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Identification of the allergenic proteins of *Ipomoea fistulosa* pollen: Partial characterization and sensitivity test

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Total soluble proteins were extracted from the pollen of *Ipomoea fistulosa* and subjected to ammonium sulphate precipitation at 80% saturation. Total protein was separated on SDS Polyacrylamide gel. Each band was recovered from the gel by electroelution avoiding the time-consuming and expensive biochemical methods of column chromatography. The allergenic proteins were identified by skin tests. The fourth and the fifth band in the protein profile of *I. fistulosa*, IF₄ (47 kDa) and IF₅ (42 kDa) were the two major allergenic components of *I. fistulosa*. The protein profiles showed slight variations in the pollen collected from different geographical regions.

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Pollen extracts are widely used both for diagnosis and treatment of allergic diseases. One of the most successful treatment for allergic rhinitis is Immunotherapy, often termed hyposensitization, involving repeated subcutaneous injections of gradually increasing concentrations of the allergen(s). Another method used for testing human reactions is the immunological cross-reactivities measured by ELISA. However a major drawback which eclipses the importance of this method is that each pollen has its own immunologic identity. For example, *Quercus alba* pollen extract will afford little relief to a patient sensitized to *Quercus nigra* pollen (Stanley & Linskens, 1974). Pollen samples collected from different source materials, stages of inflorescence, time intervals, years, from geographical places and also periods of storage, show significant variation in their allergenic components (Singh et al. 1993). For this reason, efforts are being made to standardize the pollen extracts, so that every time an antigen of the pollen of a particular species is prepared, it should have the same antigenic determinants.

Ipomoea fistulosa, belonging to the family Convolvulaceae, is a common shrub growing along road sides and waste areas and is used in India for fencing houses. According to Thommen's postulates (1931), an essential requirement for a pollen to cause an allergic reaction in human beings, is that it should come from a wind pollinated plant. However in recent years, aeropalynological surveys carried out at various places in India and abroad (Agashe 1989, Atluri et al. 1992, Chen & Huang 1980, Hyde 1950, 1959; Ong et al. 1995, Singh et al. 1973, Singh & Devi 1992), have demonstrated the occurrence of airborne pollen of typically entomophilous plants. It is also known that the abundance of a particular taxa in a particular area could be responsible for the predominance of the pollen of that taxa in the atmosphere of that region. With these points in mind,

I. fistulosa, which is an entomophilous plant but one having a high pollen production, was selected for the present study.

MATERIALS AND METHODS

Antigenic proteins can be extracted in phosphate buffered saline, which on fractionation by ion-exchange chromatography following gel filtration yield purified fractions of the antigen(s), the allergenicity of which can be identified by skin sensitivity test, bronchial provocation test and Phadezym RAST and ELISA inhibition by reaction with serum IgE (Roy Karmakar & Chatterjee 1995, Singh et al. 1993). However, to prove the allergenicity of a particular pollen type, the total extract of the pollen is usually tested (Acharya 1980, Mittal et al. 1978, Shivpuri 1980). In the present study, a different approach has been made to identify the allergenically significant proteins of the pollen of *I. fistulosa* without going into the lengthy processes of column chromatography.

Here the total crude extract was prepared at 80% saturation of solid ammonium sulphate and the precipitate dialysed against distilled water, with several changes of water, resulting in partial purification of the crude extract. It was then electrophoresed on a SDS-Polyacrylamide gel and the individual bands eluted from the gel by electroelution were tested for allergenicity by skin tests, in addition to testing the total protein extract.

Pollen Collection

Pollen grains of *Ipomoea fistulosa* were collected in bulk from the plants growing in Santiniketan, West Bengal. Two types of pollen samples were collected – one from mature buds and the other from flowers which had finished blooming, on the same day. Samples were also collected from different regions of West Bengal i.e. Burdwan, Calcutta and Siliguri (located approximately 1146 km, 145 km and 447 km away from Santiniketan respectively). Pollen grains from the anthers were sieved using different meshes (100, 200 and 300 µm). All the samples were analysed under the microscope which revealed pollen purity varying from 85% to 90%.

