

ALLERGENS OF *PARIETARIA JUDAICA* POLLEN—I.
PURIFICATION AND CHARACTERIZATION OF
A HAPTEN AND A LOW MOLECULAR WEIGHT
ALLERGENIC PEPTIDE

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(First received 24 March 1983; accepted in revised form 13 July 1983)

Abstract—A low mol. wt allergen (Pj-2) and a hapten (Pj-H₁) were purified from *Parietaria judaica* pollen by means of long-term aqueous extraction, dialysis and gel filtrations. The yield of the Pj-2 allergen was 0.94% (w/v) of the total protein present in the aqueous extract of the pollen, while its allergenic activity was about 60% of the total dialyzable activity, as verified by skin prick tests, ELISA- and RAST-inhibition experiments. The homogeneity of this allergen was demonstrated by one single sharp peak on HPLC, one single band on PAGE-SDS and by one single arc on IEF. Its mol. wt. estimated by HPLC and amino acid composition, was 10,400. The amino acid analysis showed 73 amino acid residues, and lysine was predominant, with 20 residues. The hapten Pj-H₁ was 0.2% (w/v) of the total protein found in the pollen aqueous extract. It was inactive in skin prick tests even at a protein concn of 2 mg/ml, while it was capable of inhibiting by 60% in ELISA- and RAST-inhibition experiments, suggesting an immunochemical relationship with both IgE and allergens specific to *P. judaica*. The homogeneity was demonstrated by one single sharp peak on HPLC and one single band on PAGE-SDS. The amino acid analysis showed 10 amino acid residues, with no specific traits, and the mol. wt determined by gel filtration and amino acid composition was 1000. An immunochemical relation between the allergen and the hapten was also suggested by the results of an ELISA-inhibition test, and by the ability of the hapten to partially inhibit the precipitin line between rabbit antibodies to whole *P. judaica* pollen extract and the Pj-2 allergen. The allergen and the hapten described above, purified at homogeneity and in an antigenically active state, both provide adequate material for further structural and immunological characterizations.

INTRODUCTION

An area of considerable interest in allergology is the study of the allergenic molecules which can elicit an atopic response. To study the interactions between these molecules and the human immune system an essential prerequisite is the purity of the elements considered, and an important step is the isolation and characterization of highly purified allergens.

The immunochemical characterization of allergens isolated from several sources has been carried out by many laboratories (Elsayed and Aas, 1971; Geraci *et al.*, 1978; King, 1976; King and Norman, 1962; Menegozzo *et al.*, 1976; Viander *et al.*, 1979; Lapkoff and Goodfriend, 1974; Lowenstein and Marsh, 1981) and all the results up to date confirm that no specific structural features distinguish an allergen from an antigen molecule (King, 1979).

In order to study the sequence of an allergenic molecule, or to determine the nature of the allergenic determinants, or to study the interactions between an allergen and the specific IgE, it is easier to utilize an allergenically active molecule which is as small as possible. Many laboratories have reported the isolation from pollen of low mol. wt allergens (Girard *et al.*, 1975; Lapkoff and Goodfriend, 1974; Vik *et al.*,

1982), and of fractions with hapten-like properties (Attallah and Schon, 1969; Chakrabarty *et al.*, 1980; Malley *et al.*, 1964), and several structural studies have been performed (Elsayed and Bennich, 1975; Elsayed *et al.*, 1973; Goodfriend and Capra, 1975; Klapper *et al.*, 1980; Mole *et al.*, 1975).

One other approach to obtaining such small molecules is to digest the allergens with proteolytic enzymes so that several fragments can be isolated, and their allergenic activity and structural characteristics easily determined (Apold and Elsayed, 1980; Elsayed and Apold, 1980; Elsayed *et al.*, 1973, 1974, 1980).

In addition to the immunochemical studies, the purified allergens and haptens can be also used for clinical purposes.

Several experiments to discover how to improve the immunologic management of allergic conditions have been carried out.

One of the main experimental approaches actually used is focused on the suppression of the IgE antibody response by an antigen-specific mechanism, and the final goal of using allergens or haptens is to find some means to suppress such a response. One way is the use of allergens chemically modified to reduce the allergenicity, and called "allergoids" (Marsh, 1971, 1975; Marsh *et al.*, 1970), or to use conjugations of haptens with tolerogenic carriers to tolerate B-cells or to induce antigen-specific suppressor T-cells [reviewed by Schon (1982)]. However, whatever the

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approach used, the purity of the molecules involved is once again of paramount importance.

The choice of *Parietaria judaica* (Pj) pollen as the biological material for the present study is derived from its allergological importance and geographical distribution in the Mediterranean area. In fact, an epidemiological study carried out by our group in the metropolitan area of Palermo revealed that this pollen was the main cause (53%) of the allergic reactions analyzed (1787 allergic patients tested, manuscript submitted for publication). Regarding the regional distribution in Italy of the weed *Parietaria*, the two main spp. *judaica* and *officinalis*, were generally indicated only as *officinalis* by many botanico-pharmaceutical and clinical studies. However, relatively recent studies (Accorsi and Mazzanti, 1980; Raffaelli, 1977), have pointed out that in addition to palynological differences there is also a different geographical distribution: the sp. *officinalis* is present only in the north of Italy, while the sp. *judaica* is very common in all of Italy.

The aim of the present study was to purify molecules of low mol. wt involved in the allergic reaction to pollen of Pj, to use these molecules for an immunochemical characterization and to obtain a useful material for further structural analysis.

MATERIALS AND METHODS

Skin test

Skin tests were performed using the prick test method in patients with unequivocal clinical histories of Pj allergy. Histamine-HCl at a concn of 1 mg/ml was used as a positive reference and a saline solution, 0.9% NaCl, as a negative control. The area of the wheal was read after 15 min, the largest dia (*D*) and the dia perpendicular to it (*d*) were measured in millimeters and the reaction was calculated as $D + d/2$ (Marsh, 1975). Wheals of $D + d/2 = 3$ were recorded as negative. Wheals derived from the histamine prick were used as references for the reaction of the allergenic fractions. If we consider the reaction produced with the histamine-HCl solution as 1 HEP (one histamine equivalent prick), then an active fraction has 1 HEP of allergenic potency when it elicits a prick test reaction with the same $D + d/2$ value. Using fractions at different protein concns, the HEP values were calculated for milligrams of protein.

