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Review

Analysis of olive allergens

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ABSTRACT

Olive pollen is one of the most important causes of seasonal respiratory allergy in Mediterranean countries, where this tree is intensely cultivated. Besides this, some cases of contact dermatitis and food allergy to the olive fruit and olive oil have been also described. Several scientific studies dealing with olive allergens has been reported, being the information available about them constantly increasing. Up to date, twelve allergens have been identified in olive pollen while just one allergen has been identified in olive fruit. This review article describes considerations about allergen extraction and production, also describing the different methodologies employed in the physicochemical and immunological characterization of olive allergens. Finally, a revision of the most relevant studies in the analysis of both olive pollen and olive fruit allergens is carried out.

Contents

1. Introduction

Allergic diseases have increased in industrialized countries over the last years, affecting up to 30% of the population [1]. Allergens from pollen or food origin can cause allergenic reactions in predisposed humans evoking a range of symptoms including full-blown life-threatening anaphylactic shock [2]. Consequently, the characterization of allergenic components becomes an essential task for immunological, epidemiological, and genetic studies of the allergic patients.

The olive is a long-lived tree which have been cultivated and utilized by humans for more than 5000 years, obtaining oil, fruit, and wood [3]. It is widely cultivated in the Mediterranean basin and more than 1500 different varietal denominations have been recorded in the world [4]. These olive cultivars differ both in their fruit quality and in their pollen characteristics [5–7]. The olive tree produces small yellow-white flowers with four petals which appear between the end of January and the beginning of June, depending on latitude and climatic condition [8]. Allergy to olive is mainly due to the pollen produced in these flowers, being allergy to olive fruit and olive oil less common.

Olive pollen is one of the most important causes of respiratory allergy, especially where the tree is widely distributed like California and the Mediterranean area [9–11]. The incidence of this allergy is high during the olive pollination season (from May to June), although allergic symptoms have been also reported during the rest of the year [12]. This incidence is higher in adults, but olive sensitization can be also recognized in children [11]. Moreover, the prevalence of olive allergy depends on the pollen concentration and exposure time [13–15]. In fact, the exposure of a population to a large amount of olive pollen has bad consequences on their health, although its exposure to olive pollen for generations, apparently,

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reduces the number of positive responders [6]. It has been determined by standard laboratory methods that olive pollen has up to 20 proteins with allergenic activity, observing a high variability and complexity in the responses obtained by different patients with pollen allergy [16]. However, up to date, just twelve pollen allergens, from Ole e 1 to Ole e 12, have been identified.

Apart from olive pollen allergy, some cases of allergic contact dermatitis to olive [17], olive oil [18–23], or olive wood [24,25] have also been described. Moreover, allergy associated to olive [17,26–29] and olive oil [28] ingestion has seldom been reported as well. It is significant the case of a patient presenting contact dermatitis to extra virgin olive oil but not to regular oil [19]. In this study, as in a previous one [20], patients were tested by skin tests with the major constituents of olive oil (glycerides of oleic, palmitic, linoleic, stearic and arachidic acids, squalene, phytosterol and tocopherol), obtaining negative responses in all cases. Taking into account these results and that almost all food allergens are proteins [30], it is reasonable to propose proteins as the cause of allergy. Although the knowledge on proteins present in olive fruit and oil has been increased during the last years [31], the identification of allergens in those matrices is still not resolved at all. Indeed, just one allergen, named Ole e 13, has been identified in the olive fruit [32,33].

Especially relevant are the cases of allergenic reaction due to olive fruit after pollen immunotherapy [27,29]. The co-occurrence of pollinosis with allergy to certain vegetable foods may occur in up to 70% of pollen allergic patients [34]. Those cases suggest the presence of a common allergen structure both in olive pollen and in olive fruit, being a case of cross-reactivity between plant-derived foods and pollen. Allergen cross-reactivity occurs when IgE antibodies originally induced against one allergen binds or recognizes another protein from a different biological source. Although evolutionary unrelated proteins can show homologous conserved sequences and exhibit a high degree of cross-reactivity, when there are close phylogenetic relationships between the biological sources of these allergens, the possibility of cross-reactivity increases. The family *Oleaceae*, to which the olive tree belongs, contains more than 400 species including ash (*Fraxinus*), privet (*Liqustrum*), *Phillyrea*, *Forsythia,* and jasmine (*Jasminium*), though within the genus *Olea*, only the olive tree produces edible fruits.

This work reviews the analytical methodologies used in physicochemical and immunological characterization of allergens, and its application in the study of both olive pollen and fruit allergens.

2. Extraction and production of olive allergens

Proteins have been targeted as markers for the presence of potentially allergenic pollen and foods. Nearly all allergens from natural sources have been identified as proteins or glycoproteins, showing the carbohydrate structure a lower or insignificant cell-triggering activity compared to protein determinants [35].

The quality of the allergen extract is of huge importance, affecting the outcome of the entire analysis. Two strategies can be considered at this point, to isolate the allergenic molecule from the natural source or to produce it in recombinant form in a heterologous expression system. The decision is taken in each case taking into account the advantages and disadvantages of the two approaches. If the allergen shows post-translational modifications or disulfide bridges affecting the IgE binding capacity or there is more than one isoform contributing to the overall allergenicity activity, the purification from natural sources would be appropriate. On the other hand, if allergens are exposed to active proteases during the extraction and purification procedures, are degraded upon extraction or are very low expressed in natural sources, expression in heterologous systems is an important alternative [36]. In this case, its comparison with the natural counterparts is required.

Olive pollen extracts are commercially available and commonly used for extracting natural allergens. In general, pollen extracts contain a wide variety of macromolecules along with low molecular weight metabolites, salts, and pigments. Therefore, a previous defatting step with diethyl ether or acetone is widely required. It is very common the use of an extraction buffer for the solubilization of allergens. Buffers like Tris–HCl, phosphate buffered saline (PBS), or ammonium bicarbonate at slightly basic pHs are usually employed. Sometimes it is required the addition of different additives like detergents (*e.g.*, SDS or Triton X-100), reducing agents (*e.g.*, dithiothreitol (DTT)), or additives protecting the stability of the sample (*e.g.*, chelating agents as ethylenediaminetetraacetic acid (EDTA) or ethylene glycol tetraacetic acid (EGTA), antioxidants as ascorbic acid, and protease inhibitors as phenylmethylsulfonyl fluoride (PMSF)). When a deglycosylation step is required, a strong acid as trifluoromethanesulfonic acid (TFMS) is normally used [37]. All these possibilities results in a great variability in the composition of extracts. For example, the allergenic pattern of the olive pollen proteins extracted using a short hydration time (15 min) was different from those extracted using a longer hydration time (3 h). In fact, Ole e 6 and Ole e 7 allergens are highly soluble being almost completely released in 15 min hydration time, whereas Ole e 3 requires a longer hydration time [16]. Similar results were obtained by extracting pollen with ammonium bicarbonate for different times [38]. Ole e 2 and Ole e 6 are observed just in the first extract while Ole e 3 and Ole e 7 are also present in the following extracts.

The preparation of high-quality protein extracts from fruits is typically more problematic than from other organisms. Plant tissues, like the olive fruit, have relative low protein content and, in addition, they have significant amounts of interfering compounds, in this case, fats and phenolic compounds. The optimization of an extraction procedure for olive pulp proteins that simultaneously remove interfering compounds, especially polyphenols, have been developed [39]. The extraction procedure consisted of a cleaning step to remove interfering compounds by using different trichloroacetic acid (TCA) solutions, extraction of proteins with a Tris/SDS/DTT buffer, and subsequent protein precipitation with acetone.

Recombinant allergens have potential advantages over conventional allergenic extracts, as above-mentioned. The recombinant production of several allergens from olive pollen in both bacterial and eukaryotic cells has allowed resolving problems derived from the polymporphism and scarcity of the natural forms of these allergens. The complete amino acid sequences of Ole e 1, Ole e 2, Ole e 3, Ole e 5, Ole e 6, Ole e 8, Ole e 9, Ole e 10, and Ole e 11 have been obtained from cDNA clones encoding these proteins. Two different systems have been extensively used for the recombinant expression of olive pollen allergens: the bacteria *Escherichia coli* as a prokaryotic host and the yeast *Pichia pastoris* as an eukaryotic host. In addition, one example of recombinant expression in insect cells has also been reported [40]. Methodological procedures for the recombinant expression in *E. coli* are simpler than those performed for the expression in *P. pastoris*. However, *P. pastoris* is a system able to form disulfide bonds, necessary when glycoproteins are expressed. Because of this reason, *P. pastoris* has been the most widely employed organism in recombinant production of olive allergens, although some examples in *E. coli* have been also described in proteins which do not contain carbohydrate components or disulfide bridges, like Ole e 2 [41], Ole e 3 [42,43], and Ole e 8 [42,44,45].

