

Purification of *Par j* I, a Major Allergen from *Parietaria judaica* Pollen

Roberta Cocchiara^a, Giovanni Locorotondo^a, Alessandra Parlato^a, Gabriella Guarnotta^a, Severino Ronchi^b, Giuseppe Albeggiani^a, Saverio Amoroso^c, Paolo Falagiani^d, Domenico Geraci^a

^a Istituto di Biologia dello Sviluppo, CNR, Palermo; ^b Istituto di Fisiologia Veterinaria, Università di Milano;

^c Istituto di Medicina Generale e Pneumologia, Università di Palermo; ^d Laboratorio Farmaceutico Lofarma, Milano, Italia

Abstract. A major allergen, the *Par j* I, was purified to homogeneity from *Parietaria judaica* pollen by means of ultrafiltration dialysis, preparative polyacrylamide gel chromatography and affinity chromatography through a column of Sepharose-monoclonal antibody specific for *Par j* I. The homogeneity of the *Par j* I was assessed by one single arc of immunoprecipitation both in crossed immunoelectrophoresis (CIE) and crossed radioimmunoelectrophoresis, by one single band of radiostaining after a sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transfer to nitrocellulose and by one single peak after a size exclusion chromatography on high-performance liquid chromatography (HPLC). The homogeneity was further supported by crossed Laurell immunoelectrophoretic analysis, in that only one arc of precipitation was magnified in CIE after addition of the purified allergen. The purified *Par j* I allergen was capable of interacting in vitro with 70% of the human IgE specific for a crude *P. judaica* extract, as determined by radioallergosorbent test inhibition. The purified *Par j* I was capable of inducing positive reactions in vivo in skin prick tests, and of inducing release of histamine from blood containing basophils as determined by a histamine release assay. The amino acid analysis of the *Par j* I showed 118 amino acid residues per monomer analyzed and, among other residues, three methionine residues were detected. The molecular weight of the *Par j* I estimated by HPLC and amino acid composition was 26 kilodaltons.

Introduction

The immunochemical characterization of *Parietaria judaica* pollen extract has been previously reported, and a complete picture of the antigenic and allergenic composition was showed by means of quantitative immunoelectrophoretic and gel fractionation-electroblotting methods [1-4]. Four major allergens were identified and one, named antigen 9 or band 9 [1, 2], was capable of binding IgE contained in the sera of all the *P. judaica*-allergic patients analyzed so far. These results provided a proper basis for further purification and standardization studies.

The aim of the present study was to purify to homogeneity the major allergen of the *P. judaica* pollen extract, antigen 9, here named *Par j* I using the new nomenclature suggested by Marsh et al. [5].

It would be useful to establish the amino acid sequence and other immunochemical characteristics of *Par j* I in order to determine common properties, if any, with other previously sequenced allergens. This information would greatly assist reliable studies on the allergic reaction. This allergen could be also used as a tool for more accurate standardization procedures and for clinical purposes, such as in the preparation of hyposensitization therapy.

Materials and Methods

P. judaica Extract

P. judaica pollen was obtained from Allergon AB (Angelholm, Sweden), containing less than 0.5% of foreign pollen and approximately 24% (w/w) of nonpollen debris. The pollen was extracted essentially as previously reported [1]. In short, 20 g of *P. judaica*

pollen was extracted (1:10, w/v) in 0.125 M ammonium hydrogen carbonate and 0.015 M sodium azide, pH 8.3, for 24 h at 4°C. After centrifugation (50,000 g, 30 min at 4°C), the supernatant was dialyzed against water and concentrated by means of an ultrafiltration dialysis system with a 5-kilodalton (kD) cutoff filter (Amicon, USA).

Ultrafiltration Dialysis

Crude *P. judaica* extract was primarily fractionated using a C-20 (20 kD) exclusion filter and then a YM-30 (30 kD) exclusion filter. Amicon 200-ml Diaflo units and Amicon ultrafilters were used. Filtrate from the YM-30 filter was analyzed by crossed immunoelectrophoresis (CIE) to detect the *Par j* I allergen.

