

ISOLATION BY mAb BASED AFFINITY CHROMATOGRAPHY OF TWO *Par j* I ISOALLERGENS. COMPARISON OF THEIR PHYSICOCHEMICAL, IMMUNOCHEMICAL AND ALLERGENIC PROPERTIES*

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Abstract—We report the identification and separation of two isoallergen components of *Par j* I, the major allergen from *Parietaria judaica* pollen. First, electrophoretic conditions for consistently separating both isoforms in an SDS-PAGE system were established, and mol. wt values of 13,000 (isoallergen IA) and 10,500 (isoallergen IB) were estimated. Immunoblot, after SDS-PAGE experiments, with individual *P. judaica*-sensitive human sera revealed a slightly different IgE-binding pattern for each isoallergen. Four anti-*Par j* I mAbs were obtained from BALB/c mice immunized with a purified *Par j* I preparation comprising IA and IB isoallergens. Three mAbs were directed to an epitope shared by both isoallergens, and the fourth one recognized specifically one epitope on *Par j* IB. Dot-blot experiments with the deglycosylated allergen showed that the mAbs did not recognize the carbohydrate prosthetic group of the molecules. Affinity chromatography using the mAbs allowed the separation of the isoallergens that retained their IgE-binding ability after the purification process. Amino acid composition analyses and partial N-terminal sequencing demonstrated an extensive homology and also the existence of some structural differences between *Par j* I isoallergens, which is in agreement with the high, but not complete, cross-reactivity observed in competition ELISA experiments. Finally, skin prick tests performed on 28 *P. judaica*-sensitive patients showed that all of them recognized both isoforms and that allergenic epitopes present in *Par j* IA and IB are responsible for most of the allergenic activity of the whole extract.

INTRODUCTION

Plants belonging to the genus *Parietaria*, mainly *P. judaica* and *P. officinalis* species, are the most important cause of pollinosis in the Mediterranean area (Serafini, 1975; D'Amato *et al.*, 1991). Both species possess a major allergen, *Par j* I and *Par o* I respectively, which are recognized by serum IgE from almost the totality of patients allergic to *Parietaria*. These allergens have been purified and characterized by different methods (Feo *et al.*, 1984; Corbi *et al.*, 1985a; Rubio and Brieva, 1987; Ayuso *et al.*, 1988; Polo *et al.*, 1990, 1991; Oreste *et al.*, 1991), and a certain degree of similarity in their physicochemical features as well as antigenic and allergenic cross-reactivity between them have been observed (Corbi *et al.*, 1985b, c).

As far as *Par j* I allergen is concerned, it has been reported to be a glycoprotein with mol. wt about 10,000 and it shows a considerable charge heterogeneity which is mostly due to the carbohydrate prosthetic groups (Polo *et al.*, 1991). In addition to this carbohydrate heterogeneity, the existence of distinct isoforms of *Par j*

I molecule had been suspected since, sometimes, it could be split into two bands when analyzed on SDS-PAGE (Ford *et al.*, 1986; Ayuso *et al.*, 1988). However, the conditions for consistently separating these bands had not been established.

Thus, the first goal of this work was to find an SDS-PAGE system that would allow us to separate both isoallergens and subsequently to determine, by immunoblotting, the prevalence of specific IgE to each isoallergen in individual sera from *P. judaica*-sensitive patients. Besides, mouse anti-*Par j* I monoclonal antibodies were obtained, whose specificity could be investigated by using this SDS-PAGE system. In a second stage of this work we used anti-*Par j* I mAbs, with different specificities, to isolate both isoallergens. Finally, these isoallergens were characterized by means of several physicochemical methods, and their contribution to the total allergenic activity of the whole extract, and the cross-reactivity between them were studied using *in vivo* and *in vitro* methods.

