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Melanoidins Exert a Weak Antiradical Activity in Watery Fluids

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The Maillard reaction has a dramatic impact on the overall acceptance and nutritional quality of most of the foods consumed daily in European countries. Melanoidins are polymeric structures formed in the last stage of the Maillard reaction with nearly unknown effect on the human health. The antiradical activity of several melanoidins isolated from model systems and coffee has been studied. A novel antiradical efficiency concept has been applied to describe the antiradical activity in an aqueous medium by bleaching the radical cation *N,N*-dimethyl-*p*-phenylenediamine ($DMPD^{\bullet+}$). Melanoidins exerted a significantly lower antiradical activity than classical antioxidant compounds (tannic acid, ferrulic acid, caffeic acid, gallic acid, and Trolox) in an aqueous medium. Significant differences have been observed according to the type of amino acid used as reactant during the formation of the melanoidin structure and the antiradical efficiency exerted.

KEYWORDS: Maillard reaction; melanoidins; free radical scavenging; DMPD; antiradical efficiency

INTRODUCTION

In developed countries most of the foods consumed are processed to some extent, apart from home-cooked fresh foods. Food processing induces a variety of physical and biochemical changes that could affect the overall acceptability by the consumer, as well as the nutritional quality of the product. Among the reactions taking place, the Maillard reaction (MR) has played an important role in improving the appearance, aroma, and taste, apart from its impact on the nutritional quality of foods (*1*, *2*). MR is a complex network of chemical reactions divided into three stages, the early, advanced, and final MR, during which a myriad of products that have different chemical compositions and, consequently, different properties (i.e., nutrient or contaminant binding, antioxidant, prooxidant, mutagenic, and antimutagenic) are formed (*3*−*6*). However, MR also takes place in the human body, such as in long-lived tissue proteins (collagen and lens crystallins), where these chemical modifications accumulate with age and may contribute to pathophysiologies associated with diabetes and artherosclerosis (*6*, *7*).

Despite the large amount of research that has been done on the MR, the final stage is still obscure. Melanoidins are polymeric and colored final products of the MR. Melanoidins are formed by cyclizations, dehydrations, retroaldolizations, rearrangements, isomerizations, and condensations of Maillard reaction products (MRP), but none has been fully characterized yet. Melanoidins are responsible for the color in industrially processed and home-processed foods, and they are present in widely consumed dietary components (e.g., coffee, cocoa, bread, malt, and honey). In recent years a growing interest in the effect of melanoidins of the human diet and their feasible nutritional, biological, and health implications has been pointed out. Hence, the European Union has launched a specific COST (Cooperation in Science and Technology) action titled "Melanoidins in Food and Health" to put together working groups on this topic.

It is well-known that the MRP influence the oxidative and shelf life of foods, such as cereal (*8*), milk (*9*), coffee (*10*), or meat (*11*). Different approaches have been applied to describe the antioxidative properties of MRP, such as polarographic methods, rancimat, measurement of conjugated dienes, TBARS index, or scavenging of certain radicals (e.g., DPPH radical cation) in a methanolic or chloroformic medium, among others (*11*, *12*). It is supposed that the main mechanism of action is the ability to trap positively charged electrophilic metabolites, scavenge oxygen radicals, and chelate metal to form inactive complexes, or synergies. For instance, Kim and Lee (*13*) identified imidazole and furfurals as MRP with antioxidant activity in soybean oil and explained by the resonance stabilization of the pyrrole radical that can be formed by the interaction of pyrrole with free radicals. However, melanoidins have been specifically avoided in these studies or included in the overall response recorded by the low molecular weight fraction of the MRP.

Frankel and Meyer (*14*) reviewed the different factors that affect the measurement of an antioxidant activity where food matrix is critically important. Previous studies on the overall antioxidant capacity of MRP (including high molecular weight fraction) have been mainly developed in a lipophilic medium. Because most of the melanoidins exert their biological activity in a hydrophilic food matrix, it is mandatory to study the free

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radical scavenging capacity of melanoidins in an aqueous medium. The aim of this paper is to study the antiradical capacity of isolated soluble melanoidins from model systems and coffee brew as well.

