Original paper

4-Hydroxy-5-Methyl-3(2H)-Furanone (HMFO) Contributes to Browning in the Xylose-Lysine Maillard Reaction System

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During research on the Maillard reaction between xylose and lysine (Lys), we detected a major peak showing an absorbance maximum at about 280 nm by diode-array-detection (DAD)-HPLC. In this study, the peak was isolated and identified as 4-hydroxy-5-methyl-3(2H)-furanone (HMFO). This compound accounted for 60 – 80% of the total area of HPLC peaks (280 nm), and about 20 mg/100 mL of HMFO was produced from a solution containing 200 mg/100 mL of xylose. HMFO was produced not only from xylose, but also from arabinose and ribose, and ribose was the best precursor. When HMFO was heated in a buffer solution with or without Lys, it was decomposed or polymerized, and colored and colorless polymers appeared. Diacetyl and methylglyoxal were the major decomposed dicarbonyl compounds from HMFO; these dicarbonyl compounds are considered to be the major precursors for polymers formed from HMFO.

Keywords: Maillard reaction, browning, 4-hydroxy-5-methyl-3(2H)-furanone, dicarbonyl, methylglyoxal, diacetyl

Introduction

The Maillard reaction between reducing sugars and amino acids or proteins has a critical influence on the quality of cooked or stored foods, such as their color, flavor, and taste. In addition to the qualities of foods, the Maillard reaction contributes to food function (Fogliano, 2015) and has some effects on nutrition and food safety (Friedman, 1996; Virk-Baker *et al.*, 2014).

Browning is the most apparent result of the Maillard reaction. Various heated or long-stored foods show a brown or dark color. The color of these foods is mainly attributed to melanoidins, which are brownish, hydrophilic, acidic, and nitrogen-containing compounds formed by the Maillard reaction. The chemical structures of melanoidins remain unclear because they are heterogeneous and high-molecular-weight polymers (Wang *et al.*, 2011). On the other hand, several low-molecular-weight colored compounds formed from sugars and amino acids have been reported. For example, Hayase *et al.* (1999) and Shirahashi *et al.*

(2009) reported blue and red Maillard pigments formed from a glycine-xylose model solution. Our group also reported some yellow Maillard pigments, such as furpiperate (Murata *et al.*, 2007; Totsuka *et al.*, 2009) from furfural and lysine (Lys), dilylsyldipyrrolones from xylose and Lys (Sakamoto *et al.*, 2009; Nomi *et al.*, 2011; Nomi *et al.*, 2013), and pyrrolothiazolate from glucose and cysteine (Noda *et al.*, 2015; Noda *et al.*, 2016).

In general, pentose turns brown more intensively or more rapidly than hexose in the Maillard reaction, because more open-chain form isomer is formed in pentose than hexose. We recently compared the Maillard reaction systems between xylose-Lys and glucose-Lys, and examined their contributions to browning (Mikami *et al.*, 2015). During this research, we found a major peak showing an absorption maximum at about 280 nm by HPLC equipped with Diode-Array-Detection (DAD) of a reaction solution containing xylose and lysine (Figs. 1A and 1B). Neither this peak nor a similar peak showing the same spectrum was detected in the

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Fig. 1. Typical profiles of reversed-phase HPLC of a model Maillard solution of xylose and Lys depicted by absorbance at 280 nm (A), contour map (B), and absorbance at 400 nm (C). Low-molecular-weight pigments, dilysyldipyrrolones A, B and C (DLDPs), and melanoidins appeared (C). A solution of 0.2 M phosphate buffer (pH 6.5) containing 13.3 mM xylose and 34 mM Lys was heated at 100°C for 60 min and was applied to HPLC equipped with DAD.

glucose-Lys system. The chromatogram depicted by absorbance at 400 nm or color showed that melanoidins were apparent as broad convex or trapezoidal peaks showing no specific absorption maxima in the UV-Vis region, and that low-molecular-weight Maillard pigments, dilysyldipyrrolones (Sakamoto *et al.*, 2009; Nomi *et al.*, 2011; Nomi *et al.*, 2013), were also formed (Fig. 1C; Mikami *et al.*, 2015).

The aim of this study was to identify the major compound (HMFO) showing the absorption maximum at 280 nm on DAD-HPLC, and clarify its role in browning.

