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Presence of Disulfide-Bonded Thiols in Malt and Hops as the Precursors of Thiols in Beer

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ABSTRACT: Disulfide-bonded thiols in malt and hops were first identified as possible precursors of thiols in beer. The presence of disulfide-bonded 3-mercaptohexan-1-ol (3MH) was confirmed in malt and hops by observing an 8.9−9.9 times increase in the 3MH concentration in hopped water and unhopped wort after the reduction using tris(2-carboxyethyl)phosphine (TCEP), a reducing agent specific for disulfide bonds. The presence of disulfide-bonded 4-mercapto-4-methylpentan-2-one (4MMP) was confirmed in hops by observing 2.1 and 5.1 times increase in the 4MMP concentration after reduction in hopped water. Proteins, peptides, and amino acids having sulfhydryl groups or other thiol substances were assumed to form disulfide bonds with polyfunctional thiols in malt and hops. The release of thiols by the reduction of disulfide-bonded thiols during fermentation was first identified. A 65−82% of disulfide-bonded 3MH were reduced during fermentation, and as a result, concentrations of 3MH in hopped water and unhopped wort increased by 9.5−14.2 times during fermentation.

KEYWORDS: *disulfide-bonded 3-mercaptohexan-1-ol, 3MH, precursor, reduction during fermentation, 4-mercapto-4-methylpentan-2-one, 4MMP, tris(2-carboxyethyl)phosphine, hop, unhopped wort*

■ INTRODUCTION

Polyfunctional thiols have been drawing attention as important contributors to the characteristic aroma of beer owing to their characteristics and ng/L levels of threshold values.[1−8]

Several studies on polyfunctional thiols as 4-mercapto-4-methylpentan-2-one (4MMP) and 3-mercaptohexan-1-ol (3MH) in beer and wine have investigated the presence of not only free type of compounds but also its nonvolatile precursors, such as the S-cysteinylated and S-glutathionylated forms. These precursors—present in malts and hops—are transformed by yeast to release free volatile thiols from the conjugated compounds via the activity of the *β*-lyase enzyme.[9−11] Since the S-cysteinylated precursor was first identified in Sauvignon Blanc by Tominaga et al. in 1998,[12] the release mechanism have been widely accepted as the main source of volatile thiols.[13−20]

However, the molar concentrations of these precursors in the raw materials are extremely higher than those of the free volatile thiols. A direct correlation between the concentration of the precursor in the raw materials and the free volatile thiols in the resulting beers has not been reported in previous studies. Studies on wine also reported that there is no correlation between the concentrations of precursors and corresponding thiols, and that S-cysteinylated forms are not the major precursors,[21,22] wherein the simultaneous presence of other precursors is supposed.

Considering the structure of thiols, the presence of disulfide bonds—covalent bonds derived by the coupling of two thiol groups—is expected. The disulfide bond is common in sulfhydryl group binding. The disulfide bond is the weakest link in many molecules, with a typical dissociation energy of 60 kcal/mol, which is ∼40% lower than those of C−C and C−H bonds.[23] In biology, disulfide bonds between thiol groups of cysteine residues are important components of the secondary and tertiary structure of proteins, and the bonds between cysteine amino acids are created via a process known as oxidative folding, which involves many enzymes.[24] The formation of disulfide-bonded thiols as the oxidation products were also investigated in wine and beer in previous reports.[25,26]

In this study, the presence of disulfide-bonded thiols, as the possible precursors of thiols, were examined in malt and hops. Protein-rich and amino-acid-rich cereals, such as barley hence malt, which are known to contain many disulfide bonds, are supposed to contain disulfide-bonded substances.

In the current study, 3MH was used as the indicator to examine the presence and behavior of disulfide-bonded thiols in malt and hops because 3MH exists in both malt and hops while some polyfunctional thiols such as 4MMP are found exclusively in hops.[20,27] A simple system of unhopped wort and hopped water was used in this study to prevent interference between malt- and hop-derived 3MH.

