

Isolation and characterization of an olive allergen-like protein from lilac pollen

Sequence analysis of three cDNA encoding protein isoforms

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An olive allergen-like protein has been isolated from lilac (*Syringa vulgaris*) pollen extract. The protein can be considered as an allergen since is recognized by IgE from olive hypersensitive human sera, and has been called *Syr v I* (IUIS nomenclature). This protein consists of a glycosylated polypeptide of 20 kDa, which has an amino acid composition, spectroscopic properties, and an N-terminal sequence similar to the major allergen from olive pollen, *Ole e I*. The lilac allergen is recognized by rabbit polyclonal antisera raised against olive allergen as well as by an *Ole e I*-specific monoclonal antibody. Using a polymerase chain reaction strategy, based on the similarities observed between these olive and lilac proteins, three cDNA clones encoding *Syr v I* have been isolated and sequenced. These clones code for a polymorphic protein of 145 residues with a derived molecular mass of about 16400 Da, which contains a potential *N*-glycosylation site. Comparison of the deduced amino acid sequences of these *Syr v I* isoforms to each other revealed identities of 90–97%. Moreover, these sequences showed a high degree of similarity (85.5–89.6% identity) with *Ole e I*. The structural and immunological characterization of *Syr v I* justify the cross-reactions observed between olive and lilac pollen extracts. The molecular cloning of *Syr v I* is relevant for the epitope mapping in Oleaceae allergens, and may contribute to an improvement in the design of reagents for diagnosis and therapy of IgE-dependent allergic reactions.

Type-I allergy to olive tree (*Olea europaea*) pollen is an important environmental disease in Mediterranean countries and some areas of North America [1, 2], where this Oleaceae is extensively cultivated. The major allergen from olive tree pollen (*Ole e I*) has been recently isolated and characterized [3–5]. Other Oleaceae members such as privet (*Ligustrum vulgare*), lilac (*Syringa vulgaris*) or ash (*Fraxinus excelsior*) are widely distributed in Central and North Europe, however the studies on their allergens are rare.

Antigenic cross-reactivity between olive and privet pollen proteins, detected by IgE antibodies, has been demonstrated [2, 6]. More recently, *Ole e I*-like proteins have been obtained from privet, ash and lilac pollen extracts using olive allergen-specific monoclonal antibodies coupled to a Sepharose column [7]. A preliminary characterization of these proteins showed some structural and immunological similarity with *Ole e I*, being recognized by IgE from olive pollen-

allergic sera. Therefore, the *Ole e I*-like proteins obtained from privet, ash and lilac, were called *Lig v I*, *Fra e I* and *Syr v I*, respectively [7], according to the recommended nomenclature for allergens [8]. The allergenic cross-reactivity observed between privet and olive pollen extracts [6] could be attributed to the presence of the *Ole e I*-like protein in the privet pollen saline preparations.

The characterization of protein allergens of pollens from related plants is of great interest since cross-reactions may play a role in the induction of immune responses [9]. Moreover, the cloning of allergens is an interesting strategy for the recombinant production of well defined and homogeneous proteins to be used in research and therapy [10], since it is difficult to obtain reproducible preparations of allergens from their natural sources. The cloning and sequencing of several pollen allergens have recently been reported, but no data are available from Oleaceae allergens. In this study, we describe the isolation and characterization of the olive allergen-like protein, *Syr v I*, from lilac. The complete nucleotide sequences of three cDNA encoding this polymorphic protein are also reported, and the similarities of the deduced proteins with *Ole e I* are discussed.

MATERIALS AND METHODS

Isolation of *Syr v I*

S. vulgaris pollen (Allergon AB; 1 g) was suspended in 50 ml 10 mM ammonium bicarbonate, pH 8.0, containing

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Abbreviations. HRPO, horseradish peroxidase; PCR, polymerase chain reaction.

