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Influence of Molecular Weight Fractions Isolated from Roasted Malt on the Enzyme Activities of NADPH−Cytochrome *c*−Reductase and Glutathione-*S*-transferase in Caco-2 Cells

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In the present study, water-soluble nonenzymatic browning products (melanoidins) formed in roasted malt were separated, quantified, and investigated for their effects on detoxifying mechanisms in intestinal Caco-2 cells. The melanoidins were prepared from roasted malt by hot water extraction, and the water-soluble compounds were separated into different molecular weight (MW) fractions by gel filtration chromatography. By monitoring the effluent at 300 nm, seven molecular fractions I−VII were consecutively collected, revealing that ∼2.3% of the water-soluble compounds had mean MWs between 10000 and 30000 Da. Thus, the bulk of water-soluble malt melanoidins consisted of MW > 30000 Da, among which ∼58% showed mean MWs between 60000 Da and 100000 Da, whereas ∼32% exhibited mean MWs of 200000 Da. Biotransformation enzyme activities of NADPH−cytochrome *c*−reductase (CCR) and glutathione-*S*-transferase (GST) were analyzed in Caco-2 Cells after 48 h of exposure to the different MW fractions. The low MW fraction of 10000 Da was most effective in activating the CCR and the GST activities (+122 and +33% vs control, respectively). The majority of the mid molecular weight compounds tested showed an activating effect on CCR activity and an inhibitory effect on GST activity. These effects were most pronounced for compounds of up to 70000 Da and >200000 Da but less distinct for fractions of an average molecular weight of 100000 Da.

KEYWORDS: Malt; melanoidins; glutathione-*S*-transferase; Caco-2 cells; NADPH−cytochrome *c*−reductase; CML

INTRODUCTION

Traditional malting techniques provide all of the essential substrates driving the Maillard reaction. Enzymatic degradation reactions start during germination, when the cereals' proteins and starches are broken down into smaller peptides, amino acids, and the oligosaccharides, maltose and glucose. The Maillard reactions are then promoted by the high temperatures applied during kiln-drying and optional roasting, resulting in the formation of dark-colored melanoidins (*1*, *2*). Whereas kiln-drying achieves maximum temperatures between 80 and 100 °C for up to 4 h, traditional roasting temperatures applied for the production of dark-colored, intensively flavored malt types reach a maximum between 105 and 250 °C for up to 3 h. Thus, the process of heat treatment is chiefly responsible for the formation of colored compounds deriving from nonenzymatic browning reactions (*3*−*5*). Depending on the kiln-drying and roasting conditions, various browning products, termed melanoidins, are formed in, for example, barley malt. Although the effect of the different kilning temperatures on the formation of nitrogen-containing compounds in barley wort was investigated many years ago (*6*), information on the molecular weight distributions of compounds formed during kilning and roasting of malts is still very fragmentary. In addition, it is as yet not clear how such Maillard reaction products influence xenobiotic enzymes in the human body. For example, most chemopreventive, nonendogenously formed agents act through enzyme systems by modulating phase I and phase II enzymes. Phase I metabolic transformations include reduction, oxidation, and hydrolytic reactions, whereas phase II transformations generally act through conjugation reactions of the parent xenobiotics or of phase I metabolites. The conjugation reactions facilitate transport and enhance elimination of the inactive compounds via the renal and biliary routes. Therefore, the main determinant of whether exposure to xenobiotics will result in toxicity is the balance between the activities of phase I and phase II enzymes

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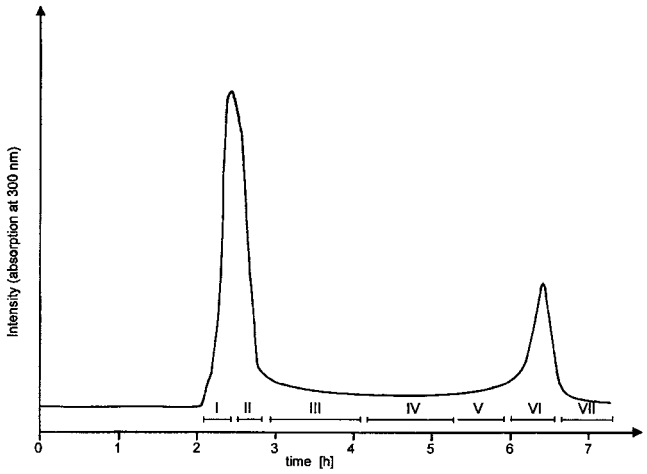

Figure 1. Separation of an aqueous roast malt extract by GFC on Sephadex G-50.

