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RAPID PURIFICATION OF THE MAIN ALLERGEN OF *LOLIUM PERENNE* BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

The main allergen from rye grass (*Lolium perenne*) pollen was purified by size-exclusion high-performance liquid chromatography. The purified allergen had a molecular weight of 32 000 daltons and was significantly more active in solid phase radioimmunoassay than the whole extract. The highly purified antigen can be obtained very rapidly and with a recovery of 30%.

INTRODUCTION

The purification of allergens is a prerequisite for up-to-date diagnosis and therapy in allergic diseases, and the need for removal of irritant, toxic or irrelevant components is well recognized[1]. Pollen of Graminiae is the most common cause of atopy throughout the world and *Lolium perenne* (rye grass) is one of the most aggressive species of this family.

High-performance liquid chromatography (HPLC) has been used by several workers for the purification of allergens from pollens[2–4]. Some excelent work has been published on the purification and characterization of the major allergen of rye grass, which binds to specific IgE from sensitive allergic patients[5–10]. Nevertheless, classical biochemical techniques such as ammonium sulphate precipitation. CM- and DEAE-cellulose ion-exchange chromatography and gel filtration using Sephadex have been used, often resulting in large losses of allergenic material and complicated purification schemes.

HPLC is a powerful and rapid method for the purification of the components of complex mixtures. Here we report the purification of the main allergen of *Lolium perenne* by using only two 15-min runs in a size-exclusion HPLC column. The pure allergen is biologically active and its recovery from crude extract is about 30%.

EXPERIMENTAL

Pollen extraction

A 1-g amount of pollen (Allergon, Sweden) was extracted with 10 ml of 0.15

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M phosphate-buffered saline (PBS) (pH 7.4) for 1 h in an ice-bath. After centrifugation at 12100 *g* for 15 min at 0°C, the extract was filtered on a 0.45 μm Millipore membrane. The extractions were carried out in the presence or absence of phenylmethylsulphonyl fluoride (PMSF) (Sigma, St. Louis, MO, U.S.A.). All protein determinations were performed by the method of Lowry *et al.*[11].

Size-exclusion HPLC

HPLC analyses were performed on a Waters Assoc. chromatography system with Protein Pak 125 columns. The system includes a Model 510 pump, a Model 440 UV detector operated at 254 nm, a loop-type U6K injector and a Waters data module.

The eluent was PBS and the injection volumes varied from 10 to 100 μl.

Solid-phase radioimmunoassay

HPLC-purified rye grass allergen was dialysed against 0.05 *M* carbonate buffer (pH 9.6). A 50-μl volume of the antigen solution was allowed to become attached to the wells of PVC plates (Flow) overnight at 37°C. Residual binding sites were further covered with 5% bovine serum albumin (BSA) for 1 h at 37°C.

Sera from allergic patients having a type 4 RAST to *Lolium perenne* were added to the wells (50 μl) and after incubation for 1 h at 37°C were washed three times with PBS containing 0.5% of Triton X-100.

The radioimmunoassay (RIA) was developed with 50 μl of rabbit anti-human IgE labelled with ^{125}I (Pharmacia, Uppsala, Sweden). Washed and cut wells were counted in a Packard Autogamma 500 C gamma counter.

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis

All electrophoresis materials was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Molecular-weight markers were obtained from Pharmacia.

Slab gels containing 0.1% of SDS were prepared and run in a system according to Laemmli[12]. The separating gel consisted of 12.5% of polyacrylamide and the stacking gel contained 4.5% of polyacrylamide. The gels were stained with 0.25% Coomassie Brilliant Blue G-250 in 45% methanol–10% acetic acid for 1 h and destained with methanol–acetic acid–water.

