Olive Oil Microemulsions as a Biomimetic Medium for Enzymatic Studies: Oxidation of Oleuropein

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ABSTRACT: Microemulsions consisting of olive oil as the nonpolar solvent, lecithin as surfactant, 1-propanol as cosurfactant, and water were prepared. The choice of the compositions of the microemulsions used was based on the pseudo-ternary phase diagrams of the system determined at 30°C, for different weight ratios of lecithin/olive oil. Lecithin solubilization and water incorporation in these microemulsion systems was limited. Tyrosinase, an oxidizing enzyme present in olives, was successfully incorporated in the water core of these microemulsions. Enzymatic oxidation of oleuropein, the most abundant olive phenolic compound, in the restricted aqueous environment of olive oil microemulsions was studied. Formation of oleuropein oxidation products was followed spectrophotometrically at 30°C for several minutes. An absorption maximum was observed at 415 nm. When the enzymatic reaction was considered at different tyrosinase and oleuropein concentrations, a rapid inactivation of the enzyme was observed. Addition of L-proline as a coupling reactor did not succeed in preventing enzyme inactivation in the microemulsions, probably owing to substrate localization and product accumulation around the entrapped enzyme molecules in the micellar interface.

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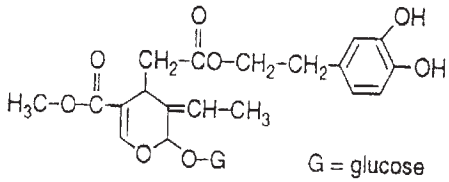
KEY WORDS: Lecithin, oleuropein, olive oil microemulsions, tyrosinase.

The health benefits of olive oil as a nutrient are universally recognized. Recent investigations suggest that phenolic antioxidant compounds found in virgin olive oil possess specific biological effects that can contribute to the prevention of cardiovascular and thrombotic diseases and reduce the risk of common cancers (1,2).

Phenolic compounds in the fruits of the olive plant are important factors to consider in evaluating virgin olive oil quality because they are partly responsible for its auto-oxidation stability and organoleptic characteristics (3). These phenolic compounds are either present in the olive fruit or produced during the oil manufacturing process (4). Among the minor phenolic components of olive fruits, oleuropein, a bitter-tasting secoiridoid glucoside (Scheme 1), is the most abundant (5). Oleuropein can greatly influence the final product properties through its enzymatic and/or chemical oxidation.

Recently, the presence of oxidizing enzyme activities, such as lipoxygenase and polyphenol oxidase, in Greek virgin olive oil samples has been reported (6).



SCHEME 1

Microemulsions are optically transparent and thermodynamically stable systems containing at least three components: water, a nonpolar solvent, and a surfactant (7). Reverse micelles or water-in-oil microemulsions are useful as microreactors for enzymatic reactions in low-water-content media (8). The catalytic reaction takes place in these systems because of the large interfacial area between water and the continuous nonpolar solvent. The aqueous interior of the reverse micelles hosts the water-soluble enzymes, and substrates can be solubilized either in the aqueous phase or in the oil external continuous phase. When amphiphilic substances are considered, solubilization can be achieved at the interface, which consists of a monolayer of oriented surfactant molecules.

Four-component microemulsions consisting of olive oil (extra virgin or refined), lecithin as surfactant, 1-propanol as cosurfactant, and water were prepared. Lecithin reverse micelles can only be obtained in the presence of cosurfactants such as alcohols with short aliphatic chains (9).

Tyrosinase (polyphenol oxidase, EC 1.14.18.1) is a widespread copper-containing enzyme in microorganisms, plants, and animals. It catalyzes the hydroxylation of monophenols to *o*-diphenols and the oxidation of *o*-diphenols to *o*-quinones in the presence of molecular oxygen. The latter products are unstable in aqueous solutions and undergo further nonenzymatic processes to form melanin-like stable compounds (10). As reported in the literature, polymerization of *o*-quinones is inhibited when the foregoing reactions are carried out in systems with low water content (11). For this reason, several systems of organic solvents with low water content containing tyrosinase immobilized on a solid support were developed for biotechnological applications. Among other applications, reverse micelles have been considered as effective microreactors for tyrosinase catalysis (12–16).

