

Identifying novel allergens from a common indoor mould *Aspergillus ochraceus*

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ABSTRACT

The increasing burden of respiratory disease is a rising concern in India. Although chronic colonisation is primarily caused by pathogenic fungi, the common environmental fungi also play an important role in developing sensitisation. This study aims to examine the allergenic potency of mycelial proteins of a common indoor fungus *Aspergillus ochraceus* to a selected atopic patient cohort as well as to identify the novel IgE-binding proteins through an immunoproteomic approach. 1-D and 2-D IgE specific western blot detected the IgE reactive proteins which were identified through MALDI-TOF/TOF and manual *de novo* peptide sequencing. The results revealed the detection of 10 cross-reactive IgE-binding proteins. Cluster analysis of 1-D immunoblot with individual patient sera identified NADP(+) dependent glycerol dehydrogenase (GldB) homologous protein as a major allergen, which was further purified and the allergenicity was assessed. Other IgE-binding proteins showed homology with allergens like short-chain dehydrogenase, NAD-dependent mannitol dehydrogenase, and subtilisin-like serine protease. GldB purified under native conditions showed IgE reactivity amongst the selected patient cohort, which is reported for the first time in this study. The identified IgE-binding proteins can act as candidate molecules for developing hypoallergenic vaccines for designing specific immunotherapeutic techniques to fungal allergy.

The significance of the study: Exposure to environmental fungal allergens is directly associated with promoting allergic response as well as complicating existing respiratory disease, leading to poor respiratory health. Amongst others, *Aspergillus* spp. contributes to the majority of the fungal derived atopic diseases. *Aspergillus ochraceus* is a common indoor mould in India, however, its allergenic potency was not explored till date. In this study, we establish *A. ochraceus* responsible to cause an allergic response to susceptible individuals and identified 10 IgE-binding proteins using an immunoproteomics approach for the first time. *A. ochraceus* being unsequenced, a homology-driven proteomics approach was used to identify the IgE-binding proteins which can be extended to identify proteins from other unsequenced species. The information on the IgE-binding proteins could be used as a step towards characterising them by molecular and structural methods to investigate the molecular basis of allergenicity. This will also help to enrich the existing database of allergenic proteins and pave a way towards developing therapeutic avenues.

1. Introduction

Fungi are ubiquitous and contribute a major fraction to ambient bioaerosol [1]. Exposure to fungi induces allergic reactions to the susceptible population that cause partial or permanent damage to the respiratory system. Nearly 10 million people are suffering from fungal allergy worldwide and it is a rising health concern [2]. Fungal sensitisation is generally associated with patients with an existing respiratory disease like asthma [3,4]. More than 33% of severe asthma cases are

linked with fungal sensitisation. They are grouped under disease phenotype such as severe asthma with fungal sensitisation (SAFS) [5,6] and allergic bronchopulmonary aspergillosis (ABPA) [7], a more severe form of pulmonary disease with fungal inflammation, affecting about 2.5% of adult asthmatics globally [8]. The highest prevalence rates of SAFS and ABPA are reported in India [2]. About 35% and 5% of adult asthmatics have a prevalence of developing SAFS and ABPA respectively [9].

The pathology of fungal allergy is poorly understood. Fungal

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allergens disrupt lung epithelial barrier and initiate a cascade of an allergic response through T helper 2 (Th2) and B cells leading to IgE production [10]. The fungal sensitisation is caused by clinically important pathogens like *Aspergillus fumigatus* [11], *Alternaria alternata* [12], *Penicillium spp.* [13], *Fusarium spp.* [14], *Scedosporium spp.* [15]. More than 80 genera of fungi have been identified to be responsible for airway allergy, thus having a greater impact on the host immune system compared to pollen and other allergenic sources [16,17]. However, the most common environmental fungal species among *Aspergillus* genus are from *A. niger*, *A. versicolor*, *A. restrictus* and *A. ochraceus* [18–20]. Additionally, many studies have confirmed the presence of important clinically relevant *Aspergillus* species such as *A. fumigatus*, *A. flavus*, *A. oryzae*, and *A. terreus* at higher concentration in the environment [21–23]. *A. fumigatus* is a recognised human pathogen and the most commonly studied species in *Aspergillus* genus. The Allergen Nomenclature Sub-Committee of the World Health Organisation (WHO) and the International Union of Immunological Societies (IUIS) (www.allergen.org) has enlisted 23 different groups of allergens from *A. fumigatus*. Recent studies have reported that indoor fungal contamination can increase the risk of fungal asthma [24,25]. It has been reported that the common allergens from fungi are highly conserved and sensitisation to one possibly lead to cross-reactivity to known fungal allergens as they might share common IgE-binding epitopes [26].

Proteomics tools have been extensively used for detecting allergens from different sources [27]. The conventional method by implementing mass spectrometry-based identification requires an existing protein database for precise detection, making it difficult to identify proteins from sources with an unsequenced genome. To overcome this limitation, *de novo* peptide sequencing followed by homology-based database search has been implemented by many studies [28,29]. This led to identifying novel allergens from unknown sources which eventually enrich the existing allergen database.

A. ochraceus is generally known as a toxic food contaminant and observed as a common fungus in indoor environments [30–32]. Although there is no strong evidence of *A. ochraceus* as an allergenic species to date, it has been reported that *A. ochraceus* increases the risk of developing childhood asthma [33]. Primary cases have suggested its association with ABPA [34], invasive aspergillosis [35] and chronic pulmonary aspergillosis [36]. Above findings pointed us to hypothesise that *A. ochraceus* allergens can induce an allergic inflammation to atopic patients. In the present study, the IgE-binding proteins from *A. ochraceus* mycelia were identified through a combined approach of immunoproteomic tools and homology-driven protein identification techniques.

2. Material and methods

2.1. Strain maintenance and antigenic extract preparation

The environmental strain of *A. ochraceus* (MTCC 1877) was maintained in potato dextrose broth (PDB) at room temperature ($25 \pm 2^\circ\text{C}$) through periodic subculturing. The 7–10 days old spore mycelium was harvested from culture and vigorously washed with sterile 0.1 M phosphate buffer (PB), pH 7.2. The mycelial mat was lyophilised overnight to remove excess water. The dried mycelia were crushed in liquid nitrogen, dissolved with 0.1 M PB, pH 7.2 at 1:5 (w/v) and incubated overnight at 4°C with constant shaking. The slurry was centrifuged twice at 20,000 g for 20 min to remove any remaining debris. The clear supernatant was obtained and stored at -20°C for further use as antigenic extract and for Skin Prick Test (SPT).

2.2. Selection of patient cohort

The atopic patients of the age group range from 18 to 55 years were selected for this study. The primary inclusion criteria for selection were high eosinophil count and serum IgE level against known fungal allergens. Patients with a smoking habit, immunodeficiency disorders and

those who were under any kind of immunotherapy trials, were excluded from the study. For SPT, the antigenic extract was diluted in PB at 1:50 (v/v) and applied with a sterile lancet on skin. Approximately 20 min after administration, the wheal reaction was visible and graded in a scale of +1 to +3 as described previously [37]. Histamine diphosphate (1 mg/mL) and PB (0.1 M, pH 7.2) were used as positive and negative controls, respectively. This part was performed by a collaborative physician.

Patients, who showed +2 / +3 responses to *A. ochraceus* crude mycelial protein with the previous medical history of existing respiratory disease were further screened for the study. Among them, 10 patients agreed to volunteer for the present study and provided their blood samples with written consent for further immunochemical experiments. The detailed information is provided in Supplemental Table 1. The serum was separated from the blood samples and stored at -80°C for further use. Sera from three non-atopic patients were collected as negative controls. The whole study was approved by the ethical committee of the concerning institute.

2.3. Specific IgE ELISA

50 μL of antigenic extract or purified major allergen with concentration 100 ng/ μL were coated in ELISA plate (Maxisorp, Nunc, USA) wells and incubated at 4°C overnight. Wells were washed with 100 μL 0.1 M PBS, pH 7.2 and 0.05% Tween-20 (PBST) and were blocked subsequently with 50 μL PBST containing 1% BSA for 3 h at 4°C . The wells were washed thrice with PBST to remove the excess blocking agent and incubated overnight with patient sera diluted at 1:10 in blocking solution (PBST-BSA) at 37°C . The antigen-specific IgE was detected by incubating with monoclonal antihuman IgE alkaline phosphatase tagged produced in mouse (Sigma, USA) at 1:1000 dilution with blocking solution for 3 h at 37°C and followed by adding 50 μL of paranitrophenyl phosphate solution (Sigma) as substrate and placed for 30 min at 37°C in dark. The reaction was stopped by adding 3 N sodium hydroxide (NaOH) and the OD was measured at 405 nm in ELISA reader (Multiskan, Labsystems). The experiments were conducted in triplets and the mean value represented.

2.4. Extraction of crude protein content from spore mycelium

For further proteomic analysis, the crude protein was extracted by tris buffered phenol and precipitated in ammonium acetate and methanol by following the protocol as described earlier [38]. Briefly, 1 mg freeze-dried mycelial mat was crushed into a fine powder and mixed with 5 mL extraction buffer containing 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.7 M Sucrose, 7 mM potassium chloride (KCl), 2% (v/v) NP-40, and incubated for 1 h at 4°C in constant shaking. The clear supernatant was recovered by centrifuging the slurry twice at 20,000 g for 20 min. An equal volume of phenol saturated with 10 mM Tris-HCl pH 8.0, 1 mM EDTA (SRL, India) was added to the supernatant and vortexed for 1 h at 4°C . The solution was centrifuged and the phenol phase collected. The total protein was precipitated by incubating overnight with 0.1 M methanolic ammonium acetate at -20°C and washing thrice with chilled acetone. The final pellet was resuspended in Urea-CHAPS buffer (7 M Urea, 2 M Thiourea, 2% CHAPS) and incubated at -20°C . The final protein was extracted by centrifuging at 20,000 g for 20 min at 4°C and the concentration was determined using Bradford reagent (Bio-Rad, USA) by following the manufacturer's protocol.

