

RESEARCH ARTICLE

Purification and immunological characterization of a 12-kDa allergen from *Epicoccum purpurascens*

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Abstract

Exposure to *Epicoccum purpurascens* is implicated in respiratory allergies and asthma. Several allergens of clinical importance were identified in *Epicoccum* extract (EE), but only one allergen has been isolated and characterized. In the present study, a 12-kDa allergen was isolated from an *Epicoccum* spore-mycelial extract by concanavalin-A sepharose, reverse-phase hydrophobic and gel filtration chromatography. The purified protein was recognized as a single 12-kDa allergen on immunoblot with a serum pool of *Epicoccum*-sensitive patients. Of the 94 respiratory allergy patients tested intradermally, 17 showed marked positive skin reactions to EE and 12 of them reacted with the 12-kDa protein, indicating a diagnostic sensitivity of 70%. More than 80% patients' sera showed immunoglobulin E (IgE) reactivity to the purified protein in enzyme-linked immunosorbent assay and immunoblot, identifying it as a major allergen. Preincubation of pooled serum with the protein led to inhibition of IgE binding to solid-phase-bound EE (effective concentration 50% = 180 ng). Twelve of the 17 serum samples showed significant basophil histamine release upon stimulation with purified protein. The protein induced significant proliferation of peripheral blood mononuclear cells of 13 patients. A high level of interleukin-4 in the culture supernatant of these cells indicated induction of a T-helper type 2 response. The purified 12-kDa protein is a clinically relevant allergen and has potential for the diagnosis and therapy of *Epicoccum* allergies.

Introduction

Sensitization to fungi accounts for c. 25–30% of allergic asthma cases (Vijay *et al.*, 2005). *Epicoccum purpurascens* is a predominant mold that induces sensitization in 5–7% of the atopic population worldwide (Hogan *et al.*, 1996; Noble *et al.*, 1997; Bisht *et al.*, 2000). This mold contains many immunoglobulin E (IgE) binding proteins in its spore-mycelial extract (Dixit *et al.*, 1992; Bisht *et al.*, 2000, 2002; Kukreja *et al.*, 2008a). A previous study demonstrated the presence of 16 IgE-reactive proteins in the spore-mycelial extract of *E. purpurascens* by two-dimensional (2-D) immunoblotting. These proteins were recognized by 50–87% individual patients' sera tested in the study (Kukreja *et al.*, 2008a). The frequency of IgE reactivity was the highest for a 25-kDa protein. This knowledge can be utilized to develop suitable molecules for component-resolved diagnosis and therapy of allergic disorders.

Currently, spore-mycelial extracts derived from various fungi including *Epicoccum* are used for the diagnosis and therapy of allergic diseases. These extracts are a heterogeneous mixture of proteins, carbohydrates and organic compounds. In addition, fungal extracts may contain mycotoxins and tremorgenic metabolites (Hussein & Brasel, 2001). The use of undefined extracts can induce a false-positive reaction in the diagnosis or adverse reactions during therapy. The repeated administration of fungal extracts can lead to the acquisition of new sensitivities due to the presence of many potent allergens. In a study on grass pollen immunotherapy for 1 year, the patients developed *de novo* immune responses to new epitopes/allergens present in the extract (Ball *et al.*, 1999). Birch pollen rush immunotherapy of patients sensitized to Bet v 1 led to the development of clinically relevant sensitizations to new allergens in 65% of the treated patients (Moverare *et al.*, 2002). Such evidences call for a reassessment of the use of a crude extract and

necessitate the need for defined allergen molecules from different sources for specific diagnosis and therapy.

The reactivity profile of patients sensitized to an allergen source differs widely. Hence, a panel of purified major allergens that can replace a crude extract is recommended. Studies have demonstrated that a mixture of a few purified allergens can reproduce the allergenic complexity of natural extracts required for the *in vitro* diagnosis of allergies (Niederberg TePas *et al.*, 2004). Previously, a 33-kDa major allergen was isolated from a spore-mycelial extract of *E. purpurascens* using concanavalin-A (Con-A) sepharose chromatography and characterized using hypersensitive patients' sera (Bisht *et al.*, 2004a). This protein was characterized as serine protease and its activity augments the lung inflammation (Bisht *et al.*, 2004b; Kukreja *et al.*, 2008b). In addition, two Con-A-binding proteins (16 and 12 kDa) were recognized by > 80% of patients' sera. The present study aimed to isolate an allergenic protein from *E. purpurascens* and characterize by immuno-biochemical methods for clinical application.

