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OLIVE (*OLEA EUROPEA*) POLLEN ALLERGENS—II. ISOLATION AND CHARACTERIZATION OF TWO MAJOR ANTIGENS

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Abstract—A dialyzed extract of olive (*Olea europea*) pollen was fractionated by anion exchange chromatography on DEAE-Sephadex CL-6B using a discontinuous gradient of ammonium bicarbonate. The most important protein allergen was obtained from the 0.3 M fraction after gel filtration on Sephadex G-100 and separation by lentil-lectin Sepharose-4B. The major allergen of olive pollen was contained in the effluent and was designated Olea Antigen I. This material inhibited the RAST activity of 15 patients' sera that were tested. Analytical IEF demonstrated a major band at pH 5.3 and two minor ones at pH 5.6 and 5.0. When these were run into SDS-polyacrylamide gel electrophoresis in a second dimension, all were separated into two bands of mol. wt 17 and 19 K. A second protein, which is the next most important allergen, Olea Antigen II, was obtained from the 0.5 M fraction by chromatofocusing in a 4–7 pH range followed by filtration on Bio-gel P-30. Olea Antigen II had a mol. wt of 8 K as assessed by SDS-PAGE. IEF analysis displayed one main band at pH 3.6 and two minor bands at pH 3.8 and 4.0, respectively. *OL-1*, an anti-*Olea europea* monoclonal antibody (MAb) previously reported by us Lauzurica *et al.* (1988) reacted with the 17 and 19 K antigens from the crude extract and with Olea Antigen I but not with Olea Antigen II.

INTRODUCTION

The isolation and characterization of highly purified IgE binding molecules are of great importance. These purified antigens can be used not only for diagnostic purposes but also as tools for dissecting cellular and humoral IgE response (Rawle *et al.*, 1984; Ekramoddoullah *et al.*, 1986b). The isolation of major antigens is thus the first step for studies leading to the identification of determinants related to the IgE response (Olson and Klapper, 1986; Elsayed *et al.*, 1986; Ekramoddoullah *et al.*, 1986a) as well as for studying the relationship with molecules such as Class II and T cell receptors which could be involved in the IgE response (Marsh *et al.*, 1982; Marsh, 1986; Roebber *et al.*, 1985).

Among the pollens of the Mediterranean area, *Olea europea* pollen is one of the more widely distributed (Bousquet *et al.*, 1985). Although several allergens have been identified (Vela *et al.*, 1982; Blanca *et al.*, 1983) little information is available regarding the molecular characteristics of the isolated antigens.

In a previous report (Lauzurica *et al.*, 1988) we analysed, by immunoblotting and CRIE, the major allergens found in the *O. europea* crude extract and we described a monoclonal antibody (MAb) *OL-1* which recognizes the 17 and 19 K bands from the whole crude extract.

This paper describes the purification and partial characterization of two proteins, considered as major allergens from *O. europea* pollen. These proteins (Olea Antigen I and Olea Antigen II) are quite different in terms of their charge, size and allergenic activity. Monoclonal antibody *OL-1* reacts only with protein Olea Antigen I.

MATERIALS AND METHODS

Extraction of pollen. *Olea europea* crude extract (C.E.) was prepared according to the procedure described elsewhere (Lauzurica *et al.*, 1988). Figure 1 shows an outline of the purification steps that followed.

Fractionation of *O. europea* crude extract. Ten ml of C.E. solution at 20 mg/ml, were applied to a 2.5 × 30 cm column of DEAE-Sephadex CL-6B (Pharmacia Fine Chemicals) which had been equilibrated with ammonium bicarbonate (0.02 M). Fractions were eluted stepwise at 30 ml/hr with ammonium bicarbonate. Discontinuous gradient separations of 0.02, 0.1, 0.3, 0.5 and 1 M of ammonium bicarbonate were performed. Fractions were concentrated over a YM-2 membrane in a Diaflo cell (Amicon, MA).

