High Molecular Weight Coffee Melanoidins Are Inhibitors for Matrix Metalloproteases

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Ⓢ *Supporting Information*

ABSTRACT: High molecular (above 10 kDa) melanoidins isolated from coffee beans of varying roasting degree were found to be efficient inhibitors for the zinc-containing matrix metalloproteases MMP-1, MMP-2, and MMP-9 with IC_{50} values ranging between 0.2 and 1.1 mg/mL in vitro. The inhibitory potential increased with roasting degree. No or only slight inhibition of other zinc-containing peptidases closely related to MMPs, namely, *Clostridium histolyticum* collagenase and angiotensin converting enzyme, was found, indicating specific structural features of melanoidins to be responsible for the interaction with MMPs. A continuous increase on the apparent molecular weight of melanoidins as well as incorporation of phenolic substances into the melanoidin structure with progress of roasting was observed, concomitant with a significant increase in the carbon/nitrogen of the melanoidins. This suggests that the melanoidins are mainly formed by incorporation of carbohydrates and phenolic compounds onto a proteinaceous backbone. As MMP-1, MMP-2, and MMP-9 play a pivotal role in pathogenesis of colorectal cancer, studies on possible physiological effects of melanoidins are mandatory.

KEYWORDS: Coffee melanoidins, roasting, Maillard reaction, glycation, polyphenols, matrix metalloproteases, bioactivity

■ INTRODUCTION

During roasting of coffee beans, nonvolatile, high molecular weight polymers with a polydisperse structure are formed due to the Maillard reaction.[1−3] These nitrogen-containing products are referred to as melanoidins, which make up to 25% of the dry mass of a coffee brew.[1,4,5] The chemical structure of melanoidins is still unknown. The involvement of proteins, polysaccharides, and chlorogenic acids and the influence of heat treatment to form polymers of varying size have been demonstrated.[6−10]

Epidemiological research suggests that coffee consumption may have positive effects on several chronic diseases, including type 2 diabetes mellitus, Parkinson's disease, and liver disease.[11] Recent meta-analysis showed an inverse association between coffee consumption and the risk of colorectal cancer.[12] The putative protective effect of coffee seems to be dose-dependent, as the risk decreased by 6% with each cup of coffee per day, reaching a 30% decreased risk for people drinking up to 5 cups per day.[13] Apparently, other components besides caffeine are responsible for explaining this phenomenon, as a significant reduction in the risk of rectal cancer also has been reported for decaffeinated coffee in a prospective cohort study.[14] However, at present, no final statement on possible health-promoting or cancer-preventing properties of coffee can be given, as some meta-analysis and prospective cohort studies showed no or only marginal effect of coffee consumption on the incidence of colon cancer.[15,16] Nevertheless, it has been suggested that, because of the high consumption of coffee worldwide, even minor effects could have considerable consequences on public health.[15]

Recently, it was observed that coffee melanoidins can inhibit angiotensin-I converting enzyme (ACE), a zinc metalloprotease involved in the regulation of blood pressure.[17] Matrix metalloproteases (MMPs) are another class of zinc-containing endopeptidases, which are able to degrade and remodel extracellular matrix and basement membrane components, and are involved in several physiological processes, including embryonic development, growth, wound healing, and other functions requiring tissue reorganization.[18] However, MMPs are also associated with various inflammatory, malignant, and degenerative diseases and are essential for tumor progression and metastasis.[19,20] MMP-1 is the only collagenase that has been consistently implicated in the pathogenesis of colorectal cancer.[21] MMP-2 and MMP-9 are gelatinases overexpressed in colorectal cancers and are positively related with the stage of disease.[22] Inhibition of the MMPs was demonstrated to decrease tumor cell invasiveness.[23] Therefore, the search for molecules capable of inhibiting the activity of this class of enzymes is growing in importance.[24] Most of the synthetic MMP inhibitors consist of a peptidic or peptidomimetic skeleton, bound to a functional group (the so-called "zinc-binding group") able to coordinate with the active zinc(II) ion in the catalytic domain,[24] promoting a competitive and reversible inhibition of the enzymatic activity.[25]

Clostridium histolyticum collagenase (ChC) is a zinc metalloproteinase able to digest triple-helical types I, II, and III collagens.[26,27] ChC is responsible for extensive tissue destruction in gas gangrene but has also been used in the clinic for the removal of dead tissue from ulcers or burns and for nonsurgical treatment of Dupuytren's disease.[28] It is generally considered that the mechanisms of action of MMPs and ChC are similar.[29]

The present work was performed to evaluate the inhibitory potential of coffee melanoidins isolated from coffee beans of varying roasting degree (RD) against selected MMPs, namely,

MMP-1, -2, and -9, ACE, and ChC. Aiming to characterize the structural modifications of the melanoidins during the roasting, melanoidins with an apparent molecular mass over 10 kDa were isolated by diafiltration and were analyzed using gel permeation chromatography (GPC). The ratio between carbon and nitrogen contents (C/N ratio) was calculated using elemental analysis, and the total phenol content of the samples was measured using Folin—Ciocalteu reagent. The inhibitory potential of the melanoidins against MMPs was evaluated using a fluorogenic assay.

