NOTE

Free Fatty Acid Level and Galactolipase Activity in a Red Tide Flagellate *Chattonella marina* (Raphidophyceae)

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Abstract: Free fatty acid (FFA) level and galactolipase activity in a marine microalga causing red tides, *Chattonella marina*, were examined. The FFA content increased greatly during the growth from 2.8% of total lipids in the mid-logarithmic phase to 15.4% in the late stationary phase. A crude enzyme preparation derived from the alga had activities hydrolyzing the acyl groups of various glycosylglycerolipids and phospholipids, especially monogalactosyldiacylglycerol (MGDG) molecules. The results suggest that FFAs in *C. marina* are released mainly from MGDG, which is a major lipid class in the alga, by galactolipase.


Key words: *Chattonella marina*, free fatty acid, galactolipase, monogalactosyldiacylglycerol

1 Introduction

*Chattonella marina* is known to be one of the most noxious red tide flagellates causing mass mortality of cultured fish as well as *Chattonella antiqua* (1-3). These species contain high amounts of free fatty acids (FFA) such as 20:5(n-3), which damage the epithelial tissues of fish gills (2-4). In experimental assay system, FFAs destroy red blood cells and show hemolytic activity (3,5). However, there are no reports on the formation and physiological importance of FFA in *Chattonella* species. In this study, we examined the FFA contents during the growth of *C. marina* and its galactolipase activity, which would release FFA from glycolipid molecules such as monogalactosyldiacylglycerols (MGDG).

2 Experimental

2.1 Materials

HPLC-grade solvents, acetonitrile and propanol-2-ol, were obtained from Kanto Chemicals (Tokyo, Japan).

Pure 1-oleoyl-2-palmitoyl-sn-glycero-3-phosphocholine (PC) and nonanedecanoic acid (19:0) were obtained from Sigma (St. Louis, MO, USA). Silica gel 60 F$_{254}$ aluminium sheets (0.25-mm thick) for analytical thin-layer chromatography (TLC) and Silica gel 60 F$_{254}$ plates for preparative TLC (20×20 cm, 0.25-mm thick) were obtained from Merck (Darmstadt, Germany). 9-Anthryldiazomethane (ADAM) was a product of Funakoshi (Tokyo, Japan).

2.2 Algal Culture

*C. marina* (NIES-3) was grown in 2 L of a sterile f/2 medium at 20°C under 4000 lux of cool-white fluorescent illumination on a 12 h light/12 h dark cycle (6). Cells in mid-logarithmic phase (1×10$^4$ cells/mL, 8 d culture), early stationary phase (2×10$^4$ cells/mL, 16 d culture) and late stationary phase (2×10$^5$ cells/mL, 61 d culture) were harvested by centrifuging at 3000 rpm for 10 min. Total lipids were extracted by the method of Bligh and Dyer (7).

2.3 Lipid Analysis

Lipid classes were separated by TLC on Silica Gel 60F$_{254}$ aluminium sheets using chloroform/methanol/water/ethyl acetate/propanol-2-ol (5:2:1:5:5, by vol) as
the developing solvent. Spots were detected by heating at 100°C after spraying an orcinol-sulfonic acid reagent (8). Lipid class composition was determined using an Iatroscan TH-10 TLC-FID analyzer on Chromarods S-III (Iatron, Tokyo, Japan) equipped with C-R6A Chromatopac Integrator (Shimadzu, Kyoto, Japan) (9). An elution system with n-hexane/diethyl ether/acetic acid (60:17:0.5, by vol) was used to separate triacylglycerols (TAG), FFA, free sterols (ST) and polar lipids (PL). MGDG, which was one of major lipid classes in the alga, was separated from PL using acetone followed by chloroform/acetone/formic acid (99:1:0.2, by vol) as the developing solvents (10).

Total lipids were separated into four fractions by preparative TLC on Silica gel F254 to give MGDG, digalactosyldiacylglycerols (DGDG), sulfoquinovosyl-diacylglycerols (SQDG) and phosphatidylglycerols (PG) (11,12). FFA fraction was also obtained from total lipids with n-hexane/diethyl ether/acetic acid (80:20:1, by vol). Fatty acid compositions of these lipid fractions were determined by gas liquid chromatography (11) on an Omegawax 320 column (30 m × 0.32 mm i.d.; Supelco, PA, USA). The column temperature was programmed from 180 to 240°C at 1°C/min. Helium was used as the carrier gas at a constant flow of 1.2 mL/min. Peaks were identified by comparison with known fatty acids from marine organisms (11,13).

2.4 Substrates and Crude Enzyme Preparation

MGDG, DGDG, SQDG, and PG, which were isolated from spinach leaf lipids by preparative TLC (11,12), and synthetic PC were used as the substrates of enzyme reaction. Acetone powders were provided from C. marina of the early stationary phase, as described by Bier (14).

