

Immunological and molecular characterization of the major allergens from lilac and privet pollens overproduced in *Pichia pastoris*

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Summary

Background The main allergens from privet and lilac pollens, Lig v 1 and Syr v 1, are proteins homologous to Ole e 1 and have been shown to be involved in cross-reactivity.

Objective To overproduce the correctly folded Lig v 1 and Syr v 1 allergens and to study their immunological properties in comparison with those of their natural counterparts.

Methods The yeast *Pichia pastoris* was used as an expression system to produce these recombinant allergens. The proteins were isolated by ion-exchange and size-exclusion chromatographies. Amino acid quantifying, Edman degradation, mass spectrometry and circular dichroism were carried out to obtain molecular properties of the recombinant proteins. Anti-Ole e 1 monoclonal and polyclonal antibodies, as well as sera from patients allergic to olive pollen, were used in immunoblotting and ELISA for immunological characterization.

Results Recombinant Lig v 1 and Syr v 1 were secreted at high yield to the extracellular medium of the yeast. The purified proteins displayed the native conformation, as deduced from their spectroscopic properties and binding ability to an IgG monoclonal antibody. The recombinant allergens behaved similarly to their natural counterparts when they were analysed against Ole e 1-specific antibodies. IgE and IgG binding properties of lilac and privet allergens to olive allergic sera and Ole e 1-specific antibodies indicated that these molecules share common B-cell epitopes with Ole e 1.

Conclusion *P. pastoris* yeast is an appropriate system for the efficient production of Ole e 1-like allergens, which could be used as analogous allergens and predictors of clinical sensitization.

Keywords: lilac, privet, olive, pollen allergy, recombinant allergens, cross-reactivity

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Introduction

Olive pollen is an important and well-determined cause of type-I allergy in countries bordering the Mediterranean sea, where the olive tree is extensively cultivated [1,2]. Although in Northern and Central Europe the olive tree is very scarce and its pollinosis has no clinical significance, other members of the *Oleaceae* family such as ash, lilac or

privet appear increasingly cultivated, thus being able to produce hay fever and asthma symptoms [3]. Lilac and privet are becoming widely used as ornamental plants in landscape areas of Mediterranean countries, making a longer pollination period of putative sensitizing *Oleaceae* species (12–14 weeks for privet vs. 6 to 8 for olive), and leading to a remarkable increase in pollen counts in the air. A high degree of cross-reactivity has been described among pollen extracts of the *Oleaceae* family [4–7] and attributed to the existence of Ole e 1-homologous proteins [8,9]. Amino acid sequences of Ole e 1-like allergens from lilac

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and privet pollens -Syr v 1 and Lig v 1-were determined [10,11]. The high structural similarity between all these proteins, including Ole e 1, could explain their recognition by IgE of sera from patients allergic to olive pollen [11], as well as their IgG-binding to Ole e 1-specific monoclonal antibodies (MoAbs) [9].

IgE cross-reactivity between crude protein extracts constitutes an important aspect in the diagnosis and treatment of allergic diseases, and so the availability of well known and structurally related allergens may be useful in such protocols [12,13]. Ole e 1-like proteins are the main components of *Oleaceae* pollen extracts, but their purification renders highly heterogeneous products because of the polymorphism of this family of allergens [10,11]. The utilization of the methylotrophic yeast *P. pastoris* has been shown to be very successful for expression of Cys-containing proteins [14]. Cyn d 1 from Bermuda grass [15] and Bla g 4 from German cockroach [16] have been produced as allergenically active in this heterologous system. Moreover, recombinant Ole e 1 (rOle e 1) has been recently purified from the secreted medium of transformed *P. pastoris* cells [17]. The protein exhibited a correct folding, and immunological properties equivalent to those of the natural allergen from olive pollen [17,18]. This expression system could be used to produce homogeneous and well-characterized Ole e 1-like allergens, some of which exhibited a reduced allergenicity, at least for a part of the hypersensitive population [11], these hypoallergenic forms being suitable derivatives for molecular immunotherapy [19,20].