After exhaustive dialysis the material was concentrated on a YM-5 (5 kD) filter and dialyzed by a C-20 filter. After the second filtration step, the retentates were collected dialyzed exhaustively and analyzed by CIE.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Electrophoresis in polyacrylamide gel containing sodium dodecyl sulfate (SDS-PAGE) was performed as previously described [2]. Preparative electrophoresis was carried out in the slab gel by loading 5 mg of the pollen extract, partially purified by the ultrafiltration steps. The electrophoretic band corresponding to the *Par j* I allergen was cut and eluted electrophoretically.

Immunoblotting was carried out by electrophoretic transfer of the separated bands to a nitrocellulose membrane. Probing and autoradiography was carried out as previously described [2].

Monoclonal Antibodies

BALB/c mice were immunized with a partially purified extract of *P. judaica* pollen. The first three boosts were carried out at 2-week intervals, by intraperitoneal injection of 500 µg of the crude extract in complete Freund's adjuvant. A month after the last injection, a mouse was boosted both intraperitoneally and intravenously with an equal amount of the pollen extract in phosphate-buffered saline (PBS). Three days later, the spleen cells were fused with the nonproducing myeloma Ag 8653 as described elsewhere [6]. After the fusion, the cells were distributed in 96-well culture trays, limiting the input of spleen cells to 2×10^4 /well in order to reduce the clonal competition. Two weeks later, supernatants with growing hybrids were initially screened for their specificity by using an enzyme-linked immunosorbent assay and coating the wells of microtiter plates with 3 µm/well of the *P. judaica* extract. Positive hybridomas were cloned by limiting dilutions. One hybridoma termed IBS-1 was selected for further studies. This monoclonal was expanded in vivo by injecting 5×10^6 cells intraperitoneally in mice pretreated with pristane. The pooled ascites were partially purified by precipitation with 40% saturated ammonium sulfate and extensive dialysis at 4°C against PBS. The class and subclass of the monoclonal was determined by immunodiffusion using specific antisera.

The monoclonal IBS-1 was characterized as an IgG2a-antibody. Further purification was achieved by affinity chromatography through protein A-Sepharose columns. The purified IBS-1 was used to prepare an immunosorbent by covalently coupling the monoclonal antibody IBS-1 (3 ml) to 0.5 g of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). The specificity of the IBS-1 monoclonal was determined by its capability of binding the *Par j* I allergen.

This was used to purify to homogeneity the allergen itself. 0.5 mg

of the fraction eluted from the preparative SDS-PAGE gel were applied to the immunosorbent column in 0.05 M sodium citrate and 0.5 M sodium chloride, pH 6.2. The column was then eluted by increasing the sodium chloride molarity to 1.0 M. The eluted material was tested by CIE and crossed radioimmuno-electrophoresis (CRIE) for the presence of the *Par j* I allergen.

Patients

Sera ($n = 100$) were collected from patients [75 males and 25 females with a mean age of 35 ± 10 ($\pm$ SD) years] with seasonal allergy towards *P. judaica* pollen. The most diffuse symptoms among these patients were rhinitis (80%) and asthma (23%). Only 5 out of 100 patients showed allergic reaction to the *P. judaica* extract only, while the remaining 95 were also allergic to, e.g., grass pollen, *Dermatophagoides farinae* and *Pteronyssinus*, and olive, as determined on the basis of clinical record and skin prick testing performed as previously reported [3]. A *P. judaica*-specific radioallergosorbent test (RAST) grade 2–4 was determined among the selected patients. No hyposensitization therapy was received by the patients, and the pooled sera were stored at -20°C until used.

Rabbit Antibodies

Rabbit anti-*P. judaica* pollen extract antibodies were a precious gift from Dr. Henning Lowenstein (Copenhagen, Denmark).

CIE, CRIE, Crossed Laurell Immuno-electrophoresis

The immuno-electrophoretic methods were performed as described elsewhere [1].

