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ARTICLE

Biscuit Melanoidins of Different Molecular Masses Protect Human HepG2 Cells against Oxidative Stress

María Ángeles Martín, Sonia Ramos, Raquel Mateos, José Angel Rufián-Henares, Francisco José Morales, Laura Bravo, and Luis Goya*

Consejo Superior de Investigaciones Científicas, Instituto del Frío - ICTAN, José Antonio Novais 10, 28040 Madrid, Spain

Soluble melanoidins from biscuits were enzymatically solubilized and isolated by sequential ultrafiltration and separated by molecular mass in three different fractions, below 3 kDa, between 3 and 10 kDa, and over 10 kDa; the latter was subsequently digested by simulating gastric plus pancreatic digestive conditions. The four fractions were investigated for their protective effect against an oxidative challenge in HepG2 cells. Pretreatment of cells for 20 h with 0.5−10 μg/mL of any of the four fractions prevented the increased cell damage evoked by the challenge but, except for the intermediate size fraction, did not suppress the increased reactive oxygen species. Antioxidant defenses were rapidly restored after the challenge, and the increase of the oxidative stress biomarker malondialdehyde was prevented by the pretreatment with all but the undigested high molecular mass fraction. The results show that treatment of HepG2 cells with concentrations of biscuit melanoidins within the expected physiological range confers on the cells a significant protection against an oxidative challenge.

KEYWORDS: Antioxidant defenses; biomarkers for oxidative stress; dietary antioxidants; Maillard reaction products; reactive oxygen species

INTRODUCTION

There is currently considerable interest in the cytoprotective effects of dietary compounds against oxidative stress and the different defense mechanisms involved. Dietary antioxidants may protect against oxidative stress, and the human diet contains antioxidants that are formed in heat-treated foods. During food processing and storage, chemical reactions among food components lead to the formation of secondary antioxidants that may play a role in protection against cell oxidative damage (*1*). Maillard reaction products (MRPs) are important candidates for food antioxidants and, therefore, are being investigated for physiological and biological activities (*2*). Although the exact nature of the products formed is not yet well-known, melanoidins are thought to be the major antioxidant compounds resulting from MRP formation (*3*). Apart from their well-known technological and organoleptic implications on the overall food acceptability by consumers (*4*, *5*), melanoidins possess in vitro antioxidant (*6*, *7*) and antimicrobial activities (*8*) and exert anticariogenic (*9*) and antitumoral properties (*10*) as well as prebiotic effects (*11*). In addition, melanoidins are widely distributed in Western diets, although a precise estimation of their physiological fate after ingestion has not been addressed yet, being a subject of high interest for future research.

The antioxidant activity of melanoidins is especially interesting because these products are naturally formed during food processing and storage and they can influence the oxidative and shelf life of foods, such as cereal (*12*), milk (*13*), coffee (*14*), meat (*15*), and tomato juice (*16*). This effect of melanoidins has been related to their effectiveness as metal chelating agents (*17*) and their peroxyl or hydroxyl radical scavenging activity (*18*, *19*). In line with their antioxidant activity, some physiological effects of melanoidins have been reported such as induction of NADPH-cytochrome *c* reductase and glutathione-*S*-transferase of the detoxification enzyme system (*5*, *20*, *21*) and the protective effect against lipid peroxidation (*22*, *23*).

Because the liver is the main target for dietary constituents once absorbed from the gastrointestinal tract, studies dealing with the effect of antioxidants at a physiological level in the liver of live animals and at a cellular level in cultured hepatic cells are required. In this line, Somoza et al. (*21*) reported an increase in the hepatic activity of chemopreventive enzymes in rats fed a 4.5% coffee beverage for 15 days. A protective effect has been reported in cultured cells against an induced oxidative stress of a model melanoidin in isolated rat hepatocytes (*22*) and of a digested coffee melanoidin in human HepG2 (*23*). In the latter studies, only high molecular mass melanoidins (over 10 kDa) were tested for the chemoprotective effect. However, both high (*24*) and low (*25*) molecular mass melanoidins have been isolated from processed foods; consequently, the biological role of the potentially highly bioavailable low molecular mass melanoidins should not be dismissed.

A high proportion of daily melanoidin intake comes from processed cereals, especially bread and biscuits (see the Discussion). Hence, this research was aimed to investigate the protective effect of three biscuit melanoidin fractions (BMFs)

*Corresponding author (telephone +34.91.544.56.07; fax +34.91.549.36.27; e-mail luisgoya@if.csic.es).

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Table 1. Biscuit Composition: Main Formula Ingredients and Parameters Related to the Thermal Load Applied

flour	wheat[a]
protein	5.58 g/100 g
fat	18.5 g/100 g[a]
carbohydrate	72.0 g/100 g[a]
fiber	5.0 g/100 g[a]
moisture	4.83 g/100 g
Color CIE-Lab	43.71 (L^*), −1.03 (a^*), 16.80 (b^*)
furosine	837 mg/100 g of protein
hydroxymethylfurfural	11.2 mg/kg
furfural	<0.03 mg/kg
acrylamide	308 μg/kg

[a] As declared on the nutritional label. See Materials and Methods for the determination of the rest of the parameters.

of different molecular masses plus a high molecular mass fraction enzymatically treated against chemically induced oxidative stress in HepG2 cells. The human hepatoma HepG2 cell line is widely used for biochemical and nutritional studies as a cell culture model of human hepatocytes because these cells retain their morphology and most of their function in culture (*26*). In addition, HepG2 is a reliable model through which many dietary antioxidants and conditions can be assayed with minor interassay variations (*23*, *26*). Cell viability by the lactate dehydrogenase (LDH) assay, reactive oxygen species (ROS), nonenzymatic [reduced glutathione, (GSH)] and enzymatic [glutathione peroxidase (GPx) and reductase (GR)] antioxidant defenses, and the biomarker of lipid peroxidation malondialdehyde (MDA) were analyzed. The results show that treatment of HepG2 cells with concentrations of biscuit melanoidin within the expected physiological range seems to prepare favorably the cell to face an oxidative challenge by modulating the antioxidant defense system.

