Content and Constituent Properties of Sphingolipid Classes in Saccharomyces kluyveri

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Abstract: The results of our previous study indicated the presence of cerebroside in Saccharomyces kluyveri and related species. The present study was performed to determine to distribution and content of ceramide, cerebroside and acidic sphingolipids in S. kluyveri. Ceramide, cerebroside, inositolphosphorylceramide (IPC), mannosylinositolphosphorylceramide (MIPC) and mannosyl-diinositolphosphoryl ceramide [M(IP)2C] as acidic sphingolipids were all clearly shown to be present in S. kluyveri. Sphingoid bases were recovered at a rate of 74% from the acidic sphingolipid fraction, while recoveries of ceramide and cerebroside, were only 10 and 16%, respectively. The major fatty acid and sphingoid bases in ceramide and acidic sphingolipid of S. kluyveri were C26 2-hydroxy fatty acid (>70%) and trihydroxy sphingoid bases (4-hydroxysphinganine and 4-hydroxyicosasphinganine, >90%), respectively. These results indicated that S. kluyveri has acidic sphingolipids as most sphingolipid classes and major constituents were essentially the same with that of S. cerevisiae.

Key words: ceramide, cerebroside, acidic sphingolipid, yeast, sphingoid base

1 Introduction

Sphingolipids are each comprised of a sphingoid base backbone, amide linked fatty acid (normal and 2-hydroxy fatty acids) and a polar headgroup, such as phosphocholine or hexose. These lipids may frequently be found in eukaryotic organisms, with the mammalian sphingolipid trans-4-sphingenate (sphingosine) being most prevalent. Other sphingoid bases such as spa...
A biochemical study was conducted on yeast sphingolipid used in the baker’s yeast, *Saccharomyces cerevisiae* having various industrial applications as a model for eukaryotic cells in biochemistry, physiology, genetics and molecular biology as reported in the literature (5, 6). Attention in particular was directed to relationships of sphingolipids to various kinds of environmental stress and the biophysical properties of membrane in yeast cells have been extensively examined using *S. cerevisiae* (7-10). Consequently, *S. cerevisiae* was unsuitable for biochemical examination of sphingolipids as a model for eukaryotic cells.

Cerebroside was previously shown present in *S. kluyveri*, *Zygosaccharomyces cidri*, *Z. fermentati*, *Kluyveromyces lactis*, *K. thermotolerans* and *K. waltii* (11, 12). The major sphingoid base in these cerebrosides were noted to be 9-Me d18:24t, 8t and its putative metabolic intermediates (11). Nevertheless the chemical compositions of ceramide and acidic sphingolipids in these yeast strains have not yet been characterized. This study examines the distribution and individual content of ceramide, cerebroside and acidic sphingolipids in *S. kluyveri*.

### 2 Experimental

#### 2.1 Yeast Strain and Culture

*S. kluyveri* NBRC 1685 from the NITE Biological Resource Center (Chiba, Japan) and *S. cerevisiae* Ni-087 from Nippon Beet Sugar Mfg., Co., Ltd. were cultured at 25°C with shaking (125 rpm) in 100 mL medium (YPD medium) containing 1% yeast extract, 2% polypeptone and 2% glucose in a 500 mL Erlenmeyer flask for 24 h. The harvested cells were lyophilized after three washings in distilled water and then stored at -20°C.

#### 2.2 Lipid Extraction and Separation of Sphingolipid Classes

Lyophilized cells were treated in methanol for 10 min with an ultrasonic disrupter (UD-200, Tomy Seiko, Tokyo, Japan). Total lipids were then each extracted twice with five volumes chloroform-methanol (2:1 and 1:2, v/v) and then chloroform-methanol-water (6:4:1, v/v). The combined extracts were evaporated to dryness to yield total lipids. For glycerolipid removal, mild alkali hydrolysis was conducted followed by dialysis of the total lipids (13). For neutral and acidic sphingolipid class separation, DEAE-Sephadex A-25 column chromatography (Pharmacia LKB Biotechnology, Sweden) was carried out on the alkali-stable product as previously reported (14, 15).

The alkali stable lipid (mainly sphingolipid and sterol lipid) was determined by TLC (Silica gel 60, Merck, Darmstadt, Germany) using chloroform-methanol-acetic acid (86:7:7, v/v) as the developing solvent for neutral lipid and chloroform-methanol-water (6:4:1, v/v) and 50% H2SO4 or Dittmer-Lester reagent as the detection reagent.

#### 2.3 Quantitation of Sphingolipid Classes and Analysis of Ceramide M moiety Constituents

The content distribution of sphingolipid classes in yeast was found based on sphingoid base quantitation. Sphingolipid classes were separated by TLC as described above and scraped from the the plate. Sphingoid base content of the fractions was computed using the methylorange method (16).

Fatty acid and sphingoid base compositions of ceramide moieties in the ceramide, cerebroside and acidic sphingolipids were clarified as indicated previously (11).

