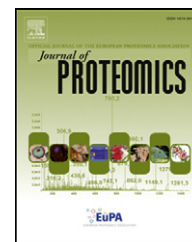


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Identification of aero-allergens from *Rhizopus oryzae*: An immunoproteomic approach

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ABSTRACT

Airborne fungal spores bearing allergens are the causative agent for inducing immediate hypersensitive reaction in sensitive individuals. In this study the potential aeroallergens have been reported for the first time from *Rhizopus oryzae* a common airborne mold. Clinical data based on SPT was further confirmed by ELISA. IgE reactive bands were revealed by one-dimensional immunoblotting. A 44 kDa major reactive band was found in all immunoblots. For precise identification of allergens, an immuno-proteomic approach was taken with a combination of 2-Dimensional gel electrophoresis and Mass-spectrometry. 2D map of spore-mycelial protein was confronted with pooled sera and several IgE reactive spots were detected, most of which were glycoproteins and except for one, which has no antigenic determinacy after metaperiodate modification. Each of those spots was identified by MALDI-TOF-TOF. Some bioinformatic approaches were taken to predict the signal peptide and subcellular localization of each protein. Major 44 kDa allergen was identified as Aspartyl endopeptidase. Sequence information was extracted from MS/MS spectra of two tryptic peptides generated from the 44 kDa endopeptidase. Multiple alignments with other reported aspartyl protease allergens showed significant homology. Allergenicity assessment of this protein was performed in silico and identified as a potential putative allergen.

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1. Introduction

Manifestation of allergic diseases, spanning from mild rhinitis to anaphylaxis is a major health problem, affecting the quality of life of millions of people all over the world, especially in the developed countries. More than 30% of the total population is affected by different kinds of allergy, caused by naturally occurring as well as synthetically produced compounds and their prevalence is increasing each day [1]. Exposure to aero-allergens in sensitized individuals is a major risk factor for the development and persistence of atopic sensitization to that specific allergen [2,3].

Unlike food allergens, inhalation through the respiratory tract is the most important route for all aeroallergens and the

airborne molds are an important contributing source in both indoor as well as outdoor environments. Airborne fungi are ubiquitous in the environment and their spore concentration is often far greater than all other allergenic bio-particles including pollen grains [4]. About 5% of individuals are predicted to have mold mediated allergic airway inflammation over their lifetime [5]. According to previous health survey reports, about 30% of the total allergic patients suffer from fungal spore induced allergy [1,6]. More than 80 fungal genera have been reported to induce IgE-mediated hypersensitive reactions in susceptible individuals and about 150 specific fungal allergens have already been identified from 23 different fungal genera [7,8]. Inhalation of airborne fungal spores carrying allergens is considered the risk factor for

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manifestation of different respiratory allergy symptoms like, severe asthma, chronic bronchitis, alveolitis, hypersensitivity, pneumonitis, aspergillosis, and seasonal rhinitis [1,6,9] in predisposed individuals.

The sensitization to aero-spora depends upon their allergenic potency, level of exposure to the aeroallergens and human genetic predisposition to the disease [10,11]. Clues to the environmental factors and gene–environment interactions are also critical to the pathogenesis of allergic disorders. There is not a single gene or environmental factor explaining the development of allergic disease, but it is probably regulated by a gene–environment interaction. There had been a major focus on the immunological and inflammatory mechanisms triggered by allergens which underlie the origin of allergy and its progression to allergic inflammation. Therefore, proper identification and characterization of the allergens are essential for successful diagnosis and subsequent therapy of atopic patients. The increasing prevalence of mold allergy has led to a corresponding increase in studies evaluating the allergenic potential of the individual proteins derived from fungal spores and mycelia. Hence, the focus of our current research was the characterization of a fungal allergome, *Rhizopus oryzae* based on proteomic approach.

R. oryzae (RO) is a prevalent fungal species in the air of Kolkata, a metropolis in India [12] but till date no research work has been conducted regarding the allergenic significance of this species, as well as identification and molecular characterization of its allergen(s). The present study is the first report of its kind, on allergenicity of *R. oryzae* by clinical investigations based on skin prick tests (SPT) and Enzyme-linked Immunosorbent Assay (ELISA) in combination with an immunoproteomic analysis to identify and characterize the allergens. This includes improved separation of spore mycelial proteins into hundreds of protein spots by 2D-PAGE following SDS-PAGE, Western blot to detect IgE antibody-specific reactive proteins and in-gel digestion of protein spots followed by mass spectrometry. This has led to the discovery of a number of previously unreported allergens from RO with special reference to a major allergen of 45 kDa; which can be further investigated in more detail to elucidate its structural and molecular features as a potent immunogen.

2. Materials and methods

2.1. Materials

Fungal cultures were prepared from airborne spores and then identified microscopically. Pure culture of RO was obtained by sub culturing an isolated young colony onto fresh media. Chemicals, kits, antibody and other reagents were procured from Bio-Rad Laboratories (Hercules, CA, USA), Sigma-Aldrich (St Louis, MO, USA), Amersham (UK), Promega (Madison, WI, USA), G Biosciences (USA) etc.

2.2. Preparation of fungal culture and crude antigenic extract

Airborne fungal spores were trapped by Andersen volumetric air sampler and impinged on a Potato Dextrose Agar (PDA) plate. Pure cultures were obtained by sub culturing individual colony on sterile PDA plates. PD Broth (Himedia, India) was

prepared, sterilized and inoculated with RO spores. Inoculated broths were maintained at 30 °C with moderate humidity in an incubator without shaking. A mycelial mat of two weeks old culture weighing about 500 mg was harvested and washed with sterile double distilled water. 1 g lyophilized spore mycelial mat was crushed in liquid nitrogen and soaked in 5 ml sterile 0.1 M Phosphate Buffer (pH 7.2) for 72 h and centrifuged at 22,000×*g* for 20 min. The clear extract was obtained by filtering the supernatant through a 0.22 µm membrane (Millipore) and used for SPT.

2.3. Clinical study, patient selection and sera collection

A total number of one hundred and thirty eight patients with allergic rhinitis and/or bronchial asthma, aged 18 to 50 years were considered as subjects of the present study. They had to visit the Allergy unit of the Institute of Child Health (ICH), Kolkata, and Department of Allergy & Asthma, Mediland Diagnostics, Kolkata in the year 2009–2011 with different symptoms like cough, breathlessness, sneezing and wheezing and asthma.

Skin prick test (SPT) is a deliberate and controlled exposure to a suspected allergen to confirm the atopic sensitivity of the allergic patients. SPT was performed on the sensitive patients with fungal crude antigenic extract (1:10 w/v) following the standard method [13,14]. Histamine diphosphate (1 mg/ml) and PBS (0.01 M, pH 7.2) was used as positive and negative controls respectively. Tests were performed with 20 µl of antigen solution placed on the ventral side of the arms and then pricked with a sterile lancet. The wheal response was measured after 20 min and graded according to Stytiš et al. [15]. Patients with other chronic illness and smokers were not included in the study.

Blood (5 ml) from each sensitive patient was collected with their given written consent; serum was then separated and stored at –20 °C for further studies. Sera from two non-atopic individuals were also collected for negative control. The entire study was approved by the Ethical Committee of the clinics.

2.4. Specific IgE estimation

Qualitative detection and quantitative estimation of specific IgE in patients' sera (+1 to +3 against RO crude extract) was performed by indirect ELISA [16]. Wells of multisorbent ELISA plates (Nunc, Thermo) were coated with 10 ng/µl crude protein extract (50 µl/well) of RO prepared with PB and incubated overnight at 4 °C. The wells were then washed with 100 µl of 0.1 M PB saline (pH 7.3) plus 0.5% Tween20 (PBS-T) for three times (15 min each) and was followed by blocking with 50 µl 1% Bovine serum Albumin (Sigma) in PBS-T for 3 h at 4 °C. After washing with PBS-T for another three times, wells were incubated with 50 µl of individual patients' sera diluted (1:5) with blocking solution (PBS-T-BSA) at 37 °C for 16 h. Further washing with PBS-T was followed by incubation with 50 µl of 'monoclonal anti human IgE-Alkaline Phosphatase conjugate' produced in mouse (Sigma, St Louis, Mo), diluted 1000 times with blocking solution at 37 °C for 3 h. After final washing with PBS-T, 50 µl of para-Nitro phenyl phosphate (pNPP) liquid substrate system for ELISA (Sigma, St Louis, Mo) was added in each well for incubation at room

temperature strictly for 25 to 30 min in the dark. After color development, the reaction was stopped by adding 3(N) NaOH and OD was taken at 405 nm in an ELISA reader (Multiscan-Labsystem, Finland). The average OD value of the replica for each patient's serum was calculated and the ratio of this patient's OD (P) and mean of OD values of all the normal subjects (N) was determined. For a particular serum, P/N ratio greater than 2.5 was considered as in vitro "positive" with markedly elevated level of specific IgE.

