
Clinical and immunobiochemical characterization of airborne *Delonix regia* (Gulmohar tree) pollen and cross-reactivity studies with *Peltophorum pterocarpum* pollen: 2 dominant avenue trees from eastern India

Jyotshna Mandal, MS*; Prasenjit Manna, MS†; Pampa Chakraborty, PhD‡; Indrani Roy, MD§; and Swati Gupta-Bhattacharya, PhD*

Background: *Delonix regia* and *Peltophorum pterocarpum* pollen are important aeroallergens for type 1 hypersensitivity in the tropics. The IgE-binding proteins of *D regia* and their cross-allergen with *P pterocarpum* pollen have not been evaluated.

Objectives: To isolate and characterize the IgE-binding proteins of *D regia* pollen for the first time and to investigate the cross-allergen with *P pterocarpum* pollen belonging to the same family (Leguminosae).

Methods: Allergenic activities were determined by in vivo and in vitro analyses. Pollen extract was fractionated by a combination of 2 columns (diethyl amino ethyl Sephadex and Sephacryl S-200). Protein components were characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, periodic acid–Schiff staining, and immunoblotting. In vitro inhibition tests were performed to evaluate the cross-reactivity.

Results: The skin prick test results of the patients with respiratory allergies in Calcutta, India, showed 31.1% positivity with *D regia* pollen. Nine IgE-reactive protein components were found in the crude extract. An optimum IgE-reactive fraction was resolved into 4 subfractions. Subfraction A, which showed maximum IgE reactivity, contained 2 (96- and 66-kDa) IgE-reactive protein components. The 66-kDa component was found to be glycoprotein. Remarkable cross-reactivity between *D regia* and *P pterocarpum* pollen was found on IgE enzyme-linked immunosorbent assay inhibition and dot blotting. Shared IgE-binding components (66, 56, 32, 28, 25, and 23 kDa) were observed between *D regia* and *P pterocarpum* pollen extracts, whereas the 96- and 43-kDa components were specific to *D regia*.

Conclusion: The purification of the IgE-binding proteins and the identification of the shared/cross-reactive proteins in these taxonomically related pollen members should be helpful for the diagnosis and therapy of patients susceptible to these pollens.

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INTRODUCTION

The prevalence of aeroallergen sensitization is increasing worldwide.^{1–3} A field study conducted in the 4 major Indian cities (Delhi, Mumbai, Calcutta, and Chennai) showed the presence of asthma in 2006 to be 2.38%.⁴ India is blessed with one of the richest flora on the earth, consequently providing considerable variation in the quality and quantity of airborne pollen in different ecogeographical regions.⁵ Airborne pollen allergens are 1 of the most important causes of asthma and rhinitis.⁶ The identification, purification, and characterization of proteins causing IgE-mediated allergies

are the main research objectives in the area of molecular allergy; they are used for establishing the components necessary to diagnose and treat type 1 allergy.⁷

The Leguminosae (Fabaceae) is a large economically important family of flowering plants. It is the third largest family (after Orchidaceae and Asteraceae), with 730 genera and more than 19,400 species. *Delonix regia* (Boj Ex Hook Raf.) or Gulmohar is a member of this family; it is a very common shade/avenue tree with bright flowers, widely grown in the tropics and subtropics of India, southeast Asia, and parts of the United States (Southwest Florida, the Rio Grande Valley of South Texas, Southern Arizona, Southern California, Hawaii, Puerto Rico, the US Virgin Islands, and Guam). *Delonix regia* pollen was found to be a potent cause of respiratory allergy in susceptible individuals in Calcutta and its suburbs (in eastern India).^{8–13} A previous report of its atmospheric presence was made from central¹⁴ and southern India.¹⁵ The pollen caused 14% to 23% sensitization in respiratory allergic cases with positive skin reactivity.^{9,12} Pollen grains of *Peltophorum pterocarpum*, another popular avenue

Affiliations: * Division of Palynology and Environmental Biology, Department of Botany, Bose Institute, Calcutta, India; † Department of Molecular Medicine, Bose Institute, Calcutta, India; ‡ Department of Botany, Sree Chaitanya College, West Bengal, India; § Allergy Department, Institute of Child Health, Calcutta, India.

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tree of Leguminosae, have already been studied for its allergen; its atmospheric prevalence has been studied from Central,¹⁴ Western,^{16,17} and Southern India.¹⁸ During the peak flowering month (May), *D regia* and *P pterocarpum* contributed to a relatively high percentage (6.3%–8%) of the total airborne pollen load in Calcutta.¹³ Microscopic studies revealed that both *D regia* and *P pterocarpum* pollen grains have tricolporate aperture with reticulate ornamentation. *Delonix regia* pollen is prolate spheroidal, whereas *P pterocarpum* grains are oblate spheroidal (Fig 1A).

Despite its abundance and proved implication in pollinosis, to our knowledge, there have been no studies addressing the characterization of the IgE-binding protein of *D regia* pollen. The objectives of this study are to characterize the IgE-binding proteins of *D regia* pollen and to identify the cross-reactive allergens it shares with *P pterocarpum* pollen (Fig 1B).

