

Isolation, cDNA cloning and expression of Lig v 1, the major allergen from privet pollen

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Summary

Background An olive allergen-like protein has been detected in privet pollen. This protein could be involved in the allergenic cross-reactivity described for privet and olive tree pollen extracts.

Objective Isolation and characterization of natural Lig v 1. Cloning and expression of its cDNA in order to assess its structural similarity with the olive allergen.

Methods Current chromatographic methods were used to isolate the privet counterpart of Ole e 1. A pool of sera from subjects allergic to olive tree pollen was used to immunodetect the protein in the elution profiles. Ole e 1-specific polyclonal antibody and allergic sera were used in immunoblotting assays of the isolated protein. Polymerase chain reaction amplification of the first strand cDNA synthesized from the privet pollen total RNA was carried out to prepare a full-length fragment encoding Lig v 1. After nucleotide sequencing, expression of one clone was performed in *Escherichia coli*, under the form of a fusion protein with glutathione S-transferase. The IgE binding capability of the recombinant protein was also analysed.

Results The major allergen from privet pollen, Lig v 1, was purified to homogeneity by two gel filtration chromatographies and one reverse-phase high-performance liquid chromatography. Its amino acid composition and N-terminal amino acid sequence were determined. Two different clones encoding Lig v 1 were sequenced. Strong sequence similarity between Lig v 1 and Ole e 1 was observed, the identity being 85 and 96%. One of the sequenced clones was expressed and the recombinant product exhibited IgG and IgE binding activities against both anti-Ole e 1 polyclonal antibodies and olive-allergic sera.

Conclusion Privet pollen contains a protein structurally and immunologically related to the major allergen of olive pollen. The similarity exhibited by these proteins could explain the cross-reactivity observed between the two pollen extracts. Since these allergens are highly polymorphic, the expression of an immunologically active recombinant Lig v 1 will permit the preparation of well defined molecules for both research and clinical purposes.

Keywords: Lig v 1, privet, major allergen, protein isolation, nucleotide sequence, recombinant pollen allergen, allergen expression.

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Introduction

Allergy to privet (*Ligustrum vulgare*) pollen is infrequent among hypersensitive individuals [1]. However, olive tree (*Olea europaea*) pollen is one of the most important causes of asthma and hay fever in Mediterranean areas [2]. Both species belong to the *Oleaceae* family, which also includes ash (*Fraxinus excelsior*), lilac (*Syringa vulgare*) and forsythia (*Forsythia suspensa*). *Ligustrum* is a genus of such a family widely occurring in Europe. Although allergic sensitization to privet pollen has a low frequency [1,3], probably due to its entomophilous (insect pollination) character, allergenic cross-reactivities between privet and olive pollens have been demonstrated [4,5]. The allergenic spectrum of the privet pollen extract has been studied by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [5] showing a great similarity to that of the olive pollen. Recently, the presence of allergens homologous to Ole e 1, the major allergen from olive pollen, has been demonstrated in other species of the *Oleaceae* family [6–8].

The analysis of the structural features of related allergenic proteins would facilitate the studies on structure–antigenicity relationships and further B- and T-cell epitope elucidation. Moreover, the knowledge of proteins homologous to significant allergens would improve the diagnosis and treatment of allergic individuals, since the contribution to allergic sensitization of cross-reacting allergens from related or non-related sources seems to be of higher occurrence than currently believed [9,10].

cDNA technology is useful for obtaining the primary structure of proteins exhibiting a significant degree of polymorphism, because it allows the analysis of individual clones. Recombinant DNA methods may lead to the production of well defined isoforms and site-directed mutants, which should facilitate the elucidation of specific amino acid residues involved in recognition by B- and T-cells [11,12].

In this work, we present the purification of Lig v 1, the major allergen from privet pollen, as well as its primary structure by cDNA cloning and recombinant production.

Materials and methods

Sera

Sera from 40 hypersensitive individuals, which exhibited a positive reaction to Ole e 1, were selected from among a population with a clinical history and positive skin-prick test to olive pollen. The sera had specific IgE to olive pollen (3.6–70 kU_λ/l, classes 3–5) determined by CAP-FEIA System (Pharmacia AB, Uppsala, Sweden). Sera from non-allergic individuals were used as a negative control.

Polyclonal serum against Ole 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.

Isolation of Lig v 1

Lig v 1 was purified from privet (*L. vulgare*) pollen (Allergon AB, Pharmacia). Pollen was suspended (5% w/v) in 50 mmol/l ammonium bicarbonate, pH 8.0, containing 1 mmol/l phenylmethylsulphonyl fluoride and extracted for 60 min at room temperature. Undissolved material was removed by centrifugation (8700 g) for 20 min at 4°C. The pellet was twice re-extracted under the same conditions. The clear supernatant fluids were collected, lyophilized, redissolved in 0.2 mol/l ammonium bicarbonate, pH 8.0, and chromatographed on a Sephadex G-75 superfine (Pharmacia) gel-filtration column (1.5 × 88.0 cm), equilibrated in the same buffer, and calibrated with standard proteins of known molecular mass. The fractions corresponding to about 20 kDa were pooled, lyophilized and rechromatographed on the same column. A reverse-phase high-performance liquid chromatography (HPLC) step on a µBondapak C-18 column (7.9 × 300 mm) was finally carried out by using a linear gradient (0–60%) of acetonitrile in 0.1% (v/v) trifluoroacetic acid. The eluent was continuously monitored at both 214 and 280 nm wavelengths. Concentration of the purified allergen was determined by amino acid analysis.

