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The Effect of pH on the Antioxidative Activity of Melanoidins Formed from Glucose and Fructose with L and D-Asparagine in the Maillard Reaction

Ji-Sang Kim and Young-Soon Lee†

Department of Food and Nutrition, Kyung Hee University, Seoul 130–170, Korea

Abstract

In this study, the effect of pH on the antioxidative activities of melanoidins formed as a result of the reaction between sugars, glucose (Glc) or fructose (Fru), and amino acids, L-asparagine (L-Asn) and D-asparagine (D-Asn) are examined. For this purpose, antioxidative activities were evaluated on the basis of reducing power, including ferric reducing/antioxidant power (FRAP) and free radical scavenging activity includes 1,1-diphenyl-2-picryl- hydrazil (DPPH) and 2,2′-azinobis(3-ethylbenothiazoline-6-sulfonic acid) diammonium salt (ABTS) and ferrous ion chelating activity. Ethylene diamine tetraacetate (EDTA) and trolox, a water-soluble analog of tocopherol, were used as reference antioxidant compounds. The antioxidative activities of the melanoidins at a pH of 7.0 were greater than those with a pHs of 4.0 and pH 10.0. Especially, it was found that the melanoidins formed from D-isomers are more effective antioxidants in different in vitro assays. The reducing power and chelating activity of the melanoidins formed from the Fru systems were higher than those of the melanoidins formed from the Glc systems. However, the ABTS radical scavenging activity of the melanoidins formed from the Glc systems were higher than those of the melanoidins formed from the Fru systems. In particular, the DPPH radical scavenging activity and the FRAP of the melanoidins showed different antioxidative activities according to pH level.

Key words: amino acids enantiomers, antioxidative activity, Maillard reaction, melanoidins

INTRODUCTION

The reaction between reducing sugars and amino acids is known as the Maillard reaction or non-enzymic browning reaction. The Maillard reaction is a complicated reaction that produces a large number of the so-called Maillard reaction products (MRPs) such as aroma compounds, ultra-violet absorbing intermediates, and dark-brown polymeric compounds named melanoidins (1). Maillard reaction products, generated through a reaction between amino acids or peptides with reducing sugars, are known to have antioxidative effects in food systems. Some of the early MRPs are known to have antioxidative properties (2). Amadori rearrangement products, the key intermediate products in the Maillard reaction are known to generate reactive oxygen, especially in the presence of transition metals.

Melanoidins are widely distributed in foods and have different functional properties such as antioxidant, antimicrobial and metal-binding activities (3). Gomyo and Horikoshi (4) reported that the melanoidins behave as anionic hydrophilic polymers, which can form stable complexes with metal cations. At pH values close to those found in most foods, melanoidins have a negative net charge and are able to bind metallic ions (5). Many approaches have been applied to study the metal binding ability of melanoidins, e.g. titration, dialysis equilibrium, and spectrometry (4). Recently, immobilized metal affinity chromatography (IMAC) has been proposed as a useful tool for the fractionation of melanoidins in homogeneous fractions according to their metal binding ability (1).

The Maillard reaction can also explain the formation of D-amino acids in food. Brückner et al. (6) have recently pointed out that D-amino acids are formed on heating aqueous solutions of L-amino acids (2.5 mM) together with an excess (278 mM) of saccharides (glucose, fructose, and saccharose) at 100°C for 24~96 h in aqueous solutions of pH 2.5 (AcOH) or pH 7.0 (NaOAc). Thus, the formation of D-amino acids in many foods of plant and animal origin are the results of non-enzymic browning since the presence of amino acids together with saccharides is common. As for the racemization mechanism, it is postulated that the reaction of amino acids with glucose or fructose starts with the reversible formation of Schiff bases. The degree of racemization depends in particular on steric and electronic properties of the amino acid side chains. It should be noted that the early stages of the Maillard reaction proceeds already under mild conditions (6) and do not re-

†Corresponding author. E-mail: yysllee@khu.ac.kr
Phone: +82-2-961-0881, Fax: +82-2-968-0260

quire alkaline or acidic condition. This new racemization mechanism based on the releatively stable Amadori compounds has been used to explain the generation of free D-amino acid in foods such as dried fruits, concentrated plant juices and fortified wines (7). Recently, heating experiments of synthetic Amadori compounds proved that they are sources of amino acid-enantiomers (8). Furthermore, convincing evidence has been recently established that D-amino acids are formed in the course of the Maillard reaction (6,8).

Therefore, the aim of this study was to evaluate the effect of pH on the antioxidative activities of melanoidins formed from glucose and fructose with L and D-asparagine in the Maillard reaction. For this, antioxidative activities were evaluated by examining reducing power, free radical scavenging activity, ferrous ion chelating activity, ferric reducing ability and ABTS radical scavenging activity. Melanoidins were, rather arbitrarily, defined as being a high molecular weight (HMW) with a lower limit of 3.5 kDa, which was the nominal cut-off value in the dialysis system used.

