Chapter 3

Detection and Quantitation of Olive Pollen Allergen Isoforms Using 2-D Western Blotting

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1. Introduction

The use of biological extracts for allergy diagnosis and immunotherapy has some disadvantages, including the high variability in their allergenic composition and the presence of allergens to which the patient is not allergic. As the result, wrong diagnosis, new sensitizations and/or systemic reactions often occur, limiting their use for specific immunotherapy. One of the strategies to overcome these problems is the standardization of biological extracts in order to control their allergenic composition. For this purpose, it is highly recommended to identify the allergenic molecules in the extract, to quantify them and to evaluate their allergenic activity in sensitized patients.

The allergenic pollen used for the preparation of natural extracts or recombinant allergens may contain different allergenic isoforms and/or variable amounts of each allergen, depending of its genetic origin among other factors (Castro et al. 2003, Hamman-Khalifa et al. 2008, Castro et al. 2010 & Jiménez-López et al. 2012). Consequently, allergic patients from different geographical areas may exhibit differential sensitization to a given allergen (Movérare et al. 2002). This fact can hinder the diagnosis of an allergic patient in response to a particular extract. Therefore, the allergenic variability in standardized protein extracts should resemble as much as possible to that observed in the natural sources in order to assure the efficiency and safety in the diagnosis and immunotherapy procedures.

Olive pollen produces seasonal respiratory allergy in the Mediterranean area, as well as in other temperate regions where it is intensively cultivated. Eleven olive pollen allergens, called Ole e 1 to Ole e 11, have been identified and characterized so far (Esteve et al. 2012). Many of these proteins exhibit a significant polymorphism as a consequence of the existence of point substitutions in the amino acid sequence, posttranslational modifications (e.g. glycosylation), and/or multimeric forms (Castro et al. 2010). In addition, some allergens

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show major quantitative differences, depending on the genetic origin (i.e. cultivar) of pollen (Castro et al. 2003). Here, we have used a 2-D electrophoresis-based immunodetection method to analyze the molecular variability of four olive allergens, namely Ole e 1, Ole e 2, Ole e 7 and Ole e 9, in the pollen of the olive cultivar 'Picual'. The advantages and putative applications of this method are also discussed.

2. Polymorphism of olive pollen allergens

The olive (*Olea europaea* L.) pollen exhibits a complex allergenic profile with numerous proteins recognized by sera of olive pollen allergic patients (Rodríguez et al. 2002 & Esteve et al. 2012). The great majority of olive pollen allergens show a highly polymorphic nature in 1-D and 2-D polyacrylamide gels, resulting in a pattern of multiple bands or spots, respectively.

Ole e 1 is the main olive pollen allergen, and affects more than 70% of patients suffering from olive pollinosis. Ole e 1 is an acidic protein that consists of a single 145 amino acid polypeptide chain, which is glycosylated at Asn^{111} (Lombardero et al. 1992). The molecular function of Ole e 1 is unknown but, on the basis of its cellular location and expression pattern, it has been suggested that it might have a role in pollen-pistil communication during pollen tube growth (Alché et al. 1999 & Alché et al. 2004). After 1-D SDS-PAGE and staining, or blotting and immunolabeling with specific antibodies or sera from allergic patients, three bands of 18.5, 20, and 22 kDa are visible, corresponding to the non-glycosylated, glycosylated and hyperglycosylated Ole e 1 isoforms, respectively (Villalba et al. 1993). Under non-reducing running conditions, a dimeric form of 40 kDa has been also detected (Villalba et al. 1993 & Morales et al. 2012). Ole e 1 is highly polymorphic due to the presence of point changes in the nucleotide sequence. These changes are extended to the expressed protein, affecting up to 59 different positions in the amino acid sequence (Villalba et al. 1994, Hamman-Khalifa et al. 2008 & Castro et al. 2010). Some of these amino acid substitutions are located within the epitope sequences involved in IgG and IgE binding (González et al. 2002). The composition of the sugar moiety is also highly variable among allergen isoforms (Castro et al. 2010). Interestingly, the N-linked glycan contributes to the final immunogenic and allergenic capacity of Ole e 1 (Batanero et al. 1994, 1999). The varietal origin of pollen represents a major source of variability for Ole e 1 (Hamman-Khalifa et al. 2008 & Castro et al. 2010). Moreover, this allergen showed conspicuous quantitative differences among olive cultivars (Castro et al. 2003).