Materials and methods

Allergic patients' blood and sera samples

Respiratory allergy patients visiting the Out Patients Department, V.P. Chest Institute, Delhi, were tested intradermally with a battery of common allergens such as pollen, insects, dust mite and fungi including *E. purpurascens* extract for allergy diagnosis (Kukreja *et al.*, 2008a). Blood/sera were obtained from patients skin test positive to *E. purpurascens*. The serum samples showing high-specific IgE were pooled and used during isolation of allergenic protein by an immunoblot. The study protocol was approved by the human ethics committee and informed consent of the subjects was obtained for participation.

Epicoccum spore-mycelial extract

Epicoccum purpurascens MTCC 2129 (Microbial Type Culture Collection and Gene Bank, Institute of Microbial Technology, Chandigarh, India) was grown in Sabouraud's broth with yeast extract medium as a stationary culture for 13 days at 25 °C. The spore-mycelial mass was harvested on day 13 and the extract was prepared as described previously (Bisht *et al.*, 2000). The protein content of the extract was determined by modified Lowry's method (Singh *et al.*, 1992). The protein profile of *Epicoccum* extract (EE) was tested by immunoblotting with sera of *E. purpurascens*-allergic patients.

Protein purification

Three chromatographic steps were used to obtain a purified 12-kDa protein.

Affinity chromatography

EE was fractionated on a Con-A Sepharose column (Bisht *et al.*, 2004a). The column was washed with equilibration buffer containing 5 mM Tris-HCl, pH 7.4, 0.02% NaN₂ and 1 mM each of MgCl₂, MnCl₂ and CaCl₂. One hundred milligram of EE was applied onto the column and the unbound proteins were removed with equilibration buffer (pH 7.4). To remove the nonspecifically bound proteins (nonspecific electrostatic and hydrophobic interactions), 4 M NaCl and 0.25% Triton X-100 in equilibration buffer, respectively, were passed through the column. The bound proteins were eluted with 0.4 M α -methyl mannopyranoside in equilibration buffer. The eluate was dialyzed extensively against distilled water and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot. The proteins were fractionated further by reverse-phase hydrophobic chromatography.

Reverse-phase hydrophobic chromatography

For this, octadecyl silica gel (0.5 g) (Sigma Chemical Co.) was packed into a 1-mL column in 100% acetonitrile. The column was washed with 50 mL acetonitrile and equilibrated with 20 mL distilled water. The dialyzed eluate of Con-A gel was incubated with equilibrated octadecyl silica resin overnight, at 4 °C. Unbound proteins were washed out with distilled water till the absorbance became zero at 280 nm. The bound proteins were eluted with 15 mL each of 20%, 30%, 40%, 50% and 60% acetonitrile in water containing 0.1% trifluoroacetic acid. The fractions eluted with different concentrations of acetonitrile were freeze dried and stored at -20 °C. The eluted fractions were analyzed by SDS-PAGE and immunoblotted using *Epicoccum*-hypersensitive patients' serum pool.

Fast protein liquid chromatography

The sample obtained after a two-step separation on Con-A sepharose and reverse-phase hydrophobic gel (30% acetonitrile fraction) was subjected to gel filtration. A Pharmacia HR 10/30 SuperdexTM 75 gel filtration column (flow rate 0.5 mL min⁻¹) was equilibrated with 20 mM phosphate buffer, pH 7.0. The column was calibrated using RNase (13.7 kDa), ovalbumin (43 kDa) and blue dextran (2000 kDa) as reference proteins. The fraction eluted with 30% acetonitrile from a reverse-phase column was resuspended in 20 mM phosphate-buffered saline (PBS), pH 7.0, and loaded for the gel filtration. Fifty fractions (0.5 mL) were collected and the protein content in each fraction was determined by a bicinchoninic acid assay (Stoscheck, 1990). Fractions containing the protein domain were concentrated and analyzed by SDS-PAGE and immunoblotting with

Epicoccum-hypersensitive patients' sera. The fractions containing the 12-kDa allergenic protein (checked by immunoblot) were pooled.

The purified protein was assessed for its allergenic potential by *in vivo* (skin-prick) tests and *in vitro* assays.

In vivo reactivity of purified protein

Patients with allergic rhinitis, asthma or both were tested intradermally using *E. purpurascens* extract (1:500 w/v in PBS) and purified protein (100 µg mL⁻¹ in PBS). Histamine diphosphate (100 µg mL⁻¹) and PBS were used as positive and negative controls, respectively. The tests were graded after 20 min based on weal size, in comparison with positive control (Dhyani *et al.*, 2006). A weal size equivalent to histamine control was considered as 2+. Weal with two pseudopodia and a size greater than histamine control was considered as 3+. Blood was collected from patients (*n* = 17) showing marked positive skin reactivity (2+ and above) with EE. Blood was also collected from nonallergic subjects (*n* = 4) with negative skin tests to EE for use as control (C). The blood/serum samples were used for evaluation of the allergenic potency of purified protein by immunoblot, enzyme-linked immunosorbent assay (ELISA), ELISA inhibition, histamine release and lymphoproliferation assays.

