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COMBINED SIZE-EXCLUSION–ION-EXCHANGE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF *PARIETARIA* POLLEN EXTRACTS WITH THE AID OF ISOELECTROFOCUSING AND A RADIO-ALLERGO-SORBENT TEST

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SUMMARY

Ion-exchange high-performance liquid chromatography (HPLC) of the total extract of the pollen of *Parietaria judaica* allows the separation of several allergens in the acidic p*I* range and could be used for enrichment procedures. Prior treatment by size-exclusion HPLC improves the efficiency of the purification slightly, allowing further fractionation of a peak at about 12 500 daltons. Isoelectrofocusing and radio-allergo-sorbent test are used for the analysis of individual fractions.

INTRODUCTION

The analysis and purification of pollens have been attempted with a variety of chromatographic methods, mainly gel filtration and ion exchange[1] and their combination. Short ragweed (*Ambrosia elatior*) pollen has been purified by diethylaminoethyl-cellulose (DEAE-cellulose) chromatography[2], alder (*Alnus incana*) pollen by column isoelectric focusing (IEF) fractionation[3], perennial rye grass (*Lolium perenne*) pollen by gel filtration[4] and birch (*Betula alba*) pollen by gel filtration[5] or, more recently, by high-performance liquid chromatography (HPLC) gel filtration[6] and cocksfoot grass (*Dactylis glomerata*) pollen by size-exclusion HPLC[7]. A combination of techniques was used in the purification of timothy (*Phleum pratense*) pollen, where gel filtration followed by ion-exchange[8] or HPLC ion-exchange and size-exclusion chromatography[9] were used. We have attempted to purify parietaria (*Parietaria iudaica*) pollen with the use of size-exclusion HPLC[10], and another group has used sodium dodecyl sulphate polyacrylamide gel electrophoresis[11] (SDS-PAGE). In the course of these studies, HPLC had proved to be the method of choice for such difficult purification problems, as it couples very high efficiency of separation and reproducible results with the possibility of using different stationary phases.

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As *Parietaria* allergenic extracts are used in therapy in the Mediterranean area, standardization of the extracts and studies of their chemical composition are needed. In particular, molecular weight determinations and enrichment procedures could lead to important improvements in the practical application of these extracts and could open the way to the laboratory preparation of substances that mimic the biological activity of pollen extracts.

EXPERIMENTAL

Parietaria pollen extract

A 10-g sample of dry pollen obtained from Allergon (Engelholm, Sweden) was extracted with 200 ml of 0.15 *M* phosphate buffer (pH 7.2) for 24 h at 4°C. The pollen grains were filtered and the extract was dialysed for 48 h against water, then filtered through a 0.45-μm Millipore membrane and lyophilized (2-ml aliquots).

HPLC analyses

Preparative HPLC analyses were performed by dissolving lyophilized pollen extracts in phosphate buffer (pH 6.8)–0.1 *M* potassium chloride (protein content 1.3 mg/ml) and injecting through a Rheodyne 10-μl sampling loop (300 μg per injection). The instrument was a Varian 5000 HPLC system equipped with a MicroPak TSK 2000 SW (30 cm × 10 mm O.D. × 8 mm I.D.) size-exclusion column, equipped with a TSK SW guard column (7.5 cm × 10 mm O.D. × 8 mm I.D.), and eluting with phosphate buffer (pH 6.8)–0.1 *M* potassium chloride at a flow-rate of 1 ml/min. The detector was a Varian UV 50 at 280 nm.

Ion-exchange HPLC with an ionic strength gradient was performed by dissolving the material in tris(hydroxymethyl)aminomethane (Tris)–acetic acid buffer (pH 6.0)–20 m*M* sodium acetate and injecting through a 10-μl Rheodyne loop (20 μg per injection). The instrument was a Varian 5500 HPLC system equipped with a TSK 3000 IEX-545 DEAE ion-exchange column (15 cm × 9 mm O.D. × 6 mm I.D.) and a guard column (see above), and eluting with a 45 min linear gradient of a mixture of (A) Tris–acetic acid buffer containing 20 m*M* sodium acetate and (B) Tris–acetic acid buffer containing 500 m*M* sodium acetate at a flow-rate of 1 ml/min.

