

Note

Purification of allergens by high-performance liquid chromatography

IV. Purification of the allergen of olive pollen (*Olea europea*)

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The olive (*Olea europea*) is an important cause of pollen allergy in Mediterranean countries. Around 50% of pollinic patients in Spain give a positive reaction to olive extracts in the skin prick test¹. The pollen grain of *Olea europea* is around 20 μm in diameter and it reaches concentrations of 50 grains/ m^3 in the pollinating season (May-June)².

No attempts to purify olive allergens have been performed to our knowledge. Here we report the purification of the main allergen of *Olea europea* using distilled water as the eluent in Protein Pak 125 (Waters Assoc.) columns.

EXPERIMENTAL

Pollen extraction

Pollen from *Olea europea* (Allergon, Sweden) was extracted at a 10% concentration in phosphate-buffered saline of pH 7.4 (PBS) containing 50 $\mu\text{g}/\text{ml}$ of phenylmethyl sulphonyl fluoride (PMSF) (Sigma) for 1 h in an ice-bath. After centrifugation at 12 000 g for 15 min at 0°C, the extract was filtered on a 0.45- μm Millipore membrane and injected into the high-performance liquid chromatographic (HPLC) system.

Size exclusion HPLC

The HPLC system used was as described previously³.

Immunological techniques

Solid-phase radioimmunoassay (SPRIA) was performed as described previously³.

NOTES

Skin testing

Intradermal skin prick tests were carried out in olive allergic patients using serial ten-fold dilutions of the HPLC-purified allergen in 50% glycerol. The skin response was measured after 20 min. The positive control was 0.1% histamine · HCl and the negative control PBS. Fifteen patients (ten males and five females) were studied. The mean ages were 9 years for males and 12 years for females.

RESULTS

Purification with PBS as mobile phase

PBS has previously been found to be an excellent mobile phase for the HPLC purification of allergens from *Lolium perenne* pollen³, *Dermatophagoides pteronyssinus* mite cultures⁴ and *Fungi imperfecti* spores and micelia⁵, so it was tried in the

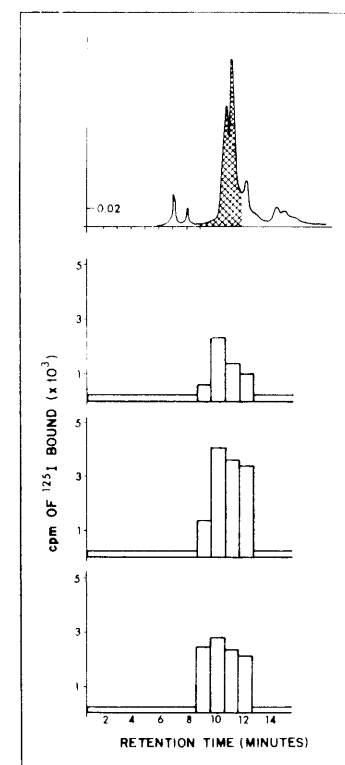


Fig. 1. HPLC separation of *Olea europea* pollen extract with a Protein Pak 125 column and PBS as eluent. Three allergic sera were tested in SPRIA against the separated fractions (histograms). The value on the y-axis (0.02) is the sensitivity of the detector.

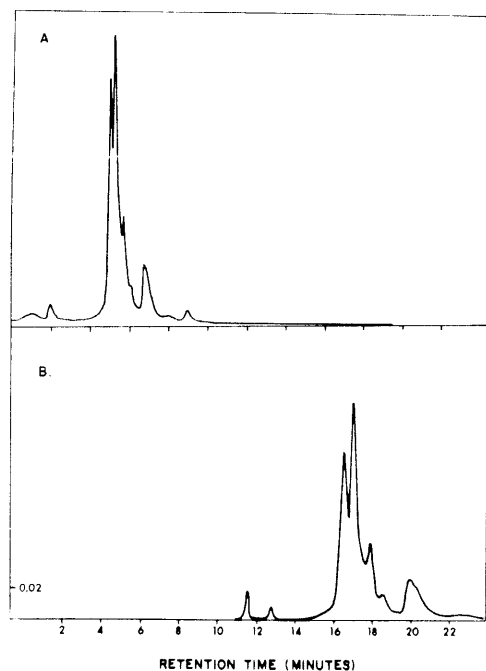


Fig. 2. Influence of flow-rate on the HPLC separation of *Olea* allergens: (A) 2 ml/min; (B) 0.5 ml/min.

separation of *Olea europaea* pollen. However, as shown in Fig. 1, PBS led to a poor resolution in the zone where the allergenic activity is detected by SPRIA (shaded area). Two unresolved peaks are associated with biological activity in the zone of mol.wt. 30 000. The distribution of immunological activity in SPRIA strongly suggests that the leading peak of the unresolved pair of peaks (retention time = 10 min) is responsible for the allergenicity. To confirm this point, we tried to improve the resolution by changing some of the parameters in the chromatographic run.

