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Effect of Dietary Melanoidins on Lipid Peroxidation during Simulated Gastric Digestion: Their Possible Role in the Prevention of Oxidative Damage

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The ability of high molecular weight melanoidins extracted from coffee, barley coffee, and dark beer to inhibit lipid peroxidation during simulated gastric digestion of turkey meat has been investigated. Results showed that melanoidins decrease the synthesis of lipid hydroperoxides and secondary lipoxidation products. Coffee melanoidins at 3 mg/mL reversed the reaction and broke down hydroperoxides to concentrations lower than the initial value. Barley coffee and dark beer melanoidins were less effective, and even at 12 mg/mL did not reverse the reaction. The proposed mechanism of action involved Fe^{2+} chelating capacity, heme-binding ability, and radical-scavenging activity. Melanoidins were characterized for their content in total proteins, carbohydrates, and phenolics, and the relationship between the chemical composition and the antioxidant activity of dietary melanoidins was investigated. Coffee melanoidins, which contain more phenolics and proteins with respect to the other melanoidins, showed greater antioxidant activity with respect to the other melanoidins tested.

KEYWORDS: Food melanoidins; lipid peroxidation; gastric fluid; free radical; antioxidants; turkey meat; heme

INTRODUCTION

Atherosclerosis and related cardiovascular diseases are the most widespread pathologies in the Western world, where they are the main cause of morbidity and mortality. The molecular mechanism of atherosclerosis is currently unknown, but it is supposed that it could be a multifactorial disease, and several risk factors, including a time-dependent response to arterial injury, low-density and remnant lipoprotein oxidation, and postprandial response to eating, are hypothesized to be involved (*1*).

Consumption of a meal containing oxidized and oxidizable lipids gives rise to increased plasma levels of lipid hydroperoxides (*2*) that can be incorporated into lipoproteins and thus act as initiators for further lipoprotein oxidation (*1*, *2*). Secondary lipoxidation products such as malondialdehyde (MDA) and 4-hydroxynonenal may be absorbed in the gastrointestinal tract and can be involved in the pathogenesis of some cardiovascular diseases (*3*). Lipid hydroperoxides and secondary lipoxidation products may be already present in oxidized food but, more interestingly, they may be generated during digestion of highly oxidizable foods such as red meat (*4*). Kanner and co-workers suggested that the stomach may act as a "bioreactor", promoting lipid peroxidation, especially in gastric fluid, which contains dissolved oxygen and has a low pH, and particularly when the meal contains catalysts, such as free iron ions, a free heme group, or heme-containing proteins such as metmyoglobin (*4*, *5*).

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The idea that the gastrointestinal tract is the location for the protective activity of antioxidants was presented by Halliwell et al. (*6*). It has been observed that the consumption of a meal rich in oxidizable fat together with a rich source of antioxidants, such as red wine (*7*) or procyanidins (*8*), reduces the absorption of lipid hydroperoxides and their secondary lipoxidation products as a consequence of the antioxidant effect of red wine polyphenols (*4*, *5*).

Recently, scientific interest in the field of high molecular weight melanoidins has increased because of their varied biological activities and functional properties (*9*). Melanoidins are polymeric brown compounds formed in the last stage of the Maillard reaction, and they are involved in the color, flavor, and texture of thermally treated foods (*10*). They are present in some widely consumed foods and beverages such as coffee, dark beer, sweet wine, traditional balsamic vinegar, cocoa, and bread (*11*−*13*). Some important biological activities of food melanoidins are their antioxidant and chelating activities (*14*, *15*) as well as antimicrobial (*15*) and antihypertensive (*11*, *15*) activities.

Aim of This Work. The aim of this work was to verify the antioxidant effect of high molecular weight melanoidins extracted from coffee, barley coffee, and dark beer during in vitro gastric digestion of turkey meat. For the first time, the protective effect of dietary melanoidins versus lipid peroxidation during simulated gastric digestion was investigated.

MATERIALS AND METHODS

Materials. Ammonium ferrous sulfate, hemin ($HmFe^{III}$), hemoglobin, pepsin, 2,6-di-*tert*-butyl-4-methylphenol (BHT), thiobarbituric acid (TBA), trichloroacetic acid (TCA), 2,4,6-tripyridyl-*S*-triazine

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(TPTZ), and xylenol orange were supplied by Sigma (Milan, Italy). 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was supplied by Calbiochem (Darmstadt, Germany). All of the other chemical reagents were from Carlo Erba (Milan, Italy). Amicon Ultra-4 and Microcon YM-10, regenerated cellulose 10 kDa, were supplied by Millipore (Italy). Whatman no. 4 filter papers were supplied by Whatman (Maidstone, Kent, U.K.). Turkey meat, coffee, barley coffee, and dark beer were purchased in a local market (Reggio Emilia, Italy). The absorbance was read using a Jasco V-550 UV−vis spectrophotometer.

Sample Preparation. Coffee and barley coffee were prepared by dissolving 2 g of soluble coffee or barley coffee in 100 mL of boiling water, then diluted 1:2 with water, and filtered with Whatman no. 4 filter papers. Dark beer was diluted 1:2 with water and filtered. The samples were diluted and filtered to avoid clogging of the ultrafiltration units.

