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Identification of *Epicoccum purpurascens* allergens by two-dimensional immunoblotting and mass spectrometry

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Abstract

Sensitization to allergens of *Epicoccum purpurascens* is reported from many places world over. Previously, the IgE binding proteins of *Epicoccum* were studied by one-dimensional immunoblotting (1-DE). However, the allergens recognized by 1-DE may be a mixture of proteins appearing at the same molecular weight. In the present study, IgE-binding components of *E. purpurascens* were detected using two-dimensional immunoblotting and mass spectrometry. Approximately 250 protein spots were identified by Coomassie staining in the range of 14–110 kDa and pI 3.5–9.5. Of these, 147 showed specific IgE reactivity with pooled patients' sera whereas 16 protein components reacted with more than 50% of the individual patients' sera. The frequency of IgE reactivity was highest (87.5%) for a 25-kDa protein (pI-7.85). Matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis of 16 protein components led to identification of 6 important allergens as phospho-glycerate kinase (PGK), RNA-dependent RNA polymerase, pyrroline-5-carboxylate dehydrogenase, 40S ribosomal protein and two proteins of unknown functions. The major allergens identified may be assessed for cross reactivity with other fungi. In conclusion, 2-DE followed by immunoblotting allowed characterization of *E. purpurascens* allergens. Six new allergens have been identified using this technique and mass spectrometry. The availability of such relevant allergens would help in component-based diagnosis and therapy of fungal allergy.

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Keywords: Two-dimensional electrophoresis (2-DE); Isoelectric focusing (IEF); *Epicoccum purpurascens*; Fungal allergens; MALDI/TOF

Abbreviations: 1-DE; one-dimensional electrophoresis; 2-DE, two-dimensional electrophoresis; CBB, coomassie brilliant blue; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; DAB, diaminobenzidine; DTT, dithiothreitol; HPLC, high performance liquid chromatography; HRP, horse radish peroxidase; IEF, isoelectric focusing; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MSDB, matrix science database; NCM, nitrocellulose membrane; NP-40, nonidet P-40; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PGK, phospho-glycerate kinase; pI, isoelectric point; SDS, sodium dodecyl sulphate; TBS, tris buffered saline; TFA, trifluoroacetic acid

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Introduction

Exposure to fungal allergens triggers respiratory allergies and asthma in predisposed individuals. Fungi such as *Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum*, *Curvularia lunata*, *Epicoccum purpurascens*, *Fusarium solani* and *Penicillium citrinum* have been implicated in respiratory allergies (Achatz et al., 1995; Bisht et al., 2000; Gupta et al., 1999; Hansen et al., 1994; Verma and Gangal, 1994). Sensitization to these molds is considered a strong risk factor for asthma (Black et al., 2000). *E. purpurascens* is a ubiquitous aeroallergen that accounts for sensitization in around 5–7% individuals worldwide (Bisht et al., 2000; Portnoy et al., 1987). The mold has also been implicated in allergic fungal sinusitis (Noble et al., 1997). Earlier the major allergens of *Epicoccum* were identified using one-dimensional (1-D) immunoblotting (Bisht et al., 2000; Dixit et al., 1992). However, 1-D immunoblotting does not explain sensitization to individual allergen because IgE reactive bands may contain more than one protein. Further, the technique allows only detection and not the identification of IgE binding components. The IgE reactivity of protein isoforms is also masked in 1-D immunoblotting (Barea et al., 1999). The identification of IgE binding components based on either molecular weight or pI requires purification and to date only a single allergenic protein of *Epicoccum* has been isolated and identified using this approach (Bisht et al., 2004a, b).

Two-dimensional electrophoresis (2-DE) is a high-resolution separation technique (Barea et al., 1999) that allows simultaneous identification of the several IgE binding components of a source when coupled with immunoblotting. The technique has been used previously to identify the IgE binding proteins of fungi, pollen and latex (Barea et al., 1999; Chardin et al., 2002; Chow et al., 1999; Fujimura et al., 2004). The allergen profile of *A. fumigatus*, *Candida albicans* and *Malassezia furfur* has been investigated by 2-DE (Barea et al., 1999; Chow et al., 1999; Onishi et al., 1999). In the present study, 2-DE in conjunction with immunoblotting was employed to elucidate the allergen spectra of *E. purpurascens*. Mass spectrometry of IgE reactive spots, most frequently recognized by patients' sera was performed to identify the individual allergens responsible for the allergic sensitization.

