Quantitation and Structural Determination of Glucosylceramides Contained in Sake Lees

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Abstract: Sake lees are solid parts filtered from the mash of sake, the traditional rice wine of Japan, which is brewed with Aspergillus oryzae and Saccharomyces cerevisiae. The moisture-holding activity of sake lees has long been recognized in Japan. However, the constituent responsible for this activity has not been elucidated. The glucosylceramides contained in sake lees were N-2’-hydroxyoctadecanoyl-L-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine (d19:2/C18:0h), N-2’-hydroxyoctadecanoyl-L-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C18:0h), N-2’-hydroxyicosanoyl-L-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C20:0h) and N-2’-hydroxyicosanoyl-L-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C22:0h), which corresponded to those of A. oryzae and rice. The glucosylceramide produced by A. oryzae constituted the most abundant species (43% of the total glucosylceramide) in the sake lees. These results will be of value in the utilization of sake lees for cosmetics and functional foods.

Key words: sphingolipid, sake lees, Aspergillus oryzae, glucosylceramide, cosmetics

1 INTRODUCTION

Sake is a traditional brewed alcoholic beverage in Japan, which has also traditionally been utilized in cosmetics. During the manufacture of sake, steamed rice is first fermented with the fungus Aspergillus oryzae to form koji mold. The koji, steamed rice, and sake yeast Saccharomyces cerevisiae are pitched into a sake mash tank to cause ethanol fermentation⁵. After fermentation, the sake mash is filtered to produce liquid and solid parts. The solid part is called sake lees. Traditionally, it has been utilized in moisture-holding cosmetics in Japan. Although ethyl alpha-D-glucoside has been reported to contribute to the moisture-holding ability of sake⁶,³, the substance responsible for the moisture-holding ability of sake lees has never been reported. This lack of knowledge about the substance responsible for its moisture-holding ability has hampered its further development for cosmetics and functional foods.

On the contrary, sphingolipids have gained attention in terms of their moisture-holding abilities. Moreover, sphingolipids are generating increasing interest because of their effect in the formation of lamella phases in the skin and in maintaining the skin barrier function⁴,⁶. Moreover, sphingolipids have roles in cell signaling and apoptosis⁵,⁷, improving the cognitive function⁶, suppressing tumorigenesis by reducing the number of aberrant colonic crypt foci by dietary intake⁶-¹³, adiponectin signaling⁴, and type 2 diabetes through mediating the loss of insulin sensitivity¹⁴,¹⁵. However, the content or molecular structures of the sphingolipids contained in sake lees have not been investigated to date.

Structural determinations of sphingolipids require complex analytical methods because they contain various chemical structures such as sphinganine (dihydrosphingosine, d18:0), sphingosine (trans-4-sphingenine, d18:1⁴), 4,8-sphingadienine (d18:2⁴,⁶), 4,8,10-sphingatrienine (d18:3⁴,⁶,¹⁰), phytosphingosine (4-hydroxysphinganine, t18:0), 4-hydroxy-8-sphinganine (t18:1⁴), 9-methyl-4,8-sphingadienine (d19:2⁴,⁶), and 9-methyl-4,8,10-sphingatrienine (d19:3⁴,⁶,¹⁰) as sphingoid bases, along with various unsaturated and hydroxylated fatty acids acylated to...
sphingoid bases and various glycosidic or ester modifications (glucose, galactose, mannose, neuraminic acid, syalid acid, inositol, mannosylinositol, phosphodiester or choline) on the 2-hydroxyl bond of ceramide. In order to facilitate the analysis of these complex chemical structures of sphingolipids, tandem mass spectrometry and fragment ion analysis coupled with fragment ion analysis have successfully been applied to determine the complex chemical structures of sphingolipids.

In this study, by applying tandem mass spectrometry and fragment ion analysis, we first determined the structure of the glucosylceramide contained in sake lees. Furthermore, we elucidated the glucosylceramide contents contained in various sake lees. This is the first study to make a structural determination and determine the source of the glucosylceramides contained in sake lees. The results will facilitate the utilization of sake lees as cosmetics and functional foods, as well as the augmentation of their moisture-holding ability.

