A Low-Molecular Weight Maillard Pigment from Beer was Identified as Perlolyrine, a Maillard Reaction Product from Tryptophan

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The color of beer, which is a typical example of the Maillard or browning reaction, is mainly attributed to melanoidins. However, as melanoidins are heterogeneous polymers formed by the Maillard reaction, there is little data on the chemical structure of the components responsible for the color of beer. To obtain chemical information on the color components of beer, we here isolated a low-molecular-weight yellow pigment from black beer and identified it using instrumental analyses and an authentic sample. As a result, the pigment was identified as perlolyrine, which is a Maillard reaction product from tryptophan; however, its contribution to the total color of beer was very low. This pigment was present in various kinds of beer at the level of 3.2-14.0 μg/100 mL.

Keywords: beer, Maillard reaction, melanoidin, perlolyrine, color, browning

Introduction

Beer shows a brilliant yellowish or gold color that is formed through the Maillard reaction. Pale beer is characterized by a pale-yellow color, while black beer shows dark brown or black. The major determinant of the color of beer is the malt. A normal malt used in the preparation of typical pale beer is dry-roasted at a temperature less than 100°C, as the malt must maintain enzyme activity as well as good storage quality. On the other hand, special malts are used for the preparation of various kinds of beer including black beer, amber ale, stout etc. in addition to pale malt; these malts are heated to more than 100°C and sometimes up to 225°C during kilning to impart special flavor and color (Lewis and Young, 1995). During the heating process, the Maillard reaction occurs between reducing sugars and amino acids, peptides and/or proteins in malts (Carvalho et al., 2016). In addition to malting, the color or pigment of beer is partly formed during other heating process including mashing, boiling, and pasteurization (Fig. 1).

The imparting of color to beer is mainly attributed to melanoidins. However, melanoidins are heterogeneous polymers formed by the Maillard reaction between carbonyl compounds such as sugars or their degradation products and amino compounds such as amino acids, peptides, and proteins. These compounds react with each other by various chemical reactions such as condensation, polymerization, degradation, cyclization etc. to form melanoidins. This complexity causes difficulties in the analysis of melanoidins (Echavarria et al., 2012), although some plausible chromophores of model melanoidins have been proposed (Hofmann, 1998; Hayase et al., 2006). In terms of the color or pigment of beer, some properties such as chromatographical characteristics and UV-Vis spectra (Kuntcheva and Obretenov, 1996) were examined; however, no clear data is available on the chemical structures. Although beer color is mainly attributed to melanoidins, and the contribution of individual low-molecular weight pigments to beer color seems to be low; we here searched for a low-molecular-weight pigment from beer to obtain chemical information regarding the color of beer. We observed that a peak having a maximum absorption wavelength in the visible region was present in the basic substance fraction of beer; thus, we described the isolation and identification of the pigment (BB1) and its distribution in various kinds of beer.

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Materials and Methods

Materials Various kinds of beer were purchased from a retail shop in Tokyo between 2016–2018 and used for experiments.

Fractionation of beer samples Beer samples (two brands of pale beer; Kirin Co. (Tokyo, Japan) and Sapporo Breweries (Tokyo, Japan) and a brand of black beer (Sapporo Breweries)) were fractionated using ethyl acetate into neutral and acidic substance fraction, basic substance fraction, and water-soluble substance fraction. The pH of each beer sample was adjusted to less than 2 with 6 M HCl. Each sample was then extracted with a similar amount of ethyl acetate three times, before being concentrated in vacuo (acidic and neutral substance fraction). Then, the pH of the residual water layer was adjusted to 9-10 with 6 M NaOH. Each water layer was extracted with ethyl acetate three times, before being concentrated in vacuo (basic substance fraction). The residual water layer was designated as the water-soluble substance fraction. The volume of each fraction was arbitrarily adjusted to detect peaks using HPLC.

HPLC analysis Beer samples and their fractions were as described above. These beer samples and the fractions during purification procedures were analyzed with a reversed-phase HPLC system equipped with a photodiode-array detection (DAD) under the following conditions: system, Chromaster 5430 (Hitachi, Tokyo, Japan); column, Mightysil RP-18GP (4.6 mm i.d. x 250 mm, Kanto Chemical, Tokyo, Japan); eluent, solution A (acetic acid:water=0.1:100, v/v) and solution B (methanol:acetic acid:water=0.1:80:20; v/v/v), 0 % B for 0-10 min and 0-100 % B (v/v) for 10-40 min with a linear gradient for beer samples, and 0-100 % B (v/v) for 0-30 min with a linear gradient for fractions during purification procedures; flow rate, 1.0 mL/min; wavelength, 230-500 nm. BB1 in beer samples and fractions during purification procedures were, respectively, detected at retention times of about 32 min and 22 min under these conditions.

