J Food Sci Nutr
Vol 15, p 287～296 (2010)
DOI: 10.3746/jfn.2010.15.4.287

J Food Science
and Nutrition

Influence of the pH and Enantiomer on the Antioxidant Activity of Maillard Reaction Mixture Solution in the Model Systems

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Abstract

This study was designed to investigate the influence of the pH and enantiomer on the antioxidant activity of Maillard reaction mixture solution in model systems. The loss of glucose in MRPs did not show different characteristics for the different amino acid enantiomers; however, the concentration of glucose decreased as the pH levels increased. The enolization of sugars was observed in all MRP samples according to increase of pH levels. In addition, D-amino acids were detected in L-amino acid systems and L-amino acids could also be observed in D-amino acid systems. Formation of the isomer was the highest in the Glc/L-Lys system. The browning development increased as pH levels increased; however, browning development did not show different characteristics based on the use of L- versus D-isomers of the same amino acid. The L- and D-isomers show different absorption values in the UV-Vis spectra, but the absorption patterns display a similar shape. The antioxidant activities of MRPs derived from the Glc/Gly, Glc/L-Asn and Glc/D-Asn systems at pH 7.0 were greater compared to those of pH 4.0 and pH 10.0. The antioxidant activities of MRPs derived from the Glc/L-Lys and Glc/D-Lys systems decreased as the pH increased. In addition, the results show that the MRPs derived from the D-isomers have similar antioxidant activities as those from L-isomer. Therefore, the MRPs have the different antioxidant activities on the basis of the pH level, but not on the basis of different amino acid enantiomers.

Key words: amino acid enantiomers, antioxidant activity, Maillard reaction products, pH level

INTRODUCTION

The Maillard reaction involved in the formation of brown pigments comprises the condensation between a carbonyl group of reducing sugars, aldehydes or ketones, and an amine group of free amino acids (such as amino acids, peptides and proteins) or any nitrogenous compound. 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). The Maillard reaction is influenced by many factors, including reactant concentration, temperature, time, initial pH and water activity (2-5). The MRPs produced from an amino acid-sugar model system have been associated with the formation of compounds with strong antioxidant activity (6,7).

The conversion of free or protein- or peptide-bound physiological L-amino acids into their mirror images (enantiomers), called D-amino acids, is of great interest from the nutritional and physiological point of view (8). This process of the change of chirality ("handedness") of amino acids is commonly referred to as racemization or epimerization if several chiral centers are involved, although, in the strict sense, racemic amino acids contain equal amounts of D- and L-amino acids (9). The Maillard reaction can also explain the formation of D-amino acids in food. Brückner et al. (9) reported that D-amino acids are formed on heating aqueous solutions of L-amino acids together with an excess of saccharides (glucose, fructose, and saccharose) at 100°C for 24～96 hr in aqueous solutions of pH 2.5 (aceic acid) or pH 7.0 (sodium acetate). 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 can proceed under mild conditions (10) and do not require alkaline or acidic conditions. Recently, heating experiments of synthetic Amadori compounds proved that they are sources of amino acid-enantiomers (11-13). Amino acid racemization, however, is very much dependent on

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temperature, pH, and the presence of catalysts (14). Furthermore, convincing evidence that D-amino acids are formed in the course of the Maillard reaction, i.e. reaction of reducing sugars and amino compounds such as amino acids, has recently been established (9,11,12). Thus, the derivatives from the glucose-lysine model system are frequently used to study diverse aspects of MRPs, such as browning development and complex formation (15). Furthermore, from the point of view of acrylamide formation, the glucose-asparagine model system is important. Therefore, the objective of this study was to evaluate the influence of the pH and enantiomer on the antioxidant activity of Maillard reaction mixture solution in the model systems.

MATERIALS AND METHODS

Materials

Glycine, L-asparagine, D-asparagine, L-lysine, D-lysine, D-glucose, ferrous chloride, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2'-azinobis(3-ethylbenothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), ethylene diamine tetraacetate (EDTA, 2 Na-salt) and 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) 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). HPLC-grade water was purchased from J.T. Baker (Phillipsburg, NJ, USA). Reagents were of highest reagent grade and used without further purification.

Preparation of Maillard reaction products (MRPs)

Glucose and amino acids were dissolved in 100 mL of 0.5 mol/L sodium acetate buffer, pH 4.0, 0.5 mol/L phosphate buffer, pH 7.0 or 0.5 mol/L sodium carbonate buffer, pH 10.0 to obtain a final concentration of 1 mol/L. Six model systems were prepared, as follows: glucose (Glc), glucose–glycine (Glc/Gly), glucose－L-asparagine (Glc/L-Asn), glucose－D-asparagine (Glc/D-Asn), glucose－L-lysine (Glc/L-Lys), glucose－D-lysine (Glc/D-Lys). The reaction mixtures were then distributed over glass, screw-capped, Schott tubes (16×160 mm), with each containing a minimum of 10 mL. A model solution was heated without pH control at 100°C for 2 hr. The heating was carried out in a silicone oil bath and the proper safety measures were taken. After being heated, the model solutions were withdrawn and immediately cooled in ice water. All model systems were prepared in triplicate.

