Enzymatic Polymerization of Phenolic Compounds Using Laccase and Tyrosinase from *Ustilago maydis*

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Flavonoids are a big group of polyphenols of low molecular weight with in vitro antioxidant properties. In this study, the laccase and tyrosinase from *Ustilago maydis* were partially characterized and their effect on the antioxidant activity of some phenolic compounds was investigated. Since enzymatic polymerization of the phenolic compounds was detected, the size of the aggregates was determined and related to their antioxidant activity. Morphology of the polymers was analyzed by atomic force microscopy. The results showed that the laccase- and tyrosinase-catalyzed polymerization of quercetin produced aggregates with relatively low molecular weight and higher antioxidant activity than the monomeric quercetin. In the case of kaempferol, the aggregates reached higher sizes in the first 2 h of reaction and their antioxidant activity was increased. In the last case, the aggregates adopted fractal-ordered shapes similar to coral in the case of the kaempferol−laccase system and to fern in the case of the kaempferol−tyrosinase system. The kaempferol and quercetin polymers at low concentration had strong scavenging effect on Reactive oxygen species (ROS) and inhibition of lipoperoxidation in human hepatic cell line WRL-68.

Introduction

Free radicals are generated in vivo for specific metabolic purposes, but they become a problem when there is an imbalance between the generation of free radicals and the defense mechanisms of the organism. In this case, oxidative damage is produced in target cells.[1] Antioxidants are the defense against free radicals and their oxidative damage, and an increased interest in the use of natural antioxidants as food additives has arisen in the last years.[2,3]

Flavonoids are an extensive group of phytochemical phenolic compounds of relatively low molecular weight and in vitro antioxidant properties which depend on the number and position of the hydroxyl groups in the molecule.[4] Flavones, flavonols (rutin, kaempferol, and quercetin), and anthocyanins belong to this group.

The corn smut *Ustilago maydis* (Persoon) Roussel, commonly known in Mexico as huitlacoche, has been widely studied both as a food source and as a phytopathogen. Several additional applications have been found for the corn smut metabolites, including environment remediation and nutraceutical uses.[5] The fungus has enzymes such as laccase and tyrosinase which have been used mainly in bioremediation, but their catalytic properties could lead to the generation of interesting food products.

Laccase (*p*-diphenol oxygen oxidoreductase, E. C. 1.10.3.2) catalyses the oxidation of *p*-hydroxyphenols and tyrosinase (monophenol dihydroxyphenylalanine oxygen oxidoreductase, E. C. 1.14.18.1) of monophenols. Both enzymes act on a great variety of substrates and their activity depends on copper.[6,7] There are several advantages in the enzymatic polymerization of phenolic compounds: (i) the reaction takes place in the absence of toxic reagents such as formaldehyde, (ii) enzymes catalyze the polymerization of a great variety of phenol monomers, (iii) the phenolic compounds with more than two reactive groups can be polymerized selectively, and (iv) the structure and solubility of the polymer can be controlled by changing the conditions of the reaction.[8]

The enzymatic oxidative polymerization of different 4-hydroxybenzoic acid derivatives has been carried out with the use of laccases,[9] and these enzymes have also been used to oxidatively polymerize rutin (quercetin-3-rutinoside) to produce poly(rutin), a flavonoid polymer with superior antioxidant properties compared with the rutin monomer.[10] There are many studies on the antioxidant activity of low-molecular weight flavonoids; however, few studies have been performed on polymeric flavonoids. Hence, the purpose of this work is the study of enzymatic polymerization of phenolic compounds and their effect on the antioxidant activity and protection effects from oxidative injury of hepatic human cell line WRL-68.

Materials and Methods

Material. Corn cobs infected with *Ustilago maydis* were bought at the Central de Abastos market in Mexico City. The galls were separated by hand and maintained at −20 °C until used. Rutin, gallic acid, cafeic

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acid, vanillin, and quercetin were obtained from Sigma Chemical Co. (St. Louis, Mo), and kaempferol was purchased from Fluka (Buchs, Switzerland).

Preparation of the Raw Enzyme Extract. The raw extract was obtained by a modification of a fractional precipitation method used for polyphenol oxidase from grapes.[11] *Ustilago maydis* galls (1000 g) were crushed in 1 L of buffer A (0.1 M sodium phosphate + 0.03 M ascorbic acid, pH 6.5 + 100 g polyvinylpolypyrrolidone) and homogenized for 15 min. After this, 2 L of buffer B (0.1 M sodium phosphate, pH 6.5 + 0.1% Triton X-100 + 25 g PVPP) were added, and the mixture was homogenized for 15 min and centrifuged at 10 000*g* for 45 min. The precipitate was discarded, and solid ammonium sulfate was added to the supernatant until 95% saturation. After centrifuging at 10 000*g* for 45 min, the new precipitate was dialyzed against 0.01 M phosphate buffer (pH 8). The dialyzed sample was concentrated at 4 °C by ultrafiltration with Amicon cells equipped with a 10-kDa cutoff membrane filter under a high purity nitrogen atmosphere at a pressure of 517.1 kPa.

Enzyme Purification. Two milliliters of ultrafiltrate were applied to a Sephadex G-150 column (15 × 500 mm) equilibrated in 0.01 M phosphate buffer (pH 8). The collected fractions were analyzed and selected on the basis of laccase activity. The laccase-containing fractions were pooled and concentrated by ultrafiltration[12] and analyzed for enzyme activity, and the protein content was measured by the Lowry method.[13]

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS−PAGE) was performed to know the number and relative molecular weight of the proteins in the extract. One milliliter of ultrafiltered enzyme extract was applied to a 10 mL Concanavaline A − Sepharose affinity column equilibrated in binding buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$, pH 7.4). The extract was injected at a 0.1 mL/min flow rate and a lineal gradient from 0 to 100% was formed with the elution buffer (0.5 M methyl-α-D-glucopyranoside, 20 mM Tris-HCl, 0.5 M NaCl, pH 7.4).[14] The fractions were selected on the basis of simultaneous laccase and tyrosinase activity for further concentration by ultrafiltration and homogeneity assay by SDS−PAGE. To increase the molecular homogeneity, the selected fractions were reintroduced into the affinity column and the new fractions were selected on the basis of higher enzyme activity and concentrated by ultrafiltration before being assayed by SDS−PAGE.

Enzyme Activity. *Laccase Activity.* It was measured by mixing 25 μL of the sample and 225 μL of freshly prepared 2 mM ABTS (diammonium 2,2′-azino-bis(3-ethyl-benzothiazoline-6-sulfonate)) in 0.01 M citrate buffer (pH 3) and recording the increase in absorbance at 420 nm every 30 s for five minutes at 20 ± 2 °C. A blank (250 μL of ABTS solution) was included also in the assay.[15]

Tyrosinase Activity. It was measured by mixing 25 μL of the sample and 225 μL of freshly prepared 50 mM L-DOPA (3,4-dihydroxy-L-phenylalanine) in 50 mM Tris-HCl buffer (pH 7.5) and reading the increase in absorbance at 475 nm every 30 s for five minutes at 20 ± 2 °C. A blank (250 μL of L-DOPA solution was also included in the determination.[16]

Molecular Weight. The relative molecular weight of the enzyme was determined by SDS−PAGE using a 12.5% gel concentration, staining with Coomasie blue G-250 and destained with methanol: acetic acid: water 3:1:6 v/v/v.[17] SDS-PAGE markers for molecular weights 14.4−97 kDa (Amersham Pharmacia Biotech, Piscataway, NJ) were used to determine the relative molecular weight of the bands. Staining with catechol and 20 mM ABTS was also used to detect the enzyme activity of the bands.

