The Role of Endogenous Amphiphiles on the Stability of Virgin Olive Oil-in-Water Emulsions

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ABSTRACT: Stable virgin olive oil-in-water emulsions were prepared using total endogenous surface-active components derived from oil as emulsifying agents, and the interfacial properties of the emulsion droplets were examined. The amount of oil extracted into the aqueous buffer increased with buffer pH, with the most stable emulsions being formed at pH 7.5. Light microscopy of the emulsions revealed the presence of spherical droplets with diameters ranging from 1.5 to 3 μm. Their surface was negatively charged at pH 7.5, as confirmed by the effect of ions and polycations. Potassium chloride, Ca^{2+}, and spermine induced rapid aggregation (as monitored by the turbidity change and by light microscopy), showing their maximal effect at 1 M, 4 mM, and 60 μM, respectively. Papain treatment of the emulsion particles rapidly induced particle aggregation, suggesting the destruction of stabilizing structural olive oil proteins. Unlike papain, treatment with phospholipase C did not result in an appreciable turbidity change. Treatment with soybean lipoxygenase slightly increased the turbidity of the emulsion. The interaction of linoleate–Tween 20 mixed micelles with emulsion droplets produced turbidity, which was maximal at a neutral pH, whereas interaction with proteolyzed and lipoxygenase-treated droplets induced both a significant increase in turbidity and a red shift to a different absorption maximum of the system as compared with those of the untreated emulsion.

Paper no. J11056 in *JAOCS 82,* 415–420 (June 2005).

KEY WORDS: Amphiphiles, interfacial properties, lipoxygenase, oil-in-water emulsion, proteolysis, virgin olive oil.

Virgin olive oil is one of the oldest known vegetable oils and is extracted from the fruit of the olive tree *Olea europaea*. It is unique among vegetable oils in that it is consumed without refining (1); consequently, olive oil contains a variety of minor components that may play a role in its health benefits (2). Olive oil extraction from the olive paste involves paste malaxation and centrifugation, a widespread continuous system (mostly two-phase or three-phase procedures—in the latter, water is added) that may give rise to the formation of oil/water emulsions. Eighty percent of the oil drops in the olive paste have a diameter greater than 30 μm after the malaxation step (1,3). On the other hand, olive oil itself can be considered a fine emulsion of generally a small quantity of polar water phase in a large quantity of lipophilic phase, i.e., glycerides (4). Both ionic and nonionic amphiphilic components transferred from the olives into olive oil can be responsible for the formation of emulsions at various stages of oil production. Part of these components may have originated from olive "oil bodies." Oil bodies found in the olive seed endosperm and embryo tissues have diameters in the range of 0.5–2.0 μm and a protein content of about 10% (w/w), whereas oil bodies present in the fruit mesocarp are large (single droplets of about 30 μm in diameter in mature fruits) with an insignificant protein content (5,6). Endogenous emulsifiers in virgin olive oil include FFA and a variety of minor constituents such as partial glycerides, polyphenols, and phospholipids (1).

Recent studies from this laboratory have shown the presence of proteins and enzymes as usual components in virgin olive oils (7,8). The protein content of various olive oils was further studied by a separate research group (9,10). Since proteins are one of the most important natural food emulsifiers (11), the role of olive oil proteins in stabilizing olive oil emulsions must be critical.

In the present paper we investigate the role of endogenous amphiphiles of virgin olive oil on the stability of olive oil-in-water emulsions. In this respect, we have probed the charge properties on the surface of the emulsion droplets by examining the effect of external ions, the proteolysis of interfacial proteins, and the oxidation of PUFA by lipoxygenase.

EXPERIMENTAL PROCEDURES

Fresh virgin olive oil samples were a gift of Elais S.A.–Unilever (Piraeus, Greece). Lipoxygenase (lipoxidase type I-B), papain (crystallized, lyophilized), phospholipase (type I), spermine tetrahydrochloride, calcium chloride dihydrate, linoleic acid, and polyoxyethylene sorbitan monolaurate (Tween 20) were obtained from Sigma-Aldrich Co. (Diesenhoven, Germany). One unit of lipoxygenase activity was defined as an increase in absorbance at 234 nm (A_{234}) of 0.001 per min at pH 9.0 at 25°C.