MATERIALS AND METHODS

Chemicals. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH), *tert*-butyl hydroperoxide (t-BOOH), dichloroflourescin, nicotine adenine dinucleotide phosphate reduced form (NADPH), 2,4-dinitrophenylhydrazone (DNPH), mercaptoethanol, EDTA, Pronase E, and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (Sigma-Aldrich, Madrid, Spain). 2,2′-Azobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was from Fluka Chemicals (Madrid, Spain). Falcon plates (100 and 60 mm) were from Cajal (Madrid, Spain), and 24- and 96-well Falcon microplates were from Biogen Científica (Madrid, Spain).

Cell Culture. Human HepG2 cells were maintained in a humidified incubator containing 5% CO_2 and 95% air at 37 °C. They were grown in DMEM F-12 medium from Biowhitaker (Lonza, Madrid, Spain), supplemented with 2.5% Biowhitaker fetal bovine serum (FBS) and 50 mg/L of each of the following antibiotics: gentamicin, penicillin, and streptomycin (all from Sigma, Madrid, Spain). Because serum added to the medium might interfere in the running of the assays and affect the results, plates were changed to FBS-free medium before the beginning of the assay (*26*). To study the cellular effects of the melanoidin fractions, different concentrations (0.5, 1, 5, and 10 μg/mL) of each fraction, dissolved in serum-free culture medium, were added to the cell plates for 20 h.

Isolation, Purification, Analysis, and Digestion of Melanoidins from Commercial Biscuits. A traditional wheat-flour biscuit sample (Fontaneda Marbú Dorada, United Biscuits Iberis, SL., Barcelona, Spain), for which the formula ingredients, composition, and parameters related to the thermal load applied are shown in **Table 1**, was purchased in a local supermarket. Protein, hydroxymethylfurfural, and furfural were measured as described by Rufian-Henares et al. (*27*); furosine was measured as in Delgado-Andrade et al. (*28*); color (CIE-Lab color system) and acrylamide were determined as described by Morales and Arribas-Lorenzo (*29*). This biscuit was used to obtain melanoidins and related fractions following the procedure described by Borrelli et al. (*20*) with some modifications. Briefly, homogenized biscuit samples (500 mg) were diluted in 25 mL of a Pronase E solution containing 0.375 mg of Pronase E (7.5 units/mg) in 0.1 M sodium borate at pH 8.2 buffer, vigorously stirred, and incubated at 37 °C for 40 h under shaking. These proportions can be scaled up, but 35 independent extractions were carried out following a formerly validated procedure (*6*). The reaction was stopped by cooling in an ice−water bath followed by the addition of 100 μL of trichloroacetic acid solution (40%, w/v) and centrifugation at 4500*g* for 10 min at 4 °C. The supernatants were filtered (0.45 μm) and stored at −20 °C until analyzed. The solubilized fractions were then subjected to sequential ultrafiltration using an Amicon ultrafiltration cell model 8400 (Amicon, Beverly, MA), equipped with a 10000 Da nominal molecular mass cutoff membrane (*6*). The retentate was made up to 200 mL with water and washed again. This washing procedure (diafiltration) was repeated at least three times. The high molecular mass fraction corresponding to melanoidins (BMF10 sample; retentate at 10 kDa) was freeze-dried and stored in a desiccator at 4 °C until analysis. The eluates obtained after 10000 Da ultrafiltration were mixed and ultrafiltered against a 3000 Da nominal molecular mass cutoff membrane. Then, two fractions were obtained: a retentate (BMF3−10) with intermediate molecular mass compounds and a filtrate (BMF3) with low molecular mass compounds. These two fractions were also lyophilized and stored at 4 °C until analysis. Analysis of melanoidins by high-performance gel permeation chromatography (HPGPC) was performed to assess the purity of the fractions as described previously (*6*). Simulated gastrointestinal digestion of the high molecular mass fraction of biscuit melanoidins (BMF10) was carried out following the method described by Ames et al. (*23*, *30*). The sample, identified as BMF10D, was lyophilized and stored at −20 °C until analysis. The fraction containing the highest molecular mass melanoidin was selected for simulated digestion because it is the most likely to undergo gastric and intestinal digestion, yielding a higher amount of products with potential biological function. In the case of degradation by gastric enzymes, it will be more relevant in structures with higher molecular mass because a higher proportion of protein in the structure is expected.

In Vitro Antioxidant Activity. *$ABTS^{\bullet+}$ Assay*. The in vitro antioxidant capacity of the biscuit melanoidins was estimated in terms of free radical scavenging activity, following the procedure described by Delgado-Andrade et al. (*6*) for Maillard reaction products, adapted to a plate reader. A Synergy HT-multimode microplate reader with automatic reagent dispenser and temperature control from Biotek Instruments (Winooski, VT) was used. Biotek Gen5 data analysis software was used. All of the reaction mixtures were prepared in duplicate, and four independent assays were performed for each sample. Limit of quantitation was set at 40 μM. Results were expressed as micromole equivalents of Trolox per gram of sample.

ORAC Assay. $ROO^{\bullet}$ scavenging activity was measured by monitoring the fluorescence decay as result of $ROO^{\bullet}$-induced oxidation of fluorescein, known as the oxygen radical absorbance capacity (ORAC) assay. The water-soluble azo initiator AAPH was applied as a clean and controllable source of thermally produced alkylperoxyl free radicals ($ROO^{\bullet}$) in aqueous media. $ROO^{\bullet}$ are generated by AAPH in a microplate reader at 37 °C. Previously, it was ensured that substrate did not affect the activity of AAPH, mainly by sequestering, at the concentrations tested. The antiradical activity against AAPH was estimated according to the procedure reported by Morales et al. (*31*). Reaction fluorescence was recorded every minute for 90 min at 485 and 528 nm excitation and emission wavelengths, respectively, and the area under the curve was calculated. Limit of quantitation was set at 5 μM. Results were expressed as micromole equivalents of Trolox per gram of sample.