METHODS

Preparation of Pollen Extracts

The pollen samples were collected from the mature anthers of the fresh flowers of the relevant trees growing around the city

during their peak flowering period.¹³ The batches used for this study contained less than 1% nonpollen impurities. They were defatted with diethyl ether, and then 10 g was extracted in phosphate-buffered saline (PBS) (0.1M sodium phosphate and 150M sodium chloride, pH 7.2) by continuous stirring at 4°C for 16 hours in a 1:10 wt/vol ratio. After centrifugation at 14,000g, the clear supernatant was dialyzed and passed through a 0.22- μ m filter (Millipore Corp, Bedford, Massachusetts). The filtrate was then lyophilized and stored at –70°C in aliquots. The protein content of the crude pollen extract (CPE) and fractions was determined by the method of Lowry et al.¹⁹

Fractionation of Pollen Extracts

Fraction A. Ten grams of the pollen grains was extracted with 50M phosphate buffer (PB), pH 7.2, at 4°C in a 1:10 wt/vol ratio. Crude pollen extract was then fractionated in the range of 0% to 90% saturation of ammonium sulfate. The salted-out fraction was dissolved in PB and dialyzed (molecular cutoff limit, 10,000) to remove traces of ammonia. The concentrated protein (18 mg) was passed through a diethyl amino ethyl column (Sephadex column [GE Healthcare Biosciences, Uppsala, Sweden]: diameter, 1.3 cm; height, 12 cm) and eluted in a linear gradient of 0M to 1M sodium chloride. The fractions were collected in 4-mL aliquots at 4°C.

Fraction B. The optimum IgE-reactive fraction (16-mg) solution was passed through a different column (Sephacryl S-200 column [GE Healthcare Biosciences]: diameter, 2.4 cm; height, 48 cm) equilibrated with 10M PB, pH 7.2. The fractions were collected in 3-mL aliquots (void volume, 74 mL; flow rate, 12 mL/h) at 4°C, and the elution was monitored at 280 nm using a spectrophotometer. The fractions were further pooled, dialyzed, and lyophilized.

Participants, Skin Prick Tests, and Serum Sample Collection

Skin prick tests (SPTs) were performed with *D regia* pollen allergen extract on 2,234 patients with respiratory allergies (age range, 18–55 years; 51.75% male and 48.25% female) attending the Allergy Unit of The Institute of Child Health, Calcutta, from June 2004 to May 2008. The allergen extracts were prepared by extracting the pollen at 95% purity with 0.1M PBS (1:50 wt/vol).²⁰ Histamine phosphate, 1 mg/mL, and PBS, 1 mg/mL, were used as positive and negative controls, respectively. According to international guidelines, positivity was defined as a mean wheal diameter of 3 mm or greater compared with the negative control.²¹ The reaction was graded from +1 to +3 (+1 indicates erythema, 20 mm in diameter; +2, erythema, >20 mm in diameter; and +3, wheal and erythema).²² Control serum samples were collected from 5 nonsensitized healthy volunteers without a history of a previous or current episode of any allergic, systemic, or joint diseases in them and their families. The serum samples of each patient were separated and stored at –20°C for future use. The study was approved by the ethics committee of the

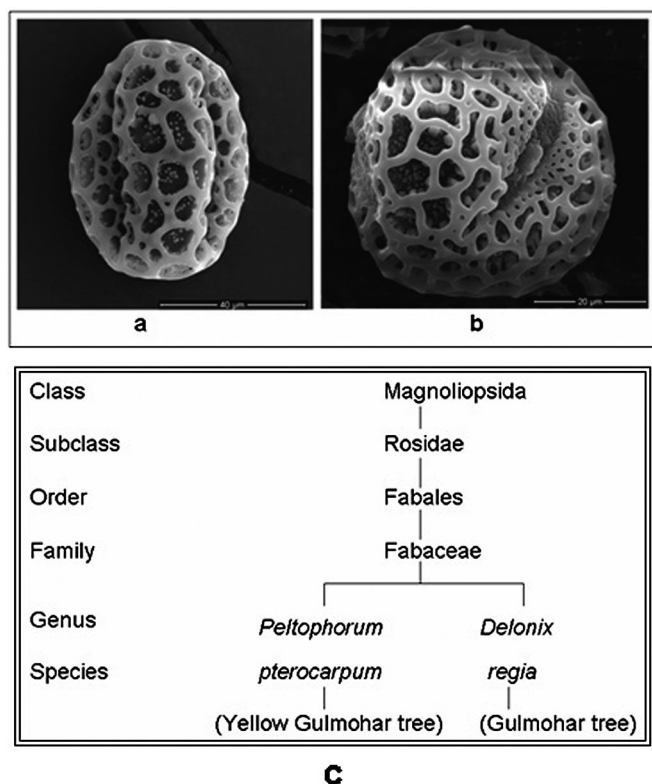


Figure 1. Scanning electron micrographs of *Delonix regia* pollen (equatorial view, original magnification $\times 1,500$) (A), *Peltophorum pterocarpum* pollen (equatorial view, original magnification $\times 2,400$) (B), and plant taxonomic classification of the Fabaceae (Leguminosae) family (C). Data are from Cronquist A. *An Integrated System of Classification of Flowering Plants*. New York, NY: Columbia University Press; 1981:1262.

hospital, and written informed consent was obtained from the participants before their participation.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) of *D regia*/*P pterocarpum* CPE was performed in 11% gel.²³ The protein samples (approximately 130 µg of protein per well) were mixed with an equal volume of reducing sample buffer and β-mercaptoethanol by boiling for 1 minute at 95°C. After completion, gel was stained at room temperature overnight by incubating with staining solution (0.25% wt/vol Coomassie brilliant blue dissolved in destaining solution: a combination of methanol, glacial acetic acid, and distilled water [9:2:9, vol/vol/vol]). Fraction A was observed by silver staining.

Glycoprotein detection was performed using the periodic acid–Schiff (PAS) technique. Chemical detection of carbohydrate after SDS-PAGE was accomplished by periodic acid oxidation, followed by reaction with Schiff reagent.²⁴

The serum total IgE level was measured using an enzyme immunoassay kit for the quantitative determination of IgE in human serum (Pathozyne IgE; Omega Diagnostics, Alva, England), according to the manufacturer's instructions.