The qualitative characteristics of specific olive oil samples used throughout this work were determined by ELAIS S.A.–Unilever using standard methodologies. These were as follows: acidity, 0.45% (% oleic acid); K_{232}, 1.68; K_{270}, 0.09; oxidative stability (Rancimat, 120°C), 6.63 h; peroxide number, 4.3 mequiv O_2/kg. The protein content of the emulsion obtained from the same olive oil sample at pH 7.5 was estimated, as described previously (7), to be 0.3 mg/mL of extract when determined by the method of Lowry *et al*. (12) and 0.05 mg/mL of extract by the method of Bradford (13). This difference

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suggests the presence of reducing phenolic components in the emulsion. Phospholipids were extracted from the above emulsion as described previously (8) and were further determined using a fluorometric microdetermination assay using PC as the standard (14); a concentration of phospholipids of about 70 µg/mL of extract was estimated.

Olive oil samples were filtered through a large, coarse fluted paper and mixed with equal volumes of 50 mM Tris-HCl buffer, pH 7.5, unless otherwise stated. The mixture was vortexed for 2 min at maximum speed, with a Vortex-Genie 2 mixer from Scientific Industries Inc. (New York, NY) and was further centrifuged with an Eppendorf 5410 centrifuge (Hamburg, Germany) for 3 min at 12,000 × *g*. The lower aqueous phase, which was separated by a thin cream layer from the oil phase, was carefully removed using a syringe.

Turbidity and absorption measurements were carried out using a Cary 1E UV-vis spectrophotometer from Varian Inc. (Harbor City, CA) with a thermostated cell holder at 25°C. The stability of the emulsions was estimated by relative turbidity measurements (15). Difference spectroscopy studies of the interaction of olive oil emulsions with linoleic acid–Tween 20 mixed micelles were performed by adding 0.02 mL of the emulsion, prepared as described above, to 0.7 mL of a final micellar solution, and their absorption spectra were recorded against a control micellar solution. For enzyme treatment, the emulsion was first mixed with the appropriate enzyme stock solution, incubated for 5 min at 25°C, and then transferred to the mixed-micellar system. A stock solution of linoleic acid was prepared in deionized water in the presence of Tween 20 (16). The final mixed-micellar mixture contained 0.4 mM linoleic acid and 0.013% (vol/vol) Tween 20 in 50 mM phosphate buffer (pH 6.2) or other buffer as indicated. When necessary, thermal inactivation of olive oil emulsions was performed at 100°C for 30 min.

Capillary electrophoresis was carried out at 25°C using a Hewlett-Packard ^{3D}CE system (Hewlett-Packard, Waldbronn, Germany). Instrument control, data acquisition, and analyses were achieved using Hewlett-Packard ChemStation software. A 50 µm × 80.5 cm fused-silica capillary column (G1600-62211; Hewlett-Packard) was used. Detection was performed using diode array detection at 280 nm. Samples were separated using a 50 mM tetraborate buffer, pH 9.5, following injection for 10 s at 50 mbar. Before electrophoresis, samples were filtered through Minisart filters (0.2 µm) (Sartorius Ltd., Epsom, Surrey, United Kingdom).

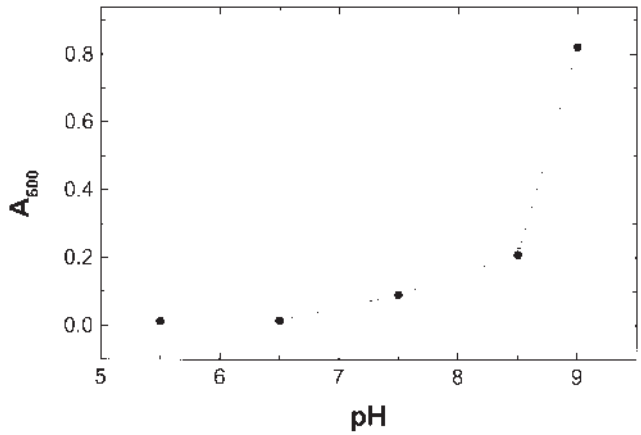

FIG. 1. Dependence on pH of olive oil emulsion formation, as measured by turbidity at 600 nm. The following buffers (50 mM) were used: sodium acetate, pH 5.5; sodium phosphate, pH 6.5 and 7.5; and Tris-HCl, pH 8.5 and 9.0. Other conditions are as described in the Experimental Procedures section. Error bars represent SD (n = 3).

