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Oxidative Stability of Glyceroglycolipids Containing Polyunsaturated Fatty Acids

Tomoyo Yamaguchi, Rumiko Sugimura, Junki Shimajiri, Masatake Suda, Masayuki Abe, Masashi Hosokawa and Kazuo Miyashita*

Laboratory of Bio-functional Material Chemistry, Division of Marine Bioscience, Faculty of Fisheries Sciences, Hokkaido University (Hakodate 041-8611, JAPAN)

Abstract: The oxidative stability of glyceroglycolipids (GLs) from spinach or brown edible seaweed (*Laminaria japonica*) was compared with those of phosphatidylcholines (PCs) from salmon roe and triacyglycerols (TAGs) from soybean oil or sardine oil. All the lipids were subjected to autoxidation after removing oxidants and/or antioxidants such as chlorophylls, tocopherols, and carotenoids. The oxidative stability of the lipids decreased with increasing number of bisallylic positions in the molecule. Due to the higher mean number of bisallylic positions, salmon roe PC and sardine oil TAG were oxidized more rapidly than soybean oil TAG. Spinach GL and brown edible seaweed GL showed the same oxidative stability as that found in soybean oil TAG, although the mean number of bisallylic positions of both GLs was much higher than that of soybean oil TAG and approached the number found in sardine oil TAG and salmon roe PC. The present study indicates the important effect of galactosyl and sulfoquinovosyl moieties on the oxidative stability of GL.

Key words: oxidative stability, glyceroglycolipids, bisallylic positions, α-linolenic acid, stearidonic acid, eicosapentaenoic acid

1 INTRODUCTION

Up to 7% of the dry weight of plant leaves is lipid, with monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) being major constituents (Fig. 1)[1]. Plant sulfoquinovosyl diacylglycerol (SQDG) is also a significant component, representing approximately 5% of the total acyl lipids (Fig. 1). MGDG, DGDG, and SQDG are primary glyceroglycolipids (GLs) of macro algae (seaweeds)[2,3]. In contrast to the abundance of the phospholipids in membranes of animals and yeast, MGDG and DGDG represent some 75% of the total membrane lipids in plant leaves. These galactosyldiacylglycerols are therefore especially important in the photosynthetic membranes of higher plants, as well as in algae and bacteria[4]. Similarly, SQDG is present in all photosynthetic plants, algae, cyanobacteria, and purple sulfur and nonsulfur bacteria[5].

The fatty acid composition of chloroplast GL is unusually rich in polyunsaturated fatty acids (PUFAs). α-Linolenic acid (LN, 18:3n-3) is the main fatty acid in the GL of plant leaves and is particularly enriched in MGDG and DGDG[1]. Stearidonic acid (SA, 18:4n-3), arachidonic acid (AA, 20:4n-6), and eicosapentaenoic acid (EPA, 20:5n-3) are major

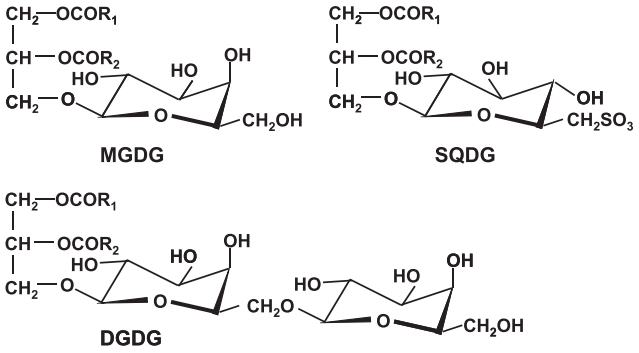

Fig. 1 Structures of MGDG, DGDG, and SQDG. MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; SQDG, sulphoquinovosyl diacylglycerol.

fatty acids in seaweeds[2,6]. These PUFAs are very easily oxidized compared with other lower unsaturated fatty acids such as oleic acid (OA, 18:1n-9) and linoleic acid (LA, 18:2n-6)[7-9]. The chloroplast GL is therefore continuously exposed to oxidative stress owing to the exceptionally high level of PUFA and the absorption of light energy for photo-

*Correspondence to: Kazuo Miyashita, Faculty of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, JAPAN
E-mail: kmiya@fish.hokudai.ac.jp
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synthesis.

Reactive oxygen species (ROS) are generated in the cell membrane and can cause oxidative damage to many cellular components, including membrane lipids, proteins, nucleic acids, and chlorophyll[10]. To control the level of ROS and protect cells, plants possess a number of antioxidants (ascorbate, glutathione, phenolic compounds, tocopherols, and carotenoids) and enzymes (superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase) scavenging ROS and regenerating the active forms of antioxidants[11–13]. Another defense system against oxidative stress in photosynthetic tissues may be related to the presence of PUFA in the form of GL. GL may have a different response to oxidation compared with other lipid classes such as triacylglycerol (TAG) and phospholipids (PLs). To better understand the role of PUFAs in photosynthetic biological tissues, it is necessary to understand the characteristic response of GL to lipid oxidation, although very few reports on GL oxidation exist in the literature. In the present study, we compared the oxidative stability of 3 lipid classes, namely, GL, TAG, and PL.

