Note

Characterization of Glycolipids in the Strain Chlorella pyrenoidosa

Shinji Yamashita1, Taiki Miyazawa2, Ohki Higuchi2, Hideo Takekoshi3, Teruo Miyazawa2 and Mikio Kinoshita1,*

1Department of Life and Food Sciences, Obihiro University of Agriculture and Veterinary Medicine, Obihiro 080–8555, Japan
2Food Biotechnology Innovation Project, New Industry Creation Hatchery Center (NICHe), Tohoku University, Sendai 980–8579, Japan
3Production and Development Department, Sun Chlorella Co., Ltd., Kyoto 600–8177, Japan

(Received March 29, 2022)

Summary Plant-derived polar lipids have been reported to exhibit various beneficial effects on human health. The green alga Chlorella is known to be abundant in nutrients, including lipophilic components, and has varying nutrient content depending on the strain. In this study, to assess the nutritional functions of the strain Chlorella pyrenoidosa, we comprehensively analyzed the composition of fatty acids, polar glycerolipids, and sphingolipids. We found that n-3 polyunsaturated fatty acids (PUFAs) comprised 45.6 mol% of fatty acids in the total lipids and 62.2 mol% of n-3 PUFAs in the total lipids occurred in the glycolipids. Monogalactosyldiacylglycerol was the primary glycolipid class, and n-3 PUFA constituted 73.5 mol% of the fatty acids. Although glucosylceramide was observed in trace amounts, highly polar sphingolipids (HPSs), including glycosyl inositol phosphoryl ceramide, were found in much higher amounts compared to rice bran, which is a common source of sphingolipids. These results suggest that the examined Chlorella strain, which is abundant in glycolipids bearing n-3 PUFAs and HPS, is potentially useful as a dietary supplement for improving human health.

Key Words GIPC, MGDG, long chain base, n-3 PUFA, sphingolipid

The incidence of intestinal impairments, such as colorectal cancer (CRC) and inflammatory bowel disease (IBD), has recently increased in East Asian countries, including Japan, while cases in Western countries continue to remain high (1, 2). Patients with IBD undergo long-term treatment and are at increased risk of developing CRC (3). Epidemiological studies have indicated that CRC is strongly associated with the Western diet (4), which contains red meat and processed meat, which are abundant in saturated fatty acids, n-6 polyunsaturated fatty acids (PUFAs), and heme iron. An excessive intake of these is believed to embrittle the gut barrier (5), accelerate intestinal inflammation (6), and damage the intestine via the oxidation of PUFAs during digestion (7).

Generally, plants do not have large amounts of lipids, but plant-derived lipids, especially glycolipids, have been reported to show various beneficial effects on human health, including the prevention of intestinal impairments. Leaves have a high ratio of n-3 PUFAs, which are anti-inflammatory substances, where the n-3 PUFAs mostly exist as glycolglycerolipids. Glycoglycerolipids bearing n-3 PUFAs are highly stable against oxidation (8). Glucosylceramide (GlcCer) is a major plant-derived sphingolipid that alleviates intestinal inflammation (9) and the formation of aberrant crypt foci, which are precursors of CRC (10). Glycosyl inositol phosphoryl ceramide (GIPC) is another major plant-derived sphingolipid, and highly polar sphingolipids (HPSs) containing GIPC are expected to exhibit suppressive effects on intestinal inflammation (11).

Chlorella is a unicellular green alga that grows in fresh water and is known to be abundant in various nutrients, including proteins and lipophilic components (e.g., n-3 PUFAs and lutein). Chlorella ingestion has been reported to increase lutein levels in human erythrocytes and reduce oxidation (12, 13). The lipid composition in Chlorella differs depending on the strain and growth conditions (14, 15), and the details of composition, especially in terms of GIPC, are poorly understood owing to the complexity of GIPC extraction and analysis.

To investigate whether the strain Chlorella pyrenoidosa has functional lipids that can prevent intestinal impairment, we analyzed the composition of fatty acids, polar glycerolipid classes, and sphingolipid classes in this study.

