

Purification and fine characterization of a major allergen from *Olea europaea* pollen extract

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Olea europaea (olive) pollen extract was prepared by aqueous extraction and characterized by biochemical and immunochemical methods. Two components, displaying respective mol. wt. of 17000 and 19000, were the most reactive allergens, being the doublet (designated *Ole e I*) recognized by most sera tested. The 19000 mol. wt. component, purified by conventional biochemical procedure and lectin-affinity chromatography from the *Ole e I* doublet, was deglycosylated and analyzed by SDS-PAGE and by ELISA inhibition. The results obtained suggest that the 19000 mol. wt. component represents the glycosylated form of the 17000 component.

Key words: allergen purification; chemical deglycosylation; ELISA; IgE; *Olea europaea*; SDS-PAGE.

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Olea europaea (olive) is one of the most common pollen allergens in the Mediterranean area (3), and several papers concerning the characterization of the extract and the purification of the relevant allergens have been published so far (1, 2, 8, 9, 14–16). Laururica et al. (9) reported the purification and characterization of the two major IgE reactive components, one displaying in SDS-PAGE two bands with mol. wt. 19000 and 17000, respectively, and the other with mol. wt. 8000; moreover, they were able to identify a third component, with mol. wt. 42000, highly reactive with the sera of their patients.

As regards the 17000 and 19000 allergenic components, the same authors demonstrated that, although the two molecules share common epitopes recognized by a monoclonal antibody, they have different behavior in a Con (concanavalin) A binding test, thus suggesting a difference in their sugar moieties. Recently, other authors (16) have suggested that these two components are linked together to constitute the mol. wt. 42000 component already described.

In the present paper, we first evaluated the reactivity of 12 sera from patients sensitive to *Olea eu-*

ropaea obtained from southern Italy, confirming with these sera the characteristics of the main reactive components (8, 9). Once the major allergen had been identified, it was purified by biochemical methods, and the pure material was used to investigate the biochemical relationship between the 17000 and the 19000 mol. wt. components.

Material and methods

Preparation of the *Olea europaea* pollen extract. *Olea europaea* pollen was kindly supplied by Lofarma (Milan, Italy). The extract was prepared essentially as described by Giuliani et al. (6).

Human allergic sera. Twelve human sera were obtained from untreated patients sensitive to *Olea europaea* only, as proved by skin test and RAST, by testing the donors against a panel of the most relevant allergens (Graminaceae, Urticaceae, Compositae, mites, trees, etc.). Highly positive sera (RAST class 4) were either used individually or pooled, and they were stored at -20°C in small aliquots until used. The sera considered for the pool were all re-

active with the major allergen OLEA I (8) (*Ole e* I) and with many other allergenic components, as evaluated by immunoblotting.

SDS-PAGE and immunoblotting. SDS-PAGE was performed, according to the method of Laemmli (7), in a vertical slab gel BIO-RAD Minigel apparatus (BIO-RAD, Richmond, CA, USA), by using a 13% w/v polyacrylamide separating gel and 3% w/v stacking gel. *N,N'*-methylene bis acrylamide (0.8%) was used as a cross-linker. The *Olea europaea* pollen extract (20 µg/well) was reduced by 5% v/v 2-mercaptoethanol before application to the gel. The run was carried out in a refrigerated system at 8°C, and the gel was stained with 0.05% w/v Coomassie Brilliant Blue in water:methanol:acetic acid (50:40:10) and destained in the same solution.

The immunoblotting was performed according to the method of Towbin et al. (13) in a BIO-RAD apparatus. The relevant components were detected by overnight incubation with 2 ml undiluted patients' sera, followed, after five washings with PBS-Tween 20, 0.05% v/v, by ¹²⁵I-labeled rabbit antihuman IgE (RAST I, Pharmacia, Uppsala, Sweden) (approximately 500 000 cpm/strip) diluted in PBS-Tween 20, 0.05% v/v. After five further washings, the reactive bands were detected by 3 days' exposure at -80°C of the strips with radiographic film (Kodak Diagnostic Film X-Omat AR, Eastman Kodak Company, Rochester, NY, USA).

Protein assay. The protein assay was performed according to the method of Bradford (4).