2 EXPERIMENTAL PROCEDURES

2.1 Standards and chemicals

Triolein and 1,2-dioleoyl phosphatidyl choline (PC) were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan) and from Avanti Polar Lipids Inc. (Alabaster, AL), respectively. Plant leaf MGDG, DGDG, and SQDG were purchased from Lipid Products (Redhill, UK), α-tocopherol was purchased from Kanto Kagaku (Tokyo, Japan), and β-, γ-, and δ-tocopherols were from Sigma-Aldrich Japan (Tokyo, Japan). Lutein, neoxanthin, violaxanthin, astaxanthin, and fucoxanthin were purchased from CaroteNature GmbH, Lupsingen, Switzeland. Silica gel (BW-60F) for column chromatography was purchased from Fuji Sylysia Chem. Ltd., Kasugai, Aichi, Japan. Activated carbon and Celite (545 RVS) are products of Nacalai Tesque Inc., Kyoto, Japan. All the other chemicals and solvents used in the study were of analytical grade, except that high-performance liquid chromatography (HPLC) grade solvents were used for HPLC analysis.

2.2 Lipid preparation

2.2.1 Crude lipid

Sardine and salmon roe oils were kindly donated by Maruha Co. (Tokyo, Japan) Soybean oil was obtained from Nacalai Tesque Inc. Edible dried powders of spinach and brown seaweed, *Laminaria japonica*, were purchased from the local market in Hakodate.

Spinach and Laminaria dried powders (approximately 2 kg) were soaked in acetone (approximately 3 L) overnight at room temperature under the dark, followed by filtration to collect the filtrate; the residue was subjected to one more overnight extraction with acetone to again collect the filtrate. These filtrates were pooled, and the solvent was removed under vacuum at 30 ± 1℃, using a rotary evaporator. Any last traces of solvents remaining in the extracts were removed under high vacuum. The green viscous liquid obtained was designated as spinach and Laminaria lipids.

2.2.2 Purification of lipid by activated carbon-Celite column chromatography

Soybean, sardine, and salmon roe oils (approximately 25 g) were passed through a column (50 × 4 cm i.d.) packed with a *n*-hexane slurry mixture of activated carbon (100 g) and Celite (100 g) to remove tocopherols and pigments by eluting with *n*-hexane (1200 mL). Carbon-Celite column chromatographic treatment of spinach and Laminaria lipids was carried out according to the procedure described earlier, except for eluents. Both lipids were eluted with 500 mL each of 90% aqueous ethanol, ethanol, and ethanol-chloroform (50:50, *v/v*). Lipids eluted with ethanol and ethanol-chloroform (50:50, *v/v*) were combined and used as pigment-free spinach and Laminaria lipids.

2.2.3 Preparation of TAG

After treatment with active carbon-Celite column chromatography, soybean and sardine oils (approximately 10 g) were further refined on a silicic acid column (50 × 4 cm i.d.) packed with a *n*-hexane slurry of silica gel BW-60F (200 g) by eluting with *n*-hexane (200 mL) and a mixture of *n*-hexane-diethyl ether (98:2 (200 mL) and 90:10 (1200 mL), *v/v*). The fraction eluted with *n*-hexane-diethyl ether (90:10) was used for the present study as soybean oil and sardine oil TAGs.

2.2.4 Preparation of PC

After removing pigments by active carbon-Celite column chromatography, salmon roe oil (approximately 21 g) was dissolved in acetone (700 mL) at room temperature and then cooled at 5℃ for 1 day under the dark to precipitate PC-rich fraction.

The fraction was dissolved in chloroform-methanol (1:1, *v/v*) and then subjected to preparative thin-layer chromatography (TLC). Preparative TLC was carried out on 1.0-mm silica gel plates developed with chloroform-methanol-water (65:25:4, *v/v/v*). Spots were visualized under ultraviolet light and a band corresponding to PC was scratched off from the plate. The PC-containing powder obtained from the plate was subjected to silica gel column chromatography by eluting with methanol.

2.2.5 Preparation and separation of GL

The pigment-free spinach and Laminaria lipids (approximately 10 g) were subjected to a silicic acid column (50 × 4 cm i.d.) packed with a n-hexane-acetone (70:30, *v/v*) slurry of silica gel BW-60F (200 g) by eluting with n-hexane-acetone (70:30 and 50:50, *v/v*) and methanol. The fraction eluted with methanol was used for the present study as

spinach and Laminaria GLs.