■ MATERIALS AND METHODS

Materials. Unless otherwise indicated, chemicals were obtained from Sigma-Adrich (Steinheim, Germany) and used without further purification. Coffee beans from *Coffea arabica* var. Santos of varying RD were obtained from a local coffee roaster (K+M Kaffee and Maschinen, Dresden, Germany). The green coffee beans (RD 0) had been roasted at increasing temperatures to obtain samples with varying RD, designated RD 1 to RD 4. RD 1 was taken from the roaster after 10 min of treatment (the roaster temperature at the time of removal was 174 °C). RD 2 was roasted for 12 min (the final temperature was 190 °C). RD 3 was treated for 14.5 min (the final temperature was 194 °C). RD 4 was the commercial product and was roasted 16 min until the temperature in the roaster reached 200 °C. The RDs of the samples were visually classified as follows: RD0, green beans without roasting; RD1, light roasting; RD2, medium roasting; RD3, dark roasting; and RD4, espresso roasting.

Isolation of Melanoidins. The coffee beans were frozen with liquid nitrogen and were ground using a Retsch GM 100 mill (Retsch GmbH, Haan, Germany) equipped with a 0.74 mm sieve at 10.000 rpm. Coffee extract was prepared by percolation as described by Bekedam et al.[5] Water heated to 90 °C (600 mL) was poured on coffee powder (100 g). After they were cooled to room temperature, the extracts were centrifuged at 4000 rpm for 10 min and filtered subsequently, using filter nos. 288 and 292 (Sartorius Stedim Biotech, Göttingen, Germany). The filtrates were freeze-dried, yielding 19.73, 17.35, 16.32, 15.21, and 16.43 g, respectively. Samples of 5 g of each dried extract were solubilized in 10 mL of water and subjected to ultrafiltration, using centrifugation spin filters with a cutoff of 10 kDa (Macrosep, Pall Life Science Corp., NY) until the filtrates showed an absorption below 0.1 at a wavelength of 405 nm.[5] The freeze-dried retentates were defatted by Soxhlet extraction[5] and pooled, yielding the pure high molecular weight melanoidins RD 0 (2.27 g), RD 1 (1.27 g), RD 2 (1.39 g), RD 3 (1.75 g), and RD 4 (2.02 g) each from 100 g of coffee powder, which were used in the following investigations.

Average Molecular Weight Distribution. GPC was performed using a pump K-1000 and a diode array detector (DAD) K-2600 (Knauer, Berlin, Germany), a BioSep-SEC-S3000 gel permeation column (300 mm × 7.8 mm), connected to a guard cartridge GFC 3000 (4 mm × 3.0 mm, both from Phenomenex, Aschaffenburg, Germany). Samples of melanoidins were dissolved in elution buffer at a concentration of 2 mg/mL, and 100 μL was injected. The column was eluted with 50 mM phosphate buffer containing 0.15 M NaCl (pH 6.5) at a flow rate of 0.5 mL/min for 10 min, followed by 0.2 mL/min for 80 min. The chromatograms were recorded at the wavelengths of 220 and 405 nm. The molecular mass was estimated after calibrating with a protein molecular marker mix (Amersham Biosciences, Little Chalfont, England), containing ovalbumin (43 kDa), bovine serum albumin (66 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa). Lysozym (14.6 kDa) and β-lactoglobulin (36.8 kDa, dimeric at pH 6.5) were obtained from Sigma-Adrich. Dextran blue (2000 kDa) was also from the calibration kit and was used for void volume determination.

C/N Ratio. The contents of nitrogen, hydrogen, and carbon were quantified using elemental analysis (Elemental Analyzer Euro EA 3000,

Eurovector, Milan, Italy). The C/N ratio (proportion between the carbon and the nitrogen contents) was calculated.

