Identification of 2,4-Dihydroxy-2,5-dimethyl-3(2H)-thiophenone as a Low-Molecular-Weight Yellow Pigment in Soy Sauce

Miki SATOH,1 Yuri NOMI,1 Shinji YAMADA,2 Makiko TAKENAKA,3 Hiroshi ONO,3 and Masatsune MURATA1,†

1Department of Nutrition and Food Science, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan
2Department of Chemistry, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan
3National Food Research Institute, 2-1-12 Kannondai, Tsukuba, Ibaraki 305-8642, Japan

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The color of soy sauce is considered to be mainly attributable to melanoids formed by the Maillard reaction. However, the chemical structure of melanoids cannot be clarified, because melanoids are high-molecular-weight heterogeneous polymers. We isolated a low-molecular-weight pigment from soy sauce and identified 2,4-dihydroxy-2,5-dimethyl-3(2H)-thiophenone as this pigment formed by the Maillard reaction, although its contribution to the total color of soy sauce was very low.

Key words: Maillard reaction; browning; soy sauce; thiophenone

Soy sauce is made from soybean, wheat, NaCl and koji (Aspergillus oryzae or Aspergillus soyaee). Macromolecules of the raw materials are decomposed during fermentation to such low-molecular-weight compounds as amino acids and sugars by the action of enzymes of koji in the presence of salt. Soy sauce is rich in salt, glutamic acid and other amino acids, and is one of the major traditional seasonings used in Japan. The color of soy sauce is formed during the production process involving heating, fermentation and pasteurization by the Maillard reaction and is an important factor of its quality. The color of soy sauce has been mainly studied by Japanese researchers. In 1926, Kurono and Katsume reported a pigment of soy sauce (soyamelanic acid and soyamelnin) for the first time.1) Kato et al. have reported the presence of 3-deoxyglucosone in soy sauce and showed that the color of soy sauce was formed by the Maillard reaction.2,3) Hashiba separated and characterized the color of soy sauce by gel filtration chromatography.4) Motai et al. examined the relationship between the oxidation of soy sauce and the change in color.5) Hayase et al. showed the presence of pyrraline, imidazolone, pentosidine, and lysyl-pyrropyridine in soy sauce.6) These studies have clarified that the color of soy sauce is mainly attributable to melanoids which are formed by the Maillard reaction and are high-molecular-weight heterogeneous polymers. Flazine, a β-carboline derivative, has been reported by Kihara as a low-molecular-weight pigment, who described the relationship between its oxidation and the browning of soy sauce,7,8) although its contribution to the color of soy sauce is unclear.

The aim of this present study is to clarify the contribution of low-molecular-weight compounds to the color of soy sauce and identify the low-molecular-weight pigments in soy sauce. We first show that the color of soy sauce was mainly due to the hydrophilic and high-molecular-weight compounds, melanoids. We then isolate and identify 2,4-dihydroxy-2,5-dimethyl-3(2H)-thiophenone as the major lipophilic and low-molecular-weight pigment in soy sauce, although its contribution to the total color of soy sauce was very low.

Materials and Methods

Materials. Soy sauce (Koikuchi, Kikkoman, Tokyo, Japan) was purchased at a local market in Tokyo.

Evaluation of color intensity by a color unit. The color intensity is expressed by a color unit which was visually estimated according to the color dilution method described by Hoffman9) with some modifications. Briefly described, each sample (200 µL) serially diluted with a 0.1 M acetate buffer at pH 5.0 was put into a 96-well microplate, and we visually determined the most-diluted point at which we could recognize the color. The color unit is defined as its dilution ratio in this procedure.

Chromatography of soy sauce by Sephadex G-25. Soy sauce (1.0 mL) was applied to a column of Sephadex G-25 (GE Healthcare Japan, Tokyo, Japan; 2.4 cm i.d. x 41.0 cm) which was developed with reverse osmosis (RO) water at the rate of 1.0 mL/min. Each fraction (2.75 mL) was collected, and its absorbance at 280 nm and 400 nm and its Na content were monitored. The Na content was determined by an atomic absorbance spectrometer (Shimadzu AA-670, Kyoto, Japan).

