

Distribution of Allergen Activity and Identification of the Main IgE-Binding Glycopeptide of *Parietaria judaica* Pollen

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Abstract. *Parietaria judaica* pollen extract was separated by preparative isoelectric focusing and showed two allergenic components (A1 and A2) heterogeneously distributed within the pH gradient (pH 3-10). By fused-rocket immunoelectrophoresis, A1 was detected from pH 3 to 10, whereas A2 was only found at pH values below 8. Both components, A1 and A2, showed IgE-binding ability throughout their pH ranges, as determined by crossed-radio immunoelectrophoresis. In addition, all the preparative isoelectric focusing fractions contained the main allergenic polypeptide of *P. judaica* (Pj10) when analyzed by SDS-PAGE. This polypeptide was shown to be a glycopeptide or to be bound to a glycoprotein with α -D-mannopyranoside or α -D-glycopyranoside residues. Implication of charged sugar residues in the wide distribution of *P. judaica* allergens in a pH gradient is discussed.

Introduction

The study of the molecules which can trigger an allergic response has become a very interesting matter in allergology. Isolation and purification of allergens is now being carried out in many laboratories with the aim of gaining information concerning the allergenic determinants and their interactions with the IgE molecules [Karlstam and Nilsson, 1982].

Parietaria judaica pollen is allergenically the most active among pollens distributed around the Mediterranean area [Ayuso and Carreira, 1984]. In fact, this pollen is the main cause of allergy in zones of Italy [Feo et al., 1984] and Spain, where patients only sensitive to *P. judaica* pollen can be found (G. Sellés, pers. commun.). In a previous paper [Corbí and Carreira, 1984], *P. judaica* pollen allergens were found distributed from pI 4 to 7 by chromatofocusing fractionation. Analysis by SDS-PAGE of these widespread allergens yielded a 10,000-dalton polypeptide (Pj10) which exhibited the highest IgE-binding ability as demonstrated by immunoblotting. In addition, the purification of two proteolytic products which were able to bind IgE from *P. judaica* sensitive patients has been reported [Feo et al., 1984].

In the present paper, the distribution of the two allergenic crossed immunoelectrophoresis (CIE) precipitation bands, A1 and A2 [Corbí and Carreira, 1984], has been investigated by preparative isoelectric focusing. The presence of Pj10 in fractions with different isoelectric points has been demonstrated. Besides, we have studied the presence of Concanavalin A (ConA)-binding allergens in this pollen. Pj10 has been proved to bind to ConA, indicating its glycoprotein nature.

Materials and Methods

Pollen Extract and Inhibition RAST

P. judaica pollen was purchased from Allergon AB (Valinge, Sweden). The pollen extract was prepared as previously described [Corbí and Carreira, 1984]. Protein determinations were done by the method of Lowry et al. [1951]. Inhibition radioallergosorbent test (RAST) was performed according to the procedure reported by Yman et al. [1975]. For comparison of allergenic activities, RAST Unit (RU) was defined as the amount of protein producing 50% inhibition. Specific activity was defined as the total RU in 100 μ g of protein.

Antisera and Immunoelectrophoretic Techniques

Rabbits were immunized by intramuscular injections of the *P. judaica* pollen extract. Each animal received 1 ml of an emulsion of

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Freund's complete adjuvant that contained 1 mg of protein, once a week for the first 2 months. After the second month, the same immunization schedule was followed using Freund's incomplete adjuvant. Rabbit antisera obtained in this way was utilized in CIE, crossed radioimmunoelectrophoresis (CRIE) and fused-rocket immunoelectrophoresis (FRIE). These techniques were performed following the procedures previously described [Svendsen, 1975; Weeke and Lowenstein, 1975]. For CRIE, a human serum pool obtained from 30 *P. judaica*-sensitive patients was used in the incubation of the CIE plates. Patients were selected by clinical history and skin prick test. The pool was made only with sera of RAST class 3 and 4 (Pharmacia, Sweden) and was also utilized for RAST assays and immunodetection of allergenic proteins.