Enzymes. Restriction endonucleases *EcoRI* and *BamHI* (EC 3.1.21.4); reverse transcriptase (EC 2.7.7.49); *Taq* polymerase, DNA polymerase from *Thermophilus aquaticus* (EC 2.7.7.7).

Note. The novel nucleotide sequence data published here have been submitted to the EMBL sequence data bank(s) and are available under accession number(s) X76539, X76540, X76541.

1 mM phenylmethylsulfonyl fluoride, and extracted for 60 min at 4°C. The pellet was reextracted twice under the same conditions. After centrifugation, the clear supernatants were lyophilized, dissolved in 2 ml 0.2 M ammonium bicarbonate, pH 8.0, and chromatographed on a Sephadex G-75 gel-filtration column (1.5 cm×80.0 cm), equilibrated in the same buffer. The fractions corresponding to a molecular mass of about 20 kDa (approximately 18 ml) were pooled and lyophilized. The sample was dissolved in 5 ml 10 mM ammonium bicarbonate, pH 8.0, and chromatographed on a DEAE-cellulose column (2 cm×20 cm) equilibrated in the same buffer. After washing, first with 10 mM, then with 50 mM ammonium bicarbonate, pH 8.0, the protein was eluted with 200 mM bicarbonate buffer.

Amino acid analysis

Native protein (1–5 nmol) was hydrolyzed at 105°C for different lengths of time (24, 48, 72 and 120 h) with 5.7 M HCl containing 0.1% (mass/vol.) phenol and 20 nmol/ml 2-aminohexanoic acid as internal standard, in sealed tubes under vacuum. Hydrolysed samples were analyzed on a Beckman system 6300 amino acid analyzer with an analog interface module of System Gold. The cysteine content was determined as carboxymethylcysteine after hydrolysis of the alkylated protein. Tryptophan was determined spectrophotometrically [11]. When pure, protein concentrations were measured by amino acid analysis.

Electrophoresis, immunoblotting and carbohydrate detection

Analytical gel electrophoresis was performed according to Laemmli [12] in 15% (mass/vol.) polyacrylamide gels. Proteins were visualized by Coomassie blue staining. Protein bands in SDS/PAGE were transferred onto nitrocellulose membranes (BioRad) using a LKB Novablot 2117 electrophoretic transfer unit (LKB) in 48 mM Tris/HCl, pH 9.0, containing 39 mM glycine, 20% (by vol.) methanol and 0.036% SDS, for 1 h at 1 mA/cm², as described in [13].

Sera from eight allergic patients with positive skin test for olive pollen crude extract and with the highest radioallergosorbent test (values 3–4) were used. Polyclonal sera against *Ole e 1* were prepared immunizing two New Zealand White rabbits over a 6-week period by weekly injection of the protein (100 µg) in Freund's adjuvant. Sera were collected after bleeding. The *Ole e 1*-specific monoclonal antibody 19G6 was kindly donated by Dr Lahoz. It was obtained by immunizing eight BALB/c mice with olive pollen crude extract as previously described [14].

For IgE-binding assays, the hypersensitive human sera [1:8 dilution in 10 mM sodium phosphate, pH 7.2, containing 140 mM NaCl, 2.7 mM KCl (NaCl/P_i buffer), and 0.1% Tween 20, and 3% non-fat milk] and horseradish-peroxidase (HRPO)-labelled goat anti-(human IgE) (Nordic Immunology) diluted 1:1000 in the same buffer, containing 0.05% Tween 20 and 1% non-fat milk, were used [5]. Alternatively, 1:5000-diluted goat anti-mouse IgG (Pierce) or 1:3000 anti-rabbit IgG (BioRad), both HRPO labelled, were used in the immunoblotting staining for monoclonal and polyclonal antibody binding assays, respectively. The peroxidase reaction was developed with the ECL Western-blotting reagent (Amersham).