Molecular characterization

Amino acid analyses were performed as described [13], after hydrolysis of the protein with 5.7 N HCl, in sealed tubes under vacuum. The cysteine content was determined as cysteic acid after oxidation with performic acid. Tryptophan content was determined spectrophotometrically [14].

N-terminal Edman degradation of the native protein was performed on an Applied Biosystems (Foster City, CA, USA) model 477A sequencer [15]. The resulting phenylthiohydantoin-amino acid derivatives were identified by using a 120A on-line analyser and the standard Applied Biosystems program.

SDS-PAGE, Western blotting and immunodetection

Analytical gel electrophoresis was performed according to Laemmli [16] in 15% polyacrylamide gels. Proteins were visualized by Coomassie Brilliant Blue staining (Merck, Darmstadt, Germany). Proteins in SDS-PAGE were transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA, USA) in 48 mmol/l Tris/HCl, pH 9.0, containing 39 mmol/l glycine, 20% (v/v) methanol and 0.036% SDS, for 1 h at 1 mA/cm². Blot strips were probed with individual sera from olive-allergic patients or with a pool of four of

these sera (both diluted 1/10) in phosphate-buffered saline (PBS), 0.1% Tween 20 (v/v), 3% non-fat milk (w/v) for 2 h. Strips were washed with 0.1% Tween 20 (v/v) in PBS and incubated for 1 h with a mouse anti-human IgE diluted 1/5000 in PBS containing 0.05% Tween 20 (v/v) and 1% non-fat milk (w/v). Strips were then incubated for 1 h with horseradish peroxidase-labelled goat anti-mouse IgG (Pierce Chemical Co., Rockford, IL, USA) diluted 1/5000. The peroxidase reaction was developed with the ECL Western-blotting reagent and ECL-hyperfilm (Amersham International, Buckinghamshire, UK), and measured by scanning the ECL-hyperfilm on an LKB (Pharmacia) model 2202 Ultrascan laser densitometer. The values obtained were normalized between 0 and 4. The sera were classified into three groups according to the intensity of the response: –, undetected reaction (value zero); +, moderate response (values between 0 and 2) and ++, strong response (values between 2 and 4).

For IgG polyclonal antibody (diluted 1/20 000) binding assays, a 1/3000 diluted horseradish peroxidase-labelled goat anti-rabbit IgG (TAGO Inc., Burlingame, CA, USA) was used as the second antibody.

For inhibition assays, the corresponding dilutions of the rabbit polyclonal antiserum, or the pool of sera from allergic patients, were preincubated with natural Ole e 1 allergen for 2 h at room temperature.

Carbohydrate detection

Electrophoresed and transferred allergen was analysed for the presence of carbohydrates essentially as described previously [17]. Biotinylated concanavalin A lectin solution and the ABC reagent (avidin, biotinylated horseradish peroxidase), diluted 1/400 in 50 mmol/l Tris/HCl, pH 7.5, containing 0.5 mol/l NaCl and 1% poly(vinylpyrrolidone), were used. The staining was developed with the chemiluminescent ECL reagent, as described above for horseradish peroxidase-labelled antibodies.

Isolation of total RNA and cDNA synthesis

Total RNA from privet pollen was extracted as described [18]. Single-stranded cDNA was synthesized from total RNA by using a cDNA synthesis kit (Pharmacia Biotech, Uppsala, Sweden) and oligo dT as primer, following the manufacturer's instructions.

Polymerase chain reaction: based cloning and sequence analysis

The oligonucleotide primers (LV-1, LV-2 and LV-3) were synthesized by using an Applied Biosystems model 381A DNA synthesizer. The LV-1 sequence was designed based on the N-terminal amino acid sequence of Lig v 1, positions 1–5:

5'-CCGGGATCCGARGAYGTGCCXCA-3'. Two primers, LV-2 and LV-3, were designed according to the C-terminal amino acid sequence of Ole e 1 [18]. LV-2 is degenerate, (5'-ATCATRTTNGGNGGRTACATNCC-3' (positions 140–145 of Ole e 1), and LV-3 is non-degenerate: 5'-CGGAATTCTCACATGTTGGGCGGGTA-3' (positions 141–145 of Ole e 1). Primers LV-1 and LV-3 contained a *Bam*HI and an *Eco*RI restriction site, respectively (underlined).

The cDNA template and 50 pmol of the primers LV-1, LV-2 and LV-3 were denatured at 95°C for 15 min in polymerase chain reaction (PCR) mixture. After adding 2.5 U *Taq* polymerase (Bioprobe, Montreuil-Sous-Bois, France), the sample was subjected to PCR amplification following the procedure described by Villalba *et al.* [18]. Under these conditions, a single PCR product of about 440 bp was obtained. The fragment was purified by using the Magic PCR Prep kit (Promega, Madison, WI, USA) and digested with *Eco*RI and *Bam*HI endonucleases. The fragment was incorporated into an *Eco*RI/*Bam*HI-digested pUC18 plasmid vector and used for transformation of competent *Escherichia coli* DH5 α F' cells, by standard procedures.

pUC18 plasmid minipreps were used as template for sequencing, which was achieved by using the dideoxy chain termination method [19] and deoxy[α -³⁵S]ATP with the Sequenase kit (U.S. Biochemical, Bad Homburg, Germany). Sequencing primers for both strands, M13mp18 universal and reverse, were from New England Biolabs (Beverly, MA, USA). The sequencing was carried out according to the manufacturer's recommendations.