In this work, we report the expression of the major allergens from the pollen of two *Oleaceae* species, lilac and privet, by using the yeast *P. pastoris*. Molecular characterization and immunological comparison between natural and recombinant forms were also performed.

Materials and methods

Pollen extracts preparation, and purification of natural allergens and rOle e 1

Lilac, privet and olive tree pollens were obtained from Allergon (Engelholm, Sweden) and were extracted as described previously [11]. Lyophilized samples were stored at -20°C . rOle e 1 was obtained according to Huecas *et al.* [17].

Sera and antibodies

Sera from hypersensitive individuals, which exhibited positive reaction to Ole e 1 in immunoblotting, were selected from a population with documented clinical history and positive skin-prick test and RAST (classes 3–6) to olive

pollen extract. Polyclonal serum against natural Ole e 1 (nOle e 1) was prepared by immunizing a New Zealand White rabbit over a 6-week period by weekly injection of the protein (100 μg) in complete Freund's adjuvant. Ole e 1-specific monoclonal antibody (MoAb m43) was kindly donated by Dr van Ree (CLB, the Netherlands).

Strains and plasmids

P. pastoris GS115 *his4* strain (Invitrogen Corp., Carlsbad, CA, USA) was used as the host for transformation. The bacterial *E. coli* strains, INV α F' and TG1, were used, respectively, as hosts for cloning the polymerase chain reaction (PCR) fragments in pCR2.1 plasmids (Invitrogen Corp.) and pPIC9 plasmid.

Construction of Syr v 1 and Lig v 1 expression vectors

The coding region of Syr v 1 and Lig v 1 genes (clones S46 [10] and L10 [11], respectively) was amplified by PCR with Vent DNA polymerase (New England Biolabs, Beverly, MA, USA) using the plasmids pUC18/SYR1 and pUC18/LIG1 as templates and two non-degenerate primers, a sense primer 5'-CGTCTCGAGAAAAGAGAGACGTGCCTCAA-3', including a *Xho*I restriction site (underlined), and an anti-sense primer 5'-GCGAATCTCACATGTTGGGCGGGTA-3', for Syr v 1, and 5'-CGTCTCGAGAAAAGAGAAGATGTCCCCCAA-3' and 5'-CGGAATTCTCACATGTTGGGCGGGTA-3', sense and anti-sense primers for Lig v 1. These primers hybridized, respectively, with the 5' and 3' ends of the protein-encoding regions and contain a stop codon and an *Eco*RI restriction site (underlined). The amplified DNA fragments were purified and ligated into a linearized pCR 2.1 vector. The resultant constructs were used to transform INV α F' *E. coli* cells. Several clones were sequenced [21], confirming the in-frame arrangement of both leader sequence and the coding DNA. The DNA fragments were digested with *Xho*I/*Eco*RI restriction enzymes and subcloned into the same sites of the plasmid pPIC9 rendering the pPIC9/SYR1 and pPIC9/LIG1 constructions. Other recombinant DNA methods were carried out according to standard procedures [22].

Transformation of P. pastoris GS115 and production of recombinant allergens

pPIC9/SYR1 and pPIC9/LIG1 (5–10 μg) were linearized with *Bgl*II restriction enzyme and the purified bigger fragment was used to transform GS115 cells by a gene replacement-type DNA integration using lithium acetate treatment [23]. Transformed cells were incubated on Minimal Dextrose plates at 30°C for 4–6 days until colonies appeared. Screening for His⁺ Mut^s phenotype,

obtained by homologous recombination at the *AOX1* locus, was performed by patching the His⁺ colonies in replicating on Minimal Dextrose vs. Minimal Methanol plates.

Production of rSyr v 1 and rLig v 1 as secretion proteins by the selected transformed colonies was performed according to Huecas *et al.* [17]. The presence of recombinant proteins in the supernatant of the culture media was followed at different times (0, 24, 48, 72, 96 h) by SDS/PAGE. Large-scale production of rSyr v 1 and rLig v 1 was carried out in similar conditions using the colony which gave the best yield in the small-scale experiments.

