

Allergenic diversity of the olive pollen

A great number of allergenic proteins have been detected in olive pollen extracts. To date, nine allergens have been isolated and characterized, which have been called Ole e 1 to Ole e 9. The most prevalent olive allergen is Ole e 1, which affects more than 70% of patients hypersensitive to olive pollen, but others, such as Ole e 2, Ole e 8, and Ole e 9, have been demonstrated to be major allergens, and Ole e 6 or Ole e 7 reach high values of clinical incidence. Many of these allergens, such as Ole e 2 (profilin) and Ole e 3 (polcalcin), are involved in cross-reactivities, which agrees with their adscription to panallergenic families. Among the many olive allergens of high molecular mass, only Ole e 9 (46 kDa) has been characterized. The allergen is a polymorphic and glycosylated β -1,3-glucanase, which belongs to a pathogenesis-related (PR-2) protein family. In addition to the polypeptide epitopes, Ole e 1 also exhibits IgE-binding determinants in the carbohydrate, which are recognized by more than 60% of the sera from patients sensitive to the whole allergen, although the level of such glycan-specific IgE seems not to be clinically relevant in the overall content of the sera. Recent advances in the elucidation of the structure of the Ole e 1-oligosaccharide component allows us to explain the antigenicity of the molecule. Finally, the recombinant production of several allergens from olive pollen in both bacterial and eukaryotic cells has allowed us to resolve problems derived from the polymorphism and scarcity of the natural forms of these allergens. The biological equivalence between the natural and recombinant forms lets us initiate studies on the design of mixtures for clinical purposes, in which hypoallergenic derivatives of these allergens could play a definitive role.

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Allergenic diversity of olive pollen proteins

In the early studies on olive allergy, a high number of allergenic components were detected by SDS-PAGE and immunoblotting of the pollen extract. From these assays, protein bands of 17, 19, 42, 32, and 65 kDa were reported by different authors (1–4). Afterwards, a component of 20 kDa, which was called Ole e 1, was recognized for many years as the main allergen from olive pollen (5–10). Ole e 1 is the most abundant protein of olive pollen extracts, representing up to 20% of the total protein content and exhibiting a prevalence ranging from 55 to 85%. In the second half of the 1990s, other allergens with different clinical significance were identified and characterized, and were named Ole e 2 to Ole e 8 (11–17). Recently, the existence of two highly significant allergens of 36 and 45 kDa has been reported (18,19) and, latterly, the isolation and characterization of Ole e 9 (46 kDa) have been achieved (20).

As already mentioned, Ole e 1 has been recognized for many years as the most important allergen from olive pollen. It is a glycoprotein of 145 amino acids, which exhibits in SDS-PAGE a pattern of multiple bands, because it is present as two main forms: glycosylated (20 kDa of apparent molecular mass) and non-glycosylated (18.5 kDa). Minor variants at 22 kDa (hyperglycosylated component) and 40 kDa (the dimer of the glycosylated form) are frequently present in preparations of Ole e 1 (9,10,21). The allergen belongs to a family of proteins, which has been suggested to be related to pollen germination, tube growth, and/or pollen hydration (22,23). The amino acid sequences of several members from species unrelated to olive pollen are known so far: tomato, maize, ryegrass, rice, birch, *Arabidopsis thaliana*, *Sambucus nigra*, and *Phalaris coarulescens*. They exhibit a sequence identity of 24–34%, indicating a low similarity in the primary structure between taxonomically

unrelated counterparts. Ole e 1-like proteins that display high similarity (>85%) have also been isolated and characterized from lilac and privet (24,25), as well as being identified in ash and forsythia (26,27); all these species belong to the *Oleaceae* family. Three Ole e 1-like proteins were reported as IgE-reactive proteins: Lol p 11 from ryegrass (28) and its relatives from lilac and privet, Lig v 1 and Syr v 1 (24,25). Lol p 11 did not cross-react with Ole e 1 (unpublished results) but a strong cross-reactivity has been detected among members of the *Oleaceae* family (24–27).

