



Identification of a precursor of 2-mercapto-3-methyl-1-butanol in beer

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ABSTRACT

2-Mercapto-3-methyl-1-butanol (2M3MB) imparts an onion-like off-flavor to beer and is believed to result from the hot aeration of brewer's wort. However, little has been reported regarding the mechanism of formation of 2M3MB. This paper investigates the precursors of 2M3MB formed during the brewing process. Our study of a cysteine conjugate as a precursor showed its negligible contribution to the formation of 2M3MB. The 2M3MB precursor was found to be a volatile compound because 2M3MB was formed in the distillate of hop extract. The precursor was purified through distillation, column chromatography, and preparative gas chromatography, and the 2M3MB-producing potential was measured as an index for estimating the concentration of the precursor. The accurate mass of the precursor was measured using gas chromatography coupled to quadrupole-time-of-flight-mass spectrometry (GC/QTOF). The molecular structure was identified from the product ion spectra by GC/QTOF to be 2,3-epoxy-3-methylbutanal.

1. Introduction

Volatile thiols are powerful odorants that contribute to the aromas of various foods and beverages (Maga & Katz, 1976). For example, 2-furfurylthiol is a compound that has a coffee-like aroma and is found in coffee (Grosch, 1998). However, there are many thiol compounds that have an undesirable odor and, due to their low threshold value, can impair food flavors. 3-Methyl-2-butene-1-thiol (MBT) is a well-known 'skunky' off-flavor in beer and is generated when sunlight strikes a beer bottle (Sakuma, Rikimaru, Kobayashi, & Kowaka, 1991). Similarly, an onion-like off-flavor in beer has long been known and was reported in 1967 to be caused by the hot aeration of wort (Liebenow & Esser, 1967). 2-Mercapto-3-methyl-1-butanol (2M3MB) has been identified in beer and has an onion-like off-flavor (Olsen, Christensen, & Madsen, 1988). 3-Mercapto-3-methyl-1-butanol (3M3MB) is an isomer of 2M3MB, imparts a similar flavor, and has been detected in beer, coffee, passion fruit juice, and wine (Holscher, Vitzthum, & Steinhart, 1992; Tominaga, Furrer, Henry, & Dubourdieu, 1998; Tominaga & Dubourdieu, 2000; Noba, Yako, Kobayashi, Masuda, & Watanabe, 2017). We previously quantified the odor threshold values for 2M3MB and 3M3MB in beer samples and showed that only 2M3MB contributes to the onion-like off-flavor in beer; the odor activity value (OAV) for 2M3MB is greater than 1.0 in most beer samples and less than 0.1 for 3M3MB in all beer samples tested (Noba et al., 2017).

In order to reduce the onion-like off-flavor in beer, it is essential to

elucidate the mechanism of formation of the causative compounds. In particular, it is important to control the formation of 2M3MB, since it has a high OAV. It is believed that the precursor of 2M3MB is present in wort, and that 2M3MB is formed by yeast, given that 2M3MB is not present in wort but its concentration in beer increases during fermentation. Furthermore, hot aeration of wort increases the concentration of 2M3MB, suggesting that the precursor of 2M3MB in wort increases as the wort is oxidized (Noba et al., 2017).

There are several reports regarding the precursors of thiols in various foods. For example, 2-methyl-3-furanthiol and 2-furfurylthiol are chemically reactive compounds formed through the Maillard reaction. The mechanism underlying their generation has been studied during the process of cooking meat, as well as in model systems using pentose and cysteine. Several compounds have been proposed as precursors of these thiol compounds. For example, hydroxyacetaldehyde is a precursor of 2-methyl-3-furanthiol, and furfural is a precursor of 2-furfurylthiol, and both precursors exhibit the highest reaction efficiency among the compounds tested (Hofmann & Schieberle, 1998). MBT imparts a skunky off-flavor to beer and its presumed precursor is iso- α acid. The exposure of beer to sunlight results in the formation of MBT by a radical reaction of iso- α acid with sulfur compounds. Furthermore, the concentration of MBT increases as the concentration of riboflavin increases (Sakuma et al., 1991).

