IMMUNOPROTEOMIC ANALYSIS OF SECRETORY PROTEINS OF *ASPERGILLUS FUMIGATUS* WITH SPECIFIC IGE IMMUNOREACTIVITY

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ABSTRACT

Allergenic/antigenic proteins are known to induce Type I and Type III hypersensitivity reactions leading to allergic bronchopulmonary aspergillosis (ABPA) in immunocompetent host. The common structural features or intrinsic properties of the allergens/antigens leading to allergenicity in a host are not well understood. In the current report, comparative analysis of proteins on two dimensional gel electrophoresis (2D-E) and specific IgE immunoblots of *A. fumigatus* secretory proteins (1st, 2nd and 3rd week culture filtrate proteins) was carried out. We observed a total of 159 proteins in 1st, 2nd and 3rd week culture filtrates of *A. fumigatus*. Specific IgE immunoreactivity was observed in 75 proteins with different intensity. Third week culture filtrate showed maximum number of proteins, 142, and specific IgE immunoreactive proteins, 65. MALDI-TOF analysis resulted in putative identification of two allergens as hypothetical protein YBL057c from *Saccharomyces cerevisiae* and unnamed protein product from *Debaryomyces hansenii* (similar to IPF14568 of *Candida albicans*). Identification of a repertoire of specific IgE immunoreactive proteins will facilitate the studies on structure-function relationship of these proteins relevant for diagnosis and pathogenesis.

KEY WORDS

Immunoproteomics, *Aspergillus fumigatus*

INTRODUCTION

Fewer than 60 species of fungi are consistently associated with human infections (1). Most of the pathogenic fungi e.g. *Alternaria*, *curvularia*, *Epicoccum* etc lead to allergic diseases but few e.g. *Candida*, *Aspergillus*, *Blastomyces*, *Coccidioides* and *Histoplasma* are common fungal agents responsible for primary systemic disease (2). Among these, *Aspergillus fumigatus*, is known to cause various allergic [ABPA (allergic bronchopulmonary aspergillosis), extrinsic allergic alveolitis, allergic fungal sinusitis, allergic asthma] and systemic diseases [CNPA (chronic necrotizing pulmonary aspergillosis), IPA (invasive pulmonary aspergillosis)] depending upon the immune status of the host depicting the complexity of pathogenesis (3-5).

In view of the medical importance of *Aspergillus fumigatus*,

its whole genome has been sequenced in 2003 (www.sanger.uk.ac, www.tigr.org). *A. fumigatus* genome sequencing data proposed approximately 10,000 genes and 30,000 proteins. A total of 326 Expressed Sequence Tags are reported in *A. fumigatus* (www.ncbi.nlm.nih.gov) (6). Currently, out of over 2334 entries of *Aspergillus* protein/protein subunit sequences [Swiss-Prot (452) and TrEMBL(1882)] in the SWISS-PROT/TrEMBL database ([http.us.expasy.org](http://us.expasy.org)), 605 are from *Aspergillus fumigatus*.

Conventional purification methods and recombinant DNA techniques has led to the identification of few functionally important proteins of *A. fumigatus* (5, 7-9). Proteomics is a rapid approach to study large number of proteins using 2D-E and mass spectrometry. Information generated from proteomics has increased an understanding of biological regulatory networks and has opened novel scientific avenues such as "Structural proteomics" (10). In a recent approach, uncleaved compact domains after proteolytic fragmentation, identified by electrospray ionization mass spectroscopic (ESI-MS) analysis are re-expressed as potential folding units and subjected to the screening processes necessary to generate

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crystals suitable for three-dimensional structure determination (11).

Immunoproteomics approach has led to the identification of immunoreactive proteins of *Schigella flexneri* 2a and *Helicobacter pylori* (12, 13). Mycelial proteins of *A. fumigatus* have already been subjected to immunoproteomics, however, the efforts were limited and resulted in identification of Asp f 13 and enolase (14, 15). It is important to note that most of the reported purified antigenic proteins of *A. fumigatus* are secretory or culture filtrate proteins (1). Asp f 1, a major allergen/antigen of *A. fumigatus* was shown to be 1000-fold higher in culture filtrate than in hyphae or spore extract (16).