Changes in flow-rate

The standard flow-rate of 1 ml/min was changed to 2 and 0.5 ml/min to try to improve the separation of the two peaks. As can be seen in Fig. 2, only a small but detectable improvement at a flow-rate of 0.5 ml was observed, accompanied by the logical changes in retention times.

Changes in pH

Acidic and basic PBS mobile phases significantly changed the profile of the chromatographic separation, demonstrating that secondary ionic and hydrophobic interactions also play a role in HPLC separations using Waters Protein Pak columns.

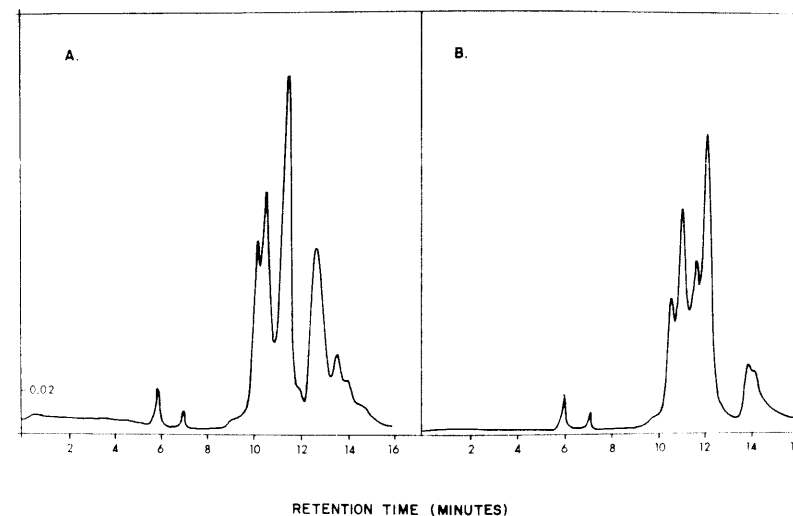


Fig. 3. HPLC separation of proteins from olive pollen using PBS adjusted to (A) pH 5.5 and (B) pH 9.

Nevertheless, these pH changes do not seem to give useful results if we compare the separation in Fig. 3 with that obtained using the physiological PBS pH of 7.4 (Fig. 1).

Changes in ionic strength

Fig. 4 demonstrates that no further improvements are obtained when the concentration in PBS is decreased to 0.1 M sodium chloride (A) or increased to 0.3 M sodium chloride (C) in comparison with the usual 0.15 M sodium chloride (B).

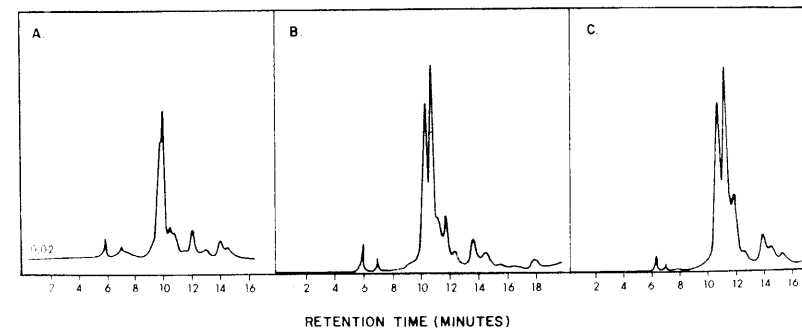


Fig. 4. Influence of ionic strength on the resolution of peaks in HPLC: (A) 0.1 M sodium chloride; (B) 0.15 M sodium chloride; (C) 0.3 M sodium chloride.