IgE-Specific Enzyme-Linked Immunosorbent Assay

The wells of the microtiter plates (Nunc-Immuno modules, Rockslide, Denmark) were coated with 0.5 µg/50 µL per well of the pollen extract in 0.1M PB, 150M sodium chloride, pH 7.2, and kept overnight at 4°C. After blocking at room temperature for 1.5 hours in 0.1M PBS containing 0.05% vol/vol Tween 20 (polyoxyethylene sorbitol), the plate was incubated with 50 µL of individual or pooled patient serum (1:10, vol/vol) overnight at 4°C, followed by incubation with anti-human IgE alkaline phosphatase conjugate (Sigma Chemical Co, St Louis, Missouri) diluted (1:1,000, vol/vol) in blocking solution. 4-Nitrophenyl phosphate was used as substrate and dissolved in PBS, 1 mg/mL. The reaction was further performed as described previously.²⁵

Specific IgE Enzyme-Linked Immunosorbent Assay Inhibition

Enzyme-linked immunosorbent assay (ELISA) inhibition was performed by overnight preincubation of pooled patient serum samples with an equal volume of the following: (1) whole extract of *D regia* and *P pterocarpum* pollen extract, (2) fractions of *D regia* pollen extract, or (3) *P pterocarpum* pollen extract with varying protein concentrations (1–10,000 ng) at 4°C.²⁶ (The baseline concentration of all the extracts and fractions was 200 µg/mL of protein, which was diluted accordingly.) The inhibited serum was then used in an ELISA with anti-human IgE alkaline phosphatase conjugate. Serum samples without inhibitor were used as a positive control. The 0% inhibition was obtained by incubating serum samples with PBS. The rest of the procedure was performed as previously described.

The percentage inhibition was calculated as follows: % Inhibition = (Optical Density of Test Sample – Optical Density of Inhibited Sample) × 100.

Histamine Release Assay

Blood samples of 25 *D regia*–hypersensitive patients and controls were processed and challenged with *D regia* CPE and purified protein fraction in the concentration range of 5 to 50 ng. The histamine standards (range, 0nM–100nM) and released histamine from the samples were acylated. The acylated histamine in the samples, controls, and standards was measured by ELISA, per the manufacturer's instructions (Immunotech, Marseilles, France). Spontaneous histamine release was measured in an unchallenged sample, and total histamine in the heparinized blood was estimated by cell lysis. Five nonallergic individuals were used as negative controls.

IgE-Specific Immunoblotting

Crude pollen extract was resolved by SDS-PAGE (11% total acrylamide–bisacrylamide monomer concentration and 5% cross-linker concentration) in the presence of β-mercaptoethanol. The resolved bands (without staining) were transferred electrophoretically onto a polyvinyl difluoride membrane by semidry blotting for 1 hour at 0.8 mA/cm². The membrane was pretreated according to the manufacturer's instructions (NEN Life Science Products, Hounslow, England). Membranes were incubated overnight with serum samples (1:10) collected from individual patients showing positive skin reactions to *D regia* pollen extract. IgE binding was observed with alkaline phosphatase–conjugated monoclonal anti-human IgE (1:1,000) using a combination of nitroblue tetrazolium chloride and 5-bromo, 4-chloro, 3-indolyl phosphate potassium salt as substrate.

IgE-Specific Dot Blotting and Immunoblotting Inhibition

Dot blotting was performed to observe the IgE-specific cross-reactions using anti-human IgE peroxidase conjugate (1:500) and diaminobenzidine as substrate.²⁷

Sodium dodecyl sulfate–PAGE–resolved *D regia* proteins were transferred to a polyvinyl difluoride membrane. After blocking the free sites, strips were kept overnight at 4°C with a preincubated mix of 500 µL of patient serum samples (1:5 vol/vol) and 500 µL of *D regia* pollen extract (200 µg) and *P pterocarpum* pollen extract. As a positive control, a *D regia* strip was incubated with sensitive pooled patient serum samples (1:10 vol/vol). Another *D regia* strip, probed with PB, served as a negative control. Strips were then developed in a manner similar to IgE-specific immunoblotting.

Statistical analyses were performed with SPSS, version 10 (SPSS Inc, Chicago, Illinois) using the *t* test. The coefficient of correlation of the paired sample was determined between the histamine release with purified protein fraction and pollen extract. *P* < .05 was considered statistically significant.

