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PURIFICATION OF *Par j* I, THE MAJOR ALLERGEN OF *PARIETARIA JUDAICA* POLLEN

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Abstract—A component of *Parietaria judaica* pollen extract, previously identified as the major allergen, then reported as Pj10 and hereafter denominated *Par j* I has been isolated by a combination of 65% ammonium sulphate salt precipitation and gel filtration and an Ultrogel AcA54 column. The purified allergen appeared essentially homogeneous on gel filtration HPLC. The mol. wt of *Par j* I was estimated by electrophoretic and chromatographic techniques. All results gave values in a range from 13 K to 25 K. Analysis in SDS-PAGE under reducing conditions revealed a broad band corresponding to a mol. wt of 10 K, which retained allergenicity when tested with a patients serum pool. CIE and CRIE patterns of the isolated *Par j* I displayed the two precipitating lines already reported as those exhibiting the highest IgE-binding ability. *Par j* I showed a specific allergenic activity about 10-fold higher than that of the whole extract and was demonstrated to be the major allergen of *Parietaria judaica* as assessed in 25 sensitive human sera.

INTRODUCTION

Most allergen preparations used in diagnosis, therapy and research of allergic diseases are complex mixtures of biological molecules inadequately defined. As was shown in a number of studies, treatments with such preparations may give rise to the production of IgE antibodies to some constituents to which the allergic individuals had not been originally sensitized (Marsh *et al.*, 1972; Richter *et al.*, 1975). This is why purified components containing the clinically relevant allergens are highly desirable.

Recently, much work has been directed towards the identification and purification of major allergens from some allergenic extracts. Thus, *Amb e* I (AgE) and *Amb e* II (AgK) (King and Norman, 1962; King *et al.*, 1964), *Phl p* I (AgB) (Malley and Harris, 1967), *Lol p* I (AgI) (Johnson and Marsh, 1965), *Der p* I (P₁) (Chapman and Platts-Mills, 1980), *Alt a* I (Alt I) (Yunginger *et al.*, 1980) *Fel d* I (Cat I) (Ohman *et al.*, 1974) are some of the most important allergens so far purified.

As far as *Parietaria judaica* pollen is concerned, in a previous paper (Corbí *et al.*, 1986) monoclonal antibodies were described which specifically recognized four polypeptides from this pollen. All of them were jointly purified by affinity chromatography on a monoclonal antibody-Sepharose column and were shown to be antigenically cross-reactive and to exhibit IgE-binding ability. The most important one, then denominated Pj10 (*Par j* I), had already been identified as the main allergenic component

of *P. judaica* pollen that exhibited the highest IgE-binding ability by immunodetection after SDS-PAGE (Corbí and Carreira, 1984).

The aim of this study was therefore to establish an isolation procedure which permitted the purification of *Par j* I, free from the other three polypeptides, in a yield and state of purity suitable for subsequent characterization and investigation of its allergenic significance by several methods.

MATERIALS AND METHODS

Preparation of the pollen extract

Parietaria judaica pollen was purchased from Allergon AB (Sweden). The extract was prepared by mixing the pollen with 50 mM ammonium bicarbonate solution at a 10% w/v ratio and stirring for 60 min at room temp, followed by centrifugation at 39,100 g for 15 min. The supernatant was removed from the pellet and dialysed for 24 hr against distilled water at 4°C. Then it was recentrifuged, filtered and freeze-dried. The lyophilized extract was stored at 4°C until use.

Isolation of the allergen

Parietaria judaica pollen extract was reconstituted with distilled water to a final protein concn of 1 mg/ml. Solid ammonium sulphate was then gradually added, at 4°C, with stirring, to 50 ml of the whole extract until a 65% concn was reached. The precipitate was separated from the supernatant by centrifugation at 12,100 g for 30 min at 4°C and resuspended in distilled water. Both supernatant and precipitate were extensively dialysed against distilled water in a

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dialysis tube with a mol. wt cut-off of 6 K, and freeze-dried.

