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Short Report

Characterization of seed storage proteins and their synthesis during seed development in *olea europaea*

WEI WANG, JUAN DE-DIOS-ALCHÉ, ANTONIO-JESÚS CASTRO and MARÍA-ISABEL RODRÍGUEZ-GARCÍA*

Departamento de Bioquímica, Biología Celular y Molecular de Plantas, Estación Experimental del Zaidín, CSIC, Granada, Spain

ABSTRACT SDS-PAGE analysis showed that seed storage proteins (SSPs) in olive mainly consisted of two hydrogen-bonded subunits, S*olea* I (41 kDa) and S*olea* II (47.5 kDa). When reduced, S*olea* I and II yielded 3 and 2 disulfide-bonded polypeptides, respectively. N-terminal sequencing confirmed that both *Solea* I and II shared a high similarity with 11S proteins. Additionally, time-course of protein synthesis in olive seeds was investigated from 25 days after anthesis (DAA) until seed ripening. Before 105 DAA, young seeds contained only trace amounts of detectable proteins. Only from 105 DAA onwards, seed proteins began to be massively synthesized. Seeds of 105 DAA and more exhibited no significant difference in protein profiles with mature seeds, especially in terms of *Solea* I and II.

Objectives

SSPs are synthesized in abundance in developing seeds and accumulate primarily in the protein storage vacuoles (PSVs) of terminally differentiated cells of embryo and endosperm (Herman and Larkins 1999). A previous report indicated that mature olive seeds contain densely packed storage protein deposits that entirely fill PSVs (Ross *et al.,* 1993). Olive (*Olea europaea* L.) is one of the most important oil-storing crops in many countries, due to its high productivity and the quality of its oil. Nevertheless, little is known about the characteristics of SSPs in this plant up to date. In this paper, we have characterized SSPs in olive, and followed their synthesis and accumulation throughout seed development.

Materials and Methods

Plant materials

Seeds were obtained from olive trees (*O. europaea* var *Picual*) located in the "Estación Experimental del Zaidín", (CSIC), Granada (Spain) 25 to 210 days after anthesis (DAA).

Protein extraction

Seeds were directly homogenized in a mortar cooled on ice using a buffer containing: 125 mM Tris-HCl, pH 6.8 (native conditions), plus 0.2 % SDS (denaturing, non-reducing conditions), and plus 0.2 % SDS, 1 % 2-mecaptoethanol (denaturing, reducing conditions). After centrifugation at 10,000 g for 5 min (4 ºC), the supernatant was boiled for 5 min and centrifuged again. Proteins in the supernatant were precipitated with 2 volumes of cold acetone and resuspended in extraction buffer. Protein concentrations were measured by a Bio-Rad microassay, using BSA as a standard.

Polyacrylamide gel electrophoresis

Native PAGE (6 % gel) and SDS-PAGE (4.75% stacking gel and 12.5% resolving gel) were performed according to Laemmli (1970). After electrophoresis, proteins were visualized with Coomassie blue or silver stain.

Partial amino acid sequencing

Polypeptides p1 (20 kDa) and p2 (22.4 kDa), derived from *Solea* II and I (see below) respectively, were purified to homogeneity and blotted onto a Sequi-Blot PVDF membrane (Bio-Rad). N-terminus sequencing was performed by the proteomics facility at the CNB (CSIC), Madrid, Spain.

Results

Electrophoretic identification of seed storage proteins

Two large proteins were resolved from crude extracts of seeds (210 DAA) under non-denaturing conditions (Fig. 1 A). After electrophoresis under denaturing, non-reducing conditions, both proteins dissociated into two identical subunits: S*olea* I and II, with molecular weights (MWs) of approximately 41 and 47.5 kDa respectively (not shown), indicating that both subunits were linked by hydrogen bonds. Therefore, the two large proteins were different forms of aggregation from native SSPs. Proteins S*olea* I and II were the major constituents in seed extracts, representing about 70 % of total proteins (Fig. 1 B,C). When dissociated and reduced, S*olea* I yielded three polypeptides in roughly equal proportion,

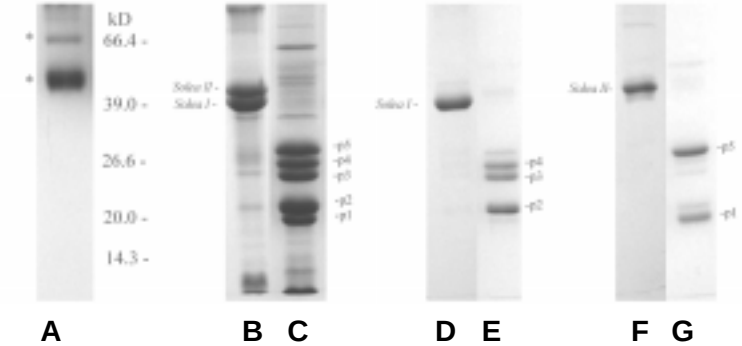

Fig. 1. Electrophoretic identification of SSPs in *Olea europaea*. *Shown are Coomassie blue–stained gels of proteins.* **(Lane A)** *Proteins of mature seeds, running under native conditions, * indicating native SSPs;* **(B)** *Seed proteins, denaturing gel without 2-mercaptoethanol (2-ME);* **(C)** *Seed proteins, denaturing gel with 2-ME; D and E, non-reduced* **(D)** *and reduced forms* **(E)** *of subunit Solea I; F and G, non-reduced* **(F)** *and reduced forms* **(G)** *of subunit Soea II. Molecular weights (in kilodaltons) are shown to the left of lane B.*

