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Comparison of Oxidative Stability of Monogalactosyl Diacylglycerol, Digalactosyl Diacylglycerol, and Triacylglycerol Containing Polyunsaturated Fatty Acids

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Abstract

Oxidative stability of three different lipid classes, namely, monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) from spinach and edible brown seaweed (Akamoku) and triacylglycerol (TAG) of linseed oil was compared. Analysis of oxygen consumption and polyunsaturated fatty acid (PUFA) composition demonstrated that spinach DGDG had the highest oxidative stability, followed by Akamoku DGDG, Akamoku MGDG, spinach MGDG, and linseed TAG. These results disagree with the order of oxidative stability expected from the average number of *bis*-allylic positions of each lipid. Additionally, DGDG constituents of both spinach and Akamoku showed higher oxidative stability than their MGDG constituents. The unusual oxidative stability of MGDG and DGDG could be conferred by the protection of *bis*-allylic positions of the PUFA against oxidative attack by the galactosyl moiety of the GL.

Keywords

Oxidative Stability, Glyceroglycolipids, Monogalactosyl Diacylglycerol, Digalactosyl Diacylglycerol, Triacylglycerol, Polyunsaturated Fatty Acids

1. Introduction

Monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), and sulfoquinovosyl diacylglycerol (SQDG) are the main lipid constituents of plant leaves (**Figure 1**) [1]. In addition, these polar lipids are known to be the major constituents of seaweeds [2] [3]. Together, MGDG and DGDG account

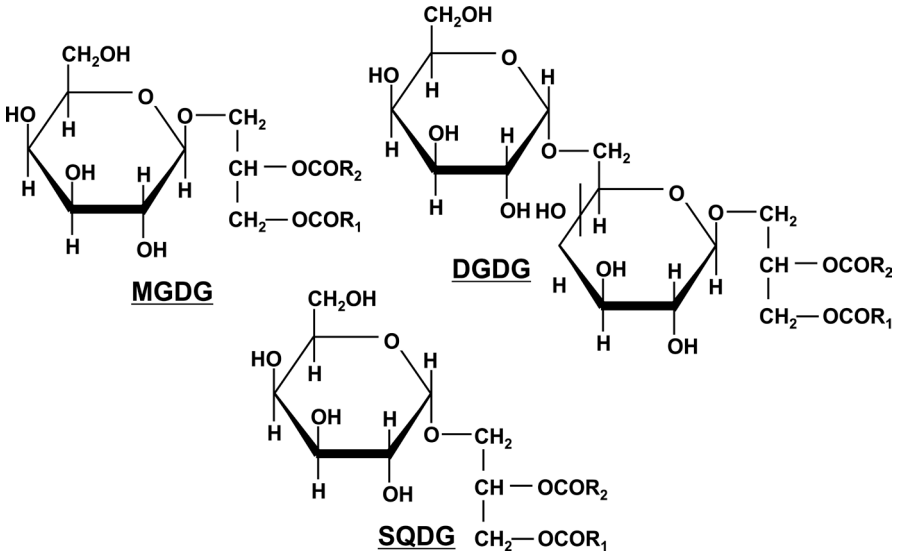

Figure 1. Structures of MGDG, DGDG, and SQDG. MGDG, monogalactosyl diacylglycerol; DGDG, digalactosyl diacylglycerol; SQDG, sulfoquinovosyl diacylglycerol.

for more than half of the total membrane lipids in plant leaves [4]. Therefore, these glyceroglycolipids (GLs) are thought to play important roles in the photosynthetic membranes of higher plants, algae, and bacteria [5].

GLs in higher plants have also been characterized as having a uniquely high content of polyunsaturated fatty acids (PUFAs), particularly *α*-linolenic acid (LN, 18:3n-3) [4]. In contrast, most PUFAs in seaweed GL are stearidonic acid (SA, 18:4n-3), arachidonic acid (AA, 20:4n-6), and eicosapentanoic acid (EPA, 20:5n-3) [2] [6]. All of these PUFAs are more easily oxidized than the more stable unsaturated fatty acids such as oleic acid (OA, 18:1n-9) and linoleic acid (LA, 18:2n-6) [7] [8] [9]. Despite the higher levels of oxidatively unstable PUFAs, GLs, a main lipid component of the chloroplast, are continuously subjected to oxidative stress because of the absorption of light energy during photosynthesis.