The objectives of the present study were to develop simple innovative microemulsions using biocompatible materials such

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as lecithin and olive oil and to study enzymatic activities when reactants were entrapped in such reverse micelles. These systems can be used as biomimetic media in studying enzymatic reactions that naturally occur in olive oil and that are of great importance for its final quality and properties. Results concerning the oxidation of oleuropein, a phenolic compound of virgin olive oil by tyrosinase, an enzyme activity that has been identified in the same medium, are presented.

EXPERIMENTAL PROCEDURES

Materials. Soybean lecithin (Epicuron 200) containing 96% PC was supplied by Lucas Meyer (Hamburg, Germany). Extra virgin olive oil and refined olive oil samples were generously donated by Elais S.A.-Unilever, Greece. Oleuropein was from Extrasynthèse (Genay, France). L-Proline was from BDH Biochemicals (Poole, United Kingdom). Mushroom tyrosinase was purchased from Sigma (2,590 units/mg solid) and used without further purification. L-Propanol was from Merck (Darmstadt, Germany). High-purity water was obtained by a Millipore Milli Q Plus water purification system.

Pseudo-ternary phase diagrams. A four-component system can be described on pseudo-ternary phase diagrams. These diagrams were constructed as follows. The oil phase (extra virgin or refined olive oil) was mixed with the surfactant (lecithin) at a constant weight ratio. 1-Propanol (cosurfactant) was added. The mixture was then titrated with water until it turned turbid. The volume of water added was recorded. The procedure was repeated several times at different alcohol concentrations. Temperature was kept constant at 30°C. The pseudo-ternary phase diagram was constructed by plotting the amounts of water (including the traces of water present in the olive oil and lecithin, as determined by Karl Fischer titrations), cosurfactant, and oil/surfactant used in the experiment. The microemulsion region (transparent solution) was identified as shown in Figure 1.

Preparation of water-in-oil microemulsions. A typical lecithin-based water-in-olive oil microemulsion was prepared as follows. A stock solution of 4% w/w lecithin in extra virgin olive oil was prepared and stored in the dark. Microemulsions were formed with the addition of 10% w/w 1-propanol and water up to 2% w/w at 30°C.

Enzymatic oxidation in aqueous solution. Enzymatic oxidation of oleuropein by mushroom tyrosinase in aqueous solution was initiated by adding a few milliliters of a concentrated enzyme solution (tyrosinase stock solution: 10 mg/mL in a mixture of 50% glycerol and 50% 0.1 M phosphate buffer solution, pH 7) in a final volume of 1.5 mL of 0.1 M phosphate buffer, pH 7, containing 0.2 mM oleuropein.

Enzymatic oxidation in water-in-oil microemulsions. Enzymatic oxidation of oleuropein by mushroom tyrosinase was started by adding a few milliliters of a concentrated enzyme solution in a lecithin/olive oil/1-propanol system containing 0.2–3 mM oleuropein. The total water content of the system was adjusted by the addition of the appropriate amount of a buffered solution (0.1 M phosphate buffer, pH 7). Final concentrations of tyrosinase and oleuropein in the microemulsions were expressed per total water volume of the system. This mode of concentration expression is referred to as local (water pool) concentration and is valid in the case of hydrophilic substances as in the present study (7). Total water content was kept constant at 1% w/w. The final volume of the reaction system was 1.1 mL.

