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Molecular species composition of glycolipids from *Sprirulina platensis*

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Abstract

The different fractions of glycolipids were separated by HPLC column. Lipase XI from *Rhizopus arrhizus* was used to hydrolyze the fatty acids esterified in sn-1. The main fatty acids in MGDG and DGDG were C16:0, C18:2n-6, and C18:3n-6; those in SQDG were C16:0 and C18:2n-6 with a small amount of C18:3n-6. The molecular species analysis of the glycolipids showed that unsaturated fatty acids, such as C18:2n-6 and C18:3n-6, mainly occupied the sn-1 position, while saturated fatty acid C16:0 occupied the sn-2 position. MGDG contained 43.6% (18:3, 16:0), 13.9% (16:0, 16:0), 9.9% (18:2,16:0), and 8.9% (18:3, 16:1). DGDG contained 37.6% (18:3, 16:0), 8.7% (16:0, 16:0), and 8.7% (18:2, 16:0). Molecular species composition of SQDG differed from those of MGDG and DGDG, and it contained 52.5% (18:2, 16:0), 11.0% (16:0, 16:0), 7.1% (16:1, 16:0), and 7.7% (18:1, 16:0).

Keywords: Gycolipid; Molecular species; *Spirulina*; Fatty acid

1. Introduction

The cyanobacterium *Spirulina platensis* is rich in nutrients, such as proteins, vitamins, minerals, carbohydrates, and gamma-linolenic acid. It has gained more and more attention, not only for foods aspects but also on the development of potential pharmaceuticals (Quoc & Pascaud, 1996). Recent work showed that this species has immuno-promoting effects, such as enhancing macrophage phagocytic function and carotenoids in algae and it has antioxidant activity in vitro and in vivo (Hayakawa, Hayashi, Hayashi, Ozawa, Nuya, & Sakugawa, 1997; Kim, Lee, & Moon, 1998; Miranda, et al., 1998; Mishima et al., 1998; Qureshi & Ali 1996). The natural sulfated polysaccharides separated from *S. platensis* inhibit replication of several viruses, such as Herpes Simples, and HIV-1 (Ayehunie, Belay, Baba, & Ruprecht, 1998; Hayashi, Hayashi, Maeda, & Kojima, 1996). Gustafson et al. (1989) reported that SQDG from the cyanobacterium had the AIDS-antivirus activities. Glycolipids are major lipid components of all chloroplast membranes and the photosynthetic membranes of cyanobacteria. MGDG and DGDG mainly occupy the thylakoid membranes of plants, and SQDG is one of the main lipid components. The fatty acid composition of *S. platensis* differs from those of other plant species. In order to elucidate the physiological functions of glycolipids with different fatty acids, the isolation and the molecular species analysis of glycolipids in *S. platensis* was attempted.

2. Materials and methods

2.1. Materials

Dried *S. platensis* was purchased from Shenzhen Yude Microalgae Development Co. Ltd. (Shenzhen,

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Nomenclature

MGDG,	monogalactosyl diacylglycerol;
DGDG,	digalactosyl diacyl glycerol;
SQDG,	sulfoquinovosyl diacylglycerol;
PG,	phosphatidylglycerol;
HPLC,	high performance liquid chromatography;
TLC,	thin-layer chromatography;
GC,	gas chromatography;
SPE,	solid phase extraction;
PUFA,	polyunsaturated fatty acids;
sn-1,	carbon 1 of the glycerol backbone;
sn-2,	carbon 2 of the glycerol backbone: (Cm, Cn), molecules species of glycolipids with fatty acids (Cm) esterified on carbon 1and fatty acids (Cn) esterified on carbon 2 of the glycerol backbone;
FAME,	fatty acid methyl ester (FAME); $CHCl_3$, chloroform; CH_3OH, methanol; HCOOH, formic acid;
CH_3COCH_3,	acetone;
HAc,	acetic acid; C_6H_6, benzene;
H_2O,	distilled water;

China). Authentic compounds of MGDG, DGDG and lipase (Type XI from *Rhizopus arrhizus*) were purchased from Sigma Chemical Co. (USA). SQDG was extracted from fresh spinach leaves.