2 EXPERIMENTAL

2.1 Materials
The sake lees and koji samples were kind gifts from sake manufacturers.

2.2 Quantitation of glucosylceramide
The glucosylceramide contents were quantitated based on the intensities of the spots on thin layer chromatography (TLC) plates. The intensities and areas of the spots for the glucosylceramides on the TLC plates were determined using image J software. By applying a known quantity of the glucosylceramides on the TLC plates, the constant k in the equation y = k × x (x represents the intensity multiplied by the area, y represents the predicted quantity of glucosylceramides, and k represents a constant) was determined by calculating the least value of the sum of the second powers of the remnant differences between the observed and predicted values. The differential equation for the sum of the second powers of the remnant differences between the observed and predicted values generated the equation: k = \frac{\sum (y_i - \hat{y}_i)^2}{\sum x_i^2}. The fitting of the standard calibration curve was judged by the correlation constant t and the t value (t = \frac{r \times (\sqrt{n-2})}{\sqrt{1-r^2}}), which followed a Student's t-distribution.

2.3 Purification of sphingolipids.
The extracted lipids were purified as described previously. Briefly, the extracted lipids were dried in a centrifugal evaporator and dissolved in 4 ml of hexane. Silicagel chromatography was applied to the sample, after which it was eluted with ethyl acetate:methanol (9:1) and fractionated by checking the elution positions of the glucosylceramide using TLC. The fractions containing glucosylceramide were dried in a centrifugal evaporator and dissolved in 4.5 mL of chloroform:methanol (2:1 v/v). Four milliliters of the sample was injected into the 500-µL injection loop of a high-performance liquid chromatography (HPLC) system. The sphingolipid purification by HPLC was performed using an Inertsil Sil 100 A column (5 μm, 4.6 mm diameter × 250 mm length). The mobile phase consisted of buffer A (chloroform) and buffer B (95% methanol), and separation was achieved using the following gradient program: 0 min A 100%/B 0%, 15 min A 75%/B 25%, and 20–40 min A 10%/B 90%. The flow rate was 0.7 mL/min, and the volume of the sample injection loop was 500 µL. Fractions were collected at 0.5 or 1 min intervals. A TLC analysis was conducted for the collected fractions, followed by orcinol-sulfate visualization.

2.4 Electrospray ionization tandem mass spectrometry (ESI-MS/MS)
Mass spectrometry was performed using an ion trap mass spectrometer (HC Ultra, Bruker Daltonics, Bremen, Germany) with an electrospray ion source. The sample was dissolved in 50 µl of chloroform:methanol (1:1 v/v), and 950 µl of methanol was added. The sample was infused into the ion trap mass spectrometer using an on-line syringe pump at a constant flow rate of 3 μL/min. Nitrogen at 4 L/min and 300°C was employed for desolvation and as a nebulizer gas at 10 psi. The instrument was set to operate in the positive ion mode with a capillary voltage of 4 kV and an end plate offset of 0.5 kV. All of the spectra were acquired in the mass range of 50–1500 m/z, with a scan speed of 4000 m/z per s. Multiple stage MS was performed by collision-induced dissociation using helium as the collision gas. The precursor ion was selected within an isolation width of 4 u. The multiple stage sequencing up to MS/MS was carried out using a fragmentation amplitude of 1.0 V, ramped from 30 to 200% within 40 ms for each single spectrum, and a fragmentation cutoff default of 27% of the precursor ion m/z.

2.5 Fragment ion analysis
Fragment ions were assigned to those of glucosylceramides using a method modified from that described earlier. Briefly, fragment ions were assigned to the Y₁, N, O, and T ions of various glucosylceramides, and the structures of the glucosylceramides were determined.

3 RESULTS AND DISCUSSION

3.1 Glucosylceramide content in sake lees
First, the amount of glucosylceramides contained in sake lees was quantitated as described in the Experimental
Glucosylceramides in sake lees section. It turned out that sake lees contain $2.2 \pm 0.42$ mg glucosylceramide/g dry weight of sake lees ($n = 6$), which corresponds to other rich sources of glucosylceramides$^{22,23}$. This result clearly indicates that glucosylceramide is one of the potential substances responsible for the moisture-holding ability of sake lees.