Specific IgE estimation

Specific IgE against purified protein (12 kDa) was measured in sera of *Epicoccum*-allergic patients (*n* = 17) and controls (*n* = 4) by ELISA. EE and purified the 12-kDa protein were coated on a microtiter plate (Nunc ELISA maxisorp, Denmark) at a concentration of 1 µg/100 µL per well and 100 ng/100 µL per well, respectively. The plate was incubated overnight at 4 °C. On the following day, the unbound antigen was washed out with PBS (0.1 M) and the free sites were blocked with 3% nonfat dry milk in PBS for 1 h at 37 °C. After washing, 100 µL of control sera or individual patients' sera (1:10 v/v in PBS) were added separately to the wells and the plate was incubated overnight at 4 °C. The plate was washed five times with PBST (PBS with 0.05% Tween 20), once with PBS and incubated with 1:1000 (v/v PBS) diluted anti-human IgE peroxidase (Sigma Chemical Co.) for 3 h at 37 °C. Following washing with PBST and PBS, color was developed using *o*-phenylenediamine in citrate-phosphate buffer, pH 5.0. The reaction was stopped after 30 min with the addition of 50 µL of 5 N H₂SO₄ and the absorbance was read at 492 nm. ODs ≥ 3 times of controls i.e. nonallergic subjects were considered ELISA positive.

Specific IgE ELISA inhibition

The serum pool of 17 patients with high IgE was used for ELISA inhibition. The serum (50 µL) was inhibited by

incubation with different concentrations (0.1–10 µg) of purified protein and EE. The inhibited and uninhibited sera were then used in ELISA as described above. Sera of nonallergic subjects were used as a control for the assay. ELISA values in wells incubated with inhibited serum samples were calculated relative to that of uninhibited serum as inhibition percentage. Allergenic activity was evaluated by establishing the 50% inhibition point.

SDS-PAGE and immunoblot

SDS-PAGE (12% gel) was performed under denaturing conditions to determine the protein profile of EE and Con-A sepharose and octadecyl silica-eluted fraction and gel filtration fraction. The gel was either stained with 0.1% Coomassie brilliant blue (CBB) or used for immunoblotting.

For immunoblot, SDS-PAGE-resolved proteins were electrophoretically transferred to a nitrocellulose membrane (Towbin *et al.*, 1979) using a mini transblot cell (Bio Rad, CA). The bands were visualized with Ponceau-S (0.1% in 1% acetic acid) to ensure the transfer of proteins. IgE-binding proteins in fractions were detected using *Epicoccum*-positive patients' serum pool/individual patients' sera (1:10 v/v in PBS). As a control, a strip blotted with purified protein was incubated with the same dilution of control serum.

Histamine release on allergen challenge

Peripheral blood was drawn from nonallergic donors and mixed (1:5 v/v) with 6% dextran in saline containing 0.01 M EDTA and 2% dextrose. After an incubation of 90 min at room temperature, the leukocyte-rich upper layer was drawn, centrifuged (300 g, 4 °C) and washed twice with saline. A 375-µL sample of cell suspension (2.5 × 10⁶ cells) was taken for each incubation. The basophils in suspension were stripped off bound IgE by incubation with lactic acid buffer for 3.5 min at 37 °C (Kleine Budde *et al.*, 2001). Following incubation, cells were washed in HEPES buffer. Subsequently, the cells were resensitized at 37 °C for 90 min with a sensitization mixture that contained sera with elevated IgE against *Epicoccum* allergen (*n* = 17). Cells sensitized with nonallergic sera (*n* = 4) served as control. The histamine release assay was standardized using a graded amount of protein (1 ng–1 µg), and the protein concentration inducing optimal histamine release (5 ng) was selected for the assay.

After passive sensitization, cells were stimulated with EE or the purified 12-kDa protein (5 ng) in HEPES buffer containing 1 mM CaCl₂. After a 1-h incubation at 37 °C, the reaction was stopped by the addition of 750 µL of ice-cold 0.9% NaCl (w/v). After centrifugation, the cell-free supernatant containing histamine was taken in a fresh Eppendorf tube and mixed with 200 µL of 12% perchloric

acid. The histamine content was determined by the fluorometric method, using *o*-phthalaldehyde (Shore *et al.*, 1959). Spontaneous histamine release was measured in the supernatant of unstimulated cells. The total histamine content was determined by lysis of cells with 3% perchloric acid (Dhyani *et al.*, 2006). The allergen-induced histamine release was calculated as a percent of the total histamine content after correcting for spontaneous release.