Chemical deglycosylation. Deglycosylation of pollen extract was performed by the method of Edge et al. (5), by using trifluoromethanesulfonic acid (TFMS). This method was chosen on the basis of preliminary experiments performed with another allergenic extract obtained from *Parietaria judaica* pollen. Briefly, the extract was dissolved in a mixture of anisole and TFMS (1:2), precooled at 0°C, and flushed with nitrogen. After stirring the solution for 1 h, we precipitated the deglycosylated protein moieties by adding precooled diethyl ether containing 10% hexane. The precipitate was dissolved in a small amount of 1 mol Tris-HCl, pH 8.6, dialyzed against distilled water, and freeze-dried.

Chromatographic purification of *Olea europaea* major allergen (*Ole e* I)

Diethylaminoethyl cellulose (DEAE-cellulose) column chromatography. We applied 100 mg of *Olea europaea* pollen extract, dissolved in 10 ml 10 mmol ammonium bicarbonate, pH 8, and dialyzed against it, to a 2 × 20-cm DEAE-cellulose (Whatman, UK)

column, previously equilibrated in the same buffer. The column was run at a flow rate of 40 ml/h, and eluted step by step with 10, 50, 300, and 500 mmol ammonium bicarbonate, pH 8, under continuous monitoring at 280 nm. All the components still absorbed after the last buffer were washed away with 200 mmol monobasic phosphate solution, pH 4.3. The content of the tubes corresponding to the peaks detected was pooled, dialyzed against water, and lyophilized. The freeze-dried material was analyzed by SDS-PAGE, and the pool displaying the strongest band of major allergen and available in the largest amount (identified as fraction III) was further purified. Although this fraction does not represent the fraction with less impurities, it was chosen for the purification procedure because of the high recovery obtained, as compared with other fractions displaying higher purity of the major allergen.

Carboxymethyl-cellulose (CM-cellulose) column chromatography. In order to purify further the major allergen obtained by DEAE-cellulose chromatography, we subjected fraction III to CM-cellulose column chromatography. Of this fraction, 16.7 mg, equilibrated in 50 mmol acetate buffer, pH 4.3, was applied to a 2 × 15 ml CM-cellulose (Whatman, UK) column, run at a flow rate of 40 ml/h, and eluted step by step with 50, 100, and 200 mmol acetate buffer, pH 4.3. The content of tubes corresponding to the peaks detected was pooled, dialyzed against 1 mmol ammonium bicarbonate, pH 8, and lyophilized. The freeze-dried material was further analyzed by SDS-PAGE, and the pools displaying the strongest band of pure major allergen (fractions IV, V, and VI) were pooled together and designated pool (IV, V, VI).

Lectin-affinity chromatography. In order to separate the 19 000 mol. wt. component from the 17 000 one, the CM-cellulose pool (IV, V, VI) was subjected to Con A column chromatography. Thus, 5 mg of pool was dissolved in 1 ml 20 mmol Tris-HCl, 0.5 mol NaCl, pH 7.4, and passed through 2 ml of Con A-Sepharose column. The effluent was collected, and the column was eluted with 0.5 mol glucose dissolved in the starting buffer. Effluent and eluate were dialyzed against water and lyophilized. The freeze-dried material was analyzed by SDS-PAGE.

Enzyme-linked immunosorbent assay (ELISA) inhibition. ELISA inhibition was performed according to the method of Mucci et al. (11). Briefly, PVC plates (Falcon, 3912, Becton Dickinson, Oxnard, CA, USA) were coated with 0.2 ml 20 µg/ml of the purified 19 000 mol. wt. component in 0.05 mol carbonate buffer, pH 9.6, eluted from the Con A-Sepharose column. Inhibition of specific IgE was carried out by incubating the pooled sera, prediluted

1:5, for 3 h with twofold serial dilutions of inhibitors at a concentration ranging from 5 µg/ml protein/ml to 0.06 µg/ml for the 19000 and the 17000 mol. wt. components, and to 0.1 µg/ml for the deglycosylated 19000 mol. wt. component. The mixture was then transferred to the plate and further incubated for 3 h at room temperature, followed, after washings, by an overnight incubation with a peroxidase-labeled, rabbit antihuman IgE (KPL, Gaithersburg, MD, USA) (1:2000). The plate was developed for 30 min by adding 0.2 ml of *ortho*-phenyl-diamine solution (0.5 mg/ml in 0.1 mol citrate/0.2 mol phosphate buffer, pH 7.2, containing 0.01% hydrogen peroxide). The reaction was stopped by adding 50 µl 0.5 N sulfuric acid, and the plates were read at 495 nm in a BIO-RAD microplate reader Mod. 3550 (BIO-RAD, Richmond, CA, USA). Data were expressed in terms of the protein concentration of each inhibitor producing 50% inhibition.