MATERIALS AND METHODS

Chemicals

D-glucose, D-fructose, L-asparagine, D-asparagine, ferric chloride, ferrous chloride, 1,1-diphenyl-2-picrylhydrazil (DPPH), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2′-azinobis(3-ethylbenothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) and ethylene diamine tetraacetate (EDTA, 2 Na-salt) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium hydrogen carbonate ($NaHCO_3$) was purchased from Shimakyu Chemical Co. (Osaka, Japan). Trichloro-acetic acid (TCA), potassium ferricyanide, sodium hydrogen phosphate, Iron(III) chloride 6-hydrate, iron(II) sulfate 7-hydrate and acetic acid were purchased from Merck Co. (Darmstadt, Germany). Reagents were of the highest reagent grade quality and used without any further purification.

Preparation of Maillard reaction products (MRPs)

Glucose, fructose and amino acids were dissolved in 100 mL of 0.5 M sodium acetate buffer, (pH 4.0), 0.5 M phosphate buffer, (pH 7.0) or 0.5 M sodium carbonate buffer, (pH 10.0) to obtain a final concentration of 1 M. Six model systems were prepared, composed of glucose (Glc), glucose-L-asparagine (Glc/L-Asn), glucose-D-asparagine (Glc/D-Asn), fructose (Fru), fructose-L-asparagine (Fru/L-Asn) and fructose-D-asparagine (Fru/D-Asn). The reaction mixtures were then distributed among screw-capped glass, Schott tube (16×160 mm), each containing a minimum of 10 mL. Model solutions, prepared at least in duplicate, were heated without at pH control at 100°C for 2 hr. The heating was carried out in a silicone oil bath and the proper safety measures are taken. After heating, model solutions were withdrawn and immediately cooled in ice water.

Dialysis

Approximately 2 mL of the reaction mixture was injected into dialysis cassettes (Mr>3,500) (Slide-Alyzer Dialysis Cassette, 3.5K MWCO, Pierce, USA) and dialyzed against distilled water. A batch dialysis was performed against 1,500 mL of double distilled water for 168 hr at 4°C. Water was changed every 3 hr for the first 12 hr, and then every 10～12 hr for the rest of the dialysis time. After dialysis, samples were freeze-dried and stored in a dessicator at 4°C until analysis. Maillard reaction product (MRP) samples after dialysis were dissolved in water before use, and their concentrations were related to the concentration of the parent melanoidins, 200 μg/mL. After dialysis, at pHs of 4.0 and 7.0, compounds by glucose were non-existent; in addition, at a pH of 4.0, compounds by fructose were also non-existent.

Total ferric ions (Fe^{3+}) reduction capability

The Fe^{3+} reducing power of the melanoidins were determined by the method of Oyaizu (9). Concentrations of melanoidins (200 μg/mL) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1%). The mixture was incubated at 50°C for 20 min. An aliquot (2.5 mL) of trichloroacetic acid (10%) was added to the mixture. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Aqueous solutions of Trolox at various concentrations were used for calibration (0.15～1.15 mM).

Ferrous (Fe^{2+}) metal ions chelating activity

The chelating of ferrous ions (Fe^{2+}) by melanoidins and standards were estimated by the method of Dinis et al. (10). A 100 μL of the melanoidins solution (200 μg/mL) was added with 600 μL of distilled water and 100 μL of 0.2 mM $FeCl_2 \cdot 4H_2O$. The mixture was allowed to rest at room temperature for 30 s. The reaction mixture thus obtained was later added with 200 μL of 1 mM ferrozine and changes in color were monitored at 562 nm with a spectrophotometer (Shimadzu UV 160A, Shimadzu Co., Kyoto, Japan), after a 10 min resting time at room temperature. Ethylene diamine tetraacetate (EDTA, 2 Na-salt) was used to compare the chelat-

ing activity. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated in the following equation:

$$\text{Ferrous ion chelating activity} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

where, $A_{control}$ was the absorbance of the control and A_{sample} was the absorbance with the sample.

DPPH radical scavenging activity

The free radical scavenging activity of the melanoidins were determined by the 1,1-diphenyl-2-picryl-hydrazil (DPPH•). This activity was measured by the procedure described by Yen and Hsieh (11) wherein the bleaching rate of a stable free radical, DPPH• is monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH• absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases. Briefly, 0.12 mM solution of DPPH• in methanol was prepared daily and protected from light. An aliquot of 2 mL of this solution was added to 80 μL of the melanoidins solution in water at a concentration of 200 μg/mL and 320 μL of distilled water. The solution was then mixed vigorously and allowed to stand at room temperature in the dark for 30 min. The absorbance of mixtures was measured at 517 nm using a spectrophotometer (Shimadzu UV 160A, Shimadzu Co., Kyoto, Japan). The antiradical activity of sample was expressed as percentage of disappearance of the initial purple color. Calibration was performed, as described previously, with Trolox stock solution.

