Sake (Rice Wine) Brewing Hydrolyzes Highly Polar Sphingolipids to Ceramides and Increases Free Sphingoid Bases

Shinji Yamashita¹, Chisato Higaki¹, Nobuhiro Kikuchi², Daisuke Suzuki³, Mikio Kinoshita¹*, and Teruo Miyazawa⁴

¹ Department of Life and Food Sciences, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-8555, JAPAN
² Fukushima Technology Centre, Aizuwakamatsu Technical Support Centre, Aizuwakamatsu, Fukushima 965-0006, JAPAN
³ Suzuki Shuzouten Co., Ltd., 10 Higashimukai, Ukedo, Namie-town, Fukushima 979-1522, JAPAN
⁴ Food and Biotechnology Platform Promoting Project, New Industry Creation Hatchery Center (NICHe), Tohoku University, Sendai, Miyagi 980-8579, JAPAN

Abstract: In plants and fungi, sphingolipids, characterized by the presence of a sphingoid base (SB), comprise neutral classes, including ceramide (Cer) and glucosylceramide (GlcCer), and acidic classes, including glycosyl inositol phosphoryl ceramide (GIPC). The major class of plant and fungal sphingolipids is GIPC; however, owing to their complicated extraction and analysis, there is still little information regarding the food characteristics of GIPC compounds. In the present study, we evaluated the content and SB composition of highly polar sphingolipids (HPS) in materials that had been obtained from our previous food processing study for GlcCer and Cer. This assessment was based on the changes that occur in HPS containing GIPC in sake rice (saka-mai) during the rice polishing and sake (rice wine) brewing process. In addition, we report a new investigation into the composition of sphingolipids in koji rice and sake yeast. HPS levels were the highest among the sphingolipid classes in brown rice cultivars and highly polished rice. Sake and sake lees (sake-kasu) were produced using three different starter cultures. In sake lees, Cer levels were the highest among the classes, while HPS was greatly reduced based on the amount of highly polished rice and koji rice, and these HPS were mainly composed of sphinganine (d18:0), which is a minor SB in highly polished rice, koji rice, and sake yeast. In addition, considerable levels of free SBs, mainly comprising d18:0, were detected in sake lees. The levels of HPS and free SBs in sake lees were dependent on the starter culture. These results suggest that HPS was hydrolyzed to Cer and that sake yeast also affected the levels of Cer and free SBs during brewing. One interesting question raised by these results is whether changes in the class and base compositions of sphingolipids during brewing contribute to taste of the final product and other food functions.

Key words: GIPC, lees, rice, sake, yeast

1 Introduction

Complex sphingolipids comprise a sphingoid base (SB) with an amide-linked fatty acid (i.e., ceramide, Cer) and a polar head group, such as phosphocholine or hexose. Plant and fungal sphingolipids contain neutral classes, mainly Cer and glucosylceramide (GlcCer), and acidic classes, including inositol phosphoryl ceramide (IPC) and glycosyl IPC (GIPC). In addition, di-, tri-, and tetra-glucosylceramides as neutral classes are also reported to exist in plants, including rice. Plant sphingolipids have a highly diverse SB composition with Δ8-unsaturation. Plant GlcCer can possess different base compositions depending on the plant species, whereas fungal GlcCer mainly comprises a fungi-specific base, 9-methyl-trans-4,trans-8-sphingadienine (9-Me d18:2Δ8Δ6). Cer, IPC, and GIPC mainly comprise the trihydroxy bases, 4-hydroxyphosphosphinganine (phytosphingosine, t18:0) and 4-hydroxy-8-sphinganine (t18:1Δ6) in plants, and t18:0 and 4-hydroxyicosasphinganine (t20:0) in fungi.

Sphingolipids play important roles in biological and food...
S. Yamashita, C. Higaki, N. Kikuchi et al.

2 Experimental Procedures

2.1 Sake rice and procedures for polishing and brewing

Six sake rice samples (Oryza sativa 'A' to 'F') were purchased from a rice wholesaler in Japan. For sake brewing, koji (Aspergillus oryzae 'Kroban-moyashi B'), 90% lactate (food grade), and sake yeast (Saccharomyces cerevisiae 'A-6') were purchased from a koji maker (Kojiya Sanzaemon Co., Ltd, Toyohashi, Japan), Showa Chemical Industry Co., Ltd, Japan, and Iwaki-Kotobuki (Suzuki Shuzouten Co., Ltd., Iwaki, Japan), respectively. For additional analysis, we purchased two dried sake yeasts (Kyokai no. 701 and no. 901) from the Brewing Society of Japan, Tokyo, Japan, and commercial koji rice from a local market in Hokkaido, Japan.