2.2. Extraction of total lipids

Total lipids were extracted from the dried *S. platensis* using the modified method of Bligh and Dyer (1959) and 0.638 g of total lipids was obtained from 10 g dried powder.

2.3. Isolation and purification of lipids

The total lipids were separated on a silica gel column with $CHCl_3$–CH_3OH–HCOOH (100:10:2, v/v/v) and $CHCl_3$-CH_3OH-HCOOH (100:20:4, v/v/v). Fractions that contained most of the MGDG, DGDG, and SQDG were combined.

MGDG was purified by further column chromatography with the elution solvent, $CHCl_3$–CH_3OH–CH_3COCH_3–HAc (73:1.5:25:0.5, v/v/v/v), and identified by two dimensional TLC and Infrared (IR) analysis, paralleled with authentic samples.

DGDG was purified by silica gel column chromatography with the elution solvent, CH_3COCH_3–C_6H_6–H_2O (91:30:4, v/v/v), and identified by two dimensional TLC and IR analysis, paralleled with authentic samples.

SQDG was purified on a silica gel column with the elution system $CHCl_3$–CH_3OH–HCOOH (100:18:3.5, v/v/v), and the fractions containing SQDG were combined. The combined mixture was applied to second column and washed with $CHCl_3$–CH_3OH (95:5, v/v), and $CHCl_3$–CH_3OH (90:10, v/v). Purified SQDG were obtained by elution with $CHCl_3$–CH_3OH (80:20, v/v).

TLC was carried out on silica gel plates (Marine Chemical Products Co., Qingdao, China) and the developing solvent was $CHCl_3$–CH_3OH–HCOOH (100:20:4, v/v/v) for one dimensional development. The two dimensional development system is as follows: (1) $CHCl_3$–CH_3OH–CH_3COCH_3–HAc–H_2O (50:10:20:10, v/v/v/v); (2) CH_3COCH_3–C_6H_6–HAc–H_2O (100:15:1.5:5, v/v/v/v). Lipids were detected by anthrone reagent for glycolipids and Dittmer-Lester reagent for phospholipids.

2.4. HPLC separation of MGDG, DGDG, SQDG molecular species

MGDG, DGDG, and SQDG, obtained after TLC, were dissolved in CH_3OH–H_2O (95:5, v/v), and the molecular species were separated on a reverse phase HPLC column (ODS, 7.8×300 mm, Dalian Chromatography Corp., China). High-performance liquid chromatography (HPLC) was performed on a Hitachi 655 Liquid System with a UV-VIS variable wavelength detector (205 nm). The elution system was CH_3OH–H_2O (95:5, v/v) at 3.0 ml/min flow rate. Each fraction identified as an HPLC peak was collected and combined after 10 injections. To obtain the objective portion, each HPLC combined fraction was passed through the SPE tube (Supelco R18, 3 ml; Sigma Co., USA) and washed with water, followed by elution with CH_3OH, CH_3OH–$CHCl_3$ (1:1, v/v), and $CHCl_3$. All the organic portions were combined, evaporated and stored in chloroform.

2.5. Enzymatic hydrolysis

Each fraction separated by HPLC was hydrolyzed with lipase (type XI) from *Rhizopus arrhizus* with the modification method described by Fisher, Heinz, and Zeus (1973). After the hydrolysis by lipase, the fatty acids were obtained from the top half of the TLC plate (R_f 0.8) with hexane–diethyl ether–formic acid (50:50:1, v/v/v), and 2-acyl-lyso-MGDG, DGDG, and SQDG were separated by means of further development with the solvent system $CHCl_3$–CH_3OH–HCOOH (80:20:4, v/v/v). The hydrolytic products were extracted from silica gel with $CHCl_3$–CH_3OH (50:50, v/v) and converted to their methyl esters for GC analysis.