***Address correspondence to:** María Isabel Rodríguez-García. Departamento de Bioquímica, Biología Celular y Molecular de Plantas, Estación Experimental del Zaidín, CSIC, Profesor Albareda 1, E-18008, Granada, Spain. e-mail: mirbicep@eez.csic.es

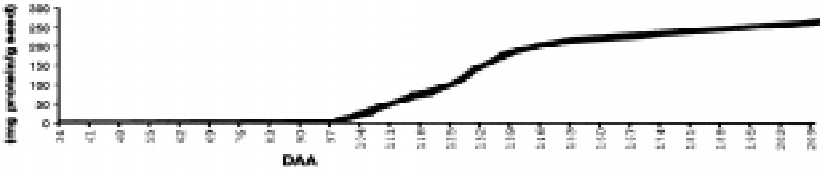

Fig. 2. Comparison of protein content of olive seeds of different ages. *Protein concentrations were measured by a Bio-Rad microassay, using BSA as standard. Represented are the mean values (mg protein/g dry seed) of 3 independent assays.*

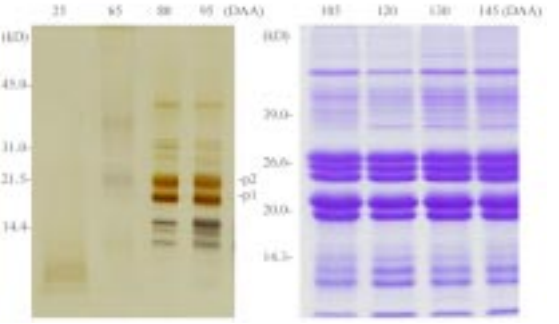

Fig. 3. SDS-PAGE analysis of seed protein synthesis at different developmental stages in *Olea europaea*. *SDS-PAGE gels stained with silver nitrate (left panel) or Coomassie blue (right panel). Proteins were extracted from olive seeds of different ages, resolved on a dry weight basis (left panel) or equal protein basis (right panel, 15 µg per lane). DAA are shown at the top. Molecular weights (in kilodaltons) are shown to the left.*

with MWs of 22.4 (p2), 23.5 (p3) and 27 (p4) kDa (Fig. 1 D,E), respectively, whereas S*olea* II yielded two polypeptides of 20 (p1) and 30 (p5) kDa (Fig. 1 F,G). The amount of p5 was approximately twice that of p1. These polypeptides were disulfide-bonded in native SSPs.

Partial amino acid sequencing of p1 and p2

N-terminal sequencing of p1 and p2 polypeptides produced the sequences GLEETLLTRLLEN and GLEESLCTNKIR, respectively. Search of the SWALL sequence database for protein homology using the FASTA 33_t program (Pearson and Lipman 1988) resulted in the following best scores: for p1, 78.5 % identity was found to legumin precursor (SWALL accession number Q41128), and 71.4 % to conglutin alpha (Q96475). For p2, 75.0 % identity to both conglutin alpha (Q96475) and globulin precursor (Q9XHPO) was found.

Protein synthesis throughout olive seed development

Olive seed development could be roughly divided into 3 stages according to the presence of differential rates for protein synthesis: (I) early synthesis period (before105 DAA), (II) rapid and massive synthesis period (105-130 DAA) and (III) slow synthesis period (from 130 DAA until ripening). Stage II was the period showing the highest rate of protein synthesis. On a dry weight basis, ca. 50 % of total protein synthesis took place within this period (Fig. 2). SDS-PAGE analysis of protein extracts at different DAA showed the following results: at stage I, young seeds contained only trace amounts of detectable proteins, which were only visualized by silver staining (Fig. 3, left) whereas no visible Coomassie blue-stained bands appeared (not shown). At late stage I (80 and 95 DAA), p1, p2 and some small polypeptides were major constituents of seed extracts. As development of seed proceeded (stage II and III), numerous polypeptides from seed extracts were resolved. Polypeptides p1 to p5 from S*olea* I and II became the major protein species, representing up to 70 % of total proteins (Fig. 3, right). However, no significant differences were found with regard to the size distribution, or the relative intensity of bands.

Conclusions

Our results indicate that SSPs in olive consist of two hydrogen-bonded subunits, S*olea* I and II, with MWs of 41 and 47.5 kDa, respectively. When reduced, S*olea* I and II yield 3 and 2 disulfide-bonded polypeptides respectively. This behaviour is typical of the disulfide bonded acidic and basic chains of 11S storage proteins (globulins) found in *Pisum* (Matta *et al.,* 1981). N-terminal sequencing further confirms that the characterized olive SSP belongs to the 11S protein family. 11S proteins are non-glycosylated proteins which form hexameric structures. Each of the subunits in the hexamer is itself composed of an acidic and a basic chain derived from a single precursor and linked by a disulfide bond (Hayashi *et al.,* 1988, Shotwell *et al.,* 1988). The endoproteolytic cleavage of 11S proteins into two disulfide-bonded chains is evolutionarily conserved in seeds of conifers, monocots and dicots (Nielsen *et al.,* 1995). It is expected that the characterized olive SSP would be very similar in structure and post-translation processing to other 11S proteins. In addition, our results reveal changes in protein synthesis during seed development. During the early period of seed development (before 105 DAA), seed proteins were synthesized only in low quantities. In contrast, most seed proteins, and particularly *Solea* I and II were rapidly synthesized and accumulated in high quantities over a short period of time (105-130 DAA, i.e., less than one month), exhibiting a strict time-dependence. Therefore a tight gene control of the developmental programme is expected. Efforts are being made to clone the genes coding for S*olea* I and II and to characterize the expression and regulation of SSPs during olive seed development.

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