Table 1. Clinical Characteristics of the Patients Selected for the Study^a

Patient No./ sex/age, y	Symptoms/ clinical history	SPT result	FEV ₁ , % predictive value)	Total IgE, IU/mL	Specific IgE ELISA, P/N value	Histamine release, %		Aeroallergen sensitization
						DR (total)	Fraction A of DR	
Group A: Patients Tested With <i>Delonix regia</i> Pollen Extract								
1/F/18	AR	++	92.0	510	3.11	33	27	AI, AA, CN, and PP
2/M/32	AR	++	86.0	656	2.14	19	11	AF, HD, and SO
3/F/34	AR	+++	79.0	725	3.33	49	37	PP, PS, and RN
4/M/47	AR	++	83.0	361	2.53	28	17	CN, PP, and SO
5/M/55	AR plus BA	++	72.0	317	2.81	31	28	CP and HD
6/M/38	AR plus BA	++	76.0	675	2.05	17	11	PP and egg
7/F/29	AR	+++	71.0	500	3.34	36	32	AI, CN, and SO
8/M/22	AR	++	74.0	219	2.63	30	21	PP and PS
9/M/31	BA	++	61.0	403	2.88	31	21	Egg, Cocos, HD, and PP
10/F/39	AR	+	83.0	150	2.61	30	23	AC, CR, and CS
11/F/45	AR plus BA	++	71.2	313	2.37	22	18	AI, AS, CN, and PS
12/M/19	AR plus BA	+++	63.0	754	3.65	54	47	AS and CS
13/M/23	AR	++	86.0	197	2.41	26	24	Egg, PP, Rohu (Labeo), and fish
14/M/40	BA	++	72.0	246	2.56	28	14	HD, PP, and PS
15/F/36	BA	++	77.0	291	2.72	29	19	AI, egg, and PS
16/F/33	AR	++	59.0	462	3.25	47	36	AI, CP, and SO
17/M/28	AR	++	64.0	362	2.30	22	13	Egg, HD, and wheat
18/M/56	AR plus BA	+++	69.0	714	3.56	52	27	CN, PP, wheat, and SO
19/F/37	AR plus BA	++	79.0	452	2.58	27	19	HD, PP, spinach, and TH
20/M/40	BA	++	80.5	612	2.89	32	19	Egg, HD, and LC
21/F/35	BA	++	68.8	635	2.30	24	18	AI and egg
22/F/38	AR plus BA	++	79.0	526	2.56	26	22	AI, CN, and SO
23/M/21	BA	++	76.0	253	2.20	21	16	Egg, HD, and PP
24/M/27	AR	+++	96.0	389	4.23	71	58	AC, CP, and PP
25/M/19	AR	+++	83.6	541	3.65	58	44	LC, PP, and tomato
Group B: Patients Tested With <i>Peltophorum pterocarpum</i> Pollen Extract								
1/M/51	BA	++	73.8	719	3.52	—	NP	AI, CN, and CP
2/F/39	BA	+++	72.0	610	3.22	—	NP	AI, CN, and EC
3/F/35	AR	++	76.0	750	2.95	—	NP	HD and CS
4/F/30	AR	+++	73.2	844	3.66	—	NP	CN and CR
5/M/18	AR plus BA	++	66.3	600	2.46	—	NP	AI, egg, and LC
Control Group								
1/F/30	AR	—	88.0	60	0.92	8	6	AC and CN
2/M/17	AR	—	74.0	412	0.76	6	5	HD and LC
3/M/36	AR plus BA	—	81.0	93	1.23	10	7	HD and egg
4/M/25	AR	—	73.0	250	1.19	9	6	AC, AI, and HD
5/F/19	BA	—	67.0	168	1.02	9	6	AI

Abbreviations: AA, *Alternaria alternata*; AC, *Areca catechu*; AF, *Aspergillus flavus*; Al, *Azadirachta indica*; AR, allergic rhinitis; AS, *Alstonia scholaris*; BA, bronchial asthma; CN, *Cocos nucifera*; CP, *Carica papaya*; CR, *Catharanthus roseus*; CS, *Cassia siamea*; DR, *Delonix regia*; EC, *Eucalyptus citridora*; ELISA, enzyme-linked immunosorbent assay; FEV₁, forced expiratory volume in 1 second; HD, house dust; LC, *Lantana camara*; NP, not performed; P/N, ratio of optical density of patient serum with respect to control; PP, *Peltophorum pterocarpum*; PS, *Phoenix sylvestris*; RN, *Rhizopus nigricans*; SO, *Saccharum officinarum*; SPT, skin prick test; TH, *Trichoderma harzianum*.

Symbols: +, no wheal and erythema of 20-mm diameter; ++, no wheal and erythema of greater than 20-mm diameter; +++, wheal and erythema; —, no wheal and no erythema.

^a For group A, serum samples of patients 1, 3, 7, 12, 16, 18, 24, 25 (P/N value >3) were chosen for immunoblotting of fraction 1 and 1A of DR pollen extract. For cross-reactivity studies with PP pollen, pooled serum samples of 7 *Delonix*-sensitive patients were taken (P/N value >2.5, showing no sensitivity to PP pollen) (patients 4, 5, 7, 12, 15, 16, and 20). For group B, pooled serum samples of 5 *Peltophorum*-sensitive patients were taken for cross-reactivity studies with DR pollen extract (they showed no sensitivity to DR pollen). The control group had a P/N value of less than 1.5. For immunoblotting with fraction A, serum samples of patients 1, 3, 7, 24, and 25 were taken. For immunoblotting with fraction B, serum samples of patients 12, 16, 18, 24, and 25 were taken.

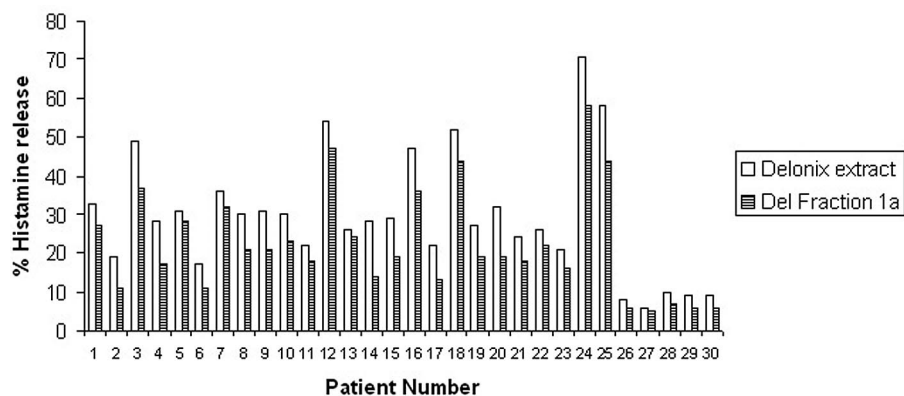


Figure 2. In vitro histamine release assay: patients 26 through 30 represent controls.

