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Identification and partial characterization of the allergenic proteins of *Ricinus communis* L. pollen - a new approach

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Identification and partial characterization of the allergenic proteins of *Ricinus communis* L. pollen — a new approach

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The pollen of *Ricinus communis* L., a potentially allergenic plant, was extracted to identify the allergenic determinants responsible for causing respiratory disorders. The soluble proteins were extracted and subjected to ammonium sulphate precipitation at 80% saturation and the total protein separated on 12% SDS-Polyacrylamide gel. In order to avoid the time consuming and expensive biochemical methods of column chromatography, each band was directly recovered from the gel by electroelution and the allergenic proteins identified directly by skin tests, without the necessity of Phadezym RAST or ELISA inhibition by reaction with serum IgE, the general procedure to identify the allergens. The fourth and the fifth band in the protein profile of *R. communis* pollen, RC4 (77 kD) and RC5 (66 kD) were the two major allergenic components. RC3 (91 kD) also induced a considerable amount of reactivity in sensitive patients. Contrary to the earlier reports of protein bands of *R. communis* ranging from 14 kD to 70 kD, 4 bands above 70 kD i.e. RC1 (123 kD), RC2 (97 kD), RC3 (91 kD) and RC4 (77 kD) are reported here for the first time. Immunodiffusion analysis with pooled sera of patients sensitive to the total extract also revealed similar results.

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Pollinosis or “hay fever” is one of the most important problems of human pathology all over the world. Clinically, hayfever is described as allergic rhinitis which is characterized by sneezing, rhinorrhea, obstruction of the nasal passages, conjunctival and pharyngeal itching and lacrimation. Although commonly seasonal, because of its relation to airborne pollen, other patterns and etiologies occur. In view of the significance of the role of pollen antigens in the diagnosis and therapy of allergic patients, identification, isolation, purification and characterization of pollen allergens is a prerequisite for proper standardization. Efforts are being made globally to standardize the pollen antigens as pollen collected from different source materials, stages of maturity, time intervals and geographical places or with different storage periods have shown significant variation in their allergenic components (Singh et al. 1993). In general, application of gel filtration and column chromatography along with electrophoresis yield purified protein fractions for chemical characterization by removing the additional irritants and toxic substances along with other unwanted components in the crude mixtures of pollen antigens.

The present study was undertaken to assess the role of *Ricinus communis* L. pollen in causing respiratory allergy and to identify the allergenic proteins. *Ricinus communis* of the family Euphorbiaceae, an economically important plant is cultivated widely for the production of castor-oil from its seeds. However, its pollen has been reported as being of an

allergenic nature (Acharya 1980, Shivpuri 1980, Singh et al. 1973, 1992, 1993). A large shrub or a small anemophilous tree, it blooms profusely during November to March releasing enormous amounts of pollen in to the atmosphere and its pollen has been recorded in the atmosphere in various parts of India (Atluri et al. 1992, Parui et al. 1998, Singh and Devi 1992, Tilak 1989).

A considerable amount of work has already been done on various aspects of allergenicity caused by *Ricinus communis* (Acharya 1980, Thorpe et al. 1988). Singh et al. (1993) have also used immunoblot analysis to identify the allergenic components of *Ricinus communis* pollen obtained from different stages of inflorescence, over different time intervals, and in different years. This has proved to be an efficient technique for investigating the specificity of IgE antibodies to different components of allergenic extracts. In this study the approach has been to identify the allergenically significant proteins of the pollen of *Ricinus communis* growing in Santiniketan by directly eluting the proteins from the gel after SDS-PAGE and then testing the allergenicity of these eluted fractions by skin tests.

Abbreviations

RC : *Ricinus communis*

SDS : Sodium dodecyl sulphate

SDS-PAGE : Sodium dodecylsulphate polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Pollen collection

Pollen grains of *Ricinus communis* were collected in bulk from plants growing in Santiniketan, West Bengal, Eastern India. The pollen samples were collected from flowers which had bloomed during the month of October. Pollen grains from the anthers were sieved using 3 different sized meshes (100, 200 and 300 µm). The samples were analysed under the microscope to check pollen purity, which was 90% to 95%.

