Foaming Power and Emulsifying Properties of the Hydrolyzates by Lipase from *Rhizopus arrhizus* on Digalactosyldiacylglycerol and Trigalactosyldiacylglycerol Extracted from Pumpkin

Takashi NAKAE, Takashi KOMETANI, Takahisa NISHIMURA, Hiroshi TAKII and Shigetaka OKADA

Biochemical Research Laboratory, Ezaki Glico Co., Ltd., Utajima 4-6-5, Nishiyodogawa, Osaka 555-8502, Japan

Received January 6, 1997; Accepted April 19, 1998

Digalactosyldiacylglycerol (DGDG) and trigalactosyldiacylglycerol (TGDG) were extracted from pumpkin and then hydrolyzed to the corresponding monoacylglycerols by L3-specific lipase from *Rhizopus arrhizus*. The hydrolyzates from DGDG and TGDG were identified as digalactosylmonoacylglycerol (DGMG) and trigalactosylmonoacylglycerol (TGMG) by TLC analysis, acid hydrolysis and GC analysis, respectively. The surface tensions of the aqueous DGMG and TGMG solutions significantly decreased up to 0.005% DGMG and TGMG, and then decreased slightly at more than that concentration. The foambilities increased with the DGMG and TGMG contents. The foambilities and the foaming stabilities of DGMG and TGMG were superior to those of the other commercial emulsifiers. The interfacial tensions at the water/kerosene interface of DGMG and TGMG significantly decreased up to 0.0019%; and then slightly decreased at more than that concentration. The types of water/kerosene (1:1, w/w) emulsions prepared with DGMG and TGMG were O/W, respectively. The creaming stabilities and the strength of the interfacial film of the emulsions increased with the DGMG and TGMG contents. The emulsifying properties of the DGMG and TGMG emulsions were almost equal to those of the commercial emulsifiers.

Keywords: glyceroglycolipid, lipase, foambility, foaming stability, emulsifying ability, emulsion stability

Recently, the demand for natural emulsifiers is increasing, because of increasing consciousness for safety in food materials. Lecithin and saponin as emulsifiers are derived from natural matter. However, their emulsifying properties are inferior to chemically synthesized emulsifiers. Accordingly, we tried to screen the surface active substance having the same emulsifying properties as the chemically synthesized emulsifiers. We found glyceroglycolipids which were expected to make emulsion because of having a hydrophobic part and hydrophilic one in the same molecule.

Glyceroglycolipids are widely found in plants, in animal tissues and in bacteria. Glyceroglycolipids consist of hydrophilic carbohydrate groups and hydrophobic fatty acid moieties which bind through a glycerol, and are known to be part of the major components of biomembranes (Kates, 1990), and have been considered to play important roles such as enhancing membrane stability (Chapman et al., 1983). Some glyceroglycolipids have also been reported to exhibit immunological (Murakami et al., 1995) or biological activities (Katsuoka et al., 1990, Sakata & Ina, 1983).

It had been reported that monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG), which are the major glycerolipids of plants, and in addition, trigalactosyldiacylglycerol (TGDG) were present in pumpkin (Ito et al., 1974). TGDG is rare in other plants and then it has been considered that TGDG would be a good emulsifier because it is more hydrophilic than MGDG and DGDG.

On the other hand, the hydrolytic enzymes have been utilized to improve the emulsifying properties. For example, phospholipase A2 has been utilized to improve the emulsifying property of lecithin (Aoi, 1990). This appears to be mainly due to raising the hydrophilicity of the emulsifier because of losing one fatty acid moiety. In the same way, it is considered that the hydrophilicity of glyceroglycolipids will be raised if it can be hydrolyzed to monoacylglycerol by lipase.

In this report, we tried to isolate DGDG and TGDG from pumpkin, and then to hydrolyze them to digalactosylmonoacylglycerol (DGMG) and trigalactosylmonoacylglycerol (TGMG) by lipase for the purpose of obtaining more hydrophilic emulsifiers. Furthermore, we investigated the foaming power and the stabilities of emulsions prepared with DGMG and TGMG by comparing them with chemically synthesized emulsifiers.

Materials and Methods

**Chemicals** Lipase (from *Rhizopus arrhizus*) and lysolecithin (from egg yolk; LPC) were purchased from Sigma Co., Ltd. (St Louis, MO). Sucrose esters of the fatty acids (F-160; SE) were purchased from Dai-ichi Kogyo Seiyaku Co., Ltd. (Kyoto). Lysolecithin (San lecithin A) and decaglycerylmonolaurate (Q-12S, PGE12) were purchased from Taiyo Kagaku Co., Ltd. (Mie). The authentic sample of DGDG was purchased from Sigma Co., Ltd. All other chemicals used were commercially available and of chemically pure grade.

