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MICROPREPARATIVE-SCALE ENRICHMENT OF SOME ALLERGENIC COMPONENTS OF *PARIETARIA* POLLEN EXTRACT

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SUMMARY

A preparative-scale enrichment of the allergenic components of the pollen extract of *Parietaria judaica*, which grow all over the Mediterranean area, has been obtained by high-performance liquid chromatography, operating in the ion-exchange mode at pH 7 with a curvilinear ionic-strength gradient.

INTRODUCTION

The analysis and purification of pollen extracts has been attempted with a variety of chromatographic methods, mainly gel filtration and ion exchange[1], alone or in combination. Short ragweed (*Ambrosia elatior*) pollen extract has been purified by DEAE-cellulose chromatography[2], alder (*Alnus incana*) pollen extract by column isoelectric focusing (IEF) fractionation[3] or horizontal IEF and two-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)[4].

High-performance liquid chromatography (HPLC) had proved to be the method of choice for such difficult purification problems. It couples high efficiency of separation and reproducible results with the possibility of using different stationary phases.

Extract of perennial rye grass (*Lolium perenne*) pollen was previously purified by gel filtration[5] and, more recently, by size-exclusion HPLC[6]; the same evolution of purification methods occurred with birch (*Betula alba*) pollen. Gel chromatography[7] or, more recently, HPLC gel chromatography[8] were used in this case. Size-exclusion HPLC was recently used with cocksfoot grass (*Dactylis glomerata*) pollen[9] and olive pollen[10].

An improvement in the separation was possible by a combination of techniques. This was the case for timothy (*Phleum pratense*) pollen, where gel chromatography and

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ion-exchange chromatography[11] or ion-exchange and size-exclusion HPLC[12] were used.

Since *Parietaria* (*Parietaria judaica*) allergenic extracts are used therapeutically in the Mediterranean area, standardization of the extracts and studies of their chemical composition are needed. In particular, enrichment procedures could lead to improvements in the practical importance of these extracts and could open the way to the laboratory preparation of substances that could mimic the biological response of pollen extracts.

We have attempted to purify *Parietaria* pollen extracts by size-exclusion HPLC[13], alone or in combination with ion-exchange HPLC[14], and another group has used gel chromatography[15], SDS-PAGE[16] and immunochemical methods[17]. Very recently, the validity of the size-exclusion HPLC purification of *Parietaria* pollen extract has been confirmed[18]. However, it is often difficult to compare analytical results obtained with different methods. This is the case with *Parietaria*, and these analytical results seem to be different from those obtained[19] by the use of crossed radioimmunoelectrophoresis (CRIE).

In a recent paper[20], we suggested that HPLC enrichment of the allergenic material could be useful for further, more refined purification methods, and this was performed by the use of ultrafiltration followed by ion-exchange HPLC on a micropreparative scale. Preparative attempts had been made with size-exclusion HPLC of cocksfoot grass pollen[9] and rye grass pollen extracts[6], and with a combination of size-exclusion chromatography and ion-exchange HPLC of timothy pollen extract[12].

Here, we report the results of the preparative enrichment of the allergenic fraction of *Parietaria* by ion-exchange HPLC with the use of different ionic-strength gradients.

MATERIALS AND METHODS

Parietaria pollen extract

A 10 g amount 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, using a Spectra/Por 3 (Spectrum medical industries, Los Angeles, CA, U.S.A.) membrane with a molecular-mass cut-off of 3500, then filtered through a 0.45-μm Millipore membrane (Millipore, Bedford, MA, U.S.A.) and lyophilized (2-ml aliquots).

HPLC analyses

Ion-exchange HPLC with an ionic strength gradient was performed by dissolving the material in 10 m*M* Tris–acetic acid buffer (pH 7.0)–20 m*M* sodium acetate and injecting through a Rheodyne 10-μl loop (50 μg per injection). The instrument was a Varian 5500 HPLC (Varian, Palo Alto, CA, U.S.A.) equipped with a TSK DEAE-5PW ion-exchange column (7.5 cm × 1.8 in. O.D., 7.5 mm I.D.) and a TSK guard column SW (7.5 cm × 10 mm O.D. × 8 mm I.D.), (Toyo Soda, Tokyo, Japan) eluting with a 45-min linear gradient from (A) a mixture of 10 m*M* Tris–acetic acid buffer containing 20 m*M* sodium acetate to (B) 10 mM Tris–acetic acid buffer containing 500 m*M* sodium acetate, at a flow-rate of 1 ml/min.