A portion of the spinach and Laminaria GLs was further subjected to preparative TLC to separate the GL into MGDG, DGDG, and SQDG. Preparative TLC was carried out using the same procedure for PC separation described earlier, except that MGDG, DGDG, and SQDG were used as TLC standards.

2.3 Analysis of purified lipids

2.3.1 Analysis of antioxidants and pro-oxidants

To confirm that all antioxidants and pro-oxidants were removed from the crude lipid samples using the purification procedures described earlier, HPLC, TLC, and peroxide value analyses were carried out.

HPLC was carried out using a Hitachi HPLC system (Hitachi Seisakusho, Co., Tokyo, Japan) equipped with a pump (L-7100) and a fluoresence detector for tocopherol analysis, whereas a Hitachi HPLC system equipped with a pump and a photo diode array detector was used for carotenoid analysis. Silica (Develosil 100-3, 250×4.6 mm i.d; Nomura Chem. Co., Seto, Aichi, Japan) and ODS columns (TSK-gel ODS 80-Ts, 250×4.6 mm i.d., 5 μm particle size; Tosoh, Tokyo, Japan) were used for the tocopherol and carotenoid analyses, respectively. Both columns were protected with a guard column (15×3.2 mm) with the same stationary phase. For the tocopherol analysis with the silica column, *n*-hexane-2-propanol (99.2:0.8, *v/v*) was used as eluent, setting the flow rate at 1.0 mL/min. The fluoresence detector was set at Ex. 298 nm and Em. 325 nm. On the other hand, when carotenoids in the sample lipid were analyzed using the ODS column, a gradient ternary mobile phase cosisting of methanol (A)-acetonitrile (B)-ethyl acetate (C) was used. The gradient program was 0 min, 55:45:0 (A:B:C, *v/v/v*); 8 min, 70:30:0 (A:B:C, *v/v/v*); 25 min, 55:45:0 (A:B:C, *v/v/v*); 27 min, 70:0:30 (A:B:C, *v/v/v*); 35 min, 30:70:0 (A:B:C, *v/v/v*). The flow rate was 0.7 mL/min from 0 to 8 min and was maintained at 1 mL/min throughout the analysis after 8 min. Chromatographic detection of all carotenoids was carried out at 450 nm.

To evaluate the lipid class purification of the separated samples, analytical TLC was carried out on 0.25-mm silica gel plates developed with *n*-hexane- diethyl ether-acetic acid (70:30:1, *v/v/v*) for TAG analysis and chloroform-methanol-water (65:25:4, *v/v/v*) for the PL and GL analyses. Lipid spots were detected with iodine vapor or 60% aqueous sulfuric acid charring. PL and GL spots were also visualized by spraying with a solution of Ditmmer's reagent or orcinol-sulfuric acid reagent, respectively[14]. Each spot was identified using standard PC (1,2-dioleoyl PC), GL (MGDG, DGDG, and SQDG) and TAG (Triolein).

GL chlorophyll was detected using a Hitachi U-2800A spectrophotometer. After dissolving an aliquot of the GL sample in ethanol, the absorption spectrum (400-800 nm) was then determined. The peroxide value of the separated lipids was determined by the AOCS official method[15].

2.3.2 Fatty acid analysis

The fatty acid composition of the sample lipid was determined by gas chromatography (GC) after conversion of fatty acyl groups in the lipid to their methyl esters. The fatty acid methyl esters were prepared using the method of Prevot and Mordret[16]. Briefly, 1 mL of *n*-hexane and 0.2 mL of 2 N NaOH in methanol were added to an aliquot of total lipid (approximately 10 mg), which was vortexed and incubated at 50℃ for 30 min. After incubation, 0.2 mL of 2-N HCl in methanol solution was added to the solution and vortexed. The mixture was separated by centrifugation at 1000×*g* for 5 min. The upper hexane layer containing fatty acid methyl esters was recovered and subjected to GC. The GC analysis was performed on a Shimadzu GC-14B (Shimadzu Seisakusho, Kyoto, Japan) equipped with a flame ionization detector (FID) and a capillary column (Omegawax-320; 30 m×0.32 mm i.d.; Supelco, Bellefonte, PA). The detector, injector, and column temperatures were 260℃, 250℃, and 200℃, respectively. The carrier gas was helium at a flow rate of 50 kPa. Fatty acid content was expressed as weight percentage of total fatty acids.