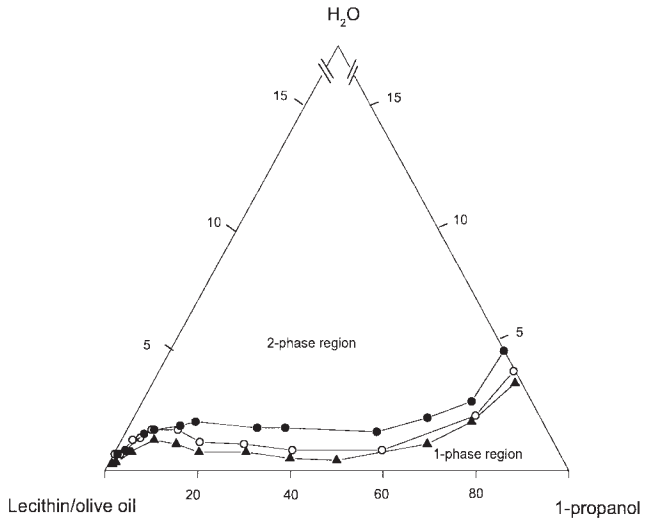


FIG. 1. Pseudo-ternary phase diagram of lecithin/olive oil/1-propanol/water system at 30°C; compositions are in weight ratios. The phase separation lines correspond to the initially fixed concentrations of lecithin in olive oil: (▲) lecithin/extra virgin olive oil 2% w/w, (○) lecithin/extra virgin olive oil 4% w/w, and (●) lecithin/refined olive oil 5.5% w/w.

Oleuropein oxidation by mushroom tyrosinase was monitored by means of successive absorption spectra for identification of absorbing species. Absorption spectra were recorded in a UV-vis Hitachi U-200 Double-beam Spectrophotometer. Temperature was controlled at 30°C using a Julabo UC-F10 circulating bath. Reference cuvettes contained all the components except the enzyme.

RESULTS AND DISCUSSION

Pseudo-ternary phase diagrams. Phase diagrams of the four-component system, lecithin/olive oil/alcohol/water, were constructed to determine the extent of the monophasic area corresponding to water-in-oil microemulsions. For simplicity and because the oil/lecithin ratio remained constant, a pseudo-ternary phase diagram was used assuming a three-component system (alcohol, water, and the mixture of oil/lecithin). As shown in Figure 1, the area of the microemulsion zone increased by increasing the lecithin concentration. Virgin olive oil microemulsions with 4% w/w lecithin at 30°C led to a more extended monophasic area than the similar system with 2% w/w lecithin. When refined olive oil was used, the solubility of lecithin increased to 5.5% w/w, allowing a further increase of the microemulsion boundaries. 1-Propanol was added in the system as a cosurfactant to reduce the interfacial tension between the oil and the aqueous phase. Maximum water incorporation in the reverse micelles was achieved at 10% w/w alcohol content. At very high alcohol

concentrations, larger quantities of water could be incorporated in the system but the reverse micellar structure is uncertain.

These results are in good agreement with previous studies (17) in which phase diagrams of the quaternary system lecithin/isooctane/alcohol/water were built. The effect of the lecithin concentration, the water added, and the nature and quantity of the alcohol used on the extent and the stability of the microemulsion region was tested. A higher water solubilization was achieved when 1-propanol was used as a cosurfactant, probably owing to its partitioning within the different microphases of the microemulsions.

Apart from isooctane, lecithin-based reverse micelles of high viscosity, also called organogels, were formed with either isopropyl palmitate or isopropyl myristate as organic solvents (18). Various spectroscopic methods were applied to investigate the structure of these systems.

In the present study, olive oil was considered as the nonpolar solvent for the formulation of lecithin-based microemulsions. It was observed that lecithin was less soluble in olive oil than in isooctane, isopropyl palmitate, or isopropyl myristate, and as a consequence the extent of the monophasic area was more limited. Nevertheless, olive oil is a natural, nontoxic, biocompatible, and inexpensive oil that can be successfully used as a constituent for the construction of various microemulsions.

The observed differences in water and lecithin solubility in the microemulsions obtained with virgin and refined olive oil are probably due to the various minor components present only in the virgin olive oil. Some of these components, such as phospholipids, phenols, and proteins (3,6), have amphiphilic properties and interfere with lecithin in the reverse micelle formation.