Evaluation of Cell Viability (LDH Leakage Assay), Reactive Oxygen Species, and Reduced Glutathione. For cell viability, cells were seeded in 60 mm diameter plates at a concentration of 1.5×10^6 per plate. Two days later the cells were treated with four different concentrations of all four melanoidin fractions (melanoidin pretreated cells) or serum-free medium (namely, controls and t-BOOH) for 20 h prior to being submitted to the t-BOOH challenge for 3 h (all except controls). Then, cell culture medium was collected, and the cells were washed with phosphate-buffered saline (PBS), collected by scraping, and assayed for LDH as in Alía et al. (*26*). The LDH leakage was estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content (*26*). Cellular ROS were quantified by the DCFH assay using a microplate

reader (*32*). For the assay, cells were seeded in 24-well multiwells at a rate of 2×10^5 cells per well and treated the day after for 20 h as above. Prior to the assay, 5 μM DCFH was added to the wells for 30 min at 37 °C. Then, the medium was discarded, the cells were washed with PBS, and fresh medium containing 200 μM t-BOOH was added for 3 h to all plates but controls. After being oxidized by intracellular oxidants, DCFH will become dichlorofluorescein (DCF) and emit fluorescence. The content of GSH was quantitated by the fluorometric assay of Hissin and Hilf with a few modifications (*33*). Cells were seeded at 2×10^6 per plate, treated the day after with melanoidins for 20 h as above, and collected afterward in PBS. The results of the samples were referred to those of a standard curve of GSH.

Determination of GPx and GR Activity. For the assay of the GPx and GR activities, cells were seeded at 2×10^6 per plate, treated the day after with melanoidins for 20 h as above, suspended in PBS, and centrifuged at 300*g* for 5 min to precipitate cells. Cell pellets were resuspended in 20 mM Tris, 5 mM EDTA, and 0.5 mM mercaptoethanol, sonicated, and centrifuged at 3000*g* for 15 min. The enzyme activity was measured in the supernatants. The determination of GPx activity is based upon the oxidation of reduced glutathione by GPx, using *tert*-butyl hydroperoxide as a substrate, coupled to the disappearance of NADPH by GR (*34*). GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione (*35*). Data are expressed as units of enzyme activity per milligram of protein. Protein was measured by using the Bradford reagent.

Determination of Malondialdehyde. HepG2 cells were seeded at 2×10^6 per plate, treated the day after with melanoidins for 20 h as above, suspended in PBS, and centrifuged at 300*g* for 5 min to precipitate cells. Cellular MDA was analyzed in supernatants by high-performance liquid chromatography (HPLC) as its DNPH derivative (*36*). An Agilent 1100 series HPLC-DAD was used, and MDA values are expressed as nanomoles of MDA per milligram of protein; protein was measured by using the Bradford reagent.

Statistics. Statistical analysis of data was as follows: prior to analysis, the data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by a Bonferroni test when variances were homogeneous or by Tamhane test when variances were not homogeneous. The level of significance was $P < 0.05$. A SPSS version 15.0 program has been used.

RESULTS

BMF Characterization. Enzymatic digestion is necessary to ensure proper solubilization of the biscuit melanoidin (*20*). After Pronase E digestion, nearly 43% of the initial cookie material (dry mass basis) was soluble. The relative mass proportions of the three BMFs were 1.9, 9.0, and 32.2% for BMF10, BMF3−10, and BMF3, respectively.

Contrary to coffee melanoidins, the chemical structure of biscuit melanoidins is not available yet, although it is generally accepted that they interact with flour proteins. Therefore, characterization of the different fractions has to be performed by indirect methods. Because color is the most obvious characteristic of melanoidins, this attribute was used to characterize the melanoidins obtained from biscuit by applying apparent absorptivity index (a_{420}, L/g/cm) at 420 nm. This parameter is similar to the specific extinction coefficient used by others (*24*), which allows quantification of the melanoidin level in a solution. The apparent absorptivity index can be deduced from the slope of Abs_{420} versus melanoidin concentration (mg/mL). Values of 0.025, 0.0158, 0.1306, and 0.1170 were recorded for BMF3, BMF3−10, BMF10, and BMF10D, respectively. As described in the literature (*17*), BMF10 has an a_{420} index higher than that of lager beer (0.014) but significantly lower than that of coffee (1.725) or sweet wine (2.027) melanoidins (10 kDa fraction).

Melanoidins are anionic structures characterized in populations according to their apparent molecular mass by gel permeation chromatography or metal affinity chromatography.