Polyacrylamide Gel Electrophoresis (PAGE) and Immunodetection of Allergenic Polypeptides

PAGE was done in the presence of sodium dodecyl sulphate (SDS-PAGE) as described by Laemmli [1970]. Electrotransfer of proteins, previously separated by isoelectric focusing or SDS-PAGE, was carried out by the method of Towbin et al. [1979]. Further immunodetection of IgE-binding proteins with *P. judaica*-sensitive human serum and ^{125} I-anti IgE polyclonal serum was accomplished as reported [Corbí and Carreira, 1984].

Isoelectric Focusing

Analytical isoelectric focusing (IEF) was performed in a pH gradient from 3.5 to 9.5 following the indications supplied by the manufacturer (LKB, Sweden). The pollen extract was fractionated by preparative IEF in the same gradient of pH. LKB Ultrodex granulated gel was used for the fractionation and the experiment was achieved in the LKB Multiphor system. After 20 h at 8 W constant power, the gel was divided into 30 equal fractions and each fraction was resuspended in distilled water and allowed to settle in a column. After eluting out the proteins and in order to remove most of the ampholytes out of the fractions, these were dialyzed against distilled water for 24 h at 4°C and freeze dried.

Affinity Chromatography on Concanavalin A-Sepharose (Con A-Sepharose)

The pollen extract (10 mg) was passed through a Con A-Sepharose column (1 ml, binding capacity 8.5 mg/ml gel), which had been previously equilibrated with 0.5 M NaCl, 0.02 M Tris-HCl, pH 7.4, containing 10^{-3} M MnCl and CaCl₂. After extensive washing with the same buffer, elution of the proteins retained by the column was carried out with 0.5 M α -methylmannoside in the same buffer. The eluted proteins were dialyzed at 4°C against PBS, pH 7.4, and then subjected to SDS-PAGE and immunodetection.

Results

Analytical Isoelectric Focusing and Fractionation of the Extract

The whole pollen extract was subjected to analytical IEF in a pH gradient from pH 3.5 to 9.5, and most of the proteins focused at isoelectric points below 7, while only some of them exhibited pIs higher than 8 (fig. 1a). Because of the good resolution obtained by

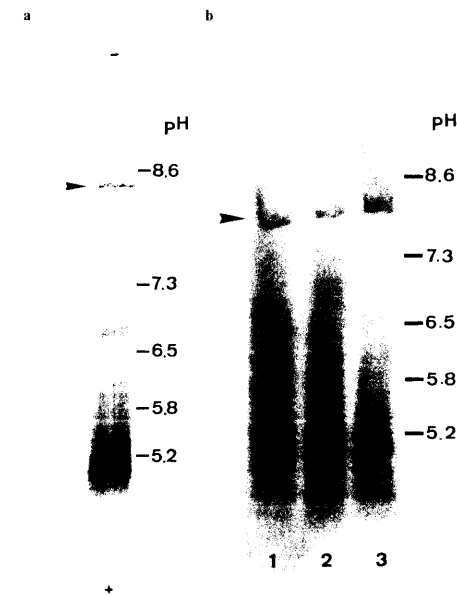


Fig. 1. a Analytical isoelectric focusing on polyacrylamide gel (pH from 3.5 to 9.5) of the whole extract. 150 μ g of pollen protein was applied on a sample application piece (5 $\times$ 10 mm). Separation was carried out for 90 min at 25 W constant power, and the gel was stained with a Coomassie blue solution. pI marker proteins were β -lactoglobulin A (pI 5.2), bovine carbonic anhydrase (pI 5.85), human carbonic anhydrase B (pI 6.55), myoglobin (basic band pI 7.35) and lentil-lectin (basic band pI 8.65). b Immunodetection of IgE-binding proteins after IEF using *P. judaica* sensitive human serum diluted 1:2 (lane 1), 1:4 (lane 2) and 1:8 (lane 3) in PBS. Allergen-bound IgE was detected with ^{125} I-anti IgE. After drying, nitrocellulose sheet was exposed to X-ray medical film for 2 days. Arrow indicates the sample application point. pI marker proteins were the same as in a.