Purification of rSyr v 1 and rLig v 1

The extracellular media obtained after the methanol induction were dialysed against 20 mmol/L ammonium bicarbonate and chromatographed in a DEAE-cellulose column by using a gradient 0.02–0.5 mol/L ammonium bicarbonate, pH 8.0. Fractions containing the recombinant proteins, judged by SDS/PAGE, were lyophilized and chromatographed onto a Sephadex G-75 size-exclusion column in 0.2 mol/L ammonium bicarbonate.

Analytical procedures

Composition and protein concentration of purified proteins were determined after hydrolysis with 5.7 N HCl at 105 °C for 24 h, in sealed tubes under vacuum, by amino acid quantifying on a Beckman 6300 amino acid analyser. Protein concentration of crude extracts was determined according to Lowry *et al.* [24]. NH₂-terminal amino acid sequence was determined using an Applied Biosystems model 477 A sequencer. Mass spectrometry analyses were carried out on a Bruker Reflex II MALDI-TOF mass spectrometer, as described [17]. SDS/PAGE was performed according to Laemmli [25] in 15% polyacrylamide gels. Alternatively, samples were analysed after reduction of disulphide bridges by treatment with 2-mercaptoethanol at 85 °C for 20 min. Proteins were stained with Coomassie blue or electrophoretically transferred onto nitrocellulose membranes.

Spectroscopic characterization

Circular dichroism spectra were obtained in the UV on a Jasco J-715 spectropolarimeter as described [17] with minor modifications. The protein concentration was in the 0.20–0.25 mg/mL range. Mean residue mass ellipticities were calculated based on 111 as the average molecular mass/residue, obtained from the amino acid composition, and expressed in terms of θ (degree $\times$ cm² $\times$ dmol⁻¹).

Deglycosylation treatment

Enzymatic deglycosylation was performed with PNGase F endoglycosidase (Boehringer-Mannheim), as described [26]. Recombinant proteins (1.5 mg/mL) in 100 mmol/L Tris-HCl, pH 8.0, containing 1 mmol/L phenylmethylsulfonyl fluoride and 1% (w/v) *n*-octylglucoside were incubated with PNGase F (6 U/mg) for 18 h, at 37 °C.

Immunological characterization

Immunodetection of transferred proteins to membranes was achieved as described [27], by using alternatively Ole e 1-specific MoAb (m43), a pool of sera from patients allergic to olive pollen, or an Ole e 1-specific polyclonal rabbit antiserum. The signal was developed by the ECL-Western-blotting reagent (Amersham Corp., Buckinghamshire, England).

For inhibition assays, the electrophorized and blotted pollen extracts were immunostained with the same antibodies mentioned above but previously incubated with 0, 5, or 20 μ g of rSyr v 1, rLig v 1 and rOle e 1 as inhibitory proteins.

ELISA inhibition assays were performed as described earlier [17]. After coating with 100 μ L recombinant antigen (1 μ g/mL), the plates were incubated alternatively, with the pool of sera (diluted 1 : 10), anti-Ole e 1 MoAb m43 (diluted 1 : 10000) or anti-Ole e 1 polyclonal antiserum (diluted 1 : 20000), which had been previously incubated with different concentrations of the inhibitory pollen extracts. Afterwards, the response was developed, respectively, by incubation with mouse antihuman IgE (diluted 1 : 5000) kindly donated by Dr Lombardero (ALK-Abelló, Madrid, Spain), and horseradish peroxidase-labelled goat antimouse IgG (Pierce Chemical Co., Rockford, IL, USA) diluted 1 : 2500, or with horseradish peroxidase-labelled goat antirabbit IgG (BioRad, Richmond, CA, USA) diluted 1 : 3000.