Isoelectric Point. The isoelectric point of the purified enzymes was determined with a Bio-Rad model 111 Mini Isoelectric Focusing cell with a set of ampholines pH 3.0−9.0.[18]

Denaturation Temperature. A high-sensitivity differential scanning calorimeter (Micro DSC III, Setaram, Saluire, France) was used to determine the transition enthalpy and the denaturation temperature of *U. maydis* laccase. A water solution (pH 7) containing 918.1 mg of the laccase purified preparation was introduced in the DSC cell. The scanning temperature range was from 5 to 120 °C at a rate of 1 °C/min. The cycle was repeated with the same sample to corroborate the irreversibility of the denaturation process.[19,20]

Surface Hydrophobicity. Ammonium 8-anilino-1-naphthalene sulfonate (ANS, Aldrich, Saint Louis, USA) was used as a probe of protein surface hydrophobicity. Each protein sample was serially diluted with 0.1 M phosphate buffer (pH 7) to concentrations ranging from 0.005 to 0.05% in a 2 mL final volume. Increasing volumes of freshly prepared 8 mM ANS in 0.1 M phosphate buffer (0.1 M) ranging from 5 to 40 μL were added to 2 mL of the solution with the highest protein concentration until the fluorescence intensity reached a constant value and the ANS volume correspondent to this constant value was added also to all the other dilutions. The fluorescence intensity was measured at the excitation wavelength of 364 nm and the emission at 484 nm using a Perkin-Elmer LS 45 luminescence spectrophotometer. The initial slope of the plot of fluorescence intensity vs protein concentration (%) was used as an index of the protein hydrophobicity.[21]

Antioxidant Activity. The antioxidant activity of the different substrates along with the raw enzyme extract were determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH•). This radical has an absorption band at 515 nm, which decreases upon reduction by an antioxidant compound. These changes in color (from intense violet to light yellow) were followed spectrophotometrically and the antioxidant activity calculated.[22]

Hepatic Cell Line Culture and Treatment. The cells used belonged to the human hepatic cell line WRL-68 and were grown and maintained in Dulbecco's modified Eagle' medium (DMEM) supplemented with 10% bovine foetal serum. The cells were grown for 48 h and then treated with H_2O_2 (30% w/v) for 10 min. The H_2O_2 medium was then removed and fresh medium without H_2O_2 was added which contained the substances to be analyzed (i.e., polymers produced enzymatically with laccase−quercetin, laccase−kaempferol, tyrosinase−quercetin, and tyrosinase−kaempferol systems). This mixture was left to act for 2 h after which the culture medium with the polymers was removed and PBS solution with 1.5% protease inhibitors was added. Using a Newbauer chamber, 10^5 cells per ROS (reactive oxygen species) assay were counted. For the membrane lipids peroxidation assay the volume containing 50 μg protein was determined. In each case the cells were recovered and stored at 4 °C.[23]

Measurement of Reactive Oxigen Species (ROS). This analysis was done using the method based on oxygenization of dihydrorodamine 123 by ROS to form the rodamine-123 fluorescent compound identified in a plate reader at 505 nm.[24]

Buffer (180 μL) containing 140 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 1.8 mM $CaCl_2$, 5 mM glucose, 15 mM HEPES, pH 7.4 and 20 μL dihydrorodamine-123 stock solution (1 μM final concentration) were added to the 10^5 cells found previously. These were then resuspended and transferred to an ELISA NUNC MaxiSorp plate, incubated at 37 °C, and the plate read (Microplate Reader, Bio-Rad mod. 5500) with a 505 nm excitation filter.

Determination of Lipid Peroxidation in Human Hepatic Cell Line WRL-68. The membrane lipid peroxidation assay was done using malondialdehyde (MDA) quantification with the thiobarbituric acid (TBA) method.[25]

To a volume equivalent to 50 μg of the previously described treated cells was added an equal volume of trichloroacetic acid (TCA) to precipitate the proteins. This was centrifuged, thiobarbituric acid (TBA) (0.375% TBA and 2% acetic acid) was added to the supernatant, and this solution was boiled for 45 min. A quantity of 200 μL of each sample was then placed on an ELISA plate and read at 532 nm (Pharmacia Biotech Ultraspec 3000).

Effect of the Laccase and Tyrosinase on Different Substrates. The antioxidant activity of different phenolic substrates (rutin, gallic acid, caffeic acid, vanillin, kaempferol, and quercetin) was measured in the presence of the raw enzyme extract at different intervals for 6 or 24 h at 30 °C. The reaction mixture contained: for the first series, 500

μL 0.01 M acetate buffer (pH 5) and 500 μL of substrate solution at different concentrations; for the second series, 500 μL of the same buffer, 440 μL of substrate solution at different concentrations, 50 μL of enzyme extract and 10 μL 0.1 mM copper sulfate, unless the contrary is indicated. An aliquot (37.5 μL) of the different samples was added to 1.5 mL of freshly prepared DPPH$^\bullet$ solution (0.025 g/L in methanol). Absorbance at 515 nm was measured immediately (time = 0 min) on a GBC UV−vis 918 spectrophotometer, and 20 min after the reaction was started at 30 °C in the dark, the decrease in absorbance was recorded. Methanol was used as the blank. Antioxidant activity was calculated with the following formula:[26]

$$\% \text{ inhibition} = \left(\frac{A_B - A_A}{A_B}\right)100$$

where A_A = absorbance at $t = 0$ min, A_B = absorbance at $t = 20$ min

Size of the Aggregates. The samples (1.5 mL) were filtered with 0.25 μm filters prior to measurement with the dynamic light scattering capability of the Zetasizer Nano System (Malvern Instruments Ltd., Worcestershire, U.K.), and the data collection and calculations of particle size and molecular weight were managed using the Dispersion Technology Software for the Zetasizer Nano System.

Polymer Isolation. The polymers obtained with quercetin and kaempferol, both with enzymatic extract and the pure enzymes, were collected, centrifuged, and washed four times with deionized water. The precipitate was lyophilized and then used in infrared spectroscopy, ROS determination and cell lipoperoxidation.

Infrared Spectroscopy. IR spectra were recorded on a Perkin-Elmer Spectrum 2000 (FTIR spectrophotometer).

Atomic Force Microscopy (AFM). These experiments were carried out using a Nanoscope IIIa AFM (Digital Instrument, Veeco, Santa Barbara, Cal). V-shaped silicon nitride tips of a 0.3 N/m force were used in contact mode. A 10 × 10 μm scanner was used for all of these measurements. The scan frequency was typically 2−10 Hz per line, and the image analyses were performed by using the software provided by Digital Instruments. The samples were deposited on mica by dropping a few microliters of protein solution. After a few minutes the mica was washed with water in order to avoid high concentrations of salt either from the buffer or additives.