Protein extraction

Protein from pollen was extracted according to the method of Singh et al. (1993) with slight modifications. The pollen was defatted with cold solvent ether and then dried in a vacuum desiccator. The defatted pollen was then used for protein extraction. Proteins were extracted in 0.2 M Tris HCl buffer, pH 7.4 by continuous stirring at 4°C for 20 h. The extract was clarified by centrifugation at 15,000 × g for 25 min at 4°C. The supernatant was collected and was subjected to fractional precipitation by solid ammonium sulphate. It was made upto 80% saturation by slow addition of the salt at 4°C by continuous stirring. The extract was then allowed to stand for half an hour before centrifugation at 10,000 × g for 15 min. at 4°C. After centrifugation the precipitate was resuspended in 0.1 M Tris HCl buffer, pH 7.4 and desalted by dialysing against distilled water for 48 hrs. at 4°C by frequent changes of the distilled water using dialysis sacs (Molecular weight cut off 12,000 d). The samples after dialysis were then stored at -20°C until further use.

Estimation of protein

The protein concentration in the extract, as well as in the various eluted fractions, was estimated by the modified method of Lowry (Lowry et al. 1951). A calibrated solution of bovine serum albumin was used as a standard.

Gel electrophoresis

The protein sample was heated with an equal amount of sample buffer [0.06 M Tris HCl (pH 6.8), 1% SDS, 10% sucrose, 0.5% β-mercaptoethanol, 0.01% Bromophenol blue] at 100°C for 3 min. 10 µl of the sample containing 84 µg of protein was loaded in the well of a 10% T mini-gel (8 × 7 cm gel) and the gel was run using Laemmli buffer system (1970) [0.05 M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.4] at room temperature for 2 hrs. 30 min, at 70 V. The gel was calibrated with a marker mixture consisting of Myosin, rabbit muscle (M.W. 205 kDa), β-Galactosidase, *E. coli* (M.W. 116 kDa), Phosphorylase b, rabbit muscle (M.W. 97.4 kDa), Albumin, Bovine (M.W. 66 kDa), Albumin, Egg (M.W. 45 kDa) and Carbonic Anhydrase, Bovine Erythrocytes (M.W. 29 kDa). After electrophoresis, the gel was stained with 0.1% Coomassie Brilliant Blue R 250 and destained with methanol:acetic acid:water (4:1:5) mixture.

Recovery of protein from the gel

Protein was eluted from the gel, following the method of Wilson & Goulding (1986). After electrophoresis, one side of the gel (covering 2 lanes) was cut vertically and stained in order to ascertain the banding positions. Only those portions corresponding to the protein bands to be recovered, were cut out with a sterile scalpel from the other half of the gel which was not stained. The gel pieces were then transferred into pre-treated dialysis bags filled with electroelution buffer (containing 0.05 M Tris and 0.192 M Glycine, pH 8.4) so that each gel piece was surrounded by small amount of buffer with no air bubbles. These dialysis bags were immersed in electrode buffer in an electrophoresis tank of a horizontal gel apparatus. Electric current (120 V) was passed through the bags for nearly 3 hrs to elute the proteins out of the gel. At the end, the current was reversed for 30 seconds in order to release the protein from the wall of the sac. The buffer containing the eluted proteins was then transferred into a cotton plugged Eppendorf tube and centrifuged for 2 min to remove contaminating gel particles. The entire process was repeated 5 times to get sufficient quantity of each fraction for loading in the gel and for further skin tests. The protein in each fraction was quantified and each fraction was again electrophoresed to check its homogeneity.