analytical IEF, preparative isoelectric focusing (pH 3-10) was used for fractionation of the extract. 30 equal fractions were cut out of the gel and their protein content and pH were measured (fig. 2). Protein distribution along the gradient was coincident with that obtained by analytical IEF. Allergenicity of the different fractions was measured by inhibition RAST and all of them exhibited IgE-binding ability (data not shown), indicating the allergenic activity was spread all along the pH gradient. This fact was further confirmed by immunodetection of allergenic proteins

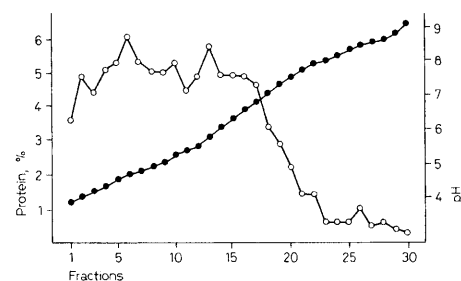


Fig. 2. Preparative isoelectric focusing of the pollen extract. Fractionation was achieved at 8 W constant power for 16 h. 30 fractions were cut out of the gel, dialyzed and their protein content measured and expressed as percentage of the recovered protein (O-O). Measurement of the fractions pH was carried out by taking a portion of each fraction into a test tube which contained 1 ml of distilled water. After shaking for 1 h, pH of the solutions in the test tubes was measured (●-●).

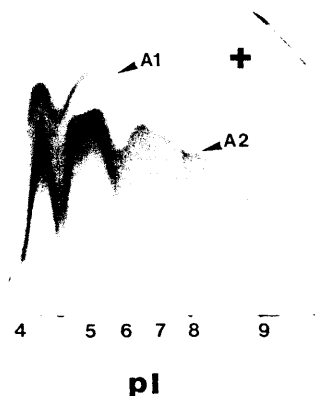


Fig. 3. FRIE of fractions from preparative isoelectric focusing. Samples were applied and allowed to diffuse for 60 min and then subjected to electrophoresis into the antibody-containing gel for 16 h at 150 V. pI of each assayed fraction is shown in the lower part of the plate.

Fig. 5. SDS-PAGE analysis of fractions from preparative isoelectric focusing. The same amount of protein from each fraction was run and the gel was stained with Coomassie blue. pI of the analyzed fractions is shown in the lower part of the gel. Molecular weight marker proteins were (from top to bottom): carbonic anhydrase (29,000 daltons), myoglobin (17,000 daltons) and cytochrome c (12,500 daltons).

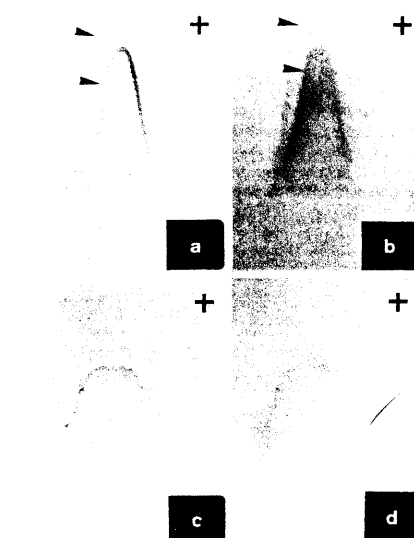
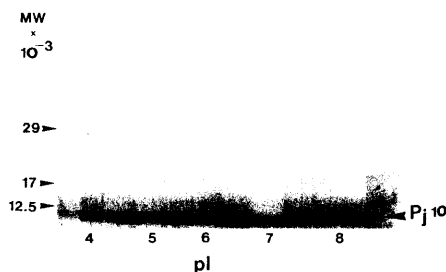