Adsorption of soy sauce pigments to ODS. Soy sauce (5.0 mL) was applied to a column of ODS (5 mm i.d. x 20 mm; Chromatex ODS, 100–200 mesh, Fuji Silysia Chemical, Kasugai, Japan). After being washed with 5 mL of water (the non-adsorbed fraction), the column was eluted with 5 mL of MeOH (the adsorbed fraction). The non-adsorbed and adsorbed fractions were dried in vacuo and each dissolved in 5.0 mL of MeOH. Soy sauce and the non-adsorbed and adsorbed fractions were analyzed by HPLC under the following conditions: system, Hewlett Packard series 1100 with a photodiode-array detector (Palo Alto, CA, USA); column, TSKgel ODS 100V (4.6 mm i.d. x 250 mm, Tosoh, Tokyo); eluent, solution A (water/MeOH = 98:2) and solution B (MeOH), with a linear gradient from solution A to a mixture of solutions A and B (30:70) in 30 min; detection, 250–500 nm.

† To whom correspondence should be addressed. Fax: +81-3-5978-5755; E-mail: murata.masatsune@ocha.ac.jp
Isolation of S1 from soy sauce. S1 was extracted with 30 L of ethyl acetate from 30 L of soy sauce. The ethyl acetate layer was concentrated in vacuo, before a crude extract (ca. 39 g) was obtained. This extract was applied to a column of ODS (6 cm i.d. x 25 cm, Chromatorex ODS, 100–200 mesh, Fuji Silysia Chemical, Kasugai, Japan) which was successively developed with water, water/MeOH (85:15, 60:40, 40:60), and MeOH. Each fraction obtained was analyzed by HPLC. The fractions eluted with water contained S1, and these fractions were combined. After S1 had been extracted with ethyl acetate from the combined fractions, the extract was concentrated in vacuo and applied to preparative HPLC (column, YMC-pack R&D ODS (20 mm i.d. x 250 mm, YMC, Kyoto, Japan); eluent, water/MeOH = 60:40; flow rate, 9.9 mL/min; detection, 370 nm). The peak at a retention time of about 12 min was collected. After being extracted with ethyl acetate and concentrated in vacuo, this material was applied to a second preparative HPLC (column, COSMOSIL SC18-MS-II (20 mm i.d. x 250 mm, Nacalai Tesque, Kyoto, Japan); eluent, 0.5% AcOH/MeOH = 60:40; flow rate, 9.9 mL/min; detection, 370 nm). The peak at a retention time of about 15 min was collected. After being extracted with ethyl acetate and concentrated in vacuo, this material was applied to a third preparative HPLC (column, COSMOSIL SC18-PAQ (20 mm i.d. x 250 mm, Nacalai Tesque, Kyoto, Japan); eluent, 0.5% AcOH/MeOH = 60:40; flow rate, 9.9 mL/min; detection, 370 nm). The peak at a retention time of about 14 min was collected, and a yellowish paste was obtained after being extracted with ethyl acetate and concentrated in vacuo. This paste was dissolved in MeOH, before a yellow platelet (ca. 40 mg) was obtained by adding CHCl3 to the MeOH solution. 

HPLC analysis of S1. Each fraction obtained during the process of purification was analyzed by HPLC. The fraction containing S1 was applied to the next step of purification under the HPLC conditions already described.

X-Ray analysis of S1. A yellow platelet crystal having approximate dimensions of 0.30 x 0.08 x 0.03 mm was mounted on a glass fiber. All measurements were made with an RAXIS RAPID imaging plate area detector (Rigaku, Tokyo, Japan) with graphite monochromated Cu-Ka radiation. Indexing was performed from three oscillations that were exposed for 300 seconds, the crystal-to-detector distance being 127.40 mm.

Instrumental analyses. Spectroscopic measurements were taken with Hitachi V-3310 (UV), Bruker Avance 600 (NMR) and Jeol MStation JMS-700 (EI-MS) instruments.

