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Biochemical Characterization and Cellular Localization of 11*S* Type Storage Proteins in Olive (*Olea europaea* L.) Seeds

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The composition of seed storage proteins (SSPs) in olive endosperm and cotyledon has been analyzed. Precursor forms of these proteins are made up of individual proteins, which have been purified to homogeneity and further named p1−p5 (20.5, 21.5, 25.5, 27.5, and 30 kDa, respectively). N-terminal sequences of p1 and p2 proteins displayed relevant homology to the basic subunit of the 11*S* family of plant SSPs (legumins). Two-dimensional polyacrylamide gel electrophoresis experiments allowed us to verify the basic character of p1 and p2 and the acidic character of p3, p4, and p5 proteins. In addition, the putative presence of highly similar isoforms or posttranslational modifications of these polypeptides was detected. As a result, a model describing the putative association of p1−p5 proteins into subunits of α(acidic)/β(basic) type has been proposed. Solubility experiments have shown that the majority of these olive seed proteins from the 11*S* storage protein family are extracted with aqueous alcohol and only partially with water and diluted saline solutions, therefore suggesting their similarity to prolamines. Moreover, no visible differences were found in either subunit composition or 11*S* proteins mass among six olive cultivars examined. This result suggests that the synthesis of storage proteins is highly conserved in this plant species. By using a rabbit antiserum raised to p1 protein, the proteins have also been immunolocalized in olive seed tissues, showing that they accumulate in conspicuous protein bodies present in both the endosperm and the cotyledon.

KEYWORDS: Cotyledon; endosperm; *Olea europaea* L.; olive tree; protein bodies; 11*S* storage protein

INTRODUCTION

Olive tree (*Olea europaea* L.) is a very important oil-producing crop in many countries, due to its high productivity and the quality of its oil (*1*). Molecular markers are regularly established in order to distinguish, characterize, or identify olive cultivars, to estimate germplasm variability, and to trace olive origin among other purposes (*2*). To develop molecular markers used as tools for breeding programs in olive, we have selected storage proteins, as they are expected to be abundant proteins in the mature olive seeds.

Storage proteins are formed during seed development and deposited predominantly in specialized storage tissues, like the cotyledon or endosperm. Correctly formed and assembled mature storage proteins are steadily accumulated, and their degradation happens only after a long period of rest when seeds germinate and seedlings start to grow (*3*, *4*). Seed storage proteins (SSPs) are classified on the basis of their solubility characteristics into albumins (water soluble), globulins (soluble in dilute saline buffer), prolamins (soluble in alcohol/water mixtures), and glutelins (soluble in dilute acid or alkali) (*5*, *6*). Globulins are the most widely distributed group of SSPs. They have been studied in most detail in legumes, notably pea (*Pisum sativum*), soybean (*Glycine max*), broad bean (*Vicia faba*), and French bean (*Phaseolus vulgaris*). Nevertheless, legumins are the major storage proteins in many other dicots (for example, in the Brassicaceae, Compositae, and Cucurbitaceae families) and some cereals such as oats and rice (*6*). They are stored as large complexes (hexameric structures) in protein bodies. Each subunit in the hexamer is itself composed of a large acidic α-protein and a small basic β-protein, derived from a single precursor (prolegumin) and linked by a disulfide bond (*6*, *7*). Disulfide bridging is a matter of controversial significance for sorting, targeting, and oligomerization of legumins (*7*). The endoproteolytic cleavage of the 11*S* proteins into two disulfide-bonded subunits is evolutionarily conserved in seeds of conifers, monocots, and dicots (*8*, *9*).

In the olive tree seed, an early report (*10*) indicated that both the mature olive embryo and the endosperm contained large protein bodies surrounded by oil bodies. However, little has been known about the biochemical and molecular characteristics of SSPs in this plant. In the present paper, we report the purification of individual components and the solubility characteristics of olive seed 11*S* storage proteins, the characterization of the reactivity of a polyclonal antiserum raised against these proteins, and the cellular localization of 11*S* proteins in the olive seed.

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MATERIALS AND METHODS

Plant Materials. Mature seeds of *O. europaea* were obtained from olive (*O. europaea* L.) trees cv. Picual grown in the "Estación Experimental del Zaidín" (Granada, Spain), 210 days after anthesis (DAA). Mature seeds (between 200 and 215 DAA) of the olive cultivars Arbequina, Frantoio, Hojiblanca, Lucio, and Manzanilla were obtained from well-characterized trees located at different places of Granada. Unless indicated, mature seeds of the Picual variety were used. Other plant seeds used in the present paper (almond, French bean, lentil, rapeseed, rice, sunflower, sesame, and hazelnut) correspond to commercially available sources.

Protein Extraction. Either whole seeds or isolated cotyledons and endosperms were directly homogenized in a mortar cooled on ice using 125 mM Tris-HCl, pH 6.8 (native conditions), 125 mM Tris-HCl (pH 6.8) plus 0.2% sodium dodecyl sulfate (SDS) (denaturing, nonreducing conditions), or 125 mM Tris-HCl (pH 6.8) plus 0.2% SDS and 1% 2-mercaptoethanol (denaturing, reducing conditions). After centrifugation at 10000*g* for 10 min (4 °C), the supernatants were boiled for 3 min and centrifuged again. Proteins in the supernatants were precipitated with 2 volumes of cold acetone and resuspended in their respective extraction buffers.

To determine solubility characteristics, proteins from mature olive seeds were extracted using the following extraction solutions: (i) distilled water, (ii) 0.5 M NaCl, (iii) 70% (v/v) 2-propanol, (iv) 60% (v/v) acetic acid, (v) 0.1 M sodium hydroxide, and finally (vi) 0.1 M sodium borate (pH 10), 1% SDS, and 50 mM dithiothreitol (DTT) (*11*, *12*). The protein concentration in each sequential extraction step was measured by using a Bio-Rad (CA) microassay.