Human sera

Sera were collected from patients allergic to Pj pollen, with positive clinical histories and RAST grade 2-4. No hyposensitization therapy was received by the selected patients. Pooled sera after collection were stored at -20°C until used.

Immunization

Antibodies against a Pj aqueous extract were raised in two rabbits weighing about 2.5 kg each. The aqueous solution was obtained by extraction of pol-

len in water for 4 hr at 4°C under continuous stirring. The suspension was centrifuged at 12,000 *g* for 10 min, filtrated and lyophilized. The aqueous extract (2 mg), was dissolved in 0.5 ml of PBS buffer, pH 7.2, and 0.5 ml of Freund's complete adjuvant was added. The suspension was emulsified prior to an intradermal injection on days 0, 14, 28 and 42. On the 10th day after the last injection, the animals were bled and killed under anesthesia. The antibody fraction was purified following a procedure previously described (Harboe and Ingild, 1973).

Crude extract of Pj pollen

Twenty grams of dry Pj pollen, 75% pure, containing less than 0.5% of foreign pollen (Allergon, Sweden), were extracted with 1 liter of bidistilled water, under continuous stirring for 2 days at 4°C in a cold room. The pollen suspension was then centrifuged in the cold at 12,000 *g* for 10 min, and the supernatant, after a filtration step to eliminate the fine debris, was added with solid ammonium sulfate to 80% saturation under gently stirring. The material precipitated was collected by centrifugation at 12,000 *g* for 10 min, and the pellet was dissolved in 25 mM sodium acetate, pH 4.0.

Isolation of allergen and hapten from pollen

The crude extract solution was exhaustively dialyzed in a cold room against 25 mM acetate buffer, pH 4.0.

The dialyzer tubing (Visking tubing, Union Carbide, Canada) retaining material with mol. wts of 12,000 and higher was boiled in 50% ethanol, then in 10 mM EDTA and stored at 4°C in bidistilled water before use. The dialyzate obtained was concentrated by lyophilization, filtrated through a Sephadex G-10 column (100 $\times$ 2.8 cm) previously equilibrated in 1 M acetic acid, pH 4.0, and then eluted at a flow rate of 40 ml/hr, using the same buffer. Fractions of 4 ml were collected and buffered with 150 μl of 1 M Tris-HCl, pH 7.6. Optical absorption at 220 nm was monitored with an LKB 2138 Uvicord S, and measured with a Beckman spectrophotometer. Protein determination was carried out using the method of Lowry *et al.* (1951), and measuring the absorbancy at 215 and 225 nm in 0.01 N HCl buffer with BSA and glucagon as standard proteins (Murphy and Kies, 1960). The allergenic activity was determined by a direct skin test, and by inhibition of the RAST test. The fractions corresponding to active peaks were collected and lyophilized. The allergenic peak was dissolved in 10 mM Tris-HCl, pH 8.0, and filtrated through a column of Sephadex G-50 (70 $\times$ 2 cm) using the same solution to equilibrate and to elute the column at a flow rate rate of 25 ml/hr. The peak of protein, determined by absorption at 220 nm, was collected and the skin prick test (SPT) ELISA-inhibition tests were performed.

The haptenic peak, concentrated and lyophilized, dissolved in 1 M acetic acid, pH 4.0, was chro-

matographed through a column of Bio Gel P2 (200 $\times$ 1 cm) at a flow rate of 3.5 ml/hr in the same buffer. Each fraction was tested for the ability to inhibit *in vitro* an ELISA test in the IgE system.

Molecular weight determination

The mol. wt of the haptenic peak was determined using a gel filtration step on a column of Bio Gel P2 (200 $\times$ 1 cm) equilibrated with 1 mM acetic acid, pH 4.0. Proteins with well-characterized mol. wts such as glucagon (3640), bacitracin (1400) and glutathion ox. (612), were used as markers. Fractions of 2.0 ml were collected at a flow rate of 3.5 ml/hr. The position of the proteins was determined by absorption at 220 nm. The mol. wt of the allergenic peak was determined by HPLC with an HPLC system consisting of a Beckman Model 340 injector with a Model 112 pump and a Model 160 spectrophotometer (Beckman Instrum., Irvine, CA), using a Spherogel-TSK 2000-SW molecular sieve column (600 $\times$ 7.5 mm) (Altex Scientific, U.S.A.). Chromatographic analyses were carried out at room temp at a flow rate of 60 ml/hr (320 PSI). The column was equilibrated with 0.1 M phosphate buffer, pH 7.0, and 0.3 M NaCl, and the eluate was monitored at 254 nm at varying sensitivities as described in the Results. The protein markers were human γ -globulin (mol. wt 167,000), transferrin (mol. wt 90,000), BSA (mol. wt 68,000), ovalbumin (mol. wt 46,000), soybean trypsin inhibitor (mol. wt 24,000), cytochrome *c* (mol. wt 12,300) and glucagon (mol. wt 3640), of analytical grade purity, purchased from Sigma (St Louis, MO). Twenty-microliter samples at 1-5 mg/ml were charged onto the column in all the experiments.

Amino acid analysis

Selected proteins were analyzed for amino acid content in collaboration with Dr A. Corrao of the Istituto Zooprofilattico della Sicilia, Palermo.

The proteins were hydrolyzed in 6 M HCl at 110°C in evacuated and sealed vials for 24, 36 and 48 hr, and the amino acid composition was calculated by a computer integrator connected to a Jeol JLC-6AH (Japan) automatic amino acid analyser.

