The Presence of Oxidizing Enzyme Activities in Virgin Olive Oil

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ABSTRACT: Samples of Greek virgin olive oils were examined for the presence of proteins and oxidative enzyme activities. All oil samples tested contained detectable amounts of protein, as well as lipoxygenase and polyphenol oxidase activities. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and size-exclusion chromatography of olive oil extracts revealed the presence of low-molecular-mass (10–40 kDa) silver-staining and ultraviolet-absorbing components, respectively. Both lipoxygenase and polyphenol oxidase catalytic activities were heat- and protease-sensitive, and they expressed Michaelis-Menten kinetics.

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KEY WORDS: Enzymatic browning, lipoxidase, lipoxygenase, *Olea europaea,* olive oil, oxidation, polyphenol oxidase, protein, vegetable oil, virgin oil.

Olive oil is one of the oldest known vegetable oils and is extracted from the fruit of the olive tree *Olea europaea.* It is almost unique among vegetable oils in that it can be consumed, without any refining treatment, in its crude form called virgin olive oil (1). One of the most severe quality problems of olive oil is oxidative rancidity due to the oxidation of unsaturated fatty acids and subsequent formation of compounds that possess unpleasant taste and odor (1). It has been suggested that lipoxygenase plays a role in the oxidation of unsaturated fatty acids and pigments of the olive fruit (2). Virgin olive oils have a high polyphenol content, which is involved in the oxidative stability of the oils (3). The enzymatic oxidation of polyphenols in olive fruits results in their browning, and it is directly related to their polyphenol oxidase content (4,5). Although a number of papers reported the detection of proteins in various vegetable oils (6,7), nothing is known about the presence of enzymatic activities in virgin olive oils. We have carried out this study in an effort to investigate the protein content of virgin olive oil and to examine whether oxidative enzyme activities are present in it, which could affect the quality of this fine food product during storage.

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MATERIALS AND METHODS

Linoleic acid, 4-methylcatechol, papain, and all fast protein liquid chromatography (FPLC) molecular mass standards, except phosphorylase kinase, were obtained from Sigma Chemical Co. (St. Louis, MO). Rabbit muscle phosphorylase kinase was prepared in our laboratory according to conventional procedures. Electrophoresis molecular weight markers, except insulin (BDH, Dorset, England), were obtained from Pharmacia Biotech (Uppsala, Sweden). The virgin olive oil samples were a gift of Elais SA (Athens, Greece) and of the National Agricultural Research Foundation, Subtropics and Olive Institute of Chania, Greece.

The olive oil samples (750 µL) were extracted with equal volumes of 50 mM Tris-HCl buffer, pH 7.5, which contained 0.1 M NaCl and 10% (vol/vol) glycerol, by vortexing for 1 min or stirring for 40 min (samples for electrophoresis) at room temperature. The insoluble material was further removed by centrifugation at $12,000 \times g$ for 2 min or at $5,900 \times g$ for 10 min (samples for electrophoresis, 50 mL). When necessary, the supernatants were filtered through a large coarse fluted paper to remove traces of suspended material.

Protein was measured by a modification of the Lowry assay (8), by the Bradford method (9), or turbidimetrically by trichloroacetic acid precipitation (10). The water content was checked by Karl Fischer titration.

Lipoxygenase (LOX) activity was determined spectrophotometrically at 25°C with linoleic acid as the substrate by measuring the increase in absorbance at 234 nm, arising from the conjugated double bonds formed by hydroperoxidation of linoleic acid (11). This method measures the total activity of the LOX isozymes, but not for the specific isozymes. The reaction mixture (0.7 mL) contained 0.4 mM linoleic acid and a quantity of the olive oil extract in 0.2 M sodium phosphate buffer, pH 6.2. One unit of LOX is defined as the amount of enzyme that catalyzes the formation of 1 µmol of product per min at 25°C. Polyphenol oxidase (PPO) activity was determined spectrophotometrically at 410 nm and 25°C (4,5) in a reaction mixture (0.6 mL) that contained 50 mM sodium citrate buffer, pH 6.2, 6.7 mM 4-methylcatechol, and a quantity of the extract. One unit of PPO activity is defined as the amount of enzyme that produces, under the above-mentioned conditions, a change in absorbance (ΔA) of 0.05 min^{-1}. Ab-

sorption measurements were carried out with a Hitachi (Tokyo, Japan) U-2000 ultraviolet (UV)-visible spectrophotometer. All activity measurements were performed in triplicate.