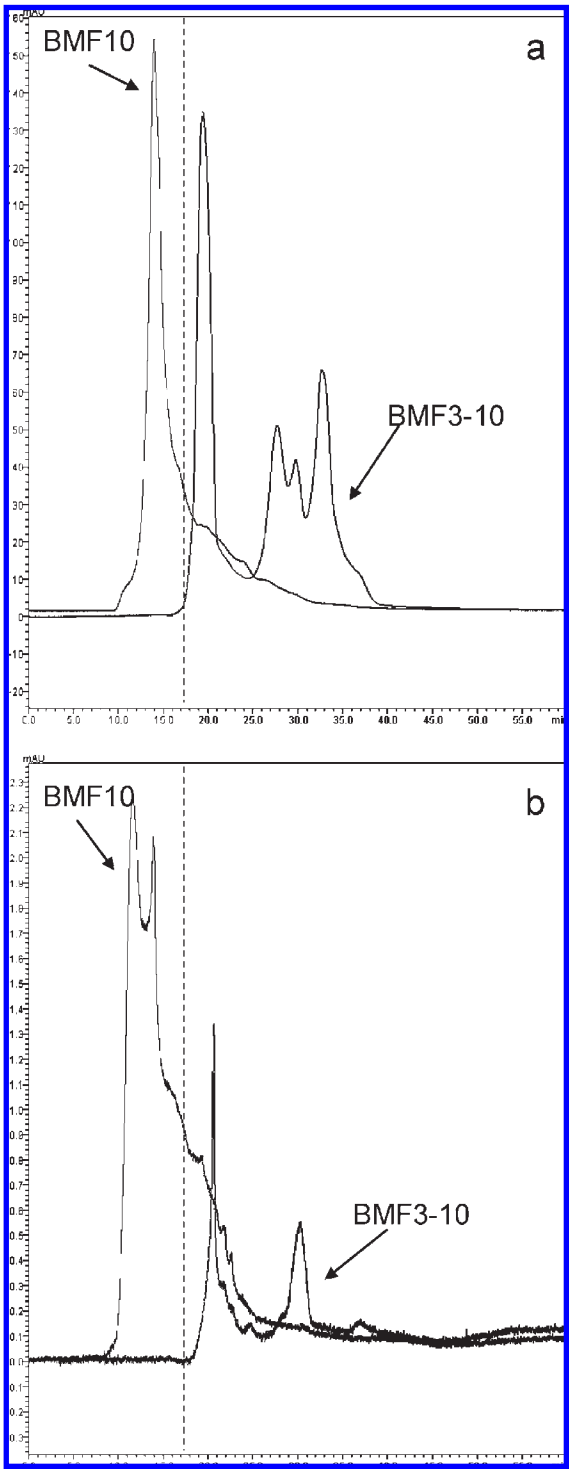

Figure 1. HPGPC chromatogram of biscuit melanoidin fraction of molecular mass over 10 kDa (BMF10) and molecular mass between 3 and 10 kDa (BMF3−10) recorded at 220 nm (**a**) and 420 nm (**b**). Dotted line denotes the nominal cutoff at 10 kDa. Sixty microliters of sample (10 mg/mL) was loaded at 0.6 mL/min in water.

Figure 1 shows the complexity of the high molecular mass fractions where some chromatographic regions present a clear absorption at 420 nm. In addition, UV spectral analysis of the BMF3−10 fraction is characterized for an important absorbance between 270 and 280 nm, apart from 220 nm.

Another characteristic assigned to melanoidins is their antioxidant properties. In vitro antioxidant capacity of the BMFs tested by ABTS and ORAC showed the highest values

Table 2. In Vitro Antioxidant Capacity of BMFs[a]

	ABTS (μmol of Trolox/g)	ORAC (μmol of Trolox/g)
BMF3	6.7 ± 0.9 a	2.5 ± 0.2 a
BMF3−10	30.5 ± 3.2 b	9.3 ± 0.7 b
BMF10	28.6 ± 2.4 bc	8.0 ± 1.1 bc
BMF10D	22.0 ± 3.1 c	6.2 ± 1.2 c

[a] Values are means ± SD of four data per condition. Means in a column without a common letter differ, $P < 0.05$.

Table 3. Direct Effect of BMFs on Cell Viability and Intracellular ROS Generation[a]

		LDH leakage (% of LDH)	ROS (fluorescence units)
control		3.5 ± 0.6 a	14695 ± 603 d
BMF3	0.5 μg/mL	3.2 ± 0.9 a	10214 ± 594 c
	1 μg/mL	4.0 ± 0.6 a	8159 ± 902 b
	5 μg/mL	4.2 ± 0.7 a	7188 ± 603 ab
	10 μg/mL	4.1 ± 0.8 a	7154 ± 802 ab
BMF3−10	0.5 μg/mL	3.9 ± 0.5 a	9642 ± 603 c
	1 μg/mL	4.2 ± 0.6 a	8033 ± 509 b
	5 μg/mL	4.0 ± 0.4 a	8124 ± 710 b
	10 μg/mL	3.6 ± 0.7 a	7970 ± 630 b
BMF10	0.5 μg/mL	2.9 ± 0.7 a	9874 ± 603 c
	1 μg/mL	2.7 ± 0.6 a	8918 ± 884 bc
	5 μg/mL	2.6 ± 0.5 a	8012 ± 732 b
	10 μg/mL	2.7 ± 0.5 a	8897 ± 701 b
BMF10D	0.5 μg/mL	2.8 ± 0.5 a	8701 ± 911 bc
	1 μg/mL	3.1 ± 0.4 a	6787 ± 530 a
	5 μg/mL	2.8 ± 0.6 a	5396 ± 562 a
	10 μg/mL	3.5 ± 0.4 a	5617 ± 683 a

[a] HepG2 cells were treated with the noted concentrations of BMFs during 20 (LDH leakage) or 2 (ROS) h. Results of LDH leakage are expressed as percent of LDH activity in the culture medium of the total activity, culture medium plus intracellular. Values are means ± SD of six to eight data. Intracellular ROS production is expressed as fluorescence units, and values are means ± SD of seven to eight different samples per condition. Means in a column without a common letter differ, $P < 0.05$.

in BMF3−10 and BMF10 followed by BMF10D, and the lowest capacity was that of BMF3 (**Table 2**).

Direct Effects of BMFs on HepG2. Cells were incubated for 20 h with 0.5, 1, 5, and 10 μg/mL (LDH, ROS, and GSH assays) and 1 μg/mL (MDA assay) of the four BMFs to evaluate the direct effect on the biomarkers. **Table 3** shows that LDH leakage to the culture medium (indicating cell damage) remained unaltered after 20 h with all BMF treatments. Additionally, treatment of cells with all concentrations of the four BMFs greatly decreased ROS production as compared to those of control cells (**Table 3**). A significant dose−response was observed with the four fractions; that is, the higher the dose, the lower the ROS generated. BMF10D was the melanoidin fraction that evoked a larger decrease in ROS. Furthermore, treatment of HepG2 cells with increasing concentrations of BMFs during 20 h generally provoked a significant increase in GSH concentration (**Figure 2**). BMF3−10 and BMF10D were the fractions that more steadily increased GSH concentration, and only cells treated with 10 μg/mL BMF3 and 5 μg/mL BMF10 did not show an increase in steady-state GSH. To condense the data and emphasize their significance and to test for MDA values as well as the protective effect against oxidative stress-induced GPx and GR activity, a single dose of each BMF (1 μg/mL) was selected. Treatment of cells with 1 μg/mL BMF3 and BMF3−10 evoked a diminution of MDA levels, whereas BMF10 and BMF10D had no significant effect on the lipid peroxidation biomarker (**Figure 3**).

Protective Effect of BMFs on HepG2 Submitted to Oxidative Stress. *Cell Viability*. Treatment with 200 μM t-BOOH for 3 h evoked a great increase in LDH activity in the culture medium, indicating prominent cell damage in HepG2 (**Table 4**). Pretreatment for 20 h of HepG2 cultures with 0.5−10 μg/mL of all BMFs completely prevented cell damage induced by t-BOOH, maintaining LDH ratios in the range of control nonstressed cells (**Table 4**).

