

B. P. Singh^a
 Jyotsna Verma^a
 Susheela Sridhara^a
 Deepak Rai^a
 Neelam Makhija^a
 S. N. Gaur^b
 S. V. Gangal^a

^a Centre for Biochemical Technology,
 Delhi University Campus, and

^b Clinical Research Centre,
 V.P. Chest Institute, Delhi, India

Immunobiochemical Characterization of *Putranjiva roxburghii* Pollen Extract and Cross- Reactivity with *Ricinus communis*

Abstract

Putranjiva roxburghii (PR) pollen has been found to be an important aeroallergen for type I hypersensitivity. In the present study, the IgE binding proteins of PR pollen have been characterized and compared with pollen allergens of *Ricinus communis* (RC) belonging to family Euphorbiaceae. On isoelectric focusing, PR pollen extract resolved into 35 bands (pI 3–9), whereas SDS-PAGE separated it into 18 protein components (MW 14–100 kD). Pooled patient's sera (ID +ve to PR) recognized 12 allergenic proteins in *Putranjiva* and five of them (MWs 92, 80, 55, 43 and 30 kD) showed immunologic reactivity to most of the sera samples tested individually by immunoblot. A number of shared allergenic proteins (MWs 92, 80, 66, 50, 43 and 14 kD) were observed between PR and RC pollen extracts on immunoblot using *Putranjiva* allergic serum pool. Inhibition in the binding for most of PR pollen allergenic proteins was obtained with higher concentration of RC extract than PR itself, depicting the presence of cross-reacting allergens in both. *Putranjiva* pollen extract was fractionated by a combination of DEAE Sephadex-A 50 and Sephadex-G 200 column chromatography. Periodate deglycosylation of western blotted PR extract and *Put* I fraction indicated the involvement of carbohydrate moieties in the allergenic activity. Of the two fractions from *Put* I (Ia and Ib), *Put* Ib was found to be the most allergenic protein by ELISA inhibition. Dot blot analysis with individual patients sera identified it as a major allergen of PR.

Key Words

Characterization
 Pollen
 Extract
Putranjiva roxburghii
Ricinus communis
 Cross-reactivity

Introduction

Allergen extracts derived from pollen and spores are usually multiallergenic system with more than one IgE binding proteins responsible for type I hypersensitivity disorders [1]. Studies have shown that allergen extracts supplied by different manufacturers show a wide variation with respect to their potency [2, 3]. Hence, the need for standardized extracts for immunotherapy has been emphasized [4].

The extracts used in Europe, USA and other western countries have been mostly standardized with procedures recommended by international societies. Though a few extracts have been characterized in India, the reference patterns are not yet available to allergen suppliers [5]. Due to this, the strength of extracts for diagnosis and immunotherapy is still expressed in w/v (g/ml), PNU or by biopotency test conducted on a group of patients.

KARGER

E-Mail karger@karger.ch
 Fax +41 61 306 12 34
 http://www.karger.ch

© 1997 S. Karger AG, Basel
 1018–2438/97/1143–0251 \$12.00/0

This article is also accessible online at:
 http://BioMedNet.com/karger

Correspondence to: Dr. B. P. Singh
 Scientist E1, Centre for Biochemical Technology
 Mall Road Near Jubilee Hall, Delhi University Campus
 Delhi-110007 (India)
 Fax (011)-7257471

Putranjiva roxburghii (PR) Eng. Indian Amulet tree from family Euphorbiaceae sheds large amount of pollen in the air during March–May, sensitizing a number of atopic patients [6–11]. Besides the natural occurrence, it has been extensively planted under the afforestation programme in the Indian subcontinent. Due to this, *Putranjiva* has been recorded amongst the 10 important tree pollen allergens in ‘Pollen Calendars’ published for different states of the country [12]. *Ricinus communis* (RC) Eng. castor bean, another important member from the family Euphorbiaceae has already been characterized for its IgE binding proteins in India and other countries [13–15], but no attempt has been made to standardize the PR pollen extract. The present study was undertaken to characterize the allergenic components of PR pollen and find out cross-reacting proteins with RC pollen extract. Efforts were also made to isolate and identify the important allergenic proteins from PR pollen extract to be used for diagnosis and immunotherapy.

Materials and Methods

Preparation of Extract. Polleniferous material of PR was collected during the peak season and processed carefully to avoid any cross contamination. Pollen samples of >98% purity (<2% other floral parts of the same plant) were defatted with diethyl ether. Pollen extracts of PR were prepared in three batches under similar conditions. The extraction of antigens (1:20 w/v) was carried out in ammonium bicarbonate buffer (0.05 M, pH 7.8) containing 1 mM of phenylmethylsulphonyl-fluoride by continuous stirring of pollen material for 18 h at 4°C. After centrifugation at 10,000 rpm for 30 min, the supernatant was brought to 90% saturation with ammonium sulphate to precipitate the proteins. The pellet was separated by centrifugation, dissolved in distilled water and dialysed with 3,500 molecular weight cutoff membrane (Spectrum, USA). The extract was dispensed in different aliquots, freeze-dried and stored at –20°C. The same protocol was followed for preparation of pollen extract of RC.

