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<td>Sugawara, Tatsuya; Duan, Jingjing; Aida, Kazuhiko; Tsuduki, Tsuyoshi; Hirata, Takashi</td>
</tr>
<tr>
<td>Citation</td>
<td>Lipids (2010), 45(5): 451-455</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2010-05</td>
</tr>
<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/2433/126699">http://hdl.handle.net/2433/126699</a></td>
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<tr>
<td>Type</td>
<td>Journal Article</td>
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Kyoto University
Identification of Glucosylceramides Containing Sphingatrienine in Maize and Rice using Ion Trap Mass Spectrometry

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Running Head: Glucosylceramides containing sphingatrienine in maize and rice

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Abstract  We characterized the glucosylceramide moieties from maize and rice using liquid chromatography-ion trap mass spectrometry. Glucosylceramides containing 4,8-sphingadienine (d18:2) acylated to hydroxy-fatty acids were detected as the predominant molecules both in maize and in rice. In addition, 4-hydroxy-8-sphingenine (t18:1) and sphingatrienine (d18:3) were found in maize and rice glucosylceramides, and in the case of rice, sphingenine (d18:1) was also detected. Glucosylceramides containing d18:3 were acylated to hydroxyl fatty acids (16 to 24 carbon atoms). Our results indicate the presence of the triene type of sphingoid base in higher plants.

Abbreviations

d18:0  Sphinganine
d18:1  Sphingenine
d18:2  Sphingadienine
d18:3  Sphingatrienine
t18:0  Phytosphingosine
t18:1  Hydoroxysphingeine
HPLC  High-performance liquid chromatography
MS    mass spectrometry

Introduction

Sphingolipids are found in a wide variety of organisms, and constitute a family of compounds that have a sphingoid base (long-chain base) with an amide-linked fatty acid and a polar head group. The hydrolyzed products of sphingolipids (ceramides and sphingoid bases) are highly
bioactive compounds that play roles as second messengers that are known to be involved in many aspects of cell regulation, such as cell growth, cell differentiation and apoptosis [1-3].

Recently, dietary sphingolipids have gained attention for their potential to protect the intestine from inflammation and cancer [4-9]. In addition, other physiological functions of sphingolipids, such as improving the barrier function of skin, lowering plasma lipids and prevention of melanin formation, have also been reported [10-12].

Diverse structures of the sphingoid base occur in nature. The most common sphingoid base of mammalian sphingolipids is sphingosine (trans-4-sphigenine, d18:1\textsuperscript{4}). Smaller amounts of other sphingoid bases, such as sphinganine (dihydrosphingosine, d18:0) and phytosphingosine (4-hydroxysphinganine, t18:0) are frequently encountered. The structures of sphingoid bases in higher plants are more complicated than in mammals [13]. Plants primarily contain \textit{cis}- and \textit{trans}- isomers of \textit{Δ}8-unsaturated sphingoid bases, such as 8-sphingenine (d18:1\textsuperscript{8}), 4,8-sphingadienine (d18:2\textsuperscript{4,8}) and 4-hydroxy-8-sphingenine (t18:1). Determination of those diverse structures including variations of the sphingoid backbone is important to understand the functional and nutritional significance of dietary sphingolipids.

Mass spectrometry is one of the most powerful methods for detecting and identifying the chemical structures of lipids including sphingolipids [14-16]. In this study, we characterized the structures of glucosylceramide, one of the predominant glycosphingolipids in plants, from rice and from maize using liquid chromatography-ion trap mass spectrometry. Our results demonstrate the presence of sphingatrienine (d18:3) in higher plants, which has been described previously in marine invertebrates [17-19] and was found in tobacco leaf [20].

**Experimental Procedures**
Materials

Glucosylceramides were prepared from maize grain and from rice grain using a silica gel column after lipid extraction and saponification as described previously [21]. All other chemicals and solvents were of reagent grade.