Immunologic techniques

ELISA was carried out as described previously (Engvall and Perlman, 1972) with minor modifications (Cocchiara *et al.*, 1981; Giallongo *et al.*, 1980). The ELISA-inhibition assay was performed using disposable polystyrene plates (Cook Microtiter System, U.K.). Two hundred microliters of an aqueous extract of Pj pollen, or of a purified allergenic peptide, at a protein concn of 20 $\mu\text{g}/\text{ml}$ in the coating buffer (15 mM pH 9.6) sodium carbonate, were added to coat each well overnight at room temp. Aliquots from a pool of 30 sera of patients allergic to Pj pollen were diluted with PBS-Tween buffer (PBS + 0.05% Tween + 0.02% sodium azide) mixed

with the allergen or hapten solution at increasing protein concn and incubated overnight at room temp (the ELISA reference values were obtained using PBS-Tween instead of the inhibitor solutions). Two hundred microliters of this solution were added to the wells coated with the allergen and incubated for 6 hr at room temp. After a washing step to eliminate the unbound material, 200 μl of a conjugate solution (anti-human-IgE/alkaline phosphatase) were added to the allergen-IgE-coated wells and incubated overnight at room temp. The unbound conjugates were washed out and 200 μl of *p*-nitrophenyl phosphate at a concn of 1 mg/ml were added. After an incubation step at room temp for 60 min, the enzymatic reaction was determined at 405 nm by a Titertek Multiskan automatic plate reading machine (Flow Laboratories, U.S.A.). The ELISA-inhibition values were expressed as percent of the ELISA reference value obtained without addition of any inhibitor molecule.

The radioimmunoassay (RAST) described by Ceska *et al.* (1972) has been used. Allergens (coupled to discs in our laboratory) were from the same preparations as those used for the skin prick tests and ELISA assay. Radiolabelled ^{125}I -rabbit anti-human IgE was obtained from Pharmacia (Uppsala, Sweden). The RAST-inhibition was carried out employing a pool of 30 sera of patients allergic to Pj pollen (RAST grade 3-4). Allergosorbent discs were prepared by linking the Pj aqueous extract to cyanogen-activated filter paper discs, 5 mm dia (Whatman 1), about 1 mg for each disc. Fifty microliters of the pooled sera solution were preincubated overnight at room temp with 200 μl of PBA buffer containing increasing quantities of a given fraction expected to inhibit. After this preincubation step, each mixture was added to an allergosorbent disc and incubated for 3 hr at room temp. After a washing step with PBS buffer, the IgE binding values were determined by addition of ^{125}I -labelled rabbit anti-human IgE. The percent of inhibition was calculated as the reduction in the uptake of the ^{125}I -anti-IgE by the allergosorbent disc in the presence of the inhibitor, in relation to the amount of radioactivity bound to the allergosorbent disc in the absence of the inhibitor.

Immunoelectrophoresis was performed as previously described (Geraci *et al.*, 1978). Agarose was dissolved in 0.14 M veronal buffer, pH 8.6, to give a 1% solution. Calcium lactate was added to 0.7% and sodium azide to 2%. This solution was used to prepare the agar slides. Immunoelectrophoresis was performed with 0.14 M veronal buffer, pH 8.6, for 30 min, at a potential difference of 8 V/cm. The precipitation patterns were developed for 24 hr in a humid chamber at room temp, and colored with 0.5% Coomassie brilliant blue G-250, in 45% methanol, 45% water and 10% acetic acid, and destained by diffusion in a methanol, water and acetic acid solution (45:45:10%).

The inhibition of the immunodiffusion was carried out as follows: the same vol of rabbit anti-Pj pollen

extract serum was preincubated overnight at room temp with increasing amount of the Pj-H₃ hapten; the reference control was PBS instead of the potential inhibitor; the final vol was the same for all the samples, to give the same dilution of the antiserum added. After the preincubation, 15 μ l of these samples were placed in four wells around a central well where 15 μ l of the allergen Pj-2 at 1 mg/ml was added, and the immunodiffusion was performed according to the Ouchterlony method (Ouchterlony, 1958).

Polyacrylamide gel electrophoresis

Electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate (PAGE-SDS) was performed essentially as described by Weber and Osborn (1969). The protein samples were denaturated using a mixture of SDS and beta-mercaptoethanol, 1% final concn bromophenol blue, 0.05% final concn, was used as the tracking dye. After a boiling step of 2 min, the samples were added with glycerol, 10% final concn, and loaded on a 15% polyacrylamide slab gel containing SDS. Electrophoresis was performed and stopped when the tracking dye reached a fixed dis-

tance from the top of the gel. For direct visualization, the gel was stained with Coomassie brilliant blue.

RESULTS

Long-term pollen extraction

The surface of many pollens contains, in addition to molecules with allergenic activity, a set of several enzymes with specific proteolytic activity (Bousquet *et al.*, 1978; Ceska *et al.*, 1973; Marsh, 1975). The pollen of Pj also contains enzymes with proteolytic activity (data not showed here, manuscript in preparation), and this endogenous activity has been used to obtain molecules of low mol. wt. Using long-term extraction (2 days) of the pollen, an endogenous enzyme with proteolytic activity was capable of reducing the size of the native allergenic molecules released during the pollen extraction. An indirect control of this enzymatic activity, using shorter times of pollen extraction in the presence of a protease inhibitor, will be discussed later. A schedule of the methods used to obtain the allergenic and haptenic molecules is showed in Fig. 1.