■ MATERIALS AND METHODS

Materials for Brewing. A pilsner-type malt, with a moisture content of 4.3%, Kolbach index (KI) of 44%, and a diastatic power of

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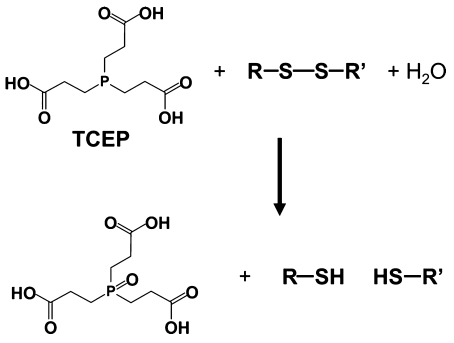

Figure 1. Reduction of the disulfide bond using TCEP.[31]

350 °WK, was purchased from Rahr Malting Co. (MN, USA). Hop varieties Citra and Simcoe were purchased from Yakima Chief (WA, USA). Bottom-fermenting sludgy yeast was purchased from Chuetsu Yeast Co., Ltd. (Niigata, Japan).

Chemicals. 3-Mercaptohexan-1-ol-d_5 (>95%), 4-mercapto-4-methylpentan-2-one-d_{10} (>95%), 3-*S*-(1-hydroxyhexyl)-cysteine (>95%), and 3-*S*-(1-hydroxyhexyl)-glutathione (>95%) were purchased from aromaLAB AG (Freising, Germany). 3-Mercaptohexan-1-ol (>98%) and 4-mercapto-4-methylpentan-2-one (>98%) were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Tris(2-carboxyethyl)phosphine hydrochloride was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). MetaSEP IC-Ag was purchased from GL science (Tokyo, Japan). 1,4-Dithiothreitol was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). D(+)-Glucose was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan).

Small-Scale Brewing of Unhopped Beer. To 2 L of distilled water, 341 g of milled pilsner malt was added, and the mixture was proteolyzed at 40 °C for 30 min. The mash was then saccharified at 68 °C for 30 min, held at 78 °C for 5 min, and filtered. Unhopped wort was boiled for 60 min and settled for 20 min until the trub had precipitated; subsequently, 1 L of the supernatant was obtained. Water was added to bring the extract concentration of the wort to 12.0%, which was then cooled to 4 °C. Sludgy bottom-fermenting yeast was pitched into the wort at a rate of 20×10^6 cells/mL, and the mixture was fermented with stirring at 1000 rpm for 7 days at 15 °C. After 7 days of fermentation, the beer was centrifuged at 10,600 × *g* for 30 min and the supernatant beer was analyzed.

Fermentation of Hopped Water. Citra (13.0% alpha acids; USA) and Simcoe (12.5% alpha acids; USA) hop pellets (10 g each) were added to 1 L of water and stirred at 1000 rpm for 12 h at room temperature to obtain hopped water, which was used for the analysis of 3MH and 4MMP. Next, 140 g of glucose was added to hopped water to bring the extract concentration of the solution to 12.6% (w/w). Sludgy bottom-fermenting yeast was pitched into hopped water at a rate of 70×10^6 cells/mL, and the mixture was fermented with stirring at 1000 rpm for 7 days at 15 °C to produce liquor with an alcohol content of 5.5% (w/w) and a real extract of 1.6% (w/w). After 7 days of fermentation, the beer was centrifuged at 10,600 × *g* for 30 min and analyzed.

Analysis of Beer Properties. Apparent extract and alcohol content was determined using an Alcolyzer ME system equipped with DMA5000M (Anton Paar GmbH, Graz, Austria). Amino acids were analyzed using an amino-acid analyzer (LA8080 AminoSAAYA, Hitachi High-Tech Corporation, Tokyo, Japan).

Quantification of 3MH and 4MMP Concentrations. A total of 30 mL of ethyl acetate was added to 100 mL of the sample including 3MH-d_5 and 4MMP-d_{10} as the internal standards. Next, the extraction of odorants was conducted by gentle stirring to prevent the formation of an emulsion. The ethyl acetate layer was collected, and specific extractions of 3MH and 4MMP from the obtained organic phase were performed using MetaSEP IC-Ag according to a previously reported method.[32] The concentrated extracts were injected into an Agilent 8890 GC system (Agilent Technologies, CA, USA) and separated using a DB-WAX UI capillary column (30 m length × 0.25 mm inner diameter; film thickness = 0.25 μm; Agilent Technologies). Ionization was performed using an MS Triple Quad system (Agilent 7000D, Agilent Technologies) operated at the electron ionization (EI) and multiple reaction monitoring (MRM) mode, with the precursor-to-product ion set at *m*/*z* 134-82 for 3MH, with a collision energy of 2 eV; *m*/*z* 139-86 for 3MH-d_5, with collision energy of 2 eV; *m*/*z* 132-89 and 132-99 for the quantitative and qualitative ionization of 4MMP, respectively, with a collision energy of 4 eV; and *m*/*z* 142-96 for the quantitative and qualitative ionization of 4MMP-d_{10}, with a collision energy of 4 eV. The standard curves were established using unhopped wort, beer, fermented glucose solution, and water. A quantification system with a calibration curve with R^2 values of >0.99 were obtained (data not shown) as described in a previous report.[32] Figures 2, 3, 5, 6, 8, and 9 indicate the averages and standard deviations of the duplicated analysis.