Determination of sugars in MRPs

The reducing sugars in MRP samples were determined using an HP 1100 liquid chromatograph (Hewlett Packard, Wilmington, DE, USA). An Agilent quaternary pump, connected to a refractive index detector (Model: G1362A, Hewlett Packard), was used with a Zorbax carbohydrate column (4.6×250 mm I.D., 5 μm particle size, Agilent Technologies, Wilmington, DE, USA). The mobile phase, consisting of acetonitrile/water (75:25, v/v), was delivered at a flow rate of 2.0 mL/min. The column temperature was 30°C and 1 μL of sample was injected into the HPLC system. The data analysis was performed using Chemstation software (Rev. A. 10.01, Hewlett Packard).

Derivatization of amino acids with FDAA in MRPs

Amino acids were derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) reagent according to Marfey's methods (16). Ten μL of MRPs in 20 μL of H_2O and 8 μL of 1 mol/L $NaHCO_3$ were mixed with 400 μg of FDAA in 40 μL acetone and incubated at 40°C for 1 hr with occasional shaking. The reaction was terminated by adding 4 μL of 2 mol/L HCl. Acetone, water and HCl were removed by evaporation under reduced pressure in a centrifugal evaporator. After evaporation, 20 μL of methanol was added to dissolve the resultant FDAA amino acid. FDAA amino acid solution (2 μL), thus prepared (0.5%, w/v) was spotted on a reversed phase precoated TLC plate (RP-18, $F_{254}S$, 5 cm ×10, Merck), and developed with acetonitrile/triethylamine-phosphate buffer (50 mM, pH 5.5) at 25/75 (v/v) in a pre-equilibrated glass chamber at 25°C. The FDAA amino acid spots were yellow and visible. When the ascending solvent front neared the top margin, the plate was removed from the chamber and dried with a hair-drier. The TLC was completed in 20 min at 25°C. A trial for quantitative analysis was made by varying the amount of D- and L-amino acids derivatives to the plate. The yellow spots were scraped off the plate after the chromatography, and extracted with methanol/water (1/1, v/v). Then FDAA amino acids (derivatized amino acids) were analyzed by HPLC. Since FDAA is sensitive to light, the FDAA amino acids were not exposed to light during all procedures.

Determination of amino acids in MRPs

The amino acids in MRPs were analyzed using HP 1100 liquid chromatograph (Hewlett Packard) with a diode array detector DAD HP 1100 operating at 338 nm

(Excitation=340 nm). Separation was carried out with a Zorbax Eclipse AAA Rapid Resolution column (150 ×4.6 mm i.d., 5 μm particle size, Agilent Technologies). A linear gradient profile of mobile phase, comprising 40 mM Na_2HPO_4, pH 7.8 (solvent A) and ACN/MeOH /water 45:45:10 (v/v) (solvent B), 0% B (0～1.9 min), 0～57% (1.9～18.1 min), 57～100% (18.1～18.8 min), 100% (18.8～22.3 min), 100～0% (22.3～23.2 min) and 0% (23.2～26 min) was applied at a flow rate of 2.0 mL/min. The column was equilibrated for 5 min under initial conditions prior to injection of the next samples. The column temperature was 40°C. In order to determine amino acids from MRPs, precolumn derivatization with *o*-phthalaldehyde (OPA) was used and 0.5 μL of prepared sample were injected into the HPLC system. The data analysis was performed using Chemstation software (Hewlett Packard). Relative quantities of amino acid enantiomers were calculated from peak areas of derivatives: %L=100L/(D+L), %D=100D/(D+L), where %L and %D represent relative amounts of L- and D-amino acids with regard to the sum of (D+L) amino acids and D and L represent the peak areas of the respective enantiomer determined by HPLC.

Measurement of browning

Browning indices of MRP samples were recorded by their absorbance at 420 nm on a spectrophotometer (Shimadzu UV 160A, Shimadzu Co., Kyoto, Japan) using a 1 cm pathlength cell after appropriate dilution with distilled water.

Wavelength spectra of MRPs

Wavelength spectra of MRPs were recorded by a UV-Vis spectrophotometer (Shimadzu UV 160A, Shimadzu Co., Kyoto, Japan), with the wavelength ranging from 200 nm to 700 nm.

