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ARTICLE

Effect of Protonation and Aggregation State of (*E*)-Resveratrol on Its Hydroperoxidation by Lipoxygenase

José Manuel López-Nicolás,* Manuela Pérez-Gilabert, and Francisco García-Carmona

Department of Biochemistry and Molecular Biology-A, Faculty of Biology, University of Murcia, Campus de Espinardo, 30071 Murcia, Spain

The protonation and aggregation states of (*E*)-resveratrol were used as tools to investigate the kinetic properties of lipoxygenase (LOX). It was found that the deprotonation of the 4′-hydroxyl group at pH values higher than the pK_{a1} of (*E*)-resveratrol produced an increase in the LOX activity, with an optimum pH of 8.5. Moreover, the results show how LOX activity is strongly affected by the aggregation state of (*E*)-resveratrol. When the enzyme uses monomers of (*E*)-resveratrol as substrate, LOX shows a Michaelian behavior and the K_m value can be determined (44.39 μM). However, when (*E*)-resveratrol concentration is increased to values higher than the critical concentration determined by fluorescence methods (35 μM at pH 8.5), LOX shows strong inhibition. These results can be interpreted as a previously unreported aggregate-induced enzyme inhibition, which can be modified by the use of different modulators of the aggregation state of (*E*)-resveratrol, such as cyclodextrins or ethanol. Finally, when the reaction was kinetically characterized in the optimum conditions of both aggregation and protonation state, a typical induction period was observed, along with a dependence of the hydroperoxidation rate with the hydrogen peroxide concentration.

KEYWORDS: Resveratrol; lipoxygenase; aggregation; protonation; cyclodextrin

INTRODUCTION

(*E*)-Resveratrol [(*E*)-3,4′,5-trihydroxystilbene] is a naturally occurring antioxidant found in grapes (*1*), grape products, including wine (*2*), and some other botanical sources, such as peanuts (*3*). In recent years, research into (*E*)-resveratrol has discovered several potentially beneficial biological effects of this compound on human health. These include anticancer activity (*4*), cardioprotection (*5*), antioxidant activity (*6*−*8*), inhibition of platelet aggregation (*9*), and anti-inflammatory activity (*10*). For these reasons, there is growing interest in its use as a functional ingredient in foods as a fortifier or nutraceutical compound (*11*). However, its propensity to oxidation and the paucity of information concerning conformational changes in its structure resulting from variations in pH and aggregation state have meant that, to date, no food has been enriched in this important antioxidant compound (*12*−*14*)

In recent years, (*E*)-resveratrol has been demonstrated to act as a potent substrate of the oxidation reaction catalyzed by lipoxygenase (LOX) (linoleate:oxygen reductase, EC 1.13.11.12) (*15*−*18*). LOXs are iron-containing dioxygenases that convert polyunsaturated fatty acids possessing one or more (1*Z*,4*Z*)-pentadiene systems into (*Z*,*E*)-conjugated hydroperoxy fatty acids (*19*). LOXs play a fundamental role in the metabolism of polyunsaturated fatty acids in plants and animals. The products of mammalian LOX are directly involved in the immune response and in inflammatory processes (*20*). The physiological role of plant LOXs is less clear. LOXs are postulated to be involved in senescence, stimulating the intracellular free radical production (*21*) that leads directly to membrane deterioration. LOX is also involved in the response of plants to wounding because hydroperoxides of linoleic acid are precursors of traumatic acid (*19*).

To date, very few papers have reported on the oxidation of (*E*)-resveratrol by LOX. Pinto and Macias (*17*) studied the oxidation of several dietary polyphenolics by the hydroperoxidase activity of LOX, showing that this enzyme, in the presence of hydrogen peroxide, produces the oxidative decomposition of quercetin, naringenin, and (*E*)-resveratrol, all known antioxidant molecules. Moreover, Fan and Mattheis (*16*) reported the inhibition of oxidative and antioxidative enzymes such as superoxide dismutase, LOX, catalase, peroxidase, polyphenol oxidase, and 1-aminocyclopropane-1-carboxylic acid oxidase by (*E*)-resveratrol, showing that this stilbene inhibited LOX activity more effectively than other LOX inhibitors, including propyl gallate, ibuprofen, ursolic acid, acetylsalicylic acid, and salicylhydroxamic acid. Furthermore, Lucas-Abellán et al. (*15*) characterized the formation of resveratrol−cyclodextrin inclusion complexes in aqueous solutions using the hydroperoxidase activity of LOX as the enzymatic system. Finally, Pinto et al. (*18*) reported that resveratrol is a potent inhibitor of the dioxygenase activity of LOX.

*Corresponding author (fax: 34 968-364765; e-mail: josemln@um.es).

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However, three main problems can be observed in these studies: (a) no exhaustive study of the effect of pH on LOX activity has been carried out, meaning that the activity was determined at different pH values in the papers; (b) no authors have borne in mind the possible effect of the aggregation of (*E*)-resveratrol on the LOX activity at high concentrations of the substrate, which is a common feature preview described for other substrates catalyzed by LOX (e.g., linoleic acid) (*22*); and (c) no research has studied the hypothetical relationship between the structure of this potent lipophilic antioxidant and its enzymatic catalysis. For these reasons, it is essential that a study be made of (*E*)-resveratrol oxidation by LOX with these important kinetic factors taken into consideration.

