

Purification and Characterization of a Cross-Reactive 45-kD Major Allergen of *Fusarium solani*

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Key Words

Allergen · Cross-reactivity · Fungus · *Fusarium* · Histamine

Abstract

Background: *Fusarium solani* (FS) is an important source of fungal allergen. A 45-kD major allergen of FS showed reactivity with patients' sera sensitive to many fungi. **Objectives:** To purify and characterize a 45-kD common allergenic protein from FS, which may be useful for the diagnosis of and therapy for fungal allergy. **Methods:** FS culture filtrate extract was separated on SDS-PAGE; 45-kD protein was electroeluted and purified on C18 column using reverse-phase high-pressure liquid chromatography (rpHPLC). The purified protein was functionally and biochemically characterized by in vitro and in vivo methods. **Results:** The 45-kD protein showed a single peak on rpHPLC. The N-terminal amino acid sequence of this protein did not show homology to enolase or known fungal proteins. It showed cross-reactivity with *Epicoccum nigrum*, *Curvularia lunata*, *Cladosporium herbarum* and *Alternaria alternata* by ELISA and ELISA inhibition using rabbit antibodies raised against these fungi. IgE ELISA inhibition with patients' sera positive to different fungi demonstrated allergenic cross-reactivity of the 45-kD protein with other fungal extracts. This 45-kD pro-

tein released a significant amount of histamine in FS-allergic patients. **Conclusion:** A cross-reactive 45-kD allergenic/antigenic protein was purified to homogeneity and characterized. It has prospects for use in allergen therapy.

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Introduction

Several species from fungi imperfecti are responsible for respiratory allergic diseases in susceptible individuals [1–3]. *Fusarium solani* (FS) is one of the most prevalent fungi throughout the world and afflicts 4% of the allergic population in India [4, 5]. FS culture filtrate (CF) extract contains 18 allergenic proteins as identified by immunoblot using pooled patients' sera [6, 7]. Of these, 8 were recognized as major allergens using serum of individual patients, who were skin test positive to FS extract. A 45-kD protein of FS bound to 80% of patients' sera tested. This protein was detected in IgE and IgG immunoblots of *Fusarium* species and other fungi using FS-allergic patients and rabbit antibodies, respectively [8, 9].

Fungal extracts contain allergenic and nonallergenic proteins. Immunotherapy with such extracts may result in hypersensitivity to irrelevant allergens. Furthermore, crude fungal extracts contain substances that provoke histamine release and, therefore, an optimum therapeutic

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dose cannot be attained during immunotherapy [10]. Thus, it is necessary to purify and characterize major allergenic proteins from fungi. Previously, Pen c 1 protein allergen from *Penicillium citrinum* was purified by sequential DEAE- and carboxymethyl-Sepharose chromatography and proteins were studied using molecular biology techniques [11]. Two major allergens, Asp f 1 and Asp f 2 purified from *Aspergillus fumigatus* bind IgE present in the serum of allergic bronchopulmonary aspergillosis patients [12, 13]. Paris et al. [14] purified a 31-kD major allergen Alt a 1₁₅₆₃* to homogeneity and characterized by immunological techniques.

The presence of cross-reactive proteins in fungi results in sensitivity to more than one extract [8, 9, 15]. This concept of cross-reactivity is important because it has implications for the diagnosis and therapy of fungal allergy. We have earlier identified cross-reactive proteins in *Fusarium* extract [8, 9]. In the present study, a 45-kD major allergen of FS was purified to homogeneity, characterized immunobiochemically and probed for cross-reactivity with other important allergenic fungi.

Material and Methods

Preparation of Culture Filtrate Extract

FS strain 3596* obtained from IARI, Delhi, India was grown in a synthetic medium at 30°C on a rotary shaker (125 rpm) for 11 days [6]. The culture medium was separated from mycelium by centrifugation. The proteins were precipitated by 90% ammonium sulfate. After centrifugation, the pellet was dissolved in a minimum amount of water and dialyzed (Spectrapore, MW cutoff 3,500). The dialyzed extract was filtered through a 0.45-µm millipore filter and lyophilized.