Expression of Lig v 1 in *E. coli*

The cDNA clone L10 was subcloned into the *Bam*HI/*Eco*RI sites of pGEX-2T expression vector (Pharmacia Biotech). Production of the recombinant protein was performed after transformation of JA221 cells with the pGEX-L10 plasmid. Overnight cultures of transformed cells were diluted 10-fold with LB medium containing 0.1 mg/ml ampicillin and grown up to an absorbance value of 0.6 at 600 nm. After induction with 1 mmol/l isopropyl β -D-thiogalactoside (Sigma Chemical Co., St. Louis, MO, USA), cells were harvested by centrifugation, resuspended in 1/10 volume of 50 mmol/l Tris-HCl, pH 8.0, containing 2 mmol/l EDTA, 1% SDS and 5% 2-mercaptoethanol, and heated for 20 min at 80°C. The obtained cell lysate was analysed by SDS-PAGE and immunoblotting.

Results

Isolation and molecular characterization of the olive allergen-like protein from privet

A highly purified olive allergen-like protein has been

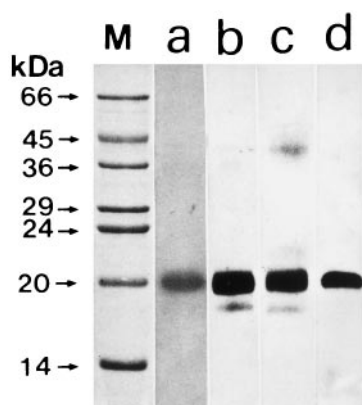


Fig. 1. Sodium dodecylsulphate polyacrylamide gel electrophoresis analysis of purified Lig v 1. (a) Coomassie Blue staining; (b) immunostaining of the protein transferred to nitrocellulose membranes with a pool of sera from patients allergic to olive pollen; (c) immunostaining of the membrane with a rabbit polyclonal antiserum raised against the olive pollen allergen; (d) Concanavalin A lectin staining for sugar detection. M, Molecular mass protein markers.

obtained from a saline extract of privet pollen, by using two chromatographic steps in a Sephadex G-75 gel permeation column, and a reverse-phase HPLC in a μ Bondapak C-18 column. IgE immunoblotting assays with a pool of olive-allergic sera were used to detect the protein. The procedure rendered 1 mg of protein per g of dried pollen. The protein exhibits an apparent molecular mass of 20 kDa on SDS-PAGE (Fig. 1). Its amino acid composition has been determined after complete acid hydrolysis and quantitative analysis (Table 1). The protein rendered a single *N*-terminal amino acid sequence by Edman degradation: EDVPQPPVSQ. This sequence fragment is identical to that obtained by Obispo *et al.* [6]. Therefore, the isolated allergen was called Lig v 1.

The purified protein was electrophoresed and transferred to nitrocellulose membranes, and then analysed for staining by reaction with concanavalin A lectin. This lectin binds to mannose terminal glycans of glycoproteins. Lig v 1 gave a positive reaction at the 20 kDa component (Fig. 1d), indicating the presence of a mannose-containing glycan moiety in this protein.

Immunological characterization of Lig v 1

The protein from privet pollen was tested, after electrophoresis

Table 1. Amino acid composition of purified Lig v 1 in comparison with those deduced from the sequenced clones (L1 and L10) and Ole e 1 (clone Ole3c) and Syr v 1 (clone S28)

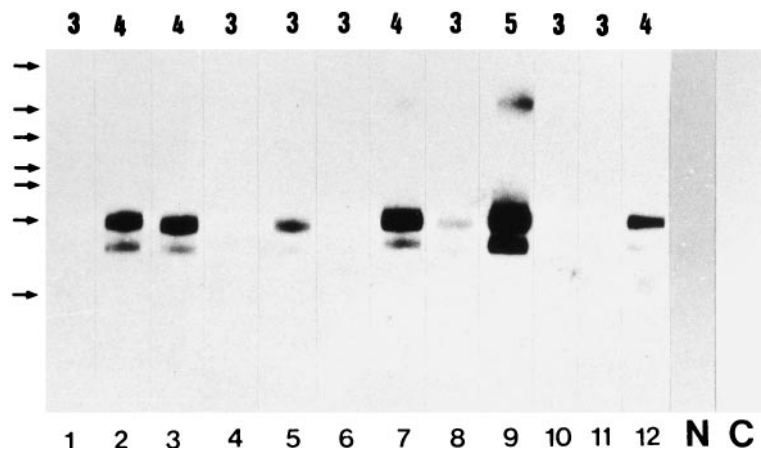
Amino acid	%*	No. of residues	L1	L10	Ole e 1	Syr v 1
Cys	4.0†	5.8	6	6	6	6
Asx	9.9	14.4	14	14	14	13
Thr	6.6	9.6	11	10	11	11
Ser	5.4	7.8	6	7	7	6
Glx	12.3	17.9	18	18	18	18
Pro	7.2	10.5	11	11	10	11
Gly	8.6	12.5	11	11	11	12
Ala	2.9	4.3	3	4	5	4
Val	5.7	8.3	11	8	9	8
Met	1.5	2.2	3	4	3	3
Ile	6.9	10.0	8	9	8	11
Leu	7.4	10.7	11	11	11	9
Tyr	2.8	4.0	5	5	5	5
Phe	5.4	7.8	8	9	8	8
His	1.4	2.1	1	1	2	2
Lys	6.9	10.0	11	10	10	11
Arg	4.5	6.6	6	7	6	6
Trp	-	1‡	1	1	1	1
Total		145	145	145	145	145

*Data from acid hydrolysis.