2.5. Proteome profiling by 1-D and 2-D gel electrophoresis

The crude protein was resolved in 12% SDS-PAGE for 1-D electrophoresis at a concentration of 150 μg /well, under reducing condition. The 2-D gels were performed in 4–7 pI range. 200 μg protein was dissolved in rehydration buffer containing 125 μL Urea-CHAPS buffer, 40 mM DTT (Himedia, India), 0.75% (v/v) IPG buffer (GE Lifesciences, USA), 0.002% (w/v) bromophenol blue and loaded in 7 cm Immobiline

Dry Strips (GE Lifesciences) placed in rehydration tray overnight at room temperature. The strips were electrofocused in the Ettan IPG Phor 3 system (GE Lifesciences) at 50 μ A/strip current up to a total of 8 KWh. Thereafter, the strips were equilibrated in equilibration buffer 1 (6 M urea, 75 mM Tris-HCl, pH 8.8, 29.3% (v/v) glycerol, 2% SDS, 1% (w/v) DTT) and equilibration buffer 2 (same as equilibration buffer with 2.5% (w/v) iodoacetamide, instead of DTT), each for 15 min. The strips were then placed on 12% SDS-PAGE gels (100 $\times$ 105 $\times$ 1.5 mm) for the second dimension, run in miniVE vertical gel apparatus (GE Lifesciences) with Laemmli buffer at 40 mA/gel [39]. The gels were further stained in Coomassie brilliant blue R 250 to visualise the proteins. The experiments were done in triplets to assure the reproducibility. The gel image was taken in GelDoc (Bio-Rad) and the images were analysed further in Imagemaster 2D Platinum (GE Lifesciences) to determine molecular weight and pI.

2.6. 1-D, 2-D IgE specific Western blot

The total protein profile in 1-D and 2-D electrophoresis was electrotransferred on to PVDF membrane (GE Lifesciences) by semi-dry transfer (Atto Corporation, Japan), applying a constant current of 1.2 mA/cm² for 1 h. The transfer efficiency was validated by Ponceau S staining. The transferred membrane was blocked using blocking solution, 1 $\times$ tris buffered saline with 0.05% tween 20 (TBST) with 3% BSA, for 3 h at 4 °C. The excess blocking agent was washed off thrice by TBST for 15 min. Sera from individual patients were used as the primary antibody for 1-D immunoblot and pooled sera were used for 2-D immunoblot in 1:10 dilution in blocking buffer (TBST-BSA) and incubated overnight at 4 °C. The membrane was washed thrice with TBST and monoclonal antihuman IgE alkaline phosphatase tagged produced in mouse (Sigma) was added at 1:1000 dilution in blocking buffer for 3 h at room temperature. The immunoreactive zones/spots were developed by incubating in nitro-blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as a substrate in the dark for 30 min. The reaction was stopped by adding 0.5 M EDTA. 2-D gel and blot were run simultaneously with an equal protein load. To identify the allergenic proteins precisely, coordinates of the IgE reactive spots in the blot were matched parallelly with the Coomassie-stained gel and Ponceau S stained membrane manually as well as using Image master 2-D platinum software (GE healthcare). Experiments were repeated thrice to increase reliability. Another 2-D immunoblot was done by pooling sera from non-atopic patients as control.

2.7. IgE specific dot blot

2 μ L of purified protein at 1 μ g/ μ L concentration was placed on a nitrocellulose membrane (Sigma, USA) cut into circular discs of 1 mm diameter and allowed to dry. The membrane was blocked by using 100 μ L of blocking buffer (TBST-BSA), followed by washing thrice with 100 μ L TBST, similar to 1-D immunoblot. 100 μ L of individual patient sera at 1:10 dilution in blocking buffer was used as primary antibody and 100 μ L monoclonal antihuman IgE alkaline phosphatase tagged produced in mouse (Sigma) at 1:1000 dilution in blocking buffer was used as a secondary antibody, exactly following steps for 1-D immunoblot. The immunoreactive zone was developed by adding 100 μ L of NBT/BCIP.

2.8. Glycoprotein staining and deglycosylation assay

Glycoproteins from the 1-D gel were identified using Glycoprotein staining kit (G Biosciences, USA) by following the manufacturer's protocol. Deglycosylation assay was performed by following previously reported protocol [40]. In short, the 2-D gel was electrotransferred to a PVDF membrane followed by incubating in 50 mM sodium metaperiodate with 10 mM sodium acetate buffer (pH 5.0) for 3 h at dark. The periodate was inactivated by adding sorbitol (0.015 mol/L) and a drop of ethylene. Finally, the membrane was incubated with sodium

borohydride (1 mg/mL) for 12 h at 4 °C, followed by acidification with 0.001% acetic acid. The membrane was then washed extensively with 1 $\times$ TBST, blocked with 3% BSA-TBST buffer, and incubated with pooled patient sera at 1:10 dilution in the blocking solution. The IgE reactive spots were identified by following 2-D immunoblot protocol as described earlier.

2.9. In-gel digestion and mass spectrometry-based analysis of IgE reactive proteins

Samples for mass spectrometry were prepared by following standard protocol [41]. In brief, the spots were excised from a coomassie stained gel using sterile scalpels and destained using 50 mM ammonium bicarbonate: ethanol mixed in 1:1 ratio. The gel pieces were dehydrated using acetonitrile, followed by reduction with 10 mM DTT and alkylation with 55 mM iodoacetamide. This was followed by alternate hydration and dehydration using 50 mM ammonium bicarbonate and 100% acetonitrile. The in-gel digestion was done by adding 12.5 ng/ μ L of trypsin (Promega, Madison, USA) in 50 mM ammonium bicarbonate and incubated overnight at 37 °C. The trypsin was removed and the peptides were extracted by adding 3% TFA and 30% acetonitrile, followed by vortexing for 20 min. The extracted peptides were dried using speed vac (Savant, Thermo Scientific, USA) and reconstituted in 5 μ L of 1:1 (v/v) 0.1%TFA and acetonitrile.

2.5 μ L peptides were mixed with an equal volume of 0.5 mg/mL of α cyano 4 hydroxycinnamic acid matrix (Bruker Daltonics, Bremen, Germany). 2.5 μ L of the mixture was spotted onto the MTP 384 ground steel target plate (Bruker Daltonics) and allowed it to air dry. The calibration was done by using peptide calibration standard II mixture (Bruker Daltonics). The spectra were acquired by Autoflex II MALDI-TOF/TOF machine by applying a pulsed nitrogen laser (λ = 337 nm, 50 Hz) in linear mode. The high-intensity peaks with good signal to noise ratio (>15) were chosen from MS data and fragmented further by MS/MS analysis in lift mode. The parent and fragment ions were analysed in Flex Analysis, version 3.4 (Bruker Daltonics). Additionally, smoothing and baseline subtraction of acquired spectra was done by Flex Analysis (Bruker Daltonics).

2.10. Homology based database search and analysis

The spectra were observed in Flex Analysis (Bruker Daltonics) and were processed through BioTools 3.2 (Bruker Daltonics). The peak data were given as direct input to MASCOT search engine version 2.4.1. (<http://www.matrixscience.com>), where the search was done in the NCBI Protein Database. The search parameters were set as follows: variable modification: methionine oxidation, fixed modification: carbamidomethylation of cysteines, maximum missed cleavage: one, a mass tolerance of parent ion: 1.2 Da and fragment ion: 0.5 Da, and taxonomy: Fungi. At least two unique peptides matched with the same protein in MASCOT beyond significance with p -value <0.05 were considered reliable. Before database search, the contaminants such as matrix, trypsin autolysis, and keratin peaks were removed by internal calibration in Flex Analysis (Bruker Daltonics).

The unmatched spectra were further manually sequenced through homology-based search using mass difference approach by implementing the annotation tool in Flex Analysis (Bruker Daltonics) by following the previously described method [42,43]. The sequences generated by *de novo* sequencing were given as input sequences in online submission for homology-based search using MS BLAST algorithm [41] (<http://genetics.bwh.harvard.edu/msblast/>) with search parameter set to Blast 2p programme; non-redundant database (nr95 2016_06); matrix 'PAM30'; Expect '100'.