2.5 Measurement of Galactolipase Activity

The acetone powder (10 mg) was added to a 0.05 M sodium phosphate buffer solution (pH 7, 1 mL) containing 1% Triton X-100 and stirred at 20°C for 1 h in a dark (500 rpm). The buffer suspension was centrifuged at 3000 rpm for 5 min and the supernatant was used for the assay of galactolipase activity. To lipids (0.1 mg) placed in a 5 mL Reacti-Vial (Pierce, Rockford, IL, USA), the supernatant (1 mL) was added and incubated for 12 h at various temperatures and pHs. The reaction was stopped by addition of methanol (100 µL) containing 33.5 nmol of 19:0 acid as an internal standard. Released fatty acids were extracted with diethyl ether and derivatized into 9-anthrylmethyl esters using ADAM for HPLC analysis (15). Galactolipase activity was assayed by measuring the amount of released fatty acids and expressed in nmol fatty acid/mg acetone powder/h.

2.6 HPLC

HPLC analysis was carried out with a Waters LC Module I (Waters, Milford, MA, USA) equipped with an ODS column (Superspher RP-18e, 250×4.6 mm i.d., Merck). The mobile phase was acetonitrile/water (98:2, v/v) for 50 min, and was changed by a linear gradient from acetonitrile/propan-2-ol (98:2, v/v) to acetonitrile/propan-2-ol (50:50, v/v) over 20 min, at a flow rate of 0.5 mL/min. The column temperature was 22°C. Peaks were monitored with a Waters 474 Scanning Fluorescence Detector. The excitation and emission wavelengths were set at 365 nm and 412 nm, respectively (15).

3 Results and Discussion

Table 1 shows the lipid class composition of C. marina in the different growth phases. The lipid content was about 2% of wet weight through the growth phases. The FFA contents in total lipids increased greatly from 2.8% in the mid-logarithmic phase to 15.4% in the late stationary phase, whereas PL decreased from 93.5% to

<table>
<thead>
<tr>
<th>Lipid class</th>
<th>Mid-logarithmic phase</th>
<th>Early stationary phase</th>
<th>Late stationary phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacylglycerols</td>
<td>0.3 ± 0.1</td>
<td>0.8 ± 0.2</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>2.8 ± 0.2</td>
<td>8.0 ± 0.6</td>
<td>15.4 ± 0.6</td>
</tr>
<tr>
<td>Sterols</td>
<td>3.3 ± 0.2</td>
<td>4.0 ± 0.2</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td>Polar lipids</td>
<td>93.5 ± 1.0</td>
<td>87.0 ± 1.2</td>
<td>80.1 ± 1.2</td>
</tr>
</tbody>
</table>

*Means ± S.D. (n = 5)
80.1%, respectively. The levels of MGDG, which was one of the major PL in the alga, decreased greatly from 27.2% to 18.3%, respectively. The contents of TAG and ST hardly changed through the growth phase. These findings imply that the FFAs arise mainly from enzymatic hydrolysis of PL, especially MGDG.

Figure 1 shows the TLC chromatogram of total lipids of *C. marina* in the late stationary phase. The glycolipids such as MGDG (Rf, 0.64), DGDG (Rf, 0.31) and SQDG (Rf, 0.20) were observed on the TLC plate as major components. The lyso-compounds, that is, monogalactosylmonoacylglycerol (MGMG; Rf, 0.40), digalactosylmonoacylglycerol (DGMG; Rf, 0.12) and sulfidoquinovosylmonoacylglycerol (SQMG; Rf, 0.08), and PC (Rf, 0.03) were also detected as lesser components. The existence of the lyso-compounds suggests that the FFAs in *C. marina* are produced by galactolipase which hydrolyzes ester bonds of the MGDG, DGDG, and SQDG molecules.

Table 2 gives the fatty acid compositions of the

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**Fig. 1** TLC Chromatogram of Total Lipids from *Chattonella marina* in the Late Stationary Phase. TLC conditions and abbreviations as given in text.

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**Fig. 2** Galactolipase Activity of the Crude Enzyme Preparation Derived from *Chattonella marina*. (A) Time-course of the activity (25°C, pH 7). (B) Effect of temperature on the activity (pH 7). (C) Effect of pH on the activity (25°C). ●: Phosphate buffer; ■: Tris-HCl buffer. Data (mean ± SE, n=3) are expressed in sum of 16:3(n-3) and 18:3(n-3) released from spinach MGDG.
Table 2  Fatty Acid Compositions of the Major Lipid Classes<sup>6</sup> of *Chattonella marina* in the Different Growth Phases (mol %).