MATERIALS AND METHODS

Pollen extract

P. judaica pollen was purchased from Allergon AB (Sweden). The extract was prepared by stirring the pollen in 50 mM ammonium bicarbonate solution, pH

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8.0, at a 10% w/v ratio for 60 min at room temperature in the presence of 1 mM phenylmethylsulfonyl fluoride, and subsequent centrifugation, dialysis against distilled water, and lyophilization according to the previously described procedure (Polo et al., 1990).

Protein content was determined by the Folin method (Lowry et al., 1951) using bovine serum albumin as a standard.

Isolation of Par j I allergen

Par j I was purified from *P. judaica* whole extract by sequential gel filtration and reverse-phase HPLC as previously described (Polo et al., 1990). This preparation consisted of a mixture (approximately 1:1) of two isoallergens, as estimated by SDS-PAGE.

Human sera

Human sera from 20 patients with hypersensitivity to *P. judaica*, as demonstrated by clinical history, cutaneous positive reaction, and presence of serum specific-IgE to *P. judaica* extract (Hamlet[®]-IgE, Alergia e Immunologia Abelló S.A., Spain), were used for *in vitro* assays in this work. None of the patients had received immunotherapy at the time of serum collection.

For some experiments, a pool was made by mixing equal volume aliquots from each serum.

Electrophoretic procedures

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed essentially as described (Fling and Gregerson, 1986), using an 8–25% acrylamide gradient in a high molarity Tris-buffer system, but excluding urea from the sample buffer and without prior reduction of the samples.

The following standards were used as mol. wt markers: phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), myoglobin (16,900), lysozyme (14,400), myoglobin (fragment I) (8200), myoglobin (fragment II) (6100), and myoglobin (fragment III) (2500).

Protein detection on the gels was done by Coomassie Brilliant Blue staining.

Immunodetection

Electrotransfer of separated polypeptides onto PVDF membranes (Immobilon, Millipore, Bedford, MA) was carried out by the method of Towbin et al. (1979). For immunodetection of allergenic polypeptides, PVDF sheets were further incubated with *P. judaica*-sensitive human serum and then with radioiodinated HE2-mAb, a mouse anti-human IgE mAb previously produced in our lab (Sánchez-Madrid et al., 1984). PVDF membranes were finally washed, dried and autoradiographed.

Dot immunoblotting

This technique was carried out following the protocol and basic equipment of Bio-Rad (Richmond, CA) Bulletin 1141. Samples were adsorbed to a PVDF membrane and, after washing, the unoccupied sites were

blocked with PBS containing 1% BSA. The membrane was washed again and further immunodetection was accomplished by sequential incubation with hybridoma culture supernatants and radioiodinated polyclonal goat antibodies against mouse Ig (GAM).

Production of mAbs against Par j I

Five male BALB/c mice were immunized with three intraperitoneal injections of HPLC-purified Par j I (50 µg/injection) at 2-week intervals. A week after the third injection, mice sera were assayed for anti-Par j I antibodies following the RIA procedure described below. The mouse with the highest antibody titer was injected intravenously with the same dose of antigen in PBS and four days later its splenocytes were fused to P3-X63/Ag 8653 myeloma cells as described elsewhere (Lombardero et al., 1986a). After 2 weeks, hybridoma culture supernatants were harvested and tested for Par j I-specific antibody production using two different immunoassays.

The first assay was carried out by incubating GAM-coated PVC wells with 50 µl of hybridoma culture supernatants for 1 hr at room temp. After washing with 0.05% Tween 20 in PBS, wells were incubated with Par j I allergen, previously radioiodinated with Bolton-Hunter's reagent (Bolton and Hunter, 1973), for 1 hr at room temp. Finally, the wells were washed and the radioactivity bound was measured in a gamma counter.

Pre-selected hybridomas were subsequently assayed using a different RIA procedure: PVC wells were coated with pure Par j I and afterwards blocked with 1% BSA in PBS. Plates were then washed and 50 µl of culture hybridoma supernatants was added per well and incubated for 1 hr at room temp. After washing, wells were incubated with ¹²⁵I-GAM for 3 hr at room temp and, finally, they were washed and the radioactivity bound was determined.