For two-dimensional (2D) electrophoresis experiments, endosperm and cotyledon samples were separately homogenized to a very fine powder in a liquid nitrogen-precooled mortar by using a pestle. Approximately 0.1 g of the resulting homogenate was put into a 1.5 mL tube with 1 mL of a lysis buffer consisting of 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), 3% (w/v) SDS, 4 mM tributylphosphine (TBP), 0.5% (v/v) Pharmalyte ampholytes (pH 3−10) (Amersham Biosciences), and 0.01% (w/v) bromophenol blue and then suspended by vigorous shaking. Proteins were precipitated in 5 volumes of 20% (w/v) trichloroacetic acid (TCA) and 0.2% (w/v) DTT in chilled acetone at −20 °C for 1 h. Precipitates were washed twice in acetone and resuspended in 0.5 mL of lysis buffer lacking SDS. Proteins were then alkylated for 2 h with iodoacetamide to a final concentration of 200 mM. Finally, samples were centrifuged at 40000*g* for 60 min at 4 °C to remove all insoluble particulates and stored in aliquots at −80 °C until use. The total protein content was estimated as described above.

SDS−PAGE under Reducing and Nonreducing Conditions. SDS−polyacrylamide gel electrophoresis (PAGE) was performed according to ref *13* on 12.5% (w/v) acrylamide gels with 4.75% stacking gels, using the Bio-Rad Mini-Protean equipment. Proteins in each sample were mixed with an equal volume of a 2× sample buffer containing 125 mM Tris-HCl (pH 6.8), 2 M urea, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, and 0.02 mg/mL bromophenol blue and boiled for 3 min before running. Mercaptoethanol was omitted from the sample buffer when nonreducing conditions were used. After electrophoresis, gels were stained with Coomassie blue.

Purification of Individual Components of 11*S* Proteins. Crude extracts of mature seeds were resolved under nonreducing conditions, and storage proteins were cut out from stained gels. The excised gel pieces were incubated for 30 min in 100 mM Tris-HCl and 0.5% (w/v) SDS, pH 8.2, and then homogenized using a mortar and pestle. The resulting homogenates were then centrifuged at 10000*g* for 10 min. The proteins in the supernatant were recovered by cold acetone precipitation, dissolved in 2 M urea, 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, and 0.02 mg/mL bromophenol blue, and then electrophoretically separated. Each individual component of 11*S* proteins was cut out from the stained gels and recovered as above.

Two-Dimensional Electrophoresis Analysis of 11*S* Proteins. For analytical 2D separations, endosperm and cotyledon samples containing approximately 75 μg of total protein were diluted to a final volume of 315 μL in solubilization buffer and subsequently applied by in-gel rehydration at 30 V for 12 h onto dried polyacrylamide gels [Immobiline DryStrip (pH 3−10) NL, Amersham-Pharmacia Biotech]. Isoelectric focusing was conducted at 20 °C in an IPGPhor apparatus (Amersham-Pharmacia Biotech) as follows: 300 and 1000 V for 1 h each followed by a linear increase from 1000 to 8000 V and finally 8000 V to give a total of 85 kVh. Focused gels were placed on top of vertical slabs of acrylamide (12% T, 2.6% C). The stacking gel was replaced by a layer of 1% (w/v) agarose, 0.15 M Bis-Tris/0.1 M HCl, and 0.2% (w/v) SDS. Electrophoretic migration along the second dimension was performed using the Laemmli buffer system (*13*) in a Protean II xi Cell (Bio-Rad) at 20 mA/gel for 1 h, followed by 40 mA/gel for 4 h. Reproducibility of 2-DE protein profiles was confirmed by first carrying out two independent experiments and second by running each protein sample in duplicate.

After completion of SDS−PAGE, the gels were fixed and silver stained (*14*). Digitized images at 84.7 μm resolution were obtained using the Power Look III scanner and the MagicScan software (UMAX Systems GmbH, Germany). The experimental molecular mass of each spot was calibrated using commercial standards (Precision Plus Protein Standards, Bio-Rad) after coelectrophoresis.

Antiserum Production and Immunoblotting. A polyclonal antiserum to the p1-purified protein was raised in rabbits using well-established procedures (*15*). Immunoblotting was performed after transferring the SDS−PAGE-separated proteins onto polyvinylidene fluoride (PVDF) membranes in a Semi-Dry Transfer Cell (BioRad, United States). Either the preimmune serum or the p1-antiserum was used to probe the membranes at a dilution 1:1000. A peroxidase-conjugated anti-rabbit IgG (Promega Co) diluted 1:10000 served as the secondary antibody, and the detection reaction was developed using diaminobenzidine as the substrate.

Light Microscopy (LM) and Transmission Electron Microscopy (TEM) Immunolocalization of Olive SSPs. Cotyledon and endosperm were dissected out from mature seeds of the olive cv. Picual and individually processed for TEM. Samples were fixed for 24 h at 4 °C with a mix of 4% (w/v) paraformaldehyde and 0.2% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). Samples were then dehydrated throughout an ethanol series and embedded in Unicryl resin (BBInternational). After ultraviolet light polymerization of samples at −20 °C for 48 h, both thin (1 μm) and ultrathin sections (70 nm) were obtained using a Reichert-Jung ultramicrotome and picked up using 200 mesh nickel grids. Thin sections were stained using toluidine blue and observed in a Zeiss Axioplan microscope. The grids were then sequentially treated with a blocking solution (5% (w/v) bovine serum albumin, 0.1% (v/v) Tween 20 in phosphate-buffered saline), a diluted (1:100) solution of the p1-antiserum in blocking solution, a 1:1000 solution of the secondary antibody (goat anti-rabbit IgG:15 nm gold, BBInternational), and finally contrasted using a 5% (w/v) uranyl acetate solution and observed in a Zeiss EM10 electron microscope. Negative control sections were treated as above but using preimmune serum instead of the p1-antiserum. Morphometric measurements were performed using the UTHSCSA ImageTool (version 3.00 for Windows) software. Statistical tests were performed using the Statistica (version 5.1 for Windows) software.