Preparation of antigenic extracts for skin prick tests

The protein fractions after electroelution were subjected to fractional precipitation by ammonium sulphate. This was made up to 100% saturation by addition of the salt at 4°C by continuous stirring. After centrifugation for 5 min at 10,000 × g in an Eppendorf tube at 4°C in a microcentrifuge, the supernatant was discarded and the pellet resuspended in 0.05 M Phosphate buffered saline, pH 7.4 (Sheldon et al. 1967), in such a way that each antigenic fraction had the concentration of 1 in 500 (w/v) i.e. 1 part by weight of protein in 500 parts by volume of the buffered saline, and again dialysed against distilled water at 4°C for 24 hrs. The total antigen was extracted according to the method of Sheldon et al. (1967) with slight modifications. Antigen from defatted pollen was extracted in 0.05 M Phosphate buffered saline, pH 7.4 in 1:10 ratio (w/v) by continuous stirring at 4°C for 20 h and then centrifuged at 15,000 × g for 25 min at 4°C and the supernatant was dialysed against distilled water. After dialysis, the sample was again centrifuged and the supernatant was collected. The antigenic extracts were then sterilized by filtering through a Millipore Filter Paper (0.22 µm). After filtration, the sterilized extracts were transferred aseptically to the sterile vaccine vials stored at 4°C. Prior to testing these extracts on patients, their sterility was checked for microorganisms by transferring a few drops of the antigenic extract to LB (Leuria broth) agar plate and incubating at 37°C for 24 hrs.

Skin prick test

Each patient was tested by placing 10 µl of each allergen, at least 5 cm apart on the volar surface of his/her forearm and each site was then pricked with a disposable hypodermic needle. Negative and positive controls were also performed. The negative control was the buffer saline in which the allergen was resuspended and the positive control was histamine acid phosphate injection diluted with buffered saline to 1:10,000 i.e. 1 µg of histamine acid phosphate. The patients were prohibited from using antihistamine, steroid and ephedrin for 48 hrs. before the skin prick tests. The skin reactions were read after 15 to 20 min from the commencement of the test. The test was quantified on the basis of the wheel diameter and graded 1+ to 4+. The skin tests were conducted at Calcutta Medical Research Institute, Calcutta. The patients were selected on the basis of their suffering from respiratory allergic disorders.

RESULTS

Protein concentration

The protein content of the extracts from pollen collected on the same day from mature buds and flowers which had finished blooming were found to be 4.6 mg ml⁻¹ and 8.4 mg ml⁻¹ respectively.

SDS-PAGE

The SDS-PAGE protein profile of the 2 different pollen samples (mature buds and flowers which had finished blooming) are shown in Figs. 1 & 2. The bands are present in the molecular weight range 31 kDa and 89 kDa with another band having a molecular weight less than 29 kDa. Eight bands were obtained which were designated IF₁ to IF₈, the molecular weights of which were estimated by SDS-Polyacrylamide gel electrophoresis by plotting the logarithms of the known molecular weights of proteins versus the relative mobilities of these proteins. The relative mobility of the antigens of unknown molecular weights were then fitted to this curve to determine their molecular weights. These are

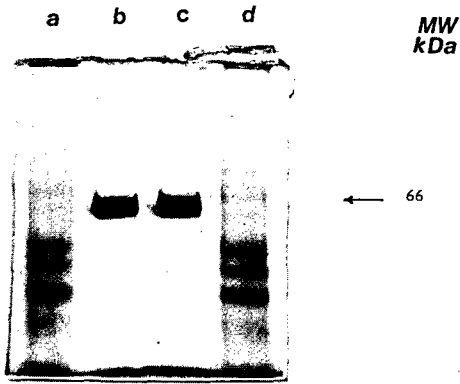


Fig. 1. SDS-PAGE of pollen extracts of *I. fistulosa* collected from mature anthers. (a,d) contain 46 μ g and 36.8 μ g respectively of 0–80% ammonium sulphate pellet fraction of crude homogenate, applied to a SDS-Polyacrylamide gel (10%). (b,c) represent 66 kDa, a protein standard.

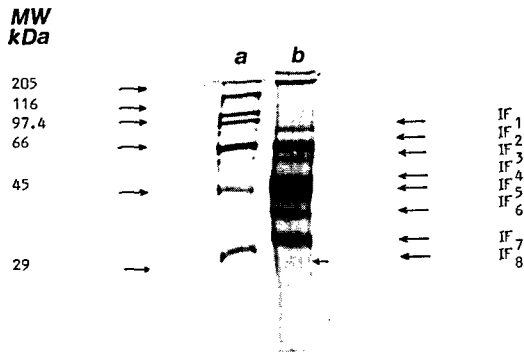


Fig. 2. SDS-PAGE patterns of pollen extracts of *I. fistulosa* collected from flowers which had finished blooming. (a) represents marker proteins. (b) contain 84 μ g of 0–80% ammonium sulphate pellet fraction of crude homogenate. It represents IF₁ to IF₇. The IF₈ band is indistinct.