Results

The SDS-PAGE analysis in reducing conditions of the *Olea europaea* pollen extract (Fig. 1, lane A) showed the presence of many components, displaying mol. wt. ranging from a few thousands to 100 000. Several of these components (14 000–70 000) were reactive with the IgE of the sera obtained from 12 patients sensitive to olive, a major allergen represented by a doublet displaying mol. wt. 17 000–19 000, hereafter designated *Ole e* I (10). The results obtained with these sera and with one negative control are shown in Fig. 1, lanes 1–13.

In order to investigate any possible relationship between the 17 000 and the 19 000 mol. wt. components, we subjected the native extract to the deglycosylating procedure with TFMS and analysis by SDS-PAGE and Coomassie staining. The results

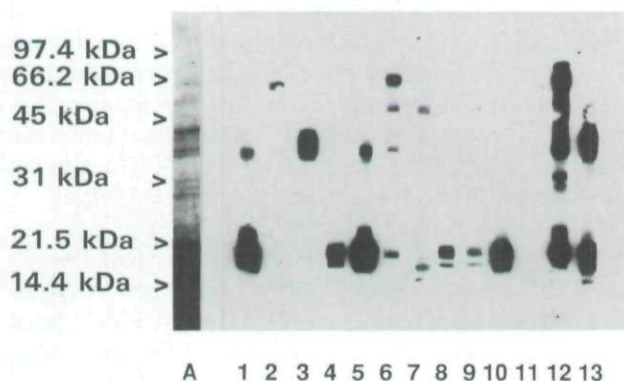


Fig. 1. SDS-PAGE in reducing conditions of *Olea europaea* pollen extract stained with Coomassie Brilliant Blue (lane A) and immunoblotting performed with sera from 12 patients sensitive to *Olea europaea* pollen only (lanes 1–10, 12–13) and one normal subject (lane 11).

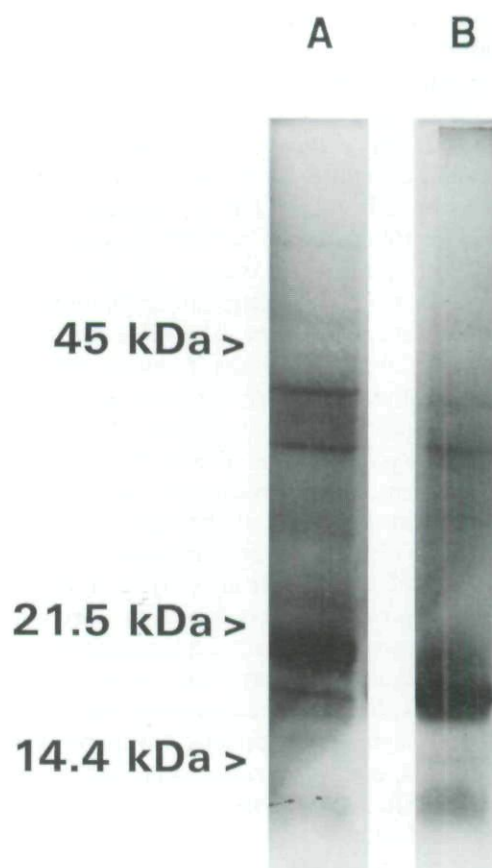


Fig. 2. SDS-PAGE of native (lane A) and deglycosylated (lane B) extract.

(Fig. 2) clearly show a dramatic decrease of the 19 000 mol. wt. band and a corresponding increase of the 17 000, together with other minor changes at the level of the other bands.

