

Purification and Characterization of a Major Cross-Reactive Allergen from *Epicoccum purpurascens*

Vandana Bisht^a Naveen Arora^a B.P. Singh^a S.N. Gaur^b
Susheela Sridhara^a

^aInstitute of Genomics and Integrative Biology, Delhi University Campus, and ^bV.P. Chest Institute, Delhi, India

Key Words

Epicoccum purpurascens · *Epicoccum nigrum* ·
Purification · Glycoprotein · Allergen · Electro-elution ·
Periodate · Cross-reactivity

Abstract

Background: *Epicoccum purpurascens* (formerly *nigrum*) (EP), is a ubiquitous saprophytic mould found both indoors and outdoors. Several studies have reported sensitization to EP in 5–7 % of different populations worldwide. The diagnosis of mould allergy requires a standardized fungal extract that contains all its important allergenic proteins. The crude allergen extract from EP was standardized earlier, however none of its allergens have been purified. **Methods:** A major allergen from spore-mycelia extract of EP was purified using concanavalin A (Con A) Sepharose chromatography, gel filtration and electro-elution. The allergen isolated was characterized for its IgE-binding ability and cross-reactivity with five well-known allergenic fungi by ELISA and immunoblot. **Results:** A 33.5-kD glycoprotein allergen of EP, Epi p 1, was purified to homogeneity. All the EP allergic patients' sera tested recognized this protein. Peri-

odate modification of Epi p 1 showed partial loss in IgE binding while proteinase K treatment caused complete loss in binding to IgE. Dose-dependent inhibition in binding of rabbit anti Epi p 1 antibodies was obtained with Epi p 1, *Aspergillus fumigatus*, *Alternaria alternata*, *Curvularia lunata*, *Cladosporium herbarum* and *Fusarium solani* in ELISA. Rabbit antibodies to all the above five fungi recognized Epi p 1 in immunoblot, confirming that Epi p 1 shares common epitopes with the fungi tested. **Conclusion:** A major glycoprotein allergen of 33.5 kD was purified from EP which cross-reacts with other fungi. Hence this glycoprotein can be exploited to reduce the panel of allergen extracts used for therapy of mould allergy.

Copyright © 2004 S. Karger AG, Basel

Introduction

Fungal spores are universal atmospheric components both indoors and outdoors and are important causative agents of respiratory allergies [1–3]. Several studies have reported sensitization to *Epicoccum purpurascens* (EP), formerly known as *Epicoccum nigrum*, in 5–7% of different populations worldwide [4–8]. The fungal extracts are

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2004 S. Karger AG, Basel
1018–2438/04/1333–0217\$21.00/0

Accessible online at:
www.karger.com/iaa

Correspondence to: Susheela Sridhara, PhD
Institute of Genomics and Integrative Biology
Mall Road, Delhi University Campus
Delhi 110007 (India)
Tel. +11 27666157, Fax +11 27667471, E-Mail susheela@igib.res.in

complex mixtures of proteins and glycoproteins. The whole mass extract used for diagnosis/therapy contains many irrelevant proteins, which may hamper skin test reactivity and cause additional sensitization to patients [9–13]. Thus, purification of allergens from crude extracts is important. In our earlier study, IgE-binding proteins were identified in standardized spore-mycelial and culture filtrate extract of EP [14, 15]. However, none of the proteins have been isolated from EP. Allergenic components of varied molecular weights have been purified notably from other fungi namely *Alternaria alternata*, *Aspergillus fumigatus*, *Fusarium solani*, *Rhizopus nigricans*, using different purification techniques [9–13]. In this study, a 33.5-kD major glycoprotein allergen of EP was isolated from its spore-mycelial extract by liquid chromatography and electro-elution. The purified protein was immunobiochemically characterized using EP-sensitive patients' sera and checked for its cross-reactivity with rabbit antibodies to *A. alternata*, *A. fumigatus*, *Cladosporium herbarum*, *Curvularia lunata*, and *F. solani* extracts.

Materials and Methods

Preparation of EP Antigen and Biochemical Analysis

EP (strain 4625, Indian Agricultural Research Institute, Delhi) was grown in Sabouraud's yeast extract medium at 25°C in stationary conditions for 13 days. The spore-mycelial mass of EP was harvested and freeze-dried. The extraction of EP antigens was carried out in ammonium bicarbonate buffer (125 mM, pH 8.0) containing 5 mM ethylenediaminetetra-acetic acid and phenylmethylsulfonyl fluoride (1 mM) for 5 h at 4°C. The extract was dialyzed using the same buffer, lyophilized and stored at –70°C until analysis [14]. The protein content of EP extract was determined using a modified method of Lowry et al. [16]. The carbohydrate content was estimated by the phenol-sulfuric acid method [17].

