Influence of Cultivar and Fruit Ripening on Olive (*Olea europaea*) Fruit Protein Content, Composition, and Antioxidant Activity

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Proteins of olive fruit mesocarp are not very well-known at present. However, they have been shown to pass, at least partially, to the olive oil during its elaboration and therefore might be contributing to some of the special characteristics of this vegetable oil. In this study, protein content and composition were determined in olive fruits, cv. Arbequina and Picual, at three stages of ripening: green, spotted, and purple. Mesocarp proteins constituted 1.3−1.8% of the dry weight of the olive fruit, and cultivar and fruit ripening did not produce important changes in mesocarp protein content or composition. In addition, this composition was also similar to the amino acid composition of a 4.6-kDa polypeptide, which is the major protein component of olive oils and of oil bodies of olive fruit mesocarp, suggesting that this polypeptide is likely to be a major component of mesocarp proteins. There was, also, a relationship between the oil content of the olive fruit and the protein content determined, suggesting a stabilizing function of these proteins in the oil bodies of the olive fruit, analogously to the role suggested for oleosins. This stabilizing function does not seem to be extended to olive oils because when the polypeptides isolated were added at 20 ppm to soybean oil, the stability of the oil increased only slightly, suggesting that if these compounds play some role in the stability of the oils, this should be mostly a consequence of the possible interactions among these protein components and other olive oil antioxidant constituents.

Keywords: *Olive fruit; proteins; amino acid analysis; olive oil; minor components*

INTRODUCTION

Olive (*Olea europaea*) trees are among the oldest known cultivated trees in the world (*1*). They were widely cultivated in southern Europe and played a significant role in the early civilizations of Egypt and Greece (*2*). Nowadays, and although the cultivation of the olive tree has been extended to many other regions of the world, olive fruits remain a typical Mediterranean crop, where they play an important role in the diet of the people in that area as well as in their economy and culture.

Because of this importance, olive fruits, and mainly olive oil, have been studied for many years from an analytical point of view (*3*−*5*) and because of their potential health benefits (*6*, *7*). However, in addition to the oil, which is ∼22% of the olive drupe, olive fruits are composed of water (50%), proteins (1.6%), carbohydrates (19.1%), cellulose (5.8%), and minerals (ash) (1.5%) (*8*, *9*). Among these components, the proteins of the mesocarp of olive drupes are not very well-known, although some studies have begun to characterize several of the enzymes that are present (*10*−*13*). Some of these enzymes seem to pass to the oil during olive oil extraction, where they may be playing a role in the stability of the oils (*14*, *15*). In addition, some proteins that are present in the oil bodies of the olive fruit mesocarp also pass to the oil during olive oil extraction, constituting the main protein component in these oils, and might also contribute to some of the special characteristics of olive oils (*16*).

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As a continuation of these studies, the present investigation was undertaken to study the influence of fruit ripening on protein content and composition in the mesocarp of olive fruits as well as on the antioxidant activity of the protein components that pass to the oil during olive oil extraction. These data may increase our understanding of the role of these new minor components in olive oils.

EXPERIMENTAL PROCEDURES

Materials. Olive fruits (*O. europaea* L.), cv. Arbequina and Picual, were harvested from our Institute's field station (Instituto de la Grasa, Sevilla, Spain). The olives were distributed in three ripening groups according to their skin color (green, spotted, or purple) for each variety. Only healthy fruits, without any kind of infection or physical damage, were selected.

Determination of Oil and Protein Content in the Mesocarp of Olive Fruits. Olive fruits were stoned, and the mesocarp tissues were ground and freeze-dried to remove moisture. The total oil content was determined by using a Soxhlet extractor for 8 h with hexane, and the results are given as percentage of dry weight. Protein content was determined by amino acid analysis after acid hydrolysis in the solid remaining after oil extraction. The solid plus D,L-α-aminobutyric acid, which was added as internal standard, was suspended in 1 mL of 6.0 M hydrochloric acid and hydrolyzed for 20 h at 110 °C. The hydrolyzed samples obtained were taken to dryness, dissolved in 3 mL of 1 M sodium borate buffer (pH 9.0), and derivatized with diethyl ethoxymethylenemalonate. Protected amino acids were, finally, fractionated by reversed-phase high-performance liquid chromatography (HPLC) with UV detection at 280 nm using a previously described gradient (*17*, *18*). Protein content was calculated from amino acid data and is given as percentage of dry weight.