Cross-reactive allergens have been identified in olive pollen. In fact, profilin (Ole e 2), polcalcin (Ole e 3), and a lipid transfer protein (LTP, Ole e 7) have been found to be remarkable allergenic components of this pollen (11,15,16). These families of proteins have been established as pan-allergens, because of their wide presence in animal and vegetable allergenic sources, and high degree of similarity (29–33). Strong cross-reactivity of olive profilin and polcalcin with their corresponding counterparts from the main allergenic pollen species has been demonstrated (11,15,34). Both families of proteins display highly conserved amino acid sequences that account for the presence of common antigenic determinants responsible for such cross-reactivities. No data on cross-reactivity are available for Ole e 7, but the existence of identity around 30–50% between the olive LTP and those from peach, apple, tobacco, sunflower, and artemisia (unpublished results) allow us to speculate on the existence of shared IgE epitopes. The presence of at least two highly cross-reactive allergens – Ole e 2 and Ole e 3 – in olive pollen could explain the low frequency of patients monosensitized to this pollen.

Olive pollen also contains allergens for which no cross-reactivities have been investigated. Some of them have been identified as belonging to known families (Ole e 4 is a superoxide dismutase, Ole e 8 is a novel four EF-hand Ca^{2+} -binding protein), but no members of these families have been reported as allergenic components in other pollens. Interestingly, a four EF-hand Ca^{2+} -binding protein, i.e. Jun o 2, has been reported as a major allergen from *Juniperus oxicedrus* (35), but its amino acid sequence does not exhibit similarity to that of Ole e 8 out of the EF-hand sites (17). Finally, Ole e 5 and Ole e 6 are allergens without homologous counterparts in other sources appearing in the sequence databases. Cross-reactivity of these allergens needs to be analysed in the future.

All the olive pollen allergens fit well with the solubility requirements assessed for standard

allergens. Most of the characterized olive allergens are actually released after very short periods of pollen hydration, because high yields of Ole e 1, Ole e 6, and Ole e 7 are obtained after 15 min of maintaining the pollen in suspension in mild saline buffers, and many others are completely extracted after 2 h. This property facilitates the accessibility of the proteins once the pollen particle has reached the epithelial cells of mucosae.

An informative analysis on the specific IgE responses of olive allergic individuals can be obtained from immunoblotting of the crude pollen extract. Most of the patients with high values of RAST (between 4 and 6) exhibit complex allergograms (Fig. 1), which show one or two bands around 10 kDa, the multiple pattern of Ole e 1 (bands at 18.5, 20, and 22 kDa), and two or three protein components between 35 and 60 kDa. It is notable that protein bands at 9–11 kDa would include Ole e 3, Ole e 6 and Ole e 7, and the profilin reactivity is frequently missing when using this technique. From this analysis, it can be deduced that a high percentage of patients with high levels of olive-specific IgE titres are sensitive to at least 4–5 allergens; data that should be taken into account for designing protocols for specific immunotherapy.

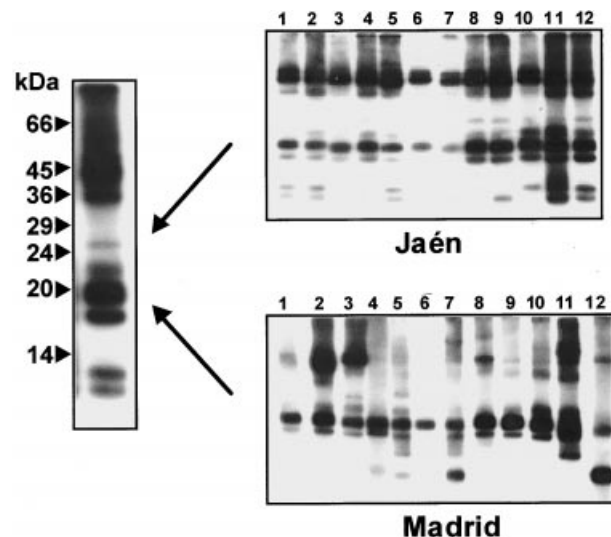


Figure 1. IgE recognition of proteins from olive pollen extract by sera from allergic patients. Saline-soluble proteins from olive pollen were electrophorized in SDS-PAGE and transferred to membranes. Afterwards, they were immunostained with sera from patients from two different areas of Spain: Jaén and Madrid. All the patients had 5–6 RAST values (by CAP). The separate panel (on the left) represents the immunostaining of the pollen extract with a pool of sera from olive allergic patients.