Purification of a 45-kD Protein

FS (CF) extract was resolved on SDS-PAGE (10% gel). After staining with CBB R-250, a 45-kD protein was eluted from the gel as described [16]. The protein was further purified on reverse-phase high-pressure liquid chromatography (rpHPLC) using Waters C18 column in isocratic conditions (50% A-50% B where A = 0.05% trifluoroacetic acid (TFA) in water and B = 0.05% TFA in acetonitrile). Chart speed and attenuation were maintained at 0.25 mm/min and 256, respectively.

N-Terminal Amino Acid Sequencing

Amino terminal sequencing was performed manually by Edman's double coupling reaction [17]. Briefly, 20 µl of pyridine (50%) with 20 µl of dimethylaminoazobenzeneisothiocyanate (DABITC) in pyridine was added to 5 ng of 45-kD FS protein. After heating the sample at 52°C for 30 min, 10 µl of phenyl isothiocyanate (PITC) was added and it was heated again. Unreacted DABITC and PITC in the upper phase were separated by extraction with n-heptane:ethyl acetate (1:1 v/v). Dimethylaminoazobenzenehydantoin amino acids were identified by two-dimensional thin layer chromatography in 30%

Table 1. Details of sera used in specific IgE ELISA inhibition

Patient No.	Positive to fungus	Skin test (ID grading)	Sera to study cross-reactivity of 45-kD protein
1	<i>Epicoccum</i>	++ ±	<i>Epicoccum</i>
2	<i>Epicoccum</i>	+++	<i>Epicoccum</i>
3	<i>Curvularia</i>	+++	<i>Curvularia</i>
4	<i>Curvularia</i>	+++	<i>Curvularia</i>
5	<i>Alternaria</i>	+++	<i>Alternaria</i>
6	<i>Cladosporium</i>	+++ ±	
	<i>Mucor</i>	++	<i>Cladosporium</i>
7	<i>Cladosporium</i>	+++	<i>Cladosporium</i>
8	<i>Penicillium</i>	+++	<i>Alternaria</i>
	<i>A. fumigatus</i>	+++	
	<i>Alternaria</i>	+++	
	<i>Pennisetum</i>	++	
9	<i>Fusarium</i>	+++ ±	<i>Fusarium</i>
	<i>Cenchrus</i>	++	
	<i>Penicillium</i>	++	
10	<i>Fusarium</i>	+++	<i>Fusarium</i>

acetic acid in water for the first dimension and toluene:n-heptane:acetic acid, 2:1:1 for the second dimension. The amino acids appeared as red colored spots and were identified with standard markers.

Enzyme-Linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was performed to study the cross-reactivity of the 45-kD protein with other fungi [18]. Briefly, 500 ng of 45-kD protein in 100 µl of carbonate buffer (pH 9.6) was coated on the microtiter plate and incubated overnight at 4°C. After washing, the free sites were blocked with 1% BSA and further incubated with rabbit antibodies against FS, *Epicoccum nigrum*, *Cladosporium herbarum*, *Curvularia lunata*, or *Alternaria alternata* (1:1,000 v/v). The plates were washed and incubated for 4 h with diluted anti-rabbit IgG peroxidase (1:8,000 v/v; Sigma). The color was developed with o-phenylene diamine in sodium citrate buffer and absorbance was read at 490 nm.