In a previous work (*23*), we studied the aggregation state of (*E*)-resveratrol at acidic and basic pH values, determining the (*E*)-resveratrol concentration value at which this stilbene changes from its monomeric to aggregate form. The results clearly showed that the aggregation mode of (*E*)-resveratrol was affected by the pH. Moreover, bearing in mind the importance of the ionization state of (*E*)-resveratrol in determining potential beneficial biological effects of this compound on human health, and due to the contradictory results existing in the literature about the three (*E*)-resveratrol acidic dissociation constants, we determined in that paper the correct pK_a of this stilbene in aqueous solution using UV and fluorescence studies. These results can modify the published works to date about the oxidation of (*E*)-resveratrol by LOX (*15*−*18*) as it has been reported by Bru and García-Carmona in another paper for other reactions catalyzed by this enzyme (*22*).

As a continuation of this study, and with the above taken into consideration, the principal aims of this work were to (i) study whether LOX had any preference for the physicochemical properties of the (*E*)-resveratrol substrate, particularly in its protonation and aggregation state; (ii) evaluate the effect of different substrate modulators such as cyclodextrins or ethanol on the LOX activity; and (iii) characterize kinetically the oxidation of (*E*)-resveratrol by LOX in the optimum conditions of protonation and substrate concentration.

MATERIALS AND METHODS

Materials. Biochemicals were purchased from Fluka (Madrid, Spain). (*E*)-Resveratrol, 2-hydroxypropyl-β-CD (HP-β-CD), hydrogen peroxide, and soybean lipoxygenase type V from soybean were obtained from Sigma (Madrid, Spain). (*E*)-Resveratrol is sensitive to light, and irradiation of solutions of the analyte induces the formation of (*Z*)-resveratrol; if the irradiation is intense, it leads to the formation of a highly fluorescent compound. Because of this, the samples were stored in darkness. The hydrogen peroxide, lipoxygenase, and (*E*)-resveratrol were freshly prepared every day, and their concentrations were calculated using ε_{240} = 39.4 M^{-1} cm^{-1}, ε_{280} = 160,000 M^{-1} cm^{-1}, and ε_{312} = 33,400 M^{-1} cm^{-1} (*15*).

Equipment and Experimental Procedure. *Fluorescence and Absorbance Spectroscopy*. A PiStar-180 spectrometer (Applied Photophysics Ltd. Leatherhead, U.K.) equipped with a xenon lamp source and quartz cell was used to perform all absorbance and fluorescence measurements. Excitation and emission bandwidths were both set at 2 nm. For both absorbance and fluorescence spectroscopy, excitation and emission spectra were recorded at 25 °C. To avoid self-absorbing (inner filter) effects, 2 mm quartz cells were used.

Enzyme Assay. LOX activity was followed spectrophotometrically in a Jasco V-650 spectrometer (Applied Photophysics Ltd.) at 25 °C equipped with thermostated cells at the absorption maximum of the oxidation product of (*E*)-resveratrol (250 nm). The reaction was started by adding the indicated volume of enzyme to a standard reaction medium that contained the indicated concentration of (*E*)-resveratrol at different pH values. The buffers used were 0.1 M sodium acetate from pH 4.0 to 5.5, 0.1 M sodium phosphate from pH 5.5 to 8.5, and 0.1 M sodium borate from pH 8.5 to 10.0.

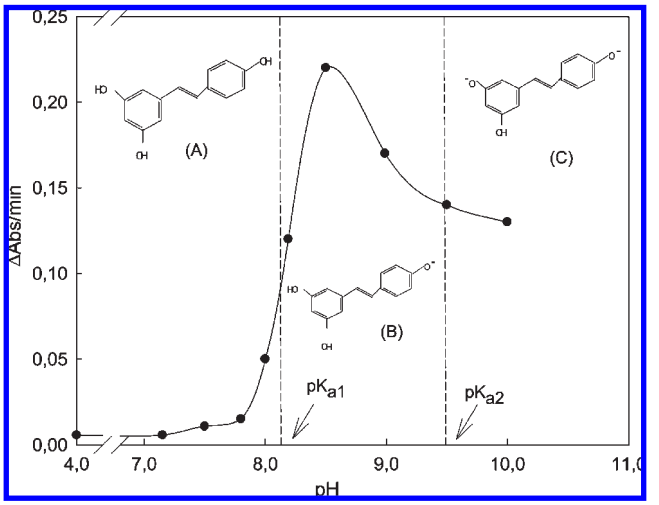

Figure 1. Effect of pH on the hydroperoxidation of (*E*)-resveratrol by LOX. The reaction medium consisted of 12 μM (*E*)-resveratrol and 4 mM H_2O_2 at different pH values: (**A**) totally protonated; (**B**) one hydroxyl group deprotonated; (**C**) two hydroxyl groups deprotonated.

RESULTS AND DISCUSSION

Effect of pH on the Enzymatic Oxidation of (*E*)-Resveratrol by LOX. Although several papers have been published on the oxidation of (*E*)-resveratrol by LOX (*14*−*18*), no research has studied the effect of medium pH on the LOX activity. Moreover, not all of the papers have studied the enzymatic reaction at the same pH: although the majority of the authors have studied this reaction at pH 9.0 (*15*, *16*, *18*), Pinto and Macias (*17*) studied the enzymatic oxidation at pH 7.0. For this reason, in this work we have studied the oxidation of this lipophilic antioxidant in a range of pH values. Moreover, due to the problems associated with measuring the enzymatic activity on substrates that can suffer aggregation phenomena in the UV region, the first step in our investigation was to select the most appropriate substrate concentration to evaluate the effect of pH on LOX activity avoiding turbidity problems.