Isolation of BB1 About 1 L of the black beer sample (Sapporo Breweries) was stirred for about 30 min to decrease the amount of foam, and then the pH was adjusted to less than 2 with 6 M HCl. The beer sample was washed with a similar amount of ethyl acetate three times, and then the pH of the residual water layer was adjusted to 9-10 with 6 M NaOH. BB1 was extracted with ethyl acetate three times. The combined ethyl acetate layer was concentrated in vacuo, and about 60 mg of brown paste was obtained. These procedures were repeated ten times. The combined paste from 5 L of black beer was dissolved into 50 mL of a mixture of methanol, acetic acid, and water (40:0.1:60; v/v/v), which was applied to a Chromatorex ODS column (i.d. 20 mm × 100 mm; Fuji Silslyca Chemical, Kasugai, Japan). The column was developed with a mixture of methanol, acetic acid, and water (40:0.1:60 and 80:0.1:20; v/v/v).
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As black beer contained more pigment than the examined pale beer, we employed black beer for the isolation of BB1. After preparing the basic substance fraction, we isolated BB1 from the fraction using the chromatographic procedures described in the Materials and Methods. Less than 1 mg of yellow paste was obtained from 10 L of dark beer.

Figure 3 shows the UV-Vis and fluorescence spectra of BB1. BB1 showed three absorption maxima at 275, 340, and 405 nm in an acidic solution, meaning that this compound is yellow in beer, as the pH of beer is about 4.2 and weakly acidic. The absorption spectra showed a hypsochromic shift in the basic solution, suggesting the presence of a dissociation group containing nitrogen atom. This pigment showed blue fluorescence.

The mass spectrum of BB1 showed its molecular weight, estimated with the triangle test, using ten panelists. Individual detection limits for perlolyrine and beer were visually estimated with the triangle test, using ten panelists. The color activity (unit) of perlolyrine in a beer sample was calculated as [perlolyrine concentration (μg/mL)]/[its detection limit (μg/mL)]. The contribution of perlolyrine to total color (%) of the sample was calculated by [color activity of perlolyrine in beer]/[detection limit of beer (dilution ratio)] × 100.

Results and Discussion

Pigment in the basic substance fraction of beer First, samples of pale and black beers were applied to ODS-HPLC equipped with DAD detection; however, no peaks showing a clear absorption maximum in the visible region could be detected. It is possible that melanoidins as the major pigment prevented the detection of small amounts of low-molecular-weight pigments in HPLC. Then, we fractionated a black beer into the neutral or acidic substance, the basic substance, and the water-soluble substance fractions using ethyl acetate. Melanoidins are generally thought to be transferred to the water-soluble substance fraction. Each fraction was analyzed with DAD-HPLC (Fig. 2). As a result, a peak (BB1) showing a specific UV-Vis spectrum (Fig. 2D) was detected in the basic substance fraction of a dark beer (Fig. 2C). We then examined whether this peak appeared in the extract of other brands of pale and black beers. All the basic substance fractions from the examined beer samples showed the same peak (data not shown). We determined that BB1 was a common pigment present in beer, and aimed to isolate and identify this pigment.

Identification of a low-molecular-weight pigment (BB1) in dark beer As black beer contained more pigment than the examined pale beer, we employed black beer for the isolation of BB1. After preparing the basic substance fraction, we isolated BB1 from the fraction using the chromatographic procedures described in the Materials and Methods. Less than 1 mg of yellow paste was obtained from 10 L of dark beer.

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The mass spectrum of BB1 showed its molecular weight, molecular formula, and desaturation degree were 265, C_{13}H_{43}N_{13}O_{12}, and 12, respectively. The unique UV-Vis spectra, fluorescence spectra, and high degree of unsaturation suggest this compound is a heterocyclic aromatic compound.

The NMR data are summarized in Fig. 4. The NMR
analyses showed 16 kinds of carbons corresponding to the molecular formula. The heteronuclear single quantum correlation (HSQC) analysis showed that a carbon (δC 57.5), eight olefinic carbons (δC 110.9, 111.0, 113.2, 115.0, 121.2, 122.2, 130.0, and 138.6), and the residual seven olefinic carbons (δC 122.6, 132.1, 132.5, 134.4, 143.0, 154.3, and 157.2) were, respectively, a methylene group, methine groups, and quaternary. The chemical shifts of the methylene group (δC 57.5 and δH 4.77) suggest that it is connected to an oxygen atom or hydroxy group. Further, the heteronuclear multiple bond correlation (HMBC) analysis showed that the singlet protons of this methylene group were correlated with the quaternary carbon at δC 157.2 and the methine carbons at δC 111.0 carrying a proton at δH 6.61. The proton at δH 6.61 was coupled with the proton at δH 7.24. Further, the HMBC analysis showed that the proton at δH 7.24 was correlated with a quaternary carbon at δC 154.3. These connections suggest the presence of a 5-hydroxymethylfurfuryl group (Fig. 5A). Moreover, the 1H-1H-correlation spectroscopy (COSY) and HMBC data showed a partial structure of =C-CH=CH-CH=CH-C= (Fig. 5A). The residual two protons (δH 8.05 and 8.31) of two methine groups were coupled to each other. In addition to these substructures, molecular formula, chemical shift, HMBC and nuclear Overhauser effect (NOE) spectroscopy (NOESY) data suggested two plausible structures for BB1 (Fig. 5B). In the
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Jeffreys (1970) reported structure X as perlolyrine. The physicochemical data, including UV-Vis absorption spectra and NMR, were very similar to those of BB1. To confirm this, an authentic perlolyrine was prepared from tryptophan and 5-hydroxymethylfurfural, and compared to the isolated BB1. Their retention times on DAD-HPLC, UV-Vis spectra, and NMR data coincided completely. Thus, BB1 was identified as perlolyrine (5-(9H-pyrido[3,4-b]indol-1-yl)furan-2-yl)methanol.