High Molecular Weight Melanoidins Extraction and Determination. High molecular weight (> 10 kDa) melanoidins were extracted from filtered samples as described by Morales and Babbel (*16*). One aliquot (4 mL) of each filtered sample was subjected to ultrafiltration with Amicon Ultra-4, at 7500*g* for 70 min at 4 °C. The retentate was refilled with water and washed again. This washing procedure (diafiltration) was repeated three times to reduce the concentration of the contaminating low molecular weight antioxidants. At the end of the separation the retentates containing high molecular weight melanoidins were filled to 4 mL with distilled water; these fractions were then utilized for the in vitro digestion procedure, the antioxidant activity analysis, and heme-binding and Fe^{2+}-chelation ability determination. To quantify melanoidins extracted from the different food samples, in some experiments the retentates were dried in a desiccator for 5 days at room temperature and the solid residues were weighed. The solid residue represents the extracted melanoidins, and the results are expressed as grams of melanoidins per 100 g of dry matter.

Chemical Characterization of High Molecular Weight Melanoidins. High molecular weight melanoidins were chemically characterized for their content in proteins, phenolic groups, and carbohydrates as well as for their spectroscopic properties.

Determination of Protein Content. The protein content of high molecular weight melanoidins was assayed with Bradford methods (*17*) using bovine serum albumin as standard.

Determination of Total Phenolic Groups Content. The total phenolic groups content of high molecular weight melanoidins was determined with the Folin−Ciocalteu reagent (*18*). Briefly, 790 μL of distilled water, 10 μL of high molecular weight solution, and 50 μL of Folin−Ciocalteu reagent were mixed. After exactly 1 min, 150 μL of 20% aqueous sodium carbonate was added, and the mixture was mixed and allowed to stand at room temperature in the dark for 120 min. Detection was achieved at 760 nm. Gallic acid was used as standard.

Determination of Total Carbohydrate Content. The carbohydrate content of high molecular weight melanoidins was assayed by the phenol−sulfuric acid method (*19*) after mild acid hydrolysis. High molecular weight melanoidins (5 mg/mL) were hydrolyzed in 1 N HCl for 2 h at 105 °C (*20*). After rapid cooling, the hydrolyzed solutions were diluted 10 times with distilled water and filtered. Briefly, 100 μL of hydrolyzed solution was added to 100 μL of 5% phenol in water; after mixing, 1 mL of concentrated H_2SO_4 was added and the solution was immediately vortexed for a few seconds before reading at 500 nm. Because mannose was the most representative sugar in coffee melanoidins (*18*), it was used as standard.

Spectroscopic Analysis. Spectroscopic analyses were performed by measuring the specific extinction coefficient (*K*) of high molecular weight melanoidins at 280 and 420 nm (*18*). The *K* value was calculated by using the law of Lambert−Beer and expressed as liters per gram per centimeter because the molecular weight of melanoidins is unknown and probably variable among the different samples.

Simulated Gastric Digestion of Turkey Meat. One part of grilled turkey meat was homogenized with three parts (w/v) of simulated gastric fluid (SGF) for 2 min in a laboratory blender and adjusted to pH 3 (*4*). SGF was prepared by adding 315 units/mL of pepsin, 2.0 g of sodium chloride, 7.0 mL of hydrochloric acid, and sufficient water to make 1000 mL of SGF. To verify the inhibitory activity of melanoidins on lipid peroxidation, they were added to SGF at concentrations ranging between 1.5 and 12 mg/mL. EDTA was used as positive control to study the role of nonheme iron on gastric lipid peroxidation. The homogenate was incubated at 37 °C in a shaking bath for 180 min, and aliquots were withdrawn at different times for further analysis.

Lipid Hydroperoxides (LHP) Measurements. LHP were extracted by 10-fold dilution in methanol HPLC grade under slow stirring for 30 min. After centrifugation at 3000*g* for 15 min at 4 °C, the hydroperoxides in the supernatants were determined with the FOX assay (*21*) at 560 nm. The FOX reagent contained 250 μM ammonium ferrous sulfate, 100 μM xylenol orange, 25 mM H_2SO_4, and 4 mM BHT in 90% (v/v) methanol HPLC grade. H_2O_2 standard solutions were prepared at concentrations ranging from 5 to 200 μM in methanol of HPLC grade. The hydroperoxides content was expressed in micromolar H_2O_2 equivalents.

Secondary Lipoxidation Products Quantification. Secondary lipoxidation products were quantified as thiobarbituric acid-reactive substances (TBA-RS) on digested samples as reported by Buege and Aust (*22*). Briefly, 200 μL samples diluted 10-fold in water were added to a 1000 μL TCA−TBA solution (final concentrations in the assay were 15% TCA and 0.375% TBA) in 0.25 N HCl. The mixture was incubated for 30 min in boiling water, cooled, and then centrifuged at 10000*g* for 5 min at 20 °C. The TBA-RS in the supernatant was determined at 532 nm, and the results were expressed as micromolar malondialdehyde (MDA), using the molar extinction coefficient of 156000.

