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Fractionation and Immunological Characterization of Allergens and Allergoids of *Prosopis juliflora* Pollen

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SUMMARY **Allergoids of *Prosopis juliflora* pollen were prepared by formalinization of crude allergen and glycoprotein. Fractionation of crude allergen and allergoids on Sephadex G-100 resulted in separation of proteins of varying molecular size and a glycoprotein of 81 to 13 KD. Allergoids prepared from the glycoprotein fractionated into two proteins of approximately 200 KD and more than 200 KD. Crossed immunoelectrophoresis indicated 12 and gel diffusion test 3 precipitating antigens incrude allergen extract; by these tests allergoids depicted 8 and 3 precipitin bands, respectively. The precipitin analysis showed heterogeneity of allergenic determinants and also variation in cross-immunogenicity of the formalinized derivatives. The skin prick and radioallergosorbent tests depicted greater activity of fractionated crude allergens than the allergoids. The above tests suggest altered and concealed antigenic determinants as result of formalinization of *P. juliflora* pollen which, however, showed reduced allergenic activity relative to the native allergen.**

Purified and improved extracts containing the clinically relevant allergens are used for the hyposensitization of human pollinosis.[1,2] The extracts used for diagnosis of allergic diseases sometimes cause local and systemic anaphylactic reactions at very low doses.[3] Allergenic preparations which possess reduced allergenicity but which retain their ability to induce the production of allergen-specific blocking antibodies could provide an improved form of diagnosis and therapy of allergic diseases. Such improved allergens, termed "allergoids", have been prepared by polymerization of allergens by treatment with formaldehyde and glutaraldehyde.[4,5] Derivatives from mixed grass allergens were prepared by Marsh *et al.*[4] using formaldehyde as the modifying agent. Patterson *et al.*[5] on other hand used glutaraldehyde for allergoid preparation which was fractionated into polymerized products with molecular weight of 200 to 20 KD. Allergoids from timothy pollen extracts were prepared by Puttonen *et al.*[6] by using formaldehyde. They obtained a very slight increase in molecular weight of the allergoid product, but these exhibited reduced allergenic activity.

P. juliflora is one of the most common trees of arid regions of the tropical and subtropical world. The wood of this tree is used as an energy source. Pollen grains of this tree are known to be allergenic in nature.[7] Its crude and purified allergens have been used for diagnosis and therapy of allergic diseases.[7]

Allergens from the pollen of *P. juliflora* have been fractionated and characterized for desensitization of human pollinosis.[8] Major allergenic activities in this system were associated with a glycoprotein of 20 KD which was isolated and its immunological response studied.[8,9] However, there are no reports of attempts to prepare the allergoids from *P. juliflora* pollen. The present study attempts to bridge this gap and provides information on fractionation and immunological

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characterization of allergoids prepared from crude extract and glycoprotein of *P. juliflora* pollen.

MATERIALS AND METHODS

Allergen extract and fractionation

The pollen allergen extract (1: 10 w/v) was prepared in 0.15 M phosphate-buffered saline (pH 7.2) and fractionated by Sephadex G-100 gel filtration as described earlier.[7,8]

Allergoid preparation

Crude pollen extract and the glycoprotein fractions were treated with 2.0 M formaldehyde to prepare the allergoids.[4] The mixture was incubated at 10° C for 14 days; pH was controlled at 7.5. The resulting allergoid solution was dialyzed at 4° C to remove the formaldehyde.

Fractionation of allergoids

The allergoid prepared from crude pollen extract and glycoprotein was fractionated on Sephadex G-100 and molecular weight was determined according to Whitaker.[10] Protein and carbohydrate contents of each fraction were estimated.[11,12] The fractions obtained by gel filtration with highest absorbance were pooled, concentrated against polyethylene glycol (mol. wt. 6000) in a dialysis bag and lyophilized.

Electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli[13] using 10.0% separating gel (pH 8.9). The gel was stained with 0.5% coomassie brilliant blue R.

Antisera

Three rabbits were used for preparation of antisera with crude allergen and its allergoid as described by Freund and McDermett.[14] Equal volumes of allergens and allergoids (protein content 2 mg/ml) and Freund's complete adjuvant were injected intraperitioneally. Initially three booster doses were given at 15 day intervals and the last two doses at 7 day intervals. Blood was collected from the marginal vein of the ear and serum was separated.

Skin test

A skin test for fractionated allergens and allergoids was assessed in 5 individuals sensitized to *P. juliflora* pollen. This was performed by injecting 0.02 ml fractions intradermally, then measuring the diameter of the wheal formed due to the skin reaction;[15] 0.02 ml of 0.1 M physiological saline served as the control.