In a recent paper, using fluorescence, we demonstrated that pH determines not only the protonation but also the aggregation state of (*E*)-resveratrol (*23*). The results obtained in that paper showed that the critical concentration at which this antioxidant forms aggregates is higher when the medium pH is increased from acid to basic pH values. At pH values of 5.5 and 10.5, (*E*)-resveratrol forms aggregates above 12.5 and 37.5 μM. Thus, when (*E*)-resveratrol concentrations below this value are used, the substrate is in monomeric form, and it is not difficult to measure the LOX activity in this region because no turbidity is present. On the other hand, when the (*E*)-resveratrol concentration is above the critical concentration, this potent antioxidant is not molecularly dispersed and forms aggregates, leading to scattering and making the spectroscopic determination of LOX activity difficult. For this reason, in this paper we have studied the effect of the pH on the LOX activity using a (*E*)-resveratrol concentration of 12 μM because this is below the critical concentration (i.e., at monomeric forms) at all of the pH values tested and no turbidity is present in the reaction medium.

The pH profile of the hydroperoxidation of (*E*)-resveratrol by LOX is shown in **Figure 1**. As can be seen, the enzymatic activity was practically zero at pH values below 7.0. However, when the medium pH was above 7.5, the LOX activity suddenly increased, with an optimum at pH 8.5, above which it fell sharply. These results are in good agreement with the pH values used by several authors to study this enzymatic reaction (*15*, *16*, *18*) but do not

coincide with the values used by Pinto and Macías (*17*). However, no researcher has explained this type of behavior. In **Figure 1**, we can observe that the strong increase in LOX activity at pH 8.5 approximately coincides with the first acidic dissociation (pK_{a1}) constant calculated by several authors for (*E*)-resveratrol (*23*−*25*). Moreover, the subsequent decrease observed in LOX activity coincided when the medium pH was near the second acidic dissociation constant (pK_{a2}) determined previously by these authors. These results show the importance of the three hydroxyl groups present in the structure of (*E*)-resveratrol for its enzymatic oxidation. Many researchers have studied the role of hydroxyl groups on the properties of (*E*)-resveratrol and found that the 4′-hydroxyl group is essential to (*E*)-resveratrol's healthy activities. However, the stability of the 4′-hydroxyl group in (*E*)-resveratrol is weak because it is more acidic than the other two *m*-hydroxyl groups (*24*). Moreover, Cao et al. (*25*) indicated that it is easier to deprotonate the 4′-OH than 3′- or 5′-OH. These authors showed that the 4′-hydroxyl group of (*E*)-resveratrol is more reactive than those in the 3- and 5-positions because the resonance effects of the 4′-radical are more stable than those of the 5-radical. Finally, López-Nicolás and García-Carmona (*23*) recently demonstrated that at pH values above the first acidic constant dissociation the 4′-hydroxyl group is deprotonated.

The fact that LOX shows the highest rate of enzymatic oxidation when the pH is situated between the first and second acidic dissociation constants reveals that the (*E*)-resveratrol form shown in **Figure 1** (structure B), where only two hydroxyl groups (in the 3′- and 5′-positions) are presented, is structure preferred by the enzyme. Moreover, structures A [(*E*)-resveratrol totally protonated] and C [(*E*)-resveratrol with two hydroxyl groups deprotonated] are not good substrates for lipoxygenase.

Effect of (*E*)-Resveratrol Aggregation on Its Enzymatic Oxidation by LOX. The critical importance of knowing the preferences of LOX for the different physical forms of its substrate has been mentioned because this is the only way to correctly analyze the kinetic data and thus perform correct extrapolations to physiological conditions. Moreover, the vegetal source of this enzyme is essential for knowing its preference for the aggregation state of the substrate. Indeed, the preference of LOX-I for monomeric LA has long been known (*26*), whereas 5-LOX prefers the aggregate form of LA (*22*). However, no research has been carried out concerning the preference of LOX for the monomeric or aggregate forms of (*E*)-resveratrol. For these reasons, and in order to characterize kinetically the oxidation of (*E*)-resveratrol by LOX, it is essential to study which aggregation state of (*E*)-resveratrol is favored by this enzyme.

To study the effect of the aggregation state of (*E*)-resveratrol on its enzymatic oxidation by LOX, we evaluated the possible influence of (*E*)-resveratrol aggregation on LOX activity at the optimum pH found in the previous section, that is, pH 8.5, at concentrations above and below the critical concentrations. To do this, the first step was determine the critical concentration at which (*E*)-resveratrol forms aggregates at pH 8.5. For this, we have followed the method described recently by us, which is based on a study of the fluorescence emission and excitation spectra of (*E*)-resveratrol at increasing concentrations (*23*). The results obtained are in good agreement with those reported by Lopez-Nicolas and García-Carmona (*23*) and show that the emission and excitation spectra differ greatly at high and low (*E*)-resveratrol concentrations, showing bathochromic displacement of the maximum excitation wavelength and a nonlinear dependence of the relative intensity of fluorescence on the (*E*)-resveratrol concentration. The dependence of the maximum excitation wavelength of (*E*)-resveratrol on the concentration is also shown in **Figure 2**. As can be seen, at concentrations lower than 35 μM,