3.2 Structural determination of glucosylceramides contained in sake lees

Next, in order to elucidate the molecular structure of the glucosylceramide contained in sake lees, glucosylceramide was purified and analyzed using electrospray mass spectrometry (ESI-MS), which generated 4 candidate singly charged, sodium adduct ions: m/z 778.7, 792.7, 764.6, and 820.7 (Table 1 and Fig. 1).

A precursor molecule having an m/z of 778.7 provided major fragment ions having m/z values of 496.4 (100.0), 616.6 (31.6), and 348.3 (8.8) (Table 1 and Fig. 2, numbers in the parentheses indicate ratio (%) compared to base peak). The values for these fragment ions coincided with

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Ions provided by tandem ESI-MS/MS of glucosylceramides purified from sake lees$^a$.</th>
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<tr>
<td><strong>MS m/z (%)</strong>$^b$</td>
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<td>301.2 (94.7)</td>
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<td>290.2 (60.0)</td>
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<td>778.7 (59.0)</td>
<td>496.4 (100.0), 616.6 (31.6), 598.5 (25.5), 760.6 (18.0), 506.4 (11.5), 463.4 (10.8), 524.4 (10.7), 695.6 (9.9), 759.6 (9.1), 348.3 (8.8), 499.3 (8.2), 497.4 (6.8), 614.6 (6.4), 758.6 (6.4), 580.4 (4.9), 696.1 (4.0)</td>
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<td>230.3 (56.4)</td>
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<td>792.7 (56.4)</td>
<td>482.4 (100.0), 630.6 (34.4), 612.6 (32.5), 709.6 (20.6), 774.7 (17.8), 710.6 (13.6), 710.1 (12.6), 449.3 (11.7), 510.4 (11.7), 492.3 (9.7), 376.4 (9.4), 513.3 (8.2), 773.6 (7.0), 711.1 (5.8), 452.3 (5.7), 541.2 (5.7), 709.1 (5.7), 464.3 (5.3), 512.3 (4.7), 262.3 (4.6), 759.5 (4.1), 583.2 (4.1), 453.3 (4.1), 453.3 (4.1), 698.1 (4.0), 772.6 (3.9)</td>
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$^a$ J. Oleo Sci. 63, (1) 15-23 (2014)
The spectra of ESI-MS and ESI-MS/MS are shown in Fig. 1 to 5.

% compared to base peak.

Underlined species indicate m/z of molecules corresponding to fragment ions of glucosylceramides.

\* The spectra of ESI-MS and ESI-MS/MS are shown in Fig. 1 to 5.
\* % compared to base peak.
Glucosylceramides in sake lees

Fig. 1  Positive ESI-MS spectrum of glucosylceramide purified from sake lees. ESI-MS was applied to glucosylceramide purified from mild alkali-resistant lipids of sake lees.

Fig. 2  Fragment ion analysis of molecule having m/z of 778.7. (A) Positive ESI-MS/MS spectra of an ion having the m/z of 778.7. (B) The predicted structure of glucosylceramide and its fragment ions. Ions of glucosylceramide trapped in ESI-MS were analyzed using ESI-MS/MS.

the m/z values of the Y0, O, and T ions of glucosylceramide with a molecular structure of N-2'-hydroxyoctadecanoyl-1-O-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine (d19:2/C18:0).

A precursor molecule having an m/z of 764.6 provided major fragment ions having m/z values of 482.4 (100.0), 602.6 (41.4), and 348.3 (10.0) (Table 1 and Fig. 3). The values for these fragment ions coincided with the m/z

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values of the Y\textsubscript{0}, O, and T ions of glucosylceramide with a molecular structure of N-2\textsubscript{L}-hydroxyoctadecanoyl-l-O-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C18:0h).