Materials and Methods

Sample and lipid preparation. The Chlorella powder used in the study, which was prepared using Chlorella pyrenoidosa, was procured from Sun Chlorella (Kyoto, Japan). The lipophilic fraction was extracted from
the powder using distilled-water/methanol/chloroform (1 : 5 : 5, v/v/v). The extract was separated into organic and water layers according to Bligh and Dyer’s method (16). The organic layer was used as the total lipid fraction, and subsequently, the total lipid fraction was separated into fractions of neutral lipids, glycolipids, and phospholipids using silica gel column chromatography (Iatrobeads; LSI Medience, Tokyo, Japan) with a solvent system in the order of chloroform, acetone, and methanol. Fractions were weighed after drying.

**Analysis of lipid classes and fatty acids.** The glycolipid fraction, obtained in the previous step, was subjected to silica thin-layer chromatography (TLC; TLC Silica gel 60, glass plate, film thickness [d] 250 μm; Merck KGaA, Darmstadt, Germany) with chloroform/methanol/distilled-water (65 : 16 : 2, v/v/v) as the solvent system and anthrone-sulfuric acid as the sugar detection reagent. The phospholipid fraction was subjected to two-dimensional TLC with a chloroform/acetone/methanol/acetic acid/distilled-water (50 : 20 : 10 : 15 : 5, all fractions by vol.) solvent system after chloroform/methanol/distilled-water (65 : 25 : 4, v/v/v) was used. The Dittmer–Lester reagent was also used for phosphorus detection. After the TLC positions of the classes in glycolipids and phospholipids were confirmed, the fractions were subjected to TLC again and separated into respective classes using iodine as a nondestructive detection reagent. The separated classes were scraped and methylated under a methanol and acetic chloride solution (9 : 1, v/v) at 100°C for 2 h, using nonadecanoic acid (Nagara Science, Gifu, Japan) as the internal standard. Fatty acid methyl esters were analyzed using a gas chromatography (GC)–flame ionization detector (GC-2010; Shimadzu, Kyoto, Japan) with a high-polarity capillary column (CP-Sil 88, 50 m×inside diameter [i.d.] 0.25 mm, dI 0.20 μm; Agilent, CA, USA).

**Sphingolipid analyses.** GlcCer, ceramide (Cer), and HPS in the samples were separated, and the long-chain base (LCB) composition of each was analyzed using previously reported methods (17, 18). The sphingolipids in

### Table 1. Contents of lipid fractions and fatty acids in *Chlorella pyrenoidosa*.1

| Lipid fraction (g/100 g dry wt.) | Total lipids | Neutral lipids | Glycolipids | Phospholipids |
|---------------------------------|-------------|----------------|-------------|--------------|
| Fatty acid (mmol/100 g dry wt.) | 16.6±0.3    | 3.4±0.3        | 5.7±0.2     | 7.2±0.3      |
| C16:0                           | 3.6±0.1     | 0.4±0.0        | 0.3±0.0     | 1.8±0.1      |
| C16:3n-3                        | 2.8±0.1     | 0.2±0.0        | 2.4±0.2     | 0.1±0.0      |
| C16:4n-3                        | 1.1±0.0     | 0.1±0.0        | 1.0±0.0     | 0.4±0.1      |
| C18:0                           | 0.6±0.0     | 0.1±0.0        | 0.2±0.0     | 0.2±0.0      |
| C18:1n-9                        | 1.3±0.0     | 0.2±0.0        | 0.3±0.0     | 0.1±0.0      |
| C18:1n-7                        | 1.3±0.0     | 0.1±0.0        | 0.1±0.0     | 0.2±0.0      |
| C18:2n-6                        | 3.3±0.1     | 0.4±0.0        | 0.8±0.1     | 0.7±0.1      |
| C18:3n-3                        | 6.5±0.2     | 0.6±0.0        | 3.1±0.2     | 0.5±0.1      |
| Others                          | 2.3±0.1     | 0.2±0.0        | 1.1±0.1     | 0.9±0.0      |
| SFAs                            | 18.1        | 20.6           | 3.8         | 40.1         |
| MUFAs                           | 16.6        | 18.1           | 7.0         | 23.8         |
| PUFAs                           | 65.3        | 61.3           | 89.2        | 36.1         |
| n-6/n-3                         | 0.32        | 0.39           | 0.13        | 0.68         |

Means±standard deviation (n=3).