Fe^{2+} Chelation Ability of Melanoidins. The Fe^{2+} chelation ability of melanoidins was determined with the method developed by Morales et al. (*14*) with slight modifications. High molecular weight melanoidins at different concentrations, diluted in 0.1 M sodium acetate buffer, pH 3, to simulate gastric conditions, were incubated with 50 mg/L $FeSO_4$ (dissolved in 0.1 M sodium acetate buffer, pH 3) at room temperature. After vortexing for 2 h, samples were allowed to stand for 22 h at room temperature, before ultrafiltration, using Microcon YM-10, at 14000*g* for 30 min at 22 °C. The quantity of chelated iron was determined by measuring the content of Fe^{2+} in the filtrate (unbound Fe^{2+}) and subtracting this value from the initial quantity of Fe^{2+} incubated with melanoidins. The assay used to measure the content of Fe^{2+} utilizes TPTZ (*23*). Briefly, 10 mM TPTZ dissolved in 40 mM HCl was diluted to 1 mM in 0.3 M sodium acetate buffer, pH 3.6. After the absorbance at 593 nm at time zero had been measured, 100 μL of standard or filtrate was added and mixed. After exactly 6 min, the absorbance was measured at 593 nm. The absorbance at time zero is subtracted from the absorbance at time 6 min. The standard curve is obtained using a solution of $FeSO_4$ in 0.1 M sodium acetate buffer, pH 3, ranging from 2.5 to 250 mg/L.

Heme-Binding Ability of Melanoidins. Hemin ($HmFe^{III}$) at a concentration of 10 μM was dissolved in a 20% DMSO solution to avoid monomer aggregation. Hemin solution was brought to pH 3 to simulate gastric conditions. Aliquots (5 μL each) of high molecular weight melanoidins at different concentrations were added to the sample (995 μL of 10 μM hemin in 20% DMSO, pH 3) as well as to the reference (995 μL of 20% DMSO, pH 3) cuvette. Melanoidins−hemin binding was assayed by measuring the absorbance at 400 nm before and after the addition of melanoidins. ΔAbs at 400 nm was defined as the difference between the absorbance at 400 nm before the addition of melanoidins and that recorded at same wavelength after the addition of melanoidins. To calculate the Hill coefficient and the value of the equilibrium binding constant (K_d), a nonlinear regression analysis method was applied (GraphPad Prism 5.0).

Radical-Scavenging Activity. The antioxidant activity of high molecular weight melanoidins was measured as radical-scavenging activity with the ABTS assay (*24*). ABTS was dissolved in distilled water to 14 mM concentration. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting to the ratio of 1:1 ABTS stock solution with 4.9 mM potassium persulfate and leaving the mixture to stand in the dark at room temperature for 12−16 h before use. The resulting blue-green ABTS radical solution was diluted in ethanol to an absorbance of 0.700 ± 0.050 at 734 nm. Forty microliters of high molecular weight melanoidins diluted in water and brought to pH 3 to simulate gastric conditions was added to 1960 μL of the resulting blue-green $ABTS^{\bullet+}$. The mixture, protected from light, was incubated in a Jasco V-550 spectrophotometer at 37 °C for 10 min; the decrease in absorbance at 734 nm was measured at the end point of 10 min. Vitamin C was used as standard. The results were expressed as VCEAC (vitamin C equivalent antioxidant capacity) value defined as milligrams of vitamin C per gram of melanoidins.

In Vitro Pepsin Assay. Pepsin activity was assayed by utilizing hemoglobin as substrate (*25*), and the peptides formed were determined

Table 1. Chemical Characteristics of Dietary High Molecular Weight Melanoidins

	K_{420}[a]	K_{280}[a]	proteins[b] (%, w/w)	carbohydrates[b] (%, w/w)	phenolic groups[b] (%, w/w)
coffee melanoidins	1.1289	5.9616	8.83 ± 0.46	69.81 ± 1.58	16.39 ± 0.71
barley coffee melanoidins	0.3672	1.2446	1.13 ± 0.02	59.57 ± 0.63	2.76 ± 0.26
dark beer melanoidins	0.5838	2.1848	5.02 ± 0.03	70.72 ± 6.46	2.91 ± 0.36

[a] K = specific extinction coefficient expressed as L g^{-1} cm^{-1}. [b] Results expressed as grams of proteins or carbohydrates or phenols in 100 g of melanoidins.

