Antioxidative Effect of Maillard Reaction Products Using Glucose−Glycine Model System

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Using a glucose−glycine system as a Maillard reaction model, the rate of inhibition toward active oxygen by Maillard reaction products (MRP) was investigated by electron spin resonance (ESR). MRP obtained through heating a glucose−glycine mixture for 1 h inhibited more than ca. 90% of active oxygen species existing in the form of hydroxyl radicals (•OH) in the sample and decreased its inhibition power as heating time increased. Studies have revealed the direct scavenging activity of MRP along with the depression of the Fenton reaction, which is brought about by the strong chelating ability of MRP with Fe^{2+} due to the inhibition of •OH. Moreover, the decrease in the inhibition activity toward •OH during the prolonged heating period can be explained by the increase in reducing power of MRP promoting the reduction of Fe^{3+} to Fe^{2+}, and thus, activating the Fenton reaction. The high molecular weight fraction obtained from MRP, because of its stronger metal-chelating capability, inhibited •OH more efficiently than the low molecular weight fraction. In addition, MRP obtained after 1 h of heat treatment exhibited ca. 14% inhibition of superoxide anion (O_2^-), indicating a lower rate of inhibition compared to that of •OH, which can be explained by the contribution of trace amounts of O_2^- formation during the early stage of glucose−glycine reaction.

Keywords: *Maillard reaction product; antioxidant; active oxygen species*

INTRODUCTION

The Maillard reaction was discovered by L. C. Maillard, and its formation mechanism has been studied by food chemists (Homma *et al.*, 1982). Most major Maillard reactions are represented by sugar−amino acid groups, and the reaction between reducing sugars and amino acid or protein produce strong reducing materials such as amino reductants during heat treatment or storage in food-related area (Kirigaya *et al.*, 1969; Homma *et al.*, 1970; Kato and Hayase, 1989; Hodge, 1953). These reducing materials are known to have a scavenging effect on active oxygen species formed during food storage and processing (Lingnert and Eriksson, 1981; Kato, 1989, 1992a; Kato and Hayase, 1989). Most recently, studies have revealed that the Maillard reaction was closely related to several degenerative diseases such as diabetes and aging (Lunec, 1990). For example, enzyme forms of protein accompanied by glucose trigger the Maillard reaction, and Maillard reaction products (MRP) are known to produce active oxygen species and to possess scavenging activity on them (Kato, 1992b). Hence, MRP produced by heating the glucose−glycine mixture as the Maillard reaction model were investigated for their scavenging effect toward hydroxyl radical (•OH) and superoxide anion (O_2^-) by electron spin resonance (ESR). Also, the metal-chelating ability and reducing power of MRP were studied in relation to the scavenging mechanism on active oxygen species.

MATERIALS AND METHODS

Materials. Glucose, glycine, sodium bicarbonate, potassium ferricyanide [$K_3Fe(CN)_6$], hydroxylamine hydrochloride, and hypoxanthine were all purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ammonium ferrous sulfate and hydrogen peroxide (H_2O_2) were obtained from Kanto Chemical Co. (Tokyo, Japan). Xanthine oxidase was purchased from Sigma Chemical Co. (St. Louis, MO). Dibenzoylmethane(DBM) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Cyanogen bromide (BrCN) was purchased from Nacalai Tesque Inc. (Kyoto, Japan).

N-(2-Hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES), 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), and tri-*n*-octylphosphine oxide (TOPO) were purchased from Dojindo Laboratories (Kumamoto, Japan). These reagents were allof ultrapure analytical grade. Dialysis membrane (size 8/32) was purchased from Sanko Pure Chemical Co. (Tokyo, Japan).

Instruments. The ESR spectrometer was Model JES-RE1X, JEOL Ltd., Tokyo, Japan. A Hitachi spectrophotometer (Model 150-20, Hitachi Co., Tokyo, Japan) was also used.

Preparation of Sample. For the preparation of the Maillard reaction model system, sodium bicarbonate ($NaHCO_3$) was added into a mixture of 1 M glucose and 1 M glycine, and the pH was adjusted to 7.4. The mixture was heated over a boiling water bath with circulating cooling system for 1−6 h. After hourly collection of the products in the solution, they were freeze-dried and stored in a desiccator. The treatment of MRP with BrCN was carried out by mixing portions of BrCN starting with 50 mg and increasing to 500 mg systematically into 3 mL of 0.5% (w/v) MRP. The solution was then allowed to stand for a day at ambient temperature, followed by dialysis and then freeze-drying.