Carbohydrate detection of transferred protein was performed essentially as described [15] by using a biotinylated

Concanavalin-A solution and the ABC reagent (avidin, biotinylated HRPO), diluted 1:400 in 50 mM Tris/HCl, pH 7.5, containing 0.5 M NaCl and 0.1% poly(vinyl pyrrolidone). The staining was developed with the chemiluminescent reagent, as described above for HRPO-labelled antibodies.

Automated sequence analysis

N-terminal Edman degradation of the native protein was performed using an Applied Biosystems model 477A sequencer [16]. The resulting phenylthiohydantoin-amino-acid derivatives were identified using a 120A on-line phenylthiohydantoin-amino-acid analyzer and the standard Applied Biosystems program.

Isolation of total RNA

Total RNA was isolated from lilac pollen (Allergon AB) according to [17] with minor modifications. Pollen (0.5 g) was homogenized on a Polytron (Brinkman) in 100 mM Tris/HCl, pH 7.5, containing 140 mM 2-mercaptoethanol, 4 M guanidine thiocyanate, 0.5% sodium *N*-lauroylsarcosine. The resulting suspension was centrifuged on a SS-34 rotor (Sorvall) at 6500 rpm and 20°C, for 10 min. The supernatant (2.0 ml) was centrifuged on top of 3.0 ml of a 5.7-M CsCl cushion in 0.01 M EDTA at 40000 rpm in a SW60 rotor (Beckman) for 12 h, at 20°C. The pellet was dissolved in 10 mM Tris/HCl, pH 7.6, containing 1.0 mM EDTA and 0.1% SDS, and precipitated with ethanol [18].

cDNA preparation

Single-stranded cDNA was synthesized from the obtained total RNA (5 µg) using the cDNA synthesis Kit (Pharmacia) and oligo dT as primer, according to [19] and the manufacturer's instructions.

Synthesis of oligodeoxynucleotides

Oligodeoxynucleotides, SV-1, SV-2 and SV-3, were synthesized using an Applied Biosystems model 381A DNA synthesizer. The SV-1 sequence was designed based on the N-terminal amino acid sequences of the olive and lilac allergens (positions 1–5): 5'-CCGGGATCCGARGAYGTGCC-XCA-3'. Two primers, SV-2 and SV-3, were designed according to the C-terminal amino acid sequence of the olive pollen allergen, whose 3'-nucleotide cDNA sequence had been previously obtained (unpublished results); SV-2 was degenerate (5'-ATCATTNGGNGGRTACATNGG-3'; positions 140–145 of the amino acid sequence) and SV-3 is non-degenerate (5'-CGGAATTCACATGTTGGGCGGGTA-3'; positions 141–145 on the protein). Primers SV-1 and SV-3 contained a *Bam*HI restriction site and an *Eco*RI restriction site, respectively (underlined).

Polymerase-chain-reaction-based cloning

The cloning method was based on the polymerase-chain-reaction (PCR) method [20]. The template cDNA pool and amplification primers, SV-1 and SV-2, were denatured at 95°C for 15 min, in 50 µl PCR mixture (2.5 mM of each dNTP in *Taq* polymerase buffer containing 10 mM Tris/HCl, pH 8.3, 50 mM KCl, 1 mM MgCl₂ and 0.01% gelatin). Afterwards, 1.25 U *Taq* polymerase (Bioprobe) was added and overlaid with 50 µl mineral oil. The sample was subjected to

30 cycles of denaturation at 94°C (1 min), annealing at 42°C (90 s) and chain elongation at 72°C (2 min) in a programmable DNA thermal cycler (Techne). The temperature was then held at 72°C for 10 min and gradually cooled to room temperature. Afterwards, a new reamplification reaction was carried out with SV-1 and SV-3, using five cycles of denaturation at 94°C (1 min), annealing at 47°C (1 min), chain extension at 72°C (90 s), and 20 cycles of 94°C (1 min), 55°C (90 s) and 72°C (90 s). A last step at 72°C for 10 min was also performed. Under these conditions, a single PCR product of about 0.4 kbp was obtained. This amplified cDNA was analyzed, eluted from a 2% agarose gel, and purified by PCR magic preps (Promega). After *Eco*RI and *Bam*HI digestion, the amplified fragment was incorporated into an *Eco*RI/*Bam*HI-digested pUC 18 plasmid vector and used for transformation of competent *Escherichia coli* DH5 α F' cells. Selected recombinants bearing inserts with the expected size were sequenced.