2.4 Oxidation and analysis

Each 20-mg sample was placed in a 2-mL aluminum-sealed vial with a butyl-gum septum and then incubated at 37℃ in the dark. Before incubation, the level of oxygen in the headspace gas of the vial was estimated using a GC[9] equipped with a thermal conductivity detector and a stainless steel column (3 m×3.0 mm i.d.) packed with molecular sieve 5A. The temperatures at the injection port, detector port, and column oven were 100℃, 100℃, and 50℃, respectively. The helium flow was 50 kPa. A small portion (20 μl) of the headspace gas was extracted using a microsyringe through a butyl gum septum after selected times of oxidation. The decrease (%) in the oxygen level was calculated from the changes in the oxygen to nitrogen ratio compared with that before incubation. Each sample in 3 separate vials was subjected to oxidation.

After oxidation, polyunsaturated fatty acid contents were analyzed by GC. After conversion of fatty acyl groups in each oxidized lipid to their methyl esters, the fatty acid methyl esters were subjected to silicic acid column chromatography and then to GC. The preparation of fatty acid methyl esters and GC analysis was performed as described earlier.

Oxidation was done separately 3 times for each lipid sample. The data value was expressed as the mean + SD.

3 RESULTS

3.1 Lipid purification and fatty acid composition

Tocopherols were detected by HPLC in all the crude

lipids used in this experiment. Carotenoids were also detected by HPLC in crude salmon roe oil(astaxanthin), crude spinach lipids(lutein, neoxanthin, and violaxanthin), and Laminaria lipids(fucoxanthin). However, complete removal of these antioxidants was confirmed by HPLC analysis after purification of each crude lipid. Furthermore, the absorption peak of chlorophyll was detected in crude spinach and Laminaria lipids. The peak disappeared after the chromatographic purification of both lipids. Purified soybean oil TAG, sardine oil TAG, and salmon roe PC gave only a single spot corresponding to each lipid class standard on an analytical TLC. Spinach and Laminaria GL gave 3 spots corresponding to MGDG, DGDG, and SQDG on the TLC, but no other spots were detected. MGDG, DGDG, and SQDG separated from spinach and Laminaria GL showed only a single spot corresponding to each GL standard on the TLC. The peroxide values of purified TAG and 2 types of purified GL, spinach and Laminaria GL, were less than 0.5 meq/kg. The peroxide value of the salmon roe PC fraction obtained after active carbon-Celite column chromatography and crystallization in acetone was less than 1.0 meq/kg. However, the peroxide value of the purified PC and MGDG, DGDG, and SQDG from the spinach and Laminaria GL could not be determined because of the small amount of each sample. The analysis of sample lipids indicated that they had only low levels of antioxidants and pro-oxidants.

The fatty acid compositions of purified TAG, PC, and GL used in the present study are shown in Table 1. The main PUFA of soybean oil TAG was LA(18:2n-6), whereas those of the 2 different fish lipids, sardine oil TAG and salmon roe PC, were EPA(20:5n-3)and docosahexaenoic acid (DHA, 22:6n-3). On the other hand, marine algal lipids, Laminaria GL, had a high level of SA(18:4n-3), EPA, 18:3n-6, and AA(20:4n-6)as major PUFA. In spinach GL, more than 70% of PUFA was LN(18:3n-3)and a high content of 16:3n-3 also characterized its fatty acid profile. The overall distribution of fatty acid in spinach and Laminaria GL was

Table 2 Composition of spinach GL and Laminaria GL.

GL class (weight % of total GL)	Spinach	Laminaria
MGDG	75.3	25.6
DGDG	19.0	29.1
SQDG	5.7	45.3

MGDG, DGDG, and SQDG were separated from spinach GL and Laminaria GL by preparative TLC and weighed. The data value was expressed as the mean of three separate experiments.
GL, glyceroglycolipids; MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; SQDG, sulphoquinovosyl diacylglycerol.

Table 1 Fatty acid composition of lipids.

Fatty acid (weight%)	Soybean oil TAG	Sardine oil TAG	Salmon roe PC	Spinach GL	Laminaria GL
14:0	0.1	5.6	1.3	trace	6.6
16:0	11.3	8.4	16.7	2.4	12.1
16:1n-7	0.3	12.4	ND	ND	1.8
16:3n-3	ND	ND	1.3	18.2	ND
18:0	3.6	0.8	9.3	0.1	0.8
18:1n-7	1.6	3.6	3.3	0.6	ND
18:1n-9	27.7	14.1	7.8	0.4	16.5
18:2n-6	46.2	1.5	2.0	2.3	7.8
18:3n-3	3.2	0.7	ND	74.0	2.9
18:3n-6	ND	ND	ND	ND	8.1
18:4n-3	ND	2.0	0.1	ND	18.0
20:4n-6	ND	1.6	1.9	ND	7.8
20:5n-3	ND	19.5	10.3	ND	12.8
22:5n-3	ND	2.4	4.7	ND	ND
22:6n-3	ND	12.3	29.4	ND	ND

The data value was expressed as the mean of duplicated analysis of each sample.
TAG, triacylglycerols; PC, phosphatidylcholine; GL, glyceroglycolipids;
ND, Not detected.