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Mid-logarithmic phase</th>
<th>Early stationary phase</th>
<th>Late stationary phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FFA</td>
<td>MGDG</td>
<td>DGDG</td>
</tr>
<tr>
<td>12:0</td>
<td>0.2</td>
<td>0.3</td>
<td>0.1</td>
</tr>
<tr>
<td>14:0</td>
<td>4.8</td>
<td>5.2</td>
<td>5.5</td>
</tr>
<tr>
<td>15:0</td>
<td>0.7</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>16:0</td>
<td>30.8</td>
<td>18.3</td>
<td>11.2</td>
</tr>
<tr>
<td>17:0</td>
<td>0.1</td>
<td>—</td>
<td>0.1</td>
</tr>
<tr>
<td>18:0</td>
<td>1.5</td>
<td>0.6</td>
<td>0.5</td>
</tr>
<tr>
<td>20:0</td>
<td>0.1</td>
<td>0.1</td>
<td>—</td>
</tr>
<tr>
<td>22:0</td>
<td>0.2</td>
<td>0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>Σ saturates</td>
<td>38.4</td>
<td>25.2</td>
<td>18.2</td>
</tr>
<tr>
<td>14:1&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.3</td>
<td>0.1</td>
<td>—</td>
</tr>
<tr>
<td>16:1&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.4</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>16:1(n-7)</td>
<td>7.0</td>
<td>11.2</td>
<td>9.2</td>
</tr>
<tr>
<td>16:1(n-13)</td>
<td>2.4</td>
<td>—</td>
<td>0.1</td>
</tr>
<tr>
<td>16:0(n-3)</td>
<td>0.9</td>
<td>—</td>
<td>9.7</td>
</tr>
<tr>
<td>18:1(n-9)</td>
<td>0.9</td>
<td>1.9</td>
<td>0.9</td>
</tr>
<tr>
<td>18:1(n-7)</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Σ monoenes</td>
<td>12.0</td>
<td>13.3</td>
<td>10.3</td>
</tr>
</tbody>
</table>

| FFA  | MGDG | DGDG | SQDG | PG | FFA  | MGDG | DGDG | SQDG | PG | FFA  | MGDG | DGDG | SQDG | PG | FFA  | MGDG | DGDG | SQDG | PG |

<sup>6</sup>FFA, free fatty acid; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol.

The position of the double bond is unknown.

major lipid classes of *C. marina*. In the different growth phases, the FFA compositions were similar to those of MGDG and DGDG, but clearly differ from those of SQDG and PG. The predominant components of FFA, MGDG, and DGDG were 16:0, 18:4(n-3), and 20:5(n-3), while those in SQDG were 14:0, 16:0, and 20:5(n-3). The predominant components of PG in the mid-logarithmic and early stationary phases were 16:0, 20:5(n-3), and *trans*-16:1(n-13), but the last component was replaced by 18:1(n-9) in the late stationary phase. The 18:1(n-9) and *trans*-16:1(n-13) in PG, and 14:1 and 16:1 (the position of the double bond is unknown) in SQDG were very minor components in FFA. These results suggest that FFAs are released from the galactolipids.

A crude enzyme preparation derived from *C. marina* of the early stationary phase released significantly amounts of 18:3(n-3) from spinach MGDG, which major molecular species (*sn-1/sn-2*) were 18:3(n-3)/16:3(n-3) (51%) and 18:3(n-3)/18:3(n-3) (45%) (16). Figure 2 shows the galactolipase activity of the enzyme preparation from *C. marina* obtained with the spinach MGDG as the substrate. The activity was assayed at pH 7 to minimize non-enzymatic acyl migration of the lyso-compounds produced during incubation. The fatty acid level increased gradually to 12 h after the incubation was started (Fig. 2A). In this study, therefore, galactolipase activity was usually estimated after 12 h incubation. The optimum temperature was 20-25°C at pH 7 when 0.05 M sodium phosphate was used (Fig.
2B) and the highest activity was observed at pH 9 when 0.05 M Tris-HCl was used at 25°C (Fig. 2C). A high phospholipase A activity in basic area (pH 9-10) has been found from plankton (17), although glyco- and phospholipid acyl hydrolases in various plant tissues have pH optima in acidic or neutral area (18-22).

**Figure 3** shows the acyl hydrolase activities towards different lipid classes of the crude enzyme preparation derived from *C. marina*. The enzyme preparation had the highest activity towards MGDG. Although a high phospholipase A activity was also observed, PC was much less compound than glycolipidolipids in the alga (see Fig. 1). These results support that FFAs in *C. marina* are released mainly from MGDG by galactolipase existed in the alga. Sajiki et al. (17) found phospholipase A activities from bivalves and their feed phytoplankton, but the latter algal species were not identified and no galactolipase activity was assayed. The lower activity towards spinach leaf PG than towards synthetic sn-1-oleoyl-2-palmitoyl PC (Fig. 3) may due to a large amount of trans-16:1(n-13) existed in the PG (30% of the total fatty acids), which would resist hydrolysis. Sahsah et al. (21) also observed that the galactolipase activity in cowpea leaves was lower towards PG with 16:1 comprising 23% of the total fatty acids than towards PC.

In conclusion, this study has for the first time reported on the production and physiological importance of FFA in red tide flagellates including *C. marina*.

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**References**