RESULTS

Purification of Individual Components of 11*S* Proteins. SDS−PAGE protein profiles of whole seeds, resolved under nonreducing and reducing conditions, are represented in **Figure 1A**. To avoid contamination from other proteins (compare lanes 1 and 2), purification of individual components of 11*S* proteins was carried out by performing sequential electrophoresis. First, two large precursors of 47.5 (Pro1) and 41 kDa (Pro2), respectively, were excised from crude extracts resolved under nonreducing conditions (**Figure 1A**) and then recovered from gel slices. Second, both precursors were resolved under both nonreducing and reducing conditions (**Figure 1B**). Under reducing conditions, Pro1 yielded two proteins of 20.5 (p1) and 30 kDa (p5), whereas Pro2 yielded three proteins of 21.5 (p2), 25.5 (p3), and 27.5 kDa (p4). Density measurements performed

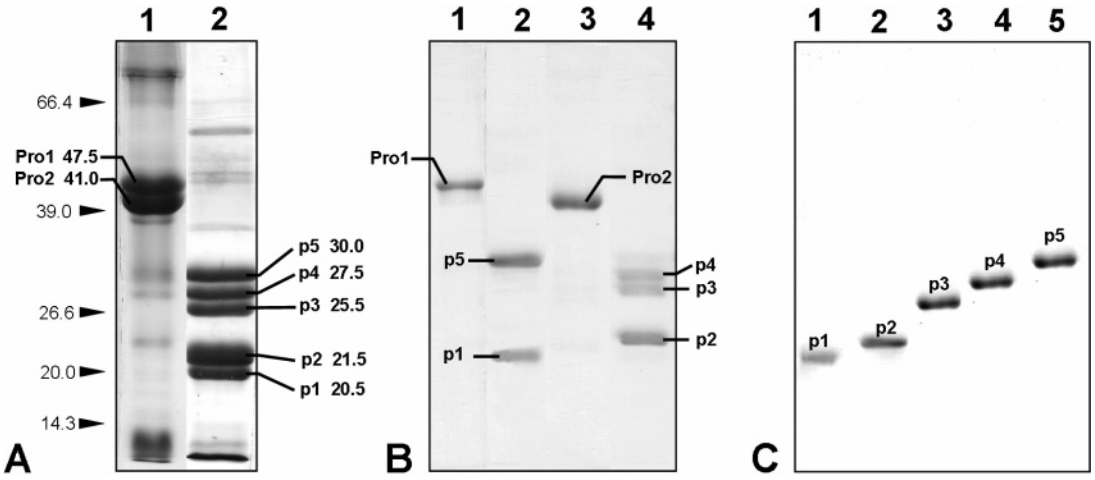

Figure 1. Coomassie-stained gels showing purification of individual components of 11*S* proteins from mature seeds of *O. europaea* cv. Picual. (**A**) Protein profiles obtained after SDS–PAGE of nonreducing (lane 1) and reducing (lane 2) crude protein extracts prepared from olive seeds. (**B**) Purification and SDS–PAGE separation of precursor forms (41.0 and 47.5 kDa) under nonreducing conditions (lanes 1 and 3, respectively). After reduction, individual components resulting from each precursor were separately resolved by SDS–PAGE (lanes 2 and 4, respectively). Molecular masses of protein standards are displayed on the left. (**C**) Reduced mature forms of 11*S* proteins after purification of individual components.

A

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p1 polypeptide O. europaea (Fragment)        GLEETLLTLRLLEN 14
Q8LK20 Castanin                              GIEETLCTLRLREN 367
Q41128 Legumin                               GIEETLCTLRLREN 312
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B

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p2 polypeptide O. europaea (Fragment)        GLEESICSAKIR 12
Q9AUD2 11S Globulin                          GLEETLCTAKLR 301
Q53I54 Legumin-like protein (Fragment)       GLEETLCTMKLR 54
Q53I55 Legumin-like protein (Fragment)       GLEETLCTMKLR 73
Q96475 Conglutin alpha (Fragment)            GLEETLCTLKLR 66
Q53153 Legumin-like (Fragment)               GLEETLCTMKLR 164
Q41036 Pea (P. sativum) legumin subu         GLEETVCTAKLR 43
P14594 Legumin B                             GLEETICSAKIR 173
O24294 Legumin (Minor small) precur          GLEETICSAKIR 401
Q43673 Legumin, legumin-related h            GLEETICSSKIR 395
P05693 Legumin K                             GLEETICSAKIR 181
P05692 Legumin J precursor                   GLEETICSAKIR 334
Q41703 Legumin B precursor                   GLEETICSAKIR 316
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Figure 2. Alignments of p1 (**A**) and p2 (**B**) N-terminal amino acid sequences after FASTA 33 homology search in the UniProt database. Asterisks show amino acid identity. Colons designate conservative changes.

in these gels (data not shown) indicate that the intensity of p2 band is approximately twice the intensity of p3 and p4 bands. Finally, each individual component (p1−p5) was recovered after excising the corresponding band and eluting the protein (**Figure 1C**). Purified-to-homogeneity proteins p1 and p2 were used for N-terminal sequencing and p1 for rabbit immunization.

N-terminal sequencing of p1 and p2 proteins brought in the sequences GLEETLLTLRLLEN and GLEESLCTNKIR, respectively. **Figure 2** describes the best alignments of both sequences after searching the UniProt library for protein homology using the FASTA 33 program (*16*). Both sequences displayed a high homology to the β-arm (basic subunit) of the 11*S* family of plant SSPs, particularly legumins.