2.11. Purification of a major allergen

Total protein was extracted in extraction buffer containing 20 mM

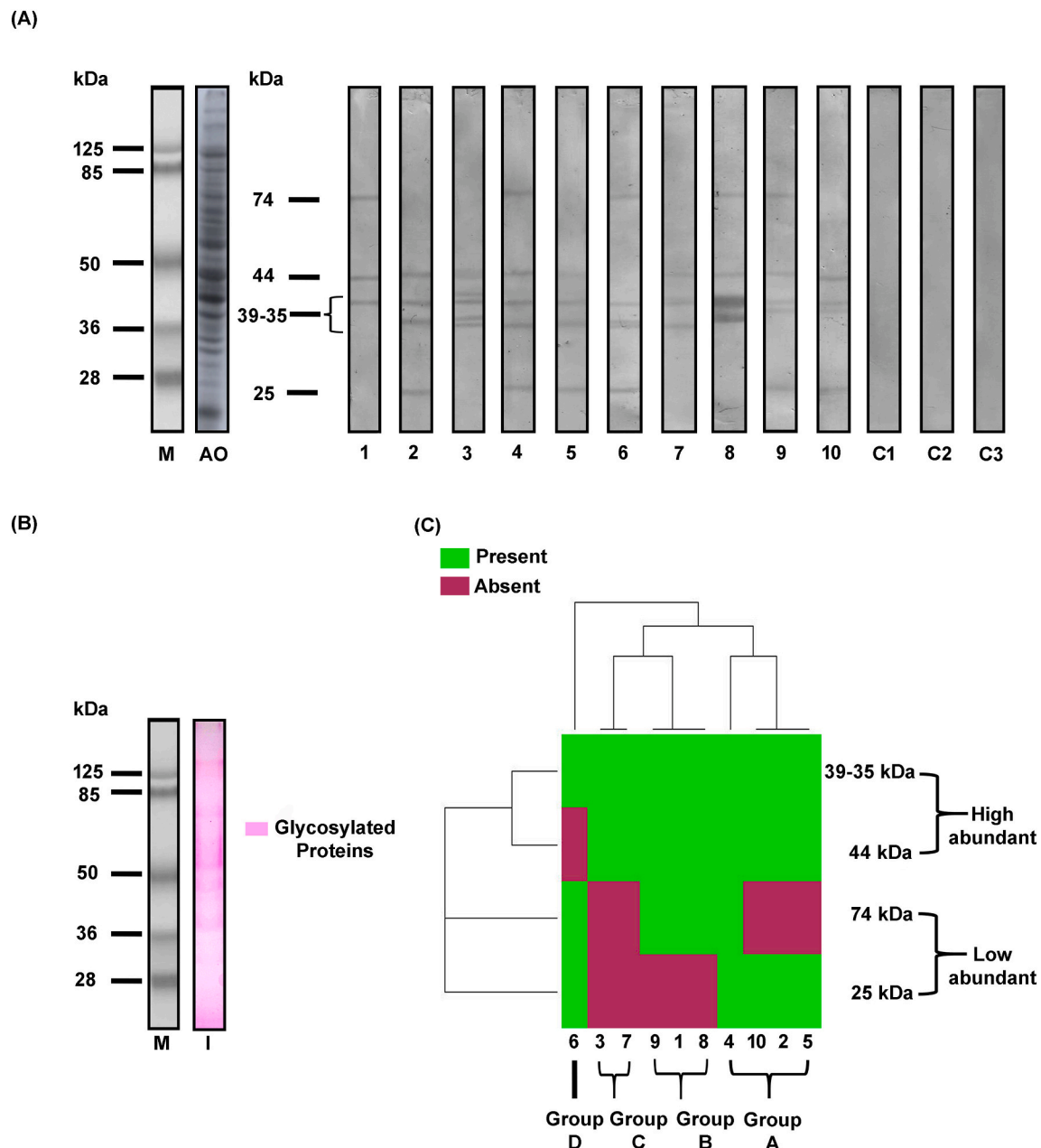


Fig. 1. The analysis of IgE reactivity to *A. ochraceus* total mycelial crude protein extract. A: Profiling of total crude protein extract and IgE specific western blot using individual serum from patients and healthy controls; M: protein molecular weight marker; AO: 12% SDS-PAGE profile of *A. ochraceus* total crude protein extract; 1–10: Immunoblots with sera from atopic patients; C1–C3: Immunoblots with sera from healthy controls. B: The glycoprotein staining of *A. ochraceus* spore mycelial crude protein extract; M: protein molecular weight marker; I: Periodic acid–Schiff (PAS) stain of 12% SDS-PAGE profile of crude protein extract. The magenta colour represents the glycosylated proteins after PAS staining of the 12% SDS-PAGE protein profile. C: Hierarchical clustering along with a heat map of the observed IgE reactive proteins.

Bis-Tris (Himedia, India) pH 6.9, 10 mM EDTA, 10 mM Sodium azide, 1% Glycerol, 1 mM PMSF. The cell lysate was centrifuged at 6000 g for 30 min twice to remove any remaining debris. The clear supernatant was then allowed to pass through ion exchange column HiTrap Q HP (GE Healthcare) (5 mL) using binding buffer (20 mM Bis-Tris, pH 6.9) in AKTA Pure FPLC system (GE Healthcare) at a maximum flow rate of 5 mL/min. Fractions were collected using 1 M sodium chloride (NaCl) gradient in binding buffer. The fractions with the protein of interest were pooled and further purified by passing through size exclusion chromatography (Superdex 200 10/300 GL column, GE Healthcare) twice. The purified protein was eluted in 1× PBS, pH 7.4 at 0.4 mL/min flowrate. The desired fractions were concentrated and buffer exchanged in Amicon Ultra 10 kDa molecular weight cutoff centrifugal filters

(Amicon, Millipore, USA). The purified fraction was visualised through silver staining (FASTsilver, G-Biosciences, USA), by following the manufacturer's protocol. The pI of the purified protein was further checked through 2-DE.

2.12. Cross-reactivity assay through ELISA inhibition

50 µL of *A. fumigatus* (MTCC 2584) and *A. ochraceus* antigenic extract at 20 µg/µL were coated in ELISA plate (Maxisorp, Nunc, USA) wells and incubated at 4 °C overnight. The wells were washed thrice with 1× PBST and blocked with 3% BSA-PBST. The purified major allergen was mixed with pooled patient sera (primary antibody) prepared at 1:10 dilution with blocking solution at a gradual increment in a dose-dependent

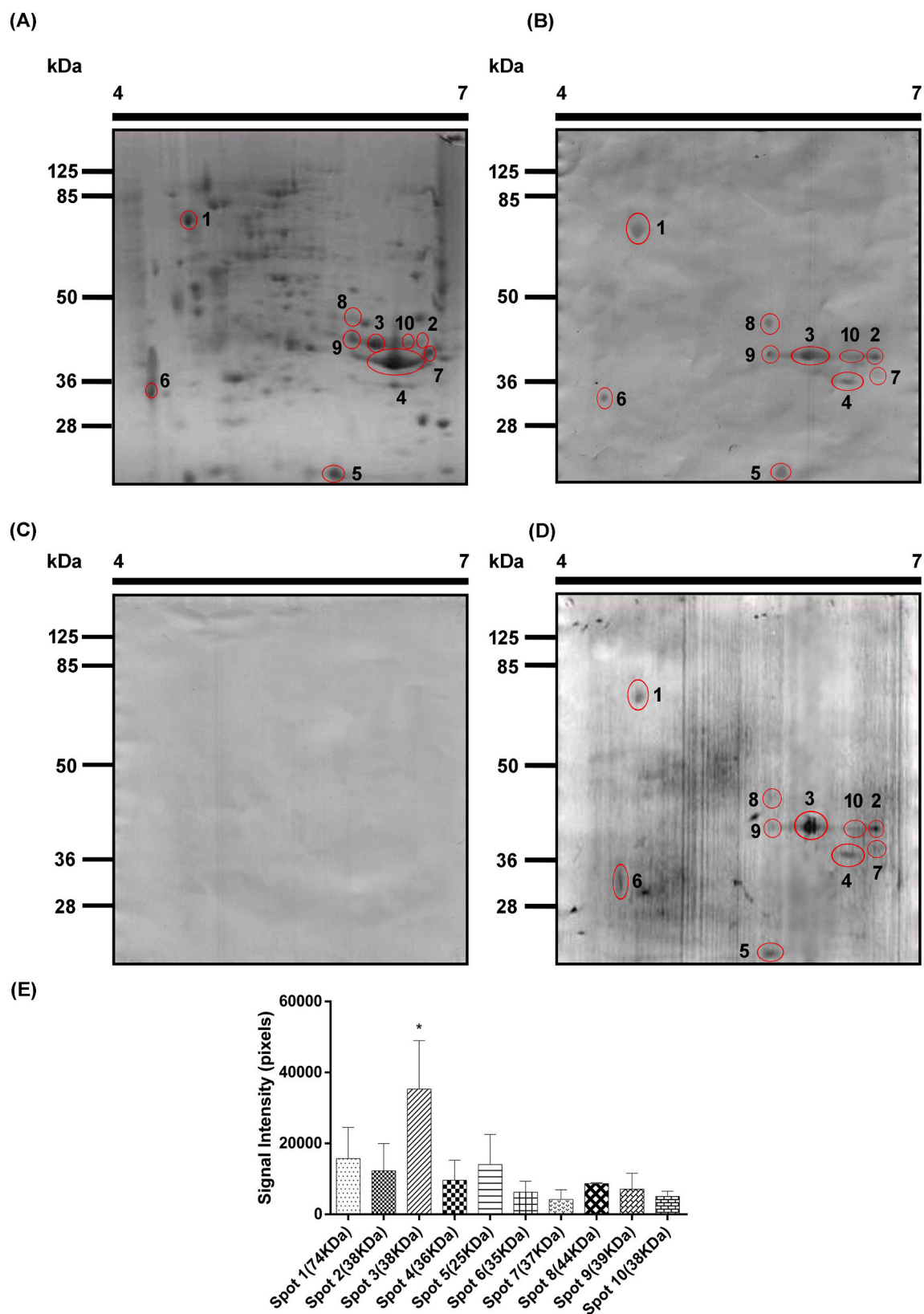


Fig. 2. Serological analysis of *A. ochraceus* spore mycelial proteome. A and B: 2-D proteome profile and immunoblot with pooled patient sera in 4–7 pI range, respectively. C: 2-D immunoblot with healthy control sera in 4–7 pI range. D: 2-D immunoblot with pooled patient sera in 4–7 pI range after deglycosylation by sodium periodate treatment. E: The signal intensity (pixels) of all 10 allergens. Bars represent mean $\pm$ SD; * $p < 0.05$; The statistical analysis was done using one-way ANOVA followed by Dunnett's multiple comparison test.

Table 1

The list of identified IgE-binding proteins through a homology-based database search. The identified peptides, matched homologous peptide sequences in MS BLAST, and corresponding scores that are underlined were identified through manual peptide sequencing. The punctual modifications in sequences of identified peptides and matched homologous peptides in MS BLAST are highlighted in bold. The reported allergens from WHO/IUIS allergen nomenclature database and Allfam database are mentioned here.

