

A clinically relevant major cross-reactive allergen from mesquite tree pollen

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ABSTRACT

Background *Prosopis juliflora* (mesquite) is one of the major sources of pollinosis in tropical and semi-arid countries of the world. The present study was undertaken to purify and characterize a major cross-reactive allergen from this tree species.

Materials and methods Mesquite pollen extract was purified using reverse-phase chromatography. Allergen characterization was done by electrophoresis, enzyme-linked immunosorbent assay (ELISA) and Western blotting. Clinical relevance of the purified protein was analyzed by *in vivo* (skin tests) and *in vitro* experiments such as ELISA, histamine release, peripheral blood mononuclear cells (PBMC) proliferation and cytokine assays. Cross-reactivity of purified protein with allergenic tree species and lima bean (food) was assessed by inhibition assays.

Results A 66-kDa protein was purified from mesquite pollen extract using octadecyl silica resin. Purified protein recognized 90% of mesquite-sensitized patients in skin test and ELISA. It induced significant histamine release in allergic patients' blood and interleukin-4 secretion in the PBMC culture supernatants. Inhibition assays suggested close allergenic relationship of this protein with *Ailanthus excelsa*, *Cassia siamea*, *Salvadora persica* pollen and *Phaseolus lunatus* (lima bean – an edible legume).

Conclusions A 66-kDa major cross-reactive allergen was isolated from mesquite pollen using single-step purification procedure. The protein seems relevant for clinical applications in allergic disorders.

Keywords Allergenicity, cross-reactivity, mesquite, pollen, protein, purification.

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Introduction

Pollen allergens from several tree species are known to afflict respiratory allergies in predisposed individuals. Cross-reactivity among various allergens further aggravates the allergies, sensitizing individuals with common proteins throughout the year. Diagnosis of pollen allergy is made by the history and *in vivo* and *in vitro* testing with allergen extracts. Immunotherapy with offending allergens is practised in properly selected cases for management of allergic disorders and has shown long-term beneficial effects [1–3]. But the current methods for allergy diagnosis/immunotherapy suffer due to the use of whole mass extracts. These extracts are a mixture of proteins and other components and the commercially available extracts show batch-to-batch variation in allergen content [4,5]. Immunotherapy with such extracts may result in additional sensitivity to irrelevant components and can hamper in reaching the optimal maintenance dose during treatment. To overcome this, use of purified and well-defined allergen preparations has been recommended [6].

Allergenic cross-reactivity can occur among phylogenetically related species or also between distantly related allergen sources [7]. Identification and isolation of these cross-reactive components (proteins) can help in understanding the mechanism of allergenic cross-reactivity and also in selection of clinically relevant purified allergens.

Prosopis juliflora (mesquite) tree is an important source of pollen allergens prevalent in tropical and arid regions of the world [8–12]. Mesquite afflicts sensitization in 6–11% cases of allergic rhinitis and bronchial asthma or both as revealed by skin testing in India. A study from Kuwait, however, showed high skin reactivity to mesquite ranging from 11% to 76% in four geographical regions [8]. Mesquite pollen extract have 16 allergenic components of which nine proteins of 24, 26, 29, 31, 35, 52, 58, 66 and 95 kDa were recognized as major allergens [11]. Of these, four proteins of 14, 41, 52 and 66 kDa were cross-reactive with other predominant tree species, whereas 20, 26, 35, 66 and

72 kDa were shared between mesquite and lima bean [10–12]. Earlier, an attempt was made to isolate allergenic proteins from mesquite pollen extract using gel filtration chromatography. The extract separated into six fractions of 13, 20, 27.5, 41, 55.5 and 81 kDa and of these, a 20-kDa fraction was characterized as a glycoprotein that demonstrated IgE binding [13,14]. However, none of the purified major allergen of mesquite was explored for cross-reactivity and component resolved diagnosis of allergy.

Previously, a 66-kDa allergen was identified in mesquite pollen extract and wood smoke using patients' sera [11,15]. In the present study, a 66-kDa protein was isolated from mesquite pollen and characterized with standard immunobiochemical methods. The purified protein was also evaluated for cross-reactivity with predominant tree pollens and lima bean (food) allergen from mesquite family Leguminosae.

