

Fractionation and Analysis of Allergenicity of Allergens from *Prosopis juliflora* Pollen

Indu Shekhar Thakur

Department of Environmental Sciences, College of Basic Sciences and Humanities, G.B. Pant University of Agriculture and Technology, Pantnagar, India

Abstract. *Prosopis juliflora* pollen allergen extract was prepared, and its crude allergen extract was fractionated by Sephadex G-100 gel filtration. Six different fractions were obtained which was confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Protein and carbohydrate content of each fraction were estimated. Fraction E (MW 20,000) showed a 25% carbohydrate concentration. The amino acid analysis indicated that this fraction was rich in glutamic acid and alanine. Antigenicity or allergenicity of fractionated allergens were checked by gel diffusion test, rocket immunoelectrophoresis, skin prick test, and radioallergosorbent test. All these tests indicated that fraction E consisted mainly of allergenic molecules (MW 20,000) of *P. juliflora* pollen.

Introduction

Prosopis juliflora belongs to the Leguminosae family and the subfamily Mimosoideae. It is a native of the arid region of Mexico, Central America, Africa, the drier parts of India, Australia, and tropical Asia [1]. *P. juliflora* plantation is under active consideration of the Government of India for wood and waste energy sources and reclaiming of desert land. It is introduced on the ridge of Delhi and New Delhi by the Horticultural Department. Its pollen grain causes allergic diseases. *P. juliflora* flowering occurs all year round and reaches its peak in April and September. Pollination causes air pollution and insidious effects on health. *P. juliflora* pollen allergen crude extract has been used for the diagnosis of allergic diseases, provoking a significant positive reaction [2]. During the past few years a great number of investigations have been reported in order to isolate and characterize pollen allergens for the hyposensitization of human pollinosis [3, 4]. Therefore, it is imperative to purify the most active allergenic molecules from *P. juliflora* pollen allergens for the hyposensitization of human pollinosis. We have reported the partial purification of *P. juliflora* pollen allergens and tested its most allergenic molecules [5-7]. In this study, we have further

purified and characterized the most allergenic and antigenic molecules from *P. juliflora* to extend the search for a more adequate isolation of major allergens, to compare these results with those of earlier isolation techniques, and to further assess its significance in the allergenic activity.

Materials and Methods

Antigen Sources

Pollen of *P. juliflora* was collected during initial flowering. The inflorescence was kept in cold acetone, and grains were harvested by rapid decantation from the pollen-rich supernatant. The pollen grains were allowed to settle, and then the acetone was decanted. Finally, the grains were air-dried at 37°C. The purity was checked by using a compound microscope and found to be 99.4%.

Allergen Extract

P. juliflora pollen allergen extract (1:10 w/v) was prepared in 0.15 M phosphate-buffered saline (pH 7.2). The suspension was slowly shaken in a glass flask for 24 h at 4°C. Insoluble pollen fragments were finally removed by centrifugation at 15,000 rpm for 30 min. The supernatant was sterilized by using 0.45-µm filters. Protein and carbohydrate contents of crude extract were estimated [8, 9].

Antisera

Three rabbits were used for immunization with pollen allergen, as described by Freund and McDermott [10]. Equal parts of al-

lergen (protein content 2 mg/ml) and Freund's complete adjuvant (Difco) were given at 15-day and the last two doses at 7-day intervals. Blood was collected from the marginal vein of the ear, and serum was separated.

Gel Filtration

Gel filtration was performed at 4°C on a column (2.5 × 90 cm) of Sephadex G-100 (Sigma), as described previously [6]. Different fractions of gel filtration were pooled, concentrated against polyethylene glycol (MW 6,000) in a dialysis bag, and lyophilized.

Amino Acid Analysis

Amino acid analysis was performed as described by Moore and Stein [11] using a Technicon sequential multisample amino acid analyzer.

Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [12] using 10.0% separating gel (pH 8.9). The gel was stained with 0.1% Coomassie brilliant blue R and scanned by using a densitometer.

Gel Diffusion

A gel diffusion test of crude allergen extract was performed as described by Ouchterlony [13]. Agarose gel (1%; Sigma) was prepared in phosphate-buffered saline (0.1 M, pH 7.5) and layered on microscopic slides. Antigen (0.01 ml; 2 mg/ml protein content of allergen) was added to the outer wells, and 0.01 ml of antibody was added to the central well. The precipitin bands were observed after 48 h against 0.1 M phosphate buffer (pH 7.5) as control added to two of the outer wells.

Immunoelectrophoresis

Rocket immunoelectrophoresis of different fractions of crude extract (0.01 ml) obtained from gel filtration was performed as described by Axelson and Bock [14]. Agarose (1%) was used containing 0.05 M barbital buffer (pH 8.2). The gel was stained with 0.1% Coomassie brilliant blue R.

Skin Test

A skin prick test of different fractions of gel filtrate was also performed in 5 individuals sensitized to *P. juliflora* pollen by injecting 0.02 ml of each fraction of gel filtrate. The diameter of wheal was measured against control (0.02 ml of 0.1 M physiological saline) [15].

Radioallergosorbent Test (RAST)

Allergens were bound to paper discs according to the method of Ceska et al. [16]. For the experiments involving the inhibition of the RAST, 0.05 ml of pooled allergenic sera, obtained from 3 allergic subjects who displayed intense skin reactivity to *P. juliflora* pollen extract, was mixed with different amounts of each pollen fraction and incubated for 3 h at room temperature and then added to the allergosorbent discs as described by Chakrabarty et al. [17]. The allergenic potency was evaluated in terms of the ability of fractionated allergens to neutralize IgE antibodies present in human allergic sera by addition of ¹²⁵I-labelled rabbit antibodies specific for human IgE immunoglobulin (Pharmacia, Uppsala, Sweden). The levels of ¹²⁵I bound to the discs were measured with a Beckman

Table 1. Amino acid composition of fraction E of *P. juliflora* pollen obtained by gel filtration on Sephadex G-100

Amino acid	Residues/1,000 residues
Aspartic acid	92
Lysine	75
Histidine	14
Threonine	84
Arginine	38
Serine	40
Glutamic acid	115
Proline	58
Glycine	93
Alanine	114
Cystine	12
Valine	76
Methionine	14
Isoleucine	49
Leucine	48
Tyrosine	30
Phenylalanine	48

gamma counter. The capacity (expressed as %) of each fraction to inhibit the RAST was calculated according to the formula

$$\frac{A-B}{A} \times 100$$

where A represents the RAST value (cycle per minute) in the absence of *P. juliflora* pollen fractions and B the RAST value (cycles/min) in the presence of *P. juliflora* pollen fractions.

Results

Fractionation of Crude Pollen Allergen Extract

Crude *P. juliflora* pollen allergen extract was fractionated by gel filtration on Sephadex G-100. The molecular size of material eluted in the different fractions was calibrated with protein of known molecular weight. The elution profile obtained by continuous monitoring at 280 nm indicated that crude allergen extract contained components with MW 81,000, 55,000, 41,000, 27,500, 20,000, and 13,000. The results indicate that fraction E (MW 20,000) contained 25% carbohydrate, whereas the other fractions had no carbohydrate. The amino acids of fraction E were determined, and the results are shown in table 1. It was found that glutamic acid and alanine levels were higher in comparison to the other amino acids.