Next to allergen extraction or recombinant production, a purification step is usually needed. For this purpose, liquid chromatography is the most suitable technique [46]. Usually, more than one chromatographic step is required, beginning with gel filtration

chromatography or size exclusion chromatography (SEC). It has been also widely employed affinity chromatography, because of its high specificity, being very useful in the cases of recombinant expression which requires a step for removing proteins becoming from the host. Affinity chromatography using columns modified with lectin [37,47–49], glutathione [41,49,50], and poly-L-proline [41] have been extensively employed in the purification of olive allergens. Columns with monoclonal antibodies (MAb) against the allergen have been also used [51–53]. Moreover, it is usual a final purification step by reversed-phase high-performance liquid chromatography (RP-HPLC).

3. Techniques employed in the physicochemical and immunological characterization of olive allergen

There are a substantial number of well-established techniques for the physicochemical and immunological characterization of allergens that have been widely employed in olive allergens analysis. Table 1 collects the studies available on olive allergens, including the main aim and the analytical methodologies used in each study.

3.1. Physicochemical characterization

The physicochemical properties influence or may even determine the immunological behavior of an allergenic molecule. Consequently, the knowledge of molecular properties is a prerequisite for an appropriate immunological characterization, and provides the basis for the generation of modified molecules.

Classically, the estimation of molecular weights or isoelectric points (p*I*s) from allergen extracts or purified proteins were performed by electrophoresis in polyacrylamide or agarose gels. The most common methods used were isoelectric focusing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). IEF has demonstrated to be more effective at detecting small differences in protein structure or size than SDS-PAGE, and it is extremely useful for the analysis of molecules with numerous isomeric forms. Nevertheless, SDS-PAGE patterns are less susceptible to buffer or sample interferences than IEF and it routinely displays good alignments of component bands [35]. In fact, SDS-PAGE has been maybe the most employed technique in olive allergen analyses, as Table 1 shows. A combination of IEF and SDS-PAGE separations into a 2D electrophoresis (2-DE) method displays a higher resolution power but remains prone to many of the same shortcomings as its component steps. Just some examples of 2-DE separations are found to olive allergens, one for Ole e 1 [47,89] and one for Ole e 11 [122]. After separation, protein spots are usually visualized by Coomassie blue or silver stain, although some example of radiostaining has also been reported [71]. In the case of glycoproteins (Ole e 1, Ole e 9, Ole e 10, and Ole e 11), proteins are transferred to a nitrocellulose membrane and detected using a bitonylated concanavalin A (ConA) solution [69,72,115,116,119,122] or the periodic acid-Schiff (PAS) technique [51]. Once it is possible the visualization of spots, a semiquantitative analysis can be performed by densitometry [70]. An alternative strategy used for the accurate molecular weight determination of olive allergens is mass spectrometry (MS). Although different ionization sources are available for the analysis of proteins, matrix assisted laser desorption ionization-time of flight (MALDI-TOF) has been the most widely employed technique for the analysis of olive allergens. MALDI ionization, unlike electrospray ionization (ESI), results very robust when salts and detergents are presented, minimizing the sample preparation. This technique has been employed to determinate the molecular weight of the intact allergens Ole e 1 [38,68,79], Ole e 2 [38], Ole e 3 [38,105], Ole e 6 [38], Ole e 7 [14,38], Ole e 8 [45], Ole e 9 [115,116], Ole e 10 [19,40,120], and the N-glycans of the allergen Ole e 1 [48,76].

MS has also been employed for the identification of pollen allergens by its previous digestion, usually with trypsin. In order to improve sequence coverage, other enzymes like the proteinases V8 protease, proteinase K, thermolysin, carboxypeptidase A, and CNBr or the glycosidases PNGase or O-glycanase have also been used. Usually, the masses of peptides resulting from the hydrolysis of an allergen are measured by MALDI-TOF and matched against protein databases for its identification. Peptide mass fingerprinting has been employed in the identification of Ole e 3 [42], Ole e 11 [122], and Ole e 13 [33]. When there is no match between peptides resulting from enzymatic digestion and data stored in protein databases, identification requires the protein sequencing.

Protein sequencing by Edman degradation [123] has been widely employed in the identification of most olive pollen allergens: Ole e 1 [14,64,74,75,78,79], Ole e 2 [98], Ole e 3 [14,42,43,105], Ole e 4 [107], Ole e 5 [107], Ole e 6 [14,109,111], Ole e 7 [14], Ole e 8 [45], Ole e 9 [114–116], Ole e 10 [40,120], Ole e 11 [122], and Ole e 13 [32]. Nowadays, MS/MS technology has also been employed for protein sequencing. For this purpose, proteins are enzymatically digested and, very often, fractionated by RP-HPLC prior to MS analysis using electrospray (ESI) ionization. Resulting fragments can be compared with databases for unequivocal identification of corresponding peptides. Although MS/MS has emerged as the preferred method for protein identification, it just has been employed in Ole e 1 analysis [68,93] and Ole e 13 [33].

Secondary structure content and conformational studies of olive allergens have been performed in many cases applying circular dichroism (CD), fluorescence emission, and nuclear magnetic resonance (NMR). Generally, five typical secondary structure elements, *i.e.*, α-helix, β-sheet, β-turn, random coil, and poly-L-proline, can be distinguished in a CD spectrum [124]. In addition to determine the molecular structure of olive allergens, CD has also been used to investigate the thermal and chemical stability of proteins and protein–ligand interactions and to monitorize protein folding and unfolding [124]. Moreover, fluorescence emission spectra have been used to analyze the influence of carbohydrate binding on the tertiary structure, for example, of Ole e 10 [121]. On the other hand, NMR enables the study of structural flexibilities and conformational changes during protein–ligand interactions [125]. However, high concentrations of allergens, isotopic protein labeling, and long data collection and analysis times are required.

The methodologies employed for the quantitation of olive allergens are the Kjeldahl method, the amino acid analysis, and different spectrophotometric-based methods (*e.g.*, Bradford, Lowry, Biuret–Lowry, and bicinchonicic acid (BCA) assay). In addition to the total protein content, it is also usual to determine the cysteine, tryptophan, and tyrosine contents.

3.2. Immunological characterization

Immunological characterization is important to study allergenicity, sources of symptom elicitors, factors contributing to cross-reactivity, and it is particularly relevant for predicting allergenicity and designing hypoallergenic molecules for diagnosis and therapy. In this section DNA-based methods will be avoided since they do not detect the compounds responsible for triggering the allergic reactions.

Immunological characterization is widely based on the binding of IgE. *In vivo* skin prik testing (SPT) and *in vitro* serological assays are available to evaluate the presence of specific IgE to certain allergens in the patients. In laboratory studies, it is very common the use of serum samples from olive allergic patients or mice for the characterization of the allergens by testing the IgE-binding or the

Table 1
Techniques and methodologies employed in the characterization of olive pollen and fruit allergens.