Two-Dimensional Electrophoresis Analysis of 11*S* Proteins in Cotyledon and Endosperm. Two-dimensional gel electrophoresis patterns obtained from olive endosperm and cotyledon are shown in **Figure 3**. Approximately 1000 and 750 spots could be detected by silver staining in the endosperm and the cotyledon 2D maps, respectively. Independently of the presence of up- and down-regulated spots in both tissues, we have focused our attention into two groups of proteins, which were the most prominent in quantitative terms. One of these groups of spots displayed experimental molecular masses close to 20 kDa and basic nature, whereas the second group of spots, of acidic character, ranged within 26−30 kDa in both tissues. In the two groups of proteins, several spots of similar masses but shifted positions in the pH gradient were detected (**Table 1**). This pattern was highly reproducible for independent extraction from the same batch of seeds.

Solubility Characteristics of 11*S* Proteins from Olive. As shown in **Figure 4**, storage proteins in olive seeds were easily solubilized in aqueous alcohol (lane 3), alkali (lane 5), and a solution containing SDS (lane 6). In comparison, only little amounts of storage proteins were recovered when water (lane 1), salt (lane 2), and acidic extracts (lane 4) were used. The exception corresponded to the p1 protein, which was enriched in the acidic extract (**Figure 4**, lane 4).

Determination of p1-Antiserum Reactivity. When compared with the corresponding SDS−PAGE Coomassie-stained gels (**Figure 1A**), immunoblotting experiments showed that p1-antiserum recognized the original antigen (p1), as well as p2, p4, and p5 proteins present in crude extracts of mature seeds

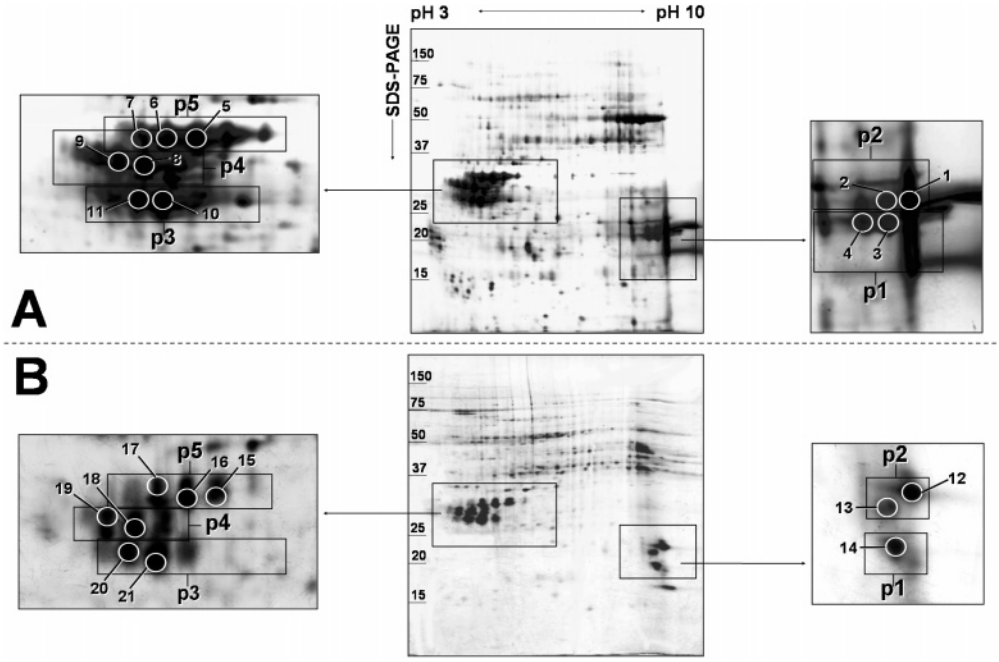

Figure 3. 2D maps of olive endosperm (**A**) and cotyledon (**B**) proteomes after silver staining. Boxes indicate the putative localization of p1–p5 proteins. The molecular masses of protein standards are displayed on the left.

Table 1. Molecular Masses Estimated for Selected Spots of **Figure 3A** (Endosperm) and **B** (Cotyledon)

endosperm			cotyledon		
	spot no.	estimated molecular mass (kDa)		spot no.	estimated molecular mass (kDa)
basic	1	21.46	basic	12	20.89
	2	21.67		13	20.42
	3	20.04		14	19.95
	4	19.73			
acidic	5	28.84	acidic	15	25.41
	6	30.90		16	25.41
	7	30.20		17	25.70
	8	28.51		18	24.95
	9	28.84		19	25.12
	10	26.92		20	23.71
	11	27.54		21	23.77

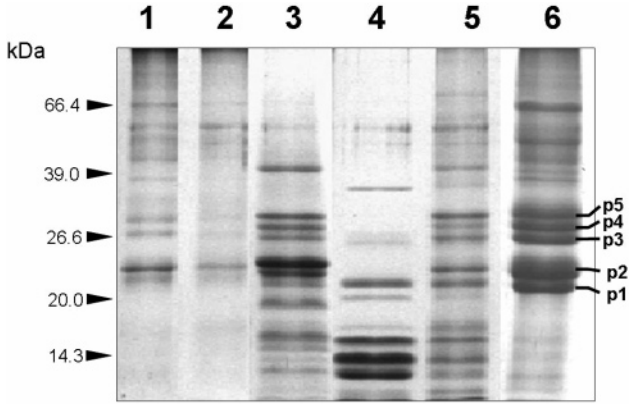

Figure 4. Coomassie-stained gel showing solubility of olive seed 11*S* proteins extracted in distilled water (lane 1), 0.5 M sodium chloride (lane 2), 70% (v/v) 2-propanol (lane 3), 60% (v/v) acetic acid (lane 4), 0.1 M sodium hydroxide (lane 5), and a buffered solution (pH 10) containing 0.1 M sodium borate, 1% (w/v) SDS, and 50 DTT (lane 6), respectively. The SDS–PAGE run was performed under reducing conditions, after loading equal amounts of protein in each lane.