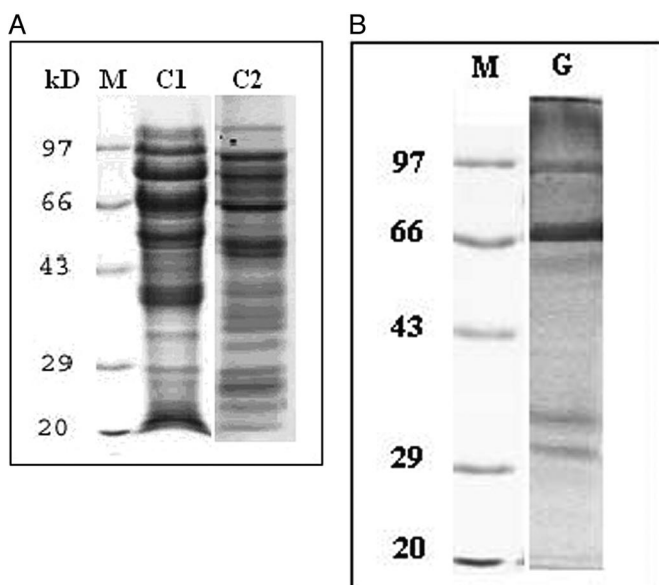


Figure 3. A, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein profile of *Delonix regia* crude pollen extract. M indicates that molecular weight markers are expressed in kilodaltons (kD). C1 indicates protein bands of *D regia* (whole pollen extract) stained in Coomassie brilliant blue; C2, protein bands of *P pterocarpum* (whole pollen extract) stained in Coomassie brilliant blue. B, Glycoprotein detection of the crude pollen extract of *D regia* pollen.

RESULTS

Patients

Of a total of 2,234 patients (mean age, 34.4 years) tested with *D regia* allergen extract, 695 (31.1%) showed a +1 and 130 (5.8%) showed a +2/+3 reaction on SPT. Among them, 25 patients were selected for serum sample collection on the basis of a higher-level reaction on SPT and a high titer of *D regia* pollen IgE-ELISA-specific results (Table 1).

Histamine Release

Blood samples from all 25 *D regia*-hypersensitive patients showed histamine release in the range of 17% to 71% (mean

[SD], 33.72 [13.63]) on challenge with 5 ng of CPE, whereas purified protein released histamine in the range of 11% to 58% (mean [SD], 25.56 [12.36]). The controls ($n = 5$) with negative skin reactivity demonstrated 8% to 10% histamine release. A statistically significant correlation was observed between histamine release with a purified protein fraction and with a pollen extract ($r = 0.03$, $P < .001$) (Table 1 and Fig 2).

SDS-PAGE and Immunoblotting

On reducing SDS-PAGE (11%), the CPE of *D regia* pollen grain resolved into more than 20 distinct bands between 16 and 109 kD (Fig 3A, lane C1). Protein fractions of 109, 96, 79, 66, 62, 54, 52, 49, 43, 40, 38, 32, 28, 25, and 21 kDa were most prominent and stained strongly by Coomassie brilliant blue. The *P pterocarpum* CPE resolved into more than 17 distinct bands between 15 and 98 kDa. Protein fractions of 76, 66, 49, 41, 38, and 20 kDa were most prominent and stained strongly by Coomassie brilliant blue (Fig 3A, lane C2).

In PAS staining, bands of 96, 66, 38, and 32 kDa were found to be glycoproteins (Fig 3B, lane G). In case of *D regia* pollen grains, IgE-specific immunoblotting with individual patient serum samples revealed 9 IgE-binding fractions (Fig 4). Six bands were the most important because they showed maximum binding with individual patient serum samples. Protein bands of 66 and 43 kDa were recognized by 13 (52%) and 12 (48%) of the individual patient samples, respectively. Other bands that were identified by sensitive patient serum were 96, 32, 28, 25, and 23 kDa.

Purification of the IgE-Binding Protein Component

The concentrated CPE (12 mg) was passed through a diethyl amino ethyl column (Sephadex), and 4 fractions were eluted (Fig 5A). Using IgE ELISA inhibition with pooled serum samples of 5 patients (P/N [ratio of optical density of patient serum with respect to control] value, >3 on IgE ELISA), the dose-response curve showed fraction A to have an optimum IgE-binding activity with respect to the crude extract (Fig 5B). Fraction A showed 50% inhibition with 80 ng of protein.

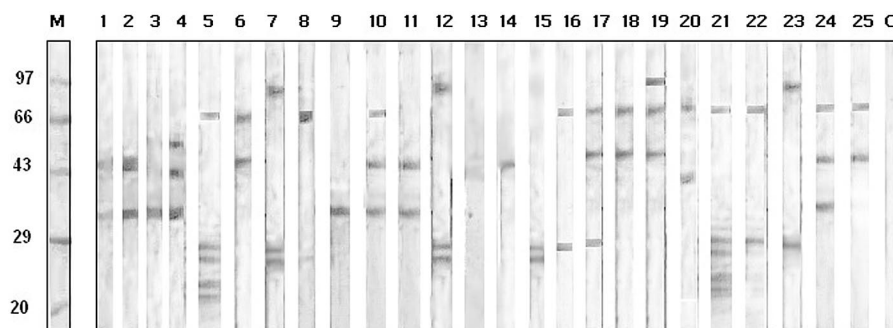
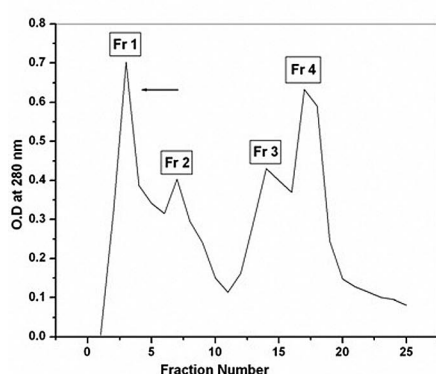
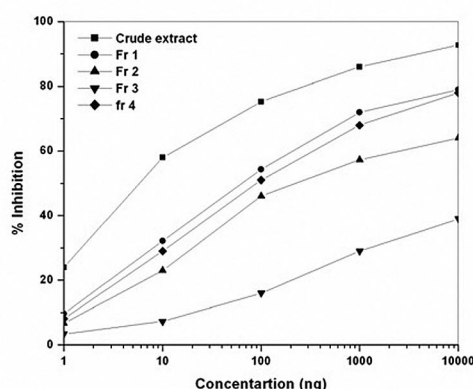


