinase	30385694	47.708/5.10	45/5.0	431	10	37
45	Chitinase	30385694	47.708/5.10	45/4.8	128	3	7
46	Hypothetical protein	70984739	40.680/4.76	45/4.7	183	2	7
47	Glycosyl hydrolase	70993214	36.790/5.14	38/5.0	43	1	5
48	Thioredoxin reductase (GltT)	70992029	36.209/5.44	38/5.6	759	8	35
49	Thioredoxin reductase (GltT)	70992029	36.209/5.44	38/5.3	825	10	40
50	β -1,3-endo-glucanase (EglC)	70985687	44.852/4.9	40/4.5	286	6	18
51	β -1,3-endo-glucanase (EglC)	70985687	44.852/4.9	40/4.6	105	3	10
52	Hypothetical protein	70985252	29.785/5.13	38/4.7	188	3	18
53	Extracellular lipase	70985264	31.823/5.59	36/4.8	375	5	22
54	Extracellular lipase	70985264	31.823/5.59	36/5.0	68	2	9
55	β -1,3-endo-glucanase (EglC)	70985687	44.852/4.9	35/4.5	641	8	24

(continued on next page)

Table 1 (continued)

Spot no.	Identified protein	NCBI ID (gi)	Theoretical MW/PI	Observed (approx.) MW/PI	Score	No. of peptides matched	Coverage (%)
56	β -1,3-endo-glucanase (EglC)	70985687	44.852/4.9	35/4.6	89	2	7
57	FG-GAP repeat protein	146322430	33.739/5.53	33/5.1	130	2	9
58	FG-GAP repeat protein	146322430	33.739/5.53	33/5.2	405	8	43
59	FG-GAP repeat protein	146322430	33.739/5.53	33/5.4	408	5	27
60	FG-GAP repeat protein	146322430	33.739/5.53	33/5.6	254	4	23
61	Aspartyl aminopeptidase	71000136	55.081/6.12	40/6.5	88	3	8
62	Uncleaved alkaline protease	2295	42.219/6.51	36/6.4	266	3	19
63	Uncleaved alkaline protease	2295	42.219/6.51	36/6.7	469	4	25
64	Conserved hypothetical protein	146323907	24.049/4.59	26/4.8	128	2	14
65	Conserved hypothetical protein	146323907	24.049/4.59	24/5.0	69	1	7

Note: The details of MS/MS data analysis are given as Supplementary material.

serine rich protein may have the predictive role in cell adhesion, as described in other fungi that adhesins contain multiple serine- and threonine-rich domain [3]. But the role of extracellular serine rich protein in *A. fumigatus* and its pathogenicity require a detailed study. These three proteins with less than 10% homology may also be exploited for diagnosis purpose.

Next in the homology ladder are beta-glucosidase, alpha-1,3-glucanase/mutanase and dipeptidyl peptidase exhibiting 15%, 19% and 33% homology respectively with the human protein (Table 2) and may also serve as a target molecule for diagnostic markers like proteins discussed above. β -glucosidase, a glycoprotein of *A. fumigatus* has been purified and characterized [20] with a major role in polysaccharide substrate utilization which acts upon β -1,4 bonds of β -D-glycoside substrates and catalyzes the hydrolysis of terminal non-reducing residues with release of glucose units. Role of α -1,3-glucanase (mutanase) has been investigated in mutan mobilization during sexual development of *A. nidulans* [21]. The glycoprotein secreted di-peptidyl peptidase V (DPP V) mainly responsible for hydrolysis of selective dipeptides that may activate the T-cell population and trigger host defense mechanisms [22].

The proteins such as mannosidase I, adenosine deaminase family protein, hydrolase with 94%, 78% and 69% similarity were found to be non immunogenic as expected. Mannosidase I has its main role in the processing of mannose containing glycans. Recently Li et. al reported that α -mannosidase I activity of MsdS gene is involved in polarity and septation in *A. fumigatus* [23]. Adenosine deaminase, a metalloenzyme catalyzes irreversible deamination of adenosine and 2-deoxyadenosine to inosine and deoxyinosine [24]. Hydrolases, such as protease, amylase, lipase etc. have the hydrolytic activity and disintegrate the polymer into its component units. Whereas the glycosyl hydrolases, such as cellulase, hemicellulase etc. have the capability of degrading the polysaccharides. These hydrolases may have significant role in causing pathogenicity to the host.

Surprisingly, aspartyl aminopeptidase, chitinase, FG-GAP repeat protein and alkaline protease having 97%, 80%, 78% and 71% similarity with corresponding human proteins turned out to be immunogenic. Aspartyl aminopeptidase specifically degrades only amino-terminal acidic amino acids from

peptides [25]. The exochitinase, endochitinase, and transglycosylation activities have been associated with one chitinase of *A. fumigatus* [26] where it may have its role in morphogenesis [13]. Predominantly found secretory FG-GAP repeat proteins may be responsible for integrin-mediated cell adhesion and may play role in the signal transduction in immune recognition [14,27]. Elastinolytic property of alkaline proteases plays a role in the invasion of lung tissue by the fungus [28]. Recently Behnsen et. al reported that the secreted alkaline protease degrades the human complement proteins which play a major role in innate immune system [29].