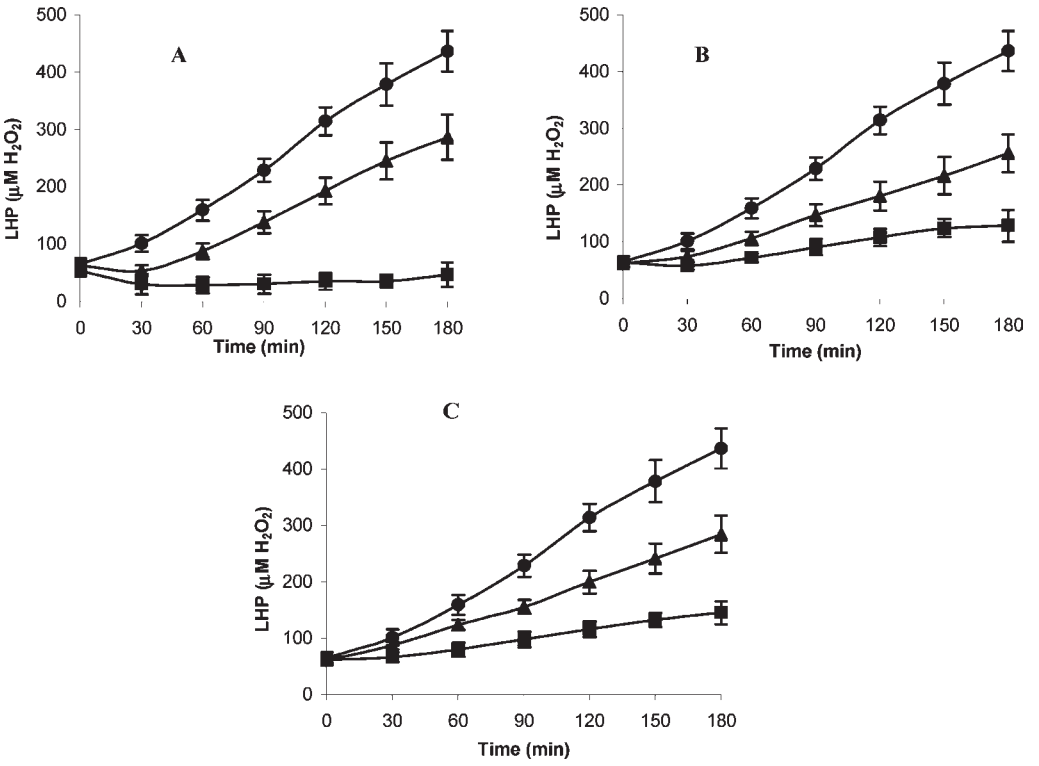

Figure 1. Changes in the level of hydroperoxides during incubation of homogenized grilled turkey meat in simulated gastric fluid in presence of coffee (**A**), barley coffee (**B**), and dark beer (**C**) melanoidins: (**A**) (●) control, (▲) in the presence of 1.5 mg/mL of coffee melanoidins, (■) in the presence of 3.0 mg/mL of coffee melanoidins; (**B**) (●) control; (▲) in the presence of 6.0 mg/mL of barley coffee melanoidins, (■) in the presence of 12.0 mg/mL of barley coffee melanoidins; (**C**) (●) control, (▲) in the presence of 6.0 mg/mL of dark beer melanoidins, (■) in the presence of 12.0 mg/mL of dark beer melanoidins. Data are presented as mean ± SD for three parallels for each prepared sample.

by recording the increase of absorbance at λ = 280 nm. The incubation mixture, in a final volume of 3 mL, contained 20 units of pepsin, 52 mM HCl, and 1.7% (w/v) of hemoglobin. To evaluate the effect of high molecular weight melanoidins on the enzymatic activity, the assay was conducted in the presence of food melanoidins at concentrations ranging between 0.05 and 0.5 mg/mL. After 10 min of incubation at 37 °C, 1 mL of 50% trichloroacetic acid was added to precipitate hemoglobin. After 10 min of incubation at room temperature, the samples were centrifuged and the peptides in the supernatants were determined by recording the absorbance at 280 nm. Control samples for pepsin autodigestion (pepsin without substrate) and substrate stability (reaction mixture with substrate but without pepsin) were included. These control reactions were treated exactly as described above. All of the reagents were preheated at 37 °C before the reaction.

Statistical Analysis. All data are presented as mean ± SD for three parallels for each prepared sample. The statistical analyses were performed using Graph Pad Prism 5 (GraphPad Software, San Diego, CA).

RESULTS

High molecular weight melanoidins (> 10 kDa) were extracted from three different samples (coffee, barley coffee, and dark beer), separating them from the low molecular components by an ultrafiltration technique. Barley coffee is the product with the highest melanoidins content (71.87 g of melanoidins/100 g of dry matter) followed by coffee (37.87 g of melanoidins/100 g of dry matter) and dark beer (20.02 g of melanoidins/100 g of dry matter). Contrary to coffee melanoidins that are carbohydrate−phenol-based structures (*18*), the chemical structure of dark beer and barley coffee melanoidins is actually unknown. In **Table 1** are reported some chemical characteristics of melanoidins extracted from coffee, barley coffee, and dark beer. **Table 1** also shows that melanoidins extracted are qualitatively different. Coffee melanoidins contain many more phenolic groups and more proteins than the other melanoidins extracted. The content in proteins, carbohydrates, and phenolics of coffee melanoidins is consistent with previously reported data (*18*). Furthermore, the sum of the content in proteins, carbohydrates, and phenolics of coffee account for about 95% of melanoidins weight, suggesting that these compounds are responsible for the main part of the chemical structure of coffee melanoidins. The same conclusions cannot be deducted for dark beer and barley coffee melanoidins, where proteins, carbohydrates, and phenolics account for 78.65 and 63.46% of melanoidins weight, respectively, suggesting that other compounds are incorporated in the structure of this type of melanoidin.

Inhibition of Lipid Hydroperoxides and Secondary Lipoxidation Products Formation during Gastric Digestion of Turkey Meat by Melanoidins. The level of lipid hydroperoxides found in simulated gastric fluid after the homogenization of turkey meat was 65.0 ± 10.0 μM. The incubation at 37 °C caused a 6.7-fold increase in the level of hydroperoxides (**Figure 1** and **Table 2**). When high

Table 2. Changes in Hydroperoxides and TBA-RS Concentration during Simulated Digestion of Turkey Meat in the Presence or Not of Coffee (CM), Barley Coffee (BCM), and Dark Beer (DBM) Melanoidins