Ole e 2 allergen belongs to the profilin family. Profilins are ubiquitous proteins, which are present in all eukaryotic cells. They control actin polymerization (Karlsson and Lindberg 2007), being key mediators of the membrane–cytoskeleton communication (Baluska and Volkmann 2002). As allergens, profilins have been identified in various plant sources, such as pollen, latex, fruits and vegetables (Santos and Van Ree 2011). They have been designed as panallergens since they are responsible for many IgE cross-reactions between unrelated pollen and plant food allergenic sources (Valenta et al. 1992 & Santos and Van Ree 2011). Ole 2 has a clinical prevalence of 24% in the allergic population (Asturias et al. 1997,

Ledesma et al. 1998a, Martínez et al. 2002 & Quiralte et al. 2007). Four Ole e 2 isoforms of 17.8, 17.0, 16.0, and 15.2 kDa were firstly determined (Asturias et al. 1997). Three isoforms with molecular weights of 13.3, 13.9 and 14.3 kDa are distinguishable after 1-D SDS-PAGE in the 'Picual' pollen (Morales et al. 2008). Two additional isoforms with apparent molecular masses of 15.7 and 14.9 kDa have been described in the cultivar 'Verdial de Huévar' (Alché et al. 2007). Under non-reducing electrophoretic conditions, a dimer form of Ole e 2 of about 32 kDa has been identified (Ledesma et al. 1998a). Recently, the polymorphism of the allergen Ole e 2 was evaluated in 24 olive cultivars (Jiménez-López et al. 2012). Data showed that profilins displayed 28.2 and 24.6% of variability among cultivars for nucleotide and amino acid sequences, respectively. Ole e 2 sequences had 130, 131 or 134 amino acids length and 39 amino acid positions were variable among the cultivars analyzed.

Ole e 3 and Ole e 8 allergens are Ca^{2+}-binding proteins involved in intracellular signalling processes (Batanero et al. 1996b & Ledesma et al. 1998b, 2000). Ole e 3 is a small (9.2 kDa) and acidic (pI 4.2-4.3) protein, which is specifically expressed in pollen (Ledesma et al. 1998b & Alché et al. 2003). It belongs to a new Ca^{2+}-binding protein subfamily named polcalcins (Ledesma et al. 1998b), which has also been established as a new panallergen. The allergenic activity of polcalcins is primarily associated with the Ca^{2+}-bound isoforms (Engel et al. 1997, Hayek et al. 1998 & Twardosz et al. 1997). The prevalence of Ole e 3 is higher than 50% of the olive pollen sensitized population (Batanero et al. 1996a). This allergen shows polymorphism at positions 43 ($L \leftrightarrow P$) and 80 ($V \leftrightarrow I$) of its amino acid sequence (Ledesma et al. 1998b). Ole e 8 consists of a single polypeptide of 20 kDa and its clinical incidence is as low as 3-4% (Ledesma et al. 2002). The molecular variability of Ole e 8 remains to be studied.

Ole e 4 and Ole e 6 allergens have no homology with other known proteins. Ole e 4 has an IgE-binding frequency by immunoblot of 80% (Boluda et al. 1998), while the prevalence of Ole e 6 depends on the geographical zone and reaches values between 10 and 55% (Batanero et al. 1997). Ole e 4 consists of a single acidic polypeptide chain with an apparent molecular weight of 32 kDa, and at least two isoforms with pIs between 4.6 and 5.1 have been described (Boluda et al. 1998). Ole e 6 is a Cys-rich small protein of 5.8 kDa and its amino acid sequence shows no microheterogeneities (Batanero et al. 1997).