Protein Estimation. Protein was quantitated in PR and CR pollen extracts by modified Lowry’s method. Phosphotungstic acid reagent was used for protein precipitation from the extracts [16].

Intradermal Tests and Collection of Sera. The PR pollen extract was subjected to skin tests on 180 patients of nasobronchial allergy (aged 18–43 years) at the Clinical Research Centre, V.P. Chest Institute, Delhi. For intradermal tests (ID), a bleb of 2–3 mm was raised by injecting PR extract (1:500 w/v) in the forearm of the patient. Simultaneously, phosphate-buffered saline (PBS) was injected as a negative control. The ID tests were graded after 20 min in comparison to negative control [17]. To assess the skin reactivity, histamine diphosphate (100 µg/ml) was also injected as a positive control. Sera were collected from 17 (9.4%) patients showing markedly positive skin reactions (2+ to 4+) to PR pollen extract. Sera were also collected from nonallergic volunteers, who showed negative ID tests to the extract. The consent of every patient was obtained prior to skin tests and collection of blood (sera). None of the patients were under immunotherapy, when the blood was collected. The sera collected were screened by enzyme-linked immunosorbent assay (ELISA) for the presence of specific IgE

[18] to PR allergens. Sera showing optical density (OD) >5 times of the normal human serum (NHS) i.e. negative control were used for various in vitro tests.

Isoelectric Focusing (IEF). IEF was carried out on readymade gel (pI 3–9) supplied by Pharmacia (Sweden). Five µg of PR pollen protein (extract) was loaded per well of the sample applicator and applied onto gel. The protein components were resolved using the Phast system and stained with silver staining kit [19].

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Electrophoresis was carried out on 10% gel using discontinuous buffer system (0.025 M Tris, 0.2 M glycine and 0.1% SDS) using the ‘midget’ apparatus supplied by LKB, Pharmacia [20]. The protein samples were boiled in sample buffer containing β-mercaptoethanol at 100°C for 5 min prior to loading on the gel. Fifty micrograms protein of PR, RC pollen extract and different fractions of PR extract were loaded separately in each lane. The separated proteins were either stained with Coomassie brilliant blue (CBB) R-250 or used for immunoblot analysis. Prestained molecular weight markers were loaded in a separate lane.

Immunoblot. The separated proteins after SDS-PAGE were transferred onto nitrocellulose paper (NCP) using Tris-glycine buffer with 20% methanol and dry blot apparatus [21]. Nonspecific sites were blocked by incubation of blotted strips with 1% BSA for 2 h at 37°C. After washing with PBS-Tween, the strips were incubated overnight at 4°C with 1:10 v/v diluted patients’ serum. As a negative control, PR and RC pollen protein blotted strips were also incubated with serum of a normal healthy person. The strips were washed with PBS-Tween and incubated with (1:500 v/v PBS-Tween) horseradish peroxidase labelled anti-human IgE (Sigma) for 3 h at 37°C. The unbound antibodies were washed off and the IgE binding components were detected by addition of freshly prepared substrate i.e. diaminobenzidine, hydrogen peroxide in sodium acetate buffer, pH 5.0. The blots were washed finally with distilled water to stop the colour development. The antigen-antibody binding was visualized as brown colour bands.

Immunoblot Inhibition. It was carried out to investigate the shared allergenic components between PR and RC pollen extracts. Protein components of PR pollen extract resolved on SDS-PAGE in separate lanes were blotted on NCP. Individual lanes were cut out and each strip was incubated overnight at 4°C with 500 µl of 1:5 (v/v PBS-Tween) diluted patients serum mixed with equal volume of either PR or RC extract, in different concentrations (250 µg, 500 µg/500 µl). As a positive control, a PR pollen proteins blotted strip was incubated with 1:10 diluted (v/v PBS-Tween) patients’ serum without the inhibitor. The strips were washed with PBS-Tween and incubated with 1:500 v/v diluted peroxidase-conjugated anti-human IgE (Sigma) for 4 h at 37°C. The colour was developed as mentioned in immunoblotting.

Periodate Oxidation. Whole pollen extract of PR and Put I fraction after SDS-PAGE were transferred on nitrocellulose membrane and treated with 0.2 mol/l sodium meta-periodate, overnight in dark at 4°C [22]. A drop of ethylene glycol was added to inactivate the periodate and the membrane was kept at room temperature for 2 min. Sodium borohydride (1 mg/ml) was added and left overnight at 4°C. The reaction was then stopped by adding one drop of acetic acid. The control was prepared by treating the membrane as described above without the addition of sodium meta-periodate. The nonspecific sites of membrane were blocked with 1% BSA and used for immunoblot.