In the present study, we subjected 1st, 2nd and 3rd week culture filtrate proteins of *A. fumigatus* to immunoproteomic analysis for the first time. The efforts resulted in a comprehensive catalogue of secretory allergens of *A. fumigatus* and two of them could be assigned with a putative identity after MALDI-TOF analysis.

MATERIALS AND METHODS

A. Preparation of culture filtrate proteins

A. fumigatus secretes different types of proteins at different intervals. In order to identify a large number of secretory allergens/antigens, proteins secreted at 1st, 2nd and 3rd week were studied using proteomic approach. *A. fumigatus* (strain 285, isolated from the sputum of an ABPA patient visiting Vallabh Bhai Patel Chest Institute, Delhi, India) was grown in a synthetic broth (L-asparagine medium) for one, two and three weeks at 37°C in a stationary culture (17, 18). The filtrate obtained after separating the mycelia was subjected to 80% (w/v) ammonium sulphate precipitation and dialysed extensively against deionised water. The dialysate was lyophilized to get the protein-enriched antigenic fraction. Protein content was estimated by bicinchoninic acid method (19).

B. Two- dimensional polyacrylamide gel electrophoresis (2D-PAGE)

The electrophoretic separation of proteins was performed essentially as described by O'Farrell et al. (20). Isoelectric focusing was carried out in glass tubes (Bio-Rad, Hercules, USA; inner and outer diameters of 1.5 and 4 mm respectively). The tubes were filled with 4.5% tube gel containing 8M Urea, 2% Triton-X-100, 6% ampholine (pH 3-9.5) (Amersham, Uppsala, Sweden). The length of the gel prepared was 11cm. A pre-run at 50V and 200V for 15 min. was given. A total of 600µg protein (1st, 2nd and 3rd week culture filtrate proteins from clinical isolate of ABPA patient)

and standard pl marker (Sigma, Saint Louis, Missouri, USA) was loaded on first dimensional gel and isoelectric focusing was conducted and run at 200V for 2h, 500V for 2h and 800V for 16h. After isoelectric focusing tubes were incubated for 30 min. at 4°C in equilibration buffer containing 6M Urea, 129.7mM DTT, 2% SDS, 20% Glycerol, 1.5M Tris. For separation of proteins in second dimension, tube gel was placed on 12% SDS-PAGE. Proteins were visualized by coomassie brilliant blue R- 250 staining (Sigma, Saint Louis, Missouri, USA).

C. Sp IgE immunoblotting

Proteins separated in 2D-E gels from 1st, 2nd and 3rd week culture filtrate (wcf) were transferred onto a nitrocellulose membrane (Schleicher and Schuell, Hahnestrabe, Dassel). The membranes were blocked for 3h with 3% skimmed milk powder in TBST (Tris buffer saline with 0.05% tween 20 + 0.005% Triton-X-100) at 37°C. Test membranes were incubated overnight with pooled sera of 10 suspected cases of ABPA [with skin prick test positive and high titre of specific IgG antibodies (with ELISA values ranging from 0.740-1.483) and IgE antibodies (with ELISA values ranging from 0.170-0.287)] diluted 1:5 in TBST + 1% BSA + 5mM CaCl₂ at 4°C. Control membranes were incubated with pooled sera of 10 healthy individuals [with skin prick test negative with specific IgG (with ELISA values ranging from 0.030-0.037) and IgE antibodies (with ELISA values ranging from 0.032-0.062)]. All the membranes were further incubated with secondary antibodies (anti-human IgE ε-chain specific-horse-raddish peroxidase antibodies from Sigma) diluted 1:1000 in TBST + 1% BSA + 5mM CaCl₂ at 37°C and developed with substrate (3, 3' Diaminobenzidine).

Comparative analysis of 2D gels and specific IgE immunoblots of 1st, 2nd and 3rd week culture filtrate proteins was carried out using a Microcal origin: version 3.

