

A recombinant isoform of the Ole e 7 olive pollen allergen assembled by *de novo* mass spectrometry retains the allergenic ability of the natural allergen

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ABSTRACT

The allergenic non-specific lipid transfer protein Ole e 7 from olive pollen is a major allergen associated with severe symptoms in areas with high olive pollen levels. Despite its clinical importance, its cloning and recombinant production has been unable by classical approaches. This study aimed at determining by mass-spectrometry based proteomics its complete amino acid sequence for its subsequent expression and characterization. To this end, the natural protein was in-2D-gel tryptic digested, and CID and HCD fragmentation spectra obtained by nLC-MS/MS analyzed using PEAKS software. Thirteen out of the 457 *de novo* sequenced peptides obtained allowed assembling its full-length amino acid sequence. Then, Ole e 7-encoding cDNA was synthesized and cloned in pPICZαA vector for its expression in *Pichia pastoris* yeast. The analyses by Circular Dichroism, and WB, ELISA and cell-based tests using sera and blood from olive pollen-sensitized patients showed that rOle e 7 mostly retained the structural, allergenic and antigenic properties of the natural allergen. In summary, rOle e 7 allergen assembled by *de novo* peptide sequencing by MS behaved immunologically similar to the natural allergen scarcely isolated from pollen.

Significance: Olive pollen is an important cause of allergy. The non-specific lipid binding protein Ole e 7 is a major allergen with a high incidence and a phenotype associated to severe clinical symptoms. Despite its relevance, its cloning and recombinant expression has been unable by classical techniques. Here, we have inferred the primary amino acid sequence of Ole e 7 by mass-spectrometry. We separated Ole e 7 isolated from pollen by 2DE. After in-gel digestion with trypsin and a direct analysis by nLC-MS/MS in an LTQ-Orbitrap Velos, we got the complete *de novo* sequenced peptides repertoire that allowed the assembling of the primary sequence of Ole e 7. After its protein expression, purification to homogeneity, and structural and immunological characterization using sera from olive pollen allergic patients and cell-based assays, we observed that the recombinant allergen retained the antigenic and allergenic properties of the natural allergen. Collectively, we show that the recombinant protein assembled by proteomics would be suitable for a better *in vitro* diagnosis of olive pollen allergic patients.

1. Introduction

Olive pollen is responsible for Type I respiratory responses in the Mediterranean countries and large areas of America, South Africa, and Australia [1]. Thirteen allergens from *Olea europaea* -Ole e 1 to Ole e 13- have been identified and characterized to date [2, 3]. Among them, Ole e 7, a non-specific lipid binding protein (nsLTP), exhibits a molecular mass of 10 kDa and a basic character with multiple isoforms

reported with pIs ranging from 8 to 10 [4]. nsLTPs are a family of proteins widely distributed throughout the plant kingdom [5]. These proteins are integrated into the protein superfamily of prolamins, which includes more than 3000 proteins from more than 250 species mainly found in plants [6]. nsLTPs contain an eight-cysteine residue conserved motif with four disulfide bonds, and an internal hydrophobic cavity where the lipid-binding site is located. Many of these nsLTPs have been described as relevant allergens from different biological sources such as

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pollen, food or latex [7–15]. Among them, Ole e 7 is a major allergen associated to severe clinical symptoms in those regions with high olive pollen counts, where olive tree is extensively cultivated such as Andalusia in Spain, or Portugal and Italy [3, 16]. Due mainly to its high degree of polymorphism or its mRNA instability, its cloning was unable, and Ole e 7 remained to be produced as a recombinant allergen from its identification in 1999 to be used in clinics [4], and mainly for diagnosis.

The primary structure of the majority of reported allergens has been commonly inferred from their corresponding nucleotide sequence obtained by cloning of their specific cDNA. However, as the genome sequence of many biological sources remains to be elucidated, and therefore if the protein of study is highly polymorphic or its mRNA is highly unstable, it might become virtually impossible to obtain its nucleotide sequence - as occurred with Ole e 7 - [4]. An alternative approach for the completion of amino acid sequences consists of *de novo* peptide sequencing, where a peptide sequence is directly obtained from a MS/MS spectrum and, therefore, a protein database is not required. Thanks to the extensive development of mass spectrometers, the primary structure of proteins from unsequenced biological sources genomes might now be reliably achieved. The combination of different fragmentation techniques and software, enabling the combination of complementary fragmentations (*i.e.* collision-induced dissociation –CID- and higher-energy collisional dissociation –HCD-), can be used for automated *de novo* peptide sequencing with high accuracy at amino acid level [17]. Indeed, these methods can now be applied not only for identifying and/or quantifying peptides and proteins, but also for determining the primary structure of allergens from unsequenced organisms [18–22].

The aim of the present study consists of the assembling of the whole Ole e 7 amino acid sequence from different peptide fragments obtained by multiple *de novo* peptide sequencing by mass spectrometry. Then, we produced Ole e 7 as a recombinant protein in *Pichia pastoris*, determining that its molecular and immunological behavior was similar to the natural allergen. Collectively, the here obtained data assessed the effectiveness of the strategy to determine the primary structure of highly polymorphic allergens from unsequenced genomes, and the usefulness of the recombinant Ole e 7 allergen assembled by proteomics for clinical patient diagnosis.

2. Materials and methods

See the Materials and methods section in this article's Supplementary data for full details about materials, expression and purification of the recombinant allergen, two-dimensional gel electrophoresis, analytical procedures, circular dichroism spectroscopic analyses, IgG and IgE immunoassays, inhibition experiments, and cell-based allergic mediator release assays.

2.1. Sera

Sera from 62 well-characterized patients sensitized to olive pollen were used to carry out immunological assays. Well-characterized sera from sensitized patients to olive pollen were obtained from two regions of Spain with high olive pollen counts: Jaén ($n = 39$) and Málaga ($n = 23$) [23–25]. Sera from non-atopic individuals were used as controls. Written informed consent was obtained from all patients. The work was performed accomplishing the Ethic Guidelines of Complutense University of Madrid.

2.2. Ole e 7 digestion, mass spectrometry analysis and *de novo* peptide sequencing

Ole e 7 (5 µg) from olive pollen was separated by two-dimensional gel electrophoresis -2DE- [23]. The main spot of the protein was in-gel digested with trypsin (Fig. 1) and peptides extracted using 100%

acetonitrile (ACN) and 0.5% trifluoroacetic acid.

Extracted peptides were dried and purified using a Zip Tip with 0.6 µL C18 resin (Millipore). Finally, samples were reconstituted in 5 µL 0.1% formic acid/2% ACN before the analysis by nLC-MS/MS in the Proteomics Facility of the Centro de Investigaciones Biológicas (Madrid, Spain). Peptides were trapped in a C18-A1 ASY-Column 2-cm pre-column (Thermo Scientific) and eluted to a reverse phase column (15 cm × 75 µm inner diameter, Nanoseparations) packed with 3 µm particle C₁₈ resin, where they were separated using a 150 min gradient from 0 to 35% buffer B (Buffer A: 0.1% Formic acid/2% ACN; Buffer B: 0.1% Formic acid in ACN) at a flow rate of 250 nL/min. The LC system, a NanoEasy HPLC (Proxeon), was coupled to an LTQ Orbitrap Velos (Thermo Scientific) via an electrospray ion source (Proxeon). The LTQ Orbitrap Velos was operated in an ion positive mode and in a data dependent manner as indicated [26, 27].