Ion-exchange HPLC with a pH gradient was performed with the above column and guard column by eluting with a 30-min linear gradient of a mixture of phosphate buffer (pH 6.35)–10 m*M* potassium chloride and phosphate buffer (pH 3.35)–1 *M* potassium chloride at a flow-rate of 1 ml/min.

Isoelectrofocusing

An acrylamide–bisacrylamide 7% gel, 1 mm thick, 25 × 23 cm, was used. Samples of the required Ampholine range were allowed to migrate for 2.5 h at 500 V. The gel was then cut into slices (5 mm) and the fractions were eluted by extraction with 0.15 *M* phosphate buffer solution (PBS) (pH 7.2) (300 μl per slice) for 18 h with continuous stirring. The pH of the eluted fractions was determined and they were analysed for allergenic content by a direct radio-allergo-sorbent test (RAST).

Radio-allergo-sorbent test (RAST) of the chromatographic fractions

The fractions (75 μl each) were bound to cyanogen bromide-activated paper discs and direct RAST was performed according to Yman *et al.*[12].

RESULTS AND DISCUSSION

The *Parietaria judaica* pollen extract was subjected to two alternative purification procedures: (1) direct ion-exchange HPLC purification with either a pH gradient or an ionic strength gradient and (2) size-exclusion HPLC pre-treatment followed by ion-exchange HPLC purification of the fractions. Combination of the purification techniques in a particular sequence had been shown to be effective in the purification of *Phleum pratense* pollen[8,9]. In both purification procedures, IEF of the fractions and monitoring of the allergenic activity with RAST was needed in order to optimize the ion-exchange chromatographic conditions.

Staining the IEF gel with Coomassie Brilliant Blue did not reveal sharply the components of every fraction, and the blotting procedure was not performed. It may in fact give ambiguous results if the appropriate selection of the antibody-containing sera is not performed.

As a first experiment, the combined IEF–RAST analysis of a *Parietaria judaica* pollen extract in Ampholine of pH range 3.0–10.0 was performed (Fig. 1). Allergenic activity was present over the entire pH range, suggesting that the extract contains several allergenic proteins with different chemical behaviours. This is in line with the observation that the allergenic pollen proteins studied up to now cover a wide range of isoelectric points, ranging from basic (9.30 from timothy) to acid (4.78 from alder), and that the same pollen may contain several allergenic proteins, both acidic and basic, *e.g.*, *Ambrosia elatior*[13] pollen.

Further purification studies concentrated on the acidic fraction, which could

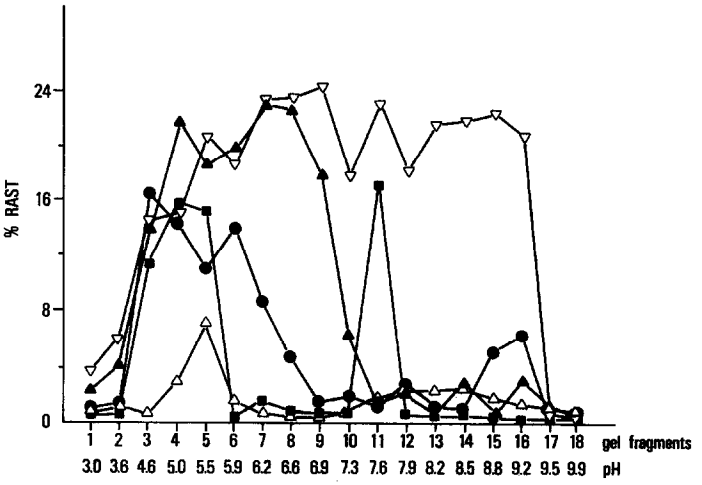

Fig. 1. IEF profile of *P. judaica* pollen total extract (▽) and of size-exclusion fractions 2 (△), 3 (▲), (4) ▲ and 5 (●) in the Ampholine pH range 3.0–10.0 and RAST analysis of the slices (% RAST is percentage of bound radioactivity).