(**Figure 5**). The purified forms of these proteins were also recognized by the p1-antiserum. No differences in the reactivity of the antiserum were detected with regard to the use of reducing or nonreducing running conditions. A faint cross-reactivity of the antiserum with p3 was also detected. The antiserum did not cross-recognize any protein in extracts of olive mesocarp, pollen, flower buds, or leaves in our immunoassays (not shown), whereas the preimmune serum did not cross-react with any protein in seed extracts (**Figure 5C**).

The p1-antiserum also recognized multiple spots within the two major groups of proteins (basic and acidic) after 2D electrophoresis analysis of protein extracts from endosperm and cotyledon (**Figure 6A**,**B**, respectively). The preimmune serum produced a negative response in parallel sets of transferred 2D gels.

p1 Cross-Reactive Proteins in Olive Cultivars and Other Oil-Storing Seeds. A comparison of the protein profiles corresponding to mature seeds from six olive cultivars (Picual, Arbequina, Frantoio, Hojiblanca, Lucio, and Manzanilla) showed no conspicuous differences in their respective protein patterns after SDS−PAGE under reducing conditions. The five bands (p1−p5), representing the reduced components of 11*S* proteins, were present in all olive cultivars and were recognized by the p1-antiserum. No visible differences in the expression levels of p1−p5 proteins were observed among the cultivars tested (**Figure 7**).

When a variety of crude protein extracts from different oil seeds were assayed, a number of immuno-related proteins were detected (**Figure 8**). Prominent bands in the range of 20 kDa were detected in the extracts corresponding to sesame, sunflower, rice, and lentil after probing the immunoblot with the p1-antiserum. Reactive bands of higher molecular masses were also observed in most extracts.

TEM Localization of 11*S* Proteins in the Mature Seed. Endosperms and cotyledons from mature seeds were used for immuno gold-labeling experiments. A general view of both tissues is observed in **Figures 9A** and **10A** after LM observation. Endosperm cells showed a uniform morphology (**Figure 9A**) with densely packed and numerous heavily stained protein

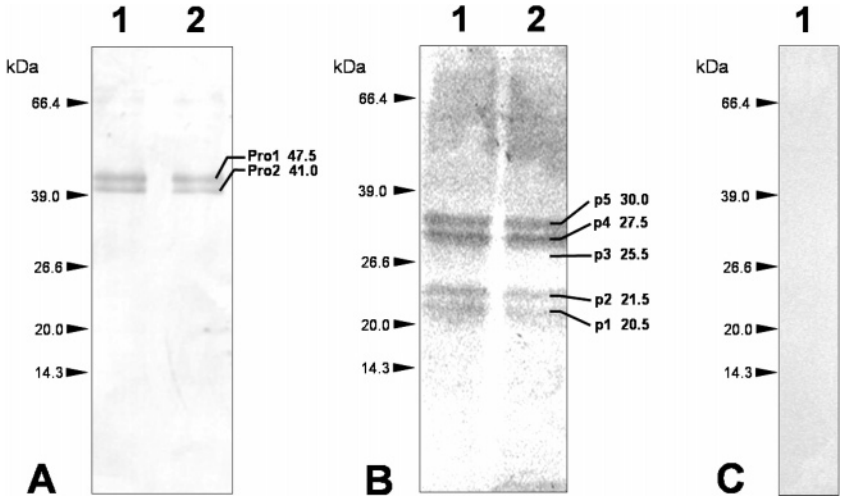

Figure 5. Immunoblots probed with the p1-antiserum (**A**,**B**) or the preimmune serum (**C**). Either crude protein extracts obtained from mature seeds (lane 1) or purified 11*S* protein precursors (lane 2) were subjected to SDS–PAGE under nonreducing (**A**,**C**) and reducing (**B**) conditions.

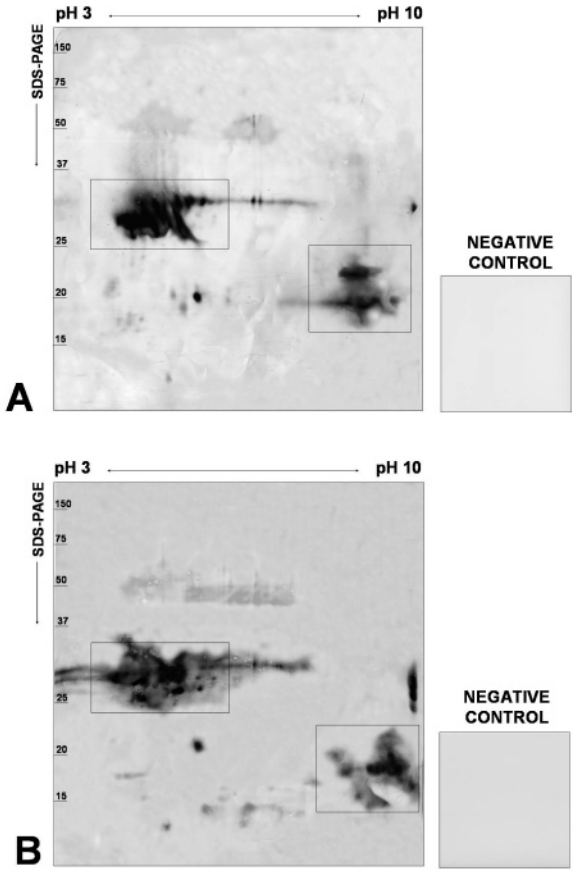

Figure 6. Immudetection of 11*S* proteins in 2D gels corresponding to endosperm (**A**) and cotyledon (**B**) protein extracts by using the p1-antiserum. Negative controls were probed with the preimmune serum.