MATERIALS AND METHODS

Chemicals and Reagents. D-Glucose, L-tryptophan, L-lysine monohydrochloride, L-glycine monohydrochloride, *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD), ferric chloride, tannic acid, ferrulic acid, caffeic acid, and gallic acid were purchased from Sigma; l-histidine, L-methionine, and L-cysteine were from Merck; and D-lactose monohydrate and l-ascorbic acid were from Panreac. 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was from Aldrich.

Preparation of Water-Soluble Melanoidins from Aqueous Maillard Reaction Model Systems. Sugar (0.1 M) and amino acid (0.1 M) were dissolved in 250 mL of 0.05 M sodium−phosphate buffer (pH 7). Twelve model systems were prepared: glucose−glycine (GG), glucose−histidine (GH), glucose−lysine (GL), glucose−tryptophan (GT), glucose−cysteine (GC), glucose−methionine (GM), lactose−glycine (LG), lactose−histidine (LH), lactose−lysine (LL), lactose−tryptophan (LT), lactose−cysteine (LC), and lactose−methionine (LM). Model solutions were heated without pH control in tightly stoppered Pyrex flasks, which were immersed in a polyethylene glycol bath kept at 100 °C. After a heating time of 24 h, the samples were rapidly cooled in ice and filtered through a Whatman no. 4 filter. A portion of the MR mixture (50 mL) was stored for further analysis. Another portion of 200 mL was subjected to ultrafiltration using an Amicon ultrafiltration cell model 8400 (Amicon, Beverly, MA), equipped with a 10000 Da nominal molecular mass cutoff membrane, to give a retentate volume of ~25 mL. The retentate was made up to 200 mL and washed again. This washing procedure was repeated at least three times. The final retentate (~20 mL) containing the high molecular mass fraction of melanoidin sample was freeze-dried. Lyophilized samples were stored at −20 °C prior to analysis.

Preparation of Water-Soluble Melanoidins from Coffee. Medium-roasted coffee powder was purchased from a local store. Ground coffee (100 g) was stirred in 300 mL of distilled water at 75 °C for 5 min. Coffee brew was filtered through a Whatman no. 4 filter and defatted with dichloromethane (2 × 200 mL). A portion of 200 mL was ultrafiltered as described above.

Sugar Analysis. Lactose, glucose, isomerization products (lactulose and fructose), and galactose (as degradation product) were analyzed by ion-exchange HPLC. A filtered sample (20 μL) was injected onto an ION-300 polymeric resin column (100 × 7.8 mm, Interaction-Lab, San Jose, CA) kept at 50 °C. A diluted sulfuric acid solution (4.5 mM) was used as mobile phase. Sugars were recorded with a refractive index detector (Erma Inc., Tokyo, Japan).

High-Performance Gel Permeation Chromatography (HPGPC) Analysis. A TSK-GEL 3000SW column (60 cm × 7.5 mm i.d., TosoHaas) was used. The injection volume was 10 μL for model solutions and melanoidin standards (3 mg/mL), and detection at 280 and 420 nm was selected. Samples were eluted in double-distilled water at 0.8 mL/min. A Kontron Instruments (Milan, Italy) chromatographic system was used. Void column volume was calculated with a standard solution of 1 mg/mL blue dextran (2000 kDa) diluted in 50 mM sodium phosphate at pH 7.

Capillary zone electrophoresis (CZE) analysis was performed as described by Morales (*15*).

Free Radical Scavenging Determination. Antiradical activity was measured by the $DMPD^{\bullet+}$ decolorization method as described by Fogliano et al. (*16*). A stock DMPD solution (100 mM) was distributed in fractions of 1 mL each and frozen. Daily, a light-protected $DMPD^{\bullet+}$ solution (0.75 mM) was prepared from the DMPD stock solution.

