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Oxidation of Dietary Polyphenolics by Hydroperoxidase Activity of Lipoxygenase

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Lipoxygenase, in the presence of hydrogen peroxide, produces the oxidative decomposition of quercetin, naringenin, and resveratrol, known antioxidant molecules. Quercetin was the molecule more efficiently oxidized, followed by resveratrol and naringenin. When this molecule was incubated in the presence of GSH, a quinoid derivative was produced. This compound was not obtained in the presence of naringenin or resveratrol, suggesting that in the presence of hydrogen peroxide and lipoxygenase, quercetin may be oxidized to a prooxidant species. When hydrogen peroxide was substituted by hydroperoxy linoleic acid, the same oxidative process was observed. This means that in food products in which lipoxygenase and linoleic acid are presents, quercetin may be oxidized to prooxidant species; in contrast, naringenin and resveratrol may constitute a valid additive for the prevention of the oxidative degradation of foods.

KEYWORDS: Oxidation; polyphenolics; hydroperoxidase; lipoxygenase; linoleic acid; quercetin; naringenin; resveratrol; antioxidants

INTRODUCTION

The most important polyphenols of the diet are flavonoids. These compounds are found in fruits and vegetables, the study of their biochemical properties being of great interest because, from epidemiological studies, it has been suggested that the consumption of polyphenol-rich foods and the prevention of diseases are directly related (*1*). One of the most relevant characteristics of flavonoids is that they prevent oxidative damage, scavenging reactive oxygen and nitrogen species. In addition, flavonoids possess inhibitory effects on prooxidant enzymes such as lipoxygenase, xanthine oxidase, or myeloperoxidase (*2*).

Among the pharmacological activities of flavonoids, an anti-inflammatory activity mediated by the inhibition of arachidonic acid metabolizing enzymes has been reported (*3*), flavonols being, in general, efficient lipoxygenase inhibitors (*4*).

Flavonoids also possess prooxidant properties that may contribute to tumor cell apoptosis and cancer chemoprevention (*5*). The mechanism for flavonoid prooxidant toxicity involves several peroxidases that catalyze the oxidation of polyphenols to phenoxyl radicals, which can cooxidize other relevant molecules, such as proteins, lipids, or nucleic acids (*6*).

One of the flavonoids more studied, by their its distribution in the nature, is quercetin. This compound possesses antioxidant and prooxidant activities, depending on concentration and on the source of free radicals; it has been suggested that the generation of reactive oxygen species (ROS) may be a common mechanism of toxicity for a variety of phenolic compounds (*7*). It is known that in the presence of lactic peroxidase, quercetin is oxidatively degraded to *O*-semiquinone and *O*-quinone and that these compounds facilitate the formation of superoxide and the depletion of GSH present in the medium (*8*). Also, it has been reported that products derived from the oxidative degradation of quercetin by polyphenol oxidase are responsible for the cooxidation of other biomolecules (*9*).

Because flavonoids, such as quercetin, and oxidases are present simultaneously in fruits and vegetables, the generation of quinoid derivatives in biological systems is plausible. This process is of great relevance from a biological point of view, because the conversion of supposed beneficial antioxidants, such as flavonoids, to electrophilic prooxidants may constitute a possible toxicological risk (*10*).

On the other hand, it is to be noted that, in addition to dioxygenase activity, lipoxygenase, a prooxidant enzyme involved in lipid peroxidation processes, possesses a peroxidase activity toward a wide range of compounds (*11*). Dioxygenase activity produces the insertion of oxygen into a polyunsaturated fatty acid containing a 1,4-*cis,cis*-pentadiene moiety, producing the corresponding lipid hydroperoxide. In the process, an intermediate peroxyl radical is generated. This compound, or the hydroperoxide, supports the cooxidase activity of lipoxygenase toward a suitable electron donor, which is transformed into a radical. This hydroperoxidase activity also can be observed in the presence of hydrogen peroxide instead lipid hydroperoxide, being related to xenobiotic oxidation processes (*12*−*14*). In addition, it is known that a variety of phenolic compounds and flavonoids with antioxidant properties are inhibitors of lipoxygenase (*15*). In this way, in previous works we reported that resveratrol, a polyphenolic with relevant antioxidant properties, produces the inhibition of dioxygenase

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activity of lipoxygenase, in a process in which resveratrol is cooxidized by the hydroperoxidase activity of this enzyme (*16*).