Total Content of Phenolic Compounds. This was determined with the Folin—Ciocalteu reagent according to[30] with modifications. Solutions (1.0 mg/mL) from all coffee samples were prepared. To 40 μL of the sample solution, 200 μL of 0.2 N Folin—Ciocalteu reagent (Sigma-Aldrich) was added. After it was mixed, 160 μL of a 0.7 M solution of sodium carbonate was added, and the samples were allowed to react at room temperature for 2 h. The absorbance at 750 nm was measured using an Ultraspec 1000 UV-spectrophotometer (Pharmacia Biotech, Cambridge, England). Chlorogenic acid was used as the standard. The concentration of phenolic compounds was calculated as chlorogenic acid equivalents.

Assay for Inhibition of MMPs. The activity of the recombinant human MMP-1, MMP-2, and MMP-9 catalytic domains (Enzo Life Sciences, Lörrach, Germany) in the absence or presence of coffee melanoidins was measured using the fluorescent substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH$_2$ [Mca = (7-methoxycoumarin-4-yl)-acetyl, and Dpa = *N*-3-(2,4-dinitrophenyl)-L-α-β-diamino-propionyl)] (Enzo Life Sciences), dissolved in dimethyl sulfoxide (DMSO) to a concentration of 400 μM. For the assay, the substrate solutions were diluted to 40 μM in assay buffer [50 mM HEPES (*N*-2-hydroxyethylpi-perazine-*N'*-2-ethanesulfonic acid), 10 mM CaCl$_2$, and 0.05% Brij-35, pH 7.5]. MMPs were dissolved to a concentration of 0.765 U/μL in assay buffer. The test samples were dissolved in assay buffer, and appropriate volumes were chosen to achieve concentrations in enzymatic assay ranging between 0.0005 and 2.5 mg/mL. As a negative control, 10 μL of assay buffer was used instead of melanoidin solution. As a positive control, 20 μL of a 6.5 μM NNGH [*N*-isobutyl-*N*-(4-methoxyphenylsulfonyl)glycyl hydroxamic acid, Enzo Life Sciences] solution was used. This control inhibitor was stored as a 1.3 mM solution in DMSO, which was diluted 1:200 in assay buffer prior to use.

Ten microliters of test solutions, 20 μL of enzyme solution, and buffer to complete 90 μL were mixed and preincubated for 60 min at 37 °C in a black 96-well plate (Brand, Wertheim, Germany). Ten microliters of substrate solution was added to start the reaction, and the increase in the fluorescence (λ_{ex} = 328 nm, λ_{em} = 393 nm) was measured for 15 min in 1 min intervals using a Tecan Infinite F 200 plate reader and Tecan-i-Control software (Tecan, Crailsheim, Germany). The curve was plotted, and the slope of the linear region of the regression curve (initial velocity) was used to compare the activities between the samples. The estimation of the inhibitory potentials was done using the software Origin 6.1 (OriginLab, Northampton, United States), for the plotting of the sigmoidal curves and obtaining the parameters of the Boltzmann equation, and Excel 2003 (Microsoft, Redmond, WA), for the calculation of the IC$_{50}$ values. All assays were performed at least in duplicate.

Effect of Zinc Addition on the Inhibition of MMP-1 by Melanoidins. To study if inhibition of MMPs by melanoidins is due to zinc sequestration, ZnCl$_2$ was dissolved in MMP-assay buffer (see above) to a concentration of 0.5 mM. This solution was diluted to 0.05 and 0.005 mM with the same buffer. The MMP-1 inhibition assay was conducted as described above, substituting 10 μL of buffer by 10 μL of each one of the zinc solutions, providing zinc concentrations of 0.5, 5, and 50 μM in the assay, respectively. The melanoidins were tested at a concentration of 0.5 mg/mL. Negative and positive controls were also measured. The inhibitory activity of each sample was calculated as described above. All assays were performed in duplicate.

Assay for Inhibition of ACE. The ACE activity was measured using the method described in[31] with some modifications. The test samples were dissolved in assay buffer, and appropriate volumes were chosen to achieve concentrations in enzymatic assay ranging between 0.005 and 1.5 mg/mL. In each well of a 96-well microtiter plate (Brand), 10 μL of an inhibitor solution was mixed with 10 μL of ACE from rabbit lung (Sigma-Adrich) dissolved to reach an activity of 0.4 mU/mL in