Materials and methods

Extraction of proteins

Inflorescence were collected during peak flowering season from mesquite (Leguminosae) and trees; namely, *Ailanthus excelsa* (Simarubaceae), *Cassia siamea* (Leguminosae), *Putranjiva roxburghii* (Euphorbiaceae), *Salvadora persica* (Salvadoraceae) and *Holoptelea integrifolia* (Ulmaceae). Polleniferous material was freeze-dried and sieved to get fine pollen powder whereas dried lima bean (*Phaseolus lunatus*; Leguminosae) seeds were ground to a fine powder using pestle and mortar. Extraction of antigens was carried out as described earlier [11,12].

Protein purification

Purification was carried out by reverse phase liquid chromatography using Octadecyl silica gel (Sigma, St. Louis, MO, USA). Briefly, 500 mg of Octadecyl silica gel was packed into 1 mL column in 100% acetonitrile. The column was washed with 50 mL acetonitrile and equilibrated with 20 mL distilled water. Mesquite pollen extract (10 mg) was loaded on the column and unbound fraction was recycled 10 times to ensure maximum binding. Elution was carried out with 10 mL each of 10–100% acetonitrile in water containing 0.01% trifluoroacetic acid. Eluted fractions were lyophilized and stored at –20 °C.

Electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli using 12% resolving gel [16]. The gel was stained with 0.1% Coomassie brilliant blue.

Skin tests and sera collection

Intradermal tests were carried out with mesquite pollen extract (1 : 500 w/v in phosphate buffer saline) and purified protein

(100 µg mL⁻¹) and other allergen extracts on 60 respiratory allergy patients at S.P. Medical College, Bikaner [17]. Blood samples were collected from patients showing marked positive skin reactions to mesquite. Blood samples were also collected from five individuals skin test negative to mesquite and other allergens, pooled and used as control. Informed consent of the subjects was obtained for participation in the study. The study protocol was approved by human ethics committee of the institute.

Specific IgE estimation

Specific IgE levels were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, microtitre plate was coated with purified protein (200 ng 100 µL⁻¹) and/or mesquite pollen extract (1 µg 100 µL⁻¹), blocked and incubated with mesquite hypersensitive individual patients' sera (1 : 10 v/v). ELISA was further carried out as described previously [11,12].

Specific IgE inhibition

Mesquite pollen extract (1 µg 100 µL⁻¹)/purified protein (200 ng 100 µL⁻¹) was coated on ELISA plate in triplicates, blocked and incubated with sera pre-incubated with 0.1 to 10 000 ng of purified protein. Sera pool of subjects (*n* = 5) with negative skin tests was used as a control and sera without inhibitor was used as a positive control, respectively. Percentage inhibition was calculated as under:

$$1 - \frac{\text{OD of sample with inhibitor}}{\text{OD of sample without inhibitor}} \times 100$$

Curvularia lunata, a fungus, was used as an irrelevant control.

IgE Immunoblot

SDS-PAGE-resolved purified protein was transferred onto nitrocellulose membrane, blocked and incubated with 17 individual patients' sera (1 : 10 v/v). The IgE binding was visualized with anti-human-IgE horseradish peroxidase using diaminobenzidine as substrate [11].

Histamine release

Histamine release assay was performed according to the manufacturer's protocol (Immunotech, Marseille, France) with some modification. Different concentrations (1, 5, 10 and 20 ng) of protein were tested to standardize the protein concentration to be used for the assay and optimal concentration was selected.

Briefly, heparinized blood samples from mesquite hypersensitive patients (*n* = 20) were challenged either with 5 ng of pollen extract or purified protein for 30 min at 37 °C. The histamine standards (0–100 nM) and released histamine in the samples were acylated and measured by ELISA. Spontaneous histamine release was determined in unchallenged sample and

total histamine content in the heparinized blood was measured by freeze-thawing of cells.

Lymphoproliferation and cytokine analysis

Lymphoproliferation assay was carried out as described elsewhere [17]. Briefly, peripheral blood mononuclear cells (PBMCs) isolated from mesquite-sensitized patients' blood were cultured in RPMI-1640 medium in a 96-well plate (10^5 cells/well) and stimulated with 5 μ g pollen extract or purified protein in triplicates. Phytohaemagglutinin was used as positive control and cells without stimulant were taken as negative control. The plates were incubated for 72 h in a CO₂ incubator and supernatant was collected for cytokine assays. The cells were washed, incubated for 2 h with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 100 μ g mL⁻¹), lysed in acidic isopropanol solution and absorbance read at 570 nm. Optical density (OD) values three times or more the negative control were considered with positive stimulation index.