Table 1. Yield of Molecular Weight Fractions Obtained by GFC on Sephadex G-50 and Their Contents of N^{ϵ}-Carboxymethyllysine (CML)

		amount/WS		CML content	
fraction	av mol wt (Da)	mg	%	ng/mg of dry matter	ng/total fraction
I	200000	633.4	31.7	0.07	44.3
II	100000	310.0	15.5	0.16	49.6
III	70000	455.8	22.8	0.22	100.3
IV	60000	326.0	16.3	0.63	205.4
V	40000	69.8	3.5	}1.27	}131.1
VI	30000	33.4	1.7		
VII	10000	11.5	0.6	3.48	6.9
Σ (I–VII)		1839	92.1		537.6
complete mixture		2000	100.0		1108

(*7*). Although phase II enzymes are hypothesized to facilitate the metabolic transit of food-derived Maillard reaction products formed in heat-treated proteins (*8*−*10*), it is still an open question whether nonenzymatic browning products formed in foods require specific detoxifying mechanisms or contribute to the chemopreventive potential of the organism via induction of phase II enzymes. Because the colon is clearly one of the most likely sites for the development of different types of dietary-induced cancers, the intestinal Caco-2 cell line is widely used to investigate the effects of dietary compounds on xenobiotic enzymes (*11*, *12*).

The aims of the present investigation were, therefore, to isolate molecular weight fractions from roasted malt and to study their effects on phase I NADPH−cytochrome *c*−reductase (CCR) and phase II glutathione-*S*-transferase (GST) activities in Caco-2 cells.

MATERIALS AND METHODS

Isolation of Molecular Weight Fractions from Dark Malt. Ground roasted malt (Caraffa, 25 g, roasted at 220 °C for 2 h) was extracted with tap water (60 °C) until no colored material could be extracted (~600 mL). The aqueous solution was extracted with dichloromethane to remove lipids and, finally, concentrated by freeze-drying. Aliquots (2.0 g) of this material were dissolved in distilled water (20 mL) and put onto the top of a glass column (75 × 5 cm i.d.) filled with Sephadex G-50 in water (Pharmacia, Uppsala, Sweden). Elution was done with distilled water at a flow rate of 4 mL/min. The effluent was monitored at 300 nm, and seven fractions were collected and freeze-dried (**Figure 1**; **Table 1**). The molecular weight ranges were approximated by means of polystyrene sulfonates as the calibration standards. For the cell culture experiments, the average molecular weight was calculated for each fraction to adjust the incubation conditions for comparable concentrations.

Quantification of N^{ϵ}-Carboxymethyllysine (CML). The CML content of each of the molecular weight fractions isolated from malt was determined after acid hydrolysis by using a reversed-phase HPLC method with *o*-phthalaldehyde precolumn derivatization (*13*).

Cell Culture Experiments. Caco-2 cells were obtained from the German Collection of Microorganisms and Cell Cultures (DMSZ). Reagents were purchased from Sigma (Deisenhofen, Germany) except for cytochrome *c*, which was obtained from Boehringer (Mannheim, Germany). Caco-2 cells (passages 11, 12, 15, and 16) were maintained at 37 °C in Dulbecco's modified Eagle's medium, containing 20% fetal bovine serum, 2% L-glutamine (200 mM), and 2% penicillin−streptomycin (5000 units of penicillin and 5 mg of streptomycin^{-1} × mL of 0.9% NaCl) in an atmosphere of CO_2/air (1:20). Cells grown in 75 cm^2 culture flasks were supplied on 30 mL of culture medium, which was exchanged (50%) twice a week. Cells were seeded at a density of 20000 cells/cm^2 to achieve confluency at day 6 after seeding. The different molecular weight fractions of malt were calculated for their average molecular weight, dissolved into the medium to final concentrations of 0.5, 5, 50, and 100 μM and were exposed to the cells for different incubation times ranging from 24 to 96 h. Each of the experiments was performed in triplicate.