Spot no.	Identified protein	Organism	NCBI Accession No.	Identified peptides	Matched homologous peptide sequences in MS BLAST	Peptide no.	Score	Method	Function	Known allergen
1	Probable beta-glucosidase L	<i>Aspergillus fumigatus</i>	Q4WGT3.1	WVVPSTGTFKVYVGSSSR	-	2	57	MS/MS	glycoside hydrolase	-
2	Cytochrome P450 subfamily protein	<i>A. terreus</i>	Q0CEF3.1	FANPVTA FPAGINAGATWDR	-	2	65	MS/MS	monoxygenase	-
3	NADP (+)-dependent glycerol dehydrogenase (GldB)	<i>A. oryzae</i> 3.042	EIT74943.1	IYPPVSPSILR	LYPPVAASLLR	2	<u>60</u>	MS/MS	aldo/keto reductase	-
		<i>Candida maltosa</i>	Q12587.1	TGGPAFMFPSTGPR	-	3	40	MS/MS		
		<i>A. fumigatus</i> A1163	EDP50537.1	GGNTLAQVLIWGLR	-	3	91	MS/MS		
		<i>A. steinii</i> IBT 23096								
		<i>A. nidulans</i>	XP_024710400.1	NGIMPEAYSPLGSQNQVPTTGER	-		98			
				GTFASEGASGETYR						
4	Short-chain dehydrogenase	<i>A. niger</i>	XP_663167.1	NTGKEFVFEDPR	GTFASEGASGETYR	2	<u>99</u>	de novo	NAD or NADP-dependent oxidoreductase	Alt a 8, Cla h 8
		<i>A. steinii</i>	GAQ45550.1	FIHLDSLQDSV	NTGKEFVFEEPR		<u>87</u>	de novo		
		<i>IBT 23096</i>	XP_024701765.1		FVHIDLSDQDSV		<u>39</u>			
5	Hypothetical Protein	<i>Schizopora paradoxa</i>	KLO19850.1	ATPPTAPCSQR	ATPPTAPPSQR	2	<u>64</u>	de novo	unknown	-
		<i>Fusarium oxysporum</i> f. sp. <i>raphani</i> 54,005	EXK78574.1	SPCSNPDA	SPCSNPDA		<u>60</u>			
6	NAD-dependent malate dehydrogenase	<i>A. oryzae</i> 3.042	EIT77523.1	GSEIVLIPAGVPR	-	2	61	MS/MS	oxidation of malate to oxaloacetate	Mala f 4
				GGPGVAADLSHINTNSTV	-		31			
7	Subtilisin-like serine protease pepC	<i>A. niger</i>	XP_001391470.1	SGYEATPSGLR	-	2	40	MS/MS	serine protease	Asp n 18, Asp f 18, Pen o 18
8	Cyanide hydratase	<i>Penicillium rubens</i>	XP_002562104.1	NAPWGLAR	-	2	158	MS/MS	carbon-nitrogen hydrolase	-
				YLYASEGGEGVDAYTIDTGINVDHVDVEGR	-	2	54	MS/MS		
9	Hypothetical protein	<i>Hirsutella</i>	KJZ77464.1	AAAVNAEPGWFDLQESVR	-	2	36	MS/MS	NAD-dependent epimerase/dehydratase	-
		<i>minnesotensis</i> 3608	CEL09873.1	LVFGDGTGDTTESVMDTEIGR	-	2	<u>60</u>	de novo		
		<i>A. calidoustus</i>		IAENGSRVWAENVR	AENGSHVWAD		<u>69</u>			
		<i>F. oxysporum</i>	EXM14107.1	PPTIYGPR	PPTIYGPR		<u>61</u>	de novo	unknown	-
10	Hypothetical protein	<i>f. sp. vasinfectum</i>	EPS96472.1	DIYDGWR	DIYDGWR	2	<u>61</u>	de novo		
		<i>25,433</i>		ASSLGMFPGSR	ASSLGMFPGSR		<u>61</u>			
		<i>Fomitopsis pinicola</i> FP-58527 SS1								

manner at concentrations of 0.001, 0.01, 0.1, 1, 10, 100, 1000, and 10,000 ng and added to the coated wells and incubated overnight at 37 °C. Bound antibodies were detected by subsequent incubation with monoclonal antihuman IgE alkaline phosphatase tagged produced in mouse (Sigma) at 1:1000 dilution with blocking solution for 3 h at 37 °C and addition of 50 µL paranitrophenyl phosphate solution (Sigma) as substrate and placed for 30 min at 37 °C in the dark. The reaction was stopped by adding 3 N NaOH, following measurement of OD at 405 nm in ELISA reader (Multiskan, Labsystems). The IgE inhibition was quantified by the following equation as described previously [44]. Experiments were repeated thrice.

$$\% \text{ of inhibition} = \left(1 - \frac{\text{OD at 405nm with inhibitor}}{\text{OD at 405 nm without inhibitor(only sera)}} \right) \times 100$$

2.13. Image analysis and statistical analysis

The signal intensity of IgE reactive spots in 2-D immunoblot was analysed in Image Studio Lite (LI-COR, Lincoln, Nebraska, USA) and determined by combining the unsaturated pixels. The statistical analysis was performed in Graphpad Prism 6.0 (San Diego, CA, USA). The comparison of IgE responses against purified and crude protein between patients and healthy negative controls as well as the signal intensity (pixels) of IgE reactive spots were analysed using One-way ANOVA followed by Dunnett's multiple comparison test. The hierarchical clustering and heat map was done in R studio 3.4.2 (Boston, MA, USA). The clustering was done based on euclidean distance along with average linkage.

3. Results and discussion

Airborne fungi have a significant role in developing airway colonisation and infection to the patients with underlying lung conditions. Apart from *A. fumigatus*, a well-studied allergenic fungus, several other *Aspergillus* spp. are common in the indoor environment such as *A. ochraceus* which is reported to exert cytotoxic effect [32]. Identifying the IgE-binding proteins is a key to develop therapeutic strategies, understand the cross-reactivity and also to study the molecular basis of allergenicity. The present study aims to identify the IgE-binding proteins from *A. ochraceus* spore mycelial proteome in a selected patient cohort for the first time.

3.1. Determination of IgE reactive regions from *A. ochraceus* total mycelial crude protein profile

Sera from the selected atopic patients showed elevated specific IgE response against *A. ochraceus* crude protein extract in ELISA (Supplemental Table 1) compared to healthy subjects. Around 21 distinctive bands were observed in the 1-D protein profile of *A. ochraceus* crude extract (Fig. 1A). 1-D immunoblot with 10 individual patient sera revealed three distinct IgE reactive bands at 74, 44 and 25 kDa. Bands with similar molecular mobility were observed between 35 and 39 kDa region. At least one band in this region of molecular weight 38 kDa was present in all patients (Fig. 1A).

3.2. Glycoprotein staining of *A. ochraceus* total mycelial crude protein profile

Several pathogenic fungi harbour allergens which contain cross-reactive carbohydrate determinants that are responsible for inducing an allergic response in the host system [45–47]. Periodic-Acid-Schiff (PAS) staining of the SDS-PAGE profile of total crude proteins showed the presence of glycoproteins (Fig. 1B). Several distinct bands were observed at the molecular weight of 125, 90, 74, 51 and 37 kDa. Some bands with similar molecular mobility were also observed within 42–48 kDa. These regions include the proteins with molecular weight 74, 44

and 37 kDa that were recognised as IgE reactive zones.

3.3. Allergenicity profile of the selected patient cohort

The seroreactivity profile of individual patients was determined through a hierarchical cluster analysis (Fig. 1C). Based on the presence/absence of IgE reactive proteins, the patient cohort was grouped into three categories. Group A was identified as a highly reactive group with four patients, showing the presence of the maximum number of IgE reactive bands. Group B and D were categorised as moderate reactive groups with three and one patient(s) respectively. Both the two groups showed absence of reactivity only to a single distinct immunoreactive band. Group C comprising of two patients was detected as the group that selectively reacts only to the most immunoreactive proteins.

Similarly, the immunoreactive zones were also clustered into two categories. The high abundant IgE-binding protein group, comprising of 35–39 kDa and 44 kDa regions, were present in all patient groups, except Group D. The low abundant IgE-binding protein group, comprising of 74 and 25 kDa regions, each was found to be reactive in five patients. While 74 kDa was found reactive in one patient of Group A and all patients in Group B and D, the 25 kDa region showed immunoreactivity only in Group A and D. All groups of patients were observed to be reactive to at least one protein from 35 to 39 kDa region.

3.4. Resolving and locating IgE-binding proteins in the total mycelial proteome

The total spore mycelial proteome profile revealed 112 ± 12 well-resolved spots in 2-DE (Fig. 2A). Immunoblot at similar pI range with pooled patient sera detected 10 distinct IgE reactive spots (Fig. 2B). Most of these were concentrated between 35 and 50 kDa region. Amongst these, spot 3 revealed the highest intensity of IgE binding, while spot 4 in spite of having higher protein expression did not show high IgE binding. No IgE reactive spots were found in immunoblot with control sera (Fig. 2C).

3.5. Deglycosylation assay and identification of a major allergen

The glycoprotein staining of 1-D total crude protein profile showed the presence of glycoproteins at IgE reactive zones with similar molecular weight. Deglycosylation followed by immunoblotting of the total mycelial crude proteome was performed to determine whether carbohydrate moiety is responsible for IgE binding. The result showed the retention of immunoreactivity of all 10 IgE reactive spots, inferring the peptidic nature of the IgE-binding epitopes (Fig. 2D).

Upon correlating with the hierarchical clustering, the 2-D immunoblot shows the presence of seven IgE-binding proteins (spot 2, 3, 4, 6, 7, 9 and 10) which falls within the 35–39 kDa region, identified as one of the highly allergenic zones. The signal intensity observed in immunoblot amongst all IgE-binding proteins was analysed through the intensity of unsaturated pixels of acquired 2-D immunoblot images. The result showed that spot 3, present in 38 kDa region of *A. ochraceus* proteome, has a higher signal intensity in terms of pixels, which is more than 8-fold than the lowest signal producing IgE reactive protein (spot 7) and approximately 2-fold higher than the second-highest signal producing IgE reactive protein (spot 1) ($p < 0.05$, Fig. 2E) and hence considered as a major allergen.

3.6. Identification of the IgE-binding proteins from *A. ochraceus* spore mycelium

All 10 detected immunoreactive proteins were identified through homology-based database search with other fungi and are listed in Table 1. Four spots were identified through direct database searching of MS/MS spectra. These are probable beta-glucosidase L (spot 1), NAD-dependent malate dehydrogenase (spot 6), subtilisin-like serine