Determination of MRP Reducing Power. Applying the method of Sasaki *et al.* (1991), which utilizes the ability of MRP to reduce Fe^{3+} of $K_3Fe(CN)_6$ to Fe^{2+}, the reducing power of MRP was determined by the decrease in absorption of $K_3Fe(CN)_6$ at 420 nm. That was carried out by adding 200 μL of 0.5% (w/v) MRP into 3 mL of 1 mM potassium ferricyanide solution, and the absorbance was measured at 420 nm after 10 min.

Dialysis of MRP. A dialytic tube made of cellulose with 24 Å pore size was used for separation of MRP by molecular weight. In the dialysis system, 100 mL of MRP solution was immersed in 1 L of water for 12 h to obtain the HMW fraction (molecular mass >14 000 Da) as well as the LMW (molecular mass <14 000 Da) of MRP.

Determination of MRP Iron-Chelating Power. The method of Gomyou and Horikoshi (1970) was applied to

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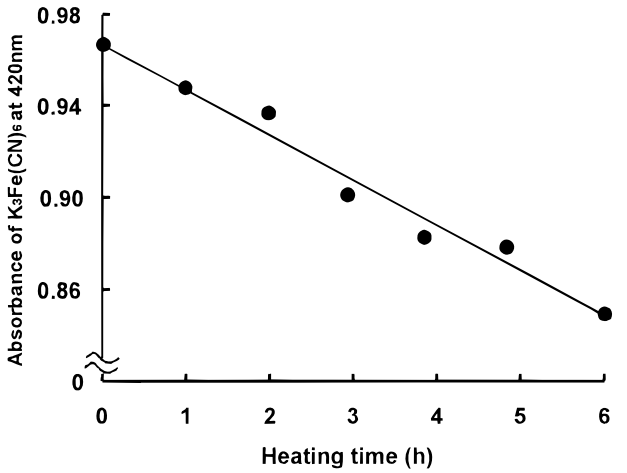

Figure 1. Change in absorbance of potassium ferricyanide over Maillard reaction time. Three milliliters of 1 mM potassium ferricyanide was added into MRP, final concentration of 0.5% (w/v).

measure MRP iron-chelating power. The MRP sample 0.5% (w/v) (final concentration) and 1 mL of the aqueous layer were prepared by adding the sample and 1 mL of 10% hydroxylamine hydrochloride into 1.4 mg of $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ (final concentration = 357 μM) and diluting to 10 mL with HEPES buffer (pH 6.7). The aqueous solution and 10 mL of benzene containing 10 mM DBM and 1 mM TOPO were mixed and shaken for 20 min. The mixture was allowed to settle, and the absorbance was determined for the benzene layer at 408 nm. The chelating ability of MRP with Fe was measured by the linear regression line of the quantity of Fe extracted into the benzene layer against absorbance at 408 nm. Samples used for this experiment were MRP produced by the reaction of glucose−glycine mixture for 6 h, HMW fraction (molecular mass >14 000 Da) as well as LMW fraction (molecular mass <14 000 Da) obtained from dialysis of MRP, and BrCN-treated MRP.

Determination of Inhibition Rate on •OH. Fifty microliters of 0.05 M HEPES buffer (pH 7.4) was mixed with 50 μL of 0.1 mM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$. The mixture was stirred with 50 μL of 0.5% (w/v) MRP, 10 μL of DMPO, and 50 μL of 0.1 mM H_2O_2. The ESR signal intensity of this mixture was then taken to measure the yield of the inhibition of •OH by MRP. The inhibition rate of •OH was determined by the ratio of peak height of the second signal from the DMPO-OH spin adduct to the third signal of Mn^{2+} and compared to the ratio of control (Yoshimura et al., 1993). The measurement conditions of an ESR were as follows: temperature, ambient; microwave power, 8 mW; magnetic field, 336 ± 5 mT; sweep time, 2 min; modulation frequency, 100 kHz; modulation amplitude, 0.063 mT; receiver gain, 2 × 100; time constant, 0.03 s.

Determination of Inhibition Rate on O_2^-. Fifty microliters of 2 mM hypoxanthine, 50 μL of 0.05 M HEPES buffer solution (pH 7.4), 50 μL of MRP, 10 μL of DMPO, and 50 μL of 0.4 unit/mL xanthine oxidase were stirred together successively to determine the ability of MRP to inhibit O_2^- (Yoshimura et al., 1993). The inhibition rate of O_2^- was determined by taking the ratio of the peak height of the first signal from the DMPO-OH spin adduct to the third signal of Mn^{2+}, and the ratio was compared to that of control value. The ESR measurement was carried out under the same conditions as described for the determination of inhibition rate of •OH.