Redox Status. Cells treated with t-BOOH showed a significant 2-fold increase in ROS generation after 2 h as compared to nonstressed controls (**Table 4**). Pretreatment of HepG2 cultures with 0.5−10 μg/mL of BMF3 or BMF10D for 20 h had no ROS-reducing effect. A considerable reduction of ROS production was observed when cells were pretreated with 5−10 μg/mL BMF10 for 20 h, but the most significant reduction of ROS production was observed when cells were pretreated for 20 h with BMF3−10 (**Table 4**).

Antioxidant Defenses. *Nonenzymatic Defense*. Addition of 200 μM t-BOOH to cells evoked a dramatic decrease in the cytoplasmic GSH, which was barely overcome by a pretreatment with 0.5 μg/mL BMF3 (**Figure 4**). Pretreatment for 20 h with BMF3−10 evoked a partial (1 and 5 μg/mL) or complete (10 μg/mL) recovery of the decreased GSH induced by t-BOOH. A partial dose-dependent recuperation of GSH values was also observed when cells were pretreated with BMF10. Finally, pretreatment with all doses of BMF10D for 20 h attenuated to some extent the diminished GSH (**Figure 4**).

Enzymatic Defenses. The presence of 200 μM t-BOOH in the culture medium for 3 h induced a significant 2-fold increase in the enzyme activity of GPx and GR (**Figure 5**). When cells were pretreated with 1 μg/mL of each BMF for 20 h, the t-BOOH-induced increase in GPx was completely blunted in all cases except in cells pretreated with BMF3. The induced increase of GR was prevented with all four BMFs. It is worth noting that the activity of GR in cells pretreated with BMF3−10 and BMF10D and that of GPx in those pretreated with BMF3−10 were even lower than that of control cells not submitted to t-BOOH (**Figure 5**).

Biomarker of Oxidative Damage. The treatment of control HepG2 cells with 200 μM t-BOOH during 3 h evoked a significant increase of about 60% in the cellular concentration of MDA, indicating permanent oxidative damage to cell lipids (**Figure 6**). Pretreatment of HepG2 with 1 μg/mL of BMF3, BMF3−10, or BMF10D for 20 h prevented the MDA increase induced by t-BOOH, indicating a reduced level of lipid peroxidation in response to t-BOOH. Only a partial reduction of MDA was observed in cells that had previously been in the presence of the undigested high molecular mass melanoidin, BMF10 (**Figure 6**).

DISCUSSION

Biological activities of melanoidins include prevention of LDL oxidation (*37*) and scavenging of active oxygen (*38*) and hydroxyl, superoxide, and peroxy radicals (*18*, *19*). All of these in vitro activities may well correspond to physiological effects in cultured eukaryotic cells and live organisms. In this line, melanoidins have been reported to induce the chemopreventive enzyme system (*5*, *20*, *21*) and to protect against lipid peroxidation in isolated rat hepatocytes (*22*). More recently, a digested coffee melanoidin has shown a relevant protection of HepG2 cells against oxidative stress (*23*). In this study, we investigated the chemoprotective effect of different molecular mass fractions of biscuit melanoidins against an induced oxidative stress. The results unequivocally

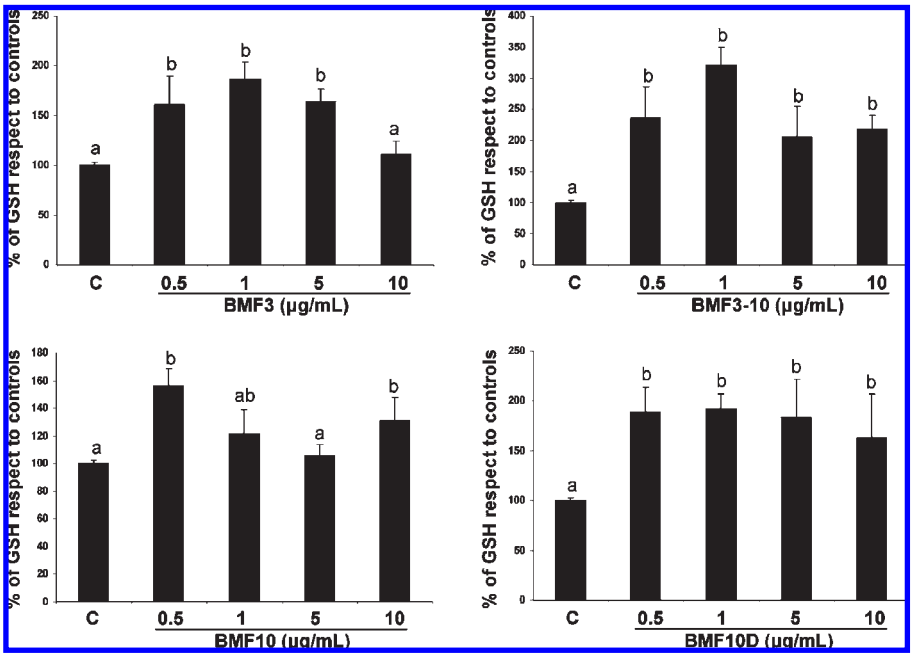

Figure 2. Direct effect of BMFs on intracellular concentration of reduced glutathione. HepG2 cells were treated with the noted μg/mL concentrations of BMFs for 20 h. The results of the fluorescent analysis for GSH are expressed as percent of control value ± SD of four or five different samples per condition. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

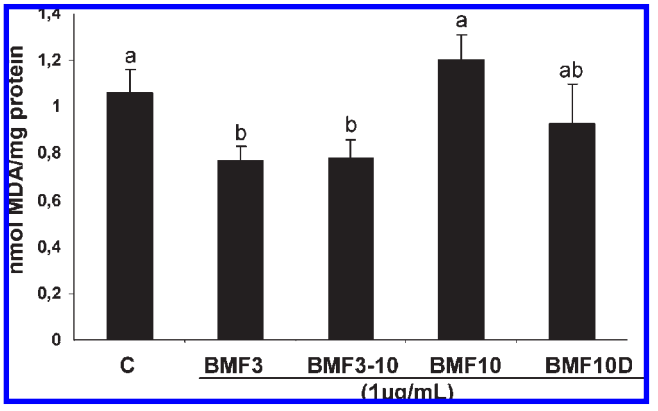

Figure 3. Direct effect of BMFs on cytoplasmic concentration of malondialdehyde. HepG2 cells were treated with 1 μg/mL of BMFs for 20 h. MDA measured by HPLC in cytoplasmatic contents of HepG2 is expressed as means ± SD, $n = 4$. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

show that pretreatment of HepG2 cells with the biscuit melanoidins offered significant protection against the oxidative challenge.