Results

Production and purification of rSyr v 1 and rLig v 1

The coding regions of Syr v 1 and Lig v 1 were amplified by PCR using the plasmids pUC18/SYR1 and pUC18/LIG1 as templates, and the primers described under Materials and Methods. Both cDNAs (EMBL data library accession numbers X76540 and X77788), which code for polypeptide chains of 145 amino acids and include one putative N-glycosylation site, were subcloned in the pPIC9 expression plasmid. pPIC9/SYR1 and pPIC9/LIG1 constructs were used to transform *P. pastoris* cells after confirming their correct sequences. The recombinant

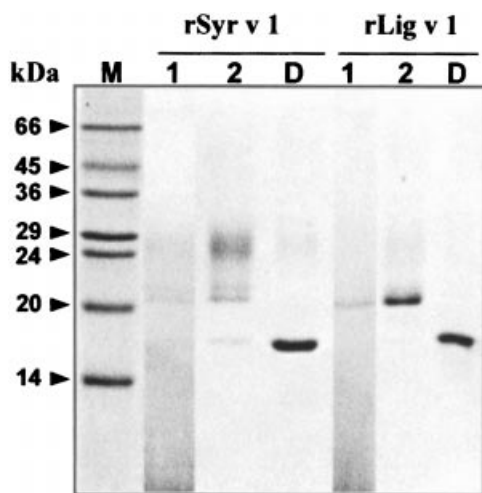


Fig. 1. SDS-PAGE analysis of the production and purification of rSyr v 1 and rLig v 1. Coomassie blue staining of culture supernatant (lanes 1); purified recombinant proteins after Sephadex G-75 column (lanes 2); deglycosylated proteins with PNGase F (lanes D). M, molecular mass markers.

proteins, rSyr v 1 and rLig v 1, were secreted to the extracellular medium, as detected by SDS-PAGE (Fig. 1). In both cases the highest level of production was reached 96 h after induction, and the yields of transformed colonies were 30 mg for rSyr v 1 and 20 mg for rLig v 1, approximately, per litre of cell culture.

Both recombinant proteins were purified after two chromatographic steps by using DEAE-cellulose ion-exchange and Sephadex G-75 size-exclusion columns. Fig. 1 shows the SDS-PAGE analysis of the obtained proteins.

Molecular characterization

Amino acid compositions of rSyr v 1 and rLig v 1 were in agreement with those obtained from the deduced amino acid sequences of the corresponding clones (data not shown). Edman degradation of the NH₂-termini of these recombinant proteins rendered the same sequence EDVPQ, which is according to those expected for the positions 1–5 of the amino acid sequences deduced from both clones, as well as to those of the allergens purified from lilac and privet pollen extracts [10,11].

rSyr v 1 and rLig v 1 were obtained as glycosylated proteins, as proved by staining with concanavalin A (data not shown). The SDS-PAGE analysis of rSyr v 1 showed several bands of 21–25 kDa, and a single of 18.5 kDa (Fig. 1). Bands of 21–25 kDa were reactive to concanavalin. Mass spectrometry analysis of the protein confirmed this result, showing a single peak at 16220 Da and

several peaks around 18417 Da. These results could be due to a heterogeneous glycosylation of the polypeptide chain. To elucidate whether the carbohydrate moiety was responsible for the heterogeneous behaviour, rSyr v 1 was treated with PNGase F endoglycosydase. The deglycosylated allergen gave a single band of 18.5 kDa in SDS-PAGE (Fig. 1), and a single peak of 16151.1 Da in the mass spectrum. These values are in agreement with those expected for the non-glycosylated chain of the recombinant protein.

Purified rLig v 1 consisted of a single glycosylated band of 20.5 kDa apparent molecular mass, which produced, after treatment with PNGase F, a 18.5-kDa molecule corresponding to the non-glycosylated form of the allergen (Fig. 1). Mass spectrometry analysis showed an heterogeneous peak around 18700 Da, whereas only a peak of 16200.7 Da was obtained for the protein treated with PNGase F. These results can be interpreted, as for rSyr v 1, on the basis of a heterogeneous glycosylation of the allergen expressed in *P. pastoris* cells, which renders the homogeneous glycan-free polypeptide chain after deglycosylating treatment.