†Determined as cysteic acid.

‡Determined spectroscopically.

Fig. 2. IgE recognition of purified Lig v 1 (1 µg) by individual sera from patients hypersensitive to olive pollen. Immunoblotting assay responses are shown for sera of 12 patients (lanes 1–12). Serum of a non-allergic individual (N) and phosphate-buffered saline (C) substituting the allergic sera were used as controls. Molecular mass markers as in Fig. 1 are indicated by arrows. Numbers above lanes indicate CAP-FEIA class of each serum.



and blotting to nitrocellulose membranes, for binding to IgE of a pool of sera from individuals allergic to olive tree pollen. Figure 1(b) shows that Lig v 1 can be recognized by these IgE antibodies. Therefore, the privet allergen shares allergenic determinants with Ole e 1.

The binding of Lig v 1 to rabbit polyclonal antiserum raised against purified Ole e 1 was also analysed. Figure 1(c) shows the strong reaction with this antibody indicating that privet and olive pollen allergens display common B epitopes which would be responsible for such a recognition. These immunostainings allowed the detection of 18.5 and 40 kDa minor components, which were also detected in Ole e 1.

Individual recognition of Lig v 1 by olive pollen allergic sera

IgE binding to purified privet allergen was analysed by immunoblotting assays with 40 Ole e 1-specific human sera (CAP-FEIA classes 3–5). A representative sample of 12 sera

is shown in Fig. 2. The IgE responses of the 40 sera were measured by densitometric scanning of the ECL-hyperfilms. Table 2 shows the results obtained as well as the total IgE and olive tree-specific IgE of these sera. The strongest response of the allergen corresponded to the sera of CAP-FEIA classes 4 and 5, while the sera exhibiting class 3 showed either no response (lanes 1, 4, 6, 10 and 11 in Fig. 2), or a very weak response (lanes 5 and 8 in Fig. 2). These data indicate a correlation between the intensity of the response of the sera from patients sensitized to olive pollen and the level of specific IgE antibodies.

Cloning and sequence of cDNA encoding Lig v 1

The cDNA encoding Lig v 1 was amplified from the total RNA isolated from privet pollen. This was achieved by means of PCR using oligonucleotide primers, which were designed on the basis of the *N*-terminal amino acid sequence of Lig v 1 (residues at positions 1–5) and the *C*-terminal

Table 2. Age and serum IgE values of 40 patients allergic to olive pollen whose sera were analysed by immunoblotting for binding to purified Lig v 1

	Group			<i>P</i>
	—*	+*	++*	
No. of cases	15	11	14	
Patient age (years)†	5–42	5–32	5–51	‡
Total IgE (kU _λ /l) (mean ± s.e.m.)†	255 ± 66	263 ± 49	341 ± 91	‡
Olive pollen specific IgE (kU _λ /l) (mean ± s.e.m.)†	5.86 ± 0.44	11.47 ± 1.27	40.11 ± 3.76	§

*Data from the densitometric scanning of the immunoblots. The areas obtained were normalized between values 0 and 4. —, no response; +, moderate response (values <2); ++, strong response (values 2–4).

†Values fit to a standard plot (Kolmogorov–Smirnov test).

‡No significant differences among the three groups.

§*P* < 0.01 between groups (–) and (+); *P* < 0.001 between groups (+) and (++)