Several important fruity aroma thiol-containing compounds in wine have been studied. For example, 3-mercapto-1-hexanol (3MH) and 4-

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mercapto-4-methyl-2-pentanone (4MMP) in wine are present as cysteine conjugates (3MH-cys, 4MMP-cys) in fruit juice. 3MH and 4MMP are enzymatically cleaved by β -lyase during fermentation (Tomimaga, Peyrot des Gachons, Dubourdiou, 1998; Peña-Gallego, Hernández-Orte, Cacho, & Ferreira, 2012). Similarly, the cysteine conjugate of 3M3MB (3M3MB-cys) has been detected in hops, and yeast β -lyase may catalyze the formation of 3M3MB during the brewing of beer (Cibaka, Gros, Nizet, & Collin, 2015).

Nonetheless, many details regarding the 2M3MB precursor remain unknown. It has previously been proposed that 3-methyl-2-buten-1-ol (3MBol) is a precursor of 2M3MB (Olsen et al., 1988). A more recent report indicated that hydrogen sulfide reacts with 3MBol to produce 2M3MB in a model system (Vermeulen, Lejeune, Tran, & Collin, 2006). Furthermore, both 2M3MB and 3M3MB were formed after the fermentation of beer at the pilot plant scale following the addition of 3MBol (Gros, Nizet, & Collin, 2009). However, recent quantitative studies revealed that 3MBol is not the major precursor of 2M3MB in beer (Noba et al., 2017). When 3MBol was added during fermentation, both 2M3MB and 3M3MB were formed but the conversion rate was very low, with an essentially negligible amount of 2M3MB being formed from the 3MBol in wort. The concentration of 3MBol did not increase upon the hot aeration of wort, in contrast to the formation of 2M3MB. 3MBol is therefore unlikely to be a factor in 2M3MB formation. In addition, 2M3MB was not formed from unhoppled wort during fermentation, whereas 3M3MB was formed from either unhoppled or hoppled wort. Consequently, the main precursors of 2M3MB and 3M3MB are likely to be different compounds.

Taken together, it appears that the precursor of 2M3MB has not yet been identified, but is present in hops. In this study, we attempted to identify the major precursor of 2M3MB in an isomerized hop extract as the first step to suppressing the formation of onion-like off-flavor during beer production. Various chromatographic techniques for volatile compounds were used to separate the components responsible for the formation of 2M3MB, followed by structural analysis using high-resolution GC/MS. The identified compound was added to wort and the conversion rate to 2M3MB by yeast was investigated.

2. Materials and methods

2.1. Chemicals

Methanol (pesticide residue analysis grade), ammonia solution (28.0%), acetonitrile (LC–MS grade), dichloromethane (pesticide residue analysis grade), diethyl ether (pesticide residue analysis grade), *n*-pentane (Cica-special grade), formic acid (98.0%), ammonium sulfate (99.5%), and d-glucose (98.0%) were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Silica Gel 60 (spherical, neutral) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Yeast extract and peptone were purchased from BD Japan (Tokyo, Japan). *S*-Benzyl-l-cysteine (benzyl-cys) (95.0%) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). *S*-2-(3-Methyl-1-butanol)-l-cysteine (2M3MB-cys) (95.0%) and 2,3-epoxy-3-methylbutanal (95.0%) were obtained from NARD Institute, Ltd. (Hyogo, Japan).

2.2. Quantification of 2M3MB-cys in wort

Wort was pretreated by solid-phase extraction as follows. Methanol (10 mL) and 10 mL of 2% formic acid in water were loaded sequentially onto a Waters MCX column (500 mg/6 mL), then 10 mL of wort containing 0.4 mL of formic acid and 100 μ L of benzyl-cys (1 μ g/mL) as internal standard (IS) were loaded onto the column. The column was washed with 5 mL of 2% formic acid in water, followed by 4 mL of methanol. 2M3MB-cys was eluted with 5 mL of 28% ammonia solution/methanol (5/95). The eluate was dried at room temperature under a nitrogen gas stream and dissolved in 1 mL of 0.1% formic acid in water. Each sample was filtered through a 0.45- μ m polytetrafluoroethylene