Major and minor allergens

Several olive proteins have been reported as major allergens, because a prevalence higher than 50% has been determined for them. In fact, Ole e 1, Ole e 4, and Ole e 9 exhibited, respectively, 55–90%, 80%, and 5%. However, we already know that prevalence data are strongly dependent on several conditions: population, technique, and pollen extract source used in these studies, among others. Interestingly, we found that the geographic area of the population in which prevalence is analysed constitutes a determinant factor for the results (36). As an example, notable differences were observed for Ole e 1 and Ole e 6 prevalences in populations living in areas with high olive pollen grain counts and those with lower values (13,36,37). In addition, results depended on the method utilized for the analysis, because conformational epitopes are usually lost when denaturing agents are used: in the earliest assays, Ole e 2 was shown to be recognized in immunoblotting by 24% of the olive allergic sera, whereas this value was increased to up to 50% when the assay was performed in enzyme-linked immunoassay (15). Finally, the marked variability that has been shown for the allergenic components of different Olea cultivars (38,39) could lead to ambiguity in the IgE-binding responses detected for the allergens insufficiently represented in some pollen extracts. Therefore, data on the prevalence of olive allergens are, to our understanding, preliminary and they should be revisited for all the known allergens under identical conditions, using different methods and with different and broad allergic populations.

Novel allergens in olive pollen

The allergenic pattern shown in Fig. 1 can be better resolved by using SDS-PAGE plus isoelectrofocussing (IEF), because it allows us to separate by charge many proteins that share similar molecular masses. Figure 2 shows the bidimensional pattern of the crude extract immunostained with a pool of sera from patients allergic to olive pollen. Ole e 1 appears as many spots of different pI values (5.5, 5.9, and 6.6) but identical molecular mass, which is in agreement with the polymorphic character of the protein. In the high molecular mass region, several proteins are well separated, with some of them exhibiting an acidic character (pIs between 4.5 and 6.8) and others with pIs around 7.3. It cannot be discounted that several spots belong to the same allergen, as a consequence of

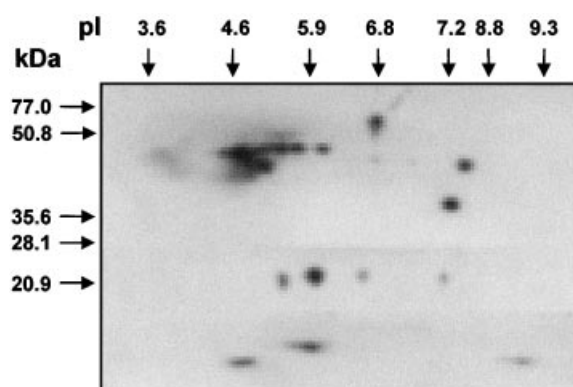


Figure 2. Two-dimensional map of the allergenic pattern of olive pollen extract. Separation by electrofocusing plus SDS-PAGE, and further immunostaining with a pool of sera from patients allergic to olive pollen. Isoelectric point (pI) and molecular mass (kDa) values of standard proteins are indicated.

its polymorphic character. Most of these allergens of high molecular mass have been neither isolated nor characterized, and their prevalence is still to be analysed.

Ole e 9: a new β -1,3-glucanase

The most recently discovered and purified olive allergen is Ole e 9 (20). It is also the allergen of highest molecular mass (46.4 kDa) reported so far from this pollen. Ole e 9 is a glycoprotein that exhibits a low but significant polymorphism: it can be separated by reverse-phase high performance liquid chromatography (RP-HPLC) as two different forms, and by IEF as four components with pI values 4.8, 4.9, 5.1, and 5.4. The cDNA encoding this allergen possesses an open reading frame of 460 amino acids, containing a putative signal peptide of 26 residues, and two potential N-glycosylation sites at positions Asn355 and Asn447. The mature protein consists of a single polypeptide chain of around 234 amino acids and at least one of the putative N-glycosylation locations should be linked to carbohydrate, because the allergen shows a positive reaction to concanavalin A lectin.

The amino acid sequence of Ole e 9 is well aligned to different plant β -1,3-glucanases, so it should belong to this 'pathogenesis-related' (PR-2) protein family (20) (Fig. 3). However, the olive protein exhibits a low amino acid sequence similarity to the most closely related known enzymes, i.e. those from wheat, willow, and *Arabidopsis thaliana*, because the sequence identity was from