The olive oil emulsions were observed with a Nikon Eclipse TE200 inverted microscope (Tokyo, Japan). A Sony CCD-Iris color video camera (Tokyo, Japan) was utilized for image acquisition. Image-Pro Plus (version 3) image analysis software (Media Cybernetics, Silver Spring, MD) was used to capture the images directly from the video camera. The size of emulsion droplets was estimated using an internal standard (8). The dispersed-phase volume fractions, ϕ, of the emulsions were determined by measuring their densities with an Anton Paar DMA 38 densimeter (Graz, Austria) and using the equation $\phi = (d_{\text{emulsion}} - d_{\text{continuous phase}})/(d_{\text{oil}} - d_{\text{continuous phase}})$ (17).

RESULTS AND DISCUSSION

Homogenization of virgin olive oil with equal volumes of various buffers of increasing pH, followed by centrifugal separation of the two phases, led to the formation of oil-in-water emulsions obtained as a turbid lower buffer phase. This phase

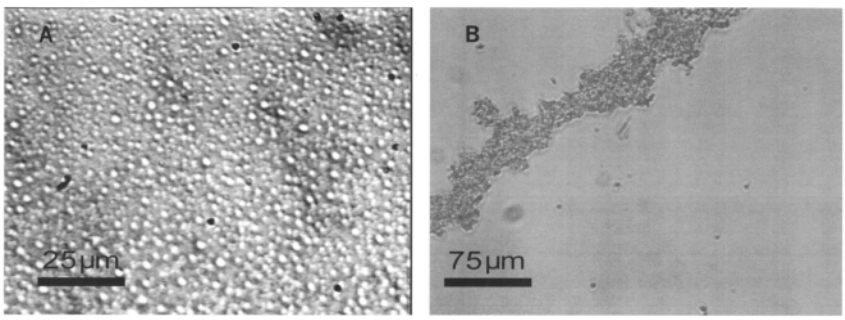

FIG. 2. (A) Light photomicrograph of an olive oil emulsion formed after extraction with 50 mM Tris-HCl (pH 9.0). (B) Light photomicrograph of an olive oil emulsion extracted with 50 mM Tris-HCl (pH 7.5) after papain treatment (0.05 mg/mL) for 10 min.