Interleukin-4/interferon- γ (IL-4/IFN- γ) levels induced by purified protein were estimated by ELISA following the manufacturer's instructions (BD Pharmingen OptEIA kits, San Diego, CA, USA) in culture supernatants as described earlier [17].

Cross-reactivity of purified protein with other allergens

To study cross-reactivity of purified 66 kDa protein with tree pollens and lima bean allergens, ELISA inhibition was carried out using mesquite hypersensitive pooled patients' sera. The microtitre plate was coated with 200 ng of purified protein. After blocking, wells were incubated with patients' sera pre-incubated with 0.1 ng to 10 000 ng of different tree pollen extracts and/or lima bean seed extract, separately.

Further immunoblot inhibition was also carried out to establish the component based cross-reactivity. Briefly, purified protein was transferred to nitrocellulose membrane and probed with pooled patients' sera hypersensitive to mesquite, pre-incubated with 100 μ g of pollen and lima bean extracts, individually. Immunoblots were then developed as described earlier.

Sequencing of purified protein by mass spectrometry

The N-terminal of the purified protein was blocked; hence, the protein was fragmented with trypsin to obtain internal peptide sequences by mass spectrometry at the Centre for Genomic Application, New Delhi, using a MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The peptide mass fingerprinting data obtained were used to search in the Matrix Science database with mascot software (<http://www.matrixscience.com>). Homology searches were also made using Blast and Swiss-Prot database.

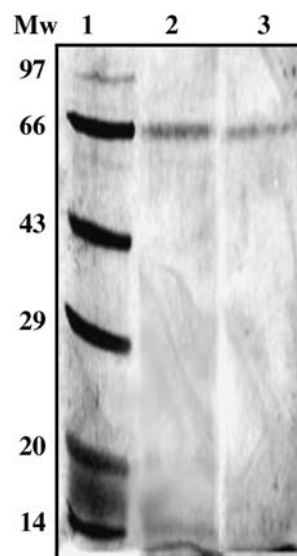


Figure 1 SDS-PAGE profile of eluted fractions of mesquite pollen extract. Lane 1: molecular weight markers (kDa); Lane 2: acetonitrile fraction (90%; 100 μ g); Lane 3: acetonitrile fraction (100%; 100 μ g). The gel was stained with Coomassie brilliant blue.

Statistical analysis

Correlation coefficients were determined by means of linear regression analysis. *P*-value less than 0.05 was considered significant.

Results

Purification of protein

SDS-PAGE analysis of eluted fractions of mesquite pollen extract with 90% and 100% acetonitrile showed a homogeneous band of 66 kDa (Fig. 1). The yield of protein was 10 mg g⁻¹ of pollen protein applied to the column.

Clinical evaluation of purified protein

Of 60 patients tested intradermally, 20 showed marked positive skin reaction with mesquite pollen extract whereas 18 elicited positive reaction with purified protein (Table 1). Thus, 90% of mesquite positive cases were recognized by purified protein on skin testing. A significant correlation was observed ($r = 0.7438$, $P < 0.0002$) between the skin reactivity with purified protein and pollen extract.

Allergenicity of purified protein

Specific IgE binding of purified protein was determined by ELISA using all 20 mesquite positive (intradermal tests) patients' sera with elevated IgE levels (ODs 0.315 to 0.580) against pollen

Table 1 Skin reactivity and specific IgE values in patients ($n = 20$) tested with *Prosopis* extract and purified protein

Patient no.	Age (year)/sex	Symptoms	ID (mm)		Specific IgE (OD)	
			<i>P. juliflora</i>	Purified protein	<i>P. juliflora</i>	Purified protein
1	32/F	AR	12	14	0.370	0.320
2	34/M	BA	10	11	0.415	0.342
3	42/M	AR	11	10	0.440	0.317
4	26/M	BA, AR	12	16	0.383	0.412
5	36/M	AR	14	16	0.332	0.350
6	32/M	AR	14	16	0.580	0.510
7	32/M	AR	10	11	0.500	0.520
8	14/M	BA, AR	12	10	0.550	0.500
9	28/M	AR	12	10	0.540	0.512
10	38/M	BA, AR	15	16	0.420	0.400
11	21/M	AR	11	12	0.470	0.450
12	28/M	BA, AR	12	12	0.450	0.390
13	45/M	AR	18	17	0.450	0.480
14	29/F	BA, AR	11	12	0.410	0.460
15	31/M	BA, AR	15	16	0.420	0.400
16	31/M	BA, AR	10	4	0.319	0.107
17	35/M	BA, A R	12	6	0.315	0.153
18	28/M	AR	12	12	0.426	0.420
19	25/F	AR	10	10	0.380	0.350
20	32/M	AR	12	12	0.410	0.320
Control (pool)					0.072	0.078