The IgE-binding ability of the molecular structures recognized by positive mAbs was assessed in a four-step RIA: PVC wells were coated with GAM and, after saturation with 1% BSA in PBS, they were successively incubated for 1 hr with: (1) hybridoma culture supernatants, (2) *P. judaica* pollen extract at 1 mg/ml, (3) serum pool (diluted 1:3 in PBS) from *P. judaica*-sensitive patients, and (4) ¹²⁵I-anti-human IgE rabbit antiserum (Pharmacia, Sweden). Hybridomas with positive reactivity were cloned twice by limiting dilution.

The isotype of the mAbs was determined by immunodiffusion using rabbit anti-mouse subclass antibodies (Nordic, Tilburg, Netherlands). All the mAbs selected were IgG₁ subclass.

Monoclonal antibodies were purified from ascitic fluid produced in BALB/c mice by precipitation with caprylic acid (McKinney and Parkinson, 1987).

Cross-competition experiments with mAbs

PVC wells were coated with 50 µl of a solution of Par j I in PBS at 5 µg/ml, and after saturation with 1% BSA in PBS, they were incubated with 25 µl of several dilutions of each purified mAb. After 2 hr 25 µl of

¹²⁵I-labeled mAb (100,000 cpm in PBS-BSA) was added and incubation continued for three more hr. After washing, the radioactivity bound to the wells was measured.

Competition experiments between mAbs and specific IgE were carried out in a similar way, by incubating Par j I-coated PVC wells with serial dilutions of the mAbs in the presence of a human serum pool from atopic patients (diluted 1:3 with PBS), and, after washing, with 50 µl/well of ¹²⁵I-anti-human IgE mAb.

Purification of Par j IA and Par j IB isoallergens by affinity chromatography

Two immunosorbent columns were prepared by coupling 19 mg of either 2.2.3 or 4.1.3 mAb, purified by caprylic acid precipitation as indicated above, to 1 g of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden).

Par j IA and IB isoallergens were purified from *P. judaica* pollen extract by sequential affinity chromatography with mAbs of appropriate specificity. Thus, *P. judaica* pollen extract (6 ml at a protein concentration of 5 mg/ml in PBS) was passed through the anti-Par j IB 2.2.3-mAb-Sepharose column. The unbound components (Par j IB-depleted *P. judaica* extract) were passed through the anti-Par j I(A + B) 4.1.3-mAb-Sepharose column. After extensive washing with PBS both columns were equilibrated with 10 bed volumes of pre-elution buffer (10 mM phosphate, pH 6.8). Bound proteins were eluted using 100 mM glycine, pH 2.5, and 1 ml-fractions were collected in tubes containing 50 µl of neutralizing buffer (1 M phosphate, pH 8.0). Fractions were pooled on the basis of protein content (as detected by absorbance at 280 nm), dialyzed extensively in Spectra/Por dialysis tubings with mol. wt cut-off 6–8 kD (Spectrum Medical Inc., Los Angeles, CA) against 50 mM ammonium bicarbonate, pH 8.0, lyophilized and stored at 4°C until use.

Preparations of Par j I(A + B) allergen, that is, containing both isoallergens, were obtained by passing *P. judaica* pollen extract through a 4.1.3-mAb-Sepharose column, and eluting as described above.

Amino acid analysis

Protein samples were hydrolyzed at 110°C for 24 hr with vapor phase 6 N HCl. The hydrolysates were derivatized with dansyl chloride (Vendrell and Avilés, 1986) and analyzed in reverse-phase HPLC, using a Dabs-Amino Acid Analysis kit and System Gold HPLC equipment supplied by Beckman Instruments Inc. (San Ramón, CA).

Analyses were performed in triplicate, and no corrections were made for destruction during hydrolysis or incomplete hydrolysis. The content of tryptophan was not determined.