Enzymatic treatment of commercial biscuits with Pronase is necessary to efficiently extract the material bound to protein as previously reported (*20*). In contrast to coffee, where melanoidins are carbohydrate−phenol-based structures, melanoidins in processed cereals are associated with the protein structure and so they are also called melanoproteins (protein-based melanoidins) (*39*). Recently, Smaniotto et al. (*40*) have investigated the role of different peptides and proteins in melanoidin formation and concluded that melanoidins are not formed from further degradations of glycated proteins. Regardless of the method applied to test for the in vitro antioxidant capacity, high (plain and digested) and intermediate molecular mass fractions showed the highest antioxidant activity. These results are in line with those obtained by Borrelli et al. (*20*), who found that the low molecular mass melanoidin fraction from bread and biscuit showed the lowest

Table 4. Protective Effect of BMFs on Cell Viability and Intracellular ROS Generation[a]

		LDH leakage (% of LDH)	ROS (fluorescence units)
control		3.5 ± 0.3 a	13154 ± 805 a
t-BOOH (200 μM)		73.0 ± 7.1 b	24652 ± 1052 b
t-BOOH + BMF3	0.5 μg/mL	1.9 ± 0.4 c	23840 ± 919 b
	1 μg/mL	1.8 ± 0.6 c	22977 ± 812 b
	5 μg/mL	2.0 ± 0.5 c	24823 ± 773 b
	10 μg/mL	2.3 ± 0.6 c	23542 ± 896 b
t-BOOH + BMF3−10	0.5 μg/mL	2.4 ± 0.2 c	16765 ± 929 c
	1 μg/mL	2.6 ± 0.2 c	16049 ± 694 c
	5 μg/mL	2.8 ± 0.3 c	16176 ± 980 c
	10 μg/mL	2.1 ± 0.5 c	16711 ± 763 c
t-BOOH + BMF10	0.5 μg/mL	2.1 ± 0.6 c	22909 ± 923 b
	1 μg/mL	2.8 ± 0.3 c	22882 ± 898 b
	5 μg/mL	2.6 ± 0.2 c	19523 ± 582 d
	10 μg/mL	5.0 ± 1.3 d	19821 ± 944 d
t-BOOH + BMF10D	0.5 μg/mL	2.6 ± 0.9 c	23122 ± 962 b
	1 μg/mL	2.0 ± 0.8 c	22958 ± 802 b
	5 μg/mL	2.6 ± 0.5 c	22879 ± 711 b
	10 μg/mL	2.1 ± 0.2 c	23131 ± 990 b

[a] HepG2 cells were treated with the noted concentrations of BMFs during 20 h, then the cultures were washed and 200 μM t-BOOH was added for 3 (LDH leakage) or 2 (ROS) h to all cultures except controls. Results of LDH leakage are expressed as percent of LDH activity in the culture medium of the total activity, culture medium plus intracellular. Values are means ± SD of six to eight data. Intracellular ROS production is expressed as fluorescence units, and values are means ± SD of seven to eight different samples per condition. Means in a column without a common letter differ, $P < 0.05$.

antioxidant activity. Although there are other sources in the original biscuit accounting for potential antioxidant activity

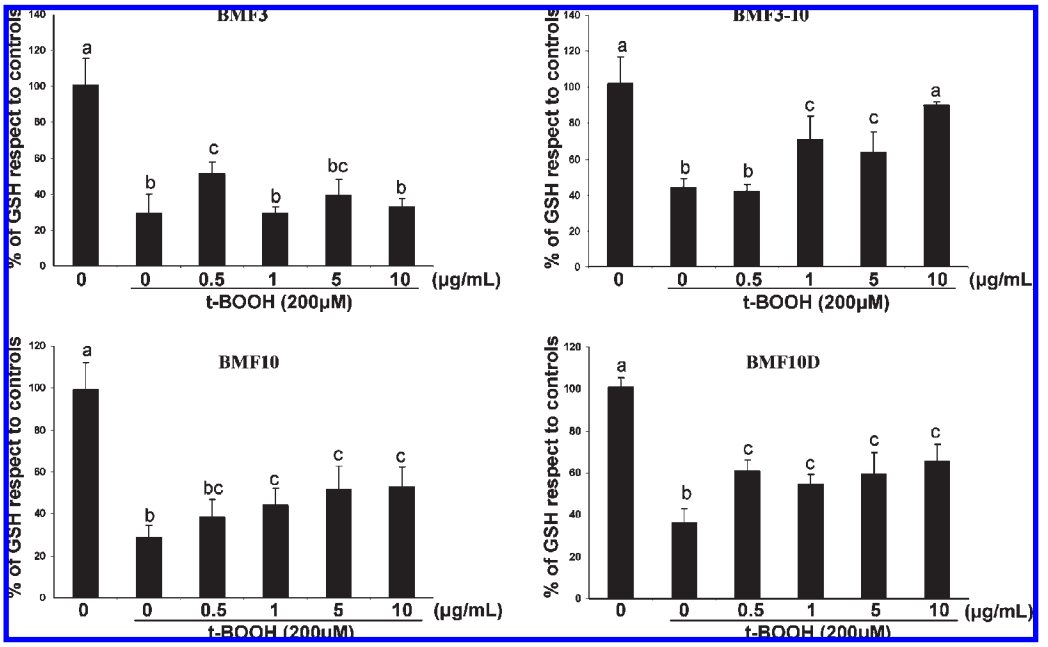

Figure 4. Protective effect of BMFs against oxidative stress on intracellular concentration of reduced glutathione. HepG2 cells were treated with the noted μg/mL concentrations of BMFs for 20 h, then the cultures were washed, and 200 μM t-BOOH was added to all cultures except controls for 3 h. The results of the fluorescent analysis of GSH are expressed as percent of control value $\pm$ SD of four or five different samples per condition. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