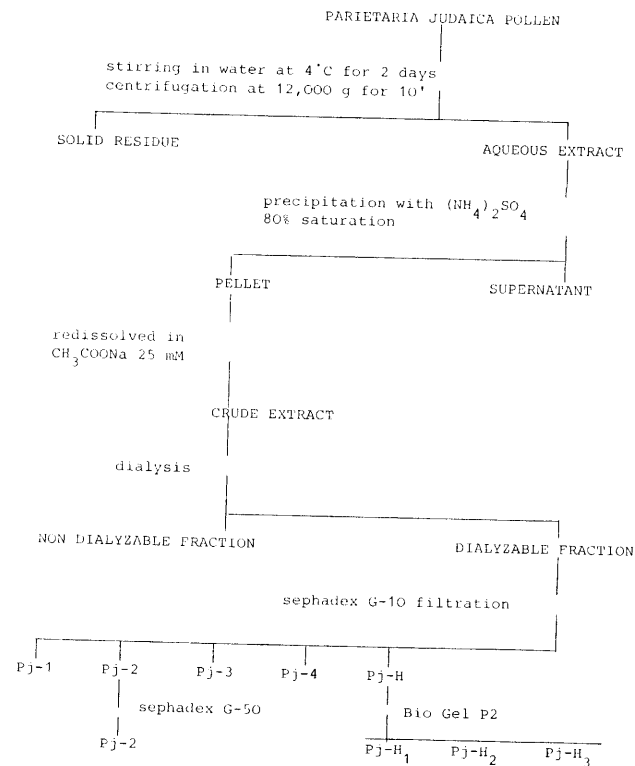


Fig. 1. Procedure used for the isolation of allergens and haptens from Pj pollen.

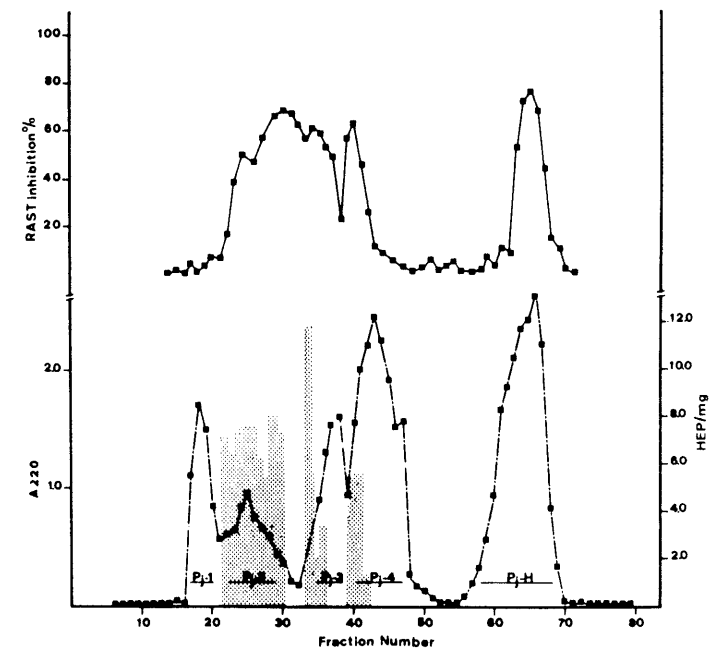


Fig. 2. Gel filtration pattern of dialyzable fraction on Sephadex G-10 (100 $\times$ 2.8 cm) equilibrated and eluted with 1 mM acetic acid, pH 4.0, at a flow rate of 40 ml/hr. Each fraction (4.0 ml) was buffered with 150 μ l of 1 M Tris-HCl, pH 7.6, and used for determination of absorption profile at 220 nm (■---■), and of protein concn using the procedures described in Materials and Methods. The allergenic activity was measured by a direct SPT, and the HEP values of each fraction were related to 1 mg of protein (□---□). The RAST inhibition (■—■) was carried out as described in Materials and Methods.

Dialysis and gel filtration of pollen extract

The supernatant obtained after centrifugation of the pollen suspension was dialysed until no more allergenic molecules crossed the dialysis bag. This was determined by changing the external buffer several times, and then testing it for SPT activity. All the SPT-active dialysis solutions were concentrated and, after a Sephadex G-10 filtration, five protein peaks were obtained (Fig. 2). The O.D., RAST and SPT values obtained gave patterns that were identical after several filtrations carried out on four different (year of collection) pollen batches, and on five extractions of each batch. Concerning the separation of the protein peaks, these are eluted from this column later than predicted from their molecular size. This adsorption effect could be explained by an interaction between the small molecules of the solute and the tightly cross-linked gel G-10 in the presence of a elution solvent at low ionic strength such as the 1 mM acetic acid used (Janson, 1967).

The allergenic activity (SPT) of each fraction was determined using 30 volunteers allergic to Pj pollen. The RAST-inhibition values were obtained using a

pool of sera derived from the same 30 Pj-allergic volunteers. The SPT activity was mainly present in the second protein peak Pj-2, while it was completely absent in the first and fifth peaks.

The RAST-inhibition activity was also present in the Pj-2 peak and absent from the first peak, but gave significant positive values in the fifth peak. As a control for both SPT and RAST-inhibition activity, the whole Pj-2 peak was tested on 10 subjects. Five of them were non-allergic, three were grass-pollen allergic and two were *Dermatophagoides* pt allergic subjects. None of these controls were positive with the SPT and the RAST-inhibition test.

This result suggested that Pj-2 was the most active allergenic peak, about 60% of the total dialyzable allergenic activity, and that Pj-H, which was not active in the SPT but was capable of inhibiting the RAST assay, was a fraction with haptenic properties. If the time of the pollen extraction was reduced to 4 hr, and if a protease inhibitor such as the phenyl-methylsulphonyl fluoride (PMSF) was added at a concn of 50 μ g/ml to the pollen suspension, a different protein profile was obtained after the

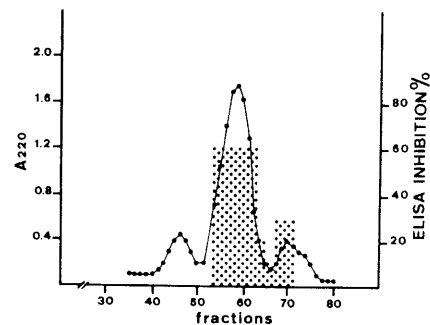


Fig. 3. Gel filtration profile of the Pj-2 fraction on a column of Sephadex G-50 (60×2 cm). Elution buffer: 1 mM acetic acid, pH 4.0; flow rate 13 ml/hr. Each fraction (2.0 ml) was buffered with 1 M Tris-HCl, pH 7.6, and used for the determination of the absorption profile at 220 nm (●—●), and as a potential inhibitor in an ELISA inhibition test (.....). A crude Pj extract at 20 μ g/ml, and a pool of 30 sera of patients allergic to the Pj pollen were used in the immunoassay.

