Journal of Chromatography, 548 (1991) 229–234
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2346

Purification of *Phleum pratense* pollen extract by immunoaffinity chromatography and high-performance ion-exchange chromatography

EZIO BOLZACCHINI, PATRIZIA DI GENNARO, GIUSEPPINA DI GREGORIO and BRUNO RINDONE*

Dipartimento di Chimica Organica e Industriale, Università di Milano, Via Venezian 21, I-20133 Milan (Italy)

PAOLO FALAGIANI and GIOVANNI MISTRELLO

Lofarma Allergeni, Reparto Ricerche, Viale Cassala 40, I-20146 Milan (Italy)

and

IB SONDERGAARD

Royal Veterinary and Agricultural University, 40 Thorvaldsenvej, DK-1871 Frederiksberg C, Copenhagen (Denmark)

ABSTRACT

Basic allergens of *Phleum pratense* pollen extract have been purified by either sequence gel filtration–ion-exchange high-performance liquid chromatography (HPIEX) and size-exclusion HPLC or sequence gel filtration–immunoaffinity chromatography and HPIEX. The second procedure seems to be suitable for preparative purposes.

INTRODUCTION

Phleum pratense (Graminaceae) pollen allergenic extracts are used in the immunotherapy of allergic diseases. Since these extracts are constituted by a mixture of heterogenous proteins, studies aimed at improving their standardisation and knowledge of their chemical composition are needed. An early attempt to characterise phleum allergens was performed using combinations of electrophoretic and immunoelectrophoretic methods [1] and 28 antigens were thus shown to be present, some of them being allergens.

A preparative approach was performed in a sequence of conventional anion-exchange chromatography followed by conventional cation-exchange chromatography [2] and four allergens were isolated. They were the acidic antigens 3 (molecular weight, MW = 10 000; isoelectric point, pI = 3.9), 19 (MW = 15 000; pI = 4.9) and 25 (MW = 15 000; pI = 4.5) and the basic antigen 30 (MW = 34 000; pI = 9.4).

Other purification sequences were gel chromatography–ion-exchange chromatography [3,4] and, more recently, gel filtration–high-performance ion-exchange high-

0021-9673/91/$03.50 © 1991 Elsevier Science Publishers B.V.

performance liquid chromatography (HPIEX) and high-performance size-exclusion chromatography (HPSEC) [5]. Some immunochemical characterisation of the basic allergen [6] and analyses of its carbohydrates content [7,8] were also performed.

The uncertainty in the comparison of phleum allergens obtained with different procedures stimulated a recent attempt of better definition of *Phleum pratense* pollen extract composition [9]. However, a preparative-scale multi-step procedure for the separation of phleum components was needed. The preparative work experienced in our laboratory in the purification of *Parietaria judaica* allergens [10–15] using multi-step procedures based essentially on high-performance liquid chromatography (HPLC) stimulated us to use a similar approach for *Phleum pratense* pollen extracts.

EXPERIMENTAL

Phleum pollen extract and gel filtration

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 supernatant obtained after centrifugation at 43 000 *g* for 40 min was gel-filtered on Sephadex G-25, and phosphate buffer 20 m*M* pH 6.8, was used as eluent. The exclusion peak corresponding to blue dextran was collected and lyophilised.

HPLC analyses

HPSEC was performed by dissolving lyophilised pollen extracts in 0.15 *M* phosphate buffer, pH 6.8, 0.5 *M* potassium chloride and injecting the sample through a Rheodyne 50-μl loop. The instrument was a Varian 5500 HPLC gradient (Varian Palo Alto, CA, USA), equipped with a Synchropack 100 (30 × 10 mm O.D. × 8 mm I.D.) SEC column, eluted with 0.15 *M* phosphate buffer, pH 6.8, 0.5 *M* potassium chloride, at a flow-rate of 1 ml/min. The detectors were a Varian UV 50 instrument at 280 nm and a Hewlett-Packard 1040 diode array detector (Hewlett-Packard, Palo Alto, CA, USA).