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

The chelating of ferrous ions (Fe^{2+}) by MRPs and standards are estimated by the method of Dinis et al. (17). 100 μL of MRP samples with 5-fold dilution was added to 600 μL of distilled water and 100 μL of 0.2 mmol/L $FeCl_2 \cdot 4H_2O$. The mixture was allowed to rest at room temperature for 30 sec. The reaction mixture thus obtained was later mixed with 200 μL of 1 mmol/L ferrozine and changes in color were monitored at 562 nm with a spectrophotometer (Shimadzu UV 160A, Shimadzu Co.), after a 10 min resting time at room temperature. Ethylene diamine tetraacetate (EDTA, 2 Na-salt) was used to compare the chelating activity. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated in the following equation:

$$\text{Ferrous ion chelating activity} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

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 MRPs was determined by the 1,1-diphenyl-2-picryl-hydrazil (DPPH·). This activity was measured by the procedure described by Yen and Hsieh (7) 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 mmol/L 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 MRP samples with 5-fold dilution 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.). The control was prepared in the same manner, except that distilled water was used instead of MRP samples. For the blank, the assay was conducted in the same manner but methanol was added instead of DPPH solution. The antiradical activity of sample was expressed as percentage of disappearance of the initial purple color. Aqueous solutions of trolox at various concentrations were used to perform the calibration curves (0.15～1.15 mM).

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

The FRAP assay was done according to the modified Benzie and Strain method (18) with some modifications. Briefly, 900 μL of FRAP reagent, freshly prepared and warmed to 37°C, were mixed with 90 μL distilled water and either 30 μL of MRP samples or standard or appropriate reagent blank. The FRAP reagent contained 2.5 mL of a 10 mmol/L 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mmol/L HCl, plus 2.5 mL of 20 mmol/L $FeCl_3 \cdot 6H_2O$, plus 25 mL 0.3 mmol/L acetate buffer pH 3.6. Readings at the absorption maximum (595 nm) were taken every 15 sec, using a spectrophotometer (Shimadzu UV 160A, Shimadzu Co.). 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 $ABTS^{\cdot+}$ radical scavenging activity of MRPs was determined according to the method described by Re et al. (19) with slight

modifications. This method is based on the reaction between ABTS and potassium persulfate resulting in blue/green ABTS radical ($ABTS^{\cdot+}$). With the addition of the antioxidants, decolorization is attained and measured spectrophotometrically at 734 nm. The results were expressed as mmol trolox per mL of MRPs. Briefly, $ABTS^{\cdot+}$ was produced by reacting 7 mmol/L ABTS stock solution with 2.45 mmol/L potassium persulfate and allowing the mixture to stand in the dark at room temperature during 12～16 hr before use. The $ABTS^{\cdot+}$ solution (stable for two days) was diluted with 5 mmol/L 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 $ABTS^{\cdot+}$ solution and 30 μL of MRP samples were mixed for 45 sec 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 means from triplicate analysis at ($p<0.05$) were determined by Duncan's multiple range tests using the statistical analysis system (SPSS 12.0 for windows, SPSS Inc, Chicago, IL, USA).

RESULTS AND DISCUSSION

The loss and enolization of sugars in MRPs

The enolization reaction known as the "Lobry de Bruyn-Alberda van Ekenstein transformation" produces enediol anion species. Glucose and fructose can isomerize into one another this transformation (20). The loss and enolization of sugars in MRPs derived from glucose/amino acid enantiomer model systems according to pH level are shown in Table 1. The results show that, after heating for 2 hr, the concentration of the reactants was decreased according to increase of pH levels. Notably, the sugars were almost completely destroyed in the MRPs that resulted from L- and D-lysine. The losses of glucose in MRPs did not show any differences between the use of D- or L-entantiomers. Higher levels of degradation of both fructose and glucose occurred at 100°C under alkaline conditions (21,22). Benjakul et al. (23) also found that sugars were lost faster in alkaline solutions than in neutral solutions. In addition, the enolization of sugars was observed to correlate with an increase in pH levels in all MRP samples except for Glc, Glc/Gly, Glc/L-Asn and Glc/D-Asn at a pH 4.0 and Glc/L-Lys and Glc/D-Lys at a pH 10.0. In Glc/L-Lys and Glc/D-Lys, enolization of sugar was found at pH 4.0. A sugar isomerization and degradation reaction also takes place at the same time as the Maillard reaction. Sugar isomerization and degradation reactions were reported to be much more important, from a quantitative point of view, than what is necessary in the Maillard reaction (24). Because these sugar reactions occur simultaneously with the Maillard reaction and the sugar reaction products subsequently take part in the Maillard reaction, the Maillard reaction becomes even more intricate. Thus, sugar isomerization also can be formed in Maillard reaction by L- and D-amino acids.