M, male; F, female; BA, bronchial asthma; AR, allergic rhinitis; ID, intradermal reaction (wheal size in mm). Positive control: histamine diphosphate 100 $\mu\text{g mL}^{-1}$. Negative control: pool of skin test individuals serum ($n = 5$). ELISA positive: specific IgE (ODs) $\geq$ three times of negative control.

extract. Of these, 18 sera showed high IgE levels against (ODs 0.317 to 0.520) 66-kDa protein (Table 1). Patient nos 16 and 17, however, did not show elevated IgE against purified protein. Specific IgE value ≥ 3 times of normal control were considered as positive cut-off for ELISA. IgE binding of 66-kDa protein was further assessed by immunoblotting with pooled/individual hypersensitive patients' sera (patients nos 1–17; Fig. 2a). Of these, 15 (88%) sera showed IgE binding with purified protein demonstrating it as a major allergen of mesquite pollen.

ELISA inhibition was carried out to investigate the biological potency of purified protein. A dose-dependent inhibition of IgE binding to solid phase mesquite extract was observed, when serum pool was incubated with increasing concentrations of extract and purified protein, separately (Fig. 2b). For self-inhibition, 70 ng of mesquite extract and 40 ng of purified protein was required to achieve 50% inhibition of specific IgE binding. For cross-inhibition, 90 ng of purified protein resulted in 50% inhibition of IgE binding to mesquite extract (solid phase).

Histamine release

Blood samples from all 20 mesquite hypersensitive patients ($n = 20$) showed histamine release in the range of 22% to 58% on challenge with pollen extract, whereas purified protein released histamine ranging from 11% to 52%. The controls ($n = 3$) with negative skin reactivity demonstrated 6–10% histamine release (Fig. 3). Statistically significant correlation was observed between histamine release with purified protein and pollen extract ($r = 0.8832$; $P < 0.0001$).

Lymphocytes proliferation on antigen stimulation

Purified protein induced significant proliferation (≥ 3 times of negative control) in 18/20 mesquite-sensitized patients' PBMCs (Fig. 4a). OD values ranged from 0.302 to 0.841 on stimulation with purified protein ($n = 18$) as compared to 0.300 to 0.820 obtained with pollen extract ($n = 20$).

Stimulation with pollen extract released significant amount of IL-4 (140–478 pg mL^{-1}) compared to normal controls (20 pg mL^{-1})