Sephadex G-10 filtration, and no fractions with haptenic activity were detected.

Purification of the allergen and hapten

The fractions of the Sephadex G-10 column corresponding to the Pj-2 allergenic peak were collected, lyophilized and chromatographed on a column of Sephadex G-50. A main peak of protein was obtained

and its allergenic activity was demonstrated by an SPT and an ELISA-inhibition assay, carried out using the pollen aqueous extract as substrate, and each fraction of the column as a potential inhibitor (Fig. 3).

The haptenic fraction Pj-H isolated by the Sephadex G-10 filtration was resolved by chromatography on a column of Bio Gel P2 into at least three peaks designated arbitrarily as Pj-H₁, Pj-H₂ and Pj-H₃ (Fig. 4). The haptenic activity determined by an ELISA-inhibition assay was almost equally distributed through the three peaks, and Pj-H₃ has been chosen for additional studies because of its lower mol. wt.

Allergenic activity

The allergenic and haptenic activities of the two molecules Pj-2 and Pj-H₃ were determined in their capacity to inhibit the ELISA test. The aqueous extract fraction adhering to the plastic wells was used as substrate, and a pool of 30 Pj-allergic sera as a source of IgE antibodies. These sera were derived from patients that after an SPT were positive for the Pj-2 allergen, and negative for the Pj-H₃ hapten, even at a protein concn of 2 mg/ml. The results given in Fig. 5 and Table 1 suggest that the allergen and hapten were capable of inhibiting 66 and 60% respectively of the ELISA reference value. As a control, unrelated proteins such as bacitracin, cytochrome *c* and BSA, at the same protein concn, were used in the ELISA-inhibition assay, and no inhibition values were obtained. The allergenic activity was also calcu-

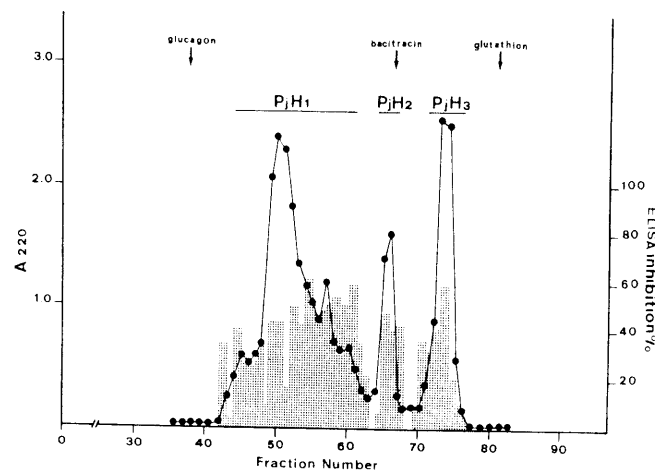


Fig. 4. Gel filtration on a Bio Gel P2 column (200×1 cm) of the haptenic fraction Pj-H. The column was previously equilibrated in 1 mM acetic acid, pH 4.0, and the haptenic material was eluted with the same solution at a flow rate of 3.5 ml/hr. Fractions of 2.0 ml were collected and the absorbance profile was determined at 220 nm in a spectrophotometer (●—●). The haptenic activity of each fraction was evaluated by inhibition of the ELISA test, using disposable polystyrene plates coated with 20 μ g/ml of an aqueous extract of Pj pollen. The ELISA-inhibition values (.....) were expressed as per cent of an ELISA reference test carried out by addition of those fractions of the column without any A_{220} absorption value.

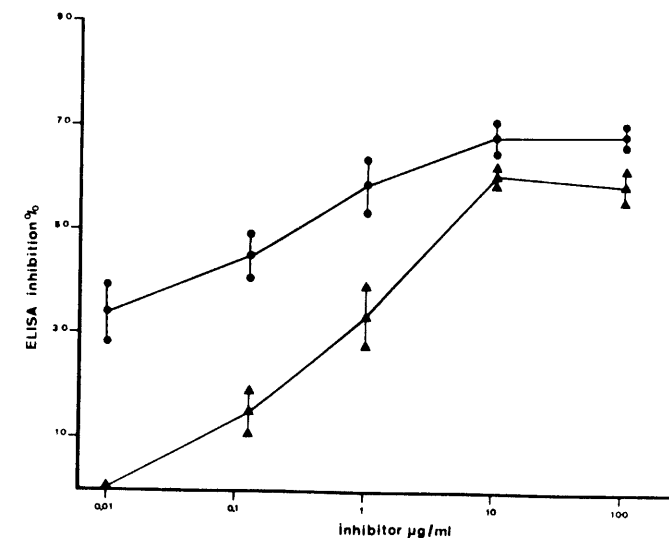


Fig. 5. Inhibition of the ELISA test by the allergen Pj-2 (●—●) and the hapten Pj-H₃ (▲—▲). The ELISA-inhibition values were the mean $\pm$ SD of triplicate experiments.

lated as HEP values determined by an SPT, and the total HEPs expressed by Pj-2 were about 12% of the total HEPs present in the whole aqueous extract, while the protein yield was only 0.94% (Table 1).

Pj-H₃ was 0.2% of the total protein present in the aqueous pollen extract and was also inactive in an SPT at a protein concn of 2 mg/ml (Table 1), when tested in a series of 82 allergic patients who gave strong SPT reactions to the aqueous extract of Pj pollen and with a RAST class grade of 3–4.

Homogeneity

The purity of the allergen Pj-2 was determined by chromatographic runs on HPLC and a single symmetrical peak was obtained (Fig. 6A). This purity was also indirectly demonstrated when Pj-2 was chromatographed on a different (molecular sieve) HPLC column to determine the mol. wt. and, as shown in Fig. 6C, a single peak was determined. The hapten Pj-H₃ was also analyzed by HPLC and only one sharp peak was detected (Fig. 6B).