	LHP (μM H_2O_2)			TBA-RS (μM MDA)		
	0 min	180 min	% of inhibition[a]	0 min	180 min	% of inhibition[a]
no addition	65.0 ± 10.0	436.3 ± 35.5		10.2 ± 0.6	25.0 ± 0.9	
without pepsin	74.4 ± 6.5	234.1 ± 26.4[b]	57.0	10.3 ± 0.7	18.3 ± 1.6[b]	45.9
CM 1.5 mg/mL	62.1 ± 9.5	286.4 ± 39.2[b]	39.9	10.0 ± 1.4	20.6 ± 1.7[b]	28.4
CM 3 mg/mL	53.3 ± 9.3	46.4 ± 21.3[b]	100.0	10.1 ± 0.3	11.4 ± 0.2[b]	91.2
BCM 3 mg/mL	54.7 ± 6.7	379.0 ± 36.9	12.7[c]	10.7 ± 0.7	23.6 ± 0.9	12.8[c]
BCM 6 mg/mL	61.6 ± 5.2	256.6 ± 33.2[b]	47.5	11.1 ± 0.8	18.4 ± 0.9[b]	50.7
BCM 12 mg/mL	63.4 ± 2.5	128.2 ± 28.3[b]	82.5	10.9 ± 0.3	14.4 ± 1.6[b]	76.4
DBM 3 mg/mL	66.1 ± 5.5	391.7 ± 28.7	12.3[c]	11.1 ± 0.5	24.1 ± 0.7	12.2[c]
DBM 6 mg/mL	61.5 ± 4.8	284.3 ± 33.3[b]	40.0	10.2 ± 1.0	20.4 ± 0.4[b]	31.1
DBM 12 mg/mL	61.0 ± 8.6	145.1 ± 20.6[b]	77.3	10.2 ± 2.0	17.7 ± 0.8[b]	62.8
EDTA 0.4 mM	58.7 ± 6.1	174.9 ± 10.7[b]	68.7			

[a] % of inhibition of LHP or TBA-RS formation calculated as difference between time 180' and time 0'. [b] $P < 0.05$ with respect to no addition. [c] Not statistically different with respect to no addition.

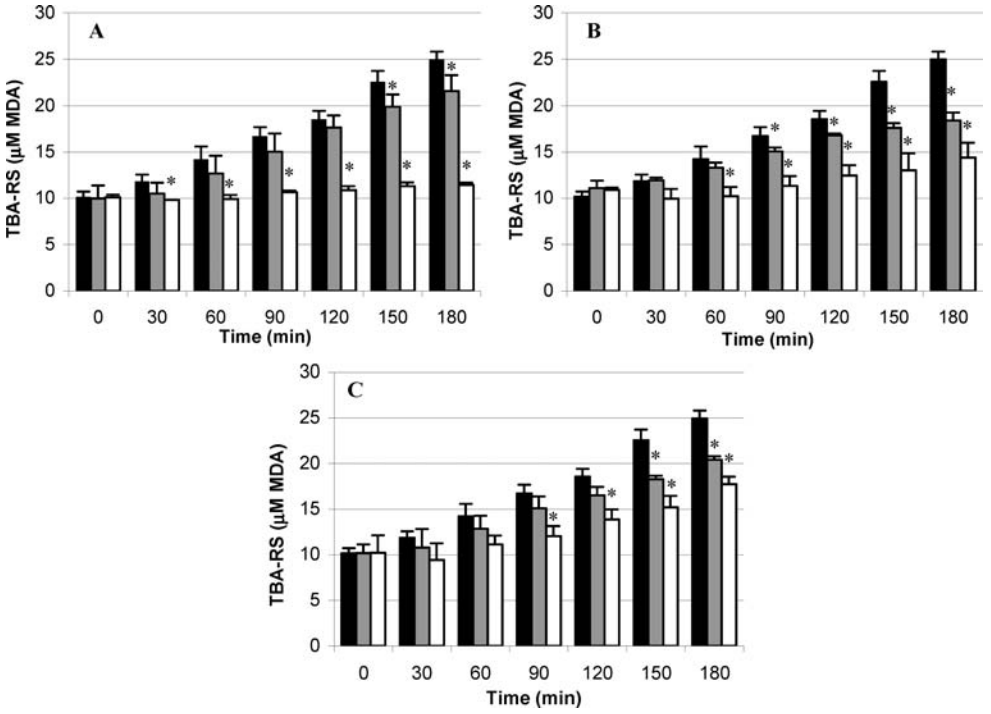

Figure 2. Changes in secondary lipoxidation products (measured as TBA-RS) concentration during incubation of homogenized grilled turkey meat in simulated gastric fluid in presence of coffee (**A**), barley coffee (**B**), and dark beer (**C**) melanoidins: (A) (black bars) control, (gray bars) in the presence of 1.5 mg/mL of coffee melanoidins, (white bars) in the presence of 3.0 mg/mL of coffee melanoidins; (**B**) (black bars) control, (gray bars) in the presence of 6.0 mg/mL of barley coffee melanoidins, (white bars) in the presence of 12.0 mg/mL of barley coffee melanoidins; (**C**) (black bars) control, (gray bars) in the presence of 6.0 mg/mL of dark beer melanoidins, (white bars) in the presence of 12.0 mg/mL of dark beer melanoidins. ∗ denotes *P* value of <0.05 with respect to the control. Data are presented as mean ± SD for three parallels for each prepared sample.

molecular weight melanoidins were incubated in simulated gastric fluid with the meat, a significant inhibition in lipid peroxidation (**Figure 1** and **Table 2**) was observed. Coffee melanoidins were the most effective and at concentration of 3 mg/mL reversed the reaction and broke down hydroperoxides to a concentration lower than the initial value (**Figure 1A**). Barley coffee and dark beer melanoidins were less effective than coffee melanoidins, and even at 12 mg/mL they did not reverse the reaction (**Figure 1B**,**C**). For both barley coffee and dark beer melanoidins at 3 mg/mL concentration, the effect was not statistically significant (**Table 2**). The inhibition of lipid peroxidation was dose-dependent with all of the food melanoidins tested.