N-terminal sequence analysis

N-terminal amino acid sequencing was performed on samples of Par j IA and IB electrophoresed and electroblotted onto PVDF membranes by using 10 mM 3-(cy-

clohexylamino)-l-propanesulfonic acid, 10% methanol, pH 11, as transfer buffer (Matsudaira, 1987). After Coomassie Blue staining, protein bands were cut out and placed on the cartridge block of an Applied Biosystems (Foster City, CA) 477A liquid-pulse sequencer equipped with an on-line PTH-analyzer model 120A, and sequenced according to the manufacturer's instructions.

Cross-competition between Par j I isoallergens

Cross-inhibition of IgE-binding between Par j I isoallergens was studied by a competition ELISA as follows. Purified proteins were adsorbed to plastic microtiter wells (Immulon II Removawells, Dynatech, Alexandria, VA) by incubating 50 µl of proteins solutions (10 µg/ml in distilled water) per well for 1 hr at 37°C. Then, plates were washed with PBS containing 0.1% Tween 20, and the remaining binding sites blocked with PBS-1% BSA for 1 hr at room temp.

The adsorbed antigens were made to react with a mixture of 25 µl of *P. judaica*-sensitive human serum pool (diluted 1:3 with PBS containing 1% BSA) and 25 µl of serial dilutions of competitor at 4°C overnight, and, after washing, with 50 µl of a stock solution of peroxidase-conjugated HE2-mAb for 1 hr at room temp. The plates were washed five times, as previously, and 50 µl of 1,2-phenyldiamine dihydrochloride (OPD) was added to each well and incubated for 30 min at room temp in the dark. The reaction was stopped with 50 µl of 8 N H₂SO₄ and the absorbance measured at 492 and 630 nm in an automatic microplate reader.

Skin prick test

Purified isoallergens were tested in 28 allergic patients, who gave voluntary consent, with clear hypersensitivity to *Parietaria judaica* pollen as demonstrated by clinical history, cutaneous positive reaction to commercially available *in vivo* diagnostics and presence of serum IgE-specific to *Parietaria judaica* pollen proteins, using commercial diagnostic kits. The concns tested were 6.0, 0.6 and 0.06 µg of purified isoallergens/ml. As controls, dilutions of a standardized extract with activities of 100, 10 and 1 BU/ml were used simultaneously. The activity of 100 BU/ml corresponds to an extract concn which produces in the population of patients allergic to *P. judaica* a geometric mean wheal of 75 mm² (Lombardero et al., 1986b). An extract at 100 BU/ml contains, 6.0 µg/ml of Par j I allergen, as measured by a sandwich ELISA using an anti-Par j I mAb and anti-*P. judaica* rabbit antiserum (Ayuso et al., 1990).

The wheal areas were measured by planimetry, and the mean wheal area of the 28 determinations was evaluated for each concn of sample. The interpatient wheal size coefficient of variation oscillated between 41% and 73%, and the homogeneity of variances was checked by Bartlett's test. Linear regression was performed with the least-square method, and the model: log(area) = intercept + slope × log (allergen concn). Regression lines were checked for linearity, and parallelism was checked by analysis of variance. The relative

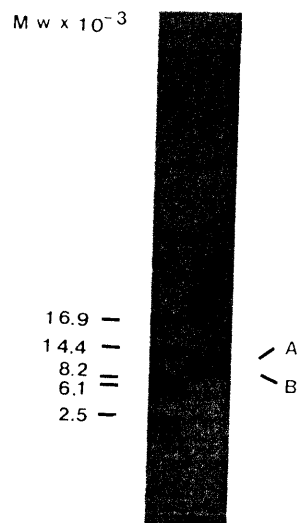


Fig. 1. SDS PAGE analysis of a partially-purified *Par j* I preparation (on the right); *Par j* IA and IB bands are indicated. Molecular weight markers were run on the same gel (on the left).

potency of each sample was calculated with the parallel-line assay (Finney, 1978).