■ RESULTS AND DISCUSSION

Minimum Effective Concentration of TCEP. Tris(2-carboxyethyl)phosphine (TCEP) hydrochloride was used to reduce the disulfide bond. TCEP is stable in aqueous solutions and selectively reduces disulfide bonds, being nonreactive toward other functional groups (Figure 1).[28,29] Furthermore, TCEP reduces disulfide bonds more effectively than 1,4-dithiothreitol (DTT) in a lower pH range at room temperature.[30] The minimum effective concentration of TCEP was examined in unhopped wort and hopped water with the Simcoe variety.

As shown in Figure 2, 10 mM of TCEP provided a sufficient molar excess for the effective reduction of disulfide-bonded 3MH and 4MMP in unhopped wort and hopped water. Because it was supposed that the concentration of disulfide bonds varies between the malt and hop varieties, for a complete reduction, 50 mM that is the maximum concentration of TCEP generally used[28,29] was adopted in this study.

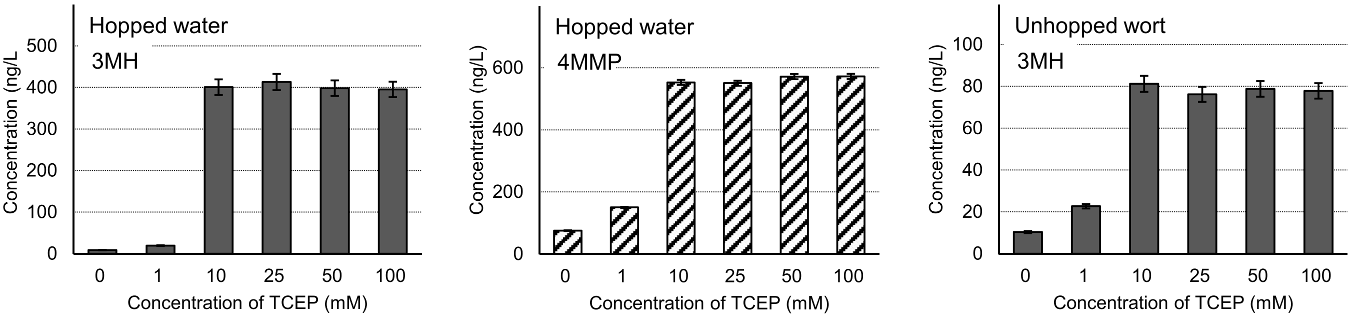

Figure 2. Effective concentration of TCEP for the reduction of disulfide-bonded 3MH in unhopped wort and 3MH and 4MMP in hopped water.

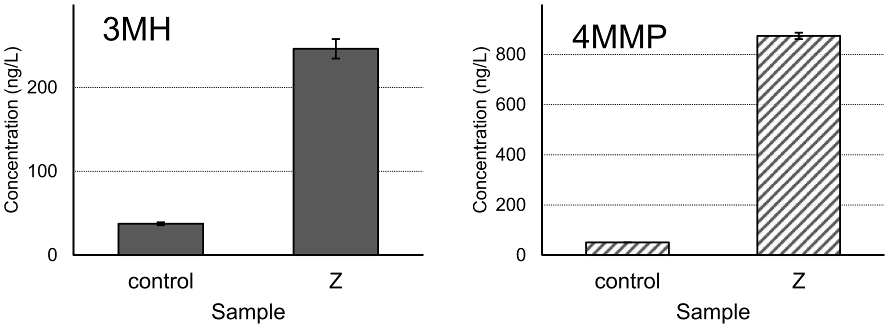

Figure 3. Increases in 3MH and 4MMP concentrations after adjusting the pH of hopped water to 8.0. Hopped water with pH adjusted to 8.0 is Sample Z, and the control is hopped water with Simcoe.