Fractionation of Proteins Extract. An aliquot of reconstituted PR pollen extract (18 mg protein) was fractionated using DEAE Sephadex A-50 column (40×1.5 cm) equilibrated with 0.05 mol/l phosphate buffer, pH 7.3. A linear salt gradient of 0.0–0.5 mol/l sodium chloride

in phosphate buffer was used for fractionation. The column was washed finally with 1 mol/l sodium chloride in phosphate buffer. Each fraction was checked by ELISA for the allergenic reactivity using pooled patients' sera positive to PR pollen extract. All the fractions of each resolved peak were pooled, dialysed and lyophilized. The fraction found most potent was further loaded on gel filtration column (Sephadex G-200).

Enzyme-Linked Immunosorbent Assay. The IgE binding capacity of each fraction (4 ml) of PR obtained from DEAE Sephadex A-50 column was determined by direct ELISA following the protocol of Jaggi et al. [23]. A volume of 50 µl of each fraction was mixed with 50 µl of carbonate buffer and coated on ELISA plate (Dynatech). The plate was incubated overnight at 4°C. After washing with PBS containing 0.1% Tween-20, the nonspecific sites were blocked with 1% w/v BSA for 2 h at 37°C. This was followed by washing and overnight incubation with 100 µl of 1:10 diluted (v/v) PBS-Tween) pooled patients' sera at 4°C. The unbound IgE (nonspecific) was washed off by PBS-Tween and the plate was incubated with 1:1,500 diluted (v/v PBS-Tween) anti-human IgE alkaline phosphatase conjugate (Sigma) for 4 h at 37°C. The substrate *p*-nitrophenyl phosphate was added after washing and incubated at 37°C for 40 min. The colour development was stopped with 3 N NaOH and the absorbance was read at 410 nm on a NUNC (Denmark) ELISA reader.

ELISA Inhibition. To check the potency of the fractions eluted from Sephadex G-200 column, 500 ng of PR pollen protein (extract) was coated per well of the ELISA plate and incubated overnight at 4°C. After washing with PBS-Tween, the unbound sites were blocked with 1% BSA. A volume of 50 µl of 1:5 diluted (v/v in PBS-Tween) pooled patients' sera was mixed with equal volumes of buffer containing various concentrations of each inhibitor i.e. *Put* Ia, *Put* Ib and crude PR extract (10 ng to 5 µg). The mixture was then poured into antigen-coated wells and incubated for 16 h at 4°C. ELISA was carried out as mentioned earlier. As a negative control, NHS was also tested. The OD value obtained with negative control was subtracted from the IgE binding value of each sample and the percent inhibition was calculated as under:

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

Dot Blot Assay. The fraction *Put* Ib was focused in equal amount on nitrocellulose paper at 10 places with the help of a capillary. The membrane was dried at room temperature and later blocked with 1% BSA. The dots were processed with individual patients sera following the procedure of immunoblot as described above.

Results

In three batches of PR pollen extract prepared under similar conditions, protein content was found to be 12.5, 14.0 and 15.0 mg, respectively per gram dry weight of pollen. On IEF, PR pollen extract separated into 35 protein bands (pI 3–9) visible with silver staining (fig. 1), whereas SDS-PAGE resolved PR (lane 2) and RC (lane 1) pollen extracts into 18 and 14 Coomassie blue-stained protein bands, respectively, with a MW range of 14–100 kD (fig. 2a). All three batches of extracts showed nearly the same pattern/number of pro-