Catalase which had 60% similarity with human protein is considered as a putative virulence factor with the capability to scavenge the H_2O_2 produced by the host phagocyte. There are three types of catalase produced by conidia and hyphae of *A. fumigatus* and their role in virulence is known [30]. Amidase hydrolyse amides and produce the corresponding carboxylic acid and ammonia. Amides can be used as sources of carbon or nitrogen in some fungi which possess amidases. There is report of amidase regulation in *A. nidulans* [31] but not in *A. fumigatus*. Beta-N-Acetylhexosaminidase from *Aspergillus oryzae* catalyzes the reverse glycosylation of N-acetylglucosamine. Lipases catalyze hydrolysis, esterification, trans-esterification etc. required for the mobilization of fatty acids, glycerol etc. as carbon source.

The identified proteins (Table 1) of *A. fumigatus* were BLAST searched with online available protein databases to search for the sequence homology with other fungi (details not shown). The results indicated that some of the proteins had significant similarities with a few other filamentous molds but none with yeast form of fungi. Therefore, in conclusion, the present study resulted in identification of 24 different proteins along with their isoforms and out of which 15 proteins were identified as immunogenic in human. Although it is likely that a few of these proteins may be common in the sera of other invasive fungal infections (IFIs) yet the reactivity of a panel of these proteins with IgG antibodies of patients of aspergillosis may form a benchmark that will be helpful in the identification of virulence factors involved in the disease and may substantiate their role in more specific diagnosis of aspergillosis.

Table 2 – SignalP and BLAST analysis of identified proteins.

Code	Protein name	NCBI ID (gi)	SignalP			NCBI BLAST				Probable functions
			Probability	Cleavage site	Cleavage probability	Score	Coverage (%)	Protein homology	NCBI ID (gi)	
Cat	Catalase	1857716, 2431866	0.952	15–16	0.870	293	60	Catalase	4557014	Scavenges oxidative defense reactions of the host phagocytes
Gta	Glutaminase A	71000415	0.999	22–23	0.374	32.7	9	Transcription factor	3090887	Amino acid catabolism
Srp	Extracellular serine rich protein	146323345	0.732	21–22	0.408	33.1	9	CHD1 protein	33392695	Adhesin
Exg	Exo- β -1,3-glucanase	70996120	0.999	24–25	0.998	32.3	7	Keratin	31074643	During hyphal morphogenesis
Glu	β -glucosidase	70990956	0.996	19–20	0.989	35.4	15	Protein kinase	119576995	Conversion of glucose polymers to glucose
Dpp	Secreted di-peptidyl peptidase V	71001112	0.998	18–19	0.873	77.4	33	N-acyl amino acyl-peptide hydrolase isoform	119585402	Metabolism of dipeptides
Hyd	Hydrolase	146323010	0.905	18–19	0.884	68.2	69	β -glucuronidase precursor	183233	Hydrolytic activity
Ada	Adenosine deaminase family protein	146323525	1.000	18–19	0.888	190	78	Growth factor adenosine deaminase type	288563186	Deamination of adenosine and 2'-deoxyadenosine
Amd	Amidase	70997715	0.062	57–58	0.046	97.4	49	Glutamyl-tRNA amidotransferase subunit A	222131590	Hydrolyse amides
Nag	β -N-acetyl hexosaminidase	70983560	0.997	19–20	0.695	201	76	Hexosaminidase β -subunit	867691	Release of N-acetylglucosamine
Msd	Mannosidase I	45826518	1.000	18–19	0.496	218	94	Mannosidase α	119577080	Involved in polarity and septation during germination
Mut	α -1,3-glucanase/mutanase	70989361	0.999	20–21	0.810	32.3	13	Hypothetical protein	169210194	Mobilizes mutan
Fda	FAD-dependant oxygenase	70985799	0.994	23–24	0.534			No match		Oxidization
Gli	Thioredoxin reductase	70992029	0.808	27–28	0.171			No match		Provide self-protection to <i>A. fumigatus</i>
Chi	Chitinase	146323424	0.995	26–27	0.860	166	80	Chitinase 1	111494006	Morphogenesis
Hp1	Hypothetical protein	70984739	0.000	–1–0	0.000	34.3	40	Centrosomal protein	150036306	Unknown
Gh	Glycosyl hydrolase	70993214	1.000	20–21	0.561	31.6	41	Uncharacterized protein	154937380	Hydrolytic activity
Egl	GPI anchored β -1,3-endoglucanase	70985687	1.000	18–19	0.959			No Match		Morphogenesis
Hp2	Hypothetical protein	70985252	0.001	25–26	0.000	32.7	23	Zinc finger and BTB domain containing factor	119589675	Unknown
Lip	Extracellular lipase	70985264	0.998	21–22	0.480	36.2	46	Di-acyl glycerol lipase	218931253	Lypolytic activity
Fg	FG-GAP repeat protein	146322430	0.999	25–26	0.961	61.6	78	Cartilage acidic protein 1	119570276	Integrin-mediated cell adhesion/signal transduction
Dap	Aspartyl aminopeptidase	71000136	0.048	27–28	0.015	418	97	Aspartyl aminopeptidase	119591136	Tissue invasion
Alp	Alkaline protease	2295	1.000	20–21	0.999	93.2	71	Preprotein convertase subtilisin/kexin type 9	31317307	Tissue invasion
Hp3	Conserved hypothetical protein	146323907	0.993	19–20	0.642	32.3	19	Unnamed protein product	194382282	Unknown

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