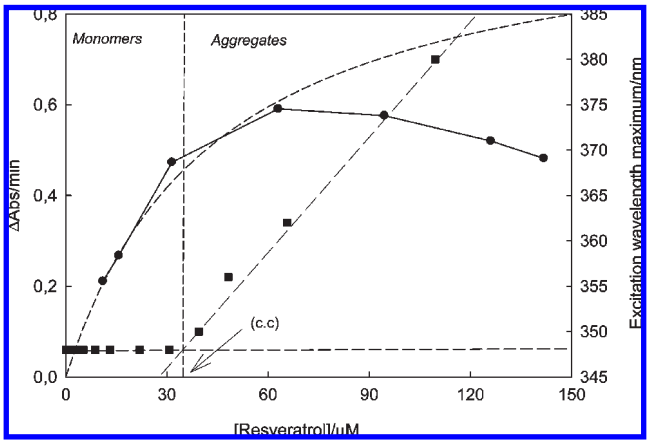

Figure 2. Effect of (*E*)-resveratrol concentration on LOX activity in the monomeric and aggregate region of (*E*)-resveratrol in the presence of 4 mM H_2O_2 at pH 8.5 (●) and effect of (*E*)-resveratrol concentration on the maximum excitation wavelength in aqueous solutions at increasing (*E*)-resveratrol concentrations at pH 8.5 (■).

(*E*)-resveratrol showed a constant maximum excitation wavelength at about 348 nm, whereas above 35 μM, a bathochromic displacement to 380 nm was observed. Using the method reported by Lopez-Nicolas and García-Carmona (*23*), we were able to determine graphically the critical concentration of (*E*)-resveratrol as the cross-point between the straight lines that define the trend of the maximum excitation wavelength in the pre- and postaggregation regions (**Figure 2**). This determination makes it possible to establish the conditions in which (*E*)-resveratrol is molecularly dispersed and does not form aggregates. According to the results published by Lopez-Nicolas and García-Carmona (*23*), a possible explanation for the dependence of (*E*)-resveratrol fluorescence on the concentration is that this molecule forms aggregates above a certain critical concentration. Therefore, this confirms that 35 μM is the highest (*E*)-resveratrol concentration value at which this potent antioxidant is molecularly dispersed and does not form aggregates at pH 8.5.

The second step of our investigation was to study the enzymatic activity of LOX at (*E*)-resveratrol concentrations lower and higher than the critical concentration at pH 8.5 (35 μM). **Figure 2** shows how the LOX activity is strongly affected by the aggregation state of (*E*)-resveratrol. When the enzyme uses monomers of (*E*)-resveratrol as substrate (monomeric region), LOX shows a Michaelian behavior (**Figure 2**) and the kinetic parameters K_m and V_{max} can be determined (44.39 μM and 1.03 $\Delta Abs min^{-1}$). However, when (*E*)-resveratrol concentration is increased above 35 μM, a strong inhibition is observed (**Figure 2**). As can be seen, the (*E*)-resveratrol concentration at which the LOX is inhibited approximately coincides with the (*E*)-resveratrol critical concentration value for this pH. For this reason, we can affirm that when aggregates of (*E*)-resveratrol are formed, a strong decrease in the enzymatic activity can be observed. The observed kinetics can be interpreted as a phenomenon of the *substrate-aggregation dependent activity* of LOX, very similar to the behavior described for 5-potato LOX (*22*) and eggplant LOX (*27*) on linoleic acid (LA) and also to that described for other enzymes. However, in the case of 5-LOX or eggplant LOX, the aggregation of LA activated in the enzymatic activity. The results presented in **Figure 2**, which also suggest that LOX catalysis is less efficient at the lipid−water interface than in solution, have been reported for other water-soluble enzymes acting on hydrophobic or membrane-bound substrates, such as phospholipase A2 (*28*−*30*) and cholesterol oxidase (*31*).

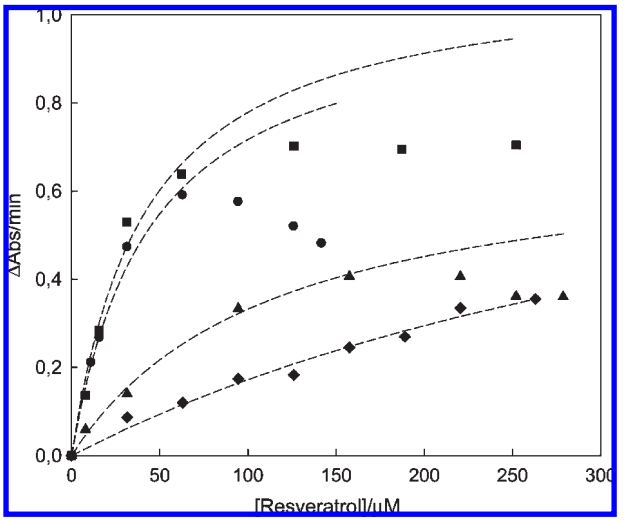

Figure 3. Effect of different agents on hydroperoxidation of (*E*)-resveratrol by LOX: (●) no agent; (■) 1% EtOH; (▲) 0.75 mMHP-β-CD; (◆) 1.5 mM HP-β-CD. The reaction medium at 25 °C contained increasing (*E*)-resveratrol concentrations and 4 mM H_2O_2 in 0.1 M sodium phosphate buffer, pH 8.5.