These findings suggested that the 19 000 component could represent the glycosylated form of the 17 000 component. Therefore, in order to test this hypothesis, we purified the 19 000–17 000 mol. wt. doublet, as described in Material and Methods. The pools from the DEAE column were tested by SDS-PAGE (Fig. 3), and pools III, IV, and V, eluted from the column at 300 mmol ammonium bicarbonate, contained the highest amount of the 19 000–17 000 mol. wt. allergen. Interestingly, by comparing the patterns in lanes B and C (Fig. 3), one can see that the 19 000 band probably consists of two components displaying very similar mol. wt., eluted in slightly different conditions from the column, and indistinguishable in the whole extract (lane A). Fraction III (lane D), obtained from the DEAE-cellulose column, was chosen and submitted to a further chromatographic step on a CM-cellulose column, in acetate buffer, using a step-by-step gradient from 50 to 200 mmol. The 19 000–17 000 mol. wt. doublet was obtained, almost purified, in the three fractions eluted

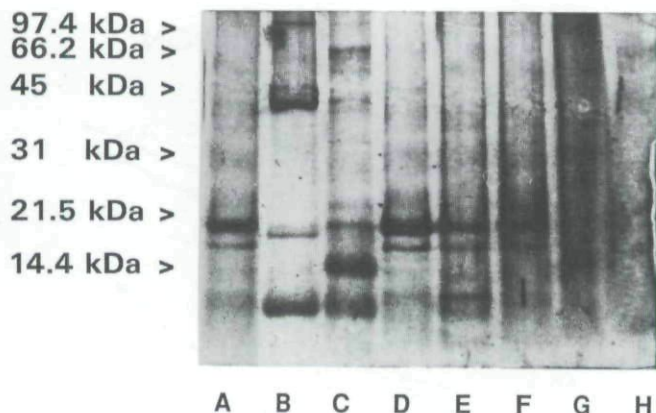


Fig. 3. SDS-PAGE of *Olea europaea* whole extract (lane A) and DEAE-cellulose fractions (lanes B-H). Column was run by step-by-step increase of ammonium bicarbonate concentration. Lane B, fraction I (10 mmol); lane C, fraction II (50 mmol); lane D, fraction III (300 mmol); lane E, fraction IV (300 mmol); lane F, fraction V (300 mmol); lane G, fraction VI (500 mmol); lane H, fraction VII (200 mmol KH_2PO_4). The gel was stained with Coomassie brilliant blue.

with 100 mmol acetate buffer (Fig. 4). Since no differences were demonstrable between the various pools (lanes E, F, and G), they were further pooled together; the SDS-PAGE pattern of the pool is represented in lane H. No material was eluted at 200 mmol, and therefore the fraction was not further analyzed.

In order to separate the 17 000 and the 19 000 mol. wt. components, on the basis of preliminary experiments performed on the whole *Olea europaea* pollen extract, we further subjected the purified major allergen *Ole e* I to an affinity chromatography pro-

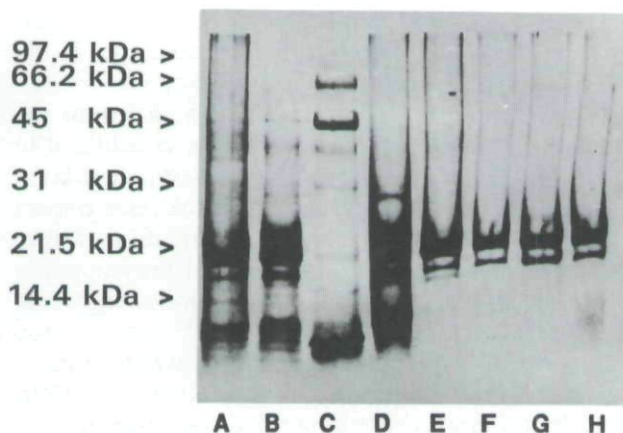


Fig. 4. SDS-PAGE of *Olea europaea* whole extract (lane A), DEAE-cellulose fraction III (lane B) and CM-cellulose fractions (lanes C-G). Column was run by step-by-step increase of acetate buffer concentration. Lane C, fraction I (50 mmol); lane D, fraction III (100 mmol); lane E, fraction IV (100 mmol); lane F, fraction V (100 mmol); lane G, fraction VI (100 ml). Lane H, pooled fractions IV, V, VI (pool IV, V, VI). The gel was stained with Coomassie brilliant blue.