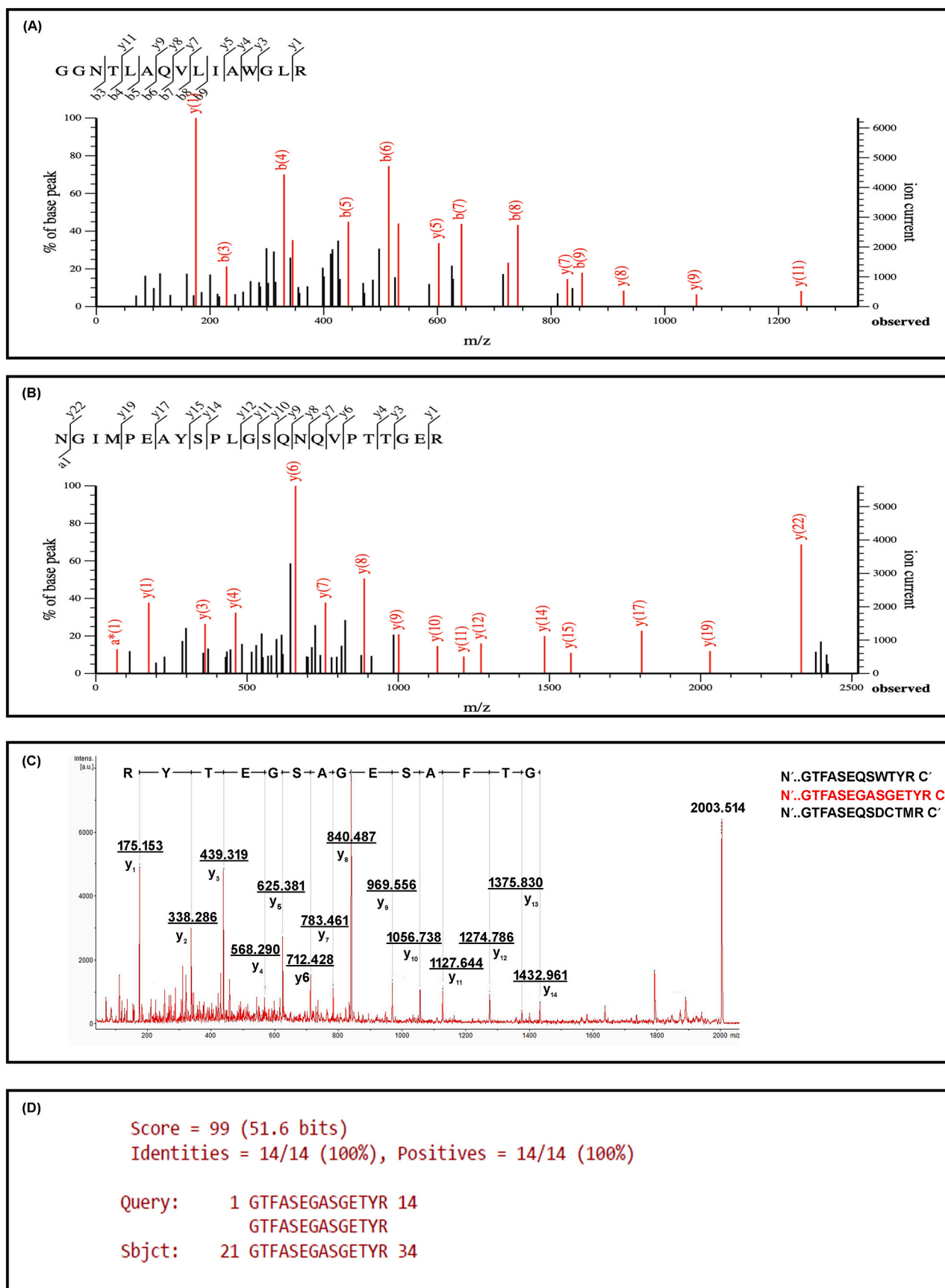


Fig. 3. MS/MS spectra of spot no 3. A and B: MS/MS spectra of peptides with molecular weights of 1568.765 and 2446.983 Da, respectively. C: *De novo* sequencing of the peptide with molecular weight 2002.794 Da. The 'y' ions were spotted and mentioned. Sequence proposals are shown adjacently and the matched sequence is highlighted in red; $p < 0.05$. D: Screenshot of the result from MS BLAST.

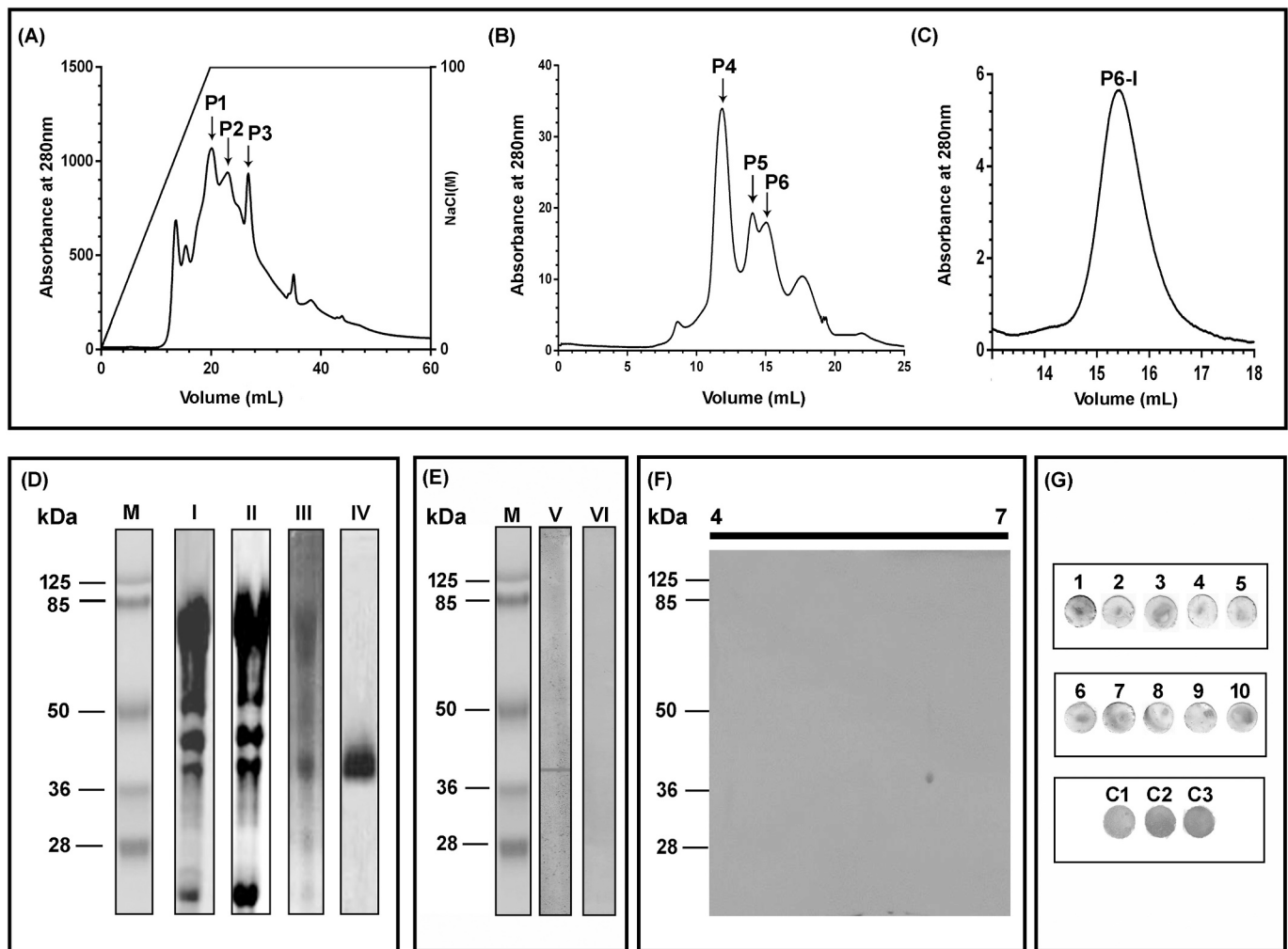


Fig. 4. Purification of a major allergen. A: ion-exchange chromatogram. B-C: gel filtration chromatogram. D: 12% SDS PAGE profile of obtained peaks; M: protein molecular weight marker; I-III: Fractions obtained from P2, P3 and P6; IV: Fraction obtained from P6-I. E: IgE specific western blot of the purified major allergen probed with pooled patient sera; V and VI: immunoblot of purified protein against pooled serum of patients and healthy controls, respectively. F: 2-D profile of the purified major allergen in 4–7 pI range. G: IgE specific dot-blot of the purified major allergen using individual serum from patients and healthy controls; 1–10: patients; C1–C3: healthy controls.

protease pep C (spot 7), and cyanide hydratase (spot 8). Two peptides were identified for NADP(+) dependent glycerol dehydrogenase (GldB) protein (spot 3) and one peptide was identified for cytochrome P450 subfamily protein (spot 2) through MS/MS. Among these four identified IgE-binding proteins, spot 1 was homologous to probable beta-glucosidase L which is an enzyme involved in cellulose degradation. Although, beta-glucosidase was reported as food allergen from wheat [48] and mustard [49], but not yet listed in the allergen nomenclature database of WHO/IUIS Allergen Nomenclature Sub-Committee. Spot 6 showed homology with NAD-dependent malate dehydrogenase, which is a major allergen from *Malassezia furfur* (Mala f 4) [50]. Spot 7 showed homology with subtilisin-like serine protease pepC from *A. niger*. The vacuolar serine protease, Asp n 18, a reported allergen from *A. niger* showed homology with serine proteinases of subtilisin family [51]. Further database search using BLAST has revealed the vacuolar serine proteases from *A. fumigatus* (Asp f 18) [52] and *Penicillium oxalicum* (Pen o 18) [53] have extensive homology with subtilisin-like serine proteases. However, spot 8 showed homology to cyanide hydratase and is reported as IgE-binding protein for the first time in the present study.

Peptides from the rest of the spots were identified through *de novo* peptide sequencing as their scores failed to reach statistical significance ($p < 0.05$) in the MASCOT database search. The probable sequences for each spot were inserted as inputs for MS BLAST search. Peptides with

significant scores were considered. Manual sequencing, though labour intensive, can be easily applied to spectra obtained from MALDI where the peaks are singly charged. In many cases, the EST database is often useful to identify proteins with minimal sequence information, which was unavailable in *A. ochraceus*. The proteins identified by database searching implies that the matching peptides were identical or highly conserved between the species. Peptides identified by the homology-based search were found to have multiple mismatches that may have restricted their identifications by traditional database search (Supplemental Fig. 1–5). Four IgE-binding proteins including spot 4, 5, 9, and 10 were identified. Additionally, one peptide from each spot 2 and 3 was also identified through *de novo* followed by MS BLAST. Spot 2 showed homology with cytochrome p450 subfamily protein and reported as IgE-binding protein for the first time in the present study. Peptides from spot 4 showed homology with short-chain dehydrogenase, a major allergen in *Cladosporium herbarum* (Cla h 8) [54] and *Alternaria alternata* (Alt a 8) [55]. Three proteins, spot 5, 9, and 10 showed homology with fungal hypothetical proteins. Using BLAST search with these hypothetical proteins, spot 5 and 10 were found to be unannotated while spot 9 showed high homology with NAD-dependent epimerase/dehydratase, which shares a similar functional domain with isoflavone reductase, an allergen from pear (Pyr c 5) [56] and white birch (Bet v 5) [57].