In this study we shows that two structurally related flavonoids (quercetin and naringenin) and a phytoalexin with relevant antioxidant properties (resveratrol) are cooxidized by the hydroperoxidase activity of lipoxygenase. Quercetin is the most efficient substrate for this oxidative process, giving rise to a potentially prooxidant quinoid species as the product of the catalytic reaction. Naringenin and resveratrol show less efficiency as substrates for hydroperoxidase activity of lipoxygenase but are potent inhibitors of the dioxygenase activity of the enzyme. Our results suggest that the efficiency of the catalytic process is mediated by the appropriate interaction between the catalytic center of the enzyme and the phenolic compound and also by the properties of these compounds as free radical scavengers.

MATERIALS AND METHODS

Chemicals. Quercetin, naringenin, resveratrol, GSH, and hydrogen peroxide were purchased from Sigma Aldrich. Lipoxygenase (9.4 units/mg) (LOX, EC 1.13.11.12) was purchased from Fluka. The hydrogen peroxide solutions were freshly prepared from a solution stock 11.7 M by dilution, and the final concentration was checked by spectrophotometric measurement at 240 nm using $\epsilon_{240} = 39.4\ M^{-1}\ cm^{-1}$.

Spectrophotometric Measurement of the Oxidation of Phenolic Compounds by Lipoxygenase. The oxidation of phenolics (quercetin, naringenin, or resveratrol) by lipoxygenase was followed spectrophotometrically by recording successive scans with a number of cycles and an interval between scans that is indicated in each experiment. The reaction was initiated by the addition of LOX (0.4 unit) to the reaction mixture containing the phenolic compound and H_2O_2 (50 μM). The cooxidative process of phenolics was carried out in the absence of hydrogen peroxide, using linoleic acid (100 μM) as cosubstrate. All spectrophotometric measurements were performed in 25 mM potassium phosphate buffer, pH 7.0, at room temperature.

Spectral Measurement of the Effect of GSH on the Phenolics Oxidation Catalyzed by the Peroxidase Activity of Lipoxygenase. Quercetin, naringenin, or resveratrol was added to 25 mM phosphate buffer, pH 7.0, in a spectrophometric cuvette, containing glutathione (195 μM), H_2O_2 (50 μM), and lipoxygenase (0.4 unit) in a final volume of 1 mL. This solution was mixed thoroughly and scanned by a UV−vis spectrophotometer.

Measurement of Dioxygenase Activity of Lipoxygenase. The standard assay mixture contained 15 milliunits of lipoxygenase and 100 μM linoleic acid in pH 7.0 potassium phosphate buffer to a final volume of 1 mL. The reaction was started by the addition of linoleic acid, and then the increase in absorbance due to the formation of hydroperoxy derivatives from polyunsaturated fatty acids was recorded. Enzymatic activity was calculated using $\epsilon = 25000\ M^{-1}\ cm^{-1}$ for the *cis,trans*-hydroperoxydiene of linoleic acid measured at 234 nm. One unit of lipoxygenase activity is defined as the quantity of enzyme that produces the oxidation of 1 μmol of linoleic acid min^{-1} (*16*).

Determination of Antioxidant Activity. The antioxidant activity of phenolics was determined using the Trolox equivalent antioxidant capacity (TEAC) method. This procedure gives a measure of the antioxidant activity by determination of the Trolox concentration which produces the same antioxidant activity that a 1 mM concentration of the studied compound does on the inhibition of the generation of the $ABTS^{\bullet+}$ radical. The method is based on the one described by Re et al. (*17*). Briefly, $ABTS^{\bullet+}$ radical was generated by persulfate oxidation of 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid (ABTS). For this, 7 mM ABTS was reacted with 2.45 mM potassium persulfate, and the mixture was allowed to stand at room temperature for 12−16 h to give a dark blue solution. This solution was diluted until 0.7 absorbance unit measured at 734 nm. One milliliter of this solution was mixed with 10 μL of the sample, and the absorbance was measured at 30 °C at 1, 4, and 6 min. The decrease of absorbance is plotted versus the antioxidant compound assayed to give a straight line. The concentration of antioxidants giving the same percentage of decrease in absorbance measured at 734 nm as 1 mM Trolox was regarded as TEAC.