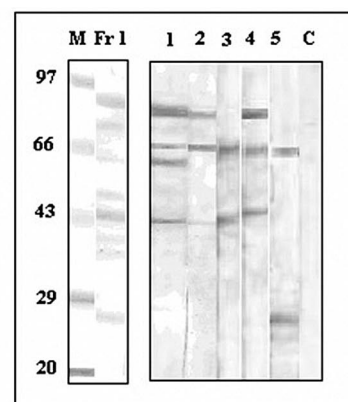
Figure 4. IgE-specific immunoblotting of *Delonix regia* pollen extract. Lane M indicates marker; lanes 1 to 25, immunoblot using *D regia*-sensitive individual patient serum samples; lane C, immunoblot using negative control.



a



b



c

Figure 5. A, Elution profile of the whole pollen extract of *Delonix regia* pollen on a diethyl amino ethyl column (Sephadex). B, IgE enzyme-linked immunosorbent assay inhibition of each eluted fraction with pooled patient serum samples using a different fraction of *D regia* pollen compared with the crude extract. C, Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of fraction A of *D regia* pollen stained in Coomassie brilliant blue. Lane M indicates marker; lanes 1 to 5, immunoblot using *D regia*-sensitive individual patient serum samples; C, control.

Other fractions failed to reach that level. Using SDS-PAGE, fraction A showed 7 prominent protein components: 28, 32, 43, 49, 66, 79, and 96 kDa (Fig 5C). On immunoblotting, fraction A showed many IgE-binding components, such as 96, 66, 43, and 28 kDa (Fig 5C, lanes 1–5 show immunoblots using *D regia*-sensitive individual patient serum samples [lane C is immunoblotting with the serum of control patients]). Other fractions exhibited insignificant IgE-binding protein components. (Figures not included.)

Fraction A was again passed through a column (Sephacryl S-200) and separated into 6 distinct parts, collected as fractions A through F (Fig 6A). Using IgE ELISA inhibition, fraction A revealed optimum IgE reactivity and 50% inhibition with 110 ng of protein (Fig 6B). This fraction exhibited the presence of 2 protein components of 96 and 66 kD using SDS-PAGE (visualized by silver staining) (Fig 6C, fraction A), among which the 66-kDa band was recognized by individual sensitive patient serum samples on immunoblotting (Fig 6C, lanes 1–5).

Cross-reactivity Studies

On ELISA inhibition of *D regia* pollen with *P pterocarpum* pollen extract (using *D regia* as the solid phase), it was observed that 50% inhibition in IgE activity was attained with 24 ng of *D regia* protein, whereas 100 ng of *P pterocarpum* protein was required for the same result (Fig 7A). For inhibition of *P pterocarpum* pollen with *D regia* pollen extract (using *P pterocarpum* as the solid phase), 75 ng of *P pterocarpum* protein was required for 50% inhibition, whereas 154 ng of *D regia* was required (Fig 7B).

Using a dot blot assay, the serum pool against each relevant pollen recognized allergenic determinants in the 2 extracts. Strong binding was observed with the homologous allergens; it was heterogeneous with the heterologous allergen extracts (Fig 8A and B).

Using immunoblot inhibition, there is clear evidence of shared IgE reactive components in the studied pollen types. In case of *D regia* pollen, bands of 66, 56, 32, 28, 25, and 23 kDa were inhibited by *P pterocarpum* pollen. In case of *P*