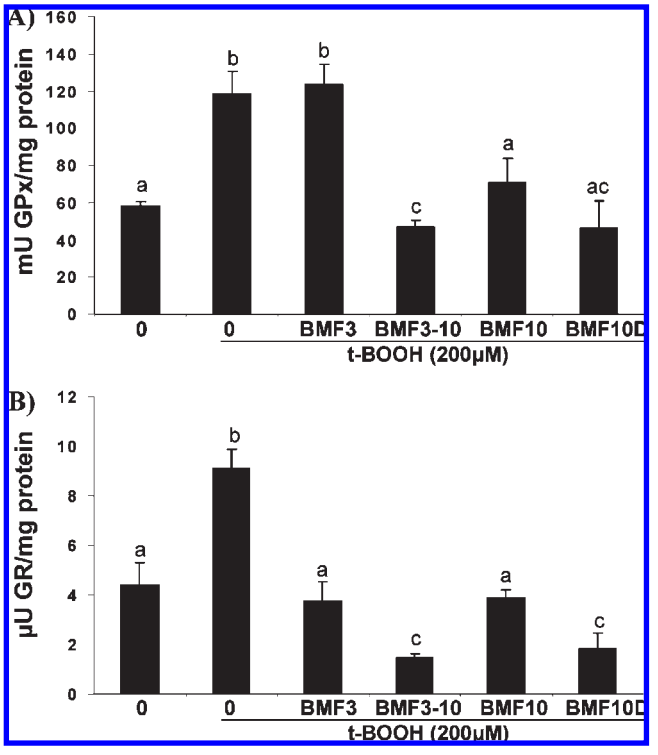

Figure 5. Protective effect of BMFs against oxidative stress on the activities of GPx and GR. HepG2 cells were treated with 1 μg/mL of BMFs for 20 h, then the cultures were washed, and 200 μM t-BOOH was added for 3 h to all cultures except controls. Values are means $\pm$ SD of four or five different samples per condition. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

such as polyphenols, fiber, and thiol residues, Borrelli et al. (*20*) did not consider most of them but speculated on the contribution of pyrrolinone reductones named pronyl-glycine and pronyl-lysine.

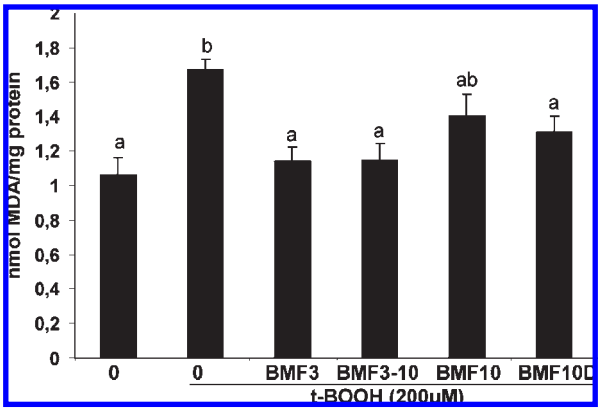

Figure 6. Protective effect of BMFs on cytoplasmic concentration of malondialdehyde. HepG2 cells were treated with 1 μg/mL of BMFs for 20 h, then the cultures were washed, and 200 μM t-BOOH was added for 3 h to all cultures except controls. MDA measured by HPLC in cytoplasmatic contents of HepG2 is expressed as means $\pm$ SD, $n = 4$. Different letters indicate statistically significant differences ($P < 0.05$) among different groups.

Although melanoidins may have potent antioxidant effects in vitro and in vivo, elevated doses of these dietary compounds may also be toxic and mutagenic in cell culture systems, and excess consumption by mammals could cause adverse metabolic reactions (*2*, *5*). Therefore, before the protective effect of the tested antioxidant is targeted, it is necessary to ensure that no direct damage is caused to the cell by the compound. Thus, cell integrity and redox status were determined in cells treated with different concentrations of BMFs in the micrograms per milliliter range. The concentration range is realistic for the evaluation of the effect at the physiological level because the daily intake of melanoidins from Western diets is relevant. It is estimated that about 322 mg of intermediate and high molecular mass melanoidins is consumed per biscuit (average biscuit weight 6.88 g) (*31*). Considering

the consumption of biscuits in Spain (4.59 kg/person/year), a realistic estimation of the daily intake of biscuit melanoidin would be of about 1 g/person/day (this value includes the sum of fractions BMF10 and BMF3−10). Because no damage in cell integrity was observed after a treatment for 20 h with BMF concentrations up to 10 μg/mL, it can be assumed that the range of concentrations finally selected (0.5−10 μg/mL) can be safely used to study the potential protective effect in vitro of BMFs against a condition of oxidative stress. It should be mentioned that mutagenicity of the fractions has not been evaluated. Through either bioavailability or gut fermentation, melanoidins or their fractions may reach the liver and other target tissues and accomplish their biological role (*21*).

Evaluation of ROS can be used as an index to quantify the overall oxidative stress in cells (*32*). The considerably reduced cellular ROS production evoked by the four BMFs is in agreement with previous papers indicating that melanoidins are effective scavengers of hydroxyl, superoxide, and peroxy radicals in vitro (*18*, *19*). A direct effect on ROS quenching has been reported in the same cell line for other natural dietary antioxidants such as flavonoid quercetin (*26*) and olive oil phenolic hydroxytyrosol (*41*).

Previous papers have indicated that treatment of HepG2 cells with natural antioxidants has resulted in both increase (*26*) and decrease (*23*) of steady-state GSH concentrations. The former has been explained by a specific effect of certain plant-derived food components stimulating glutathione synthesis (*42*, *43*) and the latter by intense conjugation of compounds to thiol groups of reduced glutathione, as in the case of green tea phenolics (*44*). Because no direct effect on GSH synthesis or repressed interactions with SH groups have been reported for melanoidins to date, no plausible explanation for the enhanced GSH concentration after 20 h with BMFs can still be offered. Other reduced soluble thiols including cysteine, γ-glutamylcysteine, and homocysteine are able to react with the fluorescent probe, and some interference with the results should not be ruled out (*26*, *45*). Finally, the significant decrease in steady-state MDA when cells were treated with BMF3 or BMF3−10 confirms a reduced oxidative damage in cell lipids.

