Preliminary Communication

Identification of Blue Pigment Formed in a D-Xylose-Glycine Reaction System

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D-Xylose (1 M), glycine (0.1 M), and sodium hydroxycarbonate (0.1 M) were dissolved in aqueous 60% ethanol at pH 8.1 and left at 26.5°C for 2 days in a dark room under nitrogen displacement. Blue pigment was isolated and purified from the blue solution by anionic exchange and gel filtration chromatographies. Blue pigment which was designated Blue-M1 (blue Maillard reaction intermediate-1) was identified as 5-(1,4,4-dicarboxymethyl)-5-(2,3-dihydroxypypropyl)-2-pyrrolo[3,2-b]pyrrolitone-1,4-(dicarboxymethyl)-2-(1,2,3-trihydroxypypropyl)-pyrrolo[3,2-b]pyrrolylum. Blue-M1 is supposed to be a dimer of yellow colored pyrrolopyrrole-2-carboxaldehyde compounds. Blue-M1 that reacts readily to yellow compounds has a polymerizing activity, suggesting it is an important Maillard reaction intermediate through the formation of melanoids.

Key words: Maillard reaction; xylose; glycine; blue compound; browning

The Maillard reaction is known to proceed nonenzymatically during storage and processing of foods, progressing also in vivo as well as in soil. Melanoids, which are the final products of the reaction, are nitrogen-containing polymeric substances that decompose with difficulty. The formation mechanisms of melanoids are complex because many reactants, such as osones, unsaturated osones, furfurals, pyrorle-2-carboxaldehydes (pyrinaline), carbonyl compounds generated by cleavage of reducing sugars, and various amino compounds are involved.1-3 Recently, postulated structures of brown pigments have been proposed. Tressl’s group has reported that pyrrole polymers are formed in the 2-deoxy-D-ribose and methyl 4-aminoobutyrate Maillard system.4 They also reported that N-substituted pyrrole, 2-furaldehyde, and N-substituted 2-formylpyrroles, formed in pentose and hexose Maillard reaction systems, were identified as components of extraordinary polycondensation activity.4 Hofmann has reported that some novel red-brown and red compounds were identified by Maillard reaction of bound arginine with glyoxal and furan-2-carboxaldehyde, pentoses and primary amino acids, and hexoses and primary and secondary amino acids. However, these colored compounds have less hydrophilic properties, indicating significant differences in hydrophilic properties of melanoids.

Miura and Gomyo,§ and Gomyo et al.,§ have found the formation of blue pigments in the Maillard reaction of D-xylose and glycine. The blue pigment is postulated to be an intermediate oligomer in the generation of melanoids. In this communication, we have reported the identification of a novel blue pigment that was designated Blue-M1 (blue Maillard reaction intermediate-1).

D-Xylose and glycine were obtained from Kanto Chemicals Co., Ltd, all other chemicals used in this study were of analytical reagent grade. D-Xylose was decolorized with activated carbon before incubation. Water was used after purification by reverse osmosis, using Milli RO 10 PLUS and Milli-Q Jr. (Millipore) membranes and ion exchanger.

D-Xylose (1 M), glycine (0.1 M), and sodium hydroxycarbonate (0.1 M) were dissolved in 1 liter of aqueous 60% ethanol, and the solution 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, and 20°C for 4 days in a dark room. A strongly blue solution was obtained.

Isolation of blue pigment was done in a cold room at 2°C. The ethanol was removed under reduced pressure, and the solution diluted to one liter with water. After this was adjusted to pH 6.8, the solution was put on a DEAE-Sephadex A-25 column (25 cm x 3.8 cm i.d., Amersham Pharmacia Biotech, Sweden), which had been equilibrated with 0.1 M Tris-HCl buffer (pH 6.8). The development was done by a linear gradient system made up 1 liter of 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 put on a Sephadex G-15 column (23 cm x 4 cm i.d., Fine grade, Amshearn Pharmacia Biotech, Sweden) to complete the desalting process. The desalted blue fractions were concentrated under reduced pressure, and further purified by a Bio-Gel-P-2 column (75 cm x 2 cm i.d., 200-400 mesh, 1800 daltons exclusion limit, Bio-Rad Laboratories, U.S.A.) that had ben equilibrated with 1 M NaCl. The main blue band was effectively isolated by developing with 1 M NaCl as shown in Fig. 1, and followed by desalting twice in the same manner as that already described. The blue fractions containing purified Blue-M1 and Blue-M2 were each lyophilized.

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The resulting substance, which was designated Blue-M1, was found to contain a small amount of a shoulder component, which was estimated to be less than approximately 4% in terms of the peak area on HPLC chromatogram. Yield of Blue-M1 was 2.5 mg. HPLC (Waters 515 HPLC pump) was done by monitoring at 625 nm with a Waters 996 Photodiode Array detector under the following conditions: Reversed-phase HPLC with a solvent system of 0.005 M tetrabutyl ammonium phosphate in water-methanol (60:40), using a Mitysyl RP-18 (Kanto Chemicals Co. Ltd., 250 mm x 4.6 mm i.d.) at a flow rate of 0.5 ml/min.

Mass spectra were recorded with a JEOL SX-102 mass spectrometer. The ionization mode was set to FAB(+) with glycerol as a matrix. 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. The ^1^H-NMR and ^13^C-NMR spectra were recorded with a JEOL GSX-500 (500 MHz) instrument, these NMR data being recorded in D2O. Sample tubes (5 mm i.d.) were passed through nitrogen and sealed before spectra were measured.