Skin Test and Collection of Patients' Sera

The detailed history of each patient was recorded. The clinicopathological tests including blood analysis (total leukocyte count/differential leukocyte count), chest and paranasal sinus X-ray, pulmonary function test, urine and stool were conducted under the supervision of a specialist in allergy treatment (one of the authors). Intradermal tests were carried out with EP spore-mycelial extract (1:500 w/v) on the patients with nasobronchial allergy at the Clinical Research Center, V.P Chest Institute, Delhi, as described earlier [14, 15]. Phosphate-buffered saline (PBS, 0.1 M, pH 7.4) was used as negative control and histamine diphosphate (100 µg/ml) as positive control. Sera were collected from 50 patients showing marked positive skin reactions (wheal size 12 mm diameter or above) to EP and/or other fungal extracts. Sera were also collected from some nonallergic individuals to be used as negative control. Nine sera (equal amount) showing ODs 10 times or more than normal human sera in EP-specific IgE ELISA were used to prepare a serum pool. Informed consent was obtained from patients and normal subjects (Indian Council of Medical Research, New Delhi) for skin tests and serum collection.

Purification of EP Glycoprotein

The crude EP spore-mycelial extract showed a high carbohydrate to protein ratio (2:1) and a large number of protein bands stained with periodic acid-Schiff (PAS). Hence the glycoproteins (glucose and mannose specific) were fractionated on a Con A Sepharose column. The column was washed with equilibration buffer containing 5 mM Tris-HCl pH 7.4, 0.02% NaN₃, 1 mM each of MgCl₂, MnCl₂ and CaCl₂ [18]. One hundred milligrams of EP spore-mycelial extract was loaded onto the column and the unbound fractions were collected. The nonspecifically bound proteins were removed by passing 4 M NaCl and 0.25% Triton X-100, respectively, in equilibration buffer. The bound proteins were eluted with 0.4 M α -methyl mannoside in equilibration buffer.

The Con-A-bound protein fraction was applied to a Sephadex G-75 column and proteins eluted in phosphate buffer (50 mM, pH 7.4). The fractions were dialyzed against ammonium bicarbonate buffer (50 mM, pH 7.4) and lyophilized.

The major allergenic protein was electro-eluted from sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using an electro-eluter (Bio-Rad®). Briefly, proteins were eluted in ammonium bicarbonate buffer (50 mM, pH 8.0) containing 0.1% SDS at 10 mA for 6–7 h at room temperature. The electro-eluted protein was retrieved, lyophilized and analyzed for purity on reverse-phase HPLC using a C-18 Waters column under isocratic conditions, with trifluoroacetate in water (solvent A) and trifluoroacetate in acetonitrile (solvent B).

SDS-PAGE

EP spore-mycelial mass extract and the protein fractions obtained at each step of purification were pooled on the basis of their absorbance at 280 nm and resolved on 12% SDS-PAGE gel under reducing conditions [19]. The resolved proteins were stained with Coomassie brilliant blue R-250 or used for immunoblot analysis.

Immunoblot

SDS-PAGE-separated proteins were electrophoretically transferred onto a nitrocellulose (NC) membrane as described by Towbin et al. [20]. The efficiency of transfer was checked by Ponceau S staining. IgE binding proteins in the fractions were detected using a serum pool of EP hypersensitive patient/individual patient's serum. Normal human serum pool was used as control.

Immunization of Rabbits

Antiserum against purified protein was prepared by immunizing rabbits with 50 µg of protein in Freund's complete adjuvant. The same amount of protein was used for monthly booster injections in Freund's incomplete adjuvant. Blood was collected on the 10th day of booster injection, the serum separated and used for immunobiochemical experiments. The protocol for raising antibodies was in accordance with the guidelines for animal research, Indian National Science Academy, Delhi [21]. Antiserum against fungal extracts was obtained by immunizing the rabbits with whole mass extract (200 µg) in Freund's adjuvant as described above.

Characterization of Purified Protein: Matrix-Assisted Laser Desorption/Ionization –Time of Flight Mass Spectrometry (MALDI-TOF)

The lyophilized electro-eluted protein was subjected to MALDI-TOF for molecular mass determination using 3,5-dimethoxy-4-hydroxy cinnamic acid (C₁₁H₁₂O₅; molecular weight 224.212) as

matrix according to the manufacturer's instruction (MALDI, Kratos International, Shimadzu).

Sodium Metaperiodate and Protease Treatment

The carbohydrate moieties of purified protein were modified by periodate oxidation with 0.02 M sodium metaperiodate and its IgE binding ability analyzed using EP-positive pooled patient's sera [22]. Briefly, the NC membrane containing electro-transferred semipurified protein (obtained after gel filtration) was subjected to 0.02 M sodium metaperiodate overnight in dark at 4°C. The reaction was stopped by adding a drop of ethylene glycol. After 2 min, 1 mg/ml of sodium borohydride was added to the incubated strip and kept overnight at 4°C. The reaction was stopped by adding a drop of acetic acid and immunoblot was performed.

For protease treatment, the electro-eluted purified protein, 50 µl (10 µg) was treated with 50 µl (100 µg) of Proteinase K (Sigma) overnight at 37°C and probed for IgE binding after adsorbing as dot on the NC membrane. The untreated protein was used as control.

Cross-Reactivity of Purified Protein with Other Fungi

The purified protein was analyzed for its cross-reactivity using rabbit antisera to *Alternaria alternata*, *Cladosporium herbarum*, *Curvularia lunata*, *Fusarium solani* and *Aspergillus fumigatus* by ELISA, ELISA inhibition and immunoblot. All these allergenic fungi were grown under standardized laboratory conditions and their extracts were prepared in ammonium bicarbonate buffer as described earlier [9, 23].