Antioxidant capacity by ferric reducing/antioxidant power (FRAP) assay

The FRAP assay was done according to the modified Benzie and Strain method (12) with some modifications. Briefly, 900 μL of FRAP reagent, freshly prepared and warmed at 37°C, were mixed with 90 μL distilled water and either 30 μL of the melanoidins solution (200 μg/mL) or standard or appropriate reagent blank. The FRAP reagent contained 2.5 mL of a 0.01 M TPTZ (2,4,6-tripyridyl-s-triazine) solution in 40 mM HCl, plus 2.5 mL of 20 mM $FeCl_3 \cdot 6H_2O$, plus 25 mL of 0.3 mM acetate buffer pH 3.6. Readings at the absorption maximum (595 nm) were taken every 15 s, using a spectrophotometer (Shimadzu UV 160A, Shimadzu Co., Kyoto, Japan). Temperature was maintained at 37°C. The readings at 30 min were selected for calculation of FRAP values. Calibration was performed, as described previously, with Trolox stock solution.

ABTS radical cation decolorization assay

The spectrophotometric analysis of $ABTS^{\bullet+}$ radical scavenging activities of the melanoidins was determined according to the method described by Re et al. (13) with slight modifications. This method is based on the reaction between ABTS and potassium persulfate forming a blue/green ABTS radical ($ABTS^{\bullet+}$). With the addition of the antioxidants, decolorization is attained and measured spectrophotometrically at 734 nm. The results were expressed as mmol Trolox per gram of melanoidins. Briefly, $ABTS^{\bullet+}$ was produced by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature during 12～16 hr before use. The $ABTS^{\bullet+}$ solution (stable for two days) was diluted with 5 mM phosphate buffered saline (pH 7.4) to an absorbance of 0.70±0.02 at 734 nm and equilibrated at 30°C. For the photometric assay, 3 mL of the $ABTS^{\bullet+}$ solution and 30 μL of melanoidins solution (200 μg/mL) were mixed for 45 s and measured immediately after 5 min at 734 nm (absorbance did not change significantly up to 10 min). Calibration was performed, as described previously, with Trolox stock solution.

Statistical analysis

All experimental data were analyzed by analysis of variance (ANOVA) and significant differences among the means from triplicate analysis at ($p<0.05$) was determined by Duncan's multiple range test using the statistical analysis system (SPSS 12.0 for windows, SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Determination of total ferric ion (Fe^{3+}) reducing ability by reduction of potassium ferricyanide

In the reducing power assay, reductants (antioxidants) in the test samples reduce the Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}), which can be monitored by measuring the formation of Perl's Prussian blue color at 700 nm (14). Reducing power is generally associated with the presence of reductones (15), which break the free radical chain by donating a hydrogen atom (16) or reacting with certain precursors of peroxide, thereby preventing peroxide formation (17). The results obtained for the reducing power of different melanoidins formed from glucose and fructose with L and D-asparagine are shown in Table 1. In the Glc systems, the reducing power of melanoidins formed from the Glc/L-Asn and Glc/D-Asn systems were increased and then decreased according to pH level. The differences in reducing power on the basis of the type of amino acid enantiomers were statistically significant ($p<0.05$), with exceptions being the Glc/L-Asn and Glc/D-Asn systems at a pH of 10.0. In partic-

Table 1. Antioxidant activity as determined by the ferric ions (Fe^{3+}) reduction capability[1)]

Sample	pH 4	pH 7	pH 10
Glc	−	−	45.14±0.34[b]
Glc/L-Asn	26.93±0.06[2)d3)]	46.18±0.06[c]	40.34±0.09[d]
Glc/D-Asn	28.25±0.12[c]	46.61±0.07[b]	40.42±0.06[d]
Fru	−	38.08±0.12[d]	52.53±0.06[a]
Fru/L-Asn	30.85±0.15[b]	47.31±0.12[a]	41.28±0.15[c]
Fru/D-Asn	32.59±0.09[a]	47.09±0.15[a]	40.02±0.18[d]

[1)]Data expressed as μmol equivalents of trolox/melanoidins released from 1 g of melanoidins.
[2)]Values are mean±standard deviation of three experiments.
[3)]Means in a column followed by different superscripts are significantly different at the $p<0.05$ level.