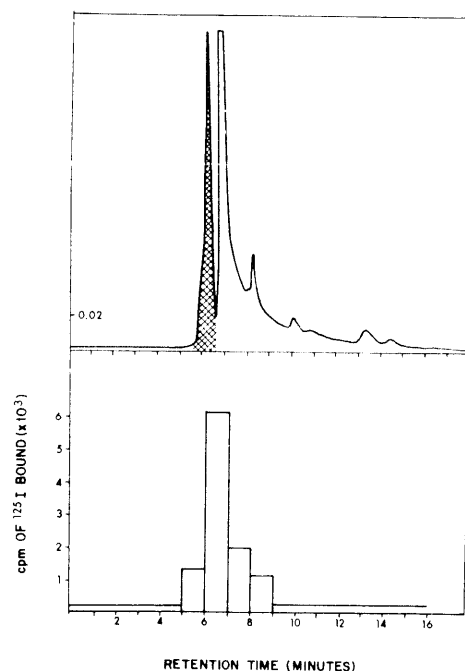


Fig. 5. HPLC separation of *Olea europaea* allergen using distilled water as eluent. The pool of the three sera used in Fig. 1 was employed to assign the allergenic activity to the leading peak by SPRIA.

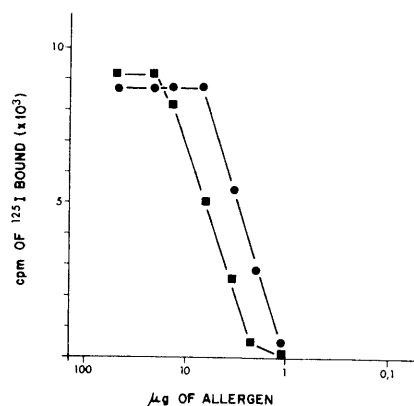


Fig. 6. Titration of olive pollen crude extract (■) and HPLC-purified main allergen (●). A pool of allergic sera (class 4 RAST) and ¹²⁵I-labelled rabbit anti-human IgE were used in SPRIA.

Distilled water as mobile phase

A high-resolution separation was obtained using distilled water as the mobile phase (Fig. 5). In this solvent, the first peak is eluted quickly (retention time, 7 min) and the second contaminant seems to be retained as a function of hydrophobic interactions with the column matrix. This effect allows us to purify the *Olea* allergen in a single simple step.

As demonstrated in the histogram depicting the activity in SPRIA, employing the pool of three sera used in Fig. 1, the allergen elutes as a sharp leading peak (shaded area). The integration data indicate a 95% purity and a recovery approaching 100%.

In vitro immunological activity of pure allergen

The crude extract and purified allergen titration in SPRIA shows a dose-dependent binding of human immunoglobulin E (IgE) antibody (Fig. 6). HPLC-purified allergen needs 4 μg and crude *Olea* extract 8 μg per polyvinyl chloride well to reach 50% binding of rabbit anti-human IgE antibody labelled with ¹²⁵I.

Hence a 2-fold purification has been performed, in accordance with the quantitative contribution of the allergen peak to the total area of the chromatogram.

In vivo allergenic activity

Skin prick tests with purified allergen were performed with fifteen untreated patients. Five different ten-fold dilutions were tested with each patient in order to determine which solution gave a mean wheal reaction of the same diameter as the positive control. That allergen solution was labelled 1000 biological units (B.U.)/ml.

The results demonstrated that the HPLC-purified allergen remains fully active *in vivo*. The speed of the separation minimizes allergen degradation by temperature, pH or enzymatic activity over long periods of time. The Lowry determination⁶ of protein from 100 000 B.U. samples indicated that in this system 1 B.U. corresponds to 0.75 ng of pure allergen.

DISCUSSION

HPLC provides rapid separations of protein and polysaccharide allergens with high recoveries of both material and bioactivity. In this work, Protein Pak 125 was used in an one-step method to purify the main allergen of *Olea europaea*. Distilled water was used as mobile phase to take advantage of the different behaviours of the allergen and a contaminant peak, which could not be resolved using medium ionic strength buffers, in contrast to *Lolium perenne* pollen allergen³. Changes in flow-rate, pH and ionic strength do not improve the resolution owing to the very similar molecular weights of the proteins involved.

The olive allergen is not retained on the column matrix (silica with a bound neutral hydrophilic moiety) and can be separated from the contaminant in a 15-min run when water is used as the solvent.

Owing to the mild conditions employed, the allergen retains its ability to bind human specific IgE as measured in an *in vitro* SPRIA, and *in vivo*, producing a dose response in the skin prick test of allergic patients.

Because of the great importance of *Olea europaea* as a cause of severe allergic