All of the experiments were performed in triplicate.

Results and Discussion

Two proteins were purified. A laccase with a 99 kDa molecular weight, an isoelectric point of 5.5, a surface of hydrophobicity of 75, and a denaturation temperature of 99.31 °C, and a tyrosinase with a 20.8 kDa molecular weight, an isoelectric point of 6.3, a surface of hydrophobicity of 217, and a denaturation temperature of 91.97 °C.

Antioxidant Activity. Figure 1 shows the effect of the raw enzyme extract addition on the antioxidant activity of 20 mM rutin at pH values of 5 and 7. It can be observed that in all cases, the enzyme improved the antioxidant activity. This is probably due to the synthesis of poly(rutin) by a laccase-catalyzed oxidative polymerization.[10] The products with the highest antioxidant activity were formed at pH 5.

The addition of Cu^{2+} (1 μM) increased the enzyme extract activity. This ion was included in the reaction mixture since a previous report[27] indicated that the flavonoids that act as tyrosinase inhibitors do so by sequestering the copper ions from the enzyme active site. When the enzyme extract was absent, the copper ion showed prooxidant activity at pH 5. This kind of activity has been studied before in the case of flavonols such as quercetin which are oxidized by Cu^{2+} in aqueous media.[28] In this study, it can be observed that the polymerization at pH 5 in the presence of the enzyme extract generates a higher

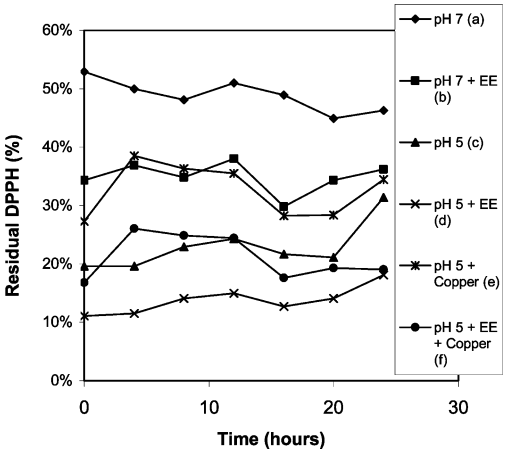

Figure 1. Effect of the raw enzyme extract (EE) addition on the antioxidant activity of 20 mM rutin at pH 5 and 7. Legends followed by different letters are statistically different ($p \leq 0.05$).

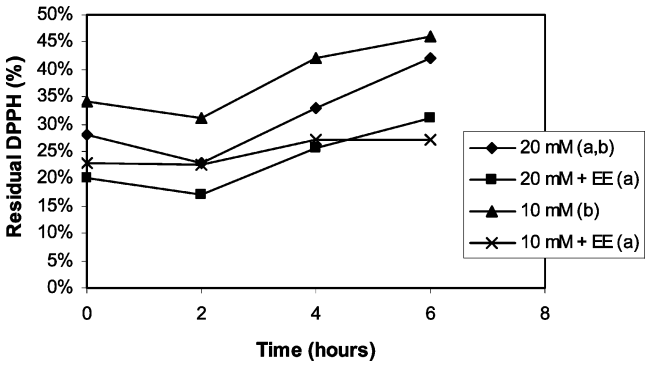

Figure 2. Effect of the raw enzyme extract (EE) addition on the antioxidant activity of gallic acid (10 and 20 mM). Legends followed by different letters are statistically different ($p \leq 0.05$).

antioxidant activity than at pH 7. Generally, flavonoids are autooxidized in aqueous media and can form highly reactive OH$^\bullet$ radicals in the presence of transition metals. This depends on the environment and the kind of flavonoid and transition metal. Rutin is able to chelate Cu^{2+} probably through its catechol structure, though this effect can be reversed by adding EDTA.[29]

Figure 2 shows the effect of the addition of the raw enzyme extract on the antioxidant activity of gallic acid at two concentrations, pH 6 and in the presence of 1 μM copper sulfate. A pH value of 6 was used since this is the pH of maximal enzyme activity when gallic and caffeic acids were used as substrates. The amount of residual DPPH$^\bullet$ was higher when 10 mM gallic acid was used compared with a 20 mM concentration; that is, the antioxidant activity was smaller. The addition of the enzyme preparation increased this activity for both concentrations. After 2 h of reaction, the antioxidant activity shows a tendency to decrease for both concentrations. This activity tended to improve when the gallic acid concentration was higher or the enzyme extract was added. In this study, in the absence of enzyme, 10 and 5 mM concentrations of gallic acid decreased the DPPH radical concentration in 66 and 72%, respectively; when the enzyme was added, the antioxidant activity was improved and the final concentrations were 77 and 80%. It has been shown that the addition of zinc up to a 1:1 molar ratio with respect to gallic acid significantly enhanced the rate constant for the reaction between gallic acid and DPPH$^\bullet$. Under these conditions, gallic acid was able to decrease the concentration of the DPPH radical by 65% after 20 min and the addition of Zn did not significantly increased the amount of radical

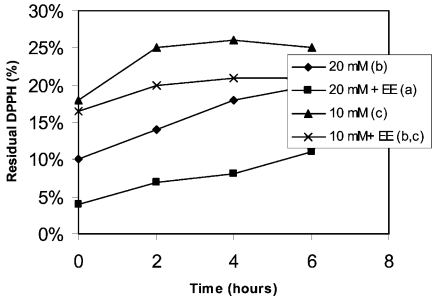

Figure 3. Effect of the raw enzyme extract (EE) addition on the antioxidant activity of caffeic acid. Legends followed by different letters are statistically different ($p \leq 0.05$).

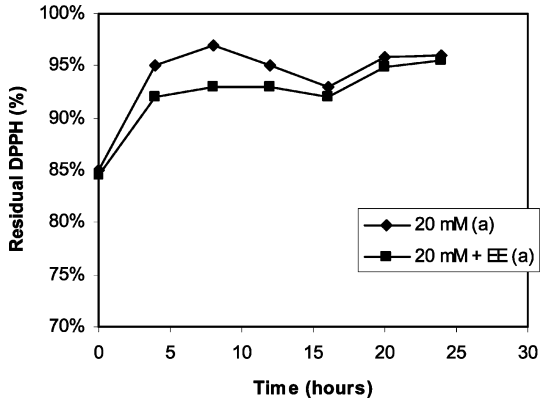

Figure 4. Effect of the raw enzyme extract (EE) addition on the antioxidant activity of vanillin. Legends followed by different letters are statistically different ($p \leq 0.05$).

scavenged.[30] In our study, the loss of antioxidant activity after 2 h could be due to the copper-catalized oxidation of gallic acid to its hydroquinone. This has been previously reported for systems in the presence of Fe^{3+} ions.[31]

Figure 3 shows the effect of the addition of the raw enzyme extract on the antioxidant activity of caffeic acid at different concentrations, pH 6 and 1 μM copper sulfate. In this case, as in the experiment with gallic acid, the highest concentration presented the highest antioxidant activity. It can also be observed that there is a decrease in antioxidant activity with time which is proportional to the acid concentration.