Molecular Weight Determination

The molecular weight of the *Par j* I allergen was determined by using gel filtration on a high-performance liquid chromatography (HPLC) TSK-2000 column equilibrated with Tris-CM (0.025 M Tris, 0.12 M NaCl, 0.005 M KCl, 6×10^{-4} M CaCl_2 , 0.001 M $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$, pH 7.4), and a flow rate of 2 ml/min. The column was calibrated for molecular weight determination with the following protein markers: bovine serum albumin (66 kD); trypsin (24 kD), and myoglobin (17 kD). The retention times of the markers and *Par j* I allergen were determined by ultraviolet absorption at 254 nm.

RAST Inhibition

RAST inhibition was performed essentially as described elsewhere [7]. Polystyrene beads, 6.4 mm in diameter (Allergosphere®, Lofarma, Italy), coated with crude *P. judaica* extract, were used for the solid phase. The IgE-binding values (pooled sera from *P. judaica* patients, $n = 100$) were determined by addition of 50 µl rabbit antihuman ^{125}I -IgE (Sferikit®, Lofarma) and the bound radioactivity was measured in a γ -counter (Packard 800). Pooled sera ($n = 50$) from *P. judaica*-nonallergic subjects were used as a control. The inhibition of the RAST values was carried out by incubation of the serum samples with increasing amount of *Par j* I allergen or with crude *P. judaica* extract for 3 h at room temperature.

Histamine Release Assay

The histamine release assay was performed by an enzymatic isotopic microassay [8, 9] and using sensitized human leukocytes, obtained from patients allergic to *P. judaica* pollen. 200-µl aliquots of the donor blood were placed in Eppendorf microfuge tubes together with different dilutions of *Par j* I in Tris-ACM buffer [7]. Af-

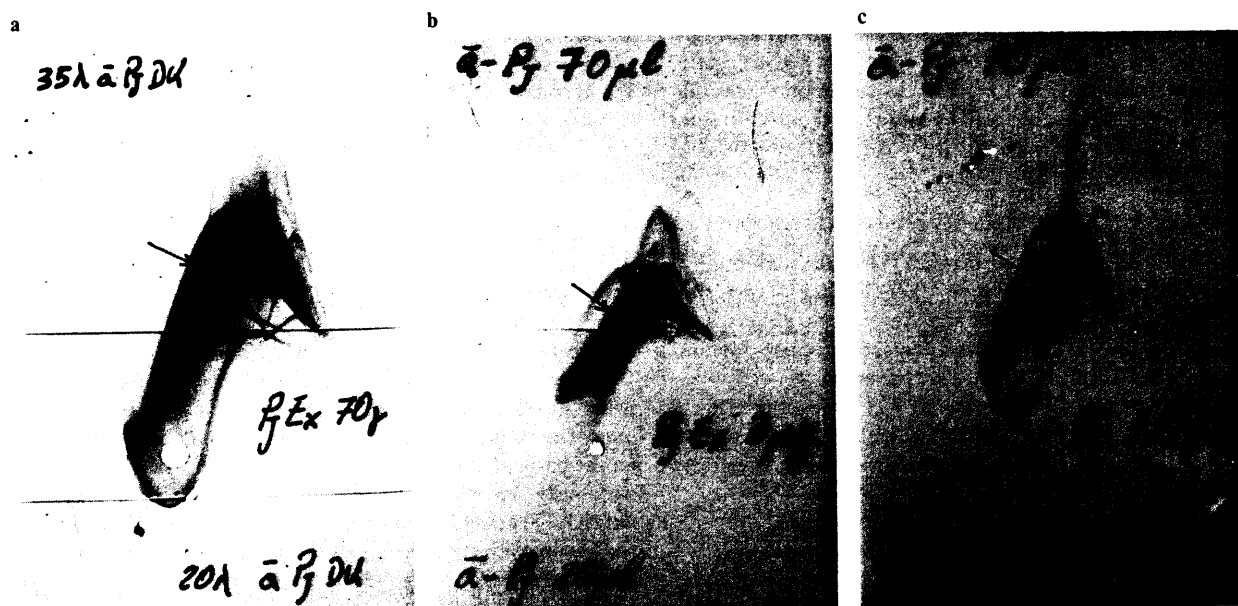


Fig. 1. CIE analysis performed by means of a high-titer antiserum of rabbits immunized with a crude *P. judaica* extract and: total crude *P. judaica* extract (a); the filtrate of the total crude *P. judaica* extract after passage through a 30-kD cutoff filter (b), and the retentate after a 20-kD cutoff filtration of the material in b (c). The arrows indicate the *Par j* I allergen after Geraci et al. [1].