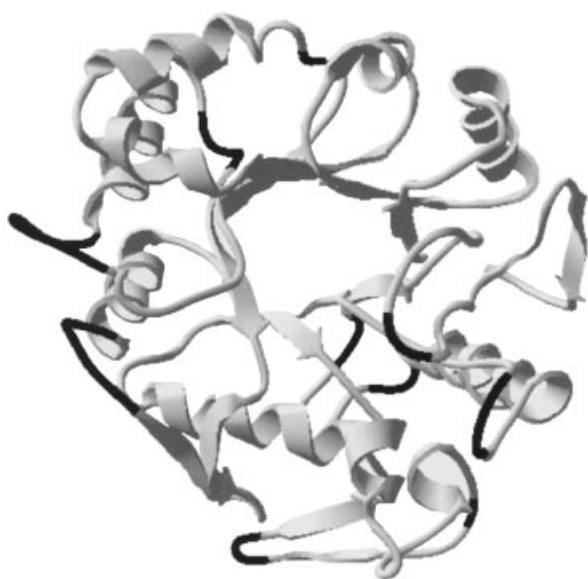


Figure 3. Modelling of the three-dimensional structure of the 323 amino acid residues of the N-terminal end of Ole e 9.

39 to 32%. This fact suggests that Ole e 9 could represent a new subfamily of β -1,3-glucanases. Ole e 9 was found to exhibit β -1,3-glucanase activity, using laminarin as a substrate, which confirms its preliminary inclusion within such a family of enzymes.

Ole e 9 has been recognized as a relevant allergen, because it is able to bind to the IgE from 65% of the sera of patients allergic to olive pollen extracts (20). This also indicates that it can be considered to be a major allergen, with a value of prevalence near to that of Ole e 1. Northern-blot analysis of total RNA obtained from several tissues (leaf, fruit, steam, and pollen) showed the specific presence of Ole e 9 transcript in the pollen. The same technique was used to detect the biological expression of mRNA corresponding to proteins homologous to Ole e 9 in different allergenic pollens. Hybridizing bands were only obtained for the pollen from species closely related to olive, indicating a low similarity of the amino acid sequences of β -1,3-glucanase-homologous proteins. It is interesting to note that β -1,3-glucanases are widespread enzymes of the vegetable kingdom, and could constitute a new source of pan-allergens, both in pollens and plant foods. Furthermore, they have been found to be allergens in latex (40), and a high content of β -1,3-glucanase has been isolated from the pulp of banana ripens (41). If these enzymes are involved in cross-reactivity processes remains to be investigated.

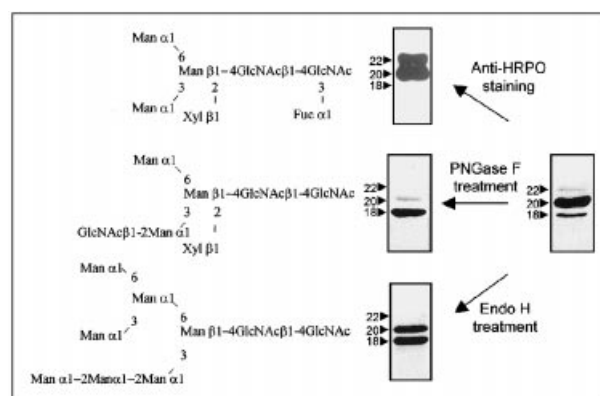


Figure 4. Structures of the main components of the glycan from Ole e 1: complex-, hybrid-, and mannose-rich-type, respectively, from top to bottom. The presence of these carbohydrates agrees with the results obtained from the corresponding treatments described in the right-hand side of the figure. Anti-HRPO, antihorse radish peroxidase antibody; PNGase F, peptide-*N*-glycosidase F; Endo H, endo- β -*N*-acetylglucosaminidase.

The carbohydrate moiety of Ole e 1: structural and immunological studies

An interesting aspect in the study of olive pollen allergens is the possible involvement of carbohydrate in the IgE response, mainly taking into account that the most prevalent allergen, i.e. Ole e 1, is a glycosylated protein. In fact, Ole e 1 exists as two variants, i.e. glycosylated and non-glycosylated, exhibiting a single N-glycosylation site located at Asn111 (42). A preliminary analysis of the N-glycan of Ole e 1 was obtained by specific enzymatic deglycosylation. SDS-PAGE analysis showed that Ole e 1 was sensitive – albeit in varying proportions – to both endo H and PNGase F deglycosidases (Fig. 4). While endo H released about 45% of the N-glycan from the glycoprotein, PNGase F removed nearly all the carbohydrate (90–95%) (43). It is known that endo H releases high mannose and/or hybrid N-glycans types (44), while PNGase F also cleaves complex-type chains but not glycans with a fucose in α -1,3-linkage to the terminal GlcNAc of the core (45). From these results and the specificity of these enzymes, it was concluded that Ole e 1 contained both N-glycans of the high mannose- and complex-type in a very similar ratio, with a minor glycoform of the latter carrying fucose α -1,3-linked to the innermost GlcNAc. This glycoform was also detected by antihorseradish peroxidase antiserum (46) and by antifucose antibody (47). However, glycosyl derivatives have been described for allergens such as Api m 1 (phospholipase A2) from bee venom (48), Art v 2

from mugwort (49), Cry j 1 from Japanese cedar (50), BG60 from Bermuda grass (51), Ara h 1 from peanuts and Lol p 11 from ryegrass (47,52).

