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Full Length Research Paper

Antioxidant activity of maillard reaction products from lysine-glucose model system as related to optical property and copper (II) binding ability

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Maillard reaction (MR) was carried out in L-lysine-D-glucose (Lys-Glu) model system heated at 120°C for 0 to 10 h without pH control. Optical property (UV-Vis absorbance and fluorescence) development of MR was monitored. Antioxidant activity of maillard reaction products (MRPs) was investigated by a series of *in vitro* tests. The UV-Vis absorbance zero and first-order derivative spectrophotometric was utilized to study the metal-binding property and chelating effect of different fractions of MRPs. MRPs had reducing power, strong free radical-scavenging activity and copper (II)-binding ability. The antioxidant activity of MRPs related well with the UV-Vis absorbance properties of maillard rection. Cu^{2+}-binding to MRPs caused the electronic rearrangement in UV-Vis absorbance intermediates, but had no effect on the chromophore structure in brown polymers. The UV-Vis absorbance compounds were responsible to the reducing power and radical-scavenging activity. The fractions of low molecular weight MRPs showed effective metal-chelating ability.

Key words: Maillard reaction products, antioxidant activity, metal-chelating effect, optical properties.

INTRODUCTION

The antioxidant activity of MRPs was first reported by Franzke and Iwainsky (1954) and has been extensively investigated thereafter (Eichner, 1981; Wijewickreme and Kitts, 1997; Benjakul et al., 2005). Some fractions were reported to have strong antioxidant properties comparable to those of commonly used food antioxidants (Lingnert and Hall, 1986). The action mechanisms are supposed to involve radical chain-breaking activity (Morales and Babbel, 2002), metal-chelating ability (Bersuder et al., 2001), active oxygen species scavenging (Yen and Hsieh, 1995; Wagner et al., 2002) and hydroperoxide-destroying ability (Wijewickreme et al., 1999). However, the compounds accounting for this effect have not been identified and the mechanism of antioxidant effect is still under studies.

There are three major stages during maillard reaction including early, advanced and final stage. Free amino groups react with carbonyl groups to form a reversible Schiff base during the early stage. Studies have showed that, the Schiff base has strong metal-binding ability to form the Schiff base complexes (Jing and Kitts, 2004). In the advanced phase, several of low molecular weight intermediate compounds are produced and these compounds may be involved in antioxidant activity of whole MRPs (Eichner, 1981; Hodge, 1953). Compounds produced during the final stage are mainly brown polymer melanoidins. Some studies have found that melanoidins have antioxidant activity via scavenging oxygen radicals and chelating metals (Franzke and Iwainsky, 1954; Wagner et al., 2002). However, it has been unclear with regard to their physical, chemical and physiological properties due to their complex structures.

MRPs from lysine are frequently present in food, as this amino acid is very sensitive to MR. Derivatives from the lysine-glucose model system are frequently used to study diverse aspects of MRPs. The studies are significant to

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Abbreviations: MR, Maillard reaction; **MRPs,** maillard reaction products; **TMM,** tetramethylmurexide; **DPPH,** 2,2-diphenyl-1-picrylhydrazyl.

the optimization of food processing conditions for human health. Different MRPs with different UV-absorbance properties, fluorescent properties and browning properties are formed under different conditions. It helps to understand the different antioxidant properties and mechanisms of different MRPs. Photometric analysis have been developed to study MRPs formation (Hodge, 1953; Ajandouz et al., 2001; Morales and Jiménez-Pérez, 2001). The UV-absorbance is an important and obvious feature of the non-color or non-fluorescent low molecular weight intermediate products in advanced stages and the fluorescence indicates the non-color low molecular weight fluorescent intermediate compounds. Browning development demonstrates the browning polymers in the final stage. So, we propose a new but more suitable methodology to study the antioxidant activities of Lys-Glu MRPs and the antioxidant effects of each kind of MRPs. This method based is on the relationship between the antioxidant activity of MRPs and maillard reaction progression via pH, UV-absorbance, fluorescence and browning analysis. The aim of this paper was to study the pH, UV-absorbance, fluorescence, browning and anti-oxidant effect development of maillard reaction products in lysine-glucose model system and analyze the anti-oxidant activities and action mechanisms of the same kind of products during each stage, especially employ the zero and first-order derivative spectrophotometric as indicators of metal complex/chelate formation of MRPs.

MATERIALS AND METHODS

Chemicals

Reagents were from the following sources: L-lysine monohydrochloride, D-Glucose, tetramethylmurexide (TMM), hexamethylene-tetramine (hexamine), copper(II) sulphate, iron(III) chloride, potassium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, potassium ferricyanide and trichloroacetic acid from Bodi chemical Co.(Tianjin, China); methanol (HPLC grade) from Merck KGaA (Darmstadt, Gemany); 2,2-diphenyl-1-picrylhydrazyl (DPPH·) from Sigma Chemical Co.(St. Louis, MO,USA). Millipore grade water (Elix 3 system, Millipore, Milford, MA) was used throughout in this study.

Preparation of MRPs from Lys-Glu model system

The model system consisted of 0.03 M Lys, 0.03 M Glu and 100 ml water with pH adjusted to 6.68 using 1 M Na_2CO_3. About 5 ml aqueous solution was poured into screw-capped tubes (15 ml) and flushed with nitrogen for 2 min, then, heated without pH control in glycerol bath kept at 120°C. After predetermined heating time (5, 10, 15, 20, 30 min, 1, 2, 3, 4, 6, 8 and 10 h), a sample was obtained and immersed in ice for rapid cooling. Aliquots were directly used to determine pH with the help of a Mettler pH instrument Delta320. The remaining part was kept at 4 until future use.