Table 3 Fatty acid composition of MGDG, DGDG, and SQDG from spinach and Laminaria GL.

Fatty acid (weight%)	Spinach GL			Laminaria GL		
	MGDG	DGDG	SQDG	MGDG	DGDG	SQDG
14:0	trace	0.1	0.2	9.4	4.4	4.3
16:0	1.3	8.0	23.5	9.3	5.8	35.8
16:3n-3	18.6	3.7	0.6	ND	ND	ND
18:1n-9	0.3	1.3	1.3	18.2	14.5	25.7
18:2n-6	2.3	4.7	7.1	7.7	9.0	8.1
18:3n-3	75.2	77.2	39.1	2.2	2.7	5.3
18:3n-6	ND	ND	ND	9.1	9.5	ND
18:4n-3	ND	ND	ND	20.7	23.6	5.4
20:4n-6	ND	ND	ND	8.5	7.5	2.0
20:5n-3	ND	ND	ND	11.0	20.0	6.0

The data value was expressed as the mean of duplicated analysis of each sample.
MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; SQDG, sulphoquinovosyl diacylglycerol; GL, glyceroglycolipids; ND, not detected.

Table 4 Average number of bisallylic positions of lipids used in this experiment.

Number of bisallylic positions	Soybean oil TAG	Sardine oil TAG	Salmon roe PC	Spinach GL	Laminaria GL	Spinach			Laminaria		
						MGDG	DGDG	SQDG	MGDG	DGDG	SQDG
Per molecule	0.55	1.71	2.11	1.93	1.60	1.93	1.70	1.16	1.58	2.02	0.64
Per g lipid ($x6.02\times10^{20}$)	0.63	1.93	2.70	2.43	1.92	2.52	1.85	1.41	2.06	2.18	0.78

TAG, triacylglycerols; PC, phosphatidylcholine; GL, glyceroglycolipids; MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; SQDG, sulphoquinovosyl diacylglycerol.

reflected by the GL class composition (Table 2) and fatty acid composition of each GL class (Table 3).

3.2 Number of bisallylic positions

It is well known that the oxidative stability of polyunsaturated lipids decreases with increasing number of bisallylic positions[17, 18]. The number of bisallylic positions per molecule of lipids can be calculated from the molar concentration of each PUFA and the mean molecular weight (MW) of each lipid (Table 4). The molar concentration of PUFA was obtained on the basis of the weight % of PUFA (Tables 1 and 3) and the MW of each PUFA. The mean MW of TAG, PC, GL, MGDG, DGDG, and SQDG was computed from the mean MW of all the fatty acyl moieties and the MW of glycerol moiety and phosphatidylcholine, galactosyl, or sulfoquinovosyl moiety. The mean MW of all the fatty acyl moieties was calculated from the mol% of each fatty acid and the MW. In the case of GL, after calculating the molar distribution of MGDG, DGDG, and SQDG from the weight % of each GL class (Table 2), the mean MW of GL was calculated. The mean MW of each lipid was as follows: soybean oil TAG, 871.6; sardine oil TAG, 885.2; salmon roe PC, 780.6; spinach GL, 793.5; Laminaria GL, 833.8; spinach MGDG, 764.7; spinach DGDG, 917.4; spinach SQDG, 822.7; Laminaria MGDG, 768.3; Laminaria DGDG, 927.2; and Laminaria SQDG, 820.2. Table 4 shows the number of bisallylic positions per gram of lipid calculated from the mean MW of each lipid and the number of bisallylic positions per molecule (Table 4).

3.3 Oxidative stability

The oxidative stability of the 2 types of TAG, salmon roe PC, and the 2 types of GL were compared in the bulk phase by measuring the decrease in oxygen in the headspace (Fig. 2). Both lipids of marine animal origin, sardine oil TAG and salmon roe PC, were oxidized most rapidly among the lipids tested in the present study. On the other hand, little decrease in oxygen level was observed in the headspace of vials containing soybean oil TAG. Although the oxidative stabilities of the 2 types of GL were a little lower than that of soybean oil TAG, their stabilities were much higher than those of the 2 marine lipids. Little difference in the oxidative stability was apparent between the spinach and Laminaria GL. Fig. 3 shows the oxidative stability of each GL class of spinach GL (A) and Laminaria GL (B). The stability of SQDG was the highest among the 3 types of spinach and