Objective	Allergen	Techniques and methodologies	Ref
Cultivar differentiation	Whole allergen extract	Optical microscopy, SPT, RAST, IEF	[54]
		SDS-PAGE-Coomassie/Immunoblot, Indirect ELISA, SPT	[55]
		MALDI-TOF-MS	[38]
Determination of cross-reactivity	Whole allergen extract	Lowry method, SDS-PAGE-Coomassie/Immunoblot/Immunoblot Inhibition	[56]
		SPT, Lowry method, Bradford method, Basophil activation assay, SDS-PAGE-Coomassie/Immunoblot/Immunoblot Inhibition, FEIA, FEIA Inhibition	[57]
		SPT, Direct ELISA, ELISA Inhibition, SDS-PAGE-Immunoblot	[58]
		Indirect ELISA, ELISA Inhibition, IEF-Immunoblot	[59]
		Kjeldahl procedure, RAST, RAST Inhibition	[60]
Allergen characterization	Whole allergen extract	SDS-PAGE-Coomassie, CIE, RAST, RIA, RIA Inhibition, Basophil activation assay	[61]
Epidemiological study	Whole allergen extract	Sandwich ELISA	[62]
Determination of cross-reactivity	nOle e 1	Lowry method, SDS-PAGE-Immunoblot/Immunoblot Inhibition, Indirect ELISA, ELISA Inhibition	[63]
		Amino acid analysis, SDS-PAGE-Coomassie/Immunoblot, Edman sequencing, Indirect ELISA, ELISA Inhibition	[64]
		Amino acid analysis, Bradford method, SDS-PAGE-Coomassie/Immunoblot, Competitive ELISA Inhibition, RAST, TEM	[65]
		BCA assay, SDS-PAGE-Immunoblot, RAST, RAST Inhibition	[15]
		SDS-PAGE-Immunoblot, ELISA	[66]
		Lowry method, SDS-PAGE-Coomassie, CRIE, Edman degradation, RAST, RAST Inhibition, Sandwich ELISA	[53]
		Bradford method, SDS-PAGE-Immunoblot, Optical microscopy, TEM	[67]
Allergen purification	nOle e 1	RIA, SPT	[46]
Allergen characterization	nOle e 1	SDS-PAGE-Coomassie, MALDI-TOF-MS and–MS/MS	[68]
		Bradford method, SDS-PAGE-Immunoblot/ConA, IEF, RIA Inhibition, CRIE, CIE	[69]
		Biuret-Lowry method, SPT, SDS-PAGE-Coomassie/Densitometry/Immunoblot, Indirect ELISA, Competitive ELISA	[70]
		SDS-PAGE-Ag/Immunoblot, MALDI-TOF-MS, ^{1}H NMR, RAST, RAST Inhibition	[48]
		Bradford method, IEF-^{125}I, SDS-PAGE-Coomassie/^{125}I, 2-DE-^{125}I, RAST, RAST Inhibition, Indirect ELISA	[47]
		SDS-PAGE-Immunoblot, IEF-Immunoblot, CIE, CRIE	[71]
		Bradford method, SDS-PAGE-Coomassie/Immunoblot, ELISA Inhibition	[37]
		Amino acid analysis, SDS-PAGE-Coomassie/Immunoblot/Con A, PD-TOF-MS, ELISA Inhibition, Dot immunoblotting, CD analysis, Edman degradation	[72]
		IEF, RAST, RAST Inhibition, SPT, SDS-PAGE	[73]
		SDS-PAGE, Edman degradation, ELISA, ELISA Inhibition	[74]
		SDS-PAGE	[52]
		Amino acid analysis, Cysteine content, Tryptophan content, SDS-PAGE-Immunoblot/ConA, Edman degradation, CD analysis	[75]
		SDS-PAGE-Coomassie, MALDI-TOF-MS	[76]
	rOle e 1	SDS-PAGE-Coomassie/Immunoblot, ELISA Inhibition	[41]
Allergen characterization and cloning	nOle e 1 and rOle e 1	ELISA Sandwich, ELISA Inhibition, SDS-PAGE-Immunoblot, Dot immunoblotting	[77]
		Amino acid analysis, Edman degradation, SDS-PAGE-Coomassie, CD analysis, Fluorescence emission, MALDI-TOF-MS, ELISA Inhibition	[78]
		Edman degradation, SDS-PAGE-Coomassie/Immunoblot/ConA, MALDI-TOF-MS, CD analysis, Fluorescence emission, ELISA Inhibition	[79]
		SDS-PAGE-Coomassie/Immunoblot	[49]
Characterization of the immune response in mice sensitized	nOle e 1	Lowry method, SDS-PAGE-Coomassie/Immunoblot, Indirect ELISA, Competition ELISA, ELISA Inhibition	[80]
	nOle e 1 and rOle e 1	Lowry method, SDS-PAGE-Coomassie, 2-DE-Immunoblot, Indirect ELISA, Competitive ELISA, Cytokine assay	[81]
Determination of the allergen location	nOle e 1	Bradford method, SDS-PAGE-Immunoblot, Optical microscopy, TEM	[82]
		TEM	[83]
		TEM	[84]
		Bradford method, SDS-PAGE-Immunoblot, Optical microscopy, TEM	[67]
Allergen immunological characterization	nOle e 1	Amino acid analysis, Edman degradation, SDS-PAGE-Coomassie, Dot immunoblotting/ConA, Basophil activation assay	[85]
	nOle e 1and rOle e 1	RAST, T-cell proliferation assay, RIA, RIA inhibition, Indirect ELISA	[86]
	nOle e 1 and rOle e 1	SPT, SDS-PAGE-Coomassie, Indirect ELISA	[87]
	nOle e 1 and rOle e 1	Amino acid quantification, SPT, Indirect ELISA, ELISA Inhibition	[88]
Evaluation of Ole e 1 variability in different olive pollen batches/cultivars/ stages of growth	nOle e 1	SDS-PAGE-Immunoblot, RAST Inhibition, Sandwich ELISA	[89]
		Lowry method, Kjeldahl method, SDS-PAGE-Coomassie/Silver, IEF, RAST Inhibition, SEC-UV	[90]
		Bradford method, SDS-PAGE-Coomassie/Immunoblot, SPT	[91]
		SDS-PAGE-Coomassie/Immunoblot, ELISA Competition, ELISA Inhibition	[92]
		2-DE-Ag/Fluorescence/Immunoblot, nanoLC-MS/MS	[93]
		Bradford method, SDS-PAGE-Coomassie/Ag/Immunoblot/Densitometry, TEM, FM	[94]

Table 1 (*Continued*)

Objective	Allergen	Techniques and methodologies	Ref
Development of detection methods	nOle e 1	BCA protein assay, SDS-PAGE-Immunoblot, Sandwich ELISA	[95]
		SDS-PAGE-Coomassie/Densitometry/Immunoblot, Sandwich ELISA, EAST Inhibition	[96]
Evaluation of new vaccines	nOle e 1	Dot immunoblotting, ELISA Inhibition, T cell proliferation assay, Cytokine assay, Optical microscopy, FM	[97]
		Amino acid analysis, BCA assay, SDS-PAGE-Coomassie/Immunoblot, ELISA Inhibition	[98]
		Indirect ELISA, Cytokine assay	[99]
	nOle e 1 and rOle e 1	T cell proliferation assay, Cytokine assay, Basophil activation assay, SDS-PAGE-Coomassie/Immunoblot, Indirect ELISA, ELISA Inhibition	[100]
Quantification of airborne antigenic activity	nOle e 1	Indirect ELISA	[101]
Obtaining of MAb	nOle e 1	Lowry method, SDS-PAGE-Coomassie/PAS technique, CIE, SPT, Basophil activation assay, RIA, RAST Inhibition	[51]
Allergen characterization	nOle e 2	Lowry method, Amino acid analysis, Edman degradation, SDS-PAGE-Ag/Immunoblot, CD analysis, Indirect ELISA	[102]
Allergen characterization and cloning	nOle e 2 and rOle e 2	SDS-PAGE-Coomassie/Immunoblot, ELISA Inhibition	[41]
Determination of the allergen location	nOle e 2	Bradford method, SDS-PAGE-Coomassie/Immunoblot, FM, TEM	[103]
Determination of the response pattern	nOle e 2	SPT, Indirect ELISA	[104]
Determination of cross-reactivity	rOle e 3	Amino acid analysis, Lowry method, Edman degradation, MALDI-TOF-MS, SDS-PAGE-Immunoblot, Indirect ELISA, ELISA Inhibition, CD analysis	[42]
Allergen characterization	nOle e 3	Amino acid analysis, Lowry method, Edman degradation, IEF-Coomassie, SDS-PAGE-Coomassie/Immunoblot, MALDI-TOF-MS, CD analysis, Indirect ELISA, ELISA inhibition	[105]
Allergen characterization and location	rOle e 3	SDS-PAGE-Coomassie/Immunblot, CD analysis, Amino acid analysis, Edman degradation, Indirect ELISA	[43]
	nOle e 3	SDS-PAGE-Immunoblot, TEM	[106]
Allergen characterization	nOle 4	Biuret-Lowry method, SDS-PAGE-Coomassie/Ag/Immunoblot, IEF-Coomassie, Indirect ELISA, ELISA Inhibition, Edman degradation	[107]
Allergen characterization	nOle e 5	Biuret-Lowry method, SDS-PAGE-Coomassie/Ag/Immunoblot, IEF-Coomassie, Indirect ELISA, ELISA Inhibition, Edman degradation	[107]
Allergen characterization and location	nOle e 5	Bradford method, PAGE, IEF, SDS-PAGE-Immunoblot, TEM	[108]
	nOle e 5 and rOle e 5	Bradford method, SDS-PAGE-Coomassie/Immunoblot/Immunoblot Inhibition, Indirect ELISA, ELISA Inhibition	[50]
Allergen characterization	rOle e 6	Amino acid analysis, Edman degradation, CD analysis, NMR, SDS-PAGE-Coomassie/Immunoblot, Indirect ELISA, ELISA Inhibition	[109]
		SDS-PAGE-Immunoblot, CD analysis, NMR	[110]
Determination of cross-reactivity	nOle e 7	Edman degradation, SDS-PAGE-Coomassie/Immunoblot, IEF, Indirect ELISA	[111]
		Double-blind placebo-controlled, SPT	[112]
Allergen characterization	nOle e 7	Amino acid analysis, Lowry method, Edman degradation, MALDI-TOF-MS, SDS-PAGE-Coomassie/Immunoblot, Indirect ELISA	[14]
Determination of cross-reactivity	rOle e 8	Amino acid analysis, Lowry method, Edman degradation, MALDI-TOF-MS, SDS-PAGE-Immunoblot, Indirect ELISA, ELISA Inhibition, CD analysis	[42]
Determination of cross-reactivity and cloning	nOle e 8 and rOle e 8	Amino acid analysis, Lowry method, SDS-PAGE-Coomassie/Immunoblot/Immunoblot Inhibition	[44]
Allergen characterization	rOle e 8	Amino acid analysis, Edman degradation, MALDI-TOF-MS, SDS-PAGE-Coomassie/Immunoblot, CD analysis, Indirect ELISA	[45]
Determination of cross-reactivity	rOle e 9	Lowry method, CD analysis, SDS-PAGE-Coomassie/Immunoblot/Immunoblot inhibition, Indirect ELISA	[113]
Allergen characterization	rOle e 9	Amino acid analysis, Edman degradation, MS, SDS-PAGE-Immunoblot, NMR	[114]
Characterization of the immune response in mice sensitized	nOle e 9	Amino acid analysis, Cysteine content, Tryptophan and tyrosine content, Carbohydrate determination, Edman degradation, CD analysis, MALDI-TOF-MS, SDS-PAGE-Coomassie/Immunoblot/Immunoblot Inhibition, Indirect ELISA, ELISA Inhibition	[115]
		Edman degradation, SDS-PAGE-Coomassie-Immunoblot, Carbohydrate detection, Amino acid analysis, Cysteine content, Lowry method, IEF-Coomassie, MALDI-TOF-MS, Indirect ELISA	[116]
		Lowry method, SDS-PAGE-Coomassie/Immunoblot, Indirect ELISA, Competition ELISA, ELISA Inhibition	[80]
Evaluation of Ole e 1 contain in different olive pollen batches	nOle e 9	SDS-PAGE-Immunoblot, RAST Inhibition, Sandwich ELISA	[89]
Study of a clinical case	nOle e 9	SPT, SDS-PAGE-Immunoblot/Immunoblot Inhibition	[117]
Determination of the diagnostic value of N- and C-terminal domains	rOle e 9	SDS-PAGE-Immunoblot Inhibition, Indirect ELISA, ELISA Inhibition	[118]