It is generally accepted that autoxidation of PUFA proceeds in a free radical chain reaction through three stages: i) initiation, ii) propagation, and iii) termination. The free radical theory of autoxidation was defined as the attack of oxygen at the allylic position leading to the formation of unsaturated hydroperoxides. These hydroperoxides can then decompose into peroxyl and alkoxyl radicals, which followed by secondary oxidation products including aldehydes, ketones, alcohols, and acids. Impaired taste, flavor, and texture of foods are among the adverse effects of secondary oxidation products, which may be toxic compounds. Thus, lipid oxidation is a major concern that can shorten the shelf life of foods.

Plants possess intrinsic antioxidant compounds for defense against oxidation, including ascorbate, glutathione, phenolic compounds, tocopherols, and carotenoids. Innate enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase are capable of scavenging reactive oxygen species and eventually protecting plants from oxidative stress [10] [11] [12].

Another defense system against oxidative stress in photosynthetic tissues may be related to the presence of PUFA in the form of GL. However, few studies have examined the resistance of GL to oxidation [4]. Therefore, it is very important to determine the role of PUFA in the form of GL and its characteristics response. This study evaluated the oxidative stability of GL by comparing the oxidative stability of 5 types of lipids: spinach MGDG, spinach DGDG, brown seaweed MGDG, brown seaweed DGDG, and linseed oil triacylglycerol (TAG).

2. Experimental Procedures

2.1. Sample

Spinach powder (GABAN Co. Ltd., Tokyo, Japan) was obtained from a local food market in Hakodate, Japan. Edible brown seaweed, *Sargassum horneri* (Turner) (common name: Akamoku), was collected from Hakodate, Hokkaido, Japan, and the powder was prepared by Cokey Co., Ltd. (Tokyo, Japan). Linseed oil was purchased from Wako Pure Chemical Ltd. (Osaka, Japan).

2.2. Referenced Compound and Reagent

Silica gel (BW-60F) for column chromatography was purchased from Fuji Sylysia Chem. Ltd. (Kasugai, Aichi, Japan). Activated Carbon and Celite (545 RVS) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). MGDG, DGDG, and SQDG standards were purchased from Lipid Products (Redhill, UK), while triolein and tricaprylin as medium chain TAG (MCT) were obtained from Wako Pure Chemical. All other chemicals and solvents used in this study were of analytical grade and high-performance liquid chromatography (HPLC)-grade solvents were used for HPLC analysis.

2.3. Sample Preparation

Spinach powder (*ca.* 2 kg) was soaked in methanol (12,000 mL) at room temperature, which was incubated in the dark overnight (approximately 16 h) [13]. The extract was filtered through a ceramic filter funnel lined with filter paper (No. 2, Qualitative Filter Paper; 150 mm; Advantec®; Tokyo, Japan). The filtrates were pooled and the solvent was removed using a pilot scale rotary evaporator at less than 40˚C. Traces of solvent remaining in the extract were completely removed in the dark under vacuum, leaving a dark green viscous liquid. This dark green viscous liquid was collected by dissolution in an equivalent volume of methanol, which was designated as crude spinach lipids.

Crude spinach lipids were further dissolved in chloroform-methanol-water (10:5:3, v/v/v) [13]. The solution was placed into a separatory funnel for liquid-liquid distribution. After shaking, the funnel was allowed to stand overnight. The lower layer, a mixed lipid layer with methanol and chloroform, was collected and again dissolved in water using a new separatory funnel. In this case, the same volume of water as that in the first separation, was added; after shaking, the funnel was allowed to stand for overnight separation. The lower layer

was then concentrated under a vacuum in a rotary evaporator. Remaining traces of organic solvents and water were removed in a desiccator (approximately 3 days) under a high vacuum, leaving the sample in an amber-colored rotary flask. Total lipids of spinach (*ca.* 20 g) were eventually collected by dissolving in an equivalent volume of chloroform and subjected to consequent analysis or stored at −30˚C in an equivalent volume of ethanol. The same procedure was used for Akamoku powder.