ter 30 min incubation at 37°C, the mixture was centrifuged in an Eppendorf 5414 centrifuge. Triplicate aliquots of 20 µl of plasma were removed for the histamine determinations. A HMT-SAM solution was prepared by adding, before the histamine assay, 250 µl of histamine methyltransferase (HMT) at a protein concentration of 4 mg/ml prepared as previously reported [9] to an Eppendorf microfuge tube containing 10 µl of ³H-SAM (S-adenosyl-L-methyl-³H-methionine, 500 mCi/mmol; Radiochemical Centre, Amersham, UK). 10 µl of HMT-SAM solution were added to each triplicate aliquots of plasma (20 µl) and the tubes incubated for 1 h at 37°C. The reaction was stopped by addition of 10 µl of 1 N NaOH. The ³H-methyl histamine formed was extracted into 250 µl of chloroform [9]. After centrifugation at 3,000 g for 5 min, the aqueous phase was removed and the organic phase was transferred to a scintillator vial and evaporated to dryness. The bound radioactivity was determined in a Beckman counter. Total blood histamine was determined by boiling for 10 min in 100 µl of whole blood diluted with 100 µl of 0.05 M phosphate buffer, pH 7.9, containing 0.01% Triton X-100. Spontaneous release was calculated by incubation of the blood with buffer alone. The amount of histamine release was expressed as percentage of total histamine after subtraction of spontaneous release.

Amino Acid Analysis

Samples were hydrolyzed with 6 N HCl, under vacuum, for 24 h at 105°C, and the amino acid content determined by means of a single column chromatography in an Erba Analyzer, equipped with high-sensitivity cuvettes. The cysteine content was quantified as cysteic acid after oxidation with dimethyl sulfoxide.

Results

Preliminary Fractionation of the *P. judaica* Pollen Extract

The crude extract of *P. judaica* pollen, after exhaustive ultrafiltration, was analyzed by CIE. The initial mixture contained at least 26 distinct antigens (fig. 1a). After filtration through a filter with a cutoff of 30 kD, at least 12 antigens including the *Par j* I allergen were detected in the filtrate (fig. 1b). This fraction with a molecular weight range less than 30 kD was filtered through a 20-kD cutoff filter. The retentate analyzed by CIE showed at least 6 antigens including the *Par j* I allergen (fig. 1c). This fraction was further chromatographed on SDS-PAGE under reducing conditions, and the protein band corresponding to a molecular weight of about 13 kD was electrophoretically eluted.

Isolation and Homogeneity Assessment of *Par j* I

An affinity chromatography column was prepared by linking the *Par j* I-specific monoclonal antibody to CNBr-activated Sepharose beads. The material eluted from the preparative SDS-PAGE that contained the

Fig. 2. CIE (a) and CRIE (b) analysis of material obtained by monoclonal antibody *Par j* I-Sepharose affinity chromatography. The rabbit antiserum used was the same as in figure 1. b CRIE control made from a pool of nonallergic subjects.

Par j I allergen plus 5 other antigens was chromatographed on the immunosorbent and the bound material was analyzed by CIE and CRIE. Only one immunoprecipitate arc was detected by CIE (fig. 2a) by using the same rabbit anti-*P. judaica* extract capable of detecting 26 antigens.

To confirm that the antigen detected was an allergen, a CRIE analysis was performed by using a pool of sera ($n = 100$) from *P. judaica*-allergic patients. Only one arc of radiostaining was detected (fig. 2c), while no radiostaining was detected if a pool of sera ($n = 50$) from nonallergic people was used (fig. 2b).

An additional analysis was performed in order to establish the homogeneity of the material bound to the immunosorbent. Chromatography on SDS-PAGE under reduced conditions followed by transfer to a nitrocellulose membrane and probing with the pool of sera from allergic patients, reported above, showed a single band of anti-IgE- ^{125} I radiostaining corresponding to an apparent molecular weight of approximately 13 kD (fig. 3).