Crude *P. juliflora* pollen extract was subjected to

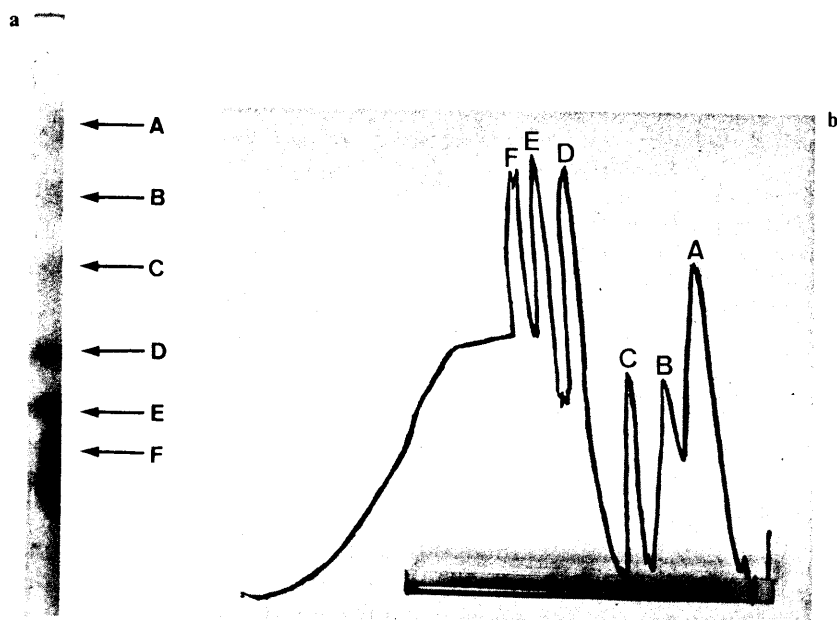


Fig. 1. SDS-PAGE of 100 µg of *P. juliflora* pollen extract. The separating gel was acrylamide (10%, pH 8.9). The gel was stained with 0.1% Coomassie brilliant blue R (a) and scanned by using a densitometer (b). Fractions are represented by letters A-F.

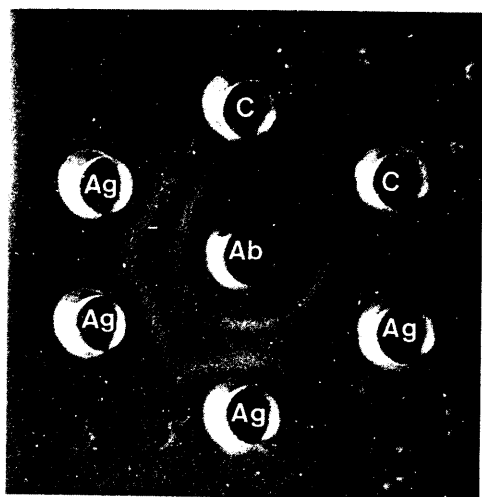


Fig. 2. Immunodiffusion analysis of crude *P. juliflora* pollen allergen extract (Ag) against *P. juliflora* antiserum (Ab) obtained from rabbits. Phosphate-buffered saline (0.1 M) was taken as control (C).

SDS-PAGE. After staining with 0.1% Coomassie brilliant blue R, six bands were seen (fig. 1). The gel was scanned using a densitometer, and six absorption peaks were observed, as shown in figure 1.

Analysis of the Allergenicity of Pollen Allergens

The gel diffusion test of Ouchterlony of crude *P. juliflora* pollen allergen was performed against rabbit

P. juliflora antiserum. As can be seen from figure 2, this step revealed at least two predominant and one thin immunoprecipitin bands after immunodiffusion in agarose.

Gel filtration fractions A-F were subjected to rocket immunoelectrophoresis. After reacting with polyspecific rabbit *P. juliflora* antiserum, the electro-separated gel filtration fraction formed an immunoprecipitin band. It was observed that fraction E formed a thick, broad-based immunoprecipitin band staining with 0.1% Coomassie brilliant blue R. Fraction C, D, and F immunoprecipitin bands were thin and deficient, as shown in figure 3, and fractions A and B (not shown) did not form precipitin bands with antiserum on rocket immunoelectrophoresis.