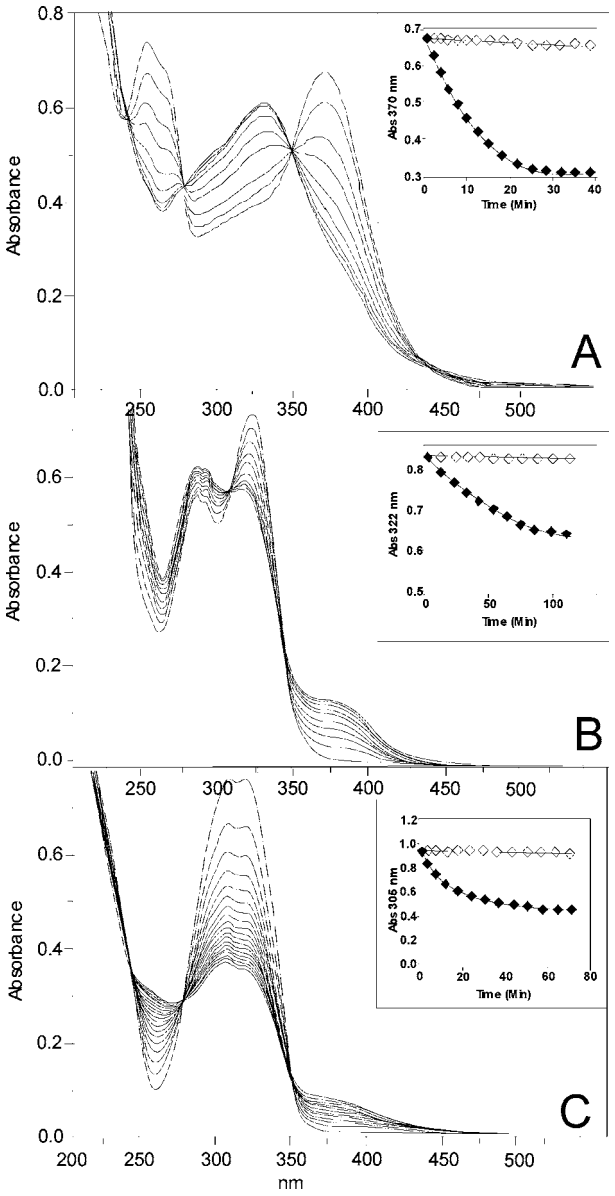

Figure 1. Oxidation of quercetin (**A**), naringenin (**B**), and resveratrol (**C**) by lipoxygenase in the presence of hydrogen peroxide. Quercetin, naringenin, and resveratrol were incubated in the presence of lipoxygenase and hydrogen peroxide in the conditions under Materials and Methods. The interval between scans was 6 min for quercetin and resveratrol and 10 min for naringenin. The inset in each panel corresponds to the time course of absorbance value measured at the maximum of absorbance (solid symbols) and the time course obtained in absence of enzyme, maintaining the same concentration of hydrogen peroxide (open symbols).

RESULTS AND DISCUSSION

When soybean lipoxygenase was incubated in the presence of hydrogen peroxide and quercetin, naringenin, or resveratrol, an oxidative decomposition of these molecules occurs. **Figure 1** shows the evolution the UV−vis spectra of quercetin (**A**), naringenin (**B**), and resveratrol (**C**) following the oxidative reaction by repetitive scans. The time course of the decrease in absorbance for each molecule, measured at the maximum of absorbance, is shown in the inset. When the reaction was carried out in the absence of lipoxygenase, the decrease in absorbance