Table 1 (*Continued*)

Objective	Allergen	Techniques and methodologies	Ref
Allergen characterization	nOle e 10	Carbohydrate detection, Amino acid analysis, Cysteine content, IEF, SDS-PAGE-Coomassie/Immunoblot, MALDI-TOF-MS, CD analysis, PCR, Indirect ELISA, ELISA Inhibition	[119]
	nOle e 10 and rOle e 10	MALDI-TOF-MS, Edman degradation, SDS-PAGE-Coomassie/Immunoblot, AGE-Coomassie, Indirect ELISA	[40]
	rOle e 10	Edman degradation, MALDI-TOF-MS, SDS-PAGE-Coomassie/Immunoblot, Indirect ELISA	[120]
Allergen characterization and location	nOle e 10	CD analysis, Fluorescence analysis, TEM, FM	[121]
Characterization of the immune response in mice sensitized	nOle e 10	Lowry method, SDS-PAGE-Coomassie/Immunoblot, Indirect ELISA, Competition ELISA, ELISA Inhibition	[80]
Determination of the response pattern	nOle e 10	SPT, Indirect ELISA	[104]
Allergen characterization	rOle e 11	SDS-PAGE-Coomassie/Immunoblot, 2-DE-Coomassie/Silver/Immunoblot, Edman degradation, MALDI-TOF-MS, PCR, Carbohydrate detection, CD analysis, Determination of enzymatic activity, Indirect ELISA	[122]
Study of a clinical case	nOle e 13	SPT, Edman degradation, SDS-PAGE-Immunoblot	[32]
Allergen characterization	nOle e 13	SDS-PAGE-Coomassie, MALDI-TOF-MS, nanoLC-MS/MS	[33]

IgG-binding respectively, to both the natural extracts and cloned allergens.

Gel electrophoresis followed by immunoblotting represents the standard procedure for allergen separation and identification, and it has been extensively applied for olive allergens, as it can be seen in Table 1. In the immunoblot procedure, sample components separated by IEF, SDS-PAGE, or 2D gels are transferred onto a nitrocellulose membrane and examined for immunological reactivities with antibodies. The presence of bound antibodies is confirmed by incubation with a second antibody specific for the antibody source. Second antibodies are usually conjugated to enzymes such as alkaline phosphatase or peroxydase, which produce insoluble colored products with particular substrates [35]. Although this technique is not quantitative in a formal sense, the intensities of individual bands on a blot are proportional to antibody or antigen concentrations. When immunoblot inhibition is employed the incubation with the antibody is performed in the presence of a similar antigen, usually the recombinant one.

Although in a lesser extent, crossed immunoelectrophoresis (CIE) and crossed radioimmunoelectrophoresis (CRIE) assays have also been employed for olive allergen detection. CIE combines conventional agarose electrophoresis in one dimension with a perpendicular second-dimension rocket immunoelectrophoresis using antibody-containing gels to produce a pattern of overlapping immunoprecipitates [51,61,69,71]. On the other hand, CRIE patterns are produced by autoradiography of unstained CIE gels after incubations with allergic human serum and radiolabeled anti-human IgE [53,69,71].

Moreover, dot immunoblotting have been employed in the detection of Ole e 1 [72,77,85,97]. In this case, sample protein extracts are spotted onto a membrane and incubated with enzyme-labeled protein specific antibodies, which bind to the target antigens. The dots are visualized by the formation of a colored product after enzyme-substrate interaction. Dots intensity is proportional to the amount of antigen. Nevertheless, these immunological methods render only qualitative or semiquantitative results.

For antigen immobilization assays, three different approaches are available depending on the order of the addition of the reactants. The indirect approach involves the immobilization of the antigen on a solid phase and its incubation with a specific antibody, usually a specific human IgE. A second labeled anti-IgE antibody is next added allowing the detection [125]. In the sandwich approach, the antibody is the one immobilized on the solid phase while the antigen is captured by the antibody and detected by a second antigen-specific labeled antibody, which binds the analyte. The concentration of antigen/antibody complex is subsequently estimated based on a standard curve generated with purified reference standards. Finally, in the inhibition approach, a reference antigen is immobilized on the solid phase. Afterwards, the labeled antibody and the target allergen are pre-incubated and added to the solid-phase antigen. If no allergen is present in the inhibitor sample, the labeled antibody will show maximal binding to the solid-phase bound antigen, resulting in a high signal. In this case, the absorbance is inversely proportional to the concentration of antigen in the sample. When two antibodies are added to compete for the binding of the antigen, the assay is carried out in a competitive mode. The most common label for antibodies employed in the analysis of olive allergens are enzymes producing colored products (enzyme-linked immunosorbent assay (ELISA)). In some occasions the reaction product of the enzyme was a fluorescent compound (fluorescence enzyme immunoassay) [57]. Nevertheless, ELISA has been by far the most widely employed technique in olive allergen quantitation, as Table 1 shows. Moreover, other techniques using antibodies labeled with a radioactive isotope (RIA or RAST) have been also extensively employed in the characterization of olive allergens [51,61,69,86]. Although the techniques based on radioactive isotopes are extremely sensitive, allowing the development of methodologies with detection limits in the nanogram range [92], they require specialized equipment and special precautions, making limited its application.

Apart from the detection and the quantitation studies, many efforts have been also focused on determining allergens cell localization, allowing in many times to determine or confirm the allergen function in the cell. Different microscopy techniques have been employed for this purpose. Optical microscopy has been used to determine the cell localization of olive allergens after immunostaining [67,82] or haemotoxylin–eosin staining [97]. Nevertheless, transmission electron microscopy (TEM) has been the most widely employed technique in the immunolocalization assays. In this technique the allergen is firstly incubated with a specific monoclonal antibody followed by incubation with an anti-IgG secondary antibody coupled with gold particles. The images obtained by TEM let to determine the presence and the position of allergens in

the cell. Instead of gold, the second incubation can be performed using a fluorescence marker, and detecting the epifluorescence (reflected light fluorescence) with a confocal laser scanning microscope (CLSM) [94,97,103,121].

