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Allergenic fragments in *Parietaria judaica* pollen extract

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ABSTRACT

High-performance ion-exchange chromatography and immunoaffinity chromatography suggest that Par jI, the principal allergenic component of *Parietaria judaica* pollen, is a very unstable molecule, which tends to fragment in solution. Several fragments were obtained from Par jI and some of them show positivity toward the anti-Par jI monoclonal antibody, suggesting that they retain the entire structure of the allergenic determinant. These fragments could be the target for sequence and conformation studies.

INTRODUCTION

Parietaria pollen allergenic extracts are used in the immunotherapy of allergic diseases. As these extracts consist of a mixture of heterogeneous proteins, studies aimed to improve their standardization and their chemical composition are needed.

A variety of chromatographic [1–7], electrophoretic and immunochemical [8–11] techniques have been attempted. In particular, the combination of sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and reversed-phase high-performance liquid chromatography (RP-HPLC) led to the isolation of an allergenic protein with a molecular weight of 10 000 dalton, called Par jI [12], which can polymerize spontaneously. On the other hand, using high-performance ion-exchange chromatography (HPIEC) we obtained at high elution volume a fraction containing a single component, probably derived from the dissociation of a homopolymer as a result of the dissociating effect of the ionic strength gradient used. This component seems similar to Par jI allergen, as demonstrated by SDS-PAGE, isoelectric focusing (IEF) and crossed immunoelectrophoresis (CIE) [13].

Recent evidence suggests the importance of studies on the molecular structure

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of allergens, in order to define epitopes that may represent targets of the human allergic response. The fragmentation with cyanogen bromide of the major allergen of *Festuca elatior* (Fes el) allowed a peptide fragment with immunoglobulin E (IgE)-binding ability to be obtained. This was also able to induce an IgG response which cross-reacted with the entire allergen when used as an immunogen in mice [14]. This could occur also with *Parietaria*, as a low-molecular-weigth peptide having allergenic activity has been isolated [15].

In order to isolate allergenic peptides from *Parietaria* pollen extract, we used alternatively a combination of HPIEC and high-performance size-exclusion chromatography (HPSEC), or a combination of affinity chromatography and HPSEC or a combination of methanol extraction and HPSEC.

EXPERIMENTAL

Preparation of Parietaria pollen extract

A 10-g sample of defatted 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. After centrifugation at 28 000 *g* for 40 min the solution was filtered on a Sephadex G-25 gel column (ratio of sample to adsorbent = 1:5) with 20 m*M* phosphate buffer (pH 6.8) as the eluent. The exclusion peak corresponding to blue dextran was collected and lyophilized.

HPLC analyses

HPSEC was performed by dissolving lyophilized pollen extracts in 0.15 *M* phosphate buffer (pH 6.8)–0.5 *M* potassium chloride and injecting the solution through a Rheodyne 100-μl loop. A Varian (Palo Alto, CA, U.S.A.) 5500 HPLC gradient instrument was used equipped with a Synchropak 100 column (SynChrom, Lafayette, IN, U.S.A.) with 0.15 *M* phosphate buffer (pH 6.8)–0.5 *M* potassium chloride as eluent at a flow-rate of 0.8 ml/min. The detector was a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 1040 diode-array detector.

HPIEC with an ionic strength gradient was performed by dissolving the material in 10 m*M* Tris–acetic acid buffer (pH 7.0)–100 m*M* sodium acetate at the required molarity and injecting the solution through a Rheodyne 5-ml loop. A Waters Delta Prep 3000 HPLC instrument equipped with a TSK DEAE-5 PW ion-exchange column (15 cm × 21.5 mm I.D.) was used, eluting with a 50-min gradient from solvent A to B, where A was 10 m*M* Tris–acetic acid buffer of the required pH plus sodium acetate of the required molarity and B was 10 m*M* Tris–acetic acid buffer of the required pH containing 500 m*M* sodium acetate. The flow-rate was 6 ml/min. The fractions were then analysed by the direct radio-allergo-sorbent test (RAST).