ular, the reducing power of the melanoidins formed from the Glc/D-Asn system was higher than that of melanoidins formed from the Glc/L-Asn system. On the other hand, in the Fru systems, the reducing power of the melanoidins formed from the Fru/L-Asn and Fru/D-Asn systems were increased and then decreased according to pH level. The differences in reducing power, on the basis of the type of amino acid enantiomers, were statistically significant ($p<0.05$), with exceptions being the Fru/L-Asn and Fru/D-Asn systems at a pH of 7.0. In particular, the reducing power of the melanoidins formed from the Fru/D-Asn system was the highest, at a pH of 7.0. In both the Glc and Fru systems, the reducing power of the melanoidins formed from the Fru/L-Asn system was the highest, while that formed from the Glc/L-Asn system showed the lowest reducing power as pH increased. The reducing power of the melanoidins formed from the Fru systems was higher than that of the melanoidins formed from the Glc systems. It was reported that the compounds responsible for reducing activity were formed during the thermolysis of Amadori products in the primary phase of Maillard reactions (18), or they could be formed as the heterocyclic products of the Maillard reaction or the caramelization of sugars (19). The following exhibited reducing power: heat-induced MRPs produced from the xylose-lysine reaction (11), glucose-glycine reaction (20), and the sugar-lysine reaction (21); a porcine plasma protein-glucose model (22) could also be used to obtain reducing power. The hydroxyl groups of the MRPs played an important role in the reducing activity (20).

Ferrous (Fe^{2+}) ion chelating activity

The production of highly reactive oxygen species (ROS)−such as superoxide anion radicals, hydrogen peroxide, and hydroxyl radicals−was catalyzed by free iron through the Haber-Weiss reaction (23):

$$O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\bullet}$$

Transition metals such as ferrous ions can stimulate lipid peroxidation by generating hydroxyl radicals through the Fenton reaction and accelerate lipid peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals, thereby initiating the chain reaction of lipid peroxidation (24). The Fenton reaction is written as follows:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$$

Fe^{2+} ions are the most powerful pro-oxidants among the various species of metal ions (25). Chelating activity occurs through the chelation of Fe^{2+} by the reagent ferrozine (10). In the presence of other chelating agents, the formation of complexes is disrupted; as a result, there is a decrease in the intensity of the red color of the complex (26). The ferrous ion chelating activities of melanoidins formed from glucose and fructose with L and D-asparagine are shown in Fig. 1. These melanoidins showed different ferrous ion chelating activities according to pH level. In the Glc systems, the ferrous ion chelating activities of the melanoidins from the Glc/L-Asn and Glc/

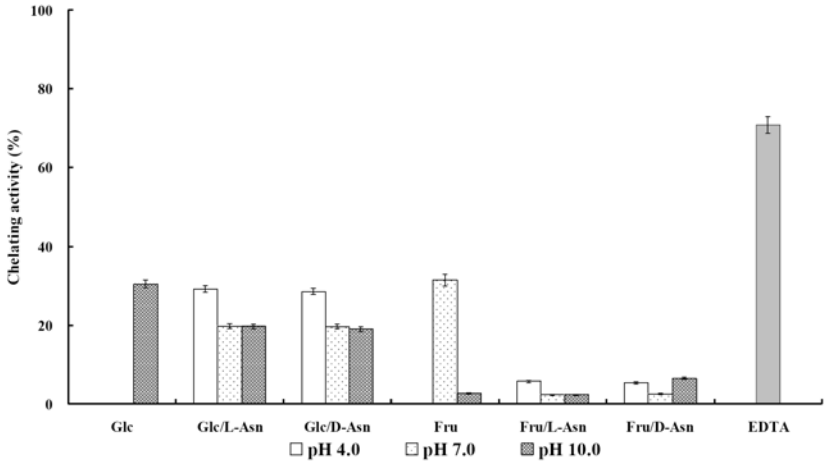

Fig. 1. Ferrous ions (Fe^{2+}) chelating activity in different melanoidins formed from the glucose and fructose with L and D-asparagine in the Maillard reaction with pH level. Data are expressed as mean±standard deviation of three experiments and data are evaluated by using one-way analysis of variance was performed by ANOVA procedures. Concentration of EDTA was 200 μg/mL.

D-Asn systems decreased from pH 4.0 to 7.0 and remained constant at pH 10.0. The difference between these chelating activities was not significant. On the other hand, in the Fru systems, the ferrous ion chelating activities of the melanoidins from the Fru/L-Asn system were decreased as pH increased. The differences in the ferrous ion chelating activities, on the basis of the type of amino acid enantiomers, were not significant ($p<0.05$). In both the Glc and Fru systems, the ferrous ion chelating activities of the melanoidins formed from the Glc/D-Asn system were the highest, while those formed from the Fru/D-Asn system showed the lowest ferrous ion chelating activities, increasing with pH. The ferrous ion chelating activities of melanoidins formed from the Glc systems were higher than those of the melanoidins formed from the Fru systems. MRPs (glucose-casein) are reported (27) as being capable of chelating divalent cations, and they resulted in a decrease in Fe^{2+} absorption from foods in the animal model. The formation of melanoidins in the late stage of the Maillard reaction was responsible for the chelating ability, which was dependent on the sugar sources as well as on the heating conditions－e.g., glucose was most favorable for the formation of copper chelating affinity－(1). Metal chelating capacity was significant, since it decreased the concentration of the catalyzing transition metal in lipid peroxidation. It has been reported that chelating agents are effective as secondary antioxidants, because they reduce the redox potential, thereby stabilizing the oxidized form of the metal ion. Therefore, melanoidins from D-isomers showed a marked capacity for iron-binding, suggesting that their main action as a peroxidation protector could be related to their iron-binding capacity.