Materials and Methods

Preparation of model solutions Buffer solutions (0.2 M phosphate-Na/K buffer (pH 5.5–8.0), 0.2 M acetate-Na buffer (pH 3.5–6.0), or tris(hydroxymethyl)aminomethane (Tris)-HCl buffer (pH 7.0–9.0)) containing 13.3 mM sugar (xylose, ribose, arabinose, or glucose) and 34 mM L-Lys monohydrochloride were put into a test tube with a cap, and heated for 30–120 min in boiling water.

Isolation of 4-hydroxy-5-methyl-3(2H)-furanone (HMFO) A solution (800 mL) of 0.2 M phosphate buffer (pH 7.0) containing 0.3 M xylose and 0.3 M L-Lys was heated for 60 min in boiling water, before being cooled to room temperature. The reaction solution was extracted with ethyl acetate. During the concentration of the ethyl acetate layer, crystals appeared. Pure crystals (about 330 mg) were obtained by filtration.

HPLC analysis Model solutions were analyzed with a reversed-phase HPLC system equipped with DAD under the following conditions: pump, L-6320 Intelligent Pump (HITACHI, Tokyo, Japan); column, YMC-Pack R&D ODS-A (i.d. 4.6 × 250 mm, 10 μm, YMC, Kyoto, Japan); eluent, solution A (0.1% trifluoroacetic acid (TFA)-water/MeOH=98/2, V/V) and solution B (0.1% TFA-water/MeOH=50/50, V/V), 0% B for 0–2 min, 0 to 50% B for 2–12 min and 50% B for 12–30 min; flow rate, 1 mL/min; detector, L-4500 Diode Array Detector (HITACHI); wavelength for detection, 250–500 nm. HMFO was detected at a

retention time of about 7.5 min under this condition.

X-ray analysis A colorless prism crystal of C₅H₆O₃ having approximate dimensions of 0.600 × 0.500 × 0.400 mm was mounted on a glass fiber. All measurements were made on a R-Axis RAPID diffractometer (Rigaku, Tokyo) using graphite monochromated Cu-Kα radiation. Indexing was performed from three oscillations that were exposed for 40 s, the crystal-to-detector distance being 127.40 mm.

Physicochemical properties of HMFO NMR (CDCl₃): δ_H: 2.27 (3H, s), 4.52 (2H, s), δ_C: 13.51, 77.03, 135.27, 175.66, 196.17. UV λ_{max}: 280 nm (in water). MS (*m/z*): 115.0388 [M+H]⁺, calcd. for C₅H₆O₃, 115.0390.

Gel permeation chromatography (GPC) analysis A solution of 0.2 M phosphate-Na/K buffer (pH 8.0) containing 1.8 mM HMFO in the presence or absence of 3.4 mM Lys was heated at 100°C for 3 h. The solutions were analyzed with GPC equipped with DAD under the following conditions: column, TSK-gel G2500PW_{XL} (i.d. 7.8 × 300 mm, TOSOH, Tokyo, Japan); eluent, 0.1 M phosphate-Na/K buffer (pH 7.0); flow rate, 0.5 mL/min; detector, L-4500 Diode Array Detector (HITACHI); wavelength for detection, 250–500 nm.

Preparation of quinoxalines of methylglyoxal and diacetyl An aqueous solution (100 mL) of 10 mM methylglyoxal and 10 mM *o*-phenylenediamine (OPD) was left overnight at room temperature. After the formed quinoxaline derivative was extracted with ethyl acetate, the ethyl acetate layer was concentrated *in vacuo* and dried. Methylglyoxal quinoxaline was purified by a Chromatorex ODS column (ODS-DM 1020T; Fuji Silysia Chemical, Kasugai, Japan; i.d. 1.5 × 17 cm), which was developed with MeOH:water = 0:100, 20:80, 50:50, 75:25, and 100:0, successively. After each fraction was analyzed with DAD-HPLC, fractions containing methylglyoxal quinoxaline were collected. After concentration, about 40 mg of pure methylglyoxal quinoxaline (MS (*m/z*): 145.0767 [M+H]⁺, calcd. for C₉H₉N₂, 145.0760; NMR (CDCl₃): δ_H: 2.74 (3H, s), 7.67 (1H, t, *J*=8.1 Hz), 7.70 (1H, t, *J*=7.5 Hz), 8.00 (1H, d, *J*=8.3 Hz), 8.03 (1H, d, *J*=8.2 Hz), 8.71 (1H, s), δ_C: 22.8,

Fig. 2. Plausible structures (A) and crystals of the objective compound (B). This compound was identified as 4-hydroxy-5-methyl-3(2H)-furanone (HMFO) by X-ray analysis.