Sequence analysis of *Syr v* I clones

pUC18 clones were sequenced (Sequenase Kit, US Biochemicals) using the dideoxy chain termination method [21] and deoxy[α^{35} -S]ATP. Sequencing primers for both strands, M13mp18 universal and reverse, were from Biolabs. The sequencing was carried out according to the manufacturer's recommendations.

RESULTS

Isolation and characterization of the olive allergen-like protein from lilac pollen

An olive allergen-like protein has been isolated from lilac (*S. vulgaris*) pollen extracts by gel filtration (Fig. 1) and ion-exchange chromatography on DEAE-cellulose. The protein obtained (4–5 mg/g dried pollen) was homogeneous by SDS/PAGE analysis (Fig. 1) and N-terminal amino acid sequencing. The isolated protein gives the electrophoretic pattern shown in Fig. 2a. Two bands, both immunoreactive, are detected at 20 kDa and 22 kDa, which account for 80% and 20%, respectively, of the total protein preparation. Both polypeptides are glycosylated since they stain after transfer to nitrocellulose membranes and treatment with biotinylated concanavalin A (Fig. 2b). This pattern is similar to that previously described for *Ole e* I, the major olive pollen allergen [5, 22, 23]. However, the relative proportions of each component are different. It has been suggested that the 20-kDa and 22-kDa bands of *Ole e* I only differ in the degree of glycosylation of the polypeptide chain, the 18-kDa form being demonstrated to be the non-glycosylated variant [23]. The N-terminal sequence data, molecular mass and carbohydrate staining of *Syr v* I suggest the existence of the two glycosylated variants (20 kDa and 22 kDa forms) as components of this allergen, but the near absence of the non-glycosylated form.

The amino acid composition of the obtained protein is given in Table 1. The N-terminal Edman degradation of the molecule gave a single sequence; Glu-Asp-Val-Pro-Gln-Pro-Pro-Ile-Pro-Gln-Phe-His-Ile-. Two amino acids, Ile (60%) and Val (40%), were obtained at position 8. This sequence is very similar, but not identical, to that reported by Obispo et al. [7] for the protein isolated by affinity chromatography. They obtained Ser and Tyr at positions 9 and 12, respectively, instead of Pro and His as in our case. Spectroscopic analysis