Protein extraction and estimation

Protein was extracted from defatted pollen in 0.2 M Tris HCl buffer, pH 7.4 following the method of Singh et al. (1993). The protein in the supernatant was subjected to ammonium sulphate precipitation at 80% saturation and the protein repelleted by centrifuging at 10,000 x g for 15 min at 4 °C. 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 using dialysis sacs (Molecular weight cut off 12,000 d) obtained from Sigma diagnostics, USA. After dialysis, the samples were stored at -20 °C until further use. The protein concentration was estimated by the method of Lowry et al. (1951).

Gel electrophoresis

SDS-PAGE was performed in 12% gel following the method of Laemmli (1970). 10 µl of sample containing 80 µgm of protein was loaded into the well of the gel and electrophoresis was performed using Laemmli buffer system [0.05 M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.4] at room temperature at 70 V (mini-gel-8 × 7 cm) for 2 hrs. 30 min. The gel was calibrated with marker protein (M.W range, 29,000 to 205,000 d) obtained from Sigma Chemical Co., USA. After electrophoresis, the gel was stained with 0.1% Coomassie Brilliant Blue R250.

Recovery of protein from gel

Protein was eluted from the gel following the method of Wilson and Goulding (1986). The protein from the gel pieces was eluted in dialysis bags filled with electroelution buffer (containing 0.05 M Tris and 0.192 M Glycine, pH 8.4) immersed in the electrophoresis tank of a horizontal gel apparatus containing the same buffer at 120 V for 3 hrs. At the end of the process, the current was reversed for 30 seconds in order to release the protein from the wall of the sacs. The buffer containing the eluted proteins was then transferred through a cotton plugged Eppendorf tube and centrifuged for 2 min to remove contaminating gel particles. The protein in each fraction was quantified and each fraction was again electrophoresed to check its homogeneity.

Preparation of antigenic extracts

The total antigenic extract was prepared by the method of Sheldon et al. (1967) while the individual eluted protein fractions after 100% ammonium sulphate precipitation were resuspended in 0.05 M Phosphate buffered saline, pH 7.4 at a concentration of 1 in 500 (w/v), dialysed against distilled water for 24 hrs at 4 °C and sterilized, prior to testing on patients.

Skin prick test

The antigenic extracts were treated on patients already suffering from naso bronchial allergy. Their age range was 18–64 years. Each patient was tested with 10 µl of each allergen. Negative and positive controls were also performed. The negative control was the buffer saline in which the allergen was extracted or 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 skin reactions were read after 15 to 20 min from the commencement of the test and the test was quantified on the basis of the wheal diameter and graded 1+ to 4+. The skin tests were conducted at Calcutta Medical Research Institute, Calcutta.

Immunodiffusion

Blood samples were collected from patients showing positive response to the total extract of *R. communis* and the antiserum prepared following the method of Sadasivam and Manickam (1992). Ouchterlony immunodiffusion was performed following the method described by Johnstone and Thorpe (1982). Equal protein loadings of each fraction (50 µg total protein) were placed into the outer wells and 25 µl of the mixture of antiserum (150 µg total protein) of *R. communis* pollen sensitive patients was placed in the central well. The plates were incubated for 48 hrs several times, rinsed thoroughly in distilled water and dried to observe the precipitin arcs.

RESULTS

SDS-PAGE

The SDS-PAGE protein profiles of the pollen of *R. communis* collected from blooming flowers are shown in Figs. 1, 2 and 3. The molecular weights of the protein bands were estimated by SDS-PAGE 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, which are presented in Table I. Eleven major bands were present in the molecular weight range from 42 kD to 123 kD, and were designated RC1, RC2, RC3 ... RC11. There were, however, a few more bands between the range of 29 kD to 40 kD which were not well developed. The major bands were RC3 (91 kD), RC4 (77 kD), RC5 (66 kD), RC6 (61 kD), RC7 (59 kD) and RC8 (56 kD).