Figure 1 shows the polishing and brewing procedures used in this study. Brown rice F (Omachi) was polished to 65%, and the rice bran was divided into three types (aka-nuka, naka-nuka, and shiro-nuka from the external bran) using a sake-rice-polishing machine. After the polished rice was steamed, it was used as koji rice (an enzyme cocktail containing amylases) and added to rice (kake-mai, a carbohydrate source). Koji rice was prepared by seeding koji (g/100 kg rice; at starter culture, 70, 60, and 60 g for sokujo-moto, ki-moto, and yamahai-moto styles, respectively; at the first stage of fermentation mash preparation, 70, 50, and 50 g; at the second stage, 50, 50, and 50 g; at the third stage, 50, 40, and 40 g) onto steamed rice and was cultured.

Sake and sake lees were produced by three starter cultures using the same materials (koji rice, adding rice, and sake yeast), while these starter cultures differed in terms of lactate source, rice-grinding process, and fermentation time. The sokujo-moto style is prepared by adding 90% lactate (700 mL/L water of starter culture). The ki-moto style is prepared from natural Lactobacillus, obtained from the air of the brewery by incubating the starter culture at low temperature for long time, with a rice-grinding process. The yamahai-moto style is prepared from natural Lactobacillus without a grinding process.

In terms of starter preparation, sokujo-moto, ki-moto, and yamahai-moto styles required 8, 29, and 27 days for growing a starter culture and 4, 31, and 26 days for conditioning (karashi), respectively. Subsequently, for the fermentation mash (moromi) preparation, koji rice, steamed rice, and water were added to the starter in three-steps. The sokujo-moto, ki-moto, and yamahai-moto styles were 210, 700, and 700 kg, respectively; the ratio of koji rice to total rice for sokujo-moto, ki-moto, and yamahai-moto styles were 20.5%, 21.9%, and 21.9%, re-
respectively. The ratio of rice used for starter preparation to total rice (shubo-buai) for sokujo-moto, ki-moto, and yamahai-moto styles were 6.67%, 7.14%, and 7.14%, respectively. The ratio of water to rice (kumimizu-buai) for sokujo-moto, ki-moto, and yamahai-moto styles were 129.0%, 135.7%, and 135.7%, respectively. The ratio of sake lees to total rice (kasu-buai) for sokujo-moto, ki-moto, and yamahai-moto styles were 53.5%, 38.1%, and 35.9%, respectively; the ratio for sokujo-moto was higher because of insufficient filtration caused by an issue with the pump. The ratio of dried sake lees to total rice were 14.3%, 14.4%, and 13.1%, respectively. The alcohol concentrations of sake were 17.2%, 16.2%, and 16.2% in sokujo-moto, ki-moto, and yamahai-moto styles, respectively. The final sake volume produced from 100 kg of total rice was 164.1, 206.1, and 229.4 L, respectively.

2.2 Lipid analysis

The GlcCer, Cer, and HPS in the samples were separated, and their SB composition was analyzed using a slight modification of previous methods. Briefly, crude lipids were extracted using a chloroform/methanol/water solvent.
system, and the dried weight of the organic layers was considered as the total lipid amount. Sphingolipids in the water layers were assumed to be HPSs. GlcCer and Cer in the organic layers were alkaline-treated and separated by thin-layer chromatography (TLC). The SBs in sphingolipid structures were prepared for fatty aldehydes and analyzed using gas chromatography–mass spectrometry (GC–MS). The GC-MS system was equipped with a GC-2030 and GC-MS-QP2020NX instrument (Shimadzu, Kyoto, Japan). For fatty acid analysis, the samples of koji rice and sake yeast were directly methylated and determined using GC–MS.

For analysis of the sake yeast sphingolipids, the fractions were treated with trimethylsilylation (TMS) and analyzed using GC–MS. Free SBs were separated using TLC (mobile phase, chloroform/methanol/2 M ammonia solution = 80/20/2 by vol.), derivatized with o-phthalaldehyde (OPA), and analyzed using a high-pressure liquid chromatography–fluorescence detector. C1P and SB-1-phosphate (SB1P) were confirmed using TLC and Dittmer reagent which detects phosphorus.

### 3 Results

#### 3.1 HPS levels and SB composition in the sake rice cultivars

The HPS amounts and SB compositions of the brown rice samples are shown in Table 1. We found that HPS contained the highest levels of sphingolipid classes in all brown rice cultivars. The HPS content was the highest in rice C, followed by B, D, F, and A. The levels and ratios of sphingolipid classes varied depending on the cultivar. The base composition of HPS in brown rice was almost the same in all the cultivars; the predominant base in HPS was t18:0.