Ole e 5 is a CuZn-superoxide dismutase (SOD) with a molecular weight of 16 kDa, which catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide, forming part of the cellular antioxidant defence system (Alché et al. 1998). This olive pollen allergen has an IgE-binding frequency of about 35% in the population assayed (Butteroni et al. 2005). Several studies have demonstrated that Ole e 5 shows a remarkable degree of polymorphism. Thus, the olive pollen of the variety 'Picual' contains at least 4 isoforms of the enzyme with pIs of 4.60, 4.78, 5.08, and 5.22, respectively (Alché et al. 1998). In addition, Boluda et al. (1998) described five isoforms with pIs between 5.1 and 6.5 in a commercial pollen sample of uncertain varietal origin from USA. More recently, Butteroni et al. (2005) reported discrepancies between the Ole e 5 amino acid sequences of the native and the recombinant forms.

Ole e 7 allergen exhibits a high homology with several lipid transfer proteins (LTPs). It is a small protein (10 kDa), which exhibits a high degree of polymorphism (Tejera et al. 1999). Thus, two isoforms with microheterogeneities at positions 4, 5, 10 and 11 of the N-terminal domain of the allergen have been described (Tejera et al. (1999). Ole e 7 is recognized by sera of 47% of patients allergic to olive pollen (Tejera et al. 1999). The ubiquitous nature of Ole e 7 and its high homology with LTPs from other plant species explains its high cross-reactivity and its designation as panallergen (Díaz-Perales et al. 2000).

Ole e 9 is a 1,3-β-glucanase and belongs to group 2 of pathogenesis-related proteins. This glycoprotein displays a molecular weight of 46.4 kDa on 1-D gels and is composed of two immunologically independent domains: an N-terminal domain (NtD) with 1,3-β-glucanase activity, and a C-terminal domain (CtD) that binds 1,3-β-glucans (Treviño et al. 2008). The clinical incidence of Ole e 9 is high, affecting the 65% of olive allergic patients. After SDS-PAGE under non-reducing conditions, a monomeric form of the allergen (46 kDa) and its dimer (91 kDa) are distinguishable. Ole e 9 shows a low but still significant level of polymorphism due to the presence of microheterogeneities in its amino acid sequence (Huecas et al. 2009). Thus, IEF analysis of the purified protein rendered four bands with pI values of 4.8, 4.9, 5.1, and 5.4 (Huecas et al. 2009). Ole e 9 has two potential N-glycosylation sites at positions Asn-355 and Asn-447. This N-linked sugar motif might also contribute to increase the molecular variability of Ole e 9, as it does for Ole e 1.

Ole e 10 is a small protein (10 kDa), which belongs to a new carbohydrate-binding-module (CBM43) family and binds specifically 1,3-β-glucans (Barral et al. 2005). The protein is composed of 102 amino acid residues and shows partial homology with the C-terminal domain of Ole e 9. Co-localization of Ole e 10 and callose in the growing pollen tube suggests a role for this protein in pollen tube wall remodelling during germination (Barral et al. 2005). Ole e 10 has been described as a major inducer of type I allergy in humans. It is involved in the allergic responses of 55% of patients suffering olive polinosis (Barral et al. 2005). However, there is no data about the polymorphism of this allergen so far.

Finally, Ole e 11 allergen is a pollen pectin methylesterase (PME) with an apparent molecular weight of 37.4 (Salamanca et al. 2010). PMEs are involved in demethylation of cell wall pectic compounds and they are key regulators of pollen tube growth (Bosch et al. 2005 & Jiang et al. 2005). The prevalence of this allergen between different populations of olive allergic patients varies from 55.9% to 75.6% (Salamanca et al. 2010). The molecular polymorphism of Ole e 11 remains to be studied.

The molecular variability of olive allergens is underrepresented in commercial protein extracts of olive pollen that are used for allergen-specific diagnosis and treatment of allergy (Morales et al. 2012). The relative abundance of individual isoforms with distinct IgE-reactivity within the protein content might affect the total allergenic potency of the extract. This, in turn, could lead to ambiguities in the IgE-binding responses detected for these allergens. Therefore, it is important to ensure that the allergenic variability in standardized protein extracts resembles as much as possible to that observed in the natural sources from which such extracts are prepared (Morales et al. 2012).