Skin prick test

The results of skin prick tests are presented in Tables II and III. A total of 48 patients were tested, out of which 19 (39.6%) showed a positive reaction to the total extract of *R. communis*. Of these 11 were male and 8 were female. Among the heterogenous population showing a positive reaction to the total extract, 63% were atopic ($\geq 2+$ skin reactivity), and 10 of them had suffered from allergic rhinitis for 8–20 years. The highest number of sensitive patients was in the age group 21–30 years. Of the 19 patients, showing positive sensitivity to the whole extract, 57.9% showed 2+ reactivity, 21.1% showed 3+ sensitivity, 15.8% showed 1+ reactivity and only 5.3% showed 4+ reactivity. The highest skin reactivity was found with fractions RC4 (77 kD) and RC5

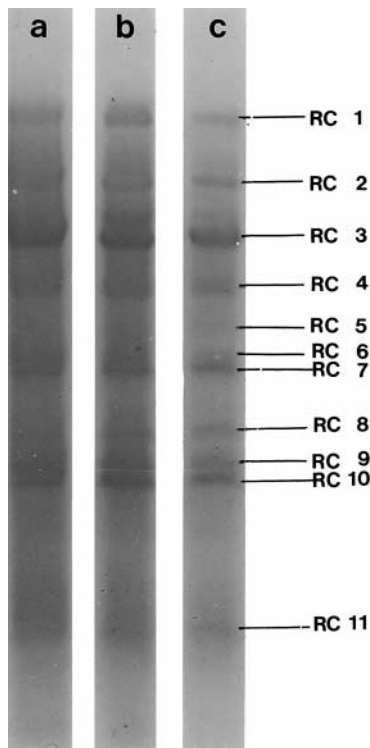


Fig. 1. SDS-PAGE of pollen extracts of *R. communis* collected from the anthers of blooming flowers. (a, b & c) contain 86 µgm, 84 µgm and 80 µgm respectively of 0–80% ammonium sulphate pellet fraction of crude homogenate, applied to a 12% SDS-Polyacrylamide gel (16 × 14 cm). This represents RC1 to RC11.

(66 kD). All the 19 patients found to be sensitive to *R. communis* pollen were sensitive to these fractions, and the majority of them showed 2+ reaction (52.6% in case of RC4

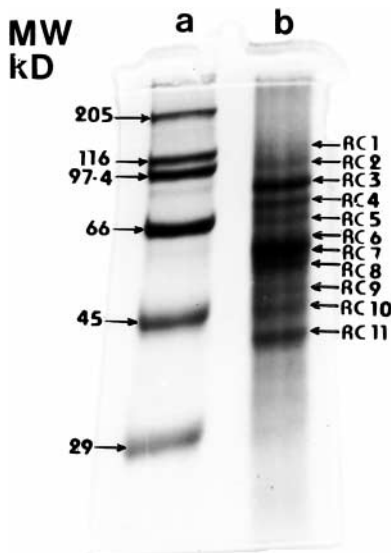


Fig. 2. SDS-PAGE patterns of pollen extracts of *R. communis*. (a) marker proteins; (b) 40 µgm of 0–80% ammonium sulphate pellet fraction of crude homogenate. It shows RC1 to RC11. A few faint bands are also present below RC11 within the molecular weight range of 29 kD to 40 kD.

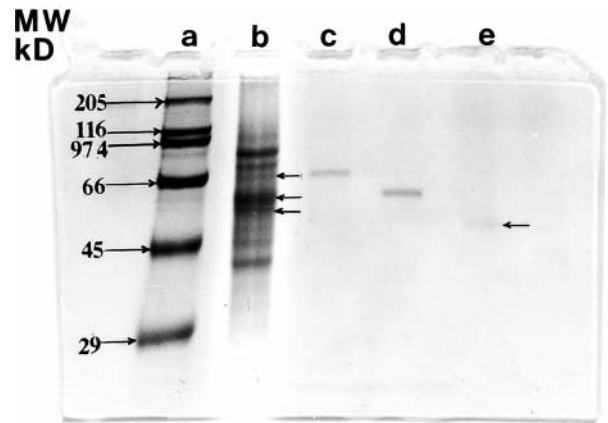


Fig. 3. SDS-PAGE of the eluted fractions of the pollen extract of *R. communis*. (a) marker proteins; (b) total protein; (c & d) 10 µgm of 0–100% ammonium sulphate pellet fractions of eluted proteins of bands RC5 and RC6 respectively; (e) RC8 (band not clear).