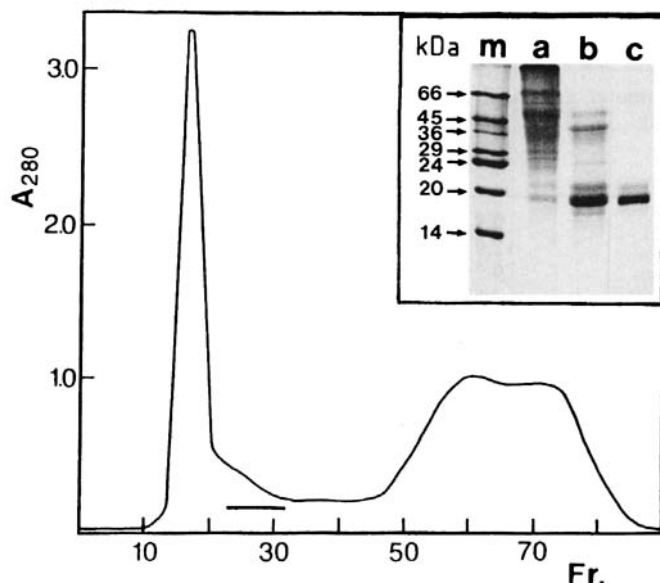


Fig. 1. Isolation of *Syr v* I. Elution profile from the gel-filtration chromatography column on Sephadex G-75 of the saline extract from lilac pollen. Fractions under the bar were pooled, lyophilized and chromatographed on DEAE-cellulose. Inset, SDS/PAGE analysis of different steps of the isolation procedure; (a) saline extract; (b) pooled fractions eluted from gel filtration; (c) protein fractions eluted with 0.2 M ammonium bicarbonate from the DEAE-cellulose column; (m) molecular-mass protein markers (BioRad).

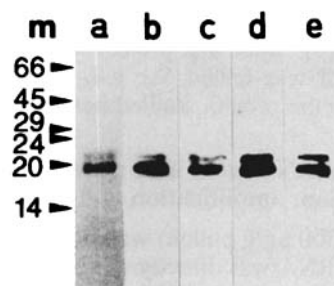


Fig. 2. SDS/PAGE analysis of *Syr v* I. (a) Coomassie Blue staining; (b) biotinylated Concanavalin-A staining; (c) immunodetection with IgE of olive-pollen hypersensitive human serum; (d) immunostaining with rabbit polyclonal antiserum raised against *Ole e* I allergen; (e) immunostaining with *Ole e* I-specific mouse monoclonal antibody 19G6. The staining of b–e was obtained after electro-transfer to nitrocellulose membranes, then using the ECL reagent to develop the peroxidase reaction of the corresponding HRPO labelled secondary antibodies (for details see Materials and Methods). 4 μ g protein were applied in lane (a), 0.35 μ g in lanes (b), (d) and (e) and 0.5 μ g in lane (c). (m) molecular-mass protein markers.

(circular dichroism and fluorescence spectra; data not shown) of the isolated lilac protein indicates identical conformational characteristics to those of olive pollen allergen [5].

The *Ole e* I-like protein isolated from lilac extracts is recognized on immunoblotting assay by the IgE of a pool of sera obtained from olive pollen allergic patients (Fig. 2c). The structural similarity between these lilac and olive pollen proteins is supported by the recognition of the latter, not only by the polyclonal sera raised against *Ole e* I, but also by the *Ole e* I-specific monoclonal antibody 19G6 (Fig. 2d, e). These results demonstrate the existence of shared antigenic and allergenic epitopes in these proteins.

Table 1. Amino acid composition of *Syr v I* allergen. Cysteine was determined as the carboxymethylated derivative.

Amino acid	Analysis	No. of residues deduced from the sequences of <i>Syr v</i> clones		
		I.1	I.2	I.3
Cys	5.7	6	6	6
Asx	12.9	13	11	13
Thr	9.7	11	10	11
Ser	7.5	6	6	6
Glx	15.8	19	18	18
Pro	10.7	11	12	11
Gly	13.0	11	12	12
Ala	6.5	5	4	4
Val	8.1	6	8	8
Met	2.2	3	3	3
Ile	9.9	12	11	11
Leu	10.3	9	11	9
Tyr	4.5	5	4	5
Phe	7.0	8	9	8
His	3.2	2	3	2
Lys	9.3	10	10	11
Arg	7.4	7	6	6
Trp	1 ^a	1	1	1
Total	145	145	145	145

^aDetermined spectroscopically.

According to the structural and immunological results obtained, it can be deduced that this purified lilac protein corresponds to that obtained through affinity chromatography by Obispo et al. [7]. Since that protein was considered as the lilac allergen and was called *Syr v I*, we shall adopt this nomenclature for the protein studied here.

