Antioxidative Activity of the Blue Pigment Formed in a D-Xylose-Glycine Reaction System

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A blue compound was prepared from 1 M D-xylose and 0.1 M glycine, and designated Blue-M1, an intermediate color product of melanoidins. As melanoidins are well known to have antioxidative activity as well as high scavenging activity against active oxygen species, the antioxidative activity of Blue-M1 against the peroxidation of linoleic acid was investigated, in addition to the scavenging activity of Blue-M1 toward hydroxyl and DPPH radicals.

Blue-M1 suppressed the peroxidation of linoleic acid as effectively as melanoidins did. The scavenging activity of Blue-M1 toward hydroxyl and DPPH radicals was also as strong as that of melanoidins. Blue-M1 showed higher activity with increasing concentration. The pyrrolopyrrole ring and a methine bridge between two pyrrolopyrrole rings in Blue-M1 could be related to the ability for radical scavenging activity, but not for carboxyl groups.

Key words: melanoidin; Maillard reaction; blue pigment; antioxidative activity; scavenging activity of hydroxyl and DPPH radicals

Melanoidins, which are the final products of the Maillard reaction, are nitrogen-containing polymeric substances that are difficult to decompose. Humans consume melanoidins daily in browning foods. Melanoidins demonstrate physiologically positive effects because of the unique partial components in the molecules such as reductones and enamines, and the pyrrole-like structure.1,2)

Melanoidins are known to have various physiologically positive effects such as antioxidative activity3,4) and strong scavenging activity against active oxygen species, e.g., hydroxyl radicals, hydrogen peroxides and superoxides,5,6) It is believed that melanoidins show their physiological effects in vivo in digestive organs because parts of melanoidins have been demonstrated to be absorbed through the gastrointestinal tracts of rats.7) The Maillard reaction is known to occur in vivo as well as in food and environmental systems.

Miura and Gomyo,8) and Gomyo et al.,9) have found the formation of blue pigments in the Maillard reaction of D-xylose and glycine. This blue pigment has been postulated to be an intermediate oligomer in the generation of melanoidins. The authors9) have recently reported the identification of a novel blue pigment designated as Blue-M1 (blue Maillard reaction intermediate I). This pigment consists of four molecules of D-xylose and glycine, and has a methine proton between two pyrrolopyrrole rings as shown in Fig. 1.

We report in this paper the antioxidative activity of Blue-M1 in comparison with that of melanoidins.

Materials and Methods

Chemicals. D-Xylose, D-glucose, linoleic acid and α-tocopherol were purchased from Kanto Chemicals Co. Glycine, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and BHA were obtained from Wako Pure Chem. Ind. 5,5-Dimethylpyrroline-N-oxide (DMPO), trimethylsilyldiazomethane, and 2,5-dihydroxybenzoic acid were purchased from Doujin Chem. Co., Aldrich Chem. Co., and Kanto Chemicals Co, respectively.

All other chemicals used were of analytical reagent grade. Water was used after purification by reverse osmosis, using Milli RO 10 Plus and Milli-Q Jr. (Millipore) membranes.

Fig. 1. Structure of Blue-M1.

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Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; BHA, 3-tert-butyl-4-hydroxyanisole; DMPO, 5,5-dimethylpyrroline-N-oxide; MALDI, matrix-assisted laser desorption ionization; TOF-MS, time-of-flight mass spectrum
Preparation of Blue-M1 and Melanoids. Blue-M1 was prepared as previously described by the authors. D-Xylose (1 M), glycine (0.1 M) and sodium hydrogen carbonate (0.1 M) were dissolved in 1 liter of aqueous 60% ethanol, and the solution was adjusted to pH 8.1 with HCl. The reaction solution was placed in a nitrogen atmosphere and left at 26.5°C for 2 days, before being left at 2°C for 4 days in a dark room. A strongly blue solution was obtained. The blue pigment was isolated in a cold room at 2°C. The ethanol was removed under reduced pressure, and the solution diluted to 1 liter with water. After this solution had been adjusted to pH 6.8, the solution was poured into a DEAE-Sephadex A-25 column (25 cm × 3.8 cm i.d., Amersham Pharmacia Biotech, Sweden) which had been equilibrated with a 0.1 M Tris-HCl buffer (pH 6.8). The compound was developed by a linear gradient system made up of 1 liter of a 0.1 M Tris-HCl buffer (pH 6.8) and 1 liter of 0.4 M NaCl in the same buffer.