Enzyme inactivation by proteolysis was performed at 25°C. The olive oil extracts were preincubated with papain (final concentration 50 µg/mL) for 60 min and then assayed for the enzymatic activities as mentioned above.

Concentration of proteins from olive oil extracts by precipitation with trichloroacetic acid and ammonium sulfate was performed as follows: The extracts remained on ice for 30 min in the presence of trichloroacetic acid (final concentration 15%) or 15 min in the presence of ammonium sulfate (final concentration 70%). Trichloroacetic acid precipitate was pelleted by centrifugation at 17,300 × *g* for 10 min at 4°C, and the pellet was washed twice with diethyl ether and further dissolved in the electrophoresis sample buffer (12). Ammonium sulfate precipitate was pelleted by centrifugation at 27,000 × *g* for 15 min at 4°C, and the pellet was washed twice with ethanol (50%) and further dissolved in the electrophoresis buffer. Alternatively, the ammonium sulfate pellets were resuspended in the extraction buffer and extensively dialyzed against the same buffer at 4°C.

Vertical-slab gel electrophoresis was performed in a Mini Protean II Electrophoresis Cell apparatus (Bio-Rad, Richmond, CA) with a discontinuous buffer according to Laemmli (12). Silver-staining of the gels was used to visualize protein bands (13). The gels were scanned by a ScanTouch image scanner of Nikon (Tokyo, Japan). Digitized images were further analyzed by Aldus (Seattle, WA) Photostyler 2.0 software.

FPLC was performed by using a Waters (Milford, MA) 650 Advanced Protein Purification System and a size-exclusion chromatography Pharmacia Superose 6 HR 10/39 column, which was previously calibrated with a set of protein molecular weight markers, including soybean LOX (94 kDa) and trypsin inhibitor (20.1 kDa).

Statistical analysis (determination of standard deviations) was performed with the Enzfitter Non-linear Regression Data Analysis Program (14).

RESULTS AND DISCUSSION

All virgin olive oil samples tested contained detectable amounts of protein when determined with two colorimetric assays. When the protein concentration was assayed with the enhanced alkaline copper (Lowry) method, the obtained values were always about one order of magnitude higher than those obtained by the Coomassie blue (Bradford) method. For seven different oil samples, the estimated mean apparent protein concentration was 0.38 mg of protein/mL of oil extracted (ranged from 0.28 to 0.52 mg/mL), when assayed with the Lowry method, and 0.03 mg/mL (ranged from 0.01 to 0.04 mg/mL), when assayed with the Bradford method. The high protein values obtained with the Lowry assay are most probably due to interfering extractable oil components (e.g., phenolic compounds or lipids) (15,16). On the other hand, the Bradford method could have led to an underestimation of the total protein concentration, owing to the specificity of the assay for arginine residues (17). Nevertheless, with the aid of a generally applicable turbidimetric procedure for protein determination, involving the use of trichloroacetic acid (10), we calculated protein values in the range of those estimated by Lowry (0.4 mg/mL). After extraction of oil extracts with petroleum ether (1:2, vol/vol), we found that, although Lowry and Bradford protein estimates did not change significantly, the values of the turbidimetric method were diminished by about 50%, suggesting the presence of hydrophobic substances that interfere with the turbidimetric assay (not shown).