The molecular weight and the homogeneity were further assessed by chromatography on a HPLC column previously calibrated with protein markers of known molecular weight. The sharp peak of optical absorbance suggested a molecular weight of approximately 26 kD with an acceptable grade of homogeneity (fig. 4). The results of a crossed Laurell immunoelectrophoretic (CLIE) analysis of the molecule bound to the column of monoclonal immunosorbent suggest that it is the *Par j* I allergen. Figure 5 shows that only the single arc corresponding to the *Par j* I arc was enhanced in its height.

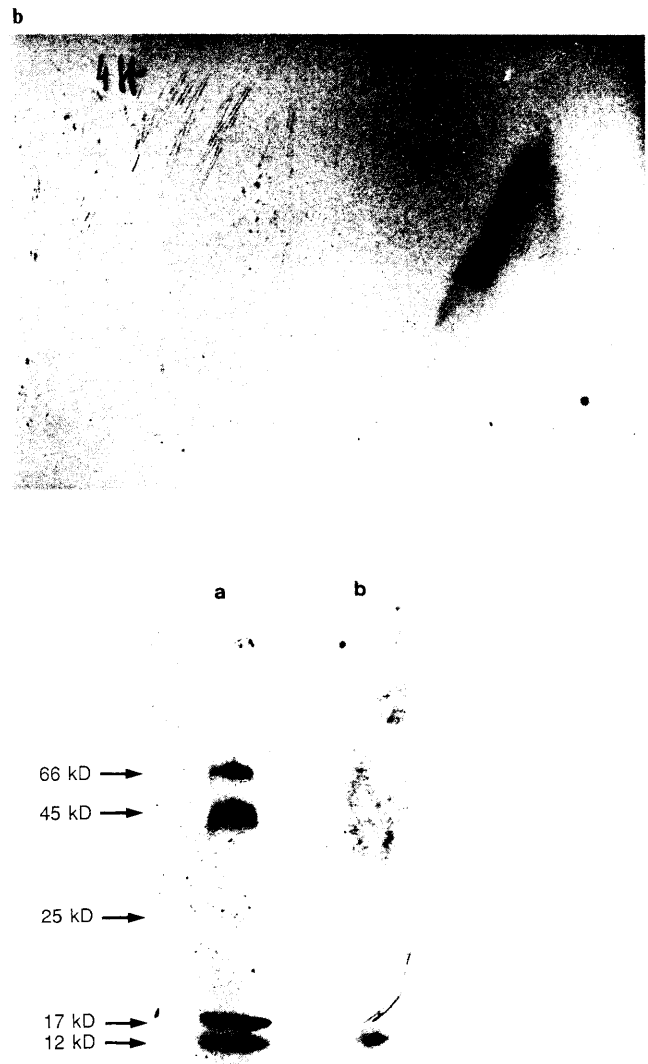


Fig. 3. SDS-PAGE chromatography made under reducing conditions. a Protein markers. b ^{125} I radiostaining after transfer on nitrocellulose membrane and probing with a pool of *P. judaica*-allergic patients sera.

Characterization of *Par j* I

In order to better characterize the *Par j* I allergen, several analyses were performed. In a RAST inhibition experiment, made by coating polystyrene beads with crude *P. judaica* extract, 50% of inhibition was obtained by using 1 μ g of *Par j* I allergen, while only approximately 125 ng of crude *P. judaica* extract was necessary to obtain the same inhibition (fig. 6). The maximum inhibition capability of the *Par j* I was

