

HPLC PURIFICATION OF THE MAIN ALLERGEN OF *PARIETARIA JUDAICA* POLLEN

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Abstract—A two-step purification procedure of *Par j* I from the whole *Parietaria judaica* pollen extract is described. The first step consisted of gel filtration HPLC using a TSKG 3000 SW column, and 0.1% trifluoroacetic acid as the eluant. By this method, proteins were separated from the highly colored material present in the extract. Then, *Par j* I-containing fractions were chromatographed on a reversed-phase HPLC column (Vydac C4) using an acetonitrile gradient. This second step yielded pure *Par j* I as assessed by SDS-PAGE and CIE. Previously reported microheterogeneity was still observed, but amino acid analysis of various RP-HPLC fractions suggested that the heterogeneity of *Par j* I might not be due to changes in its primary structure. Allergenic activity of *Par j* I was shown to be retained after the purification procedure by several immunochemical techniques.

INTRODUCTION

Parietaria judaica pollen is allergenically the most active among pollens distributed around the Mediterranean area. In fact, it is the main cause of allergy in zones of Italy (Fco *et al.*, 1984) and Spain (Corbí and Carreira, 1984) where patients only sensitive to *P. judaica* pollen can be found.

Over the past few years, several studies on the identification and characterization of *P. judaica* pollen allergens have been reported (Corbí and Carreira, 1984, 1985; Geraci *et al.*, 1985; Ford *et al.*, 1986). Although different authors differ on the number of allergens present (mainly when CRIE technique is employed), all of them agree on the identification of a 10,000 mol. wt polypeptide as the main IgE-binding component, since it can be recognized by nearly 100% of individual sera from allergic patients (Ayuso *et al.*, 1988; Ford *et al.*, 1986). This allergen (formerly named Pj10 and now *Par j* I) has been described to be a glycoprotein which shows a considerable heterogeneity in its carbohydrate prosthetic groups and in its electric charge, giving complex patterns on CIE and IEF (Corbí and Carreira, 1984, 1985).

Fractionation of *P. judaica* pollen, aimed at the purification of IgE-binding components, has been attempted using different physico-chemical approaches, such as salt precipitation, dialysis and gel filtration (Feo *et al.*, 1984), chromatofocusing (Corbí

and Carreira, 1984), affinity chromatography on concanavalin A-Sepharose (Corbí and Carreira, 1985) or size-exclusion and ion exchange HPLC (Bolzacchini *et al.*, 1988). However, the complexity of the source material and the above mentioned heterogeneity of *Par j* I have hindered the purification of this allergen in an amount sufficient to allow subsequent structural and clinical studies. In the same way, affinity chromatography with anti-*Par j* I monoclonal antibodies was not successful because of the presence, in the pollen extract, of three different molecules which bear the same antigenic determinant defined by the anti-*Par j* I MAb. Consequently, these molecules were also immunoadsorbed and then co-eluted along with *Par j* I (Corbí *et al.*, 1985, 1986). More recently, we reported a method for the purification of *Par j* I based on ammonium sulfate salt precipitation of the whole pollen extract and gel filtration of the supernatant (Ayuso *et al.*, 1988). By this method, *Par j* I was purified separately from other proteins; however, it failed to separate completely proteins from pigments contained in the pollen extract. In addition, the recovery of *Par j* I was not complete, since a part of it remained in the ammonium sulfate precipitate which was not suitable for further purification.

Therefore, additional efforts were needed in order to establish a better purification method.

In the present work, we describe a rapid and efficient procedure for the purification of *Par j* I in two chromatographic steps. This method consisted of gel filtration HPLC at an acidic pH and low ionic strength, followed by reversed-phase HPLC.