Reversed-phase (RP) HPLC was performed using a LiChrosorb RP-C_{18} column (25 cm × 4 mm I.D.) (Merck-Bracco, Milan, Italy). The material was dissolved in methanol and injected into the Waters instrument previously described using a 100-μl loop, then eluted with a 30-min linear gradient from A [0.1% aqueous trifluoroacetic acid (TFA)–acetonitrile (70:30)] to B [0.1% aqueous TFA–acetonitrile (10:90)]. The individual fractions were lyophilized and the residue was analysed by direct RAST.

Monoclonal antibody to Par jI

Cells producing monoclonal antibody to Par jI were expanded *in vivo* by injecting $2 \cdot 10^6$ cells intraperitoneally into Balb/c mice pretreated with pristane. The ascitic liquid was separated from cells by centrifugation at 1680 *g* for 10 min, precipitated with 50% saturated ammonium sulphate and extensively dialysed against 0.1 *M* sodium hydrogencarbonate (pH 8.3).

The ascitic liquid was further purified on protein Sepharose G, eluting IgG fractions with 0.1 *M* glycine hydrochloride (pH 2.7). The fractions corresponding to the peak were pooled and dialysed against 0.1 *M* sodium hydrogencarbonate (pH 8.3).

Immunoabsorbent preparation for conventional affinity chromatography

The purified immunoglobulin fraction from ascitic liquid was covalently coupled to a properly activited support (Affi-Gel 10; Bio-Rad Labs., Segrate, Milan, Italy) by continuous stirring for 4 h at room temperature. After blocking residual reactive sites by 1 *M* ethanolamine hydrochloride (pH 8), the gel was transferred to a 10 × 1.2 cm I.D. Bio-Rad column and washed with coupling buffer until the absorbance at 280 nm was close to zero.

Conventional affinity chromatography

The immunoabsorbent column prepared as above was used for the purification of 10 ml of a gel-filtered *Parietaria judaica* extract with a protein content of 0.8 mg/ml. After washing with phosphate-buffered saline (PBS) (pH 7.2) until the absorbance at 280 nm was close to zero in the eluent, the antibody-bound fraction was eluted with 2 *M* sodium chloride in PBS buffer (pH 7.2). The eluted fraction was dialysed against 10 m*M* PBS, lyophilized and tested by direct RAST, then further analysed by HPSEC.

Immunoabsorbent preparation for high-performance affinity chromatography

The purified immunoglobulin fraction from ascitic liquid was covalently coupled to a properly activated support using an Affi-prep 10 affinity cartridge (25 × 15 mm I.D. (Bio-Rad Labs., Segrate, Milan, Italy) according to the procedure recommended by the producer (Bulletin 1237).

High-performance affinity chromatography

The extract of *Parietaria judaica* (1 ml, protein content 0.8 mg/ml) was applied to the column which was eluted with 2 *M* sodium chloride in 0.15 *M* phosphate-buffered saline (PBS) (pH 7.2). The eluted fraction was dialysed against 10 m*M* PBS, lyophilized and tested with direct RAST, then further analysed by HPSEC.

Methanol extraction

A sample of 40 mg of gel-filtered *Parietaria judaica* extract was treated with 10 ml of methanol with stirring for 15 min at room temperature. The soluble fraction was then separated by centrifugation at 4200 *g* for 15 min. The solvent was evaporated at reduced pressure at room temperature and the residue was analysed by direct RAST, then analysed by HPSEC.

Radio-allergo-sorbent test (RAST)

The fractions were bound to cyanogen bromide-activated paper discs, and direct RAST or RAST inhibition was performed according to Yman *et al.* [16] using a pool of sera from 95 patients with high sensitivity to *Parietaria judaica* pollen and polystyrene beads as solid phase (Sferikit, Laboratorio Farmaceutico Lofarma, Milan, Italy).

For immunocapture RAST, the purified immunoglobulin fraction derived from ascite was bound to activated polystyrene beads, then used for a direct RAST.