IgE/IgG ELISA Inhibition

To study cross-reactivity of purified 45-kD protein of FS with other fungi, ELISA inhibitions with patients' sera and rabbit antibodies raised against different fungal extracts (*E. nigrum*, *C. lunata*, *C. herbarum*, *A. alternata* and FS) were performed. The microtiter plate was coated with 100 ng of fungal extracts. After blocking, *Fusarium*-allergic pooled patients' sera as well as sera specific to individual fungus (1:10 v/v) were mixed with different concentrations of the respective fungus or the 45-kD protein of FS for IgE ELISA inhibition (table 1). Sera of patients with multiple sensitivity were not used for inhibition studies. Similarly, rabbit antibodies raised against individual fungus (1:1,000 v/v) were mixed with different concentrations of 45-kD protein of FS, added to wells and incubated overnight at 4°C. After washing with PBS-Tween 20, human anti-IgE peroxidase (1:1,000 v/v) or rabbit anti-IgG peroxidase (1:8,000 v/v) were added, respectively. Preimmune sera from rabbits or normal human

sera were taken as controls. *Imperata cylindrica* pollen extract was used as a nonfungal control. Color developed with the substrate *o*-phenylene diamine in sodium acetate buffer and the absorbance was read at 490 nm. Percent inhibition was calculated using the following formula:

$$\% \text{ inhibition} = \frac{\text{OD of test sample} - \text{OD of inhibited sample}}{\text{OD of test sample}} \times 100$$

Histamine Release Assay

To study the physiological function of 45-kD protein, a histamine release assay was performed according to the manufacturer's instructions using a kit (Immunotech, Marseille, France). Briefly, heparinized blood was challenged either with 10 ng each of crude extract or purified 45-kD protein for 30 min at 37 °C. The optimum concentration of allergen for histamine release assay was standardized in our earlier study [19]. The histamine standards (0–100 nM), released histamine in the supernatants was acylated and measured by ELISA. Spontaneous histamine release was measured in an unchallenged sample and the total histamine content in the heparinized blood was measured by freeze thawing after cell lysis. Heparinized blood from 4 nonallergic individuals was used as control. All the assays were performed in triplicate.

Results

Purification of the 45-kD Protein

The 45-kD allergenic protein eluted from the SDS-PAGE gel showed a single peak on rpHPLC using Waters C18 column (fig. 1). N-Terminal 7-amino acid sequence of the 45-kD protein was obtained by Edman's technique. The sequence Lys-Gly-Arg-Thr-Glu-Phe-Ala did not show homology to enolase or any other known fungal proteins using BLAST search. However, the 6 amino acids showed 100% identity to TMA-N oxide reductase from *Shewanella massilia* and protein XP_162193 from *Mus musculus*.

Cross-Reactivity of 45-kD FS Protein with Proteins of Different Fungi Using Rabbit Antibodies

ELISA was performed with antisera raised in rabbit against FS, *E. nigrum*, *C. herbarum*, *C. lunata* and *A. alternata* using the 45-kD FS protein as solid phase allergen. *Alternaria* showed the lowest reactivity whereas other fungi gave a similar absorbance indicating the cross-reactivity of 45-kD. A preimmune rabbit serum was taken as a negative control (table 2).

Cross-Reactivity by ELISA Inhibition Using Patient/Rabbit Sera

IgE ELISA inhibition was performed with other fungal extracts to show cross-reactivity of 45-kD protein (fig. 2). FS required 250 ng of 45-kD self protein whereas 110 ng

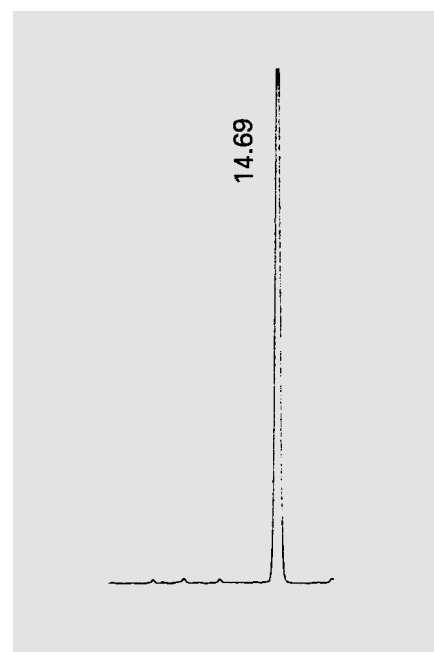


Fig. 1. Elution profile of 45-kD protein of FS on rpHPLC using Waters C18 column (program 50% A-50% B; A = 0.05% TFA in water; B = 0.05% TFA in acetonitrile; chart speed: 0.25 mm/min; AT: 256).