D. In situ tryptic digestion of proteins

Gel slices were trimmed to approx. 1 mm³ and washed 3X in 50% acetonitrile (ACN) /25 mM ammonium bicarbonate pH 8.0 (400 ml, 15 min. each time) to destain and soaked in 100%ACN for 5 min. to dehydrate the gels. ACN was removed and gel slices were dried in speed vacuum for 20–30 min. Dried gel slices were rehydrated with approx. 15ml cold trypsin solution (Sigma Sequencing Grade, Modified Trypsin 10-15ng/ml in 25mM ammonium bicarbonate pH 8.0). Rehydrated gel slices were incubated at 37°C for 16 hours followed by soaking in 25-50 µl 50%ACN/5%TFA for 30-60 min. with gentle agitation. Supernatant was lyophilized at room temperature and reconstituted by adding 3.0 µl of 50%ACN/0.1%TFA to

the bottom of the tube and gently pipetted to dissolve the extracted peptides.

E. Matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF) analysis

Peptide mass mapping was performed with 0.5 µl reconstituted extract with 0.5 µl fresh α -cyano-4-hydroxycinnamic acid (CHCA, Applied biosystem, Foster City, USA) matrix on a MALDI plate. Cal Mix 2 was spotted adjacent to sample for close external calibration. MALDI spectra were acquired in reflector mode with a method optimized for high resolution in 800-3000 Da range. The samples were analyzed by MALDI-TOF on Ultraflex II, Bruker Daltonics. Peak list obtained was calibrated with internal Trypsin peaks T7 (842.5099) and T4 (2211.1046). Peptide masses were searched in fungi using the Mascot search engine (MSDB database) (www.matrixscience.com) for protein identification. The parameters were set as follows: Mascot score above 53 were taken as the cut off, mass tolerance 100ppm, molecular mass unrestricted, charge +1 and missed cleavage 1 while performing the search.

RESULTS AND DISCUSSION

Comparative analysis of 2D-E and specific IgE immunoblots (with pooled patient sera) of 1st, 2nd and 3rd week culture filtrate protein fractions of *A. fumigatus* showed 38, 92 and 142 proteins respectively out of which 16, 49 and 65 proteins showed specific IgE immunoreactivity (Fig. 1,2,3,4, Table 1). We have observed a total of 75 proteins with specific IgE immunoreactivity in culture filtrate whereas information is available only for 27 proteins (25 secretory proteins and 2 intracellular proteins) in the public domain identified from mycelia as well as culture filtrate including 23 recombinant allergens/antigens of *A. fumigatus* (Table 1). Out of 25 secretory allergens available in literature, we observed 25 proteins with similar molecular mass and pI showing binding to IgE antibodies but their identity needs to be confirmed by MALDI-TOF and ESI-MS/MS analysis. Third week culture filtrate showed maximum number of proteins, 142, and specific IgE immunoreactive proteins, 65. It is already reported that third week culture filtrate proteins are relevant for immunodiagnosis on SDS-PAGE (18).

Figure 1.

2D gel electrophoresis (a) and corresponding specific IgE immunoblots with test sera (b) and control sera (c) of 1st week culture filtrate proteins of *A. fumigatus* (clinical isolate of ABPA patient). 2D-E (after staining with Coomassie R-250 staining) detected 38 proteins and immunoblots detected 16 with specific IgE immunoreactivity in 1st week culture filtrate. Proteins (600 µg) were separated by isoelectric focusing on tube gel, 11cm, pI 3–10, and then by SDS–PAGE, 12.0%

Figure 2

2D gel electrophoresis (d) and corresponding specific IgE immunoblots with test sera (e) and control sera (f) of 2nd week culture filtrate proteins of *A. fumigatus* (clinical isolate of ABPA patient). 2D-E (after staining with Coomassie R-250 staining) detected 92 proteins and immunoblots detected 49 with specific IgE immunoreactivity in 2nd week culture filtrate. Proteins (600 µg) were separated by isoelectric focusing on tube gel, 11cm, pI 3–10 and then by SDS–PAGE, 12.0%

Figure 3

2D gel electrophoresis (g) and corresponding specific IgE immunoblots with test sera (h) and control sera (i) of 3rd week culture filtrate proteins of *A. fumigatus* (clinical isolate of ABPA patient). 2D-E (after staining with Coomassie R-250 staining) detected 142 proteins and immunoblots detected 65 with specific IgE immunoreactivity in 3rd week culture filtrate. Two protein spots (spot no. 131 and 147) in 2D-E marked with arrow were subjected to MALDI-TOF analysis. Proteins (600 µg) were separated by isoelectric focusing on tube gel, 11cm, pI 3–10 and then by SDS–PAGE, 12.0%