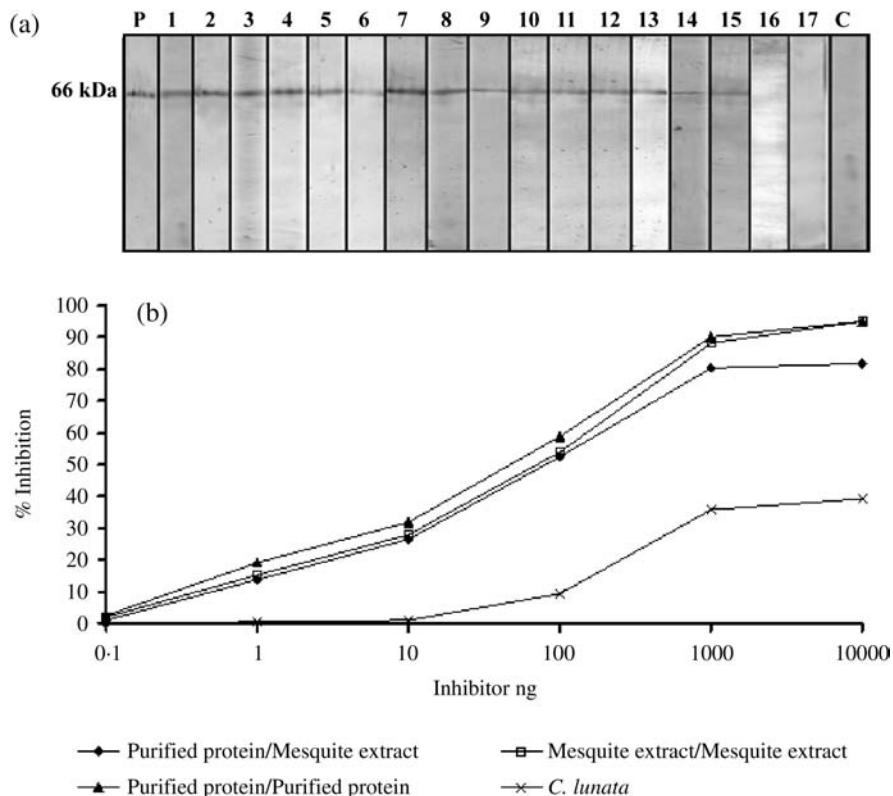


Figure 2 (a) Immunoblot of purified protein: 100 µg purified protein (66 kDa) was resolved on 12% SDS-PAGE, transferred to nitrocellulose membrane and probed with mesquite hypersensitive patients' sera (1 : 10 v/v). P: a strip probed with a pool of 17 mesquite hypersensitive individual patients' sera; Lanes 1–17: strips probed with individual patients' sera; C: control sera. (b) ELISA inhibition. IgE inhibition of mesquite extract/purified protein using *Prosopis* hypersensitive pooled patients' sera pre-incubated with 0.1–10 000 ng of mesquite pollen extract/purified protein and *Curvularia lunata* extract as inhibitors. The values represent the mean of three independent experiments.

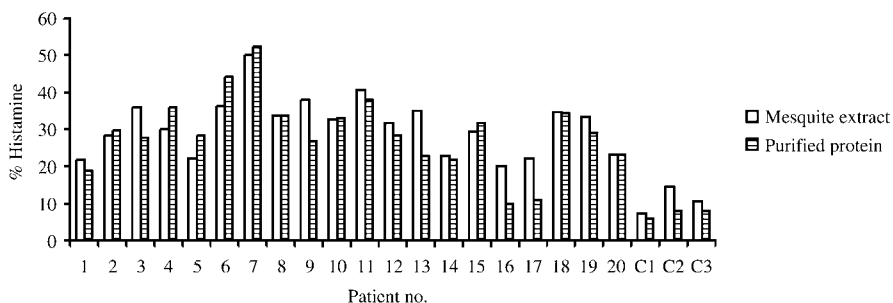


Figure 3 *In vitro* histamine release assay. Histamine released in blood samples of patients ($n = 20$) skin test positive to pollen extract on challenge with 5 ng of mesquite pollen extract and purified protein, separately. C1–C3 = controls. The values represent the mean of three independent experiments.

in culture supernatants (Fig. 4b). High IL-4 levels were detected (121–488 pg mL⁻¹) in the culture supernatant ($n = 18$) on challenge with purified protein as compared to controls (7.3–18.6 pg mL⁻¹). A significant correlation was observed ($r = 0.8593$; $P < 0.0001$) between IL-4 levels against both purified protein and pollen extract. IFN- γ levels, however, were low (20–50 pg mL⁻¹) against both pollen extract as well as purified protein (data not shown).

Cross-reactivity of purified protein with tree pollen allergens

Cross-inhibition of 66-kDa purified protein with tree pollen extracts showed allergenic relationship with mesquite and other allergens (Fig. 5a). Here 40 ng of purified protein was required for 50% inhibition of self protein bound to solid phase, whereas 95 ng

of mesquite extract was required for 50% inhibition of purified protein (66 kDa). Pollen extracts, such as *A. excelsa*, *C. siamea* and *S. persica*, required 145, 150 and 155 ng of protein, respectively, for 50% inhibition of specific IgE to purified protein. *P. roxburghii* and *H. integrifolia* could induce 50% inhibition of IgE binding to purified protein with 180 and 200 ng of protein, respectively. Whereas, 600 ng of *P. lunatus* seed extract was required to cause 50% inhibition of IgE directed towards purified protein.