After extensive dialysis of oil extracts against the extraction buffer, the protein content, estimated with the Lowry method, decreased to 12–14% of that of the initial extract, suggesting the presence of low-molecular-weight (MW), dialyzable, Lowry-reacting components. Concentration of oil extracts through ultrafiltration Amicon UM2 membranes, with a nominal MW cut-off of 1000, revealed that almost all Lowry reacting components were retained during ultrafiltration. On the other hand, ammonium sulfate (70%) treatment of the oil extracts gave precipitates that, upon dialysis,

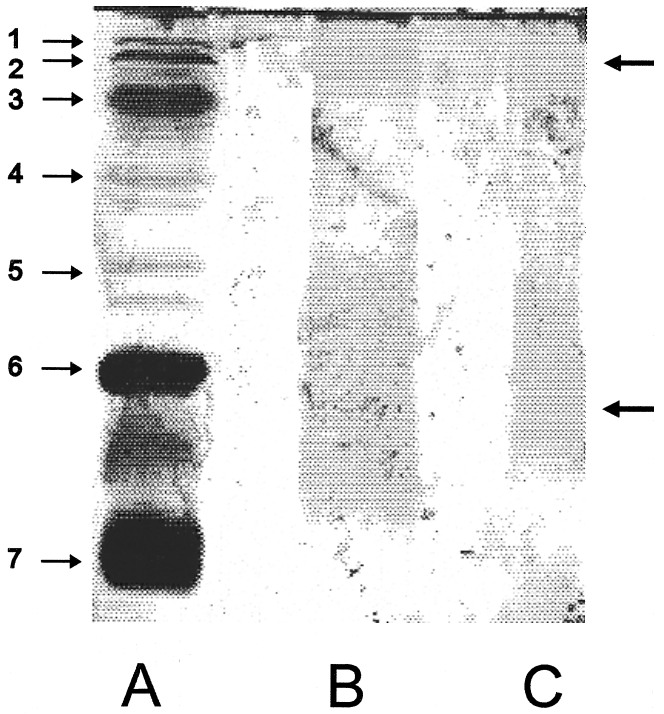

FIG. 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) of (B) trichloroacetic acid- and (C) ammonium sulfate-precipitated material from an olive oil extract; 5 mL of a virgin olive oil sample extract (sample 7 of Table 1) was treated with trichloroacetic acid or ammonium sulfate, the precipitated material was dissolved in electrophoresis sample buffer and further analyzed by 10% SDS–PAGE and silver-stained as described in the Materials and Methods section. (A) Molecular mass standards: (1) phosphorylase b (94 kDa); (2) albumin (67 kDa); (3) ovalbumin (43 kDa); (4) carbonic anhydrase (30 kDa); (5) trypsin inhibitor (20.1 kDa); (6) alpha lactalbumin (14.4 kDa); (7) insulin (5.7 kDa). Lower arrow, I; upper arrow, II.

showed a protein content (Lowry method) of about 7% of the initial total protein.

To identify and monitor proteins present in olive oil extracts, we used sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate protein species and the sensitive silver gel-staining technique to detect the polypeptides. Figure 1 shows representative SDS–PAGE profiles of trichloroacetic acid- and ammonium sulfate-precipitated material from olive oil extracts. We could not see distinct protein bands as usually observed in cell extracts. Instead, we observed two major diffused zones of protein silver-stained material (I and II), one of high molecular mass (on the top of the gel, II) and the other corresponding to molecular masses in the range of 10–20 kDa (I). Diffusion of protein bands suggests a polydispersity of protein-stained components and might be a result of partial proteolysis of the initial protein molecules present in olive fruits. Less-sensitive Coomassie blue staining was ineffective to visualize protein bands. When the olive oil extract or an ammonium sulfate-precipitated material of this extract was chromatographed on an FPLC/Superose 6 size-exclusion column under nondissociating conditions, several high-MW UV-absorbing components were separated (Fig. 2). For both, the bulk of this material (monitored at 280 or 214 nm) eluted in the range of about 10–40 kDa. Nevertheless, the elution profiles revealed several other olive oil components with higher molecular masses. Comparing the electrophoresis and FPLC results with those of protein determination, we can hypothesize that: (i) a heterogeneous population of low-MW protein molecules is present in olive oil extracts, and (ii) the small size of these polypeptides and/or their amino acid composition did not permit efficient staining, although the amount of protein loaded was not limited (13,18). Nevertheless, we cannot exclude the possibility that some of the above components, which are present in olive oil extracts and exhibit many of the properties typical of proteins and peptides, may be in fact nonprotein molecules (19).