The freeze-dried supernatant was dissolved in 50 mM ammonium hydrogen carbonate buffer (pH 8.0) and subjected to gel filtration on an Ultrogel AcA54 column (0.9 × 57 cm) pre-equilibrated with the same buffer. The column was eluted at a flow rate of 2 ml/hr and fractions of 1 ml were collected. The absorbance was monitored throughout the elution at 280 nm using an LKB Uvicord II detector. All fractions were analysed by SDS-14% PAGE, and those containing *Par j* I were pooled and freeze-dried. The entire procedure was performed at 4°C.

Protein content was estimated by the dye binding method of Bradford (Bradford, 1976), using the commercially available reagents from Bio-Rad (Richmond, California).

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 (Laemmli, 1970), under reducing conditions, unless otherwise indicated. The following standards were used as mol. wt markers: phosphorylase A (mol. wt 94 K), catalase (60 K), carbonic anhydrase (29 K), myoglobin (17 K) and cytochrome *c* (12.5 K). Electrophoretic transfer of separated polypeptides was carried out by the method of Towbin (Towbin *et al.*, 1979). Further immunodetection of IgE-binding polypeptides with *P. judaica*-sensitive human serum pool and Hamlet¹²⁵I (Alergia e Immunologia Abelló, S.A.), an iodinated mouse anti-human IgE monoclonal antibody (Sánchez-Madrid *et al.*, 1984), was performed as reported (Corbi and Carreira, 1984).

Radioallergosorbent test (RAST) and RAST inhibition assays

The allergenic activity of the different samples was measured by RAST inhibition assays basically performed as described by Yman (Yman *et al.*, 1975).

In order to compare activities based on RAST inhibition, sp. act. was defined by the amount of protein that produces a 50% inhibition of the binding of specific IgE from a human reaginic serum pool to solid-phase allergens.

Direct RAST was carried out using, as allergosorbent, either whole pollen extract or *Par j* I covalently linked to CNBr-activated paper discs (Ceska *et al.*, 1972). These were sequentially incubated with 50 µl of individual *P. judaica*-sensitive human serum and Hamlet¹²⁵I. After washing, the binding of specific IgE to the allergosorbents was determined by measuring the radioactivity bound to the discs.

Crossed immunoelectrophoresis (CIE) and crossed radioimmunoelectrophoresis (CRIE)

In the first dimension of CIE (Weeke, 1973) and CRIE (Weeke and Lowenstein, 1973), samples were

run for 30 min at 10 V/cm, in a 1% agarose gel. The second dimension against the anti-*P. judaica* rabbit serum containing gel was carried out at 150 V for 20 hr. The temp was maintained at 4°C. For CIE, the gels were stained with Coomassie blue R-250, and for CRIE, the CIE plates were incubated with *P. judaica*-sensitive patients serum pool and Hamlet¹²⁵I.

Molecular wt estimation

The mol. wt of the purified allergen *Par j* I was estimated by the following methods:

(a) *SDS-14% PAGE*. This was carried out as described above. Prior to electrophoresis, samples were incubated at 100°C for 5 min in Laemmli's buffer containing 5% 2-mercaptoethanol or, alternatively, in the absence of this reducing agent.

(b) *Size-exclusion chromatography on Ultrogel AcA54*. Elution conditions were exactly the same as described above. Dextran blue 2000, bovine serum albumin (mol. wt 67 K), ovalbumin (45 K), carbonic anhydrase (31 K), and myoglobin (17 K) were used to calibrate the column.

(c) *Gel filtration on a Spherogel TSKG 3000 SW column*. The column (21.5 × 300 mm) was supplied by Beckman. Chromatography was carried out on HPLC equipment from Waters comprising a Model 680 gradient programmer, a 510 pump, a U6K injector, and a 490 multiwavelength u.v./visible detector. Absorbance was monitored at 280 nm and 425 nm. Mobile phase consisted of 0.1 M Na₂SO₄, 0.02 M NaH₂PO₄, pH 6.8, and the column was eluted at a constant flow rate of 2 ml/min. Calibration of the column was carried out by injection of a Gel Filtration Standard from Bio-Rad containing thyroglobulin (mol. wt. 670 K), gamma globulin (158 K), ovalbumin (44 K) and myoglobin (17 K).