CID and HCD fragmentation spectra were analyzed with PEAKS Studio software (Bioinformatics Solutions Inc.) for *de novo* peptide sequencing. Amino acid sequences were aligned with DIALIGN and visualized with GeneDoc software.

2.3. rOle e 7 expression and purification

The synthetic cDNA-encoding sequence of Ole e 7 was purchased from IDT-DNA Technologies (Supplementary Fig. 1), directly subcloned in pPICZαA and transformed into KM71H *P. pastoris* strain yeast cells to express the protein in the extracellular media. Then, the extracellular media containing the recombinant protein was lyophilized and subsequently purified by chromatography and RP-HPLC.

See the methods section in this article's Supporting information for the full details about the expression and purification of rOle e 7.

3. Results

In previous reports, we identified, purified the allergen Ole e 7 from olive pollen, and analyzed its contribution to olive pollen allergy [3, 4, 28–30]. Although partial amino acid sequences of Ole e 7 were obtained by means of Edman degradation and peptide fingerprinting and deposited in protein databases, the high number of isoforms of Ole e 7 precluded its cloning and the completion of its amino acid sequence by classical techniques [4].

In this context, the main goal of this work consisted of determining the primary amino acid sequence of Ole e 7 by proteomics for its further expression and characterization in comparison to the natural protein isolated from pollen (Fig. 1).

3.1. Assembling of Ole e 7 amino acid sequence by nLC-MS/MS analysis

We performed a proteomic analysis of Ole e 7 isolated from pollen to obtain by *de novo* peptide sequencing as many peptides as possible to try to assemble the complete Ole e 7 primary sequence.

First, we separated Ole e 7 isolated from pollen by 2DE. Second, the main spot of Ole e 7 was in-gel digested with trypsin and directly analyzed by nLC-MS/MS in an LTQ-Orbitrap Velos (Fig. 1). Then, CID and HCD Fragmentation spectra were analyzed using PEAKS software. A total number of 457 *de novo* sequenced peptides were obtained (Table 1 and Supporting Table S1). *De novo* sequenced peptides with individual amino acid ALC confidence factors higher than 60% were used for the manually assembling of Ole e 7, using 3 previously reported amino acid peptides of Ole e 7 obtained by Edman degradation and peptide mass fingerprinting [4], and nsLTPs amino acid sequences deposited in databases. In total, we used for the manual assembling of the complete amino acid sequence of Ole e 7 13 out of 457 *de novo* peptides sequences (Table 1 and Fig. 2A).

Then, the assembled primary sequence of Ole e 7 was compared with allergenic LTPs amino acid sequences deposited in the WHO-IUIS database (Fig. 2B). Identity and similarity values with previously

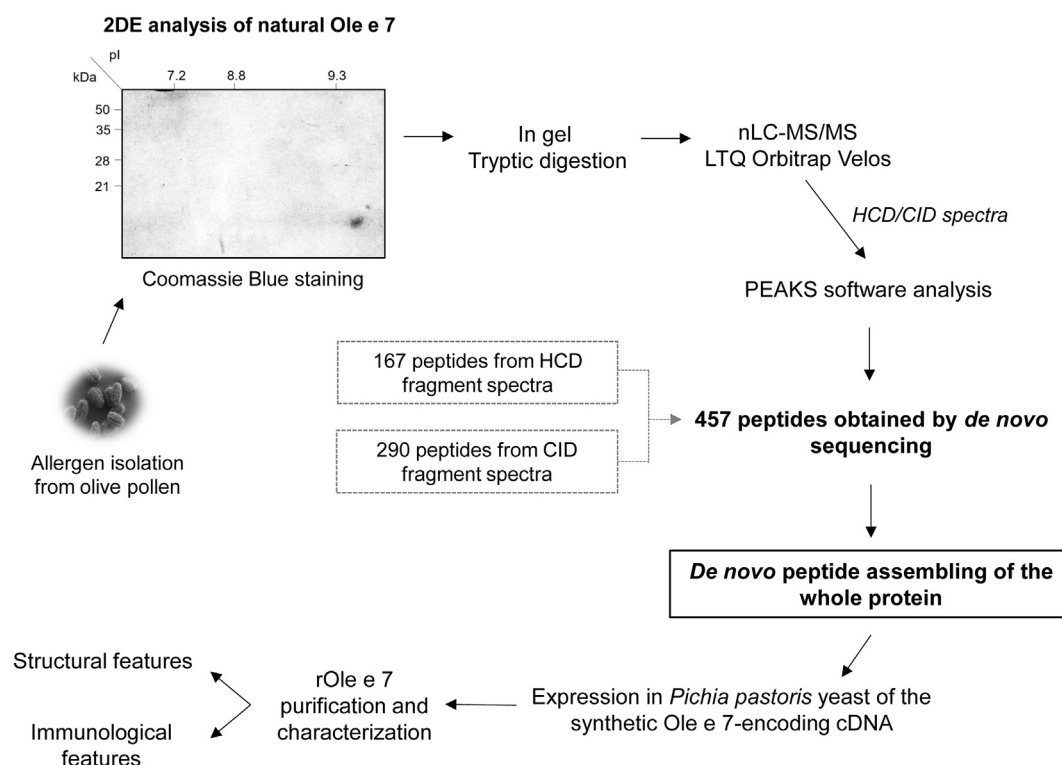


Fig. 1. Scheme of the approach followed to obtain the Ole e 7 primary structure for a subsequent Ole e 7-encoding cDNA expression, and characterization of the recombinant allergen.

reported allergenic nsLTps from pollen and plant-derived foods ranged from 21 to 48% and 21 to 57%, respectively (Fig. 2B).

3.2. Recombinant production and purification of rOle e 7

We then proceeded to the production and characterization of the assembled primary sequence of Ole e 7 for its comparison with its natural counterpart isolated from pollen.

The synthetic cDNA-encoding sequence of Ole e 7 was subcloned into the pPICZαA vector and directly used to transform KM71H *P. pastoris* yeast cells to produce the recombinant allergen as an extra-cellular secretion protein. The highest expression levels were observed at 8 days of induction (Fig. 3A–B). After purification at homogeneity

with a final yield of approximately 1.5 mg/L of cell culture, the integrity and purity of rOle e 7 was assessed by Coomassie Blue staining and immunostaining after SDS-PAGE with the nOle e 7-specific pAb (Fig. 3C). We observed a similar IgG-binding ability to both proteins suggesting rOle e 7 was well-folded and should resemble the immunological properties of nOle e 7 (Fig. 3C).