The MALDI-TOF-MS measured by linear mode for Blue-M1 showed an M^+^ ion at m/z 619. The high-resolution FAB-MS data for Blue-M1 showed as three Na adduct ions: m/z [M-2H+2Na]^+^: Calcd. for C27H29N4O11Na2: 663.1526, Found: 663.1503, m/z [M-3H+3Na]^+^: Calcd. for C27H29N4O11Na3: 685.1346, Found: 685.1372, and m/z [M-4H+4Na]^+^: Calcd. for C27H29N4O11Na4: 707.1166, Found: 707.1226, the molecular formula of [M]^+^ being estimated to be C27H21N4O11. The UV-visible spectrum of Blue-M1 showed maximum peak at 625 nm (ε=8.24 x 10^4 mol^-1 liter cm^-1) with a shoulder peak at 580 nm and small peaks (238 nm, 322 nm, and 365 nm). These results indicate that Blue-M1 contained extensive resonance structure in the molecules. Fluorescence: E_{max}=349 nm. E_{max}=445 nm.

^1^H-NMR and ^13^C-NMR measurements (completely decoupled and DEPT with 3/41 spectra) of Blue-M1 gave the following data. ^13^C-NMR δC(D2O): (numbers in parentheses show the proton position) position A(A), 32.4 (1C, i); B(B), 48.9 (1C, i); C(C), 48.9 (1C, i); D(D), 48.9 (1C, i); E(E), 49.5 (1C, i); F(F), 63.3 (1C, i); G(G), 65.9 (1C, i); H(H), 67.7 (1C,d, d); I(I), 71.2 (1C, d); J(J), 74.3 (1C, d); K(K), 90.6 (1C, d); L(L), 91.2 (1C, d); M(M), 102.6 (1C, d); N(N), 103.9 (1C, d); O(O), 123.4 (1C, d); P(no proton), 136.1 (1C, s); Q(no proton), 137.9 (1C, s); R(no proton), 138.6 (1C, s); S(no proton), 140.3 (1C, s); T(no proton), 145.6 (1C, s); U(no proton), 149.7 (1C, s); V(no proton), 150.8 (1C, s); W(no proton), 157.8 (1C, s); X(1(no proton), 175.6 (1C, s); X2(no proton), 175.9 (1C, s); X3(no proton), 176.3 (1C, s); X4(no proton), 176.6 (1C, s). ^1^H-NMR δH(D2O): A, 2.74-2.89 (2H, m); G, 3.57-3.72 (2H, m); F, 3.59-3.74 (2H, m); J, 3.99-4.02 (1H, m); I, 4.03-4.05 (1H, m); B, 4.53 (2H, s); C, 4.66 (2H, s); D, 4.66 (2H, s); E, 4.66 (2H, s); H, 4.76-4.77 (1H, d); L, 5.91 (1H, s); K, 6.09 (1H, s); O, 6.98 (1H, s); M, 7.06 (1H, s); N, 7.11 (1H, s).

Moreover, ^1^H-detected ^1^H-^13^C COSY (HMQC) and DEPT (distortionless enhancement by polarization transfer) spectra enabled assignment of the carbons bound with protons. The ^1^H-NMR and ^1^H-^13^C COSY spectra of Blue-M1 in D2O showed the presence of the ^1^H network on A-I-G and H-J-F. Moreover, the carbons bound with protons except for the quaternary carbons, could be assigned by HMQC.

The long-range coupled quaternary carbons could be assigned by ^1^H-detected long range ^1^H-^13^C COSY (HMBC) spectrum. The HMBC spectrum for Blue-M1 gave cross peaks with carbon-proton long-range coupling within two of three bonds. Figure 2 shows the connection between the unit around all carbons in Blue-M1. On the basis of these NMR and high-resolution FAB-MS data, Blue-M1 was identified as 5-{[(1,4-dicarboxymethyl)-5-(2,3-dihydroxypropyl)-2-pyrrolo[3,2-b]pyrrol]methylene}-1,4-{(dicarboxymethyl)-2-(1,2,3-tri-hydroxypropyl)-pyrrolo[2,3-b]pyrrolylum. In order to verify the structure, we measured the MALDI-TOF-MS by PSD mode (MS/MS) for a parent ion (m/z=619) of Blue-M1. Figure 3 showed a, b, c, d, and e fragments were certainly generated from Blue-M1, supporting the structure of a methine bridge between...
two pyrrolopyrrole rings. Miura and Gomyo have reported that various colored compounds including blue (Blue-M1 and Blue-M2), yellow (Yellow-M1 and Yellow-M2), and red (Red-M1) pigments were generated in the early stage of a D-xylose-glycine system. We isolated yellow pigments contained Yellow-M1 and Yellow-M2 which were eluted by DEAE-Sephadex A-25 in the same manner to purify Blue-M1. Yellow pigments showed a characteristic color reaction of pyrrolo-2-carboxyaldehyde with 2,4-dinitrophenylhydrazine. Blue-M1 was formed when the yellow pigments were incubated at 26.5°C for 24 h, indicating Blue-M1 might be generated by the decarboxylation from two molecules of yellow pigments, possibly two pyrrolopyrrole-2-carboxyaldehyde compounds (M⁺ ions at m/z = 324 and 340, respectively). Moreover, the MALDI-TOF-MS measured by linear mode for Blue-M2 showed an M⁺ ion at m/z 942 (Blue-M1 [m/z = 619] + Yellow pigment [m/z = 340] – OH [m/z = 17]). Consequently, Blue-M2 could be formed by the addition reaction of yellow pigment to Blue-M1 (Hayase, F. et al., unpublished results). In an advanced stage of the Maillard reaction, melanoidins may be formed by such a polymerization reaction.

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