ELISA

Briefly, purified protein (0.1 µg/100 µl/well) or different fungal antigens (1 µg/100 µl/well) were coated on the wells of the ELISA plate. Nonspecific sites were blocked with 3% bovine serum albumin for 3 h at 37°C. After washing with PBS containing 0.01% Tween-20, rabbit anti fungal serum (1:1,000 v/v) was added and the plate incubated overnight at 4°C. Anti-rabbit IgG horseradish peroxidase conjugate (1:1,000 v/v; Sigma, USA) was used to detect IgG binding and the color developed with substrate ortho-phenylenediamine, hydrogen peroxide in citrate buffer. Absorbance was read at 490 nm on ELISA reader (Spectramax). Preimmune rabbit serum was used as a negative control.

ELISA Inhibition

Purified protein (0.1 µg/100 µl/well) was coated onto the wells. After blocking, rabbit antiserum raised against the purified protein incubated overnight at 4°C with different concentrations (0.1–500 ng) of fungal antigens was added to the wells. The IgG binding was detected using anti-rabbit IgG peroxidase (1:1,000 v/v, Sigma) as described earlier [23].

IgG Immunoblot

The cross-reactivity of purified protein was further checked by IgG immunoblot. Briefly, the protein fraction obtained after gel filtration was transferred on NC membrane and probed with rabbit antisera to different fungi as described earlier [9, 23].

Results

Skin Tests and Specific IgE

Out of 1,092 patients with nasobronchial allergy, skin tested with a battery of allergens during the years 1995–2001, 62 (5.7%) showed markedly positive skin reactivity to EP spore-mycelial extract. However, sera could be collected from only 50 patients and analyzed by ELISA for specific IgE against EP spore-mycelial extract. Nine sera with high titres of IgE were selected for immuno-biochemical experiments.

Purification and Identification of IgE Binding Glycoproteins

The crude EP extract exhibited a high carbohydrate to protein ratio (2:1) and showed carbohydrate-stained bands on PAS staining (not shown). Hence a carbohydrate affinity column, Con A-Sepharose was used for purification to separate glucose- and mannose-specific glycoproteins from the other proteins in the extract (fig. 1a). IgE immunoblot of the crude and bound fraction using EP hypersensitive pooled patients' sera showed four prominent protein bands (30, 26, 16 and 14 kD) along with other allergenic proteins (fig. 1b). The fractions obtained with 4 M NaCl and 0.25% Triton X-100 did not show any protein bands on SDS-PAGE, suggesting that there was no loosely bound (nonspecific binding) EP protein to Con A. The Con-A-bound fraction was applied to a Sephadex G-75 column (fig. 1c). The protein profile of the four different fraction pools made on the basis of their OD showed three prominent CBB-stained bands which also bound IgE present in the EP-positive serum pool (fig. 1d). All the four proteins were identified as major allergens using 9 individual patients' sera positive to EP (fig. 2a). A protein of approximately 30 kD, which appeared as a single band on SDS-PAGE (fig. 1f), was efficiently isolated by electro-elution. The purity of the electro-eluted protein was checked by reversed-phase high-pressure liquid chromatography using a C-18 Waters column. The protein showed a single large peak (fig. 1e). The molecular weight of this major allergenic glycoprotein named Epi p 1 (as per the IUIS Allergen Nomenclature subcommittee) was 33.5 kD by MALDI-TOF (fig. 3).

Carbohydrate Modification of Allergen

The modification of carbohydrate moieties of Epi p 1 by sodium metaperiodate caused partial loss of IgE binding as visualized by reduced intensity of bands on immunoblot with EP hypersensitive pooled patients' sera (fig. 2b). However, proteinase K treatment of the protein