Olea Antigen I purification. The 0.3 M fraction was subjected to gel filtration on Sephadex G-100 (2.6 × 100 column) at 4°C. The major protein peak was pooled, dialysed, freeze-dried and redissolved in 0.5 M sodium chloride, 0.05 M Tris buffer, pH 7.5,

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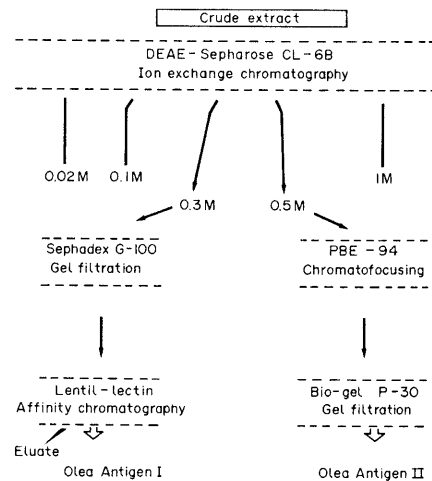


Fig. 1. Schematic diagram of the isolation of Olea Antigen I and Olea Antigen II.

containing 1.0 mM $MnCl_2$ and $CaCl_2$. The sample was then applied to a lentil-lectin Sepharose-4B (Pharmacia Fine Chemicals) 1×8 column which had previously been equilibrated with the same buffer solution, at a flow rate of 30 ml/hr at $4^\circ C$. The starting buffer was used during elution until no more u.v. absorbing material (280 nm) was detected in the effluent. Thereafter the column was washed with the same buffer containing 5% methyl-D-mannoside.

Olea Antigen II purification. Chromatofocusing of the 0.5 M fraction was performed on a 30 ml (1×30) column of PBE 94 equilibrated with 0.025 M imidazole-HCl. The fraction was eluted using a polybuffer 74-Cl (Pharmacia, Sweden). The peak eluted at pH 4 was applied to a Bio-gel P-30 (LKB, Sweden).

Isoelectrofocusing (IEF). Olea Antigen I and Olea Antigen II were labelled with ^{125}I according to the method of Greenwood *et al.* (1963). Labelled Olea Antigen I and Olea Antigen II were dissolved in 100 μ l of a sample buffer containing 8 M urea, 1% Nonidet P-40, 10% 2-mercaptoethanol and 4% (w/v) Ampholine (LKB) mixture, pH 3.5–10, 4–6 and 6–8 (1:4:4). After 3 min centrifugation at 11,600 g to remove insoluble material, IEF was performed as described elsewhere (Rodríguez de Córdoba *et al.*, 1984).

Sodium dodecyl sulphate (SDS) polyacrylamide gradient gel electrophoresis. SDS-PAGE was performed according to Laemmli (1970).

Two-dimensional electrophoresis. One strip from a running IEF gel described above was placed on top of a 7–15% polyacrylamide linear gradient and SDS-PAGE electrophoresis carried out as above. Gels were fixed in H_2O :methanol:acetic acid (10:4:1) and

analysed by autoradiography with Kodak X-OMAT GRS film.

Protein determination. The protein content of samples was estimated by the Coomassie blue assay of Bradford (1976).

Solid-phase radioimmunoassays (RAST). This was performed by incubating the different allergenic fractions at serial dilutions in PBS with nitrocellulose discs (5 mm diameter). The discs were incubated at $4^\circ C$ for 18 hr. Allergen solutions were then withdrawn and the discs washed with PBS. Unbound sites were blocked with 3% BSA (Sigma) in PBS containing 0.1% Tween-20. Finally, the discs were washed 3 times with 0.1% Tween-20 PBS. After 2 hr of incubation with 100 μ l of allergic sera, specific IgE was developed with ^{125}I -anti-human IgE (Pharmacia, Uppsala, Sweden).

Radioimmunoassay (RAST) inhibition analysis. The radioallergosorbent inhibition test was performed according to Gleich *et al.* (1975) with commercial discs coated with *Olea europea* pollen extract (Pharmacia, Uppsala, Sweden). Five-fold serial dilutions of Olea Antigen I (200 μ g/ml) and crude extract (200 μ g/ml) were used as inhibitors.