Antiradical Efficiency (AE) Determination. AE was was determined according to the procedure described by Sánchez-Moreno et al. (*17*) with some minor modifications. The percentage of remaining $DMPD^{\bullet+}$ against different standard dilutions was plotted to obtain the amount of melanoidin (milligrams pers milliliter) necessary to decrease the initial $DMPD^{\bullet+}$ concentration by 50% (EC_{50}). By plotting the time (minutes) necessary to reach the steady state against the corresponding dilution factor of the reaction mixtures or concentration of standards, the TEC_{50} (minutes) was calculated, which is the theoretical time needed by the EC_{50} (milligrams per milliliter) to reach the steady state. AE was defined as

$$AE = 1/EC_{50} \times TEC_{50}$$

Statistical Analysis. One-way analysis of variance (ANOVA), where $p < 0.05$, and the means separated by Duncan's multiple-range test were performed by applying Statgraphics v.2.3 statistical package (Statistical Graphics Corp., Rockville, MD). All of the statistical procedures were performed at a significance level of 95%. All of the analyses were performed at least in duplicate.

RESULTS AND DISCUSSION

In a first step, highly purified melanoidins were isolated form the different model systems and coffee as well. It was more convenient to use a continuous ultrafiltration process instead of the classical dialysis tube for several reasons. Ultrafiltration is faster than classical dialysis; higher volumes of sample could be treated in a few hours instead of days. Thus, the risk of microbial contamination is drastically reduced, apart from cross-reactions among the MRP formed. On the other hand, the classical dialysis procedure could improve the oxidative modification of the intermediary low molecular weight MRP or the recently formed melanoidins structures, apart from inducing a melanoidin aggregation process. Ultrafiltration will keep the preliminary melanoidin structure formed without additional aggregation or reorganization.

Both the purity of the isolated melanoidins fractions and the absence of low molecular weight compounds were tested by IE-HPLC, CZE, and HPGPC. The absence of unreacted glucose or lactose in the model systems was used as an index of efficiency of the ultrafiltration process. It was observed that after a washing cycle repeated three times, thepresence of reactants was not detected. Additional assays were conducted by applying CZE and HPGPC, where melanoidin was eluted as a unique and homogeneous peak according to its electrophoretic mobility and apparent molecular weight as well. These assays confirmed that the isolated melanoidins were free of low or intermediate molecular weight MRP. An average recovery of 1.53 ± 0.5 mg of melanoidin/mL of heated solution was obtained. Higher melanoidins recoveries were reached in model systems containing lysine and tryptophan, and lower recoveries were obtained in systems containing cysteine. As described in a previous study, the melanoidin recovery was highly related to the browning of the heated model systems (*15*).

The method proposed by Fogliano et al. (*16*) to assess the antiradical activity of different substances in a hydrophilic media was standardized to a concentration of 0.75 mM of $DMPD^{\bullet+}$ in order to get an appropriate range of inhibition. For a good extrapolation to calculate both EC_{50} and TEC_{50} parameters, it is necessary to cover the range of concentration needed to decrease the initial concentration of $DMPD^{\bullet+}$ by 50%. On the other hand, the concentration of the radical cation is not recommended to be <0.5 mM in order to avoid interference with the color of the melanoidins, which is the main limiting factor of the assay. The range of absorbance of melanoidins at 420 nm (browning index) was diverse, where melanoidins from lysine or tryptophan showed a higher browning index than melanoidins constituted by cysteine or methionine. The maximum concentration of melanoidins to get an efficient inhibition range without color sample interference was set at 2 mg/mL.