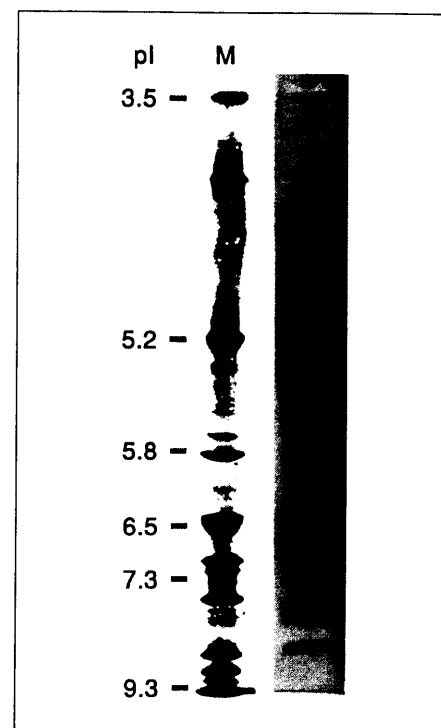
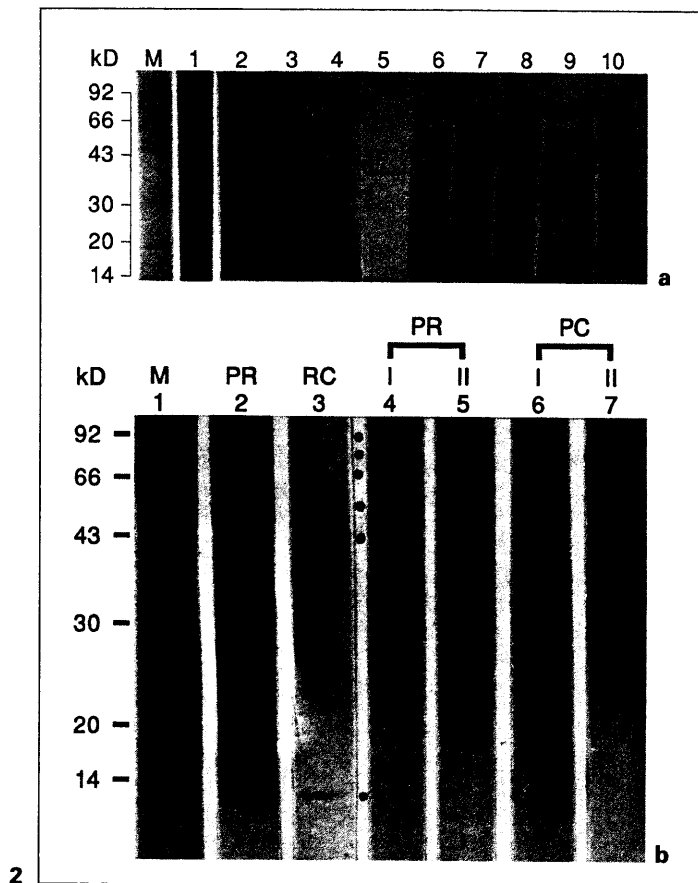


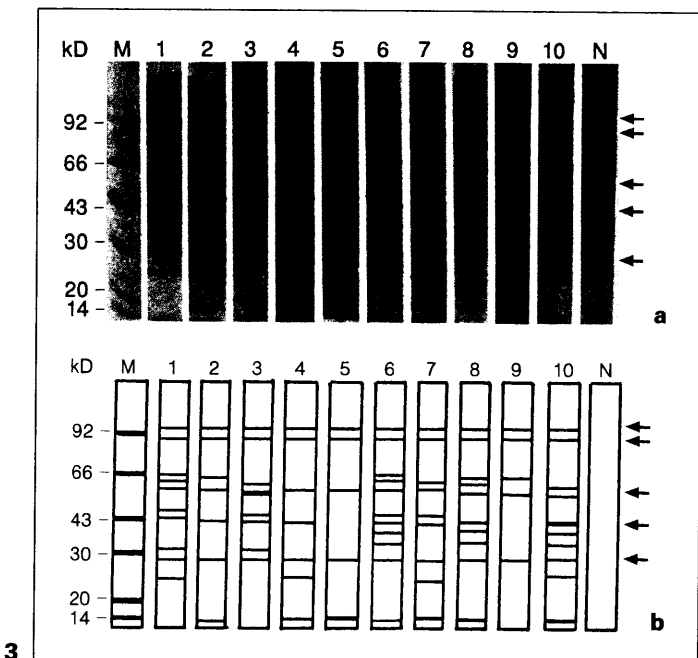
Fig. 1. IEF pattern showing silver stained bands of PR pollen extract and pI markers (M).

tein bands on IEF and SDS-PAGE. Hence, they were pooled for further characterization. The allergenicity of proteins present in PR extract was checked by immunoblotting. Of the 12 IgE bindings bands (MWs 92, 80, 66, 55, 50, 45, 43, 35, 30, 28, 20 and 14 kD) identified in *Putranjiva*, 6 allergenic proteins (MWs 92, 80, 66, 50, 43, 14 kD) were detected in RC pollen extract using pooled sera of patients sensitive to PR extract (fig. 2b). The shared allergenicity among the protein components of PR and RC pollen extracts was further confirmed by immunoblot inhibition. A high degree of inhibition in IgE binding was detected with homologous antigen (*Putranjiva*) compared to that of heterologous antigen (*Ricinus*). The IgE binding components of PR extract could be inhibited with higher concentration of RC protein (500 µg) as compared to that of *Putranjiva* (250 µg) itself (fig. 2b).