RESULTS AND DISCUSSION

The HPIEC purification of the *Parietaria* extract could give better results if performed at different pH values and with different ionic strength gradients. These could act as better dissociating conditions for the homopolymerized allergen and allow the enrichment into the monomer to be optimized.

The first experiment was performed using a pH 7 buffer and a linear gradient of ionic strength from 250 to 500 m*M* sodium acetate, in order to have the maximum dissociating power of the eluent throughout the chromatography. The result is shown in Fig. 1a. Direct RAST analysis of all the fractions showed that the allergenic activity was spread over the chromatogram. IEF analysis of the fractions indicated that most components were eluted in the first part of the chromatogram. Fractions 23 and 25 had mainly a band at p*I* 4.5, which could correspond to the major allergenic protein of *Parietaria* pollen extract, *i.e.*, Par jI. Hence this chromatographic region was analysed by HPSEC.

The result from fraction 26 is shown in Fig. 2a and a RAST test using a monoclonal anti-Par jI antibody was performed. This showed that both the main peak in Fig. 2a and the peak at a 15.6-min elution time were allergens, suggesting that Par jI was eluted in the main peak (centred at about mol. wt. 11 000 dalton), and that in the minor peak a peptide of lower molecular weight that contained the allergenic determinant was eluted. This peak was analysed with a diode-array detector and showed a sharp absorption at 278 nm.

A further experiment was effected using a pH 5 buffer and a linear ionic strength gradient from 100 to 500 m*M* sodium acetate and the fractions were analysed by direct RAST. The result is shown in Fig. 1b. The individual fractions were analysed by HPSEC and most of these had the chromatographic profile shown in Fig. 2b for fraction 8. Also in this instance, RAST analysis was performed with the monoclonal anti-Par JI antibody. Again the major peak containing Par jI was accompanied by the peak at a 15.5-min elution time. Calibration of the column suggested an apparent molecular weight slightly higher than 5000 dalton which contained the allergenic determinant and absorbed at 278 nm. Diode-array detection showed that no contaminant was coeluted in this peak in most fractions. The experiments reported in Fig. 1a and b showed that a pH 5 mobile phase allowed fractions to be obtained from which this peptide could be recovered by purification using HPSEC.

These results suggested that the structure of the allergenic determinant in Par jI could be obtained by isolating peptides positive to the monoclonal anti-Par jI antibody, instead of isolating the very elusive Par jI itself.

Confirmation of these data was obtained submitting the *Parietaria* total extract