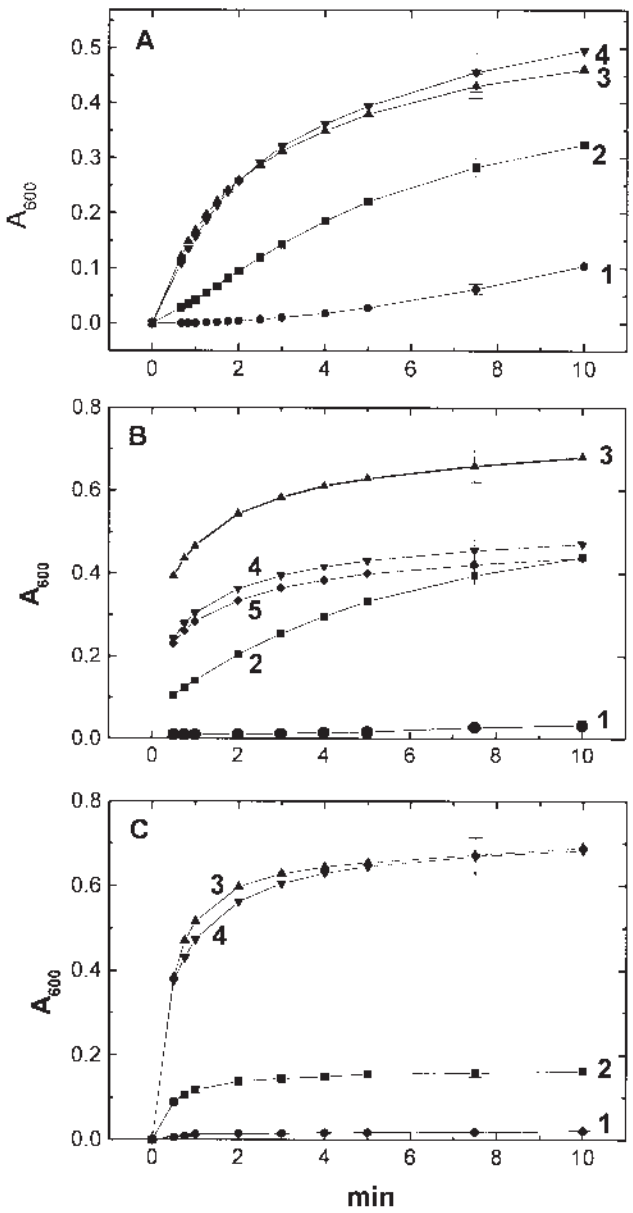

FIG. 3. Kinetics of turbidity development in olive oil emulsions after interaction with various concentrations of KCl (A), Ca^{2+} (B), and spermine (C). A 0.45-mL quantity of the emulsion was reacted with 0.45 mL of each ionic effector component to obtain a final solution at the desired effector and buffer (50 mM Tris-HCl, pH 7.5) concentrations. (A) Final KCl concentrations: 1, ●—●: 0.1 M; 2, ■—■: 0.4 M; 3, ▲—▲: 0.7 M; 4, ▼—▼: 1 M. (B) Final Ca^{2+} concentrations: 1, ●—●: 0.3 mM; 2, ■—■: 1 mM; 3, ▲—▲: 4 mM; 4, ▼—▼: 7 mM; 5, ◆—◆: 10 mM. (C) Final spermine concentrations: 1, ●—●: 0.017 mM; 2, ■—■: 0.03 mM; 3, ▲—▲: 0.06 mM; 4, ▼—▼: 0.1 mM. Error bars represent SD (n = 3).

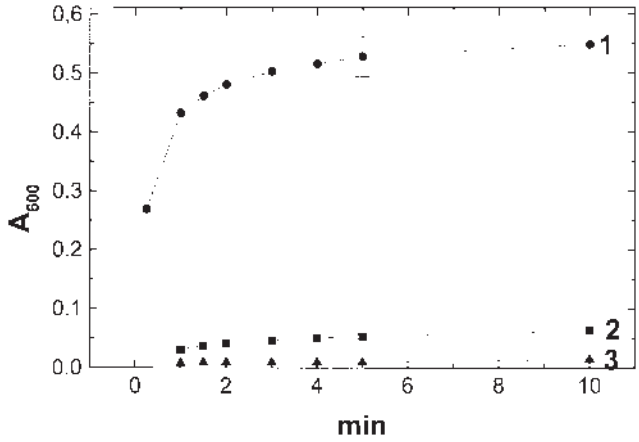

FIG. 4. Kinetics of turbidity development in olive oil emulsions after interaction with papain (0.05 mg/mL) (1; ●—●), lipoxygenase (5000 units/mL) (2; ■—■), and phospholipase C (0.05 mg/mL) (3; ▲—▲). One milliliter of emulsion was reacted with 0.01 mL of each enzyme stock solution. Error bars represent SD (n =3).

was separated from the upper oil phase by an intermediate cream layer, which became thicker with increasing pH. The turbidity of the lower emulsion phase was almost negligible at acidic pH values, but it showed a dramatic increase with increasing pH (Fig. 1). The oil content of the emulsion increased with increasing turbidity, showing dispersed-phase volume fractions ϕ of 0.05 ± 0.03 and 0.21 ± 0.05 (five determinations) at pH 7.5 and 9.0, respectively. This finding suggests that the ionization of acidic groups of endogenous amphiphilic molecules of olive oil, such as FFA and phospholipids, increases with increasing pH, thus permitting an increase in the total volume of emulsion droplets. Light microscopy of the emulsion phases, at both pH 7.5 and 9.0, as well as that of the cream layers, revealed the presence of emulsion droplets with diameters ranging from 1.5 to 3.0 μm, a number that became higher at alkaline pH and in the cream layer (Fig. 2A). For all the following experiments, we used the emulsions formed at pH 7.5 since they were more stable than those at pH 9. As revealed by light microscopy and relative turbidity measurements, the emulsions at pH 7.5 were stable for several days without the appearance of creaming, sedimentation, flocculation, or coalescence phenomena.