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the mesocarp of olive fruits were isolated according to a previously described procedure (*16*). Briefly, the mesocarp of olive fruits was homogenized in buffer with a Waring blender. The homogenization medium (4 mL/g of tissue) contained 0.4 M sucrose, 100 mM Hepes/NaOH (pH 7.5), 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, and 1% (w/v) ascorbic acid. The homogenate was then filtered through four layers of cheesecloth and centrifuged at 5000*g* for 15 min. The fat layer containing the crude oil bodies was recovered from the top of the 5000*g* supernatant. These crude oil bodies were dispersed in 5 volumes of buffer and layered beneath a further 20 volumes of buffer containing 0.1 M sucrose. This was then centrifuged at 18000*g* for 15 min, after which time the oil body fraction was again recovered from the top of the gradient. This dispersal−layering−centrifugation procedure was repeated two more times. The purified oil bodies were dispersed in 3 volumes of water and extracted with 3 volumes of chloroform/methanol (2:1) to remove lipids. The layers were separated by centrifugation at 650*g* for 5 min and then removed. The whole procedure was repeated, and the resulting solid was dried under nitrogen and stored at −28 °C. This solid was used in stability assays and for the study of amino acid composition using amino acid analysis, which was carried out as described above.

Partial Characterization of the Peptides and Proteins Obtained from Olive Fruits. Peptides and proteins obtained from oil bodies of the mesocarp of olive fruits were characterized both by electrophoresis and by amino acid analysis. Tricine−sodium dodecyl sulfate−polyacrylamide gel electrophoresis (tricine-SDS-PAGE) was performed according to the method of Schägger and Jagow (*19*) with 16.5% total acrylamide gels containing 3% of cross-linker. Gels were silver stained according to the method of Morrissey (*20*). Briefly, gels were treated successively with glutaraldehyde, dithiothreitol, silver nitrate, formaldehyde/sodium carbonate, citric acid, water, Farmer's reducer, and new silver staining. A typical calibration curve ($r = -0.987$, $p = 0.018$) was obtained with bovine serum albumin (66.0 kDa), chicken egg albumen (45.0 kDa), bovine erythrocytes carbonic anhydrase (29.0 kDa), chicken egg white lysozyme (14.3 kDa), and bovine insulin chain B (3.5 kDa).

Amino acid analysis was carried out by HPLC as described above.

Measurement of Antioxidative Activity. Oxidative stability of refined soybean oil without antioxidants was compared with refined oil samples containing 20 ppm of the protein isolated from the oil bodies of the mesocarp of olive fruits. Duplicate oil samples (10 g) were weighed into 90 × 20 mm Petri dishes and oxidized for 264 h under air in the dark at 60 °C. Peroxidation was evaluated periodically by using the thiobarbituric acid-reactive substances (TBARS) assay as described by Kosugi et al. (*21*). The obtained curves were adjusted by using the Boltzmann equation (Microcal Origin, v. 6.0, Microcal Software, Northampton, MA)

$$y = [(A_1 - A_2)/(1 + e^{(x-x_0)/dx}] + A_2 \quad (1)$$

where A_1 is the initial y value, A_2 is the final y value, x_0 is the x value at y_{50}, and dx is the width of the obtained curve. The final y values obtained (A_2) were employed for comparison purposes by using a protection index (PI), which was determined according to the following equation:

$$PI = 100 - [100 \times (A_{2,sample})/(A_{2,oil})] \quad (2)$$

PI equal to 100 meant that the compound protected completely. PI equal to 0 meant that the compound tested had no protective effect.