#### 3.2 HPS distribution during the polishing process

We compared HPS in brown rice, highly polished rice, and three types of rice bran (aka-nuka, naka-nuka, and shi-
Changes in Sphingolipid Levels During Sake Brewing

Table 3  Effects of brewing process on contents and sphingoid base (SB) profiles of highly polar sphingolipids (HPS) in sake rice F.

<table>
<thead>
<tr>
<th></th>
<th>HPS/Cer</th>
<th>HPS/GlcCer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Highly polished rice</td>
<td>1.39</td>
<td>1.49</td>
</tr>
<tr>
<td>Sokujo-moto sake lees</td>
<td>0.15</td>
<td>0.48</td>
</tr>
<tr>
<td>Ki-moto sake lees</td>
<td>0.03</td>
<td>0.12</td>
</tr>
<tr>
<td>Yamaihai-moto sake lees</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td>Sokujo-moto sake</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Ki-moto sake</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Yamaihai-moto sake</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

1 nmol/100 g dry wt. or nmol/100 mL sake.

3.3 Changes in HPS during brewing

We investigated the effect of brewing on rice HPS using three starter cultures (Table 3). Among the sake lees, the content of HPS was the highest in sokujo-moto, lower in ki-moto, and the lowest in yamaihai-moto. Compared to highly polished rice as a source, the HPS/Cer ratio in sake lees was markedly low and dependent on starter cultures. In terms of SB composition in HPS, all three sake lees had a much higher ratio of d18:0 and a much lower ratio of t18:0 than highly polished rice.

3.4 Compositions of sphingolipid classes and SBs in koji rice and sake yeasts

To clarify the contribution of lipids contained in koji rice and sake yeast to sake and sake lees, we analyzed the sphingolipid profiles (Table 4). HPS was the most predominant class of sphingolipids in koji rice, whereas HPS in sake yeasts had almost the same levels as Cer. In terms of the SB composition of koji rice, Cer and HPS mainly comprised t18:0, and GlcCer predominantly comprised fungi-specific 9Me-d18:2. With respect to the SB composition of sake yeasts, we note that all sphingolipid classes contained fungi-specific t20:0.

When the total rice used for brewing was converted to sphingolipid content of highly polished rice along with koji rice, the levels of Cer, GlcCer, and HPS were 1,904, 1,240, and 7,833 nmol in 100 g wet wt. of total rice as the source in sake yeasts and had almost the same levels as Cer. In terms of the SB composition of koji rice, Cer and HPS mainly comprised t18:0, and GlcCer predominantly comprised fungi-specific 9Me-d18:2. With respect to the SB composition of sake yeasts, we note that all sphingolipid classes contained fungi-specific t20:0.

The corresponding values for GlcCer were 1,825, 2,535, and 2,024 nmol, respectively. The levels of HPS in sake lees were 884, 299, and 59 nmol, respectively. Compared to the total rice used, the Cer level in sake lees of sokujo-moto, ki-moto, and yamaihai-moto styles were 3.2, 5.8, and 5.0 times greater, respectively, and the GlcCer levels in those lees were 1.5, 2.0, and 1.6 times greater, respectively,
whereas the HPS level was 0.11, 0.04, and 0.01 times greater, respectively.

### 3.5 Presence of free SBs during brewing

Once we understood why HPS levels were reduced during brewing, we tested the samples for the presence of C1P, SB1P, and free SB, which are GIPC fragments (apart from Cer). Using TLC and Dittmer reagent, we found that C1P and SB1P were undetectable in any of the samples. However, marked levels of free SBs were detected in sake lees, with levels in a decreasing order from sokujo-moto, ki-moto, to yamahai-moto (Table 5), whereas they were not detected or detected only at trace levels in other samples. The base composition of the free SBs was predominantly d18:0.

The free SB levels were 10,162, 8,732, and 6,933 nmol in sake lees of the sokujo-moto, ki-moto, and yamahai-moto styles, generated from 100 g wet wt. of total rice, respectively.