RESULTS

Isolation of *Par j* I

Ammonium sulphate salt precipitation of the whole extract of *P. judaica* pollen was tried out as a first step of purification of *Par j* I. Figure 1 shows the results obtained for four different concns of the precipitating agent. It can be observed that increasing percentages (w/v) of ammonium sulphate produce larger salting-out of proteins, mainly those with mol. wt higher than 20 K, so that the 60 and 67% supernatants contain a 10 K polypeptide only slightly contaminated by proteins of higher mol. wt and some pigments. In order to optimize the method, a similar assay was performed with several ammonium sulphate percentages ranging between 55 and 67% (results not shown). This experiment allowed us to select the 65% concn as the most suitable one to get the best correlation between yield and purity for the protein isolated in the supernatant.

The freeze-dried allergen from the 65% supernatant was further purified by gel filtration chro-

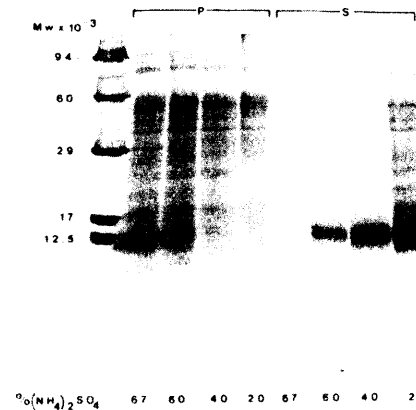


Fig. 1. SDS-14% PAGE analysis of supernatants (S) and precipitates (P) from four concns (20, 40, 60 and 67%) of ammonium sulphate. Aliquots of precipitates and supernatants were subjected to SDS-14% PAGE and subsequently stained with Coomassie blue. Molecular wt markers (MW) are as in Materials and Methods.

matography, and its elution profile is shown in Fig. 2(a). *Par j* I eluted in several fractions with an elution vol similar to that of myoglobin (17 K), but the maximum protein concn (fractions 22 and 23) did not

coincide with the highest absorbance at 280 nm. Pigments distribution seemed to be responsible for this discrepancy, thus leaving *Par j* I free from most non-protein contaminants. Figure 2(b) illustrates the

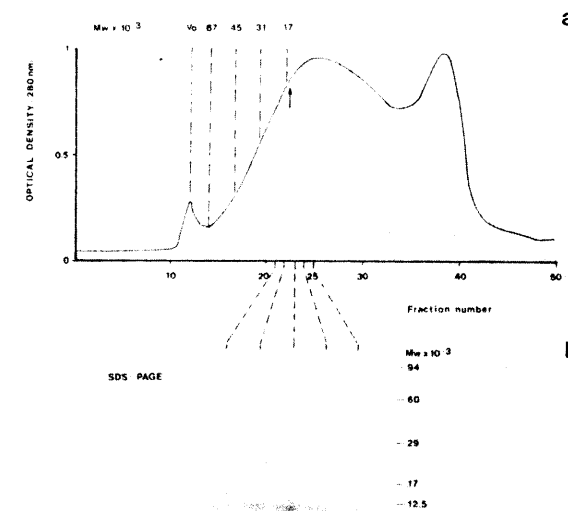


Fig. 2. (a) Elution profile of the 65% ammonium sulphate supernatant on Ultrogel AcA54 monitored at 280 nm. The void vol of the column as well as the elution vols of mol. wt markers (67, 45, 31 and 17 K) are indicated. The arrow shows the elution vol of *Par j* I. (b) SDS-14% PAGE under reducing conditions of fractions 20-25. Molecular wt markers (MW) are as in Materials and Methods.

Mw x 10⁻³

94—

60—

29—

17—

12.5—

Fig. 3. SDS-14% PAGE analysis of purified *Par j* I, under reducing conditions, stained with silver nitrate. Molecular wt markers (MW) are the same as indicated in Materials and Methods.

SDS-PAGE pattern of various fractions. After pooling and dialysis, these fractions presented a nearly homogeneous pattern following silver nitrate staining (Merril *et al.*, 1979) (Fig. 3).