The main blue fractions were concentrated to a small volume under reduced pressure. The solution was then poured into a Sephadex G-15 column (23 cm × 4 cm i.d., fine grade, Amersham Pharmacia Biotech, Sweden) to complete the desalting process. The desalted blue fractions were concentrated under reduced pressure, and further purified in a Bio-Gel-P-2 column (75 cm × 2 cm i.d., 200–400 mesh, 1800 dalton exclusion limit, Bio-Rad Laboratories, U.S.A.) that had been equilibrated with 1 M NaCl. The main blue band was effectively isolated by developing with 1 M NaCl, and then by desalting twice in the same manner as that already described. The purified blue solution was used as Blue-M1.

The melanoids were prepared as previously described by the authors. D-Glucose (2 M) or D-xylene (2 M), glycine (2 M) and sodium hydrogen carbonate (0.2 M) were dissolved in water. The mixture was refluxed in an oil bath at 95°C for 7 h. The resulting brown solution was dialyzed against water for 2 weeks through cellulose tubing (Visking), and the nondialyzable fraction was then lyophilized.

Generation and measurement of the hydroxyl and DPPH radicals. DMPO, (5,5-dimethylpyrroline-N-oxide) was used as the spin-trapping reagent for an ESR analysis.

The hydroxyl radicals were generated by a Fenton reaction. Ten microliters of 3% H₂O₂, 10 µl of 250 mM DMPO, and 10 µl of the sample in a 0.2 M phosphate buffer at pH 7.4 were mixed, and 10 µl of 5 mM FeSO₄ was then added. The mixture was poured into a borosilicate glass capillary column (100 mm × 0.58 mm i.d., Hilgenberg No. 1406119). The DMPO-OH adducts were measured by a JEOL RE1X ESR spectrometer for 5 min after the addition of FeSO₄.

DPPH (7.92 mg) was dissolved in 10 ml of ethanol. Two hundred microliters of DPPH and 10 µl of a sample in a phosphate buffer at pH 7.4 were mixed, and then after 5 min, the DPPH radicals were measured by ESR.

Mn²⁺ was used as an internal standard to calculate the relative amount from the ESR signal intensity. All experiments were performed at room temperature.

Measurement of the peroxy value. Five hundred milligrams of linoleic acid dissolved in 10 ml of ethanol was poured into a 50-ml brown test tube with a stopper. Twelve point five milliliters of a 0.2 M phosphate buffer at pH 7 and 1 ml of the test solution in water were added to this solution. The test tube was tightly stoppered and stored at 50°C for 24 h in a dark room. The oxidized linoleic acid was extracted with chloroform by using a separatory funnel, and the peroxy value (POV) was measured by iodometry. The antioxidative activity was calculated as follows: POV% = POV of the test sample/POV of control × 100. Accordingly, the lower the POV% value is, the stronger the antioxidative activity of the sample.

Preparation of methylated Blue-M1. Blue-M1 (0.5 mg) was dissolved in 0.8 ml of methanol and 2.8 ml of benzene. To the solution was added 200 µl of trimethylsilyl diazomethane, and the mixture incubated at room temperature for 30 min. The methanol and benzene were removed under reduced pressure, and the dried sample was dissolved in water. The presence of methylated Blue-M1 in this solution was confirmed by HPLC and mass spectrometric analyses.

Reversed-phase HPLC (Waters 515 HPLC pump) was conducted by monitoring at 625 nm with a Waters 996 photodiode array detector, using a solvent system of 0.005 M tetrabutyl ammonium phosphate in water-methanol (60:40), a Mightysil RP-18 column (Kanto Chemicals Co., 250 mm × 4.6 mm i.d.) and a flow rate of 0.5 ml/min.