The loss and racemization of amino acids in MRPs

The quantities of L- and D-amino acids determined by HPLC in MRPs and the relative quantities of their isomers are shown in Table 2. Except for Glc/D-Asn, the concentration of the reactants was increased according to the increase in the pH level. D-Amino acid forms were detected in L-amino acid systems, and, similarly, L-amino acid forms could also be observed in D-amino acid systems. However, the mixture of L- and D-amino acids was much greater in L-isomer systems than D-isomer systems. Formation of the isomer was the highest in the Glc/L-Lys system. The relative amounts of isomers in Glc/L-Asn and Glc/D-Asn were decreased as the pH levels increased. Brückner et al. (9) had pre-

Table 1. The loss and enolization of sugars (mmol/L) in MRPs derived from glucose/amino acid enantiomer model systems according to pH level

		Glc	Glc/Gly	Glc/L-Asn	Glc/D-Asn	Glc/L-Lys	Glc/D-Lys
pH 4.0	Glucose	724.86 ± 14.50^{aA}	614.33 ± 12.29^{bA}	563.34 ± 11.27^{cA}	558.23 ± 11.16^{cA}	75.32 ± 1.51^{dA}	81.98 ± 1.64^{dA}
	Fructose	−	−	−	−	44.46 ± 0.89^{bA}	58.44 ± 1.17^{aA}
pH 7.0	Glucose	57.55 ± 2.88^{aB}	23.91 ± 1.20^{bB}	16.41 ± 0.82^{cB}	16.54 ± 0.83^{cB}	1.11 ± 0.06^{dB}	1.95 ± 0.10^{dB}
	Fructose	18.80 ± 0.94^{aA}	5.33 ± 0.27^{bB}	2.44 ± 0.12^{dB}	3.68 ± 0.18^{cB}	2.20 ± 0.11^{dB}	2.30 ± 0.12^{dB}
pH 10.0	Glucose	10.61 ± 0.53^{bC}	9.80 ± 0.49^{cB}	9.59 ± 0.48^{cB}	13.99 ± 0.70^{aB}	−	−
	Fructose	11.16 ± 0.56^{aB}	6.90 ± 0.35^{bA}	6.76 ± 0.34^{bcA}	6.25 ± 0.31^{cA}	0.91 ± 0.05^{dC}	−

Values are means of three replicates ± standard deviation.
Different small superscripts (a-d) indicate significant differences according to the samples in the same pH level at $p<0.05$ level.
Different capital superscripts (A-C) indicate significant differences according to the pH level in the same sample at $p<0.05$ level.

Table 2. Quantities of L- and D-amino acids (mmol/L) and relative amounts of isomers (%L and %D) in MRPs derived from glucose/amino acid enantiomer model systems according to pH level

		Glc/Gly	Glc/L-Asn	Glc/D-Asn	Glc/L-Lys	Glc/D-Lys
pH 4.0	L-AAs	48.62 ± 2.43^{cC}	82.13 ± 4.11^{aC}	8.77 ± 0.44^{dB}	74.65 ± 3.73^{bC}	5.20 ± 0.26^{dC}
	D-AAs	−	8.80 ± 0.44^{dB}	85.80 ± 4.29^{bC}	39.62 ± 1.98^{cB}	190.19 ± 9.51^{aC}
	%L	−	−	9.27 ± 0.46	−	2.66 ± 0.13
	%D	−	9.68 ± 0.48	−	34.67 ± 1.73	−
pH 7.0	L-AAs	93.76 ± 4.69^{aB}	95.19 ± 4.76^{aB}	8.42 ± 0.42^{cC}	88.48 ± 4.42^{aB}	22.86 ± 1.14^{bB}
	D-AAs	−	6.89 ± 0.34^{dB}	118.72 ± 5.94^{bB}	31.19 ± 1.56^{cB}	135.58 ± 6.78^{aB}
	%L	−	−	6.62 ± 0.33	−	14.43 ± 0.72
	%D	−	6.75 ± 0.34	−	26.06 ± 1.30	−
pH 10.0	L-AAs	525.08 ± 7.88^{aA}	441.30 ± 6.62^{bA}	19.71 ± 0.39^{eA}	145.54 ± 2.91^{cA}	29.75 ± 0.59^{dA}
	D-AAs	−	23.19 ± 0.46^{dA}	436.52 ± 6.55^{aA}	93.91 ± 1.88^{cA}	197.57 ± 3.95^{bA}
	%L	−	−	4.32 ± 0.22	−	13.09 ± 0.65
	%D	−	4.99 ± 0.25	−	39.22 ± 1.96	−

Values are means of three replicates ± standard deviation.
Different small superscripts (a-e) indicate significant differences according to the samples in the same pH level at $p<0.05$ level.
Different capital superscripts (A-C) indicate significant differences according to the pH level in the same sample at $p<0.05$ level.

viously shown that heating L-amino acids together with reducing sugars leads to the formation of large amounts of D-amino acids. This reaction is known as the Maillard reaction (10) or non-enzymic browning reaction (25). Pätzold and Brückner (26) had postulated that D-amino acids are generated from relatively stable intermediates of this reaction, named the Amadori and Heyns compounds. This general route for the generation of D-amino acids has been extended to other foods rich in reducing sugars and amino acids. The Maillard reaction also explains the occurrence of D-amino acids in roasted coffee and cacao (27,28). Thus, the racemization of amino acids can also occur during the course of the Maillard reaction, the levels of which increase as the pH level increases.