Immunodetection of purified *Par j* I

The allergenic activity of the isolated *Par j* I was demonstrated by the electroblotting procedure after SDS-PAGE. Figure 4 shows that the whole extract and the 65% ammonium sulphate precipitate display several polypeptides with capacity to bind specific human IgE, although the concn of the 10 K polypeptide appears reduced in the precipitate as compared with the whole extract. On the contrary, the 65% supernatant could only demonstrate a band with IgE binding ability which was revealed to be a doublet.

Allergenic activity of the purified allergen

The allergen sp. act. of *Par j* I was evaluated by RAST inhibition assay, using a human reaginic serum pool. In the same assay, *P. judaica* whole extract and 65% ammonium sulphate precipitate activities were also estimated. The inhibition curves shown in Fig. 5 point out the fact that purified *Par j* I possesses an activity about 10-fold higher than that of the

whole extract, whereas the 65% ammonium sulphate precipitate accounts only for a half of the whole extract activity.

The capacity of *Par j* I to induce an IgE response in humans was studied in 25 *P. judaica*-sensitive patients. The results, listed in Table 1, indicate that all the patients tested possessed specific IgE to the purified allergen in large quantities, and even some patients displayed similar IgE-binding capacity to both solid phases, the whole extract and the purified *Par j* I.

CIE and CRIE analysis

The CIE and CRIE patterns of the isolated *Par j* I (Fig. 6) exhibited the two main precipitating arcs of the whole extract, already reported as A1 and A2 in a previous paper (Corbí and Carreira, 1984). These lines were found to have the highest IgE-binding ability of *P. judaica* pollen extract.

Molecular wt estimation of *Par j* I

The mol. wt determination of *Par j* I was performed by electrophoretic and chromatographic techniques.

SDS-PAGE experiments were done alternatively in the presence and absence of reducing agent. The electrophoretic patterns under both conditions are

Mw x 10⁻³

94—

60—

29—

17—

12.5—

E P *Par j* I

Fig. 4. Immunodetection of IgE-binding polypeptides after SDS-14% PAGE in *P. judaica* pollen extract (E), 65% ammonium sulphate precipitate (P) and *Par j* I isolated in the 65% supernatant, using a serum pool from *P. judaica*-sensitive patients.

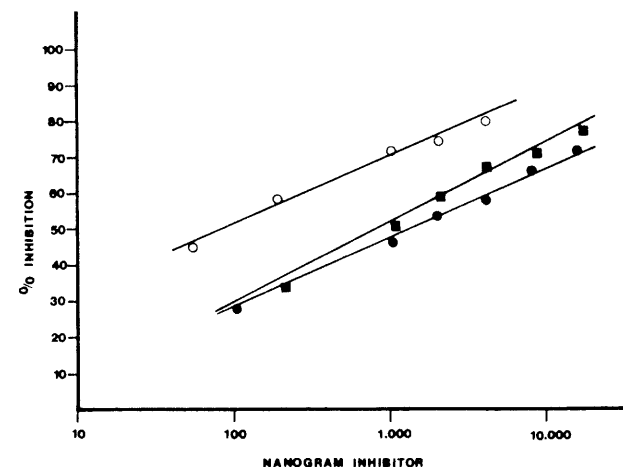


Fig. 5. RAST inhibition curves of *Par j* I (○) compared with whole *P. judaica* extract (■) and 65% ammonium sulphate precipitate (●). Paper discs were coupled with whole extract. Serum pool was from untreated patients allergic to *P. judaica* and diluted 1:3 with PBS.

shown in Fig. 7. *Par j* I exhibited a single band with a mol. wt of 10 K when 2-mercaptoethanol was added. In the absence of this reducing agent the isolated allergen gave a broad and diffuse band in a mol. wt range from 15 to 25 K.

The mol. wt of *Par j* I under native conditions was estimated by gel filtration through the Ultrogel AcA54 column used in the second purification step described above. The elution chromatogram is shown in Fig. 2. The mol. wt determined by this method and deduced in terms of its elution vol was in a region of 13.5–16 K with reference to standard globular proteins.