2.4. Lipid Class Analysis

Total lipids of spinach and Akamoku were subjected to lipid class analysis by preparative thin layer chromatography (TLC) [14]. The lipid fraction was dissolved in chloroform-methanol-water (65:25:4, v/v/v) and spotted onto a 0.25-mm silica gel plate (Silica gel 60G; Merck, Darmstadt, Germany). The plate was developed with the same cocktail mix solvents of chloroform-methanol-water (65:25:4, v/v/v) and the spots were visualized by spraying the plate with orcinol-sulfuric acid or Dittmer reagent, followed by charring. The lipid sample was also analyzed by preparative TLC with the same specification of silica gel plate mentioned previously but using *n*-hexane-diethyl ether-acetic acid (80:20:1, v/v/v) as the developing solvent. The spots were detected using 60% aqueous sulfuric acid charring.

The chromatogram was photographed with a digital camera and the image of the silica gel plate was acquired and transferred to a computer. The image was cropped and saved in bitmap format. The percentage ratio of each lipid fraction (as compared to standards) in the sample was expressed as the bitmap percentage of the total bitmap intensities [14].

2.5. Purification of GL from Spinach and Akamoku

Total lipids of spinach and Aakamoku (*ca.* 20 g) were first passed through a column (70 × 6 cm i.d.) packed with a chloroform slurry mixture of silica gel. The whole column was wrapped with aluminum foil to protect the GL from light-induced degradation. The elution was first conducted with chloroform (approximately 3000 mL) and then with acetone (approximately 13,000 mL). Fractions eluted with acetone were used as GL (through continuous elution, with appropriately adjusting the flow rate manually, for more than a week). The first whole dark green-black fraction was designated as MGDG and the consequent fractions (approximately 24 fractions) of each 500 mL collected with acetone (clear light green solution) were designated as the DGDG.

Although the absence of chlorophyll was confirmed in the DGDG, a trace amount of chlorophyll was detected in the MGDG on TLC. Therefore, MGDG (*ca.* 4 g) was further refined using a carbon column (70 × 6 cm i.d.) packed with 90% of ethanol in chloroform slurry of activated carbon (*ca.* 250 g). Carbon was first heated at 110˚C for 1.5 h prior to mixing with 90% of ethanol in chloroform. Elution was conducted with gradually decreasing concentrations of etha-

nol; 90% ethanol in chloroform (approximately 1000 mL), 50% ethanol in chloroform (approximately 1500 mL), and finally 100% chloroform (approximately 10,000 mL). The fractions eluted with 50% ethanol in chloroform and 100% chloroform were used as the MGDG. Purified MGDG and DGDG were used as Spinach MGDG, Spinach DGDG, Akamoku MGDG, and Akamoku DGDG.

2.6. Purification of TAG from Linseed Oil

Linseed oil was separated by two types of column chromatography [15]. To remove tocopherols and pigments, linseed oil (*ca.* 25 g) was first passed through a column (50 × 4 cm i.d.) packed with an *n*-hexane slurry mixture of activated carbon (100 g) and Celite (100 g). The carbon was first heated at 110˚C for 1.5 h prior to mixing with Celite and *n*-hexane. Elution was performed with *n*-hexane (1200 mL).

The obtained oil (*ca.* 10 g) was again refined using a silicic acid column (50 × 4 cm i.d.) packed with an *n*-hexane slurry of silica gel (200 g). Elution was conducted with *n*-hexane (200 mL), *n*-hexane-diethyl ether (98:2, v/v) (200 mL), and *n*-hexane-diethyl ether (90:10, v/v) (1200 mL). The final fraction eluted with *n*-hexane-diethyl ether (90:10) was used as Linseed TAG.

To confirm the absence of impurities, the TAG fraction was subjected to preparative TLC. The lipid fraction was spotted onto a 0.25-mm silica gel plate. The plate was again developed with *n*-hexane-diethyl ether (60:40, v/v) and spots were detected with iodine vapor or 60% aqueous sulfuric acid charring as compared to standard triolein.