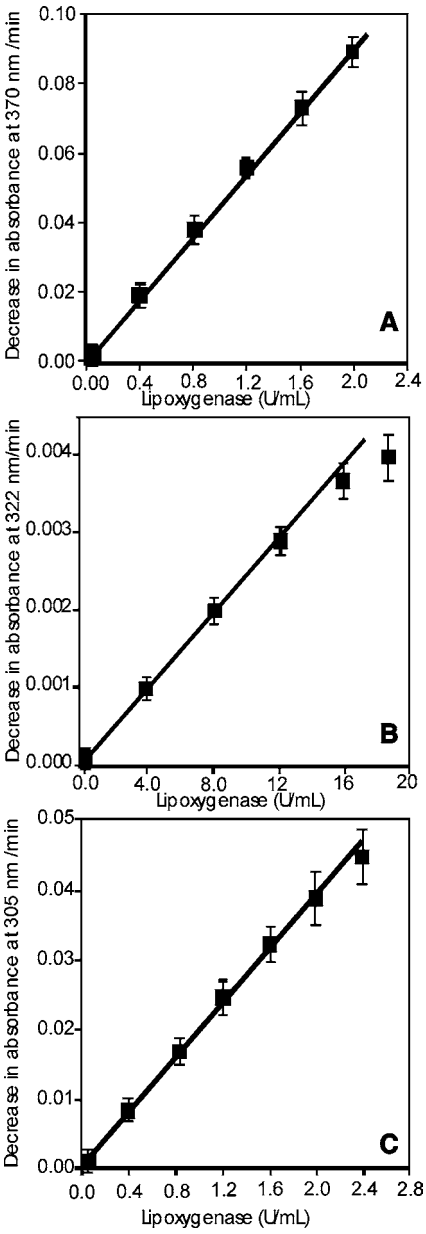

Figure 2. Effect of lipoxygenase concentration on the rate of oxidation of quercetin (**A**), naringenin (**B**), and resveratrol (**C**). Quercetin, naringenin, and resveratrol were incubated in the presence of hydrogen peroxide (50 μM) and lipoxygenase, in 25 mM potassium phosphate buffer, pH 7.0. The reaction started with the enzyme addition, and then the decrease in absorbance was measured at the wavelength indicated. Bars represent standard error of the mean ($n = 3$).

was negligible (see the open symbols in **Figure 1**). The enzymatic origin of this oxidative decomposition was confirmed by the study of the effect of the lipoxygenase concentration on the rate of substrate decomposition. **Figure 2** shows the linear correlation between lipoxygenase concentration in the incubation medium, maintaining constant the H_2O_2 concentration, and the rate of decomposition of quercetin, naringenin, or resveratrol measured spectrophotometrically at 370, 320, and 304 nm, respectively. The results obtained reveal relevant differences in the efficiency shown by quercetin, naringenin, and resveratrol as substrates of the hydroperoxidase activity of lipoxygenase. When this activity is expressed as micromoles of substrate oxidized per minute and milligram of enzyme, we can see that the rates of the oxidative decomposition of naringenin and resveratrol are 26 and 40%, respectively, of the rate shown by quercetin (**Table 1**). This behavior is coincident with the efficiency of the three molecules when they are assayed as inhibitors of dioxygenase activity of lipoxygenase. **Figure 3** shows the effect of quercetin, naringenin, and resveratrol concentrations on lipoxygenase activity. It can be see that quercetin shows the highest inhibitory potency, obtaining a 90% inhibition at concentration >10 μM. In contrast, at the same concentration, only a 40% inhibition is produced by resveratrol and only a 20% by naringenin. This is coincident with the capacity antioxidant of these molecules measured by the TEAC method (**Table 2**).

Table 1. Efficiency of Quercetin, Naringenin, and Resveratrol as Cosubstrates for Hydroperoxidase Activity of Lipoxygenase

compound	activity (μmol/min/mg)	efficiency (%)
quercetin	0.019	100
naringenin	0.005	26
resveratrol	0.008	42

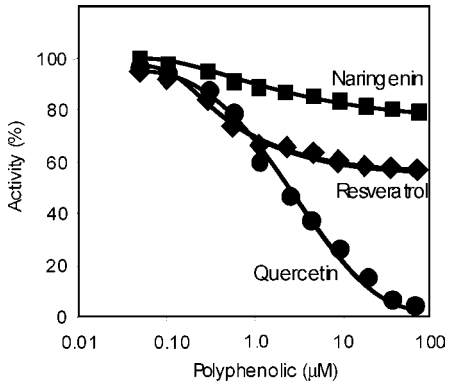

Figure 3. Effect of phenolic compounds concentration on dioxygenase activity of lipoxygenase. Dioxygenase activity of lipoxygenase was measured by recording the increase in absorbance at 234 nm using the procedure indicated under Materials and Methods. The incubation mixture contained the phenolics at the indicated concentration. One hundred percent activity corresponds to the generation of 2.6 μmol of hydroperoxy linoleic acid per minute and milligram of lipoxygenase.

Table 2. Antioxidant Capacity Measured by TEAC Method

compound	antioxidant capacity
quercetin	4.69 ± 0.07
naringenin	0.33 ± 0.01
resveratrol	2.68 ± 0.04

Taking together the results obtained, we can conclude that the peroxidase activity of lipoxygenase produces the oxidative decomposition of the known antioxidant flavonoids quercetin and naringenin and of the phytoalexin resveratrol.