Materials and methods

Preparation of extract

Spore-mycelial extract of *E. purpurascens* MTCC 2129 (microbial-type culture collection and gene bank,

Institute of Microbial Technology, Chandigarh, India) was prepared as described previously (Bisht et al., 2000). Briefly, the mold was grown as stationary culture for 13 days at 25 °C in Sabouraud's medium with yeast extract. The surface growth (fungal mat) was harvested, freeze-dried and antigens extracted 1:20 w/v in 125 mM NH₄HCO₃, pH 8.0, containing 5 mM ethylenediamine tetra-acetic acid and 1 mM phenyl methyl sulphonyl fluoride by continuous stirring for 5 h at 4 °C. Supernatant was collected after centrifugation at 12,000g and dialyzed against distilled water. The extract was lyophilized and stored at –20 °C and protein content was determined by modified Lowry's method (Singh et al., 1992). Allergen extracts of *E. purpurascens* and other inhalants were procured from the Antigen Laboratory, Institute of Genomics and Integrative Biology, Delhi for skin testing.

Subjects, skin tests and sera collection

One hundred and twenty-four patients with respiratory allergy aged 20–42 years were included in the present study at the out patients department, V. P. Chest Institute, Delhi. The institute receives referred cases of asthma and rhinitis but the diagnosis was confirmed following American Thoracic Society guidelines (1991) and Adamopoulos et al. (1995), respectively. Detailed history of patients was recorded including duration/severity, suspected allergen, family history of allergy and illnesses other than allergy. Routine clinical investigations such as chest X-ray, blood analysis and pulmonary function test were done in these patients. Smokers and patients with other chronic illnesses were not included in the study.

Intradermal (ID) tests were performed with a battery of common allergens such as pollen, insect, dust mite and fungi including *E. purpurascens* extract, on patients of suspected allergy (history). The extracts included 5 grass pollens (*Cenchrus ciliaris*, *Cynodon dactylon*, *Imperata cylindrica*, *Pennisetum typhoides* and *Sorghum vulgare*), 8 weeds (*Ageratum conyzoides*, *Amaranthus spinosus*, *Artemisia scoparia*, *Brassica campestris*, *Chenopodium album*, *Gynandropsis gynandra*, *Parthenium hysterophorus* and *Xanthium strumarium*), 5 trees (*Prosopis juliflora*, *Ricinus communis*, *Salvadora persica*, *Holoptelia integrifolia* and *Ailanthus excelsa*), house dust mite (*Dermatophagoides pteronyssinus*), 5 fungi (*Alternaria alternata*, *Aspergillus fumigatus*, *Cladosporium herbarum*, *Epicoccum purpurascens* and *Fusarium solani*) and 3 common insects (*Periplaneta americana*, *Musca domestica* and *Culex quinquefasciatus*).

For ID test, 0.02 ml of each extract (1:500 w/v) was injected intradermally into the forearm of the patient raising a bleb of 2–3 mm. Histamine diphosphate (100 µg/ml) was used as a positive control and PBS as

a negative control. The reactions were graded after 20 min based on the wheal size of positive control (Shankar et al., 2006). Blood was collected from patients showing marked positive skin reactions (wheal size equal to or more than positive control), sera were separated and stored at -70°C . Blood of 5 non-allergic volunteers with negative skin tests was collected to be used as negative control. The study protocol was approved by the human ethics committee of the institute and informed consent of subjects was obtained for participation.

Enzyme linked immunosorbent assay (ELISA)

Specific IgE to *E. purpurascens* in hypersensitive patients' sera was determined by ELISA (Voller et al., 1976). Briefly, the microtitre plate (Maxisorp, NuncTM Immunomodule, Denmark) was coated with $1\text{ }\mu\text{g}/100\text{ }\mu\text{l}$ of spore-mycelial extract (protein) in carbonate-bicarbonate buffer (pH 9.6). The unoccupied sites were blocked with 3% non-fat dry milk, $200\text{ }\mu\text{l}/\text{well}$ in 20 mM Tris, 150 mM NaCl, pH 7.5 buffered saline (TBS). After incubation for 2 h at 37°C , the plate was washed thrice with TBS. Hundred microlitre of pooled or individual sera (1:10 v/v in TBS) were added to the wells and plate was incubated overnight at 4°C . The plate was washed with TBS containing 0.05% Tween-20, 0.005%, Triton X-100 (TBST) and incubated with $100\text{ }\mu\text{l}/\text{well}$ of anti-human IgE-horse radish peroxidase (HRP) (Sigma, MO, USA) diluted 1:1000 v/v in TBS. The plate was washed with TBST and TBS and the colour was developed using o-phenylenediamine and H_2O_2 . The reaction was stopped with 4 N H_2SO_4 and the absorbance read at 492 nm. Serum from non-allergic volunteers ($n = 5$) was used as a control. Patient's sera showing five times or more ($\text{OD} \geq 0.30$) IgE binding than negative control ($\text{OD} = 0.06$) were used for immunoblotting.