Lymphocyte proliferation and cytokine assay

Peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. For this, the blood was diluted 1:1 with PBS and layered on 4 mL histopaque (Sigma Chemical Co.). After centrifugation at 400 *g* for 30 min, mononuclear cells at the interface of plasma and Ficoll layer were collected. Cells were washed twice and resuspended in complete Roswell Park Memorial Institute (RPMI) medium containing penicillin/streptomycin (100 U mL⁻¹ and 100 µg mL⁻¹, respectively) and fetal calf serum (10%). The cell count was adjusted to 1 × 10⁶ cells mL⁻¹. One hundred microliters of this suspension from each patient (10⁵ cells) was plated in 96-well flat-bottom tissue culture plates (Corning, NY) and challenged with EE or purified 12-kDa proteins at a 5 µg mL⁻¹ concentration. As a positive control, the cells were challenged with phytohemagglutinin (5 µg mL⁻¹). Wells without antigen were treated as negative control. The culture plates were incubated in a humidified 5% CO₂ incubator for 72 h and the supernatant was collected for cytokine assay. Cells were washed with RPMI and incubated for 2 h with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (100 µg mL⁻¹). The cells were lysed in an acidic isopropanol solution and the absorbance was read at 570 nm (Shankar *et al.*, 2006). OD values more than or equal to three times the negative control were considered as a positive stimulation index.

The levels of cytokines interleukin-4 (IL-4) and interferon-γ (IFN-γ) in the cell culture supernatant were determined by a sandwich ELISA following the manufacturer's instructions (BD OptEIA kit). The detection range for IL-4 and IFN-γ was 7.8–500 and 4.7–300 pg mL⁻¹, respectively. The amounts of cytokines IL-4 and IFN-γ in various samples were calibrated from the absorbance vs. concentration log-log curve for known amounts (standards) of each cytokine.

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) analysis

The purified protein was run on SDS-PAGE and stained with CBB. The stained band/protein was cut using a sterile blade and placed into a fresh Eppendorf tube. The MALDI-

TOF analysis of the trypticized sample was carried out at The Centre of Genomics Applications, New Delhi.

Statistical test

The correlation between positive *in vivo* (intradermal reactivity) and *in vitro* (ELISA reactivity) was determined by means of linear regression analysis (Microsoft EXCEL).

Results

Allergen purification

EE resolved into *c.* 26 bands on SDS-PAGE. EE (100 mg protein) was applied to a Con-A sepharose column and eluted with α-methyl mannopyranoside. The SDS-PAGE profile of Con-A-eluted proteins is presented in Fig. 1a. The eluate showed five darkly stained bands on 12% gel. IgE immunoblot of the Con-A-bound fraction with *Epicoccum*-positive pooled patients' sera identified five allergenic frac-

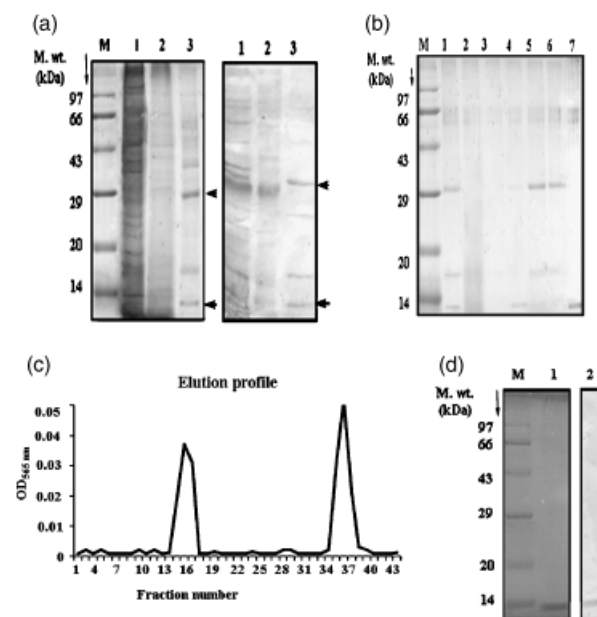


Fig. 1. (a) SDS-PAGE and immunoblot of spore-mycelial extract and Con-A fractionated proteins of *Epicoccum purpurascens*. Lane M, molecular weight marker; lane 1, *Epicoccum* extract; lane 2, Con-A unbound fraction and lane 3 fraction eluted with α-methyl mannopyranoside. (b) SDS-PAGE and immunoblot with fractionated proteins of *E. purpurascens*. Lane M, molecular weight marker; lane 1, Con-A eluted fraction; lane 2, octadecyl silica unbound; lanes 3–6, fractions eluted with 20–60% acetonitrile; lane 7, fraction eluted with 30% acetonitrile immunoblotted with *Epicoccum*-hypersensitive pooled patients' sera. (c) Elution profile of proteins separated from HR 10/30 SuperdexTM 75 gel filtration column. A volume of 0.5 mL was collected in each fraction. The protein content in different fractions was determined by bicinchoninic acid assay. (d) SDS-PAGE and immunoblot of purified 12-kDa protein with hypersensitive patients' sera. Lane M, molecular weight marker; lanes 1 and 2, purified protein on 12% gel and immunoblot.