MATERIALS AND METHODS

Preparation of the pollen extract

P. judaica pollen was purchased from Allergon AB (Sweden). The extract was prepared by mixing the

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Abbreviations: RP-HPLC reversed-phase high-performance liquid chromatography; GF-HPLC, gel filtration HPLC; SDS-PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; MAb, monoclonal antibody; PMSF, phenylmethylsulfonyl fluoride; TFA, trifluoroacetic acid; C(R)IE, crossed (radio) immunoelectrophoresis; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline.

pollen with 50 mM ammonium bicarbonate solution, pH 8.0, at a 10% w/v ratio and stirring for 60 min at room temp in the presence of 1 mM PMSF, followed by centrifugation at 39,100 *g* for 15 min. The supernatant was removed from the pellet and dialyzed for 24 hr against distilled water at 4°C, using a Spectra/Por 3 membrane with a mol. wt cut-off of 3500. Then it was recentrifuged, filtered and freeze-dried. The lyophilized extract was stored at 4°C until use.

Purification of Par j I

The lyophilized pollen extract was dissolved in 0.1% aqueous trifluoroacetic acid (TFA) and injected onto a gel filtration HPLC column (Beckman Spherogel TSKG 3000 SW, 30 × 2.1 cm, 13 µm particle size, 25 nm pore size). Elution was performed with the same solvent at a constant flow rate of 1 ml/min at room temp. Collected fractions were analyzed by SDS-PAGE and immunoblotting, and the allergenic activity measured by competition ELISA.

Then the fraction with the highest content of *Par j I* was applied onto a short alkyl chain-bonded silica column (Vydac C4, 25 × 1.0 cm, 10 µm particle size, 30 nm pore size) and eluted with a 10–60% gradient of acetonitrile in 0.1% TFA at room temp and at a constant flow rate of 1 ml/min. Collected fractions were analyzed using the same techniques as above. Fractions containing pure *Par j I* were pooled, dialyzed against distilled water and lyophilized.

Electrophoretical procedures

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) was performed with an 8–25% acrylamide gradient in a high molarity Tris buffer system (Fling and Gregerson, 1986). 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).

Electrotransfer of separated polypeptides onto nitrocellulose membrane 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 Hamlet[®]-¹²⁵I (Alergia e Inmunologia Abelló S.A., Madrid), an iodinated mouse anti-human IgE monoclonal antibody (Sánchez-Madrid *et al.*, 1984), was performed as reported (Corbí and Carreira, 1984).

Crossed immunoelectrophoresis (CIE) was done essentially as described (Weeke, 1973) using an anti-*P. judaica* rabbit serum in the second dimension. For CRIE (Weeke and Lowenstein, 1983), CIE plates were sequentially incubated with *P. judaica*-sensitive serum pool and Hamlet[®]-¹²⁵I.

HPLC equipment

All the chromatographic procedures were carried out on HPLC equipment from Waters comprising a Model 680 gradient programmer, two 510 pumps, a U6K injector, and a 490 multiwavelength u.v./visible detector set at 214 nm.

Amino acid analysis

Samples were hydrolyzed at 110°C for 24 hr using a vapour-phase hydrolysis technique with constant boiling HCl. The hydrolysates were analyzed with a PICO-TAG system (Waters). No corrections were made for destruction during hydrolysis or incomplete hydrolysis. Tryptophan and cysteine were not determined.

Protein quantitation

Protein content of the whole pollen extract and HPLC fractions was estimated by the Folin method (Lowry *et al.*, 1951). Concentration of purified *Par j I* samples was measured by the difference between spectrophotometric absorptions at 215 and 225 nm (Wolf and Maguire, 1983). This technique showed a good correlation with amino acid analysis.

Inhibition of ELISA technique

P. judaica pollen antigens were adsorbed to microtitration plates (Dynatech type II) by incubating 100 µl per well of a 0.11 mg/ml solution of the allergenic extract in 0.1 M sodium carbonate, pH 9.6, at 4°C overnight. Unbound protein was washed three times with distilled water. Unoccupied well sites were blocked with 100 µl of PBS containing 1% bovine serum albumin for 1 hr at room temp. The plates were afterwards washed three times with distilled water and once with PBS containing 0.5% Tween 20.