Browning intensity

Brown color development (*A* 420 nm) is the easiest measurable consequence of the Maillard reaction because it offers a visual estimate. Its intensity is often used as an indicator of the extent to which the Maillard reaction takes place in foods, and it symbolizes an advanced stage of the Maillard reaction (29). The browning intensity of MRPs derived from glucose/amino acid enantiomer model systems according to pH level are shown in Fig. 1. The results indicate that browning development was somewhat increased as the pH levels increased; however, the difference was not significant except for Glc/Gly. Moreover, browning development does not show any characteristic differences in the use of L- versus D-amino acid isomers.

Hofmann (30) reported that only trace amounts of compounds with molecular weights greater than 3000 Da were formed when both glucose/glycine and glucose/alanine systems were heated in phosphate buffer for 4 hr at 95°C, pH 7. A much higher percentage of color

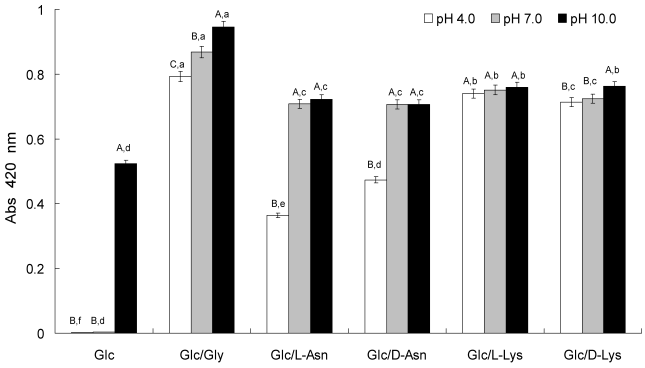

Fig. 1. Browning (as measured by absorbance at 420 nm) of MRPs derived from glucose/amino acid enantiomer model systems according to pH level. Results are mean ± standard deviation of three experiments and data are evaluated by using one-way analysis of variance performed by ANOVA procedures. Different small letters (a-f) indicate significant differences according to the samples in the same pH level at $p<0.05$ level. Different capital letters (A-C) indicate significant differences according to the pH level in the same sample at $p<0.05$ level.

was detected in the high-molecular-weight fraction ($\geq$ 70%), which is expected, since the melanoidins are attached to the protein that is high-molecular-weight by itself (30,31).

Wavelength spectra of MRPs

The wavelength spectra of MRPs derived from the glucose/amino acid enantiomer model systems according to pH level are shown in Fig. 2. The absorbance is increased as the pH levels increase. The L- and D-isomers both show different absorption in the UV-Vis spectra, but they do display a similar shape. Every peak has a stable absorbance appeared in the range between 260 nm and 320 nm, characteristic of melanoidins. This trend was also described by other authors for melanoidin-type