RESULTS AND DISCUSSION

Reduction Power of MRP. The MRP obtained from heat treatment of the glucose−glycine mixture was examined for its reducing power indicated by decrease in absorption of $K_3Fe(CN)_6$ at 420 nm, and the results are summarized in Figure 1. The absorbance of $K_3Fe(CN)_6$ at 420 nm decreases linearly with increasing heating time, which suggests the correlation between formation of reducing materials and heat treatment time of glucose−glycine mixture. To examine the contribution of the OH group originating from glucose in MRP to the reducing power, the bromination of the OH group was carried out by treating MRP from the glucose−glycine mixture with BrCN. After bromination of the OH group of MRP, the absorption of $K_3Fe(CN)_6$ increases the increase in the amount of BrCN increased. Obviously BrCN treatment retards the reducing power of MRP, and it becomes clear that the OH group of MRP plays a relevant role in reducing activity. Furthermore, constant absorbance observed after addition of >200 mg of BrCN can be explained by the development of complex stereochemical structure of the MRP macromolecule during the extended reaction time of the glucose−glycine mixture, which prevents the OH group from further bromination.

Table 1. Chelate Activity of Ferrous Ion Extracted from Maillard Reaction Products

sample	amount of Fe extracted[a,b] (μg)	chelate activity of Fe[c] (%)
glucose−glycine	185.50 ± 1.25	7.3
MRP	14.09 ± 0.98	93.0
LMW fraction[d]	17.71 ± 0.28	91.1
HMW fraction[d]	9.84 ± 0.28	95.1
MRP treated with BrCN	61.60 ± 0.18	69.2

[a] Values presented as mean ± SD (n = 5). [b] Ferrous ion was added in the final concentration of 357 mM. Benzene with TOPO−DBM complex was used to extract ferrous ion. [c] Chelate activity of Fe was estimated from amount of Fe ion extracted against 200 μg of Fe ion. [d] Molecular mass of HMW fraction is over 14 000 Da. Molecular mass of LMW fraction is below 14 000 Da.

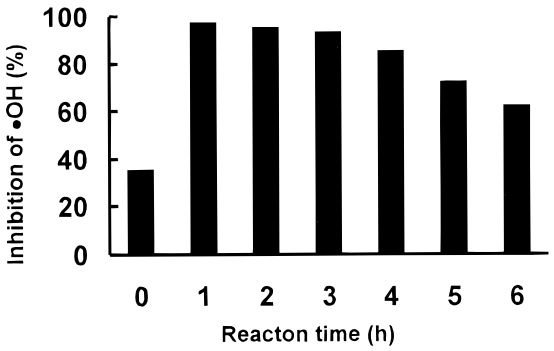

Figure 2. Change in inhibition of •OH over Maillard reaction time of heating glucose−glycine mixture. Hydroxyl radical was generated by the reaction of Fenton reagent, 50 μL of 0.1 mM of Fe^{2+}, and 50 μL of 0.1 mM of H_2O_2.

Metal-Chelating Power of MRP. The results of comparison between the metal-chelating power of MRP produced by heat treatment of the glucose−glycine mixture for 6 h and by Br-treated MRP from the same source are shown in Table 1. Furthermore, the metal-chelating power of each HMW fraction and LMW fraction obtained from the dialysis of MRP was also investigated. When 200 μg of Fe^{3+} ion was added to 0.5% (w/v) of the each HMW fraction, LMW fraction, MRP, and BrCN-treated MRP, the yields of Fe^{3+} ion extracted into organic layer were 95.1, 91.1, 93.0, and 69.2%, respectively. These results indicate the HMW fraction possesses greater metal-chelating power than the LMW fraction. The lower metal-chelating power of MRP with the bromination of the hydroxyl group suggests the great contribution of the hydroxyl group of MRP in metal-chelating power.