Gel diffusion

A gel diffusion test of crude allergen and its allergoid was performed as described by Ouchterlony.[16] 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) was added in outer wells, and 0.01 ml of antibody was added in the central well. The precipitin bands were observed after 48 hours against 0.2 M phosphate buffer (pH 7.5) as control.

Immunoelectrophoresis

Cross immunoelectrophoresis of crude allergens and allergoids was performed as described by Weeke.[17] Samples were run for 1 hour at 10 V/cm in a 1% agarose (Sigma) gel containing 0.05 M barbital buffer (pH 8.5). The second dimension run against the antiserum containing gel was carried out at 150 V for 20 hours. The gel was stained with 0.5% coomassie brilliant blue R.

Radioallergosorbent test

Allergens were bound to paper discs according to Ceska *et al.*[18] The allergenic activity of allergoid preparations in comparison to the allergen was examined by inhibition of the RAST.[9] The allergenic potency was evaluated in terms of the ability of allergens and two different preparations of allergoids to neutralize IgE antibodies present in human allergic sera by addition of ^{125}I-labelled rabbit antibodies specific for human IgE immunoglobulin (Pharmacia, Uppsala, Sweden). The level of ^{125}I bound to the discs was measured on a Beckman gamma counter. The capacity (expressed as%) of each allergen and allergoid preparation which inhibited the RAST was calculated according to the formula :

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

where A represents the RAST value (counts per minute) in the absence of allergen and allergoids preparation and B represents the RAST value (counts per minute) in the presence of allergens and allergoids preparations.

RESULTS

Fractionation of allergens and allergoids

Crude allergen from *P. juliflora* and allergoids were fractionated by gel filtration on Sephadex G-100 (Fig. 1). Six fractions thus obtained (I, II, III, IV, V and VI) were calibrated with known molecular weight proteins. The elution at 280 nm showed that crude allergen contained proteins of MW 81, 55, 41, 27, 20 and 13 KD. The highly absorbing material was eluted after the bed volume. The prominent peaks appeared in the late fractions and one of these fraction V contained visible yellow pigment of MW 20 KD. the results indicate that fraction V of the allergoid prepared from crude allergen possessed a slightly higher molecular weight (22KD). Fraction V from both crude allergen and allergoid contained 25% carbohydrate, whereas other fractions contained no carbohydrate. Allergoid prepared from the glycoprotein gave only two peaks (I and II) with molecualr weight 200 KD and more than 200 KD (Fig. 1).

Fig. 2 shows the SDS-PAGE pattern of glycoprotein fractions of crude allergen and its allergoid. A

single band was obtained on SDS-PAGE after staining with 0.5% coomassie brilliant blue R; calibration with known proteins suggested the molecular weight to be between 20 and 22 KD.

Antigenicity and allergenicity of allergens and allergoids

Skin test of different fractions exhibited that the allergen activity was localized in fraction V. Averaging the wheal formation in the five sensitized individuals, the response of fraction V from crude allergoid was less as compared to crude allergen fraction.

The gel diffusion test of the allergen gave two predominant and one thin immunoprecipitin bands (Fig. 3a). However, when the allergoids from crude extract were cross reacted with the native allergen rabbit antiserum the precipitin bands were lost. As can be seen from Fig. 3b, antiserum raised against the crude extract allergoid gave three distinct precipitin lines.

Twelve precipitin bands were detected after cross reaction of native rabbit antiserum with crude allergen in second direction (Fig. 4a); native rabbit antiserum could not however bind with the allergoid separated on agarose gel. Antiserum prepared against allergoid cross reacted with electroseparated allergoids prepared from the crude allergen (Fig. 4b). However, polymerized glycoprotein did not form any precipitin bands either with the native crude allergen antiserum or formalinized antiserum of the crude extract.

The result of RAST with the three different preparations, glycoprotein fractions of crude allergen and allergoids and allergoid prepared from purified glycoprotein fraction are shown in Fig. 5. This experiment was performed in order to assess the capacity of active fractions to inhibit the binding of allergen specific IgE to solid phase allergen discs, and to locate the allergenically most active