Effect of Different Modulators of the Aggregation State of (*E*)-Resveratrol on the LOX Activity. In the previous section, we demonstrated the importance of the aggregation state of the substrate in the enzymatic oxidation of (*E*)-resveratrol by LOX. To evaluate the effect of modulators of the structure and availability of this potent antioxidant in the enzymatic reaction, the next step was to evaluate the response of LOX to the presence of several agents such as ethanol or CDs in the reaction medium.

Ethanol is a well-known "solubilizer" of lipophilic substrates in the enzymatic reactions. As shown in **Figure 3**, the presence of 1% ethanol in the reaction medium leads to an increase of the critical (*E*)-resveratrol concentration at which molecular aggregates are formed. Consequently, the inhibition of the LOX activity at high substrate concentration is produced at higher (*E*)-resveratrol concentrations than in the absence of the organic solvents. In the presence of 1% ethanol a K_m of 41.54 μM and a V_{max} of 1.10 ΔAbs min^{-1} were calculated for the hydroperoxidation of (*E*)-resveratrol by LOX. As is shown in **Figure 3**, in the monomeric region, the enzymatic activities in both the presence and absence of EtOH are very similar. However, the presence of organic dissolvents can inhibit enzymatic catalysis, and the results obtained in these conditions cannot be extrapolated to physiological conditions. For this reason, the use of other modifiers of the aggregation state of (*E*)-resveratrol in aqueous medium is recommended.

The next modifier of the aggregation state of (*E*)-resveratrol studied was HP-β-CD. Cyclodextrins (CDs) are torus-shaped oligosaccharides made up of six to eight glucopyranose units and originated by the enzymatic degradation of starch through the action of CD-glucano-transferase (*32*). Poorly water-soluble compounds and hydrophobic moieties of amphiphilic molecules interact non-covalently with the CD cavity to form so-called inclusion complexes, which are also highly water-soluble. Several publications have reported the aggregation behavior of different enzymatic substrates such as fatty acids, phenols, and stilbenes in the presence of CD, and evidence has been presented concerning the formation of guest/CD inclusion complexes with several stoichiometries (*33*, *34*).

Figure 3 also shows the (*E*)-resveratrol dependence of LOX at pH 8.5 at two HP-β-CD concentrations. As shown, the addition of increasing concentrations of HP-β-CD also produced a change in the LOX enzymatic activity. When 0.75 mM HP-β-CD was added to the reaction medium, the LOX activity was lower than that obtained in the absence of any agent for the same (*E*)-resveratrol concentrations (V_{max} = 0.71 ΔAbs$min^{-1}$and K_m = 112.66 μM) in the monomeric region of (*E*)-resveratrol. These results are in good agreement with those obtained for the catalysis of other LOX substrates such as linoleic, linolenic, or arachidonic acid (*35*). This behavior is due to the ability of both natural and modified CDs to sequester part of the (*E*)-resveratrol to form soluble inclusion complexes (*36*−*38*), thereby reducing the concentration of the free (*E*)-resveratrol. Indeed, as it is described for the oxidation of other LOX substrates in the presence of CDs (*33*, *35*), free (*E*)-resveratrol is the only effective substrate and the oxidation of the complexed substrate requires the previous dissociation of the complex. Furthermore, in **Figure 3** it can be seen that, when 0.75 mM HP-β-CD is added to the reaction medium, the previously observed inhibition by substrate aggregation is produced at a higher (*E*)-resveratrol concentration (150 μM) than observed in the absence of any agent (*E*)-resveratrol (35 μM) or in the presence of 1% EtOH. Thus, the concentration or (*E*)-resveratrol at which the LOX activity was inhibited increased in the presence of HP-β-CD, reflecting the formation of inclusion complexes and resulting in the extension of the range of monomeric (*E*)-resveratrol. This result is due to the ability of CDs to increase the critical concentration at which the aggregation of different substrates is produced, thus widening the apparent monomer concentration range (*34*, *39*). Indeed, as deduced from the fluorescence experiments and our previous studies (*39*), free and complexed monomers of (*E*)-resveratrol exist in the premicellar region, whereas in the postmicellar region there is the additional presence of (*E*)-resveratrol aggregates. In both regions, the complexed (*E*)-resveratrol simply constitutes a pool of substrate to which enzymes might or might not have direct access (*34*).

On the other hand, when the HP-β-CD was increased to 1.5 mM, the LOX activity was less than in the presence of 0.75 mM HP-β-CD for the identical substrate concentration because lower free (*E*)-resveratrol was present in the reaction medium. However, no inhibition of LOX activity due to aggregation of substrate was observed, because the critical free (*E*)-resveratrol concentration at which LOX activity is inhibited was not reached.

Analysis of the Oxidation Kinetics of (*E*)-Resveratrol by LOX in the Optimum Conditions of Aggregation and Protonation State. The results obtained in the previous sections show that the optimum reaction conditions for characterizing the oxidation of (*E*)-resveratrol by LOX are as follows: (i) The substrate must be present in the monomeric form, and (ii) the optimum structure of (*E*)-resveratrol is that in which the 4′-hydroxyl group is deprotonated. To fulfill these objectives, a pH value of 8.5 and a substrate concentration of 30 μM were selected. **Figure 4** shows that LOX catalyzes the oxidation of 30 μM (*E*)-resveratrol to its oxidized form in the presence of 4 mM H_2O_2 in the reaction medium at pH 8.5, producing a decrease in the (*E*)-resveratrol absorbance at 312 nm, with an increase of absorbance at 250 nm. The formation of three isosbestic points at 240, 280, and 347 nm indicated that (*E*)-resveratrol was transformed in its oxidized form at a constant ratio.