3. Immunoblotting as a tool for allergen standardization

The standardization of commercial olive pollen extracts is of great importance in order to assure efficiency and safety in the allergy diagnosis and immunotherapy procedures (Morales et al. 2012). Allergen standardization in European and United States legislations is mainly based on IgE-binding potencies and not on the content of individual allergens in a protein extract. Current procedures for preparation of protein extracts from natural sources are frequently inadequate, because they are standardized by determining the IgE response of a population, which is represented by a pool of sera from allergic patients, to major allergens present in these extracts (Morales et al. 2012). On the contrary, IgE binding to minor allergens will be difficult to detect because of their relatively low content in the extract. Thus, only major allergens will be considered in biological standardization (van Ree 1997, 2007). The most common allergy diagnostic tests can be performed *in vivo*, like SPT (Skin Prick Test), or *in vitro*, by competitive assays such as RAST (RadioAllergoSorbent Test) or ELISA (Enzyme-Linked ImmunoSorbent Assay). All these methods measure the total allergenic activity, but they do not explain the contribution of each individual allergen to the allergic response (Morales et al. 2012). Therefore, in addition to the allergenic potency of a protein extract, it is necessary to determine the molecular variability of these allergens. For this purpose, methods based on electrophoretic separation of proteins and immunoblotting are particularly useful and should be used in parallel to *in vitro* competitive assays (Baldo 1983). Thus, antibodies raised against specific allergens can be used to detect and quantify the different allergen isoforms present in non-standardized extracts. Such analysis can help, in turn, to design adequate dosage schemes and to elucidate the dose-therapeutic response of major and minor allergens.

3.1. Detection of olive pollen allergen isoforms by 2-D Western blotting

Protein extracts were prepared from mature pollen grains collected from olive (*Olea europaea* L.) trees (cv. 'Picual') grown in the province of Granada (Spain). Pollen samples (0.1 g) were suspended in 2.5 mL extraction buffer consisting of 40 mM Tris-HCl (pH 7.0), 2% (v/v) Triton X-100, 60 mM DTT, and 10 μL of a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MI, USA). Proteins were allowed to elute for 2 h at 4°C under continuous stirring. The resulting supernatants were desalted and delipidated in a PD-10 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Pollen proteins were then precipitated at −20°C for 1 h in a solution of 20% (w/v) trichloroacetic acid (TCA) prepared in acetone. The resulting pellet was resuspended in 0.5 mL solubilization buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 5 mM tributylphosphine (TBP), 0.5% (v/v) Bio-Lyte 3–10 buffer (Bio-Rad, Hercules, CA, USA) and traces of bromophenol blue). Total protein content was estimated using the 2D Quant Kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

Samples containing ~75 μg of total protein were applied to 7 cm polyacrylamide strips (pH 3–10NL, Bio-Rad) by in-gel rehydration at 30 V for 12 h. Focusing was conducted at 20°C in an Ettan IPGphor 3 Cell (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as follows: 300 and 1000 V for 1 h each followed by a linear increase from 1000 to 10,000 V, and finally 10,000 V to give a total of 40 kVh. Reduction and alkylation steps were performed as

previously described (Görg et al. 1988). Protein separation in the second dimension was carried out in a MiniProtean III Cell (Bio-Rad). After completion of SDS-PAGE, the resulting gels were stained using ProteoSilver™ silver stain kit (Sigma-Aldrich, St. Louis, USA) according to the manufacturer's protocol. For Western blot experiments, 75 μg of pollen proteins were resolved in 2-D gels as described above. Then, proteins were electroblotted onto a PVDF membrane in a TransBlot Turbo Transfer System (Bio-Rad). Membranes were blocked overnight at 4°C in a solution containing 3% (w/v) BSA in TBS buffer (pH 7.4), and probed with primary and the corresponding secondary antibodies according to Table 1.

Images were acquired in a Pharos FX molecular imager (Bio-Rad) using the Quantity One v4.6.2 software (Bio-Rad). The reproducibility of results was confirmed by running each experiment in triplicate.