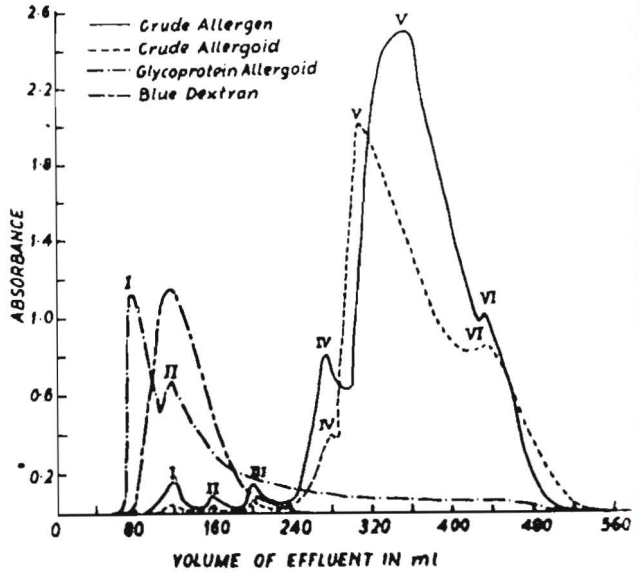

Fig. 1 Gel filtration of crude allergen, its allergoid and allergoid prepared from purified glycoprotein from *P. juliflora* pollen extract on Sephadex G—100. Effective column size was 2.5 x 75 cm, void volume 120 ml, bed volume 365 ml, and elution buffer, 10 mM phosphate buffered saline, pH 7.5. The column was calibrated with known molecular weight proteins (phosphorylase 97.4 KD, bovine albumin 66 KD, egg albumin 45 KD and carbonic anhydrase 29 KD) by Kav, partition coefficient between the liquid phase and gel phase versus the logarithm of their corresponding molecular weight.

allergen and allergoid preparations. An inhibition level of 50% was obtained after incubation of 0.1 μg of purified glycoprotein allergen; for a similar response 1 μg of purified glycoprotein allergoid was required. The allergoid prepared from purified glycoprotein showed a weak inhibitory capacity even at a concentration of greater than 10 μg.

DISCUSSION

In earlier studies we were able to isolate the major allergenic molecules from *P. juliflora* pollen allergen.[8] In the present report allergoids were prepared by treatment with formaldehyde in order to assess changes in chemical properties of allergens and allergoids and their subsequent application in therapy of allergenic diseases. Isolation of major allergens from *P. juliflora* pollen have earlier resulted in separation of five different molecular weight proteins and a glycoprotein.[8,9] Crude allergen extract and formalinized crude allergen gave similar protein profiles in this study. However, the molecular weight of formalinized glycoprotein was slightly higher. This change was also observed in SDS-PAGE. Our study in this regard is similar to that of Puttonen *et al.*[6] who observed slight increase in molecular weight of formalinized timothy pollen allergens. The most significant observation in this study was the more than ten fold increase in the molecualr size of allergoid prepared from purified glycoprotein.

This indicates that the glycoprotein was polymerized but allergoid of crude allergen either polymerized partially or its molecular structure was changed after treatment with formaldehyde. [6,19]

Skin prick test confirmed the allergenicity of fraction V but the glycoprotein modified as allergoid was less active than the native protein. Differential allergenic behaviour of glycoprotein from crude allergen, allergoid and the purified glycoprotein allergoids was confirmed using RAST. The difference in allergenic activities between glycoprotein of crude allergen and allergoid was 10 fold. But the allergenic activities between glycoprotein allergen and polymerized glycoprotein was in excess of 100 fold and compares favourably with the observations of Marsh *et al.* [4,19] and Haddad *et al.* [20] who found a 200-2000 fold reduced allergenic response of allergoids relative to the native allergen. This could be a result of change in molecular structure of formalinized allergen which might decrease avidity to allergen specific IgE and thus weaken the allergenicity of the allergoid. [6,9]

Double gel precipitin reaction confirmed the heterogeneous nature of allergen and allergoid. The loss of precipitin lines during cross reaction between native allergen antiserum and allergoid was likely result of formalin treatment resulting in alteration in antigen determinants. But when antiserum was prepared from formalinized crude allergen, it cross-reacted with modified antigen and multi-precipitin lines were formed. This could be a result of additional or altered antigenic determinants in modified materials resulting from structural changes; cross-reacted with their respective modified antiserum developed in the rabbit. [1,4] The other significant finding of this study was loss of precipitin bands during cross immunoelectrophoresis of allergoid prepared from the crude allergen. This is likely a consequence