To probe the charge of the interface of extracted lipid bodies, we examined the effect of monovalent (K^+) (Fig. 3A) and divalent (Ca^{2+}) cations (Fig. 3B), and that of the polycationic tetramine spermine (Fig. 3C) on the turbidity of the system, as monitored at 600 nm. At the same time, the aggregation or coalescence produced was examined by light microscopy. All of the compounds tested induced an increase of turbidity and aggregation, as in the case of proteolysis (see following discussion, Fig. 2B), observed by light microscopy. In the range of concentrations tested, KCl-induced turbidity increased with increasing monovalent cation concentration, whereas maximal initial rates of turbidity formation for the divalent cations and polycations were observed at 4 mM and 60 μM, respectively. Because monovalent/divalent cations and polyamines act as counter ions to acidic amphiphiles, they are expected to decrease the surface charge density and interface potential greatly. The consequent reduction of the mutual electrostatic repulsion between emulsion droplets could induce them to aggregate. In the case of calcium and polyamine ion-induced aggregation, an ion-specific effect could be involved, since the degree of destabilization did not increase with the ionic strength.

The possible structural role of surface proteins and lipids in maintaining the integrity of the emulsion droplets was probed

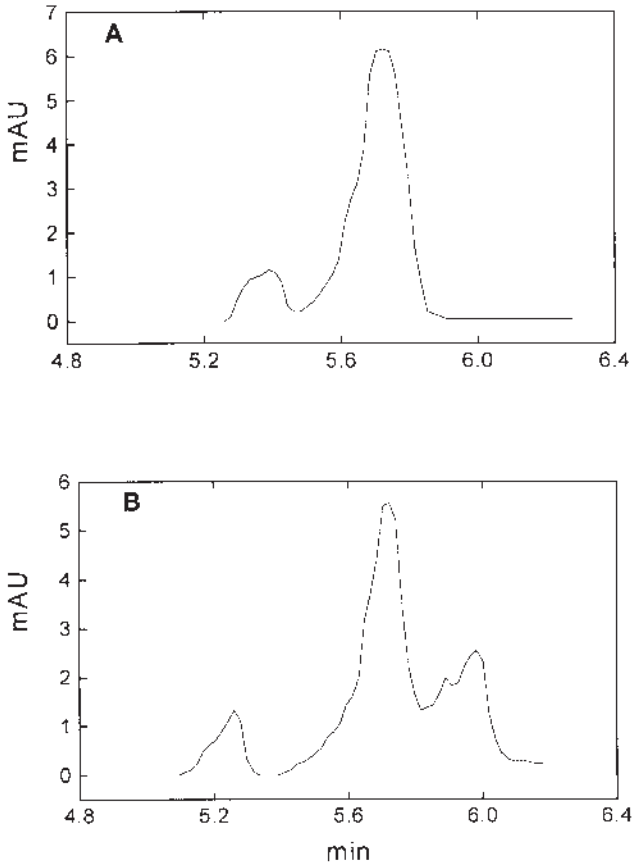

FIG. 5. Capillary electrophoresis of olive oil emulsions (A) before and (B) after treatment with papain (0.05 mg/mL) for 10 min as described in Figure 4.

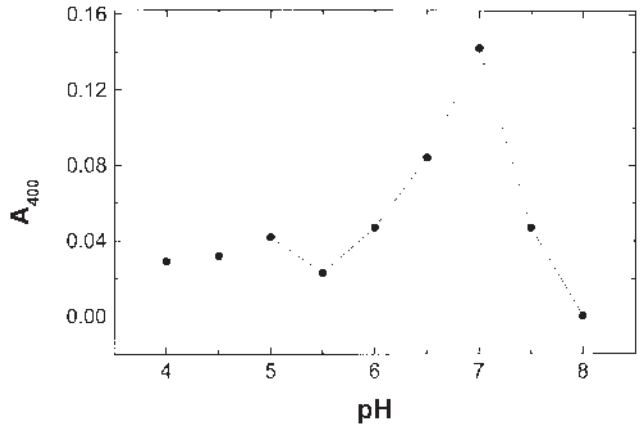

FIG. 6. Dependence on pH of turbidity formation, measured at 400 nm, during the interaction of mixed linoleic acid–Tween 20 micelles with heated olive oil emulsions. The following reaction buffers (50 mM) were used: sodium acetate from pH 4.0 to 5.5, sodium phosphate from pH 6.0 to 7.5, and Tris-HCl at pH 8.0. Error bars represent SD (n = 3).