The same trend of results was obtained when secondary lipoxidation products formation was monitored during simulated gastric digestion of grilled turkey meat in the presence of different concentrations of coffee, barley coffee, and dark beer melanoidins (**Figure 2**). It should be noted that the initial level of secondary lipoxidation products was 10.2 ± 0.6 μM MDA, and their concentration increased by about 2.5-fold during incubation in simulated gastric fluid.

Typically, lipid peroxidation in gastric fluid occurs in the presence of catalysts, such as free iron ions, free heme ($HmFe^{III}$) group, or heme-containing peptides/proteins such as metmyoglobin. We studied the role of nonheme iron on gastric lipid peroxidation by incorporating EDTA in the simulated gastric digestion of turkey meat. As can be seen in **Table 2**, EDTA inhibits the formation of hydroperoxides in the gastric fluid of 68.7%, suggesting that lipid peroxidation in turkey meat is

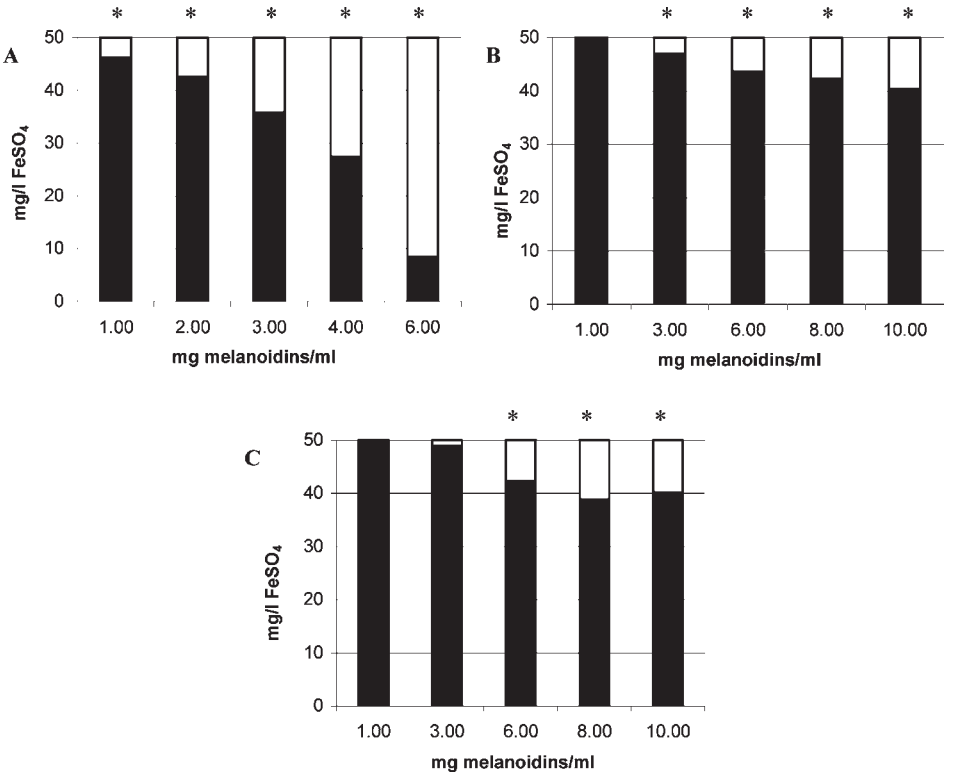

Figure 3. Fe^{2+} chelation ability of coffee (**A**), barley coffee (**B**), and dark beer (**C**) melanoidins: (black bars) free iron; (white bars) chelated iron. $FeSO_4$ (50 mg/L) was incubated with food melanoidins as described under Materials and Methods. ∗ denotes *P* value of <0.05 with respect to the control. Data are presented as mean ± SD for three parallels for each prepared sample.

Table 3. Heme-Binding Ability of Dietary High Molecular Weight Melanoidins

	heme-binding parameters	
	K_d[a]	h[b]
coffee melanoidins	0.20 ± 0.07	1.52 ± 0.30
barley coffee melanoidins	1.30 ± 0.05	3.66 ± 0.32
dark beer melanoidins	1.35 ± 0.01	3.95 ± 0.10

[a] K_d = dissociation constant expressed as mg/mL of melanoidins. [b] h = Hill coefficient.

affected especially by nonheme iron. However, a significant part of lipid peroxidation seems to be independent from free iron ion and attributable to the catalytic role of heme iron.