Proteins observed specifically in 1st, 2nd and 3rd wcf are 2, 12 and 63 respectively. Proteins observed in 1st and 2nd wcf, 2nd and 3rd wcf and 1st and 3rd wcf are 3, 46 and 2 respectively. Proteins commonly observed in 1st, 2nd and 3rd wcf are 31 (Table 2). Proteins showing high immunoreactivity with test sera (patient) were considered as specific IgE immunoreactive proteins (Fig 1, 2, 3). Immunoblot with control sera (healthy) showed negligible binding to culture filtrate proteins.

Out of a total of seventy five specific IgE immunoreactive proteins, seven proteins were observed in 1st and/or 2nd week. Among these, five proteins (spot no. 26, 74, 75, 114 and 159) were observed in 2nd week and only one protein (spot no. 123) in 1st week. One protein (spot no. 97) was commonly observed in 1st and 2nd week (Table 1).

2D-E of three week culture filtrate was observed to have proteins with similar molecular mass and different pIs at 13kD

Figure 4
Comparative analysis of total proteins and specific IgE immunoreactive proteins in 1st, 2nd and 3rd week culture filtrate of *A. fumigatus*

(a)

(b)

Figure 5
MALDI-TOF mass spectral analysis of the peptides after in situ tryptic digestion of spot no.131 and 147.

Table 1

Comparative analysis of 1st, 2nd and 3rd week culture filtrate proteins of *A. fumigatus* with specific IgE immunoreactivity