1 Total lipids and neutral lipids were analyzed as fractions, while glycolipids and phospholipids were the total of the corresponding classes shown in Tables 2 and 3.

SEAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids.

### Table 2. Composition of classes and fatty acids in glycolipids of *Chlorella pyrenoidosa*.

| Glycolipid class (mol%) | MGDG | DGDG | SQDG |
|-------------------------|------|------|------|
| Composition             | 87.0±8.2 | 9.7±0.7 | 3.3±0.0 |
| Fatty acid (mol%)       |      |      |      |
| C16:0                   | 0.7±0.0 | 8.9±0.3 | 60.5±5.0 |
| C16:3n-3                | 26.4±3.0 | 27.8±0.6 | 7.4±3.1 |
| C16:4n-3                | 12.7±1.0 | 1.9±0.0 | 1.9±0.3 |
| C18:0                   | 0.1±0.1 | 2.6±0.1 | 0.0±0.0 |
| C18:1n-9                | 3.2±0.4 | 4.7±0.1 | 2.0±0.4 |
| C18:2n-7                | 0.5±0.1 | 1.1±0.0 | 2.1±0.3 |
| C18:2n-6                | 9.0±0.9 | 12.9±0.2 | 3.5±1.1 |
| C18:3n-3                | 34.3±1.4 | 29.8±0.8 | 20.3±2.0 |
| Others                  | 12.9±0.9 | 10.3±0.3 | 2.3±1.3 |
| SFAs                    | 0.8   | 11.4  | 60.5  |
| MUFAs                   | 6.7   | 9.7   | 4.9   |
| PUFAs                   | 92.4  | 78.9  | 34.7  |
| n-6/n-3                 | 0.12  | 0.22  | 0.12  |

Means±standard deviation (n=3).

MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids.
Table 3. Composition of classes and fatty acids in phospholipids of Chlorella pyrenoidosa.

| Phospholipid class (mol%) | PE | PG | PC | PI |
|---------------------------|----|----|----|----|
| Composition               | 26.5±4.4 | 43.5±0.7 | 27.3±4.7 | 2.7±0.4 |
| Fatty acid (mol%)         |    |    |    |    |
| C16:0                     | 31.2±4.7 | 38.9±3.6 | 32.7±5.3 | 49.3±2.9 |
| C16:3n-3                  | 2.5±0.4  | 0.2±0.0  | 4.8±1.7  | 0.6±0.3  |
| C16:4n-3                  | 15.1±0.8 | 6.1±1.0  | 4.8±2.1  | 0.9±0.8  |
| C18:0                     | 3.2±1.2  | 3.1±0.3  | 6.6±1.4  | 24.0±1.8 |
| C18:1n-9                  | 2.1±0.3  | 0.1±0.1  | 4.3±0.4  | 12.9±1.3 |
| C18:1n-7                  | 1.7±0.6  | 5.7±0.6  | 6.8±1.2  | 1.5±0.5  |
| C18:2n-6                  | 15.5±2.1 | 11.0±1.8 | 18.7±5.4 | 4.0±1.8  |
| C18:3n-3                  | 10.9±2.2 | 9.8±2.5  | 13.3±3.1 | 0.7±0.4  |
| Others                    | 17.7±2.2 | 25.1±2.5 | 7.9±1.0  | 6.3±0.3  |

Means±standard deviation (n = 3).
PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylinositol; PI, phosphatidylcholine; SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Results

The Chlorella strain examined contained 16.6 wt% of total lipids in dry weight (Table 1). The fraction of neutral lipids, glycolipids, and phospholipids accounted for 20.5, 34.3, and 43.2 wt% of the total lipids, respectively. In terms of total lipids, n-3 PUFA was 45.6 mol% of fatty acids, while n-6 PUFA was 14.6 mol%; 62.2 mol% of n-3 PUFA in the total lipids were found in the glycolipids.