ELISA. This was performed according to the method of Chapman *et al.* (1984). Olea Antigen I, Olea Antigen II and crude extract were bound to microtitre plates at a concn of 10 μ g/ml and these were incubated with serial dilutions of MAb OL-1 supernatant. A goat anti-gamma mouse serum peroxidase labelled (Tago, Burlingame, CA) was used as a second antibody.

Patients sera. Sera were selected from patients with documented positive clinical history and positive cutaneous tests to olive pollen. All patient's sera were screened with Phadebas RAST and showed specific IgE binding (RAST class 3–4). Some sera were used as individual sera while others were pooled prior to use.

RESULTS

Fractionation of *Olea europea* pollen proteins

Five fractions were obtained from the pollen extract by ion exchange chromatography. Figure 2(a) shows the SDS-PAGE analysis of these fractions. Two bands of 17 and 19 K, which had previously been observed as the major proteins from the crude extract (Lauzurica *et al.*, 1988), were found in the eluates obtained with 0.1 and 0.3 M ammonium bicarbonate.

The IgE binding capacity of these fractions was tested using a solid-phase RIA with a pool of sensitized sera. The results shown in Fig. 2(b) indicate that the two most active fractions were those obtained with 0.5 and 1 M ammonium bicarbonate.

Figure 3 shows the results of a RAST inhibition analysis using the 0.3 and 0.5 M fractions. In contrast with the above results, the 0.3 M fraction was the most active fraction to inhibit IgE binding to the

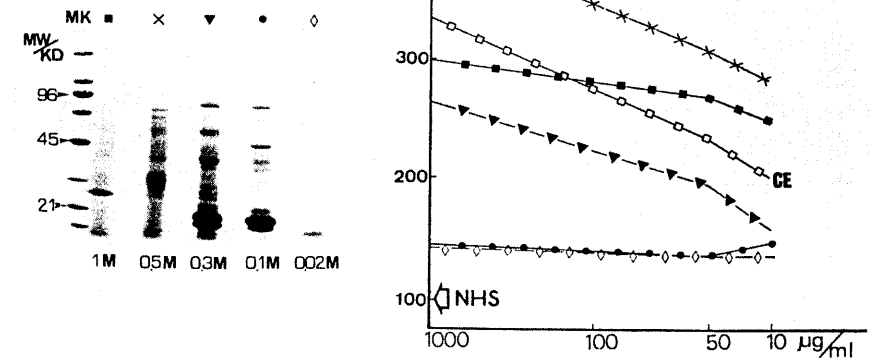


Fig. 2. SDS-PAGE and IgE binding activity of the fractions obtained from *O. europea* crude extract by anion exchange chromatography on a column of DEAE-Sephacrose CL-6B. (a) SDS-PAGE of each of the five fractions. Ten μ g of protein of each fraction were analysed on a 7–15% gradient gel. (MK) Molecular wt marker; some values are given on the left. (b) IgE binding activity of the same fractions by solid-phase radioimmunoassay using a pool of olive-sensitized sera: serially diluted crude extract ($\square$ CE), 0.02 M fraction ($\diamond$), 0.1 M fraction ($\bullet$), 0.3 M fraction ($\blacktriangledown$), 0.5 M fraction ($\times$) and 1 M fraction ($\blacksquare$).

crude extract. Results demonstrated that the 0.3 M fraction reached 50% inhibition at 2 orders of magnitude less than the 0.5 M fraction. Therefore, both the 0.3 and 0.5 M fractions were further analysed.

Purification of the 0.3 M fraction: properties of Olea Antigen I

The 0.3 M fraction was purified by gel filtration [Fig. 4(a)]. The major peak corresponding to 38 K, was further purified through a lentil-lectin column and both the glycoprotein fraction and the effluent were analysed by SDS-PAGE. Figure 4(b) shows these results compared with the unfractionated material. The effluent was designated Olea Antigen I and consisted of two bands of 17 and 19 K, of which the

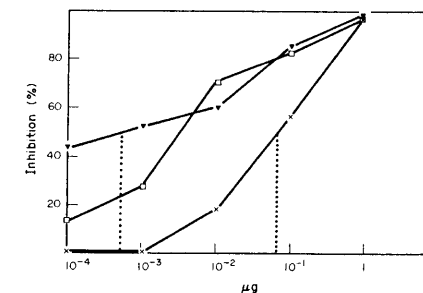


Fig. 3. RAST inhibition activity of the 0.3 M fraction ($\blacktriangledown$) and 0.5 M fraction ($\times$) compared with the crude extract ($\square$).