Each sample was determined antioxidant effects by a series of *in vitro* tests. All crude MRPs solution was clear. The concentration of crude MRPs was calculated as total concentration of initial reactants and diluted when used. Crude MRPs solution was colorful, so sample blanks were treated in spectra tests.

Monitoring optical property development

UV-Vis absorbance spectra were recorded as described by Jing and Kitts, (2004) with some modifications. The MRPs were diluted (1:100, v/v) with water and then, measured for absorbance spectra between 198 and 552 nm using a Unico spectrum spectrophotometer UV2802. Optical density (OD) at several maximum absorbance bands (λmax) was depicted as MRPs characteristic indexes.

The fluorescence spectra were also prepared by Morales and Jiménez-Pérez, (2001) with some modifications. Samples were diluted (1:100, v/v) with water and then, fluorescence at an emission spectrum (350 to 550 nm) was measured with the excitation wavelength set at 347 nm, using a Hitachi spectrofluorophotometer F-2500. Then, the fluorescent intensity at the maximum emission was noted to indicate the fluorescent compounds.

Determination of reducing power

The reducing power of MRPs was determined according to the method of Benjakul et al. (2005) with some modifications. 1 ml of MRPs samples (1:100 diluted, v/v) was mixed with 1 ml of 0.2 M sodium phosphate buffer (pH6.6) and 1 ml of 1 g/100 g potassium ferricyanide. The reaction mixture was incubated in water bath at 50°C for 20 min, followed by addition of 1 ml of 10 g/100 g trichloroacetic acid.

1 ml of the mixtures was treated with 1 ml of water and 0.2 ml of 0.1 g/100 g iron (III) chloride. The absorbance at 700 nm was determined and the increase in absorbance of the reaction mixture indicated the reducing power of samples.

Free radical-scavenging activity

Radical scavenging ability of MRPs was determined using the stable radical DPPH· by a modified method of Jing and Kitts, (2004). Reaction mixture containing DPPH· (0.1 mM) methanol solution and MRPs samples (1:100 diluted, v/v) was kept at room temperature in dark for 30 min. Mixtures containing absolute methanol and MRP samples were also prepared for MRPs color correction. The absorbance at 517 nm was recorded. The DPPH· scavenging activity was calculated according to the equation:

$$\text{Scavenging activity (\% control)} = (A_{control} - A_{sample})/A_{control} \times 100.$$

Measurement of copper (II) binding ability

Copper (II) binding ability of MRPs was determined using the dual-wavelength spectrophtometric tetramethylmurexide (TMM) method described by Wijewickreme, et al. (1997) with some modifications. 0.5 ml of MRPs samples (1:20 diluted, v/v) was mixed with 1.5 ml of hexamine buffer (10 mM, pH5) containing 10 mM KCl. The mixture was then, treated with 0.5 ml of $CuSO_4$ (1 to 5 mM) and incubated in dark for 30 min at room temperature. Blanks without MRPs and sample blanks were treated in the same conditions. 0.05 to 0.1 ml of the reaction mixture was mixed with 0.1 ml of 1 mM TMM in 2.45 to 2.4 ml hexamine buffer. The amount of free copper (II) in the solutions was obtained from a standard curve, where the absorbance ratio A_{460}/A_{530}, in a solution of 0.5 ml of $CuSO_4$ (0.02 to 0.1 mM), 2.0 ml of hexamine buffer and 0.1 ml of TMM, was plotted against the amount of $CuSO_4$ (0.02 to 0.1 mM). The amount of copper (II) bound to MRPs was calculated as the difference between the amount of copper (II) added and free copper (II) present in the solution.