cedure on Con A-Sepharose. The effluent and the eluate were collected and analyzed in SDS-PAGE; the results indicate (Fig. 5) that only the 19 000 mol. wt. component was able to bind the lectin. The 17 000 mol. wt. component was recovered in the effluent, together with several degradation products and another component displaying a mol. wt. very close to 19 000. The presence of this latter component, displaying mol. wt. slightly higher than 19 000, was not due to overloading of the Con A column, this possibility being ruled out by the fact that a second effluent, obtained by applying the first Con A effluent over the regenerated lectin column, still displayed this near 19 000 component (data not shown). Thus, these data confirm the presence, in the 19 000 region, of at least two closely related components with slightly different mol. wt. Moreover, Fig. 5 (lane C) also shows that pool (IV, V, VI), which was obtained from the CM-cellulose column, displaying within a short time after its preparation the doublet of the major allergen only (Fig. 4, lane H), was able to show (upon storage) a high mol. wt. component together with some degradation products with low mol. wt. Moreover, this component seemed to resemble the Con A behavior of the 19 000 mol. wt. component, since it bound to the column (Fig. 5, lane A), and therefore it could be considered to be an aggregation product.

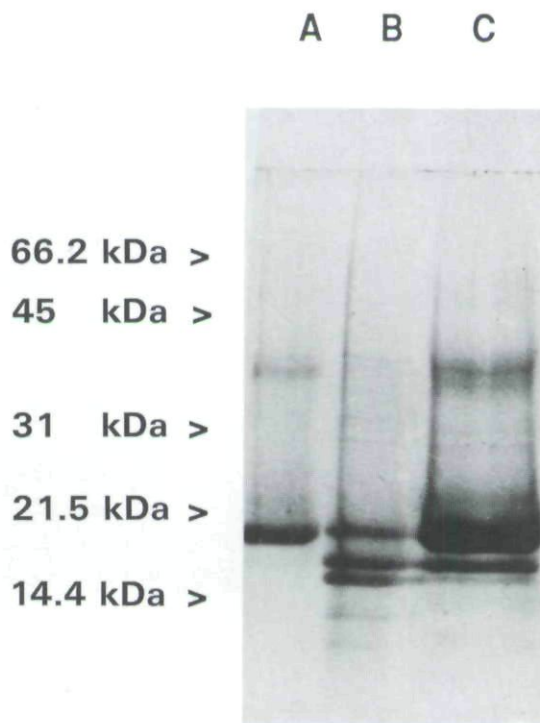


Fig. 5. SDS-PAGE of fractions obtained by concanavalin A affinity chromatography. Lane A, eluate from Con A column; lane B, effluent from Con A column; lane C, pool IV, V, VI. The gel was stained with Coomassie brilliant blue.

In order to investigate whether the 19000 mol. wt. component of the doublet *Ole e I* could be transformed into the 17000 component upon sugar removal – as suggested by the results obtained with the crude extract (Fig. 2) – the purified 19000 component (Con A eluate) was treated with TFMS acid. The results (Fig. 6) showed that the purified 19000 component was converted, after the deglycosylation, into the 17000 mol. wt. band. Interestingly, we noted that upon storage at -20°C , the Con A eluate showed aggregates (Fig. 6, lane C), displaying mol. wt. different from that of the aggregates detectable in the same sample immediately after the affinity chromatography (Fig. 5, lane A).

The finding that sugar removal produced a shift in the electrophoretic mobility of the 19000 mol. wt. component, which then displayed an apparent mol. wt. of 17000, cannot, however, be regarded as a clear-cut demonstration of the identity of the protein structure of these two molecules. In order to gain further information on this aspect, we tested the 19000, the deglycosylated 19000, and the 17000 mol. wt. components in the ELISA inhibition. Results (Fig. 7) suggested that the purified 19000 mol. wt. component is as good as the 17000 component in inhibiting the binding of human IgE to the 19000 mol. wt. component adsorbed on the plate, leading to the

