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Angiotensin-I Converting Enzyme Inhibitory Activity of Coffee Melanoidins

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Melanoidins formed at the last stage of the Maillard reaction have been pointed out to possess certain functional properties. Potential antihypertensive activity of food melanoidins (coffee, beer, and sweet-wine) has been evaluated according to in-vitro ACE-inhibitory activity. Precision of the assay (3.2% of coefficient of variation, $n = 10$) for melanoidins is similar to those reported of well-known antihypertensive peptides. Assay was applied on food melanoidins obtained from coffee (three roasting degrees), beer, and sweet-wine. All samples showed in-vitro ACE-inhibitory activity. The activity in coffee melanoidins was significantly higher at more severe heating conditions. These experiments demonstrate that food melanoidins could inhibit ACE activity. In-vitro ACE-inhibitory activity of coffee melanoidins is likely located within the melanoidin structure. But ACE-inhibitory activity is also partly due to the low-molecular-weight compound nonchemically bound to the melanoidin structure, then melanoidins can act as carrier-protecting agents. These compounds could be naturally phenolic compounds present in the green beans or intermediary Maillard reaction products with antihypertensive activity.

KEYWORDS: Melanoidin; antihypertensive activity; coffee brew; roasting degree; Maillard reaction.

INTRODUCTION

Thermal processes utilized in the food industry are applied to produce color, texture, and flavor and to sterilize the materials, assessing longer shelf life and enhancing product safety (*1*). An important reaction in thermally treated foods is the Maillard reaction that takes place between the amino group of a free or protein-bound amino acid and reducing sugars (*2*). Variables such as the reaction conditions (e.g., heating time and temperature), physicochemical properties of the system (e.g., water activity or pH), chemical composition, and nature of reagents can affect the kinetics of the reaction, and this can lead to the formation of Maillard reaction products (MRP) with different structure and chemical composition and, consequently, different properties (*3*). The high-molecular-weight compounds formed in the final stage of the Maillard reaction are called melanoidins. They are widely distributed in foods (*4*) and could exert different functional properties such as antioxidant (*5*), antimicrobial (*6*), and metal-binding activity (*7*).

In recent years, several studies have been mainly focused on the effect of melanoidins on the human diet and their possible nutritional, biological, and health implications since they are not chemically inert substances (*8*−*9*). Then melanoidins revealed as potential functional components formed during processing and their activity should be better characterized. Antihypertensive activity has not been previously studied, probably due to the high interest that the antioxidant and chelating activities has aroused in researchers and food producers in order to extend the product shelf life (*1*). Angiotensin-I converting enzyme (ACE; EC 3.4.15.1) is also known as peptidyl dipeptidase A since it removes C-terminal dipeptides from a wide variety of peptide substrates. One of its key actions is the regulation of blood pressure together with water and salt metabolism, since it cleaves angiotensin I into the potent vasopressor angiotensin II. It also inactivates bradykinin, which is a hypotensive peptide, by sequential removal of two C-terminal dipeptides. The result of ACE action is an elevation of blood pressure (*10*). In the food area, studies on functional properties have been focused on the identification of food components with different activities such as prebiotic, antioxidant, antihypertensive, and so forth. In the case of hypertension, nutrition has been reported as one of the main factors influencing blood pressure (*11*). In this sense, food intake is being considered as a source of bioactive compounds effective over hypertension, including bioactive peptides (*12*) or chlorogenic acid (*13*).

One of the major sources of daily melanoidins intake is coffee consumption, in which they play an important role as stabilizers of the aromas developed during the roasting process (*14*). It is known that coffee increases arterial pressure because of its caffeine and hydroxyhydroquinone content (*15*). However, other components of coffee such as flavonoids and chlorogenic acid (CGA) have antihypertensive activity in essential hypertensive subjects (*13*). This activity is mediated by their antioxidant

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activity (*16*), improving the endothelial function by decreasing the superoxide anions, which react with nitric oxide (an endothelium-derived relaxing factor). The roasting process affects the final composition of coffee (*17*), thus, levels of phenolic acids vary depending on the treatment of beans. While compounds with antihypertensive properties (i.e., CGA) are lost to some extent during roasting, the development of other compounds, like MRP, could maintain or even improve the antihypertensive properties of coffee brew, in the same way that has been reported for the antioxidant activity (*18*).