Although the role of carbohydrate in allergens has been controversial, its involvement in antigenicity has been repeatedly demonstrated. Preliminary analyses on the role of Ole e 1 glycan in the allergenicity of the molecule suggested the involvement of this group, because the PNGase F-deglycosylated allergen, which maintained its secondary structure, displayed a reduced IgE binding capability (42). By contrast, endo H-deglycosylated allergen exhibited identical IgE reactivity to wild Ole e 1. These data showed the role of the complex-type N-glycan of Ole e 1 in the allergenicity of the molecule. Complementary analyses demonstrated that the tryptic glycopeptide of Ole e 1 was recognized by IgE from patients allergic to olive pollen, corroborating the existence of an IgE epitope in this moiety of the allergen. The significance of the glycan in the allergenicity of the molecule was further supported by IgE binding assays performed with the purified glycan released by PNGase F digestion (43), which showed that a high percentage (65%) of sera from patients with specific-IgE response to Ole e 1 recognized the free glycan. However, there is a dominance of the polypeptide epitopes in the allergen, because a non-glycosylated mutant of Ole e 1, obtained by genetic engineering, exhibits similar immunological features to wild Ole e 1 (53,54).

To elucidate the structural basis for IgE recognition of the Ole e 1 glycan, a set of glycoproteins with known N-linked carbohydrate structures, mainly from vegetal origin, were analysed by immunoblotting against Ole e 1-sensitive sera (46). Ascorbate oxidase, bromelain, and horseradish peroxidase, but not phospholipase A₂, were recognized by IgE of sera from patients allergic to olive pollen. This IgE recognition ceased after treatment with periodate, confirming the presence of an IgE epitope in the carbohydrate, because no polypeptide sequence similarity with Ole e 1 was found. These vegetable glycoproteins have in common a β -1,2-linked xylose, to which the IgE-binding capability of the allergic sera was attributed. Therefore, the presence of such a residue in the carbohydrate of Ole e 1 was hypothesized.

The free glycan of Ole e 1 was able to induce *in vitro* histamine release from basophils of olive-sensitized patients, but not from patients sensitized to mites (43). These data showed for the first time that free N-glycans stimulated *in vitro* release from basophils of sensitized patients. In contrast, the free glycan of Ara h 1 could not elicit histamine

release (55). Taking into account that both Ole e 1 and Ara h 1 contained xylosylated-glycan, these controversial results could be explained by a steric hampering of the IgE binding to the xylose-containing epitope, owing to the degree of substitution of the core mannose.

The N-glycan structures of Ole e 1 were recently determined (47,52). Both high mannose (Man7GlcNAc2) and xylosylated complex-type N-glycans (GlcNAcMan3XylGlcNAc2) were identified as the major N-glycan components of Ole e 1 (47). Minor fucosylated structures were also found (52), confirming previous data from our group that reported incomplete activity of PNGase F on Ole e 1 glycan (43). Figure 4 illustrates the agreement between the enzymatic deglycosylation sensitivities and the structures of the glycan components of Ole e 1. Both xylose and fucose components can contribute to the antigenicity of the Ole e 1 glycan, because these residues have been largely demonstrated to be involved in antibody induction in the sera of animals (56). The presence of β -1,2-xylose and/or α -1,3-fucose in allergenic sources could explain the high degree of cross-reactivity observed among allergens from pollen, vegetable food and invertebrate animals (57). However, their clinical relevance in allergy has not yet been clearly assessed.

Furthermore, as already mentioned, Ole e 1 belongs to a family of pollen-specific proteins that could be involved in the hydration and/or germination of the pollen. Sequence information has shown that all these proteins contain at least one potential N-glycosylation site, and several studies have shown these sites to be glycosylated in Ole e 1, Lol p 11, and Zmc13. Interestingly, N-glycosylation sites are located at different positions in the polypeptide chain of different Ole e 1 counterparts, but they seem to be conserved among members of the same taxonomic family. So far, the biological function of this family is unknown, but their members might play an equivalent role in the corresponding pollen. In this sense, although variations in the protein sequences may encode specificity, it is possible to speculate that structural diversity generated through their glycans could play a role in modulation of the biological activity and/or in discrimination between species.