It has been reported (*18*) that the flavanone naringenin caused the GSH oxidation in the presence of peroxidase and hydrogen peroxide. In this process the formation of a prooxidant phenoxyl radical has been suggested. On the other hand, quercetin, in the presence of horseradish peroxidase (HRP) and hydrogen peroxide, is converted to a quercetin−SG conjugate characterized by an absorbance peak centered at 335 nm, without the formation of a thyil radical. In contrast, when lactoperoxidase was used, instead of HRP, quercetin was converted to a prooxidant semiquinone (*19*). To check if the hydroperoxidase activity of lipoxygenase produces effects similar to or different from those described for other peroxidases, we incubated quercetin and lipoxygenase in the presence of hydrogen peroxide

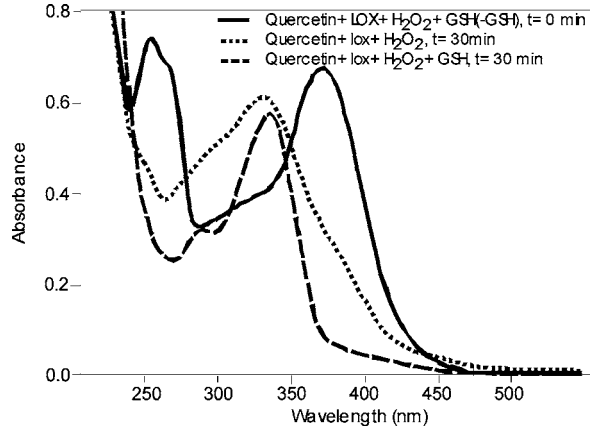

Figure 4. Effect of GSH on the quercetin oxidation by lipoxygenase in the presence of hydrogen peroxide. Quercetin (35 μM) was incubated in the presence of lipoxygenase (0.4 unit), hydrogen peroxide (50 μM), and GSH (200 μM) in 25 mM potassium phosphate buffer, pH 7.0, to a final volume of 1 mL. The continuous line reflects the initial conditions of incubation ($t = 0$ min) in the presence or absence of GSH (GSH does not modify the absorption spectrum). After 30 min of incubation, the spectrum represented by a dashed line was obtained. The dotted line corresponds to the spectrum of the mixture after 30 min of incubation in absence of GSH.

and GSH (**Figure 4**). The analysis of the spectra obtained suggests the formation of a quercetin−SG conjugate characterized by a maximum at 335 nm. These results are coincident with those obtained by Galati et al. (*19*) for quercetin oxidation in the presence of HRP/H_2O_2. It can be seen that the peak of quercetin centered at 375 nm, caused by the B-ring absorption, is shifted to 335 nm, when quercetin was incubated with lipoxygenase/H_2O_2. In addition, the peak and shoulder centered at 256 and 267 nm, respectively, produced by the A-ring and dihydroxy substitution of the B-ring of quercetin, decrease concomitantly with the 375 nm peak. It is to be noted that the peak centered in the region of 335 nm is present when quercetin is oxidized by lipoxygenase/H_2O_2 in the presence or in the absence of GSH. However, the origins of these peaks are not the same. When Galati studied the oxidation of quercetin by HRP/H_2O_2, he obtained the same pattern, the peak centered at 335 nm, obtained in the presence of GSH, being attributed to the formation of a quercetin−SG, and the peak obtained at the same wavelength, but in absence of GSH, to the formation of an *O*-quinone (*19*). To date, this is the first time that the oxidative decomposition of quercetin by hydroperoxidase activity of lipoxygenase has been reported, suggesting that in the presence of the lipoxygenase/H_2O_2 system quercetin is oxidized to a quinoid product. It is remarkable that this behavior is not shown by naringenin or resveratrol, probably due to the different redox potentials of these compounds.

It is of great interest to check if the oxidative decomposition of quercetin in the presence of lipoxygenase is produced in the presence of linoleic acid. It is known that the hydroperoxidase activity of lipoxygenase produces the cooxidation of suitable donors in the presence of the hydroperoxides of linoleic or arachidonic acid, the natural substrates for this enzyme.