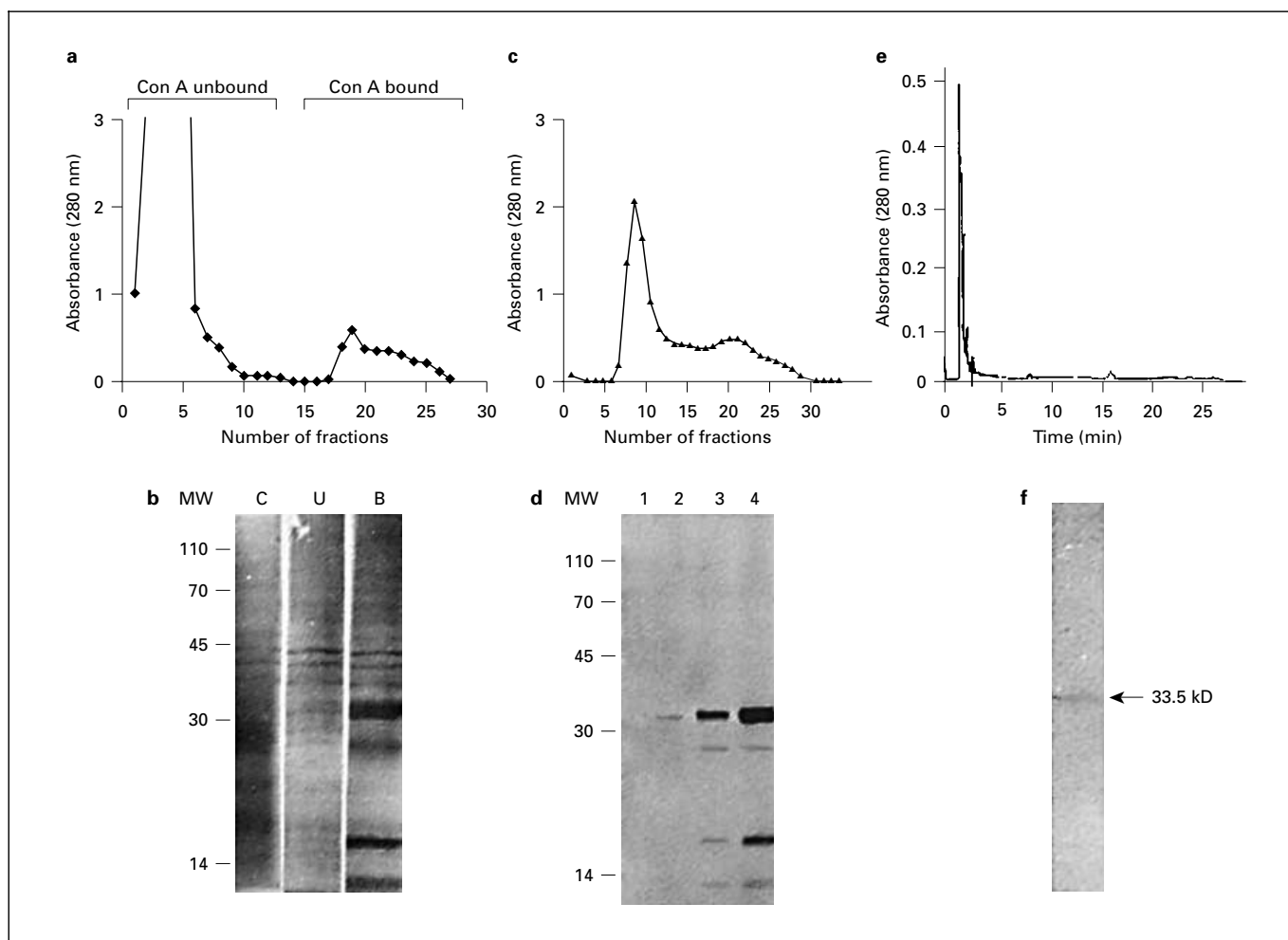


Fig. 1. Elution profiles and IgE immunoblot of EP spore-mycelial extract at different steps of chromatography. For immunoblot, the resolved proteins were transferred on NC membrane after electrophoresis and incubated with EP-positive pooled patients' serum (1:5 v/v). The IgE binding was detected using anti-human IgE peroxidase conjugate. MW represents molecular weight in kilodaltons. **a** Con A Sepharose affinity column. Column size: 2.5×10 cm; eluent: 0.4 M α -methyl mannopyranoside in Tris-HCl buffer; flow rate: 15 ml/h; fraction size: 5 ml. **b** Immunoblot of EP spore-mycelial extract (C);

U = Con A unbound fraction. **c** Con-A-bound fractions on Sephadex G-75 column. Column size: 1.5×35 cm; eluent: 50 mM phosphate buffer, pH 7.3; flow rate: 15 ml/h; fraction size: 2 ml. **d** IgE immunoblot of pooled fractions (1–4) obtained after gel filtration. **e** Electro-eluted protein on C18 Waters (HPLC) column. Condition: isocratic: solvent A: 0.1% TFA in water; solvent B: 0.1% TFA in acetonitrile, Program: 70% A; 30% B, flow rate 1 ml/min. **f** Coomassie-blue-stained electro-eluted protein (P) as seen on 12% SDS-PAGE.

resulted in complete loss of IgE binding, demonstrating that the protein part of the molecule is responsible for IgE binding activity (not shown).

Cross-Reactivity of Epi p 1

The cross-reactivity of Epi p 1 with 5 other fungi was studied by ELISA and ELISA inhibition using rabbit antibodies raised against different fungi. IgG ELISA was carried out using two approaches. In the first approach, the Epi p 1 (33.5 kD) was coated on the wells and probed with

antisera raised against Epi p 1 and other fungal extracts. The antibodies against *A. alternata* showed maximum binding to Epi p 1 followed by *C. lunata*, *A. fumigatus*, *C. herbarum* and *F. solani*. (fig. 4a). In another approach, different fungal antigens, Epi p 1 and crude EP extract were used as solid-phase antigens and probed with antibodies to Epi p 1. In this case, Epi p 1 antibodies showed higher IgG binding with extracts of *A. fumigatus* followed by *C. herbarum* (fig. 4a). Further, IgG ELISA inhibition showed that 6–9 ng of different fungal extracts were

Fig. 2. a IgE immunoblot of protein fraction pool obtained after Sephadex G-75 chromatography, using individual EP hypersensitive patients' sera. The electrophoresed protein was transferred onto NC membrane and incubated with individual (n = 9) EP-positive patients' sera (1:5 v/v). C = Control blot with normal human serum. **b** Effect of sodium metaperiodate (20 mM) on IgE binding activity of 33.5-kD purified protein present in gel-purified fraction pool. Periodate-treated NC membrane containing transferred protein was incubated with EP-positive pooled patients' sera (1:5 v/v). C = Untreated; P = periodate treated.