RESULTS

Electrophoretic separation of *Par j* I components

Different electrophoretic conditions were investigated aiming at consistently separating the isoallergenic components of *Par j* I. Finally, an SDS-PAGE system, using an 8–25% acrylamide gradient in a high-molarity Tris-buffer system without urea (Fling and Gregerson, 1986)



Fig. 2. SDS-PAGE-immunoblotting with individual sera. *P. judaica* pollen extract (200 µg per lane) was subjected to SDS-PAGE in the same conditions as in Fig. 1. Separated polypeptides were transferred to PVDF membranes, which were subsequently incubated with sera from *P. judaica*-sensitive patients (a–t) and ¹²⁵I-anti-human IgE HE2 mAb.

and in the absence of any reducing agent, was shown to be a suitable procedure for the separation of *Par j* I into two bands (Fig. 1). Estimations of apparent molecular weight under these conditions rendered values of 13,000 and 10,500 daltons for *Par j* IA and IB, respectively.

IgE-binding patterns of *Par j* IA and IB

Once the electrophoretic conditions had been set, the IgE-binding specificity of each *Par j* I component could be studied by immunoblotting with 20 individual sera from *P. judaica* allergic patients (Fig. 2).

Although most sera reacted in a similar way with both isoallergens, some differences in the radiostaining intensity of A and B were observed for a few patients. Thus, serum *i* displayed a higher IgE binding to *Par j* IA, whereas IgE from sera *a*, *g*, and *j* preferentially bound isoallergen IB.

This experiment evidenced a high homology between the IgE-reactivity patterns of both *Par j* I components, but it also suggested the presence of some different IgE-binding sites in each molecule.

Obtention and characterization of anti-*Par j* I mAbs

In order to further proceed with the study of the homologies and differences in structure and activity between *Par j* I isoallergens, an attempt was made to obtain monoclonal antibodies against them that could allow their isolation. BALB/c mice were immunized with HPLC-purified *Par j* I (containing both isoallergens) as described in Materials and Methods. After two different screenings, four monoclonal antibodies against *Par j* I were selected. The confirmation that these mAbs recognized *Par j* I in allergenically active form was accomplished by means of a four-step RIA with a *P. judaica*-sensitive patients serum pool.

Cross-competition experiments with anti-*Par j* I mAbs indicated that they defined, at least, two different epitopes. Three out of the four mAbs (1.2.3, 4.1.3 and 6.4.4) completely inhibited the binding of each other to *Par j*

Table 1. Cross-inhibition experiments between mAbs, and inhibition of IgE-binding to *Par j* I by the mAbs

Inhibitor	¹²⁵ I-labeled mAb				IgE
	1.2.3	4.1.3	6.4.4	2.2.3	
1.2.3	97	98	99	43	23
4.1.3	97	97	98	52	23
6.4.4	99	99	99	18	24
2.2.3	14	9	17	98	0

The binding of ¹²⁵I-mAbs or specific IgE to *Par j* I-coated wells was inhibited by increasing concentrations of cold mAbs as described in Materials and Methods. Figures represent the maximum inhibition percentage reached in each case.

I (Table 1). On the other hand, 2.2.3 mAb only slightly inhibited the binding of the other mAbs.

Concerning the cross-reactivity of these mAbs with specific IgE, the first three mAbs mentioned above could inhibit up to the same extent (24%) the binding of IgE to *Par j* I; however, mAb 2.2.3 did not affect this binding at all.

In order to elucidate some structural features of the epitopes defined by the anti-*Par j* I mAbs, a dot-blot experiment with deglycosylated, reduced and alkylated, and native allergen was carried out. The results obtained (Fig. 3) seem to suggest that the mAbs do not recognize carbohydrate prosthetic groups of *Par j* I, since the binding to both the native and the deglycosylated allergen was similar. In contrast, none of the mAbs bound the reduced and alkylated allergen, indicating that the epitopes defined by these mAbs are conformational.