2.6. Preparation and analysis of fatty acid methyl esters (FAME)

The acyl lipids and free fatty acids were esterified by the method of Carreau and Dubacq (1975). The FAME obtained from total lipids of *S. platensis* were purified by TLC with the solvent benzene. Analysis of the fatty acid methyl esters was performed on a HP5890A gas chromatograph equipped with a fused silica capillary column (0.32×30 m), coated with OV-225 (Angilent Technlologies, USA). The column temperature was 180 °C and the injector temperature was 250 °C. Fatty acid methyl esters were identified by standards.

2.7. Calculation of amounts of molecular species

The amounts of molecular species of fractions separated from HPLC are based on the carbon number, which is indicated as a sum of carbon atom number of acyl groups minus double bond number multiplied by 2. The molecular species of glycolipids were calculated from the amounts of fractions separated by HPLC and molecular species identified.

2.8. Statistical analysis of experimental data

Each reported value is the mean of three determinations. Significant difference among the treatment means were separated by using Duncan's (1995) multiple range test, at a level of $P<0.05$.

3. Results and discussion

3.1. Fatty acids composition of glycolipids

The total lipid content of dried *Spirulina* powder was 6.38%, and the glycolipid content was 28.2±1.28% of the total lipids by the colorimetric method. Total lipid contained MGDG (10.3±0.54%), DGDG (6.44±0.43%), and SQDG (11.4±0.71%). Fatty acid composition of the three glycolipids and total lipid in *Spirulina* are shown in Table 1.

Table 1
Fatty acid composition of total lipids and polar lipids in *Spirulina platensis*[a]

Fatty acid	Amount (%)			
	Total lipids	SQDG	MGDG	DGDG
14:0	0.1±0.1	0.2±0.2	0.2±0.1	0.3±0.1
16:0	45.6±3.5	56.1±6.5	42.0±5.5	40.4±2.5
16:1n-9	2.6±0.3	0.2±0.1	2.5±0.4	2.4±0.5
16:1n-7	5.3±0.3	2.3±0.3	5.3±1.2	5.9±1.3
16:2n-9	3.3±0.5	–	0.3±0.3	0.4±0.2
16:3n-6	0.4±0.2	0.3±0.1	0.3±0.2	0.6±0.2
17:0	0.1±0.1	0.4±0.2	0.1±0.1	0.2±0.1
17:1n-9	0.3±0.1	0.6±0.2	0.2±0.1	0.2±0.2
iso-18:0	0.3±0.1	0.6±0.3	0.2±0.2	0.3±0.2
17:3n-6(?)	0.2±0.1	–	0.3±0.2	0.3±0.1
18:0	0.7±0.2	1.2±0.4	0.7±0.2	0.8±0.3
18:1n-9	1.8±0.3	4.3±0.5	1.7±0.3	1.0±0.2
18:1n-7	0.3±0.2	0.8±0.5	0.7±0.5	0.6±0.2
18:2n-6	17.6±2.3	28.3±4.2	9.3±1.5	11.0±2.1
18:3n-6	20.3±3.0	3.6±0.5	30.9±5.1	29.7±3.6
20:2n-6	0.1±0.0	0.2±0.1	0.3±0.1	0.6±0.2
20:3n-6	0.6±0.1	0.4±0.2	0.7±0.2	0.7±0.3
C_{20} PUFA	0.4±0.1	0.2±0.1	1.0±0.3	1.2±0.5

[a] Data are meanSD for three determinations.