Target	Primary antibody (source)[a]	Dilution	Secondary antibody (source)	Dilution
Ole e 1	Mouse anti-olive Ole e 1 mAb (Lauzurica et al. 1988)	1:5,000	Goat anti-mouse IgG Ab, DyLight 633-conjugated (Agrisera, Vännäs, Sweden)	1:10,000
Ole e 2	Rabbit anti-olive Ole e 2 PoAb (Morales et al. 2008)	1:10,000	Donkey anti-rabbit IgG Ab, DyLight 549-conjugated (Agrisera)	1:10,000
Ole e 7	Chicken anti-olive Ole e 7 synthetic peptide PoAb	1:250	Mouse anti-chicken IgG Ab, DyLight 488-conjugated (Agrisera)	1:1,000
Ole e 9	Chicken anti-olive Ole e 9 synthetic peptide PoAb	1:5,000	Mouse anti-chicken IgG Ab, DyLight 488-conjugated (Agrisera)	1:1,000
Olive pollen allergens	Sera from olive allergic patients	1:100	Goat anti-human Ab, peroxidase-conjugated (Sigma-Aldrich)	1:10,000

Table 1. Primary and secondary antibodies and the corresponding dilutions used for 2-D Western blotting and IgE-binding experiments. Footnotes: a) mAb, monoclonal antibody; PoAb, polyclonal antibody.

A representative 2-D gel pattern of the olive pollen proteome (cv. 'Picual') containing approximately 1,400 spots is shown in Fig. 1. The global protein 2-D map was largely reproducible among replicas and the number of spots was similar to that previously reported in this cultivar (Castro et al. 2010). It was noticeable the presence of a string of prominent spots of about 20 kDa distributed along the whole pH range, forming the so-called "train". Densitometric studies indicated that the sum of these spots may represent about 15% of the total protein displayed in gels.

The distribution of Ole e 1 isoforms on 2-D polyacrylamide gels was studied using an anti-Ole e 1 antibody, namely 10H1 (Lauzurica et al. 1988). This antibody was able to recognize up to 12 different immunoreactive protein spots in the molecular weight range of 18-22 kDa (Fig. 2A). The Ole e 1 distribution pattern was identical to that previously reported (Castro et al. 2010), but a new non-glycosylated isoform was detected in the present work (Fig. 3A, spot 1l). A single spot matched with a 22 kDa hyperglycosylated isoform of Ole e 1 (Villalba et al. 1993 & Castro et al. 2010). Four major and four minor spots showed an apparent

molecular weight of 20 kDa and pI values between 5.1 and 8.0. They are different monoglycosylated isoforms of the allergen (Villalba et al. 1993 & Castro et al. 2010). Finally, two minor spots with pI values between 5.5 and 6.0 corresponded to two different 18.5 kDa non-glycosylated isoforms of Ole e 1 (Villalba et al. 1993 & Castro et al. 2010). Since proteins were electrophoresed under reducing conditions, we did not detect the dimer form of Ole e 1. All these immunodetected spots fitted well in their positions on the membrane with the "train of spots" visualised on 2-D gels after silver staining (Fig. 1).

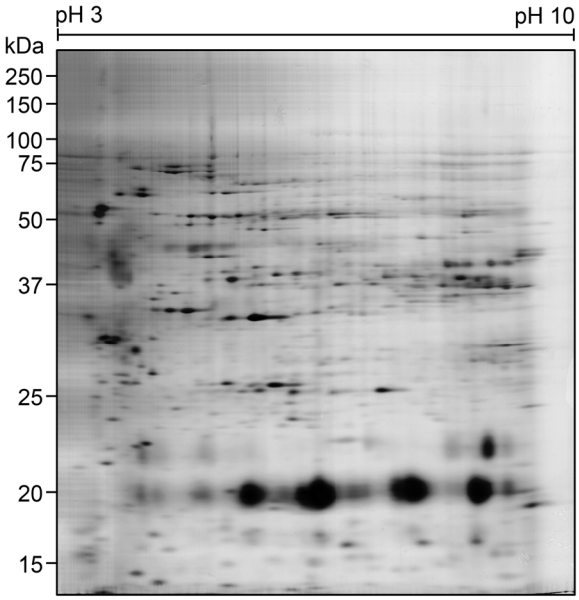

Figure 1. Representative gel of the olive pollen proteome after 2-D electrophoresis and silver staining. Approximately 75 μg of pollen total protein was loaded. Numbers at the top of the gel denote the pH gradient in the first dimension (IEF), while protein weight markers (SDS-PAGE) are shown on the left.