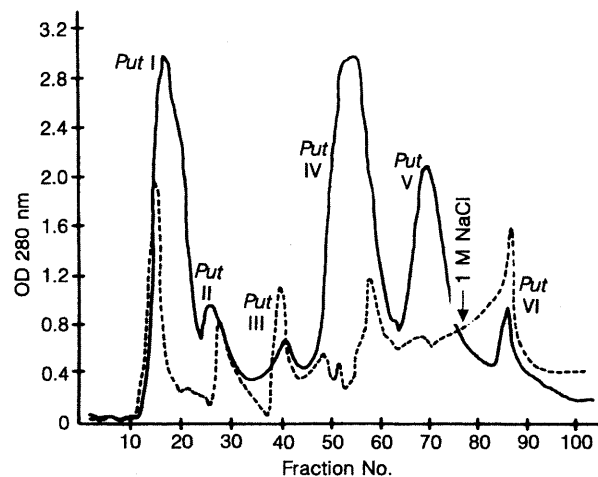
Figure 3a, b presents the IgE binding components of PR extract with individual patients' sera on immunoblot. The heterogeneity in binding patterns of the allergenic components in PR extract was observed with different sera. However, five protein components with MWs 92, 80, 55, 43 and 30 were recognized by specific IgE present in most of the patients' sera tested. Hence they were described as major allergens of PR extract.



2



3



4

Fig. 2. a SDA-PAGE pattern showing CBB-R250 stained bands; M = low molecular weight markers, 1 = RC (WPE); 2 = PR (WPE); 3 = *Put I*; 4 = *Put Ia*; 5 = *Put Ib*; 6 = *Put II*; 7 = *Put III*; 8 = *Put IV*; 9 = *Put V* and 10 = *Put VI* fractions eluted by DEAE Sephadex A-50 and Sephadex G-200 columns. **b** Immunoblot exhibiting IgE binding components in pollen extracts of PR (lane 2) and RC (lane 3) using pooled serum of patients sensitive to PR extract (● = Common allergenic proteins between both the extracts; M = molecular weight markers (lane 1). Immunoblot inhibition of PR pollen extract by PR (lanes 4–5) and RC (lanes 6–7) using allergic serum pool from PR-sensitive patients. In each set of inhibition two different concentrations – 250 (I) and 500 µg (II) – of antigens (PR and RC) were used as inhibitors. Inhibition caused by each extract in IgE binding bands of PR extract was compared with positive control i.e. PR extract without inhibitor (lane 2).

Fig. 3. a IgE binding protein components of PR with individual patients' sera (1–10) on immunoblot. Major allergenic proteins are shown as (→); N = normal human serum (negative control) and M = molecular weight markers. **b** Line diagram showing IgE binding proteins of PR extract on immunoblot.

Fig. 4. Elution profile (—) of *Putranjiva* pollen extract on DEAE-Sephadex A-50 column. Column size 40×1.5 cm; eluent, 0.5 M phosphate buffer (pH 7.3), gradient, 0.0–0.5 M NaCl followed by 1 M NaCl, Flow rate, 18 ml/h, sensitivity 0.5 AU; chart speed 0.2 mm/min and fraction size 4 ml. (---) IgE binding capacity of each eluted fraction as determined by ELISA at 405 nm.

Elution profile of PR pollen extract exhibited 6 peaks, viz. *Put I*–*Put VI* on DEAE Sephadex A-50 column (fig. 4). The first four peaks were eluted using salt gradient of 0.0–0.5 M sodium chloride, whereas the remaining two peaks with 1 M sodium chloride. Of the six peaks, *Put I* was found to be the most potent by IgE-specific ELISA using pooled sera of PR-sensitive patients. Further fractionation of *Put I* by gel filtration (fig. 5a) resulted in two peaks viz. *Put Ia* and *Put Ib*. All the eluted fractions of PR extract from

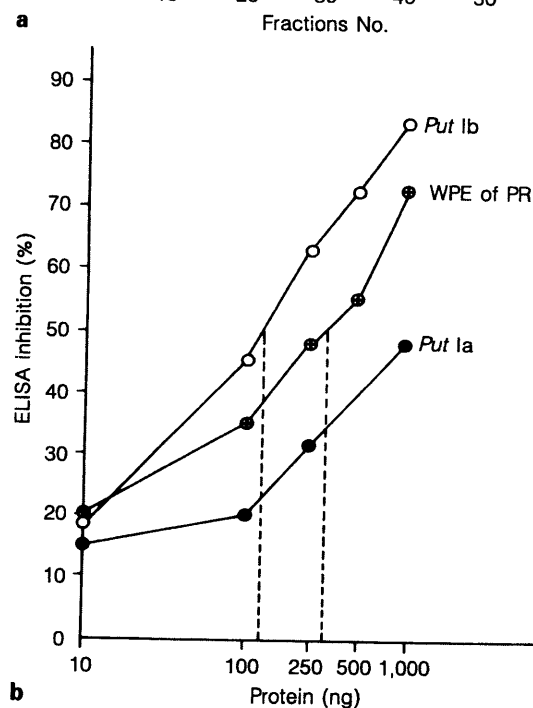
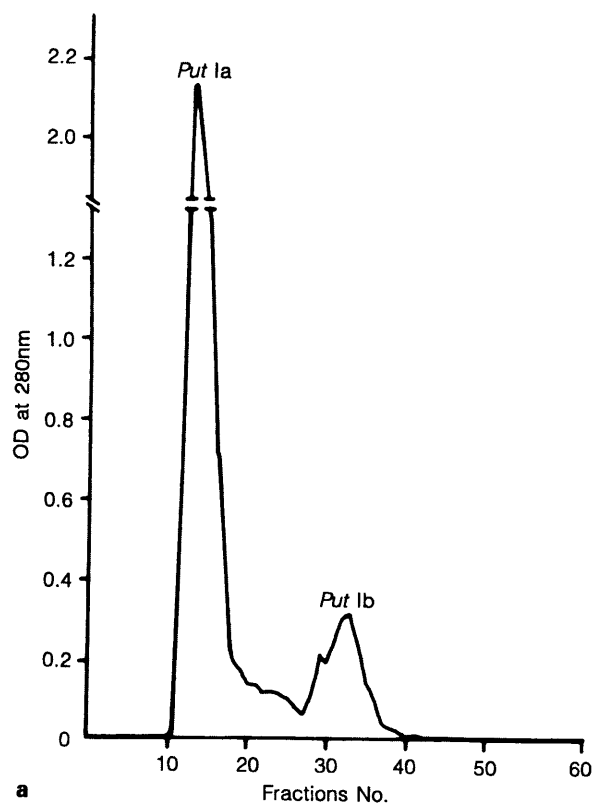