### 4 Discussion

Dietary sphingolipids have been reported to have a number of health benefits, including anti-inflammatory and anti-carcinogenic activities as well as skin moisture improvement. Given that it is becoming increasingly clear that the effect of sphingolipids varies among the different classes of sphingolipids and their SB composition, knowledge of the detailed structure of food materials would be of value. However, there is still little information regarding the changes in sphingolipids during food processing, especially in the case of GIPC compounds, which are the major sphingolipids in plants and fungi. Previously, we reported that sake brewing markedly increased Cer levels in sake lees and changed the SB composition of Cer and GlcCer when compared to that of highly polished rice. In the present study, we investigated changes in HPS containing GIPC in highly polished rice, along with koji rice and sake yeast, during polishing and brewing. In all of the brown rice cultivars used in our study, HPS

![Table 4](image-url)
showed the highest levels of all sphingolipid classes (Table 1). It was also an apparent class in all products during polishing (Table 2). In the sake lees, however, the levels of HPS were the lowest among all the sphingolipid classes, considerable levels of free SBs were detected, and the levels were dependent on the starter cultures (Tables 3 and 5).

We found that during brewing, HPS containing GIPC was dramatically eliminated. It has long been known that plants and fungi have phospholipase D activity, which can produce C1P from GIPC\(^{29, 30}\). However, C1P and SB1P could not be detected in sake lees, and levels of Cer and free SBs were markedly increased in sake lees compared to those in total rice used (Tables 2 and 5). Neutral phospholipase C for phosphosphingolipids is found in yeast\(^{31}\) and the activity of acid phosphatase is high in fungi, especially koji. The enzyme obtained from koji is utilized commercially. Sake starter and fermentation mash are acidic, and the NaOH equivalent necessary to neutralize 1 L of sake as a final product was 13 mmol for sokujo-moto style, 17 mmol for ki-moto style, and 16 mmol for yamahai-moto style sake. In addition, HPS levels in the sake lees of ki-moto and yamahai-moto styles were much lower than those in the sokujo-moto style, with a shorter brewing duration. With respect to the difference in HPS contents in sake lees of between ki-moto and yamahai-moto styles, because levels of not only HPS, but also other sphingolipids, in the sake lees of yamahai-style were lower than those of ki-moto style, the yeast number of yamahai-style is thought to be lower than that of ki-moto style. Therefore, C1P generated from GIPC may immediately be degraded to Cer by acid phosphatase, which in turn may be degraded to free SBs. In this study, oligo-glycosylceramides were not measured, and these neutral classes, similar to GlcCer, may be concentrated in sake lees.

Based on 100 g wet wt. of total rice used, the additional levels of Cer in sake lees of sokujo-moto, ki-moto, and yamahai-moto styles were 4,180, 9,356, and 7,829 nmol, respectively. In contrast, the corresponding reductional levels of HPS were 6,949, 7,966, and 8,206 nmol, respectively, in the three types of lees. Additionally, high levels of free SBs, which were not detected in highly polished rice and koji rice, were observed in sake lees. The increased levels of Cer with free SBs were much higher than the reduced levels of HPS when compared to the total rice used. Sake yeast are also used for brewing and have Cer and HPS (Table 4). When 30 mg wet wt. of dried yeast is added to 100 g wet wt. of total rice according to the manufacturer’s protocol, the yeast number increases more than 1,000-fold during brewing (sake yeast A, 3,917 nmol of Cer and 3,067 nmol of HPS; sake yeast B, 1,902 nmol of Cer and 2,157 nmol of HPS) and the survival ratio of ki-moto and yamahai-moto mash are higher than that of sokujo-moto mash\(^{32, 33}\). Additionally, sake yeast generates Cer and free SBs under stress\(^{34}\), and sake yeast is exposed to various stresses (e.g., osmotic pressure, alcohol, and high

### Table 5 Changes in free sphingoid bases (SB) contents during polishing and brewing.

<table>
<thead>
<tr>
<th></th>
<th>Free SB(^1)</th>
<th>Free SB/Cer</th>
<th>t18.0(^2)</th>
<th>d18.0(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aka-nuka</td>
<td>trace</td>
<td>-</td>
<td>n.d.</td>
<td>100.0</td>
</tr>
<tr>
<td>Highly polished rice</td>
<td>n.d.</td>
<td>-</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Sokujo-moto sake lees</td>
<td>71061</td>
<td>1.67</td>
<td>5.0</td>
<td>95.0</td>
</tr>
<tr>
<td>Ki-moto sake lees</td>
<td>60637</td>
<td>0.77</td>
<td>7.2</td>
<td>92.8</td>
</tr>
<tr>
<td>Yamahai-moto sake lees</td>
<td>52923</td>
<td>0.71</td>
<td>8.4</td>
<td>91.6</td>
</tr>
<tr>
<td>Sokujo-moto sake</td>
<td>trace</td>
<td>-</td>
<td>n.d.</td>
<td>100.0</td>
</tr>
<tr>
<td>Ki-moto sake</td>
<td>trace</td>
<td>-</td>
<td>n.d.</td>
<td>100.0</td>
</tr>
<tr>
<td>Yamahai-moto sake</td>
<td>trace</td>
<td>-</td>
<td>n.d.</td>
<td>100.0</td>
</tr>
</tbody>
</table>

\(^1\)nmol/100 g dry wt. or nmol/100 mL sake, \(^2\)mol%.