ELISA INHIBITION

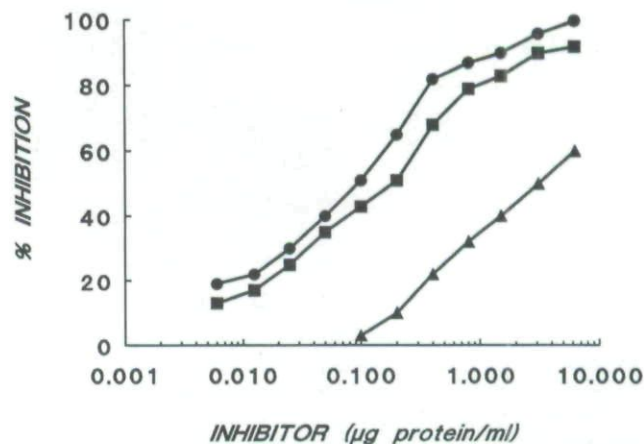


Fig. 7. ELISA inhibition. Binding of human IgE to native 19000 mol. wt. component was inhibited by 17000 (■), 19000 (●), and deglycosylated 19000 mol. wt. (▲) components.

conclusion that many epitopes recognized by the polyclonal human reagent are shared by the two components. On the other hand, it is also evident that the capacity of the 19000 mol. wt. component to inhibit IgE binding is reduced upon deglycosylation.

Discussion

In recent years, many allergens from different sources have been investigated, purified, and characterized biochemically and immunochemically. Among these, allergens from pollen have been extensively studied, with special attention to those causing the highest number of allergologic problems, such as *Ambrosia* spp. in the United States and *Parietaria* spp. in the Mediterranean area.

Recently, more attention has been given to the pollen of *Olea europaea*, which is now considered to be among the most important causes of pollinosis in the Mediterranean area. Therefore, several papers have been published on the characterization of olive pollen extract (1, 2, 8, 9, 14–16). In the present paper a further characterization of the major allergen *Ole e I* is presented.

The SDS-PAGE analysis of the whole extract, performed in reducing conditions, followed by Coomassie staining, clearly confirmed the complexity of the preparation, which contained several major components whose mol. wt. ranged from a few thousands to about 100 000. Among many allergenic components detected by immunoblotting performed with human sera, we confirmed that the major allergen *Ole e I* is composed of two closely related components, displaying, respectively, mol. wt. of

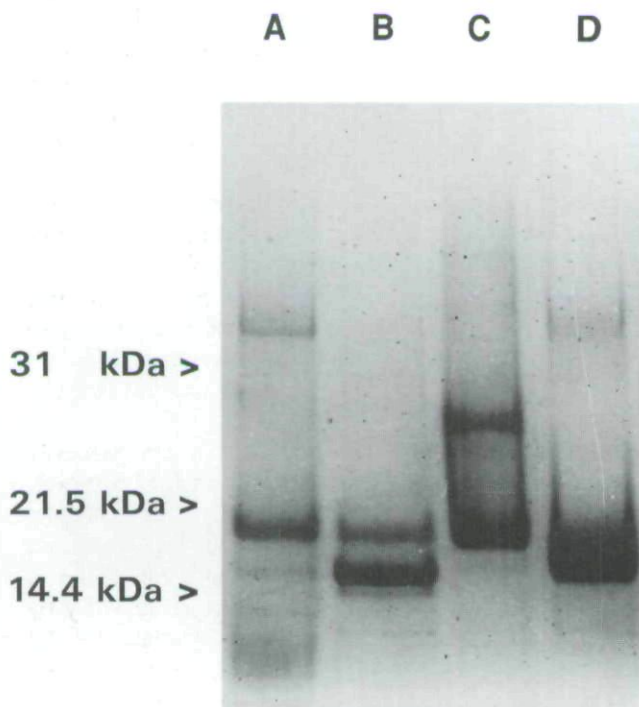


Fig. 6. SDS-PAGE of concanavalin A affinity chromatography eluate before and after deglycosylation. Lane A, pool IV, V, VI; lane B, effluent; lane C, eluate; lane D, chemically deglycosylated eluate. The gel was stained with Coomassie Brilliant Blue.