Table I. Molecular weights of various protein bands of the antigenic extract of *I. fistulosa*.

Fraction	Molecular weights (in kDa)
IF ₁	89
IF ₂	68
IF ₃	57
IF ₄	47
IF ₅	42
IF ₆	33
IF ₇	31
IF ₈	<29

presented in Table I. The 47 kDa (IF₄) and 42 kDa (IF₅) were the two most prominent bands with the other major bands being 68 kDa (IF₂), 33 kDa (IF₆) and 31 kDa (IF₇). Very little difference was observed in the banding pattern of extracts prepared from pollen collected from mature buds and fully opened flowers (Figs. 1 & 2). However, the protein profiles of the pollen extracts collected from different places showed minor differences (Fig. 3). The IF₈ band (<29 kDa) was absent and the 57 kDa (IF₆) band was not very distinct

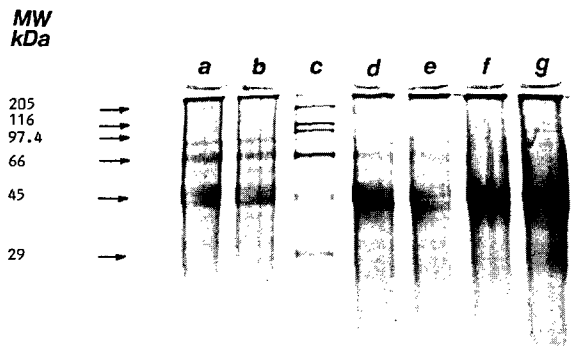


Fig. 3. SDS-PAGE of pollen extracts of *I. fistulosa* collected from different places in West Bengal. (a,b) – Burdwan; (d,e) – Calcutta; (f,g) – Siliguri and (c) – Marker proteins.

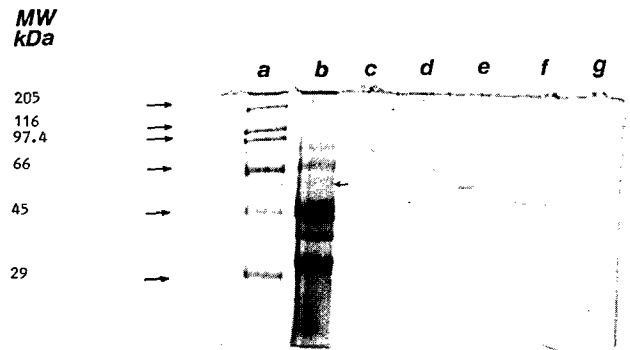


Fig. 4. SDS-PAGE of the gel eluted fractions of the pollen extract of *I. fistulosa*. (a) represents marker proteins; (b) contain 84 μ g of 0–80% ammonium sulphate pellet fraction of total homogenate; (c) to (f) contain 11 μ g of 0–100% ammonium sulphate pellet fraction of the eluted proteins of the first 5 bands i.e. IF₁, IF₂, IF₃, IF₄ and IF₅ respectively. The band 3 (IF₃) is indistinct in the total protein profile.

in the pollen samples collected from Burdwan and Calcutta while the 33 kDa (IF₃) band was not distinct in the samples collected from Siliguri and Calcutta. Apart from this, an indistinct band around the range of 26–27 kDa was obtained in the samples procured from Burdwan and Siliguri, and another indistinct band below 29 kDa (i.e. around 13–14 kDa) was observed in the sample obtained from Calcutta. Each of the fractions (IF₁–IF₈) after electroelution were again electrophoresed on a SDS-Polyacrylamide gel. Each of the fractions gave single bands (Figs. 4 & 5), thus confirming proper elution of the proteins.