tions of 66, 33, 26, 16 and 12 kDa. Of these, 33-, 16- and 12-kDa components were prominent (Fig. 1a).

The Con-A-bound fraction was dialyzed and applied on an octadecyl silica column. The unbound proteins were removed by washing with water and bound proteins were eluted using an acetonitrile gradient (20–60%). The SDS-PAGE profile showed a 66-kDa doublet in the fraction eluted with 20% acetonitrile and two prominent proteins of 66 and 12 kDa in the fraction eluted with 30% acetonitrile. The 17 and 33-kDa components were eluted in both 40% and 50% acetonitrile fractions (Fig. 1b). The fraction that eluted with 30% acetonitrile was further analyzed by immunoblotting with *Epicoccum*-positive patients' serum pool. Both the components were recognized by IgE in the serum pool.

The proteins were further separated on the gel filtration column. The protein profile of the eluted fractions showed two major peaks. The fractions corresponding to these peaks were lyophilized and analyzed by SDS-PAGE. It showed a single band of 12 kDa in the 35th fraction (Fig. 1c). The yield of protein was 0.6 mg/100 mg protein loaded on the column. Immunoblotting with *E. purpurascens*-positive patients' serum pool showed the presence of a single protein at this molecular weight (Fig. 1d). The control sera did not react to this protein component.

Allergenic potential of purified protein

Skin reactivity with purified protein

Intradermal testing was performed on 96 respiratory allergy patients with both the EE and the purified protein (12 kDa). Seventeen patients exhibited a marked positive skin reaction (2+ and above) to EE. Of these, 12 patients showed positive skin test reactivity to the purified 12-kDa protein (Table 1). The blood/serum sample of 17 *Epicoccum*-positive patients and four nonallergic individuals was used for further analysis.

Specific IgE estimation and immunoblot

The antigen-specific IgE binding was determined by ELISA using the above-mentioned sera. ODs ≥ 3 times of control serum (average OD: 0.056) were taken as a cut-off for ELISA-positive results. Serum samples from all 17 patients showed elevated IgE binding (average OD: 0.684) to EE. Of these, 14 sera showed elevated IgE binding to the 12 kDa protein (average OD: 0.496). Serum samples from patient numbers 7, 8 and 12 did not show IgE binding with the 12-kDa protein. There was a significant correlation in the positive skin test reactivity of the purified protein and specific IgE binding by ELISA ($r=0.717$) (Table 1).

Figure 2a shows the IgE binding of the 12-kDa protein by immunoblot with individual patients' sera. This 12-kDa

Table 1. Intradermal test and ELISA binding with the purified 12-kDa protein and EE in asthma and rhinitis patients

Sl. no.	Weal area (mm ²)			ELISA (OD)	
	Histamine	EE	The purified 12-kDa protein	EE	The purified 12-kDa protein
1.	12 × 14	14 × 14	12 × 13	0.781	0.515
2.	13 × 12	12 × 11	6 × 6	0.692	0.419
3.	12 × 12	12 × 12	10 × 11	0.583	0.394
4.	17 × 12	15 × 13	14 × 12	0.661	0.511
5.	15 × 14	14 × 10	14 × 14	0.762	0.494
6.	11 × 12	15 × 13	11 × 13	0.812	0.398
7.	12 × 13	16 × 15	5 × 6	1.079	0.102
8.	11 × 12	14 × 12	5 × 5	0.534	0.111
9.	12 × 12	15 × 14	11 × 12	0.803	0.650
10.	14 × 10	15 × 13	14 × 11	0.614	0.583
11.	10 × 10	11 × 12	10 × 10	0.514	0.291
12.	13 × 14	14 × 15	4 × 6	0.736	0.092
13.	12 × 11	12 × 14	12 × 11	0.492	0.692
14.	13 × 12	15 × 16	13 × 10	0.932	0.456
15.	14 × 12	15 × 13	10 × 11	0.502	0.502
16.	13 × 12	14 × 13	13 × 12	0.487	0.547
17.	11 × 11	12 × 13	6 × 5	0.650	0.169
C1	11 × 12	3 × 4	3 × 4	0.070	0.056
C2	12 × 13	4 × 3	3 × 3	0.074	0.063
C3	13 × 11	5 × 3	4 × 3	0.068	0.049
C4	13 × 12	4 × 3	3 × 3	0.060	0.058

Histamine diphosphate (100 µg mL⁻¹) was used as a positive control for intradermal testing. The tests were also carried out on four nonallergic subjects (C1–C4).