Ion-exchange preparative HPLC was performed on the material obtained from

two consecutive ultrafiltrations with the membranes Millipore minitan plates with molecular-weight cut-offs of 100 000 and 10 000 (chromatography with the linear gradient) or with ultrafiltration through the molecular-weight cut-off of 100 000 (chromatography with the concave gradient). The ultrafiltrate was lyophilized, dissolved in 1.5 ml of 10 m*M* Tris–acetic acid buffer (pH 7.0)–20 m*M* sodium acetate (eluent A), and injected. The instrument was a Waters Deltaprep 3000 HPLC (Waters Assoc., Milford, MA, U.S.A.) equipped with a DEAE-5PW ion-exchange column (15 cm × 21.5 mm I.D.) (Toyo Soda) eluting with a 45-min gradient of A to B of a mixture of eluent A and eluent B (10 mM Tris–acetic acid buffer (pH 7.0), containing 500 m*M* sodium acetate) at a flow-rate of 6 ml/min. The detector was a Waters Lambda Max Model 481 set at 280 nm. The fractions were then analysed by RAST-inhibition[21].

Isoelectric focusing

A 7% acrylamide–bisacrylamide gel, 2 mm thick, 25 × 23 cm, was used. Ampholine (LKB, Bromma, Sweden) samples, pH range 3–10, were allowed to migrate for 2.5 h at 500 V. The gel was cut into thin slices (5 mm), and the fractions were extracted with 0.15 *M* phosphate buffer (pH 7.2) (300 μl per slice) for 18 h by continuous stirring. The eluted fractions were analysed for allergenic content by direct RAST[21].

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

The fractions (75 μl each) were bound to cyanogen bromide-activated paper[22] discs, and direct RAST or RAST inhibition was performed according to Yman *et al.*[21], using a pool of sera from 95 patients with high sensitivity to *Parietaria judaica* pollen.

RESULTS AND DISCUSSION

Previous work in size-exclusion HPLC[13] and isoelectrofocusing[14] had given us information about the p*I* region in which most of the allergenic proteins appeared. This allowed us to select some analytical HPLC ion-exchange procedures for analysing the pollen extract. This had been chromatographed under three different sets of ion-exchange HPLC conditions[14,20]. In the first an aqueous buffer–acetonitrile gradient was used with a silica-based TSK IEX-545-DEAE column; the second attempt was performed with an ionic-strength gradient at pH 6, operating with a silica-based TSK IEX-545-DEAE column; the third experiment was performed with an ionic-strength gradient at pH 7 operating with a resin-based TSK DEAE-5PW column.

Since this resin-based column could survive several purification cycles, if washed with dilute sodium hydroxide after each use, the preparative experiments were likely to have practical value when performed with the same type of column. A pH 7 buffer in an ion-exchange HPLC procedure with an ionic-strength gradient could be the most appropriate separating system. Under these conditions, a possible pH-dependent denaturation of the biologically active components could also be avoided. This was tested on a sample of *Parietaria judaica* extract in an analytical scale experiment. Fig. 1 shows that the allergenic activity, monitored by direct RAST, was concentrated in a group of fractions. Thus, this procedure was selected for preparative experiments.

Two consecutive ultrafiltration procedures allowed us to separate the material

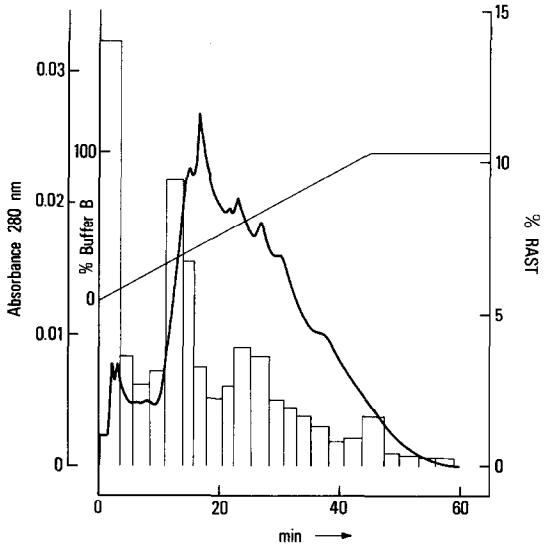

Fig. 1. Ion-exchange HPLC profile of *P. judaica* pollen extract on an analytical scale and RAST analysis of the fractions (% RAST is the percentage of bound radioactivity).

between 10 and 100 kD, and this was injected in a preparative TSK DEAE-5PW column. Elution with an ionic-strength gradient at pH 7 gave the chromatographic profile shown in Fig. 2a. Scale-up of the procedure was then performed by injecting under the same conditions a three times larger amount of proteic material. The chromatogram of this experiment (Fig. 2b) was very similar to that obtained at the lower concentration. This suggested that increasing the concentration of material did not influence the separation. Hence, a nine-fold increase in the injected material was made, giving the result shown in Fig. 2c. Again, a fair chromatographic similarity with the previous experiments was noted. The amount of material injected in this last run was 65 mg.