Isolation of total RNA from lilac pollen, cDNA preparation, amplification and sequence

Total RNA (300 µg/g pollen) was isolated from lilac pollen. 5 µg of this RNA was directly used as a template for the synthesis of single-stranded cDNA. This cDNA was used for the PCR amplification of the specific DNA encoding *Syr v I*. A mixture of degenerate oligodeoxynucleotides, SV-1 and SV-2, designed on the basis of the known amino acid sequence of the olive pollen allergen, was used as primers in the first round of PCR amplification. They were selected because of the identity observed in the five residues at the N-termini of olive and lilac allergens. The same degree of similarity at the five C-terminal amino acids was supposed. In the second round, the reaction was carried out using both the same oligonucleotide SV-1 and the non-degenerate SV-3 in order to increase the efficiency of the PCR amplification. The primers used to prepare the specific cDNA also contained *Bam*HI and *Eco*RI adaptor sites for obtaining the correct ligation of the amplified cDNA into PUC18 plasmid vector. A single PCR product of about 0.4 kbp was obtained (Fig. 3). The clones were obtained in *E. coli* DH5aF⁺ cultures after transformation with the above construction, and were used for sequencing. Three different DNA sequences were obtained. The complete nucleotide sequences are composed of 444 bases, with a reading frame (Fig. 4) corresponding to polypeptides of 145 amino acids. The primary sequences are shown in Fig. 5. The amino acid composition derived from the deduced amino acid sequences agrees well with that obtained by acidic hydrolysis of the purified protein (Table 1).

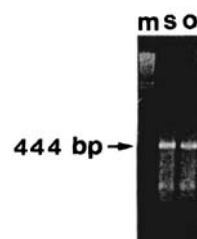


Fig. 3. PCR amplification of lilac allergen cDNA. (s) cDNA PCR was performed on 5 µg total RNA from lilac pollen in a volume of 50 µl. The product was purified on 2% agarose gel. (o) PCR product obtained from olive pollen under identical conditions. (m) λDNA digested with *Hind*III (US Biochemicals) was used as marker (bp, base pairs).

Taking into account the high similarity observed among the cloned sequences, identity values ranging over 89.6–97.2% (4–15 different amino acids), they have been considered as polymorphic forms of the lilac pollen protein and, according to the IUIS nomenclature, they can be called *Syr v I.1*, *Syr v I.2* and *Syr v I.3*. The similarity of these sequences with *Ole e I* allergen, ranging over 85.5–89.6% (15–21 different amino acid residues), is enough to justify the recognition of native *Syr v I* by *Ole e I*-specific antibodies (Fig. 2).

The deduced polypeptide sequences show a single putative *N*-glycosylation site in the three isoforms located at Asn111. Therefore, the positive reaction of the electrotransferred *Syr v I* with biotinylated concanavalin A (Fig. 2b) suggests that this Asn111 is glycosylated. This residue has been demonstrated to be glycosylated in the olive allergen [14].

DISCUSSION

Cross-reactivity among allergens may play an important role in the induction of type-I allergies. It is known that clinical symptoms often appear when patients allergic to certain tree pollinosis are exposed to related pollens [24]. This is expected to be more pronounced for pollens from trees belonging to the same botanical order or family. In fact, there is evidence for cross-reactivity among pollen extracts of the order Fagales (alder, birch, hazel and hornbeam) [25], as well as among common cypress and Japanese cedar pollens (Pinales) [26]. These cross-reactivities have been associated with the presence of common epitopes in their major allergens, and are correlated with either similar amino acid sequences of polypeptide segments or common carbohydrate determinants [9, 27, 28]. For the Oleaceae family, immunological cross-reactions have been described between the saline extracts of pollen from privet and olive tree [6]. However, recent data about the epitope mapping of olive allergen and cross-reactivity with other Oleaceae pollens showed differences in the immunological behaviour of the lilac, privet, ash and forsythia extracts against *Ole e I*-specific monoclonal antibodies (unpublished results). Therefore, a more detailed structural and immunological analysis of these *Ole e I*-like proteins is necessary.