RESULTS

Oil and Protein Content of Olive Fruit Mesocarp as a Function of Fruit Ripening. The total oil content determined did not show big changes as a function of the ripening state, in accordance with previous studies (*22*). Thus, the oil content increased from 53 to 57% in the Arbequina cultivar and decreased from 53 to 52% in the Picual cultivar (Figure 1). A similar behavior was observed for the total protein content determined in the Arbequina and Picual cultivars. Thus, protein content exhibited also only some changes as a function of the ripening stage for the Arbequina cultivar (it increased from 1.27 to 1.60%) and was almost constant for the Picual cultivar (1.76%).

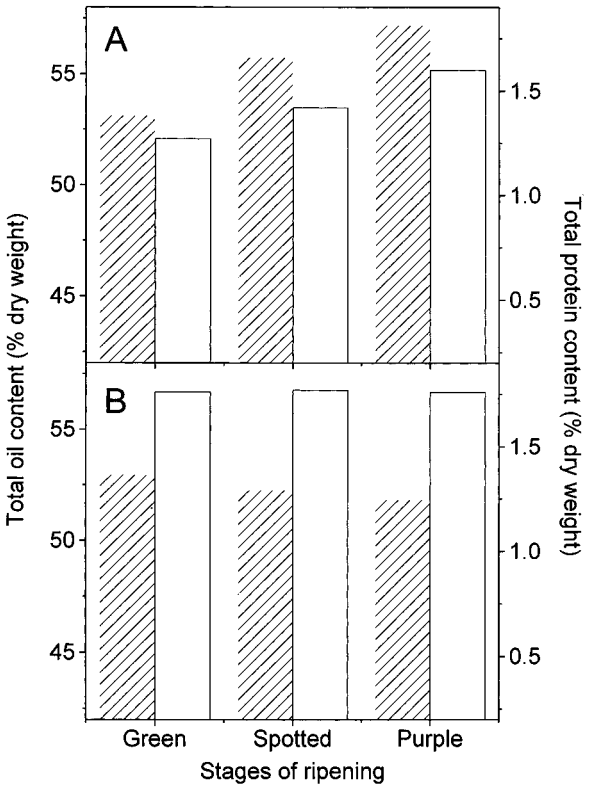

Figure 1. Oil (striped bars) and protein contents (open bars) as a function of the stages of ripening in olive fruits of the varieties (A) Arbequina and (B) Picual.

Despite the increase in protein content as a function of the ripening stage observed in the Arbequina cultivar, the amino acid composition of olive fruit mesocarp proteins was almost constant with independence of the variety of the olive fruit analyzed or the ripening stage studied (Table 1). Only the amino acid lysine in both varieties seemed to increase as a function of the ripening stage and changed from 0.85 to 1.68% in cv. Arbequina and from 2.35 to 2.88% in cv. Picual.

Isolation and Study of the Proteins Present in the Oil Bodies of the Mesocarp of Olive Fruits. The proteins present in the oil bodies of the mesocarp of olive fruits were isolated by acetone precipitation. These proteins were found, by tricine-SDS-PAGE (data not shown), to be always mostly composed only by a 4600 kDa polypeptide with independence of the variety of the olive fruit or the stage of ripening, in accordance with previous studies (*16*). This similarity among cultivars and degrees of ripening was also observed by amino acid analysis after acid hydrolysis. Table 2 collects the amino acid composition of protein isolates from the oil bodies of the mesocarp of olive fruits of Arbequina and Picual cultivars at the three stages of ripening analyzed. The results obtained were also very similar to the amino acid composition of olive fruit mesocarp proteins (Table 1), therefore suggesting that the polypeptide isolated from

Table 1. Amino Acid Composition (Mole Percent) of Olive Fruit Mesocarp Proteins[a]