HPIEX 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 the sample 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 40-min gradient of A to B (A: 10 m*M* Tris–acetic acid buffer, pH 7.0, sodium acetate of the required molarity; B: 10 m*M* Tris–acetic acid buffer, pH 7.0, containing 500 m*M* sodium acetate). The flow-rate was 6 ml/min. The fraction was then analysed by radio allergosorbent test (RAST) and RAST-Inhibition (RAST-I).

Immunoabsorbent preparation for affinity chromatography

Purified immunoglobulin fraction from serum of rabbit immunised against *Phleum pratense* pollen extract was coupled to Minileak medium gel (Kem Entek, Copenhagen, Denmark). The mixture constituted 1 g of gel in 10 ml of water containing 1 mg of antibodies and 3 ml of a 3% PEG solution and was left at room temperature for one night. The gel was then transferred into a Pharmacia column and washed with 0.1 *M* phosphate buffer, pH 7.0 and 0.5 *M* NaCl.

Affinity chromatography

The immunoabsorbent column was loaded with 10 ml of gelfiltered *Phleum pratense* with protein content 1.2 mg/ml. After 24 h of recycling, specific fractions were eluted with glycine–HCl buffer, pH 2.5, and collected into two samples (A and B) which were neutralised with 0.1 *M* phosphate buffer pH 6.8, containing 0.5 *M* KCl, dialysed against water and lyophilised.

Radioallergo sorbent test

The fractions were bound to cyanogen bromide-activated paper discs, and direct RAST or RAST-I was performed according to the method of Yman *et al.* [16] using a pool of sera from 95 patients with high sensitivity to *Phleum pratense* 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 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 [17–18].

Fused rocket immunoelectrophoresis (FRIE)

A 1% (w/v) agarose gel (10 cm × 1.5 mm) containing 11 $\mu l/cm^2$ Lofarma anti-phleum rabbit antibody (Lofarma Allergeni, Milano, Italy), was used. The buffer was Tris–tricine (pH 8.6, ionic strength 0.1 *M*). Electrophoresis was performed at 2 V/cm for 18 h at 15°C [19]. 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, ionic strength 0.1 *M*). 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 $\mu l/cm^2$ Lofarma anti-phleum rabbit antibody and operating at 15°C and 2 V/cm for 18 h [20]. Detection was performed by the Coomassie brilliant blue R-250 method (0.5% in water–ethanol–acetic acid, 45:45:10).

RESULTS AND DISCUSSION

The first purification procedure tested was performed with the gel-filtered extract in a preparative HPIEX experiment at pH 7.0 using an ionic strength gradient with an anion-exchange column. The result is shown in Fig. 1. Most basic and neutral protein components were eluted first in the basic exchanger used. Direct RAST of the fractions showed that the biological activity was spread over the whole chromatogram, and that much activity was present in the first chromatographic fractions, *i.e.* the basic and neutral part of the chromatogram. This was confirmed by RAST-I experiments.

The individual fractions were analysed by electrophoretic and immunochemical methods. Two families of antigens were apparent in FRIE analysis, the first occurring in fractions 1–15 (*i.e.* in the region containing basic and neutral components) and the other in fractions 20–30 (*i.e.* in the region containig acidic components).

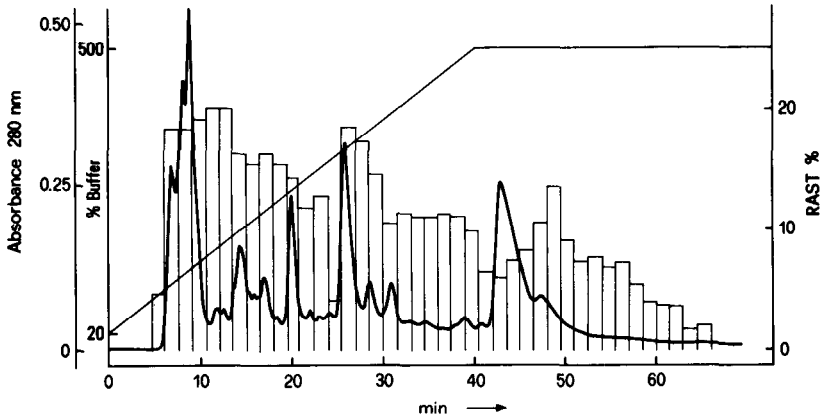

Fig. 1. HPIEX separation of *Phleum pratense* pollen extract and RAST analysis of the fractions.