2.5. Total protein extraction and profiling

2 g of lyophilized spore-mycelial mat of RO was crushed in liquid N₂ and mixed with 10 ml Y-cell lysis kit (Sigma) containing Protease inhibitor cocktail (Sigma, St Louis, MO) and was incubated for 3 h at 4 °C with continuous stirring periodic sonication. Clear supernatant was collected after centrifugation of the mixture at 15,000×g for 20 min at 4 °C and then extensively dialyzed in dialysis membrane (10 kDa cut-off, Sigma) against sterile MiliQ water for 24 h. Total protein was precipitated with 95% ammonium sulfate cut and then the protein solution was salted out by dialysis again in the same dialysis membrane. After which the proteins were pelleted by centrifugation at 15,000×g for 30 min at 4 °C, which was finally dissolved in 3 ml 0.1 M PB (pH 7.2) and was dialyzed for 2 h using Tube-O-Dialyzer (G-Biosciences, USA) to remove the trace of ammonium sulfate. Protein content was estimated using Bradford reagent (Bio-Rad Laboratories).

About 80 µg of total protein was resolved in 12% SDS-polyacrylamide gel electrophoresis under denaturing condition using a vertical gel electrophoresis apparatus (Amersham, Sweden) along with a medium size protein molecular weight marker (GeNei, Bangalore, India). The protein bands were observed by CBB-R250 staining.

2.6. IgE specific Western blot analysis

First dimensional specific IgE immunoblotting was used to detect the allergenic components in crude protein mixture and to determine their apparent molecular weight [17,18]. Resolved total protein profile was transferred from (7 cm×10 cm) SDS-PAGE to a High Bond LFT PVDF membrane (GE Lifesciences, USA) by semi dry transfer method at a constant current of 1.2 mA/cm² for 1 h using Amersham TE-70 ECL Semi-Dry Transfer unit (Amersham, Sweden). Membrane was air dried and cut into 1 mm strips. The transfer was verified by staining a strip with Swift membrane stain (G Biosciences, USA). Unstained membrane strips were blocked with 3% BSA in Tris buffer saline (pH 7.5) plus 0.05% Tween-20 (TBS-T) for 3 h at 4 °C followed by washing with TBS-T three times, for 15 min each. Primary antibody incubation was done using ten individual patient's sera with elevated level of specific IgE in 1:10 dilution in blocking solution for overnight at 4 °C. Repeated washing with TBS-T was followed by incubation with secondary antibody, i.e., monoclonal anti-human IgE-alkaline phosphatase conjugate (Sigma), diluted with blocking solution (1:1000). Reactive bands were observed by addition of NBT-BCIP (Sigma) and the reaction was stopped by adding 0.5 M EDTA.

2.7. Periodic Acid Schiff staining

Total protein was resolved in 12% SDS-PAGE and PAS staining was performed to detect glycoprotein using a Glycoprotein Staining Kit (G Biosciences, USA) following the manufacturer's protocol.

2.8. Protein isolation for fungal proteome analysis

For proteomic study, total protein was isolated following a combination of TCA-acetone [19] precipitation method and Tris-phenol extraction method [20]. 500 mg of lyophilized spore mycelia mat was homogenized in 6 ml of lysis buffer (0.05 M Tris-HCl pH 8.0, 2% SDS, 50 mM DTT, 5 mM EDTA, 0.001% PMSF and 100 µl/10 ml Protease inhibitor cocktail (Sigma, St Louis, Mo)), mixed thoroughly for 1 h at 4 °C and heated at 95 °C for 10 min. This mixture was then centrifuged at 20,000×g for 20 min and the supernatant was collected. The cellular pellet was again resuspended in same amount of lysis buffer and previous step was repeated. All the supernatants were pooled and mixed with 40 ml 20%TCA in pre-chilled acetone and 0.01% DTT. Proteins were precipitated for overnight at –20 °C. Protein pellet was obtained by centrifugation at 40,000×g for 30 min. The pellet was washed twice with chilled washing buffer (acetone+0.01% DTT) and finally the protein pellet was dissolved in 1 ml of buffer containing 0.5 M Tris-HCl (pH 8.3), 2% (v/v) Nonidet P-40, 20 mM MgCl₂, 2% (v/v) β-mercaptoethanol, 700 mM sucrose and 1 mM PMSF. Insoluble materials were removed by centrifugation. This sample was then mixed with an equal volume of Tris-HCl (pH 7.8) buffered phenol. The mixture was homogenized for 30 min at 4 °C and finally centrifuged at 10,000×g for 30 min. The proteins in the upper phenol phase were precipitated with five volumes of 0.1 M ammonium acetate in methanol overnight at –20 °C and pelleted by centrifugation at 15,000×g for 30 min at 4 °C. The protein pellets were washed twice with ice-cold methanol followed by multiple pre-chilled acetone washes. Final pellet was dissolved in 100 µl of rehydration (IEF) buffer (without DTT and ampholytes) containing 7 M Urea, 2 M thiourea and 4% CHAPS and stored at –80 °C. Protein content was estimated using Bradford based 2D quantification reagent (Bio-Rad Laboratories).

2.9. Two dimensional gel electrophoresis

120 µg IEF sample protein was processed thoroughly using Focus Perfect 2D Clean up Kit (G Biosciences, USA) and reconstituted freshly in 125 µl IEF buffer. 1% immobilized pH gradient (IPG) pH 3–10 linear buffer (v/v) (GE Healthcare, Uppsala, Sweden), 25 mM 1,4-dithiothreitol and traces of Bromophenol blue was added freshly. A short spin at 40,000×g was given to the sample to remove the insoluble material, if any. Rehydration loading was done with 7 cm Immobiline Dry Strips (IPG strip, pH 3–10 L) (Amersham Biosciences, San Francisco, CA, USA) in a re-swelling tray for overnight at room temperature. On the next day, Isoelectric focusing was performed using Ettan IPGphor 3 isoelectric focusing system (GE Healthcare) at 18 °C in gradient mode. Briefly, 7-cm strips were focused at 0–200 V for 1 min, 200–3500 V for 1.30 h and 3500 V for 1.15 h, with a total of

8 kVh accumulated. After focusing, the strips were stored at -80°C for future use. Before second dimension electrophoresis, the strips were equilibrated in equilibration buffer-I (6 M Urea, 75 mM Tris-Cl pH 8.8, 30% glycerol, 2% SDS and 1% w/v DTT) for 15 min followed by equilibration buffer-II (same as equilibration buffer-I with 2.5% w/v iodoacetamide instead of DTT). Finally, strips were run in 12% SDS-PAGE with miniVE Vertical Electrophoresis System (GE Healthcare). Gels were stained with Coomassie Brilliant Blue-G250 and photographed in a Bio-Rad Versa Doc (Bio-Rad Laboratories) system.

2.10. 2D immunoblot

A 7 cm $\times$ 10 cm 2D gel was prepared following the above protocol, only skipping the equilibration step with equilibration buffer-II. Semi dry transfer on PVDF membrane was done following the same procedure as described for the 1D blot. After transfer, the 2D membrane was immunoblotted with pooled sera collected from atopic patients and another blot was developed with sera from healthy subjects as negative control (see Supplementary Fig. 1).

Molecular weight of IgE reactive bands and spots were determined using Quantity One® software (version 4.6.3, Bio-Rad Laboratories, USA).