ELISA positivity was determined with reference to negative control. Sera showing OD ≥ 3 times the average value of control serum (0.056) were considered positive.

Correlation test was carried out between weal size of positive (2+ and above) skin tests and positive specific IgE values (ODs) in ELISA for the purified 12-kDa protein.

Correlation coefficient (r) = 0.717.

protein was recognized by 13/17 (76.4%) of the *Epicoccum*-hypersensitive patients' sera, suggesting it to be a major allergen (Fig. 2a).

ELISA inhibition

To check the specificity of IgE binding and the allergenic potency of the purified 12-kDa protein, ELISA inhibition was carried out with hypersensitive pooled patients' sera. Here, a maximum of 68% inhibition was achieved with the highest amount (1 µg) of the purified 12-kDa protein used as an inhibitor for the solid-phase-bound EE. For 50% inhibition of the solid-phase-bound EE, 180 ng of purified protein was required, while 40 ng of EE was required for the same (Fig. 2b).

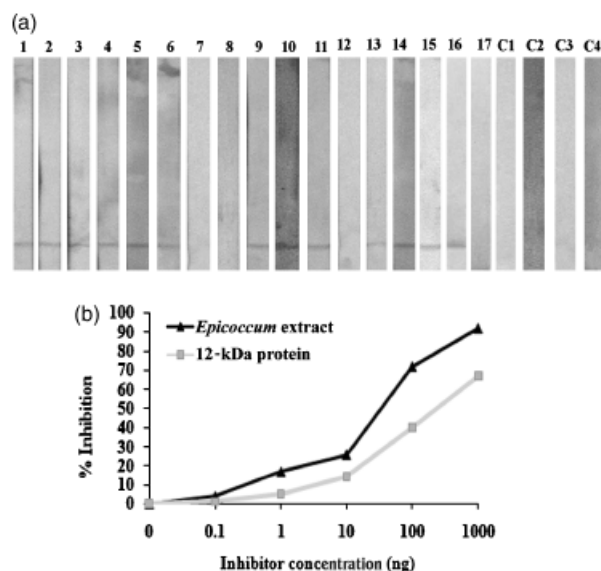


Fig. 2. (a) IgE binding of purified 12-kDa protein with individual patients' (1–17) sera on immunoblot; C1–C4, IgE reactivity with control sera. (b) IgE inhibition of *Epicoccum* extract (solid phase) with *Epicoccum*-hypersensitive pooled patients' sera. The serum was incubated with 0.1–1000 ng of *Epicoccum* extract or purified protein as inhibitor.

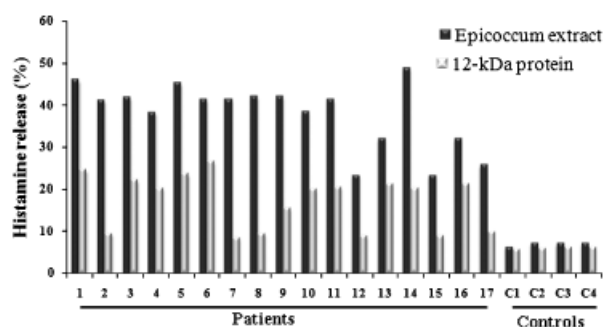


Fig. 3. Histamine release assay. Basophils in leukocyte suspension were stripped off the bound IgE. Stripped basophils were sensitized with sera of *Epicoccum*-hypersensitive patients. The sensitized basophils were stimulated with *Epicoccum* extract or 12-kDa purified protein. The histamine released in cell supernatant was quantified and expressed as percentage of total histamine content.

Histamine release assay with purified protein

The biological potency of purified protein was assessed by its ability to induce histamine release from sensitized basophils. Basophils sensitized with 17 patients' sera (IgE) individually showed a positive histamine release on challenge with 5 ng EE in the range of 23–45%. On challenge with the purified 12-kDa protein (5 ng), 12 patients' samples showed significant histamine release in the range of 12–28%. Stimulation with the 12-kDa protein induced low levels (< 10%) of histamine release in patient samples 2, 7, 8, 12 and 17. Basophils sensitized with control subjects' sera showed 6–8% histamine release in response to these antigens

(Fig. 3). Here, histamine release $\geq 10\%$ was taken as the cut-off to grade the positive results (Dhyani *et al.*, 2006).