Different assays are also employed to study the cell response to allergens presence. T-cell proliferation assay has been used to study cell mediated immune responses. T-cells are the most abundant type of white blood cells and are specialized in binding to antigens. In this assay, the cell is stimulated with the allergen, [^{3}H]-thymidine is next added to label the stimulated cell, and cells are detected by radiation using a scintillation counter [86]. On the other hand, basophils are white blood cells involved in the processes of allergy and asthma. The activation of basophils by olive allergens have been measured both by histamine and β-hexosaminidase release assays confirming the identification of the allergens or doing an allergy screening. In the histamine release assay, a little volume of freshly collected blood sample is added to an allergen extract and the released histamine is measured by fluorescence [51,57,61] or radioactive immunoassay [85]. For a given human sample, the released histamine is proportional to the concentration of a specific allergen in the extract [125]. β-Hexosaminidase from basophil leukemia cells has also been used, but just in one occasion, for the detection of olive allergens [100]. The immune cell response has been also evaluated by cytokine measuring. In the same way, cells were exposed to the olive allergen and the cytokine released due to the cell–antigen interaction was measured by ELISA [97,100].

4. Olive pollen and olive fruit studies

The presence of at least 20 proteins with allergic activity has been demonstrated in olive pollen extracts despite only 12 of them have been characterized (Ole e 1 to Ole e 12) [73]. Among them, Ole e 1 has been identified as the major allergen of olive pollen. Regarding the olive fruit, just one allergen has been described, Ole e 13, being included in the thaumatin-like family.

Most studies have been focused on the study of one or few allergens although there are also some works in which the whole pollen extract was employed. Indeed, pollen extracts have been employed for olive cultivar differentiation, since olive cultivars display wide differences in the expression levels of many allergens and in the number and molecular characteristics of the expressed allergen isoforms. In this sense, IEF [54], SDS-PAGE-Commassie or Immunoblot [55], and MALDI-TOF-MS [38] have demonstrated to be useful tools. Furthermore, total allergen extracts have also been employed for studying cross-reactivity between olive pollen and other species of the Oleaceae family such as ash (*Fraxinus exselsior*) [57,59,60], privet (*Lingustrum vulgare*) [57,59,60], lilac [57], *Phillyrea angustifolia* [60], and Russian olive [58,59]. The results showed a high degree of cross-reactivity between species, finding common allergens in all species. Nevertheless, the presence of allergens characterizing every species made there were no total identity between them [60]. Moreover, the cross-reactivity between olive pollen and pollen from phylogenetically unrelated species, including at least, birch, mugwort, and pine pollens has also been demonstrated [56], indicating that olive pollen can be included in the family of cross-reactive pollens.

5. Characterization of olive allergens

First studies focused on olive pollen date from the early 80 s [61,73]. In these works, a preliminary fractionation of an olive pollen extract showed different allergenic fractions and first approximations to the physicochemical and immunological characterization were performed in these works.

Ole e 1 was the first determined pollen allergen [73], although it was not until 1988 when it was named as Ole e 1 [69]. Its function is still unknown although some authors have suggested it is a constitutive protein involved in reproductive functions [75] or in the control of the osmotic gradient in the hydration process [82]. It is the most abundant protein in the olive pollen extracts, representing up to 5–20% of the total olive pollen proteins [70]. It is considered the most prevalent olive allergen affecting more than 80% of patients suffering hypersensitivity to olive pollen [69,71]. This allergic activity has been also tested in mice [80,81]. Indeed, the removal of Ole e 1 from the whole extract results in the nearly total disappearance of its allergenic activity [51]. These facts make Ole e 1 the best characterized olive allergen so far, as Table 1 shows.

Ole e 1 is a glycoprotein that exhibits an electrophoretic pattern consisting of two bands corresponding to the glycosylated (20 kDa) and nonglycosyltaed (18.5 kDa) forms (see Fig. 1a) [37,51,74,126]. MALDI-TOF analysis of Ole e 1 also showed the presence of these two forms being the glycosylated one the most abundant (see Fig. 1b). During sample treatment or storing, those two components can produce either aggregates of about 40 kDa [71,73] or degradates of about 8 kDa [47]. Both the cDNA [52] and the amino acid chain of Ole e 1 have been completely sequenced [61,71,126], observing extensive microheterogeneity [75]. Moreover, sequencing also showed a putative N-glycosylation site at position 111–113 [52,75] and the presence of six cysteine residues linked by disulfide bonds [75,94]. Results also demonstrated that p*I* ranged from 5.0 and 6.0 [47,71]. Furthermore, the allergen showed a relatively high content of periodically ordered secondary structure with 22% α helix, 21% β bend, 17% β turn, and 40% of periodic conformation, being a glycosylation site located in the β turn conformation [75].

The distribution of Ole e 1 transcripts (the complementary RNA copy of a sequence of DNA) has been observed by optical microscopy in both the microspores and the sporophytic tissue [82]. The immunolocalization of Ole e 1 and its transcripts was determined by immunogold labeling showing that it is placed throughout the rough endoplasmic reticulum, where it is stored and synthesized [65,82–84,91,94]. On the other hand, the expression of the allergen is controlled at the transcriptional level [82,83,91], increasing after its emergence from the pollen tube during *in vitro* germination and releasing into the culture medium throughout pollen germination [94].

The proliferative response of blood cells of allergic patients induced by Ole e 1 synthetic peptides allowed to determine that the IgE and IgG response is fundamentally directed against peptides located in the C-terminal half of the protein [86,87]. Besides this, antigenicity prediction showed the existence of four main regions which may contribute to B-cell epitopes [75].

Ole e 1 was also the first cloned olive allergen. Despite first clones were obtained in *E. coli* [49], nowadays *P. pastoris* [79] has become the most employed host. The comparison of the natural allergen and the recombinant allergen obtained from *P. pastoris* by their spectroscopic properties and immunological reactivities showed they were indistinguishable [79,88].

Glycosylated Ole e 1 has been the target of many studies focused on the carbohydrate moiety and its possible role in IgE response. Although results on the role of this carbohydrate moiety in allergenicity are controversial, its involvement in antigenicity is demonstrated [77]. The carbohydrate component of Ole e 1 has a molecular mass of 1.3 kDa, representing 5% of the total mass of the glycoprotein [72]. Tryptic digestion of Ole e 1 revealed the presence of a single carbohydrate-containing peptide [72]. The primary structure of the N-glycan was determined by NMR [48] and MALDI-TOF-MS [76], identifying it as $GlcNAc_{1-2}Man_3XylGlcNAc_2$. The analysis of the peptide containing the carbohydrate moiety was performed both by MALDI-MS and MS/MS allowing the characterization of the Ole e 1 allergens [68]. Fig. 2 shows, as a

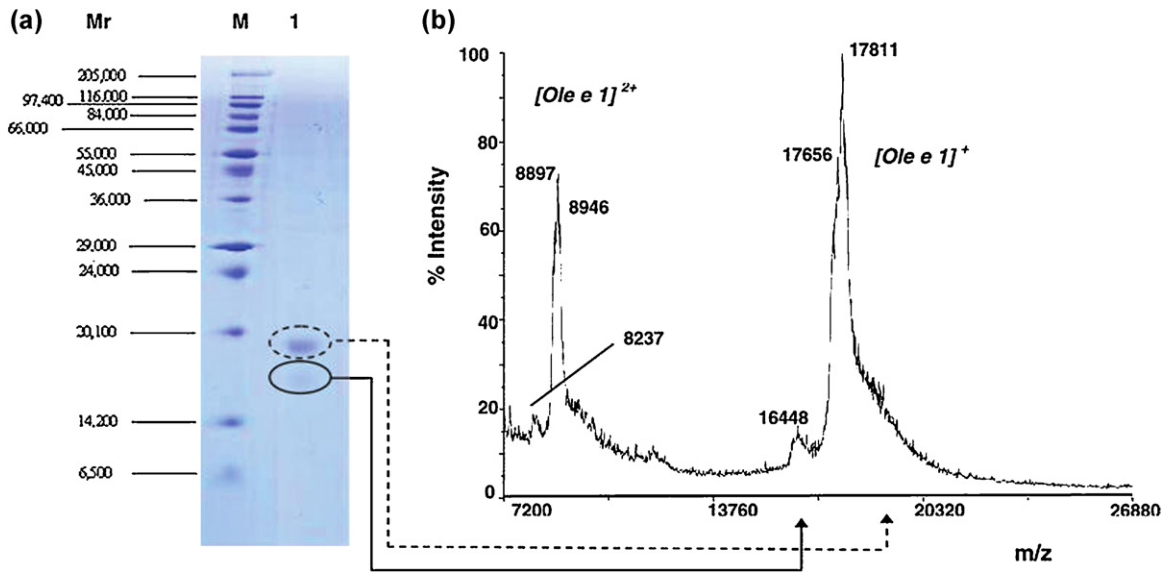

Fig. 1. (a) SDS-PAGE (lane M, marker, lane 1, Ole e 1 pure fraction); (b) linear MALDI spectrum of Ole e 1 chemical fraction. From [68].

representative example, the MS/MS spectrum of one glycopeptide from Ole e 1. It showed fragments originating on either side of the glycosylation side and fragments belonging mainly to the cross ring cleavages of the glycan moieties. Moreover, N-glycan profiles can also varied depending on the olive pollen cultivar [93].