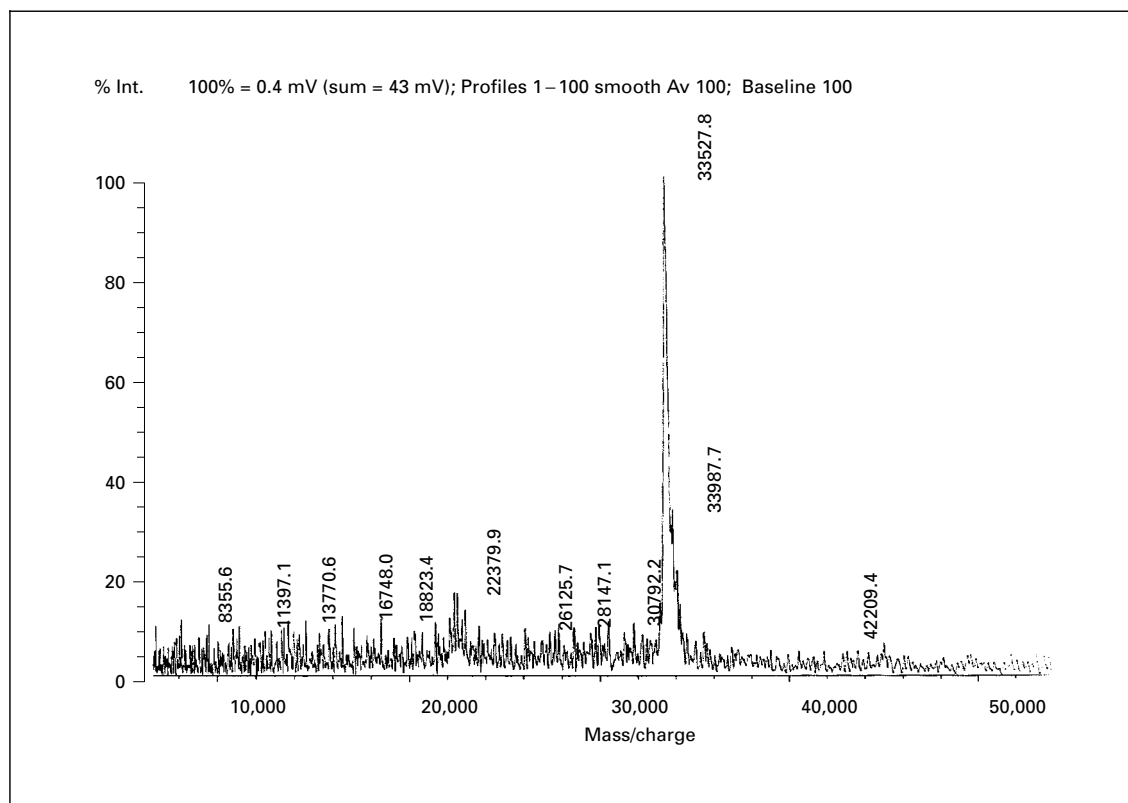
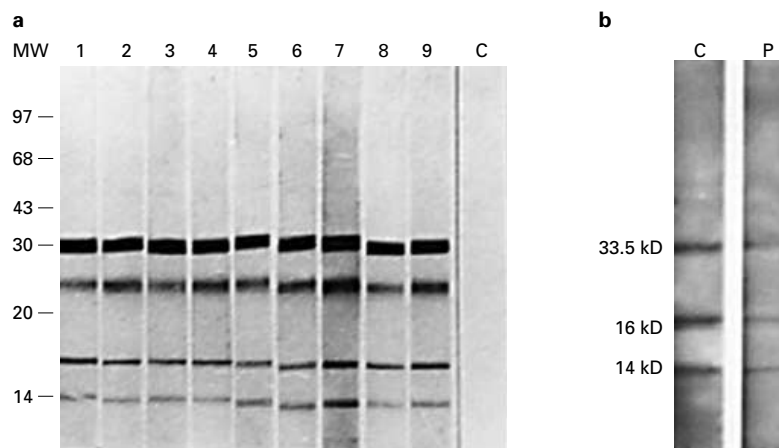


Fig. 3. MALDI-TOF analysis of purified protein using automated MALDI analyzer. The single largest peak shows the molecular weight (33.5 kD) of purified protein.