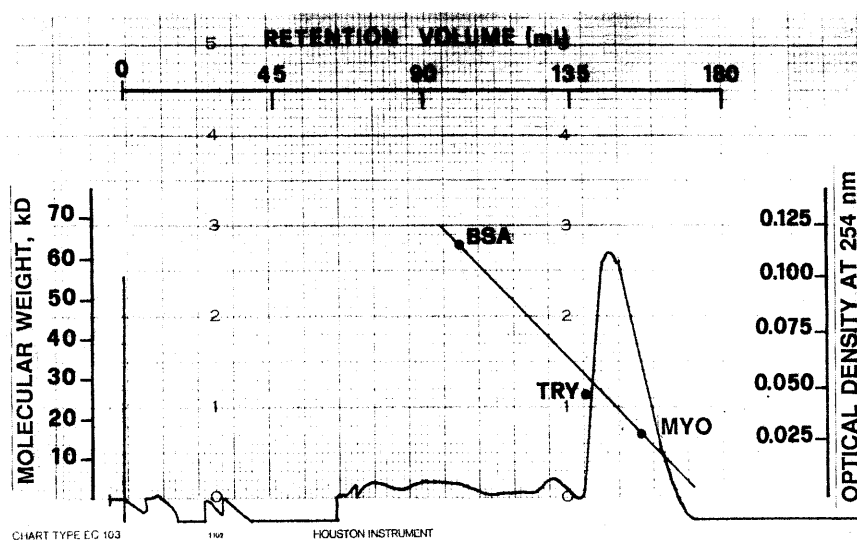


Fig. 4. Size exclusion HPLC analysis of the material obtained after the monoclonal-Sepharose affinity chromatography. Bovine serum albumin (BSA), trypsin (TRY) and myoglobin (MYO) were the markers used to calibrate the column.

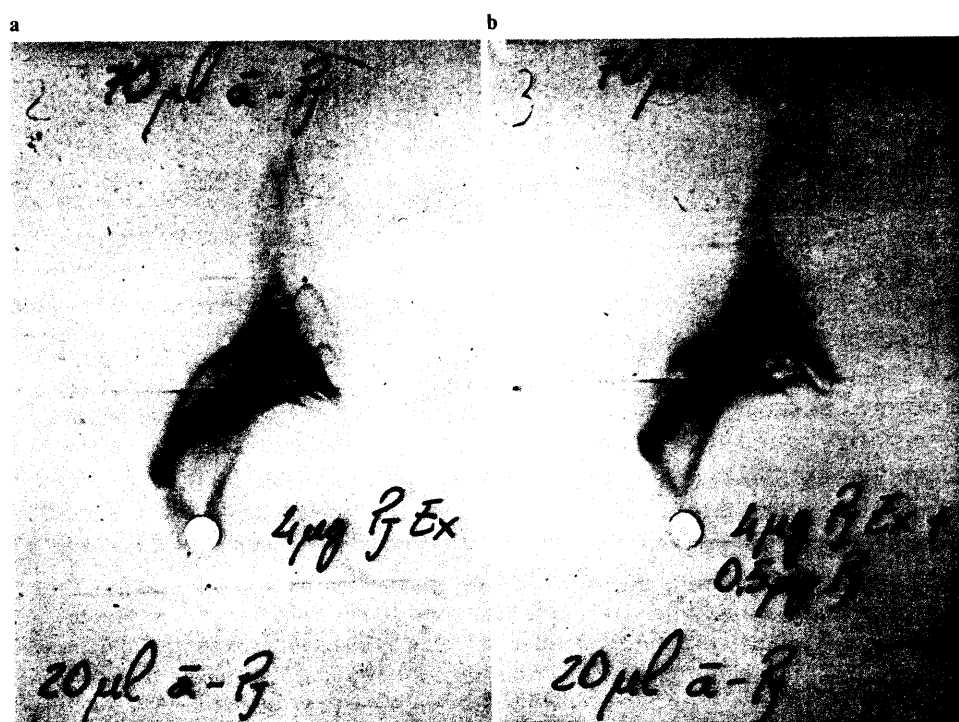


Fig. 5. CLIE analysis. **a** CIE reference. **b** CLIE made by adding 0.5 µg of the material obtained after the monoclonal-Sepharose affinity chromatography into the intermediate gel.

about 70% (fig. 6). The *Par j* I allergen purified as reported above was biologically active as suggested by its capability to induce positive reactions in skin prick tests on *P. judaica*-sensitive subjects. Moreover, blood basophils prepared from these subjects were challenged in a histamine release assay with increasing amounts of the purified allergen. The results reported in table 1 support the fact that, also after several puri-

fication steps, the *Par j* I allergen retains a significant allergenic activity. Table 2 shows the results obtained from an amino acid determination of the *Par j* I allergen. 118 amino acid residues were detected and the molecular weight determined was 12.6 kD per monomer. The hydrophilic amino acid residues were in equal proportions as hydrophobic ones, with 3 methionine residues present for each monomer analyzed.