Using an anti-Ole e 2 polyclonal serum, we studied the number and distribution of Ole e 2 isoforms on 2-D polyacrylamide gels. Thus, two major spots of 14.3 and 13.9 kDa and a minor spot with an apparent molecular weight of 13.3 kDa were detected on 2-D blots (Fig. 2B). The three Ole e 2 isoforms are acidic and show pI values between 4.5 and 5.1.

The anti-Ole e 7 antibody revealed the presence of two different isoforms of this allergen in the olive pollen (Fig. 2C). Both proteins showed a molecular weight of approximately 10 kDa and exhibited pI values slightly acidic (6.6-6.8).

The anti-Ole e 9 antibody was able to recognize six immunoreactive protein spots of about 46 kDa and pI values between 5.8 and 6.7 (Fig. 2D). The number of spots is higher and pIs are different compared with other Ole e 9 isoforms previously described (Huecas et al. 2009). This is likely because the genetic origin of pollen samples used by Huecas et al. (2009) is different. Control reactions using the corresponding preimmune sera lacked of reactivity in all cases (results not shown).

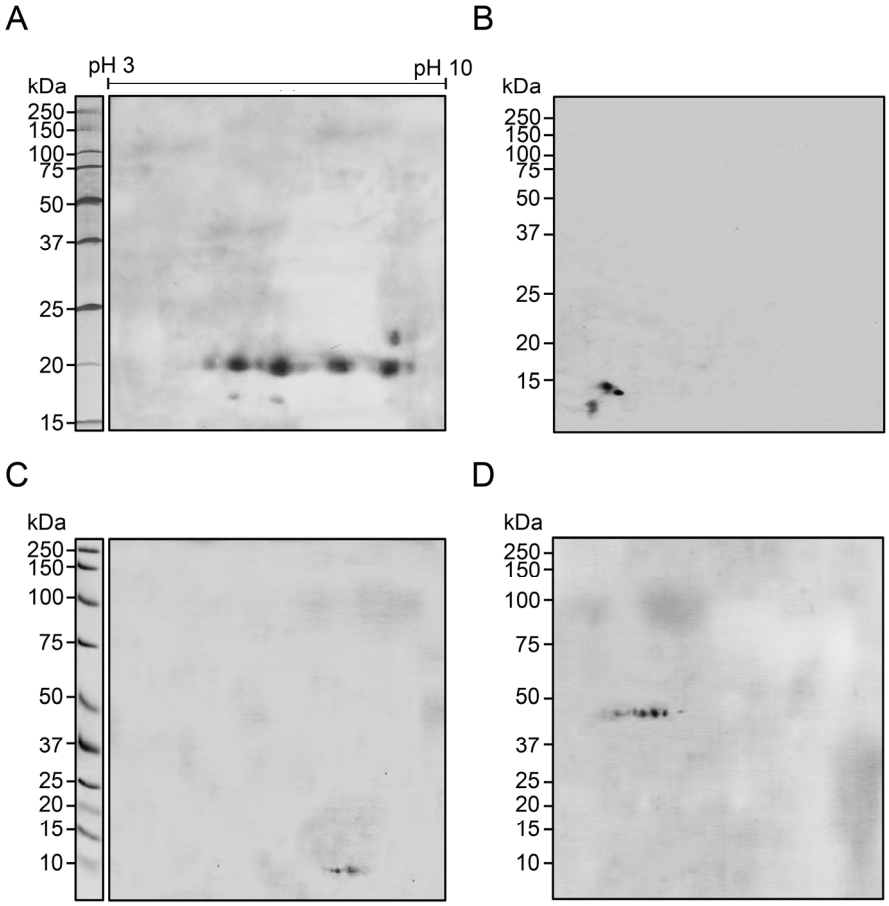

Figure 2. Detection of olive pollen allergens Ole e 1 (A), Ole e 2 (B), Ole e 7 (C) and Ole e 9 (D) on 2-D Western blot. Approximately 75 µg of pollen total protein was loaded per each gel. Numbers at the top of the gel denote the pH gradient in the first dimension (IEF), while protein weight markers are shown on the left.