1-D electrophoresis and immunoblot

Electrophoresis was carried out on 10% gel using discontinuous buffer system containing 0.025 M Tris, 0.2 M glycine and 0.1% sodium dodecyl sulphate (SDS) (Laemmli, 1970). Spore mycelial protein ($60\text{ }\mu\text{g}$ per lane) was boiled for 5 min in two-fold concentration of sample buffer containing 2-mercaptoethanol. The proteins were electrophoresed at 100 V. The resolved proteins were stained with 0.1% Coomassie brilliant blue (CBB) R-250 or transferred to nitrocellulose membrane (NCM). The transfer of resolved proteins was carried out in mini trans-blot electrophoretic transfer Cell (Bio-Rad, CA) in Tris-glycine buffer (pH 8.4) containing 25% methanol, at 200 mA for 3 h. The non-specific sites were blocked with 3% non-fat dry milk in TBS for 1 h at 37°C . Following washing with TBST, the membrane was

incubated with *Epicoccum* positive patients' serum pool or individual patients' sera (1:10 v/v) overnight at 4°C . IgE binding proteins were detected using anti-human IgE-HRP. The blots were developed with diaminobenzidine (DAB, 15 mg/50 ml) in 50 mM sodium acetate buffer (pH 5.0) and hydrogen peroxide (15 $\mu\text{l}/50\text{ ml}$).

Isoelectric-focusing (IEF)

For sample preparation, *Epicoccum* extract was incubated 10:1 v/v (30 μl extract to 3 μl solution), with a solution containing 10% SDS and 3% dithiothreitol (DTT) at 100°C for 5 min. The resulting sample was solubilized with two volumes (66 μl) of buffer containing urea (10 M), 4% detergent, 50 mM Tris, 65 mM DTT and 0.65% carrier ampholine (Bio-Rad, CA). The detergents used were Triton X-100, CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate) and Nonidet P-40 (NP40).

The IEF was performed in tube gels using a rod gel electrophoresis system (Bangalore Genei, India). Acrylamide gels (5%) were polymerized in a solution of 8 M urea, 2% Triton-X-100, 6% ampholine (pH 3.5–9.5) (Amersham, Sweden). The length of the gel prepared was 7 cm. Electrofocusing was performed in 20 mM NaOH (cathode) and 20 mM orthophosphoric acid (anode). A prerun at 50 and 200 V for 15 min each was given to establish a pH gradient. Six hundred microgram protein was loaded. IEF reference protein markers pH 3–10 (Amersham, UK) were run as the standard. The proteins were focused for 2 h each at 200 and 500 V followed by 14 h at 800 V. Subsequently tubes were removed; gels extruded and equilibrated in buffer containing 0.5 M Tris (pH-6.8), 10% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.05% bromophenol blue for 15 min. The procedure was repeated with fresh equilibration buffer for another 10 min.

2-D separation and immunoblot

For the 2D separation, the tube gel was laid horizontally above stacking gel and separated by polyacrylamide gel electrophoresis (PAGE) on mini-protein II apparatus (Bio-Rad, CA). Proteins were resolved at 100 V for 75 min on 10% gel and were stained with CBB or Periodic acid-Schiff stain or used for immunoblot analysis. Immunoblotting was carried out as described above with the same sera which were used for 1-D immunoblotting.

Periodic acid-Schiff (PAS staining)

SDS-PAGE separated proteins were fixed with acetic acid-methanol solution. The carbohydrate moieties were

oxidized by treatment with 0.7% periodic acid in 5% acetic acid on a shaker for 2 h. The gel was incubated in freshly prepared 0.2% w/v sodium metabisulfite in 5% acetic acid for 45 min and the solution was changed several times. Finally, the gel was placed in Schiff's reagent.