bidistilled water. Each vertical row of the plate had a negative control, which was prepared by replacing the inhibitor solution by 10 μL of water. As a positive control, captopril was used. The mixture was preincubated for 10 min at 37 °C. Eighty microliters of a 5 mM solution of the synthetic substrate hippuryl-histidyl-leucine (HHL) dissolved in HEPES buffer [50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid and 300 mM NaCl at pH 8.3] was added. The system was incubated for 120 min at 37 °C. The reaction was terminated by adding 100 μL of 1 M HCl. All assays were performed in duplicate. The amount of enzymatically liberated hippuric acid was measured via reversed-phase high-performance liquid chromatography (RP-HPLC). The HPLC system was Smartline, composed by Manager 5000, Pump 1000, UV Detector 2600, Autosampler 3950, and column oven (Knauer). The column was a C18-Eurosphere 100, 5 μm, 250 mm × 4.6 mm, from the same company. The column temperature was set at 25 °C. A total of 30 μL of the sample was injected. Elution was achieved by a gradient of a 0.1% formic acid aqueous solution (solvent A) and 0.1% formic acid in methanol (solvent B) at a flow rate of 1 mL/min. The gradient was as follows: 2 min at 15% solvent B, 15−25% solvent B in 8 min, 25−80% solvent B in 11 min, 1 min at 80% solvent B, 80−15% solvent B in 2 min and equilibration for 3 min at 15% solvent B. Hippuric acid was detected at 228 nm (8.9 min elution time). The evaluation software was ChromGate V3.3.1 (Knauer). The product peak was integrated to calculate the ACE activity in the absence or presence of inhibitors. To calculate the inhibitor concentrations needed for 50% inhibition of ACE (IC$_{50}$ values), the activity of ACE was plotted against the inhibitor concentration (in logarithmic scale). The IC$_{50}$ was calculated as described above.

Assay for Inhibition of ChC. A chromatographic method was developed based on a spectrophometric method.[32] In each well of a 96-well microtiter plate (Brand), 25 μL of a inhibitor solution was mixed with 10 μL of ChC (type VII, Sigma-Adrich) dissolved to an activity of 0.4 FALGPA mU/mL [FALGPA = N-(3-[2-furyl]acryloyl)-Leu-Gly-Pro-Ala] in TRIS buffer [50 mM 2-amino-2-(hydroxymethyl)-1, 3-propanediol, pH 7.5]. Each vertical row of the plate had a negative control, which was prepared by replacing the inhibitor solution by 25 μL of water. As a positive control, ethylenediaminetetraacetic acid (1 mM) was used. The mixture was preincubated for 10 min at 37 °C. Sixty-five microliters of a 4 mM solution of the synthetic substrate 4-phenylazo-benzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg-OH dehydrate (PzPLGL$_D$R) dissolved in TRIS buffer was added. The system was incubated for 15 min at 37 °C. The reaction was terminated by adding 100 μL of methanol. All assays were performed in duplicate. The amount of enzymatically liberated 4-phenylazobenzyloxycarbonyl-Pro-Leu-OH (PzPL) was measured via RP-HPLC. The HPLC system and the column were as described above for measurement of ACE activity. The column temperature was set at 25 °C. A total of 30 μL of the sample was injected. Elution was achieved by a gradient of H$_3$PO$_4$ at pH 3.0 (solvent A) and methanol (solvent B) at a flow rate of 0.75 mL/min. The gradient was as follows: 15% B for 2 min, 15−25% solvent B in 6 min, 25−80% solvent B in 4 min, 18 min at 80% solvent B, 80−15% solvent B in 4 min, and equilibration for 3 min at 15% solvent B. PzPL was detected at 320 nm (23.1 min elution time). The evaluation software was ChromGate V3.3.1 (Knauer). The product (PzPL) peak was integrated and used to calculate the enzymatic activity. IC$_{50}$ values were calculated as described above.

Statistical Analysis. The significance of the observed differences was calculated by one-way analysis of variance (ANOVA) using the software Origin 6.1 (OriginLab).

■ RESULTS AND DISCUSSION

Inhibition of MMPs by Melanoidins. The aim of the study was to investigate the inhibitory potential against selected human
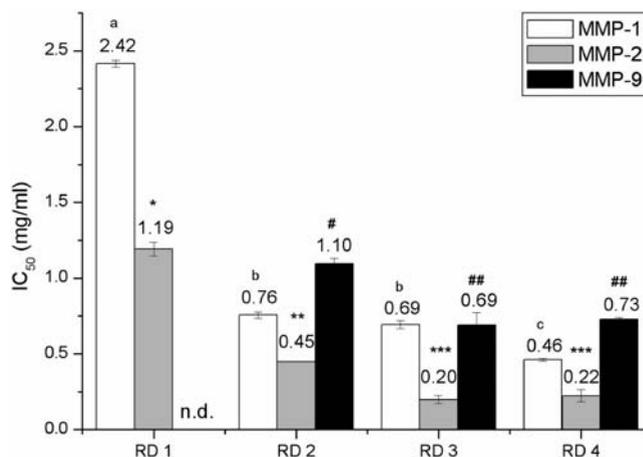
Figure 1. IC$_{50}$ values against MMP-1, MMP-2, and MMP-9 of high molecular mass (>10 kDa) coffee melanoidins obtained from coffee of varying RDs (n.d., not determinable, above 2.5 mg/mL). Different letters or symbols within each MMP indicate significant differences between RDs (one-way ANOVA, $p < 0.05$).