Purification and physico-chemical characterization of *Par j* I isoallergens

Immunoblotting after SDS-PAGE demonstrated that three out of the four mAbs (1.2.3, 4.1.3, and 6.4.4) recognized both *Par j* I isoallergens, whereas 2.2.3 mAb only recognized the isoallergen *Par j* IB (data not shown). This different specificity allowed the purification

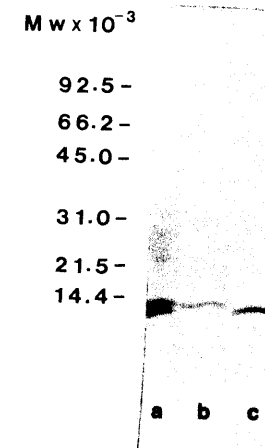


Fig. 4. SDS SAGE analysis of purified *Par j* I isoallergens. Lane a: a partially-purified allergen preparation containing both isoallergens as a reference; lane b: affinity-purified *Par j* IA; lane c: affinity-purified *Par j* IB.

of IA and IB isoallergens by sequential affinity chromatography with 2.2.3 and 4.1.3 mAbs, as described in Materials and Methods. Purity of the isolated isoallergens was assessed by SDS-PAGE (Fig. 4). Averaged recoveries were 1% of total *P. judaica* extract protein for isoallergen IA, and 3% for isoallergen IB.

The results from amino acid composition analyses (Table 2) showed that *Par j* IA and IB are distinct proteins, although they are highly homologous. The most remarkable differences were observed in the content of Glx, Gly, Pro and Ile, and the absence of Tyr in *Par j* IB.

A similar conclusion can be reached from *N*-terminal amino acid sequencing (Fig. 5). Thus, the two first amino acid residues of *Par j* IA were different from those found

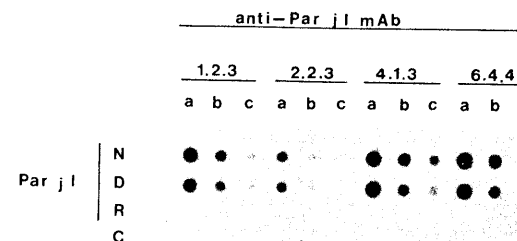


Fig. 3. Dot-blot analysis of samples from different *Par j* I preparations. N: native, D: deglycosylated and R: reduced and alkylated allergen, were adsorbed to a PVDF membrane, at serial 1:3 dilutions from 10 µg/ml (columns a, b and c). After a blocking step, the adsorbed samples were incubated with radiolabeled anti-*Par j* I mAbs. Row C: BSA was adsorbed to the membrane as a negative control.

Table 2. Amino acid composition of *Par j* 1 isoallergens

Amino acid	<i>Par j</i> 1A	<i>Par j</i> 1B
Lys	12.1	13.1
His	3.3	3.6
Arg	3.0	2.4
Asx	5.8	5.9
Thr	7.2	7.6
Ser	5.9	6.3
Glx	15.6	12.2
Pro	5.7	7.2
Gly	7.0	9.1
Ala	7.4	6.7
Cys	3.7	3.7
Val	6.9	7.5
Met	1.7	2.6
Ile	7.1	5.4
Leu	4.8	5.4
Tyr	0.9	--
Phe	1.8	1.3

Results are expressed as the number of residues per 100 residues.

in 1B; but the rest of the sequences, from the position 3 of 1A and the position 4 of 1B, were identical, except that they were shifted by one position.

Allergenic activity of *Par j* 1 isoallergens

The cross-reactivity between the isolated isoallergens was investigated by a competition ELISA, using a serum pool from *P. judaica* allergic patients (Fig. 6). *Par j* 1A and 1B showed extensive cross-reactivity since both of them strongly inhibited the binding of IgE to each other (1A reached 80% inhibition; 1B, 90%), thus indicating that most allergenic epitopes are common to both isoallergens. However, these results also seem to suggest that each isoform, especially *Par j* 1B, might bear some unique allergenic determinants.