DPPH radical scavenging activity

DPPH is a chromogen-radical-containing compound that can directly react with antioxidants (28). When the DPPH radical is scavenged by antioxidants through the donation of hydrogen to form a stable DPPH-H molecule, the color is changed from purple to yellow (16). Stable radical DPPH has been widely used for the determination of primary antioxidant activity－that is, the free radical scavenging activities of pure antioxidant compounds, plant and fruit extracts, and food materials (29). The results obtained from the DPPH assay using different melanoidins formed from glucose and fructose with L and D-asparagine are shown in Table 2. In the Glc systems, the DPPH radical scavenging activity of melanoidins formed from the Glc/L-Asn and Glc/D-Asn systems were increased and then decreased, according to pH level. The DPPH radical scavenging activity of the melanoidins formed from the Glc/L-Asn system was the highest at a pH of 7.0. The differences in the DPPH radical scavenging activity, on the basis of the type of amino acid enantiomers, were statistically significant ($p<0.05$). Especially, at a pH of 4.0, the DPPH radical scavenging activity of the melanoidins formed from the D-isomers was higher than that of melanoidins formed from L-isomers. Otherwise, at a pH of 7.0 and 10.0, the activity of the melanoidins formed from the L-isomers was higher than that formed from the D-isomers. On the other hand, in the Fru systems, the DPPH radical scavenging activity of the melanoidins from the Fru/L-Asn and Fru/D-Asn systems was increased and then decreased with increasing pH. The DPPH radical scavenging activity of the melanoidins formed from the Fru/L-Asn system was the highest, at a pH of 7.0. The differences in the DPPH radical scavenging activity, on the basis of the type of amino acid enantiomers, were statistically significant ($p<0.05$); especially, at a pH of 4.0 and 10.0, the DPPH radical scavenging activity of the melanoidins formed from the D-isomers was higher than those formed from the L-isomers. Otherwise, at a pH of 7.0, the activity of the melanoidins formed from the L-isomers was higher than that formed from the D-isomers. In both the Glc and Fru systems, at a pH of 4.0, the DPPH radical scavenging activity of the melanoidins formed from the Fru systems were higher than those of the melanoidins formed from the Glc systems. Otherwise, at a pHs of 7.0 and 10.0, the DPPH radical scavenging activity of the melanoidins formed from the Glc systems were higher than those of the melanoidins formed from the Fru systems. In a recent study, Del Castillo et al. (30) pointed out that bread-derived melanoidins had a higher peroxyl radical scavenging activity than a low molecular weight (LMW) compounds, which were previously reported to be bound to the melanoidins skeleton (3,31), thereby contributing to the final antioxidative activity. This observation is in agreement with the hypothesis formulated by Delgado-Andrade and

Table 2. Antioxidant activity as determined by the DPPH method[1)]

Sample	pH 4	pH 7	pH 10
Glc	−	−	102.47 ± 0.87^{a}
Glc/L-Asn	$53.20 \pm 0.65^{2)c3)}$	100.45 ± 0.44^{a}	84.91 ± 0.66^{b}
Glc/D-Asn	60.28 ± 0.55^{b}	97.41 ± 0.65^{b}	80.44 ± 0.76^{c}
Fru	−	79.06 ± 0.76^{e}	101.24 ± 0.76^{a}
Fru/L-Asn	55.73 ± 1.41^{b}	89.46 ± 1.31^{c}	68.52 ± 0.76^{e}
Fru/D-Asn	67.22 ± 0.76^{a}	81.81 ± 0.78^{d}	70.83 ± 0.66^{d}

[1)]Data expressed as μmol equivalents of trolox/melanoidins released from 1 g of melanoidins.
[2)]Values are mean ± standard deviation of three experiments.
[3)]Means in a column followed by different superscripts are significantly different at the $p<0.05$ level.