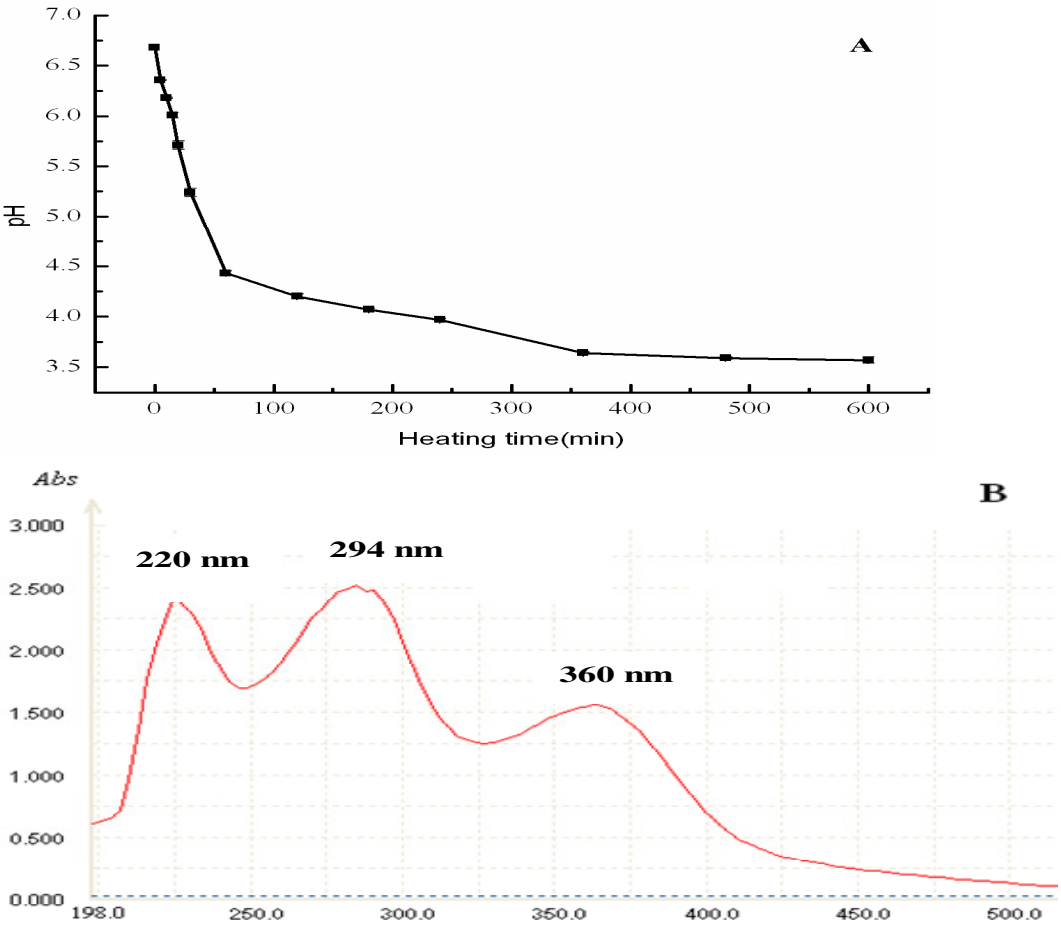

Figure 1. Maillard reaction progression in heated Lys-glu model system: (A) development of pH as function of heating time (min). Bars indicate the standard deviation from triplicate determinations; (B) the UV-Vis absorbance spectrum of MRPs from Lys-glu model system heated for 8 h; (C) optical properties of MRPs as function of heating time (min): MRPs were used at 1.413 mg/ml for optical property measurements.

Spectrophotometric monitoring the characteristic of complex/chelate formation of MRPs with copper (II)

Absorbance spectrum of MRPs (1:100 diluted, v/v) with different Cu^{2+} concentration was recorded between 198 and 552 nm. The OD of several absorption bands was analyzed as complex/chelate formation characteristic indexes. First-order derivative spectrum of MRPs with different Cu^{2+} concentration was recorded to determine the possible chelation of different MRPs fractions.

Effect of copper (II) on the antioxidant properties

The effect of copper (II) on the antioxidant properties of MRPs was investigated by reduce power and 2,2-diphenyl-1- picrylhydrazyl radical (DPPH·) test described previously.

Statistical analysis

All the tests were done at least in triplicate and data were averaged. Standard deviation was also calculated. Hypothesis testing method included one-way analysis of variance (ANOVA), followed by least significant difference (LSD) test $P < 0.05$ and $P < 0.01$ indicates statistical significance.

RESULTS AND DISCUSSION

Evaluation of pH of the MRPs samples

The initial value was adjusted to 6.68 to obtain neutral values. The MR is greatly influenced by the pH of medium (Ajandous et al., 2001; Cämmerer and Kroh, 1996) and the rate and profile of MRPs formed depends on the pH of the medium. Figure 1a depicted the time course of pH along with maillard reaction progression. A marked decrease in the pH values, being 2.25 units, was observed during the first 60 min of treatment. Then, the drop was not so obvious (about 0.86 of a unit) at prolonged heating. The MR itself has a strong influence on pH and pH falls through the reaction due to the disappearance of basic amino groups at early stage. The facts is that Amadori product and its dicarbonyl derivatives tended to produce low molecule weight

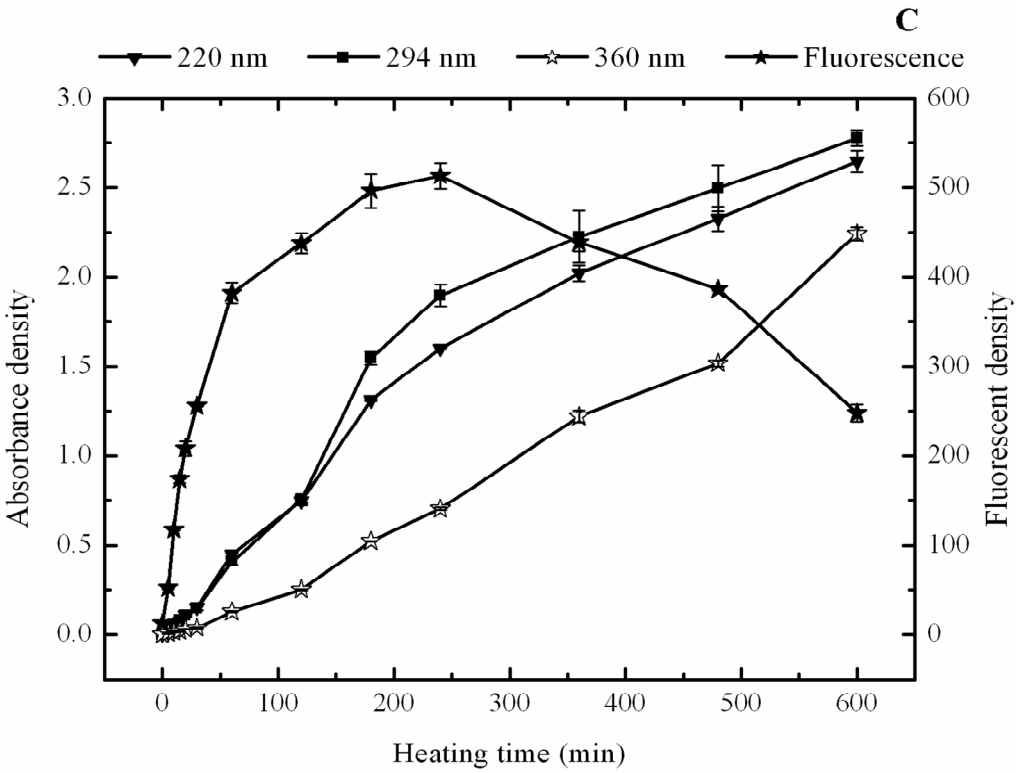

Figure 1. Continued.

organic acids at advanced stage might also account for the pH decline (Tressl et al., 1995).