S. No.	Protein spots (MW, pI)	Presence in 1 st /2 nd /3 rd wcf	S. No.	Protein spots (MW, pI)	Presence in 1 st /2 nd /3 rd wcf	S. No.	Protein spots (MW, pI)	Presence in 1 st /2 nd /3 rd wcf	S. No.	Protein spots (MW, pI)	Presence in 1 st /2 nd /3 rd wcf
1.	109.8, 4.3	2	41.	56.2, 4.3	3+	81.	35.5, 4.8	2+, 3+	121.	26.4, 9.8	2+, 3+
2.	98.8, 4.5	2	42.	56.2, 4.5	3+	82.	35.5, 5.0	1+, 2+, 3+	122.	26.4, 10.0	1+, 2+, 3+
3.	97.4, 4.7	2, 3	43.	56.0, 4.9	3+	83.	35.1, 5.2	1+, 2+, 3+	123.	26.3, 10.8	1+, 2
4.	84.5, 5.1	3	44.	54.1, 3.0	3+	84.	34.5, 4.3	2, 3	124.	26.0, 4.5	2
5.	85.5, 5.4	3	45.	54.1, 4.1	2, 3+	85.	34.5, 4.5	3	125.	25.4, 3.0	1, 2+, 3+
6.	85.5, 5.9	3	46.	54.1, 4.5	2, 3+	86.	33.4, 6.5	3+	126.	25.4, 4.1	2
7.	84.5, 5.4	3	47.	54.2, 5.3	1, 2+, 3+	87.	33.4, 7.0	1+, 3+	127.	25.4, 4.3	2
8.	82.5, 6.1	3	48.	54.2, 5.4	1+, 2+, 3+	88.	33.4, 7.3	3	128.	24.6, 3.0	2, 3
9.	79.1, 4.3	3	49.	54.2, 5.6	2+, 3+	89.	33.4, 8.5	1	129.	24.6, 3.8	3
10.	79.1, 4.6	3	50.	54.2, 5.8	3+	90.	33.3, 9.0	1, 2+, 3+	130.	24.6, 5.1	3
11.	79.1, 8.0	3	51.	54.4, 6.1	2+, 3+	91.	33.3, 9.4	1, 2+, 3+	131.	24.1, 5.1	1+, 2, 3+
12.	79.1, 8.5	3	52.	54.4, 6.2	2, 3+	92.	33.3, 10.5	1	132.	24.6, 5.8	3
13.	79.1, 8.9	3	53.	54.4, 6.4	2+, 3+	93.	32.0, 3.0	3	133.	24.0, 4.1	3
14.	78.2, 5.2	2, 3	54.	54.4, 6.7	2, 3+	94.	32.0, 3.2	3	134.	22.8, 4.0	3
15.	78.3, 5.4	2+, 3+	55.	54.4, 6.9	2+, 3+	95.	32.0, 4.1	3	135.	22.8, 4.3	3
16.	78.6, 5.7	2+, 3+	56.	54.2, 8.2	2+, 3+	96.	32.0, 4.7	3	136.	22.6, 6.3	3
17.	78.2, 5.9	2+, 3+	57.	54.2, 8.5	2, 3+	97.	31.1, 3.6	1+, 2+, 3	137.	22.4, 6.9	2
18.	78.1, 6.1	2+, 3+	58.	49.5, 5.2	2+, 3+	98.	29.8, 3.5	1, 2+, 3+	138.	22.4, 7.5	1, 2, 3
19.	78.5, 6.3	2+, 3+	59.	49.5, 5.5	2+, 3	99.	29.8, 3.9	2, 3	139.	22.4, 8.8	2
20.	78.5, 6.7	2+, 3+	60.	48.3, 6.4	3	100.	29.4, 4.6	3	140.	21.4, 3.1	2, 3
21.	78.4, 4.3	2, 3+	61.	47.0, 5.0	3	101.	29.0, 4.3	3	141.	19.5, 3.0	1, 2, 3
22.	78.4, 4.4	1, 2, 3	62.	47.0, 5.3	3	102.	29.9, 5.1	3+	142.	19.1, 4.1	1+, 2+, 3+
23.	78.4, 5.9	3	63.	46.5, 5.4	3	103.	29.6, 4.3	3	143.	18.6, 3.2	1+, 2+, 3+
24.	78.0, 4.3	1, 3+	64.	46.3, 5.6	3	104.	28.8, 3.5	3	144.	18.6, 4.3	1+, 2+, 3+
25.	78.0, 4.5	1, 2, 3	65.	45.5, 5.8	3	105.	28.6, 4.1	3	145.	18.5, 4.5	1+, 2+, 3+
26.	72.6, 5.7	2+, 3	66.	44.4, 4.2	3	106.	28.3, 5.2	3	146.	18.5, 4.9	1+, 2+, 3+
27.	70.6, 5.9	2, 3+	67.	44.3, 4.3	3	107.	28.1, 6.5	1+, 2+, 3	147.	18.0, 7.7	3+
28.	63.8, 4.5	2, 3	68.	44.3, 5.4	3	108.	28.3, 7.0	1+, 2+, 3	148.	17.9, 7.5	2, 3+
29.	63.2, 5.9	3	69.	44.3, 6.3	3+	109.	27.2, 8.9	2, 3	149.	17.7, 4.1	1, 2, 3
30.	62.1, 6.1	3	70.	44.0, 7.0	3	110.	27.3, 9.9	2+, 3+	150.	17.9, 5.5	2+, 3+
31.	61.7, 6.3	3	71.	44.0, 7.3	3	111.	27.3, 10.2	2+, 3+	151.	17.0, 9.1	2
32.	61.7, 6.5	3	72.	42.5, 4.1	3	112.	27.3, 10.5	2+, 3+	152.	13.2, 6.8	2+, 3+
33.	61.1, 4.3	3	73.	42.5, 4.3	3	113.	27.3, 10.8	2	153.	13.0, 3.5	2+, 3+
34.	61.1, 5.4	2, 3	74.	42.1, 7.0	2+	114.	26.6, 5.9	1, 2+	154.	13.1, 3.9	2, 3+
35.	60.8, 5.6	3	75.	42.1, 7.9	2+	115.	26.6, 6.3	1, 2+, 3+	155.	13.0, 7.0	2+, 3+
36.	59.5, 5.9	3	76.	41.6, 3.9	3+	116.	26.6, 7.7	1+, 2, 3+	156.	13.0, 8.5	1, 2+, 3+
37.	59.1, 4.1	2, 3	77.	41.6, 4.2	3+	117.	26.6, 8.6	1, 2+, 3+	157.	13.0, 10.1	1, 2
38.	59.1, 4.3	1, 2, 3	78.	38.1, 4.4	3	118.	26.4, 9.2	1, 2+, 3+	158.	13.0, 10.8	2
39.	58.4, 5.3	2, 3	79.	38.1, 4.6	3	119.	26.4, 9.4	1+, 2+, 3+	159.	11.0, 6.4	2+, 3
40.	57.1, 5.3	2, 3	80.	35.5, 4.1	2, 3	120.	26.4, 9.6	1, 2+, 3+			