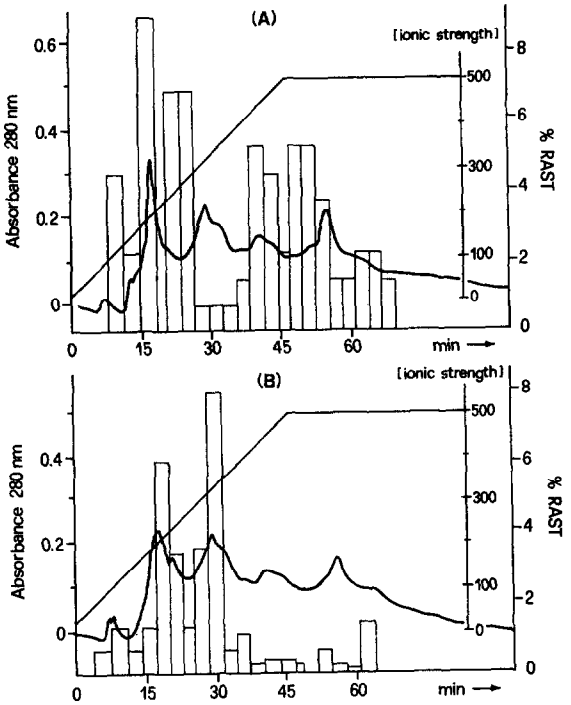

Fig. 2. Ion-exchange HPLC profile of (A) fraction 5 from the size-exclusion HPLC separation of the *P. judaica* pollen extract and (B) *P. judaica* pollen total extract, in ionic strength gradient and RAST analysis of the fractions (% RAST is percentage of bound radioactivity).

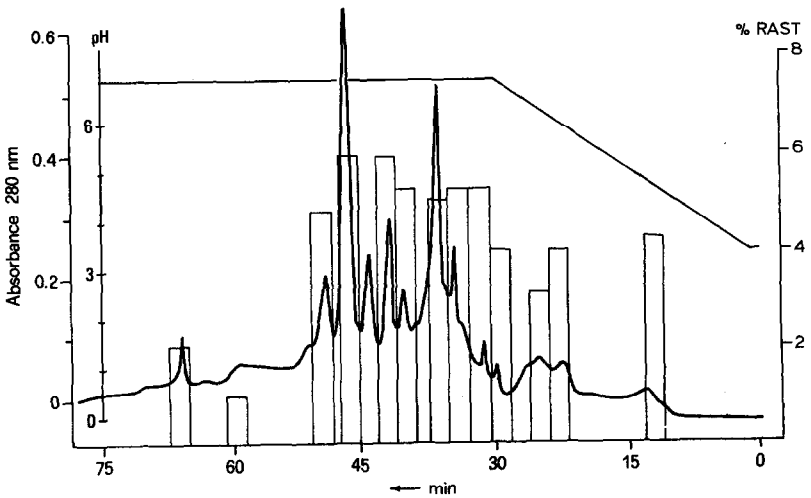

Fig. 3. Ion-exchange HPLC profile of *P. judaica* pollen total extract in pH gradient and RAST analysis of the fractions (% RAST is percentage of bound radioactivity).

contain the Antigen 9 isolated from a *P. judaica* pollen extract by SDS-PAGE and blotting on nitrocellulose membranes[11] and shown to possess the highest frequency of IgE binding. This component seemed to be similar to Antigen AZ, isolated from the same source by another group[14]. The analysis of the acidic fraction could help to distinguish among these allergenic components or could perhaps demonstrate that they were identical. Therefore, purification by direct ion-exchange HPLC was tested.

The ion-exchange HPLC profile of the *P. judaica* total extract obtained with a TSK 3000 IEX-545 DEAE column is shown in Fig. 2B. The elution was performed with a buffer of pH 6.0 and the ionic strength was increased linearly. Several groups of peaks appeared and RAST analysis showed that most of the allergenic activity was concentrated in two regions consisting of at least four peaks. Hence, two groups of chemically similar allergenic components had to be present in the pollen extract.