In order to demonstrate the role of this carbohydrate moiety in IgE recognition, the reactivity of sera from allergic patients were analyzed against the free carbohydrate isolated from the allergen, the carbohydrate-containing peptide from the allergen, and the synthetic peptide corresponding to the same fragment [85].

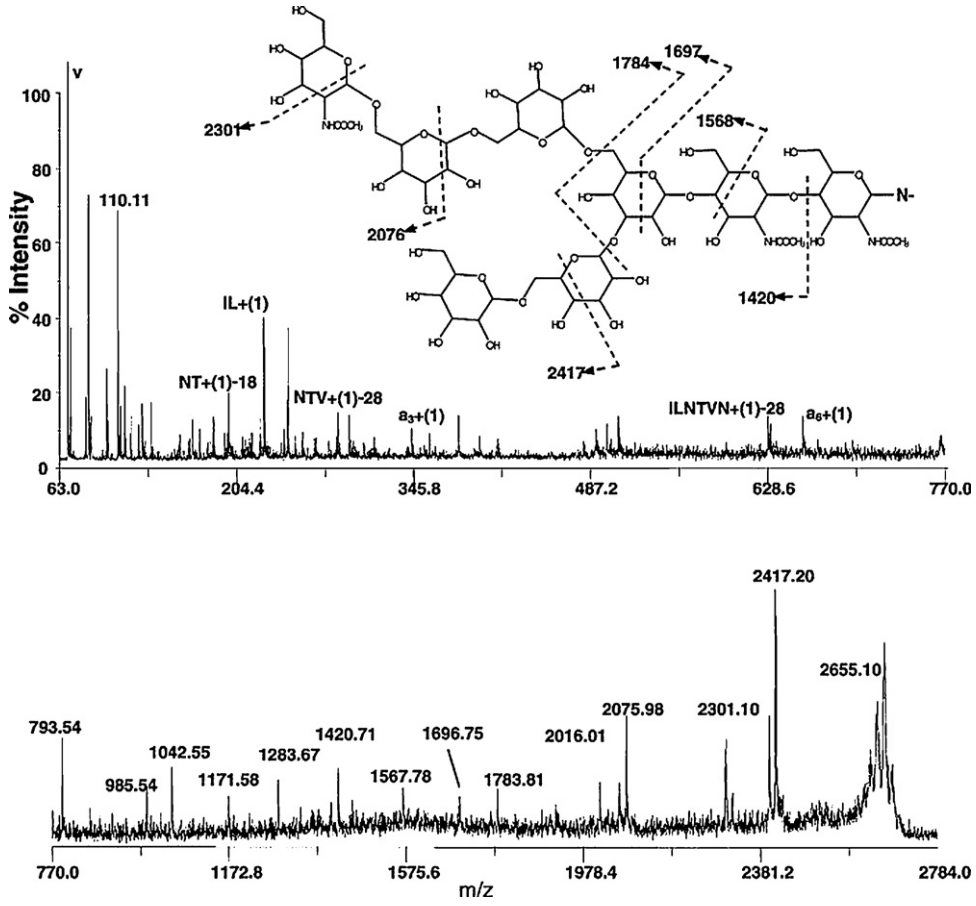

Fig. 2. CID spectrum of ion species at *m*/*z* 2655.10. From [68].

Only the unglycosylated synthetic peptide yielded negative results demonstrating the determining role of the carbohydrate fraction in the antigen–antibody recognition. The analysis of protease digestion extracts of the allergen by RAST also demonstrated that both α(1,3)-fucose and β(1,2)-xylose were involved in IgE binding [48].

Polymorphism is a general feature in pollen allergens. It has been demonstrated that the differences in allergen composition among olive cultivars is responsible for the important differences in the allergenic potency of the extracts. The study analyzing pollens collected in different Spanish geographic areas and in different pollen seasons showed a considerable variability in its biochemical and immunochemical properties [62,90]. Moreover, other works analyzing proteins [91,92] and RNA [127] from different varieties of olive pollen also demonstrated great differences in allergenic potency and Ole e 1 content [38,55,91,92]. These allergenicity differences were observed in five consecutive years, demonstrating that they are mainly due to genetic differences among olive varieties [92]. Nevertheless, the climatic effect has also demonstrated to influence on the Ole e 1 content, existing a positive correlation between rainfall in the winter months and the total allergenicity of the pollen [88].

Different studies have been focused on determining the allergenic cross-reactivity of Ole e 1 and other proteins belonging or not to the *Oleaceae* family [128]. Cross-reactivity of Ole e 1 and Ole e 4 [66] has been determined using different immunological methodologies. This cross-reactivity could be explain taking into account they share allergenic epitopes of Ole e 1 and Ole e 4, explaining the cross-reactivity found between both of them. Furthermore, the cross-reactivity of Ole e 1 with species belonging to the Oleaceae family such as ash (*Fraxinus excelsior*) [53,63,65], privet (*Ligustrum vulgare*) [15,53,65], lilac (*Syringa vulgaris*) [53,65], and forsythia (*Forsythia suspensa*) have also been evaluated [65]. Ole e 1 homologues of *Oleaceae* species have shown a very similar amino acid sequence and *in vivo* and *in vitro* reactivities indicating a relevant role in cross-reactivity [53,65]. Apart from the olive family, the cross-reactivity with other allergens from phylogenetically unrelated species has also been studied. It has been demonstrated cross-reactivity between olive Ole e 1 and ryegrass pollen, couch grass (Bermuda) pollen [15], and English plantain (*Plantago lanceolata* L.) [67]. The role of the N-linked glycan on cross-reactivity has been also determined [64]. For that purpose, standards of glycoproteins as ascorbate oxidase, horseradish peroxydase, bromelain, ovalbumin, and honeybee venom phopholipase A_2 were employed. Ascorbate oxidase, peroxidase, and bromelain yielded a positive recognition by Ole e 1 antiserum. This response disappeared when they were deglycosylated suggesting a high relevance of glycan in IgE recognition.

Several MAbs against Ole e 1 have been produced and purified [51], finding five of them reactive to Ole e 1, and two of them (called OL 2 and OL 7) able to recognize two different epitopes on the allergen. MAbs have been used in the development of sensitive ELISA tests for the measurement of Ole e 1 for clinical purposes [95,96]. The developed methods demonstrated to be reproducible, specific, reliable, and sensitive, achieving detection limits between 0.5 and 1 ng/mL, and demonstrating to be a useful tool in routine clinical analysis. The ELISA methodology has been also employed in the quantification of airborne Ole e 1. This study demonstrates that both the allergenic activity as well as the pollen particles followed in a similar curve, except in the periods before or succeeding the main olive pollen season [101].

Finally, many efforts have been done in the development of new therapies for the treatment of patients with olive pollen allergy. The main part of the developments is based on the use of the allergen Ole e 1, due to its high presence in the olive pollen and its high prevalence in patients. Recombinant mutants of Ole e 1 with lower allergenicity have been performed by genetic engineering [100]. Three allergen mutants containing changes in the C-terminal end were produced and immunologically tested both by *in vitro* and *in vivo* methodologies. Only one of them was considered interesting as immunotherapeutic agent for treating allergic patients. Ole e 1 has been also employed in the development of intranasal vaccines using biodegradable poly (DL-lactide glycolide) microparticles as vehicle [97–99]. In this studies, the whole allergen [98,99] or the peptide with the major T cell epitope [97] were encapsulated in the biocompatible and biodegradable polymer and their viability to serve as a vehicle for the administration of the allergen was evaluated. The results demonstrated the effectiveness of this type of immunization in preventing the allergic sensitization to Ole e 1.

Ole e 2 has been described as a significant allergen that belongs to the profilin family. Profilin is an ubiquitous protein found in a large number of vegetable and animal biological sources that control actin polymerization in eukaryotic cells [129]. They have been established as pan-allergens (allergens widely distributed in nature) due to its high cross-reactivity [102]. The presence of profilins as cross-reacting components in various plants could explain the existence of allergic patients to pollen and food from distantly related plants [129]. Indeed, Ole e 2 shows structural and immunological similarity with other pollen sources such as birch, ash, and grass [102,130]. Different studies of olive-pollen profilin have established its molecular mass range between 15 and 18 kDa [38,102]. More specifically, four isoforms with 17.8, 17.0, 16.0, and 15.2 kDa molecular masses were firstly determined [41]. Nevertheless, other authors have found two isoforms with an average molecular weight of 14.4 kDa [130] and three isoforms of 13.3, 13.9, and 14.3 kDa by SDS-PAGE analysis [103]. The p*I* of Ole e 2 was 5.1 [130]. The analysis of this allergen by CD revealed a secondary structure consisting of 15% α-helix, 33% β-strand, 20% β-turn, and 32% random conformation, which is in agreement with molecular data of other profilins [102]. The prevalence of this olive allergen has been estimated between 24 and 27% in olive-hypersensitive patients [102,104,130], being related with asthma as the major IgE response in sensitized patients, tighter with Ole e 10 [104]. In fact, the risk of developing asthma in patients sensitized against both allergens increases in comparison with the patients only sensitized against one of them [104]. The main immune response of Ole e 2 is related with asthma increasing this response when patients show hypersensitivity against Ole e 2 and Ole e 1 allergens [104]. Ole e 2 displays high cross-reactivity, showing structural and immunological similarity with other pollen sources such as birch, ash, and grass [102,130].