Physico-chemical properties of S1. UV/vis (in 0.01 M HCl) nm (ε): 365 (3,300); UV/vis (in water) nm (ε): 365 (3,200); UV/vis (in 0.01 M NaOH) nm (ε): 9 (1,900) (Fig. 1B). 1H-NMR δ (ppm): 1.68 (s, 3H), 2.19 (s, 3H), 13C-NMR δ (ppm): 14.0 (CH3), 25.5 (CH3), 83.9 (C), 136.7 (C), 147.8 (C), 196.9 (C). EI-MS (m/z): 160 (M+), 149, 132, 117, 101, 43.

Determination of 2,4-dihydroxy-2,5-dimethyl-3(2H)-thiophenone (S1) of soy sauce. Soy sauce (50 mL) was extracted five times with 50 mL of ethyl acetate. 2,4-Dihydroxy-2,5-dimethyl-3(2H)-thiophene-none was analyzed by HPLC. After the extract had been concentrated in vacuo and dissolved in 5 mL of MeOH. The HPLC conditions have already been described, using a wavelength of 400 nm for quantification.

Results and Discussion

Analysis of soy sauce color by Sephadex G-25 and ODS

It is well-known that the color of soy sauce can be separated into three fractions by Sephadex G-25 chromatography. These fractions usually being monitored by the absorbance in a visible region such as 400–500 nm. However, we did not know that the strength of absorbance at a wavelength would correspond to our sensory evaluation. Hence, the color intensity of each fraction visually estimated by the color dilution method was compared here with the absorbance. As shown in Fig. 1A, the color of soy sauce monitored by the absorbance at 400 nm was divided into three fractions of P1, P2 and P3, with P1 being the major peak. The apparent molecular weights of P1, P2 and P3 were respectively more than 5 kDa, 1.2 kDa, and 0.7 kDa. The chromatogram of soy sauce depicted by the color intensity (Fig. 1B) was similar to that by the absorbance (Fig. 1A). P1 was also the major peak, and P1, P2 and P3 respectively contributed 58%, 23%, and 17% of the total color intensity. These results show that the strength of absorbance almost corresponded to the sensory evaluation estimated by a color unit.

Lipophilic and low-molecular-weight compounds are often separated in an ODS column, so soy sauce was directly applied to ODS-HPLC. However, we could not detect any peaks showing a clear absorption maximum in the visible region. The adsorption ratio of the soy sauce pigments by ODS was therefore examined by the absorbance at 400 nm and a color unit. Both data were similar. As shown in Fig. 2A, about 70% of soy sauce color was not adsorbed to ODS, only 20–30% of the color being adsorbed to ODS and eluted with MeOH. The non-adsorbed and adsorbed fractions were analyzed by ODS-HPLC (Fig. 2B). No clear peak, except for a void volume, was apparent in the adsorbed fraction, and we could not detect any peaks showing a clear absorption maximum in the visible region. These results show that the color of soy sauce was mainly constituted by the high-molecular-weight and hydrophilic compounds, melanoids.
Low-molecular-weight pigments of soy sauce

Although the color of soy sauce was mainly derived from the hydrophilic and high-molecular-weight compounds, melanoids, it was difficult to identify the chemical structure of the melanoids. We therefore decided to isolate and identify a lipophilic and low-molecular-weight pigment in soy sauce. After extracting soy sauce with ethyl acetate, the remaining aqueous layer was analyzed by Sephadex G-25 chromatography. Figure 3 shows that the color of P1 and P2 was not extracted at all with ethyl acetate, while that of P3 was partially extracted. The ethyl acetate extract of soy sauce was then analyzed by ODS-HPLC which was monitored by the absorbance at 400 nm (Fig. 4). Although several peaks appeared, only one peak (S1) with a retention time of about 17 min showed a clear absorption maximum at 365 nm. We therefore decided to isolate and identify S1. S1 was purified by ODS column and preparative HPLC to obtain a yellowish platelet. The X-ray crystal data were as follows: C_{28}H_{30}O_{13}S, \( M_r = 196.22 \), monoclinic, space group \( P 2_1/n \), \( \mu (\text{Cu-K}\alpha) = 3.523 \text{ mm}^{-1} \), \( a = 5.81367(13) \AA, \ b = 17.2478(4) \AA, \ c = 8.03506(18) \AA, \ \beta = 115.0698(12)^\circ, \ V = 729.80(3) \text{ A}^3, \ T = 298 \text{ K}, \ Z = 4, \ D_c = 1.458 \text{ g cm}^{-3}, \ F(000) = 336. \) A total of 6983 reflections were collected, with 1266 being unique (\( R_m = 0.052 \)). \( R_1 \) and w\( R_2 \) were respectively 0.0436\([I > 2\sigma(I)]\) and 0.1630 (all data). Further details of the crystal structure investigation are deposited in the Cambridge Crystallographic Data Center as supplementary publication no. CCDC 780906. 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]. The X-ray analysis of S1 showed its chemical structure to be 2,4-dihydroxy-2,5-dimethyl-3(2H)-thiophenone (Fig. 5). The \(^1\)H-NMR data showing two singlet peaks (\( \delta_c 14.0 \) and 25.5 ppm) considered to be derived from two methyl groups, and the \(^{13}\)C-NMR data showing one carbonyl group (\( \delta_c 196.9 \text{ ppm} \)), two olefinic carbons (\( \delta_c 136.7 \) and 147.8 ppm) and an aliphatic carbon (\( \delta_c 83.9 \) ppm) support this structure.