Recombinant production of olive pollen allergens: validation of recombinant allergens

One of the main goals in the study of allergens concerns the design and preparation of mixtures

of allergenic proteins to be used in diagnosis and specific immunotherapy of allergy. Current procedures make use of natural extracts obtained from the biological sources, but they are frequently inadequate, because of the lack of standardization, variable concentration and damage of allergens, and the presence of large amounts of undesirable products, such as glycans, lipids or pigments, which interfere in the process. As an alternative, the utilization of mixtures of purified allergens has been proposed (58–60). These mixtures will have quantified and equilibrated amounts of immunologically active allergens, whose stability would be preserved. Two approaches can be considered to obtain the purified allergens: (1) isolation from their natural sources or (2) their recombinant production. The first approach frequently involves difficulties in the purification procedures and often renders quite a limited amount of protein. Olive pollen allergens have been isolated by current procedures of protein manipulation. However, very different yields have been obtained for different olive allergens, mainly depending on their levels in the pollen. Whereas Ole e 1 can be isolated in large amounts (10–12 mg/g of dried pollen), only around 100 µg of Ole e 3 and Ole e 7 can be obtained per gramme of pollen (10,11,16). These low quantities of natural allergens lead us to consider other possibilities to obtain unlimited amounts of the products.

Molecular biology has been a very useful tool for the production of properly folded and functionally active proteins. It allows very well-defined products to be obtained at almost unlimited levels and free of irrelevant molecules, proteases or oxidant agents. This system also overcomes problems derived from the existence of polymorphism in polypeptides, because one clone is selected to be expressed among those encoding different isoforms of the protein. In addition, it is possible to introduce sequence changes into the specific cDNA molecules using genetic engineering, in order to modify functional properties or the stability of their protein products. In the past decade, procedures for cloning, sequencing, and recombinant expression have been applied to the study of a large number of allergens, and most of the olive pollen allergens have been treated under such approaches. The complete amino acid sequences of Ole e 1, profilin, Ole e 3, Ole e 6, Ole e 8, and Ole e 9 have been obtained from cDNA clones encoding these proteins (12,13,17,20,21,34,61). The analysis of the putative polymorphism of these allergens has been one of the goals in these studies, because such data are very difficult to resolve

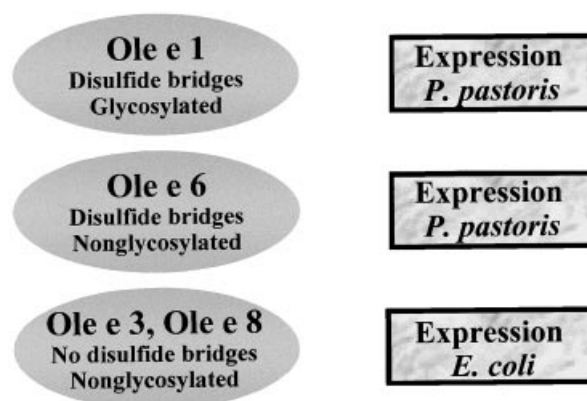


Figure 5. Olive pollen allergens produced by genetic engineering, showing properties and expression systems.

from the polypeptide sequences. In addition, these allergens – except Ole e 9 – have been recombinantly produced, as well as the very related counterparts of Ole e 1 from lilac and privet, Syr v 1 and Lig v 1, respectively (24,25), and the products of expression fit well with their natural forms.

Two different heterologous systems have been used for the recombinant expression of olive pollen allergens: *Escherichia coli* as a prokaryotic host and the methylotrophic yeast *Pichia pastoris* as an eukaryotic system (Fig. 5). The bacteria *E. coli* has resulted in a successful system to produce proteins that do not need to go through postranslational maturation. Allergens such as profilin, Ole e 3, and Ole e 8, which do not contain carbohydrate components or disulphide bridges, were properly folded into the host cell during their recombinant expression in *E. coli*. The products obtained were structurally and immunologically analysed, and they were found to reproduce the features of the natural proteins (12,17,34). Methodological procedures for the recombinant expression in *E. coli* are simpler than those performed for the expression in *P. pastoris*, so it should be chosen when possible. However, bacteria do not have adequate mechanisms for attaching glycans to polypeptide chains, and glycoproteins are expressed as their sugar-free derivatives. Moreover, it is difficult to arrange the correct disulphide bridges successfully, because of the reducing environment of the *E. coli* cytoplasm. Therefore, allergens such as Ole e 1 (glycoprotein with three disulphide bonds) and Ole e 6 (containing four disulphide bridges in a polypeptide chain as short as 50 amino acid residues) must be produced in eukaryotic cells.