The adsorbed antigens were made to react with a mixture of 50 µl of *P. judaica*-specific human serum pool (diluted 1:3 with PBS) and 50 µl of serial dilutions of the sample to be tested, for 2 hr at room temp. Afterwards the plates were washed three times with PBS containing 0.5% Tween 20 and incubated with 100 µl per well of a stock solution of a mouse anti-human IgE monoclonal antibody, conjugated with β-galactosidase (Hamlet[®], Alergia e Inmunologia Abelló S.A.), for 2 hr at room temp. The plates were washed six times, as previously, and 100 µl of a solution of *o*-nitrophenyl-β-D-galactopyranoside in PBS, with 10 mM MgCl₂ and 100 mM 2-mercaptoethanol, were added to each well and incubated for 2 hr at 37°C. The reaction was stopped with 100 µl of 0.1 M sodium carbonate solution and the absorbance measured at 405 and 620 nm in an automatic microplate reader.

To evaluate the activity of the samples an arbitrary value of 1 was given to the amount of protein inhibitor (in µg) needed to inhibit up to 50% the binding of specific IgE to *P. judaica*-coated wells.

RESULTS

Purification of Par j I

Purification of *Par j* was achieved in two chromatographic steps using HPLC. First, the lyophilized whole pollen extract was dissolved in 0.1% TFA and subjected to GF-HPLC, using the same solvent as the eluant. The use of 0.1% TFA did not precipitate any protein from the whole extract, as can be observed in lanes 1 and 2 in Fig. 2. The GF-HPLC elution profile (Fig. 1) showed two main well-resolved peaks. Allergic activity was only present at retention times between 45 and 75 min (fractions a–e), corresponding to the highest allergenic activity with the first maximum absorbance peak at 67 min (fraction d). Moreover, protein could only be found at retention times shorter than 80 min, whereas for longer times the eluted material mainly contained colored compounds. Therefore, this method enable the separation of proteins from the highly colored pigments present in the extract.

Analysis of fractions a–e by SDS-PAGE under reducing conditions revealed (Fig. 2) that fraction d was essentially composed of a 10,000 mol. wt polypeptide, slightly contaminated with some polypeptides of mol. wts ranging from 17,000 to 30,000. By immunodetection after SDS-PAGE (Fig. 3), fraction d again revealed the highest IgE-binding ability, mostly due to its high content of the 10,000 mol. wt polypeptide (*Par j* I allergen). On the other hand, the weak radiostaining of the 10,000 mol. wt band in fraction e seems to indicate that most of the protein in this fraction would be a different protein, with a mol. wt similar to that of *Par j* I but with no IgE-binding ability. Consequently, fraction d was selected as the starting material for a second chromatographic purification step, consisting of RP-HPLC. As can be deduced from the elution profile

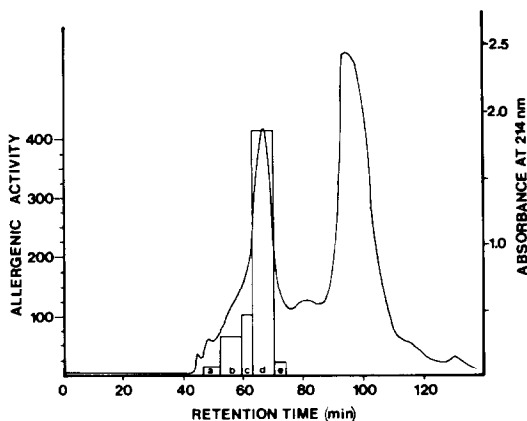


Fig. 1. Elution profile of *P. judaica* pollen extract on GF-HPLC monitored at 214 nm. Pollen extract (6.3 mg) was dissolved in 0.1% TFA and applied to a Spherogel TSKG 3000 SW column equilibrated and eluted with the same solvent. The bar graph depicts the allergenic activity of each fraction evaluated by a competition ELISA as described in Materials and Methods.