2.7. Tocopherol Analysis of Spinach GL, Akamoku GL, and Linseed TAG

Tocopherol analysis was performed for all 5 types of purified lipids, Spinach MGDG and DGDG, Akamoku MGDG and DGDG, and Linseed TAG, with a Hitachi HPLC system equipped with a pump (Hitachi L-2130, Hitachi Seisakusho, Co., Tokyo, Japan) and fluorescence detector (Hitachi L-2485) [4]. Analysis was conducted on a silica column (Si 60, 250 × 4.6 mm i.d.; Kanto Chemical Co., Tokyo, Japan) protected with a guard column (15 × 3.2 mm) with the same stationary phase. The mobile phase was *n*-hexane-2-propanol (99.2:0.8, v/v) at a flow rate of 1.0 mL/min. The fluorescence detector was set at Ex. 298 nm and Em. 325 nm.

2.8. Fatty Acid Composition of Spinach GLs, Akamoku GLs, and Linseed TAG

The fatty acid composition of all 5 purified lipids was determined by gas chromatography (GC) after conversion of fatty acyl groups in the lipid to their methyl esters as described by Prevot and Modret [16], with slight modifications. Briefly, to an aliquot of total lipid (*ca.* 20 mg for GLs and 10 mg for TAG), 1 mL of *n*-hexane and 0.2 mL of 2N NaOH in methanol solution were added, vortexed for 10 s, and incubated at 50˚C for 30 s. Next, 0.2 mL of 2N HCL in methanol

was added to the solution followed by vortexing for 1 min. 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. GC analysis was performed using Shimadzu GC-2014 (Shimadzu Corporation, Kyoto, Japan) equipped with a flame-ionization detector and capillary column (Supelco™ Column; Omegawax-320; 30 m × 0.32 mm i.d.; Sigma-Aldrich, St. Louis, MO, USA). The detector, injector, and column temperatures were 260˚C, 250˚C, and 200˚C, respectively. The carrier gas was helium at a flow rate of 50 kPa. The fatty acid content was expressed as a weight percentage of total fatty acids.

2.9. Oxidation Analysis of Purified Lipids

Each purified lipid (100 mg) was placed in a 2-mL aluminum sealed vial with a butyl gum septum (GL Science; Tokyo, Japan) and then incubated at 50˚C in the dark. The level of oxygen and nitrogen in the headspace gas of the vial was estimated using a GC system (Shimadzu GC-14B) equipped with a thermal conductivity detector and stainless steel column (3 m × 3.0 mm i.d.) packed with a molecular sieve 5A (GL Science) as described by Cho *et al.* [9]. The temperatures at the injection port, detector port, and column oven were 120˚C, 120˚C, and 70˚C, respectively. The helium flow was 50 kPa. Three separate vials containing similar samples were prepared and incubated. A small portion (20 μL) of the headspace gas was taken from each vial using a microsyringe through the butyl gum septum at selected times during oxidation. Before the incubation, the ratio of peak area of oxygen to nitrogen was around 0.26. The ratio decreased with the progress of lipid oxidation. The percentage decrease (%) in oxygen could be calculated from the changes in the oxygen to nitrogen ratio compared to the ratio before incubation. In Three replicate measurements of all data and value at different oxidation times of the stored samples were expressed as the mean ± SD (n = 3).

After oxidation, PUFA contents were again analyzed by GC. The preparation of fatty acid methyl esters and GC analysis was performed as described above.

3. Results

3.1. Lipid Class Analysis

When each lipid composition of both Spinach and Akamoku GLs was visually analyzed based on the spot intensities of TLC (**Figure 2**), the overall distribution of their main lipid classes was determined and is shown in **Table 1**. Only MGDG and DGDG from both Spinach and Akamoku were analyzed, while SQDG was not analyzed because it generally showed low levels in Spinach and thus was not the focus of this research.

3.2. Purification of GL and Linseed TAG

Purified MGDG and DGDG from Spinach and Akamoku showed spots corresponding to MGDG and DGDG standards on TLC, respectively. In addition,

Figure 2. Representative TLC of spinach lipids. Sample solution was spotted on a 0.25-mm silica gel plate. The plate was developed with chloroform-methanol-water (65:25:4, v/v/v) and the spots were visualized by charring.

Table 1. Lipid class composition of Spinach GL and Akamoku GL.