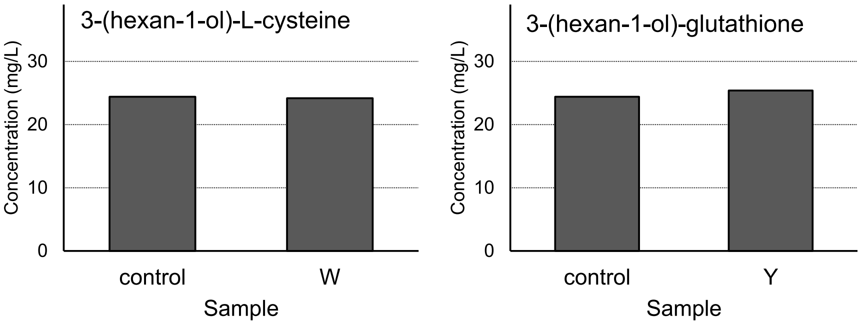

Figure 4. Concentrations of TCEP-treated 3-*S*-(1-hydroxyhexyl)-cysteine (Sample W), TCEP-treated 3-*S*-(1-hydroxyhexyl)-glutathione (Sample Y), and controls containing 24 mg/L of corresponding pure substances.

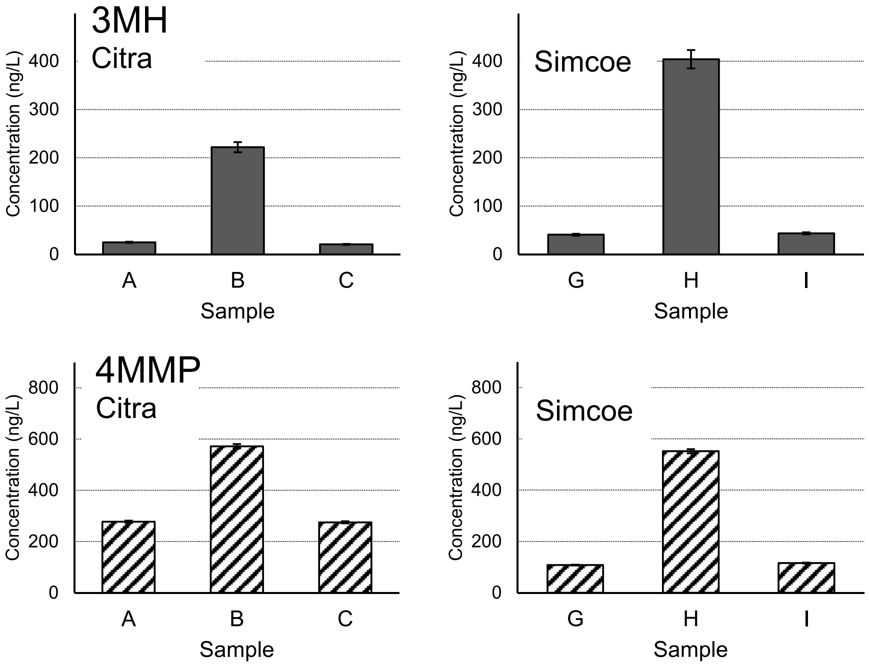

Figure 5. Concentrations of 3MH and 4MMP in hopped water (control; Samples A, G), TCEP-treated hopped water (Samples B, H), and hopped water with pH adjusted to 2.3 (samples C, I).

For the treatment, 50 mM of TCEP was added to each of the 100 mL samples and incubated at 20 °C for 3 h.

Another reducing agent DTT was also examined. However, DTT can exhibit its reducing power under alkaline conditions. As shown in Figure 3, the concentrations of 3MH and 4MMP increased significantly by simply adjusting the pH value of hopped water to 8 and incubating at 20 °C for 3 h (Sample Z). In this case, it was not possible to identify if the increases in 3MH and 4MMP concentrations were due to the dissociation of disulfide bonds. Furthermore, quantitative chromatograms and results were not obtained in the analysis of the sample including DTT and β-mercaptoethanol; because the commonly used reducing agents as DTT and β-mercaptoethanol contain sulfhydryl groups, it was supposed that the higher concentration of DTT in the sample interfered the adsorption of polyfunctional thiols to the MetaSEP IC-Ag column on the

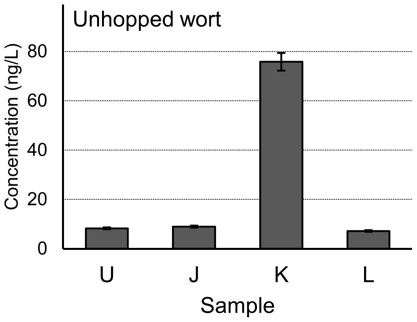

Figure 6. Concentrations of 3MH in unhopped wort before boiling (sample U) and after 60 min boiling (control; sample J), TCEP-treated unhopped wort before boiling (sample K), and unhopped wort with pH adjusted to 2.4 (sample L).