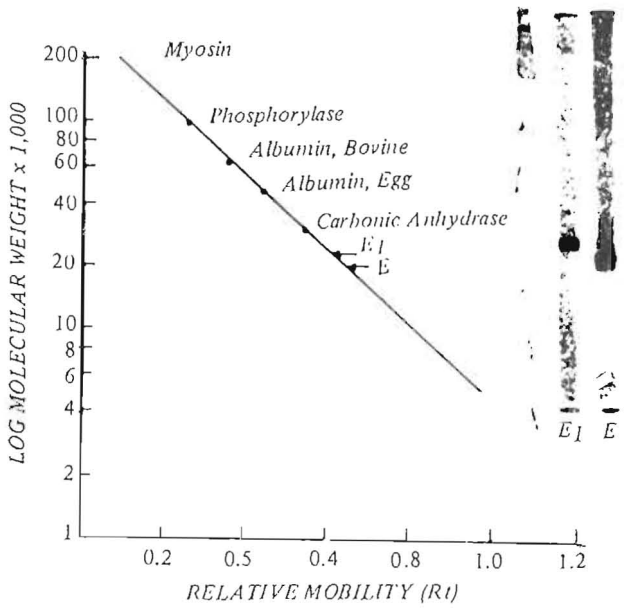

Fig. 2 SDS–PAGE of glycoprotein of crude allergen (E) and allergoid (E1). The separating gel was acrylamide (10%, pH 8.9). The gel was stained with 0.5% coomassie brilliant blue R and molecular weight was determined. Sample size was 100 μg.

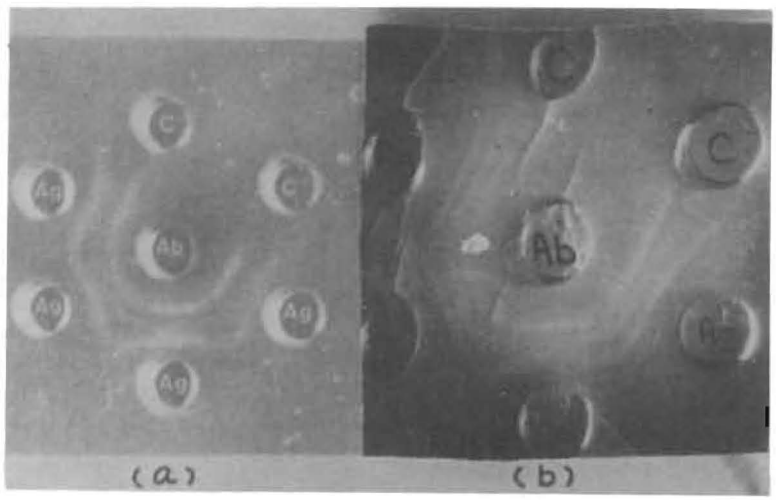

Fig. 3 Immunodiffusion analysis of crude allergen (a), and allergoid (b) of crude allergen of *P. juliflora* pollen anainst *P. juliflora* crude allergen antiserum and formalinized crude allergen antiserum respectively. Phosphate buffer (0.1 M) was taken as control (C); antigen indicated as Ag and antiserum as Ab.

of partial polymerization of some antigens or allergens after treatment with formaldehyde. However no precipitin bands were detected when polymerized glycoprotein was cross reacted with modified antiserum in agarose gel run in second direction. This may be due to altered molecular structure along with the determinants of glycoprotein polymerized with formaldehyde. Characterization of the antigenicity of polymerized purified glycoprotein is currently being investigated.

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REFERENCES

1. Marsh DG, Lichtenstein LM, Norman PS. Induction of IgE-mediated immediate hypersensitivity to group I Rye grass allergen and allergoids in non-allergic man. Immunology 1972; 22 : 1013-28.
2. Patterson R, Suszko IM, McIntire FC. Polymerized ragweed antigen E. Part I. J Immunol 1973; 110 : 1402-12.
3. Puttonen E, Pilstrom L, Wahn W, Maasch HJ. Studies on allergen and allergoid preparations from purified timothy (*Phleum pratense*) pollen extracts. II. Anaphylaxis studies in rats and histamine release from human leukocytes. Int Arch Allergy Appl Immunol 1982; 68 : 7-12.
4. Marsh DG, Lichtenstein LM, Chambell DH. Studies on 'allergoids' prepared from naturally occurring allergens. Assay of allergenicity and antigenicity of formalinized rye group I component. Immunology 1970; 18 : 705-20.
5. Patterson R, Suszko IM, Pruzansky JJ, Zeisscr, Metzger WJ, Robert M. Polymerization of mixtures of grass allergens. J Allergy Clin Immunol 1977; 59 : 314-19.
6. Puttonen E, Massch HJ, Pilstrom L. Studies on allergen and allergoid pre-

