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Ion-exchange high-performance liquid chromatographic isolation of the major allergen of parietaria pollen extract

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ABSTRACT

The analysis and purification of a parietaria pollen extract were attempted using an ion-exchange high-performance liquid chromatographic method on a preparative scale. A homopolymerization equilibrium was observed to occur in solution. A combination of electrophoretic methods and immunoelectrophoretic methods was also used and showed that a small amount of the major allergenic component was isolated.

INTRODUCTION

Parietaria (*Parietaria judaica*) allergenic extracts are used therapeutically in the Mediterranean area. Therefore, standardization of the extracts and studies of their chemical composition are needed. In previous papers we reported attempts to purify parietaria pollen extracts with the use of high-performance size-exclusion chromatography (HPSEC)[1] or of a combination of HPSEC and high-performance ion-exchange chromatography (HPIEC)[2]. Other groups used HPSEC[3], gel chromatography[4] and ammonium sulphate precipitation followed by HPSEC[5].

Electrophoretic methods have also been used for the isolation of minute amounts of parietaria allergens, involving sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)[6], immunochemical methods[7] or crossed radioimmunoelectrophoresis (CRIE)[8]. In these attempts, low-molecular-weight allergens were also isolated[9], and a pure allergen was obtained in a very low amount[10] and was named Par j I, using the new allergen nomenclature[11].

Chromatographic methods may lead to small-scale purification of allergens if combinations of techniques is used. This has been performed with timothy (*Phleum*

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pratense), where gel chromatography and IEC[12] or HPIEC and SEC[13] were used. With parietaria pollen extract we suggested the use of HPSEC and HPIEC[2] and, more recently[14,15], ultrafiltration and HPIEC. This method was further developed on a micropraparative scale[16].

The molecular weight of the major allergen from parietaria obtained by chromatographic purification experiments seemed to be different from that obtained from small-scale electrophoretic or immunochemical purification experiments, suggesting that the purification technique modified the composition of the allergenic mixture orginally present in parietaria pollen extract. Hence clarification of this point was important both for the standardization of parietaria pollen extracts to be used in therapy and for chemical studies.

EXPERIMENTAL

Parietaria pollen extract

A 10-mg 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 lyophilized (2-ml aliquots). Other samples were dialysed for 48 h against water, using a Spectra/Por 3 membrane (Spectrum Medical Industries, Los Angeles, CA, U.S.A.) with a molecular weight cut-off of 3500, then filtered through a 0.45-μm membrane (Millipore, Bedford, MA, U.S.A.) and lyophilized (2-ml aliquots).

High-performance liquid chromatographic analyses

The lyophilized material was dissolved in a 10 m*M* Tris–acetic acid buffer (pH 7.0)–20 m*M* sodium acetate (eluent A) to obtain a concentration of 6 mg/ml and injected into a Rheodyne 5.1-ml loop. The instrument was a Waters Deltaprep 3000 high-performance liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with a DEAE-5PW ion-exchange column (15 cm × 21.5 mm I.D.) (Toyo Soda, Tokyo, Japan) eluting with a 45-min gradient from A to B of a mixture of eluents A and B [10 m*M* Tris–acetic acid buffer (pH 7.0) containing 500 m*M* sodium acetate] at a flow-rate of 6m ml/min. A second linear gradient to 800 m*M* sodium acetate was then added. The detector was a Hewlett-Packard (Palto Alto, CA, U.S.A.) Model 1040 diode-array detector. The eluate was then divided into four pools (A, 72 ml; B, 132 ml; C, 84 ml; D, 204 ml). Pools A–C were analysed by HPIEC under the same conditions, then tested by radio allergo sorbent test (RAST) inhibition for allergenic content. Pool D was analysed with a curvilinear ionic strength gradient.

Radio allergo sorbent test

The fractions were bound to cyanogen bromide-activated paper discs and RAST inhibition was performed according to Yman *et al.*[17] using a pool of sera from patients with high sensitivity to parietaria pollen.

Isoelectric focusing (IEF)

A 5% polyacrylamide gel (18 × 9 cm) containing 2 *M* urea, 0.5 mm thick, was used. Ampholine (LKB, Bromma, Sweden), pH range 3–10, was used. The anodic solution was 1 *M* phosphoric acid and the cathodic solution was 1 *M* sodium

hydroxide. The samples were allowed to migrate at 15°C for 1.5 h at 2500 V and 7 W. Detection was performed by silver staining[18,19].