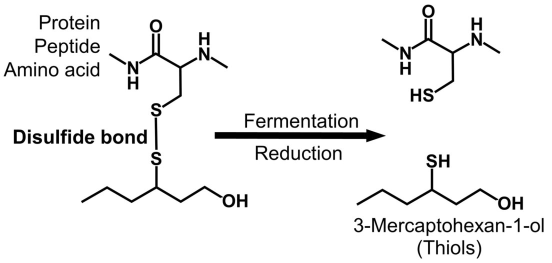

Figure 7. Precursor of thiols proposed in this study.

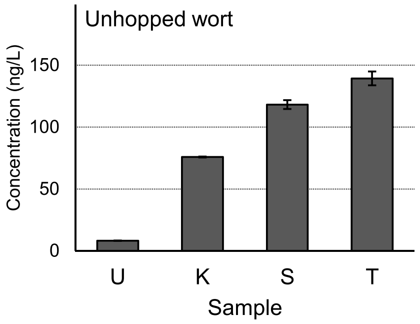

Figure 9. Concentrations of 3MH in unhopped wort before boiling (control; Sample U), TCEP-treated unhopped wort before boiling (Sample K), fermented unhopped wort (Sample S), and TCEP-treated fermented unhopped wort (Sample T).

extraction step. Therefore, DTT and β-mercaptoethanol could not be used as the reducing agent in this study.

For the preliminary experiment, 10 mL of distilled water containing 24 mg/L of 3-*S*-(1-hydroxyhexyl)-cysteine (Sample W) and 3-*S*-(1-hydroxyhexyl)-glutathione (Sample Y) were prepared; the solutions were treated with 50 mM of TCEP and incubated at 20 °C for 3 h, and then, degradation was analyzed. As shown in Figure 4, the degradations of the S-cysteinylated and S-glutathionylated 3MH were not observed after the TCEP treatment.

TCEP Treatment of Hopped Water Containing Citra, Simcoe, and Unhopped Wort. The TCEP treatment was applied to hopped water and unhopped wort. As shown in Figure 5, a substantial increase in the 3MH concentrations was observed in TCEP-treated hopped water, by 8.9 and 9.9 times for Citra (sample B) and Simcoe (sample H) varieties compared to the control samples (A, G), respectively. An 9.1 times increase in the 3MH concentration was also observed for unhopped wort (sample K), as shown in Figure 6. No increase in the 3MH concentration was observed from before (sample U) to after (sample J) boiling of unhopped wort, indicating that malt-derived disulfide-bonded 3MH was not affected by the boiling process.

Because the pH of hopped water and unhopped wort dropped to 2.3 and 2.4 upon the addition of TCEP, the pH-adjusted wort samples (Samples C, I, and L) were prepared using HCl. An increase in the 3MH concentration was not observed in the pH-adjusted samples, indicating that the increases were not caused by the pH decrease but by the reduction of the disulfide-bonded 3MH upon TCEP addition. For reference, a substantial increase, 2.1 and 5.1 times increase in the 4MMP concentration, was observed in TCEP-treated hopped water, as shown in Figure 5. These proved that disulfide-bonded 3MH was present in both malt and hops, and disulfide-bonded 4MMP was present in hops.

Disulfide Bond Reduction During Fermentation. Many types of proteins, peptides, and amino acids containing sulfhydryl groups or other thiol substances were expected to form disulfide bonds with polyfunctional thiols in malt and hops and expected to undergo reduction, as shown in Figure 7. The reduction of both malt- and hop-derived disulfide-bonded 3MH during fermentation was investigated by analyzing the 3MH concentration.

To proceed with the fermentation while eliminating the influence of malt-derived 3MH, glucose was added as the sugar source to hopped water with Citra and Simcoe, which were then fermented for 7 days at 15 °C. After the fermentation, the liquor with an alcohol content of 5.5% (w/w), a pH value of 3.4, and a real extract of 1.6% (w/w) were obtained, which indicated that the fermentations were successfully completed.