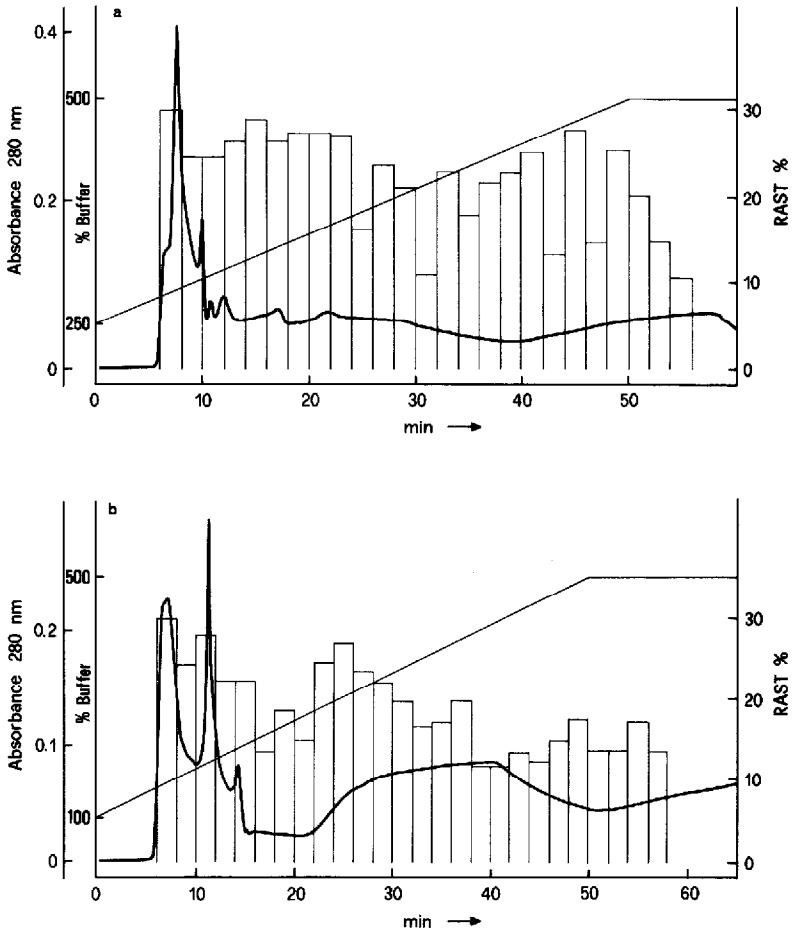

Fig. 1. Preparative HPIEC separation of *Parietaria* pollen extract and RAST analysis of the fractions. Column: DEAE-5PW. Ionic strength gradient: (a) 10 m*M* Tris–acetic acid buffer (pH 7.0)–250 m*M* sodium acetate (eluent A), 10 m*M* Tris–acetic acid buffer (pH 7.0) containing 500 m*M* sodium acetate (eluent B); (b) 10 m*M* Tris–acetic acid buffer (pH 5.0)–100 m*M* sodium acetate (eluent A), 10 m*M* Tris–acetic acid buffer (pH 7.0) containing 500 m*M* sodium acetate (eluent B). Flow-rate, 6 ml/min; 12-ml fractions were collected.

to immunoaffinity chromatography. In a first attempt, a monoclonal anti-Par jI antibody purified by ammonium sulphate precipitation was coupled to the stationary phase in conventional affinity chromatography. Elution with sodium chloride solution gave an eluate which was analysed by HPSEC. Several peaks appeared in the chromatographic region where Par jI was expected.

In order to improve the separation efficiency of the immunoaffinity approach, a further immunoaffinity chromatographic experiment was then performed using an HPLC column, and the same monoclonal anti-Par jI antibody was used. Elution with sodium chloride solution gave an eluate which was analysed by HPSEC. Several components were present in the eluate; these were collected, submitted to immuno-