Finally, the mol. wt of *Par j* I was evaluated by gel filtration on an HPLC TSKG 3000 SW column. Figure 8 shows the elution profile monitored at 280 and 425 nm. *Par j* I eluted as a single symmetrical and sharp peak at a retention time of 43 min. Then, from the calibration curve, a mol. wt of 13 K could be deduced, under native conditions. Pigments accompanying *Par j* I could not be separated from the protein in these conditions of elution, and it might be the reason for the observed absorbance at 425 nm.

DISCUSSION

In a previous paper we reported the purification of *P. judaica* main allergenic structure, by means of affinity chromatography, using monoclonal antibodies specific for *P. judaica* allergens (Corbí *et al.*, 1985). The complex allergen then purified consisted of, at least, four different allergenic components, not covalently linked, which shared an identical epitope for the monoclonal antibody (Corbí *et al.*, 1986). One

Table 1. Binding of human IgE from *Parietaria judaica*-sensitive patients to *Par j* I and *P. judaica* whole extract

Patients sera	<i>Par j</i> I (cpm)	<i>P. judaica</i> (cpm)
1	5396	10844
2	3244	6944
3	6206	9656
4	7124	11906
5	13770	22134
6	5906	10384
7	13874	18940
8	9056	13060
9	15720	16314
10	10724	17062
11	7750	12584
12	8032	11418
13	4958	10428
14	7722	13356
15	6446	12678
16	12582	19308
17	5796	9462
18	7926	13956
19	8016	13642
20	22516	25678
21	22704	22666
22	11470	19026
23	15394	16972
24	7800	10696
25	4902	7630
N1	690	712
N2	868	756

Results from the RAST screening with 25 individual patients' serum. cpm represent Hamlet[®],¹²⁵I bound to the allergosorbent discs prepared with either *Par j* I or *P. judaica* whole extract, as described in Materials and Methods. Two negative sera (N1 and N2) were used to determine the non-specific binding.

of them, then denominated Pj10 and now *Par j* I, was reported as a glycopeptide, with mol. wt 10 K, and exhibited the highest IgE-binding ability (Corbí and Carreira, 1984).

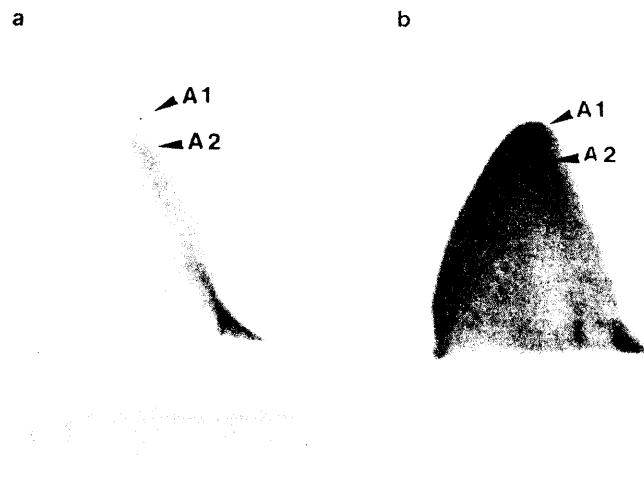


Fig. 6. CIE (a) and CRIE (b) analysis of *Par j* I. Fifty μ g of protein were run as described in Materials and Methods. The arrows indicate A1 and A2 allergenic precipitation bands.

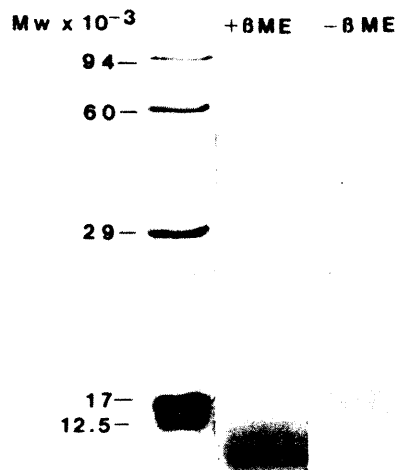


Fig. 7. Evaluation of the mol. wt of *Par j* I by SDS-14% PAGE under both reducing (+ β ME) and non-reducing (- β ME) conditions. Proteins were stained with Coomassie brilliant blue R-250.