3.3. Structural characterization of rOle e 7

To address this question, we first performed a CD analysis of rOle e 7 in comparison to nOle e 7 isolated from pollen (Fig. 3D–E). We observed for both proteins the main features previously reported for nsLTps with highly dominant α-helix content [31–33]. However, we

Table 1

De novo sequenced peptides obtained from HCD or CID fragmentation spectra on a LTQ-Orbitrap Velos used for the assembling of the complete Ole e 7 primary amino acid sequence.

Scan	Peptide	TLC	ALC (%)	m/z	z	RT	Mass	ppm	PTM	Local confidence (%)	Fragmentation
4815	APSC(+57.02)STVTAK	7.1	71	511.2535	2	35.29	1020.491	1.5	Yes	93 90 89 90 88 73 59 50 53 21	CID
3554	APSC(+57.02)STVTAK	6.7	67	511.2535	2	24.59	1020.491	1.4	Yes	92 88 87 79 71 71 67 47 46 21	CID
2426	LTSC(+57.02)VSYLDM(+15.99) EQPTSDDDDTTK	17.1	74	675.7791	4	29.23	2699.1101	−8.4	Yes	94 94 94 92 93 94 92 89 83 66 44 48 48 90 90 90 91 92 41 41 41 70	HCD
2202	KLAYNLDDR	6.9	77	554.2877	2	18.95	1106.572	−10	No	93 93 92 91 68 70 71 91 21	CID
3698	TVLAQATSK	6.8	75	459.7665	2	25.49	917.5182	0.4	No	55 64 95 96 80 91 88 89 21	CID
2209	TVLAQSTSKPDK	8.6	71	637.8516	2	21.73	1273.6877	0.7	No	49 53 79 86 74 83 90 91 88 70 71 21	CID
2478	LATC(+57.02)NC(+57.02)LK	6.3	78	490.2384	2	20.42	978.4626	−0.3	Yes	94 94 92 90 75 50 79 55	CID
2325	SALALVGDKVDTGR	9.6	69	467.9278	3	28.02	1400.7622	−0.5	No	35 35 47 76 90 94 94 92 90 90 82 52 51 35	HCD
2347	SALALVAGNKVDTGR	9.4	63	491.2796	3	27.74	1470.8154	1	No	41 45 77 95 96 85 66 48 46 51 81 82 48 47 34	HCD
3913	SALANLGDKVDTGR	9.2	66	708.876	2	26.19	1415.7368	0.4	No	23 26 38 88 88 94 94 92 89 89 87 46 44 25	CID
4656	WSKSALALVGNK	8.6	71	637.3694	2	29.61	1272.719	4.2	No	35 35 48 94 93 95 93 91 77 62 55 78	CID
3861	VDTGEVSSLPK	7.4	68	566.2987	2	25.99	1130.5818	1	No	67 67 78 87 87 89 85 49 45 68 21	CID
5538	QC(+57.02)GMSVNLPLDK	8.9	69	729.8544	2	37.79	1457.7007	−4.4	Yes	38 41 77 89 93 94 85 92 93 49 43 58 40	CID