Fig. 5. a Elution profile of *Put I* fraction of *Putranjiva* on Sephadex G-200. Column size = 15 × 0.5 cm, chart speed = 0.2 mm/min, eluent 0.05 M phosphate buffer (pH 7.3), flow rate 12 ml/h, sensitivity = 0.2 and fraction size 3 ml. **b** ELISA inhibition - dose response curves of sub-fractions of *Put I* purified on Sephadex G-200, whole pollen PR extract, *Put Ia* and *Put Ib*.

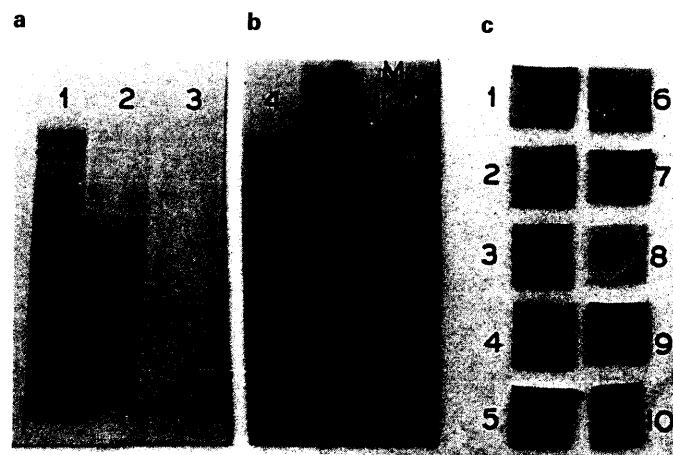


Fig. 6. a Immunoblot exhibiting IgE binding proteins in fractions 1 = *Put I*; 2 = *Put Ia*; 3 = *Put Ib* of PR extracts. **b** Immunoblot showing the effect of periodate on IgE binding capacity of blotted proteins on NCP. 4 = *Put I* fraction and 5 = WPE of PR. **c** Dot blot assay of *Put Ib* with individual patients' sera (1-9). 10 = Negative control (NHS).

DAEA-Sephadex A50 and gel filtration chromatography resolved in a MW range of 14-100 kD on SDS-PAGE (fig. 2a). *Put I* fraction of PR extract exhibited a greater number of bands than the other fractions. The subfractions *Put Ia* and *Put Ib* were not homogeneous on SDS-PAGE. *Put Ia* revealed a number of bands while *Put Ib* showed 2 bands at MW 40 and 30 kD. On immunoblot, *Put I* fraction of PR extract showed more allergenic bands (fig. 6a, lane 1) while other fractions (*Put II*-*Put VI*) exhibited few IgE binding bands (photograph not included). The subfraction *Put Ia* (lane 2) demonstrated more allergenic bands while subfraction *Put Ib* (lane 3) contained only one allergenic band at approximately 30 kD (fig. 6a). Of these, *Put Ib* was found to be more allergenic than *Put Ia* by ELISA inhibition as *Put Ib* required 160 ng of protein for 50% inhibition of specific IgE binding, compared to > 1,000 ng and 300 ng of *Put Ia* and WPE, respectively to achieve the same degree of inhibition (fig. 5b).

Periodate deglycosylation experiment was also performed with the western blotted WPE of PR and the fraction *Put I* to study the involvement of carbohydrate in constituting the IgE epitopes (fig. 6b). Some IgE-binding bands were missing while some showed low IgE binding in comparison to blot performed without periodate treatment (fig. 2b, lane 2). Dot blot analysis with individual patients sera showed that *Put Ib* was a major allergen as all the 9 patients sera showed specific IgE to this allergen (fig. 6c).