128.6, 129.0, 129.1, 130.1, 146.0 (2C), 147.0, 153.8) was obtained. For the preparation of diacetyl quinoxaline, diacetyl was used instead of methylglyoxal. Diacetyl quinoxaline was purified by a Chromatorex ODS column (ODS-DM 1020T; Fuji Silysia Chemical; i.d. 1.5×17 cm), which was developed with MeOH:water = 0:100, 20:80, 50:50, and 100:0, successively. About 30 mg of diacetyl quinoxaline (MS (m/z):159.0918 [M+H]⁺, calcd. for C₁₀H₁₀N₂, 159.0917; NMR (CDCl₃): δ_H : 2.73 (6H, s), 7.66 (2H, dd, $J=3.3, 6.3$ Hz), 7.98 (2H, dd, $J=3.3, 6.3$ Hz). δ_C : 23.4, 141.3, 128.5, 129.1, 153.8) was obtained.

Analyses of quinoxaline Quinoxaline derivatives of dicarbonyl compounds formed from heated HMFO were prepared according to the method of Gobert and Glomb (2009) with some modifications. A solution of 0.2 M phosphate buffer (pH 8.0) containing 1.8 mM HMFO was heated at 100°C for 0.5, 1, 3, and 6 h, and then reacted with the same amount of 1.8 mM OPD solution for 5 h at 20°C. Formed quinoxalines were analyzed with DAD-HPLC (column, YMC-Pack ODS-A (i.d. 4.6×250 mm, 10 µm, YMC); eluent, solution A (0.1% trifluoroacetic acid (TFA) water/MeOH=98/2, V/V) and solution B (0.1% TFA water/MeOH=50/50, V/V), 0% B for 0 – 10 min, 0 to 100% B for 10 – 40 min and 100% B for 40 – 45 min; flow rate, 1.0 mL/min), and LC-MS (column, YMC-Pack ODS-A (i.d. 2.0×150 mm, 5 µm, YMC); eluent, solution A (0.1% formic acid water/MeOH=98/2, V/V) and solution B (0.1% formic acid water/MeOH=50/50, V/V), 0% B for 0 – 10 min, 0 to 100% B for 10 – 40 min and 100% B for 40 – 50 min; flow rate, 0.2 mL/min).

Instrumental analyses Spectroscopic measurements were performed using the following instruments: spectrophotometer (Multispec-1500; Shimadzu, Kyoto, Japan), NMR (Avance 600; Bruker Biospin, Karlsruhe, Germany), and MS (Triple TOF 4600;

AB Sciex, Foster City, CA).

Results and Discussion

Isolation and identification of HMFO As described in the introduction, a major peak showing an absorption maximum at about 280 nm appeared by DAD-HPLC of a reaction solution containing xylose and Lys (Figs. 1A and 1B). This peak occupied 60 – 80% of the total area on the chromatogram.

The target compound (HMFO) was extracted from a reaction solution with ethyl acetate. Pure crystals were obtained during the concentration of the ethyl acetate layer.

The MS data of this compound showed its molecular weight and molecular formula to be 114 and C₅H₆O₃, respectively. The NMR spectra of this compound showed the existence of protons of a methyl and methylene groups at δ_H 2.27 ppm (3H, s; CH₃-C=) and 4.52 ppm (2H, s; -O-CH₂-), and five kind of carbons, two which were methyl and methylene carbons and the other three carbons were quaternary (one carbonyl and two olefinic carbons). Considering the molecular formula, C₅H₆O₃, these results suggest that this compound is furanone carrying methyl and hydroxy groups (Fig. 2A). Among these compounds, 4-hydroxy-5-methyl-3(2H)-furanone (HMFO) was the most plausible, because only this compound maintained a skeleton of five carbons of xylose.