Linoleic acid is the natural substrate for plant lipoxygenase and is present in many vegetal foods together with flavonoids and the enzyme. For this, it is of interest to check if lipoxygenase or linoleic acid produces the oxidative decomposition of flavonoids in a mode similar to the one observed in the presence of H_2O_2. The pattern of the spectral changes observed for the cooxidation of quercetin, naringenin, and resveratrol (**Figure 5**, panels **A**, **B**, and **C**, respectively) is coincident with the one obtained for the oxidative process in the presence of hydrogen peroxide (**Figure 1**), although the efficiency of the process is less for quercetin and naringenin when linoleic acid is present than in the presence of hydrogen peroxide. In contrast, the results obtained for resveratrol when linoleic acid or hydrogen peroxide is used as cosubstrate are close. When quercetin is incubated in the presence of lipoxygenase and linoleic acid (**Figure 6**), a decrease of the band centered at 375 nm is produced, together a slight increase of absorbance in the region of 330−340 nm. When the incubation was carried out in the presence of GSH, the peak at 375 nm was displaced to 335 nm. This pattern is

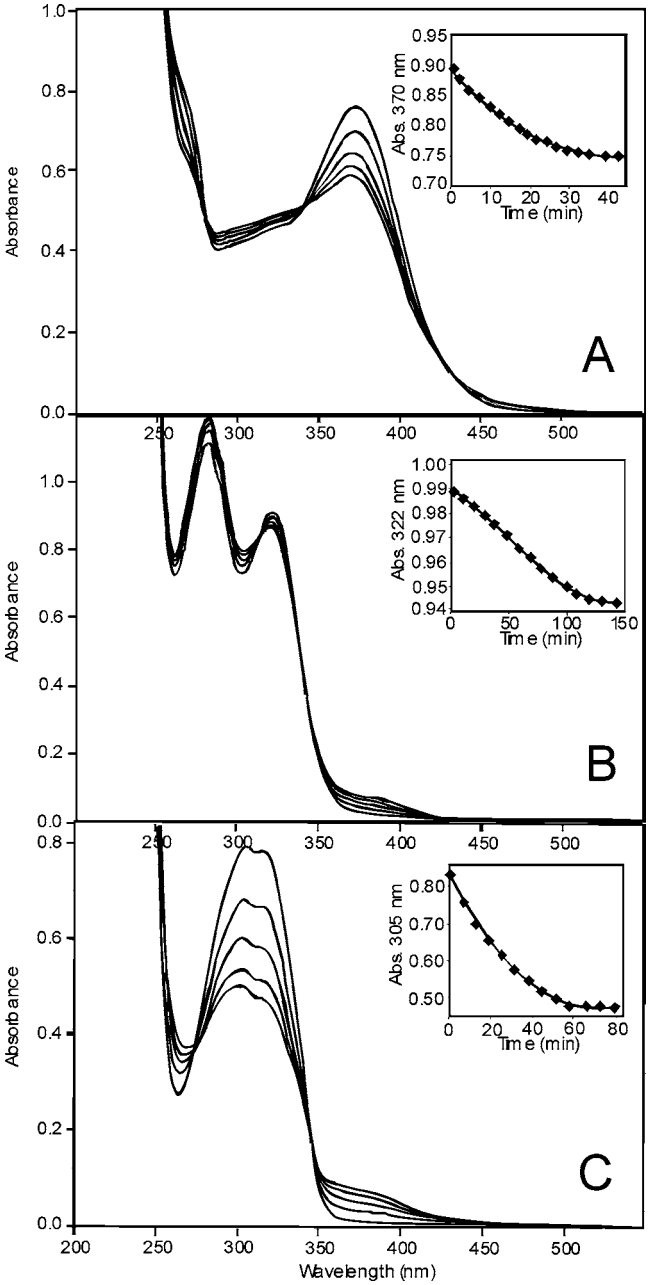

Figure 5. Oxidation of quercetin (**A**), naringenin (**B**), and resveratrol (**C**) by lipoxygenase in the presence of linoleic acid. Quercetin, naringenin, and resveratrol were incubated in the presence of lipoxygenase and 100 μM linoleic acid in the conditions described under Materials and Methods. Spectra were recorded at 6 min intervals for quercetin and resveratrol and at 10 min for naringenin. The inset in each panel corresponds to the time course of absorbance value measured at the maximum of the initial spectrum.