MMPs of the high molecular weight melanoidins isolated from coffee beans of varying RDs. Figure 1 shows the IC$_{50}$ values (concentration needed for 50% inhibition) obtained for each melanoidin preparation against MMP-1, MMP-2, and MMP-9. The high molecular fraction obtained from green coffee beans (RD 0) showed no significant inhibitory activity against any of the studied enzymes at concentrations up to 2.5 mg/mL. RD 1, the melanoidin isolated from slightly roasted coffee, showed a weak inhibitory potential against MMP-1 (IC$_{50}$ = 2.416 ± 0.023 mg/mL), more pronounced activity against MMP-2 (IC$_{50}$ = 1.193 ± 0.045 mg/mL), and no inhibitory activity against MMP-9 (IC$_{50}$ > 2.5 mg/mL). As compared to this, an increase in the inhibitory potential depending on the RD was observed for samples RD 2 to RD 4. For the melanoidin isolate from the final roasted product (RD 4), IC$_{50}$ values of 0.461 ± 0.006 mg/mL against MMP-1, 0.224 ± 0.040 mg/mL against MMP-2, and 0.728 ± 0.014 mg/mL against MMP-9 were determined. These results indicate that the thermal treatment during roasting has a direct impact on the formation of MMP-inhibiting structures formed within the complex melanoidins. The observation that high molecular melanoidins are inhibitors for zinc-containing peptidases are in line with a recent report by Rufian-Henares and Morales,[17] who had found an inhibitory activity of coffee melanoidins of different roast intensities against ACE, another metallopeptidase containing a catalytic zinc(II) ion in the active center. The authors found that at a concentration of 2 mg/mL, the high molecular coffee melanoidins showed an inhibition of ACE ranging from 36.8 (light roasted) via 43.1 (medium roasted) to 45.1%. This points to the fact that concentrations higher than 2 mg/mL are necessary to inhibit ACE to 50%. Using the assay conditions mentioned in the Materials and Methods, the high molecular weight melanoidins isolated in our study did not show significant inhibition against ACE in concentrations up to 1.5 mg/mL (data not shown). Furthermore, the inhibition against ChC, another zinc-dependent peptidase, was tested. Like for ACE, up to concentrations of 1.25 mg/mL, no inhibition of ChC was observed (data not shown). Therefore, concentrations needed to inhibit MMPs found in our study are significantly lower as compared to concentrations needed to inhibit the
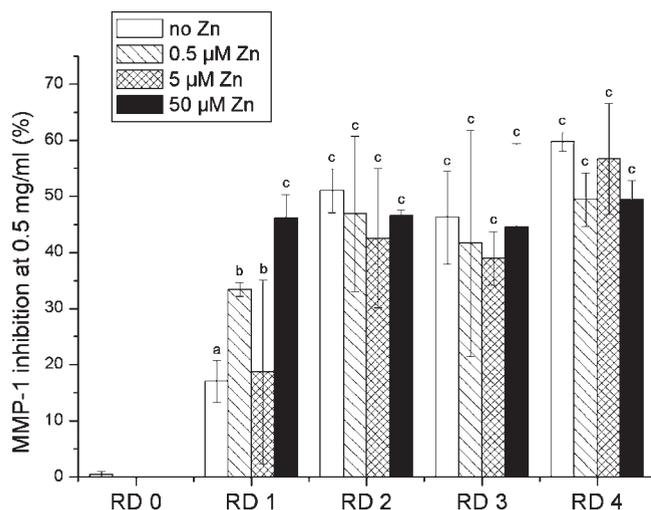
Figure 2. Influence of zinc addition on the inhibition of MMP-1 by coffee melanoidins (at 0.5 mg/mL). Different letters indicate significant differences between different RDs (one-way ANOVA, $p < 0.05$).

zinc-containing peptidases ACE and ChC, suggesting a specific inhibition mechanism based on a molecular interaction between the melanoidins and the catalytic center of the MMPs.