In order to study the clinical relevance of *Par j* 1 isoforms, whole *P. judaica* pollen extract, and purified isoallergens, prepared as indicated in Materials and Methods at three different concentrations, were assayed by skin prick test in 28 *P. judaica*-allergic patients. All patients tested were sensitive to the purified isoallergens, and the size of the wheals produced in most patients with these preparations were similar to those obtained with the whole extract. Thus, the mean wheal size at the highest concn tested for *Par j* 1A and 1B were, respectively, 80% and 93% of that of the whole extract. The

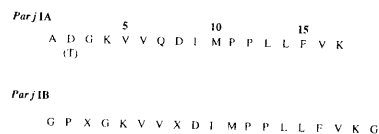


Fig. 5. NH₂-terminal amino acid sequences of *Par j* 1 isoallergens. Sequencing was performed on samples blotted onto PVDF membranes after SDS-PAGE. Amino acids indicated in brackets could substitute at these positions.

results obtained with each preparation were compared by parallel-line assay and are indicated in Fig. 7. *Par j* 1A demonstrated a parallel response to the whole extract preparation and a potency estimate of 80%. In contrast, *Par j* 1B did not show a parallel response.

DISCUSSION

In this work, electrophoretic conditions have been set to separate *Par j* 1 isoallergens. The SDS-PAGE system employed was essentially as described by Fling and Gregerson (1986). The main innovations of the method reported here compared to those used previously (Ayuso *et al.*, 1988; Ford *et al.*, 1986) are the higher concn of Tris-buffer of the resolving gel and running buffer, which brings about an increased sharpness of the bands and a better resolution, and the absence of 2-mercaptoethanol in the sample buffer. The latter condition could imply, however, that the difference in the apparent mol. wt values observed might be, at least, partially due to a conformational dissimilitude between both isoallergens when disulphide bridges are not disrupted.

The use of this SDS-PAGE system followed by immunoblot with individual patients' sera yielded additional information about the IgE-binding profile of *P. judaica* pollen. Thus, although most patients had specific IgE that recognized both isoallergens, some of them had IgE antibodies that mainly bound to only one isoallergen. These results, with the exception of the inconvenience inherent to this technique for the possible loss in the activity of some labile epitopes, strongly suggest a certain degree of singularity of some allergenic determinants in each molecule. In addition, this uniqueness of certain epitopes does not seem to be restricted to human IgE, as a murine IgG monoclonal antibody that recognizes only *Par j* 1B has been obtained from mice immunized with a *Par j* 1 preparation consisting of a mixture (approx. 1:1) of isoallergens 1A and 1B. The specificity of this mAb was not related to possible differences in the carbohydrate moiety, according to the results obtained in a dot-blot experiment with the deglycosylated allergen (Fig. 3) and hence implies a singularity in the polypeptide part of the molecule.

Nevertheless, it is worth mentioning here that the immune response, both human IgE and murine IgG, seems to be mostly directed to epitopes shared by *Par j* 1A and 1B isoallergens. For instance, three out of the four mAbs obtained in this work recognized an epitope present in both isoallergens which was demonstrated to be localized in the protein moiety and to be conformation dependent (Fig. 3), and that must be related to an IgE-binding site in *Par j* 1 (Table 1). These results indicate a close homology between *Par j* 1 isoallergens in spite of the differences remarked above. In fact, this homology had previously precluded the separation of these isoallergens. Thus, size-exclusion-HPLC failed to resolve them under different conditions (Ayuso *et al.*, 1988; Polo *et al.*, 1990, 1991), and even they eluted together when chromatographed on a reversed-phase HPLC column (Polo *et al.* 1990). In the same way, mAbs