In this sense, the aim of the present work is to evaluate the in-vitro antihypertensive properties of melanoidins in coffee brews with different roasting degrees. Moreover, the antihypertensive activity of other beverage-derived melanoidins—such as beer and wine—is studied. These findings will help to understand the potential contribution of melanoidins, as Maillard reaction products, to the in-vitro antihypertensive effect in some foods and subsequently their possible health implications.

MATERIALS AND METHODS

Samples. Instant coffees produced from roasted coffee beans (blend of 80% Arabica and 20% Robusta) were supplied by the Nestlé Research Center (Lausanne, CH) in three different roasting degrees (roasting color, roasting loss): light (CTn 110, 14.5%), medium (CTn 85, 16.2%), and dark (CTn 60, 18.9%). Three widely distributed commercial brands of beer in Europe with different elaboration procedures were selected. A Pilsener-style beer from a Spanish brewery (sample LB), an Abbeys-style beer from a Belgian brewery (sample TB), and a dry-stout beer from an Irish brewery (sample BB) were used. A widely consumed Spanish sweet-wine, "Pedro Ximenez", was also used (sample W). This Spanish sweet-wine is elaborated from dry-grapes by a process called "Soleo".

Preparation of Coffee Brews. One gram of the different instant coffees was resuspended in 100 mL of hot water (50−60 °C). The coffee brew (CB) aqueous solutions obtained were then filtered (Whatman Filter Paper no. 40, ashless, Whatman, UK) and stored at 4 °C until analysis was shortly performed (CB110, CB85, and CB60 samples, respectively).

Preparation of Melanoidins and Related Fractions from Coffee Brews. An aliquot of each sample described above was subjected to ultrafiltration using an Amicon ultrafiltration cell model 8400 (Amicon, Beverly, MA), equipped with a 10000 Da nominal molecular mass cutoff membrane. The retentate was filled up to 200 mL with water and washed again. This washing procedure (diafiltration) was repeated at least three times. The water-soluble high-molecular-weight fraction corresponding to melanoidins was freeze-dried and stored in a desiccator until analysis. Melanoidins (M) isolated from these systems were identified as M110, M85, and M60, respectively.

So-called pure melanoidins (PM) were isolated from solutions containing 5, 2, and 1 mg/mL melanoidin (to obtain a representative amount of product) in 2 M NaCl. NaCl was used to release potential low-molecular-weight (LMW) compounds ionically attached to the melanoidin skeleton, such as CGA. After overnight incubation, solutions were again ultrafiltered (Microcon YM-10, regenerated cellulose 10000 Da, Bedford, MA) at 14000*g* for 50 min. Retentates, containing PM, were resuspended in water and then freeze-dried and stored in a desiccator at 4 °C until analysis. PM obtained were named PM110, PM85, and PM60, respectively. Filtrates containing bound melanoidin compounds (BMC) were also freeze-dried and conserved at 4 °C until antioxidant analysis and named as BMC110, BMC85, and BMC60, respectively. Samples PM and BCM were resuspended in water before use and their concentration is related to the concentration of the parent melanoidin, 5, 2, or 1 mg/mL. A diagram−chart of the extraction procedure for melanoidin and related fractions is depicted in **Figure 1**.

Preparation of Melanoidins from Beer and Wine. Beer and sweet-wine melanoidins were prepared in a way similar to that for coffee melanoidins. One hundred milliliters of beer or sweet-wine were diluted with 100 mL of water; the solutions obtained were filtered and also treated with dichloromethane to mimic the same extraction conditions applied to coffee in order to avoid any possible artifact.