Circular dichroism analysis give a measure of protein folding. Fig. 2 shows the circular dichroism spectra of rLig v 1 and rSyr v 1, indicating a similar contribution of the secondary structure elements in these allergens, such as the natural forms of Ole e 1-like proteins contained [10,18].

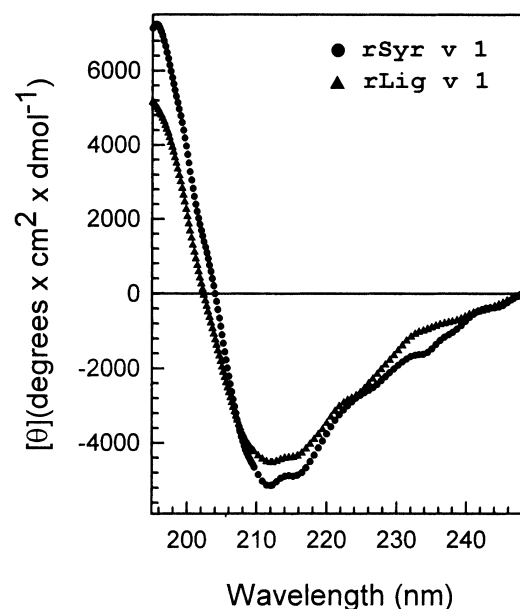


Fig. 2. Circular dichroism spectra of rLig v 1 and rSyr v 1 in the far-UV region.

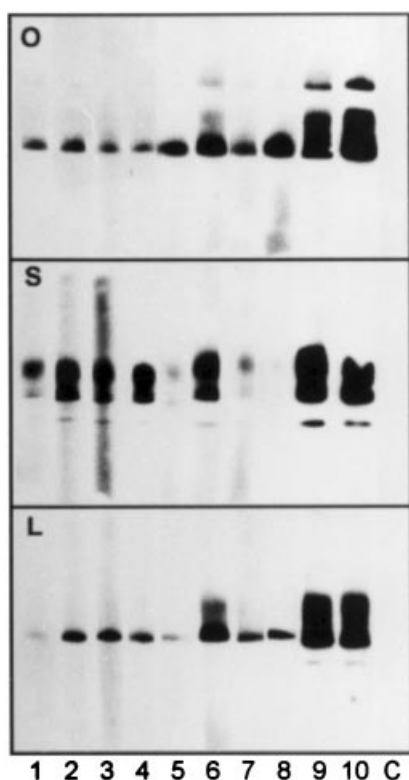


Fig. 3. IgE-binding analysis by immunoblotting of rOle e 1 (O), rSyr v 1 (S) and rLig v 1 (L) with individual sera (lanes 1–10) from patients hypersensitive to Ole e 1. A pool of non-allergic sera was used as negative control (lanes C).

Immunological characterization

Purified rSyr v 1 and rLig v 1 were recognized in immunoblotting by the sera from Ole e 1-hypersensitive patients. rOle e 1 allergen was included in the study as a positive control of binding for each serum, and a serum pool from non-allergic patients was used as negative control. As Fig. 3 shows, all of the sera, except serum 8 for rSyr v 1, contained IgEs able to bind these recombinant proteins. However, when the responses of each serum against the three proteins are compared, significant differences can be observed, indicating that minimal changes in the amino acid sequence of these polypeptides can be responsible for a decreased allergenicity against some individuals.

Immunoblotting of rSyr v 1, rLig v 1 and rOle e 1 was performed by using anti-Ole e 1 rabbit polyclonal antiserum and Ole e 1-specific MoAb m43. The latter was selected because it only binds to Ole e 1 in native conformation. All of the recombinant proteins were recognized by these two antibodies (Fig. 4 A, B). These results indicated the presence of Ole e 1 IgG epitopes in the recombinant lilac and privet allergens. After treatment of