As it is known that melanoidins are able to bind metal ions via chelation, it had to be elucidated whether "simple" zinc complexation by melanoidins, resulting in a removal of the zinc ion from the active center of the metallopeptidases, might be responsible for the observed inhibition rather than specific interactions of the melanoidins with the enzymes. To check this hypothesis, inhibition of MMP-1 by the melanoidin isolates was measured in the presence of varying amounts of zinc (Figure 2). At a melanoidin concentration of 0.5 mg/mL, which is in the range of the IC_{50} values of the coffee melanoidins RD 2 to RD 4, the inhibitory potential of the melanoidins RD 1 to RD 4 was independent of the concentration of zinc. Similar results were obtained for MMP-2 and MMP-9 (data not shown). In other words, the decrease in activity of MMPs in the presence of melanoidins cannot be restored by zinc addition, pointing to specific molecular interactions between the inhibitor and the active center of the enzymes. As for other MMP-inhibiting compounds, a part of the melanoidin molecule with high affinity to zinc, such as carboxyl or *ortho*-hydroxy phenols, may interact with the zinc ion in the active center, and other parts of the melanoidin molecule could "fix" this zinc-binding groups into the active pocket of the enzyme via noncovalent interactions. Inhibition of MMPs by melanoidins, therefore, is a result of chemical reactions occurring during roasting, for which the precise nature at present is unclear. To chemically characterize the isolated melanoidins, to obtain information about the structural changes that happen during the roasting, and to suggest ideas for possible molecular interactions, some exploratory investigations on the melanoidin structure were performed.

Chemical Characterization of Melanoidins. GPC was performed to obtain data about changes in the molar masses during roasting. Protein standards were used to estimate the molecular weight of the melanoidins. The chromatographic profiles of the individually roasted samples obtained via detection at 220 and 405 nm, respectively, are shown in Figure 3. The high molecular weight isolate obtained from the green coffee beans (RD 0) showed three distinct peaks with apparent molecular masses of
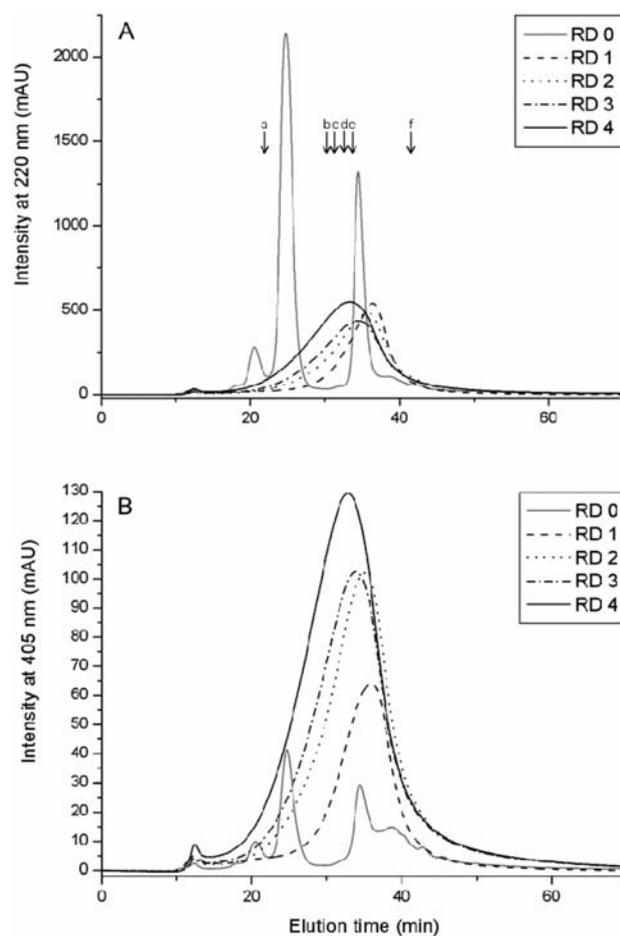
Figure 3. GPC with UV detection at (A) 220 and (B) 405 nm of high molecular mass (>10 kDa) coffee melanoidins isolated from coffee beans of increasing RD. Letters represent the elution position of protein standards used for calibration [a = thyroxin (669 kDa), b = aldolase (158 kDa), c = bovine serum albumin (66 kDa), d = ovalbumin (43 kDa), e = β-lactoglobulin, dimeric (36.8 kDa), and f = lysozyme (14.6 kDa)].