The detection of protein molecules in virgin olive oils prompted us to search for contaminating oxidizing enzyme activities, which could affect the quality of the final food product during storage. As shown in Table 1, significant PPO and LOX catalytic activities were present in all virgin olive oil samples examined, which could not be correlated with the moisture content of the oil samples. It was interesting that filtration of oil samples resulted, in several cases, in up to threefold increase of both activities (not shown), suggesting that the filtration process removed some inhibitory components.

After enzyme activity determination in the FPLC fractions, LOX always eluted in the void volume of the column as a high-MW aggregate (Fig. 2), most probably representing a membrane-bound form of the enzyme (20). We obtained the same results when chromatographing extracts of green olives (Georgalaki, M.D., T.G. Sotiroudis, and A. Xenakis, unpublished results). On the other hand, PPO activity eluted as a component of ~10 kDa (Fig. 2), although catechol oxidase from olive fruits has a molecular mass of 42 kDa (21). This discrepancy may be explained by assuming that PPO activity present in olive oil extract is a result of an active proteolytic fragment of the native enzyme present in olive fruits.

To examine whether the catalytic activities detected are ex-

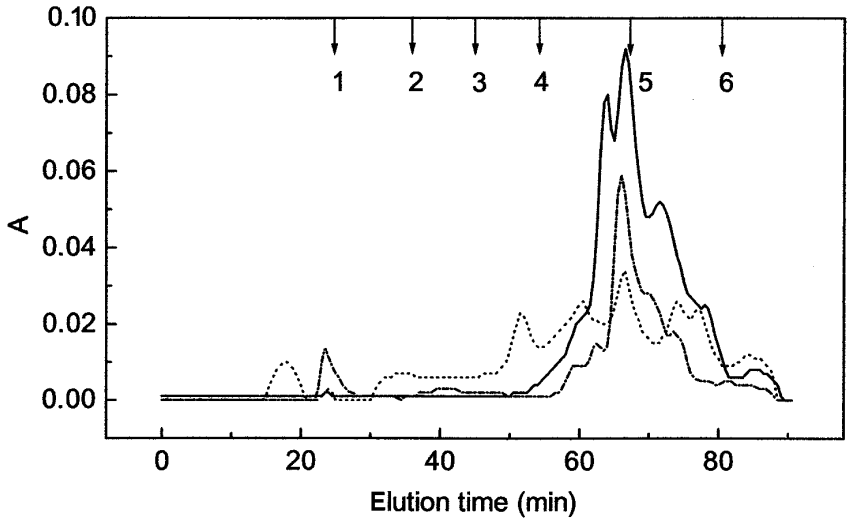

FIG. 2. Fast protein liquid chromatography size-exclusion chromatography of an olive oil extract and its ammonium sulfate-precipitated material on a Superose 6 HR 10/30 column; 240 μL of an olive oil extract (sample 7, Table 1) (—), (·–·–·–·) or of its ammonium sulfate-precipitated material (dissolved and dialyzed as described in the Materials and Methods section) (....) were chromatographed on a Superose 6 HR 10/30 column equilibrated with 50 mM Tris-HCl, 1 mM dithiothreitol, pH 7.5, at a flow rate of 0.3 mL/min. The elution profile was recorded at 280 (·–·–·–·) or 214 nm (—), (....). Arrows denote the elution time of molecular mass standards: (1) phosphorylase kinase (1300 kDa); (2) thyroglobulin (669 kDa); (3) β-amylase (200 kDa); (4) soybean lipoxygenase (94 kDa); (5) trypsin inhibitor (20.1 kDa); (6) cytochrome c (12.4 kDa).

TABLE 1
Moisture Content, PPO and LOX Specific Activities of Several Olive Oil Samples[a]