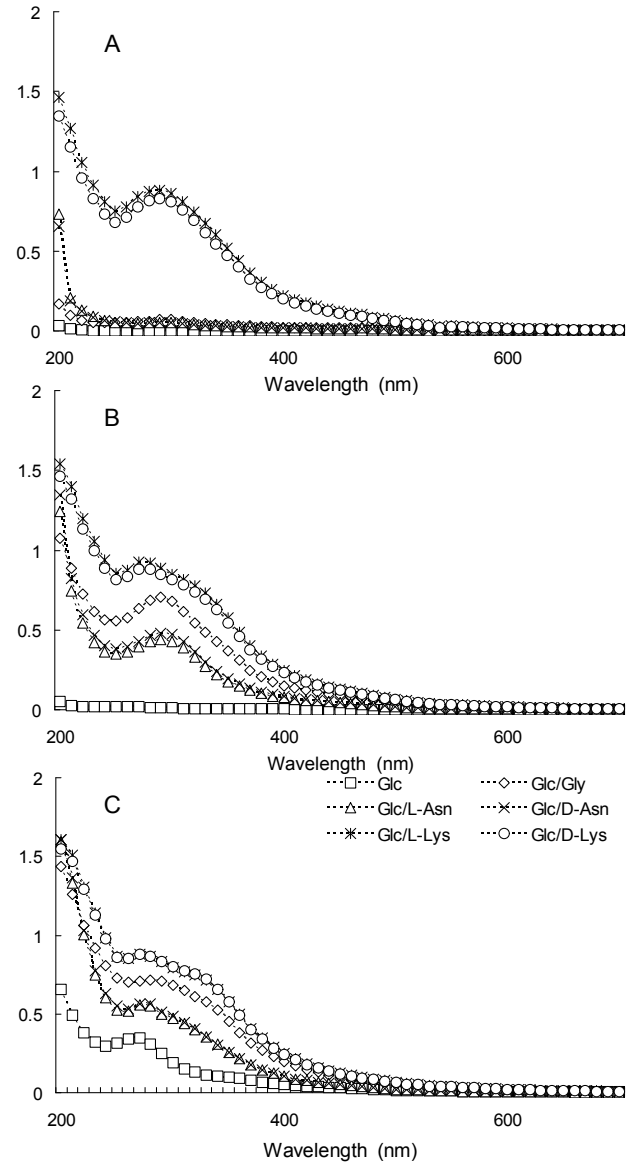

Fig. 2. Comparison of the UV-Vis spectra of MRPs derived from glucose/amino acid enantiomer model systems according to pH level. A, pH 4.0; B, pH 7.0 and C, pH 10.0.

colorants (32,33). At pH 4.0 and 7.0, the band intensities for the two isomers are different: the low absorption curve is formed for the L-isomer, whereas the higher absorption curve is formed for the D-isomer in Glc/L-Asn and Glc/D-Asn. However, the low absorption curve is formed for the D-isomer whereas the higher absorption curve is formed for the L-isomer in Glc/L-Lys and Glc/D-Lys. The highest absorption curve is formed for the L-isomer in lysine model system. However, the band intensities for the two isomers showed little difference at a pH of 10.0.

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

MRPs are known metal chelators, and their metal ion binding affinity has been proposed as a possible mechanism for their antioxidant activity because transition metals, especially iron and copper, are implicated in the generation of free radicals by Fenton reactions (34,35). The changes in the ferrous ion chelating activity of MRPs derived from glucose/amino acid enantiomer model systems according to pH level are shown in Fig. 3. Except for Glc/L-Asn system, the ferrous ion chelating activities of MRPs from the Glc/Gly, Glc/D-Asn, Glc/L-Lys and Glc/D-Lys systems were increased with increasing pH levels, The differences in the ferrous ion chelating activities became less significant according to amino acid enantiomers ($p<0.05$). Notably, the ferrous ion chelating activities of MRPs derived from the Glc/L-Lys system were the greatest. There is now strong evidence that MRPs have the ability to bind transition metals (1,36). Previously, other studies had shown the ability of different sugar-amino acid model MRPs to chelate iron, and equations for the effect of MRPs on the chelation of iron have been proposed (6). However, in the study of Morales et al. (37), no relationship was found between browning and iron-binding ability of MRPs. This observation was in agreement with the present study (Fig. 1, 3). In addition, iron solubility in the presence of glucose-lysine heated mixtures has been shown to be affected by the lower initial pH and destruction of free lysine in the model system (34,38). The results from this study indicate that MRPs have different chelating activities according to the pH level. MRPs derived from the D-isomers presented ferrous ion chelating activities similar to those exhibited by MRPs derived from the L-isomers.

DPPH radical scavenging activity

DPPH is a chromogen-radical-containing compound

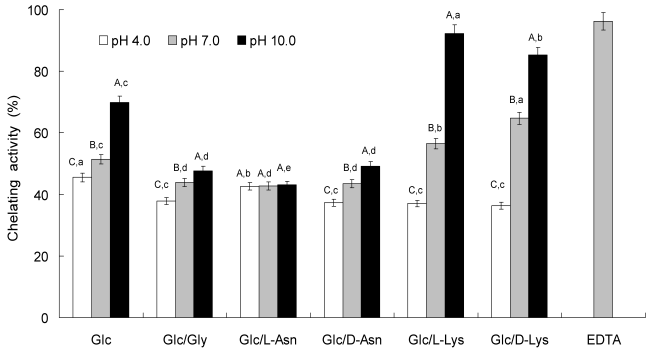

Fig. 3. Ferrous ions (Fe^{2+}) chelating activity of MRPs prepared at different pH levels. Data are expressed as mean±standard deviation of three experiments and data are evaluated by using one-way analysis of variance performed by ANOVA procedures. Concentration of EDTA is 200 μg/mL. Different small letters (a-e) indicate significant differences according to the samples in the same pH level at $p<0.05$ level. Different capital letters (A-C) indicate significant differences according to the pH level in the same sample at $p<0.05$ level.