Therefore, the results indicate that treatment for 20 h with the tested concentrations did not affect cell viability, but decreased lipid peroxidation and evoked changes in the basal redox status, such as reduced ROS and increased GSH, that place the liver cells in favorable conditions to face an oxidative challenge. The response of the melanoidin-conditioned cells to that challenge, the potent pro-oxidant t-BOOH, was subsequently tested.

The response of the antioxidant defenses to different oxidative challenges was investigated in cultured HepG2 cells, and the results demonstrate that treatment of these cells with t-BOOH yields an excellent model of oxidative stress in cell culture (*45*). In these conditions, the complete inhibition of cytotoxicity induced by t-BOOH when HepG2 cells were pretreated with BMFs for 20 h indicates that integrity of the conditioned cells was fully protected against the potent oxidative insult. Because the intimate structure and molecular mechanism of action of melanoidins remain unknown, the complete protection of cell viability induced by melanoidin moieties of highly different molecular masses might suggest an unspecific binding to cell membrane proteins and lipids by noninternalized melanoidins, rather than a precise biochemical effect through specific membrane receptors and/or defined signaling pathways by in-cell BMFs. Whatever the mechanism, the presence of BMFs permits all necessary exchange of the living cell with the milieu, and it does not hinder the entrance of t-BOOH to the cell and the consequent ROS enhancement, which is not prevented by the BMF pretreatment.

The mechanism by which melanoidins scavenge oxygen free radicals is still unclear. It has been proposed that these products contain at least one hydroxyl group, which might act as a hydrogen donor and therefore have the ability to scavenge free radicals (*18*, *19*). Contrary to a previous study with the flavonoid quercetin (*46*), but similar to what was observed with other dietary antioxidants such as the olive oil hydroxytyrosol (*41*) and a digested coffee melanoidin (*23*), the t-BOOH-induced increased ROS generation was not generally prevented in cultured cells pretreated with BMFs. Some authors have found that the high molecular mass fractions were more effective as antioxidants than all other fractions due not only to the direct scavenging ability of radicals but also to their stronger metal-chelating capability (*17*). However, in this study we found that the BMFs that partly prevented the t-BOOH-induced ROS generation were BMF3−10 and BMF10. These data suggest that high levels of ROS generated during the stress period are being more efficiently quenched by the antioxidant defenses of cells pretreated with BMF3−10 and BMF10, resulting in reduced cell damage.

As the main nonenzymatic antioxidant defense within the cell, GSH depletion reflects intracellular oxidation, whereas an increase in GSH concentration could be expected to prepare the cell against a potential oxidative insult (*42*, *43*, *45*, *46*). The decrease in the concentration of GSH induced by t-BOOH was partly prevented by pretreatment for 20 h with all four BMFs. A similar response has been reported for a coffee melanoidin (*23*) and other dietary antioxidants (*41*, *46*). The best recovery of GSH values was again observed with BMF3−10, one of the fractions with higher in vitro antioxidant capacity, and the worst with BMF3. Overall, the results suggest that, despite the increased consumption of GSH in the enzymatic and nonenzymatic quenching of ROS generated by t-BOOH, the increased levels of GSH in the BMF-treated cells before exposure to the oxidative challenge prevent the complete depletion of the intracellular GSH stock.

GPx and GR play a crucial role in the intracellular quenching of cell-damaging peroxide species and the effective recovery of the steady-state concentration of reduced glutathione. Enhanced activity of its enzyme defenses is a cell requirement to face the increasing generation of ROS induced by the potent prooxidant t-BOOH (*21*, *22*, *41*, *46*). However, a rapid return of the antioxidant enzyme activities to basal values once the challenge has been surmounted will position the cell in a favorable condition to deal with a new insult. Accordingly, we have previously reported that realistic concentrations of a digested coffee melanoidin prevent cell damage by preventing the permanently increased activity of GPx and GR induced by t-BOOH (*23*). In line with those results, in the present study we show, for the first time, that a long-term treatment of human liver cells with realistic concentrations of BMFs of molecular mass over 3 kDa prevents the long-lasting increase in the activity of GPx and GR induced by oxidative stress. These results indicate that at the end of an induced stress period the antioxidant defense system of cells that had been pretreated with biscuit melanoidin has more efficiently returned to a steady-state activity, diminishing, therefore, cell damage and enabling the cell to cope in better conditions with further oxidative challenges. As in the case of ROS and GSH, BMF3 was the fraction less effective in the recovery of GPx activity, which also coincides with the lowest in vitro antioxidant capacity of this fraction. However, BMF3 confers on HepG2 cells a complete protection against cell damage, similar to that of the other BMFs; consequently, biochemical mechanisms different from those here studied might be involved in the BMF3-induced cell protection.

Because MDA, a three-carbon compound formed by scission of peroxidized polyunsaturated fatty acids, has been found to be

elevated in various diseases thought to be related to free radical damage, it has been widely used as an index of lipoperoxidation in biological and medical sciences (*47*). By using a sensitive method (*36*) we have found that the t-BOOH-induced increase of MDA was completely avoided when cells were pretreated for 20 h with BMF3, BMF3−10, and BMF10D. This protection by different molecular mass fractions of a biscuit melanoidin against an induced lipid peroxidation in a cell culture is in line with previous studies that showed a similar effect by model melanoidins in primary cultures of hepatocytes (*22*) and a digested coffee melanoidin in cultured HepG2 cells (*23*). Therefore, the rapid recovery of the redox homeostasis evoked by the pretreatment with BMFs would ensure an insignificant lipid peroxidation and negligible cell damage.

In summary, our results demonstrate that different molecular mass fractions of biscuit melanoidin have the ability to protect human HepG2 cells against an oxidative challenge by reducing free radical activity and enhancing antioxidant defenses. Interestingly, the fraction that evidenced a higher antioxidant capacity in vitro, BMF3−10, also showed the best recovery of the antioxidant defenses in cultured cells. These results give more insight on the biological activity of food melanoidins and extend the previous data on the chemoprotective effect of synthetic and coffee melanoidins to those contained in a highly consumed foodstuff, biscuits. The underlying molecular mechanisms involved in the effect of BMF3−10 in HepG2 are currently being investigated following our previous approaches reported in Martín et al. (*48*).

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