Iron- and Heme-Binding Ability of Melanoidins. Dietary high molecular weight melanoidins were able to interact with Fe^{2+} and $HmFe^{III}$ catalysts by chelating free Fe^{2+} ions and binding $HmFe^{III}$. With regard to the iron-chelating ability, food melanoidins showed different affinities (**Figure 3**), with coffee melanoidins having greater affinity toward free Fe^{2+} ions with respect to the other melanoidins tested. Dietary melanoidins also interacted with $HmFe^{III}$. The binding of high molecular weight melanoidins with heme caused a reduction in the absorbance of the Soret band at 400 nm. The binding parameters are reported in **Table 3**. The K_d values showed that coffee melanoidins had greater affinity toward heme in comparison to the other melanoidins tested. A Hill coefficient of >1 indicates a positive cooperativity in the binding, suggesting the presence of more than one binding site for heme in food melanoidins.

Radical-Scavenging Activity of Melanoidins. Coffee melanoidins were more effective in scavenging ABTS radical cation with a VCEAC value of 186.96 ± 11.05 mg of vitamin C/g of melanoidins. Barley coffee and dark beer melanoidins showed a VCEAC value of 16.63 ± 0.41 and 24.09 ± 0.45 mg of vitamin C/g of melanoidins, respectively.

Effect of Melanoidins on in Vitro Pepsin Activity. Pepsin activity is important for gastric lipid peroxidation because the abolition of pepsin from the simulated gastric fluid caused an inhibition of about 55% in the level of hydroperoxides (**Table 2**) and of about 45% in the level of TBA-RS (**Table 2**) after 180 min of the digestion of turkey meat. High molecular weight melanoidins had no effect on in vitro pepsin activity. In the in vitro assay the highest ratio of melanoidins to pepsin tested was 0.075 mg of melanoidins/units of pepsin. This fact suggests that in the simulated digestion procedure, in which the highest ratio of melanoidins to pepsin tested was 0.038 mg of melanoidins/units of pepsin, melanoidins did not inhibit pepsin at concentrations showing antioxidant activity.

DISCUSSION

Our results clearly show that high molecular weight melanoidins (> 10 kDa) extracted from coffee, barley coffee, and dark beer have antioxidant activity inhibiting lipid peroxidation during simulated gastric digestion of turkey meat. Other authors have already reported the inhibition of lipid peroxidation by food melanoidins in different systems such as human hepatoma HepG2 cells (*26*), rat liver microsomes (*27*), and isolated rat hepatocytes (*28*).

Melanoidins Inhibit Lipid Peroxidation by Means of Different Mechanisms. Lipid peroxidation during meat digestion in simulated gastric fluid is typically catalyzed by an iron-redox cycle that involves ferrous ions and free or bound $HmFe^{III}$ (*29*). **Figure 4** shows the putative mechanism of action for the antioxidant activity of melanoidins. As shown, melanoidins may act at different levels. In the first step of the reaction (**Figure 4A**) they

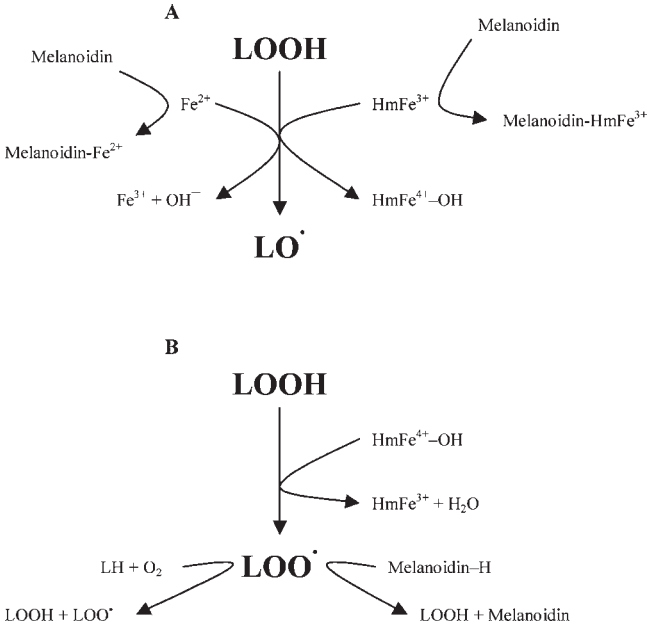

Figure 4. Simplified scheme showing the mechanism of Fe^{2+}- and heme-initiated peroxidation and the proposed mechanisms for the antioxidant activity of food melanoidins (Hm, may represent the free heme moiety or the heme-containing peptides/proteins such as metmyoglobin).

may sequester Fe^{2+} ions and $HmFe^{III}$, preventing the formation of alkoxyl radical and the protonated ferryl species ($HmFe^{4+}-OH$) of heme. Turkey meat contains large amounts of endogenous catalysts, such as free Fe^{2+} ions and free or bonded heme group. Concerning Fe^{2+}, it has been reported that turkey meat can contain up to 0.8 mg of Fe^{2+}/100 g of muscle tissue (*30*), corresponding to a theoretical concentration in simulated gastric fluid of 2 mg of Fe^{2+}/L under the assay condition used. Results showed that high molecular weight melanoidins at concentrations that inhibit lipid peroxidation may bind >2 mg/L of Fe^{2+} suggesting that, under the assay conditions, most of the Fe^{2+} present in simulated gastric fluid could be bound to melanoidins. Similarly, turkey meat contains heme at concentrations up to 0.5 mg of heme/100 g of muscle tissue (*30*), corresponding to a theoretical concentration in simulated gastric fluid of 1.25 mg of heme/L (≈1.9 μM considering a molecular weight of 652 for the heme group) under the assay condition used. In the experiment carried out the heme concentration was set at 10 μM, and given that high molecular weight melanoidins may bind heme with a dissociation constant (K_d) that is 10-fold lower than the concentration that inhibit lipid peroxidation, most of the heme present in the simulated gastric fluid should be bonded to melanoidins. The fact that iron chelation by EDTA did not cause complete inhibition of lipid peroxidation suggested that both of these mechanisms are important in determining the antioxidant activity of food melanoidins.