Ole e 3 has been identified as a polcalcin belonging to the buffering type Ca^{2+}-binding protein subfamily [43]. It is a low molecular mass allergen of 9.2 kDa [105], consisting of a single chain of 84 residues [43]. Two isoforms have been detected [38] with p*I* values of 4.2 and 4.3 determined by IEF [105]. The secondary structure of this protein, established by CD, consisted of 52% α-helix, 10% β-strand, 29% β-turn, and 9% random conformation [105], not finding differences for either natural Ole e 3 and recombinant Ole e 3 [43]. In fact, it was demonstrated that the recombinant form retained the allergenic and antigenic epitopes of the natural allergen [43]. The prevalence of Ole e 3 was established in 20–50% of allergic patients to olive pollen [42,43,105,131,132] depending, in some cases, on the geographical area. Moreover, a decreased reactivity of the sera from allergic patients in the absence of Ca^{2+} was observed for Ole e 3 [42], indicating that IgE epitopes are affected by the conformational change induced by the binding/releasing of Ca^{2+}. Allergen location was established by transmission electron microscopy (TEM) observing it is mainly in the vicinity of membrane systems and in the aperture regions of the mature pollen grain [106]. Ole e 3 cross-reactivity is lower than that of Ole e 2, due to it has been observed just in other pollens, not finding reactivity with fruits, seeds, or insects [131]. Only some cases of cross-reactivity with pollens from

rapeseed, Bermuda grass, and birch were detected observing the most significant cross-reactivity with the *Oleaceae* pollens [42] and grass pollens [132].

Ole e 4 has been scarcely studied finding just one work focused to this allergen. In this work, Ole e 4 showed a high IgE binding capacity [107]. It displays an apparent molecular mass of 32 kDa and p*I*s ranging from 4.65 to 5.1 [133]. The N-terminal extreme is blocked and the analysis of two internal amino acid sequences showed no homology with any known protein.

Ole e 5 shows a molecular weight of 16 kDa and has, at least, five isoforms with p*I*s ranging from 4.6 to 6.5 depending on the study [107,108]. Ole e 5 shows homology up to 90% with superoxide dismutase (SOD) of several plant species [50,107], being the first Cu/Zn SOD identified as an allergen in a pollen source and suggesting a function in the protection against oxidative stress during pollen development [108]. The Ole e 5 enzymatic activities were measured by nitro blue tetrazolium chloride reduction with $O_2^{\bullet -}$ radicals photochemically generated [108]. Moreover, Ole e 5 from different olive varieties were cloned and expressed, showing some differences in the amino acid sequence, probably due its high degree of polymorphism. The percentage of recognition by pollen-allergenic patients against rOle e 5 was of about 39% [50]. Unlike other pollen allergens, Cu/Zn SOD have been also found in different olive tissues such as the olive fruit pulp [33,134] and olive leaves [135,136]. This could be a case of non-specific gene transcription due to the presence of the same protein in different tissues becoming from the same tree, and its presence in the olive pulp could explain the cases of cross-allergy between olive pollen allergy and olive fruit allergy.

Ole e 6 is the smallest allergen found in olive pollen so far. Although the protein exhibits an apparent molecular weight of 10 kDa by SDS-PAGE, it consists of a single highly acidic polypeptide chain of 50 amino acids (5.83 kDa) [38,109,111] with an p*I* of 4.2 [109,111]. This difference in molecular mass could be attributed to the strong acidic character of Ole e 6 decreasing the binding of SDS to the protein and due to the fact that non-globular molecules can give abnormal results by SDS-PAGE [109]. This allergen shows no homology with any protein contained in the nucleotide sequence database [109,111]. Ole e 6 is little available in the olive pollen, making difficult its extraction and being a case where recombinant production facilitates its availability. Its cloning renders two identical forms presenting the same structure as Ole e 6 but with different molecular mass determined by SDS-PAGE, 10 and 14.2 kDa [109]. Ole e 6 consists of two nearly antiparallel α-helices, bound by a short loop and maintained by three disulfide bonds [110]. In fact, based on the results obtained by NMR and CD, five epitopes have been proposed for this olive-pollen allergen [110]. It is recognized by up to 50% of allergic patients sera depending on the geographical area and olive tree presence [111].

Ole e 7 shows similarities with nonspecific lipid transfer proteins (LTP) from plant tissues [112]. These proteins play a role related to the ability of binding fatty acids and possess antibacterial and antifungal activities [137]. Ole e 7 presents high degree of polymorphism and it is formed by four isoforms differing in the position of three amino acids [38]. Fig. 3 shows the spectra obtained by MALDI-TOF of three different lipophilic fractions obtained from olive pollen employing different extraction times. These different extraction procedures allowed observing four different isoforms, ranging from 9.6 to 10 kDa, differing in the position of three amino acids. The prevalence of Ole e 7 has been determined in 47% in patients with olive allergy [14] and it has been associated with an increased risk of food anaphylaxis [112]. However, the risk degree depended on the geographical area, ranging from 60% in patients living in areas with intensive olive cultivation to 20% in patients living in areas with low olive cultivation [14]. Moreover, Ole e 7 could also be involved in allergenic cross-reactivity between fruit and olive pollen, due to patients with anaphylactic reaction after eating fruit are also sensitized to Ole e 7 [112]. Furthermore, Ole e 7 sensitization was more frequent when symptoms were induced after eating fresh fruit from other families such as *Rosaceae* and *Cucurbitaceae* [112].

Ole e 8 has been identified as a Ca^{2+}-binding protein like Ole e 3, but displaying a different biological role: it develops a regulatory function involved in signal transduction pathways [45] and belongs to different subfamilies of Ca^{2+} binding-proteins [42]. Ole e 8 consists of a single polypeptide chain of 171 amino acid residues displaying an apparent molecular mass of 20 kDa by SDS-PAGE [45]. Although the allergenic potential of Ole e 3 and Ole e 8 are different, all sera reactive to Ole e 8 were also reactive to Ole e 3 [42]. Possible differences on the prevalence between Ole e 3 and Ole e 8 has been established due to the different amount of each protein expressed in the pollen, much higher Ole e 3 (∼500 μg/10 g dried pollen) than Ole e 8 (15–40 μg/100 g dried pollen) [42]. In fact, the low presence of this allergen in the olive pollen makes necessary the use of recombinant expression to obtain manageable levels of this protein [45]. Using the recombinant Ole e 8, which was demonstrated to be immunologically equivalent to Ole e 8, cross-reactivity with other pollens from species of the *Oleaceae* and *Juniperus communis* families were detected [45].

Ole e 9 is the allergen of highest molecular mass, 46.4 kDa, reported from olive pollen [115,116]. Ole e 9 is a glycosylated β-1,3-glucanase, which belongs to the pathogenesis-related (PR-2) protein family [116]. It exhibits a low but significant polymorphism. Indeed, two different isoforms of Ole e 9 can be separated by RP-HPLC exhibiting a molecular mass of 46.431 kDa and 46.307 kDa by MALDI-TOF analysis. On the other hand, IEF can differentiate four components with p*I* values between 4.8 and 5.4 [116]. Ole e 9 secondary structure, determined by CD, consisted of 16% α-helix and 30% β-sheet [115]. The enzymatic activity of Ole e 9 was measured using the polysaccharide laminarin as substrate [116]. Ole e 9 prevalence significantly varied from 10% to 65% in patients with olive pollinosis [62,116] observing similar results with mice [80]. This allergen is constituted by two independents domains, a N-terminal portion of 36 kDa and a C-terminal domain of 10.6 kDa, both immunologically active [114,115]. The C-terminal domain presents high IgE-binding capability against sera from Ole e 9-sensitive individuals [115] and it is homologous to Ole e 10 olive allergen. The N-terminal domain contains the enzymatic activity and shows cross-reactivity with vegetable foods and latex proteins [113]. As with Ole e 8, the low presence of Ole e 9 in the olive pollen (less than 0.3%) makes necessary the use of recombinant technology to obtain both C-terminal and N-terminal domains separately [113,115]. The production of the whole allergen has not been successfully achieved so far. Nevertheless, 94% of Ole e 9 allergic patients can be identified using both recombinant fragments [118]. Due to the low Ole e 9 concentration, the content of this olive allergen is not well established. A high variability was found in Ole e 9 content in different olive pollen commercial batches using a developed immunoassay [89]. A special clinical case of allergy to Ole e 9 was reported in a man who worked with this allergen developed adverse reactions to it as a consequence of the daily overexposure [117]. The β-1,3-glucanase olive allergen shows 39, 33, and 32% of sequence homology with β-1,3-glucanases from wheat, willow, and *Arabidopsis thaliana*, respectively [116]. Some homologous counterparts to olive glucanase in ash and birch pollen [113] and cross-reactivity to pollens, vegetable foods, and latex proteins has also been reported [115,138].