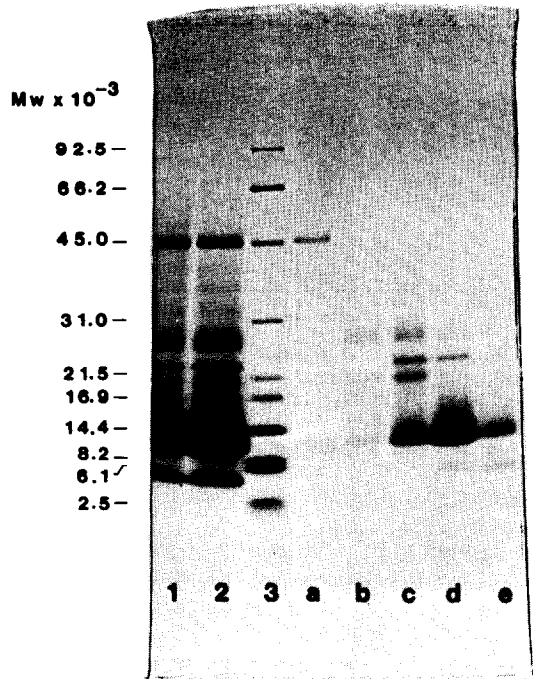


Fig. 2. SDS-PAGE under reducing conditions of GF-HPLC fractions. Lyophilized *P. judaica* pollen extract was dissolved in water (lane 1) or in 0.1% TFA (lane 2), and 300 μ g of protein were applied per well. Molecular weight markers (lane 3) are as in Materials and Methods. Lanes a–e show the electrophoretic patterns of fractions from GF-HPLC (Fig. 1). These fractions were lyophilized, redissolved in water at 1/10 of their initial volumes, and 15 μ l of each were applied per well. Proteins were stained with Coomassie blue.

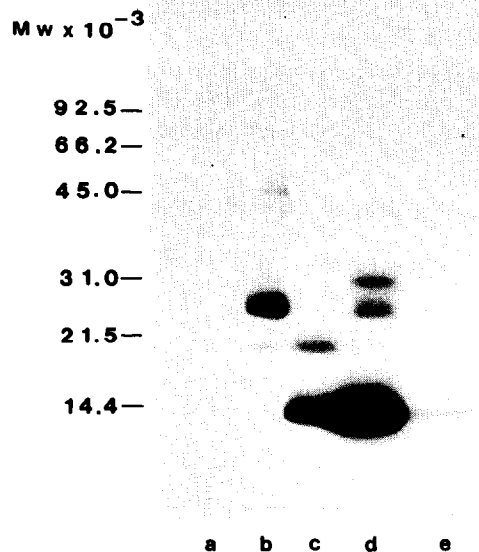


Fig. 3. Immunodetection of IgE-binding polypeptides in fractions from GF-HPLC. Samples a–e were subjected to SDS-PAGE as in Fig. 2, and electrotransferred onto a nitrocellulose membrane. The membrane was sequentially incubated with a *P. judaica*-sensitive human serum pool and ^{125} I-anti-IgE. Finally, the nitrocellulose membrane was dried and autoradiographed for 72 hr.

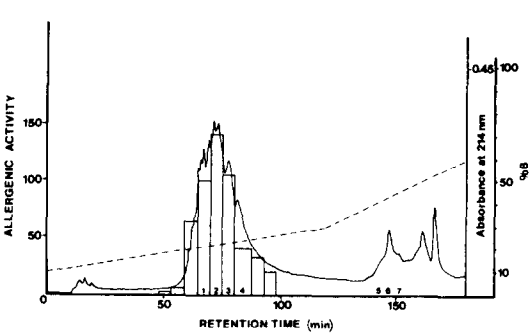


Fig. 4. RP-HPLC of fraction d from gel filtration chromatography. Protein (1.0 mg) from fraction d was injected into a Vydac C4 column. Elution was performed as described in Materials and Methods. The dashed line depicts the gradient shape, and the bar graph shows the allergenic activity.

(Fig. 4), most of the protein, as well as the allergenic activity, was eluted as a complex peak at acetonitrile percentages from 19 to 26. Proteins eluted at higher acetonitrile percentages (fractions 5–7) did not show any allergenic activity. No protein could be detected by the Folin method (Lowry *et al.*, 1951) in the last two peaks. In addition, absorbance of these peaks at 280 nm was higher than that at 214 nm (data not shown) which confirms its non-proteid nature.