Elution of the same column with a pH gradient from 6.30 to 3.25 was less efficient. Fig. 3 shows the chromatographic profile, where a good separation had occurred. However, the RAST analysis showed that allergenic activity of similar intensity was present in almost all the fractions. Hence this system was not suitable for purification purposes.

The purification by size-exclusion followed by ion-exchange chromatography was tested, taking into account that we have previously shown the *P. judaica* pollen extract to contain an allergenic fraction of 12 500 daltons[15]. This value was very

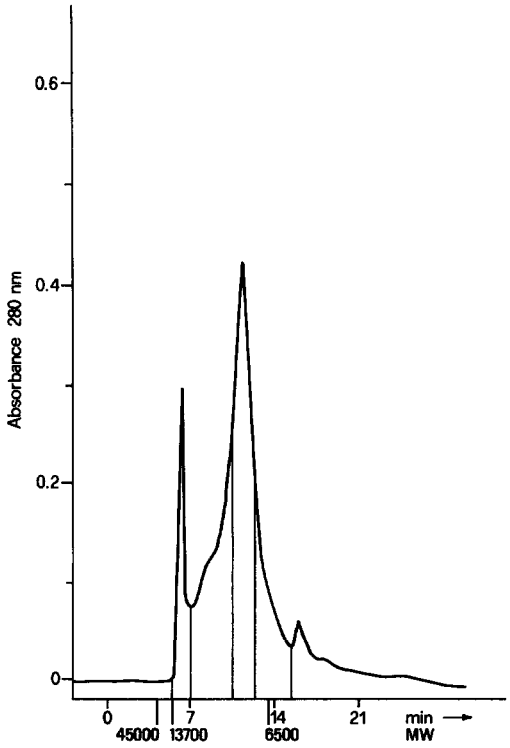

Fig. 4. Size-exclusion HPLC separation of *P. judaica* total pollen extract. MW = Molecular weight.

similar to that found by Ford *et al.*[11] for the Antigen 9 (10 000 daltons). Hence purification by size-exclusion could help to eliminate inactive components in a preliminary purification. Therefore, the pollen extract was chromatographed on a size-exclusion HPLC column and four fractions in a narrow MW range (fractions 2–5) were found by RAST to contain allergenic activity and were collected (Fig. 4).

IEF and RAST of these fractions allowed the analysis of the purification obtained. Fig. 1 shows the IEF profile of the total extract and of fractions 2–5. The size-exclusion procedure had eliminated most of the basic allergens originally present. All the fractions, in fact, showed a strong RAST activity in the acidic region (pH 3.6–7.0), whereas in only one instance (fraction 3) was RAST activity also present in the basic region.

As much of the allergenic activity of the *P. judaica* pollen extract seemed to be in the acidic region, and both Antigen 9 and Antigen AZ appeared to be acidic, a more detailed IEF–RAST investigation in a narrower pH interval was then performed (Fig. 5). Fraction 2 did not show sharp peaks of biological activity. It was, in fact, a higher-molecular-weight fraction, resulting from the size-exclusion chromatography, with a molecular weight probably above 50 000 daltons, which is the maximum value found for allergenic pollen proteins. It was not investigated further. IEF of fraction 5 in the pH range 4–6 showed that it contained at least four regions having allergenic activity. These bands seemed to be present also in fraction 3 and could be some of the most important acidic allergens in *P. judaica* pollen. Fraction 4 consisted of a very complicated and unresolved mixture of materials. Hence size-exclusion chromatography seems to elute, first, an almost inactive high-molecular-weight component (fraction 2), and then some basic allergens and a mixture of at least four acidic allergens (fraction 3). Fraction 4 contains these components, together with other material, in a very complex mixture and fraction 5 contains the same group of acidic allergens previously noted in fraction 3.

An ion-exchange HPLC separation was performed on the basis of these results.