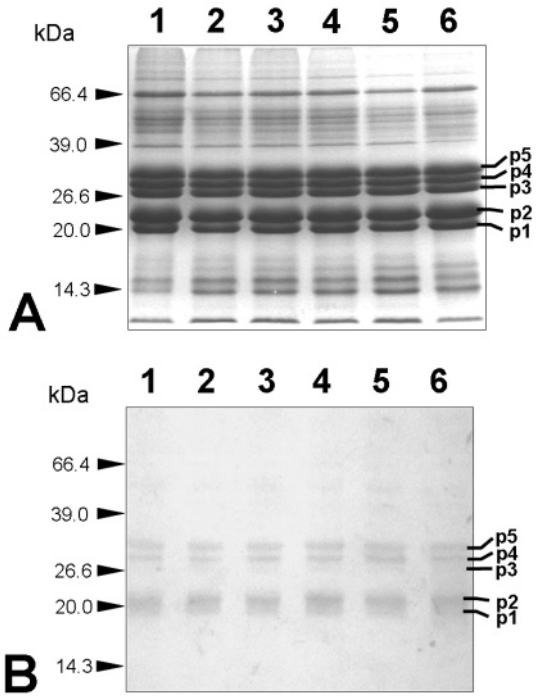

Figure 7. SSP profiling in six olive cultivars. SDS–PAGE (**A**) and immunoblot probed with the p1-antiserum (**B**) of crude protein extracts obtained from mature seeds. Gels were run under reducing conditions. Key: 1, Picual; 2, Arbequina; 3, Frantoio; 4, Hojiblanca; 5, Lucio; and 6, Manzanilla.

bodies and unstained lipid bodies. On the contrary, cotyledon cells were present in several morphological populations, although most of them presented a cytoplasm containing fewer protein and lipid bodies as compared to the endosperm cells (**Figure 10A**). Protein bodies in these cells were larger than those of the endosperm and were not homogeneously stained. Nuclei were frequently observed in these cells, while they were not detected in the endosperm cells.

When observed with TEM (**Figure 9B**,**C**), endosperm cells showed protein bodies with their electron dense content. Several layers of lipid bodies exhibiting lower electron density surrounded these protein bodies. TEM observation of cotyledon cells revealed the presence of protein bodies with different degrees of electron density (**Figure 10B**). Lipid bodies were frequently limited to a single layer surrounding the protein bodies. Morphometric analysis indicated that the protein bodies present in the cotyledon were larger than those present in the endosperm ($p = 0.022$). On the contrary, lipid bodies in the cotyledon were smaller than their counterpart in the endosperm ($p = 0.0000$) (**Table 2**).

Immunolocalization studies using the p1-antiserum yielded an intense labeling by gold particles specifically located in the protein bodies. Three categories, characterized by low, medium, and high labeling intensity (and, respectively, electron density) were detected in the cotyledon (**Table 2**). The intensity of the labeling by p1-antiserum was significantly higher in the protein

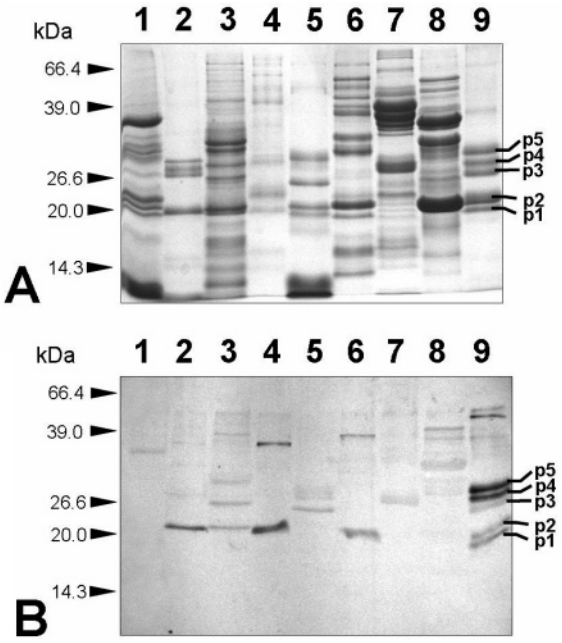

Figure 8. SDS–PAGE (**A**) and immunoblot probed with the p1-antiserum (**B**) of crude protein extracts obtained from mature seeds of several plant species. Key: 1, hazelnut; 2, sesame; 3, sunflower; 4, rice; 5, rapeseed; 6, lentil; 7, French bean; 8, almond; and 9, olive.

bodies present in the endosperm when compared to all three categories of protein bodies from the cotyledon ($p = 0.0000$ in all cases). Moreover, the differences in the intensity of labeling by p1-antiserum among the three categories of protein bodies present in the cotyledon were statistically significant ($p = 0.0000$). Labeling in the lipid bodies, any other cell structures, and in the negative controls (**Figures 9B** and **10B**) was insignificant.

DISCUSSION

Interest in SSPs is increasing because of the importance of regulation of gene expression as a matter of fundamental research and the high biological value of these proteins representing a major parameter of nutritional quality in commonly used crops (*17*). Although SSPs are the plant proteins most abundantly consumed by humans, those corresponding to olive seeds (one of the major crops in Mediterranean countries) are not in general destined for human consumption, mainly because in general, whole olive fruits are processed for oil production. A few works are dealing with the putative influence of olive seed components on oxidative stability and organoleptic quality of olive oils (*18*, *19*). Preliminary data are also emerging with regard to the potential use of oil extraction byproducts (including olive skins, pulp, and stones) for animal feeding (*20*). However, no basic knowledge of the presence of SSPs and their biochemical characteristics in olive seeds exists to date.