	<u>Glu</u>	<u>Asp</u>	<u>Val</u>	<u>Pro</u>	<u>Gln</u>	<u>Pro</u>	<u>Pro</u>	<u>Val</u>	<u>Ser</u>	<u>Gln</u>	<u>Phe</u>	<u>Tyr</u>	<u>Ile</u>	<u>Gln</u>	<u>Gly</u>	<u>Gln</u>	<u>Val</u>	<u>Tyr</u>	<u>Cys</u>	<u>Asp</u>	20
L1	GAA	GAT	GTT	CCG	CAA	CCT	CCA	GTT	TCA	CAA	TTC	TAC	ATT	CAG	GGA	CAA	GTT	TAT	TGC	GAC	60
L10			C	C		C	G		T		T						G				
	<u>Thr</u>	<u>Cys</u>	<u>Arg</u>	<u>Ala</u>	<u>Arg</u>	<u>Phe</u>	<u>Ile</u>	<u>Thr</u>	<u>Glu</u>	<u>Leu</u>	<u>Ser</u>	<u>Glu</u>	<u>Phe</u>	<u>Ile</u>	<u>Pro</u>	<u>Gly</u>	<u>Ala</u>	<u>Gly</u>	<u>Val</u>	<u>Arg</u>	40
L1	ACG	TGT	CGA	GCT	CGA	TTC	ATT	ACT	GAA	CTT	AGC	GAG	TTC	ATC	CCA	GGT	GCC	GGT	GTA	CGC	120
L10	A		T							C							T		T		
	<u>Leu</u>	<u>Gln</u>	<u>Cys</u>	<u>Lys</u>	<u>Asp</u>	<u>Gly</u>	<u>Glu</u>	<u>Asn</u>	<u>Gly</u>	<u>Lys</u>	<u>Val</u>	<u>Thr</u>	<u>Phe</u>	<u>Thr</u>	<u>Glu</u>	<u>Val</u>	<u>Gly</u>	<u>Tyr</u>	<u>Thr</u>	<u>Lys</u>	60
L1	CTC	CAA	TGC	AAA	GAC	GGT	GAG	AAC	GGG	AAA	GTA	ACA	TTT	ACT	GAG	GTC	GGT	TAC	ACG	AAA	180
L10	T					G		T	A	G	A					A			A	G	Arg
										Ile											
	<u>Ala</u>	<u>Glu</u>	<u>Gly</u>	<u>Leu</u>	<u>Tyr</u>	<u>Asn</u>	<u>Met</u>	<u>Leu</u>	<u>Ile</u>	<u>Glu</u>	<u>Arg</u>	<u>Asp</u>	<u>His</u>	<u>Lys</u>	<u>Asn</u>	<u>Glu</u>	<u>Phe</u>	<u>Cys</u>	<u>Glu</u>	<u>Ile</u>	80
L1	GCA	GAA	GGA	CTC	TAC	AAC	ATG	CTC	ATT	GAA	CGA	GAT	CAC	AAG	AAT	GAG	TTT	TGT	GAA	ATC	240
L10	G			T	T	G					C					A		C			
						Ser															
	<u>Thr</u>	<u>Leu</u>	<u>Ile</u>	<u>Ser</u>	<u>Ser</u>	<u>Ser</u>	<u>Arg</u>	<u>Lys</u>	<u>Asp</u>	<u>Cys</u>	<u>Asp</u>	<u>Glu</u>	<u>Ile</u>	<u>Pro</u>	<u>Thr</u>	<u>Glu</u>	<u>Gly</u>	<u>Trp</u>	<u>Val</u>	<u>Lys</u>	100
L1	ACA	CTT	ATT	TCA	AGT	AGC	AGA	AAA	GAT	TGT	GAT	GAA	ATT	CCT	ACT	GAA	GGA	TGG	GTA	AAA	300
L10		T	G	C	C								A		T						
			Leu												Ile						
	<u>Pro</u>	<u>Ser</u>	<u>Leu</u>	<u>Lys</u>	<u>Phe</u>	<u>Val</u>	<u>Leu</u>	<u>Asn</u>	<u>Thr</u>	<u>Val</u>	<u>Asn</u>	<u>Gly</u>	<u>Thr</u>	<u>Thr</u>	<u>Arg</u>	<u>Thr</u>	<u>Ile</u>	<u>Asn</u>	<u>Pro</u>	<u>Leu</u>	120
L1	CCA	TCA	TTG	AAA	TTT	GTA	CTC	AAT	ACA	GTA	AAT	GGC	ACC	ACA	CGC	ACG	ATA	AAT	CCT	CTT	360
L10						A	G		T			T			T	A					
						Met															
	<u>Gly</u>	<u>Phe</u>	<u>Leu</u>	<u>Lys</u>	<u>Lys</u>	<u>Glu</u>	<u>Val</u>	<u>Leu</u>	<u>Pro</u>	<u>Lys</u>	<u>Cys</u>	<u>Pro</u>	<u>Gln</u>	<u>Val</u>	<u>Phe</u>	<u>Asn</u>	<u>Lys</u>	<u>Leu</u>	<u>Gly</u>	<u>Met</u>	140
L1	GGA	TTC	TTG	AAG	AAA	GAA	GTT	CTT	CCA	AAA	TGT	CCA	CAA	GTC	TTT	AAT	AAG	TTA	GGA	ATG	420
L10		T	C				C											G			
			Phe				Ala														
	<u>Tyr</u>	<u>Pro</u>	<u>Pro</u>	<u>Asn</u>	<u>Met</u>	145															
L1	TAC	CCG	CCC	AAC	ATG	435															
L10																					

Fig. 3. Nucleotide sequences of two cDNA clones, L1 and L10, encoding Lig v 1. For L10, only differences with respect to the L1 sequence are indicated. The deduced amino acid sequences are shown above and below the corresponding nucleotide sequences. The data obtained by Edman degradation are underlined.

amino acid sequence of Ole e 1 (residues 141–145) [18]. The latter was considered assuming the same degree of similarity along the whole sequence of both allergens, on the basis of the *N*-terminal sequence and amino acid composition data (Table 1). Polymerase chain reaction amplification resulted in a fragment with an estimated size of about 440 bp, which must contain the whole genetic information for encoding Lig v 1. This fragment was isolated from agarose gels, reamplified and cloned into pUC18. Two cDNA clones were sequenced (Fig. 3), 45 nucleotide differences being determined between them (35 of these substitutions are silent). Some of the substitutions appeared at positions which are also heterogeneous in Ole e 1 and Syr v 1 allergens [9,18].

The two amino acid sequences deduced contain 145 residues and exhibit differences at eight positions. Six of these changes account for conservative substitutions (Val/Ile, Lys/Arg, Ile/Val, Val/Met, Leu/Phe, Val/Ala). The amino acid compositions deduced from these sequences are in agreement with the natural protein isolated from the pollen (Table 1). The polypeptides obtained can be considered as isoforms of the major allergen from privet. The two sequences contain a putative *N*-glycosylation site (NXT/S) at Asn-111. This position was demonstrated to be glycosylated in Ole e 1 [20]. The existence of a glycan moiety at this position would account for the positive response of Lig v 1 to concanavalin A. Figure 4 shows the comparison of sequences of privet, olive tree and