17 K band was the most relevant. In contrast, the eluate showed the similar pattern as the unfractionated material.

The ability of the different components to inhibit the specific IgE binding of a pool of reaginic sera to whole crude extract, was investigated by RAST inhibition experiments. The results shown in Fig. 4(c) demonstrate that Olea Antigen I was the most active component, reaching 50% inhibition at a protein concn of less than 1 μ g/ml.

Olea Antigen I was further analysed by IEF and 2-dimensional electrophoresis and the results are shown in Fig. 5. The three bands focused at pH 5.0, 5.3 and 5.6 were obtained by IEF. The densitometric analysis demonstrated that the pH 5.3 band represented more than 75% of the whole molecule. When these bands were electrophoresed in a second dimension by SDS-PAGE, each of them rendered two bands corresponding to 17 and 19 K mol. wt.

To test whether Olea Antigen I was a major allergen of *O. europea*, a RAST inhibition assay was performed using commercial discs. As seen in Table 1, 1 μ g/ml of Olea Antigen I inhibited 14 out of 15 sera tested to more than 50%.

Purification of the 0.5 M fraction: properties of Olea Antigen II

As mentioned above, these fraction was obtained with 0.5 M ammonium bicarbonate by ion exchange chromatography of the crude extract. Figure 6(a) shows the profile obtained after chromatofocusing of the 0.5 M fraction. Two acidic peaks were eluted with

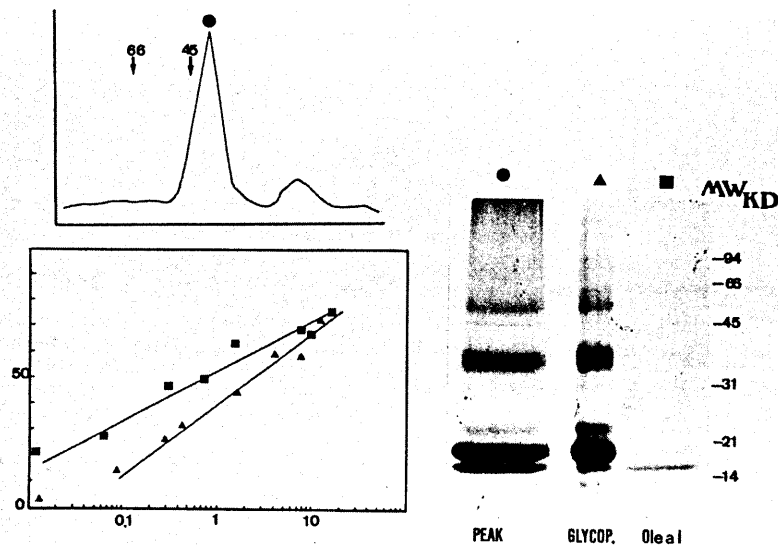


Fig. 4. Characterization of Olea Antigen I. (a) Gel filtration chromatography of 0.3 M fraction on a Sephadex G-100 column. (b) SDS-PAGE of the main peak (●) compared with its two components separated by affinity chromatography, Olea Antigen I (■) and glycoprotein (▲). (c) RAST inhibition test: serially diluted Olea Antigen I (■) and glycoprotein (▲) fractions were used as inhibitors.