Sample number	Moisture (%)	PPO (units/mg protein)	LOX (units/mg protein)
1	0.16 ± 0.02	2.01 ± 0.34	0.21 ± 0.05
2	0.24 ± 0.03	1.39 ± 0.39	0.18 ± 0.01
3	0.20 ± 0.02	0.24 ± 0.15	0.11 ± 0.02
4	0.22 ± 0.03	0.84 ± 0.04	0.15 ± 0.02
5	0.22 ± 0.02	1.00 ± 0.34	0.18 ± 0.03
6	0.18 ± 0.01	0.55 ± 0.13	0.19 ± 0.07
7	0.31 ± 0.02	3.22 ±1.33	0.83 ± 0.10
8	0.21 ± 0.02	1.54 ± 0.49	0.85 ± 0.12
9	0.23 ± 0.01	0.45 ± 0.18	0.90 ± 0.06
10	0.24 ± 0.01	0.60 ± 0.25	0.85 ± 0.28
11	0.28 ± 0.04	1.08 ± 0.07	0.95 ± 0.24
12	0.23 ± 0.03	0.71 ± 0.06	0.77 ± 0.02
13	0.22 ± 0.01	5.08 ± 0.68	1.08 ± 0.17
14	0.11 ± 0.01	3.89 ± 0.62	1.68 ± 0.21
15	0.17 ± 0.01	0.54 ± 0.29	0.79 ± 0.06
16	0.09 ± 0.01	1.55 ± 0.15	0.57 ± 0.07
17	0.19 ± 0.01	1.25 ± 0.38	0.92 ± 0.29
18	0.08 ± 0.01	1.38 ± 0.08	0.44 ± 0.03

[a]For determination of enzyme-specific activities, the oil samples were filtered. Values are the means ± SD (n = 3). PPO, polyphenoloxidase; LOX, lipoxygenase.

pressed by protein molecules, we tested the stability of these activities against protease and heat treatment. Preincubation of olive oil extracts with papain, a protease used for the complete endopeptidic cleavage of proteins, or heat treatment of the extract at 100°C (Fig. 3) drastically reduced both catalytic actions. Concerning heat inactivation, LOX loses 50% of its activity in about 4 min and PPO in 9 min. It has been reported that soybean LOX-I has a half-life of survival of 25 min at 69°C, and LOX-II is at least 36 times less stable (22), whereas PPO loses half of its activity in 19 min at 75°C (21). Obviously, these results strongly suggest that both PPO and LOX activities are catalyzed by heat-sensitive polypeptide molecules.

To further investigate the catalytic characteristics of olive oil PPO and LOX, we determined: (i) the progresss curves of enzyme reactions (Fig. 3), (ii) the dependence of reaction velocities on enzyme concentrations (Fig. 4), and (iii) the substrate-dependence (Fig. 5) of PPO and LOX.

Both PPO and LOX showed acidic pH optima with a drastic decrease of the activity at alkaline pH values (not shown), in accordance with previous results concerning the activities of olive fruits (21,23). The LOX activity likely corresponds to the LOX-2 isozyme, which has an acidic pH optimum (24,25); however, differentiation of isozymes based primarily on pH optima can be misleading. Instead, it has been suggested to classify plant lipoxygenases into two categories, based upon structural features of the proteins rather than enzymatic characteristics (24,25). As shown in Figure 3, the progress curves of PPO and LOX differed markedly. Thus, although the progress curve of PPO was linear during the early stage of the reaction, that of LOX showed a rapid decrease of velocity as the reaction proceeded. The autocatalytic destruction of LOX during oxygenation of the fatty acid substrate (26) may be a cause that contributes to this deviation from linearity.

Under initial velocity assay conditions, an increase of the amount of olive oil extract produced a linear increase in the velocity of PPO (Fig. 4). However, this was not true for LOX. If the amount of extract was increased, there was a falling off from the linear relationship (Fig. 4). This effect could be explained by assuming that the olive oil extract contains a reversible inhibitor of LOX.

When initial velocity is plotted against substrate concentration, a Michaelis-Menten hyperbolic curve is obtained for