GL class (% of total GL)	Spinach	Akamoku
MGDG	50	62
DGDG	43	8
SQDG	7	30

visible spots with small streaks of chlorophyll were observed during TLC analysis with Spinach MGDG and Akamoku MGDG, while both Spinach DGDG and Akamoku DGDG were free from spots containing small streaks of chlorophyll after the first purification. However, after both MGDGs were subjected to a second purification with a carbon column, only a single spot corresponding to MGDG standard was detected on the TLC for each type of sample.

Purified Linseed TAG, in contrast, showed only a single spot corresponding to the lipid standard triolein on analytical TLC. No other impurities such as free fatty acids, monoacylglycerol, or diacylglycerol were detected.

3.3. Tocopherol Analysis

HPLC analysis either showed complete removal of tocopherols for nearly all types of lipids or only a very small amount of this antioxidant compound remained for the remaining few types of lipids. Complete removal was confirmed after double purification of all GL samples. Minute and negligible amounts of tocopherol, however, were detected for samples of Linseed TAG, as reported by Shimajiri *et al.* [17], which do not affect comparisons of the oxida-

tive stability results.

3.4. Fatty Acid Composition

The overall fatty acid composition of purified GLs and Linseed TAG before and after oxidation is shown in Table 2. The main fatty acid in Linseed TAG and both Spinach MGDG and DGDG before oxidation was LN (18:3n-3), which showed weight percentages of 43.47%, 48.96%, and 77.53%, respectively. Only Spinach GL contained 16:3n-3, which accounted for 11.56% and 2.91% for Spinach MGDG and DGDG in weight percentage. In contrast, Akamoku MGDG and DGDG contained palmitic acid (PA; 16:0) as their main fatty acids, making up 35.92% and 24.09% of the overall weight percentage. In addition, Akamoku GLs were abundant in OA (18:1n-9), AA (20:4n-6), SDA (18:4n-3), and EPA (20:5n-3).

Table 3 shows the number of *bis*-allylic positions per molecule of each lipid. The number of *bis*-allylic positions per molecule or gram of each lipid was determined from the molar concentration of each PUFA and the mean molecular

Table 2. Major fatty acid composition of oxidation substrates before and after the oxidation.

	Spinach MGDG		Spinach DGDG		Akamoku MGDG		Akamoku DGDG		Linseed TAG	
Fatty acid (wt%)	Before oxidation	After oxidation	Before oxidation	After oxidation	Before oxidation	After oxidation	Before oxidation	After oxidation	Before oxidation	After oxidation
14:00	0.28 ± 0.00	1.00 ± 0.09	0.06 ± 0.00	0.08 ± 0.00	10.23 ± 0.21	11.07 ± 0.27	5.56 ± 0.10	5.86 ± 0.01	0.04 ± 0.01	3.08 ± 5.05
16:00	5.25 ± 0.10	18.82 ± 1.12	11.41 ± 0.07	12.25 ± 0.09	35.92 ± 0.35	35.88 ± 0.73	24.09 ± 0.30	23.40 ± 0.18	5.38 ± 0.06	18.46 ± 4.22
16:01	0.26 ± 0.03	0.84 ± 0.05	0.16 ± 0.03	0.17 ± 0.02	8.90 ± 0.08	9.06 ± 0.14	5.15 ± 0.06	5.36 ± 0.01	0.07 ± 0.00	0.16 ± 0.06
16:03	11.56 ± 0.22	6.12 ± 0.63	2.91 ± 0.07	3.83 ± 0.02	ND	ND	ND	ND	ND	ND
18:00	0.40 ± 0.01	1.26 ± 0.10	0.70 ± 0.03	0.76 ± 0.01	0.89 ± 0.01	0.93 ± 0.01	0.33 ± 0.01	0.36 ± 0.01	3.62 ± 0.02	11.55 ± 2.46
18:1n-9	1.47 ± 0.07	3.87 ± 0.23	0.86 ± 0.04	0.97 ± 0.01	14.80 ± 0.13	14.58 ± 0.13	7.26 ± 0.04	7.25 ± 0.16	26.18 ± 0.06	40.61 ± 10.34
18:2n-6	3.60 ± 0.09	4.07 ± 0.10	3.60 ± 0.07	3.83 ± 0.02	5.88 ± 0.03	5.36 ± 0.06	6.43 ± 0.02	6.34 ± 0.04	16.78 ± 0.04	3.03 ± 2.57
18:3n-3	48.96 ± 1.37	26.08 ± 2.31	77.53 ± 0.14	75.28 ± 0.12	1.13 ± 0.02	1.02 ± 0.02	5.47 ± 0.02	5.35 ± 0.00	43.47 ± 0.03	1.75 ± 1.75
18:4n-3	ND	ND	ND	ND	0.67 ± 0.01	0.56 ± 0.02	11.29 ± 0.05	10.64 ± 0.06	ND	ND
20:00	0.35 ± 0.24	0.45 ± 0.05	0.02 ± 0.00	0.02 ± 0.00	0.11 ± 0.02	0.15 ± 0.01	0.05 ± 0.00	0.07 ± 0.00	0.16 ± 0.01	ND
20:4n-6	ND	ND	ND	ND	3.04 ± 0.06	2.61 ± 0.16	12.15 ± 0.11	11.30 ± 0.01	ND	ND
20:5n-3	ND	ND	ND	ND	0.46 ± 0.08	0.42 ± 0.03	9.64 ± 0.15	9.20 ± 0.45	ND	ND