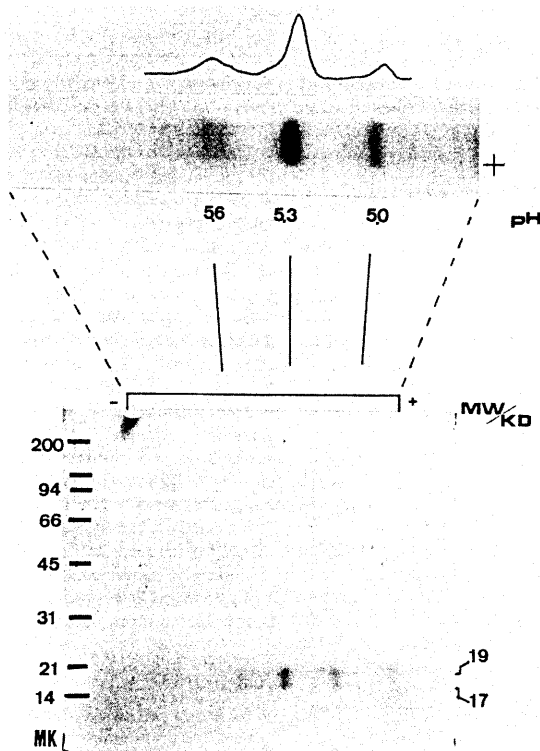


Fig. 5. Two-dimensional gel electrophoresis of ^{125}I -labeled Olea Antigen I. Top: IEF. Olea Antigen I was separated according to its isoelectric point in the first dimension. The densitometric analysis is shown over the gel picture. Bottom: second dimension SDS-PAGE. Molecular wt marker values are shown on the left.

Table 1. RAST inhibition by Olea Antigen I of 15 individual allergic sera from olive-sensitized patients

N Sera	Olea Antigen I	Control	% Inhibition
1	2147	11394	81.0
2	709	1652	57.0
3	1115	1963	43.0
4	805	2094	62.0
5	815	1878	67.0
6	1868	9093	80.0
7	1003	3222	69.0
8	708	2400	70.5
9	1143	3213	64.4
10	2009	5466	63.3
11	693	4839	85.7
12	560	5664	90.2
13	557	3800	85.7
14	704	3995	82.4
15	882	3500	75.0

One μg of Olea Antigen I was used on *O. europaea*-coated commercial discs from Pharmacia. Results are expressed in cpm. Control: results obtained using PBS instead of inhibitor.

$$\% \text{ Inhibition} = \frac{\text{cpm control} - \text{cpm problem}}{\text{cpm control}} \times 100$$

a polybuffer 74. The main peak, obtained in the 4.5 pH range was designated Olea Antigen II. The IgE binding capacity of Olea Antigen II was compared with that of the crude extract by solid-phase RIA using a pool of sensitized sera. As shown in Fig. 6(b), Olea Antigen II retains a high IgE binding ability at a protein concn of 5 $\mu\text{g}/\text{ml}$.

Figure 7 shows the results of SDS-PAGE and IEF analysis of Olea Antigen II. Gel filtration in non-denaturing conditions yielded one single peak of 10 K (data not shown). Similarly, the SDS-PAGE analysis rendered one single band of 8 K [Fig. 7(a)]. Three bands corresponding to pIs of 3.6, 3.8 and 4 were obtained by IEF [Fig. 7(b)]. The densitometric analysis demonstrated that the 3.6 band represents a major component of the whole molecule.

To test whether Olea Antigen II was a major allergen of *O. europaea*, a solid-phase RIA was per-

formed using individual sera from sensitized patients (Table 2). IgE binding activity was detected with the 12 sera used.

Reactivity of MAb OL-1 with Olea Antigen I and Olea Antigen II

Olea Antigen I and Olea Antigen II were both tested for binding with OL-1, a MAb anti-*O. europaea* previously reported by us (Lauzurica *et al.*, 1988). This MAb identifies the 17 and 19 K antigens from the crude extract. Serial dilutions of OL-1 supernatants were used in an ELISA analysis. As seen in Fig. 8, OL-1 reacted strongly with both Olea Antigen I and the crude extract (titre $>1/100,000$) whereas Olea Antigen II did not react.

DISCUSSION

The crude extract of *O. europaea* was fractionated by ion exchange chromatography. Two main protein bands of 17 and 19 K, were obtained using a buffer of both 0.3 and 0.1 M ammonium bicarbonate [Fig. 2(a)]. In addition, fractions eluted with 0.5 and 1 M ammonium bicarbonate retained most of the allergenic activity as measured by solid-phase RIA [Fig. 2(b)].