Cer, ceramide; n.d., not detected; SB, sphingoid base.
pressure) during brewing. Overall, sake lees with a larger number of sake yeasts had higher levels of Cer. In contrast, the free SB level in the sake lees of the sokujo-moto style was higher than that of other sake lees. The levels of reducing sugars in sake lees of the sokujo-moto, ki-moto, and yamahai-moto styles were 27.3 g, 17.5 g, and 17.2 g in 100 g dry wt., respectively. Free SBs have an amino group, is well known that the Maillard reaction occurs between reducing sugars and amino groups contained in proteins and lipids. Therefore, it is expected that sake lees of the sokujo-moto style, with a shorter brewing duration, would show higher levels of free SBs and reducing sugars than those of other styles.

The GlcCer levels in sake lees also increased based on the total rice used; the additional GlcCer levels were 585 nmol in sokujo-moto style, 1,280 nmol in ki-moto style, and 769 nmol in yamahai-moto style and increased as the duration of starter culture increased. Sake yeasts had lower levels of SBs in the GlcCer fractions (Table 4) and may increase the GlcCer levels in sake lees. When GlcCer fractions of sake yeasts were treated with TMS, a peak containing Cer (t20:0/26h:0) and a hexose was detected (Fig. S1), and t20:0 is a fungi-specific SB. However, many reports have shown that there is no GlcCer in Saccharomyces cerevisiae and that sake yeasts do not produce GlcCer synthase. GlcCer has also been reported to be translated from koji to sake yeast during starter culture; therefore, GlcCer translated from the culture medium may be isomerized in yeasts. Overall, sake yeasts along with koji or GlcCer synthase may be related to the increase in GlcCer levels during brewing; however, further studies are required to confirm this.

The ratio of d18:0 was markedly increased in sphingolipids containing HPS and free SBs of sake lees when compared to that in highly polished rice, and d18:0 was the major component not only in koji rice but also in sake yeasts (Table 2). Sake yeast increases proton concentration in the cytoplasm and vacuoles, which decreases the pH during alcohol fermentation under high pressure. Because various SB compounds are generated from d18:0, the base synthesis may stop at the d18:0 stage under stress, and the sphingolipids present in highly polished rice and koji rice may undergo enzymatic and chemically sphingolipid-specific reactions (e.g., hydration and hydroxylation) during brewing.

The results of our study demonstrated that sphingolipids, especially HPS and free SBs, changed during polishing and brewing. Levels of Cer and free SBs markedly increased in sake lees and they have a higher absorption compared to complex sphingolipids. Currently, free SBs along with α-tocopherol are reported to greatly suppress oxidation of fish oil and show a stronger effect at d18:0 than at d18:1. In addition, different SBs show different absorption ratios and different effects on skin and adipocytes in vitro. In contrast, HPS protects intestinal cells from inflammatory stress in vitro compared to Cer. Dietary extracts of sake rice and sake lees suppress the formation of aberrant crypt foci in the mouse colon, and sake lees with higher levels of Cer and GlcCer show lower effects than sake rice, which may be due to the decrease in HPS in sake lees. Although sphingolipids in plant cells cannot easily exert nutritional functions when consumed, extracted sphingolipids show nutritional functions at low intake levels. Thus, sake lees, which are plant cells digested during brewing, may show highly beneficial effects by sphingolipids.

In conclusion, food processing, especially brewing, can change the composition of sphingolipid classes and SBs. These data may aid in the selection of appropriate sphingolipid sources for specific applications.

Conflict of Interest
The authors have no conflicts of interest to declare.

Author Contributions
S.Y. wrote the manuscript and supervised the experiment; C.H. performed research and analyzed data; N.K. analyzed data; D.S. performed research; M.K. designed research and supervised the experiment; T.M. supervised the experiment.

Acknowledgement
Part of this research was supported by The Public Foundation of Elizabeth Arnold-Fuji.

Supporting Information
This material is available free of charge via the Internet at doi: 10.5650/jos.ess21125

References


26) Yamashita, S.; Shinada, K.; Sakurai, R.; Yasuda, N.; Oikawa, N.; Kamiyoshiihara, R.; Otoki, Y.; Nakagawa, K.; Miyazawa, T.; Kinoshita, M. Decrease in intramuscular levels of phosphatidylethanolamine bearing arachidon-