and 57.9% in case of RC5). RC3 (91 kD) also induced 1+ to 3+ reactivity in 14 patients. RC2 (97 kD), RC8 (56 kD) and RC9 (51 kD) did not show any skin reactivity while the remaining 5 bands RC1 (123 kD), RC6 (61 kD), RC7 (59 kD), RC10 (45 kD) and RC11 (42 kD) induced only 1+ and 2+ sensitivity in 7, 10, 11, 11 and 11 patients respectively.

Immunodiffusion

Immunodiffusion of the pooled antiserum of the 19 patients helped to identify the allergenic fractions as well as to compare their degree of allergenicity (Figs. 4 and 5). The results were similar to those of the skin tests, with precipitin arcs obtained with fractions RC1, RC3, RC4, RC5, RC6, RC7, RC10 and RC11. No precipitation reaction was observed with RC2, RC8 and RC9.

DISCUSSION

The pollen of *R. communis* was found to contain a potent but complex mixture of allergens. The results of skin tests showed that fractions RC4 (77 kD) and RC5 (66 kD) were

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

Fraction	Molecular weights (in kD)
RC 1	123
RC 2	97
RC 3	91
RC 4	77
RC 5	66
RC 6	61
RC 7	59
RC 8	56
RC 9	51
RC10	45
RC11	42

Table II. Results of skin tests against the total antigenic extract of *R. communis* on patients with naso-bronchial allergy and on control patients (normal persons).

Total number of patients tested	Patients					Total number of normal persons tested	Normal persons		
	–ve %	1+ %	2+ %	3+ %	4+ %		–ve %	1+ %	2+ %
48	60.4	6.3	22.9	8.3	2.1	25	92.0	8.0	0.0

Table III. Frequency distribution of allergenicity of the total extract and individual allergens of *R. communis*.

Antigen tested	Number of +ve patients	% showing			
		1+	2+	3+	4+
Whole	19	15.8	57.9	21.1	5.3
RC 1	7	71.4	28.6	0.0	0.0
RC 2	0	0.0	0.0	0.0	0.0
RC 3	14	50.0	42.9	7.1	0.0
RC 4	19	36.8	52.6	10.5	0.0
RC 5	19	36.8	57.9	5.3	0.0
RC 6	10	60.0	40.0	0.0	0.0
RC 7	11	81.8	18.2	0.0	0.0
RC 8	0	0.0	0.0	0.0	0.0
RC 9	0	0.0	0.0	0.0	0.0
RC10	11	90.9	9.1	0.0	0.0
RC11	11	100.0	0.0	0.0	0.0

the major allergen species followed by RC3 (91 kD) while RC1 (123 kD), RC6 (61 kD), RC7 (59 kD), RC10 (45 kD) and RC11 (42 kD) were minor contributors to the allergenic determinants of *R. communis* pollen. Among the tests conducted with the total extract on 25 normal persons, two showed 1+ reaction. This incidence of positivity could be due to contaminating constituents unrelated to the actual allergen to which a person has been actually sensitized. Total pollen extracts are heterogeneous and contain

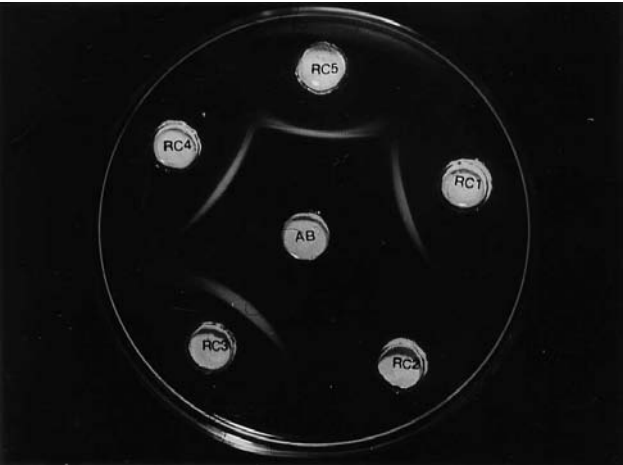


Fig. 4. Serological precipitation reaction between pooled antiserum of *R. communis* pollen sensitive patients and individual protein fractions of *R. communis*. AB represents pooled human antiserum (25 µl containing 150 µg total protein) and RC1 to RC6 contains 50 µg of protein fractions.