Immunoblotting

Immunoblotting was carried out as described by Towbin *et al.*[13]. Proteins were transferred electrophoretically to nitrocellulose strips (0.45 μm) (Schleicher & Schüll, Dassel, F.R.G.) in a Trans Blot Cell (Bio-Rad Labs.) for 2 h at 250 mA. After transfer, the strips were soaked in 3% human serum albumin (HSA) in PBS for 1 h to saturate additional protein binding sites and then incubated overnight with a pool of atopic sera diluted four times. The strips were washed in PBS containing 1% of Triton X-100 and incubated again with anti-IgE ^{125}I ($1 \cdot 10^6$ cpm) in PBS containing 0.3% of HSA. After extensive washing, the nitrocellulose was air-dried and exposed to Kodak X-OMAT R film for 4 days.

RESULTS

Mobile phase

As distilled water and aqueous buffers are the usual solvents used for the extraction of allergens from pollens, water, Tris and phosphate buffers were used as mobile phases in the size-exclusion HPLC purification.

Fig. 1A shows that the use of distilled water led to a very poor resolution, with the presence of two peaks in the void volume of the columns, probably aggregates. An ionic strength similar to the normal physiological conditions (0.15 *M* NaCl) was checked at pH 8 (Tris buffer) and pH 7.2 (PBS). Both systems (Fig. 1B and C) gave good resolutions of the components of the crude extract and PBS (both for extraction and as the mobile phase) was used throughout the work because of its similarity with the natural medium in the nose and throat, target organs in allergy.

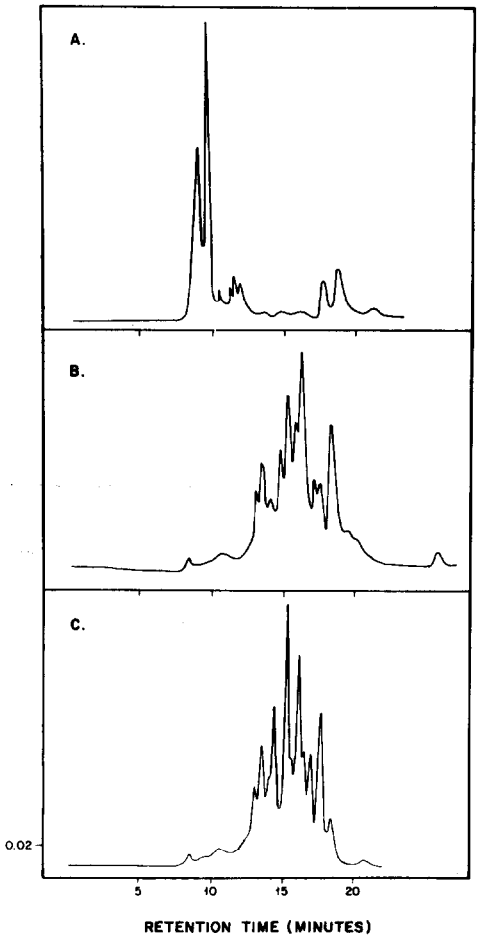


Fig. 1. HPLC of crude extract of *Lolium perenne* pollen on a Protein Pak 125 column. Eluent: (A) distilled water; (B) 0.15 *M* NaCl–0.01 *M* Tris (pH 8); (C) phosphate-buffered saline (pH 7.2).

Flow-rate

As low flow-rates usually lead to the best resolution, three different flow-rates, 1 ml/min (the highest recommended by the manufacturer of the column), 0.75 ml/min and 0.5 ml/min, were tested.

Low flow-rates produce the broadest peaks and therefore a flow-rate of 1 ml/min, which gave rapid resolution and sharp peaks, was adopted in further experiments.

Use of protease inhibitors

During the extraction procedure some enzymes are expected to be released from the pollen membranes[14,15]. This was demonstrated for *Lolium perenne*. Fig. 2B shows that the peak with a retention time of 14.44 min (downward arrow) is digested and the product(s) appears in the peak with a retention time of 16.78 (upward arrow). This digestion is prevented if phenylmethylsulphonyl fluoride (PMSF) was added at a level of 50 μg/ml to the extraction buffer (Fig. 2A).