After exposure to the different molecular weight fractions, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS), harvested with trypsin−EDTA (0.5 g of porcine trypsin and 0.2 g of EDTA × 4 Na per liter of Hank's balanced salt solution) solution (incubation time = 10 min), centrifuged (5 min, 1000*g*), washed (DPBS), and centrifuged (5 min, 1000*g*) again. Cells were diluted 1:5 in 20 mM Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose and 1.4 mM dithiothreitol, subsequently homogenized in a glass/Teflon homogenizer (*12*), and centrifuged (60 min, 105000*g*). The cytosolic 105000*g* supernatant was used for the GST analysis. The pellet containing the microsomal fraction was resuspended in 5 mL of 0.9% NaCl. Microsomal CCR activity was determined in the remaining pellet according to the method of Masters et al. (*14*). GST activity with 1-chloro-2,4-dinitrobenzene (Merck) as substrate was performed as described by Habig et al. (*15*), and protein was determined according to the method of Lowry et al. (*16*).

Data obtained from triplicate experiments is reported as means and standard deviations. Means of each treatment were compared with untreated control cells by Student's *t* test. The level of significance was set at $P < 0.05$ (*).

RESULTS AND DISCUSSION

To isolate the dark brown melanoidins from roasted malt, a dark-colored hot water extract was prepared, and then the extractables were separated into different molecular weight fractions by means of gel filtration chromatography (GFC) on Sephadex G-50 as the stationary phase. Monitoring the effluent at 300 nm, the GFC chromatogram displayed in **Figure 1** was recorded, and the seven fractions I−VII were collected separately. Estimation of the molecular weights of these fractions by using sodium polystyrene sulfonates as the calibration standards revealed molecular weights up to 200000 Da for the compounds present in fraction I, whereas fraction VII contained compounds with an average molecular weight of 10000 Da (**Table 1**).

To investigate whether these different MW fractions, which are characteristic for roasted malt but presumably not formed by living organisms, might act as xenobiotics, enzyme activities of phase I CCR and phase II GST were analyzed in Caco-2 cells after up to 96 h of exposure to the different MW fractions. Reference enzyme activity data obtained for nonexposed cells ($n = 21$) was 2.71 ± 0.29 and 343 ± 33.5 nmol·min^{-1}·mg of protein^{-1} for CCR and GST, respectively, and did not differ among the experiments.

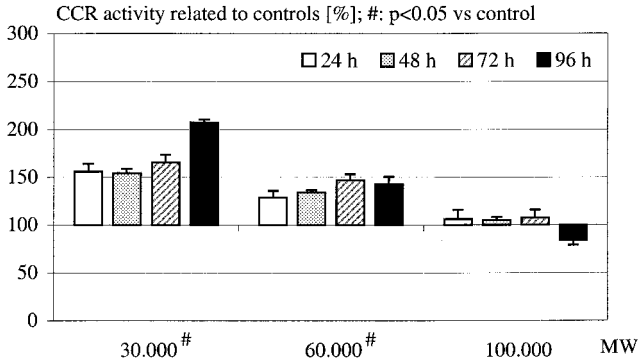

Figure 2. Time-dependent effects of different molecular weight fractions (MW) of roasted malt on the NADPH–cytochrome *c*–reductase activity (CCR) in Caco-2 cells relative to nonexposed controls (=100%). Treated Caco-2 cells were exposed to 50 μM of each of the MW fractions for 24, 48, 72, and 96 h.