	10	20	30	40	50	60	70
L1	EDVPQPPVSQFYIQGQVYCDTCRARFITE	SEFIPGAGVRLQCKDGENGKVTFTEVG	YTKAEGLYNMLIERD	HK			
L10	-----	-----	-----	-----	I-----	R-----	S-----
OLE3c	-----H-----	G-----	SL-----	K-----	D-----	R-----	S-----V-----
OLE5c	-----H-----	-----	S-----	SI-----	R-----	S-----	-----
S28	-----IP--H-----	-----	SI-----	R-----	I-----	I-----	R-----S-----V-----G-----
S46	-----P--H-----	-----	SI-----	I-----	I-----	R-----	S-----V-----G-----

	80	90	100	110	120	130	140
L1	NEFCEITLISSSRKDCDEIPTEGWVKPSLKFV	LNTVNGTTRTINPLGFLKKEVL	PKCPQVF	NKLGMYPPNM			
L10	-----L-----	I-----	M-----	-----	F-----	A-----	-----
OLE3c	-----G-----	N-----	I-----	V-----	F-----	A-----	A-----Y-----
OLE5c	D-----	-----	V-----	M-----	F-----	A-----	-----
S28	-----G-E-----	V-----	A-----	K-----	I-----	F-----	A-----T-----Y-----
S46	-----G-----	V-----	K-----	-----	I-----	F-----	A-----T-----Y-----

Fig. 4. Alignment of the two deduced amino acid sequences of Lig v 1 cDNA clones with clones encoding Ole e 1 and Syr v 1. —, identity to L1.

lilac major allergens, which could be aligned without introducing gaps.

Expression of Lig v 1

In order to obtain the expression of Lig v 1 in *E. coli*, L10 cDNA clone was inserted into the pGEX-2T plasmid. This clone was selected because of the similarity of its amino acid composition with that of the natural protein (Table 1). The construction obtained produced a fusion protein (GST-Lig v 1) composed of glutathione *S*-transferase and the recombinant allergen, both linked through a hexapeptide that contains a thrombin cleavage site [21]. *Escherichia coli* cells transformed with the recombinant plasmid expressed, in a high yield, a polypeptide of a molecular mass of 43 kDa (Fig. 5a). This molecular size is in agreement with the molecular mass of 26 kDa for glutathione *S*-transferase and 16.5 kDa for the polypeptide chain of Lig v 1. When transferred to nitrocellulose sheets, this protein was recognized by a rabbit polyclonal serum raised against natural Ole e 1, as well as by a pool of four sera (CAP-FEIA classes 4 and 5) from olive pollen-allergic individuals with positive reaction against Ole e 1 (Fig. 5b). Both rabbit IgG and human IgE binding were inhibited when the corresponding serum was preincubated with Ole e 1.

Discussion

A new major pollen allergen has been isolated from privet, a

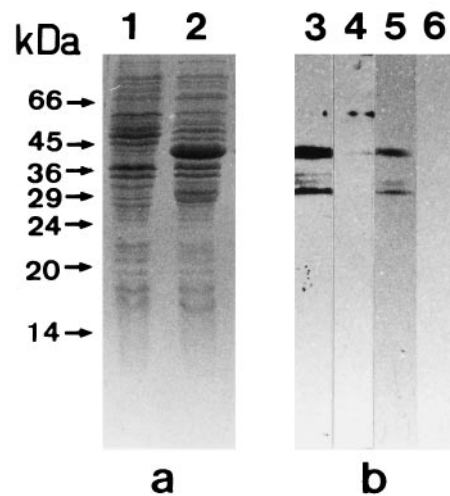


Fig. 5. Analysis of recombinant Lig v 1 expression. (a) Coomassie Blue staining of the sodium dodecyl sulphate polyacrylamide gel electrophoresis of a total cell lysate from a *Escherichia coli* strain (50 µl of culture) with pGEX-2T/L10 insert; lane 1, without isopropyl β-D-thiogalactoside (IPTG) induction; lane 2, after 3 h of induction with 1 mmol/l IPTG. (b) Western blotting of the same lysate as in lane 2, immunostained with: lane 3, rabbit polyclonal antiserum raised against Ole e 1; lane 4, same as lane 3, but the serum was preincubated with Ole e 1 (40 µg); lane 5, human IgE from a pool of sera from patients allergic to olive pollen; lane 6, same as lane 5, but the serum was preincubated with Ole e 1 (10 µg). The position of molecular mass markers is indicated.

member of the *Oleaceae* family. Although privet (*L. vulgare*) is a shrub growing in woods, a cultivated species (*L. ovalifolium*) is commonly used as a garden hedge in houses and parks of Europe and North America. Privet pollen is usually present at very low concentration and rarely develops pollinosis symptoms. However, under conditions of local and temporal exposure, it can induce allergic-like symptoms [22,23]. Lig v 1 has been obtained from the saline extract of privet pollen. The yield was lower than for Ole e 1, its counterpart in the olive pollen: 1 mg/g of dried privet pollen vs 5 mg from the olive pollen. Since the isolation procedure was essentially identical for both allergens, the different yield could be explained by a lower amount of Lig v 1 in the privet pollen, or a higher solubility and extractability for Ole e 1. These facts, in addition to the general low level of the airborne privet pollen grains, could contribute to the infrequent sensitization of hypersensitive individuals to this species [1,3].