A precursor molecule having an m/z of 792.7 provided major fragment ions having m/z values of 482.4 (100.0), 630.6 (34.4), and 376.4 (9.4) (Table 1 and Fig. 4). The values for these fragment ions coincided with the m/z values of the Y\textsubscript{0}, O and T ions of glucosylceramide with a molecular structure of N-2\textsubscript{L}-hydroxyicosanoyl-l-O-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C20:0h).

A precursor molecule having an m/z of 820.7 provided major fragment ions having m/z values of 481.3 (15.5) and 658.6 (11.1) (Table 1 and Fig. 5). The values for these fragment ions coincided with the m/z values of the Y\textsubscript{0}, O, and T ions of glucosylceramide with a molecular structure of N-2\textsubscript{L}-hydroxydocosanoyl-l-O-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C22:0h).

Glucosylceramides with the structures N-2\textsubscript{L}-hydroxyoctadecanoyl-l-O-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C18:0h), N-2\textsubscript{L}-hydroxyicosanoyl-l-O-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C20:0h), and N-2\textsubscript{L}-hydroxydocosanoyl-l-O-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C22:0h) coincide with those contained in rice\textsuperscript{24}. Glucosylceramide with the structure N-2\textsuperscript{L}-hydroxyoctadecanoyl-l-O-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine (d19:2/C18:0h) coincides with that contained in Aspergillus oryzae\textsuperscript{25}. Therefore, these results indicated that glucosylceramides contained in sake lees are derived from rice and Aspergillus oryzae.

Rice has been reported to contain various glucosylceramide structures containing t18:1/C22:0h, t18:1/C24:0h, d18:3/C22:0h, and d18:3/C24:0h\textsuperscript{24}. However, no peaks corresponding to t18:1/C22:0h (the m/z of the sodium ion adduct is 822), t18:1/C24:0h (the m/z of the sodium ion adduct is 850), d18:3/C22:0h (the m/z of the sodium ion adduct is 800), or d18:3/C24:0h (the m/z of the sodium ion adduct is 844) were detected in the ESI-MS spectrum, which may have been because these species were below the detection limit.

It was interesting that the glucosylceramide derived from Aspergillus oryzae constituted the most abundant species in the sake lees (43% of the total glucosylceramide of the sake lees). Therefore, it was concluded that to increase the amount of glucosylceramide in sake lees, it is important to increase the glucosylceramide produced by Aspergillus oryzae. Because this is the first insight into

**Fig. 3** Fragment ion analysis of molecule having m/z of 764.6. (A) Positive ESI-MS/MS spectra of an ion having the m/z of 764.6. (B) The predicted structure of glucosylceramide and its fragment ions. Ions of glucosylceramide trapped in ESI-MS were analyzed using ESI-MS/MS.
The moisture-holding constituent of sake lees, it has an impact on the manufacture of sake lees for cosmetics and functional foods.

These results clearly indicate that sake lees contain glucosylceramides, and provide the first evidence on the substances and components responsible for the moisture-holding ability of sake lees. From these results, it is now possible to monitor the moisture-holding ability of sake lees by quantitating the amount of glucosylceramides. These insights will be of value in the development and utilization of sake lees as cosmetics and functional foods.

**4 CONCLUSION**

In conclusion, we have elucidated, for the first time to the best of our knowledge, the molecular structure of the glucosylceramide contained in sake lees. The glucosylceramides contained in sake lees were N-2'-hydroxyoctadecanoyl-1-O-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine (d19:2/C18:0h), N-2'-hydroxyoctadecanoyl-1-O-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C18:0h), N-2'-hydroxyicosanoyl-1-O-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C20:0h), and N-2'-hydroxyicosanoyl-1-O-β-D-glucopyranosyl-4,8-sphingadienine (d18:2/C22:0h), which corresponded to those of rice and *A. oryzae*. The glucosylceramide produced by *A. oryzae* constituted the most abundant species (43% of the total glucosylceramide) in the sake lees. This novel data will provide information to facilitate the manufacture of sake lees with high glucosylceramide contents for the development of cosmetics and functional foods from sake lees.

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