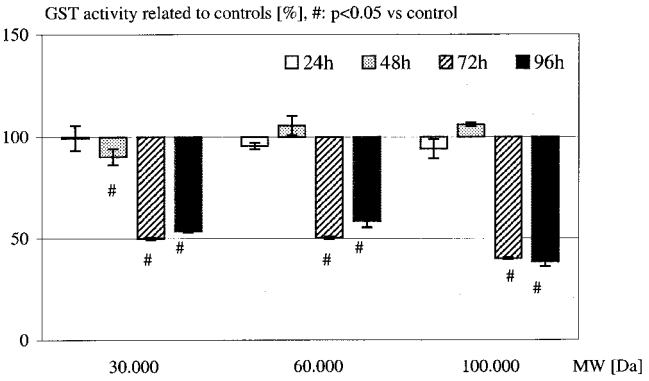

Figure 3. Time-dependent effects of different molecular weight fractions (MW) of roasted malt on glutathione-*S*-transferase (GST) activity in Caco-2 cells relative to nonexposed controls (=100%). Treated Caco-2 cells were exposed to 50 μM of each of the MW fractions for 24, 48, 72, and 96 h.

In the experiment on the time-dependent effect on CCR and GST activity, it was shown that changes in the catalytic activities were more pronounced after 72 and 96 h of exposure compared to activities analyzed after incubation times of 24 and 48 h (**Figures 2** and **3**). However, the number of living cells after harvesting decreased with the duration of the incubation time (data not shown). Therefore, all of the subsequent experiments were carried out at 48 h of exposure, when the numbers of living nonexposed (control) and exposed cells after harvesting were equal. Thus, any influence of different cellular viabilities on the data presented can be excluded.

After 48 h of exposure to the molecular weight fractions of 30000 and 60000 Da, CCR activity increased by 54 and 34% (**Figure 2**), but GST activity was decreased by the 30000 Da fraction by 9.8% (**Figure 3**). These results do not support the hypothesis that malt fractions of ≥30000 Da contribute to health-promoting effects through inhibition of CCR and activation of GST enzyme activities.

To test whether the observed enzyme modulating effects are dose-dependent, perhaps not being as distinct at lower concentrations, the Caco-2 cells were exposed to molecular weight fractions of 40000, 60000, 70000, 100000, and 200000 Da at concentrations from 0.5 to 50 μM (**Figures 4** and **5**). Indeed, the highest CCR activities were analyzed for 50 μM concentrations of the molecular weight fractions ranging from 40000 to 70000 Da, whereas for the 200000 Da malt compounds the exposure to 0.5 and 5 μM resulted in 64.8 and 65.5% increases of the CCR activity as well. However, in contrast, no effect was observed for the 100000 Da fraction (**Figure 4**).

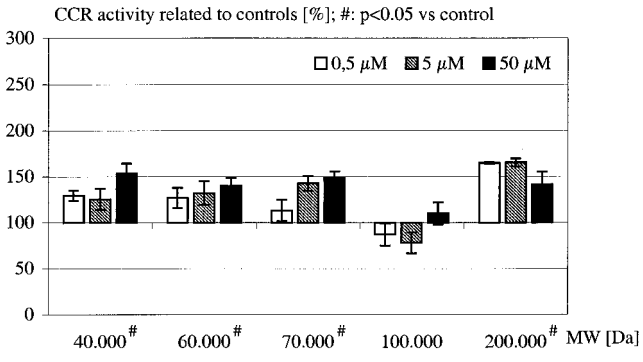

Figure 4. Dose-dependent effects of different molecular weight fractions (MW) of roasted malt on the NADPH–cytochrome *c*–reductase activity (CCR) in Caco-2 cells relative to nonexposed controls (=100%). Treated Caco-2 cells were exposed to 0.5, 5, and 50 μM of each of the MW fractions for 48 h.

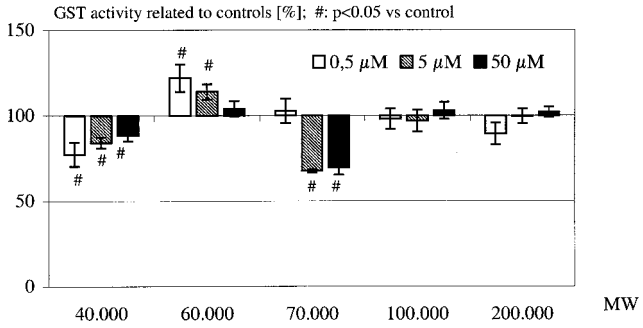

Figure 5. Dose-dependent effects of different molecular weight fractions (MW) of roasted malt on the glutathione-*S*-transferase (GST) activity in Caco-2 cells relative to nonexposed controls (=100%). Treated Caco-2 cells were exposed to 0.5, 5, and 50 μM of each of the MW fractions for 48 h.