2.11. Deglycosylation study of allergens

Periodate modification of 2D blot was performed following the method of Peterson et al. [21]. For periodate modification of the carbohydrate moiety, the PVDF membrane of the 2D blot was incubated in 10 mM sodium acetate buffer (pH 5.0) containing 50 mM sodium meta periodate for 3 h in the dark. Inactivation of periodate was done by adding sorbitol to a final concentration of 0.015 mol/l with a drop of ethylene glycol, after which the membrane was incubated with sodium borohydride solution (1 mg/ml) for 12 h at 4°C . The membrane was acidified with 0.001% acetic acid followed by repeated washing in TBS-T and then blocked with 3% BSA. Western blotting was done with pooled patient sera in 1:10 dilution in blocking solution.

2.12. Sample preparation for mass spectrometry

For MALDI analysis sample preparation was performed as previously described by Shevchenko et al. [22] with a slight modification. Briefly, the spots corresponding to IgE reactive spots on 2D blot were picked up from 2D gel along with a blank piece of gel devoid of protein spot and destained in 50 mM Ammonium bicarbonate (pH 8.0) with 50% ethanol followed by periodic dehydration and rehydration in Acetonitrile and reduction buffer containing DTT as well as alkylation buffer containing Iodoacetamide (Sigma, St Louis, MO, USA). Digestion was carried out in 12.5 ng/ μl modified sequencing grade Trypsin Gold (Promega, Madison, WI, USA) at 37°C for 16 h. Tryptic fragments were eluted from gel pieces by vigorous vortexing in extraction buffer containing 1% TFA. Final volume of the sample was reduced 10 times to remove Acetonitrile in Speed Vac (Thermo Fisher, USA). 1.5 μl of peptide digests were mixed with 5 volumes of 0.5 mg/ml α -cyano-4-hydroxycinnamic acid (HCCA) matrix solution

(Bruker Daltonics, Germany) and was spotted on MTP 384 ground steel target plate (Bruker Daltonics, Germany) and air dried.

2.13. MALDI-TOF MS analysis

Each gel spot was analyzed on separate MALDI spots. Mass spectra were obtained in Autoflex II MALDI TOF/TOF (Bruker Daltonics, Germany) mass spectrometer in linear mode equipped with a pulsed N₂ laser ($\lambda=337\text{ nm}$, 50 Hz) at 54% power in the positive ion mode. Peptide monoisotopic signals were analyzed in the FlexAnalysis software (version 2.4, Bruker Daltonics). The processed peaks were transferred through the MS BioTools™ (version 3.0) program as inputs to the MASCOT (<http://www.matrixscience.com>) search engine version 2.2 (Matrix Science, Boston, MA, USA) for protein identification. The following parameters were set for MASCOT-search: database — NCBI nr (1,438,958 sequences) and Swissprot (30,735 sequences); taxonomy — Fungi; proteolytic enzyme — trypsin; peptide mass tolerance of 100 ppm; global modification — carbamidomethylation of cysteine; variable modification — oxidation of methionine; and peptide charge state (1+) and maximum missed cleavage of either 1 or 2. Entries from NCBI and Swissprot were downloaded on December 2011. Matrix and keratin peaks were manually eliminated. External calibration was performed using the standard peptide mixture from Bruker-Daltonics (m/z 1046.54, 1296.68, 1347.73, 1619.82, 1758.93, 2093.08, 2465.19, 2932.59, 3494.65). Internal calibration was performed using autolysis peaks from trypsin (m/z 842.509 and 2211.104).

2.14. MALDI-TOF MS/MS analysis

This analysis was done to confirm protein identification with the matched peptides. All known contaminant ions (trypsin- and keratin-derived peptides) were excluded for later MS/MS analysis. MS/MS was performed to confirm the identification with most intensive and matched peptides, selected based on their suitability for fragmentation by Collision Induced Dissociation (high signal to noise ratio and relative isolation). The parent and daughter ions were analyzed using SNAP algorithm using the FlexAnalysis software (version 2.4, Bruker Daltonics). After MS spectra acquisition, the instrument was switched in LIFT mode and precursor ions for fragmentation were selected manually. MS/MS spectra were acquired with a minimum of 4000 and a maximum of 8000 laser shots using the instrument calibration file. The precursor mass window was set automatically after the precursor ion selection. Spectra baseline subtraction, smoothing [23] and centroiding was performed by FlexAnalysis software v3.0 (Bruker Daltonics). For MS/MS, peaks with a signal/noise ratio of >20 were transferred through the MS BioTools™ program as inputs to MASCOT for peptide identification. Database search was performed setting the following criteria: maximum of one missed cleavage was allowed, the mass tolerance of precursor ions and fragments were set to 1.2 Da and 0.5 Da, respectively; allowed variable modifications were methionine oxidation and carbamidomethylation on cysteine as fixed modification, and the taxonomy was restricted to Fungi.

The confidence of identification of protein was based on following criteria: (1) probability score for identification, (2) number of unique matched peptides specific to the particular protein and (3) percentage of sequence coverage. Protein identification was validated if the ion scores of peptide matches were associated with a significance level that exceeded the threshold value of $p < 0.05$, which indicates a 95% confidence level of identification. Further, the concordance between experimental and theoretical molecular weights and/or pI was also used to validate the identifications.

De novo sequences from two tryptic peptides generated from 44 kDa allergen were deduced using BioTool 2.2 software with a RapiDeNovo extension (Bruker Daltonics49). Top high-scoring candidate sequences for MS/MS spectra were searched for similarity with proteins from the UniProt Knowledgebase (release 2012_06) using the MS BLAST search engine (<http://dove.embl-heidelberg.de/Blast2/msblast.html>) with the following settings: program — blast2p; database — Swiss-Prot; matrix — PAM30MS; expect — 100.

2.15. Bioinformatics study

2.15.1. Annotation of biological function

Sequences of all ten identified and predicted allergens of RO were acquired and functional annotation to all identified proteins was given by matching their accession numbers, obtained amino acid sequences at protein databases UniProt (www.uniprot.org) and Kognitor³⁶ [24] (www.ncbi.nlm.nih.gov/COG/grace/kognitor).

2.15.2. Prediction of signal peptide and cellular localization

The presence and location of the signal peptide cleavage site in our query sequences were checked using SignalP 4.0 server [25] (www.cbs.dtu.dk/services/SignalP/). “Subcellular Localization Prediction Tool, version 3.0”, PSORTb [26] (<http://www.psort.org/psortb/>) is a web based in-silico sequence analysis tool, which was used to analyze the amino acid sequences of identified proteins for prediction of their cellular localization.

2.16. Allergenicity assessment in silico

An efficient and comprehensive web tool, Allermatch™ developed by Fiers et al. [27] was used to analyze the potential allergenicity of the sequences of certain spots. Allermatch™ provided mainly two search methods. We used the first mode which was the sliding window approach that divided the query protein sequence into an 80-amino acid window using a sliding window with a shift of a single residue. Each of these windows was compared with all sequences in the allergen database of choice. For the major allergen we identified, a number of such web based tools were used to assess its allergenicity in silico. SDAP is a repository of structurally characterized allergenic proteins which offers a flexible interface to check the FAO/WHO allergenicity rules against all allergens from the SDAP database [28] (www.fermi.utmb.edu/SDAP). Full FASTA alignment of this protein was done with all known allergens. T-coffee (www.tcoffee.org) [29] is a multiple sequence alignment package in which the query sequence, i.e., the sequence of the allergen we identified, can be aligned with known allergens.