Our extraction conditions are drastic (1 h at 0°C) but longer periods at 4°C led to increased proteolytic digestion (data not shown).

Other small changes such as the appearance of shoulders were detected but the more dramatic effect in the absence of PMSF during the extraction was the conversion of the material of retention time 14.44 min into the product with a retention time of 16.78 min.

By using integration in the data module we observed a mean decrease of 22.6 ± 1.5% peak area to 13.8 ± 0.8% in the case of the 14.44 min peak. A parallel increase in the 16.78 min peak from 5.4 + 0.3% to 16.8 ± 0.9% was observed.

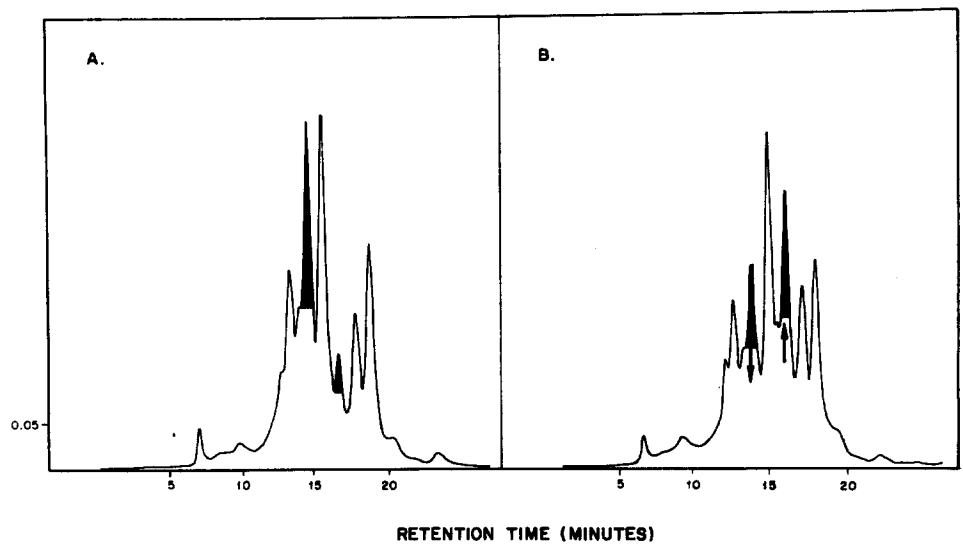


Fig. 2. Effect of protease inhibitor PMSF on the separation of *Lolium perenne* extract. Extractions and runs on the HPLC columns were performed (A) with or (B) without the presence of 50 μg/ml of PMSF in PBS. Shaded peaks indicate material digested (downward arrow) and digestion products (upward arrow).

As we subsequently demonstrated, the main allergen does not appear at this retention time (14.44 min) but, as a rule, we included PMSF during all extractions.

Final purification schedule

Two purification cycles in the Protein Pak 125 column were effective in obtaining the main allergen from *Lolium perenne* (Fig. 3). The position of the allergenic material was determined by performing SPRIA analysis of each fraction. Material from the shaded area in Fig. 3A was dialysed overnight against distilled water at 4°C, lyophilized and run again in the same column. The concentration of the sample after freeze-drying was about 10-fold.

SPRIA analysis of the fractions from the second run demonstrate that the shaded area in Fig. 3B contained the main allergen concentration in a pure form, as demonstrated in Fig. 3C. The molecular weight of this peak, as determined by plotting log (molecular weight) *versus* elution time for some pure protein markers was 32000, in very good agreement with results obtained by other workers[9]. The yield of purified allergen was about 300 mg/g, around twelve times higher that previously reported in purification by conventional biochemical methods[9].

SDS polyacrylamide gel electrophoresis further demonstrated the purity of the allergen, with an apparent molecular weight of 33000 (Fig. 4, lane C). When the crude extract from lane B was blotted to nitrocellulose paper and incubated with a pool of allergic sera, all the IgE-binding activity was associated with the pure allergen band (Fig. 4, lane D).