Periodate modification of blot

The carbohydrate moieties of transferred proteins were subjected to periodate oxidation following Bisht et al. (2004b). Briefly, the NCM was incubated in 0.02 M sodium metaperiodate in 50 mM sodium acetate and kept in the dark at 4 °C, overnight. A drop of ethylene glycol was added to inactivate the periodate and membrane was incubated overnight at 4 °C in sodium borohydride solution (1 mg/ml). The reaction was stopped by adding a drop of acetic acid. The membrane was rinsed in TBS and blocked with 3% non-fat dry milk. Immunoblotting with pooled patients' sera was carried out as described above.

MALDI-TOF MS analysis

Protein spots corresponding to 16 allergens identified on immunoblot were excised from CBB-stained 2-DE gels. Mass spectrometry of these was performed at The Centre for Genomic Application, New Delhi using a MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The proteins were reduced, alkylated and digested with trypsin. The resulting peptide mixture was separated by reverse phase-high perfor-

mance liquid chromatography (HPLC) on C-18 column (Zorbax 2.1 × 150 mm) using 0.1% trifluoroacetic acid (TFA, Solvent A) and 0.085% TFA in acetonitrile (Solvent B). The digested peptide fragments were analysed in a MALDI-TOF mass spectrometer in a positive reflectron ion mode using a Biflex III apparatus (Bruker-Daltonica, Bremen, Germany). The peptide mass fingerprinting data was used to search in the Matrix Science database (MSDB) with mascot software (<http://www.matrixscience.com>), allowing one missed cleavage of trypsin per peptide and accuracy level of 100 ppm and a mass tolerance of 1 Da.

Tandem mass spectrometry (MS/MS) was performed for selective peptide fragment that did not yield significant matches in MALDI analysis. For MS/MS of spot 15, peak 1281.708 (not shown) was subjected to further fragmentation. The sequence was deduced from the mass of generated fragments.

Results

Skin reactivity and specific IgE

Out of 124 respiratory allergy patients skin tested, 13 showed marked positive reactions to *E. purpurascens* extract. Of these, 10 patients showed significantly elevated specific IgE levels against *Epicoccum* extract compared to control (Table 1). Serum pool of 5 non-allergic volunteers was used as control. The serum from 8 patients with raised specific IgE (OD ≥ 5 times the control) was used individually for analysis of *Epicoccum* proteins by immunoblotting.

Table 1. Clinical characteristics, skin reactivity and specific IgE values

S. no.	Age/sex	Clinical history ^a	ID grading ^b	Specific IgE (OD value)
Patients				
1.	22/F	AR	++	0.702
2.	34/M	AR	+++	1.020
3.	29/F	AR	++	1.208
4.	42/F	AR + BA	++	0.600
5.	38/M	AR	++	0.327
6.	25/F	AR	++	0.532
7.	40/M	AR	++	0.456
8.	29/F	AR	++	0.339
Controls				
9.	30/F	BA	—	0.043
10.	26/M	AR	—	0.071
11.	35/F	AR	—	0.066
12.	41/M	AR + BA	—	0.048
13.	36/M	AR	—	0.072

^aAR, Allergic rhinitis; BA, bronchial asthma; ID, intradermal; OD, optical density.

^b++, equivalent to histamine (positive control); +++, more than histamine (positive control). Positive control: histamine diphosphate 100 µg/ml. Sera showing OD ≥ 5 times the average value of control serum (0.06) were considered positive.

Identification of IgE reactive bands by 1-DE

Epicoccum extract separated by 1-D PAGE showed 26 protein bands upon Coomassie brilliant blue staining (Fig. 1a). Of these, 25 protein bands showed IgE reactivity with pooled patients' sera positive to *Epicoccum* (Fig. 1b). Individual patients' sera ($\geq 75\%$) detected IgE reactive bands of 100, 75, 62, 56 and 30 kDa as major allergenic components in *Epicoccum* (Fig. 2, lanes a–h). A 25 kDa band reacted with more than 50% of patients' sera. None of the *Epicoccum* proteins showed binding with control serum pool (Fig. 2, lanes i and j).