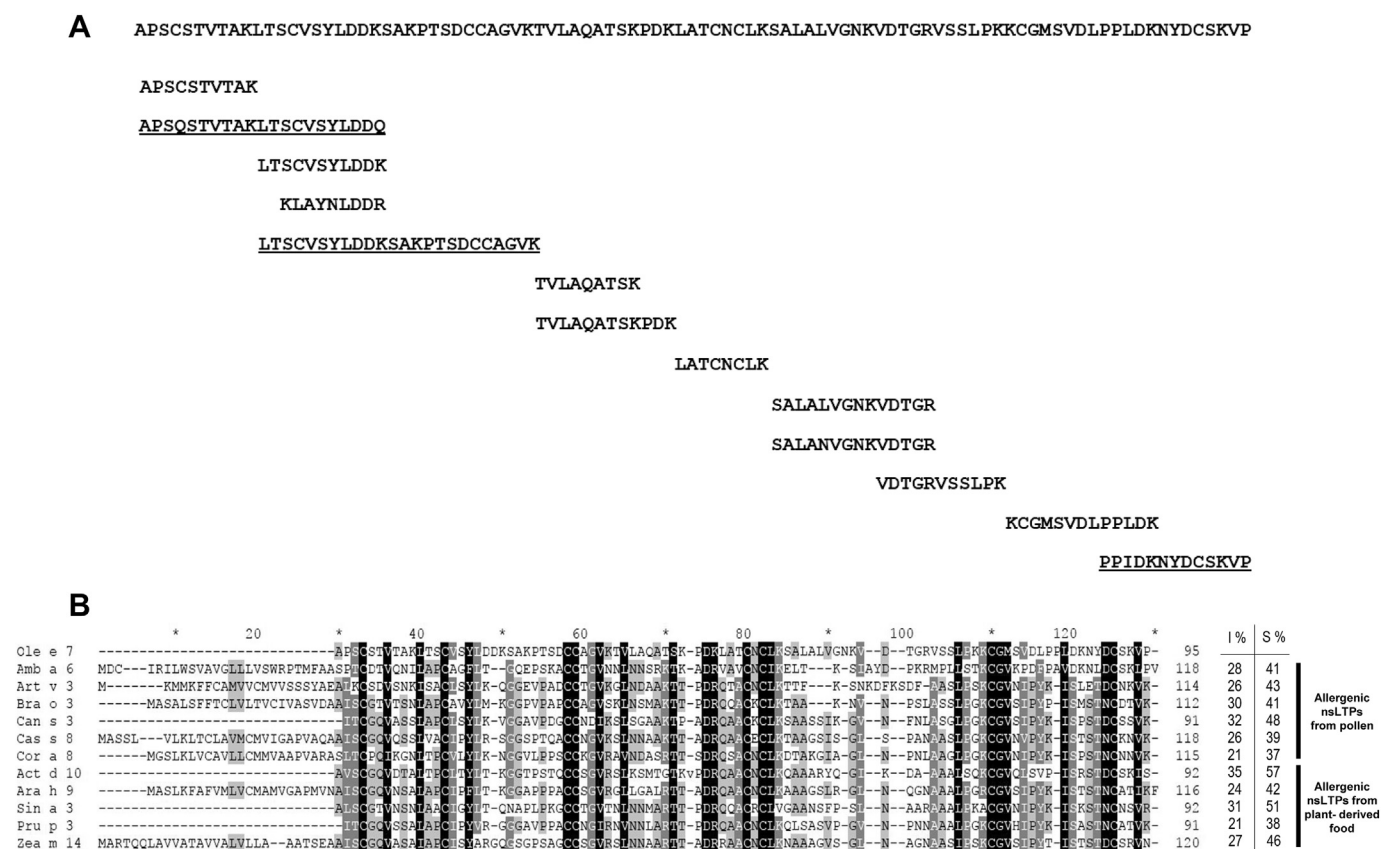


Fig. 2. Assembling of Ole e 7 by *de novo* peptide sequencing by proteomics and confirmation of its correct primary structure. (A) A total of 13 out of 457 *de novo* peptides obtained by nLC-MS/MS were used to get the 10 amino acid sequences needed for completing the Ole e 7 primary amino acid sequence based on similarity with previously reported amino acid sequences from Ole e 7 and other nsLTPs deposited in databases [4]. Peptides obtained by EDMAN degradation and peptide mass fingerprinting in previous reports are underlined [4]. Missed cleavage events were observed in four of the thirteen used peptides (Table 1). In the case of amino acid discrepancies in different peptides observed at the same position in the primary sequence, we selected the most repeated amino acid residue in the identified peptides, comparing at the same time the amino acid sequences with that derived from other allergenic LTPs deposited in databases. (B) To determine the correct assembling of the primary sequence of Ole e 7, the final sequence of Ole e 7 was aligned with those reported from allergenic LTPs from pollen and plant-derived food. The amino acid sequence of Ole e 7 obtained by proteomics showed values of identity and similarity ranging from 21 to 48% and 21 to 57%, respectively, with allergenic LTPs from indicated pollens and plant-derived food.

observed small differences between them. A 9% higher content in α -helix, and a 10% lower β -content for nOle e 7 was observed, with a similar random structure for both proteins. In addition, we analyzed their thermal stability (Fig. 3D–E). After heating to 85 °C and cooling afterwards, rOle e 7 and nOle e 7 retained the secondary structure observed at 85 °C, with a higher α -helix content for nOle e 7. However, both proteins were unable to recover their initial secondary structure, which has been reported from other nsLTPs [32, 33]. These differences in secondary structure content were attributed to the presence in nOle e 7 of multiple isoforms with a different secondary structure content respect to rOle e 7.

3.4. Immunological characterization

We next analyzed the immunological properties of rOle e 7 by ELISA and WB to determine if rOle e 7 was able to span the antigenic and allergenic properties of the natural protein isolated from pollen (Fig. 4).

Prevalence of both proteins was determined using sera from *O. europaea* sensitized patients from two different regions of Spain –Jaén and Málaga–, where rOle e 7 is a major allergen (Fig. 4A). Recognition frequency was almost equivalent for both proteins: rOle e 7 (85%) and nOle e 7 (88%). In addition, the correlation value comparing the IgE-binding of rOle e 7 and nOle e 7 was observed to be 0.93 (Fig. 4B). However, a small different IgE-binding behavior was observed for both allergens. rOle e 7 showed a lower recognition than nOle e 7 between

15% and 23%, depending on the analyzed population of allergic patients. By WB under reduction conditions, we observed a similar IgE-binding to both proteins for the analyzed sera (Fig. 4C).

Next, we performed a comparative inhibition analysis of the IgG- and IgE-binding activities of rOle e 7 and nOle e 7 by WB and ELISA (Fig. 5). By WB, we observed that rOle e 7 was not able to completely inhibit the IgG-binding to nOle e 7. However, a highly and equivalent inhibition to the recombinant allergen using nOle e 7 or rOle e 7 as inhibitors was observed (Fig. 5A). Regarding the IgE-related inhibition experiments (Fig. 5B), we observed by ELISA that nOle e 7 was able to inhibit the IgE-binding regardless of the well-coated protein, with values around 95% and 90%. However, differences in the IgE-binding inhibition were detected when rOle e 7 was used as inhibitor. We found a lower inhibition capacity by rOle e 7 when the natural allergen was coated in the ELISA wells. By WB, we observed a slightly higher IgE-inhibition capacity when nOle e 7 was used as inhibitor, regardless of the coated protein, in comparison to rOle e 7 (Fig. 5C).

3.5. Cell-based allergic mediator release assays

To further investigate the equivalence between both proteins, we analyzed if rOle e 7 produced the release of allergic mediators using functional cell-based tests at similar extents than nOle e 7. We first performed BAT using the blood of four individuals and the recombinant and natural proteins (Fig. 5D). We observed an equivalent behavior of