3. Result and discussion

3.1. Clinical study and specific IgE

A few preliminary studies were available on the allergenic significance of airborne RO [12,30], but those studies were based on skin tests and a few other clinical studies only. The present study represents the detailed analysis of allergenicity of RO and identification of its major and minor allergenic proteins. Here, positive cutaneous responses were elicited in sixty three (48%) of the 132 human subjects tested. Among the patients with positive cutaneous response, 95% had previous reports of Bronchial asthma together with or without allergic rhinitis and the remaining with allergic rhinitis only. +2 grade of positivity was found to be predominant followed by +3 and +1. Only one patient was found to be in the +4 category of skin reaction. Markedly, positive skin reactions were not observed in healthy volunteers, whose sera were used as negative control. Twenty two patients with positive skin response to RO extract had given their written consent to use their blood for experimental purposes. In specific IgE ELISA, significantly elevated IgE levels were observed in ten of those patients as compared to normal controls ($P/N > 2.5$) [see Table 1]. This result was significant enough to infer the presence of strong IgE binding proteins in this fungus. Sensitization to RO could occur in mold allergic patients by exposure to indoor and outdoor aeroallergens derived from this mold, as a consequence of extensive cross reactivity suggesting homologous IgE binding epitopes in different mold species [31–33]. In the present study, sera of these 10 patients with history of anaphylaxis to mold were used for blotting.

3.2. SDS-PAGE, immunoblot and PAS staining

Total protein profile in 12% SDS-PAGE revealed more or less forty bands within a molecular weight ranged between 14.3 and 97.4 kDa. Six IgE reactive zones viz. 15, 17, (31 to 34), (44 to 45) (both considered as a single zone), 53 and 67 kDa [Fig. 1A] were identified by IgE specific Western blotting with sera positive to RO extract. In the case of the second and third immunoreactive zones, the IgE reactive bands with the nearest molecular weight were not properly resolved in the mono-dimensional gel. Frequency of these immunoreactive bands in all the ten atopic subjects is graphically represented in Fig. 1B. First four reactive zones were found to be present in more than 70% of the total patients' sera screened by immunoblotting and hence these allergens can be considered as predominant. Overwhelming majority of sera (>90%) revealed 44 kDa IgE reactive zone in all immunoblots, hence this IgE reactive band has been considered as the immunodominant major allergen. Summing up this result more precisely, a cluster of high affinity proteins ranging from 15 kDa to 44 kDa and less pronounced clusters at 52 to 67 kDa were revealed. No clear correlation was found between reactivity in ELISA and the number of IgE reactive bands. However serum of patient nos. 8 and 9 which were highly reactive in ELISA, exhibited optimum intensity and highest number of IgE reactive bands. It is evident from the previous studies that carbohydrate moieties of allergenic glycoproteins

Table 1 – Clinical characteristics of patients with severe allergic reactions to mold and IgE reactivity to RO Antigen Extract in ELISA. AR: allergic rhinitis, BA: bronchial asthma, CU: chronic urticaria, AS: anaphylactic shock, AEDS: atopic eczema dermatitis syndrome.

Patient no.	Age (year)	Sex	Symptoms	Total IgE IU/ml	specific IgE P/N
1.	36	F	AR+BA	221	3.83
2.	42	F	AR+BA	342	2.91
3.	28	M	BA	309	3.00
4.	52	M	BA+CU	322	4.20
5.	45	M	BA+AR+AS	298	4.00
6.	43	F	AR+AEDS	466	2.90
7.	56	M	AR	456	3.10
8.	38	M	BA+AR	548	5.60
9.	25	M	AR+CU	612	4.83
10.	46	F	BA+AR+AS	386	4.42

Total IgE was estimated using 'PATHOZYME® Total IgE Estimation kit'.

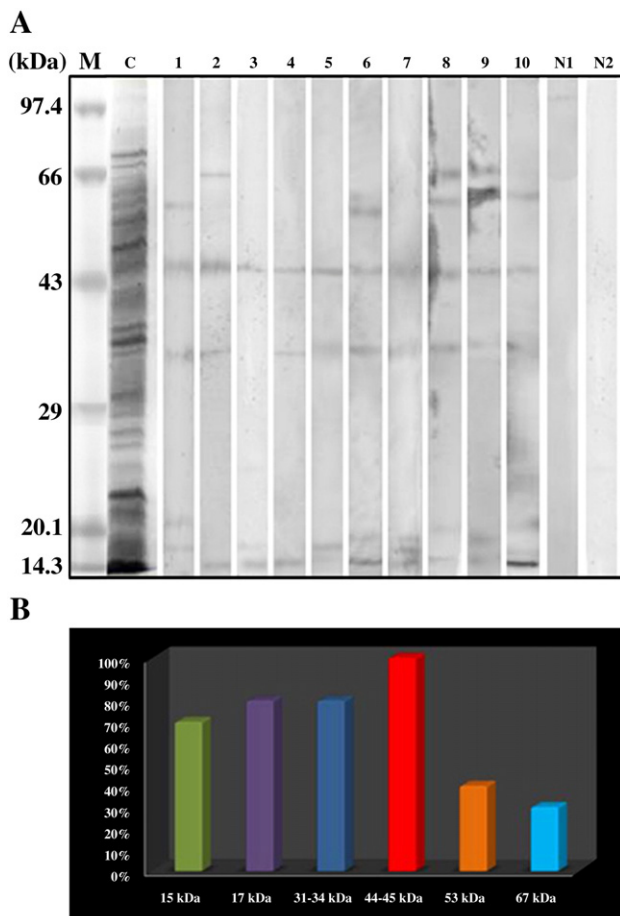


Fig. 1 – A: Total protein profiling and mono-dimensional immunoblot analysis with individual serum of ten atopic patients. Lane M — molecular weight marker, Lane C — crude protein profile of *Rhizopus oryzae* in 12% SDS-PAGE, lanes 1 to 10 — IgE specific immunoblots with ten patient's sera, lanes N1 and N2: negative control with sera of non atopic patients. **B:** Graphical representation showing the frequency of six IgE reactive bands: 44 kDa was the highest frequency and therefore considered the major allergen.

are the most abundant environmental immune determinants [34,35]. Therefore, PAS staining (Fig. 2) was customarily done in this study to detect the glycopeptidic nature of the IgE reactive proteins. Several high molecular weight proteins along with a very few low molecular weight proteins, ranging from 14 to 15 kDa turned purple upon PAS staining. Comparing the PAS stained gel with blots, it was found that the 15 kDa and 44 to 67 kDa immunoreactive bands were present in PAS stained region of the gel.

3.3. Reactivity of IgE antibodies with spots on 2D gel

Fig. 3A shows a typical 2D map of spore mycelia proteins of RO, resolved into about 310 spots within an IPG range between pH 3 and 10 horizontally and within a molecular wt. range between 14.3 and 97.4 kDa in the second dimension as visualized by CBB staining. In the present study, we developed a new method of protein extraction from filamentous fungi, which was a combination of the TCA-acetone and Tris-phenol procedure that had given better resolution and less streaking. Fig. 3B is the Western blot analysis confronted with pooled sera of 10 patients showing a specific response to the RO extract and revealing 17 IgE reactive spots. Among them, we concentrated on 14 immunoreactive zones, which were in agreement with the results of immunoblotting of one dimensional SDS-PAGE [Fig. 3A–B]. Certain IgE reactive spots were found on the 2D blot, for which no clear corresponding blue spots were observed in the 2D gel and therefore could not be identified. Due to the less availability of the serum, IgE immunoblotting of the 2D membrane could not be done with individual sera. Proteomic techniques have been proved to be a useful tool for the precise identification of allergens [36]. Recently scientists have started to use 2D maps for allergen profiling, most of the earlier works done having been executed in one-dimensional gels. It is a useful technique in which the numerous proteins would be scattered in the pI/Mr plane, thus increasing the chance of finding individual species in each excised spot and subsequent analysis by mass spectrometry. In the present experiment six immunoreactive bands as visible in one dimensional immunoblots ranging

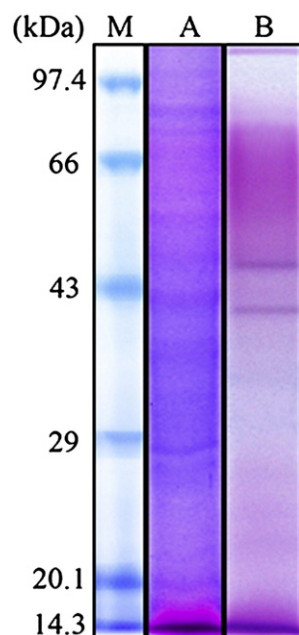


Fig. 2 – Periodic Acid Schiff staining (PAS) of total protein in 12% SDS-PAGE to detect glycoproteins. Lane M — molecular weight marker, Lane A — glycoproteins stained purple upon Periodic Acid Schiff's staining and non-glycosylated proteins stained blue by CBB-G250, lane B: only glycoprotein bands stained purple.

from 15 kDa to 67 kDa were resolved into fourteen IgE reactive spots. These are — spots 1 and 2 (mol wt. 67 kDa, pI 4.7 and 5.3), spots 3A to 3C, 4 and 13 (mol wt. 52 kDa, pI 7, 5.3 and 6), spots 5, 6, 10 and 14 (mol wt. ~43 to 44 kDa, pI 4.1, 7.5, 5 and 5.5), spots 7A to 7C, 8 and 9 (31 to 34 kDa, pI 5 to 7) and spots 11 and 12 (15–17 kDa, pI 5.9 and 6.8). Immunoblots performed with the control serum did not reveal any spot.