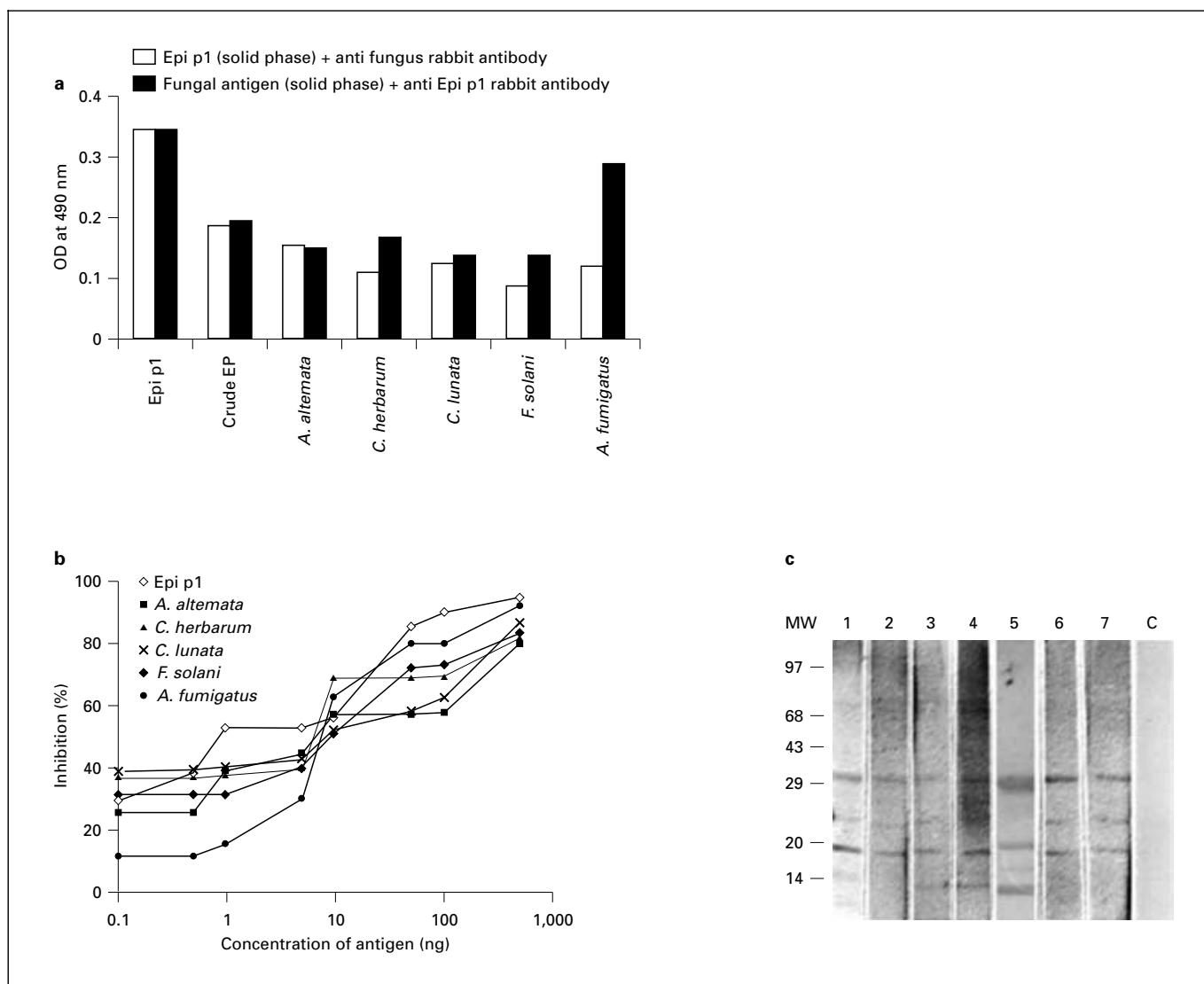


Fig. 4. **a** IgG ELISA depicting shared Epi p 1 epitopes with other fungi. Epi p 1 (100 ng) and other fungal extracts (1 µg) were coated on a microtiter plate and incubated with rabbit antifungal sera as shown in the figure. Preimmune rabbit serum was used as negative control (OD ranging from 0.02 to 0.05). **b** Inhibition ELISA showing percentage inhibition of the binding of Epi p 1 antibodies. The purified protein (0.1 µg) was coated on a microtiter plate and incubated with antisera (1:1,000 v/v) to purified protein preincubated with different concentrations (0.1–500 ng) of fungal antigens, as inhibitor. **c** Immu-

noblot showing cross-reactive IgG bands in Sephadex G-75 fraction pool recognized by antibodies raised in rabbits against different fungal extracts. The electrophoresed proteins of the gel filtration fraction were transferred on NC membrane and incubated with antisera (1:1,000 v/v) to (1) Epi p 1, (2) *A. fumigatus*, (3) *A. alternata*, (4) *C. lunata*, (5) 31 kD purified protein from *C. lunata*, (6) *C. herbarum* and (7) *F. solani*. The IgE binding was detected using anti IgG peroxidase conjugate. C = Gel-purified fraction probed with preimmune sera. MW represents molecular weight in kilodaltons.

needed to achieve 50% inhibition of Epi p 1 antibodies as compared to 0.9 ng of Epi p 1 (fig. 4b). The cross-reactivity of Epi p 1 was further confirmed by IgG immunoblot using antibodies against different fungal antigens. The antibodies against all the fungal antigens tested were able

to recognize 33.5-, 26-, 16- and 14-kD proteins of EP present in gel filtration protein fraction, indicating that all these fungi share some epitopes of Epi p 1. However, as different antibodies were used, the background and the band intensity showed variation (fig. 4c).