Table 2. Cross-reactivity of 45-kD protein with other fungi

Rabbit antibodies against	Absorbance (490 nm)
<i>F. solani</i>	1.839
<i>E. nigrum</i>	1.091
<i>C. herbarum</i>	1.095
<i>C. lunata</i>	1.072
<i>A. alternata</i>	0.901
Control (NHS)	0.026

For ELISA, 500 ng of 45-kD purified protein was coated on the plate overnight and different rabbit antibodies raised against the specific fungi (1:1,000 v/v) were added to each well. Plates were incubated overnight, washed and labeled rabbit anti-IgG peroxidase was added and color developed.

of *A. alternata* gave 50% inhibition of IgE binding. *C. herbarum*, *E. nigrum* and *C. lunata* required 400–490 ng of purified 45-kD protein to achieve 50% inhibition. ELISA inhibition using rabbit sera showed cross-reactivity among fungal extracts tested (fig. 3). *E. nigrum* required

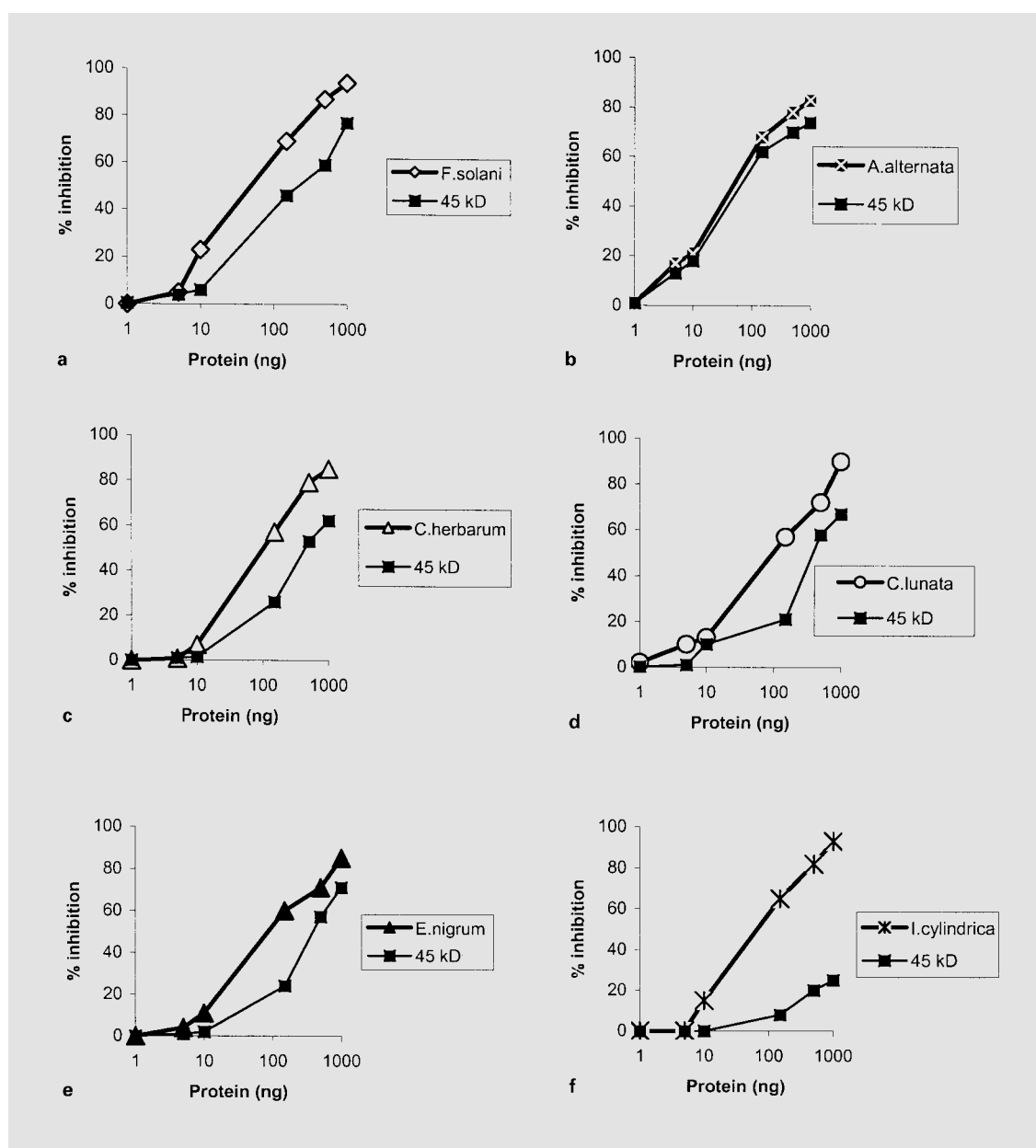
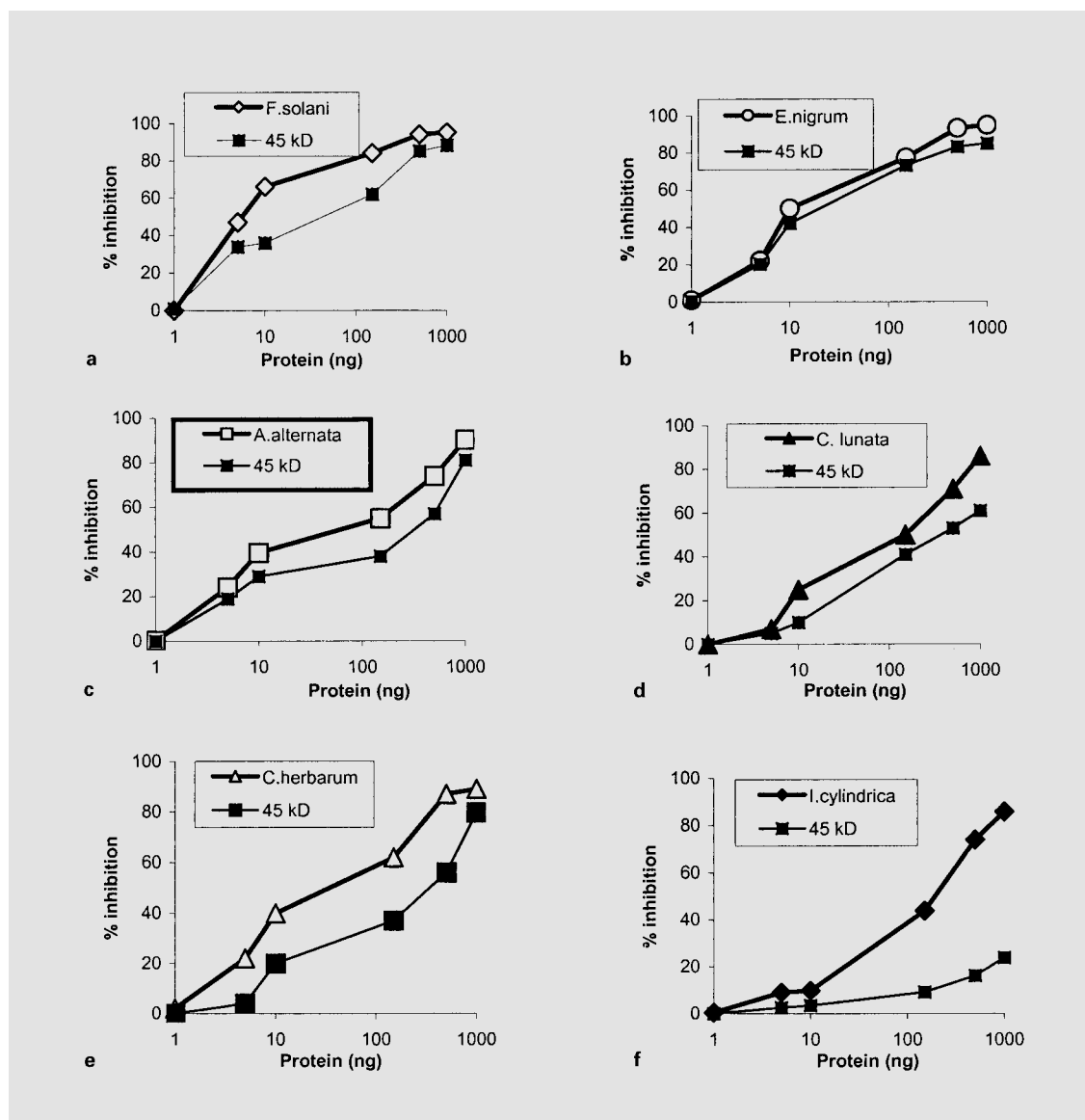