The homogeneity of the two molecules was also demonstrated by using PAGE-SDS chromatography (Fig. 7A) and a single band was obtained for both Pj-2 and Pj-H₃. To establish if the allergen Pj-2 was also immunochemically pure a preliminary immunoelectrophoresis was carried out using different concns of the allergen. The results reported in Fig. 7B would suggest this possibility, but additional evidence is needed and work using CIE and CRIE analyses is in progress.

Molecular weight determination

The mol. wt of Pj-2 was calculated by means of chromatography on HPLC using the relative retention time of the allergen and of several proteins with known mol. wt ($3.6\text{--}167 \times 10^3$) as references (Fig. 6C). From this analysis the mol. wt was calculated to be 10,400.

To determine the mol. wt of the hapten Pj-H₃, the elution vols of the hapten and protein markers (range

Table 1. Yield and biological activity of allergen and hapten from *Parietaria judaica*

Fractions	Protein (mg/ml)	Total protein (mg)	Yield (%)	Specific HEP ^a	Total HEP	ELISA-inhibition (%)
Aqueous extract	0.412	370.8	100	3.9	1446	93
Crude extract	10.6	207.5	56	6.0	1245	88
Dialyzable fraction	4.1	41	11	7.7	316	51
Pj-2	1.0	3.5	0.94	53.5	187	66
Pj-H ₃	0.73	0.73	0.2	^b	^b	60

^aHEP/mg of protein.

^bPj-H₃ was also not active at a protein concn of 2 mg/ml.

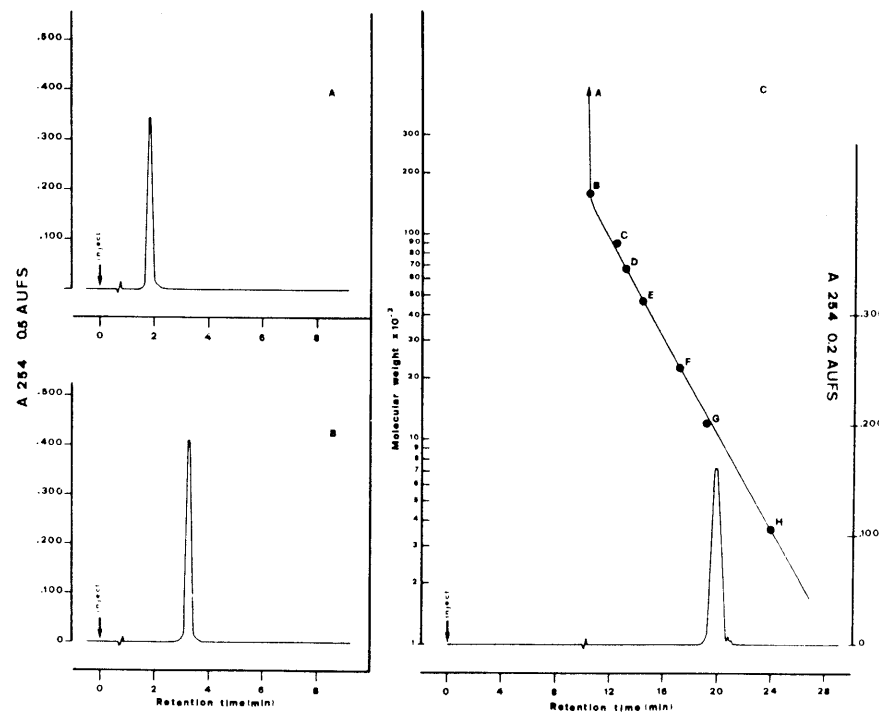


Fig. 6. HPLC analysis used to determine the homogeneity of the allergen Pj-2 (A) and the haptens Pj-H₁ (B), and the mol. wt of the allergen Pj-2 (C). The analysis of the homogeneity of the two molecules was carried out on a 5- μ Ultrasphere ODS column (150 $\times$ 4.5 mm) using 0.1 M NaH₂PO₄, pH 2.2, buffer, at a flow rate of 60 ml/hr. The vol of the samples injected was 20 μ l at varying protein concns. The detection of the protein peaks was made by a spectrophotometer at 254 nm and 0.5 AUFS. The mol. wt estimation of the allergen Pj-2 was carried out using a Sphergel-TSK 2000-SW molecular sieve column (600 $\times$ 7.5 mm), eluted with 0.1 M phosphate buffer, pH 7.0, plus 30 mM NaCl, at a flow rate of 60 ml/hr (320 PSI). About 250 μ g of sample were injected and the position of the protein peaks was detected at 254 nm and 0.2 AUFS. Proteins with known mol. wts (values listed in Materials and Methods) were used for the calibration of the column: (A) blue dextran; (B) human globulin; (C) transferrin; (D) BSA; (E) ovalbumin; (F) soybean trypsin inhibitor; (G) cytochrome *c*; (H) glucagon.

612–3640) were obtained on a column of Bio Gel P2, and the value was determined to be 1000 (Fig. 4).

Amino acid composition

The amino acid compositions of Pj-2 and Pj-H₃ were calculated on the basis of the mol. wts previously mentioned. All of the common amino acids in a total of 73 residues were present in the Pj-2 molecule, and lysine was the most abundant, with 20 residues.

The Pj-H₃ was made up of 10 amino acid residues and no predominant or specific traits were detected (Table 2).

Relation between Pj-2 and Pj-H₃

An immunochemical relationship was suggested by the results shown in Fig. 8. Using an ELISA-inhibition test, the haptens Pj-H₃ was capable of

inhibiting the reaction between the allergen Pj-2 bound to the plastic wells and the specific IgE of 30 pooled sera of subjects allergic to Pj pollen, SPT-positive for the Pj-20 allergen, and SPT-negative for the Pj-H₃ haptens (Fig. 8A).