Oleuropein oxidation by tyrosinase. The enzymatic activity of tyrosinase was studied both in aqueous media and in water-in virgin olive oil microemulsions. Formation of oleuropein oxidation products was followed spectrophotometrically at 30°C for several minutes in both media. As can be seen from Figure 2, an absorption maximum was observed at 415 nm when oxidation was considered in water-in-olive oil microemulsions. The same reaction in aqueous solution resulted in an absorption maximum at 390 nm. The red shift observed in the microemulsion is known to be due to the polarity differences of the solvents. The appearance of a second peak at 450 nm in the microemulsion is attributed to additional oxidized product(s) of oleuropein.

Previous works have shown that tyrosinase can be solubilized in the aqueous core of various reverse micellar systems and retain catalytic activity. In most cases, the synthetic dioctyl sulfosuccinate sodium salt (AOT) was used as surfactant and either isooctane or cyclohexane or octane as the nonpolar solvent. In an early study (12), commercial tyrosinase was incorporated in reverse micelles of AOT in isooctane. Enzyme activity was not much dependent on the water content of the system. The apparent kinetic constants for the oxidation of 4-methylcatechol were comparable to those observed in aqueous solutions. Recently, Rodakiewicz-Nowak *et al.* (14) investigated the oxidation of 4-*t*-butylcatechol by tyrosinase in

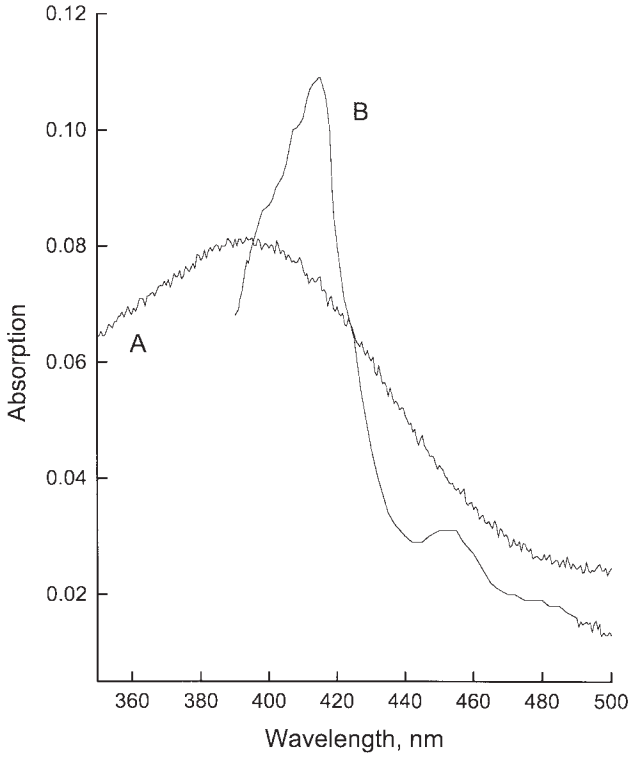


FIG. 2. Absorption spectra for the oxidation of oleuropein by mushroom tyrosinase in aqueous solution (A) and in lecithin/extra virgin olive oil microemulsions (B) at 30° C. Experimental conditions were: (A) oleuropein 0.2 mM, tyrosinase 12 µg/mL, 0.1 M phosphate buffer, pH 7 (aqueous solution), and (B) oleuropein 0.2 mM, tyrosinase 12 µg/mL (local concentrations), lecithin/extra virgin olive oil 4% w/w, 1-propanol 10% vol/vol, aqueous phase 1% vol/vol, 0.1 M phosphate buffer, pH 7 (microemulsions).

AOT/isooctane reverse micelles and showed that the surfactant concentration and water content of the system affected the enzyme activity. Tyrosinase was active in highly concentrated AOT microemulsions even at a low water content (2.1% vol/vol). These effects were discussed in terms of 4-*t*-butylcatechol partitioning between the water pool, interfacial layer, and isooctane phase. The oxidation reaction was assumed to proceed effectively in the microemulsion water pools only.

In another study, mushroom polyphenol oxidase was found to catalyze the oxidation of several phenols and catechols in AOT/cyclohexane reverse micelles (13). Catalytic efficiency of the enzyme was also related to substrate partitioning between the different microdomains of the system.