As we could obtain a colorless prism crystal of this compound (Fig. 2B), the crystal was applied to X-ray analysis. The X-ray crystal data were as follow: C₅H₆O₃, M_r = 114.10, orthorhombic, space group $Pna2_1$ (#33), μ (Cu-K α) = 10.602 cm⁻¹, T = 123 K, a = 18.7149(3) Å, b = 4.0175 Å, c = 6.87513(10) Å, V = 516.93(2) Å³, Z = 4, D_c = 1.466 g cm⁻³, $F(000)$ = 240.00. A total of 4697 reflections were collected, with 927 being unique (R_{int} = 0.0429). R_1 , R , and wR_2 were respectively 0.0387 [$I > 2\sigma(I)$], 0.0392 (all data),

Fig. 3. Effect of pH (A), heating time (B), and pentoses (C) on the HMFO formation. Buffer solutions (0.2 M phosphate-Na/K buffer, pH 5.5–8.0) containing 13.3 mM xylose and 34 mM Lys were heated at 100°C for 60 min (A). Buffer solutions (0.2 M phosphate-Na/K buffer, pH 6.0, 7.0, and 8.0) containing 13.3 mM xylose and 34 mM L-Lys were heated at 100°C for 30 min, 1 h, and 2 h (B). Buffer solutions (0.2 M phosphate-Na/K buffer, pH 6.0, 7.0, and 8.0) containing 13.3 mM sugar (xylose, ribose, or arabinose) and 34 mM L-Lys were heated at 100°C for 1 h. The concentrations of 100% HMFO at A, B, and C were 24, 20, and 33 mg/100 mL, respectively. (n=3)

and 0.1146 (all data). Further details of the crystal structure investigation are deposited in the Cambridge Crystallographic Data Center as supplementary publication no. CCDC1500343. Copies of the data can be obtained free of charge by an application to CCDC at 12 Union Road, Cambridge CB2 1EZ, UK [Fax: +44-1223-336-033, E-mail: deposit@ccdc.cam.ac.uk]. X-ray analysis of this compound showed its chemical structure to be HMFO (Fig. 2). This structure is consistent with all of the instrumental data. Although HMFO is known as a flavor and an intermediate compound of the Maillard reaction from pentose (Severin and Kronig, 1972; Nunomura *et al.*, 1979; Feather, 1981; Blank *et al.*, 1996), there were no reports showing that HMFO was a major compound in the reaction mixture.

Formation of HMFO in model systems Conditions for HMFO formation were then examined in model systems. Figure 3 shows the effect of pH and heating time on HMFO formation. The formation of HMFO was the highest at pH 6.5–7 (Fig. 3A). The amount of HMFO decreased with longer heating. Maximal concentrations of HMFO were reached with 1 h heating at pH 7 or 8 (Fig. 3B), compared to 4 h heating at pH 6 (data not shown). It appeared that HMFO was formed and decomposed more slowly at pH 6 than at pH 7–8. Under these conditions, about 20 mg/100 mL of HMFO was formed from 200 mg/100 mL of xylose, the yield of HMFO from xylose being about 10%. Next the effect of pentoses on HMFO formation was examined using arabinose, ribose, and xylose. HMFO was formed from all three pentoses. Ribose was the best precursor (Fig. 3C); about 30 mg/100 mL of HMFO was formed from 100 mg/100 mL of ribose.

Relationship between HMFO and browning We observed the decomposition of HMFO and the formation of viscous brown materials during the process of HMFO purification from the reaction mixture. The concentration of HMFO reached maximum with heating for 1 h at pH 7.0, and decreased thereafter (Fig. 3B). These results suggested that HMFO was decomposed or

polymerized during heating. Then, we examined the relationship between HMFO and browning. HMFO was dissolved in phosphate buffer at pH 6, 7, and 8, before being heated in the presence or absence of Lys. Figure 4A shows the browning of the HMFO solution. The alkaline pH stimulated the browning. Lys seemed to repress the colorization. Figure 4B shows the UV-vis spectra of heated solutions at pH 8. The maximal absorption at 280 nm of HMFO gradually decreased and the absorption of the visible region above 380 nm increased. These results showed that the color was formed with the decomposition of HMFO.