An olive pollen allergen-like protein, *Syr v I*, was isolated from lilac pollen extracts in high yield, although in lesser yields than that obtained for the olive counterpart. This could be explained based on two experimental observations; more difficult extraction of the protein from the lilac pollen wall than from olive pollen, and/or a greater tendency of the lilac

S28	<u>GAG GAC GTG CCC CAA</u> CCC CCA ATT CCT CAA TTC CAC ATT CAA GGA	45
S38	G	
S46	T	
S28	CAA GTT TAT TGT GAT ACA TGT CGT GCT AGA TTC ATT ACA GAA CTT	90
S38	C	
S46	C G	
S28	AGC GAG TTT ATC CCA GGT GCT AGC ATA CGC CTC CAA TGC AAA GAC	135
S38	G T	
S46	C T	T
S28	AGG GAG AAT GGT AAA ATA ACA TTT ACT GAG ATA GGT TAC ACG AGA	180
S38	G C	
S46	G C	
S28	GCA GAA GGA CTC TAC AGC ATG CTT GTT GAA GGT GAT CAC AAG AAC	225
S38		
S46	G	
S28	GAG TTT TGC GAA ATC ACA CTG ATT TCA AGT GGC AGA GAA GAT TGT	270
S38	T C	A
S46		A
S28	GAT GAA ATT CCT GTT GAA GGA TGG GCA AAA CCA TCA TTA AAA TTT	315
S38	A C A	T G
S46		TC G
S28	AAA CTT AAT ACG GTA AAT GGT ACC ACA CGC ACG ATA AAT CCT ATT	360
S38	T A A	C
S46		
S28	GGA TTC TTC AAG AAA GAA GCT CTT CCA AAA TGC ACA CAA GTC TAT	405
S38	T	T C
S46		T
S28	AAT AAG TTG GGT <u>ATG TAC CCG CCC AAC ATG</u>	435
S38	A C	
S46	A	

Fig. 4. Nucleotide sequences of three cDNA clones at the *Syr v* I-encoding region. Dashes indicate identities of nucleotides with the upper sequence. Partial sequences of the primers are underlined.

S28	<u>E D V P Q P P I P Q F H I Q</u> G Q V Y C D T C R A R F I T E L	10	20	30
S38	V			
S46	V			
<i>Ole e</i>	I V S			G
S28	S E F I P G A S I R L Q C K D R E N G K I T F T E I G Y T R	40	50	60
S38	G	G H		
S46		G		
<i>Ole e</i>	L K D V V			
S28	A E G L Y S M L V E G D H K N E F C E I T L I S S G R E D C	70	80	90
S38			L S	K
S46				K
<i>Ole e</i>	R			K
S28	D E I P V E G W A K P S L K F K L N T V N G T T R T I N P I	100	110	120
S38	E I V	I		L
S46	V			
<i>Ole e</i>	N T			V L
S28	G F F K K E A L P K C T Q V Y N K L G M Y P P N M	130	140	145
S38	V	P F		<i>Syr v</i> I.1
S46				<i>Syr v</i> I.2
<i>Ole e</i>	A			<i>Syr v</i> I.3

Fig. 5. Deduced amino acid sequences of the three *Syr v* I isoforms. The sequence of *Ole e* I is shown for comparison. The N-terminal sequence obtained by Edman degradation of the isolated protein is underlined.

protein to aggregate, forming poorly soluble polymeric structures, which are excluded on Sephadex G-75 gel filtration. A different behaviour in terms of solubility has been observed for *Syr v* I and *Ole e* I, mainly when they are chromatographed on a reverse-phase HPLC column, after which *Syr v* I becomes insoluble (data not shown). Many substitutions in

the amino acid sequence of *Syr v* I in comparison with *Ole e* I are of non-conservative character (e.g. positions 9, 48, 50, 71, 91, 95, etc). If some of these positions were located at particularly exposed regions, it could be enough to create solubility differences. It is well known that punctual amino acid substitutions in a polypeptide chain can induce dramatic

changes on physical and functional properties of a protein. The slow release of *Syr v* I from the lilac pollen wall and the low solubility, in addition to the non-anemophilous (non-wind pollinating) character of *S. vulgaris* [29], could also explain the low incidence of this allergen in the human population, even under conditions of local exposure to risk concentrations of pollen grains.