The presence of an induction period prior to the steady state, during which the product accumulates linearly, is a common feature of LOX-catalyzed reactions. **Figure 4** (inset) shows how such a reaction was also observed for the oxidation of (*E*)-resveratrol by LOX. The induction period, determined graphically by extrapolation of the linear part of the product accumulation curve to the time axis, decreased when the enzyme

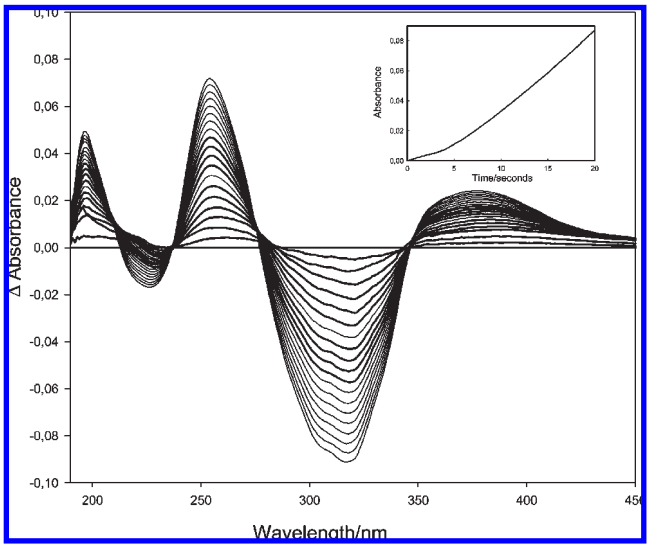

Figure 4. Difference scans of the hydroperoxidation of (*E*)-resveratrol by LOX in the presence of H_2O_2. The reaction medium at 25 °C contained 30 μM of (*E*)-resveratrol in the presence of 4 mM H_2O_2 at pH 8.5. The scans were carried out every 0.5 min. (Inset) Progress curve of LOX-catalyzed (*E*)-resveratrol hydroperoxidation.

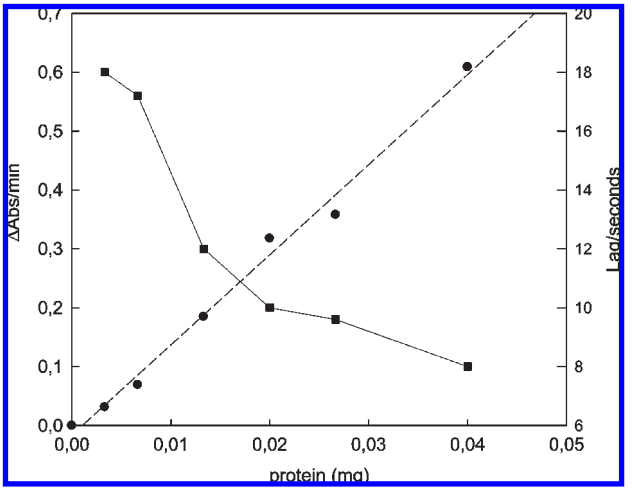

Figure 5. Effect of enzyme concentration on reaction rate (●) and induction period (■) on the hydroperoxidation at 25 °C of 30 μM (*E*)-resveratrol by LOX at pH 8.5 in the presence of 4 mM H_2O_2.

concentration increased (**Figure 5**), although it was not completely eliminated at the highest enzyme concentration tested. In the same **Figure 5**, it can be seen that the reaction rate in the linear part of the curve depends linearly on the enzyme concentration. However, the typical induction period that appears in the LOX reaction progress curves is reduced if micromolar amounts of hydrogen peroxide are added to the reaction medium (**Figure 6**). In a previous paper (*40*), we proposed a mechanism to explain the induction period during which the inactive ferrous form of the enzyme is converted to the active ferric enzyme upon addition of an equimolecular amount of hydroperoxide product, whereas the latter ferric form is maintained during the steady-state turnover of the enzyme.

Finally, as is shown in **Figure 6**, (*E*)-resveratrol oxidation was also dependent on the concentration of H_2O_2. The K_m for H_2O_2 (50 μM) is one of the lowest described for such hydroperoxide activity, especially compared with that found in the oxidation of phenothiazines or for the oxidation of other phenolic compounds such as isoproterenol or tetramethylbenzidine (*40*). However,

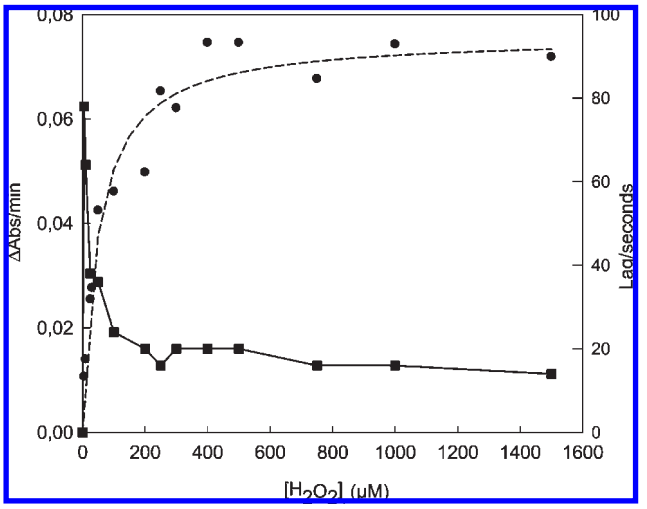

Figure 6. Effect of H_2O_2 concentration on (●) hydroperoxidation of (*E*)-resveratrol by LOX and on (■) induction period. The reaction medium at 25 °C contained 0.1 M phosphate buffer, pH 8.5, 5 μM (*E*)-resveratrol, and increasing concentrations of H_2O_2 from 0 to 1.5 mM.

contrary to the results published for several substrates oxidized by LOX (*40*), which showed that activity decreased when the H_2O_2 concentration was increased, in our conditions the activity did not decrease at higher concentration (**Figure 6**).