Caffeic and gallic acids are structurally similar, since both have a catechol skeleton though gallic acid has an additional hydroxyl group. This fact could lead to similar reaction mechanisms as in the case of the reaction with iron(III) consistent with the formation of a 1:1 complex which subsequently decays through an electron-transfer reaction as in gallic acid.[32] In this study, it is possible that a quinone could be formed with the copper which could lead to a polymerization reaction.

Figure 4 shows the effect of the addition of the enzyme raw extract on the antioxidant activity of vanillin at pH 5 and 1 μM $CuSO_4$. The first 5 h of reaction are characterized by a sharp increase in the residual DPPH•, reaching an almost constant value after that. This means that the enzyme addition did not keep or improve the initial antioxidant activity of the DPPH•.

Figures 5 and 6 show the effect of the addition of the enzyme raw extract on the antioxidant activity of kaempferol and quercetin at different concentrations, pH 5 and 1 μM copper sulfate. It can be observed in both cases that the higher the concentration of the phenolic compound, the higher the antioxidant activity. The higher concentrations (20 mM) resulted in 20−30% residual DPPH•, the 10 mM concentrations resulted

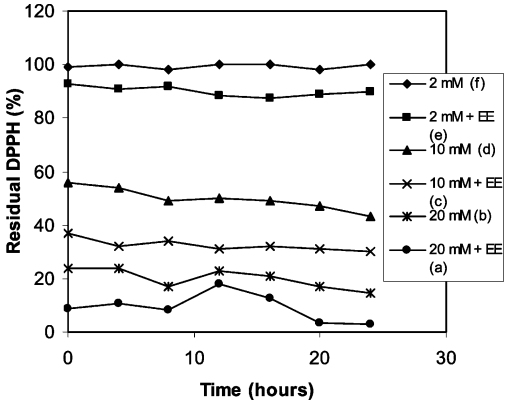

Figure 5. Effect of the raw enzyme extract (EE) addition on the antioxidant activity of kaempferol. Legends followed by different letters are statistically different ($p \leq 0.05$).

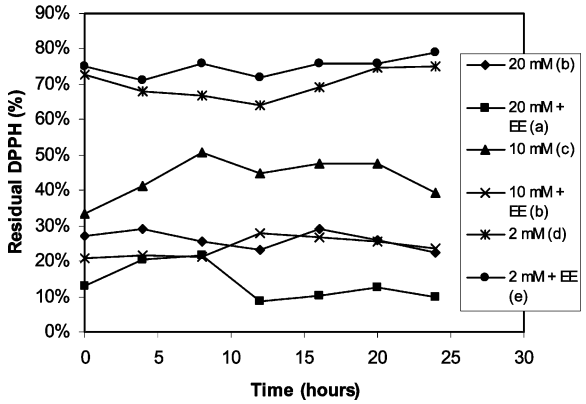

Figure 6. Effect of the raw enzyme extract (EE) addition on the antioxidant activity of quercetin. Legends followed by different letters are statistically different ($p \leq 0.05$)

in 40−60%, and the 2 mM concentrations resulted in values higher than 75%. Quercetin, as reported in many other studies, has the highest antioxidant activity. In both cases, the addition of the enzyme extract increased the antioxidant capacity. There are some reports indicating that the degradation of quercetin in the presence of copper is concentration-dependent and that the interaction of quercetin with transition metals is stronger than the interaction with rutin, with the hydroxyl group in position 3 being the key element for the copper-mediated degradation. This interaction is nonreversible and has additional effects on the hydroxyl groups of ring B.[29,33] It has also been proposed that the oxygen of the hydroxyl group at the C3 position and the oxygen of the carbonyl group at the C4 position can bind the copper at the active site of the enzyme to inhibit it.[27] The interaction of copper with kaempferol is weaker than its interaction with quercetin or rutin due to the absence of a catechol-like structure, which is very important for the metal chelation.[29] Some research has been conducted on the antioxidant activity of protein-bound quercetin; however, free quercetin had a higher antioxidant activity.[34] In this study, it is more likely that the flavonoids and phenolic compounds have acted as substrates for the enzymes and that no covalent binding between them took place, since in all cases the antioxidant activity was improved.

Figure 7 shows the effect of the addition of purified laccase and tyrosinase on the antioxidant activity of 10 mM kaempferol at pH 5 in the presence of 1 μM copper sulfate. An increase in antioxidant activity can be observed for both enzymes at all

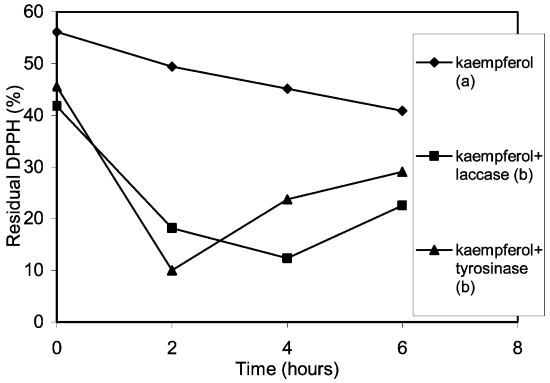

Figure 7. Effect of purified laccase and tyrosinase addition on the antioxidant activity of kaempferol. Legends followed by different letters are statistically different ($p \leq 0.05$).

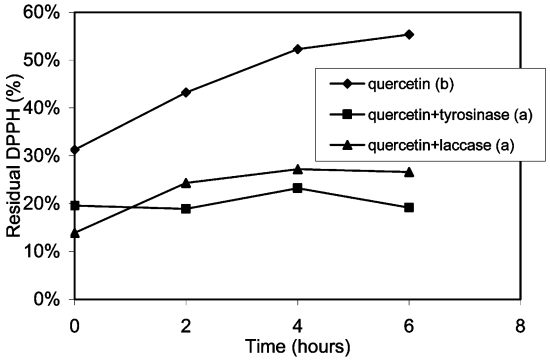

Figure 8. Effect of purified laccase and tryosinase addition on the antioxidant activity of de quercertin. Legends followed by different letters are statistically different ($p \leq 0.05$).

times. The reaction products generated after a 2 h reaction time had a higher antioxidant activity than the products formed after 4 and 6 h.

Figure 8 shows the effect of the addition of purified laccase and tyrosinase on the antioxidant activity of 10 mM quercetin at pH 5 in the presence of 1 μM $CuSO_4$. Again, a noticeable increase in antioxidant activity can be observed for both enzymes when compared with the activity of the quercetin with no enzyme added. In the case of laccase, the antioxidant activity decreases, reaching a constant value after 2 h. This value is better than the one obtained with the quercetin alone. In the case of tyrosinase, the antioxidant activity remained constant along the experiment; however, this value also was better than the value for the quercetin alone.