Immunoblot inhibition demonstrated the component-based cross-reactivity of purified protein with tree pollen extracts (Fig. 5b). Purified protein (66 kDa) was completely inhibited by 100 µg of the *P. juliflora*, *A. excelsa*, *C. siamea*, *S. persica* pollen and *P. lunatus* seed extracts while *P. roxburghii* and *H. integrifolia* caused partial inhibition of the protein at this concentration.

Figure 4 (a) *In vitro* lymphoproliferation assay. PBMCs from mesquite hypersensitive patients demonstrated significant proliferation when challenged with 5 μ g of pollen extract or purified protein, whereas PBMCs from healthy controls (C1–C3) did not show significant proliferation. PBMCs stimulated with 5 μ g phytohaemagglutinin and without stimulation were taken as positive and negative controls. The values represent the mean of three independent experiments. (b) Cytokine analysis. Levels of IL-4 in the culture supernatants on stimulation with mesquite extract and purified protein estimated by ELISA. C1–C3: controls. The values represent the mean of three independent experiments.

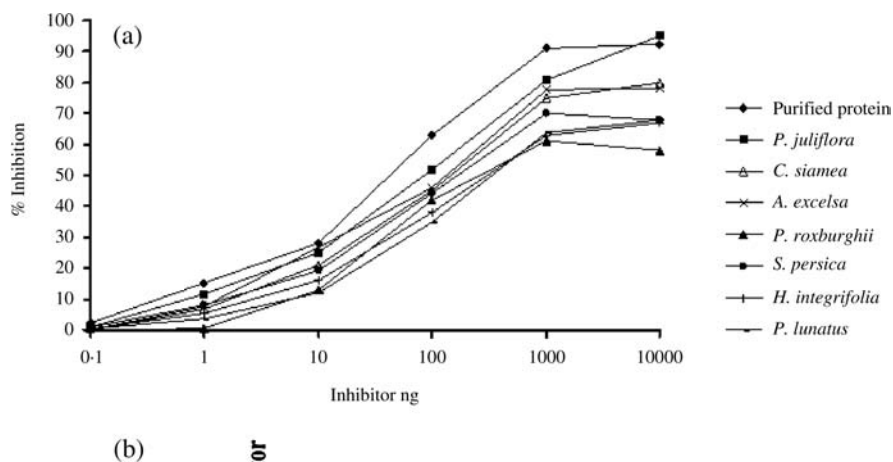
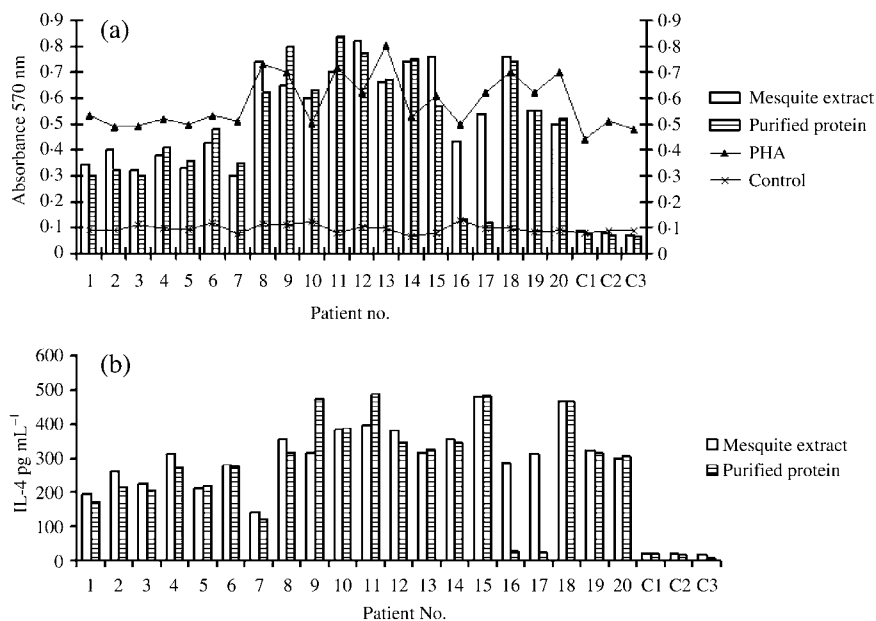


Figure 5 (a) IgE ELISA inhibition showing cross-reactivity of 66-kDa protein of mesquite with lima bean and other tree pollen extract(s) as inhibitors. (b) Immunoblot inhibition of 66-kDa protein on incubation of mesquite positive patients' sera with lima bean and other tree pollen extract(s) as inhibitors.