The SDS-PAGE pattern of Lig v 1 is similar, but not identical, to those of its counterparts Syr v 1 from lilac and Ole e 1, the main component of Lig v 1 being the 20.0 kDa glycosylated form [8,13]. The components of 18.5, 22 and 40 kDa cannot be detected when staining for proteins. The non-glycosylated 18.5 kDa variant is relatively significant in Ole e 1, and the 22 kDa form in Syr v 1. This fact suggests a different degree of glycosylation depending on the species. The amino acid compositions of natural Lig v 1, Ole e 1 and Syr v 1 show a close similarity supporting a potential homology between the *Oleaceae* major allergens.

The amino acid sequences of two isoforms of Lig v 1 have been determined by cDNA cloning. These polypeptides show 94% identity. The finding of microheterogeneity in privet allergen suggests a high degree of polymorphism, as is also displayed by the homologous allergens Ole e 1 and Syr v 1 [9,18]. Polymorphism seems to be a general property of the most clinically relevant pollen allergens, such as Lol p 1, Amb a 1, Bet v 1 and Bet v 2. Microheterogeneity is a serious limitation for the isolation of homogeneous forms of allergens, as well as for the study of the structure-immunogenicity relationships in these proteins. Moreover, as has been recently discussed, isoallergens can display significant differences in reactivity towards antibodies and T-cells [24]. The expression of cDNAs encoding individual and related isoforms can be a valuable tool for research on their B- and T-cell epitopes.

As an initial step in the study of the structural and immunological relationships among *Oleaceae* allergens, the expression of a cDNA encoding Lig v 1 has been carried out. The recombinant allergen was obtained as a fusion protein, which is recognized by antibodies raised against Ole e 1 as well as by olive pollen allergic sera. In both cases the binding is completely inhibited by the Ole e 1 allergen. Therefore, the immunological analyses performed with both

the natural and the recombinant Lig v 1 allergens confirmed the data obtained for privet crude extracts [6,7]; this protein shares IgE as well as IgG epitopes with Ole e 1. In this regard, comparison of the sequences of Lig v 1 and Ole e 1 reveals a very high degree of similarity which can explain the immunological behaviour of privet protein against the anti-Ole e 1 antisera. On the other hand, some of the amino acid substitutions found in Lig v 1 in relation to Ole e 1 create a dramatic change in the character of their side-chains (e.g. positions 12 [Tyr/His], 25 [Arg/Gly], 38 [Gly/Ser], 46 [Gly/Lys] and 50 [Lys/Asp,Ser]; Fig. 4). These changes could explain the lack of binding of privet pollen extracts to the G1 family of Ole e 1-specific monoclonal antibodies [7], if some of the substituted residues are involved in the binding of the IgG to Ole e 1.

Recently, a pollen-specific protein from *Arabidopsis thaliana* (GenPept Data Bank accession no. Z26223), as well as Lol p 11 (a major allergen from ryegrass pollen [25]), have been sequenced. On the basis of the conserved amino acids and cysteine positions, and in spite of the low identity level (around 30%) with Ole e 1, these proteins have been suggested to be homologous to the olive allergen. All these molecules belong to a family of pollen specific proteins that also includes PS1 from rice [26], LAT52 from tomato [27] and Zm13 from maize [28]. These proteins are specifically expressed in masculine reproductive tissues of the plant, and their involvement in pollen germination and/or pollen tube growth has been suggested. The protein LAT52 has been shown to be essential for the hydration of tomato pollen during its germination [29]. Therefore, Ole e 1-like proteins could be general components of pollen, and certain cross-reactivities between pollen extracts of different species could be due to their structural similarity.

All the Ole e 1-like proteins exhibit an *N*-glycosylation consensus sequence, although its localization in the polypeptide chain seems to be family-dependent (position 29 in *Gramineae*, 45 in *Solanaceae*, 92 in *Brassicaceae* and 111 in *Oleaceae*). In *L. perenne* and *Oleaceae* species the actual presence of a carbohydrate moiety has been demonstrated. The oligosaccharides from Ole e 1 and Lol p 11 have been shown to be involved in the IgE binding to the allergens and are, therefore, the possible causes of cross-reactivity [20,25,30]. However, the binding of IgE from olive pollen-allergic patients to non-glycosylated recombinant Lig v 1 indicates that the cross-reactivity between olive and privet pollen proteins cannot reside exclusively in the carbohydrate moiety. Further studies on recombinant forms of these allergens will help to elucidate the precise role of the sugar and polypeptide chain in cross-reactivity.

The data obtained corroborate the existence of a common major allergen in the *Oleaceae* family. Due to their high

similarity, they could be involved in cross-sensitization of patients never exposed to a specific pollen, as well as in developing pollinosis symptoms. Therefore, cross-reactivity should be considered in the diagnosis and immunotherapy of type I allergy to *Oleaceae* pollens.