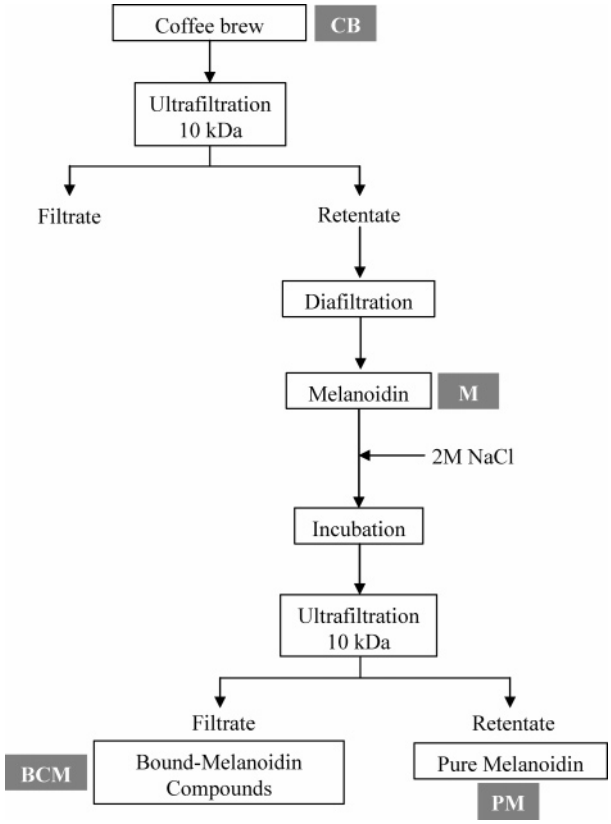

Figure 1. Flowchart for obtaining different melanoidins fractions.

Chemicals. Angiotensin-converting enzyme (ACE) of rabbit lung (EC 3.4.15.1), hippuryl-histidyl-leucine (HHL), and captopril were purchased from Sigma (St. Louis, MO). Dichloromethane and NaCl were purchased from Panreac (Barcelona, Spain).

Antihypertensive Assay. ACE-inhibitory activity was assayed with the method of Cushman and Cheung (*19*). In-vitro ACE-inhibitory activity is quantified by means of the hippuric acid formation by causing hippuryl-histidyl-leucine (HHL) to react with the angiotensin-converting enzyme in the presence and absence of inhibitor, measuring the absorbances at 228 nm (λ maximum absorption of hippuric acid). The decrease in absorbance is proportional to the inhibition exercised by the assayed inhibitor. Each 150-μL assay mixture contained the following components at the indicated final concentration: 50 μL of ACE solution (20 mU) in a 150 mM borate buffer (pH 8.3) with 300 mM sodium chloride, 50 μL of the sample solution, and 50 μL of 20 mM HHL as substrate, added in that order. This assay mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 1 mL of 1.75 M HCl. The hippuric acid formed by action of ACE was extracted with 1 mL of ethyl acetate. The solvent was then removed by heat evaporation and redissolved in deionized water. The amount of hippuric acid was measured spectrophotometrically at 228 nm on a Shimadzu UV−VIS 1603 (Duisburg, Germany) spectrophotometer using a 1-cm-pathlength cell. The % of inhibition was calculated from the following equation:

$$\text{inhibition (\%)} = [(E_c - E_s)/(E_c - E_b)] \times 100$$

where E is the absorbance, s is the reaction mixture (sample), c is the buffer (control), and b is the blank when stop solution was added before reaction occurred.

Statistical Treatment. All of the analyses were performed at least in triplicate. The Statgraphics v5.1 statistical procedures were performed at a significance level of 95%.