#### 2.4 Polar Head Partitions in Acidic Sphingolipids

##### 2.4.1 Preparation of inositol phosphate from acidic sphingolipids (15)

Purified acidic glycolipids were hydrolyzed with 2M HCl at 100°C for 100 min for sugar and inositol phosphate determination. The acidic reaction mixture thus obtained was dried in vacuo in the presence of small amounts of toluene. The residue was trimethylsilylated in a mixture of bis (trimethylsilyl) acetamide-trimethylchlorosilane-pridine (10:2:5, v/v) at 60°C for 1 h and analyzed by GC and GC-MS.

##### 2.4.2 Methylation of neutral sugar

The permethylation of acidic sphingolipids was carried out by the method of Ciucanu and Kerek (17). The peak on the gas chromatogram was identified by mass spectrometry and a comparison of the retention time with that of partially methylated alditol acetate as reference, prepared from authentic mannose (15).
3 Results and Discussion

Figure 1A and B indicate the TLC profiles of the neutral and acidic lipid fractions in *S. kluyveri*. In the neutral lipid fraction, ceramide and cerebroside spots could be seen. The dark spot with Rf 0.8 was free sterol (lane 2 in Fig. 1A). In the acidic lipid fraction, spots the same as those of Rf in the case of inositolphosphorylceramide (IPC), mannosylinositolphosphorylceramide (MIPC) and mannosyl-diinositolphosphoryl ceramide [M(IP)₂C] in *S. cerevisiae* (lane 1 in Fig. 1B) were evident. All the spots were found to be phosphorus positive through application of Dittmer-Lester reagent. The TLC (Fig. 1A and 1B) profiles clearly demonstrated the presence of ceramide, cerebroside and acidic sphingolipids in *S. kluyveri*.

To identify the constituent in the polar head group on acidic sphingolipids fractions, sugar compositions in putative IPC, MIPC and M(IP)₂C fractions (Fig. 1B, spot a, b and c) were determined. Spot a was apparent for only inositol. Spot b and c were apparent for inositol and mannose, respectively. The sugar linkage of spot b was determined based on analysis of partially methylated alditol acetate derivatives of spot b by GLC. 2,3,4,6-tetra-(O-methyl) 1,5-di-(O-acetyl)mannitol due to a non-reducing terminal mannose residue was detected at spot b. However, the content of spot c was too low to make determination of sugar linkage. *S. kluyveri* is thus shown to possess IPC (Fig. 1B spot a), MIPC (Fig. 1B spot b) and M(IP)₂C (Fig. 1B spot c) all putative as acidic sphingolipids.

To confirm the distribution of sphingolipid class content in *S. kluyveri*, differences in sphingoid base content in ceramide, cerebroside and acidic sphingolipid fractions were examined using methylorange (Table 1). Sphingoid base content in several sphingolipid classes was 0.22 in ceramide (0.49 ± 0.01 as ceramide), 0.34 in cerebroside (0.82 ± 0.05 as cerebroside) and 1.57 mg/g dry cell in the acidic sphingolipids fraction (5.39 ± 0.11 as MIPC). Sphingoid bases were recovered at 74% from the acidic sphingolipids fraction while ceramide and cerebroside, only at 10 and 16%, respectively.

For constituent determination in sphingolipids classes of *S. kluyveri*, sphingoid base and fatty acid composition in ceramide, cerebroside and acidic sphingolipids were examined (Table 2). The major fatty acid and

![Figure 1](image_url)

**Fig. 1** TLC Profiles of Sphingolipid Classes in *S. kluyveri*. A: Neutral lipid fraction developed by chloroform-methanol-acetic acid (86:7:7, v/v) and detected by 50% sulfuric acid. Lane 1, ceramide and cerebroside fraction from *Flammulina velutipes* (4); lane 2, neutral sphingolipids from *S. kluyveri*. B: Acidic lipid fraction developed by chloroform-methanol-water (6:4:1, v/v) and detected by 50% sulfuric acid. Lane 1, authentic acidic sphingolipids from *S. cerevisiae*; lane 2, Acidic sphingolipids from *S. kluyveri*; CM, Ceramide; CMH, Ceramide monohexoside (Cerebroside); FS, Free sterol; IPC, Inositolphosphorylceramide; MIPC, Mannosylinositolphosphorylceramide; M(IP)₂C, Mannosyl-diinositolphosphorylceramide.

**Table 1** Sphingoid Bases Content in Each Sphingolipid Class in *S. kluyveri* (mg/g dry cell).

<table>
<thead>
<tr>
<th>Sphingolipid Classes</th>
<th><em>S. kluyveri</em></th>
<th><em>S. cerevisiae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Ceramide</td>
<td>0.22 ± 0.01</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>Cerebroside</td>
<td>0.34 ± 0.05</td>
<td>ND¹</td>
</tr>
<tr>
<td>Acidic sphingolipids²</td>
<td>1.57 ± 0.11</td>
<td>1.54 ± 0.09</td>
</tr>
</tbody>
</table>

Values are means ± SD (n=3).