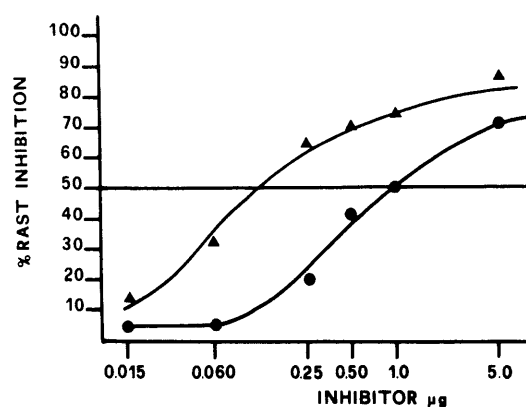


Fig. 6. RAST inhibition curve made by adding increasing amounts of the material obtained after the monoclonal-Sepharose affinity chromatography (●) and of the crude *P. judaica* extract (▲).

Table 1. Histamine release from *P. judaica*-sensitized human leukocytes after challenge with *Par j* I

Leukocyte incubation	Histamine release	
	cpm (mean $\pm$ SD)	%
Boiled (total)	1,940 $\pm$ 0.25	-
Buffer alone	345 $\pm$ 0.10	-
Anti-IgE	1,450 $\pm$ 0.13	69
<i>Par j</i> I, 0.1 μ g	1,519 $\pm$ 0.18	74
<i>Par j</i> I, 0.01 μ g	1,490 $\pm$ 0.09	72
<i>Par j</i> I, 0.001 μ g	854 $\pm$ 0.06	32

Discussion

Several methods have been previously described in the literature to obtain pure allergen molecules in a simple and reliable way. We report here the methodology used to isolate to purify the *Par j* I allergen. Since the molecular weight of the allergen to be purified was previously known, it was possible to commence the purification using simple ultrafiltration dialysis and various molecular weight exclusion filters.

Using these procedures it is possible to efficiently reduce the number of antigens in two steps. We reduced the initial crude extract to only 6 antigens, including *Par j* I, using these procedures. The initial extract contained 26 antigens. This mixture of partially purified antigens was used to induce monoclonal antibodies specific for the *Par j* I allergen. By using the

Table 2. Amino acid composition of *Par j* I purified from *P. judaica* pollen

Amino acid	Residues per molecule
Alanine	12
Arginine	6
Aspartic acid ^a	12
Cysteine ^b	2
Glutamic acid ^a	14
Glycine	11
Histidine	2
Isoleucine	4
Leucine	10
Lysine	7
Methionine	3
Phenylalanine	4
Proline	7
Serine	8
Threonine	7
Tryptophan	ND
Tyrosine	1
Valine	8
Total	118

ND = Not determined.

^a Aspartic acid and glutamic acid values include those of asparagine and glutamine.

^b Cysteine was determined as per cysteic acid after oxidation of the sample with performic acid prior to acid hydrolysis.

monoclonal antibody linked to a Solis support of Sepharose beads it was possible to obtain a homogeneous allergen in a single simple step. The homogeneity of the molecule absorbed to the affinity column was assessed by different methods based on either immunological or biochemical properties. The results of CIE and CRIE revealed one single arc, this evidence supporting the suggestion that the allergen was homogeneous. To avoid the possibility of the presences of some contaminant molecules, not recognized by the rabbit antiserum, further analysis, using an SDS-PAGE followed by blotting methodology, was performed to assess homogeneity. Once again, only one single band of radiostaining was obtained. This procedure also provided additional information on the molecular weight of the labeled molecule under reduced conditions. The molecular weight value

of 13 kD confirmed the value determined by previous reports [1, 2].

Homogeneity and molecular weight were further analyzed and confirmed using HPLC. To be sure that the molecule bound to the monoclonal immunosorbent was effectively the *Par j* I, a CLIE analysis was performed. The results showed an enhancement of the *Par j* I arc in the standard CIE picture. This evidence confirms that the bound molecule was *Par j* I.