Ole e 1 was first produced in *E. coli* as a fusion protein linked to the glutathion S-transferase (21). This procedure had the presumed advantage of

convenient detection of the recombinant allergen and its easy purification by affinity chromatography, both because of the presence of the linked protein. A specific digestion with thrombin released free Ole e 1 from the fusion protein. As is the case with other disulphide-bonded proteins, rOle e 1 was mainly obtained as high-molecular-mass aggregates, and only a small portion in soluble monomeric form. The low yield (100–200 µg per litre of culture) of the immunologically active and properly folded allergen forced us to design a procedure to express Ole e 1 by an alternative system, and *P. pastoris* was supposed to be an attractive host.

A pPIC9 expression plasmid, which carried a cDNA encoding Ole e 1, linked to a secretion peptide from *Saccharomyces cerevisiae*, was used to transform *P. pastoris* GS115 cells (62). rOle e 1 was efficiently secreted to the extracellular medium as a single polypeptide chain at a concentration of 60 mg/l. A simple purification procedure rendered 35 mg/l of culture of a pure and homogeneous protein, which was highly soluble and properly folded, as deduced from the spectroscopic analysis of the secondary and tertiary structures (62). Both structural and immunological features of rOle e 1 were assessed to be equivalent to those of the natural allergen. IgE- and IgG-binding *in vitro* capabilities were found to be identical for natural and recombinant forms of Ole e 1. However, the recombinant protein did not exhibit in SDS-PAGE the band corresponding to the non-glycosylated variant of the natural allergen. In fact, rOle e 1 was completely glycosylated by *P. pastoris* at the correct position. Nevertheless, the structure of the incorporated glycan was not that of the natural allergen isolated from the pollen, because the yeast lacks the enzymes to supply type-complex glycans with xylose and fucose residues, which are characteristic of plant glycoproteins (56). The carbohydrate linked to rOle e 1 was polymorphic (as a result of different degrees of glycosylation) and was of the mannose-enriched type. Although the glycan moiety of Ole e 1 has been demonstrated to be involved in IgE responses, this structural difference did not lead to significant differences in the allergenic behaviour of rOle e 1 in comparison with that of the natural allergen.

Nevertheless, the evaluation of the immunological activity of the recombinant allergens should be performed in a population of patients hypersensitive to olive using *in vivo* analyses such as skin prick tests or histamine release assays. Recently, it has been demonstrated that the recombinant form of Ole e 1 expressed in *P. pastoris* possesses an immunological activity in skin prick test

comparable with its natural counterpart in a population of patients selected because of their sensitivity to nOle e 1 (53). In addition, the accuracy in establishing the diagnosis to olive pollinosis in individuals with a positive response to nOle e 1 was 100% when rOle e 1 was used in the prick test. The specificity of this result was corroborated by the fact that the sera from patients with negative skin response to olive pollen did not recognize rOle e 1 (53).

P. pastoris has also been used to produce homogeneous Ole e 1-like allergens from species related to olive, such as those from lilac and privet, Lig v 1, and Syr v 1 (63). These proteins have been reported to be major allergenic components from these pollens (24,25), which belong to the *Oleaceae* family, and exhibited reduced allergenicity, at least for a significant percentage of hypersensitive individuals (25). Because these homologous forms of Ole e 1 could be suitable derivatives for specific immunotherapy, Lig v 1 and Syr v 1 were expressed in the yeast using procedures similar to those employed for Ole e 1. Recombinant proteins displayed the native conformation and capability to bind both IgE and IgG when they were analysed against Ole e 1-specific antibodies. These highly similar but not identical homologous counterparts are being used to identify B- and T-cell epitopes of Ole e 1, and to elucidate at the molecular level the basis of their cross-reactivity.

Ole e 3 and Ole e 8 have been expressed in *E. coli* (17,34). These allergens are contained in the olive pollen cell at very low levels, and their recombinant production allowed us to improve their yield. These Ca²⁺-binding proteins do not possess Cys residues or disulphide bridges, and were properly folded in the intracellular medium of the bacteria. They were produced as free polypeptide chains and with high yield (25 mg and 20 mg, respectively, per litre of culture). In both cases, the recombinant products were assessed for their structural and immunological integrity, and their functional capability to bind calcium ions was also demonstrated (17,34).