ND: not detected; PUFA: polyunsaturated fatty acids; MUFA: monounsaturated fatty acids; SFA: saturated fatty acids.

Table 3. Average number of *bis*-allylic positions of substrate lipids.

Total number of *bis*-allylic positions	Spinach		Akamoku		Linseed
	MGDG	DGDG	MGDG	DGDG	TAG
Per molecule Per g lipid (×6.02 ×1020)	1.73	1.71	0.29	1.54	1.08
	2.18	1.8	0.37	1.62	1.24

weight (MW) of each lipid. The molar concentration of PUFA was calculated based on the weight percentage of PUFA before oxidation (Table 2). The mean MW of each lipid was obtained from the mean MW of all fatty acyl moieties and MW of the galactosyl and glycerol moieties. MWs were as follows: Spinach MGDG, 796.03; Spinach DGDG, 949.03; Akamoku MGDG, 776.97; Akamoku DGDG, 950.38, and Linseed TAG, 875.16. Based on the MW of each lipid and molar concentrations of PUFAs, the highest average number of *bis*-allylic positions per molecule was observed for Spinach DGDH, followed by Spinach MGDG, Akamoku DGDG, Linseed TAG, and Akamoku MGDG (Table 3).

3.5. Stability of Purified Lipids in Bulk Phase

When the oxidative stability of different types of lipids was compared by measuring the decrease in oxygen concentration in the bulk phase (Figure 3), the stability was the highest for Spinach DGDG, followed by Akamoku DGDG and MGDG, and Spinach MGDG. Linseed TAG was shown to have the lowest oxidative stability. The oxidative stability of polyunsaturated lipids is decreased with an increasing number of *bis*-allylic positions [18]. Based on the number of *bis*-allylic positions of each lipid (Table 3), Spinach DGDG was expected to be easily oxidized as compared with Akamoku DGDG, Linseed TAG, and Akamoku MGDG. However, the oxidative stability results, shown in Figure 3, were quite different from the expected average number of *bis*-allylic positions. In addition, both GLs with the DGDG constituent were shown to be more stable than their MGDG constituent when they showed a mild decrease in oxygen concentration over time (Figure 3). Although Spinach and Akamoku MGDG were