3.2. Quantitation of olive pollen allergen isoforms after 2-D Western blotting

After scanning the resulting blots in a Pharos FX Plus Molecular Imager (Bio-Rad), the relative amount (%) of each allergen isoform was calculated using the Quantity One v. 4.6.2 software (Bio-Rad). Spots reactive to each antibody were defined with the help of the tool "contour" and quantitation of each spot was calculated using the "volume" parameter, defined as the total signal intensity of the pixels × area of a single pixel, inside the defined boundary drawn for each spot (Morales et al. 2012). This intensity was compared with that of the background, in a previous calibration procedure. Densitometric data of Ole e 1, Ole e 2, Ole e 7 and Ole e 9 allergen isoforms are shown in Fig. 3.

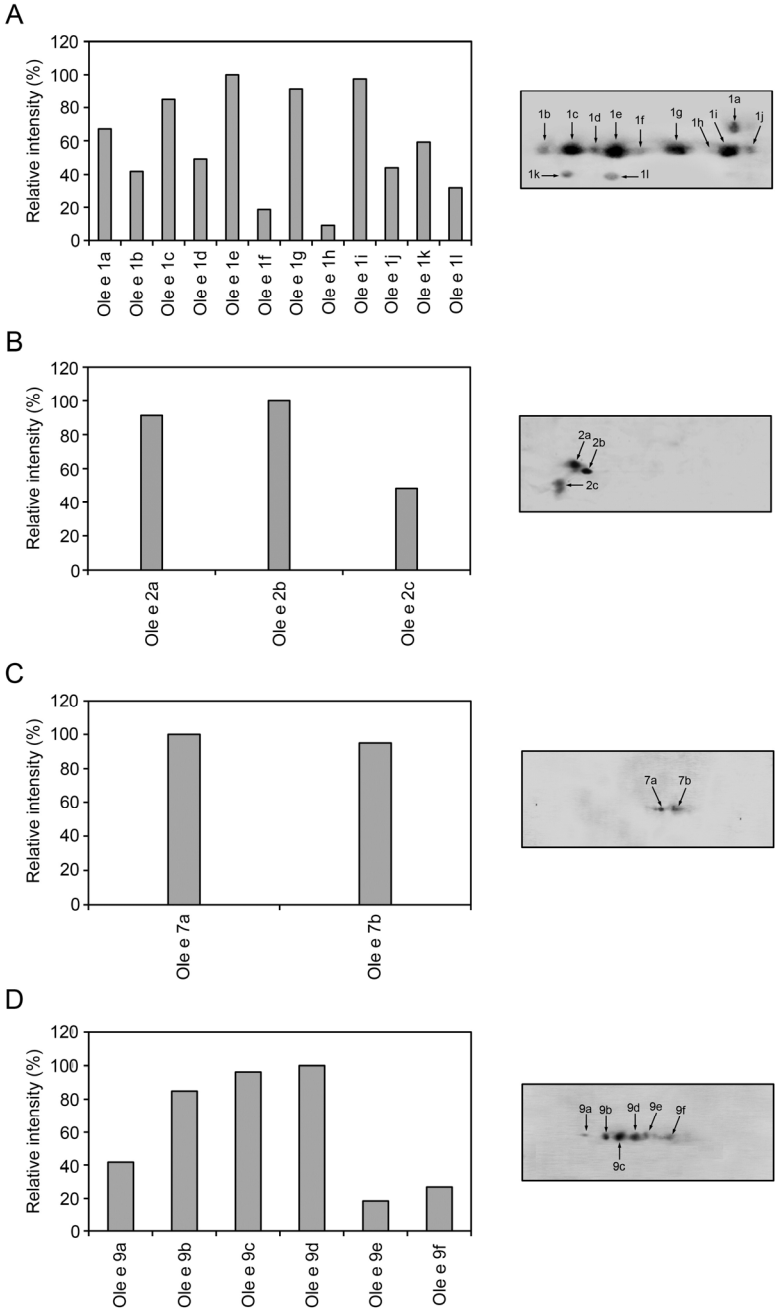

Figure 3. Quantitative analysis of Ole e 1 (A), Ole e 2 (B), Ole e 7 (C) and Ole e 9 (D) isoforms detected on 2-D blots from Fig. 2.