UV-Vis absorbance development

The UV-Vis absorbance development is an important and obvious feature of the MR after the initial stage (Hodge, 1953). Absorbance at 294 nm has been used to determine the non-colored and non-fluorescent intermediate compounds (Wijewickreme and Kitts, 1997; Ajandouz et al., 2001). The optical density at λmax between 470 and 360 nm frequently measures the rate and extent of brown compound formation (Davidek et al., 2002).

The UV-Vis spectroscopy showed that three characteristic absorbance bands developed reading 220, 294 and 360 nm (Figure 1b). Continuous increase of the peaks was observed as heating time increased (Figure 1c). Similar with other studies, absorbance developments at 294 nm showed shorter induction time than browning (Benjakul et al., 2005; Ajandouz et al., 2001), indicating the intermediate compounds produced in the advanced stage. The absorbance at 220 nm has not been reported previous. It might be caused by osones, acids and furaldehyde compounds produced through Amadori product degradation (Davidek et al., 2002), as even no lag time was observed for the optical developing. Based on the slopes of the absorbance curves at 294 nm during different heating time, being 0.0081 for 0 to 240 min and 0.0023 for 240 to 600 min, the kinetic behavior of the non-color intermediate compounds formation was different. The difference might be due to the transformation of some intermediate products into brown polymers, showing some elimination rate.

In this study, the high molecular brown polymers in MRPs showed characteristic absorbance at 360 nm. The pH of the reaction medium might influence the chromophore structure in the brown polymers. According to Tressl et al. (1995), Amadori products degradation and carbohydrates dehydration were favored in acid media, leading to furaldehyde compounds, reductones and other cyclic compounds. All of these compounds could react to produce brown polymers (Zheng and Xu, 2005). The polymers were soluble, as crude MRPs solution was clear and transparent.

Fluorescence development

Fluorescence intensity reached a maximum at the heating time of 240 min. Thereafter, the fluorescence decreased as the heating time increased (Figure 1c). The result was in line with previous researches (Benjakul et al., 2005; Ajandouz et al., 2001). Fluorescent compounds developed prior to the formation of brown pigments in MR (Morales et al., 1996), suggesting that another kind of

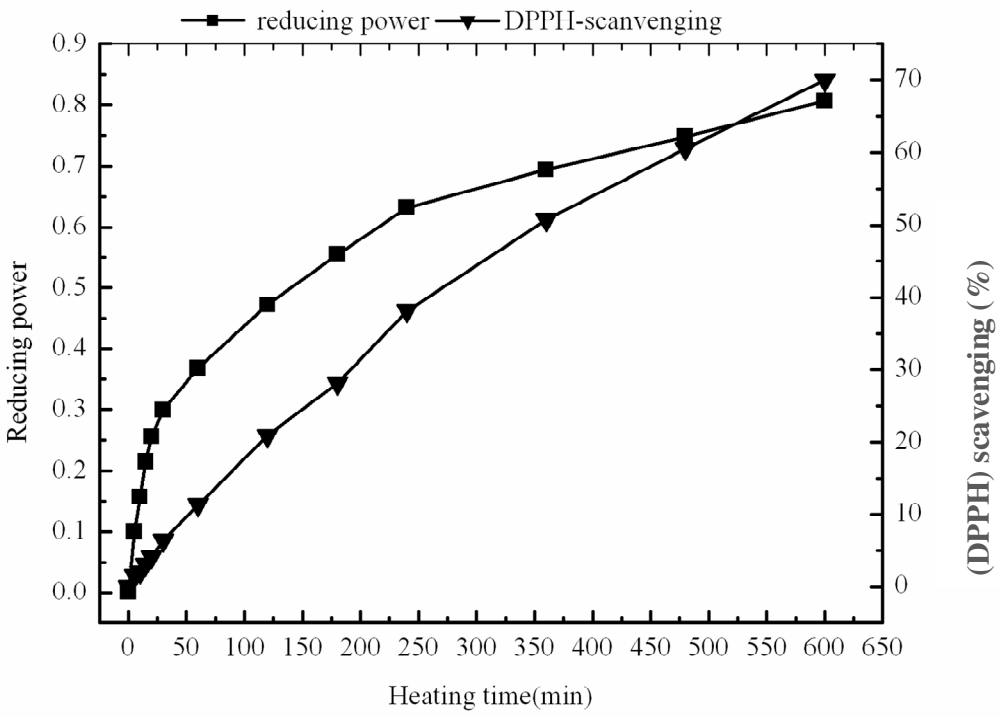

Figure 2. Antioxidant activity development of MRPs as function of heating time (min).

intermediate products different from non-color intermediates were formed, being fluorescent intermediates. However, the pattern between UV-absorbance and fluorescence was different, suggesting that non-fluorescent and fluorescent intermediates underwent the final reaction at different rates. The fluorescent intermediates seemed to be more reactive in formation of brown polymers than non-fluorescent compounds, as shown by the decrease in fluorescent intensity as heating time increased.