Fig. 4. CIA (a) and CRIE (b) preparation of fraction pI 6.5. 20 μ g of protein was run at 400 V for 30 min in the first dimension. The second dimension against the antibody-containing gel was accomplished at 150 V for 20 h. For CRIE, the CIE plate was dried, incubated with *P. judaica*-sensitive patients serum pool, washed and incubated with ^{125} I-anti human IgE. The radiostaining shown was produced after 2 days of exposure to the film. The arrows indicate A1 and A2 allergenic precipitation bands. For tandem CIE (c) and tandem CRIE (d) of fractions pI 8.2 (left well) and pI 8.5, 15 μ g of protein of each fraction were applied on two adjacent wells. After 30 min diffusion, they were developed as for CIE and CRIE.



after analytical IEF, where most of the focused proteins were able to bind *P. judaica* specific IgE (fig. 1b). Non-specific binding was rejected by running a parallel experiment with *Dermatophagoides pteronyssinus* sensitive patients serum, which yielded no radiostaining. These results demonstrate that *P. judaica* allergenic components are distributed in a wide range of pH. On the other hand, most of the pollen proteins have been shown to possess pIs below 7.

Distribution of the Allergenic Components

To determine the number of IgE-binding proteins giving rise to the broad distribution of the allergenicity, a FRIE of the IEF fractions was performed. Two precipitation bands (A1 and A2) were detected throughout the FRIE-plate, indicating their presence in fractions with different isoelectric points. Nevertheless, at pH values above 8 only the lower precipitate (A1) was observed (fig. 3). Incubations of the immunoplate with *P. judaica* sensitive patients serum pool and ^{125} I-anti IgE demonstrated that the two widespread bands possessed IgE-binding capability. For a better analysis of the distribution of these allergenic complexes, several IEF fractions were subjected to CIE (fig. 4). It was shown that fractions with pI below 8 yielded both A1 and A2 bands, while fractions whose pIs were higher than 8 only exhibited A1 band. CRIE of the selected fractions (fig. 4) showed A1 and A2 with IgE-binding ability regardless of the pI of the fractions yielding the two bands. These results demonstrate that the wide distribution of the allergenic activity along the pH gradient is mainly due to the ubiquitous presence of IgE-binding proteins which give rise to A1 and A2 bands when subjected to CIE.

Detection of Con A-Binding Proteins

In a previous paper [Corbi and Carreira, 1984], Pj10 polypeptide was found to be related with A1 and/or A2 precipitation bands. To determine whether Pj10 was detected in the allergenic fractions, SDS-PAGE analysis of all the IEF fractions was accomplished (fig. 5). All the IEF fractions yielded the allergenic 10,000-dalton polypeptide. The presence of the same polypeptide in fractions with different isoelectric points suggested that different charged sugar residues linked to the protein could be responsible for the isoelectric point heterogeneity. In order to elucidate whether a sugar moiety was attached to this allergenic polypeptide (Pj10), the whole pollen extract was assayed for lectin-binding ability. Several glyco-

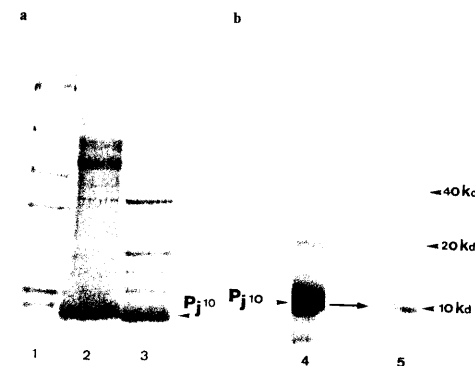


Fig. 6. a SDS-PAGE of the whole extract (lane 2) and Concanavalin A-binding polypeptides (lane 3). Lane 1 shows the molecular weight markers (from top to bottom) phosphorilase (94,000 daltons), catalase (60,000 daltons), aldolase (40,000 daltons), carbonic anhydrase (29,000 daltons), myoglobin (17,000 daltons) and cytochrome c (12,500 daltons). b Immunodetection of IgE-binding polypeptides of the whole extract (lane 4) and Concanavalin A-binding proteins (lane 5). For details, see figure 1b.