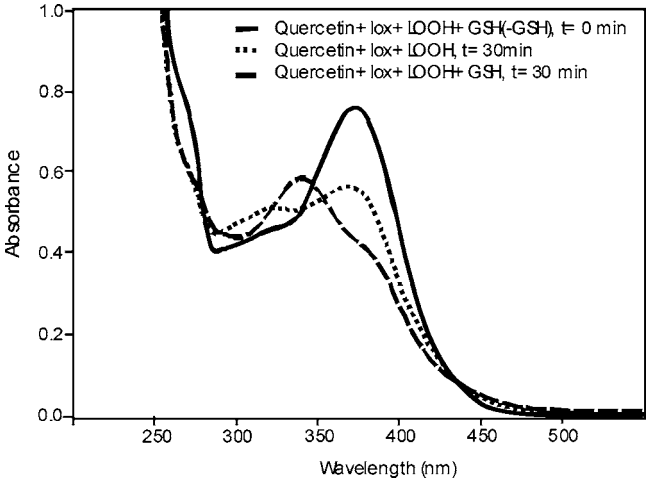

Figure 6. Effect of GSH on the quercetin oxidation by lipoxygenase in the presence of linoleic acid. Quercetin (35 μM) was incubated in the presence of lipoxygenase (0.4 unit), hydrogen peroxide (100 μM), and GSH (200 μM) in 025 mM potassium phosphate buffer, pH 7, in a final volume of 1 mL. The continuous line reflects the initial conditions of incubation (t = 0 min). After 30 min of incubation, the spectrum represented by dashed line was obtained. The dotted line corresponds to the spectrum of incubation mixture after 30 min of incubation in the absence of GSH.

similar to the one obtained when H_2O_2 was present in the incubation medium. However, the catalytic efficiency of the process is lower than that obtained in the presence of hydrogen peroxide, probably due to the inhibition of dioxygenase activity of lipoxygenase by quercetin (*2*). In addition, the hydroperoxidase activity of the enzyme is inhibited by the presence of quercetin near the active site, hindering the correct interaction of lipoxygenase and linoleic acid. This behavior is coincident with the previously reported effect of resveratrol on dioxygenase and the hydroperoxidase activity of lipoxygenase (*16*). Although hydroperoxidase activity was not inhibited by resveratrol, the rate of oxidative decomposition of resveratrol by the lipoxygenase/H_2O_2 system was higher than that obtained when linoleic acid hydroperoxide was present in the medium.

On the basis of the results obtained we can conclude that in the presence of hydrogen peroxide or hydroperoxylinoleic acid, lipoxygenase produces a quinoid product as a result of the enzymatic oxidation (in the presence of hydrogen peroxide) or cooxidation (in the presence of linoleic acid) of quercetin.

To check if the cooxidation process in the presence of linoleic acid is comparable with the oxidation in the presence of hydrogen peroxide, lipoxygenase was incubated in the presence of linoleic acid until total conversion of the substrate to the hydroperoxylinoleic acid; then, the phenolic compound was added to the medium, and the time course of the oxidative decomposition was measured at 370, 320, or 304 nm for quercetin, naringenin, or resveratrol, respectively. In conditions of reaction similar to those used when hydrogen peroxide was the cosubstrate, the time course of the oxidation of the phenolics was recorded (**Figure 7**). The results obtained when linoleic acid, lipoxygenase, and the assayed phenolics are simultaneously present when the reaction starts (**Figure 7A**) were compared with those obtained when phenolics were added after the conversion of linoleic acid to hydroperoxylinoleic acid (**Figure 7B**). It can be seen that for quercetin and naringenin the process is more efficient when linoleic acid has been totally converted to the corresponding hydroperoxy by the dioxygenase activity of lipoxygenase. In contrast, for resveratrol, the results obtained