The *Par j* I antigen obtained through purification so far was still allergenically active as demonstrated by its ability to induce positive reactions in vivo, skin prick tests, by inhibiting a RAST standard assay and by inducing release of histamine when used to challenge blood basophils from *P. judaica*-allergic patients. The allergenic activity of the *Par j* I allergen was about 8 times less active than the total crude *P. judaica* extract. The amino acid composition of the *Par j* I allergen does not reveal any peculiar feature. As calculated from the amino acid composition, the molecular weight of the reduced allergen is in good agreement with that determined by SDS-PAGE. The number of hydrophilic residues is almost equal to that of hydrophobic ones and the percentage of these two groups of amino acids is not different from the percentage reported for other molecules of the same biological properties. Only the methionine residue content seems to differ, there being 3 methionine residues per monomer. This value is quite high when compared with the amino acid composition of other allergens. The presence of 3 methionine residues could be useful as breaking points in order to obtain shorter allergenic molecules via CNBr hydrolysis. The *Par j* I allergen so far purified to homogeneity could be a useful tool for several purposes in either clinical or basic research.

It will be possible to perform studies on the IgE-specific determinants present on the major allergen. It may also help to elucidate the possibility that these determinants are shared with other allergens present on the same pollen. The purified *Par j* I could also be used to prepare specific antibodies which in turn will give more accurate diagnosis and standardization studies.

References

- 1 Geraci, D.; Bache-Billesbolle, K.; Cocchiara, R.; Lowenstein, H.; Ipsen, H.: Immunochemical characterization of antigens of *Parietaria judaica* pollen: identification of allergens by means of crossed radioimmuno-electrophoresis. *Int. Archs Allergy appl. Immun.* 78: 421-428 (1985).
- 2 Ford, S.A.; Baldo, B.A.; Geraci, D.; Bass, D.: Identification of *Parietaria judaica* pollen allergens. *Int. Archs Allergy appl. Immun.* 79: 120-126 (1986).
- 3 Feo, S.; Cocchiara, R.; Geraci, D.: Allergens of *Parietaria judaica* pollen. I. Purification and characterization of a hapten and a low molecular weight allergenic peptide. *Molec. Immunol.* 21: 25-36 (1984).
- 4 Ruffilli, A.; Orest, U.; Santonastaso, V.; Scotto D'Abusco, A.; Sacersoti, G.: Low molecular weight allergens of the pollen of *Parietaria officinalis*. *Molec. Immun.* 2: 305-312 (1987).
- 5 Marsh, D.G.; Goodfriend, L.; King, T.P.; Lowenstein, H.; Platts-Mills, T.A.: Allergen nomenclature. *Int. Archs Allergy appl. Immun.* 85: 194-200 (1988).
- 6 Guarnotta, G.; Parkhouse, R.M.E.: Hybridization of lymphocytes: techniques and applications; in Lederer, Weetall, *Clinical cellular immunology*, pp. 333-358 (Humana Press, Clifton 1982).
- 7 Amoroso, S.; Cocchiara, R.; Locorotondo, G.; Parlato, A.; Lampiasi, N.; Albeggiani, G.; Falagiani, P.; Geraci, D.: Antigens of *Euparipha pisana* (snail). I. Identification of allergens by means of in vivo and in vitro analysis. *Int. Archs Allergy appl. Immun.* 85: 69-75 (1988).
- 8 Cocchiara, R.; Di Trapani, G.; Azzolina, A.; Albeggiani, G.; Geraci, D.: Isolation of a histamine releasing factor from two-cell human embryo. *Int. Archs Allergy appl. Immun.* 84: 321-323 (1987).
- 9 Shaff, R.E.; Beaven, M.A.: Increased sensitivity of enzymatic-isotopic assay of histamine: measurement of histamine in plasma and serum. *Analyt. Biochem.* 94: 425-431 (1979).

Received: January 6, 1989

Correspondence to: Dr. Roberta Cocchiara
Istituto di Biologia dello Sviluppo
CNR
Via Archirafi
I-90139 Palermo (Italy)