Protein profile of *Epicoccum* by 2-DE

The extract was separated by 2-DE and subjected to PAS staining for glycoprotein analysis or transferred to NCM and probed with patients' sera. Protein profile with good resolution and distinct spots was obtained by pretreatment of spore-mycelial extract (proteins) with SDS and DTT followed by solubilization with buffer containing NP-40 (Table 2). Approximately 250 protein spots were observed on CBB stained 2-D gel (Fig. 3a). The number of spots was reduced when buffer contain-

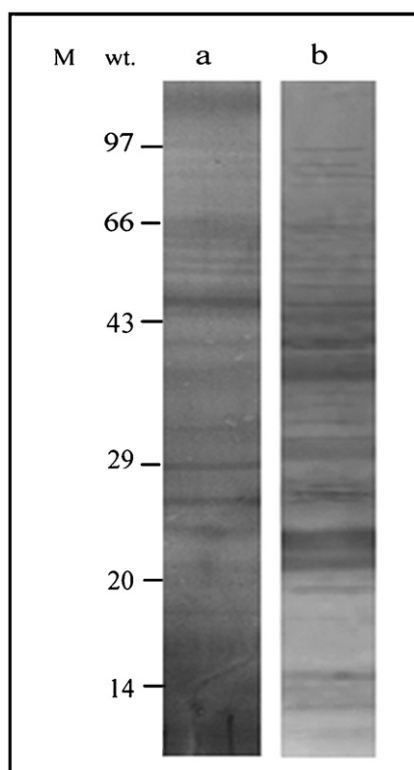


Fig. 1. 1-D analysis of *Epicoccum* spore-mycelial extract (60 µg/well) on 10% SDS-PAGE after (a) CBB staining, (b) immunoblot with *E. purpurascens* allergic patients' serum pool (1:10 v/v) followed by anti-human IgE-HRP (1:1000, Sigma, USA).

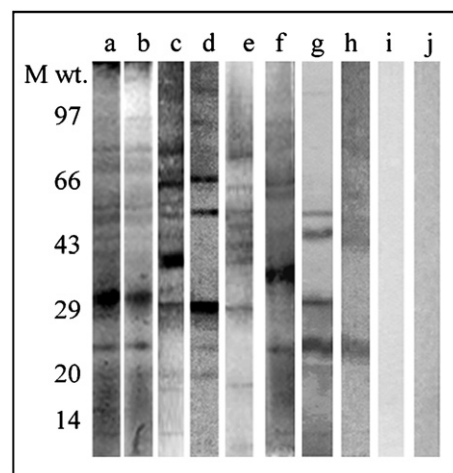


Fig. 2. Immunoblot with individual patients' sera (lanes a–h), after 1-D electrophoresis. Lanes 'i' and 'j' were probed with control sera. *Epicoccum* proteins separated on SDS-PAGE were transferred to NC membrane. NC strips were probed with individual patients' sera followed by incubation with anti-human IgE-horseradish peroxidase. The colour was developed with DAB and H₂O₂.

Table 2. Effect of detergents used for solubilization of proteins in 2-DE

S. no.	Detergent	Profile obtained	
		Resolution	Number of spots
1	CHAPS	Streaking	Spots not distinct, ~80
2	Triton X-100	Less resolution	150 spots
3	Nonidet P-40	High, spots clear	~250

All detergents were used in 4% concentration.

ing CHAPS (~150 spots) or Triton X-100 (~80 spots) was used for solubilization.

Identification of IgE reactive spots by 2-DE

To identify the allergenic proteins, 2-DE separated components were immunoblotted with sera from *Epicoccum* allergic patients. Immunoblot with pooled patients' sera showed 147 IgE reactive protein spots in *Epicoccum* extract (Fig. 3b). Periodic acid-Schiff staining identified 50 glycoproteins on gel in *Epicoccum* extract (Fig. 3c). Immunoblotting of periodate-treated membrane with pooled patients' sera recognized 41 protein spots (Fig. 3d) showing reduction in reactivity to some spots after carbohydrate modification. None of the proteins showed reactivity with control serum pool