Evaluation of antioxidant activity

To evaluate the antioxidant activity of Lys-Glu MRPs, reducing power and DPPH·-scavenging activity were determined. For the measurement of the reductive ability, $Fe^{3+} \rightarrow Fe^{2+}$ transformation in the presence of MRPs was investigated. Reducing power of MRPs increased as the heating time increased, as shown by increase in absorbance at 700 nm (Figure 2). The free radical-scavenging activity was investigated by DPPH·. MRPs showed a significant DPPH· radical-scavenging activity as the heating time increased (Figure 2). However, the increased pattern between reducing power and radical-scavenging activity was not identical; in the first 30 min of heating, the reducing power increased linearly with slope being 0.0098 ($n = 6$, $R^2 = 0.9247$, $P < 0.01$), then, the linear increase was not so drastic with slope being 0.0008 ($n = 8$, $R^2 = 0.9103$, $P < 0.01$); while, the radical-scavenging activity increased linearly during the whole heating ($n = 13$, $R^2 = 0.9799$, $P < 0.001$).

The non-color and non-fluorescent intermediate products and brown polymers accumulated almost linearly with the heating time, as depicted by the increase of UV-Vis absorbance density. These compounds might relate to the antioxidant activity. Fluorescent compounds were not related to the antioxidant effects. The results were different with Morales and Jiménez-Pérez, (2001), which indicated that fluorescence measurement was more effective that browning to follow the formation of maillard reaction products (MRPs) with free radical scavenging activities. The reason might be the different reactants and reacting conditions.

Copper (II)-binding ability

Copper (II)-binding ability (as calculated as quantity of Cu^{2+} bounded by 1 mg MRPs) of MRPs was directly investigated by dual-wavelength spectrophtometric TMM method and the results were shown in Figure 3a. MRPs exhibited the similar copper (II)-chelating property within the heating time of 180 min. Then, a significant decrease ($P < 0.05$) was observed as the heating time increased when 1 mM and 2 mM Cu^{2+} were used. The decrease was not so obvious when 5 mM Cu^{2+} was used. However, copper (II)-binding ability increased as increased Cu^{2+} concentration used. Therefore, efficiency of copper binding ability was dependent on the ratio of copper to