Fig. 3C shows the result of the deglycosylation study by metaperiodate treatment. Sodium metaperiodate is a strong oxidizing agent and exposure of a glycoprotein to this oxidant for at least 6 h may lead to complete disruption of the sugar moiety leaving the protein part unmodified. However, longer exposure to metaperiodate can also modify the protein structure. Therefore the duration of treatment was optimized for 3 h. This experiment was designed to test whether the protein part of the glycoproteins alone was able to bind with IgE antibody or whether the antigenicity was determined by the sugar moiety. As evident from the result of Periodic Acid Schiff staining, proteins having molecular weights in a range from 44 to 67 kDa along with low molecular weight proteins (15–17 kDa) were detected as glycoproteins. Therefore in the 2D map, except spots 7, 8, and 9, the remaining eleven spots had their mol. wt. corresponding to glycoproteins detected in one dimensional SDS-PAGE. We tried to stain the glycoproteins on a 2D gel but no significant result was obtained, possibly due to chemical modification of the glycan parts by TCA and/or phenol during sample preparation, which might have interfered with staining of aldehyde groups by Schiff's reagent. A membrane after periodate modification, was immunoblotted with pooled sera and it was found that except

for spot 13, the remaining ten spots (1–6, 10–12 and 14), in spite of losing glycol structure, were still retaining their IgE binding properties. This implicated the presence of non-glycosidic but peptidic nature of the epitopes. Spot 13 (53 kDa) could not be detected in immunoblotting after periodate modification. Potential loss or reduction in reactivity of this protein could be attributed to modification of carbohydrate cross reactive determinant and changes induced upon metaperiodate treatment. Periodate treatment of a major mugwort glycoprotein, Art v1, resulted in a loss of IgE binding property [37]. Carbohydrate conjugates can also promote specific Th-2 responses, resulting in increased IgE production [38]. Spot 8 was a non-glycosylated, IgE reactive protein. So theoretically, metaperiodate should not have had any effect on its IgE binding property. However this 31 kDa protein was not detected by immunoblotting. This observation led us to hypothesize that there had been periodate mediated modification of the protein per se, resulting in a deformed peptide epitope. Spots 7 and 9 are non-glycosylated proteins, so as expected these proteins were nicely detected in the immunoblot even after periodate treatment. During the deglycosylation study, the signals of non-glycosylated proteins as well as the glycoproteins with peptidic epitopes in the immunoblots were in general weaker than what we observed in the one-dimensional or normal two-dimensional immunoblot. According to Fasoli et al. [39], polypeptides assume a random coil conformation due to the strong denaturing effects of urea and thiourea, present in the rehydration buffer. Moreover the strong oxidizing effect of metaperiodate results in the loss of structural motifs of proteins which in turn reduces their ability to interact with IgE and as a consequence the signal intensity was considerably reduced.

3.4. Characterization of allergens by mass spectrometry and bioinformatics

Mass spectrometric identifications of labeled spots (Fig. 3D) are listed in Table 2. Table 2 is basically derived from a Master Table (see Supplementary Table 1) which represents the top three hits identified in each run with each of the 14 spots. The best hit was selected as a valid identification based on the highest probability score (lowest expectation value), the highest percentage of sequence coverage and the highest number of unique matched peptides (minimum 2). The upper panel of Table 2 comprises ten spots identified as proteins having homology with proteins from RO and some with proteins from other fungal species. Their biological function, subcellular localization and presence or absence of signal peptides was listed in Table 2. MASCOT search of the sequenced files from available databases (NCBI, SwissProt) led to the identification of a total of 10 proteins with significant probability scores. Sequence coverage and number of unique peptides in the identified proteins ranged between 12–44% and 2–4 respectively.

The lower panel of Table 2 represents spots 1, 4, 7 and 12 identified as hypothetical protein. Possible reason for getting unidentified immuno-reactive proteins may be that we used a less explored complex proteome of RO for immuno-proteomics studies. The sequences of these four proteins were retrieved from the NCBI database and sequence alignment was done with all known well characterized allergens

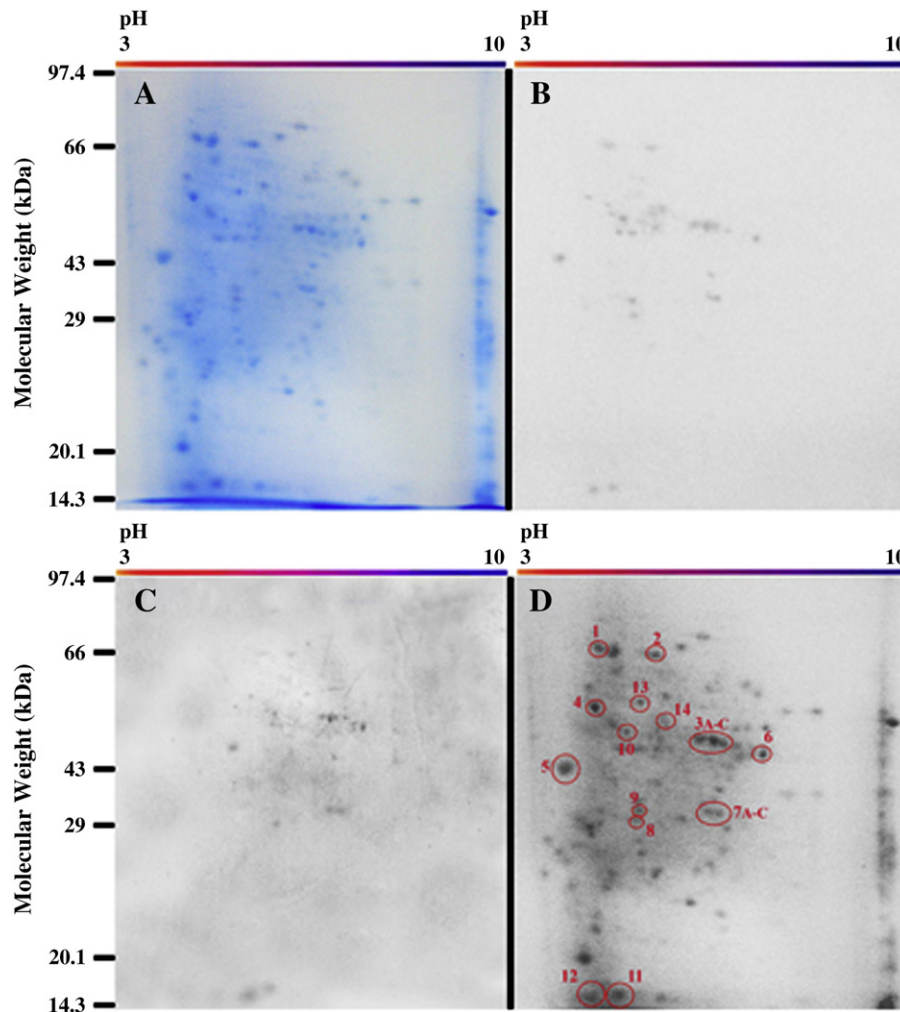


Fig. 3 – Identification of IgE reactive proteins from the two dimensional map of *Rhizopus oryzae*. A: 2D gel profile of the total protein extracted from *R. oryzae* stained with CBB-G 250. B: IgE specific immunoblots revealed IgE reactive spots. C: Periodate modification of IgE specific immunoblots for deglycosylation study. D: A preparative 2D gel with IgE reactive spots identified and marked with red circles.

using the Allermatch™ algorithm with an 80 amino acid sliding window and a 35% cutoff value. The alignment search revealed a significant percentage of ‘hits identity’ for only spot 7 with a 29 kDa IgE binding protein Cand a 3 from *Candida albicans* [40]. Although the protein score of other three spots were significant, due to lack of homology with any protein of RO or known allergens, these were not considered for further analysis.