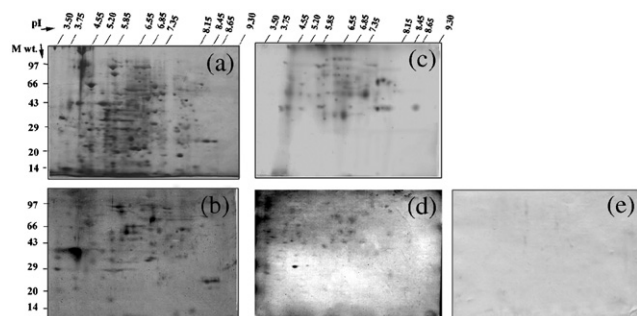


Fig. 3. 2-D analysis of *Epicoccum* spore-mycelial extract. (a) Protein profile of spore-mycelial extract of *Epicoccum* by 2-DE. (b) Allergen profile in 2-DE immunoblot using pooled patients' sera. (c) Periodic acid Schiff staining. (d) Immunoblot after periodate modification. (e) Blot probed with control serum pool. Spore mycelial proteins (600 µg) were fractionated on IEF tube gel in pI range 3.5–9.5. IEF tube was then equilibrated in SDS and laid horizontally on stacking gel. Proteins were separated on 10% resolving gel and visualized using Coomassie brilliant blue. For blot, proteins separated by 2D-PAGE were transferred to NCM and probed with pooled hypersensitive patients' sera or control sera. The IgE reactive spots were recognized using anti human IgE-HRP (Sigma, USA).

(Fig. 3e). The individual patients' sera showed IgE reactivity to 4–55 protein spots (Fig. 4 a–h). Inter-individual variation was observed in terms of both relative intensity and number of spots. A 25 kDa (pI 7.85, spot no. 1) protein spot was recognized most by 7/8 sera followed by the same molecular weight protein spot at pI 8.1 (Fig. 4 a–h). This was followed by components of 56 kDa (pI 8.10), 28 kDa (pI 5.85), 20 kDa (pI 3.70), 28 kDa (pI 5.25), 43 kDa (pI 7.00), 29 kDa (pI 7.00), 39 kDa (pI 3.70), 32.5 kDa (pI 5.20; 5.90), 36 kDa (pI 5.80), 34 kDa (pI 7.3), 67 kDa (pI 5.80), 32.5 kDa (pI 7.60) and 12 kDa (pI 6.90). The percentage reactivity of each spot is presented in Table 3.

MALDI/MS

Protein spots ($n = 16$) corresponding to identified allergens (Fig. 4 a–h) were excised from the gel and analyzed by MALDI-TOF (Table 4). This resulted in identification of 6 distinct proteins, assigned as phospho-glycerate kinase (43 kDa; 7.00; spot 7), RNA-dependent RNA polymerase (67 kDa; 5.80, spot 14), pyrroline-5-carboxylate dehydrogenase (32.5 kDa; 5.20, spot 10), 40S ribosomal protein (36 kDa; 5.80, spot 12) and one protein (32.5 kDa; 5.90, spot 11) of unknown function. The peptide mass fingerprinting data of the sixth spot (34 kDa; 7.3, spot 13) did not show a significant match. The peptide peak 1281.708 was thus

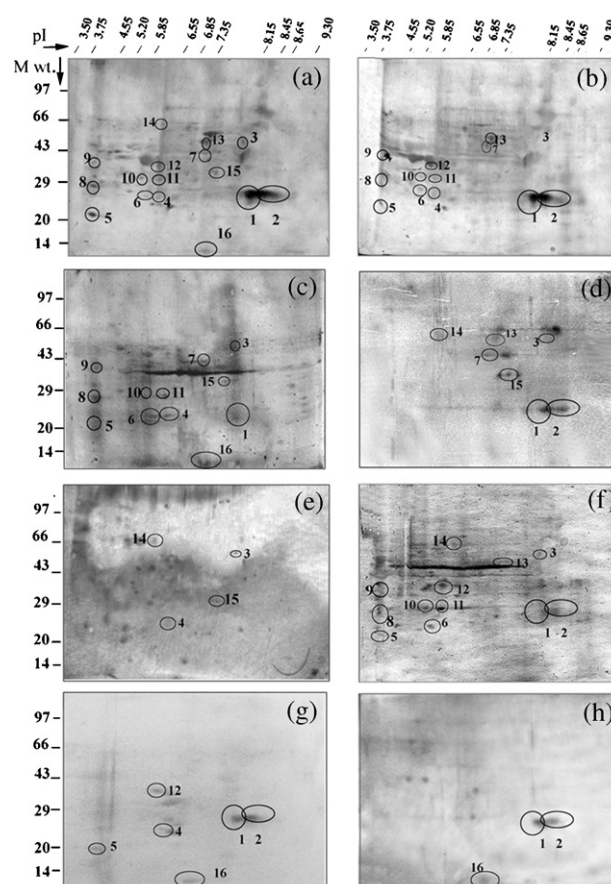


Fig. 4. 2-DE immunoblots with individual patients' sera (a–h). Proteins fractionated on 2-D PAGE were transferred to NCM and probed with individual patients' sera. Allergenic protein spots recognized by more than 50% patients' sera are marked.

subjected to further fragmentation. MS/MS generated an internal peptide sequence as 'asn-phe-val-pro-ala-his-glu-gln-val-ile-lys'. Homology search for this sequence showed similarity to hypothetical protein of *Debaryomyces hansenii*. Significant expectation values were not achieved for the remaining spots to suggest a clear identification. Some of the proteins exhibited a difference in observed and theoretical molecular weight value.