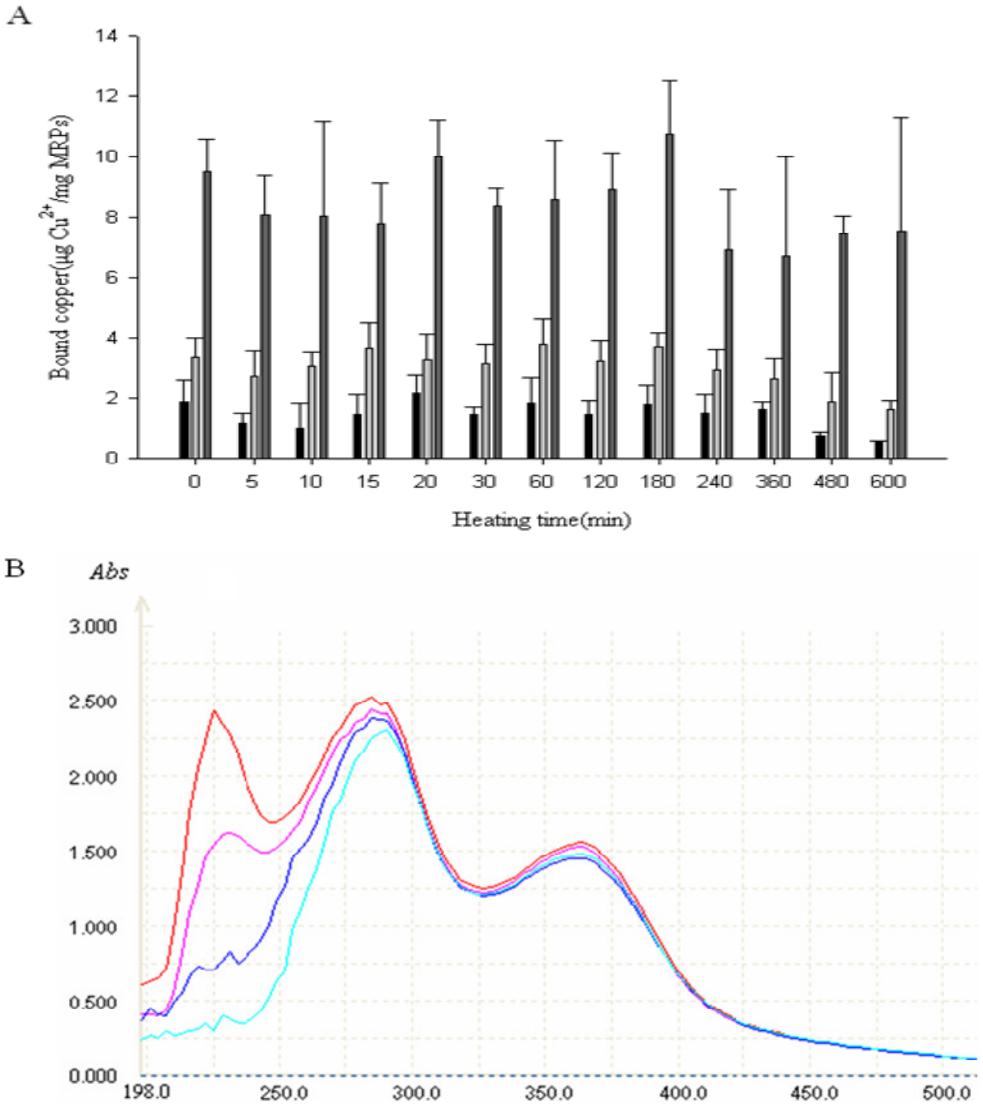

Figure 3. Copper(II) chelation of MRPs: (A) amount of copper bound to MRPs; calculated as quantity of Cu^{2+} (μg) bound by 1mg MRPs. Bars indicate the standard deviation from triplicate determinations; ■, 1 mM/L Cu^{2+}; ■, 2 mM/L Cu^{2+}; ■, 5 mM/L Cu^{2+}. (B)effects of different Cu^{2+} concentration on the absorbance spectra of MRPs from Lys-Glu model system heated for 8 h: from the top down: MRPs + 0 mM/L Cu^{2+}, MRPs + 1 mM/L Cu^{2+}, MRPs + 2 mM/L Cu^{2+}, MRPs + 5 mM/L Cu^{2+}.

MRPs. The key products in early stage are Schiff base, which is a type of amino reductone that has strong metal-binding ability to form the Schiff base complexes (Jing and Kitts, 2004).

Characteristic of complex/chelate formation

Bersuder et al. (2001) observed that Cu^{2+}-binding to MRPs from histidine-glucose model system resulted in systematic spectral changes. The effects of Cu^{2+} to the optical properties of Lys-Glu MRPs in this study were carried out by spectra measurement as characteristic analysis of complex/chelate formation (Figure 3b).

According to Bersuder et al. (2001) and Maillard et al. (2007), metals binding to MRPs produced immediate changes on the spectrum of MRPs. As shown in Figure 3b, UV-absorbance intensity changes (hypochromic) were involved, with the disappearance of the maximum absorbance band at 220 nm. The result was absolutely different from Maillard et al. (2007), likely because of the different reacting conditions. However, no changes were observed at λmax=360 nm, which were approved to be the maximum absorption bands of melanoidins. The results indicated that, Cu^{2+}-binding to maillard reaction products caused the electronic rearrangement, but had

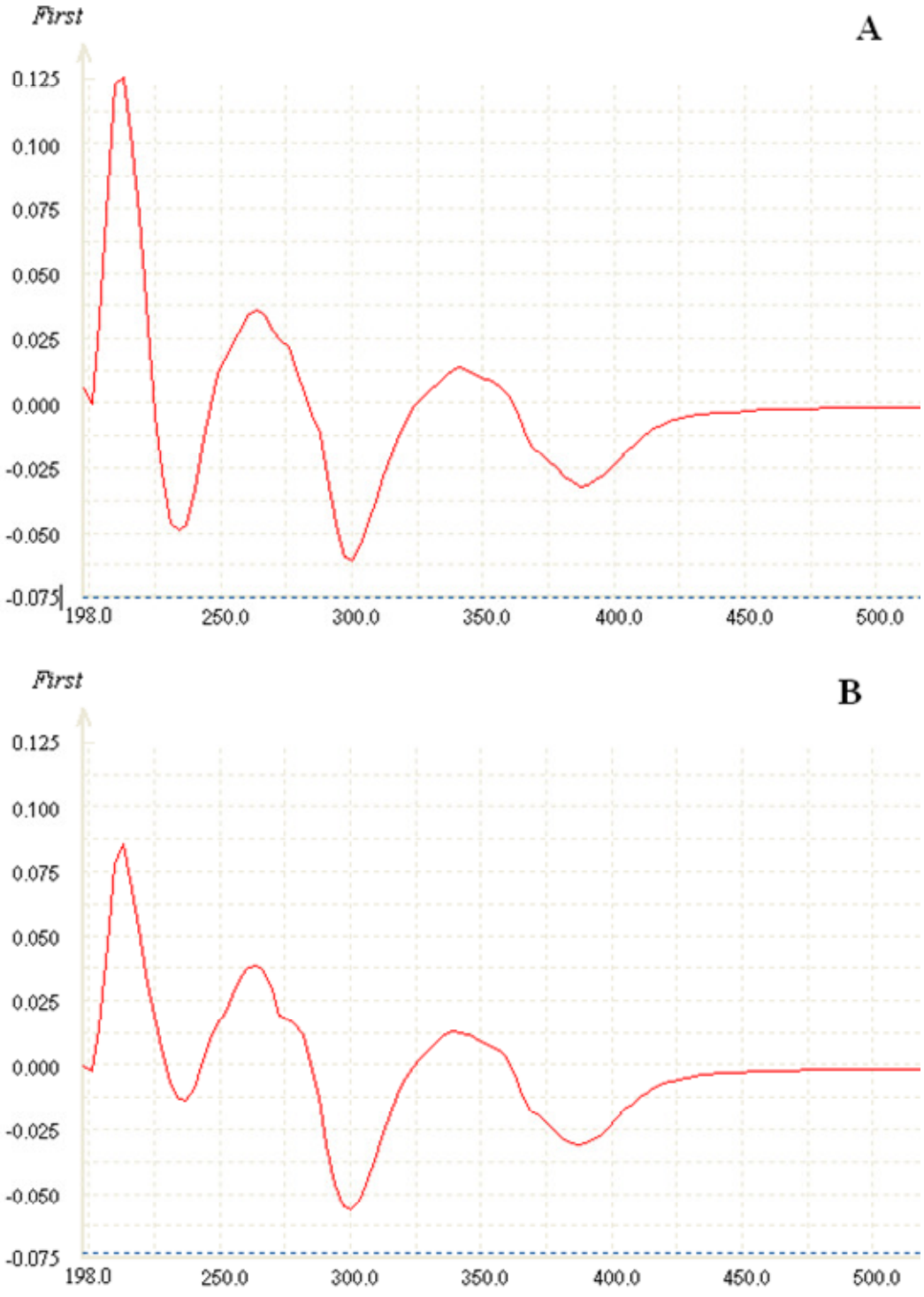

Figure 4. First-order derivative spectra of MRPs from Lys-glu model system heated for 8h with different Cu^{2+} concentration. A, MRPs + 0 mM/L Cu^{2+}; B, MRPs + 1 mM/L Cu^{2+}; C, MRPs + 2 mM/L Cu^{2+}; D, MRPs + 5 mM/L Cu^{2+}.