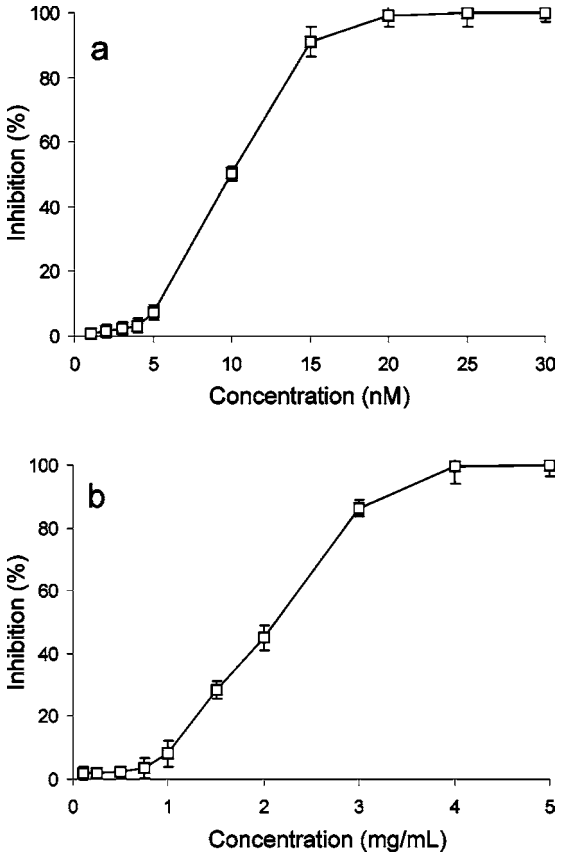

Figure 2. Dose–response inhibition of ACE activity by captopril (**A**) and coffee melanoidin M60 (**B**).

RESULTS AND DISCUSSION

The method applied to evaluate the in-vitro antihypertensive properties of melanoidins is based on that of Cushman and Cheung (*19*) and its update by Ma et al. (*20*). The method is based on the release of hippuric acid from the synthetic peptide HHL by means of the action of ACE. Then, in the presence of ACE inhibitors, less hippuric acid is released, giving rise to a lower absorbance at 228 nm (λ_{max} of hippuric acid). This approach has been previously applied for different peptides (*21*−*23*), but it has not yet been described for an application for MRP. In a first step, the procedure was evaluated for routine use for melanoidins analysis. Melanoidins themselves absorb at 228 nm, which could be a drawback since it could reduce the range of analysis, but concentrations of the studied melanoidins did not show significant interferences. However, a blank assay was performed for each melanoidin or melanoidin fraction in order to subtract any residual absorbance, if recorded.

The method was evaluated for linearity, sensitivity, and precision. A calibration curve was made by increasing concentrations of the HHL peptide (0−100 mM) at a fixed enzyme concentration (20 mU at the reaction media). A lineal relationship ($r^2 = 0.9995$) was obtained under the conditions established for the assay, the generated absorbance (0.850 absorbance units at the 0% of inhibition level) being adequate and sensitive enough for the measurement of potential inhibitory actions of melanoidins on ACE activity. In addition, a standard curve of captopril from 1 to 30 nM (**Figure 2A**) was assayed with the fixed assay conditions (20 mU of ACE and HHL 20 mM). Nowadays, captopril is a widely used antihypertensive drug, and it is routinely used as a control for ACE-inhibition studies (*24*, *32*). Half of the total enzyme activity was inhibited with 10 nM of captopril, whereas complete inhibition was achieved at 20 nM of captopril. These results are in agreement with those previously published by Sentandreu and Toldrá (*24*). Finally, in order to check the most appropriate concentration of melanoidin, a calibration curve with the M60 sample was performed, with concentrations ranging between 0.1 and 5.0 mg/mL (5 and 250 μg in the reaction vessel) (**Figure 2B**). No inhibition was recorded up to 0.75 mg/mL, where a lineal range ($r^2 = 0.9992$) was obtained from 0.75 to 3 mg/mL. Since 2 mg/mL showed an ACE-inhibitory activity close to 50%, this concentration was selected as the reference for further experiments. The reproducibility, expressed as CV ($n = 10$) was assayed with the same sample M60, obtaining a coefficient of variation of 3.2% for a mean value of 45.1%, similar to those reported by other authors for the antihypertensive activity of different peptides (*12*, *20*, *24*).