Electrophoretic analysis of fractions eluted from the RP-HPLC column is shown in Fig. 5. Every fraction of the complex peak (1–4) contained pure *Par j* I, whereas proteins that were contaminants of *Par j* I in fraction d eluted in the other fractions (5–7).

Immunodetection after SDS-PAGE of fractions 1–4 (Fig. 6) confirmed that *Par j* I retained IgE-binding ability after reversed-phase chromatography and that there were no allergenic contaminants in these fractions. Besides, after 1-month exposure of the X-ray film, no other IgE-binding proteins could be

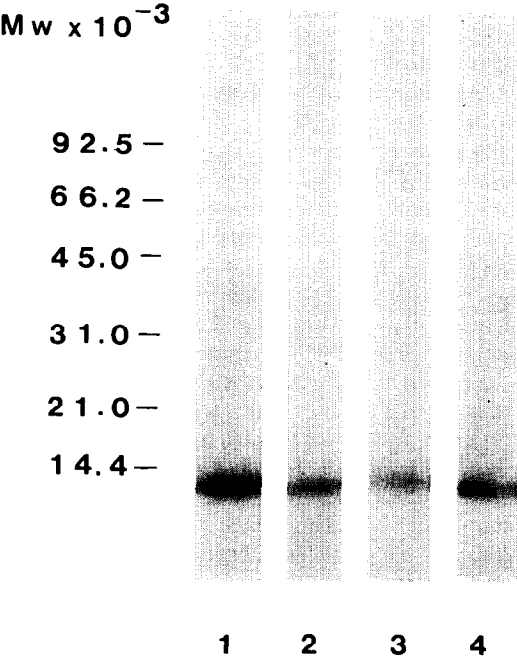


Fig. 6. Immunodetection of IgE-binding polypeptides in RP-HPLC fractions 1–4 (Fig. 4). Samples subjected to SDS-PAGE were processed as indicated in Fig. 3, and the nitrocellulose membrane autoradiographed for 24 hr.

visualized, even in fractions 5–7 (data not shown), as might be expected from the results of inhibition ELISA (Fig. 4).

After pooling the fractions containing pure *Par j* I (including the one before fraction 1 and the one after fraction 4, which showed the same pattern as fractions 1–4 in SDS-PAGE and immunoblotting), the overall protein yield after the two-step purification process was around 7%, since 440 µg of pure *Par j* I were obtained from 6.3 mg of protein of the whole pollen extract.

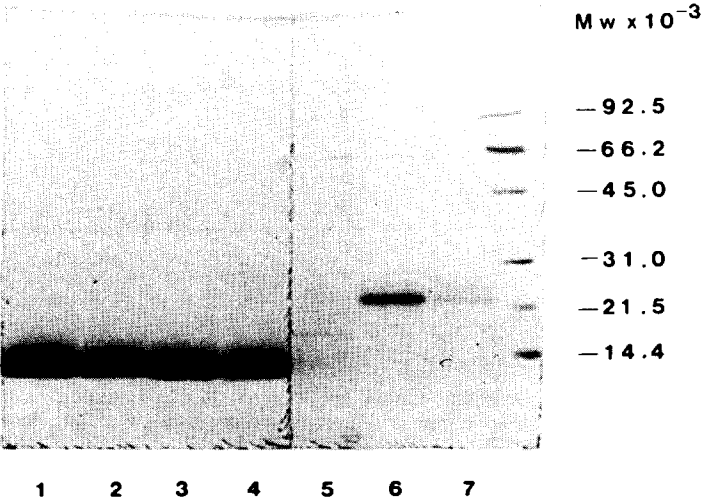


Fig. 5. SDS-PAGE analysis of fractions 1–7 from RP-HPLC (Fig 4) using the same conditions as described in Fig. 2.