Fig. 2. IgE ELISA inhibition showing allergenic cross-reactivity of 45-kD protein of FS with other fungal extracts. Solid phase antigens are *F. solani* (a), *A. alternata* (b), *C. herbarum* (c), *C. lunata* (d), *E. nigrum* (e), and *I. cylindrica* (pollen) (f). Table 1 gives the details of patients' sera and inhibitor is given within the boxes.

9 ng of 45-kD protein for 50% inhibition of IgG binding whereas FS, *C. herbarum*, *C. lunata* and *A. alternata* required 50, 450, 450 and 480 ng protein, respectively, for the same. ELISA inhibition with *I. cylindrica* pollen extract (taken as a control) showed no reactivity. This study indicated that the 45-kD cross-reactive protein is present in other fungal extracts.

Histamine Release

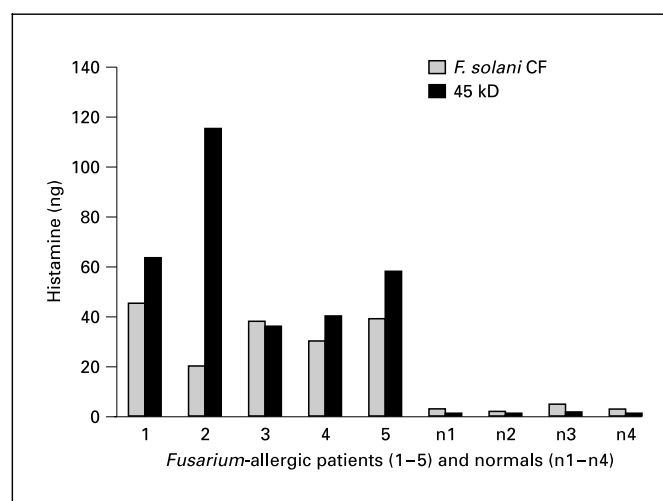
The 45-kD protein and crude FS extract released histamine from heparinized blood in *Fusarium*-allergic patients (fig. 4). Most of the patients released more histamine on challenge with the 45-kD protein in comparison to FS CF extract. Patients 1, 4 and 5 released 1.5–2 times and patient 2 released 5 times higher histamine with the



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Fig. 3. IgG ELISA inhibition showing cross-reactivity of 45-kD protein of FS with other fungal extracts using rabbit antibodies raised against respective fungus. Solid phase antigens are *F. solani* (a), *E. nigrum* (b), *A. alternata* (c), *C. lunata* (d), *C. herbarum* (e) and *I. cylindrica* (pollen) (f).

Fig. 4. Histamine release on challenge with 45-kD allergen and crude extract of FS. Heparinized blood of patients (1–5) and nonallergic individuals (n1–n4) was used. Histamine release was calculated in nM from the dose response curve with standards. Statistical analysis (2-tailed t test) showed that release of histamine by both 45-kD allergen ($p = 0.007$) and crude FS extract ($p = 0.002$) was significantly different ($p < 0.01$) in normal and FS-allergic patients.