Morales (3) with regards to the existence of melanoidins in a dynamic equilibrium in the food matrix, where LMW compounds are non-covalently linked to the core structure. In any case, the mechanism of the antioxidant effect of the melanoidins is still unclear, because the chemical structure of the melanoidins is unknown; this mechanism is presently being studied. It is assumed that the mechanism is based on the ability of melanoidins to trap positively charged electrophilic species, scavenge oxygen radicals, or carry out metal chelation to form inactive complexes (31). In relation to these assumptions, Bersuder and Hole (32) have characterized the structure of an antioxidant from a heated histidine-glucose model system. 2-acetylpyrido [3,4-*d*]imidazole ($C_8H_7ON_3$) is considered one of the compounds involved in the DPPH antiradical activity of the histidine-glucose Maillard system; however, there have been no reports showing radical scavenging activity by melanoidins formed from the D-isomers. Therefore, the results show that the melanoidins formed from the D-isomers has exhibit radical scavenging activities similar to those exhibited by melanoidins formed from the L-isomers.

Determination of antioxidative activity using ferric reducing/antioxidant power (FRAP) Assay

Several assays have been introduced to measure the antioxidant activity of single compound and/or complex mixtures (12). The ferric reducing antioxidant power (FRAP) assay measures the reduction of ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}) in the presence of antioxidants, which are reductants with half-reaction reduction potentials above Fe^{3+}/Fe^{2+}. From a mechanistic standpoint, FRAP is an electron transfer (ET)-based assay like Folin, ABTS/TEAC, or cupric ion reducing antioxidant capacity (CUPRAC), in the sense that the oxidant probe accepts an electron from the antioxidant analyte, to be converted into the reduced probe that is colored (33). In the presence of a chromogenic ligand like tripyridyltriazine (TPTZ) that is rather selective for Fe (II), Fe (III) acts as an oxidant toward the antioxidants in the sample, and is itself reduced to Fe (II)－which readily chelates with the chromogenic ligand to form a colored species. The increase in absorbance at 593 nm－due to an Fe (II)－TPTZ complex formation－is proportional to the combined (total) FRAP of the antioxidants in the sample (34). The FRAP assay has been claimed to be a robust and potentially useful test, using inexpensive reagents and equipment and a speedy reaction applicable over a wide concentration range (12). The FRAPs of the melanoidins formed from glucose and fructose with L and D-asparagine are shown in Table 3. In the Glc systems, the FRAPs of melanoidins formed from the Glc/L-Asn system were the highest, at a pH of 7.0. The differences in the FRAPs, on the basis of the type of amino acid enantiomers, were not significant with exceptions being the Glc/L-Asn and Glc/D-Asn systems at a pH of 4.0. Especially, at a pH of 4.0, the FRAPs of melanoidins formed from the D-isomers were higher than those formed from the L-isomers. On the other hand, in the Fru systems, the FRAPs of the melanoidins from the Fru/L-Asn and Fru/D-Asn systems were increased and then decreased with increases in pH. The FRAPs of these melanoidins were the highest, at a pH of 7.0. However, the differences in FRAPs, on the basis of the type of amino acid enantiomers, were not significant ($p<0.05$) at pHs of 4.0 and 7.0. In both the Glc and Fru systems, the FRAPs of the melanoidins formed from the Glc systems were higher than that of melanoidins formed from the Fru systems, with exceptions being the melanoidins, at a pH of 4.0.

Table 3. Antioxidant activity as determined by the FRAP method[1)]

Sample	pH 4	pH 7	pH 10
Glc	−	−	373.81±18.31[a]
Glc/L-Asn	215.68±6.94[2)c3)]	376.34±18.31[a]	313.88±13.90[b]
Glc/D-Asn	239.44±9.15[b]	372.55±17.98[a]	306.73±13.25[b]
Fru	−	331.11±1.49[c]	406.41±18.93[a]
Fru/L-Asn	260.89±9.15[a]	337.72±1.68[b]	317.25±11.99[b]
Fru/D-Asn	273.93±10.41[a]	338.20±1.45[b]	293.91±10.73[c]

[1)]Data expressed as μmol equivalents of trolox/melanoidins released from 1 g of melanoidins.
[2)]Values are mean±standard deviation of three experiments.
[3)]Means in a column followed by different superscripts are significantly different at the $p<0.05$ level.

ABTS radical scavenging activity

The generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods that has been applied to the measuring the total antioxidative activity of solutions of pure substances, aqueous mixtures, and beverages (24). The method for screening antioxidative activity has been reported as a decolorization assay applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants; a more appropriate format for the assay is the decolorization technique, in which the radical is directly generated in a stable form prior to the reaction with putative antioxidants. An improved technique for the generation of $ABTS^{\bullet+}$, which is described here, involves the direct generation of a blue/green $ABTS^{\bullet+}$ chromophore through the reaction between ABTS and potassium persulfate. The ABTS radical scavenging activities of the melanoidins formed from glucose and fructose with L and D-asparagine are shown in Table 4. In the Glc systems, the ABTS radical

Table 4. Antioxidant activity as determined by the ABTS method[1)]