In a more recent work (15), tyrosinase was entrapped in AOT/octane reverse micelles. Optimal catalytic activity was observed both at high water content, when the volume of the aqueous core of the reverse micelles was comparable to the size of a tetrameric tyrosinase, a more stable enzyme form, and at low water content, where a monomeric form was present.

In the present study, lecithin-based water-in-olive oil microemulsions were used for the entrapment of tyrosinase. As mentioned above, water incorporation in such a system is quite limited, and as a consequence the size of the reverse micelles of the system is quite small. The volume of the aqueous cores

corresponds to the molecular volume of the monomeric form of tyrosinase (15). Although the monomer is considered to be rather unstable in aqueous media, it retained its catalytic activity in the olive oil microemulsions studied here.

When water-in-oil microemulsions are considered as a medium for enzymatic catalysis, substrate polarity is of great importance since it affects solubility, partitioning, and diffusion of the substrate to the entrapped enzyme molecules.

It is known that oleuropein possesses a lipophilic character that leads it to be anchored within biomembranes (19). Accordingly, in the present system oleuropein is expected to partition favorably in the reverse micellar lecithin monolayer.

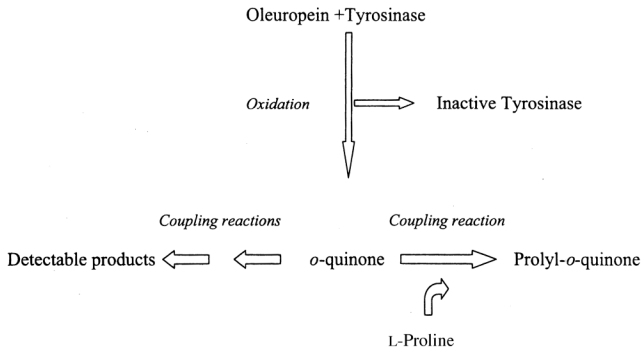
The kinetic behavior of tyrosinase is very complex because the enzymatic oxidation of oleuropein to the corresponding *o*-quinones occurs simultaneously with the coupled nonenzymatic reactions of the latter to form stable polymer products (Scheme 2).

When lecithin/refined olive oil reverse micelles were used for the oxidation of oleuropein by tyrosinase, no significant reaction was observed. Similarly, the enzyme did not exhibit activity when tested in lecithin/isooctane reverse micelles. It seems that, although refined olive oil or isooctane can yield reverse micelles, the enzyme activity is inhibited. Apparently, the absence of the minor components present in the virgin olive oil influences the accessibility of the enzyme by oleuropein.

Figures 3 and 4 show the evolution of oleuropein oxidation products in lecithin/virgin olive oil microemulsions at different tyrosinase and oleuropein concentrations, respectively. In both cases, oxidized oleuropein is produced on initiation of the reaction. The enzyme reaction stops almost immediately for all concentrations of both enzyme and substrate. Interestingly, the plot of the initial product formation, after 30 s, with increasing tyrosinase concentration (inset of Fig. 3) clearly shows a biphasic behavior of enzyme activity in the reverse micellar system. At low tyrosinase concentrations, the enzyme is more active than in higher ones. This decrease of enzyme activity could be due to changes of the structure of the tyrosinase-containing microemulsion that may occur at high enzyme concentrations (20).

The almost immediate termination of the enzyme reaction could be due either to the denaturating effect of the micellar system or to a possible enzyme inactivation reaction known to occur in presence of *o*-diphenols (21). The second possible explanation seems to be in accordance with a recent work, in which the catalytic behavior of tyrosinase toward oleuropein in aqueous solution was investigated (22). Substrate inhibition was less intense, and as a consequence initial velocities were measured and kinetic constants were determined.

When the catalytic behavior of tyrosinase toward 4-methylcatechol was studied in AOT/cyclohexane reverse micelles, substrate inhibition was not observed (13). In addition, Yang and Robb (16) studied the oxidation catalyzed by tyrosinase of 4-methylcatechol and *t*-butylcatechol in AOT/isooctane and CTAB (hexadecyl trimethylammonium bromide)/hexane/chloroform reverse micelles. In both systems and with both substrates, suicide inactivation was not observed and kinetic constants were calculated.