In a previous report, *O. europaea* crude extract was analysed by immunoblotting with individual allergic sera, and the results indicated that the most active fractions from the allergenic point of view were those of 17 and 19 K (Lauzurica *et al.*, 1988). However, these components were contained in the 0.3 M fraction which did not display the highest RAST activity. This suggested that (1) either the isolation procedures had affected the capacity of the 0.3 M fraction to bind IgE or else, (2) the coating of the antigen onto discs had interfered with such reactivity. To investigate these possibilities, the allergenic activity of the 0.3

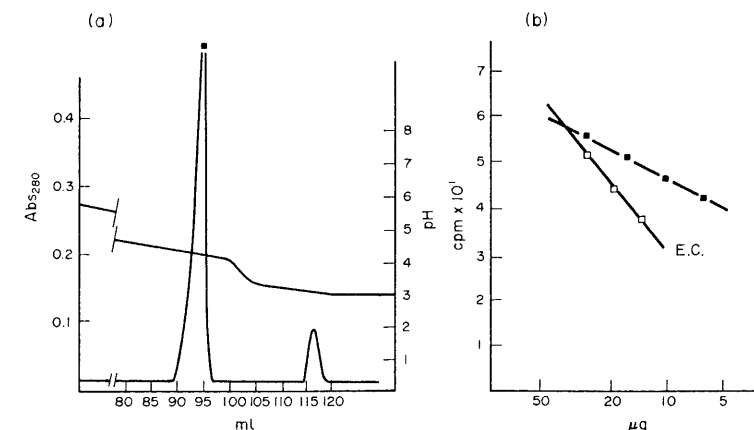


Fig. 6. Characterization of Olea Antigen II. (a) Chromatofocusing of the 0.5 M fraction in a pH range of 4-7 (b) Solid-phase radioimmunoassay of Olea Antigen II (■) compared with crude extract (□CE), using a pool of sensitive sera.

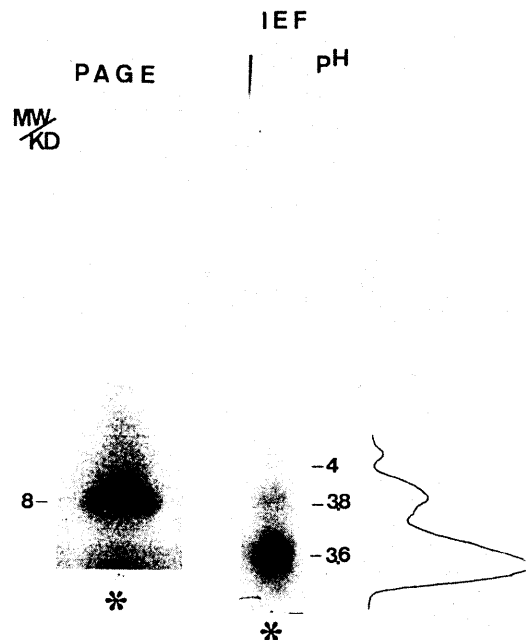


Fig. 7. (a) SDS-PAGE analysis of ^{125}I -labelled Olea Antigen II. (b) Analytical IEF of ^{125}I -labelled Olea Antigen II. The corresponding densitometric analysis is represented on the right.

and 0.5 M fractions were compared using RAST inhibition analysis. A high IgE binding activity of the 0.3 M fraction was observed by this procedure. The same results were obtained when Olea Antigen I, isolated from the 0.3 M fraction, was analysed. Therefore, the IgE binding epitopes of the 0.3 M fraction had not been denaturated by the isolation procedure since the IgE binding capacity of the 0.3 M fraction, by RAST inhibition assay, was retained.