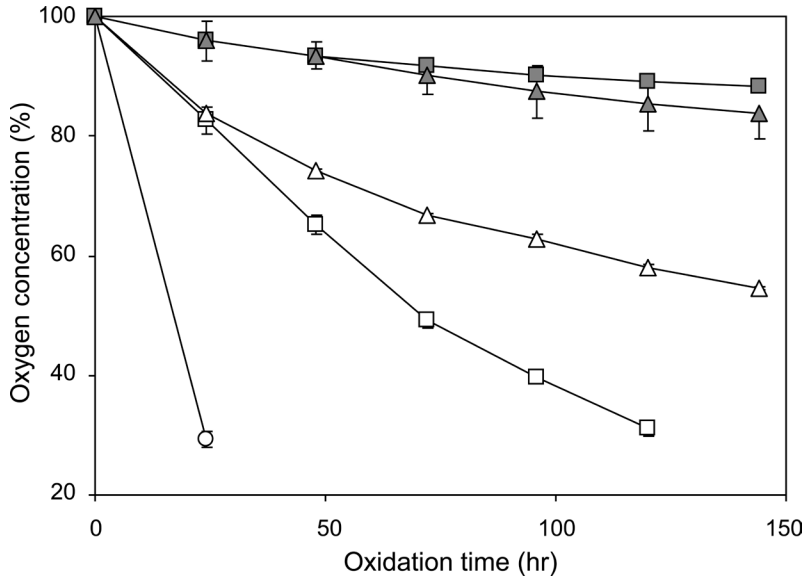

Figure 3. Oxidative stability of Spinach MGDG (open square), Spinach DGDG (closed square), Akamoku MGDG (open triangle), Akamoku DGDG (closed triangle), and Linseed TAG (open circle). Stability was analyzed by measuring the decrease in oxygen in the headspace gas of the vial. Oxidation was performed at 50˚C in the dark. The data are expressed as the mean ± SD of three independent experiments.

more easily oxidized than the corresponding DGDG, they were still much more stable compared to Linseed TAG.

The characteristic oxidative stability of MGDG, DGDG, and TAG was also confirmed by analyzing the changes in the fatty acid composition of these substrates before and after oxidation (Table 2). Table 2 shows that LN was the major PUFA in Spinach MGDG, Spinach DGDG, and Linseed TAG. After oxidation, the amount of LN in Linseed TAG was greatly reduced to only 1.75% in weight percentage, showing a nearly 96% loss in the content ratio. In contrast, the LN content in Spinach MGDG was reduced by nearly half (a content loss of approximately 47%), whereas its DGDG counterpart showed only a slight loss of approximately 3%.

All samples showed a reduction in the total content of PUFA after oxidation. The differences were calculated from Table 2. Spinach MGDG showed a difference of 36.92%, while Spinach DGDG showed a difference of only 4.96% in weight percentage. Akamoku MGDG exhibited a difference of 1.13% and Akamoku DGDG showed a minimal difference of 2.42% in weight percentage. Linseed TAG showed the greatest difference of 55.50% in weight percentage. In general, the total loss in content ratio with respect to the difference calculated in weight percentage was approximately 51% (Spinach MGDG), 6% (Spinach DGDG), 10% (Akamoku MGDG), 5% (Akamoku DGDG), and 92% (Linseed TAG). The maximal loss in content ratio was observed for Linseed TAG, followed by Spinach MGDG, Akamoku MGDG, and then Spinach DGDG and finally Akamoku DGDG. Both DGDG from Spinach and Akamoku showed the lowest reduction in the content ratio as compared to their MGDGs constituent.

4. Discussion

PUFA oxidation generally proceeds through a free radical chain reaction [19] [20]. In this reaction, the rate-limiting step is the abstraction of hydrogen radical (H•) from the substrate PUFA to form the PUFA free radical. Because this hydrogen abstraction occurs at the *bis*-allylic positions (CH=CH-CH_2-CH=CH) present in PUFA and the susceptibility of PUFA to oxidation depends on the availability of *bis*-allylic hydrogens, the oxidative stability of each PUFA is inversely proportional to the number of *bis*-allylic positions in the molecule. Therefore, among the relative oxidative stabilities of major PUFAs contained in Spinach GLs, Akamoku GLs, and Linseed TAG, EPA (20:5n-3) is most rapidly oxidized, followed by SDA (18:4n-3), AA (20:4n-6), 16:3n-3, LN (18:3n-3), and LA (18:2n-6) [7] [9] [21].

GL from marine lipid sources, both Akamoku MGDG and DGDG, showed high percentages of EPA. Particularly, Akamoku DGDG showed a higher percentage of EPA with a higher mean number of *bis*-allylic positions per molecule than that of Linseed TAG. Upon comparing the difference in the mean number of *bis*-allylic positions per molecule and the level of EPA, the oxidative stability of Akamoku DGDG was expected to be lower than that of Linseed TAG and

Akamoku MGDG. However, the stability of Akamoku DGDG was higher than that of Linseed TAG and Akamoku MGDG (**Table 2** and **Figure 3**).