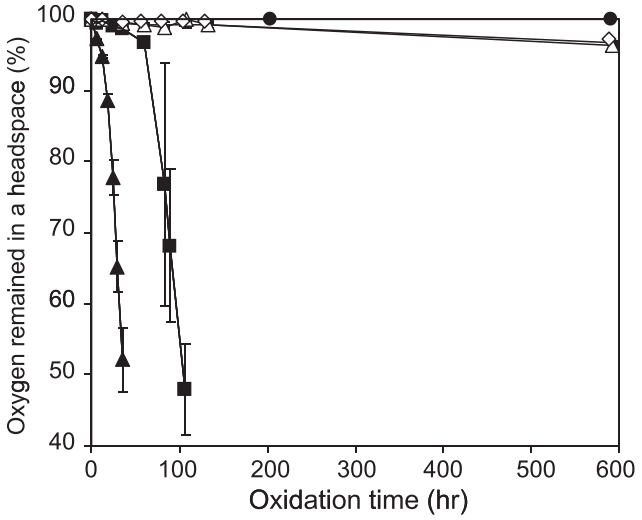

Fig. 2 Comparison of the oxidative stability of soybean oil TAG, sardine oil TAG, salmon roe PC, spinach GL, and Laminaria GL. Each lipid (20 mg) was put in a 2-mL alminium sealed vial and then incubated at 37°C in the dark. The decrease (%) in oxygen concentration was estimated by GC from the changes in the oxygen ratio to nitrogen in the headspace gas. The data value was expressed as the mean of three separate experiments. Soybean oil TAG (solid circle), sardine oil TAG (solid square), salmon roe PC (solid triangle), spinach GL (open triangle), Laminaria GL (open diamond). TAG, triacylglycerols; PC, phosphatidylcholine; GL, glyceroglycolipids.

Laminaria GL.

The higher oxidative stabilities of soybean oil TAG, spinach GL, and Laminaria GL were confirmed by GC analysis of PUFA after the incubation for 593 hr (Table 5). As shown in Table 5, the proportions of EPA and DHA decreased and that of palmitic acid (PA, 16:0) increased after oxidation of sardine oil TAG and salmon roe PC. From the ratio of both PUFA to PA, it could be calculated that approximately 50% of EPA and approximately 40% of DHA were lost during the oxidation. PUFA proportions decreased after the oxidation of Laminaria GL; however, the decrease was small compared with that in sardine oil TAG and salmon roe PC. Based on the ratio of each PUFA to that of PA before and after the oxidation of Laminaria GL, the loss of PUFA was calculated to be 4.0%, 7.5%, 7.7%, 5.2%, and 6.2% for LN, 18:3n-6, SA, AA, and EPA, respectively. Moreover, there was little difference in the composition of PUFA and PA before and after the oxidation of spinach GL, suggesting little loss of PUFA during the oxidation of spinach GL.

3.4 Discussion

Lipid oxidation proceeds through a free radical chain reaction consisting of chain initiation, propagation, and termination processes[17, 18]. The rate-limiting step in the reaction is the abstraction of hydrogen radical ($H^{\cdot}$) from substrate lipids (LH) to form lipid-free radicals ($L^{\cdot}$). Given that this hydrogen abstraction occurs at the bisallylic positions ($CH=CH-CH_2-CH=CH$) present in PUFA and the susceptibility of PUFA to oxidation depends on the availability of bisallylic hydrogens, the oxidative stability of each PUFA is inversely proportional to the number of bisallylic posi-