¹Not detected.
²Including inositolphosphorylceramide, mannosylinositolphosphorylceramide and mannosyl-diinositolphosphorylceramide.
sphingoid bases in ceramide and acidic sphingolipids of *S. kluyveri* were C26 2-hydroxy fatty acid (26h:0, >75%) and trihydroxy sphingoid bases (4-hydroxysphinganine and 4-hydroxyicosasphinganine, >90%). These observations indicate that nearly all detectable free ceramide is possibly a degradation product of acidic sphingolipids by phospholipase C-like enzyme (18). On the other hand, the principal constituents in cerebroside were C18 2-hydroxy fatty acid (18h:0) and 9-Me d18:24t,8t as previously reported (11). The content of 18h:0 in ceramide and acidic sphingolipids was much less than cerebroside. 9-Me d18:24t,8t and possible metabolic intermediates, 4-unsaturated and 4,8-diunsaturated sphingoid bases (4-trans-sphingenine and 4-trans, 8-trans-sphingadienine) as components of ceramide and acidic sphingolipids were detected in small amounts. Moreover, fatty acid and sphingoid base compositions in ceramide and acidic sphingolipids were essentially the same as noted for *S. cerevisiae* (19).

Sphingolipid class levels in cerebroside-containing yeast and fungi have not yet to be reported. The present study shows major sphingolipids in cerebroside-containing yeast, *S. kluyveri* to be acidic sphingolipids [IPC, MIPC and M(IP)2C] whose content in *S. kluyveri* is the same as in *S. cerevisiae* (Table 1). In *S. cerevisiae*, acidic sphingolipids are essential for cell growth (9). Inactivation of sphingolipid biosynthesis, such as by disrupting the serine palmitoyltransferase gene (LCB2) or adding the inhibitor of ceramide phosphoinositol transferase, aureobasidin, would be lethal (20, 21). Acidic sphingolipid content as determined in this study (Table 1) may thus possibly be essential for yeast cell growth.

The distribution of sphingolipid classes and sphingolipid class constituents in cerebroside-containing yeast, *S. kluyveri* have been fully clarified by the present study. In general, the major sphingoid bases in acidic sphingolipids were comprised of phytosphingosine and related sphingoid bases, as also noted for ceramide. The major sphingoid bases in fungal cerebroside were 9-Me d18:24t,8t along with related dihydroxy type sphingoid bases, as indicated in the previous study (11). The present and other studies (11, 12, 22) indicate two independent ceramide groups to be present in fungal cells to synthesize cerebroside and other sphingolipids from different ceramide pools. The significance of structure in ceramide moieties for cell growth remains unclear. Tani *et al.* found that, in sphingolipid-

### Table 2  Constituent Compositions in Sphingolipid Classes of *S. kluyveri* (mol%).

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Ceramide</th>
<th>Cerebroside</th>
<th>Acidic sphingolipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Hydroxy fatty acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16h:0</td>
<td>1.0 ± 0.2</td>
<td>2.2 ± 0.0</td>
<td>1.5 ± 0.7</td>
</tr>
<tr>
<td>18h:0</td>
<td>19.9 ± 0.4</td>
<td>95.6 ± 0.2</td>
<td>7.0 ± 1.0</td>
</tr>
<tr>
<td>20h:0</td>
<td>&lt;0.1</td>
<td>0.3 ± 0.0</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>22h:0</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>24h:0</td>
<td>4.1 ± 0.4</td>
<td>0.2 ± 0.0</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td>25h:0</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>26h:0</td>
<td>75.0 ± 0.8</td>
<td>1.7 ± 0.1</td>
<td>86.8 ± 0.6</td>
</tr>
</tbody>
</table>

Sphingoid bases1

| d18:0^2            | 4.3 ± 0.6 | 0.3 ± 0.3 | 4.5 ± 0.1 |
| d18:1^4t           | 2.2 ± 0.4 | 4.3 ± 2.5 | 0.6 ± 0.1 |
| d18:2^4t,8t        | 0.8 ± 0.0 | 26.0 ± 0.7| 0.6 ± 0.1 |
| 9-Me d18:2^4t,8t   | 0.1 ± 0.1 | 67.9 ± 2.6| ND^3      |
| t18:0              | 56.3 ± 2.4| 0.9 ± 0.5 | 59.1 ± 1.5|
| t20:0              | 36.3 ± 3.0| 0.6 ± 0.2 | 35.2 ± 1.5|

Values are means ± SD (n=3).

1 d18:0, sphinganine; d18:1^4t, 4-trans-sphingenine; d18:2^4t,8t, 4-trans, 8-trans-sphingadienine; 9-Me d18:2^4t,8t, 9-methyl-4-trans, 8-trans-sphingadienine; t18:0, 4-hydroxysphinganine; t20:0, 4-hydroxyicosaphinganine.

2 Including trace amount of 4-hydroxynonadecasphinganine (t19:0).

3 Not detected.
deficient \textit{S. cerevisiae} (20), the requirement for sphingoid base can be satisfied by exogenous sphingosine, an unnatural sphingoid base for yeast, although such a supplement would cause failure of lipid microdomain formation. Thus, for normal cell growth, the ceramide moiety may be essential in yeast cells.

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References