(3.5-10.8, number of spots, n=7), 18kD (pI range 3.2-9.1, n=10), 20kD (pI range 3.0-8.8, n=8), 26kD (pI range 4.5-10.8, n=11), 29kD (pI range 3.5-7.0, n=11), 33kD (pI range 4.1-10.5, n=13), 45kD (pI range 4.2-7.3, n=7), 55kD (pI range 3.0-8.5, n=15) and 78kD (pI range 4.3-8.9, n=12). Some of these proteins with similar molecular mass and different pI were also observed in first week (18kD, 26kD, 33kD and 78kD) and second week (13kD, 18kD, 26kD, 33kD, 55kD, 78kD) culture filtrate. The molecular masses of these proteins corresponds to already known allergens e.g. 18kD (ribonuclease), 20kD (peroxisomal protein), 26kD (superoxide dismutase), 28kD (with unknown biological activity), 33kD (serine protease), 45kD (protease), 55kD (fibrinogen binding protein) and 78kD

Table 2
Differential protein expression in 1st, 2nd and 3rd week culture filtrates of *A. fumigatus*

Proteins present in	Number of proteins
1 st wcf only	2
2 nd wcf only	12
3 rd wcf only	63
1 st and 2 nd wcf	3
1 st and 3 rd wcf	2
2 nd and 3 rd wcf	46
1 st , 2 nd and 3 rd wcf	31

Table 3
Homology search results for the identification of two specific IgE immunoreactive proteins from the 3rd week culture filtrate of *A. fumigatus*

S. No.	Spot No.	Accession no.	Protein name	Function	% sequence coverage	Experimental Mr(kDa) ; pI / Theoretical Mr(KDa) ; pI	Mascot Score	Allergenicity
1.	131	S39831	Hypothetical protein YBL057c (<i>Saccharomyces cerevisiae</i>)	Not known	24.3%	24.1kD, 5.1 / 23kD, 5.2	55	Not known
2.	147	Q6BLU6	Unnamed protein product, similar to IPF14568 of <i>Candida albicans</i> (<i>Debaryomyces hansenii</i>)	Not known	28.6%	18kD, 7.7 / 44kD, pI 4.7	64	Not known

(catalase) (1). The possibility of these proteins, with similar molecular mass and different pI, to be isoforms needs to be examined by MALDI-TOF and ESI-MS/MS analysis.

A number of isoforms are reported at 33kD (serine protease) (6 spots, pI range 5.2-8.2) and 47kD (enolase) (3 spots, pI range 6.0-6.5) (21, 15). We have also observed 7 spots at 33kD (pI range 6.5-10.5) and 3 spots at 47kD (pI range 5.0-5.4) in 2D-E of culture filtrate proteins (Table 1).

MALDI-TOF analysis of two protein spots from 3rd week culture filtrate led to identification of significant matches (Table 3). Fig. 5a and 5b shows the map of MALDI-TOF spectra obtained for spot no. 131 and 147. MALDI-TOF analysis resulted in putative identification of two allergens as hypothetical protein YBL057c from *Saccharomyces cerevisiae* and unnamed protein product from *Debaryomyces hansenii* (similar to IPF14568 of *Candida albicans*). The identity of these proteins will be established by internal peptide sequences using MS/MS analysis.

At present, proteomics-based studies applied to *Aspergillus fumigatus* are still in their infancy. In this study, an attempt has been made to screen large number of specific IgE immunoreactive proteins secreted by *A. fumigatus* (from a clinical isolate of ABPA) by immunoproteomics approach. MALDI-TOF and ESI-MS/MS analysis of these proteins would confirm their identity and facilitate the studies related to structure-function relationship leading to a better understanding of the biology of the fungus. The proteomic database can be of use to study differential protein expression in *A. fumigatus* under various conditions and during host-pathogen interactions.

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