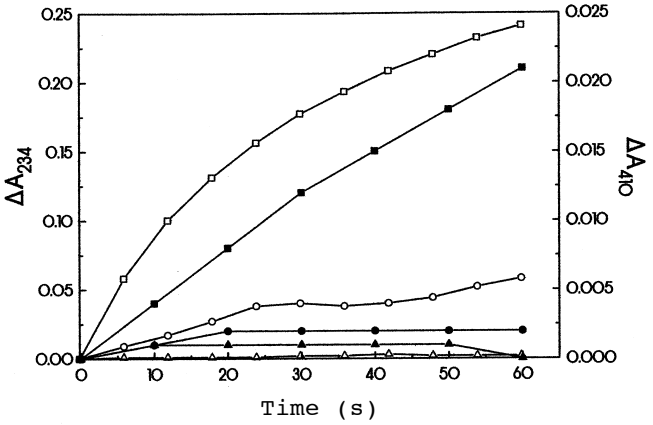

FIG. 3. Kinetics of polyphenol oxidase (PPO) and lipoxygenase (LOX) in olive oil extract. Inactivation of the enzyme activities by heat treatment and papain digestion. PPO (■, ●, ▲) and LOX (□, ○, △) activities were assayed with 100 or 20 µL of an olive oil extract (sample 7, Table 1), respectively, as described in the Materials and Methods section, before (■, □) and after heat treatment (●, ○) or papain digestion (▲, △). Heat treatment of the olive oil extract was performed at 100°C for 30 min. The extract was treated with papain (50 µg/mL) for 60 min at 25°C. The data represent means ± SD from three determinations. ΔA, change in absorbance.

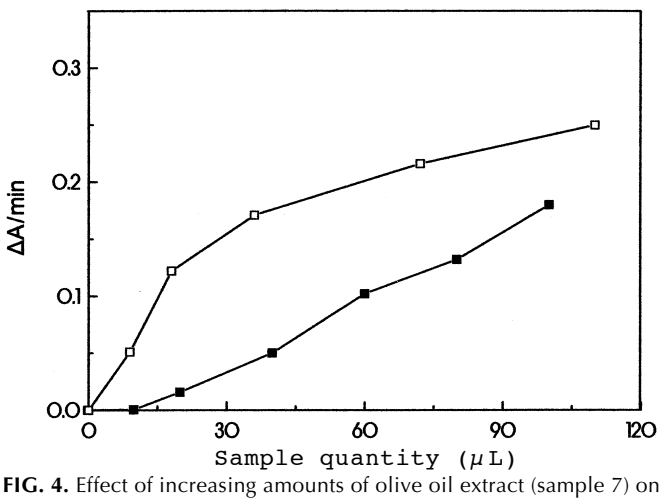

FIG. 4. Effect of increasing amounts of olive oil extract (sample 7) on the initial velocity of PPO (ΔA_{410}/min) (■) and LOX (ΔA_{234}/min) (□) activities. PPO was assayed with 143 mM 4-methylcatechol. Other conditions are as described in the Materials and Methods section. The data represent means ± SD from three determinations. See Figure 3 for other abbreviations.

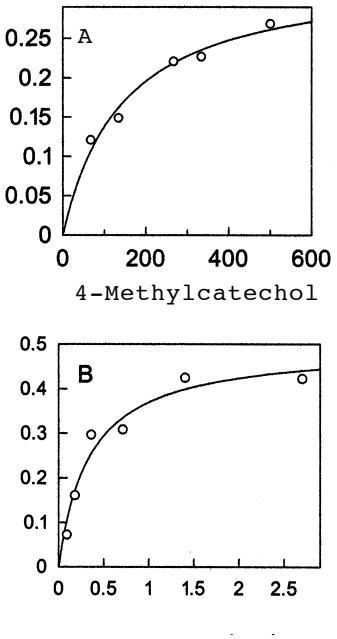

FIG. 5. Effect of substrate concentration on PPO (A) and LOX (B) activities. The assays were performed with 100 µL of an olive oil extract of sample 7 (PPO) or sample 5 (LOX) as described in the Materials and Methods section. The data represent means ± SD from three determinations. See Figure 3 for other abbreviations.

both LOX and PPO reactions (Fig. 5). The K_m value of LOX for linoleic acid was 0.5 ± 0.1 mM (mean ± SD, $n = 3$), which is in the range of values reported for other plant lipoxygenases (27). However, the Michaelis constant of PPO for the substrate 4-methylcatechol was 152 ± 34 mM (mean ± SD, $n = 3$), which is about 40-fold higher than that of catechol oxidase partially purified from green olives (21). This apparent increase of K_m is probably due to the presence of polyphenols in the olive oil enzyme extract (3), which could competitively inhibit the oxidation of the externally added substrate of PPO.

In general, the presence of oxidizing enzyme activities in virgin olive oil may influence its oxidative stability during storage, thus affecting the quality of the oil.

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