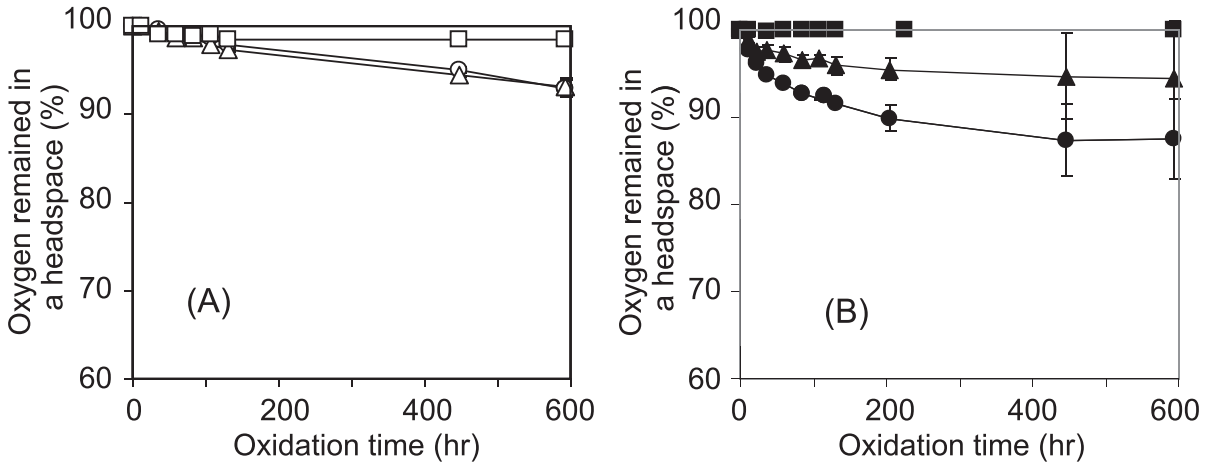

Fig. 3 Comparison of the oxidative stability of MGDG, DGDG, and SQDG of spinach GL (A) and Laminaria GL (B). The oxidation and analytical procedures were the same as those described in Fig. 2. The data value was expressed as the mean of three separate experiments. Spinach MGDG (open circle), spinach DGDG (open triangle), spinach SQDG (open square), Laminaria MGDG (solid circle), Laminaria DGDG (solid triangle), Laminaria SQDG (solid square). MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; SQDG, sulphoquinovosyl diacylglycerol; GL, glyceroglycolipids.

Table 5 Changes in 16:0 and PUFA composition of lipids before and after oxidation.

Fatty acid (wt%)	Soybean oil TAG		Sardine oil TAG		Salmon egg PC		Spinach GL		Laminaria GL	
	Before	After	Before	After	Before	After	Before	After	Before	After
16:0	11.3	11.9	8.4	11.0	16.7	24.1	2.4	2.2	12.1	12.6
16:3n-3	ND	ND	ND	ND	1.3	1.3	18.2	18.0	ND	ND
18:2n-6	46.2	49.5	1.5	1.6	2.0	2.4	2.3	2.4	7.8	7.8
18:3n-3	3.2	3.2	0.7	0.7	ND	ND	74.0	73.9	2.9	2.9
18:3n-6	ND	ND	ND	ND	ND	ND	ND	ND	8.1	7.8
18:4n-3	ND	ND	2.0	2.3	0.1	0.1	ND	ND	18.0	17.3
20:4n-6	ND	ND	1.6	1.3	1.9	1.5	ND	ND	7.8	7.7
20:5n-3	ND	ND	19.5	13.1	10.3	7.5	ND	ND	12.8	12.5
22:5n-3	ND	ND	2.4	1.6	4.7	3.4	ND	ND	ND	ND
22:6n-3	ND	ND	12.3	6.7	29.4	17.2	ND	ND	ND	ND

The data value was expressed as the mean of three separate experiments.
PUFA, polyunsaturated fatty acids; TAG, triacylglycerols; PC, phosphatidylcholine; GL, glyceroglycolipids; ND, Not detected.

tions in the molecule. As shown in Table 1, the lipids originating in marine animals contained high percentages of EPA and DHA and showed higher mean numbers of bisallylic positions per molecule of lipid than did soybean oil TAG (Table 4). Accordingly, salmon roe PC and sardine oil TAG were oxidized very rapidly, whereas there was little oxygen consumption found in the oxidation of soybean oil TAG during the incubation (Fig. 2).

The difference in the mean number of bisallylic positions per molecule between soybean oil and sardine oil TAG or salmon roe PC was 1.16 or 1.56, respectively (Table 4). A comparable difference was also found between soybean oil TAG and spinach GL (1.38) and Laminaria GL (1.05; Table 4). Judging from the difference in the mean number of bisallylic positions, the oxidative stability of spinach and Laminaria GL was presumed to be as low as those found in sardine oil TAG and salmon roe PC, although the oxidative stabilities of both lipids were almost the same as that found in soybean oil TAG (Fig. 2 and Table 5). MGDG and DGDG from spinach and Laminaria GL had relatively high oxidative stability (Fig. 3) compared with sardine oil TAG and salmon roe PC (Fig. 1), although both GL had similar number of bisallylic positions per molecule of lipid with both marine animal lipids (Table 4). The oxidative stability of SQDG from spinach GL and Laminaria GL was higher than those of MGDG and DGDG. This could be attributed to the lower number of bisallylic positions in SQDG than in MGDG and DGDG.

Studies on the oxidation of GL have been limited[19, 20]. Hirayama and Oido[19] reported the changes of lipid and pigment composition in spinach leaves under a variety of storage conditions. The contents of GL and PL in fresh leaves decreased after oxidation more rapidly than those of pigments, and the decrease was suppressed by the deactivation of spinach leave enzymes by heating at 100℃. Lee *et al.*[20] extracted total lipids from freeze-dried spinach and separated the different lipid classes, namely, neutral lipids (NL), PL, and GL by silicic acid column chromatography. Each lipid class was then exposed to autoxidation at 40℃, photo-oxidation at 10℃, lipoxygenase-catalyzed oxidation at 15℃, and iron-catalyzed oxidation at 25℃. The decrease in PUFA contents showed that NL was oxidized rapidly in autoxidation, and more than 65% PUFA was lost by the oxidation, whereas PL and GL were oxidatively more stable, with 0% and less than 10% loss of PUFA for PL and GL, respectively. On the other hand, PL was most rapidly oxidized by photo-oxidation and lipoxygenase-catalyzed oxidation. Little oxidation was observed in the iron-catalyzed oxidation of all the lipid samples.

