Antiadhesion and Antibiofilm Activities of High Molecular Weight Coffee Components against <i>Streptococcus mutans</i>

MONICA STAUDER,† ADELE PAPETTI,‡ DORA MASCHERPA,‡ ANNA MARIA SCHITO,§ GABRIELLA GAZZANI,‡ CARLA PRUZZO,‡ AND MARIA DAGLIA*‡

†Department of Biomedical Sciences, Section of Microbiology, Polytechnic University of Marche, Via Tronto 10/A, 60020 Ancona, Italy, ‡Department of Pharmaceutical Chemistry, School of Pharmacy, University of Pavia, Via Taramelli 12, 27100 Pavia, Italy, §DISC, Section of Microbiology, University of Genova, Largo R. Benzi 10, 16132 Genova, Italy, and †DIBIO, Genova University, Corso Europa 26, 16132 Genova, Italy

In previous studies we demonstrated that green and roasted coffee contains low molecular weight (LMW) compounds capable of inhibiting the ability of <i>Streptococcus mutans</i>, the major causative agent of human dental caries, to adhere to hydroxyapatite (HA) beads. This study addressed the ability of the whole high molecular weight coffee fraction (cHMW) and of its melanoidin and non-melanoidin components (GFC1–5), applied at concentrations that occur in coffee beverages, to (i) inhibit <i>S. mutans</i> growth; (ii) affect <i>S. mutans</i> sucrose-dependent adhesion to and detachment from saliva-coated HA beads (sHA); and (iii) inhibit biofilm development on microtiter plates. The results indicated that only cHMW is endowed with antimicrobial activity. The cHMW fraction and each of the five GFC components inhibited <i>S. mutans</i> adhesion, the strongest effect being exerted by cHMW (91%) and GFC1 (88%). <i>S. mutans</i> detachment from sHA was four times greater (~20%) with cHMW and the GFC1 and GFC4 melanoidins than with controls. Finally, biofilm production by <i>S. mutans</i> was completely abolished by cHMW and was reduced by 20% by the melanoidin components GFC2 and GFC4 and by the non-melanoidin component GFC5 compared with controls. Altogether these findings show that coffee beverage contains both LMW compounds and HMW melanoidin and non-melanoidin compounds with a strong ability to interfere in vitro with the <i>S. mutans</i> traits relevant for cariogenesis.

KEYWORDS: Coffee; melanoidins; HMW non-melanoidin components; <i>Streptococcus mutans</i>; adhesion; biofilm

INTRODUCTION

Melanoidins are a large family of polymeric compounds that form in the last stage of the Maillard reaction taking place after thermal processing, household cooking, and storage of foods and beverages containing reducing carbohydrates and amino compounds. Melanoidins are widely distributed in common commodities (e.g., coffee, cocoa, barley coffee, dark beer, balsamic vinegar and bakery products) (1), on which they confer sensory attributes (such as color, flavor, taste and texture), and a longer shelf life through their antioxidant and antimicrobial properties.

Despite their widespread occurrence, the chemical structure of melanoidins is not clearly understood, due to their complexity and to the fact that various types of melanoidins are formed from parent reactants depending on reaction conditions such as pH, temperature, reaction duration and water activity (2). The most widely studied melanoidins are those of coffee, which account for ~25% of the beverage dry matter and include high and low molecular weight (respectively HMW and LMW) components (3–4). Recent studies describe HMW melanoidins as heterogeneous brown, amino-carbonyl compounds with a net negative charge that can contain covalently bound phenolic compounds, probably deriving from chlorogenic acids, and galactomannan- or arabinogalactan-like carbohydrates (5, 6). Their metal chelating ability is directly related to their carbohydrate content. In addition, recent investigations have disclosed that the HMW melanoidins of coffee inherit the elemental composition, spectroscopic properties (light absorbance at 405 nm) and electrophoretic behavior of the amino acids from which they derive. LMW melanoidins account for about 40% of total coffee melanoidins; the majority of these components have been found (i) to have an apolar nature, (ii) to contain about 3% nitrogen, indicating that amino acids and proteins are involved in melanoidins synthesis, and (iii) to incorporate glucose and phenolic groups in the melanoidin structure (4).

Part of the interest in coffee melanoidins is related to their nutritional and functional properties, e.g. <i>in vitro</i> antibacterial activity, metal chelating ability, and antihypertensive activity, <i>in vitro</i> and <i>ex vivo</i> antioxidant capacity, and <i>in vivo</i> prebiotic effect (7–12). Their most extensively studied and best-established property is their antioxidant activity, which has been documented in chemical and biological assays (4, 13–16).