SCHEME 2

The lack of substrate inhibition in the reverse micellar systems just discussed may be explained by substrate partitioning among the different microdomains: organic solvent, aqueous core, and surfactant layer. In addition, the reaction products, *o*-quinones, rapidly diffuse toward the organic solvent, and therefore accumulation in the vicinity of the protein molecules does not occur.

In the present study, when tyrosinase was entrapped in the more restricted aqueous core of lecithin-based olive oil microemulsions and oleuropein oxidation was considered, substrate

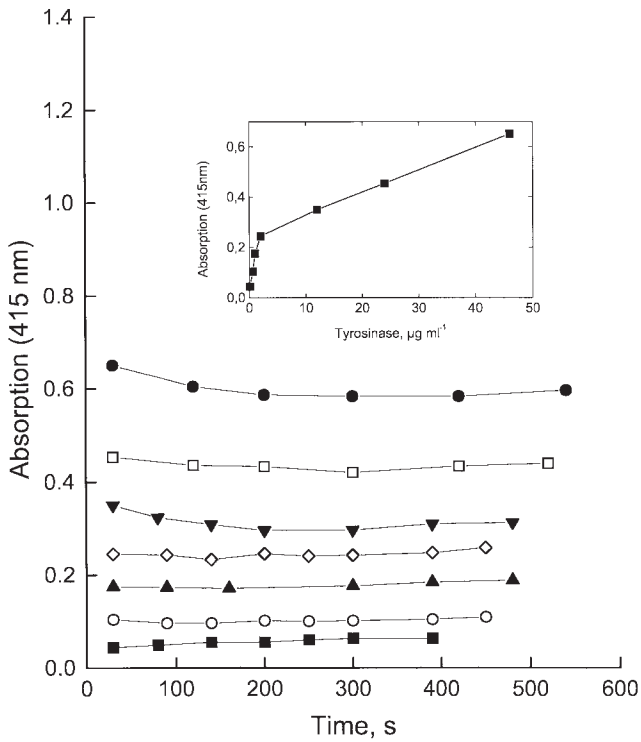


FIG. 3. Evolution of oleuropein oxidation product at different enzyme concentrations in lecithin/extra virgin olive oil microemulsions. Conditions were oleuropein 0.3 mM, tyrosinase: (■) 0.1, (○) 0.6, (▲) 1, (◇) 2, (▼) 12, (□) 24, and (●) 46 µg/mL (local concentrations), lecithin/extra virgin olive oil 4% w/w, 1-propanol 10% vol/vol, aqueous phase 1% vol/vol, pH 7, 30°C. The inset shows the variation in the initial absorption as a function of enzyme concentrations.

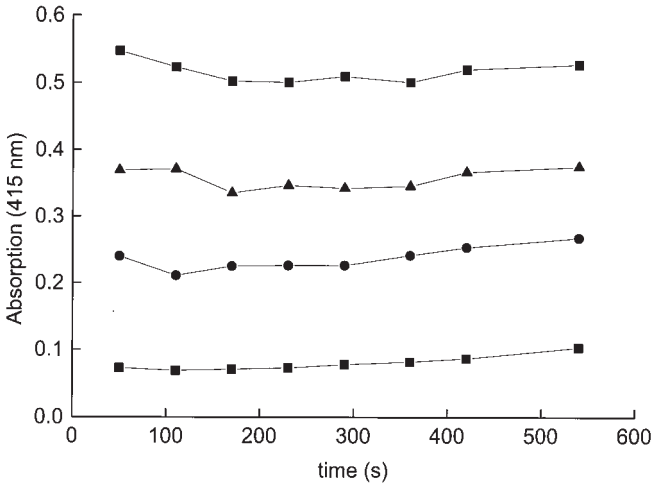


FIG. 4. Evolution of oleuropein oxidation product catalyzed by tyrosinase at different oleuropein concentrations in lecithin/extra virgin olive oil microemulsions. Conditions were tyrosinase 12 µg/mL (local concentration), oleuropein: (■) 0.2 mM, (●) 0.8 mM, (▲) 1.5 mM, (▼) 3 mM, lecithin/extra virgin olive oil 4% w/w, 1-propanol 10% vol/vol, aqueous phase 1% vol/vol, pH 7, 30°C.

inhibition was very intense. A possible explanation for this quick enzyme inactivation could be the eventual accumulation of oleuropein and the corresponding *o*-quinone products around the protein molecule and within the amphiphile monolayer.