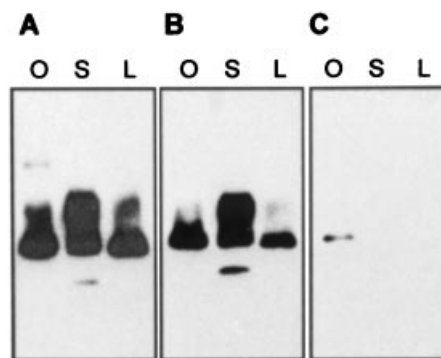


Fig. 4. Immunodetection of rOle e 1 (lanes O), rSyr v 1 (lanes S) and rLig v 1 (lanes L) with a polyclonal antiserum raised against nOle e 1 (A), and with the Ole e 1-specific MoAb m43, in non-reduced conditions (B) and under reducing conditions (C).

rOle e 1, rSyr v 1 and rLig v 1 with 2-mercaptoethanol, they lose the ability of binding to m43 MoAb (Fig. 4, C), confirming the correct three-dimensional folding of rSyr v 1 and Lig v 1.

The immunological equivalence of natural and recombinant forms of the two allergens, Syr v 1 and Lig v 1, was analysed in immunoblotting by inhibition of the binding of IgG and IgE antibodies to lilac and privet pollen extracts by using rSyr v 1 and rLig v 1 as inhibitors. The pre-incubation of Ole e 1-specific polyclonal antiserum (Fig. 5, A), m43 MoAb (Fig. 5, B), and a pool of allergic human sera (Fig. 5, C), with rSyr v 1 or rLig v 1 inhibited the recognition of their corresponding natural allergens present in the pollen extracts, including their oligomers which can be observed at high molecular masses. The remaining signal observed (10.1% of the non-inhibited signal, determined by densitometric analysis of the bands) for rLig v 1 could be due to epitopes, which are present in the polymorphic natural allergen and missing in the expressed isoform.

Quantifying of the inhibitory capabilities of lilac and privet pollen extracts on the antibody binding to the corresponding recombinant allergens were measured by IgE and IgG ELISA inhibition experiments. As Fig. 6 shows, the natural allergens present in pollen extracts reached 98% of inhibition for the polyclonal antiserum, whereas MoAb-binding was completely inhibited. The two human sera showed different affinity, but both lost their IgE binding capability to recombinant allergens in the presence of high levels of pollen extracts.

Discussion

The availability of purified allergens is essential for research and clinical purposes. Determination of B- and T-cell epitopes of allergens are the main objectives in the

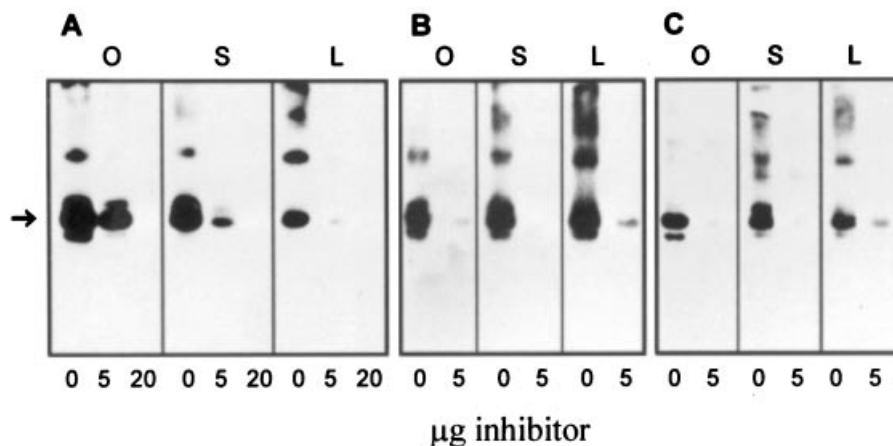


Fig. 5. Inhibition assays of the binding of IgG and IgE antibodies to natural allergens using their recombinant counterparts as inhibitors.