Discussion

Purified native or recombinant allergens are recommended for safe and reliable diagnosis and therapy of allergic disorders (Wihl et al., 1988). The identification of relevant allergens, i.e. specific proteins using appropriate techniques is required for this purpose (Chardin et al., 2002). The introduction of proteomic techniques has paved a way to identify the important allergenic proteins from different sources. In the present study, IgE binding proteins of *E. purpurascens* were identified by 2-DE immunoblotting and mass spectrometry.

Table 3. IgE reactivity (%) of important protein spots with individual patients' sera

Spot no.	Mol. wt (kDa)	pI	Patient								Control	% reactivity
			a	b	c	d	e	f	g	h	i	
1.	25.0	7.85	+	+	+	+	—	+	+	+	—	87.5
2.	25.0	8.1	+	+	—	+	—	+	+	+	—	75.0
3.	56.0	8.10	+	+	+	—	+	+	+	—	—	75.0
4.	28.0	5.85	+	+	+	—	+	—	+	—	—	62.5
5.	20.0	3.70	+	+	+	—	+	+	—	—	—	62.5
6.	28.0	5.25	+	+	+	—	—	+	—	—	—	50.0
7.	43.0	7.00	+	+	+	+	—	—	—	—	—	50.0
8.	29.0	7.00	+	+	+	—	—	+	—	—	—	50.0
9.	39.0	3.70	+	+	+	—	—	+	—	—	—	50.0
10.	32.5	5.20	+	+	+	—	—	+	—	—	—	50.0
11.	32.5	5.90	+	+	+	—	—	+	—	—	—	50.0
12.	36.0	5.80	+	+	—	—	—	+	+	—	—	50.0
13.	34.0	7.30	+	+	—	+	—	+	—	—	—	50.0
14.	67.0	5.80	+	—	—	+	+	+	—	—	—	50.0
15.	32.5	7.60	+	—	+	+	+	—	—	—	—	50.0
16.	12.0	6.90	+	—	+	—	—	—	+	+	—	50.0

The symbols '+' and '—' indicate presence and absence respectively, of a spot in the blot with mentioned sera.

Table 4. Peptide mass fingerprint database search of selected spots from 2-DE of *E. purpurascens* proteins

S. no	Spot	Gel M; pI	Mascot score	Protein name	Access key	Species	Proposed function
1	14	67; 5.8	71.9	Putative RNA-dependent RNA polymerase (Fragment)-CMW 5588.	Q8X1C2	<i>Phomopsis</i> sp.	RNA polymerase
2	12	36; 5.8	65.8	AF052483 NID	AAC15834	<i>Blumeria graminis</i> f. sp. <i>Hordei</i>	40S ribosomal protein S12
3	15	34; 7.3	53.7	Hypothetical protein. Ms data sequence: NFVPAHEQVIK-homologous to hypothetical protein DEHA0A01793 g (e value=0.021)	Q7S807	<i>Neurospora crassa</i> <i>Debaryomyces hansenii</i> CBS767	Not known
4	7	43; 7.0	64.1	ABPGKA NID	CAA66195	<i>Agaricus bisporus</i>	3-Phospho-glycerate kinase
5	10	32; 5.2	82.9	1-pyrroline-5-carboxylate dehydrogenase (EC 1.5.1.12) precursor	RDBYC	<i>Saccharomyces cerevisiae</i>	de novo synthesis of ornithine
6	11	32; 5.9	70.1	YGL028 protein	Q9P307	<i>Saccharomyces cerevisiae</i>	

In a previous study by 1-DE and immunoblotting, 44 IgE binding proteins were detected in spore-mycelial extract of *Epicoccum*. Of these, five components of 114, 82, 77, 54 and 42 kDa were IgE reactive to more than 50% of patient's sera (Dixit et al., 1992). Bisht et al. (2000) identified six bands of 105, 100, 75, 62, 56 and 30 kDa as the major IgE reactive components (>80% patients sera) in *Epicoccum* extract. The IgE reactive bands identified by 1-DE may be a mixture of proteins

and hence the need for 2-DE immunoblotting was emphasized to determine the true number of allergens (Dixit et al., 1992).