The results shown in this paper clearly demonstrate that the most abundant proteins in the mature olive seeds belong to the 11*S* protein family, accounting for approximately 70% of total seed proteins. The 11*S* proteins in olive seeds occur as two precursors of 41 and 47.5 kDa, which consist of three and two disulfide bond subunits, respectively. The information described in this paper regarding 11*S* proteins from olive seeds is in good agreement with the basic model for legumin-like 11−12*S* proteins from other plant species. These 11−12*S* proteins accumulate as hexameric complexes, the monomers of which consist of a larger, more acidic α-protein linked via disulfide

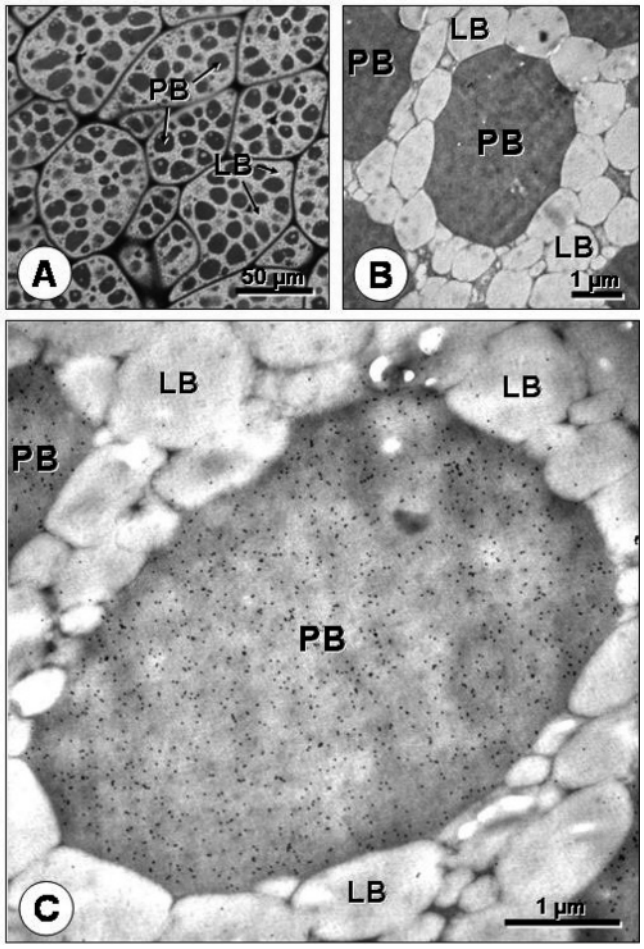

Figure 9. TEM immunolocalization of 11*S* SSPs in endosperm cells using the p1-antiserum. (**A**) LM observation of a thin section stained with toluidine blue showing a general view of the tissue. (**B**,**C**) Negative control and large magnification images of the TEM localization, respectively. Numerous gold particles decorate the electron-dense protein bodies, surrounded by several layers of elliptical, electron-translucent lipid bodies of large size. No significant labeling is present in either the lipid bodies or the negative control section. LB, lipid body; PB, protein body.

bridges to a smaller, more basic β-protein, producing an α/β-structure (*6*, *17*, *21*, *22*). According to this widespread model and considering the sequence alignments of p1 and p2 to the basic subunits of legumins shown in this paper (*23*−*25*) and their mobility after 2D gel electrophoresis, the proteins p1 and p2 correspond to the β-arms (basic subunits) of the 47.5 and 41 kDa proteins, respectively. To fit into an α/β model and in good agreement with the results of 2D electrophoresis, p5 should thus correspond to the α-arm (acidic subunit) of the 47.5 kDa protein. On the other hand, p3 and p4 (which are also acidic) may correspond to alternative α-arms of the 41.0 kDa precursor on the basis of their similar molecular masses and the relative intensity of the p2 band when compared to p3 and p4 bands. This proposed model (**Figure 11**) would indicate that the 41.0 kDa precursor (Pro2) may not correspond to a single protein but to at least two forms (Pro2 and perhaps Pro3) not resolved by the methods used in this work because of their similar molecular masses. The presence of multiple spots attributed to p1−p5 after 2D separation might stem from highly similar isoforms or posttranslational modifications of these proteins. This variability and the heterogeneity of the pairs of acidic−basic subunits are general characteristics of many legumin-like proteins, i.e., glycinin (*26*), which is also present in the p1

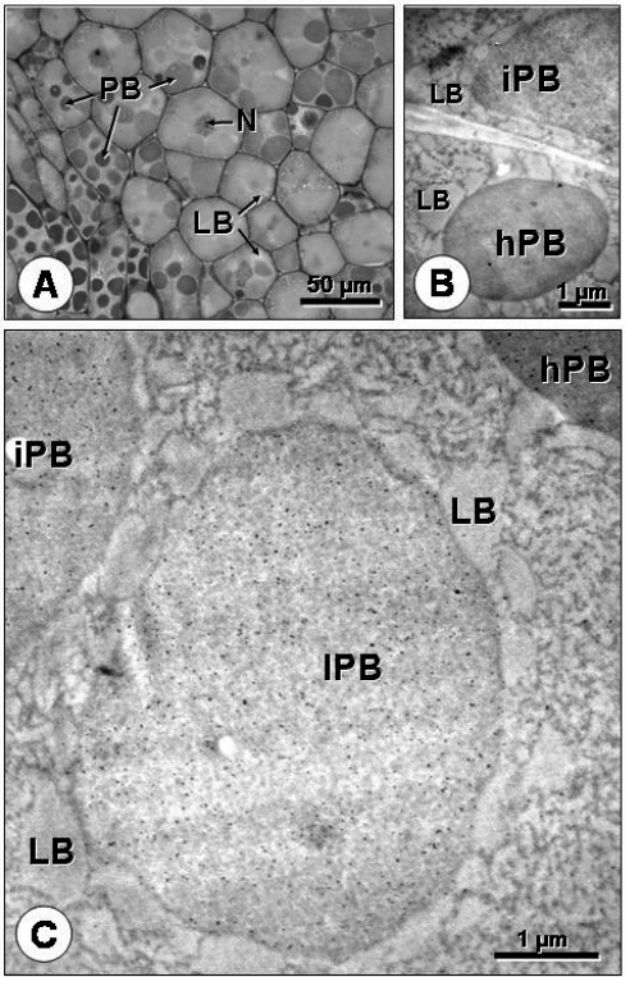

Figure 10. TEM immunolocalization of 11*S* SSPs in cotyledon cells using the p1-antiserum. (**A**) LM observation of a thin section stained with toluidine blue showing a general view of the tissue. Note the presence of several morphological cell types and different labeling intensities in the protein bodies. (**B**) Negative control showing two protein bodies of low and high electron density, respectively. (**C**) Large magnification image of the TEM localization. Numerous gold particles decorate a protein body of low electron-density, surrounded by a single layer of irregularly shaped, lipid bodies of small size. Both a high and an intermediate electron density protein body are also partially observed. No significant labeling is present in either the lipid bodies or the negative control section. LB, lipid body; N, nucleus; and hPB, lPB, and iPB, high, low, and intermediate electron density protein bodies.