	Arbequina			Picual		
amino acid	green	spotted	purple	green	spotted	purple
alanine	8.72	8.75	8.58	8.17	8.29	8.72
arginine	5.44	5.50	6.07	6.65	6.67	7.20
Asx[b]	12.92	12.82	11.94	11.82	12.80	12.04
cysteine	0.57	0.48	0.51	0.97	0.75	0.70
Glx[b]	11.09	9.91	10.95	10.82	11.18	11.09
glycine	10.16	10.13	9.88	9.94	9.86	9.61
histidine	1.49	2.38	2.22	1.84	2.04	2.13
isoleucine	6.44	6.40	6.48	6.38	6.36	6.21
leucine	9.34	9.41	9.55	9.53	9.30	9.12
lysine	0.85	1.05	1.68	2.35	2.63	2.88
methionine	1.51	1.71	1.50	1.45	1.43	1.45
phenylalanine	4.81	4.75	4.76	5.16	4.91	4.78
serine	7.26	7.47	6.91	6.49	6.20	6.42
threonine	6.38	6.48	6.63	6.33	6.12	6.32
tyrosine	4.07	3.98	3.86	3.26	3.22	3.09
valine	8.95	8.78	8.53	8.83	8.26	8.25

[a] Values are the mean of two independent determinations. [b] Abbreviations: Asx, asparagine/aspartic acid; Glx, glutamine/glutamic acid.

Table 2. Amino Acid Composition (Mole Percent) of Proteins Isolated from the Oil Bodies of Olive Fruit Mesocarp[a]

	Arbequina			Picual		
amino acid	green	spotted	purple	green	spotted	purple
alanine	8.07	8.08	8.04	8.38	8.16	8.00
arginine	5.04	4.90	5.09	5.64	5.39	5.73
Asx[b]	11.35	11.65	11.99	11.80	12.62	11.86
Cysteine	0.43	0.82	0.54	1.13	0.68	1.05
Glx[b]	12.00	11.19	11.80	11.31	11.90	12.05
glycine	9.09	9.36	8.34	9.81	8.93	9.02
histidine	1.74	2.16	1.95	1.65	1.72	1.74
isoleucine	6.90	6.62	6.68	6.37	6.87	6.23
leucine	9.20	9.15	9.06	9.01	8.78	8.71
lysine	0.62	1.13	2.00	1.95	1.20	2.42
methionine	2.24	2.15	1.96	2.00	1.95	2.01
phenylalanine	5.70	5.05	5.16	5.17	5.51	5.50
serine	8.05	7.78	7.86	6.78	7.72	7.40
threonine	6.68	6.41	6.62	6.27	6.58	6.55
tyrosine	4.47	4.90	4.46	4.63	3.65	4.01
valine	8.43	8.66	8.45	8.10	8.36	7.73

[a] Values are the mean of two independent determinations. [b] Abbreviations: Asx, asparagine/aspartic acid; Glx, glutamine/glutamic acid.

the oil bodies of the mesocarp of olive fruits seems to be the main component of olive fruit mesocarp proteins.

Measurement of Antioxidative Activity. In an attempt to investigate one of the functions that such proteins may be playing in olive oils, the antioxidant activity of the isolated proteins was evaluated. The proteins were added to refined soybean oil at 20 ppm, and the stability of the obtained oils was compared with the stability of the untreated soybean oil employed by heating the oils at 60 °C for 216 h and determining lipid oxidation products by using the TBARS method. Figure 2 shows the results obtained for the control oil and an oil treated with 20 ppm of protein obtained from purple olive fruit, cv. Arbequina. Although the effect of the isolated fractions at the concentration tested was small, all of the isolated protein fractions protected the oil by decreasing the TBARS produced. This effect was better observed when the data obtained were adjusted by using the Boltzmann equation (eq 2). Table 3 collects the A_2 values obtained for the adjustment of the seven curves obtained as well as the corresponding PI derived from the A_2 values. According to these data, the PI values