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

Sample	pH 4	pH 7	pH 10
Glc	36.82 ± 0.20^{cA}	35.15 ± 0.20^{fC}	36.17 ± 0.24^{dB}
Glc/Gly	36.02 ± 0.18^{dC}	38.92 ± 0.16^{cA}	37.96 ± 0.29^{bB}
Glc/L-Asn	35.39 ± 0.22^{eC}	37.66 ± 0.18^{eA}	37.01 ± 0.20^{cB}
Glc/D-Asn	36.83 ± 0.16^{cC}	38.38 ± 0.16^{dA}	38.03 ± 0.16^{bB}
Glc/L-Lys	42.21 ± 0.16^{aA}	40.70 ± 0.16^{aB}	38.35 ± 0.29^{abC}
Glc/D-Lys	41.54 ± 0.13^{bA}	40.16 ± 0.24^{bB}	38.78 ± 0.29^{aC}

[1)]Data expressed as μmol equivalents of trolox per 1 mL of MRPs.
Values are mean±standard deviation of three experiments. Different small superscripts (a-f) indicate significant differences according to the samples in the same pH level at $p<0.05$ level.
Different capital superscripts (A-C) indicate significant differences according to the pH level in the same sample at $p<0.05$ level.

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

Sample	pH 4	pH 7	pH 10
Glc	46.87 ± 1.70^{bB}	37.05 ± 2.36^{cC}	56.43 ± 3.28^{bA}
Glc/Gly	47.15 ± 1.70^{bC}	73.08 ± 2.88^{aA}	67.62 ± 3.31^{aB}
Glc/L-Asn	42.78 ± 1.89^{bC}	63.53 ± 2.06^{bA}	56.70 ± 3.31^{bB}
Glc/D-Asn	46.87 ± 2.06^{bC}	61.62 ± 2.88^{bA}	52.60 ± 2.88^{bB}
Glc/L-Lys	78.54 ± 2.95^{aA}	72.26 ± 2.88^{aAB}	68.17 ± 4.12^{aB}
Glc/D-Lys	82.36 ± 3.69^{aA}	75.81 ± 2.88^{aA}	67.62 ± 4.51^{aB}

[1)]Data expressed as μmol equivalents of trolox per 1 mL of MRPs.
Values are mean±standard deviation of three experiments. Different small superscripts (a-c) indicate significant differences according to the samples in the same pH level at $p<0.05$ level.
Different capital superscripts (A-C) indicate significant differences according to the pH level in the same sample at $p<0.05$ level.

that can directly react with antioxidants. 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 (39). 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 (40). The changes in the DPPH radical scavenging activity of MRPs derived from glucose/amino acid enantiomer model systems according to pH level are shown in Table 3. The DPPH radical scavenging activity of MRPs derived from the Glc/Gly, Glc/L-Asn and Glc/D-Asn systems were increased and then decreased according to pH level. The MRPs derived from the Glc/L-Lys and Glc/D-Lys systems decreased with the increase of pH. The differences in DPPH radical scavenging activity became less significant according to amino acid enantiomers ($p<0.05$). The DPPH radical scavenging activity of MRPs derived from the Glc/L-Lys system was the highest at a pH of 4.0. These results were also in agreement with those of Yen and Hsieh (7), Morales and Jimenez-Perez (29) and Benjakul et al. (41) who found that MRPs had DPPH radical scavenging activity. The results also showed that the MRPs derived from the D-isomers presented radical scavenging activities similar to those exhibited by MRPs derived from the L-isomers. Bersuder and Hole (42) 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 to be one of the compounds involved in the DPPH antiradical activity of the histidine-glucose Maillard system.

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

The 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+}. This assay is also commonly used for the routine analysis of single antioxidants and total antioxidant activity (43). The changes in the FRAP of MRPs derived from glucose/amino acid enantiomer model systems according to pH level are shown in Table 4. The FRAP of MRPs derived from the Glc/Gly, Glc/L-Asn and Glc/D-Asn systems were increased and then decreased according to pH level. Otherwise, the MRPs derived from the Glc/L-Lys and Glc/D-Lys systems were decreased as the pH increased. The differences in ferric reducing ability became less significant according to amino acids enantiomers ($p<0.05$). These results are in agreement with those obtained for the antioxidant activity determined by the DPPH radical scavenging assay (Table 3). Rufian-Henares and Morales (44) pointed out that the ferric reducing ability of MRPs was in parallel with the data from the DPPH method. Compounds responsible for reducing activity are formed during the thermolysis of Amadori products in the primary phase of Maillard reactions or they could be formed as heterocyclic products of the Maillard reaction or caramelization of sugars (45,46). The result revealed that MRPs could function as electron donors. The hydroxyl groups of MRPs play an important role in reducing activity (6). Additionally, the intermediate reductone compounds of MRPs were reported to break the radical chain by donation of a hydrogen atom (47). Moreover, the results indicated that the MRPs derived from the D-isomers have similar ferric reducing antioxidant power, such as those derived from L-isomers.