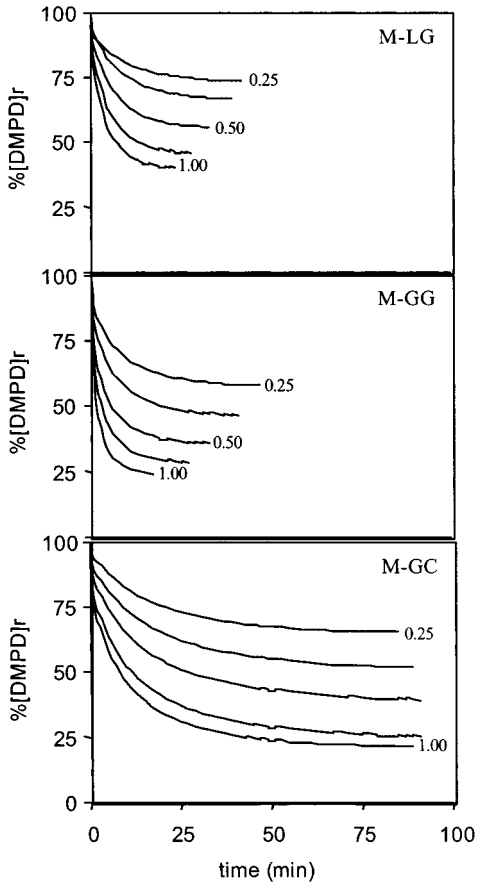

Figure 1. Classical $DMPD^{\bullet+}$ inhibition curves of isolated melanoidins from LG, GG, and GC model systems. Numbers describe melanoidin concentration (mg/mL). Data were recorded up to the steady state.

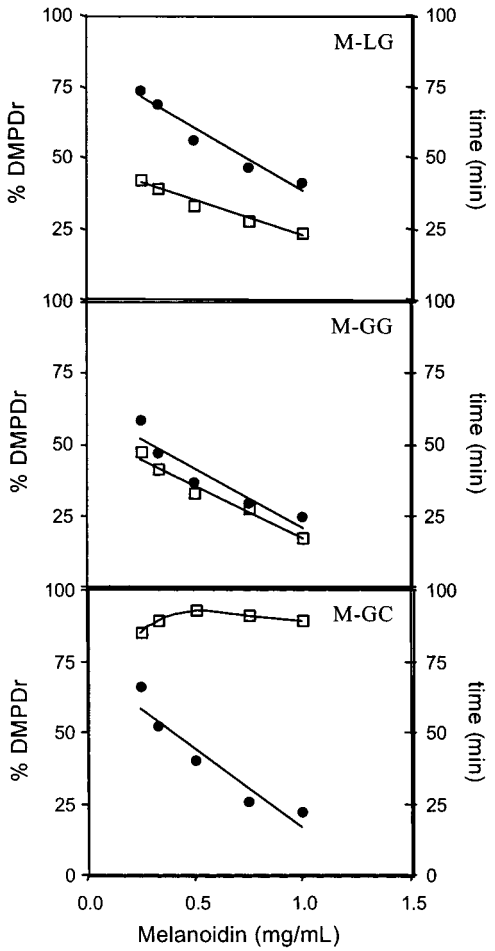

Figure 2. EC_{50} and TEC_{50} plots of isolated melanoidins from LG, GG, and GC model systems: (□) time at steady state; (●) percentage of residual $DMPD^{\bullet+}$.

A kinetic approach has been applied to the different model melanoidins and melanoidin from coffee in order to get more insight into their radical scavenging properties. The depletion of the color of the radical cation by the ability to donate a hydrogen atom by a wide range of melanoidin concentration (up to 2 mg/mL) was followed until the reaction reached a plateau. Then, for each melanoidin concentration the time to reach steady state was recorded, as well as the residual concentration of $DMPD^{\bullet+}$ at that time.