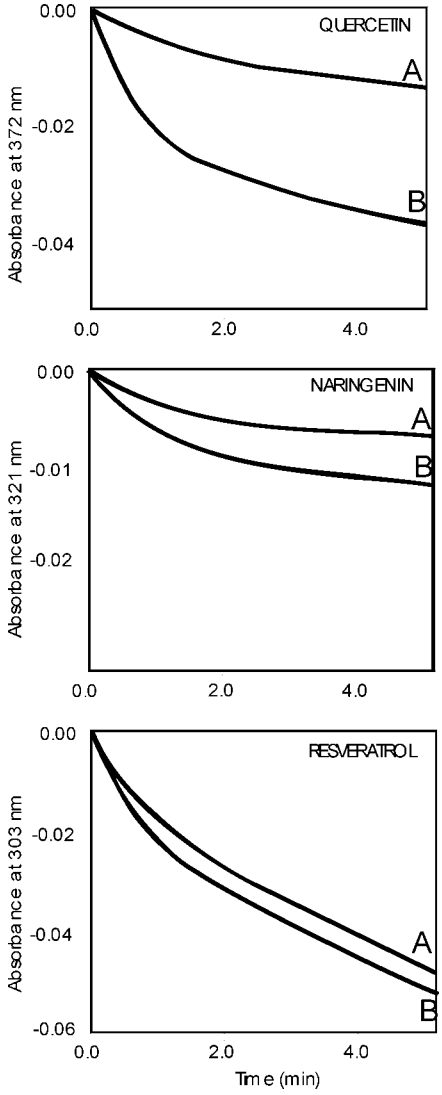

Figure 7. Effect of hydroperoxylinoleic acid. Lipoxygenase (0.4 unit) and linoleic acid (100 μM) were incubated in the presence of 35 μM quercetin, naringenin, or resveratrol in 25 mM phosphate buffer, pH 7.0. In each figure, plot A was obtained when all components of the incubation mixture were present simultaneously from the start of the reaction. Plot B was obtained when quercetin, naringenin, or resveratrol was added to the medium after the total conversion of linoleic to the corresponding hydroperoxylinoleic acid (5 min of preincubation).

in both conditions are very close. The great differences observed for quercetin between the processes carried out in the presence of linoleic acid (**A**) or in the presence of the hydroperoxiderivative (**B**) confirm that the low efficiency obtained in the presence of linoleic acid is caused by the inhibitory effect of quercetin on dioxygenase activity. This suggests that lipoxygenase in the presence of linoleic acid can cooxidize flavonoids in a process with characteristics similar to those described for other peroxidases (*8*).

The differences shown by quercetin, naringenin, or resveratrol in this cooxidative process may be attributed to the differences in their molecular structures. In previous works (*20*) it has been reported that the efficiency of flavonoids as lipoxygenase inhibitors is related with the planarity of the molecule, showing that quercetin possesses a marked character planar and a high efficiency as lipoxygenase inhibitor. In addition, the character planar of resveratrol has been also reported (*21*). Likewise, the nonplanar structure of the stable form of naringenin has been

reported. This molecule shows a slight torsion and bending between the double-hexagon ring part and the single-hexagon ring part (*22*). It is to be noted the correlation obtained between the efficiency of quercetin, naringenin, and resveratrol as substrates of hydroperoxidase activity of lipoxygenase (see **Table 1**) and the efficiency of these compounds as inhibitors of dioxygenase activity of lipoxygenase (see **Figure 3**). These results strongly suggest that both effects are mediated by the correct interaction between phenolics and the active site of the enzyme, the character planar being, probably, one of the more determinant factors that conditionins this effect. Another factor to consider is the OH position in these molecules: quercetin, the more efficient inhibitor of dioxygenase activity and the best substrate for hydroperoxidase activity, possesses a catechol ring and similar substitution of OH in the A ring (positions 5 and 7). From the results obtained for quercetin and naringenin we could deduce that the catechol group may be determinant in the efficiency of the assayed molecules, but resveratrol, with an OH distribution close to that shown by naringenin, is, in opposition to this molecule, more efficient as a dioxygenase inhibitor and a better substrate for hydroperoxidase activity than naringenin.

In conclusion, the results obtained in this work reveal that hydroperoxidase activity of lipoxygenase produces the oxidative decomposition of phenolic compounds in the presence of hydroperoxides (hydrogen peroxide or hydroperoxylinoleic acid). In this process the dioxygenase activity of lipoxygenase is inhibited, quercetin being the more efficient lipoxygenase inhibitor.

The results obtained suggest that the antioxidative mechanism of assayed phenolics compounds is mediated by this oxidative decomposition. Because of the high content of lipoxygenase in vegetables and the fact that this enzymatic activity is a pathway for oxidative decompositions, the use of phenolics as natural antioxidant additives for foods from vegetal sources may be a valid alternative to other chemical additives. However, the possible generation of prooxidant species derived from the oxidative decomposition of flavonoids by the hydroperoxidase activity of lipoxygenase needs further research to determine the potential toxicological consequences of these reactive species in foods.

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