In previous studies we demonstrated the antibacterial activity and <i>ex vivo</i> protective action exerted by coffee beverage against microsomal lipid peroxidation in rat hepatocytes (11, 17). The strong protective effect exerted by the HMW fraction, found in...
the latter study, led to isolation and purification of its five HMW components. The results showed that they all contain nitrogen with different spectra and element compositions, and that three of them exhibit absorption at 420 nm and can therefore be ascribed to the melanoidin family. We also defined the specific properties, reducing power, and metal chelating ability of the five melanoids and non-melanoids, each contributing to the overall protective action exerted by the HMW fraction.

The present work further explores the biological properties of each HMW coffee component, focusing on their effect on the health of the oral cavity, since our previous data showed that the melanoidin components of green and roasted coffee (18) and of barley coffee (19) exert an antiadhesive action against Streptococcus mutans, which is considered as the major causative agent of human dental caries. The cariogenic potential of S. mutans is related to its ability to adhere to the tooth surface, produce extracellular polysaccharides from sucrose, form a biofilm, and rapidly ferment sucrose to lactic and other organic acids. In turn, acid production contributes to tooth enamel demineralization, leading to caries formation (20, 21). We also showed that the antiadhesive action of coffee beverage may be ascribed both to natural coffee components, such as trigonelline and chlorogenic acids, and to compounds induced by roasting, such as nicotinic acid and melanoids, whose concentration in coffee beans depends on the degree of roasting (18).

A number of other studies have shown that several plant foods and beverages, such as cranberries (22, 23) and tea (24), possess antiadhesive and antibiofilm properties. Given the large demand for natural substances to replace synthetic agents that inhibit bacterial adhesion and biofilm development (22), we explored the ability of the whole HMW coffee fraction (cHMW) and of each individual melanoidin (GFC1, GFC2, and GFC4) and non-melanoidin (GFC3 and GFC5) component to affect S. mutans sucrose-dependent adhesion to and detachment from salivacoated hydroxyapatite (sHA) beads and to inhibit biofilm development on microtiter plates.

**MATERIALS AND METHODS**

**Reagents and Chemicals.** All chemical reagents were analytical grade. All were purchased from Sigma-Aldrich (St. Louis, MO).

**Coffee Brew Preparation.** Green C. robusta beans from Java were roasted in a pilot roaster apparatus (STA Impianti S.r.l., Bologna, Italy). The degree of roasting was measured by the weight lost due to vapor formation and cell fragment loss. Weight loss was about 12%, corresponding to a medium degree of roasting. Roasted coffee beans were ground in a laboratory scale mill and sieved through a no. 30 sieve. Coffee brew was prepared by the following method. Briefly, 6 g of roasted coffee powder were extracted with 60 mL of hot water (50 °C) and filtered through a 0.45 μm filter. The extract (100 mL) was filtered through a 0.45 μm filter. The retentate was freeze-dried; dry residue was determined for 6 h at 4 °C. The retentate was freeze-dried; dry residue was determined for 6 h at 4 °C.

**Gel Filtration Chromatography (GFC).** All experiments were performed using a 1100 HPLC system (Agilent, Waldbronn, Germany) equipped with a gradient quaternary pump, and a diode array detector system. The Agilent ChemStation software was used for HPLC system control and data processing. A SuperPerformance Universal glass-cartridge system (300 mm × 10 mm) (Merck, Darmstadt, Germany) was used for GFC separation of the coffee brew retentate (cHMW). The stationary phase was TSK gel Toyopearl HW-40F (exclusion limits 100–10000 Da). The mobile phase was Millipore grade water at a flow rate of 0.5 mL min⁻¹, with an injected volume of 1 mL. UV spectra were recorded in the 190–600 nm range, and chromatograms were acquired at 280, 324, and 420 nm. GFC fractions were freeze-dried, and the dry residues were reconstituted to the initial volume of coffee brew retentate. They were then subjected to the microbiological assays.

**Growth Conditions of S. mutans.** S. mutans ATCC 700610 and S. mutans S4, a clinical strain isolated from a saliva sample, were used for this study. Bacteria were cultured in brain heart infusion broth (BHIB) and brain heart infusion agar (BHIA) endowed with 0.2% sucrose at 37 °C in the presence of 5% CO₂. For the radiolabeling procedure streptococci were grown in BHIB containing 10 μCi [methyl-³H]thymidine (25 Ci mol⁻¹) for 1 h. Cells were then washed twice with 10 mM phosphate buffer (pH 7.0) and freeze-dried at 5000g for 10 min and washed twice with an equal volume of 10 mM phosphate buffer (PB), pH 7.0. Cell labeling efficiency varied among strains and ranged from 200 to 1400 cells cpm⁻¹.