LITERATURE CITED

(1) Creasy, L. L.; Coffee, M. J. Phytoalexin production of grape berries. *J. Am. Soc. Hortic. Sci.* **1988**, *113*, 230–234.
(2) Siemann, E. H.; Creasy, L. L. Concentration of the phytoalexin resveratrol in wine. *Am. J. Enol. Vitic.* **1992**, *43*, 49–52.
(3) Sanders, T. H.; McMichael, R. W.; Hendrix, K. W. Occurrence of resveratrol in edible peanuts. *J. Agric. Food Chem.* **2000**, *48*, 1243–1246.
(4) Jang, M. S.; Cai, E. N.; Udeani, G. O.; Sllowing, L. V.; Thomas, C. F.; Beecher, C. W. W.; Fong, H. S.; Farnsworth, N. R.; Kinghorn, A. D.; Metha, R. G.; Moon, R. C.; Pezzuto, J. M. Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* **1997**, *275*, 218–220.
(5) Hung, L. M.; Chen, J. K.; Huang, S. S.; Lee, R. S.; Su, M. J. Cardioprotective effect of resveratrol, a natural antioxidant derived from grapes. *J. Cardiovasc. Res.* **2000**, *47*, 549–555.
(6) Frankel, E. N.; Waterhouse, A. L.; Kinsella, J. E. Inhibition of human LDL oxidation by resveratrol. *Lancet* **1993**, *341*, 1103–1104.
(7) Fremont, L.; Belguendou, L.; Delpal, S. Antioxidant activity of resveratrol and alcohol-free wine polyphenols related to LDL oxidation and polyunsaturated fatty acids. *Life Sci.* **1999**, *64*, 2511–2521.
(8) Alarcón de la Lastra, C.; Villegas, I. Resveratrol as an antioxidant and pro-oxidant agent: mechanisms and clinical implications. *Biochem. Soc. Trans.* **2007**, *35*, 1156–1160.
(9) Bertelli, A. A. E.; Giovannini, L.; Gianness, D.; Migliori, M.; Bernini, W.; Fregoni, M.; Bertelli, A. Antiplatelet activity of synthetic and natural resveratrol in red wine. *Int. J. Tissue React.* **1995**, *17*, 1–3.
(10) Pace-Asciak, C. R.; Hahn, S. E.; Diamandis, E. P.; Soleas, G.; Goldberg, D. M. The red wine phenolics *trans*-resveratrol and quercetin block human platelet aggregation and eicosanoid synthesis: implications for protection against coronary heart disease. *Clin. Chim. Acta* **1995**, *235*, 207–219.
(11) López-Nicolás, J. M.; Núñez-Delicado, E.; Pérez-López, A. J.; Carbonell, A.; Cuadra-Crespo, P. Determination of stoichiometric coefficients and apparent formation constants for β-cyclodextrin complexes of *trans*-resveratrol using reversed-phase liquid chromatography. *J. Chromatogr., A* **2006**, *1135*, 158–165.