Immunodetection of olive (O), lilac (S) and privet (L) pollen extracts with: (A) Ole e 1-specific polyclonal antiserum, (B) m43 MoAb, and (C) a pool of sera from Ole e 1-allergic patients. The antibodies and sera were pre-incubated with 0, 5 or 20 µg of the corresponding inhibitor. The arrow indicates the position of the main components of Ole e 1-like allergens at 20 kDa.

study of the molecular basis of the allergic mechanisms, and well-defined molecules should be employed for these analyses. Moreover, diagnosis and therapy protocols can be improved by using purified and well-characterized allergens as well as hypoallergenic derivatives, which avoid most of the inconveniences caused by the whole extracts [13]. It has been shown that a limited number of recombinant proteins can be enough for diagnosis of grass and tree pollen allergy [28,29]. Allergens can be isolated and purified from their natural sources. However, the low level of most allergens in the biological sources and the complexity of the isolation procedures, frequently renders scarce amounts of purified proteins.

An additional problem comes from the polymorphism exhibited by many allergens. The production of a number of allergens was solved by the recombinant expression of their encoding cDNAs, although folding and/or post-translational processing of the produced proteins are often different to those of their natural counterparts. Bacterial systems have been the most frequently used for these purposes, but proteins that remain in the cytoplasm after their expression are usually incorrectly folded, mainly when they possess cysteine residues involved in disulphide bonds. Recombinant allergens, such as those from grasses and *Fagales*, produced in *E. coli*, did not equal the IgE-binding capacity of their natural counterparts [30,31].

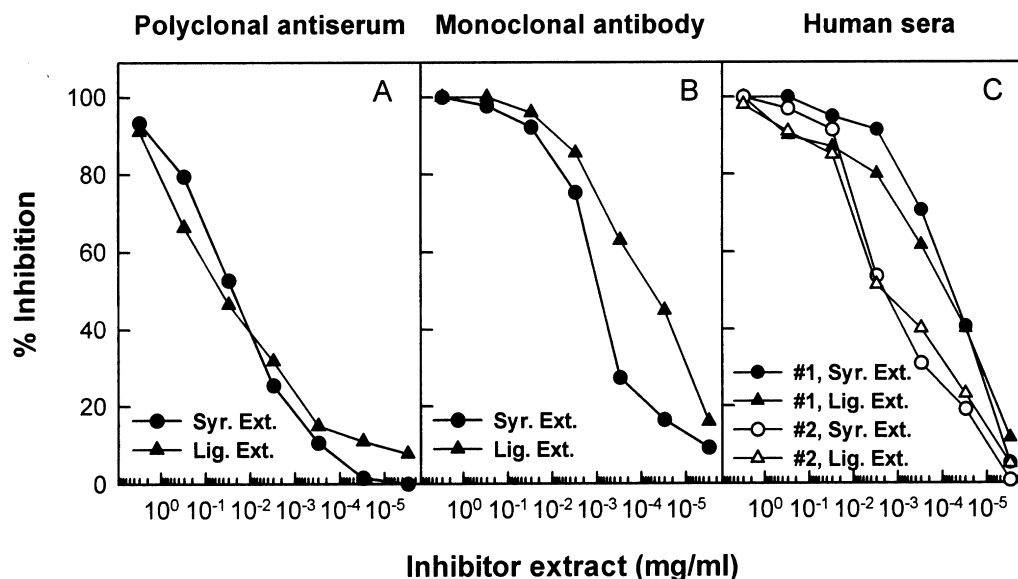


Fig. 6. ELISA inhibition of natural and recombinant lilac and privet allergens. Plates were coated with rSyr v 1 or rLig v 1 and assayed the binding to (A) IgG polyclonal antiserum, (B) IgG m43 MoAb, and (C) two olive allergic sera (black symbols: serum #1; empty symbols: serum #2). Inhibition of the binding was performed with the corresponding lilac (●) and privet (Δ) pollen extracts.