Morales and Babbel (*18*) observed a common pattern of behavior of the antiradical activity between MR mixtures (including the high molecular weight fraction). Higher MRP concentration exerted higher antiradical activity and lower time to reach the steady state, but the antiradical activity of isolated melanoidin in aqueous media was revealed to be much more complex. The two experiments cannot be fully compared because the antiradical activity of the MR mixtures was described as a dilution factor, but the antiradical activity of isolated melanoidins was expressed in terms of weight. Moreover, the recovery of melanoidins from MR mixtures is not the same, and it depends on the type of reactants, concentration, pH, temperature, and heating time. **Figure 1** depicts some representative $DMPD^{\bullet+}$ quenching curves of melanoidins isolated from model systems. Five different melanoidin concentrations were assayed in duplicate for every run. Higher melanoidin concentration exerted a rapid quenching of the radical cation in solution for all of the melanoidins analyzed, but important differences between melanoidins were observed in the time to reach the steady state. Most systems gave lower times to reach the steady state at higher melanoidin concentration except for the systems LC and GC, where this time was not affected by the melanoidin concentration. **Figure 2** represents the respective EC_{50} and TEC_{50} plots. Surprisingly, melanoidins isolated from cysteine did not show a relationship between melanoidin concentration and time to reach the steady state at the range of concentration assayed (up to 2 mg/mL). **Table 1** summarizes the kinetics parameters calculated for each melanoidin isolated from model systems or coffee. **Table 2** summarizes the kinetics parameters obtained for some reference antioxidant compounds analyzed at the same conditions.

To date, we are not able to describe a common melanoidin structure for foods, although good approximations for understanding the oligomerization pathway of melanoidins have been described by using MALDI-TOF-MS, GC-MS, and NMR in a 2-deoxy-D-ribose/methyl 4-aminobutyrate Maillard system (*19*). The final melanoidin structure depends highly on the type of constituents, water activity, or processing conditions. Therefore, the studies of the biological activity of melanoidins have been focused on the use of standard melanoidins obtained at controlled laboratory conditions. In this sense ultrafiltration is appropriate in the production of high-quality melanoidins. Then, a great limitation is the impossibility to relate the chemical structure or functional groups present in each isolated structure with the antioxidant effect recorded.

As discussed in the Introduction, most of the literature on the overall antioxidant activity of the MR has been reduced to the MRP (including the melanoidin fraction). The lack of a

Table 1. Kinetics Parameters for Assessing the Antiradical Efficiency (AE) of the Isolated Melanoidins from Model Systems As Compared with Coffee Melanoidin in an Aqueous Medium[a]

system	$EC_{50} \pm SD$ (mg/mL)	$TEC_{50} \pm SD$ (min)	data (*n*)	AE
GG	0.43 ± 0.16	41.8 ± 9.2	7	0.069 ± 0.013
GH	1.50 ± 0.08	18.2 ± 3.7	7	0.039 ± 0.008
GL	1.17 ± 0.41	31.0 ± 11.0	6	0.038 ± 0.019
GT	1.74 ± 0.06	55.0 ± 7.1	8	0.011 ± 0.001
GC	0.51 ± 0.08	87.8 ± 5.4	19	0.023 ± 0.003
GM	1.11 ± 0.08	51.5 ± 9.5	9	0.018 ± 0.002
LG	0.82 ± 0.14	28.0 ± 5.4	6	0.045 ± 0.003
LH	1.11 ± 0.55	10.4 ± 2.5	10	0.064 ± 0.022
LL	1.34 ± 0.05	39.0 ± 3.8	8	0.085 ± 0.037
LT	1.70 ± 0.12	39.0 ± 8.3	7	0.017 ± 0.006
LC	0.97 ± 0.07	78.5 ± 7.4	7	0.013 ± 0.001
LM	0.96 ± 0.05	21.1 ± 2.2	9	0.050 ± 0.004
coffee	2.06 ± 0.16	32.2 ± 5.4	8	0.015 ± 0.002

[a] EC_{50} describes the concentration (mg/mL) needed to decrease by 50% the initial $DMPD^{\bullet+}$, and TEC_{50} is the time to reach the steady state at the EC_{50}. Range of melanoidin concentration from 0.25 to 2.00 mg/mL. $P < 0.05$.