![Fig. 4](image)

**Fig. 4.** NMR spectral data of BB1 (perlolyrine) in MeOD (A), and its structure and numbering (B). *

![Fig. 5](image)

**Fig. 5.** Partial structures (A) and plausible structures (B) of BB1. In A, single arrows and dotted two direction arrows show HMBC and 1H-1H coupling, respectively. In B, single arrows and dotted two direction arrows show HMBC and NOE, respectively.
Perlolyrine was first isolated from perennial rye-grass (*Lolium perenne*) as an alkaloid showing fluorescence (Jeffreys, 1970), and subsequently isolated from Japanese sake as a yellowish green fluorescent compound (Oba et al., 1978) and from *dangshen* (*Codonopsis pilosula*), a traditional tonic medicine in China (Tun et al., 1988). This compound was also isolated from soy sauce as a yellow and weakly mutagenic β-carboline derivative (Nakatsuka et al., 1986; Oshita et al., 1991). Perlolyrine is known to have biological or physiological activities such as a phase-II enzyme induction *in vitro* (Li et al., 2011), antiproliferative activity against human stomach cancer cell lines (Lee et al., 2016), and transient receptor potential vanilloid 1 activation *in vitro* and taste modification *in vivo* (Oshida et al., 2017). Perlolyrine is also known to be formed from tryptophan and 5-hydroxymethylfurfural or sugars through the Maillard reaction (Oshita et al., 1993; Lee et al., 2016).

**Distribution of perlolyrine in various kinds of beer** To the best of our knowledge, this is the first identification of perlolyrine in beer. We then examined the amount of perlolyrine in various kinds of retail beer (Fig. 6). The 10 kinds of beer samples examined contained 3.2-14.0 μg/100 mL of perlolyrine (average ± standard deviation; 7.1±3.7 μg/100 mL). Among them, seven kinds of pale or light-colored beer (A-G in Fig. 6) contained 3.2-8.0 μg/100 mL (5.5±1.5 μg/100 mL) of perlolyrine, while three kinds of black or dark-colored beer (H-J in Fig. 6) contained 4.8-14.0 μg/100 mL (10.7±5.1 μg/100 mL) of perlolyrine. Although black beer contained more perlolyrine than pale beer, the difference was not large (7.1 vs 10.7 μg/100 mL). Beer-like alcoholic beverages contained various amount of perlolyrine (0-22.9 μg/100 mL).

Perlolyrine is formed from tryptophan and 5-hydroxymethylfurfural by the Maillard reaction (Oshita et al., 1993; Lee et al., 2016). Figure 7 shows a schematic of perlolyrine formation during beer production. During mashing, tryptophan is formed from malt peptides or proteins, while 5-hydroxymethylfurfural is formed from glucose or maltose derived from starch. The two substrates react with each other to form perlolyrine during mashing, boiling and other subsequent processes, including fermentation, pasteurization, and storage. We aim to further examine the effect of mashing and boiling conditions on the formation of perlolyrine, as we did not detect perlolyrine in malt extracts in a preliminary experiment (data not shown).

The absorbance of pale beer at 400 nm was about 0.5, while the absorbance derived from perlolyrine at 400 nm was estimated to be about 0.002 according to its content (0.055 μg/mL beer). This result indicated that perlolyrine represented about 0.4 % (0.002/0.5 × 100 = 0.4) of the absorbance at 400 nm of pale beer. Next, the sensory contribution of perlolyrine to the overall color of pale beer was estimated using the color dilution method (Hoffman, 1998). A sodium acetate buffer (pH 4.2) was used as the solvent here, as the pH of beer is about 4.2. The detection limit for the visual estimation of perlolyrine was 50 μg/mL. On the other hand, a pale beer sample contained 0.051 μg/mL of perlolyrine, and the color of this beer was visually detected at a 2-fold dilution. The rate of color contribution of perlolyrine in this beer was calculated to be only 0.05 % according to this data (0.051/50/2 × 100 = 0.051). This estimation means that the contribution of perlolyrine to the total color of beer is very low and that the major contributors to beer color are melanoids, heterogenous
high-molecular-weight pigments. However, we must acknowledge that the recognition of color is cumulative, and even if the individual contribution of a pigment is low, the total or cumulative contribution will be meaningful in the context of the multifarious pigments in beer.

In conclusion, we isolated a low-molecular-weight Maillard pigment from beer and identified it as perlolyrine. This is the first identification of a Maillard pigment in beer; however, its contribution to the total color of beer is very small.

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References