The molecular and immunological properties of the lilac allergen are very similar to those shown by *Ole e* I [5, 23]. Nevertheless, the olive pollen allergen have three variants of the same 145-amino-acid polypeptide chain, non-glycosylated (18 kDa) and glycosylated (20 kDa and 22 kDa) forms, whereas *Syr v* I would be composed of a main component, a glycosylated polypeptide of about 145 amino acids in length (20 kDa), and a variant with a greater degree of glycosylation (22 kDa).

Current PCR techniques are particularly useful for cloning and sequencing cDNA corresponding to structurally related proteins which differ in a few nucleotides. The specific cDNA amplification from total RNA preparations only depends on the knowledge of partial amino acid sequences of the protein under study [30–33]. Considering the total identity of the five N-terminal amino acid residues of these olive and lilac allergens, and assuming a similar degree of similarity at the C-terminal ends of these proteins, oligonucleotide primers were designed in order to obtain the PCR amplification of the lilac cDNA. The experiment allowed the isolation of a DNA fragment, which exhibits the expected size to encode an *Ole e* I-like protein. Three different cDNA clones were analyzed, the first nucleotide sequences of Oleaceae allergens being obtained. The sequence differences observed for the *Syr v* I isoforms are unlikely to be due to PCR artefacts because of many reasons. The expected error frequency of the *Taq* polymerase ($1/10^{-5}$ nucleotides per cycle) [34] is much lower than the observed exchanges. High amounts of the observed nucleotide exchanges are silent; 48, 63 and 73%, respectively, for *Syr v* I.1, *Syr v* I.2 and *Syr v* I.3 isoforms, which are not expected values for the *Taq* polymerase activity. Reamplification of a specific cDNA clone rendered a nucleotide sequence identical to the original template. Finally, some of the nucleotide heterogeneities detected in lilac cDNA lead to amino acids appearing at the same positions in the olive allergen, which is far from the statistical probability of error.

The microheterogeneity observed in both olive [5] and lilac allergens is not exclusive to the Oleaceae family since it is also exhibited by a remarkable number of allergens from different natural sources. The most important pollen allergens, from a clinical point of view, show a polymorphic character; tree pollen allergens such as *Cor a* I [35], ragweed pollen allergens, such as *Amb a* I and *Amb a* II [36] and grass allergens, such as *Poa p* IX and *Lol p* I [32, 37].

The amino acid sequence differences observed between olive and lilac pollen allergens isoforms could result in different immunological behaviours. It has been demonstrated that recombinant *Cor a* I isoallergens from hazel pollen display different IgE-binding and IgG-binding properties [35]. Thus, it seems that a few amino acid differences in the protein sequence would be enough to show selective recognition by individual allergic sera or monoclonal antibodies. Therefore, the differences observed when assaying the IgG binding of lilac, ash, privet and forsythia pollen extracts against several *Ole e* I-specific monoclonal antibodies would be justified by the existence of amino acid substitutions at the antigenic determinants of their closely related, but not identical,

allergens. The knowledge of the complete amino acid sequences of the Oleaceae allergens, as well as the cloning and expression of the distinct isoforms will allow important advances in finding the structure/antigenicity relationships for these molecules and will improve the design and production of well-defined antigens for clinical purposes.

Finally, the *Ole e* I-like proteins are of additional interest since they exhibit more than 30% sequence identity with the proteins encoded by *Zmc13* and *LAT52* genes, obtained from maize and tomato pollens, respectively [38, 39]. The alignment of their primary sequences allows the identification of conserved segments, which could be involved in the biological role suggested for these proteins in the pollen germination process [38, 39].

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