LC-MS/MS analyses

An HPLC system coupled to LCMS-IT-TOF equipped with an electrospray ionization interface (Shimadzu, Kyoto, Japan) was used. A TSK gel ODS-100Z column (2.0 x 50 mm, 3μm, Tosoh, Tokyo, Japan) was eluted with acetonitrile/water (93:7, v/v) at a flow rate of 0.2 mL/min. The MS was operated with the following conditions: probe voltage of 4.50 kV, CDL temperature of 200ºC, block heater temperature of 200ºC, nebulizer gas flow of 1.5 L/min, ion accumulation time of 100 msec, MS range of m/z 650 to 900, MS² range of m/z 200 to 300, and CID parameters as follows: energy, 60%; collision gas 60%. For the structural analysis of glucosylceramide, [M+H-18]⁺ (loss of water) in the positive scan mode was used for MS/MS analysis to obtain the product ions. The typical signals which are characteristic for the sphingoid base moieties were observed as the product ions using the auto MS/MS detection mode. In this system, product ions corresponding to d18:1, d18:2 and d18:3 were m/z 264.3, m/z 262.3 and m/z 260.2, respectively [14-16]. In the case of glucosylceramide molecules consisting of t18:1, the loss of glucose [M+H-162]⁺ was used as the precursor ion and the product ions corresponding to t18:1 were m/z 280.3 and m/z 262.3 [16]. Pairs of the structurally specific product ions of sphingoid bases and their precursor ions were used for the identification of glucosylceramide molecules.

Results and Discussion
In the positive full scan mode, [M+Na]^+, [M+H]^+ and [M+H-H_2O]^+ were the predominant signals in each peak. It is well known that the sugar moiety of glycosylceramides in plants is mostly glucose [22]. In the case of molecules consisting of t18:1, the loss of glucose [M+H-162]^+ was also clearly detected. Glucosylceramide molecules containing d18:2 and t18:1 were determined both in maize and in rice as described previously (Fig. 1A and 2A) [16]. In the case of rice glucosylceramide, molecules consisting of d18:1 were also identified. Detection of glucosylceramide consisting of d18:2 and t18:1 was separated into two peaks, cis- and trans- isomers of Δ8-unsaturated sphingoid bases. Cis-isomer was detected earlier than trans-isomer by separation of reverse phase [20]. Predominantly hydroxy fatty acids containing 16 to 26 carbon atoms were detected both in maize and in rice glucosylceramides.

We verified that the characteristic product ion at m/z 260.2 corresponding to d18:3 was detected in maize glucosylceramide using the auto MS/MS detection mode (Fig. 1A). Five peaks in the total ion chromatogram of maize glucosylceramide showed the product ion at m/z 260.2 (peak 1-5 in Fig. 1A). The MS spectra of those 5 peaks are shown in Figure 1B-F. As the precursor ion of m/z 260.2, [M+H-18]^+ ions at m/z 694.5, 722.5, 750.6, 778.6 and 806.6 were detected. The identification of each peak component is summarized in Table 1. The acylated fatty acid moieties were hydroxy fatty acids with 16-24 carbon atoms. In the case of rice, glucosylceramide consisted of d18:3-C18:0h and d18:3-C20:0h (Fig. 2 and Table 1).

It has been reported that the sphingoid bases in marine invertebrates are quite different from those in mammals and in plants [13]. Triene bases with conjugated diene, such as 2-amino-4,8,10-octatriene-1,3-diol (d18:3) and 2-amino-9-methyl-4,8,10-octatriene-1,3-diol (d19:3), were identified in marine invertebrates including ascidians [17], starfish [18, 19] and squid [23] and also some fungi [24]. We have also reported that sea cucumber
glucosylceramide has sphingoid bases with three double bonds [25]. Sperling et al. described the presence of sphingatrienine in tobacco leaf by HPLC analysis of sphingoid base derivatized with dinitrophenyl [20]. In this study, we identified several molecular species of sphingatrienine-containing glucosylceramides in maize and rice by LC-MS/MS system. However, the locations of double bonds in sphingatrienine structure have not been identified. Sphingolipids of plant organisms contain primarily d18:1, d18:2,4,8 and t18:1 as sphingoid bases and sphingolipid Δ4-desaturase and sphingolipid Δ8-desaturase have been identified in plants [22]. It has been reported that the composition of sphingoid bases differs between chilling sensitive and tolerant plants [26]. Details of tissue distribution, synthetic pathways and functions of plant sphingatrienines remain to be elucidated.