Discussion

EP is one of the important fungi responsible for inducing respiratory allergy disorders. Its spore-mycelial extract is used for allergy diagnosis and immunotherapy in allergy clinics worldwide [1–10]. The crude extract of EP contains a large number of major/minor allergenic proteins, which are yet not individually characterized. Currently, purified and recombinant allergens are recommended for allergy diagnosis, but their use is limited to few selected allergens [12, 24–26]. In this study, a major allergen of EP was purified to homogeneity and characterized for its cross-reactive nature.

A number of chromatography techniques either alone or in combinations have been employed to purify major/minor allergens from different fungi. The crude EP extract exhibited a high carbohydrate to protein ratio and also showed carbohydrate-stained bands. Hence, in the present study, a Con A Sepharose affinity column followed by gel filtration chromatography and electro-elution were employed to purify a major glycoprotein allergen of EP. Previous studies have reported isolation of glycoproteins from *A. fumigatus* and *C. albicans* extract using Con A Sepharose chromatography [12, 18, 27, 28]. MALDI-TOF analysis of purified protein from EP obtained after electro-elution showed its molecular weight to be 33.5 kD. Earlier, Verma et al. [11] had successfully purified a 65-kD protein of *Fusarium equiseti* by electro-elution.

Periodate modification of Epi p 1 resulted in partial loss of binding to IgE whereas proteinase K treatment caused complete loss of IgE binding, indicating that both protein and carbohydrate moieties of the protein are required for the binding to IgE. Previous studies have reported the use of 0.02 M sodium metaperiodate for

modifying the carbohydrate moiety of glycoprotein changing its allergenic/antigenic activity [18, 29, 30]. Studies with *A. fumigatus* had shown that the IgE-binding ability of 30-, 58- and 90-kD proteins were affected by periodate treatment [18, 30].

The use of cross-reacting proteins in reducing the panel of allergen extracts for diagnosis was already emphasized [15, 23, 31]. In the present study, the cross-reactivity of Epi p 1 was analyzed using antibodies raised against the well-known allergenic moulds *A. alternata*, *C. herbarum*, *A. fumigatus*, *C. lunata* and *F. solani*. The Epi p 1 antibodies recognized all the fungal extracts tested confirming the presence of similar epitopes in all these fungi. Cross-reactive 33- to 34-kD major allergens have also been observed in different species of *Penicillium* and *Aspergillus* [31]. In an earlier study, we observed a cross-reactive allergen of similar molecular weight in different fungi including *Epicoccum nigrum* [23]. It would thus be interesting to study and characterize Epi p 1 for its other properties.

To summarize, a 33.5-kD major glycoprotein allergen of EP, Epi p 1 was purified. This protein showed extensive cross-reactivity with *A. alternata*, *C. herbarum*, *A. fumigatus*, *C. lunata* and *F. solani*. The data generated would be helpful in evolving new strategies for diagnosis and therapy of EP allergy.

Acknowledgements

We thank Dr. Sunita Sharma, IGIB for providing help in carrying out MALDI-TOF analysis. Thanks are also due to Council of Scientific and Industrial Research, Delhi and the Department of Biotechnology, Government of India, for financial support in carrying out the study.

References

- 1 Burge HA: Fungi: Toxic killers or unavoidable nuisances? *Ann Allergy Asthma Immunol* 2001;87:52–56.
- 2 Bush RK, Portnoy JM: The role and abatement of fungal allergens in allergic diseases. *J Allergy Clin Immunol* 2001;107:S430–S440.
- 3 Kurup VP, Shen HD, Banerjee B: Respiratory fungal allergy. *Microbes Infect* 2000;2:1101–1110.
- 4 Hogan MB, Patterson R, Pore RS, Corder WT, Wilson NW: Basement shower hypersensitivity pneumonitis secondary to *Epicoccum nigrum*. *Chest* 1996;110:854–856.
- 5 Noble JA, Crow SA, Ahearn DG, Kuhn FA: Allergic fungal sinusitis in the southeastern USA: Involvement of a new agent *Epicoccum nigrum*. *J Med Vet Mycol* 1997;35:405–409.
- 6 Portnoy J, Chapman J, Burge H, Muilenberg M, Solomon W: *Epicoccum* allergy: Skin reaction patterns and spore/mycelium disparities recognized by IgG and IgE ELISA inhibition. *Ann Allergy* 1987;59:39–43.
- 7 Chrzanowski RR, Rupp NT, Kuhn FA, Phillips AE, Dolen WK: Allergenic fungi in allergic fungal sinusitis. *Ann Allergy Asthma Immunol* 1997;79:431–435.
- 8 Black PN, Udy AA, Brodie SM: Sensitivity to fungal allergens is a risk factor for life threatening asthma. *Allergy* 2000;55:501–504.
- 9 Bisht V, Singh BP, Gaur SN, Arora N, Sridhara S: Allergens of *Epicoccum nigrum* grown in different media for quality source material. *Allergy* 2000;55:274–280.
- 10 Bisht V, Singh BP, Kumar R, Arora N, Sridhara S: Culture filtrate antigens and allergens of *Epicoccum nigrum* cultivated in modified semi-synthetic medium. *Med Microbiol Immunol* 2002;191:11–15.