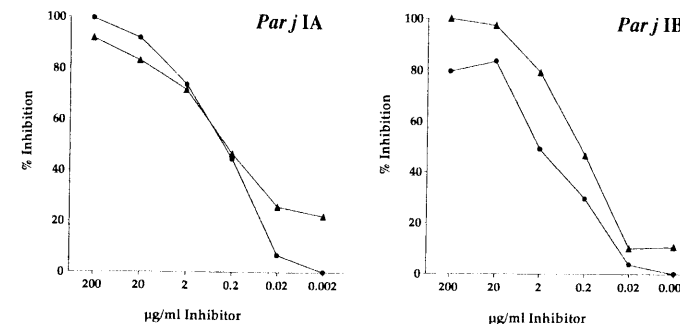


Fig. 6. Competition ELISA curves. Purified isoallergen (indicated in the graphic) was adsorbed to plastic microtiter wells and the binding of specific IgE from a *P. judaica* patients serum pool was inhibited by the addition of increasing concentrations of *Par j* 1A (●) or *Par j* 1B (▲).

previously produced in our lab (Corbi *et al.*, 1985a) recognized both isoforms and it was not possible to isolate them by affinity chromatography.

Structural data obtained with the purified isoallergens confirmed the strong homology between them, and also some differences were revealed. In fact, they only differed in the three first N-terminal amino acids out of the 17 residues sequenced and, besides, the percentages obtained in the amino acid composition analyses were only remarkably different for four amino acids. Nevertheless, these variations must be sufficient to originate the allergenic differences observed with the distinct *in vitro* and *in vivo* techniques used in this work.

The N-terminal sequence of *Par j* 1A coincides with the sequence reported by Ruffilli *et al.* (1991) for the first twelve amino acid residues of *Par o* 1, then confirming the close relationship between the major allergens of these *Parietaria* species. In agreement with this finding, some preliminary experiments using the anti-*Par j* 1

mAbs described in this article seem to indicate that *Par o* 1 bear the same or related epitopes to those in *Par j* 1 (Ayuso R., unpublished results). On the other hand, no homology between the sequence of *Par j* 1 and other known protein sequences, apart from that of *Par o* 1, was found when a sequence homology search was carried out.

The clinical relevance of *Par j* 1 isoforms has been proved from the results of *in vivo* assays. In effect, a positive cutaneous reaction was developed by all the subjects in a group of 28 *P. judaica*-sensitive patients when skin prick tested with the purified isoallergens, and, in addition, these isoallergens produced a mean wheal size comparable to that obtained with the whole extract. Consequently, it can be deduced that most of the allergenic potency of *P. judaica* pollen is caused by *Par j* 1A and 1B. Nevertheless, a distinct behaviour of these isoallergens could also be observed in this experiment when the results were analyzed by parallel-line assay, so that the response of *Par j* 1A was parallel to that of the whole extract whereas *Par j* 1B response was not. This fact is mainly due to the high values of the wheal size produced in some patients by *Par j* 1B at the lowest concn. As a possible explanation, it might happen that certain determinants, unique to *Par j* 1B, elicited a strong IgE response in some individuals, and, on the contrary, the rest of the allergenic epitopes would cause a weaker but more widespread response in the allergic population. In this way, the effect of the former epitopes in skin prick test at higher concentrations of allergen would be statistically minimized.

In conclusion, two isoforms of the major allergen from *P. judaica* pollen have been identified, and mAbs against them have been produced. These isoallergens have been purified by affinity-chromatography using the mAbs, and subsequently characterized as for their physico-chemical and allergenic properties. From the results presented in this work, it can be concluded that proper *P. judaica* pollen extracts intended for clinical uses must contain both *Par j* 1 isoallergens.

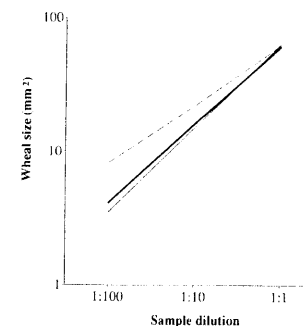


Fig. 7. Skin test activity of whole *P. judaica* extract (—), and purified isoallergens *Par j* 1A (—) and 1B (---), in 28 *P. judaica*-allergic patients. The figure shows the regression line obtained with each preparation as indicated in Materials and Methods.

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