Mitogenic potential of purified protein

PBMCs from patients ($n = 17$) were isolated and stimulated with EE and the purified 12-kDa protein separately. The response was measured in terms of *in vitro* proliferation and cytokine release. Lymphocyte proliferation in *Epicoccum*-sensitized patients with 5 μg allergen concentration is presented in Fig. 4a. OD values ≥ 3 times of the control were considered as a positive stimulation index. PBMCs from all the patients showed significant proliferation (three to seven times higher than control) upon challenge with EE (Fig. 4a). A positive stimulation index was recorded for 13/17 patients' PBMC samples upon challenge with the 12-kDa protein. The remaining four samples showed proliferation rate *c.* 2 times that of controls.

Antigen challenge induced higher IL-4 secretion from patient's PBMCs than controls. Mononuclear cells stimulated with EE and the 12-kDa allergen secreted IL-4 in the range of 175–750 and 38–600 pg mL^{-1} , respectively. IL-4 levels were, however, low in the culture supernatant of cells from patient numbers 2, 7, 8, 12, 15 and 17 (Fig. 4b). IFN- γ levels were low on stimulation with EE and the 12-kDa protein (15–43 and 10–33 pg mL^{-1} , respectively) similar to controls (Fig. 4c).

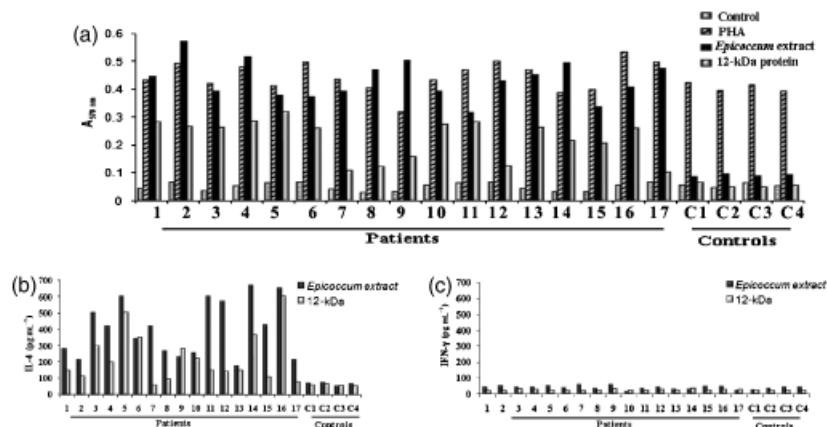
MS of protein

To investigate the biological function of the 12-kDa protein, it was subjected to tandem mass spectrometric analysis. The peptide sequences were searched in the matrix science database. This showed the highest match with sp/QO6010 *Saccharomyces cerevisiae* YLR389c. The sp/QO6010 of yeast is a metalloprotease of 116 kDa with endopeptidase activity.

Discussion

Allergen immunotherapy is an accepted form of treatment for allergic rhinitis and asthma. Although crude fungal extracts are currently used for the purpose of allergy diagnosis and therapy, purified allergens present the advantage of good sensitivity and specificity (Frostad *et al.*, 1983; Asturias *et al.*, 2005; Kurup *et al.*, 2005). Furthermore, purified proteins are also required to elucidate the functional aspect of the protein in driving allergic reactions. Earlier, a purified preparation was shown to be superior to the corresponding crude aqueous extract in the diagnosis of IgE-mediated allergy to timothy pollen (Frostad *et al.*, 1983). Alt a 1, a purified major allergen, showed a diagnostic efficacy comparable to that of crude *Alternaria alternata* extract in a skin-prick test (Asturias *et al.*, 2005). The spore-

Fig. 4. (a) Analysis of mitogenic potential of purified 12-kDa protein, by lympho-proliferation assay. PBMCs from different subjects were stimulated with allergen extract or 12-kDa protein for 3 days. The proliferative index was measured after 72 h using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. (b and c) Analysis of cytokines (IL-4 and IFN- γ) secreted in response to *Epicoccum* extract, and 12-kDa protein in the culture supernatant of PBMCs from different subjects. The cytokine levels were measured by ELISA using appropriate antibodies. Amount of cytokine in different samples was calibrated from standards of known concentration. 1–17, Patients' sample; C1–C4, controls.



mycelial extract of *E. purpurascens* demonstrated multiple IgE-reactive proteins upon immunoblotting (Bisht *et al.*, 2000; Kukreja *et al.*, 2008a). For a complete representation of this allergenic source, a panel of allergens needs to be developed from *Epicoccum*. In the present study, a 12-kDa protein was isolated from EE and evaluated by standard *in vitro* and *in vivo* methods.