Here we describe the isolation of this unique polypeptide (*Par j* I), in a high degree of purity and immunoreactive state, by a simple method that com-

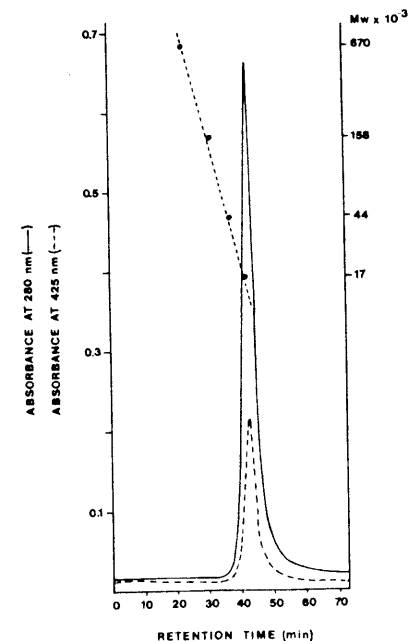


Fig. 8. Chromatography of *Par j* I on an HPLC TSKG 3000 SW column previously calibrated with the standard proteins indicated in Materials and Methods. (●---●) represents mol. wt $\times 10^{-3}$ vs elution vol.

bines ammonium sulphate precipitation and gel filtration. The protein so purified seems to be homogeneous by gel filtration HPLC and polyacrylamide gel electrophoresis in the presence of SDS and 2-mercaptoethanol. However, following immunodetection (Fig. 4) *Par j* I was revealed as a doublet. This result is not new since, every so often, two very close bands were detected after using the SDS-PAGE technique (results not shown). In the same way, others (Ford *et al.*, 1986) reported that a polypeptide of 10 K from *P. judaica* pollen (named band 9) sometimes exhibited doublet behaviour on SDS-PAGE. We do not yet have a conclusive explanation for this fact, but as a matter of speculation we can assume that differences in sugar composition might be responsible for the, so far, unpredictable appearance of this doublet. Support for this assumption comes from previous work (Corbí and Carreira, 1985) where it was shown that a part of *Par j* I polypeptides could easily bind Con A, while other *Par j* I populations could not, and also that the charge heterogeneity of the sugar components was supposed to account for the wide distribution of *Par j* I along a pH range from 4 to 7.

This sugar heterogeneity of *Par j* I might also explain the results obtained by CIE and CRIE techniques, in which *Par j* I yielded the two main

allergenic bands (A1 and A2) of the whole extract (Corbí and Carreira, 1984). According to the results described above, the possibility for A1 and A2 arising from independent components of the SDS-PAGE doublet cannot yet be ruled out. Anyway, this fact will be discussed extensively elsewhere (manuscript in preparation).

By SDS-PAGE under reducing conditions, the mol. wt of *Par j* I was estimated as 10 K. However, in the absence of 2-mercaptoethanol a diffuse and broad band was observed ranging from 15 to 25 K. These results are in agreement with previous experiments of our laboratory (Corbí *et al.*, 1985) and indicate that *Par j* I consists of a complex structure formed by two polypeptides of 10 K, covalently linked by disulphide bridges.

Gel filtration of *Par j* I under non-denaturing conditions gave lower mol. wt estimates than SDS-PAGE. This is not surprising since glycoproteins have been reported to exhibit abnormally high mol. wts when estimated by SDS-PAGE (Leach *et al.*, 1980).

The chromatographic procedures, including HPLC, used in this work, failed to separate completely proteins from pigments. We do not know yet whether this failure comes from the presence of a chromophore covalently linked to *Par j* I or from various pigments strongly and unspecifically attached to the allergen. Work is in progress with the intention of removing these contaminants using mild detergents along the purification steps, a procedure which has already proved to be successful in the purification of mite allergens (Sáiz, 1987).

The allergenicity of isolated *Par j* I was not lost during the purification process since it maintained the ability of binding specific IgE from sensitive subjects. All the experiments performed with human serum pools show that *Par j* I is the most important allergenic component of *P. judaica* and that it has a sp. act. at least 10 times higher than that of the whole extract. On the other hand, 100% of individual sera from allergic patients proved to have specific IgE to this allergen in very high quantities. Taken together these results claimed for *Par j* I the category of *P. judaica* major allergen.

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