Amino acid analysis

The elution profile from RP-HPLC (Fig. 4) showed that electrophoretically pure *Par j* I (Fig. 5, lanes 1–4) was resolved into multiple overlapped peaks. To test the hypothesis that the heterogeneous behaviour of *Par j* I might not be due to amino acid sequence variation, an amino acid analysis was performed on fractions 1–4. The results are listed in Table 1 and seem to show that the polypeptide composition of these fractions is the same. Although little variability was observed in these data and amino acid sequence variation cannot be completely ruled out until the complete primary structure of *Par j* I is known, the results suggest that secondary biochemical modifications rather than primary sequence variability might be responsible for the microheterogeneity of *Par j* I.

Immunochemical characterization of purified *Par j* I

Immunochemical identification of purified *Par j* I was done by CIE and CRIE (Fig. 7) that displayed the two precipitation lines (A1 and A2) previously described as the main allergenic ones of *P. judaica* pollen (Corbí and Carreira, 1984). In addition, CRIE demonstrated that *Par j* I retained its IgE-binding ability after the purification process.

The allergenic activity of purified *Par j* I, evaluated by an inhibition ELISA (Fig. 8), was about 10-fold higher than that of the whole extract, and it could reach an IgE-binding maximum inhibition similar to the pollen extract.

DISCUSSION

Purification of allergens is an important goal in the study of the immunogen structure–activity relationship in human atopic allergy. However, this is often impaired by the complex nature of the sources they come from. In fact, a rather small number of them have been purified so far.

A common preliminary step in fractionation of allergenic extracts consists of size-exclusion chromatography. Nevertheless, when *P. judaica* pollen

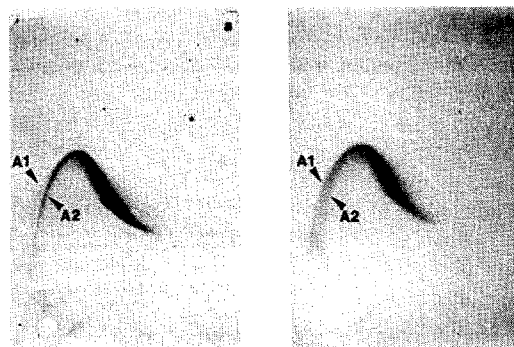


Fig. 7. CIE (a) and CRIE (b) patterns of purified *Par j* I. Protein (1 μ g) was run at 400 V for 30 min in the first dimension. The second dimension against the antibody-containing gel (75 μ l of anti-*P. judaica* rabbit serum in 3.5 ml of 1% agarose) was accomplished at 150 V for 20 hr. (a) The CIE plate was stained with Coomassie blue. (b) The CIE plate was dried and sequentially incubated with *P. judaica*-sensitive patients' serum pool and ^{125} I-anti-human IgE. The gel was then washed, dried and autoradiographed.

extract was subjected to GF-HPLC under the usual buffer conditions of neutral pH and moderate ionic strength, poor resolution was achieved because of the pollen pigments which eluted all along the chromatogram. Such behavior had been previously observed on the Ultrogel AcA54 column (Ayuso *et al.*, 1988) that failed to separate *Par j* I from pigments. Therefore, alternative conditions, that might be advantageous, exploiting electrostatic or hydrophobic interactions between solute molecules and the matrix (Irvine and Shaw, 1986), were investigated. After several attempts, the use of aqueous 0.1% TFA as the mobile-phase succeeded in separating proteins from pigments. This solvent has the additional advantage of allowing the injection of the eluted fractions onto the RP-HPLC column used in the second purification step without further handling. Moreover, it allows an easy scaling-up of the procedure since large volumes of protein solution could be directly pumped through the RP-HPLC column before starting the acetonitrile gradient.