Spot no. 7 appeared as a string of three spots in the Coomassie stained gel, (and gave immuno-reactivity in immuno-blots) and an additional spot (labeled as 7A) to the left of the originally excised spot 7B, was further analyzed and identified as an identical protein. The same applies to spot no. 3 which also appeared as a string of three spots. In this case two additional zones (marked 3A and 3C), to the left and right of the originally excised spot 3B, were also analyzed and identified as citrate synthase (see also Table 2). It is reasonable to assume that, in both cases, these strings of spots represent a sort of heterogeneity arising from genetic variants and/or post-translational modifications of the same gene product [39], although the

precise nature of the modification had not been ascertained here.

The present study had added a noticeable number of allergens to the meager list of mold allergens reported so far, namely: fructose-bisphosphate aldolase, mannitol dehydrogenase, mitogen-activated protein kinase, citrate synthase, ubiquitin-protein ligase, pre-mRNA-splicing factor, 40S ribosomal protein s6-like, eukaryotic aspartyl endopeptidase, eukaryotic translation initiation factor 2-gamma subunit and cyclophilin; some of them are known allergens or members of allergen families in a variety of different fungal systems and others have been reported for the first time in this study. These newly reported allergens include a 53 kDa subunit of eukaryotic translation initiation factor, product of a house-keeping gene involved in protein synthesis. An elongation factor beta subunit was reported in a previous study to be IgE reactive [41]; however another new component of translation machinery has been identified to have the same property. The theoretical pI value of this gamma subunit is 8.52 whereas in the 2D gel this spot was observed at pI-4. This discrepancy led

Table 2 – Identification of IgE reactive proteins by mass spectrometry (MS/MS) from RO proteome; for all hypothetical proteins the source organism is *Rhizopus oryzae*.

Spot no.	Accession no. (NCBI)	Accession no. (UniProt)	Protein name (organism)	Unique peptides	Sequence cov. (%)	Probability score [*]	Gel mass/pI	Th. mass/pI	Biological function	Cellular location
2	gi 242777061	B8M317	Ubiquitin-protein ligase Ufd4 (<i>Talaromyces stipitatus</i>)	2	21	1E–30	67/5.1	204.5/5.15	Ligase	Cyt
3	gi 300078724	D9IWB3	Citrate Synthase (<i>Rizopus oryzae</i>)	4	44	9.3e–06	52/7.0	52.4/7.25	Transferase	Mt
4	gi 164427695	Q8WZV6	Eukaryotic translation initiation factor (<i>Neurospora crassa</i>)	2	26	2.10E–11	52/5.3	55.1/8.52	GTPase	Cyt
5	gi 384498765	I1CLC6	Endopeptidase (<i>Rizopus oryzae</i>)	4	44	0.0017	43/4.1	44.0/4.70	Hydrolase,	Non-cyt
6	gi 24637970	Q4PC06	Mitogen activated protein Kinase (<i>Ustilago maydis</i>)	2	19	2.02E–12	45/7.5	41.0/5.2 6	Transcription factor	Cyt
8	gi 341038837	G0RY43	40S Ribosomal protein S6 like (<i>Chaetomium thermophilum</i>)	3	13	2.12E–11	28/5.2	27.5/10	Ribonucleo-protein	Cyt
9	gi 384496603	I1CF64	Fructose-bisphosphate aldolase (<i>Rizopus oryzae</i>)	3	24	2E–11	31/5.0	36.37/5.19	Lyase	Cyt
12	gi 384499442	POC1I3	Cyclophilin (<i>Rizopus oryzae</i>)	4	22	1E–30	17/6.8	19.8/6.96	Protein folding	Cyt
13	gi 229890359	A1CLZ5	Mannitol 2-dehydrogenase (<i>Aspergillus clavatus</i>)	3	18	7E–10	52/6.0	56.8/6.0	Oxidoreductase	Mt
14	gi 18202094	O59800	Pre-mRNA-splicing factor slt11 (<i>Schizosaccharomyces pombe</i>)	2	17	3E–10	44/5.5	40.23/9.0	mRNA processing	Nu
1	gi 384496365	I1CEH6	Hypothetical protein	2	15	5e–19	67/5.1	58/6.56	Unknown	Nu
7 (A, B)	gi 384496952	I1CG63	Hypothetical protein	2	12	5e–13	36/7.0	32/6.9	Unknown	Cyt
10	gi 384496803	I1CFR4	Hypothetical protein	2	14	7e–12	44/5	43/5.9	Unknown	Nu
11.	gi 384497157	I1CGR8	Hypothetical protein	5	38	4e–17	15/5.9	15/5.96	Unknown	Cyt

^{*} Probability score: probability of protein identification in terms of expectation value.