If a different system of analysis was used (immunodiffusion), Pj-H₃ was again capable of partially inhibiting the precipitin arc between the allergen Pj-2 and a rabbit anti-aqueous pollen extract serum (Fig. 8B). When a protein of comparable mol. wt, bacitracin (1400) was added as a control to the two assays, no inhibition or reduction of the precipitin arc was obtained.

DISCUSSION

In this paper it was demonstrated that it is possible to isolate haptenic molecules and allergens of low

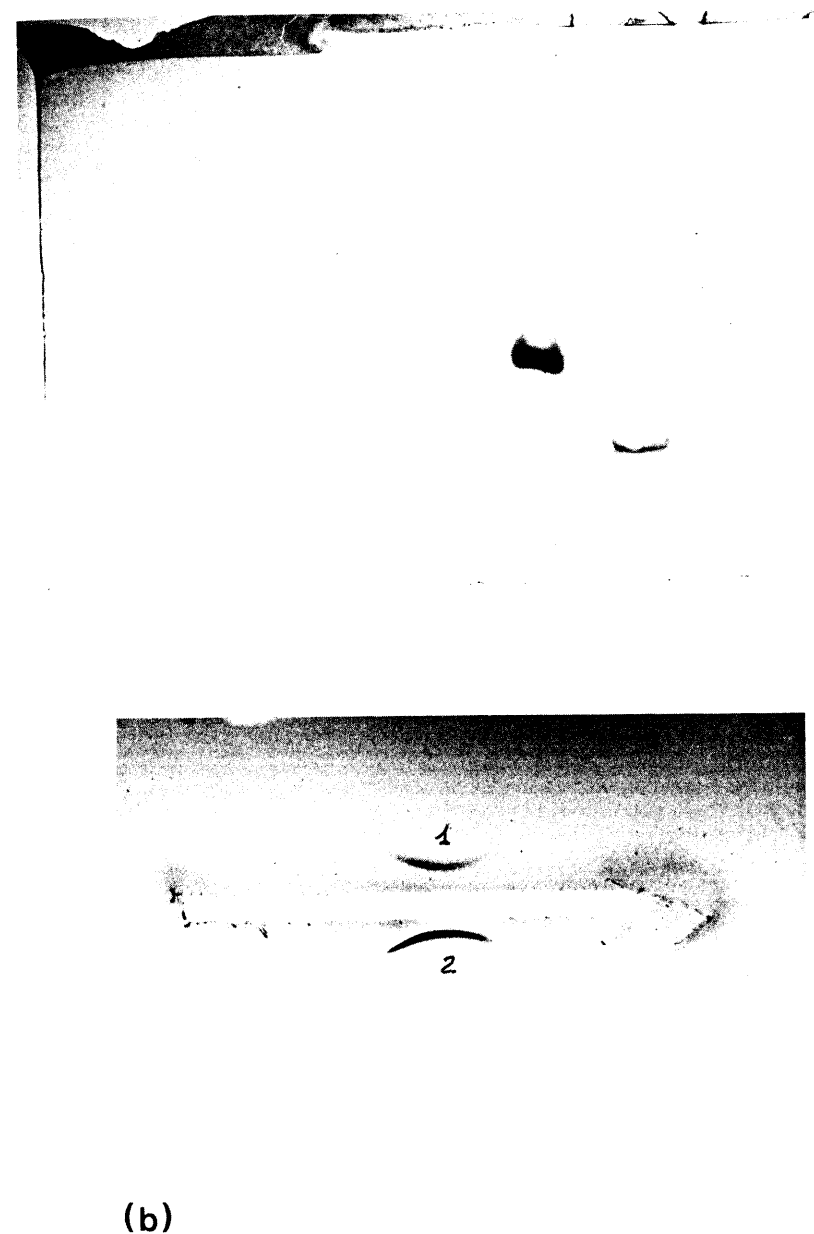


Fig. 7. (A) 15% PAGE-SDS chromatography of 10 μ g of allergen Pj-2 (left), and 25 μ g of haptens Pj-H₃ (right). The protein bands were stained with 0.5% Coomassie brilliant blue G-250 in a solution of methanol-water-acetic acid (45:45:10%), and destained overnight in the same solution. (B) Immunoelectrophoresis in 1% agarose. Fifteen microliters of Pj-2 allergen at two different protein concns (20 μ g in 1 and 40 μ g in 2) were added to the wells. In the central well, 100 μ l of rabbit antiserum against the whole Pj pollen aqueous extract were added.

Table 2. Amino acid composition of Pj-2 allergen and Pj-H₁ hapten isolated from Pj pollen

Amino acid	Residues per molecule	
	Pj-2	Pj-H ₁
Alanine	5	1
Arginine	4	—
Aspartic acid ^a	8	1
Cysteine ^b	—	—
Glutamic acid ^c	8	1
Glycine	6	2
Histidine	3	—
Isoleucine	2	—
Leucine	3	1
Lysine	20	—
Methionine	1	2
Phenylalanine	1	—
Proline	3	—
Serine ^d	2	1
Threonine ^e	3	1
Tryptophan ^f	ND	ND
Tyrosine	1	—
Valine	3	—
Total	73	10

The proteins were hydrolyzed in 6 M HCl *in vacuo* and in sealed glass vials at 110 °C for 48 hr. The mol. wt. was used for the calculations were allergen Pj-2 10,400, and hapten Pj-H₁ 1000.

^aAspartic acid and glutamic acid were comprehensive of asparagine and glutamine.

^bCysteine was determined as cysteic acid following oxidation of the sample with performic acid prior to acid hydrolysis (Hirs, 1956).

^cThreonine and serine were extrapolated to zero-time hydrolysis.