proteins were retained by a column of Con A-Sepharose (fig. 6a). Immunodetection of allergenic polypeptides was carried out with the Con A-bound proteins, and a single band of 10,000 daltons (fig. 6b), corresponding to Pj10, was observed. This result can be interpreted in two ways: (a) The main allergenic polypeptide of this pollen is a glycopeptide. (b) Pj10 is bound to a glycoprotein(s). Whichever hypothesis is correct, both of them provide an explanation for the presence of Pj10 in fractions with different isoelectric points, provided that its sugar moiety contains charged sugar residues. This feature would also explain the presence of two bands (A1 and A2) along the pH gradient established in IEF.

Discussion

In the present paper evidences are shown supporting the wide distribution of the *P. judaica* allergenic components in a pH gradient upon separation of the pollen proteins by isoelectric focusing. The two main allergenic precipitation bands of *P. judaica*, A1 and A2 [Corbi and Carreira, 1984], are reported to be distributed differently across the pH gradient established in the preparative isoelectric focusing. A1 was found

from pH 3 to 10, while A2 was spread between pH 3 and 8. All along their distribution ranges they possessed IgE-binding ability as demonstrated by FRIE and CRIE of the IEF fractions. Furthermore, when all the IEF fractions were analyzed by SDS-PAGE, the Pj10 polypeptide was yielded by all of them. Inhibition RAST also evidenced the ubiquitous presence of the allergenic activity along the pH gradient. On comparing the specific allergenic activities of the IEF fractions, differences were observed. Nevertheless, all the fractions were able to inhibit more than 50% of the specific binding of *P. judaica* specific IgE to the whole pollen extract coupled to paper discs.

In a previous report [Corbi and Carreira, 1984], A1 and A2 were described to contain the Pj10 polypeptide. The finding of their different distribution in a pH gradient suggested that, in spite of the presence of a common polypeptide in both CIE precipitation bands, some other distinct molecules could be attached to the Pj10-containing proteins, thus giving rise to distinct CIE bands. In this context, the ability of Pj10 to bind other polypeptides by disulfide bridges has been previously reported [Corbi and Carreira, 1984].

On the other hand, different charged sugar residues attached to the same polypeptidic sequence have been reported to cause a wide distribution of a single protein in a pH gradient [Kornfeld and Kornfeld, 1980]. For this reason, the appearance of sugar residues in the Pj10 structure was tested. The Con A-Sepharose column retained a 10,000-dalton polypeptide with ability to bind IgE, i.e. Pj10. Nevertheless, this polypeptide was also detected among the proteins not retained by the column. As the affinity column was not saturated with protein, it is suggested that either Pj10 or a Pj10-bound glycoprotein might contain different sugar residues (or a different number of sugars attached to them). At present, attempts to identify charged sugar residues in the Pj10 polypeptide are being carried out.

The appearance of two CIE precipitation bands with a common polypeptide can be explained by any of the two exposed hypotheses (Pj10 bound to distinct proteins or to different sugar residues). Nevertheless, the possibility of two polypeptides, each one being a component of A1 or A2, cannot be ruled out. In this last case, these two polypeptides would share properties such as molecular weight, IgE-binding ability and intermolecular disulfide bridges.

Differences in the sugar moieties linked to a pro-

tein has been already described [Parham et al., 1974], and can actually occur in *P. judaica* allergens. Moreover, a number of allergens are known to be glycoproteins [Karlstam and Nilsson, 1982]. For this reason and although the use of lectins has been claimed as a useful method for the isolation of the allergenic molecules [Krill et al., 1981], we consider that monoclonal antibodies against the proteins bearing the allergenic determinants are more advantageous reagents because they bind specifically to the recognized structures, regardless of the polysaccharides linked to them. At present, monoclonal antibodies to *P. judaica* allergens are being characterized in our laboratory and they will facilitate the isolation and knowledge of the molecular relationships among the IgE-binding structures of this pollen.

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