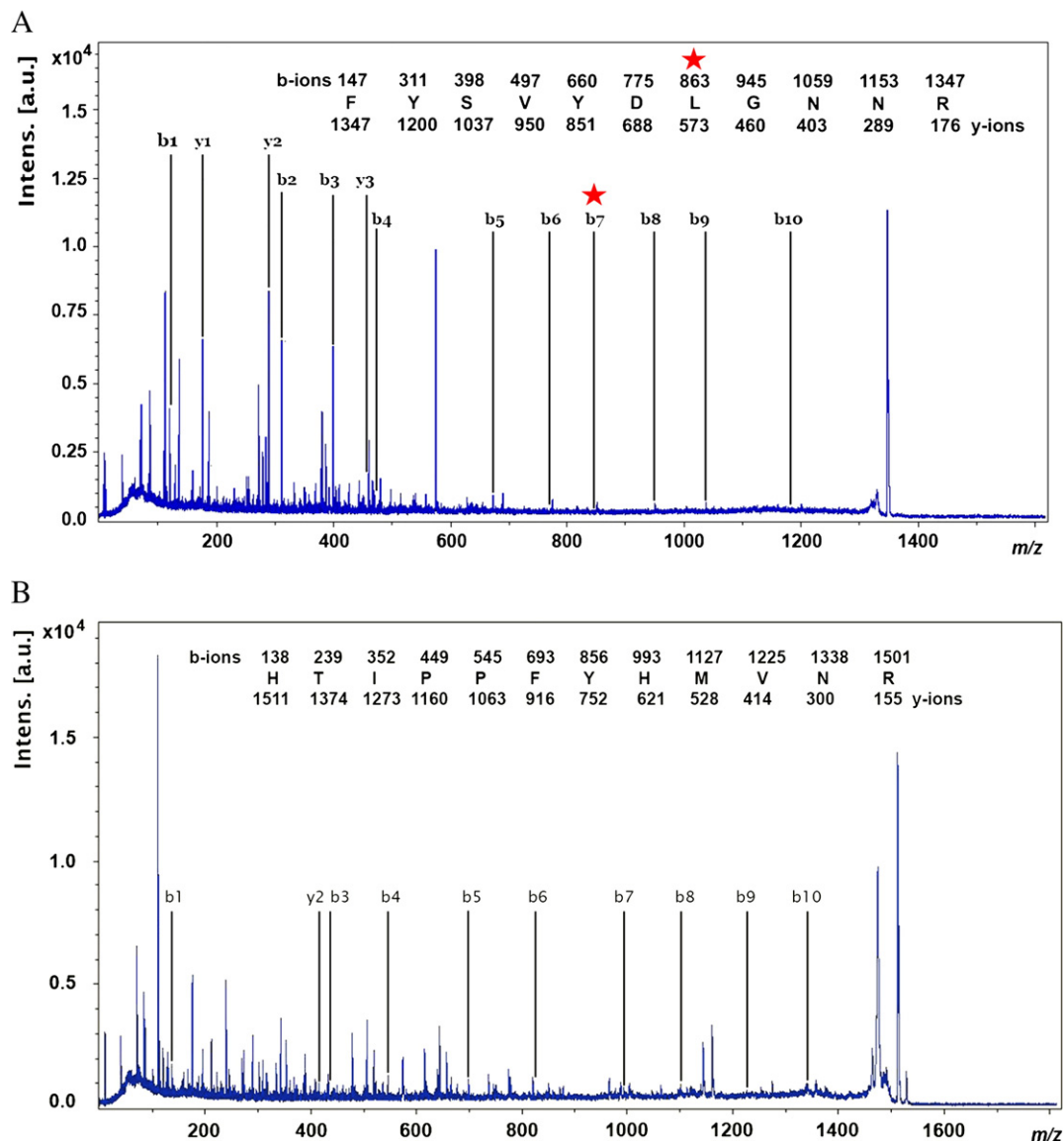


Fig. 4 – MS/MS spectra with m/z A. 1347.691 and B. 1511.842 — generated by trypsin digestion of the 45 kDa major allergenic spot (aspartyl endopeptidase). Loss of ammonia indicated by (*). Amino acid sequences deduced by de novo sequencing are shown above the two spectra. All ions were observed in spectral analysis.

us to hypothesize that the subunit has undergone post translational modification such as phosphorylation [42] required for its function like, positive regulation of translational fidelity. A 68 kDa protein showed significant probability score and acceptable sequence coverage with a putative ubiquitin-protein ligase from *Talaromyces stipitatus*, a member of HECT domain family (PF00632) [43] which modifies a protein by poly or rarely mono-ubiquitination making the substrate protein for proteasomal degradation. Spot 3 appeared as a string of three spots all of which were IgE reactive. We analyzed two of them by MS and both were identified as citrate synthase. This enzyme bears a carbohydrate moiety, which is not an antigenic determinant. Identification of more than one spot as the same protein implies the presence of expressed isoforms of this enzyme. Spots 9 and 13 were identified as fructose-bisphosphate aldolase and mannitol 2-dehydrogenase with significant probability scores.

Mannitol dehydrogenase is a member of the short-chain dehydrogenases/reductases family (AllFam: AF028) [44] from which this protein was earlier reported as having an IgE reacting property such as Alt a 8 from *Alternaria alternata* [45] and Cla h 8 from *Cladosporium herbarum* [46]. Fructose-bisphosphate aldolase is a glycolytic enzyme that catalyses the reversible aldol cleavage or condensation of fructose-1,6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde 3-phosphate [47]. This is a class II aldolase (AllFam: AF169), predominant in fungi. One such aldolase allergen (Cand a FPA₁) was identified from *C. albicans* [48]. Spot-13 was a glycoprotein and its allergenicity was determined by the carbohydrate structure. Chemical modification of the glycan part of this molecule resulted in complete loss of IgE binding property. Cyclophilins are important pan-allergens described from a number of mold and pollen sources [49]. A 17 kDa IgE reactive cyclophilin was identified from RO proteome. BLAST analysis of this cyclophilin



respectively [51]. A 53 kDa RO protein with homology to 40S ribosomal-S6 like protein from *Chaetomium thermophilum* was detected showing up to 13% sequence coverage in 3 matched peptides. Until now, this protein has been reported only once as a potent allergen associated with *Aspergillus* mediated asthma [52], where an S3 form of this protein binds to the IgE

of ABPA patients. Anticipated potential of these two proteins as allergens could be the subject of thorough validation studies.

A 31 kDa spot (no. 7) was identified as a hypothetical protein RO3G_07202 (gi|384491301) with 30% sequence coverage in 4 matched peptides. Sequence alignment search of this protein with known allergens using the Allermatch™ tool revealed significant homology with a 29 kDa IgE binding protein (wa_Cand_a_3) from *C. albicans*. The sequence of this recombinant allergen 'wa_Cand_a_3' [40] was analyzed in silico and a conserved domain was found which suggests that this protein could belong to the Thij/PfpI family.

A 44 kDa IgE reactive protein (spot 5) was detected at the acidic end of the 2D gel. Based on one dimensional immunoblotting, we considered a 44 kDa immunoreactive band as a major allergen and for that reason, special emphasis was given to this 44 kDa spot to assess its allergenicity in silico. We identified this protein as an endopeptidase from *R. oryzae* (NCBI entry gi|384498765) with a sequence coverage of 44% in four matched peptides. It belongs to a known allergen protein family as specified by PFAM (<http://www.sanger.ac.uk/software/pfam>) [53] and AllFAM (<http://www.meduniwien.ac.at/allergens/allfam>) [44]; and this protein was reported as a member of aspartic protease family. Results of the analysis by SignalP 4.0 server predicted that the protein contains a signal peptide which is cleaved off at a position between the 20th and the 21st residue resulting in a matured peptide. The sequence of this protein was retrieved from the NCBI database; and a PFam database search was performed. This protein was found to have significant matching (E value: 2e-114) with Eukaryotic Aspartyl Protease (PF00026 and AllFam:AF004).

A number of aspartyl proteases, which contain two conserved aspartate residues in their catalytic centers [54] have been reported from several fungal sources including *Aspergillus fumigatus* [55], *Aspergillus saitoi* [56], *C. albicans* [57], *Cryphonectria parasitica* [58], *Rhizomucor miehei* [59] and also from the German cockroach [60] and Japanese cedar [61]. Through an 80-amino-acid, sliding window approach this protein sequence was used for alignment with known allergens using the Allermatch™ tool [27] and a significant match (more than 35% homology) was found with aspartic protease Bla g 2 from *Blattella germanica*. Full FASTA alignment was done for a novel allergen with SDAP database which contains structurally characterized food and respiratory allergens. Acceptable similarity was observed with two allergens viz Bla g2 and Asp f10.

Cross-reactivity is caused by homologous proteins containing conserved sequence motifs, which are IgE-binding epitopes [62] and cross-reactive allergen species can be identified by homology based search with peptides identified in MS/MS analysis of the tryptic digests. However ambiguities are present for protein sequences obtained from MS/MS spectra as a result of C-terminal proline missed cleavage of trypsin and incomplete series of 'b'-type and 'y'-type fragment ions. Therefore, in our study, sequence assignment of two tryptic peptides generated from 44 kDa allergen was critically inspected [Fig. 4A and B]. Two precursor ions [M+H]⁺ at *m/z* 1347.691 and 1511.842 were selected for de novo sequencing. The de novo sequencing software confirmed the peptide

sequences (HTIPPFYHVMNR) and (FYSVYDLGNRR) respectively, with high scores (de novo score 8204 and 7718 respectively). MS-BLAST analysis was done in the UniProt database with these two short peptides and both of which showed a perfect match with *R. oryzae* (strain RA 99-880) endopeptidase. The first one is a characteristic peptide fragment which spans from 205 to 217 in the amino acid sequence of *R. oryzae* aspartyl endopeptidase and the later fragment is a conserved sequence found in other homologous allergens with its amino acid position starting from 383 to 394. Both of these peptides were predicted to be potential B-Cell epitopes as analyzed by ABCpred server (<http://www.imtech.res.in/raghava/abcpred/>) [63]. Amino acid sequence of this protein was aligned to known allergens of similar type from fungal and insect origins such as Asp f10, Asp sa AP and Rhi m AP respectively using T-Coffee (www.tcoffee.org), Jalview Version 2 tool [Fig. 5]. This cluster alignment showed good coverage especially at the C-terminus of the protein.

As an additional remark, it must be noted that, for some of the proteins theoretical pI values are distinct enough from what we observed in gel. Post-translational modifications, such as phosphorylation, deamidation or other charge-modifying variations along the polypeptide chain should be taken into account to explain this observation.