GST activities were not changed by the high molecular weight compounds of 100000 and 200000 Da at any concentration tested (**Figure 5**). However, a dose-dependent inhibition of GST activity was observed after exposure to 0.5, 5, and 50 μM of the 40000 Da compounds. Following exposure to the 60000 Da compounds, GST activity was highest at 0.5 μM and decreased to the level of the controls at a concentration of 50 μM. For the 70000 Da compounds, no distinct dose-dependent effect was observed, but GST activity was markedly decreased compared to controls at 5 and 50 μM (**Figure 5**).

To compare the efficacy of each of the molecular weight fractions in enzyme modulation directly and to confirm the results obtained so far, another experiment was conducted in which the Caco-2 cells were exposed to compounds of 10000, 40000, 60000, 70000, 100000, and 200000 Da for 48 h at a concentration of 50 μM (**Figures 6** and **7**). The most prominent elevation of CCR activity (by 222%) was observed for the fraction of 10000 Da, whereas 100000 Da compounds showed a modest effect of a 5% increase compared to controls (**Figure 6**). GST activity was markedly increased by 33% after exposure to the molecular weight fraction of 10000 Da, whereas 40000 and 70000 Da compounds decreased the enzyme activity by 12 and 30%, respectively (**Figure 7**).

Comparison of all of the effects demonstrated on phase I CCR and phase II GST activities revealed the low molecular weight fraction of an average MW of 10000 Da to be the most effective in activating the GST activity, which catalyzes the transformation of toxic compounds into inactive forms. Although there was an activating effect on phase I CCR by low molecular

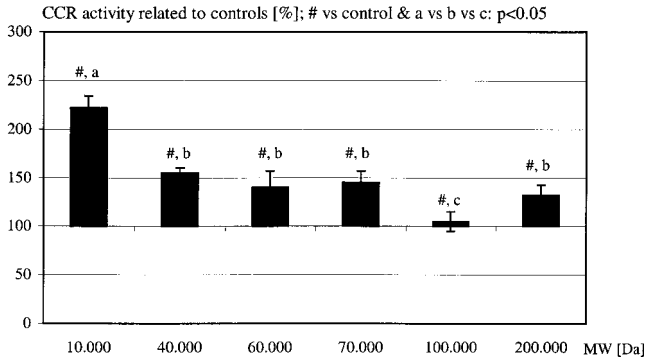

Figure 6. Comparison of different molecular weight fractions (MW) of roasted malt modulating the NADPH–cytochrome *c*–reductase activity (CCR) in Caco-2 cells relative to nonexposed controls (=100%). Treated Caco-2 cells were exposed to 50 μM of each of the MW fractions for 48 h.

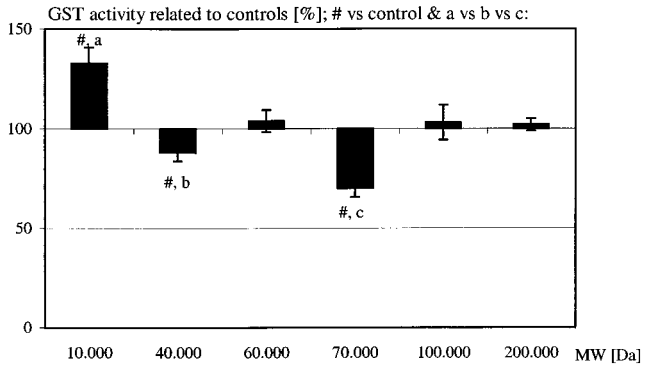

Figure 7. Comparison of different molecular weight fractions (MW) of roasted malt modulating the glutathione-*S*-transferase (GST) activity in Caco-2 cells relative to nonexposed controls (=100%). Treated Caco-2 cells were exposed to 50 μM of each of the MW fractions for 48 h.

weight compounds as well, the low molecular weight compounds are clearly the more promising compounds with respect to health-promoting properties. The majority of the mid molecular weight compounds tested showed an activating effect on CCR activity and an inhibitory effect on GST activity. These effects were more pronounced for compounds ranging from 30000 to 70000 Da and less distinct for compounds of average molecular weights of 200000 Da.