Table 1. Antihypertensive Activity (% ACE Inhibition) of Different Coffee Brews and Melanoidins Fractions[a]

	% inhibition for coffee sample		
	CTn60	CTn85	CTn110
CB	62.1 ± 2.0 A	65.0 ± 3.2 A,B	79.7 ± 1.2 B
M	45.1 ± 1.4 A	43.1 ± 3.8 A,B	36.8 ± 2.9 B
PM	58.9 ± 3.5 A	55.8 ± 2.4 A,B	53.2 ± 1.0 B
BMC	13.0 ± 2.7 A	12.1 ± 1.2 A	20.2 ± 3.2 B

[a] Coffee brew (CB); melanoidin (M); pure melanoidin (PM); bound to melanoidin compounds (BCM). Values are mean ± S.D. Data are expressed as % of ACE inhibition at a concentration of 2 mg/mL. Different letters in the same line indicate significant differences between the different coffee samples (One Way Anova and Duncan Test, $p < 0.05$).

ACE-Inhibitory Activity of Coffee Samples. The mean values and standard relative deviation of ACE-inhibition percentages of the coffee brews and related melanoidin fractions are shown in **Table 1**. ACE-inhibitory activity values ranged from 62.1 to 79.7% for coffee brew samples, showing higher ACE-inhibitory activity at lower roasting conditions (sample CB110). In the case of melanoidins, ACE-inhibitory activity was lower than their respective CB fractions, ranging from 36.8 to 45.1%. Melanoidins from coffee at the most severe roasting conditions (M60) showed a significantly higher ACE-inhibitory activity. A discussion of these apparent controversial results follows.

Chemical structure of food melanoidins has not yet been elucidated, but there are three main proposals which are based on model systems that could coexist in the coffee melanoidins (i.e., *25*). In order to gain more insight into the ACE-inhibitory activity of melanoidins, coffee melanoidins were further treated and two additional fractions were obtained (PM and BMC). It is noteworthy to mention that PM and BCM do not exist in the coffee matrix as is. **Table 1** also showed the results obtained for melanoidin fractions such as PM and BMC. Reported values for PM and BCM are obtained from the original melanoidin (2 mg/mL). PM values ranged between 53.2 and 58.9%, whereas for BMC they ranged from 13.0 to 20.2% of ACE-inhibitory activity. As described for coffee melanoidins, there was an effect related to the severity of the roasting conditions. ACE-inhibitory activity of PM110 was lower than that for PM60 and PM85, but the behavior of BMC fractions was similar to their respective coffee brews, CB110 showing the highest ACE-inhibitory activity. The highest ACE-inhibitory activity for BMC110 could be explained if we take into account that this coffee has been submitted to a light roasting step, giving rise to a lower destruction of natural compounds from green coffee beans, such

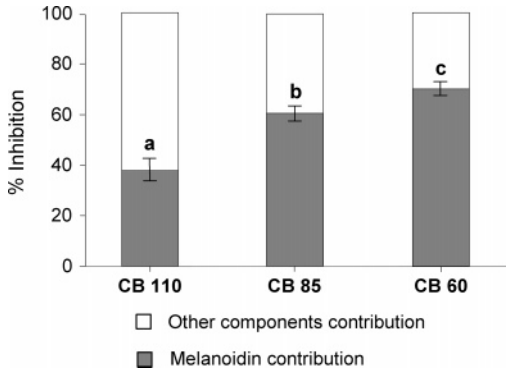

Figure 3. Contribution of melanoidins to the total in-vitro ACE-inhibitory activity of respective coffee brews. Different letters indicate statistically significant differences between the melanoidin contribution in each coffee brew.

as phenolic compounds, then allowing the ionic binding of a larger amount of these compounds to the melanoidin structure. During roasting, phenolic compounds are partially degraded and/or bound to melanoidins, depending on the roasting conditions (*4*). Literature reports that melanoidins behave as polyanionic compounds in model systems (*26*). On the other hand, involvement of phenolic compounds or their degradation products into melanoidin structure or linkage to polymeric material is also report recently in the literature (*4*, *27*). Then, the BCM fraction could be made up mainly of related phenolic compounds and/or low-molecular-weight MRP.