Lee *et al.*[20] showed that the ratio of saturated fatty acids (SFA) to unsaturated fatty acids (UFA) was 0.2027, 0.4694, and 0.3529, for spinach GL, PL, and NL, respectively. Despite having the lowest ratio of SFA to UFA, GL had a relatively high oxidative stability in this study. This result might suggest characteristics of GL oxidation distinct from that of other lipid classes such as NL and PL. However, each lipid class obtained from spinach TL contained several kinds of oxidants and/or antioxidants such as chlorophylls, carotenoids, and tocopherols[20]. Chlorophylls and carotenoids are well known to play important roles in lipid oxidation, and chlorophyll promotes lipid oxidation acting as photosensitizer[21] and as an antioxidant[22, 23]. Carotenoids are the most efficient natural scavengers of $^1O_2$ formed during photooxidation[24, 25] and can retard oxidation by scavenging of free radicals. The electron-rich status of carotenoids makes them more suitable for reaction with the

free radicals[26]. The interaction of chlorophyll and carotenoids on lipid oxidation has also been reported in edible oils and foods containing lipids as minor constituents[27].

To study the characteristic oxidative stability of GL, it is necessary to compare the stability of GL and other lipid classes without any trace of pro-oxidants and antioxidants in the sample. In the present study, lipids were purified using combinations of different chromatographic treatments, which confirmed low levels of impurities in the lipid samples. The higher oxidative stability of spinach and Laminaria GL we found in this study could be due to the chemical properties of GL or to the presence of pro-oxidants and antioxidants in the lipids as impurities. Although our study has not clarified the chemical factors affecting the oxidation of GL, galactosyl and sulfoquinovosyl moieties may play an important role in the higher oxidative stability of GL.

Lipid oxidation is most effectively inhibited by the suppression of hydrogen abstraction from a bisallylic position by a free radical. The presence of other molecules near double bonds can protect the bisallylic positions against free radical attack, as has been reported in the aqueous oxidation of DHA[28, 29]. While DHA is oxidized more rapidly than LA owing to its higher numbers of bisallylic positions, it is much more oxidatively stable than LA in aqueous micelles[30]. This unusual oxidative stability of DHA has been attributed to a specific conformation of DHA in aqueous phase[28, 29]. NMR analysis indicated that DHA molecules in micelles are packed more loosely than LA molecules, increasing the susceptibility of water molecules to permeate into DHA micelles. The penetration of water molecule into the double bonds of DHA molecules has been suggested to inhibit hydrogen abstraction from the bisallylic positions of DHA.

In the present study we have found that PUFA of GL were oxidatively more stable than those of TAG and PC, suggesting that galactosyl and sulfoquinovosyl moieties may protect the bisallylic positions of PUFA in GL by interaction with the double bonds of the PUFA. On the other hand, several papers have reported that glucose, fructose, and sucrose promote methyl linoleate oxidation in emulsion[31]. In bulk food systems, it has been also reported that addition of carbohydrates to food complexes accelerates the oxidation of lipids in the complex[32, 33]. On the contrary, the effect of sugar addition to oil in water emulsion systems has been reported to inhibit lipid oxidation by scavenging free radicals in solution in the water phase[34]. The effect of carbohydrates on lipid oxidation as shown in the studies discussed earlier may yield basic information for understanding the characteristic oxidative stability of GL. However, the sugar molecule is chemically bonded to the glycerol moiety in GL, and the characteristic oxidative stability of GL found in the present study cannot be explained solely by the experiment of corresponding sugar addition to lipid. Further studies using synthetic pure GL are needed to clarify the process of GL oxidation.

Saturated and monounsaturated fatty acyl moieties and phosphatidylcholine groups exist in PC and TAG that may affect the oxidation of PUFA in the same molecule or in other molecules. Monounsaturated fatty acyl moieties such as OA(18:1n-9)have been reported to retard the oxidation of PUFA in TAG. Neff *et al*.[35] compared the oxidative stability of each molecular species of purified soybean oil TAG at 60℃ in the dark and reported that resistance of soybean oil TAG molecule to oxidation increased with increasing OA level. They also found that replacement of PA(16:0)in dilinoleoyl-palmitoyl TAG with OA improved its oxidative stability, whereas PA was less reactive to oxidation than OA in its free fatty acid or methyl ester form owing to the absence of a double bond in the PA molecule. Further study is required to study the effect of different molecules bonded to glycerol moieties on the oxidation of PUFA in different types of aclyglycerols.

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