(12) Walle, T.; Hsieh, F.; Delegge, M. H.; Oatis, J. E.Jr.; Walle, U. K. High absorption but very low bioavailability of oral resveratrol in humans. *Drug Metab. Dispos*. **2004**, *32*, 1377–1382.
(13) Trela, B. C.; Waterhouse, A. L. Resveratrol: isomeric molar absorptivities and stability. *J. Agric. Food Chem*. **1996**, *44*, 1253–1257.
(14) Wenzel, E.; Somoza, V. Metabolism and bioavailability of *trans*-resveratrol. *Mol. Nutr. Food Res*. **2005**, *49*, 472–481.
(15) Lucas-Abellán, C.; Fortea, I.; López-Nicolás, J. M.; Núñez-Delicado, E. Cyclodextrins as resveratrol carrier system. *Food Chem*. **2007**, *104*, 39–44.
(16) Fan, X; Mattheis, J. P. Inhibition of oxidative and antioxidative enzymes by *trans*-resveratrol. *Food Chem. Toxicol*. **2001**, *66*, 200–203.
(17) Pinto, M. C.; Macias, P. Oxidation of dietary polyphenolics by hydroperoxidase activity of lipoxygenase. *J. Agric. Food Chem*. **2005**, *53*, 9225–9230.
(18) Pinto, M. C.; García-Barrado, J. A.; Macias, P. Resveratrol is a potent inhibitor of the dioxygenase activity of lipoxygenase. *J. Agric. Food Chem*. **1999**, *47*, 4842–4846.
(19) Baysal, T.; Demird, A. Lipoxygenase in fruits and vegetables: a review. *Enzym. Microb. Technol*. **2007**, *40*, 491–496.
(20) Yamamoto, S. Mammalian lipoxygenases: molecular structures and functions. *Biochim. Biophys. Acta* **1992**, *1128*, 117–131.
(21) Fobel, M.; Lynch, D. V.; John, E. T. Membrane deterioration in senescing carnation flowers: coordinated effects of phospholipid degradation and the action of membranous lipoxygenase. *Plant Physiol*. **1987**, *85*, 204–211.
(22) Brú, R.; García-Carmona, F. Potato (*Solanum tuberosum* var. Desiree) tuber 5-lipoxygenase selectivity for the physicochemical properties of linoleic acid. *J. Agric. Food Chem*. **1997**, *45*, 28692–2875.
(23) López-Nicolás, J. M.; García-Carmona, F. Aggregation state and pK_a values of (*E*)-resveratrol as determined by fluorescence spectroscopy and UV−visible absorption. *J. Agric. Food Chem*. **2008**, *56*, 7600–7605.
(24) Caruso, F.; Tanski, J.; Villegas-Estrada, A.; Rossi, M. Structural basis for antioxidant activity of *trans*-resveratrol: ab initio calculations and crystal and molecular structure. *J. Agric. Food Chem*. **2004**, *52*, 7279–7285.
(25) Cao, H.; Pan, X.; Li, C.; Zhou, C.; Deng, F.; Li, T. Density functional theory calculations for resveratrol. *Bioorg. Med. Chem. Lett*. **2003**, *13*, 1869–1871.
(26) Galpin, J. R.; Allen, J. C. The influence of micelle formation on lipoxygenase kinetics. *Biochim. Biophys. Acta* **1977**, *488*, 392–401.
(27) López-Nicolás, J. M.; Pérez-Gilabert, M.; García-Carmona, F. Eggplant lipoxygenase (*Solanum melongena*): product characterization and effect of physicochemical properties of linoleic acid on the enzymatic activity. *J. Agric. Food Chem*. **2001**, *49*, 433–438.
(28) Verger, R.; de Haas, G. H. Interfacial enzyme kinetics of lipolysis. *Annu. Rev. Biophys. Bioeng*. **1976**, *5*, 77–117.
(29) Dennis, E. A. Phospholipases. In *The Enzymes*; Boyer, P. D., Ed.; Academic Press: New York, 1983; Vol. *16*, pp 308−357.
(30) Menashe, M.; Romero, G.; Biltonen, R. L.; Lichtenberg, D. Hydrolysis of dipalmitoylphosphatidylcholine small unilamellar vesicles by porcine pancreatic phospholipase A2. *J. Biol. Chem*. **1986**, *261*, 5328–5333.
(31) Randolph, T. W.; Clark, D. S.; Blanch, H. W.; Prausnitz, J. M Enzymic oxidation of cholesterol aggregates in supercritical carbon dioxide. *Science* **1988**, *239*, 387–390.
(32) Szente, L.; Szejtli, J. Cyclodextrins as food ingredients. *Trends Food Sci. Technol*. **2004**, *15*, 137–142.
(33) Bru, R.; López-Nicolas, J. M.; Nuñez-Delicado, E.; Nortes-Ruipérez, D.; Sánchez-Ferrer, A; García-Carmona, F. Cyclodextrins as hosts for poorly water-soluble compounds in enzyme catalysis. *Appl. Biochem. Biotechnol*. **1996**, *61*, 189–198.
(34) Bru, R.; López-Nicolás, J. M.; García-Carmona, F. Aggregation of polyunsaturated fatty acids in the presence of cyclodextrins. *Colloids Surf*. **1995**, *97*, 263–269.
(35) López-Nicolás, J. M.; Bru, R.; García-Carmona, F. Kinetic characteristics of the enzymatic conversion in presence of cyclodextrins: study of the oxidation of polyunsaturated fatty acids by lipoxygenase. *Biochim. Biophys. Acta* **1997**, *1347*, 140–150.
(36) López-Nicolás, J. M.; García-Carmona, F. Rapid, simple and sensitive determination of the apparent formation constants of *trans*-resveratrol complexes with natural cyclodextrins in aqueous medium using HPLC. *Food Chem*. **2008**, *109*, 868–875.
(37) López-Nicolás, J. M.; Núñez-Delicado, E.; Pérez-López, A. J.; Carbonell, A.; Cuadra-Crespo, P. Determination of stoichiometric coefficients and apparent formation constants for β-cyclodextrin complexes of *trans*-resveratrol using reversed-phase liquid chromatography. *J. Chromatogr., A* **2006**, *1135*, 158–165.
(38) Bertacche, V.; Lorenzi, N.; Nava, D.; Pini, E.; Sinico, C. Host−guest interaction study of resveratrol with natural and modified cyclodextrins. *J. Inclusion Phenom. Macrocyclic Chem*. **2006**, *55*, 279–287.
(39) López-Nicolás, J. M.; Bru, R.; Sánchez-Ferrer, A.; García-Carmona, F. Use of "soluble lipids" for biochemical processes: linoleic acid: cyclodextrin inclusion complexes in aqueous solutions. *Biochem. J*. **1995**, *308*, 151–154.
(40) Nuñez-Delicado, E.; Pérez-Gilabert, M.; Sánchez-Ferrer, A.; García-Carmona, F. Hydroperoxidase activity of lipoxygenase: a kinetic study of isoproterenol oxidation. *Biochim. Biophys. Acta* **1995**, *1293*, 17–22.

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