2,4-Dihydroxy-2,5-dimethyl-3(2H)-thiophenone has been reported as a flavor compound formed by the Maillard reaction (Fig. 6) from cysteine and furanone\(^{11} \) and glucose,\(^{12} \) although there is no report on the color of this compound. We isolated this compound here as a yellow pigment in soy sauce, although there is no other report that soy sauce contained this compound as far as we know. This compound is considered to have been formed by the Maillard reaction during the soy sauce manufacturing process as we could not detect it in non-heated soy bean or wheat (data not shown). This compound has recently been isolated from heated garlic as an antioxidant and anti-inflammatory substance,\(^{13,14} \) although the authors used the different name, thiacremonone.

Properties of 2,4-dihydroxy-2,5-dimethyl-3(2H)-thiophenone

The aqueous solution of 2,4-dihydroxy-2,5-dimethyl-3(2H)-thiophenone was yellow. The UV-visible spectra
of 2,4-dihydroxy-2,5-dimethyl-3(2H)-thiophenone are shown in Fig. 7. This compound shows an absorption maximum at 365 nm in water and an acidic solution, while 360 nm and 390 nm in an alkaline solution. The color contribution of this compound in soy sauce was then estimated, giving a respective detection limit and content of 25 μg/mL and 5.9 μg/mL. The color of soy sauce was visually detected at a dilution of 1024-fold. The rate of color contribution of this compound in soy sauce was calculated to be only 0.02% from these data, although the content of this compound in *koikuchi* soy sauce (the regular type of soy sauce) was 6–9 μg/mL. This compound showed a burnt-like aroma reminiscent of soy sauce. 2,5-dimethyl-4-hydroxy-3(2H)-thiophenone and 2,5-dimethyl-4-hydroxy-3(2H)-furanone have been reported as structurally relevant flavor compounds. The latter is known as a caramel-like flavor and contributes to the aroma of soy sauce and beef broth.

**Fig. 5.** Structure of 2,4-Dihydroxy-2,5-dimethyl-3(2H)-thiophenone (S1).

**Fig. 6.** Plausible Formation Scheme for 2,4-Dihydroxy-2,5-dimethyl-3(2H)-thiophenone (S1) from 2,5-Dimethyl-4-hydroxy-3(2H)-furanone (DMHF) and Glucose in the Presence of Cysteine by the Maillard Reaction. 1-DG, 1-deoxyglucosone; 3-DG, 3-deoxyglucosone.

**Fig. 7.** UV-Visible Spectrum of 2,4-Dihydroxy-2,5-dimethyl-3(2H)-thiophenone. The sample was dissolved in 0.01 M HCl, water, and 0.01 M NaOH at a concentration of 10 μg/mL.
It has been reported that soy sauce contained about 1.98 mg/L of this compound, this level being several times lower than that of 2,4-dihydroxy-2,5-dimethyl-3(2H)-thiophenone. In conclusion, 2,4-dihydroxy-2,5-dimethyl-3(2H)-thiophenone was identified as a lipophilic and low-molecular-weight pigment in soy sauce, although the major pigment in soy sauce was hydrophilic and high-molecular-weight melanoidin.

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References