Inhibition Effect on •OH by MRP. Figure 2 shows the change in inhibition rate of •OH over the Maillard

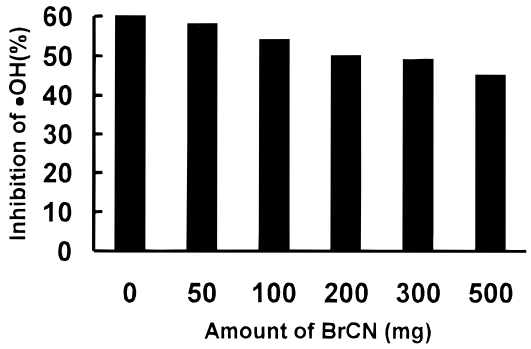

Figure 3. Inhibition of •OH of Maillard reaction products treated with BrCN. Hydroxyl radical was generated by the reaction of Fenton reagent, 50 μL of 0.1 mM of Fe^{2+}, and 50 μL of 0.1 mM of H_2O_2.

Table 2. Comparison of the Ability To Inhibit •OH by LMW Fraction, HMW Fraction of MRP, and MRP

sample	inhibition rate of •OH (%)
MRP	61.5
LMW fraction	55.4
HMW fraction	68.9

reaction time by heating the glucose−glycine mixture. MRP without heat treatment exhibited 36% inhibition rate of •OH, whereas MRP exhibited 97% inhibition of •OH after 1 h of heating, and the inhibition rate of •OH decreased with increasing heat treatment time.

The lowest inhibition rate of 60% was exhibited even after a mixture was heated for 6 h, indicating a high rate of •OH inhibition by MRP. Figure 3 presents the rate of •OH inhibition by MRP sample treated with BrCN. The inhibition rate of •OH decreased as the amount of BrCN added into MRP increased; in other words, bromination of the hydroxyl group of MRP with BrCN reduced the rate of •OH inhibition. This can be conceived by considering not only the mechanism of •OH inhibition by MRP, which works to inhibit •OH directly, but also the strong chelating power of MRP with Fe^{2+}, which depresses •OH formation, indirectly retarding the Fenton reaction where the reaction of Fe^{2+} with H_2O_2 proceeds to generate •OH. Moreover, slow inhibitory activity on •OH observed with increasing reaction time of the glucose−glycine mixture was considered due to increase in the formation of reducing materials in the glucose−glycine mixture as the reaction time increased, as shown in Figure 1 and, therefore, causing the greater reducing power of MRP to reduce Fe^{3+} derived from the Fenton reaction to Fe^{2+}, resulting in the promotion of the Fenton reaction. Upon comparison of the inhibitory effect on •OH by both the HMW and the LMW fractions obtained from MRP (Table 2), the LMW fraction exhibited a 55.4% inhibition rate, whereas the HMW fraction exhibited a rate >68.9% inhibition of •OH. This can be explained by the greater metal-chelating ability of the HMW fraction as shown in Table 1.

Inhibition Effect on O_2^- by MRP. The change in the rate of O_2^- inhibition by MRP over time is illustrated in Figure 4. MRP exhibited ca. 14, 38, and 55% inhibition of O_2^- when a glucose−glycine mixture was heated for 1−4, 5, and 6 h, respectively. Since MRP possess a reducing power equally as strong as that of ascorbic acid (Kato, 1989), they have a direct inhibitory effect on O_2^-. The generation of O_2^- by autoxidation of sugar during the early stage of Maillard reaction might be responsible for the lower rate of inhibition of O_2^- compared to that of •OH (Hayashi and Mase, 1986).

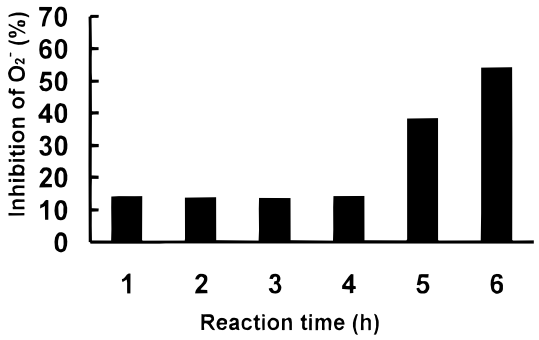

Figure 4. Change in inhibition of O_2^- over Maillard reaction time. Superoxide anion was generated by the reaction between 50 μL of 2 mM hypoxanthine and 50 μL of 0.4 unit/mL of XOD.