SPRIA analysis

In order to characterize further the purified allergen from an immunological point of view, we performed allergen titration in the solid-phase radioimmunoassay. As can be seen in Fig. 5, a dose-dependent rabbit anti-human IgE ^{125}I binding is obtained with both crude pollen extract and HPLC-purified allergen. Pure allergen needs about 0.3 μg and the extract 0.9 μg per PVC well to reach 50% radioactivity

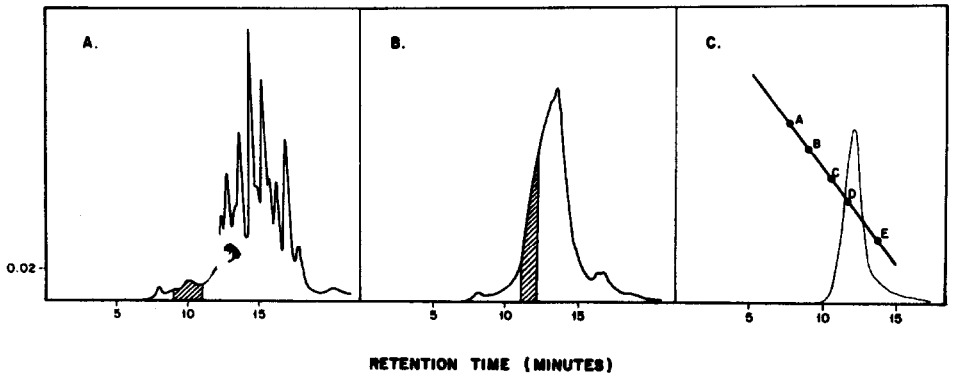
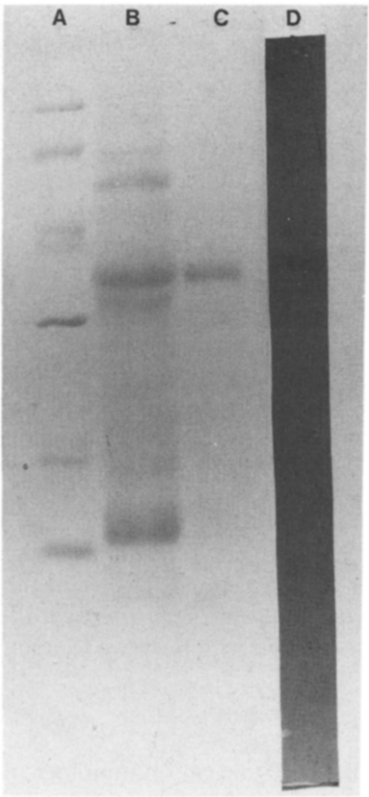


Fig. 3. Final allergen purification scheme. Shaded area in A was lyophilized and recycled in the run shown in B. Shaded area in B contains the allergen the purity of which was checked in C. The column was calibrated with (A) aldolase (MW 161000), (B) BSA (67000), (C) OVA (43000), (D) bovine pancreas DNase (31000) and (E) cytochrome *c* (12500).