Skin prick test

Tables II & III shows the results of skin prick tests which were performed on 35 patients already suffering from respiratory allergic disorder. Of these 15 (43%) showed positive reaction to the total extract of *I. fistulosa* and were analysed by age distribution (Table IV). Among these 15 patients, 6 were female within the age range 21–62 yrs and the other 9 were male within the age range 9–71 yrs. Table II shows that the highest number of sensitive patients was in the 21–30 and 41–50 age group. Among the heterogeneous population

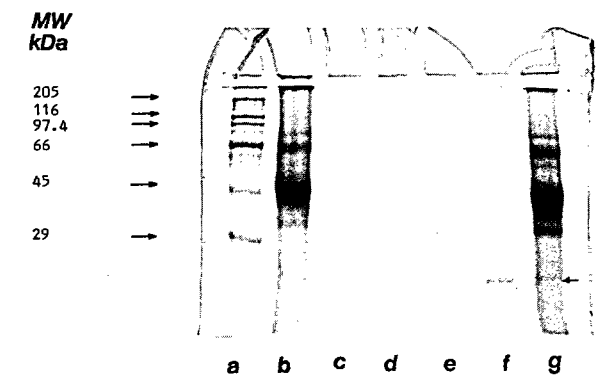


Fig. 5. SDS-PAGE of the eluted fractions of the pollen extract of *I. fistulosa*. (a) represents marker proteins; (b,g) contains 92.4 µg and 84 µg of 0–80% ammonium sulphate pellet fraction of the total crude homogenate; (b,d,e,f) contains 0–100% ammonium sulphate pellet fractions of eluted proteins of the last 4 bands (IF₅ – 11 µg, IF₆ – 8 µg, IF₇ – 8 µg, IF₈ – 11 µg).

showing a positive reaction to the total extract, 66% were atopic (≥2+ skin reactivity) and of these 3 patients had suffered from asthma for 17–28 yrs and 5 from allergic rhinitis for 9–14 yrs. Of the 8 fractions of *I. fistulosa* extract (IF₁ to IF₈), the highest incidence of skin reactivity was found with IF₄ (47 kDa) and IF₅ (42 kDa). All the 15 patients found to be sensitive to *I. fistulosa* antigen (whole extract) were sensitive to these fractions. Among them 53% and 40% showed 1+ reaction to IF₄ and IF₅ respectively and 47% and 40% showed 2+ to IF₄ and IF₅. 3+ reaction was not observed in IF₄ but 20% showed 3+ sensitivity to IF₅. A lower incidence of skin reactivity was found with other fractions. 3 patients were sensitive to IF₁ (89 kDa), and all of them gave 1+ reactivity. Three protein components, IF₂ (68 kDa), IF₆ (33 kDa) and IF₇ (31 kDa) induced 1+ and 2+ sensitivity in 9, 7 and 4 patients respectively. IF₃ (57 kDa) and IF₈ (<29 kDa) did not produce any skin reactivity.

Table III. Frequency distribution of allergenicity of the total extract and individual allergens of *I. fistulosa*.

Antigen tested	Number of + ve patients	% showing		
		1 +	2 +	3 +
Whole	15	33	47	20
IF ₁	3	100	0	0
IF ₂	9	56	44	0
IF ₃	0	0	0	0
IF ₄	15	53	47	0
IF ₅	15	40	40	20
IF ₆	7	43	57	0
IF ₇	4	75	25	0
IF ₈	0	0	0	0

Diameter of: erythema (mm)	0	Reactivity: 0
1–5		1 +
6–10		2 +
11–15		3 +

Table IV. Age distribution of patients who showed positive skin response to *I. fistulosa* pollen antigen.

Age group	Male	Female	Total
1–10	1	–	1
11–20	1	–	1
21–30	1	3	4
31–40	1	–	1
41–50	3	1	4
51–60	1	1	2
61–70	–	1	1
71–80	1	–	1
Total	9 (60%)	6 (40%)	15 (100%)

DISCUSSION

The present study aimed to detect sensitization to *I. fistulosa* pollen. Despite the fact that 43% patients showed incidence

Table II. Results of skin tests against total extract and individual antigenic fractions of *Ipomoea fistulosa* sensitive patients.