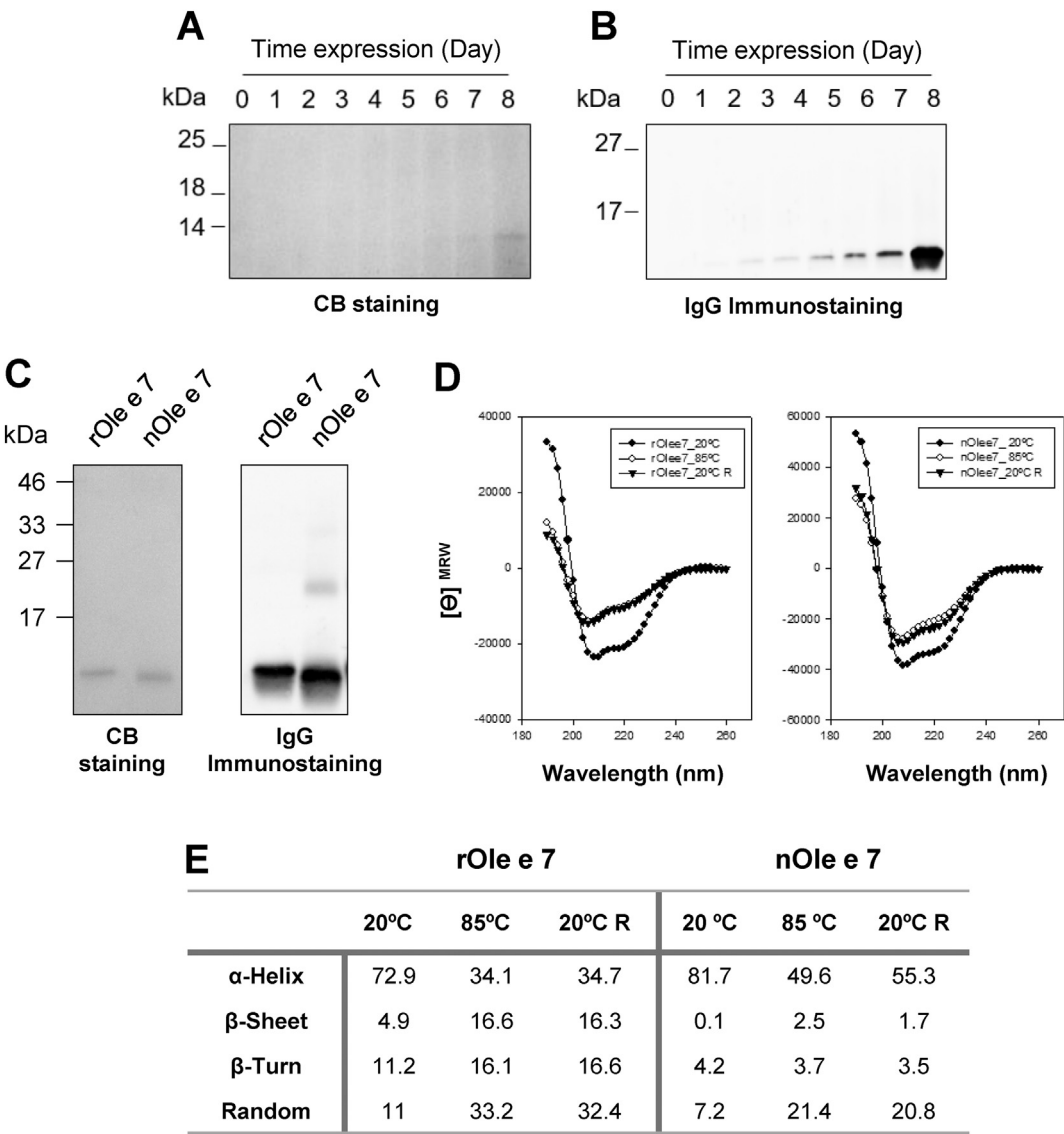


Fig. 3. Recombinant expression, purification and structural characterization of rOle e 7. Time course of the expression of rOle e 7 monitored by Coomassie Blue staining (A) and immunoblotting with a pAb raised against natural Ole e 7 allergen (B) after SDS-PAGE. Analysis of the purified rOle e 7 (50 ng) to homogeneity by Coomassie Blue staining and immunostaining after SDS-PAGE of nitrocellulose-blotted in comparison to 50 ng of nOle e 7 (C). CD spectra of recombinant Ole e 7 in comparison to nOle e 7 at a concentration of 200 μ g/mL in 20 mM sodium phosphate buffer, pH 7.5, at indicated temperatures (D). Percentages of secondary structure of rOle e 7 and nOle e 7 were obtained by deconvolution at 20 $^{\circ}$ C, after thermal treatment -85 $^{\circ}$ C and cooling -20 $^{\circ}$ C R- (E).

both proteins. In addition, four different allergic patients were analyzed for the ability to release β -hexosaminidase from the RBL-2H3 mast cell model (Fig. 5E). Both proteins triggered a highly similar release of β -hexosaminidase at 2.5 μ g/mL with values ranging from 20 to 48% and 23–39%, respectively (Fig. 5E).

Despite the natural protein contains multiple isoforms with differences in their antigenic and allergenic properties, the immunological characterization and the cell-based assays showed that rOle e 7 was practically able to span the antigenic and allergenic properties of nOle e 7. Collectively, the results derived from the comparison between rOle e 7 and nOle e 7 showed that rOle e 7 allergen assembled by *de novo* peptide sequencing by proteomics behaves similarly to the natural allergen regarding its antigenicity and allergenicity.

4. Discussion

Among the thirteen olive allergens described, Ole e 7 is a major allergen in those regions with high pollen counts such as Jaén or Málaga [28, 30, 34]. Ole e 7 is the only allergen apart from Ole e 1 that

has been described as the primary sensitizer of olive pollen [28], and it has been associated with severe reactions especially during immunotherapy treatments. Although, nsLTPs have been reported in pollen-pollen and plant-derived food-food cross-reactivity processes [35], no clear associations have been detected between natural Ole e 7 and other nsLTPs from pollens and plant-derived foods. These processes have not been deeply studied in individual patients due to the low yield of Ole e 7 isolated from pollen [4], what can be overcome now by using the recombinant allergen. Moreover, the identification of multiple sequences of this polymorphic allergen delayed its cloning and the subsequent obtaining of a suitable recombinant isoform. Due to the clinical relevance of Ole e 7, and the need to obtain information about its potential role in cross-reactions with other nsLTPs in further studies, the main aim of this work was to obtain the primary structure of Ole e 7 by proteomics to further produce, purify and characterize the recombinant protein in comparison to the natural isoform isolated from pollen to assess its usefulness for clinical management of allergic patients.

The proteomic approach started with the 2DE separation of the natural allergen [4], followed by in-gel tryptic digestion and nLC-MS/

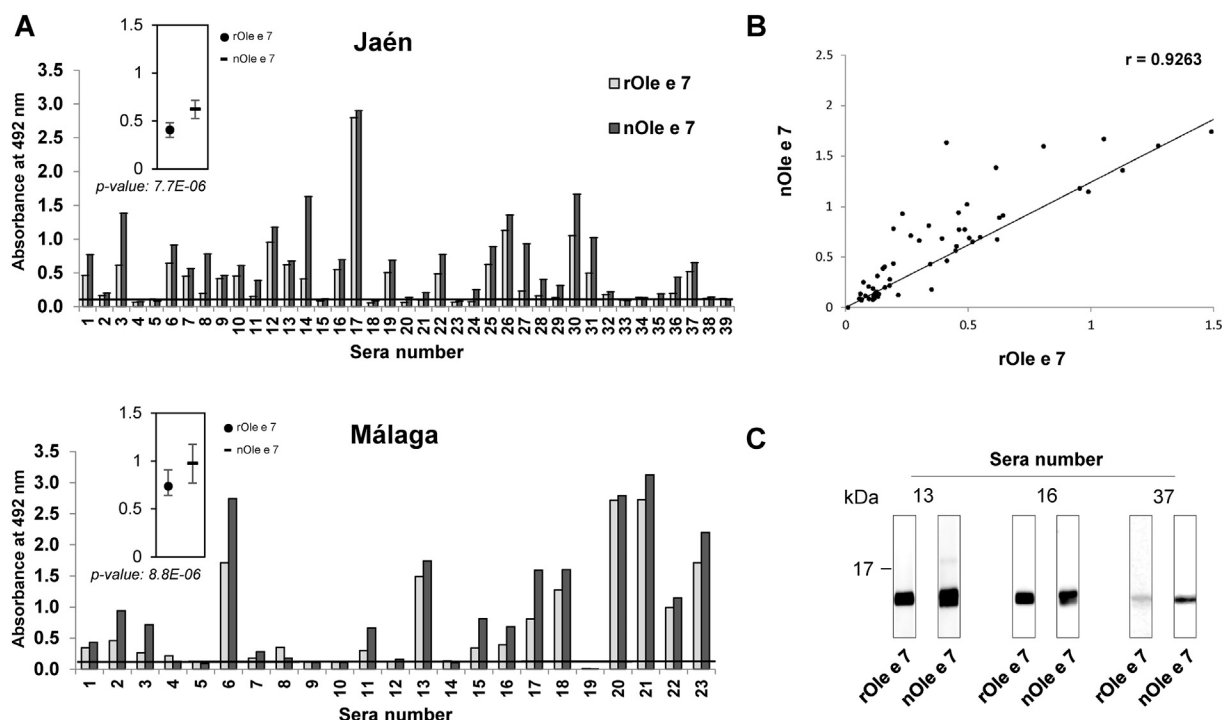


Fig. 4. Analysis of the IgE-binding to rOle e 7. IgE-binding ability to rOle e 7 (100 ng/well) was analyzed in comparison to nOle e 7 (100 ng/well) by ELISA using two populations of sera from Spain from *O. europaea* pollen sensitized patients (A). Mean $\pm$ SEM (standard error of the mean) and *p*-values of the IgE-binding to rOle e 7 and nOle e 7 for all positive serum from Jaén and Málaga are represented in the inset. Correlation coefficient (*r*) between rOle e 7 and nOle e 7 shows its highly immunological equivalence in both analyzed populations (B). Detection of the specific IgE-binding of three individual sera (13 from Málaga, and 16 and 37 from Jaén) to 50 ng of nitrocellulose-blotted rOle e 7 or nOle e 7 after SDS-PAGE (C).