Sample	pH 4	pH 7	pH 10
Glc	−	−	1057.63±38.00[a]
Glc/L-Asn	564.26±19.00[2)a3)]	958.13±34.25[a]	804.73±37.32[b]
Glc/D-Asn	568.41±28.50[a]	898.01±43.53[a]	771.56±34.25[c]
Fru	−	767.41±2.24[d]	983.00±3.43[a]
Fru/L-Asn	555.97±3.11[b]	871.06±3.43[b]	669.98±3.07[e]
Fru/D-Asn	566.33±3.43[a]	810.95±3.11[c]	711.44±1.87[d]

[1)]Data expressed as μmol equivalents of trolox/melanoidins released from 1 g of melanoidins.
[2)]Values are mean±standard deviation of three experiments.
[3)]Means in a column followed by different superscripts are significantly different at the $p<0.05$ level.

scavenging activities of the melanoidins from the Glc/L-Asn and Glc/D-Asn systems were increased and then decreased according to pH level. The ABTS radical scavenging activities of these melanoidins were the highest, at a pH of 7.0. The differences in the ABTS radical scavenging activities, on the basis of the type of amino acid enantiomers, were statistically significant at a pH of 10.0. On the other hand, in the Fru systems, the ABTS radical scavenging activities of the melanoidins from the Fru/L-Asn and Fru/D-Asn systems were increased and then decreased as pH increased. The differences in the ABTS radical scavenging activities, on the basis of the type of amino acid enantiomers, were statistically significant ($p<0.05$). In particular, the ABTS radical scavenging activities of the melanoidins formed from the Fru/L-Asn system were the highest at a pH of 7.0. The ABTS radical scavenging activities of the melanoidins formed from D-isomers were higher than those of the melanoidins formed from L-isomers, with exceptions being the Fru/L-Asn and Fru/D-Asn systems at a pH of 7.0. In both the Glc and Fru systems, the ABTS radical scavenging activities of the melanoidins formed from the Glc systems was higher than that of melanoidins formed from the Fru systems. Therefore, the results show that melanoidins from D-isomers have radical scavenging activities similar to those of ABTS, such as those from L-isomers.

In conclusions, on the basis of the data obtained in this study, the antioxidative activities of these melanoidins at a pH of 7.0 were greater than those with a pHs of 4.0 and pH 10.0. Especially, it is found that the melanoidins formed from D-isomers are found to be effective antioxidants in different in vitro assays. The reducing power and chelating activity of the melanoidins formed from the Fru systems were higher than those of the melanoidins formed from the Glc systems. However, the ABTS radical scavenging activity of the melanoidins formed from the Glc systems were higher than those of the melanoidins formed from the Fru systems. In particular, at a pH of 4.0, the DPPH radical scavenging activity and the FRAP of the melanoidins formed from the Fru systems were higher than those of the melanoidins formed from the Glc systems; otherwise, at a pHs of 7.0 and 10.0, the DPPH radical scavenging activity and the FRAP of the melanoidins formed from the Glc systems were higher than those of the melanoidins formed from the Fru systems. Therefore, the melanoidins have different antioxidative activities according to type of sugars, pH level and by using different antioxidant assays, but not amino acid enantiomers.

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REFERENCES

1. Wijewickreme AN, Kitts DD, Durance TD. 1997. Reaction conditions influence the elementary composition and metal chelating affinity of nondialyzable model Maillard reaction products. *J Agric Food Chem* 45: 4577-4583.
2. Lingnert H, Eriksson CE. 1981. Antioxidatvie effects of Maillard reaction products. In *Maillard reaction in food −chemical, physiological and technological aspects.* Ericksson CE, eds. Oxford, Pergamon Press. p 453.
3. Delgado-Andrade C, Morales FJ. 2005. Unraveling the contribution of melanoidins to the antioxidant activity of coffee brews. *J Agric Food Chem* 53: 1403-1407.
4. Gomyo T, Horikoshi M. 1976. On the interaction of melanoidin with metallic ions. *Agr Biol Chem* 40: 33-40.
5. Migo VP, del Rosario EJ, Matsumura M. 1997. Flocculation of melanoidins induced by inorganic ions. *J Ferment Bioeng* 83: 287-291.
6. Brückner H, Justus J, Kirschbaum J. 2001. Saccharide induced racemization of amino acids in the course of the Maillard reaction. *Amino Acids* 21: 429-433.
7. Pätzold R, Nieto-Rodriguez A, Brückner H. 2003. Chiral gas chromatographic analysis of amino acids in fortified wines. *Chromatogr Suppl* 57: S207-S212.
8. Pätzold R, Brückner H. 2006. Gas chromatographic detection of D-amino acids in natural and thermally treated bee honeys and studies on the mechanism of their formation as result of the Maillard reaction. *Eur Food Res Tech* 223: 347-354.
9. Oyaizu M. 1986. Antioxidant activity of browning products of glucosamine fractionated by organic solvent and thin-layer chromatography. *J Food Sci Tech* 35: 771-775.
10. Dinis TCP, Madeira VMC, Almerida LM. 1994. Action of phenolic derivatives (acetoaminophen, salycilate and 5-aminosalycilate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavenges. *Arch Biochem Biophys* 315: 161-169.
11. Yen GC, Hsieh PP. 1995. Antioxidative activity and scavenging effects on xylose-lysine Maillard reaction products.