**Evaluation of Coffee Brew Retentate (cHMW) and Its Fractionated Components (GFC Component) Minimal Inhibitory Concentration (MIC).** cHMW and its five GFC components were 2-fold serially diluted in 96 well microtiter plates in BHIB, which was the medium used in the biofilm assay. The wells were inoculated with S. mutans cells (1 × 10⁶ cells mL⁻¹, final concentration), and the plates were subsequently incubated for 24 h at 37 °C in 5% CO₂ atmosphere. The MIC was defined as the lowest concentration of the fractions that inhibited visible bacterial growth (25).

**Bacterial Adhesion to sHA Beads.** Fifty milligram aliquots of spheroidal HA beads (mean size 250–875 μm) were washed twice in 1 mL PB, pH 7.4, autoclaved and then equilibrated for 1 h in the same buffer. Beads were then treated with 200 μL of saliva that was collected, clarified, sterilized and used undiluted, as described previously (26). cHMW and its five GFC fractions at 2 × 1, 1 × 1 and (1/4) × concentrations and the radiolabeled bacterial suspensions (final concentration: 6–8 × 10⁸ cells mL⁻¹) were added simultaneously to saliva-coated HA (sHA) beads in polypropylene microfuge tubes and incubated at room temperature (RT) on a Wheaton Mini Drum Roller (Wolf Laboratories Ltd., Pocklington, U.K.) at 20 rpm.

Control samples without the tested components were included in the assay. After 1 h incubation the beads were collected by centrifugation (200 g, 5 min, 4 °C), washed three times with 10 mM PB to remove nonadherent bacteria, and transferred to PICO-FLUOR TM 15 scintillation fluid (Packard Instruments Company Inc., Downers Grove, IL). Radioactivity was assayed with an LS 1801 scintillation counter (Beckman Instruments, Fullerton, CA). Cell labeling was used to measure the number of bacteria adsorbed to the sHA beads. The inhibitory activity of the materials was gauged by comparing treated samples to the respective untreated controls (100%).

**Bacterial Detachment from sHA Beads.** The ability of cHMW and its five GFC to detach S. mutans cells from sHA beads was determined as described by Tarsi et al. 1998 (27). The radiolabeled bacterial suspension (1 mL) was added to the sHA beads, which, after 1 h incubation, were collected by centrifugation (200 g, 5 min, 4 °C) and washed three times with 10 mM PB, to remove nonadherent bacteria. A separate sample was used to assess cell viability; cHMW and its GFC components were then added to bacteria treated beads. After 1 and 2 h incubation at RT on the Wheaton Mini Drum Roller at 20 rpm, the mixtures were centrifuged and labeled bacteria in the supernatants were counted. Untreated control samples were also included.

**Biofilm Formation Assay.** The ability of cHMW fraction and its five GFC components to inhibit biofilm formation was assessed by growing S. mutans cells in 96 well, flat bottom microtiter plates (Greiner Bio-one Cellstar, Frickenhausen, Germany) as described previously (28). Briefly, overnight cultures of S. mutans were transferred to BHIB and grown at 37 °C in an atmosphere containing 5% CO₂ to an OD600 of ca. 0.5. Cultures were diluted 1:100 in fresh BHIB, with 0.2% sucrose; 200 μL aliquots containing different concentrations (2 × 1, 1 × 1 and (1/4) × 1) of the tested compounds were inoculated into the wells of the plates. Negative controls, i.e., wells containing inoculated growth medium; positive controls, i.e., wells containing inoculated growth medium without the tested components, were included. Plates were incubated at 37 °C in
5% CO₂ atmosphere for 24 h. For biofilm quantification, the plates were slowly immersed in deionized water and shaken to remove any remaining planktonic bacteria or loosely bound cells. After doing this twice, the plates were blotted on paper towels and air-dried. Adherent bacteria were stained with 100 μL of 0.01% crystal violet for 15 min at RT; the plates were then slowly immersed in deionized water twice, to rinse the wells. The bound dye was extracted from stained cells by adding 200 μL of ethanol.

Biofilm formation was then quantified by measuring the absorbance of the solution at 540 nm. Biofilm inhibitory activity (BIA%) was evaluated as a proportion of untreated controls (100%). Results were statistically significant for P < 0.05.

Effect of cHMW and of the Five GFC Components on Bacterial Adhesion to and Detachment from sHA Beads. The effect of cHMW and the GFC components on S. mutans sucrose-dependent adsorption to sHA beads was evaluated at 2×, 1× and (1/4)× concentrations. All samples induced a significant (P < 0.05) reduction in S. mutans adhesion to the beads that was also dose-dependent; the strongest activity was exerted by cHMW and GFC1 (∼90% inhibition) at 2× concentration (Figure 1).