using various enzyme treatments. With papain treatment, the lipid bodies aggregated rapidly (Fig. 4) without coalescence, as revealed by light microscopy (Fig. 2B), suggesting that the integrity of specific olive oil proteins is necessary for particle stability. Unlike the papain treatment, the phospholipase C treatment did not result in an appreciable change in the turbidity of lipid bodies, suggesting that phospholipids present on the surface of lipid droplets were not accessible to the enzyme because of a shielding effect of proteins or other olive oil amphiphiles (Fig. 4). Interestingly, soybean lipoxygenase treatment induced a slight increase in turbidity, suggesting that the production of unsaturated FA hydroperoxides destabilized the emulsion droplets (Fig. 4). The structural changes of protein-containing emulsion droplets induced by proteolysis were further verified by capillary electrophoresis of both unproteolyzed and papain-treated lipid droplets (Fig. 5). Electrophoresis revealed that a major peak absorbing at 280 nm, which appeared at 5.7 min, was split after proteolysis to three new peaks with lower mobility. These peaks most probably represent papain-modified polypeptides. Ten other peaks that eluted at higher elution times (up to 12 min) were unaffected (not shown).

It is known that cloudy olive oil produced in the form of an emulsion or dispersion contains material in suspension–dispersion, which, after storage of the oil for several months, can form a colored deposit containing protein in admixture with polymerized phenols (1). Moreover, it has been established that oxidized PUFA can form colored protein adducts (18). In this respect, we have examined whether the interaction of olive oil endogenous amphiphiles with linoleic acid, the major olive oil PUFA (1), can lead to the formation of colored products.

Our experimental system consisted of transparent stable linoleate–Tween 20 mixed micelles containing the FA olefinic chromophore, which can function as an internal structural probe component, and the extracted amphiphiles. The interaction of the mixed micelles with emulsion particles in the absence or in the presence of papain and lipoxygenase was examined by difference spectroscopy. In both cases, the emulsion particles were previously heated to inactivate the endogenous enzyme activities (7). As shown in Figures 6 and 7A, although the interaction with untreated particles induced only a small increase in turbidity, which was maximal at pH 7.0 (Fig. 6), the presence of the proteolytic enzyme induced both a significant increase in turbidity and a large red shift of the UV difference absorption maximum of the system, reaching 285 nm after 4 min of reaction (the high turbidity values produced at higher reaction times did not permit spectral characterization of the system) (Fig. 7B). The maximum in turbidity observed at pH 7.0 with unproteolyzed droplets can be explained by assuming that at neutral pH, the Tween 20–linoleate mixed micelles were reorganized in the presence of olive oil amphiphiles to form turbid—larger—structures, whereas at alkaline conditions, linoleate was released in solution from the micelles; thus, the uncharged Tween 20 micelles could not interact with the amphiphiles. On the other hand, the spectral shift and the increased turbidity formation observed with the proteolyzed emulsion droplets is consistent with an increase in the polarity of the environment of the chromophores of the olefinic bonds (19) during a reorganization of mixed micelles in the presence of the new polypeptide components produced during proteolysis.

When the mixed-micellar system containing the olive oil emulsion was treated with lipoxygenase (Fig. 7C), a significant increase in turbidity was also observed. In addition, after a 4-min