Table 2
FA composition (%) in sn-1 and sn-2 positions of the total MGDG, DGDG, and SQDG of *Spirulina platensis*[a]

Fatty acid	FA composition in SQDG			FA composition in MGDG			FA composition in DGDG		
	sn-1	sn-2	a	sn-1	sn-2	a	sn-1	sn-2	a
14:0	0.0±0.0	0.2±0.1	0.1	0.0±0.0	0.4±0.2	0.2	0.7±0.3	0.5±0.2	0.6
16:0	18.0±2.0	95.3±6.5	56.7	9.9±1.5	83.9±7.0	46.9	18.1±1.9	73.3±6.2	45.7
16:1n-9	0.0±0.0	0.0±0.0	0.0	0.2±0.1	2.6±0.5	1.4	0.3±0.1	4.9±1.5	2.6
16:1n-7	5.0±0.8	1.6±0.2	3.3	2.2±0.3	6.8±1.6	4.5	4.2±1.3	9.4±2.2	6.8
16:2 n-9	0.0±0.0	0.0±0.0	0.0	0.0±0.0	0.2±0.1	0.1	1.4±0.2	0.0±0.0	0.7
16:3n-6	0.3±0.1	0.0±0.0	0.2	0.6±0.1	0.0±0.0	0.3	1.0±0.2	0.0±0.0	0.5
17:0	0.0±0.0	0.8±0.3	0.4	0.0±0.0	0.0±0.0	0.0	0.0±0.0	0.0±0.0	0.0
17:1 n-9	1.9±0.2	0.0±0.0	0.9	0.0±0.0	0.1±0.1	0.1	0.0±0.0	0.0±0.0	0.0
iso-18:0	1.4±0.5	0.0±0.0	0.7	0.6±0.2	0.1±0.1	0.4	0.0±0.0	0.0±0.0	0.0
17:3 n-6	0.0±0.0	0.0±0.0	0.0	1.2±0.3	0.0±0.0	0.6	1.4±0.5	0.0±0.0	0.7
18:0	1.6±0.3	0.5±0.1	1.0	1.2±0.2	0.7±0.2	0.9	3.4±0.4	0.0±0.0	1.7
18:1n-9	8.1±1.8	0.0±0.0	4.1	5.3±0.9	0.8±0.2	3.1	1.7±0.5	0.5±0.1	2.1
18:1n-7	2.0±0.3	0.0±0.0	1.0	0.0±0.0	0.0±0.0	0.0	0.0±0.0	0.0±0.0	0.0
18:2 n-6	58.5±4.8	1.2±0.2	29.8	9.9±1.2	2.2±0.3	6.1	15.2±1.6	7.4±2.0	11.3
18:3 n-6	2.2±0.3	0.3±0.1	1.3	66.9±3.9	1.6±0.2	34.3	55.8±6.3	4.5±1.5	31.0
20:2 n-6	0.4±0.2	0.0±0.0	0.2	0.9±0.2	0.2±0.1	0.5	0.3±0.1	0.3±0.1	0.3
20:3 n-6	0.5±0.1	–	0.2	0.0±0.0	0.0±0.0	0.0	0.3±0.1	0.0±0.0	0.2

[a] The values were calculated as follows: (FA content in sn-1) / 2 + (FA content in sn-2)/2. Data are mean±S.D. for three determinations.

C16:0, C18:3n-6, and C18:2n-6 were the main fatty acids in glycolipids of MGDG and DGDG, while C16:0, and C18:2n-6 were the main fatty acids in SQDG. In all glycolipids separated from *Spirulina*, C16:0 represented more than 40% of total fatty acids. There were also some fatty acids of C20 and C17 in small amounts. In molecular species analysis, these fatty acids were always previously neglected in *Spirulina* (Imbs, Nershurpova, & Pham, 1998). The fatty acid composition of glycolipids varied with the marine algae. In SQDG of the red alga, *Gelidium amansii*, high levels of C16:0 (56%), C20:4 (19%), and C20:5 (16%) were found, while C18:2, and C18:3 were not found (Araki, Sakurai, Kawaguchi, & Murato, 1989). On the other hand, in SQDG of one green algae, *Enteromorpha* sp., C16:0, C18:1, C18:2, C18:3, and C18:4 were observed, while no C14:0 and C20 were found (Araki, Takemaro, Tuosi, Mitsu, & Mauki, 1991). The significant difference was supposed to originate in the growth temperature, as the cyanobacterium had a characteristic membrane lipid composition while phase transitions in membrane lipids were the major factors in this microalga (Quou, Dubacq, Justin Demandre, & Mazliak, 1993). Among the lipid fatty acids, gamma-linolenic acid was unstable. In the lipid of *Spirulina maxima*, C18:3n-6 was mainly contained in MGDG and DGDG (Kataoka & Misaki, 1983). Despite this, in the present paper, C18:3 was found in all three glycolipids isolated from *S. Platensis*.