Likewise, the ACE-inhibitory activity of PM was higher than those reported for their respective M, whereas the BMC activity was lower than that of M and PM (**Table 1**). It has been described recently (*4*−*6*) that melanoidins bind different low-molecular-weight compounds (LMW) such as chlorogenic acid and other reactive molecules from the MR which have not yet been characterized (*27*). Furthermore, the presence of a significant amount of phenolic compounds in coffee melanoidins has been reported (*28*). LMW compounds are linked to the melanoidin structure in a noncovalent way, and these compounds are released after high-ionic-strength treatment. LMW compounds exert a lower in-vitro antihypertensive activity than that reported for the PM, the behavior of M being similar to that of the PM. In this sense we could hypothesize that LMW are linked to the melanoidin structure in some places where part of the melanoidin ACE-inhibitory activity is located, then blocking the melanoidin activity, which is recovered when LMW compounds are released from the melanoidin structure. This behavior agreed with previous investigations for the antimicrobial activity of melanoidins obtained from amino acid−glucose model systems, where the LMW compounds exerted lower activity than their related PM (*6*).

As reported previously (*4*), variable amounts of melanoidin are obtained according to the degree of roasting, being 16.4%, 18.4%, and 19.5% for CB110, CB85, and CB60, respectively. The highest yield of melanoidin was obtained at more severe roasting conditions. From the results of **Table 1** it can be stated that the ACE-inhibitory activity of melanoidins fractions increased with the roasting degree. This behavior is clearly depicted in **Figure 3**, where the contribution of melanoidins to the ACE-inhibitory activity of the whole coffee brew (taken as 100%) is depicted. The values reflected in the figure take into account both the ACE-inhibitory activity of each melanoidin and its amount on the different coffee brews. A reduction in the contribution of melanoidin to the total ACE-inhibitory activity is obtained for mild roasting processes, being 70.8, 61.0, and 37.9% for M60, M85, and M110, respectively. Taking together the results from **Table 1** and **Figure 3**, it can be concluded that the increase in melanoidin formation and activity due to the more severe roasting conditions did not compensate for the decrease in ACE-inhibitory activity of coffee brews. This could be explained by taking into account that part of the ACE-inhibitory activity of CB could be due to natural compounds in coffee (such as peptides or phenolic acids) that are partially destroyed during the roasting process. Furthermore, this hypothesis could explain the higher antihypertensive activity of CB110, which could have the highest proportion of LMW compounds because of the milder roasting conditions.

In the food area, studies have been mainly focused on the identification of food components, principally peptides, able to inhibit ACE activity with the aim of controlling hypertension and then preventing cardiovascular diseases through diet. Several peptides, from a wide variety of foods with ACE-inhibitory activities—consisting of 2−12 amino acid residues (*21*)—have been recently reported (*22*, *23*). In the case of melanoidins, their basic skeleton is composed of amino-branched sugar degradation products such as deoxyosones, which come from Maillard reaction carbonyl compounds that react mainly via the Amadori product (*29*). In this sense, only Maillard-derived compounds could be responsible for the ACE-inhibitory properties of M and PM.