homologue, castanin (*27*). Further characterization (i.e., by protein sequencing of the p1−p5 spots excised from 2D maps) would be necessary in order to delve into this question.

Storage proteins of 11−12*S* type, related to the widely distributed "legumin" type, occur in most dicotyledonous species and normally belong to the globulin family (*28*). However, olive is an exception in which the major 11*S* proteins present in seeds are similar to prolamines in terms of solubility. Several other exceptions have been described, such as the rice storage proteins, which are not soluble in dilute salt solutions and hence have been defined as glutelins, although they clearly belong to the 11−12*S* globulin family (*29*).

The absence of visible differences regarding number, distribution, sizes, and relative intensities of the corresponding 11*S* bands among the six olive cultivars examined suggests that 11*S* storage protein synthesis in olive is highly conserved. However, the presence of 11*S* variants cannot be completely discarded,

Table 2. Morphometric Analysis of Protein Bodies, Lipid Bodies, and Labeling by p1-Antiserum in Endosperm and the Cotyledon Cells

	area mean (square μm)	standard deviation	labeling intensity (particles/ square μm)	standard deviation
endosperm protein bodies	18.73	4.65	69.18	1.42
endosperm lipid bodies	1.73	0.64	negligible	
cotyledon protein bodies	24.66	10.85	16.33 20.43 25.81	0.66 0.79 0.84
cotyledon lipid bodies	0.34	0.11	negligible	

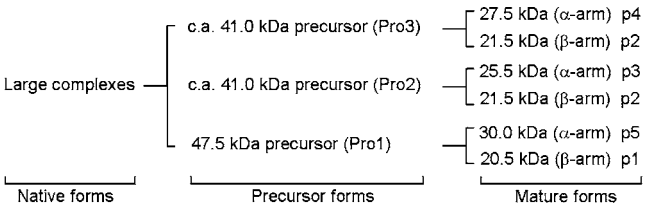

Figure 11. Proposed model showing the composition of the subunits of the different 11*S* proteins found in olive mature seeds.

as more resolutive techniques may help to identify them, particularly the use of 2D electrophoresis coupled to mass spectrometry analysis. Indeed, conspicuous differences with regards to 11*S* and other SSPs composition have been described within species, and within lines and cultivars of the same species, including the presence of naturally occurring mutants and electrophoretic variants (*30*−*34*). This polymorphism arises from the presence of multigene families and, in some cases, proteolytic processing and glycosylation (*6*), events yet to be characterized in olive seeds. The presence of an extremely wide germplasm in the olive, with about 2500 different cultivars over the world (*35*), makes differences in 11*S* SSPs likely to occur.

Cross-reaction of p1-antiserum to SSPs from other seeds from plant is an evidence of the close relationship among these proteins in different plant species. As examples, in the case of almond extracts, p1-antiserum cross-reacts with several bands, which might well match to the amandin proteins corresponding to the storage protein prunin-2 (41.8 and 38.6 kDa) (*36*, *37*). In the case of rice extracts, cross-reaction is observed with proteins displaying apparent molecular masses in a good agreement with the described forms of rice SSPs (20, 22, 28, and 31 kDa) (*29*). The deduced molecular mass of the subunit β of 11*S* globulin in sesame (24 kDa) (*24*) is also in good concordance with the major p1-antiserum cross-reactive band from sesame extracts.

Both endosperm and cotyledon constitute storage tissues, which contain the reserves of the seed. Protein bodies of both endosperm and cotyledon were densely labeled by p1-antiserum, despite their morphological differences, and those of the corresponding tissues. In all cases, labeling of p1-antiserum was uniformly amorphous, with no apparent subdomains that may contain different kinds of proteins as demonstrated for several other SSPs (*24*, *38*). Differences in electron density and 11*S* protein content among the protein bodies present in both tissues, as well as among protein bodies within the cotyledon, may reflect purely quantitative differences in the content of 11*S* proteins or (according to the model proposed in this paper for olive 11*S* forms) qualitative differences in the proportion of the different α- and β-arms described in this paper. At this point, it is necessary to remark the triploid nature of the endosperm

in comparison to the diploid cotyledon. The uniformity of sizes and electron/labeling density of protein bodies in the endosperm may also reflect that this tissue is fully developed at the developmental stage in which the present study was made. On the contrary, the cotyledon may be not fully developed at the same time or perhaps differences in the protein body compactness may reflect an evolutionary character addressed to a rapid mobilization of resources upon germination.

Transport of SSPs to the vacuole and their assembly into protein bodies, as well as mobilization of proteins upon germination, constitute important topics that will be studied in detail in the future. Moreover, isolation and characterization of full-length cDNAs for p1−p5 proteins will allow comparison of the deduced amino acid sequences with those from different plant sources and further classification of SSPs from olive seed according to their amino acid content.

ABBREVIATIONS USED

CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate; DAA, days after anthesis; DTT, dithiothreitol; LM, light microscopy; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; SDS, sodium dodecyl sulfate; SSP, seed storage proteins; TBP, tributylphosphine; TCA, trichloroacetic acid; TEM, transmission electron microscopy.

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