Ole e 10 is considered a major olive allergen and patients sensitized to this allergen have been reported to suffer from severe asthma [40,104]. It has a molecular weight of 10.789 kDa and a p*I*

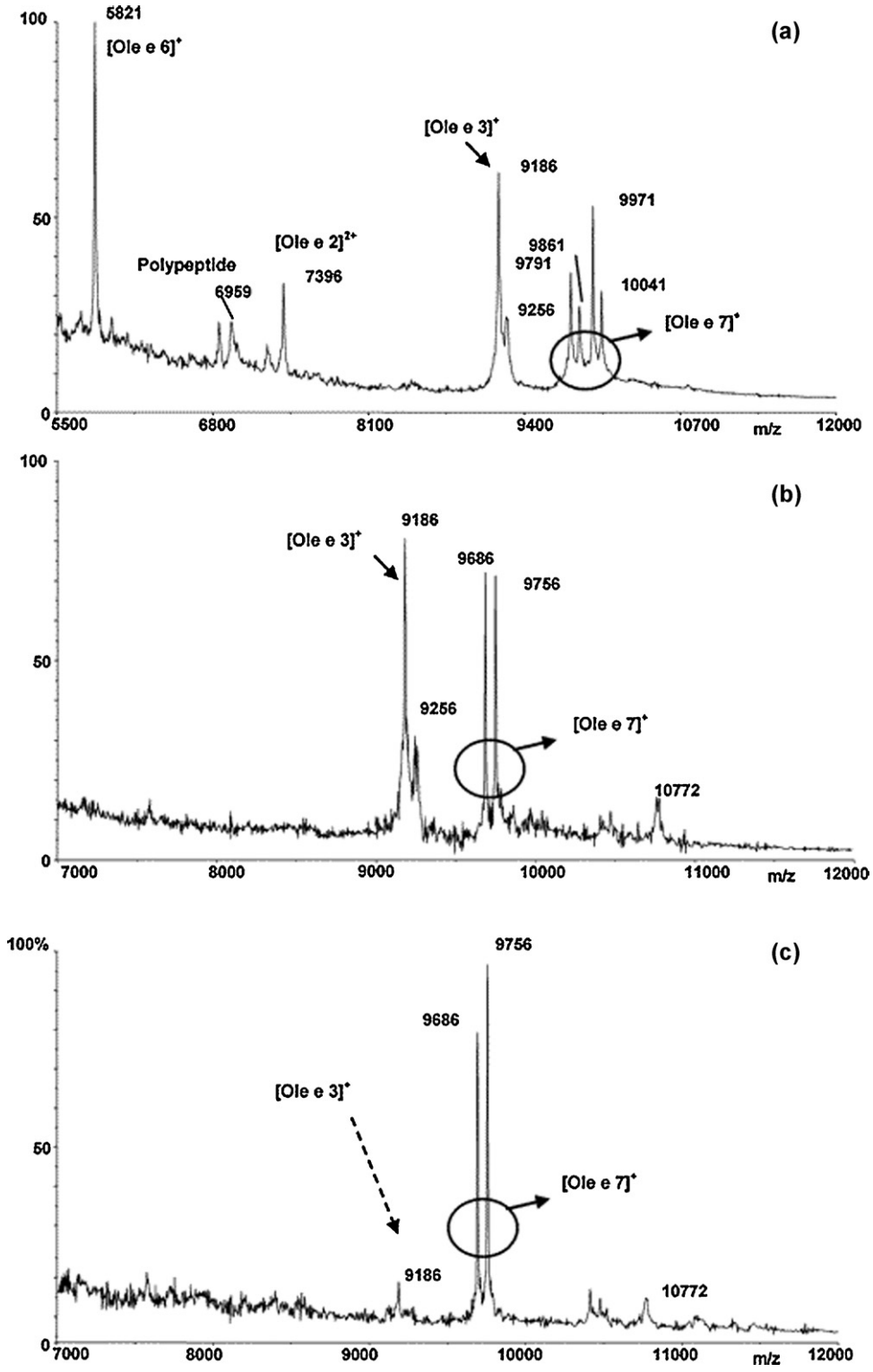

Fig. 3. MALDI spectra of the three different fractions obtained from olive pollen.

From [38].

of 5.8 [119]. Its presence in pollen is low (80 μg/g pollen) being necessary the use of recombinant technologies for its isolation [120]. However, problems with proteolysis, chemical modifications, and slight differences in IgE and IgG recognition of recombinant Ole e 10 by *P. pastoris* have been detected [120]. Total immunological and molecular equivalence of rOle e 10 and nOle e 10 was, nevertheless, demonstrated using Sf21 insect cells [139]. The secondary structure of Ole e 10 determined by CD analysis is 17% α-helix, 33% β-sheet, and 21% β-turn [120]. Moreover, a 53% of homology with the C-terminal domain of Ole e 9 could explain the remarkable cross-reactivity detected between these two allergens [104,119]. The localization of Ole e 10 in the growing pollen tube together with the presence of callose molecules (a β-glucane) which are major components in pollen tubes wall, suggested a possible role

of this allergen in the wall re-formation during germination and in the metabolism of carbohydrates [121,139]. The prevalence of Ole e 10 in olive allergic patients was 55% and 79% in mice [80]. Ole e 10 shares IgE B cell epitopes with proteins from *Oleacea*, *Gramineae*, *Chenopodiaceae*, *Cupressaceae*, *Ambrosia*, and *Parietaria* pollens, latex, and vegetable foods [119]. Its high prevalence and cross-reactivity make Ole e 10 to be considered as the major allergen in olive pollen.

Up to date, there is only one research article with information about Ole e 11 [122]. This allergen has been recently detected as a pectin methylesterase of 342 amino acid length polypeptide with a molecular mass of 37.4 kDa, and a p*I* of 7.8 [122]. The secondary structure of Ole e 11 was determined by CD spectroscopy: 3% α-helix, 50% β-sheet, and 27% β-turns. It shows low homology with other foods containing molecules from the pectin methylesterase family and higher identity with *A. thaliana* and *Salsola kali* pollen [122]. The prevalence of this olive pollen allergen ranged between 55.9% and 75.6% [122].

There is no published work on Ole e 12. Nevertheless, its DNA was sequenced by Castro et al. (www.uniprot.org), determining a sequence of 308 amino acids forming a protein of 34,068 Da. This protein belongs to the family of the isoflavone reductases, which are involved in the biosynthesis of plant defense metabolites.

Ole e 13 is the only allergen described, until now, in the olive fruit. It belongs to the family of the thaumatin-like proteins which are involved in host defense processes. It is a protein of 24,727 Da which was first partially sequenced by Edman degradation [32] and next cloned and totally sequenced by Villalba et al. (www.uniprot.org). Its analysis by nanoLC-MS/MS has permitted to detect variations in its sequence among olive varieties, even, within a single variety suggesting the presence of microheterogeneity [33]. Limited information about the allergenic reactivity of olive pulp is available, appearing just one example of immunoblotting with the allergenic serum of a patient allergic to olive fruit [32]. In this study just one intensive band corresponding to Ole e 13 appeared suggesting it as the main allergen in olive pulp.

6. Conclusions

Olive pollen is a major cause of both rhinoconjunctivitis and asthma in those regions, such as the Mediterranean area, where large amounts of airborne olive pollen result from intensive cultivation. In addition to olive pollen allergy, olive foods have also been reported to cause allergy. The search for therapies to fight these allergies requires a better understanding of the allergen molecular nature and the structures involved in cell activation. Despite there is an important research work on the characterization of some olive allergen such as Ole e 1, in most cases the knowledge is scarce and results are contradictory. Another aspect to take into account in olive pollen and food allergenicity is the high cross-reactivity observed in some of them. This fact could elicit great hypersensitivity reactions and synergistic effects. Another limitation is the low presence of some of these allergens which makes more difficult its isolation and further study. Innovative analytical methods and novel applications of available techniques are required to face the allergenicity problems due to olive allergens in an integrated manner.

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