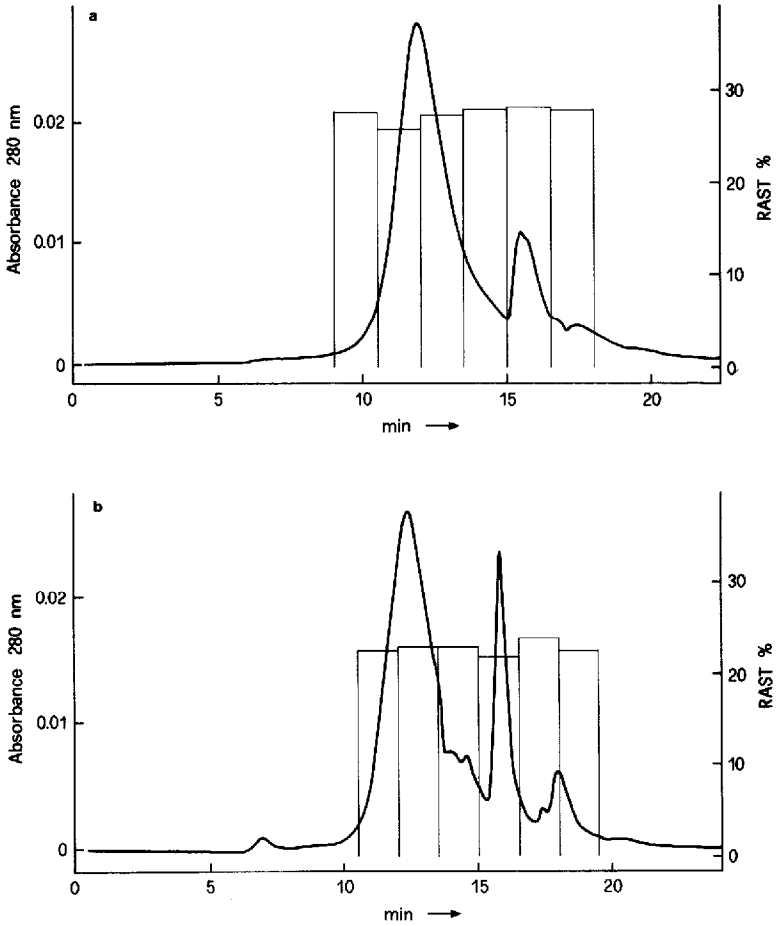

Fig. 2. (a) HPSEC analysis of fraction 26 from the run in Fig. 1a and RAST analysis of the fractions. (b) HPSEC analysis of fraction 8 from the run in Fig. 1b and RAST analysis of the fractions. Column, Synchropak 100; eluent, 0.15 *M* PBS (pH 6.8)–0.5 *M* KCl; flow-rate, 0.8 ml/min; 1.2-ml fractions were collected.

capture RAST and all the fractions were positive to RAST except one. Hence the allergenic determinant of Par jI was contained in a family of fragments.

Small peptides are sometimes extracted from complex proteic material by solvent extraction. Hence, some of these allergenic fragments could possibly be obtained by methanol extraction from the crude extract.

The gel-filtered extract of *Parietaria* pollen was extracted with methanol and centrifuged in order to eliminate the insoluble part of the extract. The solution obtained was analysed by HPSEC. The chromatogram showed a major peak at 18 min, which was positive in the RAST with monoclonal antibodies. Moreover, diode-array detection showed only a shoulder at 270 nm. The fractions containing this peak were further analysed using a reversed-phase C_{18} column with a 30-min linear gradient

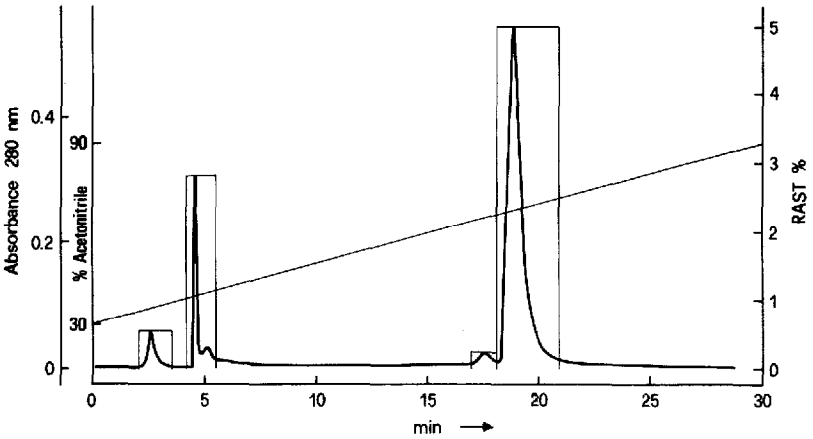

Fig. 3. RP-C_{18} HPLC analysis of the methanolic extract of *Parietaria* pollen and RAST analysis of the fractions. Column, Merck LiChrosorb RP-C_{18}; 30-min gradient from 0.1% aqueous TFA–acetonitrile (70:30) (eluent A) to 0.1% aqueous TFA–acetonitrile (10:90) (eluent B).

from 0.1% aqueous TFA–acetonitrile (70:30) to 0.1% aqueous TFA–acetonitrile (10:90). In this way we isolated the peak at a 20-min elution time, which was positive to the RAST test (Fig. 3). Diode-array detection of the peak showed a high purity.

In conclusion, these chromatographic experiments suggest that Par jI is a very unstable allergen, which tends to fragment when in solution. Several peptides were obtained from Par jI, and some of them show positivity toward the anti-Par jI monoclonal antibody, suggesting that they retain the entire structure of the allergenic determinant. These peptides could then be the target of further studies and the optimization of their isolation could lead to sufficient material for sequence and conformation studies.

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