no effect on the chromophore structure in brown polymers.

First-order derivative spectra were also recorded with different Cu^{2+} concentrations to investigate the chelating effect of maillard reaction products (Figure 4). Several specific signals have been singled out for the main components (non-color intermediates and browning compounds) of MRPs in zero-order spectra. First-order

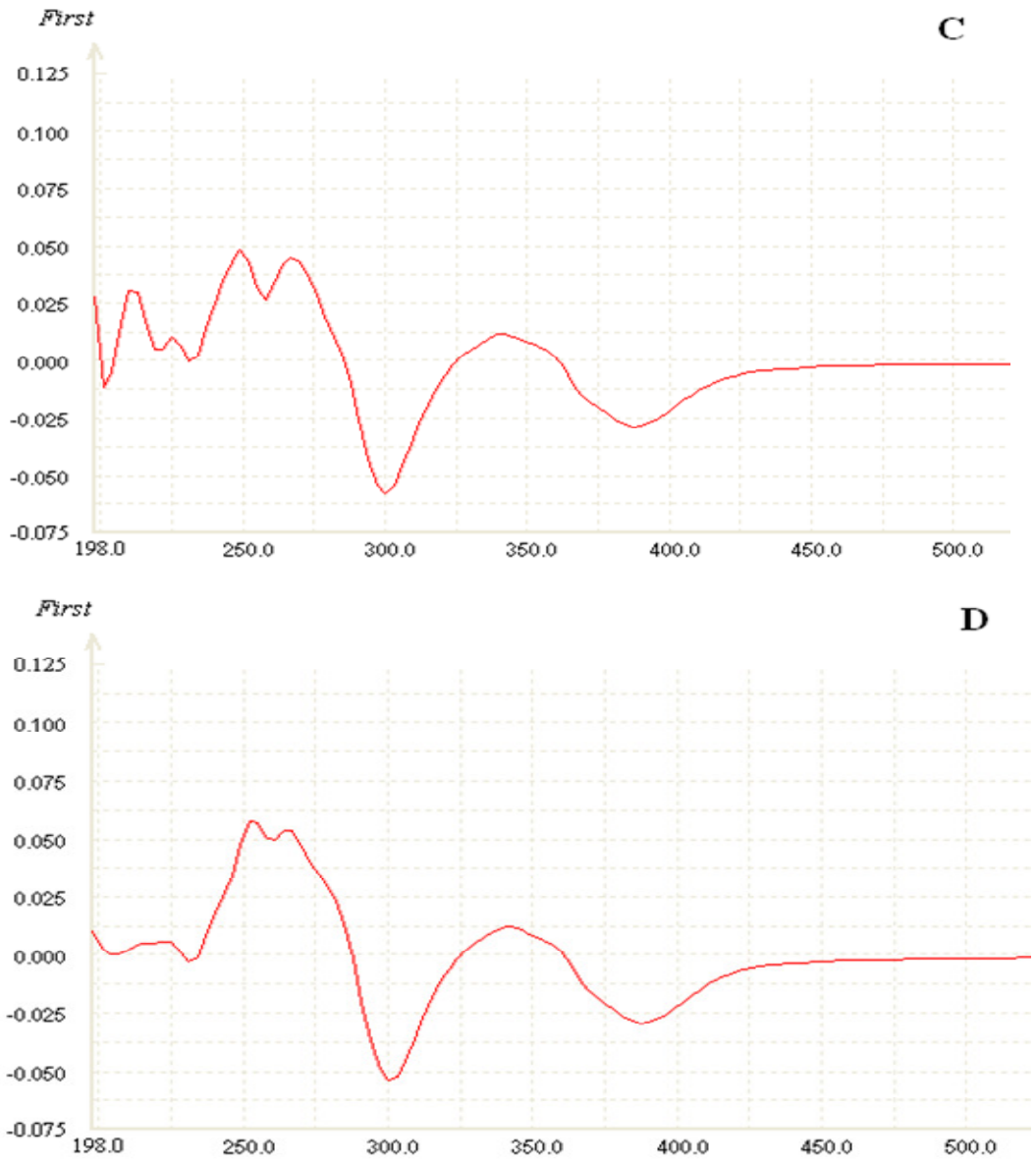

Figure 4. Continued.

derivative spectra of MRPs without Cu^{2+} showed three amplitudes $D_{210\sim230}$, $D_{270\sim300}$ and $D_{340\sim390}$, where the absorbance value was due to intermediate MRPs and melanoidins, in accordance with the specific signals of zero-order spectra. In this sense, the components of MRPs in this study were simple, possibly because the MR involved a simple model and no oxygen joined the reaction. The absorbance value of $D_{340\sim390}$ amplitude was not affected by the presence of Cu^{2+}, at whatever concentration. All MRPs showed a maximum absorbance at 300 nm, no matter what concentration of Cu^{2+} used. The changes observed from the derivative spectra were the disappearance of the $D_{210\sim230}$ amplitude and the enhancement of the maximum absorbance at 260 nm.