The study of the effect of the different concentrations of polymers produced enzymatically with laccase−quercetin, laccase−kaempferol, tyrosinase−quercetin, and tyrosinase−kaempferol on ROS scavenging (Figure 9) shows that quenching of free-radicals is dose-dependent. A prooxidant effect was observed at the highest concentrations of the four polymers; the effect was very clear (200−250%) for the polymers produced with laccase−quercetin at 180 μg/mL and tyrosinase−kaempferol at 255 μg/mL. A prooxidant effect, albeit a lower one (22%), was also observed for tyrosinase−kaempferol at 85 μg/mL, unlike the other intermediate concentrations, which had an antioxidant effect. Significant antioxidant effects were observed at the lower concentrations: over 50% for laccase−quercetin at 60 μg/mL and laccase−kaempferol at 71 μg/mL and over 100% in the other concentrations.

The study of the effect of the different concentrations on membrane lipoperoxidation in the WRL-68 cell line (Figure 10) shows that the studied polymers had a significant effect at all

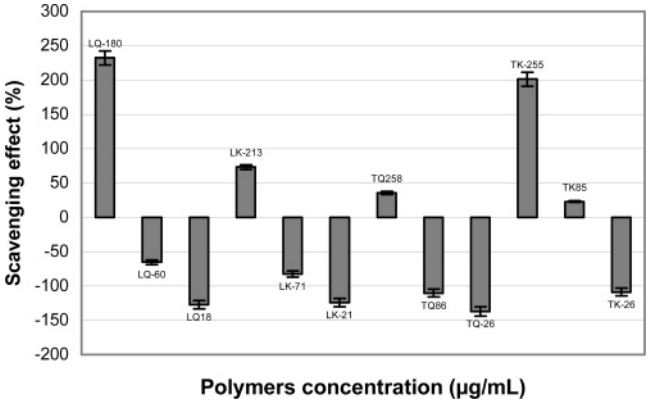

Figure 9. The scavenging activity of flavonoid polymers on reactive oxygen species. LQ180; LQ60 y LQ18: polymers (180, 60, and 18 μg/mL, respectively) produced with laccase−quercetin/$CuSO_4$; LK255; LK85; LK21: polymers (255, 85, and 21 μg/mL, respectively) produced with laccase−kaempferol/$CuSO_4$; TQ180; TQ60 y TQ18: polymers (180, 60, and 18 μg/mL, respectively) produced with tyrosinase−quercetin/$CuSO_4$; TK255; TK85; TK21: polymers (255, 85, and 21 μg/mL, respectively) produced with tyrosinase−kaempferol/$CuSO_4$.

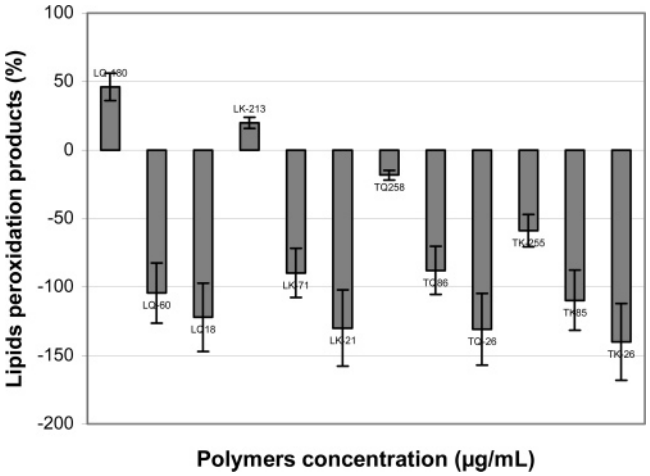

Figure 10. Inhibitory effect of flavonoid polymers on oxidation of lipids in Hepatic cell line WRL-68. LQ180; LQ60; LQ18: polymers (180, 60, and 18 μg/mL, respectively) produced with laccase−quercetin/$CuSO_4$; LK255; LK85; LK21: polymers (255, 85, and 21 μg/mL, respectively) produced with laccase−kaempferol/$CuSO_4$; TQ180; TQ60; TQ18: polymers (180, 60, and 18 μg/mL, respectively) produced with tyrosinase−quercetin/$CuSO_4$; TK255; TK85; TK21: polymers (255, 85, and 21 μg/mL, respectively) produced with tyrosinase−kaempferol/$CuSO_4$.

concentrations save laccase−quercetin at 180 μg/mL and laccase−kaempferol at 213μg/mL. The polymer produced with tyrosinase−quercetin at 255 μg/mL had a slight inhibitory effect, that produced with tyrosinase−kaempferol at 255 μg/mL had 60% inhibition, and the remaining concentrations had between 90 and 140% inhibition. Different types of reactions are known to generate ROS, causing peroxidative damage to membranes and modulating the cytosol[35] signal cascade. Molecular level damage like lipid peroxidation is believed to cause structural and functional changes in the membrane giving particular importance to those compounds that inhibit this kind of damage, such as those in the present study.

Figures 11 and 12 show a plot of the average size of the aggregates formed by kaempferol and quercetin vs time during the incubation with laccase and tyrosinase. In the absence of enzymes, kaempferol aggregated spontaneously in such a way that the molecular weight of the aggregates increased with time

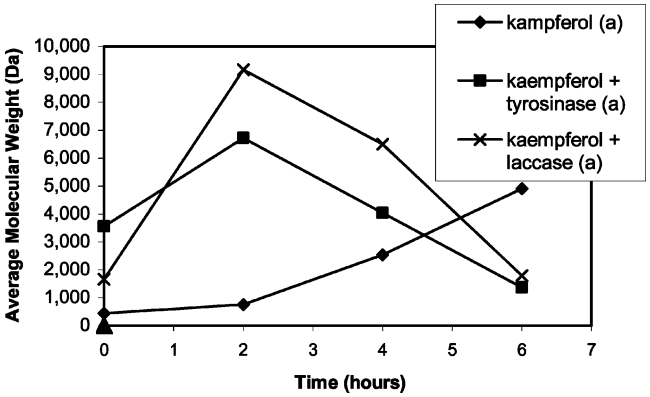

Figure 11. Effect of purified laccase and tyrosinase over average size of the aggregates formed by kaempferol through the time. Legends followed by different letters are statistically different ($p \leq 0.05$).

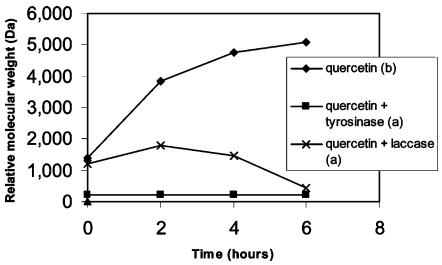

Figure 12. Effect of purified laccase and tyrosinase over average size of the aggregates formed by quercetin through the time. Legends followed by different letters are statistically different ($p \leq 0.05$).

following a first-order kinetics with a rate constant of 0.402 h^{-1}. The equation for the aggregation with time was the following:

$$PM_{agg} = PM_0 \exp(0.402t) r^2 = 0.9887$$

where PMagg is the molecular weight of the aggregates, PM_0 is the initial molecular weight (500 Da), and t is the aggregation time in h. In this case, the aggregates had more antioxidant activity than the original compound (see Figure 7).