Overall, the results for the oxidative stability of GLs and TAG obtained in the present study were very different from those expected from the average number of *bis*-allylic positions and fatty acid composition of each lipid. Our results showed that GL was oxidatively more stable than the TAG form. Furthermore, compared with different types of GLs, DGDG showed a higher oxidative stability than MGDG.

A study by Lee *et al.* [22] showed that Spinach GL contained a low ratio of saturated fatty acid to unsaturated fatty acid, but the GL had greater oxidative stability than did lipids of other classes such as neutral lipid and phospholipids. We also reported a higher oxidative stability for GL than TAG, although the average number of *bis*-allylic positions of GL was much higher than that of the TAG [4]. These results indicate that GL has unique characteristics and that the mechanisms involved in oxidation of this class of lipids are unique.

Minor contaminant compounds in the GL prepared from photosynthetic organisms such as plant and seaweeds are known to affect the oxidative stability of GL. Chlorophyll, a representative impurity, has been reported to have both antioxidant [23] [24] and pro-oxidant [25] activities. However, chlorophyll was removed successfully from the samples by double purification, first using silicic acid and then a carbon column as described in the experimental procedures. Additionally, experiments were conducted in the dark, minimizing the opportunity for traces of chlorophyll to react with light. Carotenoids and tocopherols may also have contaminated the GL. These antioxidants were also completely removed from the purified GL used in the present study. Furthermore, Linseed TAG was completely free from antioxidants and pro-oxidants such as oxidation products.

PUFA oxidation essentially proceeds via free radical chain reaction, where the rate-limiting step is the abstraction of H• from the *bis*-allylic positions of the substrate, PUFA. Therefore, the susceptibility of PUFA to oxidation depends on the availability of *bis*-allylic hydrogens. In contrast, physical and stereochemical protection of *bis*-allylic hydrogens by other molecules has been also regarded as an important factor for understanding the oxidative stability of PUFA, particularly in oil in water emulsion, food, and biological systems [26].

In food emulsions, various molecules are distributed according to their polarity and surface activity between different phases, which include the oil phase, water phase, and interfacial region. Lipid oxidation in this system is an interfacial phenomenon that is greatly influenced by the nature of the interface. In this case, emulsifiers greatly affect the oxidative stability of PUFA. At the droplet interface, emulsifier molecules arrange themselves in an interface with PUFA and water so that the polar head-groups are located at the surface and non-polar tails are in the interior; *bis*-allylic positions of PUFA can be protected by the interactions with emulsifiers [26].

In phosphatidylcholine liposomes, the model system of biological membranes, DHA showed higher oxidative stability than LA [27] [28]. These results can be explained by the protective conformation of the *bis*-allylic positions of DHA against hydrogen abstraction. The characteristic structure of the DHA (22:6n-3) moiety has been observed in several membrane models. A molecular modeling approach showed that DHA of diacylglycerol may uniquely influence acyl chain packing arrangements in cell membranes [29] [30] [31]. When in these conformations, DHA hexanes can form tightly packed arrangements to protect the *bis*-allylic positions of DHA from oxidative attack.

Therefore, the characteristic oxidative stability of GL found in the present study did not result from contamination, but rather from differences in the chemical structure of each lipid molecule. MGDG and DGDG contain both polar (galactosyl moiety) and non-polar (fatty acyl group) regions on the same molecule, which can form a self-assembly structure [32]. In this structure, galactosyl moieties may protect the *bis*-allylic positions of PUFA in GL through various interactions. The present study revealed the higher oxidative stability of DGDG than that of MGDG for the first time. This may be because of the stronger protective effect of di-galactosyl moieties on PUFA against oxidative attack than that of mono-galactosyl moieties.

5. Conclusion

In the present study, we found that PUFAs in the form of GLs were oxidatively more stable than those of TAG. In addition, when the oxidative stability of MGDG and DGDG from Spinach and Akamoku was compared, DGDG showed higher oxidative stability than its corresponding MGDG. These results improve the understanding of the oxidation of GL containing PUFA in biological systems and may be useful for preventing PUFA from undergoing oxidative deterioration. Further studies are required to elucidate the detailed mechanism of the unusually high oxidative stability of GLs.

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