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45-kD protein as compared to the FS CF extract. Patient 3 released an almost similar amount of histamine with crude extract and purified protein. Nonallergic control subjects (n1–n4) did not release histamine on challenge with either crude extract or the 45-kD protein. The variation in histamine release by allergy patients may be due to differences in their reactivity to fungi. Most of the patients had positive skin reactions to multiple fungi while patient 3 was positive to FS only. Statistical analysis (2-tailed t test) showed that histamine release by the 45-kD allergen ($p = 0.007$ and FS extract ($p = 0.002$) was significantly different ($p < 0.01$) in normal and FS-allergic patients. However, there was no significant difference in histamine release when the 45-kD allergen was compared with the crude FS extract.

Discussion

Fungal allergies are prevalent in about 30% of the allergic individuals [1]. More than 50 genera of fungi are responsible for atopic disorders. There is a need to isolate and purify cross-reactive major allergenic proteins to reduce the number of fungal extracts used for diagnosis and therapy of allergy disorders. The cross-reactive allergenic proteins may serve as a better therapeutic product and reduce the number of allergens without compromising the efficacy of treatment. FS crude extract contains cross-reactive allergenic proteins of 65, 45 and 35 kD amongst many fungi [9]. Previously, a 65-kD major glycoprotein of FS was purified to homogeneity and characterized by in vitro methods [7]. This 65-kD protein was cleaved to obtain allergenic peptides. Peptide IV–I showed IgE binding activity but did not release histamine indicating its therapeutic potential [19]. In the present study, a 45-kD cross-reactive protein was purified from FS and characterized by immunobiochemical methods. ELISA with rabbit antibodies raised against different fungal extracts using the 45-kD FS protein on solid support demonstrated the cross-reactivity with fungi, e.g. *E. nigrum*, *C. herbarum*, *C. lunata* and *A. alternata*. Further, ELISA inhibition showed a significant cross-reactivity of the 45-kD protein with patient and rabbit sera.

It is known that allergenic activity is dependent on the ability of proteins to interact with specific IgE antibodies bound to FcεRI receptors on the mast cells and basophils. This trigger results in a release of inflammatory mediators and cytokines that induce and perpetuate the allergic state [20]. Since the 45-kD protein is cross-reactive with proteins of other fungi, a histamine release assay was per-

formed using blood of fungal-sensitive patients. Of 5, 4 patients released more histamine with the 45-kD protein as compared to crude extract of FS. This may be due to the 45-kD protein not only reacting with specific IgE to FS but also specific to other fungi and, hence, this protein is relevant in fungal-allergic patients.

Studies conducted earlier identified allergenic proteins from different fungi and other sources [1–3, 16]. However, few studies on cross-reactivity with purified proteins from fungi have been performed. *Candida albicans* enolase showed cross-reactivity with *Saccharomyces cerevisiae* [21]. The rHev b 9-enolase allergen cross-reacts with *C. herbarum* and *A. alternata* [22]. IgE-reacting B cell epitopes of *C. herbarum* enolase were analyzed and a 69-residue peptide reacted with all the patients' sera indicating cross-reactivity [23]. Amino terminal sequence and Western blot using monoclonal antibodies (PCM39) showed cross-reactive proteins in various *Penicillium* species [24]. Alkaline serine protease allergen isolated from *Penicillium notatum* showed cross-reactivity with *P. citrinum* [25]. In our study, N-terminal sequence analysis of 7 amino acids of 45-kD protein did not show homology to enolase or any other known fungal proteins. However, 6 amino acids showed 100% identity to TMA-N oxide reductase from *S. massilia* and protein XP_162193 from *M. musculus*.

It was observed that fungal-allergic patients show sensitivity to multiple fungi. This is due to cross-reactivity of IgE antibodies with structurally and immunologically related components present in allergens. The cross-reactive fungal proteins can be used as representative allergens for other fungal types. In the present study, a cross-reactive major allergen (45-kD protein) of FS was purified to homogeneity and characterized. This 45-kD protein may be generated in bulk amount using chromatographic or recombinant methods and tested for efficacy in allergen immunotherapy.

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