Table 2. Kinetics Parameters of Some Antioxidant Compounds (Units as in **Table 1**)

compound	EC_{50} (mg/mL)	TEC_{50} (min)	AE
gallic acid	0.0119	55.7	1.51
caffeic acid	0.0144	34.5	2.02
ferrulic acid	0.0186	20.1	2.67
Trolox	0.0781	38.3	0.34
tannic acid	0.1081	13.3	0.69

reliable tool for the isolation of melanoidins from a complex maillarized solution may complicate the study of its antioxidant properties. Furthermore, the composition of the food matrix was skipped in previous investigations, but recently its importance has been stressed. For instance, Krings and Berger (*20*) compared the antioxidative potential of some roasted foods (wheat germ, coffee, sweet almond, etc) by the classical method of bleaching of the stable DPPH radical and an accelerated oxidation experiment using stripped maize oil. MRP (including the melanoidin fraction) plays an important role, but results in both experiments could not be correlated. In our experiments, an aqueous medium is much more reliable for assessing the antiradical activity of melanoidins in liquid foods such as coffee, beer, or sweet wine.

Coffee is a widely consumed beverage in which MR is dramatically present. Therefore, melanoidins from coffee are a good reference of food melanoidins. It is expected that melanoidin isolated from coffee is structurally more complex than melanoidins isolated from a single combination of sugars and amino acid reaction mixtures. The main antiradical activity of the crude coffee is due to the phenolic compounds, such as caffeic acid and chlorogenic acid, but during roasting of coffee most of the phenolic compounds are destroyed or incorporated into the browning products (*21*). Then, we have to consider that chlorogenic acid donates a carbonyl residue in the early stage of the MR, and different melanoidins could be formed apart from reaction of the main free amino acids, such as cysteine and methionine.

From the results obtained it could be plausible to build a useful classification of the antiradical efficiency of the isolated melanoidins. According to the parameter EC_{50}, it is able to describe three groups: high (<1.0 mg/mL), intermediate (1−1.5 mg/mL), and low (1.5−2.0 mg/mL) activity. Melanoidins formed from glycine and cysteine, independent of the type of sugar used as reactant, exerted the higher antiradical activity, as compared with melanoidins obtained from tryptophan systems. Melanoidin isolated from medium-roasted coffee gave the lowest antiradical activity. However, when the EC_{50} values of some classical antioxidant compounds are compared, the activity is nearly not significant. With regard to the TEC_{50} parameter, there was observed a high variability among samples, and it could not be compared. This effect could be correlated with the main active structure in the melanoidin, but AE values obtained for classical antioxidant are of ∼10−100 times higher than AE values of isolated melanoidins.

The results obtained for melanoidins are in line with those described by Anese et al. (*22*), who suggested that the highest oxygen scavenging properties of a heated glucose−glycine model system are located in the first stages of the MR. Then, the antiradical activity of the MRP is focused in the low molecular weight MRP formed in previous stages of the melanoidin oligomerization. Data reported also agree with those of Rivero-Pérez et al. (*23*), who stated that melanoidins isolated from several Spanish sweet wines play an important role in the color, but not in their antioxidant activity, of wine. This study was also carried out by the DMPD method in a hydrophilic medium.

In conclusion, it is extremely difficult to correlate the antiradical properties of melanoidins to their structure because this is unknown. However, these results are important to describe the biological activity expected by the melanoidins in a foodstuff. It is important to highlight that amino acid plays a determinant role in the final antiradical activity of the melanoidins. On the other hand, melanoidins exert a low antiradical activity in aqueous systems as compared with traditional antioxidants. Then, the main antiradical power of the heat-processed food described in the literature is due to intermediate or low molecular weight MRP. The results support the hypothesis that the overall antioxidant effect of melanoidins could be mainly due to their metal-chelating properties. Future studies should focus on how the accessibility of the radical center of $DMPD^{\bullet+}$ to the radical scavenging activity of MRP and melanoidins is affected.

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