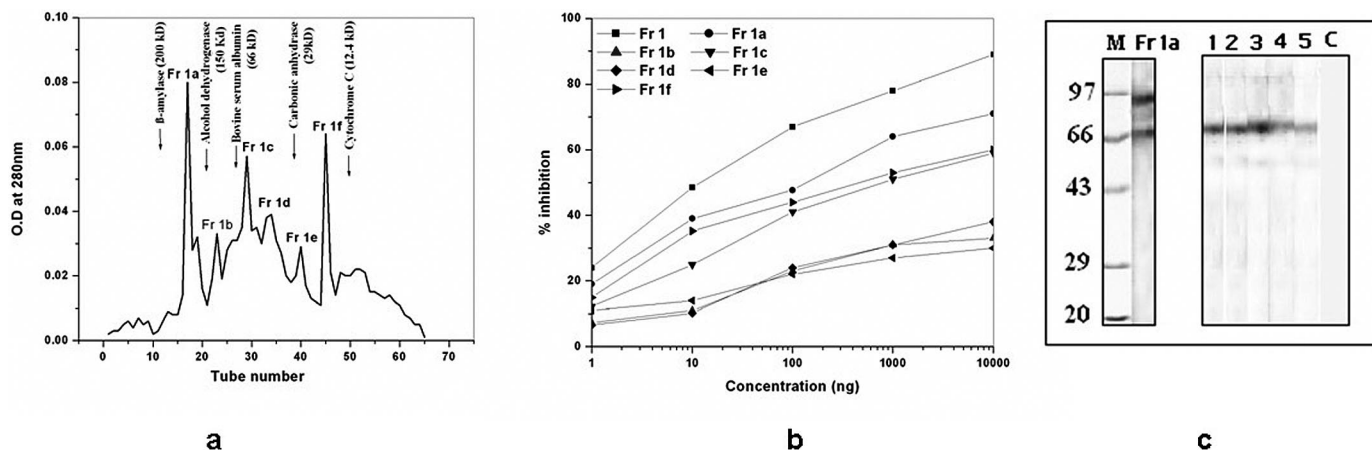


Figure 6. A, Elution profile of the whole pollen extract of *Delonix regia* pollen on a column (Sephacryl S-200) of fraction A of *Delonix regia* pollen extract. B, IgE enzyme-linked immunosorbent assay inhibition of each eluted fraction with pooled patient serum using a different fraction of *D regia* pollen compared with fraction A. C, Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) of fraction A of *D regia* pollen (silver stained). Lane M indicates marker; lanes 1 to 5, immunoblot using *D regia*-sensitive individual patient serum samples along with a control (C).

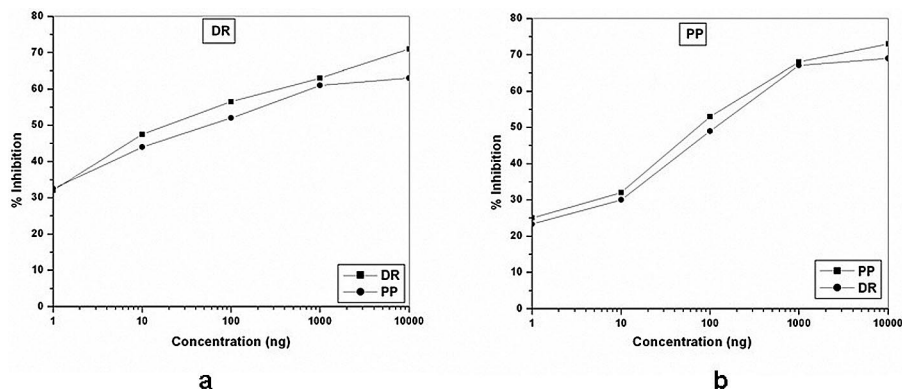


Figure 7. Enzyme-linked immunosorbent assay inhibition demonstrating allergenic cross-reactivity between *Delonix regia* (DR) and *Peltophorum pterocarpum* (PP) pollen extracts using pooled serum sensitized to both the pollen grains. The solid phase allergens are *Delonix* (A) and *Peltophorum* (B).

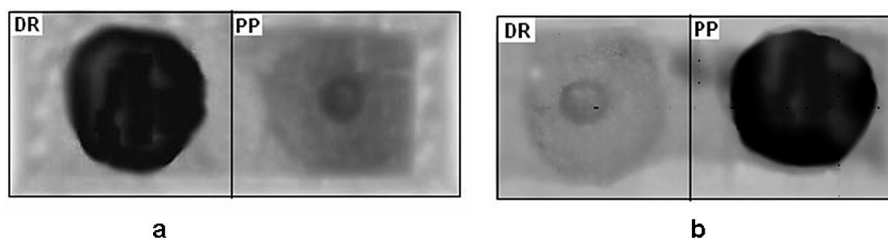


Figure 8. Detection of the allergenic relationship between *Delonix regia* and *Peltophorum pterocarpum* pollen extracts by IgE-specific dot blotting with pooled serum collected from *D regia*- and *P pterocarpum*-sensitive patients. The solid phase allergens are *Delonix* (A) and *Peltophorum* (B).

pterocarpum pollen, bands of 76, 56, 49, 28, and 23 kDa were inhibited by *D regia* pollen (Table 2 and Fig 9A and B).

DISCUSSION

Delonix regia is a common avenue tree all over India. It is very popular for decorative landscaping and commonly planted by the forest department of the Indian government.

Although the pollination of *D regia* pollen is mediated by both air and insect, it releases a considerable number of pollen grains during the flowering season (April-July), which has been established from different zones of India.^{14,15} It has already been reported to be allergenic by SPT and IgE-specific ELISA, and its allergenic components were found by immunoblotting. *Delonix regia* pollen

Table 2. Results of Immunoblotting and Immunoblotting Inhibition Analyses for the 2 Relevant Avenue Tree Pollen Extracts Indicating Shared IgE-Binding Components

Pollen type	No. of protein bands using SDS-PAGE	No. of IgE-reactive components (molecular weight)	Molecular weight of IgE-reactive components inhibited by pollen extracts
DR	23–25	8 (96, 66, 56, 43, 32, 28, 25, and 23 kDa)	66, 56, 32, 28, 25, and 23 kDa (PP)
PP	17–22	9 (76, 63, 56, 49, 40, 32, 28, 25, and 23 kDa)	76, 56, 49, 28, and 23 kDa (DR)

Abbreviations: DR, *Delonix regia*; PAGE, polyacrylamide gel electrophoresis; PP, *Peltophorum pterocarpum*; SDS, sodium dodecyl sulfate.