The spot 3, corresponding to the only protein proven to be a major

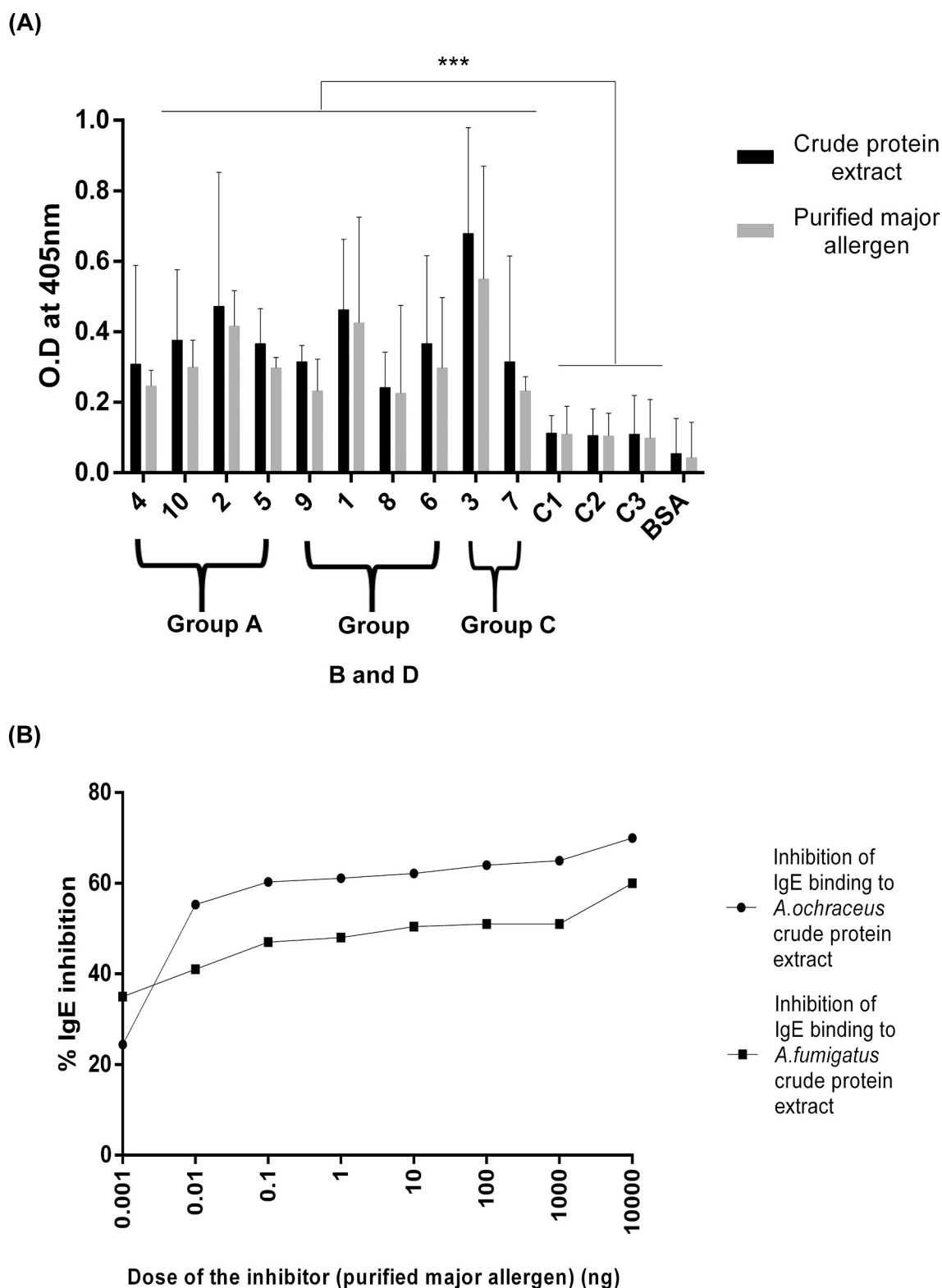


Fig. 5. *In vitro* allergenicity assay using the purified major allergen. A: The comparative analysis of the specific IgE ELISA using *A. ochraceus* crude protein extract and the purified major allergen; 1–10: patients; C1–C3: healthy controls; Bars represent mean $\pm$ SD; *** p < 0.001; The statistical analysis was done using one-way ANOVA followed by Dunnett's multiple comparison test. B: The analysis of cross-reactivity of the purified major allergen (the inhibitor protein) through IgE inhibition ELISA at an increasing dose-ranging 0.001 ng/mL to 10,000 ng/mL with crude protein extract of *A. ochraceus* and *A. fumigatus* (the inhibiting protein) in the solid phase.

allergen, was identified as 'NADP(+)-dependent glycerol dehydrogenase (GldB)' from *A. fumigatus*, *A. steinii* and *A. nidulans* (Fig. 3). Two peptides in this protein could be identified through the MASCOT search. Another peptide with sequence proposal of GTFASEGASGETYR from

spot 3 was identified through *de novo* sequencing showed a significant match GldB from *A. nidulans* helping to reinforce the identification. Glycerol dehydrogenase is an enzyme mainly involved in carbohydrate metabolism and belongs to the aldo-keto reductase family [58]. This

protein also has not been identified as an allergen from any sources.

3.7. Purification of the 38 kDa protein

The screening of IgE reactive proteins from total proteome does not alone confirm the allergenicity of a potential allergen. Further purification of the IgE-binding protein in native or recombinant form, as well as in vitro validations, are required to determine its ability to react with serum IgE in a given patient cohort. Though 1-D immunoblot provided a blueprint of this reactivity, the proteins could be identified precisely using the 2-D gel. Upon investigating the signal intensity of 2-D immunoblots, spot 3, one of the proteins with a molecular weight of 38 kDa, showed the highest, implying that this could be a major allergen that contributes towards the allergenicity of the species. In other spots, reduced IgE binding intensity could be attributed to the lower number of epitopes or low sensitivity in the investigated cohort.

The first level of purification was done by ion exchange column using HiTrap Q HP column using a 1 M NaCl gradient elution. The chromatogram showed three broad peaks at 1 M NaCl concentration (Fig. 4A). Fractions from these three peaks were resolved in SDS-PAGE and the presence of 38 kDa band was observed along with other proteins in both P2 and P3. Further polishing was done in gel filtration column twice (Fig. 4B, 4C). In the first one, fractions collected from P2 and P3 were pooled, injected and fractionated in the gel filtration column. The P6 fraction showed a high-intensity band of 38 kDa with less number of contaminant proteins. Further polishing with P6 fraction in gel filtration column revealed the presence of a single band at 38 kDa region in P6-I fraction with a single peak, observed at 15.3 mL. The 12% SDS-PAGE profile revealed the presence of native purified protein with a monomeric form of 38 kDa (Fig. 4D). In a previous study, preliminary purification of a similar protein from *A. nidulans* and *A. niger* displayed a molecular weight of 38 kDa [59].

1-D immunoblot with pooled patient sera confirmed the IgE reactivity of the purified protein (Fig. 4E). After resolving purified protein in the 2-DE, the spot was observed at 38 kDa region with 6.0 pI, similar to spot 3 in the total proteome profiling (Fig. 4F). Further, dot blot with individual protein sera validated the IgE reactivity of the purified protein in all ten patients (Fig. 4G). Mass spectrometry of the 38 kDa protein from purified fraction identified NADP(+)-dependent glycerol dehydrogenase (GldB) in accordance with previous results.

3.8. Specific IgE ELISA and cross-reactivity assay

The comparative analysis of specific IgE reactivity among selected patient cohort against purified major allergen and crude allergen of *A. ochraceus* revealed an elevation in antigen-specific IgE reactivity compared to healthy controls, indicating that the purified protein can induce a significant antigenic response to the sensitive patients than the control group ($p < 0.001$) (Fig. 5A). The specific IgE levels corroborated with the clustering of patients based on western blots where high IgE titers were prevalent in the Group A with patients which showed IgE response to the maximum number of proteins and Group C with patients showing reactivity against only high abundant IgE-binding proteins while lower IgE titers were found in Group B and D patients (Fig. 5A).

Earlier studies showed that *A. fumigatus* antigenic extract was found to be highly cross-reactive to *A. ochraceus*, *A. fischeri*, *Neosartoria aureola*, and *Neosartoria stramenia* but was absent in other clinically important fungi [60]. This led us to hypothesise that allergens from *A. ochraceus* might share cross-reactive IgE-binding epitopes to that of *A. fumigatus*, as the allergen orthologues are highly conserved across fungi kingdom and the cross-reactivity between fungal proteins with 40–70% homology was reported [26]. The cross-reactivity with *A. fumigatus* extract was assessed through inhibition of IgE activity upon incubating sera with increasing concentration of the purified protein. Inhibition of IgE activity of self-antigen was checked using *A. ochraceus* crude protein extract. 1 ng purified protein could inhibit 50% IgE binding to

A. fumigatus crude extract, whereas, the same was achieved at 0.01 ng for *A. ochraceus* crude extract. The maximum IgE inhibition of 60% and 70% for *A. fumigatus* and *A. ochraceus*, respectively, was observed at 10 μ g, which is the highest test concentration of the purified protein, indicating that the allergens from *A. fumigatus* and *A. ochraceus* might possess cross-reactive IgE-binding epitopes (Fig. 5B). These observations also point that this protein can act as a potential candidate molecule for developing a hypoallergenic variant in treating patients allergic to both *A. ochraceus* and *A. fumigatus*.

4. Conclusion

The present study has demonstrated an elaborate report that indicates *A. ochraceus* is a potent source of aeroallergens and has the capability of inducing an allergic response in atopic patients. An immunoproteomic approach assisted in detecting 10 IgE-binding proteins from *A. ochraceus* spore mycelium proteome. A combined approach of conventional database dependent and homology-based search by manually analysing the acquired MS/MS spectra has helped to identify all the detected allergens. A major allergen showed homology with NADP(+) glycerol dehydrogenase (GldB) protein and cross-reactivity with *A. fumigatus* crude extract. Epitope mapping, recombinant protein expression and cytokine profiling through the interaction of the major allergen with primary cell-line could give an insight to its allergenicity. Current diagnostic approach to determine fungal sensitisation does not include *A. ochraceus* for fungal specific IgE assay. These observations can be used as a piece of primary information to develop improved diagnostic approaches to investigating fungal allergy, especially the patients sensitised with non-*A. fumigatus* species.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jprot.2021.104156>.