446, 181, and 22 kDa. On the basis of literature facts, we suggest that the first peak is composed by arabinogalactan proteins and the two other peaks with apparent molecular masses of 181 and 22 kDa are basically of a proteinaceous nature.[9,33−36]

After roasting, a distinct change can be observed for the chromatographic profiles. Already for RD 1, only one broad peak with a mean molecular mass of approximately 14 kDa was detectable, indicating transformations and degradation of the high molecular polymers from the green coffee beans. Denaturation and aggregation of proteins and polysaccharides, leading to insoluble products caused by the high temperatures, occurred.[37] With progress of roasting, a gradual increase in the apparent mean molecular mass of this "melanoidin peak" can be noticed, increasing from 14 (RD 1) to 17 (RD 2), 21 (RD 3), and 28 kDa (RD 4). In addition to the increase on the mean molecular mass, a progressive increase of the peak areas was shown, which is due to a progressive increase in the absorption coefficient of the melanoidin isolate. This confirms findings by Wen et al., who correlated the molecular size of polymers from coffee with the progress of roasting.[38]

The ratio between the contents of carbon and nitrogen (C/N ratio) was used to estimate which major chemical transformations

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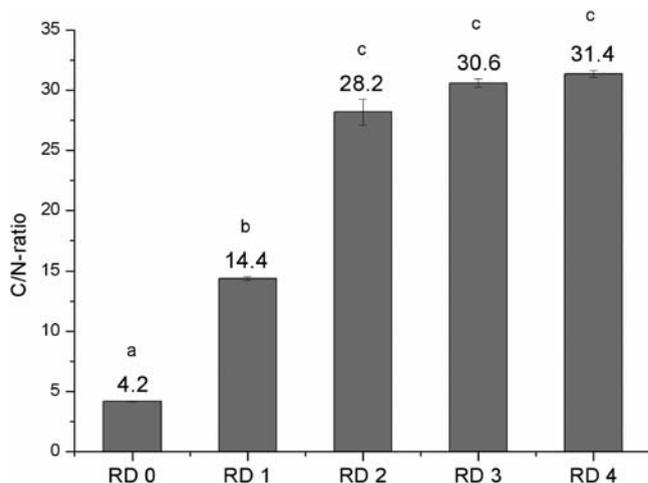
Figure 4. Relation between contents on carbon and nitrogen of coffee melanoidins obtained from coffee of increasing RD. Different letters indicate significant differences between different RDs (one-way ANOVA, $p < 0.05$).

Figure 5. Total phenol content of coffee melanoidins obtained from coffee of increasing RD, determined using Folin–Ciocalteu reagent and using chlorogenic acid as a calibration standard. Different letters indicate significant differences between different RDs (one-way ANOVA, $p < 0.05$).

take place during the roasting to explain the changes observed via GPC. As shown in Figure 4, the changes in the C/N ratio are significant when RD 0 and RD 1 as well as RD 1 and RD 2 are compared. The drastic increase in the carbon content relative to nitrogen suggests a sudden loss or degradation of nitrogen-rich substances, such as amino acids, peptides, and proteins via reactions such as deamidation. Furthermore, an incorporation of carbon-rich substances, such as carbohydrates and polyphenols, could occur to result in the final polymeric melanoidin structure. With further roasting progress (from RD 2 to RD 3 or from RD 3 to RD 4, respectively), the increase in the C/N ratio is less pronounced. The principal reaction in the formation of a high molecular melanoidin structure, therefore, should be coupling of carbon-rich compounds such as carbohydrates, polyphenols and their degradation products, to a nitrogen-rich protein skeleton. Similar results were found by Bekedam et al.,[37] who observed a positive correlation between the roasting and the content of galactomannans for the melanoidin fraction of a coffee brew. In model studies, Maillard reaction products with high C/N ratios have been related to higher metal affinity when compared to counterparts of lower C/N ratios.[39] It is, therefore, conceivable that the zinc-binding structures of the melanoidins may in part result from the Maillard reation.