Skin prick tests were performed with Sephadex G-100 fractions and *P. juliflora* pollen extract in 5 pollen-sensitized subjects and in nonatopic controls. Pooled peak E fractions showed a 3.5-fold increase in wheal diameter with respect to controls, whereas peak C and D fractions showed a 2-fold increase. Peak A, B, and F fraction responses were almost equal to that of controls.

The allergenic activity of fractionated allergens on Sephadex G-100 of crude *P. juliflora* allergen extract was tested by applying the RAST (fig. 4). The allergenicity of fractionated and crude allergens was analyzed on the basis of the inhibitory capacities of puri-

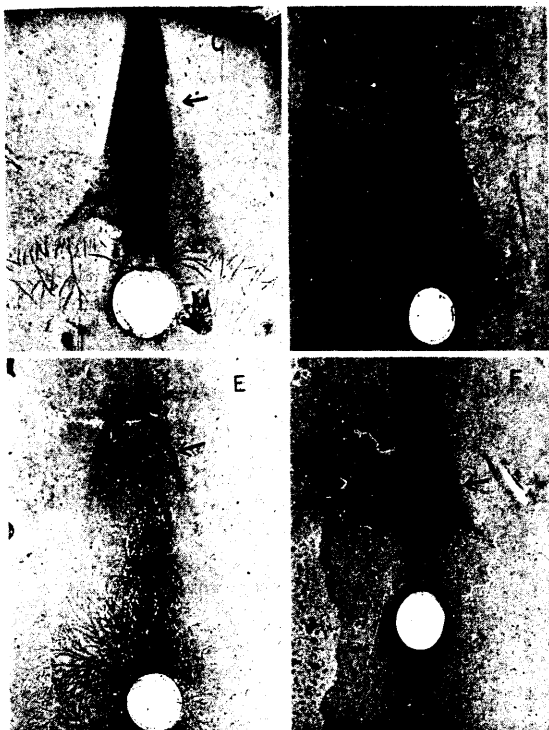


Fig. 3. Rocket immunoelectrophoresis of gel filtration fractions C-F of *P. juliflora* pollen extract obtained on Sephadex G-100 against rabbit *P. juliflora* antiserum.

fied allergens after incubating with human serum obtained from individuals sensitized with *P. juliflora* pollen grains. It was observed that fraction E of the gel filtrate was most effective to neutralize *P. juliflora* antiserum, followed by fractions C, D, B, F, and A. Further, it was observed that increasing amounts of *P. juliflora* fractionated pollen allergens resulted in increasing RAST inhibition. It has been shown that 50% inhibition was obtained after incubation of more than 0.1 μ g of purified fraction E of *P. juliflora* pollen allergen with human antiserum.

Discussion

In previous studies [6, 7], we were able to demonstrate the partial fractionation of *P. juliflora* pollen allergens and tested its allergenicity. In the present