So far, no data exist on the effects of malt melanoidins on xenobiotic enzymes in vitro. In model systems, Kitts et al. (*8*) have demonstrated that glucose−lysine-derived Maillard reaction products (MRPs) decreased phase I aryl hydrocarbon hydroxylase (AHH) activity in the small intestine of mice which were fed an experimental diet containing 2% MRPs for a total of 10 weeks. In this study, no distinct effect could be observed for the activities of phase II enzymes (*8*). In our own previous experiments, the effect of casein-linked CML on phase I and phase II enzymes was investigated in rats fed on 6% casein-linked CML for 10 days (*10*). Phase II GST activity in the kidneys isolated from rats fed on the diet containing the casein-linked CML was found to be increased by 25% compared to control animals fed on diets containing equal amounts of nonheated casein (*10*).

Since food-derived CML was demonstrated to be one of the GST-modulating MRPs at high concentrations in vivo, its content was analyzed in the different molecular weight fractions isolated from the roasted malt investigated in the present study (**Table 1**). Interestingly, the highest CML content was found in the low molecular weight fraction of 10000 Da, which was

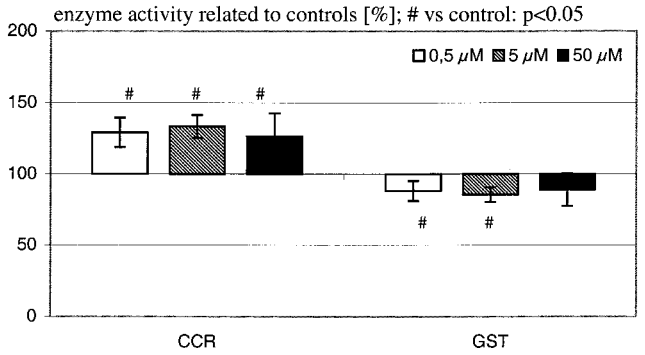

Figure 8. Dose-dependent effect of a 48 h incubation with 0.5, 5, and 50 μM of N^{ϵ}-carboxymethyllysine (CML) on the NADPH–cytochrome *c*–reductase (CCR) and glutathione-*S*-transferase activities (GST) in Caco-2 cells relative to the control (=100%).

demonstrated to be most effective in inducing the GST activity. Decreasing CML contents were associated with increasing molecular weights of the fractions isolated from the roasted barley malt.

These results are in accordance with the data reported by Narziss and Röttger (*6*), who demonstrated that the content of nitrogen-containing compounds isolated from barley wort was high in low molecular weight fractions (~30% in compounds of >2600 Da and ~15% in compounds of >4600 Da) and low in high molecular weight fractions (~3% in compounds of 30000 Da up to >60000 Da).

To test whether CML contributes to the inductive effect on GST activity, Caco-2 cells were exposed to 0.5, 5, and 50 μM CML for 48 h (**Figure 8**). The results revealed that CCR activity was induced by CML, but GST activity was not, indicating that the CML content present in the different molecular weight fractions may contribute mainly to CCR-modulating effects. However, the effect of each fraction on changes of CCR and GST cannot be specifically explained by the CML, and it seems to be likely that the modulations of CCR and GST herein demonstrated mainly for the low molecular weight compounds strongly account for a sum of different effects mediated by various reaction products formed during kilning and roasting of barley malt, such as Maillard reaction products (*2*, *17*, *18*), and by native polyphenols (*19*, *20*).

To gain a more comprehensive picture of the physiological effects of malt Maillard reaction products, further studies are necessary to characterize the chemical nature of these melanoidins and to systematically investigate their effects on xenobiotic enzymes using chemically defined and purified materials.

ABBREVIATIONS USED

MRPs, Maillard reaction products; MWs, molecular weights; CCR, NADPH−cytochrome *c*−reductase; GST, glutathione-*S*-transferase; CML, N^{ϵ}-carboxymethyllysine; GFC, gel filtration chromatography.

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