In the case of quercetin, the aggregation followed a dose−response kind of curve with a Hill slope of one (hyperbolic behavior). This curve, widely used in pharmacodynamics, has an equation of the type

$$PM_{agg} = PM_0 + \frac{k}{1 + \frac{t_{50}}{t}}$$

where k is a constant which is very near the molecular weight of the last experimental measurement and t_{50} is the time necessary for the aggregates to reach a molecular weight which is half the maximal molecular weight attainable under the experiment conditions. The fitted equation for quercetin in the absence of enzyme was

$$PM_{agg} = 1400 + \frac{4800}{1 + \frac{2}{t}} r^2 = 1.0000$$

This equation predicts that after a long enough time, the maximal molecular weight of the quercetin aggregates would be around 6200 Da. In this case, the aggregates had less antioxidant capacity than the initial compound (see Figure 8).

When the enzymes are added to the kaempferol (see Figure 11), bigger aggregates of 9000 and 6500 Da for laccase and tyrosinase are formed after a 2 h reaction period. These aggregates had a higher antioxidant activity than the original compound. After the 2 h period, the polymers underwent disaggregation, thus revealing an unstable structure. In the case of quercetin (Figure 12), the addition of the enzymes prevented the formation of large size aggregates. After 2 h, aggregates with molecular weights of 1785 and 236 Da were obtained when laccase and tyrosinase were added to the reaction mixture. This inhibition could be due to the formation of enzyme−quercetin complexes which could be so stable that they will not allow the quinone formation reaction to proceed at its normal rate.

The molecular weight of the polymers depends mainly on the reaction medium conditions, the kind of substrate used, and the enzyme source. In similar studies, the laccase-catalyzed polymerization of rutin produced a poly(rutin) with a molecular weight of 11 000 Da after 24 h at room temperature.[10] The influence of the enzyme source was clearly demonstrated when the peroxidase-catalyzed polymerization of cardanol (a phenol derivative with a C15 unsaturated hydrocarbon chain with mostly 1−3 double bonds at meta position) was successfully performed with soybean peroxidase and failed when the horseradish enzyme was used unless redox mediators were employed.[8]

Several mechanisms have been suggested for flavonoid polymerization, but in all of them, the intermediate step involves the formation of a quinone whose structure will depend on the particular flavonoid. According to this, it is possible that an *o*-quinone in C3′ and C4′ is the compound previous to polymerization in the case of quercetin[24,34] and that a quinone in C3 and C4 is the intermediate in the case of kaempferol.[36] The enzyme-catalyzed polymerization in the presence of copper is by far a more complex reaction which might involve several different mechanisms.

Considering the system we used to evaluate the antioxidant activity with DPPH, there are several possible mechanisms to be considered in the polymerization of polyphenols. Apart of the expected DPPH oxidation process, it has been demonstrated that Cu^{2+} ions promotes the oxidation of flavonols through the formation of reactive species of oxygen. The interactions between this metal and the poliphenols in some cases could be the cause of the food browning.[33] Therefore, it is likely that a similar mechanism could take place in the case of our experiments in the presence of quercetin and kaempferol, in which the interaction of the cupric ion with the hydroxy groups at C-4′ and C-5′ positions of the B ring of quercetin, **A**, leads to the oxidation to the corresponding *o*-quinone, **B**, along with the reductive elimination of Cu(0) (Figure 13). Alternatively, the chelation of Cu^{2+} ions with the 3-hydroxy group and the oxygen atom of the 4-carbonyl group, **C**, might promote the oxidation to the *p*-quinone, **E**.[29] Chelate **C** could be stabilized by electronic delocalization to the anthocyanin intermediate **D**. This second pathway might be rather followed by kaempferol, which lacks the chatechol structure.

It is worth noticing that a parallel effect of the formation of Cu(0) could be associated with the inhibition of the oxidative activity of DPPH, as shown by a decrease in the activity curve after 2 and 4 h with lacasse−kaempferol and after 2 h with tyrosinase−kampferol (Figure 7).

Although the oxidation of the flavonoids can be promoted by Cu^{2+} ions, it seems that this process is not carried out in the mixtures of quercetin and kaempferol with the solution of cupric sulfate.

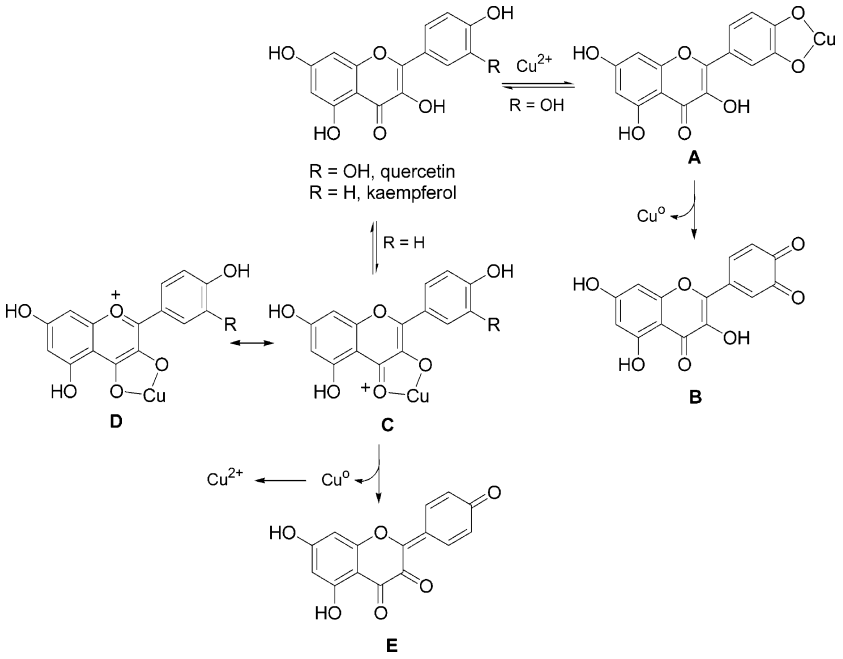

Figure 13. Possible mechanisms in the polymerization of polyphenols by Cu^{2+}.

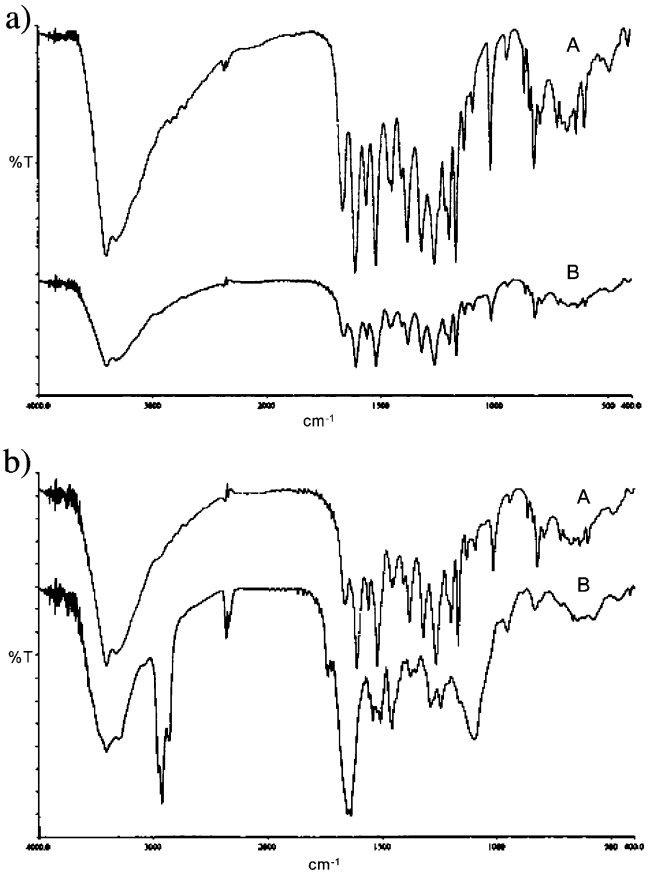

Figure 14. FT-IR spectra of (a) quercetin (A) and polymer of quercetin formed by $CuSO_4$ (B) (b) polymer of quercetin formed by $CuSO_4$ 1 μM (A) with polymer of quercetin formed by EE (B).