Finally, high molecular weight melanoidins may act as radical scavengers (**Figure 4B**) by reacting directly with peroxyl radical, preventing the propagation step. Melanoidins could act by donating a hydrogen atom to the lipid peroxyl radicals as suggested by other authors (*16*).

Pepsin activity is important for the formation of hydroperoxides during meat digestion because proteolytic activity releases catalysts such as iron and heme from proteins as well as heme-binding fragments of proteins (such as metmyoglobin) with prooxidative activity (*31*). Indeed, omission of pepsin from simulated gastric fluid gives rise to a reduction in the hydroperoxides formation as well as in the presence of pepsin inhibitors (i.e., ethanol) during the digestion of meat (*4*). However, melanoidins failed to inhibit pepsin activity at concentrations showing antioxidant activity, suggesting that inhibition of pepsin activity is not important in the mechanism of lipid peroxidation inhibition by food melanoidins.

Relationship between the Chemical Composition of and the Antioxidant Effect of Dietary Melanoidins. The molecular structure of melanoidins is largely unknown; however, it is possible to recognize the presence of substituents as, for example, proteins and phenolics. These substituents may explain some of the biological properties of melanoidins. The results demonstrated that the radical-scavenging activity of high molecular weight melanoidins is related to their phenolic content. Similarly, phenolics may act as Fe^{2+} chelators, and their presence could explain partly the iron-chelating ability of melanoidins. Also in this case, the iron-chelating ability is related to their phenolic content (**Figure 3**). However, we cannot exclude that also protein is involved in determining the iron-chelating ability of high molecular weight melanoidins. Protein may be involved in another important biological property of melanoidins: the heme-binding ability. Coffee melanoidins that have the highest protein content show the highest heme-binding ability. Therefore, coffee melanoidins that contain more phenolics and proteins with respect to the other melanoidins show greater antioxidant activity with respect to the other two types of melanoidins tested.

Possible Role for Dietary Melanoidins in the Prevention of Oxidative Damages. Our findings support the idea that dietary melanoidins may have a role in the prevention of oxidative damages. High molecular weight melanoidins may be protective at different levels.

First, they inhibit lipid peroxidation during simulated gastric digestion of meat, possibly resulting in a lower synthesis of hydroperoxides in the stomach. It has been shown that the absorption of lipid hydroperoxide generated during gastric digestion is decreased when meat is consumed along with antioxidants (*7*, *8*). It is plausible to think that also dietary melanoidins that are able to inhibit lipid hydroperoxides formation during simulated digestion of meat could decrease the absorption of these compounds, therefore providing protection against oxidative damages.

Second, dietary melanoidins inhibit the formation of secondary lipoxidation products during simulated gastric digestion. These secondary lipoxidation products may react with proteins, generating stable products that can be detected as constituents of oxidized low-density lipoproteins and also found in animal models of atherosclerosis and in human patients with increased risk factors or clinical manifestations of atherosclerosis. The formation of reactive aldehydes and modification of proteins have been shown to be involved in the oxidative modification of LDL in vitro and in vivo (*3*). It is plausible to think that dietary melanoidins that are able to inhibit secondary lipoxidation product formation during simulated digestion of meat could decrease the absorption of these cytotoxic compounds, providing protection against oxidative damages.

Finally, dietary melanoidins are able to bind heme under gastric conditions potentially preventing its absorption. Recent studies pointed out the prooxidant and cytotoxic effects of circulating heme of consumed red meat that can act as a catalyst of oxidative damages (*32*). Therefore, the binding of heme contained in meat and the prevention of its absorption may be considered an important mechanism of oxidative damage prevention by melanoidins.

An important issue concerning the antioxidant activity of melanoidins could be the incidence of the low molecular weight compounds (such as polyphenols and low molecular weight Maillard reaction products) ionically linked to the melanoidins skeleton. These compounds show antioxidant activity that can be

higher or lower than that determined in the melanoidins skeleton, depending on the food analyzed, but a lower metal-chelating ability (*33*, *34*). Because these compounds are released during in vitro gastrointestinal digestion (*33*) they could contribute to the antioxidant activity of high molecular weight melanoidins.

Although melanoidins may have positive biological activities, elevated doses of these dietary compounds may also be cytotoxic and mutagenic (*9*). However, more detailed studies are needed about the topic because it seems that the cytotoxic and mutagenic effects of Maillard reaction products are especially due to the low molecular weight (< 10 kDa) compounds (*9*).

In conclusion, we suggest a possible role for dietary melanoidins in the prevention of oxidative damages. Because high molecular weight melanoidins are poorly absorbed in the gastrointestinal tract (*9*), the present findings suggest that the gastrointestinal tract itself might constitute the main biological site of action of these antioxidant compounds. However, we need more information to better understand if the reduction in the synthesis of hydroperoxides and secondary lipoxidation products observed in vitro produces a decrease in the plasmatic level of these toxic compounds in vivo.

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