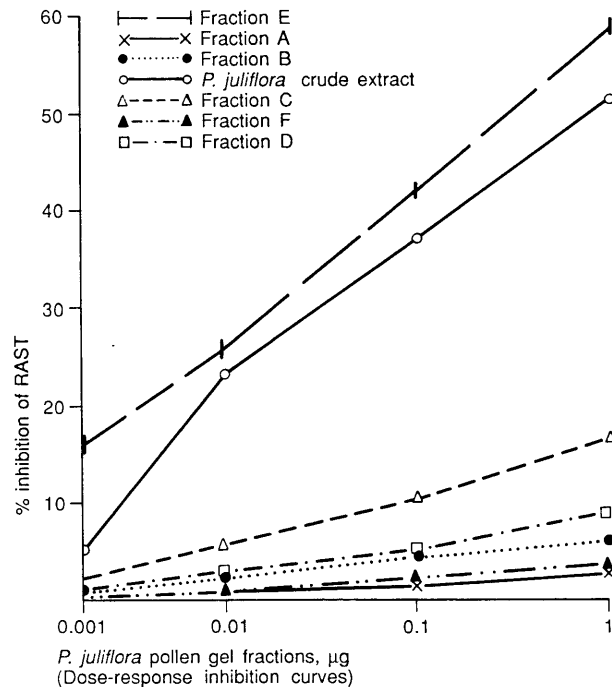


Fig. 4. RAST inhibition assay of crude allergen extract and different fractions of *P. juliflora* pollen obtained on Sephadex G-100. The results are expressed as percent inhibition of binding of specific IgE to solid-phase allergens achieved by addition of different amounts of fractionated allergens and human *P. juliflora* antiserum. Each value is an average of five experiments.

study, we have fractionated the crude allergen extract in order to obtain more data for the standardization of *P. juliflora* pollen allergenic molecules for desensitization of human pollinosis and to extend the search for a more adequate isolation of major allergens. The results indicate that crude allergen extract contained one glycoprotein and five proteins with MW 81,000–13,000. Crude allergen extract was electroseparated in six different bands on SDS-PAGE. This banding was further confirmed by densitometer gel scanning, showing the homogeneous nature of fractionated allergens. The significant finding emerging from this study was fraction E of MW 20,000 with a carbohydrate content of 25%. This suggests that fraction E is a glycoprotein, whereas the other fractions are proteins. Thus, our results are similar to those of our earlier report [6] using periodic acid-Schiff staining of purified allergens on electrophoresis and com-

parable to the results of Belin [18] investigating birch pollen and Underdown and Goodfriend [19] investigating short ragweed extract. The amino acid composition obtained in this study indicates that the glutamic acid and alanine contents were high in the glycoprotein fraction. Thus, our results are comparable to those of Vik et al. [20] observing higher glutamic acid and glycine contents in birch pollen extract and similar to those of Shafiee et al. [21] investigating Russian thistle pollen and those of Haavik et al. [22] investigating timothy pollen.

In the present work, testing of the antigenicity of crude *P. juliflora* pollen extract by using the gel diffusion test showed a multiprecipitin band. This could be due to the heterogeneous nature of allergen having two prominent antigen molecules. Our rocket immunoelectrophoresis data demonstrate maximum cross-reactivity between fraction E, glycoprotein, and rabbit *P. juliflora* antiserum, resulting in broad and thick precipitin bands. Data of this investigation suggest antigenicity and fair grade of homogeneity of glycoprotein fraction E of *P. juliflora* pollen allergens. These observations were further substantiated by skin prick test and RAST.

In the RAST inhibition experiment, the purified and crude allergen exhibited a fair grade of inhibitory capacity after incubation with human serum obtained from *P. juliflora* sensitized individuals. It is important to note that allergosorbent discs prepared with crude allergen extract achieved maximum inhibition of the binding of human IgE antibodies by fraction E of glycoprotein. RAST inhibition curves of fraction E of *P. juliflora* and its crude allergens showed maximum inhibition, and two curves were almost parallel, indicating qualitative allergenic identity. The crude allergen extract showed less inhibitory capacity in comparison to glycoprotein on RAST because of a competition between the allergenic and nonallergenic components for the active group during the coupling procedure. Thus, our investigations demonstrate the homogeneity and allergenicity of the glycoprotein fraction of *P. juliflora* pollen allergens. These results are similar to those of our earlier reports [6, 7] and comparable to those of Puttonen et al. [23] investigating timothy and Ekramoddoullah et al. [24] investigating Kentucky pollen allergens. Further investigations of monosaccharide composition and mode of linkage in the carbohydrate part of the molecule, to study the allergenic properties of *P. juliflora* pollen, are under way.

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Correspondence to: Dr. Indu Shekhar Thakur
Department of Environmental Sciences
College of Basic Sciences and Humanities
G.B. Pant University of Agriculture and Technology
Pantnagar (Nainital) UP-263145 (India)