At present, the mechanism of action for ACE-inhibitory activity of melanoidins remains unknown, but several hypotheses could be drawn. ACE is a Zn-dependent enzyme; it could be plausible that part of the inhibitory activity of melanoidins could be related to their metal-chelating properties (*7*). On the other hand, melanoidins, because of their "size-volume", could bind to the enzyme in an area other than the active center, deform the enzyme, and hinder binding to the substrate (*30*, *31*). In addition, this inhibition type usually occurs for enzymes containing a functional group, such as the zinc group of ACE—necessary to maintain its catalytically active three-dimensional form. Recently, Actis-Goretta et al. (*32*) reported that the extent of ACE-inhibition could be also associated with the number of hydroxyl groups available to establish hydrogen bonds with the ACE protein. However, the activity of BMC should be different since its metal-chelating property is limited (*4*). BMC could act as a competitive inhibitor, but this aspect is being clarified in additional studies by applying specific competitive assays by our research group.

ACE-Inhibitory Activity of Other Beverage-Derived Melanoidins. For the analysis of melanoidins derived from other heat-treated beverages, beer and wine were selected. In the case of beers, three brands were selected—lager, abbey-type, and dry-stout beer—which differ mainly in the heat treatment applied to the malts (lager < abbey-type < dry-stout). In the case of wine, a widely consumed Spanish sweet-wine, "Pedro Ximenez", was selected The results obtained for the different melanoidins are shown in **Figure 4**. They ranged from 36.8 to 60.1% of inhibition for CB 110 and BB. In general, the ACE-inhibitory activity of coffee melanoidins was lower than that of melanoidins obtained for wine and beers (except for MLB). No significant differences between MLB and MTB were found, but MBB had higher ACE-inhibitory activity. As previously stated for coffees, the stronger the heat treatment is, the higher the ACE-inhibitory activity of beer melanoidins.

In summary, it has been stated for the first time that melanoidins from different heat-treated beverages such as coffee, beer, and sweet-wine exert in-vitro ACE-inhibitory activity over angiotensin-converting enzyme. ACE-inhibitory activity of

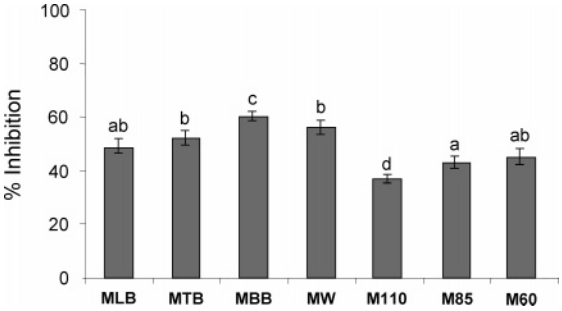

Figure 4. In-vitro antihypertensive activity of different food melanoidins. Lager beer (MLB), abbey-style beer (MTB), black beer (MBB), wine (MW), light-coffee (M110), medium-roasted coffee (M85), and hard-roasted coffee (M65). Data expressed as % ACE inhibition at a concentration of 2 mg/mL. Different letters indicate significant differences between melanoidins (One Way Anova and Duncan Test, $p < 0.05$).

melanoidins and related fractions support the previously reported relationship between antihypertensive activities of coffee and its antioxidant properties (*13*, *15*). So, it could be plausible that the sum of the ACE-inhibitory activity of melanoidins and its own antioxidant activity (*4*, *5*) could be beneficial for essential hypertensive subjects, apart from the fact that melanoidins acts as carrier-protecting agents (*4*) of antioxidant-antihypertensive substances such as chlorogenic acid. In the case of coffee brew, melanoidins could contribute up to 70% of the in-vitro antihypertensive activity, depending on the roasting degree. Moreover it is hypothesized that the ACE-inhibitory activity is located preferentially within the melanoidin structure, represented as PM fraction, which in the LMW compounds linked to them, is represented as BCM. Although melanoidins contribute to overall coffee constituents about 25% (i.e., *4*, *27*, *28*), their contribution to the total antihypertensive activity has not been studied. This research shows for the first time that coffee melanoidins, and probably food melanoidins, showed an in-vitro antihypertensive activity. Further studies are being carried out in order to understand the mechanisms of ACE inhibition by melanoidins, but from data presented in this investigation an association of melanoidins and reduction in blood pressure in terms of theirs ACE-inhibitory activity is plausible.

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