Table 1. Amino acid composition of RP-HPLC fractions

Amino acid	1	2	3	4
Lysine	15.4	13.8	14.1	13.6
Histidine	1.8	1.9	1.9	2.0
Arginine	2.3	2.3	2.6	2.7
Aspartic acid	3.9	3.8	2.7	4.1
Threonine	8.4	8.2	8.4	8.4
Serine	6.8	7.6	6.7	6.3
Glutamic acid	14.8	14.1	10.9	11.9
Proline	7.4	7.6	8.6	8.1
Glycine	8.2	9.5	10.3	10.3
Alanine	6.8	6.7	6.7	6.5
Valine	7.5	7.8	8.6	8.4
Methionine	1.3	1.6	2.5	2.6
Isoleucine	5.3	6.0	7.0	6.7
Leucine	7.3	6.5	6.2	5.8
Tyrosine	0.3	0.5	0.8	0.8
Phenylalanine	2.5	2.1	2.0	1.8

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

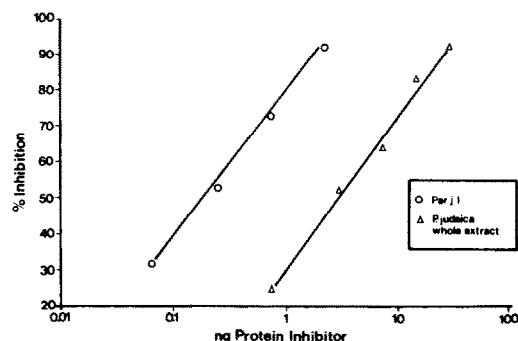


Fig. 8. Competition ELISA curves of purified *Par j* I (○) compared with whole *P. judaica* pollen extract (△). The assay was performed as described in Materials and Methods.

Previously reported microheterogeneity of *Par j* I on IEF and CIE was also observed in RP-HPLC, since *Par j* I eluted from the Vydac C4 column as a complex peak (Fig. 4). Subsequent amino acid analyses of several fractions from this peak seemed to show that the microheterogeneity may not arise from primary sequence variability but from the prosthetic groups linked to the polypeptide chain. These results are consistent with the previous observation that a population of *Par j* I allergen easily binds to Con A while another part does not (Corbí and Carreira, 1985), demonstrating some variability in the carbohydrate moiety. This observation would also explain the two precipitation bands observed in the CIE pattern of *Par j* I (Fig. 7). The same doublet behavior has been observed in some other glycoprotein allergens, such as *Api m* I (phospholipase A2 from honeybee venom) which, "although apparently homogeneous with respect to the polypeptide chain, gives two approximately equal precipitation bands on immunodiffusion analysis" (Shipolini *et al.*, 1974). In the same way, *Par j* I can sometimes be resolved into two very closely migrating bands on SDS-PAGE; however, the conditions for consistently separating these bands on every gel have not yet been established (Ford *et al.*, 1986; Ayuso *et al.*, 1988).

It is known that conformational changes of proteins upon RP-HPLC, induced by interactions of the protein with the alkyl-silica matrix and with the organic solvent (Katzenstein *et al.*, 1986), may cause complete or partial loss of biological activity. This appears to be the case with the two allergenic polypeptides (mol. wts 24,000 and 30,000) which contaminated *Par j* I in the sample applied to the RP-HPLC column (fraction d, Fig. 3). These proteins eluted at a high concn of acetonitrile (fractions 6 and 7, Fig. 4) and did not retain their allergenic activity.

However, the IgE-binding ability of *Par j* I which eluted at a lower concn of organic solvent, was not lost upon RP-HPLC, being similar to that of the allergen purified by other procedures (Ayuso *et al.*, 1988; Corbí *et al.*, 1985). This means that the conformational changes, if any, of *Par j* I were reversible.

On the other hand, purified *Par j* I could inhibit the IgE-binding up to an extent similar to that of the whole extract (Fig. 8). This fact could be explained, taking into account that, as has been proven previously (Polo *et al.*, 1987), there are three other allergenic molecules in *P. judaica* pollen (with approximate mol. wts of 20,000, 30,000 and 40,000) which share antigenic and allergenic determinants with *Par j* I, and that these molecules have not any allergenic determinants differing from those of *Par j* I. Thus, it could be possible that only a very small number of IgE molecules of the serum pool used were recognizing epitopes not located on *Par j* I.

In conclusion, we have established a simple and rapid chromatographic procedure for the purification of the main allergen of *P. judaica* pollen in an allergenic active state. The good yield, and the possi-

bility of an easy scaling-up, make this procedure a useful tool for further structure-activity relationship studies.

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