Recently, dietary sphingolipids have gained attention for their potential to protect the intestine from inflammation and cancer [4-9]. We reported that the daily intake of plant-origin glucosylceramides in Japan is estimated to be 50 mg due to their presence in foodstuffs [27] and we investigated the digestion and absorption of plant-derived sphingolipids [21]. Our findings indicate that the metabolic fate of plant-derived sphingoid bases, such as 4,8-sphingadienine, within enterocytes differs from that of sphingosine. Sphingoid bases, except for sphingosine, appear to be transported out of cells across the apical membranes of enterocytes by P-glycoprotein after absorption and consequently the intestinal uptake is quite poor [28, 29]. Thus, the determination of sphingolipid structures, including variation of the sphingoid backbone, from dietary sources is important to understand their functional and nutritional significance.

In this study, we analyzed the chemical structures of glucosylceramides from maize and from rice using liquid chromatography-ion trap mass spectrometry. Our results indicate the presence of sphingatrienine (d18:3) in higher plant sphingolipids. MS/MS analysis is a
powerful method to identify the molecular structures of sphingolipids from biological sources.

Acknowledgement  This work was supported by the Program for Promotion and Applied Researches for Innovations in Bio-oriented Industry (BRAIN).


Table 1  Glucosylceramides containing sphingatrienine (d18:3) from maize and from rice identified by HPLC-MS/MS analysis.

<table>
<thead>
<tr>
<th>Peak No. in Fig. 1 and 2</th>
<th>Precursor ion m/z</th>
<th>Product ion m/z</th>
<th>Molecule</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[M+H]+</td>
<td>[M+H-18]+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>712.5</td>
<td>694.5</td>
<td>260.2</td>
<td>d18:3-C16:0h</td>
</tr>
<tr>
<td>2</td>
<td>740.5</td>
<td>724.5</td>
<td>260.2</td>
<td>d18:3-C18:0h</td>
</tr>
<tr>
<td>3</td>
<td>768.6</td>
<td>750.6</td>
<td>260.2</td>
<td>d18:3-C20:0h</td>
</tr>
<tr>
<td>4</td>
<td>796.6</td>
<td>778.6</td>
<td>260.2</td>
<td>d18:3-C22:0h</td>
</tr>
<tr>
<td>5</td>
<td>824.6</td>
<td>806.6</td>
<td>260.2</td>
<td>d18:3-C24:0h</td>
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Figure Legends

FIG. 1. Total ion and selected ion chromatograms of maize glucosylceramide (A) and mass spectra of peak components (B-F).

FIG. 2. Total ion and selected ion chromatograms of rice glucosylceramide (A) and mass spectra of peak components (B and C).
A. TIC (1.00)

MS/MS 260.2 (100.00)

MS 712.5 (50.00)

MS 740.6 (10.00)

MS 768.6 (10.00)

MS 796.6 (10.00)

MS 824.6 (10.00)

B. Peak 1

C. Peak 2

D. Peak 3

E. Peak 4

F. Peak 5

Relative ion abundance (%)

{\[\text{[M+H]}^+\] 734.5}

{\[\text{[M+Na]}^+\] 712.5}

{\[\text{[M+H-H}_2\text{O]}^+\] 694.5}

{\[\text{[M+H]}^+\] 740.6}

{\[\text{[M+Na]}^+\] 762.6}

{\[\text{[M+H-H}_2\text{O]}^+\] 722.5}

{\[\text{[M+H]}^+\] 768.6}

{\[\text{[M+Na]}^+\] 790.6}

{\[\text{[M+H-H}_2\text{O]}^+\] 750.6}

{\[\text{[M+H]}^+\] 796.6}

{\[\text{[M+Na]}^+\] 824.6}

{\[\text{[M+H-H}_2\text{O]}^+\] 806.6}

{\[\text{[M+H]}^+\] 846.6}

{\[\text{[M+Na]}^+\] 881.6}

P. Peak 5

d18:2-18:0h d18:2-20:0h

d18:2-16:0h

118:1-22:0h
d18:2-22:0h t18:1-24:0h
d18:2-24:0h
d18:2-25:0h
d18:2-26:0h

d18:2-25:0h
d18:2-26:0h

d18:2-16:0h
A

Relative ion abundance (%)

B. Peak 2

[C+Na]+ 762.6
[M+H] 740.6
[M+H-H2O] 722.5

C. Peak 3

[M+Na]+ 790.6
[M+H-H2O]+ 750.6
[M+H]+ 768.6