No. of patient	Age (yrs.)	Sex	Antigen tested								
			Total protein	IF ₁	IF ₂	IF ₃	IF ₄	IF ₅	IF ₆	IF ₇	IF ₈
1	42	M	2+	-ve	1+	-ve	1+	1+	-ve	-ve	-ve
2	41	M	1+	1+	-ve	-ve	1+	1+	1+	-ve	-ve
3	27	F	2+	-ve	-ve	-ve	2+	2+	1+	-ve	-ve
4	21	F	1+	-ve	-ve	-ve	1+	1+	1+	1+	-ve
5	46	F	1+	1+	1+	-ve	1+	1+	-ve	-ve	-ve
6	52	F	2+	-ve	2+	-ve	2+	2+	-ve	-ve	-ve
7	31	M	3+	-ve	2+	-ve	2+	3+	2+	-ve	-ve
8	9	M	1+	-ve	-ve	-ve	1+	1+	-ve	-ve	-ve
9	11	M	1+	-ve	-ve	-ve	1+	1+	-ve	-ve	-ve
10	51	M	2+	-ve	2+	-ve	2+	2+	2+	1+	-ve
11	27	F	2+	-ve	1+	-ve	2+	2+	2+	2+	-ve
12	62	F	2+	-ve	1+	-ve	1+	2+	-ve	-ve	-ve
13	71	M	2+	-ve	-ve	-ve	1+	2+	-ve	-ve	-ve
14	49	M	3+	1+	1+	-ve	2+	3+	2+	1+	-ve
15	25	M	3+	-ve	2+	-ve	2+	3+	-ve	-ve	-ve

of *I. fistulosa* pollen sensitivity, this pollen has not yet been considered an important allergenic source. A common shrub in different regions of this country, the high pollen productivity [ca 96,000 pollen/anther, (Mondal & Mandal 1997)] and the high skin reactivity in patients suffering from asthma and allergic rhinitis, suggest that it is an aeroallergen. Protein concentration varied in the antigenic extracts of pollen collected from mature anthers (4.6 mg ml^{-1}) and those collected from the anthers of flowers which had finished blooming (8.4 mg ml^{-1}). Variation in protein patterns were also observed in the pollen samples procured from different geographical regions. There was variation in the occurrence of the 33 kDa and 57 kDa bands and the band below 29 kDa. The differences in the protein profile of these samples could be due to the different climatic conditions prevailing in these areas, soil conditions and also the stage of maturation of the inflorescence from which the pollen was collected. This is in conformity with the works of Singh et al., 1993. Of the 8 major protein bands (IF₁–IF₈) tested, the patients showed sensitivity to 6 of them, while IF₃ and IF₈ produced no skin reactivity. The majority of the patients (all 15) showed sensitivity to IF₄, a low molecular weight protein (47 kDa) and IF₅, also a low molecular weight protein (42 kDa), suggesting that these are the most important allergens of *I. fistulosa* pollen. The other proteins (89 kDa, 68 kDa, 53 kDa and 31 kDa) also induced 1+ and 2+ sensitivity but in a smaller number of patients, proving that they, too, are minor contributors to the allergenic content of this pollen.

CONCLUSION

The present study reveals that *I. fistulosa* pollen is capable of inducing hypersensitivity is one of the aeroallergens and should be considered to be a cause of respiratory allergic diseases in India. This study also helps us to conclude that IF₄ and IF₅ are two major allergenic components of *I. fistulosa* isolated from the total proteins. The isolation of different proteins from the gel by electroelution has been the easiest way to test a number of particular protein bands for allergenicity within a few days.

One of the future aims of this study is to unravel the amino acid sequences, 3-dimensional structure of these antigenic proteins and to elucidate the reaction between antigenic proteins and antibody which might help to understand the molecular basis of hypersensitivity. This approach of isolating particular proteins from a mixture of total proteins for the detection of allergenic proteins is reported here for the first time.

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