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References

- 1 D'Amato G, Mullins J, Nolard N, Spieksma FThM, Wachter R. City-spore concentration in the European Economic Community (EEC). VII. *Oleaceae* (*Fraxinus*, *Ligustrum*, *Olea*). *Clin Allergy* 1988; 18:541–7.
- 2 Bousquet J, Guerin B, Hewitt B, Lim S, Michel FB. Allergy in the Mediterranean area. III. Cross-reactivity among *Oleaceae* pollens. *Clin Allergy* 1985; 15:439–48.
- 3 Vela C, Platas C, Gurbindo C *et al.* Fractionation and biological characterization of *Olea europaea* pollen extract. *Int Arch Allergy Appl Immunol* 1982; 68:289–94.
- 4 Kennerman SM, McCullough J, Green J, Ownby DR. Evidence of cross-reactivity between Olive, Ash, Privet and Russian olive tree pollen allergens. *Ann Allergy* 1992; 69:493–6.
- 5 Baldo BA, Panzani RC, Bass D, Zerboni R. Olive (*Olea europaea*) and Privet (*Ligustrum vulgare*) pollen allergens. Identification and cross-reactivity with grass pollen proteins. *Mol Immunol* 1992; 29:1209–18.
- 6 Obispo TM, Melero JA, Carpizo JA, Carreira J, Lombardero M. The main allergen of *Olea europaea* (*Ole e I*) is also present in other species of the *Oleaceae* family. *Clin Exp Allergy* 1993; 23:311–6.
- 7 Martín-Orozco E, Cárdaña B, del Pozo V *et al.* *Ole e I*: Epitope mapping, cross-reactivity with other *Oleaceae* pollens and ultrastructural localization. *Int Arch Allergy Immunol* 1994; 104:160–70.
- 8 Batanero E, Villalba M, López-Otín C, Rodríguez R. Isolation and characterization of an olive allergen-like protein from lilac pollen. *Eur J Biochem* 1994; 221:187–93.
- 9 Pham NH, Baldo BA, Bass DJ. Cypress pollen allergy. Identification of allergens and cross-reactivity between divergent species. *Clin Exp Allergy* 1994; 24:558–65.
- 10 van Ree R, Aalberse RC. Pollen-vegetable food crossreactivity: serological and clinical relevance of crossreactive IgE. *J Clin Immunol* 1993; 16:124–30.
- 11 Nishiyama C, Yuuki T, Iwamoto N, Okomura Y, Okudaira H. Effects of amino acid variations in recombinant *Der f II* on its human IgE and mouse IgG recognition. *Int Arch Allergy Immunol* 1994; 105:62–9.
- 12 Chua KY, Greene WK, Kehal P, Thomas WR. IgE binding studies with large peptides expressed from *Der p II* cDNA constructs. *Clin Exp Allergy* 1991; 21:161–6.
- 13 Villalba M, Batanero E, López-Otín C *et al.* The amino acid sequence of *Ole e I*, the major allergen from olive tree (*Olea europaea*) pollen. *Eur J Biochem* 1993; 216:863–9.
- 14 Beaven GH, Holiday ER. Ultraviolet absorption spectra of proteins and amino acids. *Adv Protein Chem* 1952; 7:319–86.
- 15 Hewick RM, Hunkapiller MW, Hood LE, Dreyer W. A gas-liquid solid phase peptide and protein sequencer. *J Biol Chem* 1981; 256:7990–7.
- 16 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–5.
- 17 Hsi K-L, Chen L, Hawk DH, Zieske LR, Yuang P-M. A general approach for characterization glycosylation sites of glycoproteins. *Anal Biochem* 1991; 198:238–45.
- 18 Villalba M, Batanero E, Monsalve RI *et al.* Cloning and expression of *Ole e I*, the major allergen from olive tree pollen. *J Biol Chem* 1994; 269:15217–22.
- 19 Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain termination inhibitors. *Proc Natl Acad Sci USA* 1977; 74:5463–7.
- 20 Batanero E, Villalba M, Rodríguez R. Glycosylation site of the major allergen from olive tree pollen. Allergenic implications of the carbohydrate moiety. *Mol Immunol* 1994; 31:31–7.
- 21 Smith DB, Johnson KS. Single step purification of polypeptides expressed in *Escherichia coli*. *Gene* 1988; 67:31–40.
- 22 Macchia L, Caiaffa MF, D'Amato G, Tursi A. Allergenic significance of *Oleaceae* pollen. In: D'Amato G, Spieksma FThM, Bonini S, eds. *Allergenic pollen and pollinosis in Europe*. Oxford: Blackwell Scientific, 1991;87–93.
- 23 D'Amato G, Liccardi G. Pollen-related allergy in the European Mediterranean area. *Clin Exp Allergy* 1994; 24:210–9.
- 24 Lowenstein H, Sparholt SH, Klynser SS, Ipsen H, Larsen JN. The significance of isoallergenic variations in present and future immunotherapy. *Int Arch Allergy Immunol* 1995; 107:285–9.
- 25 van Ree R, Hoffman DR, van Dijk W *et al.* *Lol p XI*, a new major grass pollen allergen, is a member of a family of soybean trypsin inhibitor-related proteins. *J Allergy Clin Immunol* 1995; 95:970–8.
- 26 Zou J, Wong H, Wu H, Cheung AY. EMBL Data lib. S31710, 1992.
- 27 Twell D, Wing R, Yamaguchi J, McCormick S. Isolation and expression of an anther-specific gene from tomato. *Mol Genet* 1989; 217:240–5.
- 28 Hanson DD, Hamilton DA, Travis JL, Bashe DM, Mascarenhas JP. Characterization of a pollen-specific cDNA clone from *Zea mays* and its expression. *Plant Cell* 1989; 1:173–9.

- 29 Muschietti J, Dircks L, Vancanneyt G, McCormick S. LAT52 protein is essential for tomato pollen development: pollen expressing antisense *LAT52* RNA hydrates and germinates abnormally and cannot achieve fertilization. *Plant J* 1994; 6:321–38.
- 30 Batanero E, Villalba M, Monsalve RI, Rodríguez R. Cross-reactivity between the major allergen from olive pollen and unrelated glycoproteins: evidence of an epitope in the glycan moiety of the allergen. *J Allergy Clin Immunol* 1996; 97:1264–71.