SDS-PAGE

A 9–27% linear gradient gel was used. The samples were treated with 10% β-mercaptoethanol, 2% SDS and Tris–HCl buffer (pH 6.7) containing glycerol for 1 h, then boiled for 10 min. The electrophoresis was performed using a Tris–glycine buffer (pH 8.3) containing SDS at 200 V for 17 h at 15°C[20]. Detection was performed by silver staining[18,19].

Fused rocket immunoelectrophoresis (FRIE)

A 1% (w/v) agarose gel (10 cm × 10 cm × 1.5 mm) containing 11 μl/cm^2 of a Lofarma anti-parietaria rabbit antibody (Lofarma Allergeni, Milan, Italy) was used. The buffer was Tris–tricine (pH 8.6, $I = 0.1$). Electrophoresis was performed at 2 V/cm for 18 h at 15°C[21]. Detection was performed by the Coomassie Brilliant Blue R-250 method (0.5% in water–ethanol–acetic acid, 45:45:10).

Crossed immunoelectrophoresis (CIE)

A 1% (w/v) agarose gel (10 cm × 10 cm × 1.5 mm) was used. The buffer was Tris–tricine (pH 8.6, $I = 0.1$). Electrophoresis in the first dimension was performed at 10 V/cm for 25 min and in the second dimension using 3.75 ml of a 1% agarose gel containing 14 μl/cm^2 of Lofarma anti-parietaria rabbit antibody and operating at 15°C and 2 V/cm for 18 h[22]. Detection was performed with the Coomassie Brilliant Blue R-250 method [0.5% in water–ethanol–acetic acid (45:45:10)].

CRIE

The incubation buffer for the monoclonal CRIE was freshly prepared[23] by addition of 5 g of bovine γ-globulin and 100 ml of normal rabbit serum to 900 ml of glycine buffer containing 0.1 *M* glycine, 0.65 *M* NaCl, 10 ml/l Tween 20 and 15 m*M* NaN_3 at pH 9.2. The washing solution contained 0.65 *M* NaCl, 1 ml/l Tween 20 and 20 mg/l chlorohexidine acetate in distilled water.

The rabbit anti-mouse immunoglobulin was purified by immunosorption against Sepharose-coupled polyclonal human immunoglobulin G (IgG) and by affinity chromatography on Sepharose-coupled mouse IgG[24]. Labelling with ^{125}I was done using a modification of the chloramine-T methods[24,25]. The specific activity of the labelled antibody was *ca.* 4 mCi/mg.

A CIE experiment was performed[26] using 1% agarose in Tris–barbital buffer (0.024 *M* sodium barbital, 0.073 *M* Tris and 0.01 *M* NaN_3, pH 8.6). The first dimension was run for 30 min at 10 V/cm and the second for 18 h at 2 V/cm. The plates were washed, pressed and dried in cold air, then incubated[27,28] for 3 h with the monoclonal antibodies against Par j I obtained as reported in ref. 10, diluted 1 + 1000. The plates were then washed four times and incubated overnight with ^{125}I-labelled rabbit anti-mouse IgG ($4 \cdot 10^5$ cpm), then washed four times, dried in hot air and placed in a X-ray cassette for autoradiography.

RESULTS AND DISCUSSION

The chromatographic purification of a parietaria pollen extract was performed using an anionic preparative HPIEC column eluting with a pH 7 linear ionic strength gradient. The results are shown in Fig. 1. The fractions were then collected in four pools (A, fractions 1–6; B, fractions 7–18; C, fractions 19–24; and D, fractions 25–41) and evaluated by RAST inhibition (RAST-I) for allergenic activity. This was spread all over the chromatogram, suggesting that allergen purification could not be followed simply monitoring the elution profile.

These pools were analysed using the same HPIEC column and the same pH 7 ionic strength gradient. In order to perform this, the material was dialysed with a cut-off membrane 3500 dalton. RAST-I analysis showed that dialysis gave a loss of a small amount of low-molecular-weight allergenic material, probably formed by some protease activity in the fractions.