4. Conclusion

In conclusion, the present study describes the allergenic significance of airborne *R. oryzae* and its proteins with respect to their IgE binding property. A panel of 14 allergens has been identified from its proteome and their in vitro immunoreactivity was tested by various means. All these potent new allergens should be further studied as single proteins for clinical use and to identify their B and T-cell epitopes. In this proteomic investigation, a 44 kDa IgE-reactive protein was detected as major allergen and identified as aspartyl endopeptidase, based on mass spectrometry. Furthermore, purification, recombinant expression and immunological characterization of this 44 kDa allergen will improve the diagnosis and desensitization therapy of mold allergy.

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SUUPPLEMENTARY TABLE:1 Proteins identified as first three Hits in each run with each of the 14 spots

Spot No.	Hits	accession no. (NCBI)	protein name (Organism)	Unique peptides	sequence cov. (%)	Score
1	A	gi 384496365	Hypothetical Protein (<i>Rhizopus oryzae</i>)	3	15	5e-19
	B	gi 400599323	Hypothetical protein BBA_04320 (<i>Beauveria bassina</i>)	1	8	2.5
	C	gi 396483506	Similar to nucleolar essntial protein1 (<i>Leptosphaeria maculans JN3</i>)	1	7	3.9
2	A	gi 242777061	ubiquitin-protein ligase	2	21	1E-30
	B	gi 336371371	Hypothetical protein SERLA73DRAFT_179855 (<i>Serpula lacrymans</i> var. <i>lacrymans</i>)	1	8	3.8
	C	gi 3478322410	Hypothetical protein (<i>Botryotinia fuckeliana</i>)	1	4	6.4
3	A	gi 300078724	Citrate Synthase (<i>Rhizopus oryzae</i>)	4	44	9.3E-06
	B	gi 387510900	Hypothetical protein TBLA_0A05490 (<i>Tetrapisispora blattae</i> CBS 6284)	1	15	18
	C	gi 226290147	U1 zinc finger domain-containing protein (<i>Paracoccidioides brasiliensis Pb18</i>)	2	2	74
4	A	gi 164427695	Eukaryotic translation initiation factor (<i>Neurospora crassa</i>)	2	26	2.10E-11
	B	gi 3933220298	Chord domain containing protein (<i>Fomitoporia mediterranea</i>)	1	12	4.6E+02
	C	gi 396494637	Predicted protein (<i>Leptosphaeria maculans JN3</i>)	1	6	3.8E+02

5	A	gi 384498765	Endopeptidase (<i>Rhizopus oryzae</i>)	4	44	0.0017
	B	gi 388579913	Hypothetical protein WALSEDRAFT_20975, partial (<i>Wallemia sebi</i>)	2	14	9.3
	C	gi 396494637	4 amino-4-deoxychrosimate lyase (<i>Schizosaccharomyces pombe</i>)	1	8	18
6	A	gi 24637970	Mitogen activated Protein kinase (<i>Ustilago maydis</i>)	2	19	2.02E-12
	B	gi 388579913	Hypothetical protein WALSEDRAFT_20975, partial (<i>Wallemia sebi</i>)	1	10	9.3
	C	gi 396494637	4 amino-4-deoxychrosimate lyase (<i>Schizosaccharomyces pombe</i>)	1	11	18
7	A	gi 384496952	Hypothetical Protein (<i>Rhizopus oryzae</i>)	2	12	5e-13
	B	gi 344230216	PH domain like protein (<i>Candida tenuis</i>)	1	4	10
	C	gi 164427540	Hypothetical protein NCU03015 (<i>Neurospora crassa</i> OR74A)	1	4	46
8	A	gi 341038837	40S Ribosomal protein S6 like (<i>Chaetomium thermophilum</i>)	3	13	2.12E-11
	B	gi 255946634	Pc22g00390 (<i>Penicillium chrysogenum</i>)	1	5	29
	C	gi 14164385	NCS homolog (<i>Magnaporthe grisea</i>)	2	2	4.3E+02
9	A	gi 384496603	fructose-bisphosphate aldolase (<i>Rhizopus oryzae</i>)	3	24	2E-11
	B	gi 375165994	hypothetical protein PGTG_21328 (<i>Puccinia graminis</i>)	2	11	5.9
	C	gi 336390103	Hypothetical protein SERLADRAFT_456126 (<i>Serpula lacrymans</i> var. <i>lacrymans</i> S7.9)	1	9	8.5

10	A	gi 384496803	Hypothetical Protein (<i>Rhizopus oryzae</i>)	2	14	7e-12
	B	gi 342865733	Hypothetical protein FOXB_17592 5.9 (<i>Fusarium oxysporum</i>)	1	11	4.5E+02
	C	gi 323349701	Tat1P (<i>Saccharomyces cerevisiae</i> Lalvin QA23)	1	4	6.9E+02
11	A	gi 384497157	Hypothetical Protein (<i>Rhizopus oryzae</i>)	5	38	4e-17
	B	gi 402078309	Hypothetical protein GGTG_07430 (<i>Gaeumannomyces graminis</i> var. <i>tritici</i>)	1	12	2.1e+02
	C	gi 310799019	C2H2 type zinc finger domain-containing protein (<i>Glomerella graminicola</i>)	1	7	4.8e+02
12	A	gi 384499442	Cyclophilin (<i>Rhizopus oryzae</i>)	4	22	1E-30
	B	gi 302416223	Hard surface induced protein (<i>Verticillium albo-atrum</i>)	2	6	5.9E+03
	C	gi 359377998	Piso0_003798 (<i>Millerozyma farinosa</i>)	1	4	2E+03
13	A	gi 229890359	Mannitol 2-dehydrogenase (<i>Aspergillus clavatus</i>)	3	18	7E-10
	B	gi 157932750	beta tubulin (<i>Amanita muscaria</i>)	1	5	1.7e+02
	C	gi 50420393	DEHA2D06314p (<i>Debaryomyces hansenii</i> CBS767)	1	2	8.1E+02

14	A	gi 18202094	Pre-mRNA-splicing factor slt11 (<i>Schizosaccharomyces pombe</i>)	2	17	3E-10
	B	gi 367017640	Hypothetical protein TDEL_0H02480 (<i>Torulaspora delbrueckii</i>)	1	6	1.3E+03
	C	gi 330912484	Hypothetical protein PTT_04139 (<i>Pyrenophora teres f. teres</i> 0-1]	2	3	1.7E+03

Supplementary Table-2: Identification of Peptide sequences from all the Proteins reported in upper panel of Table-2 using MALDI-TOF-TOF

Peptide Sequences	Protein Accession No.	Ion Score
RLREILGQLRMK	B8M317	42
RVVEQACLCVSRIVESFRNKPEK		38
KYLWDTLNGGQVVPGYGHAVALR	D91WB3	54
KEYGSKVLGE VTVDQAYGGMR		56
KTLHPMSQFSLAI NALQHESQFAK		62
KVLGEVTVDQAYGGMR		64
KALLPIANRPMVWYPLDFCYR	Q8WZV6	28
RKVAYPEGVKPRTTITKQDSLVAADNVTQVEK		32
KGYWEITM ENIK	I1CLC6	53
KFYSVYDLGNR		69
KHTIPPFYHVMNR		64
KEPGLTFFAK		54
RGLKYVHSAGVVHR	Q4PC06	32
RERVPFSSQKFKNADPMALDLLEK		34
RKLLNYAKENK	I1CF64	53
KAIDNKNQEASILGAVAGAQQYVR		58
RRFSEITDLFSIAAAFGNVHGVYAPGNVK		60
KSNQVERPVVFFDISIGDVPVGR	P0C1I3	50
RVIKDFMVQGGDFIK		58
KHVVFGRLLVDGLLTLR		62
KGDGTGAMCIYGGDR		64
RDALASQDNLYTVIERSAK	A1CLZ5	48
KEHPDIQFDLNPVNENKPR		36
KIPQFIMPSIAEAIWVTAPFR		38
KTQICVGCAR	O59800	32
RLKVQWGKPRSLGGPEGEVRNAK		34
REVQPKGEGK	G0RY43	36
KLRHFYDKR		45



Supplementary Figure 1: 2D Immunoblot with sera from healthy subject as negative control