Mass spectrometry

Peptide mass finger printing by MALDI MS/MS generated five fragments showing homology with conserved domains of serum albumin precursor from humans, whereas three fragments

showed homology with bovine serum albumin precursor (Bos d 6). Furthermore, homology searches using BLAST and Allermatch databases revealed that these fragments show homology with serum albumin allergens from diverse sources (data not shown).

Other peptides obtained did not show significant homology with any known proteins.

Discussion

Exposure to allergens from tree pollens is a significant cause of immediate hypersensitivity. Allergenic cross-reactivity among pollens and/or with foods can be exploited for reducing the number of allergens without compromising the diagnostic and therapeutic efficacy. Studies suggest that purified natural and recombinant allergens show promise for use as diagnostics and therapeutics. Purified allergens are also preferred for cross-reactivity studies as they represent the disease causing components [7]. Allergens such as Bet v1 from *Betula verrucosa*, Ole e 1 from *Olea europaea* and Cup a1 from *Cupressus arizonica* tree pollen were studied extensively. Bet v 1, a major allergen, showed extensive cross-allergenicity with pollen and food allergens [18–21]. Immunotherapy with cross-reactive allergens was protective against the common allergens besides the original source [18,22,23]. Hence, the present study was undertaken to isolate and characterize a cross-reactive allergen from mesquite, an important inhalant allergen source for allergic rhinitis and asthma.

Earlier, a 66-kDa protein was demonstrated as major IgE-binding component in pollen extract and in wood smoke of mesquite [11,15]. Allergenic proteins of similar molecular weight range (60–70 kDa) were reported from other plant species such as mugwort, birch, hazelnut, timothy, peanut, apple and celery [24,25]. Here, the purified protein from mesquite appeared as a single band at 66 kDa on SDS-PAGE. Purified protein showed skin reactivity ($r = 0.7438$, $P < 0.0002$) comparable with pollen extract in 90% (18/20) of the mesquite-sensitized patients tested and showed IgE binding with $> 80\%$ of mesquite allergic patients' sera in ELISA and immunoblot. Hence, it was designated as a major allergen. Similar to our results, no significant difference was observed in skin reactivity between purified nPla 2 and *Platanus acerifolia* pollen extract. Pla a 1 and Pla a 2 were responsible for 79% of the IgE-binding against *Platanus* pollen extract [26].

In vitro allergen-specific histamine release demonstrates the functional properties of allergens leading to allergic effector mechanism and also provides information on the *in vivo* situation of allergic patients [7]. In the present study, purified protein induced significant histamine release ($P < 0.05$) in mesquite-sensitized patients' blood, suggesting its strong biological potency. Significant lymphocyte proliferation and IL-4 secretion further suggests that purified protein is allergenic.

Cross-reactivity among certain pollen allergens from same genus or different plant family has been reported earlier [27–29]. Cross-reactions are also reported between pollen allergens and plant-derived foods [30–31]. In the present study, the 66 kDa major allergen from mesquite was investigated for cross-reactivity. Data from the inhibition analysis revealed that tree pollen extracts and

lima bean seed extract(s) could significantly inhibit the serum IgE binding to a 66-kDa purified allergen of mesquite pollen extract. Purified protein showed a close allergenic relationship with *A. excelsa*, *C. siamea* and *S. persica* tree pollens and with lima bean, an edible legume. This cross-reactive purified protein could therefore be used as a representative for multiple pollen/food allergens and may have implication for component-based therapy of allergy [21,32].

Human serum albumin-like protein in plants was reported for the first time by Lait and Zwiazek in 2001. They have isolated a 70-kDa protein from spinach leaves which shared 100% identity with the first 15 residues of human serum albumin [33]. In our study, the partial amino acid sequences obtained for 66 kDa protein by mass-spectrometry showed high homology with serum albumin precursors from various animal sources including human. However, further extensive studies are required to confirm the biological function of the purified 66 kDa.

In conclusion, a 66-kDa major allergen was isolated from mesquite pollen that contributes significantly towards cross-reactivity among predominant tree pollens and lima bean, a legume allergen. This cross-reactive protein seems clinically relevant for allergy disorders.

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