Direct RAST measurements for the first two experiments showed that allergenic activity was present in most fractions. However, a more precise evaluation of this was needed. This was accomplished by performing RAST-inhibition analyses, which are known to allow a better evaluation of the allergenic activity. Fig. 2c also shows the RAST-I profile, which indicated that most of the allergenic activity was concentrated in the first part of the chromatogram. The eluted material in fractions 1–22 contained 19 mg of protein.

An IEF experiment performed on some significant fractions of this chromatographic run allowed us to separate the components of every fraction. The gel was then cut, and the slices were eluted in order to perform a direct RAST evaluation of their content of allergenic components. The results are shown in Fig. 3. This showed that a first group of components occurring in the basic p*I* range was concentrated in the first group of fractions, having low interaction with the type of ion-exchange column used, and that a second group of components, occurring in a more acidic p*I* range, was then eluted. Acidic allergens in *Parietaria* pollen extract have been previously demonstrated[14–20], but the presence of basic allergens had not been firmly

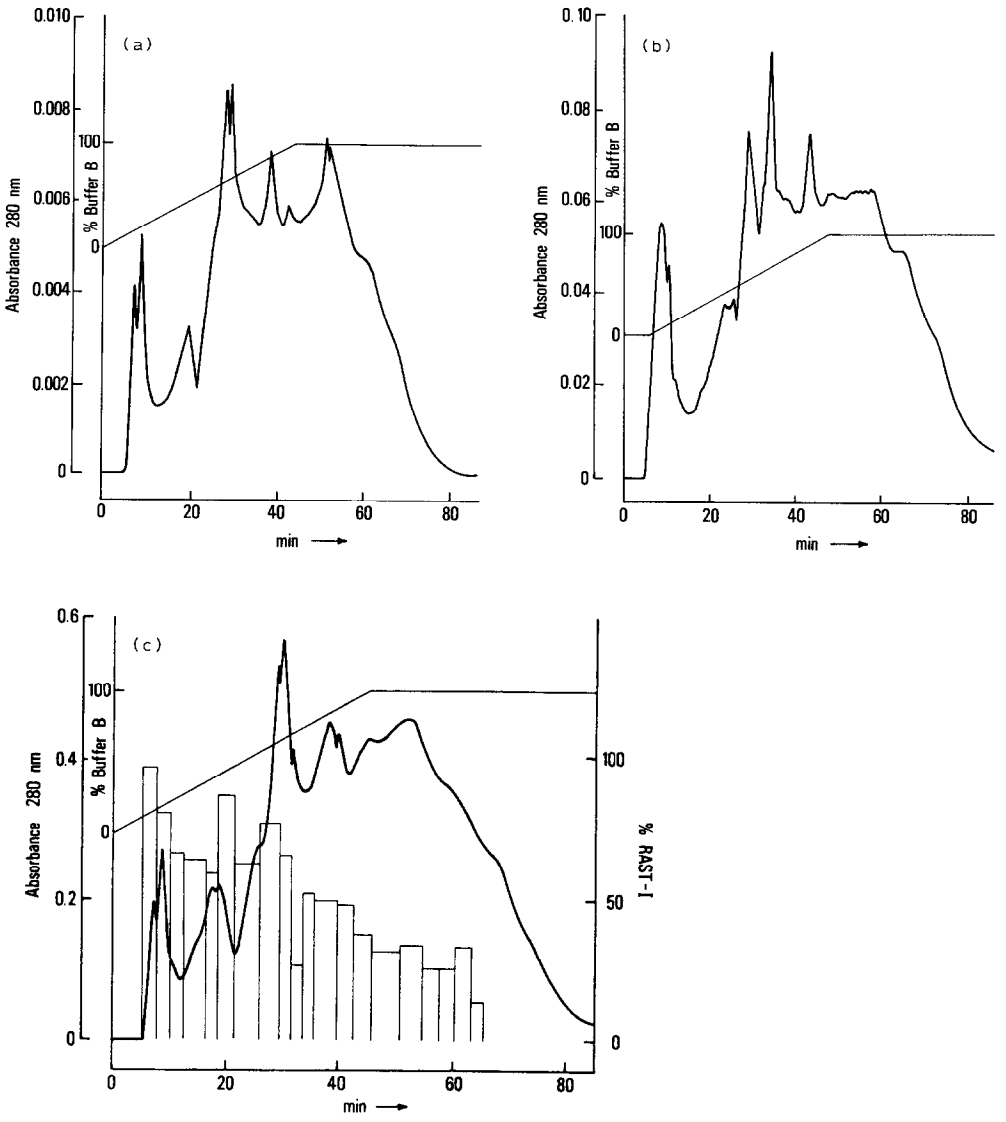

Fig. 2. (a–c) Ion-exchange HPLC profile of *P. judaica* pollen extract with different concentrations of material. RAST-I analysis of the fractions is also shown in (c).

established. The micropreparative-scale procedure could then allow the isolation of materials that often escape analytical-scale experiments, owing to their low concentration or instability.