It is known (21) that *o*-quinones can possibly undergo nucleophilic attacks by amino and thiol groups of enzyme molecules, leading to their quick inactivation. Figure 5 shows the spectrophotometric recordings of oleuropein oxidation by tyrosinase, in the presence of L-proline, in aqueous solution and in microemulsions. L-Proline is a nucleophilic reagent that can form stable chromophoric adducts with the *o*-quinone products

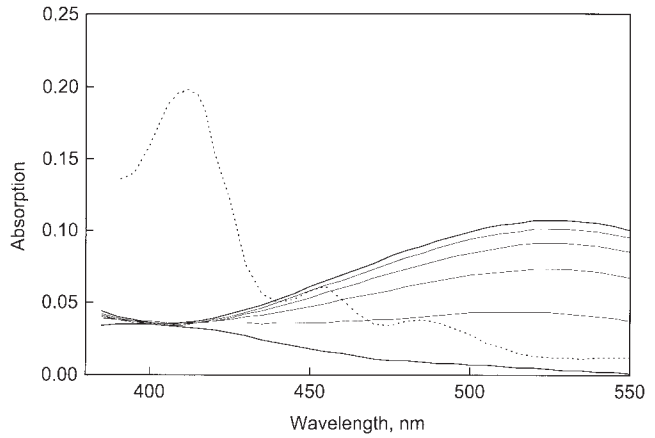


FIG. 5. Oxidation of oleuropein by tyrosinase in the presence of L-proline in aqueous solution and in lecithin/extra virgin olive oil microemulsions (dotted line). Experimental conditions were oleuropein 0.2 mM, tyrosinase 12 µg/mL, L-proline 2 mM, phosphate buffer 0.1 M, pH 7, 30°C. Reaction times from bottom to top: 10, 135, 270, 400, 535, and 670 s.

of the enzymatic oxidation as soon as they are produced (23) (Scheme 2).

When the reaction was carried out in aqueous solution, a broad new absorption peak at about 520 nm could be seen (Fig. 5), which corresponded with the evolution of prolyl-*o*-quinone stable adducts. The disappearance of the absorption maximum at 390 nm (Fig. 2) indicates a quick coupling reaction between L-proline and oleuropein *o*-quinone products.

When this reaction was carried out in olive oil microemulsions, the fast inactivation of tyrosinase could not be prevented by addition of L-proline. The recorded spectrum after 1 min is shown in Figure 5 (dotted line). The spectrum did not significantly change with time (up to 10 min), and no peaks above 500 nm, corresponding to coupled reaction products such as prolyl-*o*-quinone adducts, could be observed.

A possible explanation for this behavior could be that L-proline is localized in a different microdomain, i.e., the aqueous core, of the olive oil/lecithin microemulsion as compared with oleuropein, which is partitioned in the interfacial layer of the surfactants. As the oxidation of oleuropein proceeds, the *o*-quinone reaction product is also incorporated in the interface and thus coupling with the hydrophilic amino acid L-proline does not occur.

In conclusion, water-in-olive oil microemulsions can be obtained using lecithin as surfactant. Such novel biocompatible and food-grade microemulsions could be applied in many biotechnological applications involving enzymes. Tyrosinase, an oxidizing enzyme present in olives and virgin olive oil, can be incorporated in these microemulsions and retain catalytic activity with regard to the oxidation of oleuropein, one of the major antioxidant components of these products. This study proved to be a suitable model for understanding the oxidation process occurring in olive oil, which affects its quality and shelf life.

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