IEF analysis of the chromatographic fractions showed that most fractions contained complex mixtures of components. Fraction 3 essentially constituted a single component, having an p*I* value of *ca.* 9.0. The HPSEC analysis of this fraction showed a peak at 11 min retention time which was positive to RAST.

The amount of allergenic material recovered was very low, and this sequence of HPIEX–HPSEC seemed to be unsuitable for preparative purposes.

As an alternative procedure we chose affinity chromatography followed by HPIEX. Polyclonal immunoglobulins were used in order to purify all the antigenic material. The gel-filtered extract was loaded into the affinity column where it was recycled for 24 h to maximise specific interaction. The antigens were then eluted using a pH 2.5 buffer. Two peaks (A and B) were eluted (Fig. 2) and were positive to RAST.

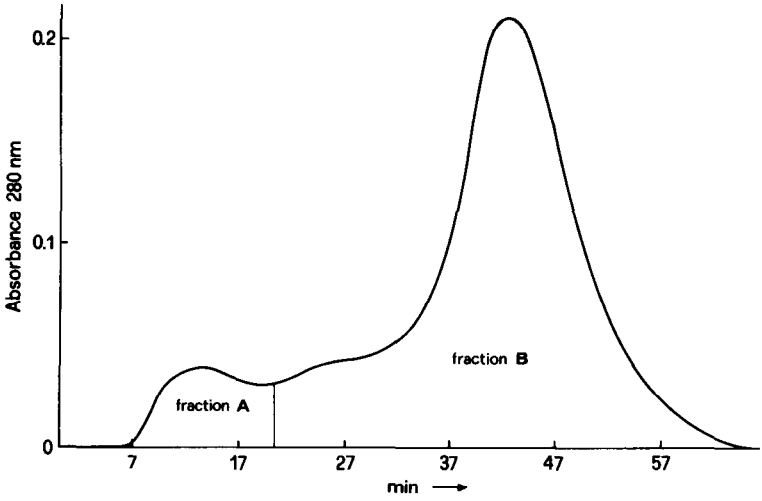

Fig. 2. Immunoaffinity separation of *Phleum pratense* pollen extract.

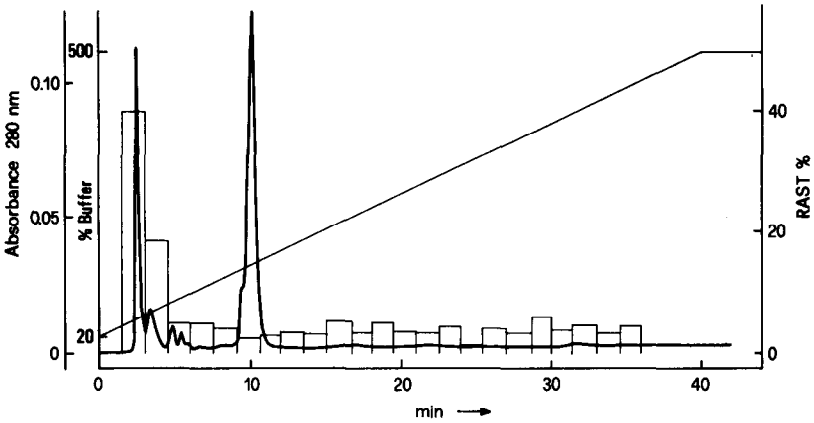

Fig. 3. HPIEX separation of fraction B of *Phleum pratense* pollen extract purified by immunoaffinity chromatography and RAST analysis of the fractions.

The HPIEX analysis of the major fraction (fraction B) using the same anion-exchange column is shown in Fig. 3. Three peaks constituted the fraction, and RAST and RAST-I analysis showed that the two peaks eluted first were allergens. HPIEX showed that these two peaks were the sole components of fraction B.