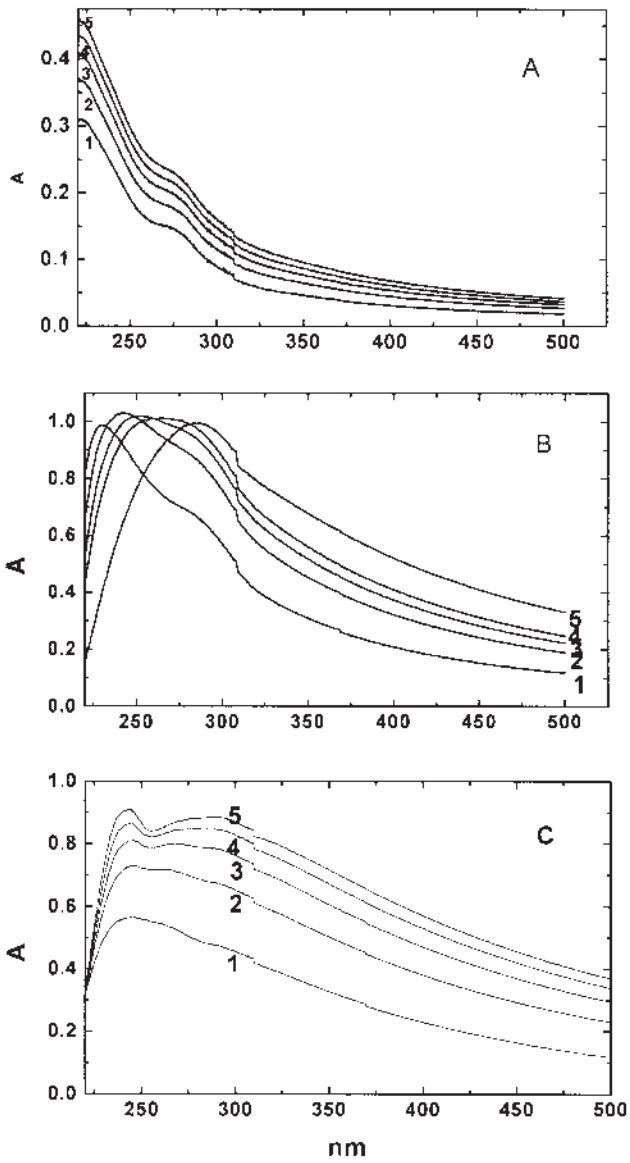

FIG. 7. Kinetics of the appearance of difference spectra after interaction of mixed linoleic acid–Tween 20 micelles, in 50 mM phosphate buffer, pH 6.2, with heated olive oil emulsions (A) in the absence or (B) in the presence of papain (0.05 mg/mL) or (C) lipoxygenase (800 units/mL). Spectra were recorded at 30 s (1); 1 min (2); 2 min (3); 3 min (4); and 4 min (5). Other conditions are as described in the Experimental Procedures section.

reaction period two different absorption maxima were observed, one at about 243 nm and the other at about 285 nm. In contrast, the oxidation of the mixed-micellar system in the absence of the olive oil emulsion was accompanied by the appearance of a difference absorption maximum at 235 nm that increased with reaction time without any significant increase in turbidity (not shown). In the first case, the absorption maximum at 243 nm was due to the production of the conjugated FA chromophore, and it was red-shifted (from 235 nm) because of the reorganization of the micellar system. On the other hand, the absorbance maximum at 285 nm may have been due to the formation of oxo-derivatives of linoleic acid (20). It is also possible that lipid hydroperoxides produced in the system by lipoxygenase oxidized polyphenolic components of the olive oil emulsion during a lipoxygenase-catalyzed reaction, producing secondary products such as polyphenol polymers (21) or polyphenol–PUFA–protein adducts absorbing at 285 nm (18,21). Thus, the oxidation of linoleic acid and polyphenolic components may affect the structural organization of the micellar/emulsion system, thereby leading to an increase in turbidity.

Accordingly, one can conclude that the interfacial properties of post-pressing emulsions prepared using virgin olive oils are drastically affected by external ionic and/or enzymatic modifications and can destabilize the dispersed emulsion droplets. This effect, in turn, may influence the quality characteristics of the oil (3).

Taken together, the results described here show that amphiphilic components of virgin olive oil, including polypeptide molecules, stabilize oil-in-water emulsion droplets, which are formed when the oil is extracted with a pH 7.5 aqueous buffer. Both surface charges and the integrity of surface polypeptide components are critical factors for maintaining emulsion droplets as stable individual entities, since the addition of potassium chloride, Ca^{2+}, and spermine cations or papain treatment induce aggregation of the emulsion droplets. Moreover, interaction of the emulsion with linoleate micelles in the presence of papain or lipoxygenase highly destabilizes the system, producing high turbidity and new chromophores absorbing at higher absorption wavelengths. These phenomena may play a critical role in the formation of post-pressing emulsions or dispersions in freshly processed virgin olive oil.

ACKNOWLEDGMENTS

We thank ELAIS S.A.–Unilever (Piraeus, Greece) for the generous gift of olive oil samples and their chemical determinations. We also thank Dr. M. Georgalaki for carrying out the capillary electrophoresis experiments.

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[Received February 9, 2005; accepted May 14, 2005]