Discussion

Allergen extracts are composed of proteins and glycoproteins, besides certain nonallergenic components. The extracts for diagnosis and immunotherapy should contain all the important antigens/allergens present in the source material. In vitro methods like thin layer isoelectric focusing, SDS-PAGE, RIE, CIE, Immunoblot and RAST/ELISA have been recommended for standardization of allergen extracts [3, 21]. In the present study, all three batches of PR pollen extract gave reproducible patterns on IEF (35 bands, pI 3–9) and SDS-PAGE (18 bands, MWs 14–100 kD). Of the 12 allergenic proteins detected on immunoblot with pooled patients' sera, 5 protein components with MWs 92, 80, 55, 43 and 30 kD were recognized in PR extract by most of the patients sera tested (fig. 3a, b). Previous studies in India with *Cocos nucifera* (MWs 10–200 kD), *Holoptelea integrifolia* (MWs 50–70 kD) and *Prosopis juliflora* (MWs 10–20 kD) tree pollen have shown the presence of wide range of allergenically active proteins [23–25]. In case of weeds viz. *Ageratum conyzoides*, *Artemisia scoparia* and *Ricinus communis* pollen, glycoproteins of MWs 10–70 kD were found to be important IgE-binding components [15, 26, 27].

Various workers have demonstrated the cross-reactivity among different pollen species [28, 29]. In the present study, shared allergenicity between the number of components of PR and RC extracts was studied by IgE specific immunoblot. The protein components of MWs 92, 80, 66, 50, 43 and 14 kD were observed in both the extracts by PR-sensitive pooled patients sera indicating the shared allergens among them. The shared allergenicity between PR and RC pollen components has been further confirmed by immunoblot inhibition (fig. 2a). RC extract could inhibit the binding of these 6 allergenic components of PR extract. However other bands of PR extract were also inhibited by the higher concentration of RC pollen extract which were not present in RC extract itself. This may be due to the presence of common allergenic epitopes in them. Cross-reacting allergenic proteins have also been reported among other phylogenetically related pollen species [30, 31].

An attempt has been made to isolate the major allergenic proteins from PR pollen by the combination of ion exchange and gel filtration chromatography. *Put I* obtained from DEAE Sephadex A-50 was found to be more potent than the other fractions in IgE-specific ELISA and immunoblot. Further fractionation of *Put I* on Sephadex G-200 resulted in two peaks i.e. *Put Ia* and *Put Ib*. They were not homogeneous on SDS-PAGE. On immunoblot, *Put Ia* exhibited a number of IgE binding bands while *Put Ib* gave a

30-kD allergenic band (fig. 6a). *Put Ib* was observed as the most potent/allergenic protein since it required 160 ng of protein for 50% ELISA inhibition compared to crude extract (300 ng) for the same degree of inhibition (fig. 5b). In dot blot assay, *Put Ib* was recognized by most of the patients sera, hence, it can be designated as a major allergen of PR extract (fig. 6c).

After periodate oxidation, decrease in IgE-binding capacity in the number of components of PR extract and in the most potent fraction *Put I* was observed. This may be due to the periodate treatment causing destruction of carbohydrate moieties thereby changing the protein conformation, thus indirectly reducing IgE binding capacity of protein bands (fig. 6b). Similar results were obtained by other workers [22, 32].

In conclusion, the important allergenic proteins of PR extract have been identified. A significant allergenic cross reactivity was also observed between PR and RC pollen extracts. The present findings will be useful in comparing future batches of PR pollen preparations to maintain the quality of the extracts used for immunotherapy.

Acknowledgments

The authors are thankful to the Department of Biotechnology, India for financial assistance to this project. We also extend our thanks to Sh. D.D. Sharma, Sh. Indu Bhusan and Sh. R. Prasad for help in various ways.