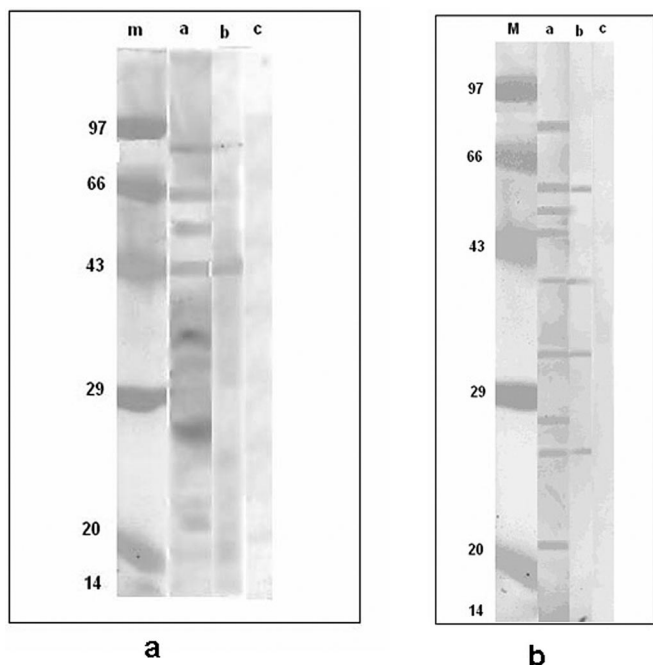


Figure 9. A, Immunoblot inhibition of *Peltophorum* pollen extract with *Delonix*-sensitive patients (a, uninhibited IgE-reactive components; b, inhibition with *Delonix* pollen extract; c, self-inhibition) B, Immunoblot inhibition of *Delonix* pollen extract with *Peltophorum*-sensitive patients (a, uninhibited IgE-reactive components; b, inhibition with *Peltophorum* pollen extract; c, self-inhibition).

has regularly been used in different allergy diagnostic clinics in crude form. To our knowledge, this study was the first attempt to isolate, characterize, and partially purify the important IgE-binding components present in the *D regia*-CPE, after conducting a basic survey on 2,234 adult patients with respiratory allergies in Calcutta. Along with clinicoimmunochemical analyses of *D regia* pollen, its cross-reactivity has been studied with another important avenue tree (*P pterocarpum*).

In the clinical survey on patients with respiratory allergies, *D regia* pollen showed 31.1% positive skin reactivity. In a previous report from Calcutta,¹² there was a 22% positive reaction; such an increase may be the result of the new set of patients and greater population studies, which are more than 3 times larger. Along with SPT and ELISA, in vitro analysis by a histamine release assay on 25 *D*

regia-sensitive patients demonstrated the functional properties of the *D regia* pollen extract—leading allergen effector mechanism.

Allergen extracts are composed of proteins and glycoproteins in addition to certain nonallergic components.²⁸ The pollen allergen extracts used for diagnostic purposes should contain the important IgE-reactive components present in the source material. Using SDS-PAGE, of more than 20 protein components, 7 were found to be very distinct among 9 IgE-reactive components on immunoblotting. The result reflects the authenticity of the previous study,¹² using fewer patient serum samples for the same pollen. Previous studies in India, with *Cocos nucifera*,²⁹ *Prosopis julifera*,³⁰ and *Putranjiva roxburghii*²⁸ tree pollen, showed the presence of a wide range of allergenically active proteins. Among the 7 IgE-binding components, 66 kDa was glycoprotein and IgE reactive in 52% of patient serum samples. The structural diversity of allergenic glycoproteins of plant sources is very limited, and it can be expected that they share common molecular features. Because of the presence of the same antigenic surface moieties, the glycoallergens with cross-reactive determinants often become panallergens, with shared allergenicity between different sources of allergens, such as pollen, vegetable, or food.^{31,32}

In the 2-step purification of *D regia* pollen extract, fraction A was found to be optimum IgE reactive, as confirmed by IgE ELISA inhibition; this fraction contained 2 important IgE-reactive components (96 and 66 kDa). In vitro cross-reactivity seems to have an in vivo expression because 13 (52%) of 25 patients in the study had a positive SPT result to *P pterocarpum* pollen. The partially purified fraction may be helpful for clinicodiagnostic purposes, instead of the crude extract used in clinics. Studies have demonstrated that a mixture of a few purified allergens can reproduce the allergenic complexity of natural extracts required for in vitro diagnosis of allergies.³³

Cross-reactivity is an important aspect of pollen allergy diagnosis and the formulation of allergen immunotherapy vaccines. Various researchers³⁴ have demonstrated cross-reactivities among different pollen species from Arecaceae,^{35,36} Asteraceae,³⁷ Poaceae,^{27,38} Oleaceae,³⁹ and Leguminosae.³⁰

In the present study, *D regia* pollen showed a well-demonstrated and marked cross-reaction with *P pterocarpum* pollen of the same family by IgE ELISA inhibition, dot blot, and immunoblotting inhibition. Using immunoblotting inhibition for *D regia* pollen, 6 bands were inhibited by *P*

pterocarpum pollen; in case of *P pterocarpum* pollen, 5 bands were inhibited by *D regia* pollen. In case of an isolated fraction with optimum IgE reactivity from *D regia* pollen, the 66-kDa glycoprotein has been found to be inhibited by *P pterocarpum* pollen extract. Immunotherapy with cross-reactive allergen was protective against the common allergens in addition to its original source.⁴⁰ Therefore, further analyses of this cross-reactive glycoprotein allergen can throw some more light on the prospect of allergen sensitization of both *D regia* and *P pterocarpum* pollen. In conclusion, *D regia* pollen is a potent allergen source and the isolated fraction A may be a useful component of allergen immunotherapy. Observations suggest that the cross-reactive 66-kDa glycoprotein of fraction A of *D regia* pollen has potential for further use in allergen immunotherapy of both *D regia* and *P pterocarpum* pollen.

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- Requests for reprints should be addressed to:*
Swati Gupta-Bhattacharya, PhD
Division of Palynology and Environmental Biology
Department of Botany
Bose Institute
93/1 Acharya Prafulla Chandra Rd
Calcutta 700 009, India
E-mail: swati@bosemain.boseinst.ac.in or swatigb2929@yahoo.com
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