MS and subsequent *de novo* peptide sequencing analyses. With the peptide sequences obtained, we manually assembled the primary structure of the most repeated peptide sequences of Ole e 7. It is important to highlight that during the development of this work, the genome of a millennial olive tree was reported [36], which allowed us confirming the presence in the genome of the primary sequence assembled by proteomics (Supplementary Fig. 2). Three Ole e 7 isoforms highly similar to that assembled were detected. Those nsLTP isoforms displayed an identity and similarity values ranging from 71 to 82% and 82–94%, respectively, which confirms the usefulness and accuracy of this proteomic approach for the primary sequence assembling of new allergenic proteins. At present, to our knowledge, there is only one allergen reported by *de novo* sequencing by using a different strategy [22]. Here, we have used a directed proteomics pipeline based on a classical bottom-up approach and only using the PEAKS software to get the sequence of Ole e 7, which matched well with that of Ole e 7 DNA sequences present in the olive tree genome deposited in databases. Remarkably, we only observed three amino acid changes and the absence of 4 amino acids in the C-terminal end in comparison to the amino acid sequences derived from the olive tree genome [36].

The other goal of the work consisted of the expression, purification and confirmation that the immunological and physicochemical properties of the recombinant allergen were equivalent to those of the natural allergen isolated from pollen. The recombinant Ole e 7 was expressed in *Pichia pastoris* yeast because of the presence of disulfide bonds in the structure and because it has been successfully used as expression system for other allergenic nsLTPs. The folding of rOle e 7 was assessed by CD-spectra in comparison to nOle e 7. Although rOle e 7 and nOle e 7 showed the typical structure of nsLTPs with α -helix content besides 70% [33], slight differences in about 10% in α -helix and β -content were observed between them. On the other hand, both proteins were unable to recover their native secondary structure after heat treatment. This low structural stability has been observed in other inhaled nsLTPs [32, 33]. Remarkably, after an extensive immunological

characterization and cell-based assays using rOle e 7, we could confirm the high IgG equivalency between the recombinant and the natural Ole e 7 allergens. No significant differences were observed in the IgE-binding of both proteins by WB and ELISA, with a similar IgE-binding ability to both proteins. The lower IgE recognition between 15% and 23% to rOle e 7 in comparison to nOle e 7 could be attributed to amino acid heterogeneity in the different isoforms of nOle e 7, and the presence of other allergenically relevant isoforms containing IgE epitopes in the natural allergen isolated from pollen not present in rOle e 7. This heterogeneity should also be the cause of the small different content in secondary structure between rOle e 7 and nOle e 7. Indeed, this polymorphism was observed by *de novo* peptide sequencing where 41 different sequences from the N-terminal end of Ole e 7 were obtained accomplishing the ALC criteria used in PEAKS (Supplementary Table S1).

Remarkably, despite the slight differences observed between rOle e 7 and nOle e 7, BAT and RBL-2H3 cellular experiments using blood and sera from olive pollen allergic patients, which take into account functional crosslinking between specific anti-Ole e 7 IgE molecules at the cell surface, confirmed the close immunological equivalence between nOle e 7 and rOle e 7. Stimulation with rOle e 7 did not produce significant differences regarding the response against nOle e 7. Taking together, these results confirmed that both allergens share most IgG and IgE conformational and linear epitopes, apart from its effectiveness for *in vitro* allergy diagnosis.

5. Conclusions

In summary, the here presented work shows that rOle e 7 assembled by *de novo* peptide sequencing by mass spectrometry is able to mostly span the antigenic and allergenic features of the natural allergen isolated from *O. europaea* pollen. This was demonstrated through immunological assays using sera and blood of olive pollen allergic patients and by the release of mediators of the allergic response in cell-based