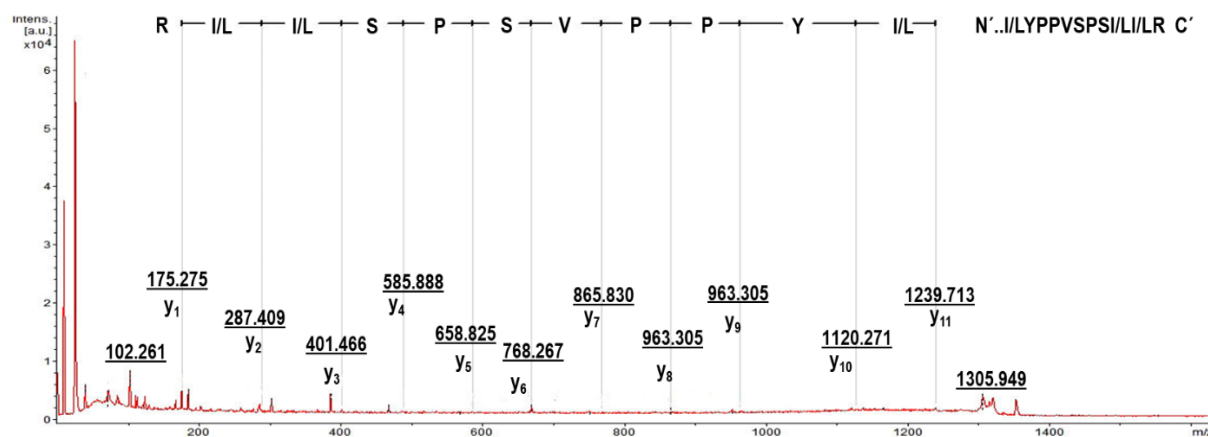
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Supplemental Figure 1: MS/MS spectra of spot 2 along with *de novo* peptide sequencing. The generated sequence proposal that showed a significant match in MS BLAST is mentioned; the identified protein: Cytochrome P450 subfamily protein, peptide m/z:1305.949 Da.



Score = 60 (29.9 bits) Identities = 7/11 (63%), Positives = 9/11 (81%)

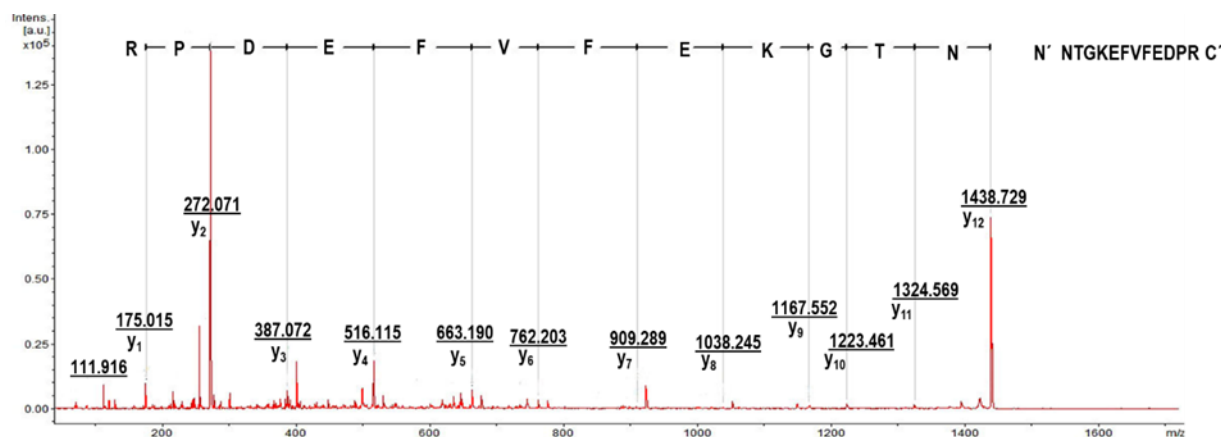
Query: 1 IYPPVSPSILR 11

+YPPV S+LR

Subject: 473 LYPPVAASLLR 483

Supplemental Figure 2: MS/MS spectra of spot 4 along with *de novo* peptide sequencing. The generated sequence proposal that showed significant match in MS BLAST is mentioned; The identified protein: Short-chain dehydrogenase; A: peptide m/z :1438.729 Da and B: peptide m/z :1746.947 Da.

(A)



Score = 87 (43.8 bits)

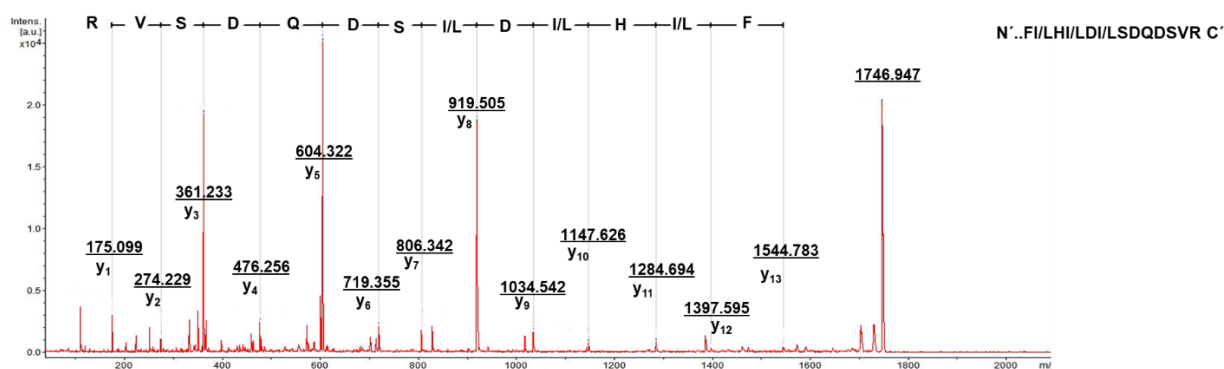
Identities = 11/12 (91%), Positives = 12/12 (100%)

Query: 1 NTGKEFVFEDPR 12

NTGKEFVFE+PR

Subject: 246 NTGKEFVFEEPR 257

(B)



Score = 77(35.8 bits)

Identities = 10/12 (83%), Positives = 11/12 (92%)

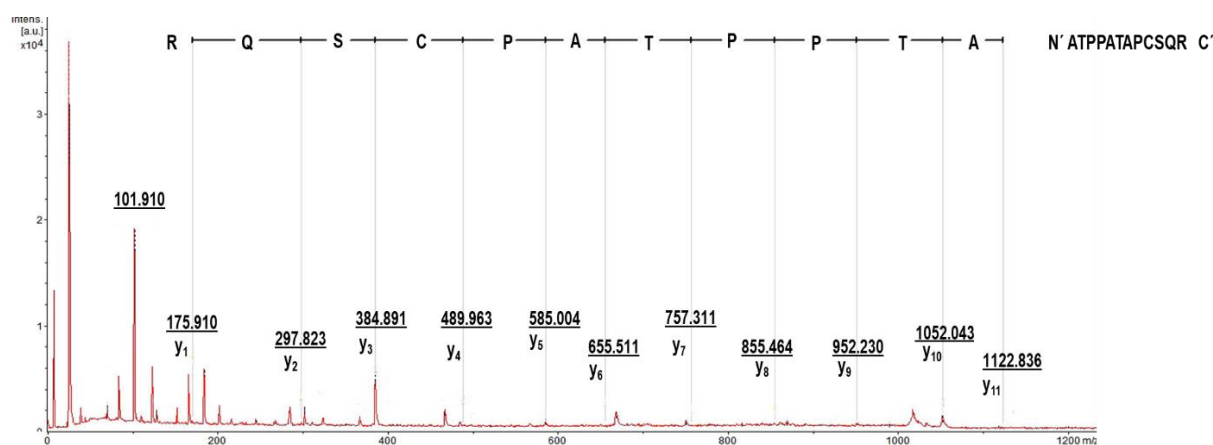
Query: 1 FIHLDSLSDQDSV 12

F+H DLSDQDSV

Subject: 80 FVHIDLSDQDSV 91

Supplemental Figure 3: MS/MS spectra of spot 5 along with *de novo* peptide sequencing. The generated sequence proposal that showed significant match in MS BLAST is mentioned; The identified protein: Hypothetical Protein; A: peptide m/z:1122.836 Da B: and peptide m/z:1165.680 Da.

(A)



Score = 64 (34.7 bits)

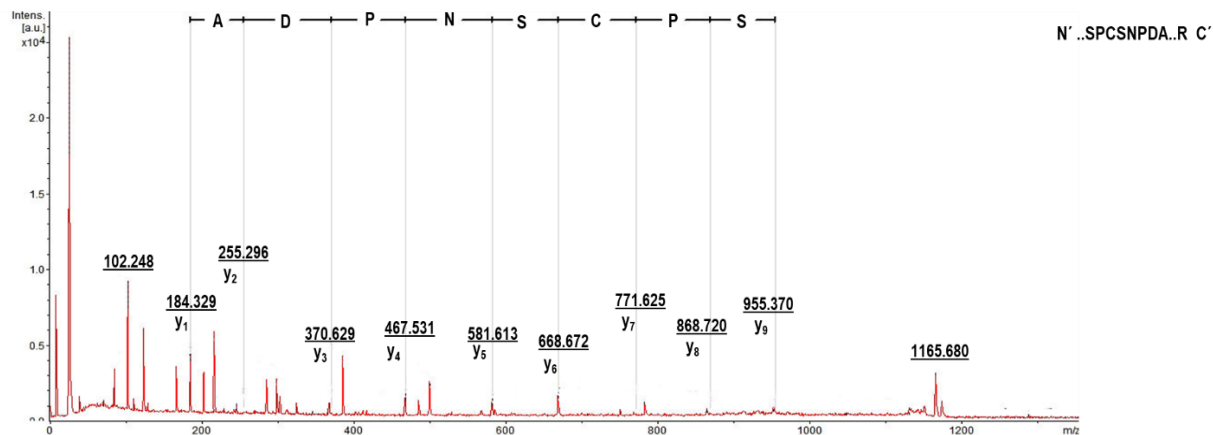
Identities = 10/11 (90%), Positives = 10/11 (90%)

Query: 1 ATPPTAPCSQR 11

ATPPTAP SQR

Subject: 422 ATPPTAPPSQR 432

(B)



Score = 60 (32.0 bits)