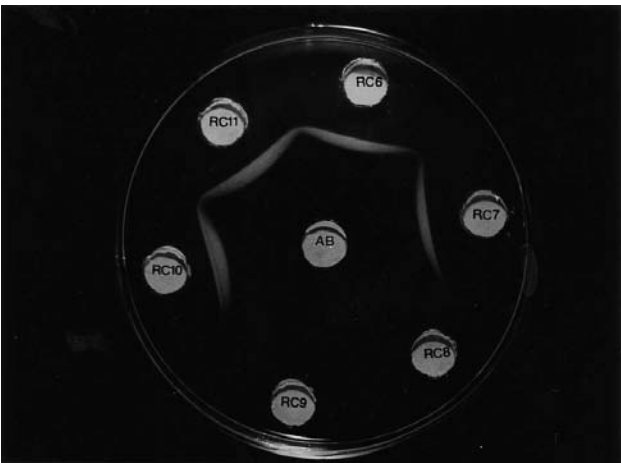


Fig. 5. Serological precipitation reaction between pooled antiserum of *R. communis* pollen sensitive patients and individual protein fractions of *R. communis*. AB represents pooled human antiserum (25 µl containing 150 µg total protein) and RC6 to RC11 contains 50 µg of protein fractions.

proteins, enzymes, lectins, glycoproteins, lipoproteins etc. They may contain the relevant allergen in minute irreproducible amounts as pollen allergens generally comprise 0.5 to 1.0% of the total extractable proteins (Stanley and Linskens 1974). These contaminating constituents may lead to the induction of IgE antibodies with new specificities (Mohapatra 1994). However, skin prick tests with individual protein fractions on normal persons induced no skin reactivity in any of them. A comparison of the present study with that of Singh et al. (1992 and 1993) showed some disparities, which were in some cases very pronounced. Contrary to our observations which showed major bands in the molecular weight range 42 kD to 123 kD with very faint, not well developed bands the range of 29 kD to 40 kD, Singh and his co-workers (1992) have reported bands in the molecular weight range of 14 kD to 70 kD. Their bands of low molecular weight (20 kD to 29 kD) become more sharp and distinct from December onwards. However, no bands in this range (20 kD–29 kD) were observed in this study. Since pollen proteins show great variation depending on the time of collection, storage periods, difference in source materials, stages of maturity and geographical location, the difference in the time of collection (October) and geographical region could be the reason behind these differences, i.e. the absence of these bands. On the other hand, bands above 70 kD have not been reported by Singh et al. (1992), while the present study showed 4 bands above 70 kD, i.e. RC1 (123 kD), RC2 (97 kD), RC3 (91 kD) and RC4

(77 kD) of which RC3 and RC4 are very prominent. With regard to the allergenic components, skin tests of individual protein bands revealed that 77 kD and 66 kD protein were the major allergenic components while 123 kD, 91 kD, 61 kD, 59 kD, 45 kD and 42 kD proteins were the other contributors. In contrast to these results, immunoblot studies for the specificity of IgE antibodies to different components of *R. communis* pollen performed by Singh et al. (1993) have shown bands 66 kD and 70 kD to be the major allergenic bands while 64 kD, 60 kD, 50 kD, 45 kD, 36 kD, 22 kD and 14 kD are the other prominent allergenic bands. The 70 kD and 64 kD, bands were not observed in the present study.

CONCLUSIONS

The protein profile of the pollen of *R. communis* growing at Santiniketan showed a considerable amount of variation in comparison with earlier reports. Hence, prior to preparation of a reference standard for this allergenic pollen, it would be desirable and essential to investigate the allergen extracts from the same material obtained from different geographical regions and with different times of collection. Further, following purification and characterization of the allergens, standardized test extracts could be prepared for adequate diagnosis and immunotherapy.

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