The second possibility appears unlikely, since the same antigens, bound to the same solid matrix

in blotting experiments (Lauzurica *et al.*, 1988) yielded good positive results. Similar test-dependent differences in activity have been reported by other

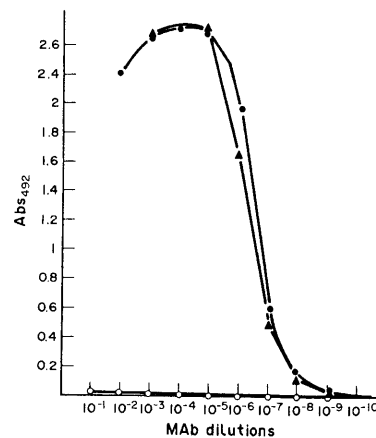


Fig. 8. Reactivity of MAb OL-1 with Olea Antigen I and Olea Antigen II by ELISA. Microtitre plate wells were coated with C.E. (▲), purified Olea Antigen I (●) and purified Olea Antigen II (○). Ten times serial dilutions of OL-1 supernatant were used.

Table 2. RAST activity by Olea Antigen II, bound to nitrocellulose matrix using individual allergic sera

N Sera	Olea Antigen II activity	RAST
1	1006	++++
2	434	++++
3	419	+++
4	392	+++
5	673	+++
6	186	+++
7	3122	+++
8	177	+++
9	180	++
10	159	++
11	449	++
12	1925	++

Results are expressed by ratio between cpm allergic sera and normal sera.

investigators (Vijay *et al.*, 1985; Olson and Klapper, 1986). These results emphasize the importance of the test chosen to characterize active allergen fractions.

Olea Antigen I is a protein that appears to be composed of two polypeptides of 17 and 19 K which can be isolated in a final step by lentil-lectin chromatography. Nevertheless these two polypeptides were also detected among the glycoproteins retained by the column. Since the affinity column was not saturated with protein, these results indicate that the 17 and 19 K polypeptides contained in Olea Antigen I are different from the 17 and 19 K peptides of the bound glycoprotein in that they do not have any attached sugar residues or that they contain different sugar residues (or different number of sugars attached to them). Attempts to identify sugar residues in Olea Antigen I protein are currently being carried out.

The IEF analysis of Olea Antigen I resulted in one main band of 5.3 and two less relevant of 5.6 and 5.0 pH units. This diversity was not observed in the 2-dimensional SDS-PAGE analysis since each of the three components yielded the 17 and 19 K polypeptides. This suggests that the different charged proteins are isoallergens representing a minor polymorphism of the same molecule.

The physicochemical characteristics of Olea Antigen II are quite different from those of Olea Antigen I. Olea Antigen II is a major allergen isolated from the 0.5 M fraction which gave a single band of 8 K when analysed in SDS-PAGE. A 10 K mol. wt was determined by gel filtration in non-denatured conditions. Similar discrepancies between these two methods have been described elsewhere (Sward-Nordmo *et al.*, 1985; Lambin, 1978). The IEF also demonstrated a polymorphism as shown by the presence of minor isoallergens.

A high RAST activity was also observed with Olea Antigen II protein, isolated from the 0.5 M fraction. Furthermore MAb OL-1, which reacted with Olea Antigen I, did not react with Olea Antigen II. Therefore at least two independent major allergens can be isolated from the *O. europaea* pollen.

There are few studies describing the isolation of *Olea europaea* antigens. Blanca *et al.* (1983), described an antigen by one single purification step, which could be related with Olea Antigen I. This antigen contains two non-covalently associated polypeptide chains of 15 and 17 K, with an isoelectric point of 6 pH units. Since Olea Antigen I consists of two dimers of 17 and 19 K, some relation may exist between the two antigens although the isoelectric point of Olea Antigen I is 5.3 pH units. Unfortunately, it has not been possible to test the antigen of these other workers with MAb OL-1.

Vela *et al.* (1982) obtained a 60 K fraction with allergenic activity by gel filtration of *O. europaea* crude extract on Sephadex G-100. In a recent study, an allergenic fraction of 55–60 K mol. wt range was obtained from crude extract by separation on a TSK

G3000 HPLC column. This material reacts with monoclonal OL-1 and rendered three bands of 17, 19 and 21 K (Wheeler *et al.*, 1987). This suggested further heterogeneity of allergenic components of *O. europaea*. However in view of our results we considered that this 60 K material might easily be formed by polymerization of 17 and 19 K monomers.

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