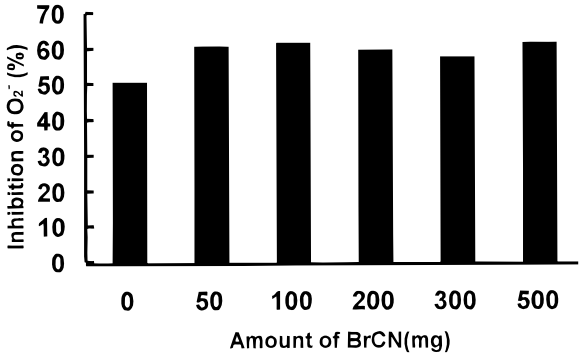

Figure 5. Inhibition of O_2^- of Maillard reaction products treated with BrCN. Superoxide anion was generated by the reaction between 50 μL of 2 mM hypoxanthine and 50 μL of 4 units/mL of XOD.

For this reason, MRP appear to exhibit a lower rate of inhibition on O_2^-. Notably, as shown in Figure 5, MRP exhibited approximately 60% inhibition of O_2^- upon treatment with BrCN, regardless of the amount of BrCN added. Imidocarbonation of the unreacted hydroxyl group of glucose induced by BrCN played an important role with regard to decreased O_2^- formation by autoxidation of sugar (Kato, 1989).

Conclusions. The results obtained from ESR clearly proved MRP produced by the thermal treatment of glucose−glycine mixture inhibited >90% of •OH. It has been revealed that this inhibition mechanism works not only by the direct scavenging of •OH but also by suppressing •OH formation through chelation with Fe^{2+}, which is involved in the Fenton reaction. There were obvious differences in metal-chelating ability between the HMW and LMW fractions of MRP, with a greater ability to inhibit •OH shown by the HMW fraction. The low, ca. 14%, rate of inhibitory effect on O_2^- by MRP seems to be due to direct attribution of the strong reducing power of MRP.

ABBREVIATIONS USED

MRP, Maillard reaction products; HMW, high molecular weight; LMW, low molecular weight; ESR, electron spin resonance; DBM, dibenzoylmethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; TOPO, tri-*n*-octylphosphine oxide.

LITERATURE CITED

Gomyou, T.; Horikoshi, M. On the interaction of meranoidine with metallic ions. *Agric. Biol. Chem.* **1970**, *40*, 33−40.

Hayashi, T.; Mase, D. Formation of three-carbon sugar fragment at an early stage of the browning reaction of sugar with amines or amino acid. *Agric. Biol. Chem*. **1986**, *50*, 1959−1964.

Hodge, J. E. Browing reactions in model systems. *J. Agric. Food Chem*. **1953**, *1*, 928−943.

Homma, S.; Suzuki, N.; Kato, H. Study on the browing of Kori-Tofu. Part III Occurrence of free amino acid and peptides accompanied with changes of proteins during browing process. *Agric. Biol. Chem*. **1970**, *34*, 523−531.

Homma, S.; Tomura, T.; Fujimaki, M. Fractionation of non-dialyzable melanoidin into components by electrofocusing electrophoresis. *Agric. Biol. Chem*. **1982**, *46*, 1791−1796.

Kato, H. Scavenging of active oxygen by melanoidin. *Agric. Biol. Chem*. **1989**, *53*, 3383−3385.

Kato, H. Maillard reaction in biological system and disease. *Farumashia* **1992a**, *28*, 466−470.

Kato, H. Scavenging of active oxygen species by glycate protein. *Biosci., Biotechnol., Biochem*. **1992b**, *56*, 928−931.

Kato, H.; Hayase, F. Chemical analysis of the compounds produced by amino-carbonyl reaction in food and biological systems. *Yukagaku* **1989**, *38*, 865−875.

Kirigaya, N.; Kato, H.; Fujimaki, M. Antioxidant activity of nonenzymatic browning reaction products. *Nihon Nogei Kagaku* **1969**, *43*, 484−491.

Lingnert, H.; Eriksson, C. E. Antioxidative Maillard reaction products I. Products from sugars and free amino acids. *Prog. Food Nutr. Sci*. **1981**, *5*, 453−466.

Lunec, J. Free radicals their involvement in disease processes. *Ann. Clin. Biochem*. **1990**, *27*, 173−182.

Sasaki, K.; Matsumoto, I.; Beppu, M. Cyanogen bromide treatment. In *Affinity Chromatography*; Tokyo Kagaku Dojin: Tokyo, 1991; pp 117−130.

Yoshimura, Y.; Matsuzaki, Y.; Uchiyama, K.; Watanabe, T.; Ohsawa, K.; Imaeda, K. Formation of 8-hydoxydeoxyguanosine from deoxyguanosine by Fe^{2+}/Ascorbic acid/EDTA/H_2O_2 system. *J Clin. Biochem. Nutr*. **1993**, *15*, 155−162.

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