17000 and 19000, both reactive with almost all the sera tested. The most reactive component of *Olea europaea* pollen extract was first identified by Vela et al. (14), who reported that the major allergens, purified and analyzed by gel permeation chromatography, had, respectively, mol. wt. of 165000 and 65000. Different results were published by Blanca et al. (2), who were able to purify by isoelectric focusing and to characterize by several techniques a major allergen, represented, in SDS-PAGE and reducing conditions, by two bands displaying, respectively, mol. wt. of 15000 and 17000. The discrepancy between the two results was explained by the last-named authors by considering the high-mol.-wt. allergens to be aggregates of acidic and basic proteins. However, papers published by Lauzurica et al. (8, 9) and more recently by Barber et al. (1) and Wheeler et al. (16) have confirmed that when the mol. wt. determination was carried out in reducing conditions by SDS-PAGE, the major allergen was represented by a doublet whose components had, respectively, mol. wt. close to 17000 and 19000, values that are very close to those obtained, by a similar technique, by Blanca et al. (2).

Our data indicated that the removal of the sugars from the whole extract by chemical deglycosylation modified the SDS-PAGE pattern of the extract, the most interesting finding being the almost complete disappearance, upon deglycosylation, of the 19000 mol. wt. band, with the simultaneous increasing of intensity of the 17000 band. Therefore, in order to investigate this aspect further, we separated by lectin-affinity chromatography the two components of the glycosylated purified major allergen. The deglycosylation of the 19000 mol. wt. band produced the 17000 mol. wt. component, thus suggesting that the two bands are essentially the same molecule with a different degree of glycosylation. When these two components were tested by ELISA inhibition, the results obtained indicate that they largely share common epitopes, some of them being destroyed upon deglycosylation. Although the obvious explanation of these data is that some of the IgE-binding epitopes are of glycidic nature, several other interpretations cannot be excluded. One is that the acidic conditions and the presence of anisole are, per se, able to denature some epitopes; to disprove this hypothesis, we tried to perform some controls, such as incubation with anisole only and with acids. As regards the former control, we were not able to demonstrate any effect on the extract (data not shown); as regards the latter point, we were not able to set up a good control for the acidic conditions produced by the TFMS, and no data are available from the literature to suggest such a peculiar control (12). It has to be considered that this procedure has been already applied to allergens by other authors (11, 12) and has yielded

no strong denaturing effects on the molecules investigated.

Another possible explanation is based on the fact that sugar removal can induce changes in the conformation of the allergen with a subsequent impairment of the IgE binding caused by an indirect mechanism.

The possibility that the presence of the two components was related to a different degree of glycosylation of the same molecule was first considered by Lauzurica et al. (9), who demonstrated a different behavior of the two components with Con A and lentil lectin, the former being able to bind the 19000 mol. wt. component only and the latter able to bind both the 19000 and the 17000 components. Moreover, they were also able to produce a monoclonal antibody recognizing both the bands; this indicates the presence of at least a common epitope. Altogether, these data suggest that the 19000 component is clearly glycosylated, while the 17000 component bears only a few sugar residues, whose presence has been demonstrated by the binding of the component with lentil lectin and whose removal by chemical deglycosylation does not modify their apparent mol. wt. in SDS-PAGE.

Another interesting finding reported in the present paper is that the SDS-PAGE analysis of the samples separated by the affinity chromatography procedure could suggest a certain degree of heterogeneity of the 19000 mol. wt. component. In fact, this component can be sometimes split, by SDS-PAGE, into two very closely related bands, generally indistinguishable; therefore, we have considered the possibility that the 19000 mol. wt. band was not due to one component only, but to at least two components with very similar mol. wt. This hypothesis was confirmed by the presence of a component which bound to and could be eluted from the Con A affinity column, and of another one, with mol. wt. slightly higher, which could be recovered in the effluent and whose mol. wt. did not change upon deglycosylation, thus suggesting that two components, different at least in sugar moieties, were present in the 19000 mol. wt. region.

Finally, our data suggest that the two components of the pure major allergen (19000 and 17000 mol. wt.) from *Olea europaea* can produce – for example, upon storage or lyophilization – either high-mol.-wt. aggregates (about 40000), confirming the hypothesis suggested by Blanca et al. (2) and more recently by Wheeler et al. (16), or low-mol.-wt. degradates (8000; Lauzurica et al. (9)). These findings suggest that part of the heterogeneity and complexity of *Olea europaea* pollen extract, as reported in the literature (1), is due to either aggregation or degradation processes occurring in the preparation and during the storage of the extracts.

Acknowledgments

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