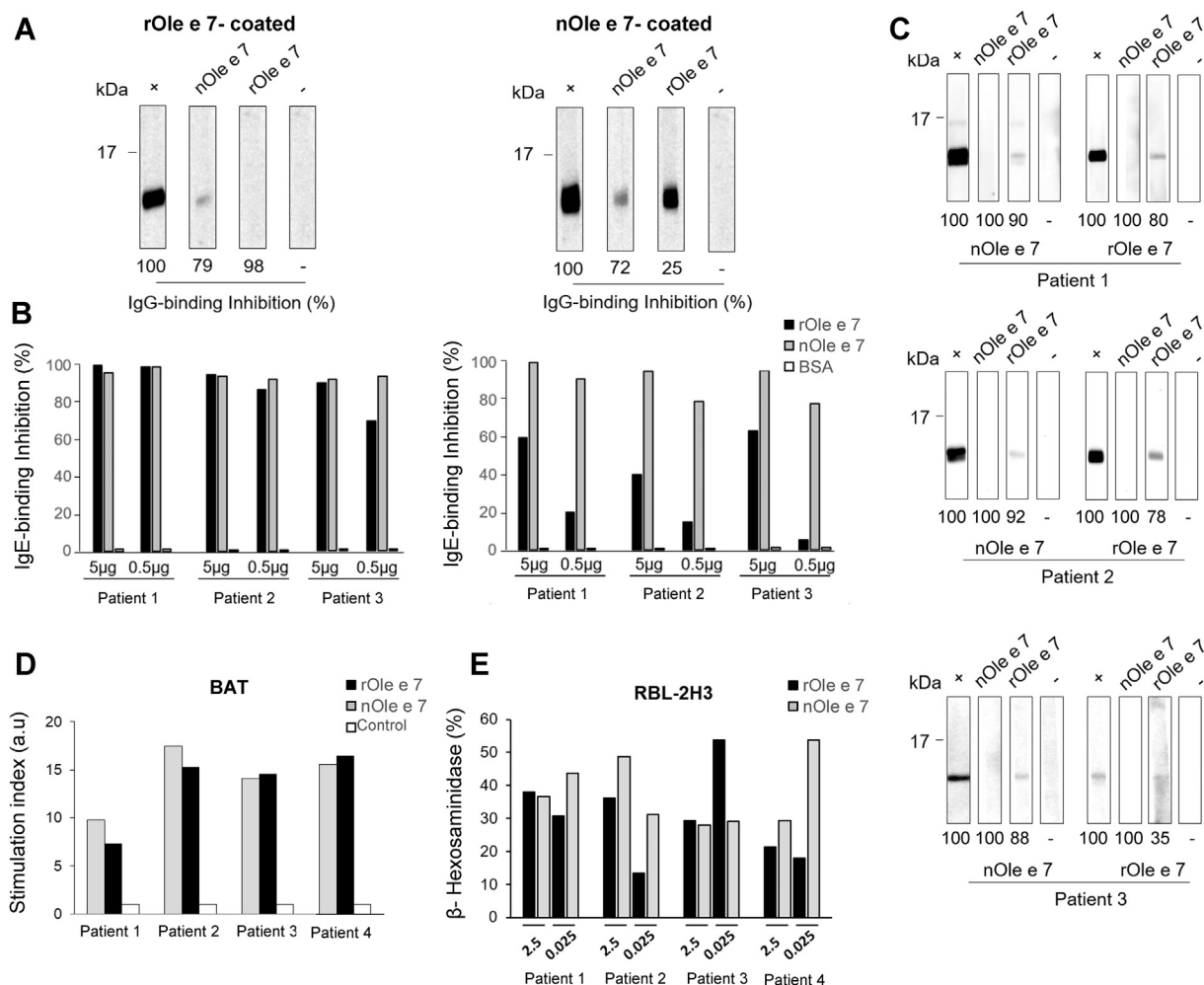


Fig. 5. Analysis of the IgG- and IgE-binding inhibition capacity of rOle e 7 and the release of allergic mediators by rOle e 7 in comparison to the natural allergen. IgG-binding inhibition assay by immunoblotting with the Ole e 7-specific pAb, using 2.5 µg of the purified natural or recombinant protein as inhibitor. (+) Positive control without inhibitor, (–) negative control (A). Inhibition of the IgE-binding of three individual sera from Málaga and Jaén was tested by ELISA (B) and WB (C). An amount of 0.5 or 5 µg of nOle e 7 or rOle e 7 were used as inhibitor in the ELISA (B), whereas 5 µg of nOle e 7 or rOle e 7 were used by WB. BSA was used as control protein in ELISA. Basophil activation test (D). Stimulation index of four patients allergic to *Olea europaea* pollen are shown. A fixed concentration of 1 µg/mL of natural and recombinant allergen was used for cell stimulation. Basophils stimulation with BSA was used as negative control, whereas as positive control, patient sera were incubated with a monoclonal anti-IgE antibody. β-hexosaminidase release assay of Hum RBL-2H3 cells activated with two different concentrations (µg/mL) of the natural and recombinant allergen (E). Absence of β-hexosaminidase release was observed without allergen stimulation (negative control, data not shown).

assays. Finally, the here presented data provide evidences about the usefulness of rOle e 7 assembled by proteomics to be used in component-resolved and *in vitro* diagnosis of olive pollen LTP sensitized patients. In addition, we showed here the effectiveness of this proteomics approach to get the primary sequence of polymorphic allergens from unsequenced genomes, when homologous proteins are deposited in databases.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jprot.2018.06.001>.

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Author contributions

C.O.S., S.M., M.V., and R.B. designed research; C.O.S., S.M., S.B., M.L.-L., J.Q., M.B., C.M., and R.B. performed research; J.Q., M.B., and C.M. contributed reagents, sera and/or blood from sensitized patients; M.V. and R.B. funded the research; C.O.S., S.M., and R.B. wrote the paper. All the authors reviewed and approved the manuscript.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Supplementary Information

Materials and methods

Protein extracts, nOle e 7 purification and antibodies

Pollen from *Olea europaea* was obtained from ALK-Abelló (Madrid, Spain). Other pollens were from Allergon. Olive tree pollen extract was obtained as previously described [1]. Other pollen and plant-derived food extracts were prepared as indicated [2, 3]. Protein extract concentration was determined according to Lowry et al. [4].

Rabbit polyclonal antiserum against Ole e 7 protein was obtained accomplishing the Ethic Guidelines of Complutense University of Madrid. Horseradish peroxidase-labeled goat polyclonal antibody against mouse IgG was purchased from Pierce Chemical Co. (Rockford, IL, USA). Horseradish peroxidase-labeled mouse monoclonal anti-IgE antibody (Alk-Abelló) against rabbit IgG was obtained from Bio-Rad (Richmond, CA, USA).

Two-dimensional gel electrophoresis (2DE)

Isoelectric focusing was achieved under reducing conditions in the presence of 3 mM tributylphosphine in a PROTEAN IEF Cell (Bio-Rad) using 7 cm length, pH 3-10 linear ReadyStrip IPG gels (Bio-Rad). After isoelectrofocusing, proteins were separated by SDS-PAGE in 17% polyacrylamide gels under reducing conditions in the presence of 50 mM dithiothreitol and 3.7% iodoacetamide and stained with colloidal Coomassie Blue (CB).

Analytical procedures

17% polyacrylamide SDS-PAGE gels were stained with Coomassie Blue R-250 (Sigma-Aldrich) or transferred to nitrocellulose membranes (Amersham Biosciences, Barcelona, Spain) according to Towbin method [5] for WB analysis. Molecular mass determinations were performed with unstained protein markers SM0431 (Fermentas) or with pre-stained protein molecular weight markers (BIO-RAD).

The concentration of the purified proteins was calculated by spectroscopy in a DU-7 spectrometer (Beckman, Barcelona, Spain) using the Ole e 7 theoretical extinction coefficient of 0.355 calculated with the ProtParam Software [6].

rOle e 7 expression and purification

The synthetic cDNA-encoding sequence of Ole e 7 optimized for its expression in *P. pastoris* yeast was purchased from IDT DNA Technologies (Supporting Figure 1). The restriction sites for *Xho*I and *Sac*II were included into the 5' and 3' ends of the Ole e 7-encoding cDNA, respectively, to directly clone the cDNA in pPICZαA vector previously digested with both restriction enzymes to subsequently express the protein in KM71H strain of *P. pastoris* yeast according to established protocols [7].

To produce rOle e 7, selected transformed KM71H cells, growing under YPD/zeocin, were cultured for 48 h in buffered minimal glycerol medium (BMG) at 30°C, supplemented with 25 µg/mL zeocin. Then, cells were centrifuged at 5000 rpm during 20 min and resuspended in one-fifth of the original volume with buffered methanol minimal medium (BMM), enhancing the induction of the AOX1 promoter. Cultures were induced for 8 days, adding 0.5% methanol every 24 h. After induction, supernatant containing the recombinant allergen was clarified by centrifugation at 5000 rpm during 20 min, dialyzed, and used to purify rOle e 7.

rOle e 7 was purified by two consecutive chromatographic steps in medium and superfine Sephadex G50 (Amersham Biosciences). Then, a last step by reverse-phase HPLC in

a Nucleobond column (5 μ m x 4.6mm x 25cm) was used to obtain the protein to homogeneity. Fractions were analyzed by Coomassie Blue R-250 (Sigma-Aldrich) staining or alternatively by WB after SDS-PAGE. Purified rOle e 7 was pooled, lyophilized and resuspended in 20mM ammonium bicarbonate and stored at -80°C until use.