The utilization of *P. pastoris* yeast cells as a host for the expression of foreign genes has become an attractive alternative since proteins are usually secreted to the extracellular culture medium in a high yield, with native conformation and without too many contaminant proteins [14,32]. As the present work shows, cDNAs encoding the major allergens Lig v 1 and Syr v 1 from privet and lilac pollens have been efficiently expressed in *P. pastoris* cells and secreted to the extracellular media. Since the yeast extracellular media were essentially enriched in the recombinant proteins, they were purified by a simple and profitable procedure. The production of single clones of rSyr v 1 and rLig v 1 will avoid ambiguities in immunological analysis inferred from the polymorphism of the natural allergens.

The data obtained from the molecular and immunochemical analyses of rLig v 1 and rSyr v 1 were in agreement with the native conformation of their natural counterparts. Lig v 1 and Syr v 1 have a single N-glycosylation consensus site at position 111 of the polypeptide chain, which was demonstrated to be glycosylated in the natural allergens [10,11]. Structural characterization strongly indicated that the recombinant proteins were glycosylated by the yeast in an heterogeneous manner, as demonstrated for Ole e 1 [17]. These results can be explained because *P. pastoris* has a glycosylation machinery specialized in producing high-mannose-type N-glycans, the length of the added chains ranging between 8 and 14 mannoses [33]. The glycan moieties linked to these recombinant allergens did not interfere with their polypeptide epitopes since they are able to bind to IgE and IgG antibodies in a similar manner as rOle e 1 (Figs 3,4,5,6).

The high cross-reactivity previously found between the natural forms of Ole e 1 and Syr v 1 or Lig v 1 [8–11] is confirmed, at both IgE and IgG levels, in the present work for their recombinant counterparts obtained in yeast (the expressed clones of Syr v 1 and Lig v 1 show 90% and 89% identities with rOle e 1, respectively). This cross-reactivity suggests that hypersensitive patients can be allergenic cross-sensitized by *Oleaceae* pollens, since privet and lilac are being increasingly cultivated. Although the levels of privet pollen grains in the air are not high (no data are available for lilac pollen), they have increased in populated areas in the last few years; for instance, they got a seven-fold rise from 1995 to 1999 in Madrid (central Spain), reaching levels which are comparable to those of olive pollen at the same place (Red Palinológica de la CAM; url: <http://www.comadrid.es/madrid/palinologica/>). Thus, the possible cross-sensitization between *Oleaceae* pollens should be taken into account when performing IgE-mediated allergy diagnosis.

Although polyclonal and monoclonal antibodies did not show significant differences on their binding to rOle e 1,

rSyr v 1 and rLig v 1, which displayed most of the IgG epitopes of their natural counterparts (Fig. 4), the IgE from several patients were able to distinguish between these proteins. Similar results, although to a lesser extent, were previously obtained with natural lilac and privet allergens [10,11]. A plausible explanation is that few changes in the amino acid sequence of these polypeptides can be responsible for a decreased allergenicity, and the isoforms can display this property since microheterogeneity is avoided. In fact, isoallergens can display significant differences in the reactivity towards antibodies and T-cells [19,34,35], and specific isoforms could lose the ability to diagnose 100% of patients sensitive to the natural allergen. In contrast, this property may be useful for designing genetically engineered allergens with low IgE reactivity to be used in immunotherapy [19,20,36].

No data have been obtained regarding the equivalence of cellular responses to natural and recombinant Lig v 1 and Syr v 1, or with Ole e 1. Immunodominant T-cell epitopes of Ole e 1 have been described at regions 91–102, and 109–130 of its polypeptide chain [37]. The amino acid sequences of these regions are highly conserved, Ole e 1 exhibiting 88% and 91% identity to Syr v 1 and Lig v 1, respectively. As most of the changes at these segments are of a conservative character, the similarity rises to 97% since only Thr at position 95 of Ole e 1 changed to Ile and Val in Lig v 1 and Syr v 1, respectively [11]. The implication of these changes in T-cell activation should be evaluated in further studies.

Finally, the production of the recombinant forms of this family of *Oleaceae* major allergens and chimerical derivatives can help to identify the amino acid residues involved in their B- and T-cell epitopes, to elucidate the molecular basis of their high cross-reactivity, and to measure immunological and biological responses to these defined molecules.

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