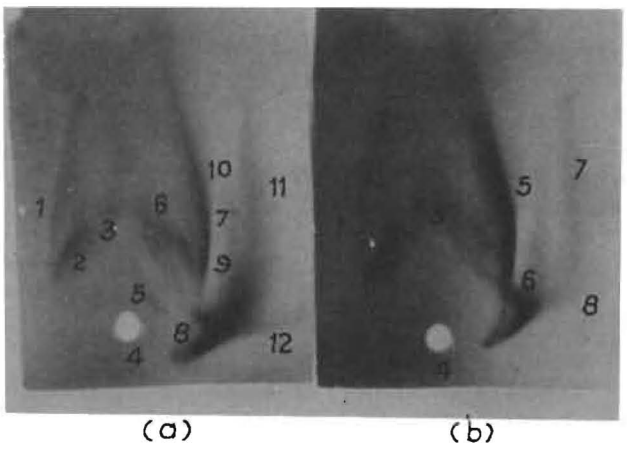

Fig. 4 Crossed immunoelectrophoresis of the *P. juliflora* crude allergen extract (a), and allergoid prepared from crude extract (b). Numbers indicate different antigenic fractions of the extract.

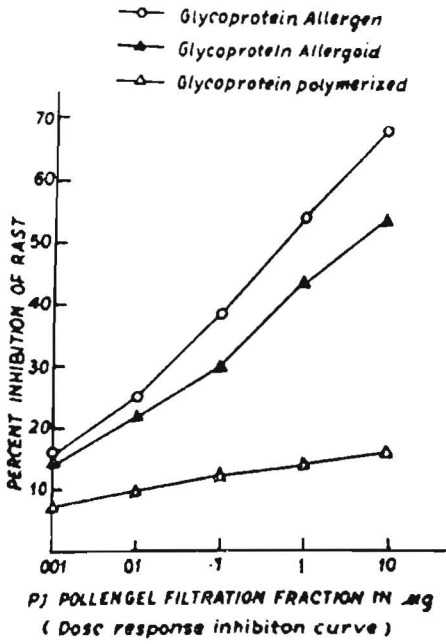

Fig. 5 RAST inhibition assay of different preparations of *P. juliflora* pollen. The results are expressed as percent inhibition of binding of specific IgE to solid phase allergens. This was achieved by addition of different amounts of glycoprotein allergen of crude extract glycoprotein of allergoid prepared from crude extract and formalinized purified glycoprotein obtained after gel filtration, and human *P. juliflora* antiserum obtained from sensitized individuals. Each value is an average of five experiments.

parations from purified timothy *(Phleum pratense)* pollen extracts. I. Physico-chemical characteristics and binding to allergen-specific human IgE. Int Arch Allergy Appl Immunol 1982; 68 : 1-6.

7. Thakur IS, Sharma JD. Analysis of allergenicity and hypersensitivity, of *Prosopis juliflora* pollen grains in guinea pigs. Curr Sci 1987; 56 : 210-12.
8. Thakur IS, Sharma JD. Isolation and characterization of allergens of *Prosopis juliflora* pollen grains. Biochem Int 1985; 11 : 903-12.
9. Thakur IS. Fractionation and analysis of allergenicity of allergens from *Prosopis juliflora* pollen. Int Arch Allergy Appl Immunol 1989; 90 : 124-29.
10. Whitaker JR. Determination of molecular weight of protein by gel filtration on Sephadex. Anal Chem 1963; 35 : 1950-9.
11. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193 : 265-75.
12. Scott TA, Melvin EH. Determination of dextran with anthrone. Anal Chem 1953; 25 : 1656-61.
13. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970; 227 : 680-5.
14. Freund J, McDermett K. Sensitization to horse serum by means of adjuvants. Proc Soc Exp Biol Med 1942; 49 : 548-53.
15. Voorhorst R, Van Krieken H. Atopic skin testing reevaluated. I. perfection of skin testing technique. Ann Allergy 1973; 31 : 137-42.
16. Duchterlony O. Diffusion-in-gel methods of immunological analysis. Prog Allergy. Basel, Karger, 1958; Vol 5, pp 1-78.
17. Weeke B. Crossed immunoelectrophoresis. Scand J Immunol 1973; 2 suppl 1 : 47-8.
18. Ceska M, Eriksson R, Varga JM. Radioimmunosorbent assay of allergens. J Allergy Clin Immunol 1972; 49 : 1-9.
19. Marsh DG, Norman PS, Roebber M, Lichtenstein LM. Studies on allergoids from naturally-occurring allergens. III. Preparation of ragweed pollen allergoids by aldehyde modifications in two steps. J Allergy Clin Immunol 1981; 68 : 449-59.
20. Haddad ZH, Marsh DG, Campbell DH. Studies on allergoids prepared from naturally-occurring allergens. II. Evaluation of allergenicity and assay of antigenicity of formalinized mixed grass pollen extracts. J Allergy Clin Immunol 1972; 49 : 197-209.