The ability of cHMW and of the GFC components to promote bacterial detachment from the beads was studied by adding different coffee samples to the beads after radiolabeled bacteria had been allowed to adsorb in the presence of sucrose. Percent bacterial detachment was determined by measuring radioactivity in the supernatant after 1 and 2 h incubation (see Materials and Methods). All samples displayed a low but consistently significant (P < 0.05) effect on both S. mutans strains at 2× concentration. As shown in Figure 2, which reports the results obtained with S. mutans ATCC 700610, the strongest activity was exerted by cHMW and by the GFC1 and GFC4 components, which induced 3- or 4-fold bacterial detachment from sHA after 2 h incubation compared to untreated controls. The GFC2, GFC3 and GFC5 components induced about 2-fold bacterial detachment. None of the samples exerted a significant effect at 1× and (1/4)× concentration.

Effect of cHMW and of the Five GFC Components on Biofilm Formation by S. mutans. Biofilm formation by the two S. mutans strains in the presence of cHMW and of the GFC components was evaluated by microtiter plate assays at 2×, 1× and (1/4)× concentrations (Figure 3). Its development was assessed after 24 h incubation at 37 °C, corresponding to the plateau accumulated phase of the biofilm formation process. Only the highest (2×) cHMW concentration, which also displayed antimicrobial activity, inhibited biofilm formation (100%) by both strains. Of the five GFC components, none of which possessed antimicrobial activity, GFC2, GFC4 and GFC5 induced a low (∼20%) but significant (P ≤ 0.05) inhibitory effect compared to controls at 2× concentration.

The data reported above show that the HMW components forming during coffee bean roasting, which include three melanoids and two non-melanoids, interfere with important virulence traits of S. mutans, such as its ability to grow in the oral cavity, to adhere persistently to tooth surfaces, and to produce biofilm that contributes to dental plaque formation, eventually causing caries.
The antimicrobial activity assays showed that cHMW inhibited the growth of both \textit{S. mutans} strains, whereas no individual HMW component was endowed with antimicrobial activity. The findings from adhesion experiments demonstrated that cHMW consistently inhibited the adhesion to sHA beads of both \textit{S. mutans} strains; interestingly, a similar antiadhesive action was also exerted at sub-MIC concentrations. The individual HMW components all showed strong antiadhesive activity, the GFC1 melanoidin component being the most active and exerting a similar inhibitory action to cHMW (Figure 1). These data suggest that the antiadhesion properties of the various HMW components may be impaired by mutual interference.

cHMW exhibited the ability to induce \textit{S. mutans} detachment from sHA beads (~20%). All GFC components also possessed this property; the GFC1 and GFC4 melanoidin components showed the strongest activity; this activity was similar to that of cHMW, again suggesting that mutual interference can reduce the antiadhesion properties of each compound. Although the detachment percentages measured with cHMW and its five compounds are ostensibly not very high, they are up to four times higher than the control values (Figure 2). Therefore the five GFC components likely play an important role, inhibiting cariogenesis also by detaching \textit{S. mutans} from tooth surfaces.

The biofilm production experiments indicated that cHMW was able to abolish \textit{S. mutans} biofilm development on microtiter plates, while the GFC2 and GFC4 melanoidins and the GFC5 non-melanoidin compound induced decreases of about 20% (Figure 3). The strong antibiofilm action of cHMW is probably to be ascribed to its antimicrobial properties. On the other hand the lower, though significant ($P < 0.05$), antibiofilm activity of the individual GFC compounds lacking antimicrobial action may stem from the combined effects of the coffee components on both the bacterial cell and the substrate. Although elucidation of the mechanisms of action of coffee melanoidins and non-melanoidins is beyond the scope of this study, they are conceivably related to interference with the adhesion/coaggregation mechanisms, and/or inhibition of the activity of key enzymes in biofilm development, and/or modulation of the quorum sensing
system (29, 30). These options are currently being examined in our laboratory. Altogether the present findings show that both the melanoidin and the non-melanoidin HMW components can interfere in vitro with crucial steps in the caries formation process.

These data and those from our previous investigations of the antiadhesive and antioxidant activities of coffee (11, 17–19, 31) suggest that consumption of coffee beverages, which contain compounds that strongly curb important S. mutans virulence factors, might restrain caries development and exert a protective effect against the free radicals involved in the cariogenic inflammation process.

ABBREVIATIONS USED

HMW, high molecular weight; LMW, low molecular weight; GFC, gel filtration chromatography; MWCO, molecular weight cutoff; BHIB, brain heart infusion broth; BHIA, brain heart infusion agar; PB, phosphate buffer; SHA, saliva-coated hydroxyapatite; RT, room temperature; BIA, biofilm inhibitory activity.

LITERATURE CITED


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