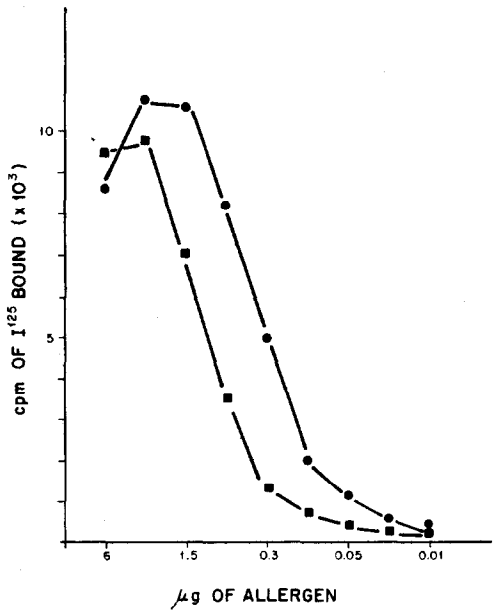


Fig. 4. SDS-PAGE of *Lolium perenne* extract and HPLC-purified main allergen. Lane A, molecular weight markers: phosphorylase *b* (MW 94000), BSA (67000), OVA (43000), carbonic anhydrase (30000), STI (20100) and α-lactalbumin (14400). Lane B, *Lolium perenne* crude extract. Lane C, HPLC-purified main allergen. Lane D, identification of IgE-binding band by immunoblotting.

Fig. 5. Titration of crude extract (■) and pure rye grass allergen (●) in solid-phase radioimmunoassay. A class 4 RAST serum and rabbit anti-IgE labelled with ^{125}I were used to detect the antigenic material.

binding. This indicates a 3-fold purification, an expected rate taking into consideration that the main allergen is a major component of the extract (Fig. 4).

When 1 µg of pure allergen is bound per well and titration of two selected RAST class 4 allergic sera was performed, the results in Fig. 6 were obtained. Serum from a subject allergic to egg white (class 3 RAST) showed a baseline under the same test conditions.

The above findings demonstrate that our SPRIA acts a dose-dependent test and that our allergen retains its specific IgE binding properties after purification. This was also demonstrated by *in vivo* skin-prick tests in which the pure allergen acted with the same specificity as crude *Lolium perenne* extracts.

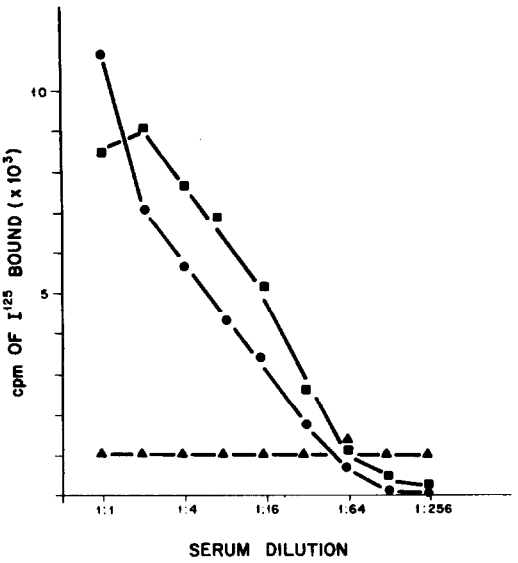


Fig. 6. Allergic sera titration on PVC wells each covered with 1 μg of HPLC-purified rye grass allergen. Two class 4 RAST sera against *Lolium perenne* (●, ■) and a class 3 RAST serum against egg white (▲) were employed.

DISCUSSION

The advantages of purification of the main allergen of *Lolium perenne* by using Protein Pak 125 HPLC columns are that there is no need to defat the pollen or remove pigments from the crude extract, coupled with high speed and good yields of the purified allergen. In addition, the evidence of common allergens in phylogenetically related species of grasses opens the field for the rapid obtaining of purified allergens than can be used for a wide range of therapeutic allergic treatment.

We must stress the use of buffers with an ionic strength equivalent to 0.15 *M* NaCl in order to avoid the formation of aggregates and to minimize the ionic interactions between the proteins and the silica gel stationary phase of the column. We advise the use of shorter periods of extraction than those employed by other workers because 1 h is sufficient for the release of most allergenic material from pollen. Careful control of the temperature of extraction at 0°C in an ice-bath avoids the production of low-molecular-weight products of protease digestion.

The allergenic activity of the purified protein is fully retained, as expected with the mild conditions of separation used.

We conclude that HPLC is a valuable technique for the purification of allergens from crude extracts of Graminiae pollen. The speed of the separation and the high yield obtained suggest applications in industrial-scale processes.

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