^fTryptophan was not determined (ND).

mol. wt from the dialyzable fraction of Pj pollen, using a time of pollen extraction longer than that usually adopted. The rationale of this long-term extraction was to allow the proteolytic digestion of the naturally occurring allergens by the enzymes solubilized from the same pollen membranes. This proteolytic activity has been indirectly confirmed in the present paper by the addition to the pollen suspension of PMSF, a proteolytic inhibitor that prevented the formation of such small molecules. Thus, it is possible to suggest that the hapten Pj-H₁ and the allergen Pj-2, which may occur bound to other high mol. wt components of the Pj pollen, may originate from these components by enzymatic or other chemical degradation processes. From this we deduce that, when allergenic molecules must be isolated in the native state from a pollen source, particular attention must be devoted to avoiding any proteolytic digestion.

Filtrations on Sephadex G-10, G-50 and Bio Gel P2 were chosen as the most useful separation procedures, because of the low mol. wt of the dialyzable allergenic molecules. The reproducibility of the Sephadex G-10 profile was confirmed by several extractions of pollen batches collected in different years, and the protein, allergenic and haptenic patterns were identical after separate runs, suggesting that a reproducible enzymatic reaction was involved.

The protein content was determined by the Lowry method and by measurement of the O.D. at 215 and 225 nm. There was a good correlation between the two methods, and through the purification steps the Lowry method was used in the earlier steps while the 215/225 ratio was used in the final steps because of the low size and yield of the material.

The allergenic activity was determined by means of SPTs and by ELISA- and RAST-inhibition experiments. The results of the two *in vitro* tests correlated well, and we used one or the other without any particular reason.

To follow the purification of the haptenic fraction, only ELISA- or RAST-inhibition tests were used, because the SPT values were also negative at high protein concentration.

The purity of the two molecules Pj-2 and Pj-H₁ has been demonstrated by the HPLC, IEF and PAGE-SDS analyses, and up to date this is the first report on the purification of an homogeneous haptenic molecule from Pj pollen.

The amino acid composition of Pj-2 reveals a higher content of lysine residues, while the 10 amino acid residues detected in the hapten Pj-H₁ did not reveal any specific features, and this is in agreement with the data reported on the amino acid sequence of other allergens (Habermann, 1972; Klapper *et al.*, 1980; Malley *et al.*, 1964; Marsh *et al.*, 1970; Mole *et al.*, 1975).

The analysis of the amino acids of Pj-H₁ suggested that this molecule contains only 10 amino acids, that it would be highly unlikely that the molecule would possess two allergenic determinants, and that the allergenic determinant(s) is (are) not representative of the total allergenic determinants present in the whole pollen extract.

A good immunochemical correlation has also been found between the allergen Pj-2 and the hapten Pj-H₁. This was demonstrated using two different systems and two different antibody classes. In fact Pj-H₁ was capable of interacting with the human IgE specific for the Pj-2, giving an inhibition the same as the ELISA reference value, and, furthermore, was capable of interacting with the rabbit-IgG specific for Pj-2, giving a reduction of the precipitin arc in an immunodiffusion assay between Pj-2 and a specific rabbit anti-whole pollen extract serum.

In conclusion, it would appear that the two molecules represent suitable starting materials for additional studies to better characterize, with amino acid sequence analysis and more sophisticated immunologic analyses such as CIE and CRIE, the chemical and immunological composition of at least one of the allergenic determinants expressed by the Pj pollen.

The use of these molecules in the desensitization therapy of allergic diseases needs more data to determine their potential usefulness *in vivo*, although the very low recovery of Pj-H₁ and Pj-2 represents a serious obstacle to purifying enough material for a clinical approach.

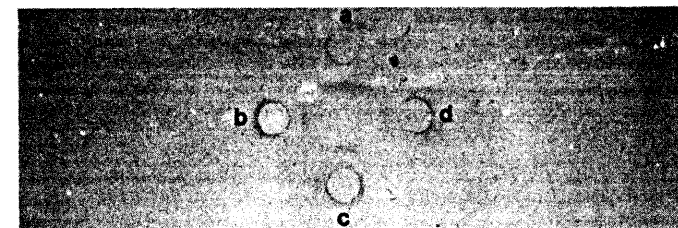
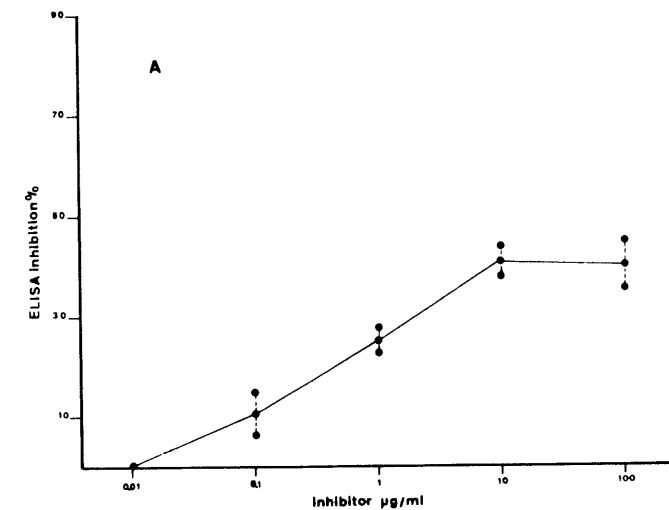


Fig. 8. ELISA-inhibition test (A) and immunodiffusion (B) to test the ability of the hapten Pj-H₁ to inhibit the interaction between the allergen Pj-2 and the human IgE (A), and the rabbit IgG (B). For the ELISA-inhibition test, a pool of 30 sera of patients allergic to Pj pollen, SPT-positive to Pj-2, and SPT-negative to Pj-H₁ were used. In the immunodiffusion the samples were: (a-d) 15 µl of antiserum (rabbit anti-Pj pollen aqueous extract) diluted 1:10 with PBS buffer. (a) No addition of Pj-H₁. (b) Addition of 9.4 µg of Pj-H₁. (c) 18.8 µg of Pj-H₁. (d) 37.6 µg of Pj-H₁. In the central well 15 µl of 1 mg/ml Pj-2 were added.

Acknowledgements—We wish to thank Prof. G. Giudice for his interest in this work. The skilful technical assistance of Antonina Azzolina and Giovanni Locorotondo is gratefully acknowledged.

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