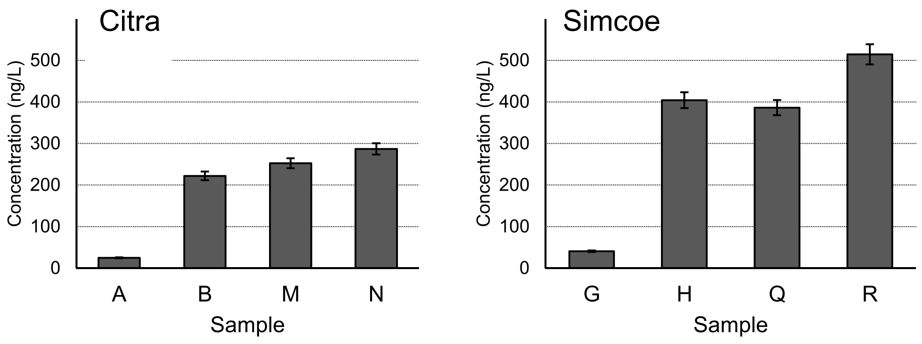

Figure 8. Concentrations of 3MH in hopped water (control; Samples A, G), TCEP-treated hopped water (Samples B, H), fermented hopped water (Samples M, Q), and TCEP-treated fermented hopped water (Samples N, R).

Table 1. Concentrations of 3MH in Various Samples and Reduction Rates of the Disulfide Bonds during Fermentation

	unit	formula	Citra		sample	Simcoe		sample	unhopped wort		sample
(1) free 3MH in hopped water or unhopped wort	ng/L		24.9	(±1.2)	A	40.7	(±1.9)	G	8.3	(±0.4)	U
(2) free 3MH in TCEP-treated hopped water or unhopped wort	ng/L		222.0	(±10.5)	B	404.1	(±19.1)	H	75.8	(±3.6)	K
(3) free 3MH derived from disulfide-bonded 3MH in hopped water or unhopped wort	ng/L	(2)-(1)	197.1			363.4			67.5		
(4) free 3MH in fermented liquor	ng/L		252.4	(±11.9)	M	386.3	(±18.3)	Q	118.2	(±5.6)	S
(5) free 3MH in TCEP-treated fermented liquor	ng/L		287.0	(±13.6)	N	514.5	(±24.3)	R	139.3	(±6.6)	T
(6) free 3MH increased from sources other than reduction of disulfide bonds during fermentation	ng/L	(5)-(2)	65.0			110.4			63.5		
(7) free 3MH derived from the reduction of disulfide bonds in the liquor	ng/L	(4)-(6)-(1)	162.5			235.2			46.4		
(8) Reduction ratio of disulfide bonds during fermentation	%	(7)/(3) × 100	82.4			64.7			68.7		

As shown in Figures 8 and 9, a substantial increase in the 3MH concentration from before (samples A, G, and U) to after fermentation (samples M, Q, and S) was observed by 10.1, 9.5, and 14.2 times for Citra, Simcoe, and unhopped beer, respectively.

The TCEP treatment was then applied to the fermented hopped water and unhopped beer. A slight increase in the 3MH concentration was observed for TCEP-treated liquor hopped with Citra (sample N), Simcoe (sample R), and unhopped beer (sample T). This result, the slight increase, indicated that the large part of disulfide-bonded 3MH were already reduced during fermentation.

The concentrations of 3MH in the samples shown in Figures 8 and 9 are listed in Table 1. The reduction rates of the disulfide-bonded 3MH during fermentation could be estimated, as shown in Table 1, although precipitation, volatization of 3MH, or adsorption of 3MH to the yeast cell surface during fermentation may fluctuate the rates. Disulfide-bonded 3MH in the Citra- and Simcoe-hopped water (82.4 and 64.7%) and 68.7% of disulfide-bonded 3MH in unhopped wort were reduced during fermentation.

Future Studies. The presence of disulfide-bonded precursors of other polyfunctional thiols is examined in future studies. In the current study, the presence of disulfide-bonded 3MH and 4MMP could be confirmed even in the hop plant, which is not considered rich in protein and amino acids. The presence of disulfide-bonded polyfunctional thiols in other raw materials, as grapes, would be investigated in future research. Yeast strains may differ in their ability to reduce the disulfide bond; therefore, a detailed examination of them would also be necessary for the development of attractive beer products.

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Notes

The authors declare no competing financial interest.

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