Time-of-flight mass spectra (TOF-MS) were recorded with a Voyager RP mass spectrometer (PE Biosystems). The ionization mode was set to matrix-assisted laser desorption ionization (MALDI) at an accelerating voltage of 20 kV with 2,5-dihydroxybenzoic acid as a matrix.

Results and Discussion

Antioxidative activity

Figure 2 shows the antioxidative activity of BHA, α-tocopherol, the melanoids, and Blue-M1. POV of the linoleic acid control solution incubated at 50°C for 24 h was 106.2 and POV% of Blue-M1 at a concentration of 230 µM (0.014%) was 35. Therefore, 65% suppression of lipid peroxidation was observed.
Fig. 2. Effect of Blue-MI, Melanoidins, BHA and \( \alpha \)-Tocopherol on the Peroxide Value of Linoleic Acid.

Mel(Glc-Gly): melanoidins from the glucose-glycine reaction system, Mel(Xyl-Gly): melanoidins from the xylose-glycine reaction system, POV\% = (POV with sample/POV without sample) \times 100. The concentrations of Blue-MI, BHA and \( \alpha \)-tocopherol were each 230 \( \mu \)M. The concentration of melanoidins was 0.025%.

Fig. 3. Effect of the Concentration of Blue-MI on the Peroxidation of Linoleic Acid.

in Blue-MI compared with the control. POV\% of the melanoidins (0.025%) prepared from the Glc-Gly and Xyl-Gly reaction systems were 22 and 30, respectively. POV\% of BHA and \( \alpha \)-tocopherol were 5.3 and 22, respectively. These results indicate that Blue-MI had antioxidative activity as strong as that of the melanoidins and \( \alpha \)-tocopherol. Figure 3 indicates POV\% of Blue-MI at various concentrations, the value decreasing with increasing concentration of Blue-MI.

In order to clarify the mechanism for antioxidative activity, we studied the reaction of Blue-MI with hydrogen peroxide as a model of a lipid hydroperoxide. Preliminary results by \( ^{1}H \)-NMR showed that the chemical shift of protons on the methine bridge in Blue-MI was to the low-field side by the reaction with hydrogen peroxide, indicating the possibility of the addition of hydroperoxide to the methine carbon (Hayase, F. et al., unpublished results).

**Scavenging activity of hydroxyl radicals**

Figure 4 shows ESR spectra of DMPO spin adducts with hydroxyl radicals in the presence of Blue-MI, the melanoidins, BHA, and \( \alpha \)-tocopherol. These samples effectively scavenged hydroxyl radicals. Figure 5 shows the relative ESR signal intensity of the DMPO spin adducts with hydroxyl radicals in the presence of Blue-MI, the melanoidins, BHA, and \( \alpha \)-tocopherol. The authors\(^5\) have also shown the strong scavenging activity of melanoidins against hydroxyl radicals formed by \( \gamma \)-irradiation (10 kGy). Blue-MI at a concentration of 3 mM (0.19%) scavenged 69% of the hydroxyl radicals, while 0.2% melanoidins (Glc-Gly) scavenged 54% of the hydroxyl radicals. Therefore, Blue-MI had hydroxyl radical scavenging activity as strong as that of the melanoidins. The hydroxyl radical scavenging activity increased with increasing concentration of Blue-MI as shown in Fig. 6.
Hydroxyl radicals are well known to react readily with various organic compounds such as proteins, carbohydrates and lipids. The authors have previously shown that melanoidins at concentrations of 0.3% and 0.03% respectively scavenged 86% and 47% of the hydroxyl radicals generated by 10 kGy of Co-60 gamma rays. However, fructose and mannitol as known scavengers of hydroxyl radicals at a concentration of 0.5% scavenged 20% and 0%, respectively. The higher hydroxyl radical scavenging activity by melanoidins than by known scavengers may depend on the unique partial structure, excepting the hydroxyl groups. We have proposed that Blue-M1 as a possible precursor of melanoidins might be generated by the decarboxylation from two molecules of yellow pigments, and speculated two condensed pyrrolopyrrole-2-carboxylanddehyde compounds between N-glycylpyrrole-2-carboxylanddehyde and 3-deoxyxylosone or xylosone. Blue-M2, having polymerizing activity, is thought to be formed by the addition reaction of a yellow pigment to Blue-M1. In an advanced stage of the Maillard reaction, melanoidins may be formed by such a polymerization reaction. Therefore, we speculate that the strong scavenging activity of Blue-M1 was not due to hydroxyl groups in the sugar moiety, but to another structure.