Identities = 8/8 (100%), Positives = 8/8 (100%)

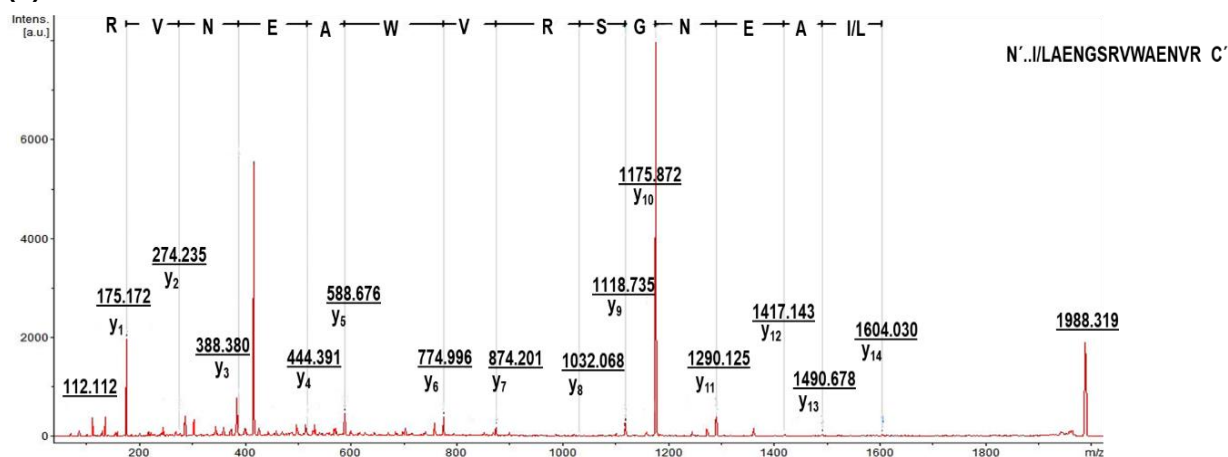
Query: 4 SPCSNPDA 11

SPCSNPDA

Subject: 64 SPCSNPDA 71

Supplemental Figure 4: MS/MS spectra of spot 9 along with *de novo* peptide sequencing. The generated sequence proposal that showed significant match in MS BLAST is mentioned; The identified protein: Hypothetical Protein, A: peptide m/z :1988.319 Da and B: peptide m/z :1988.319 Da.

(A)



Score = 60 (30.1 bits)

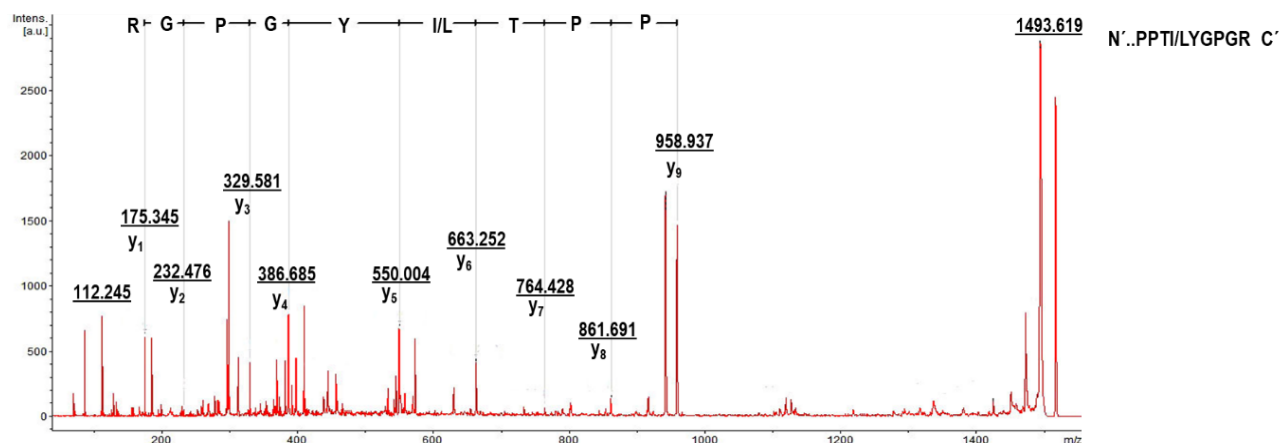
Identities = 8/10 (80%), Positives = 9/10 (90%)

Query: 2 AENGSRVWAE 11

AENGSRVWA+

Subject: 255 AENGSHVWAD 264

(B)



Score = 69 (36.8 bits)

Identities = 9/9 (100%), Positives = 9/9 (100%)

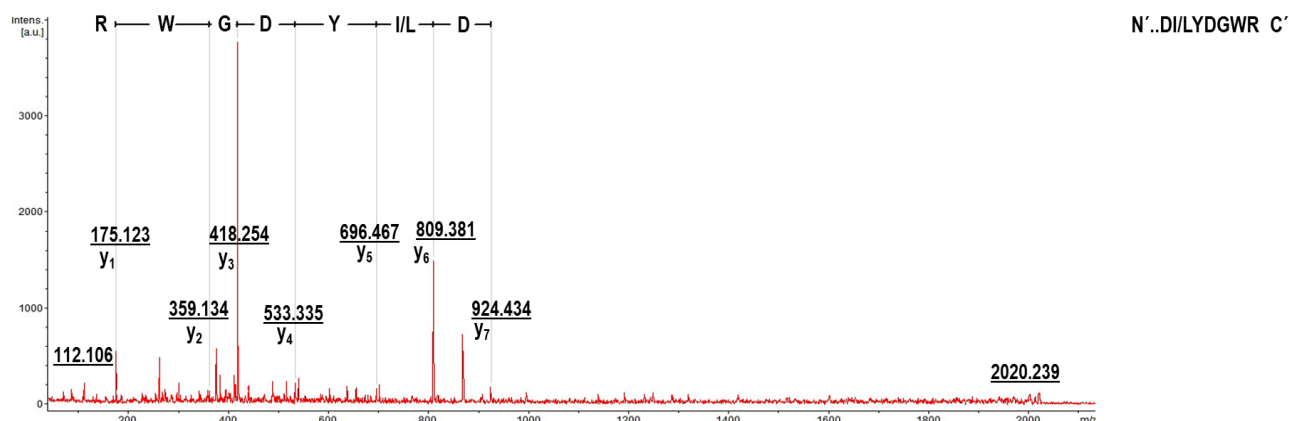
Query: 1 PPTIYGPGR 9

PPTIYGPGR

Subject: 175 PPTIYGPGR 183

Supplemental Figure 5: MS/MS spectra of spot 10 along with *de novo* peptide sequencing. The generated sequence proposal that showed significant match in MS BLAST is mentioned; The identified protein: Hypothetical Protein, A: peptide m/z :2020.239 Da and B: peptide m/z :2705.206 Da.

(A)



Score = 61 (30.5 bits)

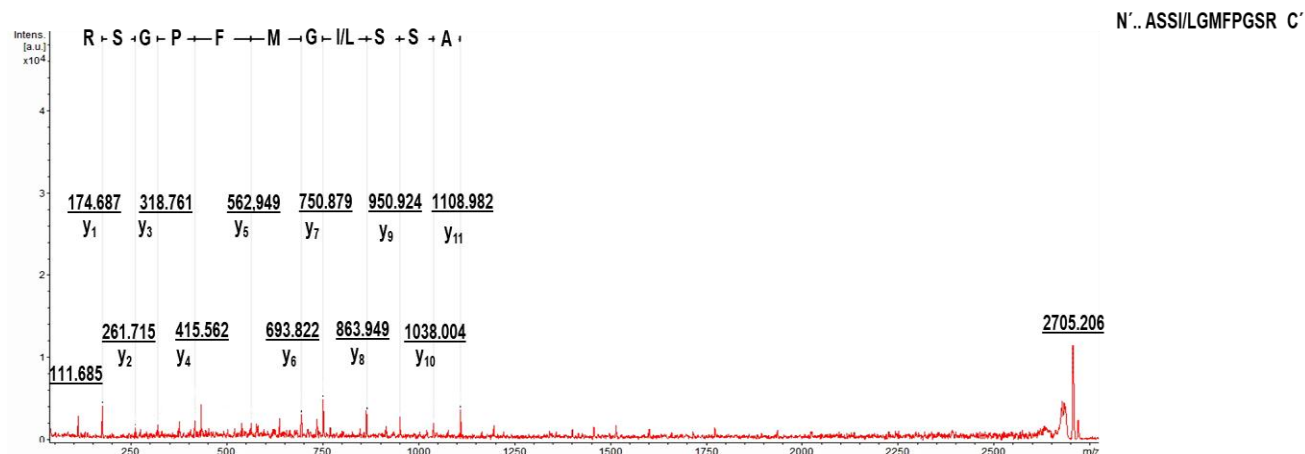
Identities = 7/7 (100%), Positives = 7/7 (100%)

Query: 2 DIYDGWR 8

DIYDGWR

Subject: 152 DIYDGWR 158

(B)



Score = 61 (33.1 bits)

Identities = 9/10 (90%), Positives = 9/10 (90%)

Query: 1 ASSLGMFPGS 10

ASSLGMFP S

Subject: 625 ASSLGMFPSS 634

Patient Number	Age (Years)	Sex	Symptoms	SPT Grades to <i>A. ochraceus</i>	Specific IgE titer (P/N value)
1	32	F	AR+ BA	+3	4.24
2	51	M	BA	+3	4.33
3	19	F	AR+ BA	+3	6.23
4	27	M	BA	+2	2.82
5	26	F	BA	+2	3.36
6	30	F	AR	+2	3.36
7	21	F	AR+ BA+AS	+2	2.89
8	35	F	AR+BA	+2	2.22
9	39	F	BA+AS	+3	2.89
10	35	M	BA	+2	3.45
C1	27	M	NS	-	-
C2	33	F	NS	-	-
C3	29	M	NS	-	-

Supplemental Table 1: Clinical characteristics of the selected patient cohort with corresponding specific IgE reactivity to *A. ochraceus* antigenic extract in ELISA; 1-10 are atopic patients and C1, C2 and C3 are healthy negative controls; AR: Allergic Rhinitis, BA: Bronchial Asthma, AS: Anaphylactic Shock, NS: No Symptom. The specific IgE titre is the ratio of the OD at 405 nm value of patient (P) to healthy controls (N). P/N value >2.0 was considered significant.