The glycolipid fraction comprised three classes (Table 2): monogalactosyldiacylglycerol (MGDG), which was a significantly large fraction, digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG), in decreasing order of proportion. Among the glycolipids, MGDG had the highest ratio of unsaturated fatty acids (99.1 mol%) and had n-3 PUFA as 73.5 mol% of fatty acids. The phospholipid fraction comprised four classes: phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylcholine (PC), in decreasing order of proportion. Among the glycolipids, MGDG had the highest ratio of unsaturated fatty acids (99.1 mol%) and had n-3 PUFA as 73.5 mol% of fatty acids.

Table 4. Contents and structures of sphingolipids in Chlorella pyrenoidosa.

| Phospholipid class (mol%) | PE | PG | PC | PI |
|---------------------------|----|----|----|----|
| Composition               |    |    |    |    |
| Fatty acid (mol%)         |    |    |    |    |
| C16:0                     | 31.2±4.7 | 38.9±3.6 | 32.7±5.3 | 49.3±2.9 |
| C16:3n-3                  | 2.5±0.4  | 0.2±0.0  | 4.8±1.7  | 0.6±0.3  |
| C16:4n-3                  | 15.1±0.8 | 6.1±1.0  | 4.8±2.1  | 0.9±0.8  |
| C18:0                     | 3.2±1.2  | 3.1±0.3  | 6.6±1.4  | 24.0±1.8 |
| C18:1n-9                  | 2.1±0.3  | 0.1±0.1  | 4.3±0.4  | 12.9±1.3 |
| C18:1n-7                  | 1.7±0.6  | 5.7±0.6  | 6.8±1.2  | 1.5±0.5  |
| C18:2n-6                  | 15.5±2.1 | 11.0±1.8 | 18.7±5.4 | 4.0±1.8  |
| C18:3n-3                  | 10.9±2.2 | 9.8±2.5  | 13.3±3.1 | 0.7±0.4  |
| Others                    | 17.7±2.2 | 25.1±2.5 | 7.9±1.0  | 6.3±0.3  |

Means±standard deviation (n = 3).
PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PC, phosphatidylinositol; PI, phosphatidylcholine; SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Discussion

In this study, we analyzed the levels and structures of functional lipids in Chlorella pyrenoidosa. The Chlorella strain had n-3 PUFA that comprised 45.6 mol% of fatty acids and more than half of the Chlorella n-3 PUFA were found in the glycolipids, which predominantly contained MGDG. Compared to oils (triacylglycerol) such as perilla oil and linseed oil, which are used as general plant sources for n-3 PUFA (approximately 60 mol% of fatty acids), this Chlorella strain had a lower ratio of n-3 PUFA. However, this Chlorella strain has a higher stability to oxidation due to the glycolipid form (8, 20). n-3 PUFA with high stability are expected to exhibit anti-inflammatory effects on the intestine and barely generate mutagenic aldehydes. Dietary MGDG has been reported to suppress colon tumor growth in mouse models (21). Additionally, the HPS amount in the Chlorella strain was approx-
imately twice that of rice bran (35.5 mg/100 g), which is a typical source of sphingolipids (18). HPSs are also expected to reduce in intestinal inflammation (11).

The n-3 PUFAs in the examined Chlorella strain comprise C16:3, C16:4, and C18:3 fatty acids. In a previous study, three samples of Chlorella pyrenoidosa tested did not contain C16:4 fatty acids (15), and the n-3 PUFA ratio was found to be approximately half of that reported in the present study. Although their growth conditions cannot be compared with those in previous study because they have not been described (15), deficiencies of trace elements such as iron increase the ratio of PUFAs in Chlorella (22), while their excess addition decreases the ratio and increases oxidative stress and the activities of antioxidant enzymes (23): supplementation of glucose as the carbon source decreases the ratio of PUFAs compared to that under autotrophic conditions (24). Because PUFAs act as antioxidants on thylakoid membranes during photosynthesis (25), the development of thylakoid membranes may be correlated with desaturase activity for synthesis of PUFAs.