References

- Suphioglu C, Singh MB, Simpson RJ, Ward LD, Knox RB: Identification of canary grass (*Phalaris aquatica*) pollen allergens by immunoblotting: IgE and IgG antibody binding studies. *Allergy* 1993;48:273–281.
- Agarwal MK, Jones BS, Yunginger JW: Immunochemical and physiochemical characterization of commercial *Alternaria* extracts: A model for standardization of mold allergen extracts. *J Allergy Clin Immunol* 1982;70:432–436.
- Ingermann L, Formgren H, Lowenstein H, Ipsen H: The use of a reference allergenic extract in the evaluation of allergen products. *Allergy* 1985;40:273–281.
- Backman A, Belin L, Dreborg S, Halvorsen R, Malling HJ, Weeke B: Standardization of allergenic preparations. Comments with reference to the second edition of the common nordic guidelines for registration of allergenic preparations. *Allergy* 1991;46:81–84.
- Gangal SV, Jaggi KS, Sridhara S: Standardization of allergens in India. *Arbeiten aus dem Paul-Ehrlich-Institut*. Stuttgart, Fischer, 1991.
- Vishnu Mittre, Khandelwal A: Airborne pollen grains and fungal spores at Lucknow during 1969–1970. *Palaeobotanist* 1975;22(3):177–185.
- Gaur RD: Aeropalynology of Meerut I-pollen grains. *J Indian Bot Soc* 1978;57:353–365.
- Acharya PJ: Skin test response to some inhalant allergens in patients of nasobronchial allergy from Andhra Pradesh. *Aspects Allergy Appl Immunol* 1980;13:14–18.
- Shivpuri DN: Clinically important pollen, fungal and insect allergens for nasobronchial allergy patients in India. *Aspects Allergy Applied Immunol* 1980;13:19–23.
- Nautiyal DD, Midha M: Aerobiology of Allahabad 1973–1979; in Pant DD (ed): *Phytomograph*. Allahabad, The Society of Plant Taxonomists, 1984, vol 2, pp 1–100.
- Singh AB: Airborne pollen types of allergenic significance in India. *Adv Aerobiol (Experientia)* 1987;51:61–64.
- Singh BP, Singh AB, Gangal SV: Pollen Calendars of different States, India. Delhi, CSIR Centre For Biochemicals, 1992.
- Layton LL, Yamanaka E, Lee S, Green TW: Multiple allergies to the pollen and seed antigens of *Ricinus communis* (castor bean). *J Allergy* 1962;33:232–235.
- Thorpe SC, Kemeny DM, Panzani R, Lessof MH: Allergy to castor bean I. Its relationship to sensitization to common inhalant allergens (atopy). *J Allergy Clin. Immunol* 1988;82:62–66.
- Singh AB, Malik P, Parkash D, Gangal SV: Identification of IgE binding proteins in castor bean (*Ricinus communis*) pollen obtained from different source materials. *Grana* 1993;31:376–380.
- Singh BP, Sridhara S, Arora N, Gangal SV: Evaluation of protein assay methods for pollen and fungal spore extracts. *Biochem Int* 1992; 27:474–484.
- Shivpuri DN, Agarwal MK: Studies on the allergenic fungal spores of Delhi, India, metropolitan area – clinical aspects. *J Allergy* 1969; 44:204–213.
- Voller A, Bidwell D, Barlett A: Enzyme linked immunosorbant assay; in Rose NR, Friedman H (eds): *Manual of Clinical Immunology*. Washington, American Society for Microbiology, 1980.
- Anonymous: Phast System Owner's Manual. Uppsala, Pharmacia AB, 1986.
- Laemmli UK: Cleavage of structural proteins during assembly of the head of the bacteriophage T4. *Nature* 1970;227:680–685.
- Towbin H, Gordon J: Immunoblotting and dot immunobinding: Current status and outlook. *J Immunol Methods* 1984;72:313–340.
- Verma J, Pasha S, Gangal SV: Purification and characterization of Fus sl_{3596} a 65 kDa allergen of *Fusarium solani*. *Mol Cell Biochem* 1994; 131:157–166.
- Jaggi KS, Arora N, Niphadkar PV, Gangal SV: Immunochemical characterization of *Cocos nucifera* pollen. *J Allergy Clin. Immunol* 1989; 84:378–385.
- Malik P, Singh AB, Gangal SV, Babu CR: Comparison of antigenic and allergenic components of *Holoptelea integrifolia* pollen collected from different source materials. *Allergy* 1991;46: 284–291.
- Sridhara S: Purification and standardization of allergens; PhD thesis, University of Delhi, 1985.
- Jaggi KS, Gangal SV: Isolation and identification of allergens and characterization of *Ageratum conyzoides* whole pollen extract. *Indian J Allergy Appl Immunol* 1987;1(1):39–48.
- Jaggi KS, Gangal SV: Isolation and identification of pollen allergens of *Artemisia scoparia*. *J Allergy Clin Immunol* 1987;80:562–572.
- Aalberse RC: Clinically significant cross reactivities among allergens. *Int Arch Allergy Immunol* 1992;99:261–264.
- Bousquet J, Hewitt B, Guerin B, Lims S, Michel FB: Allergy in the Mediterranean area. III. Cross reactivity among Oleaceae pollens. *Clin Allergy* 1985;15:439–448.
- Singh MB, Knox RB: Grass pollen allergens. Antigenic relationship detected using monoclonal antibodies and dot blotting immunoassay. *Int Archs Allergy Appl Immunol* 1985;78:300–304.
- Fernandez C, Martin-Esteban M, Fiandor A, Pascual C, Serrano CL, Alzamora FM, Jose M, Pena D, Ojeda Casas JA: Analysis of cross reactivity between sunflower pollen and other pollens of the Compositae family. *J Allergy Clin Immunol* 1993;92:660–667.
- Howlett BJ, Clark AE: Role of carbohydrate as an antigenic determinant of a glycoprotein from rye grass (*Lolium perenne*) pollen. *Biochem J* 1981;197:707–714.