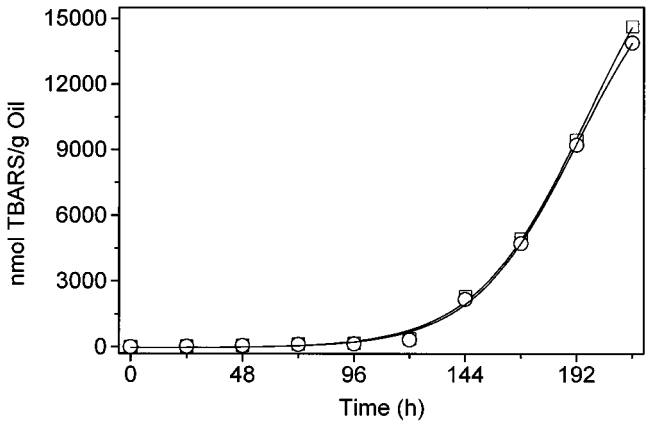

Figure 2. Effect of oil body proteins (○) obtained from the olive fruit mesocarp on soybean oil oxidation (□). Protein was added at 20 ppm, and oils were heated at 60 °C.

Table 3. Antioxidant Activity of Proteins Isolated from the Mesocarp of Olive Drupes

protein added				
cultivar	stage of ripening	A_2[a]	correlation [b]	PI[c]
none		21041	0.9995	0
Arbequina	green	18663	0.9996	11.3
Arbequina	spotted	18977	0.9995	9.8
Arbequina	purple	18369	0.9995	12.7
Picual	green	20622	0.9996	2.0
Picual	spotted	20313	0.9994	3.5
Picual	purple	19618	0.9995	6.8

[a] A_2 is the final TBARS value (in nmol/g of oil) calculated using the Boltzmann equation (see Experimental Procedures). [b] The data shown are the correlation obtained in the adjustment of the experimental data to the Boltzmann equation. [c] Protection indices (PI) were calculated as described under Experimental Procedures.

exhibited by proteins isolated from the Arbequina cultivar were 10−13 and were 2−7 for proteins isolated from the Picual cultivar.

DISCUSSION

Olive oil has gained popularity in recent years not only because of its superior flavor when compared to other vegetable oils but also because of reports of potential health benefits, which have been related to both its fatty acid composition and the presence of minor components with antioxidant effect (*7*, *23*, *24*). Among these minor components, the presence of low molecular weight polypeptides has been recently detected in olive oils. These polypeptides are present in the oil bodies of the olive fruit mesocarp and pass to the oil during olive oil extraction (*16*).

The results obtained in the present study suggest that not only are these polypeptides the main components of the oil bodies of the olive fruit mesocarp, but they seem to be also major constituents of the proteins of the mesocarp because the average amino acid composition obtained for the mesocarp proteins was very similar to the amino acid composition of the isolated polypeptides.

Mesocarp proteins constituted 1.3−1.8% of the dry weight of the olive fruit, and there was a relationship between the oil content of the olive fruit and the protein content determined. Thus, if the oil content increased with the ripening (for example, in the Arbequina cultivar assayed), there was also an increase in protein content, suggesting a stabilizing function of these proteins in the oil bodies of the olive fruit, analogously to the role suggested for oleosins (*25*, *26*).

An additional confirmation of this similarity among the polypeptides isolated from the oil bodies of the mesocarp of olive fruits and the oleosins was the amino acid composition determined. Thus, the 4.6 kDa polypeptide had a small number of basic and sulfur-containing amino acids, a high number of amino acids with hydrophobic groups, and a high content in asparagine/aspartic acid and glutamine/glutamic acid, analogously to that described for oleosins (*27*−*29*).

Cultivar and fruit ripening did not produce important changes in mesocarp protein content or composition. Therefore, it is not expected that protein content in olive oils may change considerably as a function of the variety or the ripening stage of the olive fruit employed in the elaboration of the olive oil.

In addition, when the polypeptides isolated from the oil bodies of olive fruit mesocarp were tested for antioxidative activity in vegetable oils, they exhibited small antioxidant properties at the assayed concentration (20 ppm). However, this concentration is much higher than the concentration at which they are present in the oils: <1 ppm for virgin olive oils (Hidalgo et al., unpublished results). Therefore, these results suggest that polypeptides present in olive oils are not expected to play a significant role, by themselves, in the antioxidant properties of the oils, although they might influence the shelf life of olive oils because of interactions with other olive oil antioxidant components.

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