The Chlorella strain had trace levels of GlcCer, the same level of Cer, and twice the level of HPS compared to rice bran. Regarding sphingolipids contained in Chlorella, the structures of GIPC and oligoglycosylceramides have been reported earlier (26, 27); however, to the best of our knowledge, there are no articles on the quantitative data for GIPC, Cer, and GlcCer. According to earlier reports, the GIPC structures identified in Chlorella sorokiniana and Chlorella variabilis are inositol phospholipid ceramide with two hexoses; their primary LCB is t18:0, and their primary 2-hydroxy fatty acid is C24h:0 or C24h:1, depending on the strain. This information on GIPC supports the composition of HPS, including GIPC, observed in this study.

To conclude, the Chlorella pyrenoidosa strain examined in this study was found to be rich in glycolipids bearing n-3 PUFAs and HPS; furthermore, it is potentially useful as a dietary supplement for improving human health, including the suppression of intestinal impairments.

**Authorship**

SY performed experiments and drafted the manuscript. TM (second author) and OH performed analyses. HT edited the manuscript. TM supervised this study. MK conceptualized the study and advised on the analytical methods.

**Disclosure of state of COI**

The authors have no potential conflicts of interest to disclose.

**REFERENCES**

1) Center MM, Jemal A, Smith RA, Ward E. 2009. Worldwide variations in colorectal cancer. CA Cancer J Clin 59: 366–378.

2) Molodecky NA, Soon IS, Rabi DM, Ghali WA, Ferris M, Chernoff G, Benchimol EI, Panaccione R, Ghosh S, Barkema HW, Kaplan GG. 2012. Increasing incidence and prevalence of the inflammatory bowel diseases with time, based on systematic review. Gastroenterology 142: 46–54.

3) Triantafillidis JK, Nasioulas G, Kosmidis PA. 2009. Colorectal cancer and inflammatory bowel disease: epidemiology, risk factors, mechanisms of carcinogenesis and prevention strategies. Anticancer Res 29: 2727–2737.

4) IARC. 2015. IARC Monographs Evaluate Consumption of Red Meat and Processed Meat. The International Agency for Research on Cancer, Lyon, France.

5) Ghoshal S, Witta J, Zhong J, de Villiers W, Eckhardt E. 2009. Chylomicrons promote intestinal absorption of lipopolysaccharides. J Lipid Res 50: 90–97.

6) Scialli E, Liverani E, Belluzzi A. 2017. The imbalance between n-6/n-3 polyunsaturated fatty acids and inflammatory bowel disease: A comprehensive review and future therapeutic perspectives. Int J Mol Sci 18: 2619.

7) Sessink ALA, Termont DSML, Kleibeuker JH, Van der Meer R. 1999. Red meat and colon cancer: The cytotoxic and hyperproliferative effects of dietary heme meat and colon cancer. Cancer Res 59: 5704–5709.

8) Yamaguchi T, Sugimura T, Shimaji Y, Suda M, Abe M, Hosokawa M, Miyashita K. 2012. Oxidative stability of glyceroglycolipids containing polyunsaturated fatty acids. J Oleo Sci 61: 505–513.

9) Arai K, Mizobuchi Y, Tokuji Y, Aida K, Yamashita S, Ohnishi M, Kinoshita M. 2015. Effects of dietary plant-origin glucosylceramide on bowel inflammation in DDS-treated mice. J Oleo Sci 64: 737–742.

10) Yamashita S, Sakurai R, Hishiki K, Aida K, Kinoshita M. 2017. Effects of dietary plant-origin glucosylceramide on colon cytokine contents in DMH-treated mice. J Oleo Sci 66: 157–160.

11) Jutanom M, Higaki C, Yamashita S, Nakagawa K, Matsu-moto S, Kinoshita M. 2020. Effects of sphingolipid fractions from golden oyster mushroom (Pleurotus citrinopileatus) on apoptosis induced by inflammatory stress in an intestinal tract in vitro model. J Oleo Sci 69: 1087–1093.