The $D_{210\sim230}$ amplitude and the maximum absorbance at 260 nm were due to the low molecular fractions of MRPs, which were advanced maillard reaction products. So, for MRPs produced in our experiments, the Cu^{2+} seemed to bind to low molecular fractions such as family of pyridiones (Davidek, 2002).

The effect of copper (II) on the antioxidant properties of MRPs

The effect of copper (II) on the antioxidant properties of MRPs was investigated using the reducing power and DPPH·-scavenging activity (Figure 5). The presence of Cu^{2+} in MRPs inhibited the reducing potential and the free radical-scavenging activity. Similar results were shown by

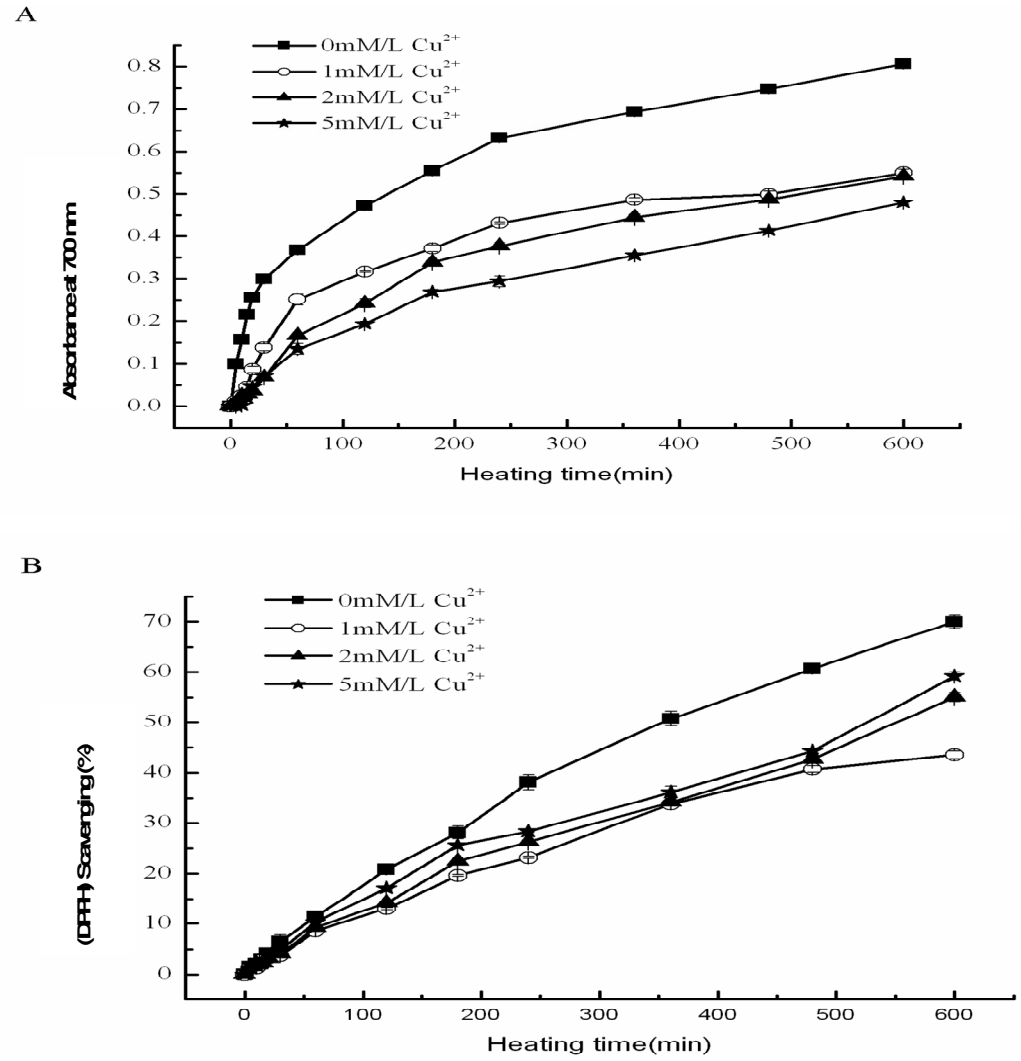

Figure 5. Effect of Cu^{2+} (0 to 5 mM/L) on the reducing power (A) and DPPH· scavenging activity (B) of MRPs. Bars indicate the standard deviation from triplicate determinations.

Bersuder et al. (2001) and Maillard et al. (2007). Inhibition effect on MRPs antioxidant activity increased with increasing copper (II) concentration. Hence, Cu^{2+}-binding sites must take effect in reducing power and free radical-scavenging activity of maillard reaction products and its inhibition occurred through a chelating mechanism. However, at least 50% of reducing power and free radical-scavenging activity was preserved for MRPs in spite of an excess of Cu^{2+}. So, metal-chelating function might be one aspect of the antioxidant mechanisms of maillard reaction products.

Conclusions

Maillard reaction products from Lys-Glu model system heated at 120℃ for 0 to 10 h without pH control showed reducing power, strong free radical-scavenging activity and copper (II)-binding ability. The antioxidant activity of MRPs related well with the UV-Vis absorbance properties of maillard rection. Low molecular weight MRP fractions showed effective metal-chelate ability. The antioxidant function of MRPs involves electron donor, free radical-scavenger and metal-chelator.

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