Glycoproteins have been isolated from various allergenic sources viz. fungi, pollens, insects, etc., and detected as important/major allergens (Hino *et al.*, 1995; Okano *et al.*, 2001; Bublin *et al.*, 2003; Gupta *et al.*, 2004). A previous study with *Epicoccum* has revealed a high glycoprotein content in its spore-mycelial extract (Bisht *et al.*, 2000). Bisht *et al.* (2004a) reported four Con-A-binding allergens in EE that were recognized by IgE of all the *E. purpurascens*-positive patients' sera tested. In another study, a 12 kDa component was detected as a major allergen by > 50% of patients' sera tested on 2-D immunoblot (Kukreja *et al.*, 2008a).

Studies suggest that a single chromatographic method may not yield the protein of desired purity. Thus, multistep chromatographic methods are used to isolate proteins from crude extracts. Yunginger *et al.* (1980) isolated a 29-kDa (Alt a 1) major allergen of *A. alternata* by acetone precipitation and ion exchange chromatography. A 33-kDa serine protease allergen Pen c 1 was isolated from *Penicillium citrinum* using DEAE sepharose and carboxymethyl sepharose gel in combination (Su *et al.*, 1999). Ion-exchange chromatography, followed by size-exclusion chromatography, was used to purify a major allergen (65 kDa) of *Fusarium equiseti* (Verma *et al.*, 1998). A combination of Con-A sepharose and gel filtration chromatography was used to isolate a major allergen (Cur l 1, 31 kDa) from *Curvularia lunata* (Gupta *et al.*, 2004). In the present study, a combination of Con-A Sepharose, reverse-phase hydrophobic and gel filtration chromatography was used to isolate an allergenic protein (12 kDa) from *E. purpurascens*. The yield of protein was 0.6 mg/100 mg of crude extract applied initially to the column.

Allergens mediate the biological effect by inducing the release of mediators (e.g. histamine) from effector cells (Chou *et al.*, 2002). The mediators released produce an allergic response, which can be examined *in vivo* by skin testing with the allergen in question (Verma *et al.*, 2003). Earlier, the biopotency of allergenic molds namely, *A. alternata*, *Aspergillus fumigatus*, *Candida albicans*, *Fusarium solani* and *Penicillium chrysogenum*, etc. has been determined by a histamine release assay (Zimmermann *et al.*, 1983; Akiyama *et al.*, 1996; Mari *et al.*, 2003; Shankar *et al.*, 2006). Epi p 1(33.5 kDa), a major allergen of *E. purpurascens*, was shown to be a potent stimulator of histamine release from *Epicoccum* allergic patients' blood samples (Kukreja *et al.*, 2008a). In the present study, the 12-kDa protein induced a markedly positive skin test reactivity in 12 of 17 *Epicoccum*-sensitive patients (70%) (Fig. 3). In an *in vitro* assay, 12-kDa protein induced significant histamine release from basophils sensitized with the serum of 12 of 17 *Epicoccum*-sensitive patients' samples. However, IgE-binding assays (ELISA and immunoblot) revealed the presence of specific IgE against the 12-kDa protein in 14 and 13 *Epicoccum*-positive patient serum samples, respectively (Fig. 2a and b). There was a discordance between IgE levels and histamine release for the sample from patient 2. This may be because histamine release depends not only on IgE levels but also on the cross-linking of effector cell-bound IgE by allergen.

The environmental allergens induce a T-helper type 2 (Th2) cell response in allergic individuals with mediation of IL-4 and IL-5 cytokines. These cytokines drive the hyperproduction of allergen-specific IgE and eosinophil infiltration (Rolland *et al.*, 2000). Studies with a purified allergenic protein from *A. alternata* showed *in vitro* mitogenic activity and Th2 cytokine secretion in patients' PBMCs (Shankar *et al.*, 2006). In the present study, the purified 12-kDa protein induced lymphocyte proliferation and IL-4 secretion, indicating a Th2-dominant immune response. The IFN- γ levels were low in the culture supernatant of cells

treated with the 12-kDa allergen (Fig. 4a). Mass spectrometric analysis of the 12-kDa protein identified it as a metalloproteinase fragment.

Crude allergen extract has side effects when used for diagnosis and therapy. Component-based diagnosis/therapy requires purified proteins. These purified proteins are the disease-causing components for allergic diseases. Moreover, multiple allergens are required for diagnosis/therapy. These purified proteins are required to elucidate the allergy mechanism, identify B and T cell epitopes and study the cross-reactivity and functional aspects.

In conclusion, a 12-kDa clinically relevant allergen was purified from *E. purpurascens*. It will facilitate diagnosis and treatment of *Epicoccum*-allergic diseases and may help in elucidating the allergy mechanism.

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