IEF analysis showed that peak A contained basic proteins and peak B contained both basic and acidic proteins. This is in accord with the elution time of the peaks in the HPIEX column. CIE showed that peak A gave only one precipitation arch and peak B showed two.

The conclusion is that, providing that the antibodies used have affinity against all the antigens in the extract, affinity chromatography of *Phleum pratense* pollen extract is a tool for the first purification step of its allergenic content. HPIEX may be the final purification step of the allergens.

ACKNOWLEDGEMENTS

We thank our students Sonia Poli, Francesca Farina and Monica Odiardo. This work was supported by a CNR grant, Progetto Finalizzato Chimica Fine II, by the Danish Natural Science Research Council and the Danish Agricultural and Veterinary Research Council.

REFERENCES

1 B. Weeke, H. Lowenstein and L. Nielsen, *Acta Allergol.*, 29 (1974) 402.
2 H. Lowenstein, *Allergy*, 33 (1978) 30.
3 E. Puttonen and H. J. Maasch, *J. Chromatogr.*, 242 (1982) 153.
4 D. Meineke, R. Wahl and H. J. Maasch, *Allergopharma*, 25 (1988) 242.
5 S. Haavik, B. Smestad Paulsen and J. K. Wold, *J. Chromatogr.*, 321 (1985) 199.
6 S. Haavik, A. Bryhni Frostad, B. Smestad Paulsen and J. K. Wold, *Int. Arch. Allergy Appl. Immunol.*, 83 (1987) 182.
7 S. Haavik, B. Smestad Paulsen and J. K. Wold, *Int. Arch. Allergy Appl. Immunol.*, 78 (1985) 197.
8 S. Haavik, B. Smestad Paulsen and J. K. Wold, *Int. Arch. Allergy Appl. Immunol.*, 83 (1987) 225.

9 D. G. Marsh, L. Goodfriend, T. P. King, H. Lowenstein and T. Platts-Mills, *Int. Arch. Allergy Appl. Immunol.*, 85 (1988) 194.
10 P. Falagiani, E. Cavallone, M. Nali, B. Rindone, S. Tollari, G. Riva and G. Crespi, *J. Chromatogr.*, 328 (1985) 425.
11 E. Bolzacchini, G. Di Gregorio, M. Nali, B. Rindone, S. Tollari, P. Falagiani, G. Riva and G. Crespi, *J. Chromatogr.*, 397 (1987) 299.
12 E. Bolzacchini, G. Di Gregorio, M. Nali, B. Rindone, S. Tollari, P. Falagiani, G. Riva and G. Crespi, *Allergy*, 42 (1988) 743.
13 A. Bassoli, F. Chioccara, G. Di Gregorio, B. Rindone, S. Tollari, P. Falagiani, G. Riva and E. Bolzacchini, *J. Chromatogr.*, 444 (1988) 209.
14 A. Bassoli, E. Bolzacchini, F. Chioccara, G. Di Gregorio, B. Rindone, S. Tollari, P. Falagiani and G. Riva, *J. Chromatogr.*,, 446 (1988) 179.
15 I. Sondergaard, P. Falagiani, E. Bolzacchini, P. Civaroli, G. Di Gregorio, V. Madonini, A. Morelli and B. Rindone, *J. Chromatogr.*, 512 (1990) 115.
16 L. Yman, G. Ponterius and R. Brand, *Dev. Biol. Standards*, 29 (1975) 171.
17 C. R. Merril, D. Goldman, S. A. Sedman and M. H. Ebert, *Science (Washington D.C.)*, 211 (1981) 1438.
18 J. Guevara Jr., D. Johnston, L. S. Rawagali, B. A. Martin, S. Capetillo and L. K. Rodriguez, *Electrophoresis*, 3 (1982) 197.
19 N. M. G. Harboe and P. J. Svendsen, *Scand. J. Immunol.*, 17 (Suppl. 10) (1983) 107.
20 P. J. Svendsen, B. Weeke and B. G. Johansson, *Scand. J. Immunol. Methods*, 62 (1983) 283.