The IR spectra of these mixtures show no significant differences with respect to those of flavonoids alone (Figure 14 a). However, when the Cu^{2+} ions are bound to the enzyme, the IR spectra of the mixtures were completely modified, with a new strong band appearing at 1639 cm^{-1}, which is probably due to the quinone moiety absorption (Figure 14 b). In contrast with the solution of $CuSO_4$, the effect with the enzyme could

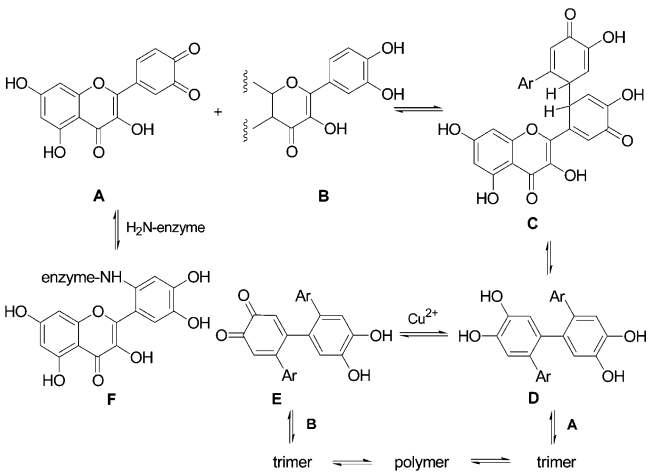

Figure 15. Possible mechanisms in the polymerization of polyphenols by a conjugate addition of nucleophiles.

be associated with the presence of "soluble" (lipophilic) Cu^{2+} ions, which can be readily bound to the flavonoids. Consequently, when the latter occupy the active site of the enzyme, they inhibit the oxidation of the substrate.

Once the quinone **A** is formed, a conjugate addition of diverse nucleophiles can take place. If the nucleophilic attack comes from another flavonoid phenolic ring, **B**, the adduct **D** can undergo a further oxidation from the enzyme of the oxidative agents to provide a new quinoid species **E**, which is capable of suffering a new addition, leading to polyphenols (Figure 15). Biaryl chatechol intermediates **D** could polymerize at once by adding to a quinone molecule **A**.[37] The strong IR bands at 3423, 1169, and 1091 cm^{-1} of a sample of the polymerization of quercetin with laccase suggest the presence of multiple O−H and C−O bonds, respectively (Figure 16).[38] Alternatively to this polymerization process, quinones are prone to be added by a protein amino group forming a covalent bond between the enzyme and the antioxidant, giving adduct **F**.[39]

All of the polymers were water-insoluble but soluble in methanol and ethanol.

The morphological characterization of the laccase-catalyzed aggregates obtained from quercetin and kaempferol was done

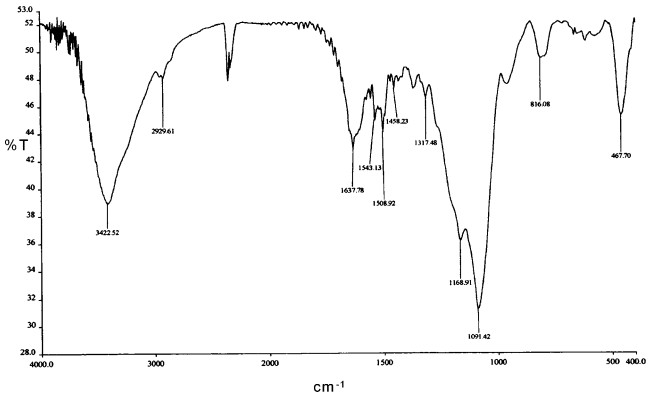

Figure 16. FT-IR spectra of quercetin polymer formed by laccase.

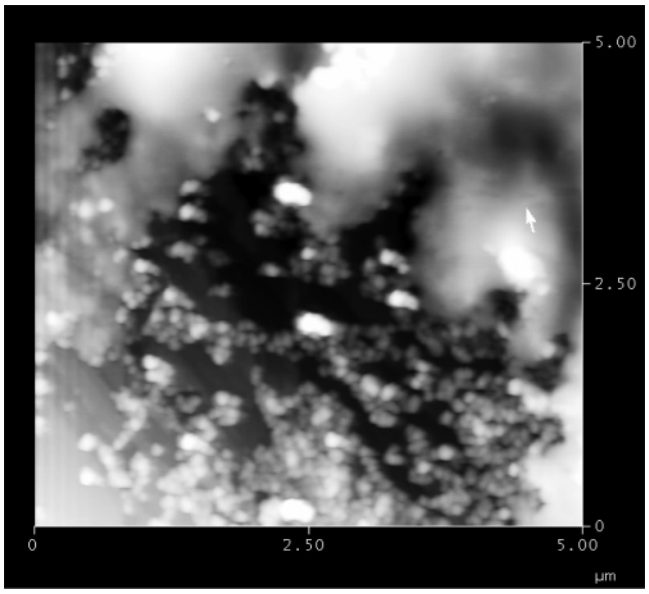

Figure 17. Quercetin aggregates atomic force micrograph with 5 × 5 μm scan area.