- 11 Verma J, Sridhara S, Rai D, Gangal SV: Isolation and immunobiochemical characterization of a major allergen (65 kD) from *Fusarium equiseti*. *Allergy* 1998;53:274–280.
- 12 Banerjee B, Kurup VP, Greenberger PA, Hoffman DR, Nair DS, Fink JN: Purification of a major allergen Asp f2 binding to IgE in allergic bronchopulmonary aspergillosis from culture filtrate of *Aspergillus fumigatus*. *J Allergy Clin Immunol* 1997;99:821–827.
- 13 Sridhara S, Gangal SV, Joshi AP: Immunobiochemical investigations of allergens from *Rhizopus nigricans*. *Allergy* 1990;45:577–586.
- 14 Portnoy J, Pacheco F, Ballam Y, Barnes C: Separation of *Alternaria* into protein and carbohydrate fractions with phenyl sepharose. *J Allergy Clin Immunol* 1991;87:789–793.
- 15 Verma J, Singh BP, Sridhara S, Gaur SN, Arora N: Purification and characterization of a cross-reactive major allergen of *Fusarium solani*. *Int Arch Allergy Immunol* 2003;130:193–199.
- 16 Singh BP, Sridhara S, Arora N, Gangal SV: Evaluation of protein assay methods for pollen and fungal spore extracts. *Biochem Int* 1992;27:477–484.
- 17 Dubois M, Gillis KA, Hamilton JK, Roberts RA, Smith F: Colorimetric method for the determination of sugars and related substances. *Analyt Chem* 1956;28:350–356.
- 18 Fratamico PM, Buckley HR: Identification and characterization of an immunodominant 58-kD antigen of *Aspergillus fumigatus* recognized by sera of patients with invasive aspergillosis. *Infect Immun* 1991;59:309–315.
- 19 Laemmli UK: Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.
- 20 Towbin H, Staehelin T, Gordon J: Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some application. *Proc Natl Acad Sci USA* 1979;76:4350–4354.
- 21 Anonymous: Guidelines for care and use of animals in Scientific Research. New Delhi, Indian National Science Academy, 1992.
- 22 Verma J, Pasha S, Gangal SV: Purification and characterization of *Fus s I₃₅₉₆**, a 65-kD allergen of *Fusarium solani*. *Mol Cell Biochem* 1994;131:157–166.
- 23 Bisht V, Singh BP, Gaur SN, Arora N, Sridhara S: Antigenic and allergenic cross-reactivity of *Epicoecum nigrum* with other fungi. *Ann Allergy Asthma Immunol* 2002;89:285–291.
- 24 Cramer R: Recombinant *Aspergillus fumigatus* allergens: From the nucleotide sequences to clinical applications. *Int Arch Allergy Immunol* 1998;115:99–114.
- 25 Achatz G, Oberkofler H, Lechenauer E, Simon B, Unger A, Kandler D, et al: Molecular cloning of major and minor allergens of *Alternaria alternata* and *Cladosporium herbarum*. *Molecular Immunol* 1995;32:213–227.
- 26 Shen HD, Wang CW, Chou H, Lin WL, Tam MF, Huang HM, Kuo LM, et al: Complementary DNA cloning and immunologic characterization of a new *Penicillium citrinum* allergen (Pen c3). *J Allergy Clin Immunol*, 2000;105:827–833.
- 27 Savolainen J, Viander M, Einarsson R, Koivikko A: Immunoblotting analysis of concanavalin A-isolated allergens of *Candida albicans*. *Allergy* 1990;45:40–46.
- 28 Gomez MJ, Torosantucci A, Arancia S, Maras B, Parisi L, Cassone A: Purification and biochemical characterization of a 65-kilodalton mannoprotein (MP 65), a main target of anti-*Candida* cell-mediated immune responses in humans. *Infect Immun* 1996;64:2577–2584.
- 29 Teshima R, Ikebuchi H, Sawada J, Miyachi S, Kitani S, Iwama M, et al: Isolation and characterization of a major allergenic component (gp55) of *Aspergillus fumigatus*. *J Allergy Clin Immunol* 1993;92:698–706.
- 30 Hansen MY, Wold JK, Smestad Paulson B, Cohen EH, Karlsson-Borga A: Allergens in *Aspergillus fumigatus*. I. Characterization of two different allergen extracts and evaluation of their stability and the importance of carbohydrate for IgE binding. *Allergy* 1994;49:235–241.
- 31 Shen HD, Lin WL, Tam MF, Wang SR, Tsai JJ, Chou H, Hans SH: Alkaine serine proteinase: A major allergen of *Aspergillus oryzae* and its cross-reactivity with *Penicillium citrinum*. *Int Arch Allergy Immunol* 1998;116:29–35.