3.2. The distribution of fatty acids in sn-1 and sn-2

The distribution of fatty acids esterified on sn-1 and sn-2 was analyzed by an enzymatic method (Araki et al., 1989). Fatty acid compositions at different positions in MGDG, DGDG, and SQDG are shown in Table 2.

C18 fatty acids mainly existed on the sn-1 and C16 on the sn-2 position in all glycolipids. This is supported by the fatty acid distribution (C18/C16) of glycolipids in prokaryotic photosynthetic organisms. The unsaturated fatty acids, such as C18:2, and C18:3, were distributed mainly on sn-1, and saturated fatty acid, C16:0, mainly on sn-2. Quoc and Dubacq (1997) found that modification of lipid species in cultured *S. platensis* can be achieved by providing the culture with exogenous fatty acids, such as oleic acid. The values in Table 2 indicate that the fatty acid distribution, after enzymatic cleavage, is similar to the total original fatty acid composition of glycolipids examined. The difference between calculated and actual fatty acid compositions is clear and the enzymatic effects can be observed.

3.3. Molecular species of MGDG, DGDG, SQDG in s. platensis

Seven fractions from SQDG and nine fractions from MGDG and DGDG were separated by HPLC. The corresponding carbon number from 28 to 32 was used to calculate the molecular species of glycolipids. According to the distribution of fatty acids at different positions of the glycerol backbone (Table 2), molecular species in SQDG should be mainly (18:2, 16:0), (16:0, 16:0), and (18:1, 16:0), while, in MGDG and DGDG, they should be (18:3, 16:0), (18:3, 16:1), (16:1, 16:0), and (16:0, 16:0). The molecular species is calculated in Table 3.

Table 3
Composition of molecular species of MGDG, DGDG, SQDG of *Spirulina platensis*[a]