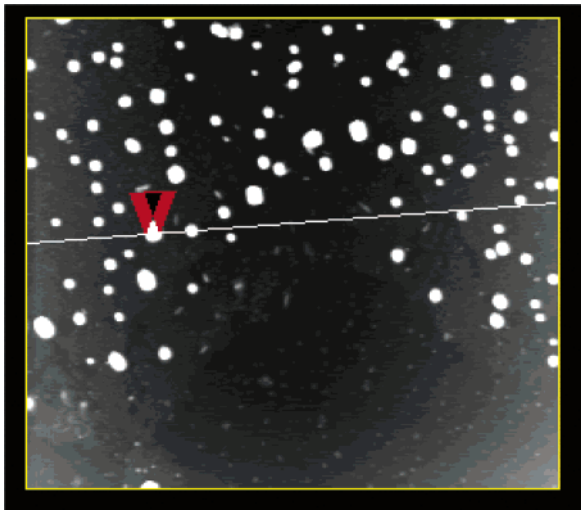

Figure 18. Laccase catalized aggregates obtained from quercetin at a magnification 10 × 10 μm^2.

by AFM. Figure 17 shows an atomic force micrograph with a 5 × 5 μm scan area, where the spheric shape aggregates of quercetin, in the absence of enzymes, with an 82.6 nm mean diameter can be observed. Figure 18 shows at a magnification of 10 × 10 μm, querecetin aggregates formed in the presence of laccase. These aggregates, which are also spherical in shape,

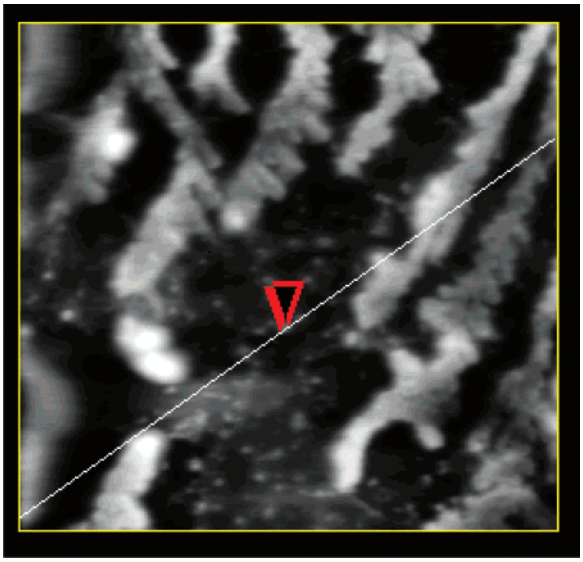

Figure 19. Tyrosinase catalized aggregates obtained from quercetin at a magnification 5 × 5 μm^2.

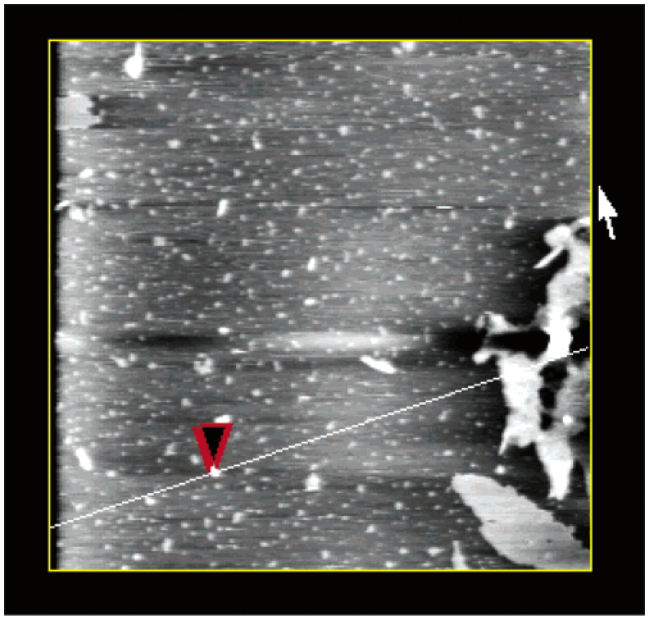

Figure 20. Kaempferol aggregates atomic force micrograph with 5 × 5 μm scan area.

exhibit different diameters (187, 234, and 327.6 nm). Figure 19 shows at a magnification of 5 × 5 μm, quercetin aggregates formed in the presence of tyrosinase. It can be observed that there are zones with small (spherical with a 87.9 nm diameter) and large (elongated) aggregates.

Figure 20 shows the spherical aggregates of kaempferol (no enzyme added) at a magnification of 5 × 5 μm. The values of diameter range from 54 to 164 nm. Figure 21 shows the image of the kaempferol aggregates formed in the presence of laccase. It is interesting to observe the formation of stem-like structures with a branching where the unit structure formed by 3 or 4 units 76 nm wide. This gives origin to dendrite-like structures of the fractal type. Figure 22 shows the aggregates formed by kaempferol in the presence of tyrosinase. In the case of kaempferol, in the presence of any of the enzymes, a stochastic fractal form called diffusion-limited aggregate produces coral or fern-like shapes or structures such as the ones observed in Figures 21 and 22. It could be possible that, according to this theory,[40] the kaempferol molecules suspended in the liquid drift

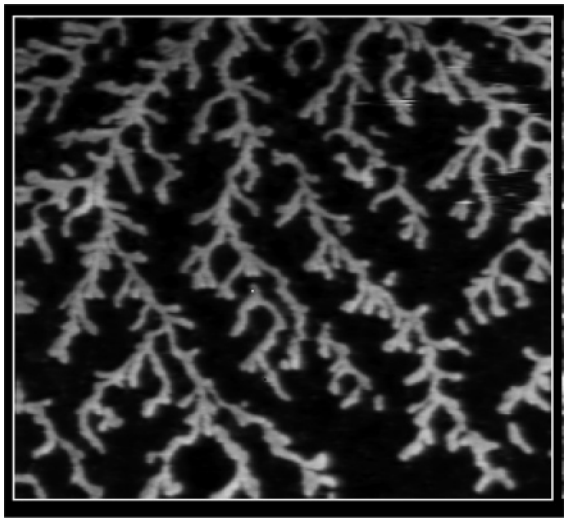

Figure 21. Laccase catalized aggregates obtained from kaempferol at a magnification 10 × 10 μm^2.

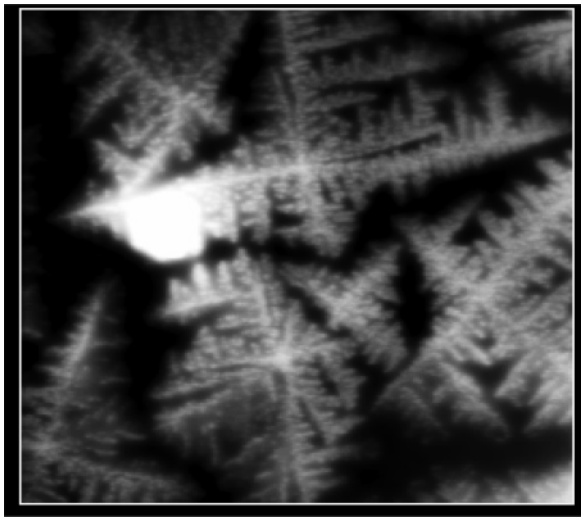

Figure 22. Tyrosinase catalized aggregates obtained from kaempferol at a magnification 10 × 10 μm^2.

toward the enzyme with a movement similar to Brownian motion caused by very light collisions with surrounding molecules and that this procedure is repeated until a branch evolves. In this case, the minimal aggregation units had a diameter of 70 nm.

Conclusions

The enzyme-catalyzed aggregation process for quercetin produced small-sized structures with a good antioxidant activity, whereas in the case of kaempferol, the same process produced larger-sized aggregates in the first reaction hours. In both cases, the aggregation was a very orderly process leading to the formation of fractal structures with the shape of a coral in the kaempferol−laccase system and of a fern in the kaempferol−tyrosinase system. The kaempferol and quercetin polymers at low concentration had a strong scavenging effect on reactive oxygen species (ROS) and inhibition of lipoperoxidation in human hepatic cell line WRL-68.

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