A better enrichment procedure could be derived from a narrower band of allergenic material in the first part of the chromatogram. Experiments in that direction were performed with a convex ionic-strength gradient, in which a strong increase in ionic strength was obtained in the early fractions. In a first attempt 60 mg of material,

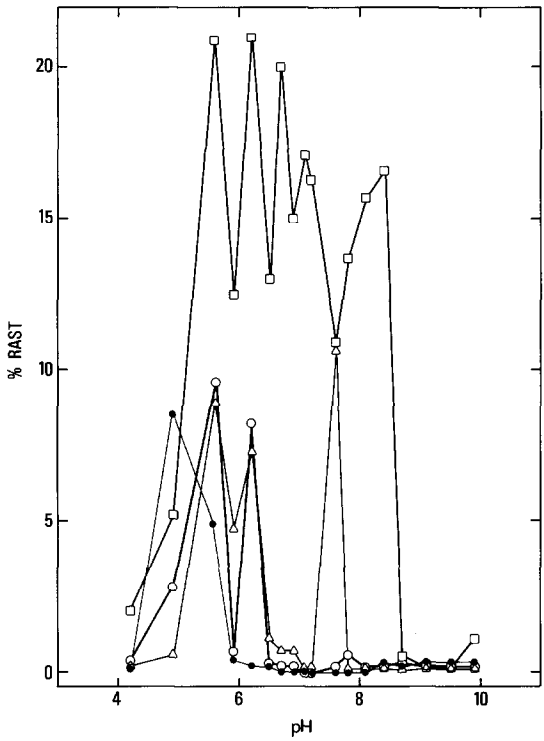

Fig. 3. IEF profile of HPLC ion-exchange fractions 2 (□), 6 (△), 10 (○) and 19 (●) from the chromatogram in Fig. 2c in the Ampholine pH range 3.0–10.0, and RAST analysis of the slices.

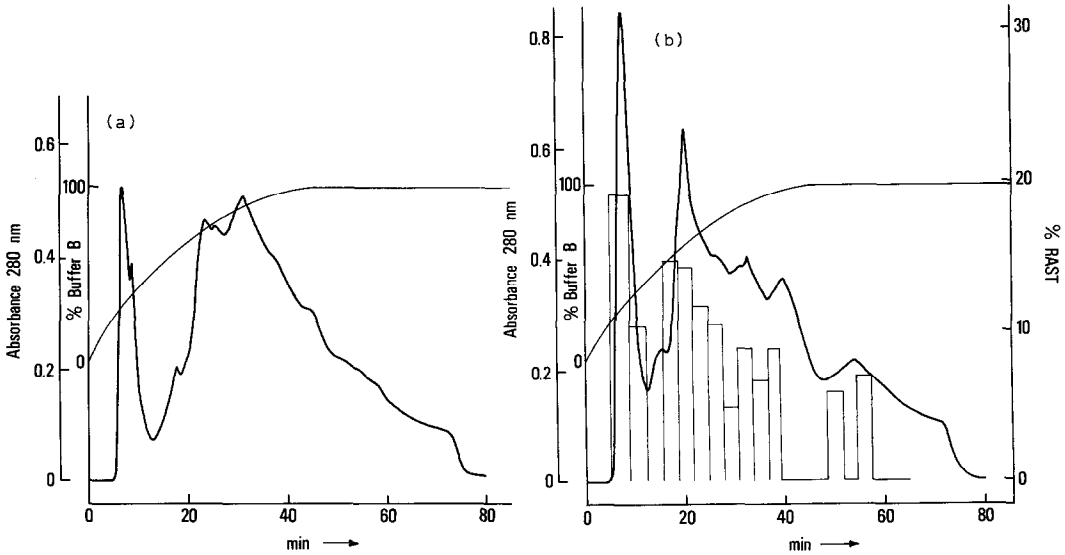

Fig. 4. (a, b) Ion-exchange HPLC profile of *P. judaica* pollen extract with different concentrations of material. RAST-I analysis of the fractions in is also shown in (b).

which had undergone ultrafiltration with a cut-off of 100 000, were injected. The chromatogram was very different from those obtained with linear gradients (Fig. 4a). Thus, a scale-up experiment was performed and 160 mg of allergenic extract were injected. A chromatographic and RAST-I profile substantially similar to that of the analytical-scale experiment was obtained (Fig. 4b).

In conclusion, ion-exchange HPLC of an ultrafiltered *Parietaria judaica* extract at pH 7 with a convex ionic-strength gradient gives a preparative-scale enrichment of allergenic components. This opens the way to further purification steps, which could lead to the separation of the major allergens in the pollen.

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