Number	Molecular species		MGDG (%)	DGDG (%)	SQDG (%)
	sn-1	sn-2			
1	18:3	16:0	43.6±3.5	37.6±2.9	2.2±1.0
2	18:2	16:0	9.9±1.2	8.7±1.0	52.5±3.4
3	16:0	16:0	13.9±0.8	8.7±0.6	11.0±1.3
4	16:0	16:1	0.8±0.2	5.3±0.5	0.0±0.0
5	16:1	16:0	1.5±0.3	3.9±0.7	7.1±0.3
6	16:0	18:3	1.4±0.5	0.7±0.2	0.0±0.0
7	16:1	16:1	0.4±0.2	0.5±0.2	0.0±0.0
8	18:3	16:1	8.9±1.7	0.6±0.3	0.0±0.0
9	16:1	18:3	0.3±0.1	0.0±0.0	0.0±0.0
10	16:0	16:2	0.1±0.1	0.0±0.0	0.0±0.0
11	16:1	14:0	0.2±0.1	0.0±0.0	0.0±0.0
12	16:1	18:0	0.4±0.2	0.6±0.4	0.2±0.1
13	16:1	18:1	0.3±0.1	0.3±0.1	0.0±0.0
14	16:3	18:0	0.1±0.1	0.0±0.0	0.4±0.2
15	16:0	20:2	0.3±0.2	0.6±0.3	0.0±0.0
16	14:0	14:0	0.1±0.1	0.2±0.1	0.0±0.0
17	14:0	16:0	0.1±0.1	0.2±0.1	0.2±0.1
18	14:0	16:1	0.1±0.1	0.0±0.0	0.0±0.0
19	i-18:0	18:3	0.3±0.1	0.0±0.0	0.0±0.0
20	17:1	16:0	0.1±0.1	0.3±0.1	1.1±0.6
21	14:0	18:0	0.1±0.1	0.0±0.0	0.0±0.0
22	18:0	16:0	0.5±0.2	0.0±0.0	0.0±0.0
23	18:1	16:0	1.3±0.5	0.7±0.2	7.7±0.4
24	18:1	14:0	0.1±0.1	0.4±0.2	0.2±0.1
25	18:0	18:2	0.3±0.2	0.0±0.0	0.0±0.0
26	17:1	14:0	0.2±0.1	0.0±0.0	0.0±0.0
27	18:2	18:0	0.4±0.1	4.5±0.4	0.3±0.1
28	18:2	18:1	0.3±0.1	0.1±0.1	0.0±0.0
29	18:2	16:1	1.0±0.2	0.7±0.3	0.1±0.1
30	18:2	18:2	0.4±0.2	0.0±0.0	0.5±0.3
31	18:1	18:1	0.1±0.1	0.6±0.2	0.0±0.0
32	18:3	18:1	0.6±0.2	0.0±0.0	0.0±0.0
33	16:0	17:3	1.0±0.3	0.0±0.0	0.0±0.0
34	18:3	18:0	0.3±0.1	0.6±0.2	0.0±0.0
35	20:2	18:0	0.2±0.1	0.0±0.0	0.0±0.0
36	16:1	18:2	0.0±0.0	5.5±0.6	0.0±0.0
37	16:0	18:2	0.0±0.0	0.6±0.3	5.1±0.5
38	18:1	16:1	0.0±0.0	0.0±0.0	0.6±0.3
39	20:2	16:0	0.0±0.0	0.1±0.1	0.4±0.2
40	20:3	16:0	0.0±0.0	0.6±0.2	0.2±0.1
41	Unknown	Unknown	10.4±2.5	17.4±3.6	10.2±3.1
Total			100.0	100.0	100.0

[a] Data are mean±S.D. for three determinations.

Forty molecular species of glycolipids were found existed in MGDG, DGDG, and SQDG. The dominant

molecular species in MGDG were (18:3, 16:0) (43.6%), (18:2, 16:0) (9.9%), (16:0, 16:0) (13.9%), (18:3, 16:1) (8.9%), and (16:1,16:0) (1.5%). In DGDG, the major molecular species were similar to those of MGDG except for (18:2, 18:0) (4.5%) and (16:1, 18:2) (5.5%). SQDG mainly contained (18:2, 16:0) (52.5%), (16:0, 16:0) (11.0%), (16:1, 16:0) (7.1%), (18:1, 16:0) (7.7%), (16:0, 18:2) (5.1%), and (18:3, 16:0) (2.2%). From Table 3, it is interesting that the molecular species of glycolipids in *Spirulina*, cultured in Shenzhen, China, had the typical molecular species (C18:0, C16:0) of *S. platensis*. In contrast, in higher plants, such as squash, highly saturated molecular species (C18:0, C16:0) were reported to be contained in phosphatidylglycerol instead of glycolipids (Xu and Siegenthaler, 1996). Smaller amounts of (C16:1, C18:0) and (C18:0, C18:2) were also determined. (17:1,16:0), (20:2, 16:0), (20:3, 16:0), and (16:0, 17:3) are now reported for the first time in *Spirulina* in the present paper. Molecular species of (C18:2, C18:0), (C18:0, C18:2), corresponding to the eukaryotic plants, have also been found in *Spirulina* (Quoc & Dubacq, 1997).

To check our results, we recalculated fatty acid compositions of total glycolipids on the basis of molecular species in MGDG, DGDG, and SQDG, using data obtained by direct analysis (data not shown here). The fatty acid composition in molecular species was very close to the direct analysis data, but with a slight difference of C18:3n-6.

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