(PTFE) filter immediately before LC–MS/MS analysis. 2M3MB-cys analysis was conducted using an API 4000 tandem mass spectrometer (SCIEX, Framingham, MA) equipped with a Nexera liquid chromatograph (Shimadzu Corp., Kyoto, Japan), a SunShell RP-AQUA analytical column (3 mm i.d. \times 150 mm, 2.6 μ m; ChromaNik Technologies, Osaka, Japan), and operated at 40 °C. The mobile phase consisted of (A) 0.1% v/v formic acid-water and (B) 0.1% v/v formic acid-acetonitrile. A linear gradient profile with the following proportions (v/v) of solvent B was applied at a flow rate of 0.3 mL/min: (*t* (min), % B: (0, 0), (1, 0), (11, 60), (11.1, 95) and (15, 95)). The total run time, including the conditioning of the column to the initial conditions, was 25 min. An autosampler was used to inject sample volumes of 10 μ L. Quantification by MS/MS was carried out in multiple reaction monitoring (MRM) mode using the following conditions: electrospray-ionization (ESI) source, positive ion mode; curtain gas, 50 psi; ion source gas 1, 80 psi; ion source gas 2, 80 psi; ionspray voltage, +4500 V; collision gas, 8 (arbitrary units); declustering potential, 51 V; and temperature, 500 °C. Transitions (*m/z*), collision energy (CE (V)), and the collision cell-exit potential (CXP (V)) were as follows: 208 \rightarrow 87, 25 V, 6 V (2M3MB-cys for quantification), 208 \rightarrow 69, 15 V, 8 V (2M3MB-cys for confirmation), 212 \rightarrow 91, 21 V, 4 V (benzyl-cys, IS). A calibration curve for quantification was generated using the standard addition method. Specifically, 2M3MB-cys was added to wort at 0–20 ng/mL, and the ratios of the peak areas of 2M3MB-cys and the IS were determined.

2.3. Brewing processes

Wort preparation was carried out based on the method of Analytica-EBC (EBC Analytica, Method 4.5.1) and fermentation was carried out as previously reported (Noba et al., 2017). Nugget (11.6% alpha acid; John I. Haas, Inc., Yakima, WA) was used for hopped wort. Unhopped wort was prepared similarly except that hop addition and boiling were omitted. The concentration of 2M3MB in beer was analyzed as reported previously (Noba et al., 2017). For the 2M3MB-cys addition test, 2M3MB-cys dissolved in ethanol was added to wort at concentrations of 20, 200 and 2000 ng/mL. Samples at 200 ng/mL were tested in triplicate.

2.4. Measurement of the producing potential of 2M3MB using yeast extract-peptone-dextrose (YPD) medium

Yeast extract (2%), peptone (4%), and glucose (4%) were added to distilled water (all w/v); then the solution was sterilized at 121 °C for 15 min to prepare a 2-fold concentrated YPD medium. Using a 200-mL plastic graduated cylinder, 100 mL of 2-fold concentrated medium and fractions obtained by purifying isomerized hop extract were mixed together, and the liquid volume was adjusted to 200 mL with sterilized water. Brewer's yeast (20×10^6 cells/mL) was added to the medium. Static fermentation was conducted at 15.0 °C for 3 days in a 200-mL plastic graduated cylinder, then the fermented liquid was centrifuged at 9000g for 15 min. The obtained supernatant was used for the quantification of 2M3MB.

2M3MB was quantified as described previously for the analysis of beer (Noba et al., 2017). The obtained 2M3MB concentration was used as the 2M3MB-producing potential of the purified fraction.

2.5. Purification of the 2M3MB precursor from hop extract

The 2M3MB precursor was purified from isomerized hop extract (30%; Hopsteiner, Inc., New York, NY). Purification was carried out by the following steps: oxidation pretreatment of hop extract by hot aeration \rightarrow atmospheric distillation \rightarrow fractionation by normal-phase column chromatography \rightarrow fractionation by preparative GC.

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