The authors have shown that glycated protein also scavenged the hydroxyl radicals formed by y-irradiation (10 kGy). The scavenging ability of glycated proteins against hydroxyl radicals is considered to depend on Maillard reaction products in the advanced stage, such as N\(^{-}\)-lysyl-5-hydroxymethylpyrrole-2-carboxylanddehyde (called pyrraline) and melanoidins.

Our findings reveal that the Blue pigment changed to a yellow color after the reaction between Blue-M1 and hydroxyl radicals. We suggest that the long conjugated system expressing the blue color may have been broken by hydroxyl radicals. Accordingly, we speculate that the hydroxyl radical scavenging activity by Blue-M1 might have been due to the pyrrolopyrrole ring and methine bridge.

Scavenging activity toward the DPPH radical
Figure 7 shows ESR spectra of the DPPH radicals for each sample. Blue-M1 more effectively scavenged the DPPH radicals than the control. Figure 8 shows the relative ESR signal intensity of the DPPH radicals with each sample. BHA and \(\alpha\)-tocopherol strongly scavenged the DPPH radicals. Blue-M1 at a concentration of 3 mM (0.19%) scavenged 66% of the DPPH radicals, while 0.2% melanoidins scavenged 58% of the DPPH radicals. Blue-M1 had as strong scavenging activity toward DPPH radicals as that of melanoidins. Figure 9 shows that the scavenging activity of DPPH radicals by Blue-M1 increased with the concentration of Blue-M1. The scavenging activity of Blue-M1 might have been dependent on the protons in the pyrrolopyrrole ring or the methine proton. An NMR experiment has revealed that these protons were readily reactive in Blue-M1 and yellow pigments (Hayase, F. et al., unpublished results). Murakami et al. have recently reported that the radical-scavenging activity in the early stage of the Maillard reaction between xylose and glycine was derived from uncolored reaction products smaller than the brightly colored blue pigments. It would be very interesting to identify the uncolored reaction products. However, both Blue-M1 and Blue-M2, which could have been formed by the addition reaction of the yellow pigment to Blue-M1 (Hayase, F. et al., unpublished results) showed fairly strong scavenging activity against DPPH radicals. The oxidation accelerating substances might
have existed in the fraction containing blue colored pigments that was separated by Murakami et al.\textsuperscript{14}

Scavenging activity against hydroxyl radicals by methylated Blue-M1

Melanoidins have been proposed to have scavenging activity against active oxygen species due to their pyrrole-like and reductone structures, and chelating ability against transition metals due to their anionic structure.\textsuperscript{2,13} Methylated Blue-M1 was derived from Blue-M1 (Mw = 619) in order to investigate the effects of four carboxyl groups on the generation of antioxidative activity. The HPLC pattern of methylated Blue-M1 indicates that peak 1 was methylated Blue-M1 as shown in Fig. 10. These peaks have a maximum absorbance at 626 nm. The MALDI-TOF-MS data measured in the linear mode for methylated Blue-M1 showed the presence of the M$^+$ ion at m/z = 674.97. These results indicate that Blue-M1 was completely methylated.

Figure 11 shows the scavenging activity against hydroxyl radicals of methylated Blue-M1 in comparison with that of Blue-M1. The results indicate strong scavenging activity of methylated Blue-M1 like that of Blue-M1. It is considered that the four carboxyl groups in Blue-M1 did not take part in the scavenging activity against hydroxyl radicals by Blue-M1. We speculate that the four carboxyl groups do not have strong chelating activity. Consequently, the pyrrolopyrrole ring and methine bridge between the two pyrrolopyrrole rings could be related to the ability for radical scavenging activity. Studies on the mechanism are now in progress.

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References

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