12) Miyazawa T, Nakagawa K, Kinuma F, Nakashima Y, Maruyama I, Higuchi O, Miyazawa T. 2013. Chlorella is an effective dietary source of lutein for human erythrocytes. J Oleo Sci 62: 773–779.

13) Miyazawa T, Nakagawa K, Takekoshi H, Higuchi O, Kato S, Kondo M, Kinuma F, Miyazawa T. 2013. Ingestion of chlorella reduced the oxidation of erythrocyte membrane lipids in senior Japanese subjects. J Oleo Sci 62: 873–881.

14) Murakami C, Takahashi J, Shimoto K, Maruyama T, Niiya I. 1997. Lipids and fatty acid compositions of Chlorella. Nikko Yukiwakaru Kaishi (J Jpn Oil Chem Soc) 46: 423–427.

15) Ötes S, Pire R. 2001. Fatty acid composition of Chlorella and Spirulina microalgae species. J AOAC Int 84: 1708–1714.

16) Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37: 911–917.

17) Yamashita S, Higaki C, Kanai A, Kikuchi N, Suzuki D, Kinoshita M, Miyazawa T. 2021. Sphingolipid properties in sake rice cultivars and changes during polishing and brewing. J Oleo Sci 70: 263–273.

18) Yamashita S, Higaki C, Kikuchi N, Suzuki D, Kinoshita M, Miyazawa T. 2021. Sake (rice wine) brewing hydro-
Glycolipids in the Strain Chlorella pyrenoidosa

lyzes highly polar sphingolipids to ceramides and increases free sphingoid bases. *J Oleo Sci* **70**: 263–273.

19) Fujino Y, Ohnishi M, Ito S. 1985. Molecular species of ceramide and mono-, di-, tri-, and tetraglycosylceramide in bran and endosperm of rice grains. *Agric Biol Chem* **49**: 2753–2762.

20) Maruya N, Shirasugi-Kataoka N, Okamoto Y, Taniguchi T, Hattori M, Nakao Y, Tsukuda T, Hayasaki H. 1998. High-temperature stability of refined perilla oil and effects of adding some food components. *Nippon Eigo Shokuryo Gakkaishi (J Jpn Soc Nutr Food Sci)* **51**: 323–332.

21) Maeda N, Kokai Y, Hada T, Yoshida H, Mizushina Y. 2013. Oral administration of monogalactosyl diacylglycerol from spinach inhibits colon tumor growth in mice. *Exp Ther Med* **5**: 17–22.

22) Sakurai Y, Iwata I. 1965. Kurorera no C16 huhouwashibousan nitsuite. *Hissu-aminosan-kenkyu* **26**: 1 (in Japanese).

23) Mandal MK, Saikia P, Chauhuri NK, Chaurasia N. 2019. Modulation of lipid content and lipid profile by supplementation of iron, zinc, and molybdenum in indigenous microalgae. *Environ Sci Pollut Res Int* **26**: 20815–20828.

24) Rath SK, Babu S, Renuka N, Prasanna R, Prasad RBN, Saxena AK. 2013. Exploring nutritional modes of cultivation for enhancing lipid accumulation in microalgae. *J Basic Microbiol* **53**: 440–450.

25) Fields MW, Hise A, Lohman EJ, Bell T, Gardner RD, Corredor L, Moll K, Peyton BM, Characklis GW, Gerlach R. 2014. Sources and resources: importance of nutrients, resource allocation, and ecology in microalgal cultivation for lipid accumulation. *Appl Microbiol Biotechnol* **98**: 4805–4816.

26) Rezanka T, Mares P. 1990. Preparative separation of sphingolipids and of individual molecular species by high-performance liquid chromatography and their identification by gas chromatography-mass spectrometry. *J Chromatogr* **509**: 333–346.

27) Rose S. 2015. A lipidomics approach to the viral-host dynamics of the unicellular, eukaryotic alga *Chlorella variabilis* and its viral pathogen, PBCV-1. Dissertations Theses Biol Sci, University of Nebraska–Lincoln.