Circular Dichroism spectroscopic analyses

The CD spectrum of rOle e 7 was recorded in the far -UV (190–250 nm wavelength) on a JASCO J-715 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) using an optical-path cell. CDNN CD spectra deconvolution software was used to analyze the CD spectra obtained.

Thermal unfolding was followed by monitoring the ellipticity at 220 nm on heating (25–85°C) or cooling (85–25°C) conditions at 0.5 °C/min with a computer-controlled water bath.

Immunological characterization

For WB analysis, immunodetection on membranes was achieved by incubating with individual human sera (diluted 1:10), anti-human IgE monoclonal antibody (diluted 1:5000) and anti-mouse IgG horseradish peroxidase-labeled antibody (1:2500). Alternatively, either a mouse polyclonal antiserum (pAb) raised against Ole e 7 (diluted 1:10000) followed by an anti-rabbit IgG horseradish peroxidase-labeled polyclonal antibody (1:3000) was used. For WB inhibition assays, individual sera (diluted 1:10) or the specific mouse polyclonal antisera raised against Ole e 7 (diluted 1: 20000) were pre-incubated with PBS and 5 μ g of natural or recombinant proteins. Chemiluminiscent signal was developed using ECL-Western blotting reagent (Amersham Bioscience) or WesternBright™ QUANTUM (Advansta) reagents.

Indirect ELISA was performed in 96-well plates (Costar) coated with 0.1 μ g natural or recombinant protein per well. For inhibition assays, individual human sera (n=3) (diluted 1:10) or the specific polyclonal antiserum raised against Ole e 7 (diluted 1:20000) were previously incubated with 0.5 μ g or 5 μ g of both natural or recombinant proteins as inhibitors. Indirect

ELISA inhibition tests were performed as previously described [8], using 20 or 200µg of pollen or plant-derived food extracts as inhibitors.

Binding of human IgE was detected by an anti-human IgE monoclonal antibody (diluted 1:5000) followed by goat anti-mouse IgG horseradish peroxidase-labeled antibody (diluted 1:2500). Binding of the specific polyclonal antisera for the natural or recombinant Ole e 7 was detected by goat anti-rabbit IgG horseradish peroxidase-labeled antibody (1:3000). Signal was measured at 492 nm in an iMark microplate absorbance reader (Bio-Rad). Peroxidase reaction was detected by using 50 µL per well of a developing solution (0.63 mg/mL o-Phenyldiamine in 0.1 M sodium citrate 4% methanol containing 1.6 µL/mL 30% H₂O₂). The reaction was stopped with 50 µL 3N H₂SO₄.

Cell-based allergic mediator release assays

The release of mediators of allergic response induced by recombinant and natural Ole e 7 was analysed in basophils and RBL-2H3 cells. Basophil Test Activation (BAT) was performed as previously described [9]. After blood-cell separation, 50 µL of each patient's cell suspension were incubated with 50 µL of a fixed protein concentration of 1 µg/mL. To evaluate background basal values without stimulation (negative control), we added 50 µL of stimulation buffer (cRPMI), containing IL-3 (2 ng/mL) in the cell suspension. A positive response was concluded for values ≥15%, and stimulation index (antigen-specific response/basal level) ≥2.

RBL-2H3 cells were cultured in minimum essential medium (MEM) containing 5% foetal bovine serum (FBS) with 100 UI/mL penicillin and 100 µg/mL streptomycin. Cells were cultured in 75 cm² tissue culture flasks, incubated at 37 °C in a humidified incubator under 5% (v/v) CO₂. Twice a week the medium was changed using EDTA 0.01 M. All cell cultures were confluent prior to being harvested for use in experiments. Cells were sensitized overnight at 37°C with 5% of sera from allergic patients. After 12 h, cells were stimulated with either natural or recombinant Ole e 7 at two different concentrations (0.025 µg/mL and 2.5 µg/mL). To

increase and stabilize the secretion of β -hexosaminidase, 50% D₂O was added to the Tyrode's buffer used for the dilution of allergens [10]. For detection of the granular enzyme β -hexosaminidase, an enzymatic colorimetric assay was used as described previously [11]. Briefly, 1 h after stimulation of 100 μ L of cells, 30 μ L of supernatant was transferred to a 96-well plate and mixed with 50 μ L of substrate solution (3.5 mg/mL p-nitrophenyl-N-acetyl- β -D-glucosaminide dissolved in 40 mM citric acid, pH 4.5). To calculate the total β -hexosaminidase activity, cells were lysated with 100 μ L of 0.1% Triton X-100 solution and incubated for 60 min at 37°C. Then, 100 μ L of glycine 400 mM, pH 10.7, was added to each well, and the absorbance at 405 nm was measured. The percentage of β -hexosaminidase released was calculated as a percentage of the total β -hexosaminidase content in the cells.

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Legend to Supplementary Figures

Fig. S1. Nucleotide sequence of Ole e 7 derived from the proteomics assembling optimized for the expression of Ole e 7 in *Pichia pastoris* yeast. The *Xho*I and *Sac*II restriction sites used to clone the cDNA in pPICZ α A are underlined. The stop codon is highlighted in bold. In addition, the nucleotide sequence AAA AGA highlighted in italics was introduced after *Xho*I to regenerate the Kex2 signal from the pPICZ α A vector to express Ole e 7 as a secreted protein.

Fig. S2. Alignment of the Ole e 7 amino acid sequence obtained by proteomics with LTPs from the sequenced olive tree genome. Three different amino acid sequences were found in the recently reported olive tree genome [12], with an identity and similarity values ranging from 70-80% and 80-90% respectively. Amino acid sequences were aligned with DIALIGN [13] and visualized with GeneDoc software.

aaa ctcgaq aaa aga

GCCCCATCTTGCTCAACTGTGACAGCCAAGTTAACCAGTTGCGTCTCATACCTGGAC
GATAAGTCTGCCAAACCAACTTCCGATTGTTGTGCTGGTGTTAAAACTGTCCTTGCT
CAAGCTACCTCCAAACCAGATAAATTAGCTACATGCAACTGCTTGAAGTCTGCATTG
GCCCTAGTCGGTAATAAAGTAGATACCGGAAGGGTGAGTTCTCTGCCCAAGAAATGT
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TGA ccgcgc aaa

SUPPORTING INFORMATION

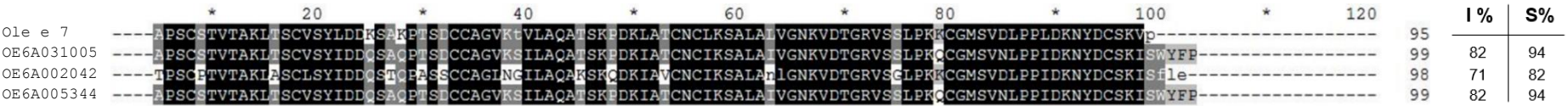


Figure S2