The HPIEC profiles of pools A–C (Fig. 2a–c) suggested equalization of the fractions. Hence a modification of the composition of the fractions had occurred during the chromatography or while in solution. Pool D, analysed with a different ionic strength gradient, showed the same effect (Fig. 2d). This behaviour could derive from the occurrence of a homopolymerization equilibrium of allergenic and non-allergenic proteins. This had been recently suggested to occur with Par j I purified HPIEC[29] or by immunoaffinity chromatography[30] and is a general behaviour with glycoproteins, owing to hydrophobic interactions[31].

The interpretation of this HPIEC required electrophoretic and immunochemical analysis of the individual chromatographic fractions. Thus, scaling-up of HPIEC purification was performed and a FRIE experiment with all the chromatographic fractions, using antibodies anti-total parietaria extract, showed the presence of several

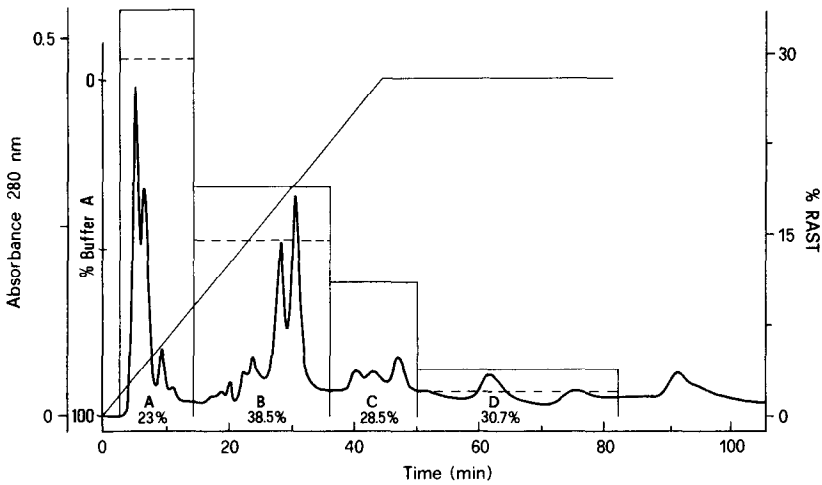

Fig. 1. Preparative HPIEC separation of parietaria pollen extract and RAST-I analysis of the fractions before (solid line) and after (dashed line) dialysis. Column, DEAE-5PW; ionic strength gradient, 10 m*M* Tris–acetic acid buffer (pH 7.0)–20 m*M* sodium acetate (eluent A); 10 m*M* Tris–acetic acid buffer (pH 7.0) containing 500 m*M* sodium acetate (eluent B).