Finally, recombinant production of olive allergens can also be useful for research on their three-dimensional structure, as was performed for Bet v 1 or Der p 2 (64,65), because it is a way to obtain large amounts of well folded polypeptides.

Looking for epitopes to design hypoallergenic molecules

Recombinant production and genetic manipulation of olive pollen allergens offer exciting new

opportunities, because of the possibility of designing hypoallergenic variants that would retain T-cell epitopes but lose their IgE-binding capability, so constituting suitable tools for clinical applications, such as the diagnosis and treatment of allergic disease. This hypothesis has been demonstrated for several allergens and could be checked for Ole e 1 and other recombinantly produced olive allergens. Nevertheless, epitope mapping of these molecules should first be carried out, in order to know what type of specific alteration is needed to reach the desired functional properties. As a simple example, the IgE reactivities of Ole e 3 and Ole e 8 are known to be dependent on the presence of Ca^{2+} ions; if we could eliminate many calcium-binding amino acid residues without perturbing the global folding of these allergens, then we could decrease the IgE-binding capability of these allergens and, therefore, diminish the subsequent cellular events that lead to the allergic response.

As mentioned throughout this paper, Ole e 1 is the best known olive allergen. A preliminary mapping of its B-cell antigenic determinants was performed by Martín-Orozco and colleagues using several families of monoclonal antibodies (27). These analyses showed the existence of four regions of antigenic dominance in the surface of Ole e 1, but they were not defined in the polypeptide sequence of the allergen. More recently, our group has found seven overlapping IgG epitopes, most of which display a conformational character, because they lose the ability to bind the IgG antibodies after SDS-treatment and/or reduction of the disulphide bridges of the allergen (66). However, the polymorphic character of Ole e 1 makes it difficult to carry out this kind of study, and the expression of homogeneous isoforms of the allergen will help to elucidate the precise antibody-antigen interaction areas.

We have also found that homologous counterparts of Ole e 1, which are present in *Oleaceae* pollens, are not able to bind to all the sera of patients sensitive to Ole e 1 (Fig. 6). Although they are structurally very similar to the olive allergen, they failed to be recognized by the IgE, because a limited number of differences in the amino acid sequence of these proteins are responsible for the specificity in the antibody recognition.

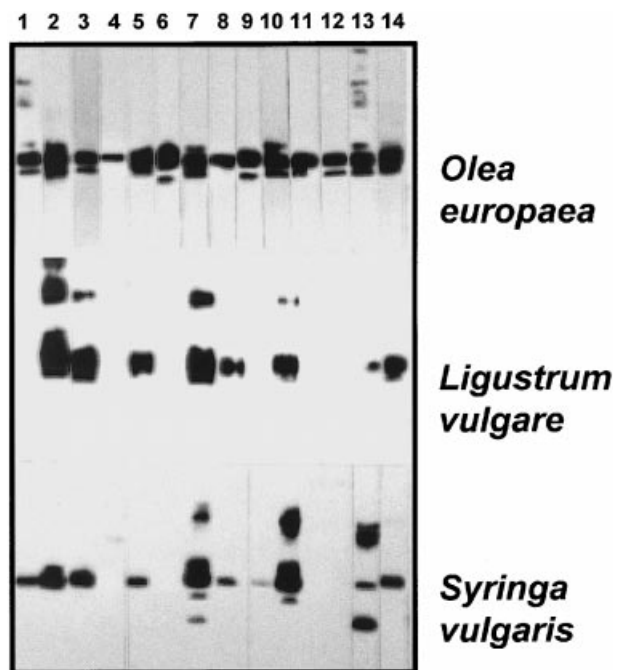


Figure 6. IgE-binding patterns of 14 patients allergic to olive pollen against pollen extracts obtained from *Olea europaea*, *Ligustrum vulgare* and *Syringa vulgaris*.

This fact will be analysed in depth in the near future, because pure and well-defined forms of Ole e 1-like proteins are already available (63).

A precise study of the T-cell epitopes of Ole e 1 was recently performed (67). From this analysis, the polypeptide segments of the allergen involved in the stimulation of T-cells were defined. When the B-cell epitopes, which are required for the IgE interaction, are definitely elucidated, it will be possible to design more effective hypoallergenic variants of the olive allergen Ole e 1. Their construction by genetic engineering and their recombinant production in *P. pastoris* will make available unlimited amounts of the modified protein for clinical purposes.

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