Ole e 1 isoforms showed the highest quantitative differences (Fig. 3A). Thus, the 12 Ole e 1 isoforms detected on 2D blots could be grouped on the basis of their intensities into 3 quantitative classes: high (>50% intensity; isoforms Ole e 1a, c, e, g and i), medium (20-50%; Ole e 1b, d, j, k and l) and low abundant (<20%; Ole e 1f and h). On the other hand, the six Ole e 9 isoforms were discriminated as high (Ole e 9a, b, c and d) or low (Ole e 9e and f) abundant (Fig. 3D). Ole e 2a and b isoforms showed similar intensities, while the amount of Ole e 2c was lower but still significant. Finally, the two Ole e 7 isoforms identified showed similar quantities.

3.3. IgE-binding analysis by 2-D Western blotting

The IgE reactivity of 'Picual' pollen proteins was assayed on 2-D blots using a pool of three olive allergic patients' sera and an anti-human IgE peroxidase-conjugated secondary antibody. Chemiluminiscent detection of IgE-binding was carried out with the Immun-Star™ WesternC™ Chemiluminescence Kit (Bio-Rad) according to the manufacturer´s instructions. Chemiluminiscent spots were imaged with a ChemiDoc XRS Molecular Imager (Bio-Rad).

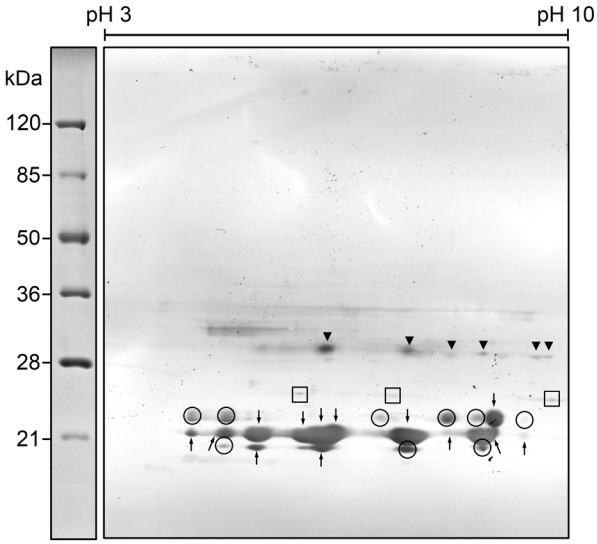

Figure 4. IgE-binding analysis by 2-D Western blotting of olive (cv. 'Picual') pollen total proteins. The blot was probed with a pool of sera from three patients allergic to olive pollen. Reactive spots were visible on the 2-D blot after detection by chemiluminiscence. Protein markers are displayed on the left.

The IgE-binding pattern is shown in Fig. 4. Incubation of the 2-D blot with patient's sera revealed up to 31 IgE-reactive spots with molecular weights ranging from 18,5 to 32 kDa. Allergic patients' IgEs recognized the 12 Ole e 1 isoforms detected by 10H1 antibody (Fig. 4, arrows). In addition, 9 new spots that might correspond to other Ole e 1 isoforms were also

detected by sera (Fig. 4, circles). The remaining spots (Fig.4, squares and arrowheads) did not match with any of the Ole e 2, Ole e 7 and Ole e 9 isoforms identified previously by 2-D Western blotting (Fig. 2).

4. Conclusions

Two-dimensional Western blotting is a suitable method for olive pollen allergen isoform profiling, and might help in the standardization of protein extracts used for allergy diagnosis and immunotherapy. The 2-D electrophoresis-based immunodetection of allergens has a few advantages over classical 1-D Western-blotting, since they provide: 1) detailed information about individual isoforms of polymorphic allergens (i.e. number of isoforms, pIs and their positions on the 2-D map), and 2) relative quantitative data of each isoform. In addition, using a pool or individual sera from an allergic population, it is possible to identify which are the most reactive isoforms of a given allergen. In the present work, four allergens, namely Ole e 1, Ole e 2, Ole e 7 and Ole e 9, have been studied in a single variety (i.e. 'Picual'). These analyses should extend to other olive allergenic proteins and a higher number of cultivars. Moreover, this method can be also applied to other allergenic pollens and other type of allergies (e.g. food allergens).

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