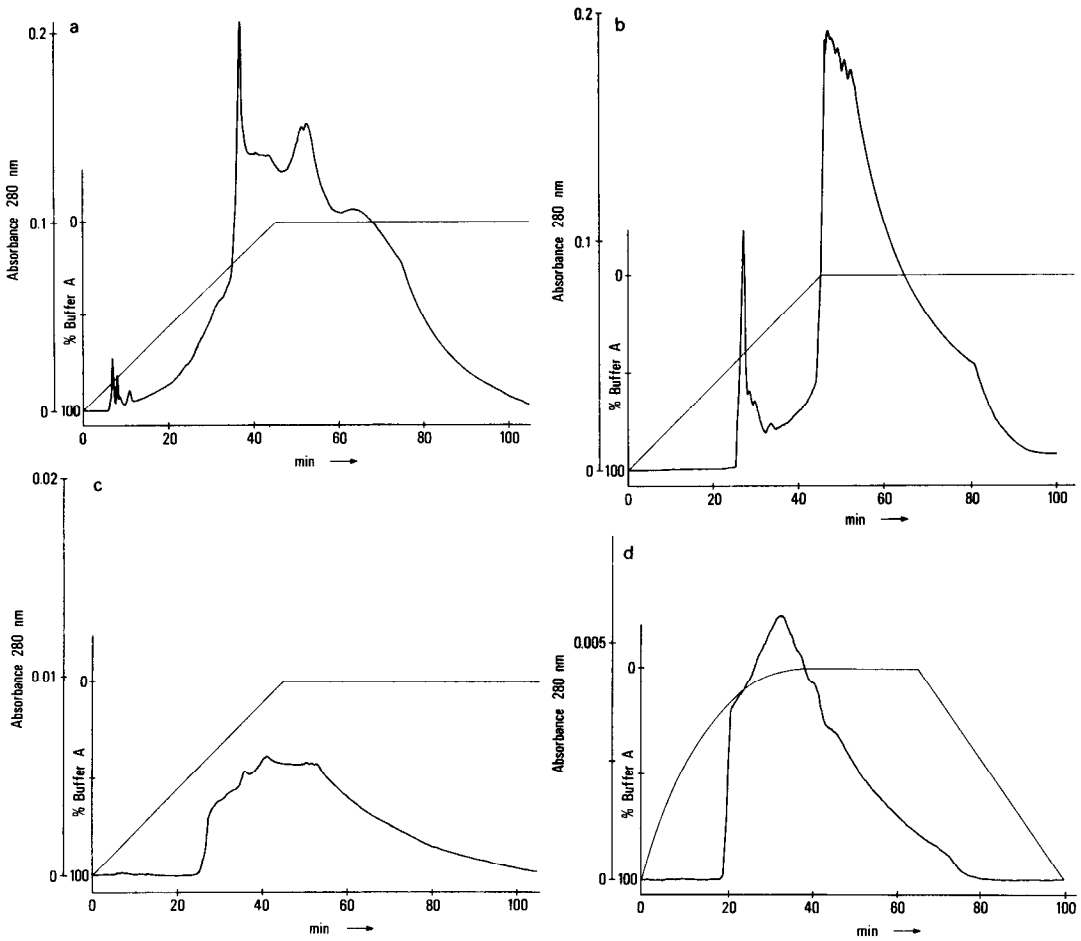

Fig. 2. HPIEC analysis of pools (a) A, (b) B, (c) C and (d) D, collected in the run shown in Fig. 1. Conditions as in Fig. 1.

antigens. One of these was present in all the fractions and was accompanied by a band with very similar characteristics. These two antigens were present as the only components of fraction 31.

An CIE experiment on fraction 31 using the same anti-parietaria antibody confirmed that two precipitation arcs were present in this fraction. A check of the purity of fraction 31 was obtained using a diode-array detector as the UV spectrum recorded by this detector did not change during elution. IEF analysis in the pH range 3.0–10.0 showed that fraction 31 had a single band located at the isoelectric point value of 3.5. An SDS-PAGE experiment was performed and showed that fraction 31 contained a single component which had a molecular weight of 11 000 dalton.

Thus, fraction 31 had a single band in IEF and SDS-PAGE and two precipitation arcs in CIE and FRIE. IEF was performed in the presence of urea and SDS-PAGE in the presence of the surfactant and β-mercaptoethanol, which are

conditions that dissociate homopolymeric material, whereas CIE and FRIE were run in the absence of dissociating agents. Hence, the two components of fraction 31 giving one band in IEF and SDS-PAGE and two in CIE and FRIE could be the monomeric protein and an oligomer.

A CRIE experiment performed on parietaria pollen extract using a monoclonal antibody against Par j I showed that the major allergens were two components with very similar characteristics, which were very similar to the components contained in fraction 31. Their reactivity toward the monoclonal antibody suggested that they had the same allergenic determinant. This fact was in line with the suggestion that they were Par j I and one oligomer.

These observations explain the uncertainty in the molecular weight of Par j I. Geraci and co-workers suggested valus of 10 000 (ref. 6), 22 000 (ref. 7) and 26 000 (ref. 10) dalton; the first value resulted from the allergen obtained from SDS-PAGE effected under dissociating conditions and could be attributed to the monomeric form. Corbi and Carreira[8] suggested that Par j I had a molecular weight of 10 000 dalton and gave a dimer under non-reducing conditions. Our results confirmed the early report[2] that Par j I is an 11 000-dalton protein which has a tendency to give homopolymers. Homopolymerization of this allergen has been also reported by another group[5], and the microhetereogeneity of Par j I has been suggested recently[30].

The HPIEC behaviour of parietaria pollen extract is probably due to the fact that the increase in the ionic strength of the eluent during the chromatography gradually results in dissociation of oligomers. Hence the composition of the mixture to be separated changes during elution.

In conclusion, preparative HPIEC followed by diode-array detection allowed the isolation of the major allergenic component shown to be in equilibrium with its homopolymer. Scaling-up of this technique is expected to allow the isolation of enough material for the solution of the uncertainty about its molecular weight and for chemical studies.

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