In 2-DE, the sample preparation is a crucial step, which involves treatment with denaturants, detergents and reducing agents. Pretreatment of *Epicoccum* extract with SDS and DTT was employed here to solubilize proteins. High concentration (4%) of non-ionic (NP-40/Triton X-100) or Zwitterionic (CHAPS)

detergent was used to replace SDS bound to proteins (Ames and Nikaido, 1976). NP-40 treatment gave the best results and approximately 250 spots were identified in *E. purpurascens* extract using this approach.

In the present study, 2-DE analysis of *Epicoccum* extract showed several protein spots corresponding to proteins identified earlier (Bisht et al., 2000). Immunoblotting showed 147 IgE reactive spots with pooled patients' sera and 4–55 spots with different individual patients' sera. There was marked difference in IgE reactivity of individual patients' sera with *Epicoccum* proteins. Such variation in IgE reactivity may be due to differential exposure to allergenic proteins and/or different genetic background of the subjects. The IgE reactivity may be attributed to both protein and carbohydrate part of the allergens. The 2-DE separated allergens were subjected to periodate treatment and probed with pooled patients' sera. This resulted in partial and/or complete loss of IgE reactivity to some spots. Thus, only 41 spots reacted to pooled sera after the periodate treatment. The reduction in reactivity can be attributed to modification of carbohydrate structure and to changes induced in protein structure/epitopes upon periodate treatment. In a previous study, periodate treatment of a purified allergen (Epi p 1) of *Epicoccum* led to partial loss of IgE reactivity, indicating the involvement of carbohydrates in allergenicity (Bisht et al., 2004b). However, the reduced reactivity upon periodate treatment may also result from other than carbohydrate modifications induced by the treatment. Periodate treatment of Art v 1, the major mugwort pollen allergen, resulted in a loss of IgE binding, even to peptide moieties of the protein (Leonard et al., 2005). Apart from role in IgE binding, carbohydrates can also promote specific Th2 response, resulting in increased IgE production (Okano et al., 2004).

Two-dimensional electrophoresis immunoblotting has been used for identification of new allergens from fungi namely *M. furfur* (Mal f 4, 35 kDa) and *P. citrinum* (Pen c 2, 39 kDa) (Chow et al., 1999; Onishi et al., 1999). Further, major allergens from *A. flavus* (33 kDa, Asp fl 1), *P. notatum* (Pen n 18, 33 kDa) and IgE-binding proteins of *Cryptomeria japonica* pollen (Yu et al., 1999, 2002; Fujimura et al., 2004) were identified using 2-DE. In the present study, 16 important allergens identified by 2 DE immunoblotting were subjected to MALDI-TOF analysis. Six of these allergens showed significant matches to protein profiles in database. Most of the proteins identified seem essential for cellular functions of the mold. Peptide spectra of 32.5 kDa protein (pI-5.90, spot 11) showed significant homology with a protein of the aldehyde dehydrogenase family. Aldehyde dehydrogenases from *Cladosporium* and *Alternaria* are reported as important allergens (Achatz et al., 1995). Ribosomal proteins have also been designated as important allergens (Hoff et al., 2003). RNA polymerase

is reported as an allergen for the first time in this study. A 43 kDa phosphoglycerate kinase was identified as a major IgE reactive protein from *C. albicans* (Ishiguro et al., 1992). Phosphoglycerate kinase identified from *E. purpurascens* can be explored for cross-reactivity among various fungi. The 34 kDa allergen (pI-7.3, spot 13) showed homology to a protein of unknown function. The internal amino acid sequence data can be used for fishing out the gene encoding this allergen. The identification of these allergens will allow development of well-defined reagents for component-based diagnosis of allergy. Monoclonal antibodies against such allergens can help in determining patient's exposure to these environmental triggers.

In conclusion, 2-DE immunoblotting and proteomic analysis enabled identification of important IgE reactive proteins from spore mycelial extract of *E. purpurascens*. Six important allergens reacting with more than 50% patients' sera were identified using mass spectrometric approach. The major allergens identified can be explored for cross-reactivity among fungal allergens. Further, the knowledge of these allergens can contribute to designing safer and more effective immunotherapy antigens.

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