Figure 5 shows the changes in the content of total phenolic substances with progression of roasting, measured with Folin–Ciocalteu reagent and using chlorogenic acid as standard. We observed a rapid increase in this apparent polyphenol content with the progress of roasting, from 2 g/100 g in the green beans (RD 0) to 11 g/100 g in the final melanoidin (RD 4). This increase can be explained by covalent incorporation of chlorogenic acids into the melanoidin structures. When plotting the apparent polyphenol content against the measured C/N ratio for each melanoidin (see the graph in the Supporting Information), a linear correlation ($y = 0.00358x + 0.00036$, $r^2 = 0.9947$) was observed, strengthening the interpretation that a progressive incorporation of chlorogenic acid into the melanoidin polymer occurs with roasting. However, as the Folin–Ciocalteu reagent may also give positive results with reductones or other reducing or metal-chelating substances formed during glycation
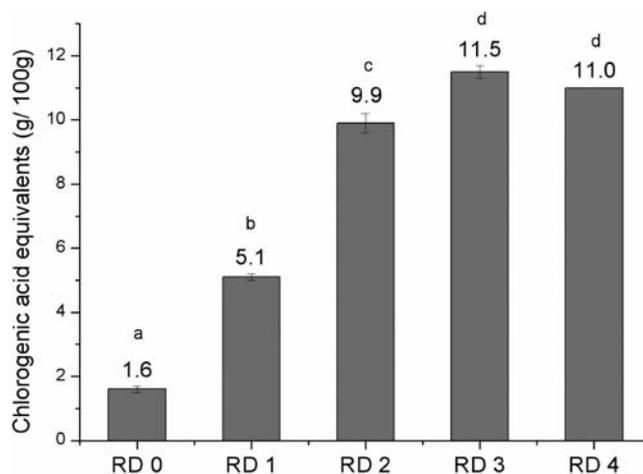
reactions,[3,8,40] one must be aware of the fact that data obtained using this standard assay do not exclusively include "polyphenols".

Taking these analytical aspects together, structural changes during the roasting of coffee beans must be responsible for the increase on the inhibitory activity of coffee melanoidins against human MMPs. The thermal treatment leads to a functionalization of macromolecular fraction by incorporation of low molecular compounds with efficient "zinc-binding groups" such as carboxylic or catechol (1,2-dihydroxy benzene) groups.[10,41] The formation of (volatile) di- and trihydroxybenzenes during roasting as a result of the degradation of caffeoylquinic acids as well as of the Maillard reaction was recently described.[42] This suggests that incorporation of polyphenols to the melanoidin structure can have been at least partially responsible for the bioactivity, although protein modifications resulting from the Maillard reaction, such as carboxyalkyl side chains (as known from N-ε-carboxymethyllysine) or pyridinones are also known to have metal-binding properties and may, therefore, also contribute to the observed inhibition of MMPs as possible zinc-binding groups. However, as no reduction of the inhibitory potential of melanoidins was observed by the addition of zinc(II) in to the assay solution, a complex and interdependent interplay of the zinc-binding group on the melanoidin and further, yet unknown structural features of the polymer are necessary to explain the MMP-selective inhibition observed her. The detailed structures of this inhibitor remain to be elucidated.

With the present study, it was shown for the first time that high molecular melanoidins formed during roasting of coffee are selective inhibitors for MMPs. Concerning possible physiological consequences, some preliminary statements are possible. The concentration of melanoidins on coffee brew depends on the degree of roast, the type and conditions of extraction, and the strength of brew.[43] Furthermore, the amount of ingested melanoidins will also depend on the serving size and drinking habits.[43] According to the isolation method used in the present study, 100 g of roasted coffee contains approximately 2 g of soluble high molecular weight melanoidins. Therefore, a cup of coffee brew (200 mL), prepared from 50 g of roasted beans per liter of water, contains around 200 mg of high molecular melanoidins.

Considering the fact that the colon accumulates its content over at least 24 h within its maximum volume of 2 L,[44] the concentration of melanoidins following the ingestion of 2—3 cups of this coffee brew may already result in an estimated melanoidin concentration of 0.2—0.3 mg/mL in the large intestine, considering the amounts obtained under the extraction conditions of this paper. This is a range of concentration comparable to the IC_{50} values measured for MMPs (Figure 1). In other words, even "conventional" drinking habits can result in intestinal melanoidin concentrations, which may lead to a significant inhibition of MMPs. This estimation concerning an uptake of melanoidins is comparable with recently published data.[43]

We are aware of the fact that a direct transfer of enzyme inhibition data obtained from in vitro tests to biological systems is definitely not possible. This comparison, however, underlines the need for further research on possible chemoprotective properties of melanoidins. In particular, their role in MMP-related physiological processes should be clarified. As already mentioned in the Introduction, MMP-1, MMP-2, and MMP-9 play a pivotal role in pathogenesis of colorectal cancer.[21,22] Further studies will have to show whether melanoidins are biologically active in vivo. From a fundamental point of view, the result of our study confirms the statement that the nutritional consequences resulting from heat-induced reactions occurring during food processing should be discussed objectively and unbiased.[45]

■ ASSOCIATED CONTENT

ⓈSupporting Information. Figure of C/N ratio to chlorogenic acid equivalents. This material is available free of charge via the Internet at http://pubs.acs.org.

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