Then, the colored solution was analyzed with GPC equipped with DAD. Figure 5A shows the profiles of heated solutions of HMFO monitored by absorbance at 280 nm (top), 250 nm (middle), and 400 nm (bottom). Peaks 1–4, in addition to residual HMFO, were detected at 280 nm. Peaks 3 and 4 were also detected by absorbance at 400 nm and had some absorption in the visible region (Fig. 5B), showing that these peaks were brown polymers or pigments. Meanwhile, peaks 1 and 2 were not detected by absorbance at 400 nm and had no absorption in the visible region (Fig. 5B), indicating colorless polymers. The chromatogram depicted with absorbance at 400 nm shows a dull or trapezoidal shape, having no specific absorption maximum like melanoidins, in addition to peaks 3 and 4. Peaks 1 and 2 were detected in both the absence and presence of Lys, while peaks 3 and 4 were detected only in the presence of Lys. The latter two peaks were thought to be formed by the reaction between the decomposition products of HMFO and Lys. The formation of these peaks might cause the repression of the total browning of HMFO, as shown in Fig. 5. Regardless, these results showed that colorless polymers and brown pigments like melanoidins were formed from HMFO in the presence and absence of Lys.

Decomposition products of HMFO As polymers were formed from HMFO, we examined dicarbonyl compounds formed from HMFO by trapping them with OPD to form quinoxalines. Figure

Fig. 4. Browning (A) and UV-Vis spectra (B) of heated HMFO in the presence or absence of Lys. Buffer solutions (0.2 M phosphate-Na/K buffer, pH 6.0, 7.0, and 8.0) containing 1.8 mM HMFO were heated at 100°C in the presence or absence of 3.4 mM Lys for 0, 0.5, 1, 3, and 6 h.

Fig. 5. Profiles (A) of GPC equipped with DAD of heated solutions of HMF and Lys, and UV-Vis spectra (B) of peaks 1–4. Buffer solutions (0.2 M phosphate-Na/K buffer, pH 8.0) containing 1.8 mM HMFO and 3.4 mM Lys were heated at 100°C for 3 h. Peaks 1 and 2 were detected in the absence or presence of Lys, while peaks 3 and 4 were detected only in the presence of Lys.

Fig. 6. Reversed-phase HPLC of quinoxalines from heated solution of HMFO (A) and changes of each quinoxaline during heating. HMFO (1.7 mM) was dissolved in a phosphate buffer (pH 8.0) and heated at 100°C for 1 h (A) and for 0.5 – 6 h (B). After heated solutions were reacted with OPD, formed quinoxalines were analyzed with reversed-phase HPLC equipped with DAD.

Fig. 7. HMFO and browning. HMFO was formed 1-deoxyxylosone (1-DX) by cyclization and dehydration. Orange low-molecular-weight pigments, dilysyldipyrrolones, seemed to be formed from Amadori compound and 1-DX (Nomi *et al.*, 2013). Dicarbonyl compounds such as methylglyoxal (MGO) and diacetyl were formed from HMFO. These dicarbonyl compounds were polymerized in the presence or absence of Lys to form brown or colorless polymers. Dicarbonyl compounds might be also formed directly from 1-DX.

6A shows the chromatogram of formed quinoxalines in the heated solution of HMFO. Two major quinoxalines, peaks I and II, showing absorption maxima at 238 and 316 nm, respectively, were detected. The LC-MS analyses suggested that the molecular formulas of these peaks (C₉H₈N₂ for peak I and C₁₀H₁₀N₂ for peak II) were those of quinoxalines of methylglyoxal and diacetyl, respectively. Moreover, these peaks coincided with the authentic

samples of quinoxalines prepared from methylglyoxal and diacetyl, respectively. With the decrease in HMFO during heating, methylglyoxal and diacetyl were formed and then decreased (Fig. 6B). These results showed that HMFO was decomposed to dicarbonyl compounds, such as methylglyoxal and diacetyl, which seemed to be polymerized to form polymers. Further examination of how these dicarbonyl compounds were formed from HMFO, or

if other dicarbonyl compounds were formed is necessary.

To the best of our knowledge, there are no reports describing the relationship between HMFO and browning, although hydroxymethyl-furanone and hydroxyethyl-furanone were reported to be reactive intermediates in the Maillard reaction (Glomb *et al.*, 1991). Considering the yield of HMFO from xylose is about 10%, HMFO seemed to partly contribute to the browning of Maillard reaction of the xylose or pentose system. The presence of HMFO in soy sauce was reported (Nunomura *et al.*, 1979), and whose color is a typical example of Maillard reaction (Kato, 1960). It will be necessary to estimate HMFO's contribution to browning in the future.

In conclusion, HMFO was identified as a major intermediate compound in a reaction system containing xylose and Lys. HMFO was decomposed to dicarbonyl compounds such as methylglyoxal and diacetyl, which were polymerized to form brown pigments (Fig. 7).

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