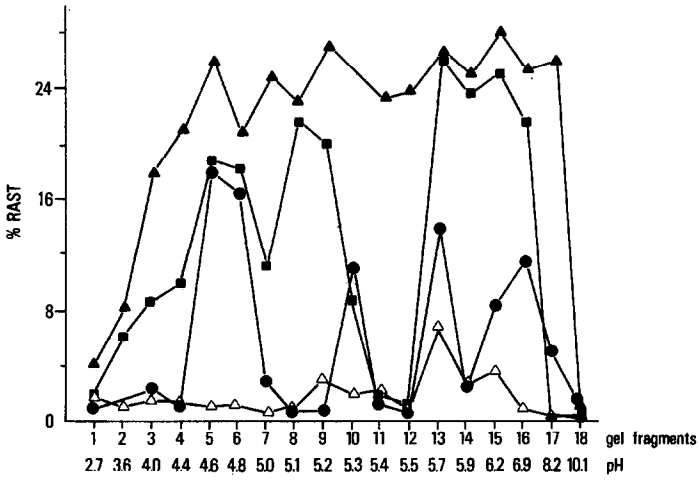

Fig. 5 IEF profile of fractions 2 (△), 3 (■), 4 (▲) and 5 (●), from the size-exclusion HPLC separation, in the Ampholine pH range 4.0–6.0, and RAST analysis of the slices (% RAST is percentage of bound radioactivity).

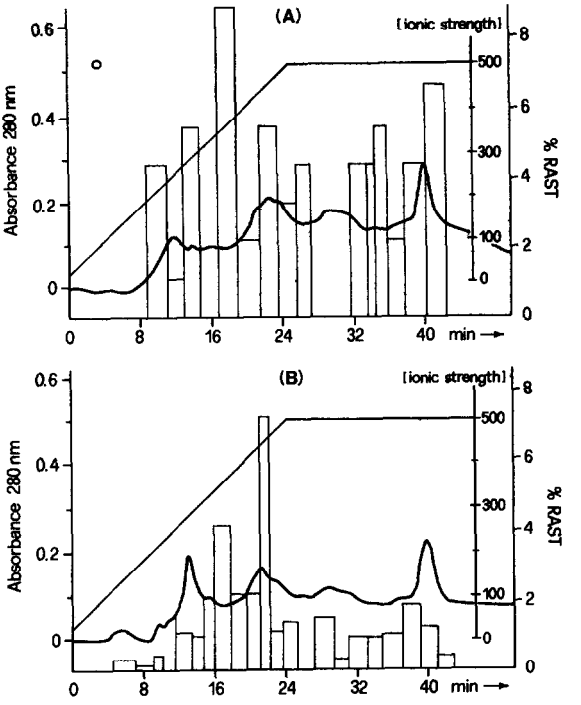

Fig. 6. Ion-exchange HPLC profile of (A) fraction 3 and (B) fraction 4, from the size-exclusion HPLC separation and RAST analysis of the fractions (% RAST is percentage of bound radioactivity).

Individual fractions were injected into the same column as that used for the total *P. judaica* extract and eluted at a constant pH of 6.0 with a gradient of linearly increasing ionic strength. This procedure gave better results than elution with a pH gradient. Fractions 3 and 4 (Fig. 6A and B) appeared less resolved, and the allergenic activity was accordingly spread all over the chromatogram. Fraction 5 (Fig. 2A) exhibited a more defined elution profile, and four regions of high RAST activity appeared. This was expected from the IEF profile shown in Fig. 5 and suggests good correspondence between IEF and ion-exchange HPLC. This result could be useful in further studies.

A comparison between the results of the chromatographic and RAST analysis of the allergens in fraction 5, derived from size-exclusion purification followed by ion-exchange chromatography, and the results of the ion-exchange HPLC–RAST analysis of the total pollen extract is shown in Fig. 2A and B and showed a close similarity between the two chromatographic profiles, at least regarding the two main allergenic regions. The first procedure seemed to increase the amount of allergenic material with a higher elution volume in the sample, probably owing to some chemical modifications during the size-exclusion chromatography.

In conclusion, ion-exchange HPLC of the total extract of the pollen of *P. judaica* allows the identification of several allergens in the acidic p*I* range and could be used for enrichment procedures. Preliminary purification by size-exclusion HPLC

slightly improves the efficiency of the purification, allowing further fractionation of the peak of MW *ca.* 12 500 daltons, but also causes modifications of the sample. Nevertheless, this procedure could lead to the purification of some allergenic acidic components for further chemical studies.

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