J Sci Food Agr 67: 415-420.
12. Benzie IFF, Strain JJ. 1996. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power the FRAP assay. *Anal Biochem* 239: 70-76.
13. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med* 26: 1231-1237.
14. Chung YC, Chang CT, Chao WW, Lin CF, Chou ST. 2002. Antioxidative activity and safety of the 50% ethanolic extract from red bean fermented by *Bacillus subtilis* IMR-NK1. *J Agric Food Chem* 50: 2454-2458.
15. Duh PD. 1998. Antioxidant activity of burdock (*Arctium lappa Linne*): its scavenging effect on free radical and active oxygen. *J Am Oil Chem Soc* 75: 455-461.
16. Shon MY, Kim TH, Sung NJ. 2003. Antioxidants and free radical scavenging activity of *Phellinus baumii* extracts. *Food Chem* 82: 593-597.
17. Xing R, Liu S, Guo Z, Yu H, Li C, Ji X, Feng J, Li P. 2006. The antioxidant activity of glucosamine hydrochloride in vitro. *Bioorg Med Chem* 14: 1706-1709.
18. Hwang JY, Shue YS, Chang HM. 2001. Antioxidative activity of roasted and defatted peanut kernels. *Food Res Int* 34: 639-647.
19. Charurin P, Ames JM, Castiello MD. 2002. Antioxidant activity of coffee model systems. *J Agric Food Chem* 50: 3751-3756.
20. Yoshimura Y, Ujima T, Watanabe T, Nakazawa H. 1997. Antioxidative effect of Maillard reaction products using glucose-glycine model system. *J Agric Food Chem* 45: 4106-4109.
21. Wijewickreme AN, Krejpcio Z, Kitts DD. 1999. Hydroxyl scavenging activity of glucose, fructose and ribose-lysine model Maillard products. *J Food Sci* 64: 457-461.
22. Benjakul S, Lertittikul W, Bauer F. 2005. Antioxidant activity of Maillard reaction products from a porcine plasma protein-sugar model system. *Food Chem* 93: 189-196.
23. Haber F, Weiss J. 1934. The catalytic decomposition of hydrogen peroxide by iron salts. *Proc R Soc London Ser A* 147: 332-351.
24. Miller DD. 1996. Mineral. In *Food chemistry*. Fennema OR, ed. Marcel Dekker, New York. p 618-649.
25. Halliwell B, Gutteridge JMC. 1984. Oxygen toxicology, oxygen radicals, transition metals and disease. *Biochem J* 219: 1-14.
26. Chandrika M, Liyana P, Fereidoon S. 2007. Antioxidant and free radical scavenging activities of whole wheat and milling fractions. *Food Chem* 101: 1151-1157.
27. Miller J. 1979. Effect on growth and hemoglobin regeneration in anemic rats of interactions between glucose and minerals during heating. *Nutr Rep Int* 19: 679-688.
28. Ozcelik B, Lee JH, Min DB. 2003 Effect of light, oxygen, and pH on the absorbance of 2,2-diphenyl-1-picrylhydrazyl. *J Food Sci* 68: 487-490.
29. Shih PW, Lai PL, Jen HWK. 2006. Antioxidant activities of aqueous extracts of selected plants. *Food Chem* 99: 775-783.
30. Del Castillo MD, Ferrigno A, Acampa I, Borrelli RC, Olano A, Martínez-Rodríguez A. 2006. In vitro release of angiotensinconverting enzyme inhibitors, peroxyl-radical scavengers and antibacterial compounds by enzymatic hydrolysis of glycated gluten. *J Cereal Sci* 45: 327-334.
31. Delgado-Andrade C, Rufián-Henares JA, Morales FJ. 2005. Assessing the antioxidant activity of melanoidins from coffee brews by different antioxidant methods. *J Agric Food Chem* 53: 7832-7836.
32. Bersuder P, Hole M. 2003. Antioxidants from a heated histidine-glucose model system. In *Melanoidins in food and health*. Office for Official Publications of the European Communities, Luxemburg. Chapter 4, p 158-166.
33. Huang D, Ou B, Prior RL. 2005. The chemistry behind antioxidant capacity assays. *J Agric Food Chem* 53: 1841-1856.
34. Benzie IFF, Szeto YT. 1999. Total antioxidant capacity of teas by ferric reducing/antioxidant power assay. *J Agric Food Chem* 47: 633-636.

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