ABTS radical scavenging activity

Generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods that has been

Table 5. Antioxidant activity determined by the ABTS method[1)]

Sample	pH 4	pH 7	pH 10
Glc	33.33 ± 0.00^{bB}	38.06 ± 2.36^{dB}	120.80 ± 10.83^{dA}
Glc/Gly	44.37 ± 1.36^{bC}	319.39 ± 22.55^{aA}	257.13 ± 15.38^{aB}
Glc/L-Asn	46.73 ± 5.46^{bC}	224.82 ± 15.50^{cA}	188.57 ± 14.44^{cB}
Glc/D-Asn	42.79 ± 2.36^{bC}	228.76 ± 16.77^{cA}	193.30 ± 14.44^{cB}
Glc/L-Lys	288.65 ± 22.55^{aA}	265.01 ± 22.55^{bB}	238.22 ± 19.11^{abB}
Glc/D-Lys	277.62 ± 22.72^{aA}	257.13 ± 23.80^{bcB}	222.46 ± 20.20^{bB}

[1)]Data expressed as μmol equivalents of trolox per 1 mL of MRPs.
Values are mean ± standard deviation of three experiments.
Different small superscripts (a-d) indicate significant differences according to the samples in the same pH level at $p<0.05$ level.
Different capital superscripts (A-C) indicate significant differences according to the pH level in the same sample at $p<0.05$ level.

applied for the measurement of the total antioxidant activity of solutions of pure substances, aqueous mixtures and beverages (48). The method for the screening of antioxidant activity is 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 a decolorization technique in which the radical is generated directly in a stable form prior to reaction with putative antioxidants. The changes in the ABTS radical scavenging activity of MRPs derived from glucose/amino acid enantiomer model systems according to pH level are shown in Table 5. The ABTS radical scavenging activity of MRPs derived from the Glc/Gly, Glc/L-Asn and Glc/D-Asn systems were increased and then decreased according to pH level. Otherwise, the MRPs derived from the Glc/L-Lys and Glc/D-Lys systems were decreased with increasing pH. The differences in ABTS radical scavenging activity became less significant according to the amino acid enantiomers ($p<0.05$). These results are in agreement with those obtained for the antioxidant activity determined by the DPPH radical scavenging assay and FRAP assay (Table 3, 4). However, the values obtained with the ABTS assay were higher than those obtained with the FRAP assay in each sample analyzed. The difference in the antioxidant activity obtained with the FRAP and ABTS assays could be due to the different reaction mechanisms involved. Moreover, results differed within those obtained with the DPPH assay. It is assumed that the difference of radical scavenging activity is due to different reaction media; aqueous and methanolic for ABTS and DPPH, respectively (44). The FRAP assay detects compounds that act only by the single electron transfer (SET) mechanism, whereas the ABTS assay detects compounds that act either by direct reduction via electron transfer or by radical quenching via the hydrogen atom transfer (HAT) mechanism (49).

In conclusion, on the basis of the data obtained in this study, the loss of glucose in MRPs did not show different characteristics for the different amino acid enantiomers; however, the concentration of glucose decreased as the pH levels increased. In addition, the enolization of sugars was observed in all MRP samples, in response to the increase of pH levels. D-Amino acids were detected in L-amino acid systems. Similarly, L-amino acid could also be observed in D-amino acid systems. Formation of isomers was the highest in the Glc/L-Lys system. The relative amounts of isomers in Glc/L-Asn and Glc/D-Asn were decreased as the pH levels increased. The browning development was increased with increasing pH levels; however, browning development does not show different characteristics between the use of L- and D-isomers of the amino acid. The L- and D-isomers both show different absorptions in the UV-Vis spectra, but the absorption patterns have a similar shape. Every peak has a stable absorbance in a range between 260 nm and 320 nm, characteristic of melanoidins. The MRPs derived from the D-amino acids are found to be effective antioxidants in different *in vitro* assays, including ferrous ion chelating activity, ABTS and DPPH radical scavenging activity, and ferric reducing antioxidant power, when they are compared to standard antioxidant compounds, such as EDTA and trolox. Notably, the antioxidant activities of MRPs derived from the Glc/Gly, Glc/L-Asn and Glc/D-Asn systems at pH 7.0 were greater compared to those of pH 4.0 and pH 10.0. In addition, the results show that the MRPs derived from the D-isomers have similar antioxidant activities as those from L-isomers. Therefore, the MRPs have the different antioxidant activities on the basis of the pH level, but not on the basis of different forms of the amino acid enantiomers.

ACKNOWLEDGEMENTS

This study was supported by Kyungnam University Research Fund, 2010.

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(Received September 3, 2010; Accepted November 22, 2010)