**Extraction of DGDG and TGDG** Pumpkin (*Cucurbita maxima*, var. Ebisu; fresh weight 150 g) was suspended
in 300 ml of chloroform-methanol (2:1, v/v) and the mixture was then homogenized using a Physcotron mixer (MICROTECH NITION Co., Ltd., Chiba) for 5 min. The mixture was extracted by stirring for 4 h at room temperature. After filtration, the residue was re-extracted in 300 ml of chloroform-methanol (2:1, v/v) as previously described. The extracts were then combined, and all water-soluble contaminants removed from the extracts using the previously reported method of Folch et al. (1957). The lipid was subjected to silica gel (Wakogel C-200, 15 g) column chromatography by sequential elution with chloroform, acetone, and methanol (Vorbeck & Marinetti, 1965). The glycolipids were eluted with acetone. They were subjected to silica gel column chromatography by stepwise elution with chloroform-acetone. Pigments and MGDG were eluted with chloroform-acetone (40:60, v/v). DGDG was also eluted with chloroform-acetone. Pigments and MGDG were eluted with chloroform-acetone. DGDG and TGDG were eluted with only acetone. Purities of the fractions containing DGDG or TGDG were confirmed by thin-layer chromatography (TLC) (Merck, art. 5626, Darmstadt, Germany) using chloroform-methanol-H₂O (65:25:4, v/v/v), and were detected by spraying 50% (v/v) H₂SO₄ followed by heating at 130°C for 5 min. The Rf values of DGDG and TGDG coincided with the result previously reported by Ito et al. (1974).

Hydrolases of DGDG and TGDG by lipase. Preparation of the reaction mixture containing 1.0% DGDG (or TGDG), 0.4% Triton X-100 and 4000 units/ml of lipase (Rhizopus arrhizus) in 25 mM Tris-HCl buffer (pH 7.5) was stirred at 37°C for 16 h. The reaction mixture of DGDG was subjected to silica gel column chromatography by elution with chloroform-methanol-H₂O (70:30:10, v/v/v, lower layer). That of TGDG was subjected to silica gel column chromatography by elution with chloroform-methanol-H₂O (65:35:10, v/v/v, lower layer). Each elute was analyzed by TLC (Merck, art. 5626) using chloroform-methanol-H₂O (65:35:10, v/v/v, lower layer), and it was detected by spraying 50% (v/v) H₂SO₄ followed by heating at 130°C for 5 min.

Fatty acid analysis. Preparation of the methyl esters from each glycerolipid was followed by a slight modification of the method previously reported by Metcalfe and Schmitz (1961). The glycerolipids were deacylated with 0.5 N sodium methoxide in methanol at 80°C for 15 min. Fatty acids were methylated with 14% boron trifluoride in methanol at 80°C for 10 min. Fatty acid methyl esters were extracted into hexane and analyzed by gas chromatography (GC) (GC-14A; Shimazu Co., Ltd.) using a DEGS (25% chromsorb W AW-DMCS 80/100 mesh) column with nitrogen gas as the carrier at the flow rate of 40 ml/min at 170°C with detection using a flame ionization detector (FID). Methylpentadecanoate was used as the internal standard.

Acid hydrolysis of lipase-treated DGDG and TGDG. In order to examine the structures of the hydrolyzates by lipase from DGDG and TGDG, each sample (5 mg) was dissolved in 2 ml of 0.2 N H₂SO₄ and boiled at 100°C for 4 h. After cooling to room temperature, each prepared sample was added to 4 ml of chloroform. The prepared sample solution was separated into two phases. Galactose was released in the water phase and fatty acids were released in the chloroform phase. The amounts of released galactose were measured by high performance liquid chromatography (HPLC) on a Sugar SC1011 and SC1821 (Shodex) eluted with H₂O at a flow rate of 1.0 ml/min at 70°C, with detection by refractive index (RI).

In this system, the retention time of galactose was approximately 18.3 min. The amounts of released fatty acids were measured by GC as already described.

Determination of surface and interfacial tensions. Surface and interfacial tensions at the water/kerosene interface were measured using a Wilhelmy type interfacial tensiometer (CBVP-A3; Kyowa Interface Science Co., Ltd., Saitama) at 25°C.

Determination of foaming power. Preparation of the foam was followed by a slight modification of the method previously reported by Yamano et al. (1984). Each aqueous solution sample (1.0 ml) was vigorously stirred in a tube (13×100 mm) with a homogenizer (Physcotron mixer) at 18,000 rpm for 60 s at 25°C and then kept at 25°C. The foamability was determined measuring the height of the foam formed immediately after preparation. The foaming stability was done by measuring the height of the foam as a function of time.

Determination of water phase separation. Preparation of the emulsion was followed by a slight modification of the method previously reported by Shimbo et al. (1993). The mixtures of kerosene (0.5 g) and each aqueous solution sample (0.5 g) with DGMG or TGMG were vigorously stirred in a tube with a homogenizer (Physcotron mixer) at 18,000 rpm for 30 s at 25°C.

Water phase separation of the oil in water (O/W) emulsion is an indicator of creaming due to the density difference between the dispersed and continuous phases. Therefore, the prepared emulsion was transferred to a graduated tube and kept at 25°C, and the water phase separation was determined with an image analyzer (SUPER ASPECT; Mitani Corporation, Fukui) by the previously reported method of Chen et al. (1993). The emulsion image transferred to the image analyzer via a CCD video camera was processed in the image analyzer and computer for the measurement of the water phase separation.

Determination of oil phase separation. The mixtures of kerosene (0.5 g) and each aqueous solution sample (0.5 g) were vigorously stirred in a tube with a homogenizer (Physcotron mixer) at 18,000 rpm for 60 s at 25°C. The prepared emulsions were centrifuged at 3000×g at 25°C for 30 min and the ratio of the separated oil weight was expressed as a percentage of the overall emulsion weight.

Results

Hydrolases of DGDG and TGDG by lipase and their structures. Figure 1 shows the TLC analysis results of the hydrolyzates of DGDG and TGDG by lipase. Lines 1 and 3 show DGDG and TGDG, respectively. Lines 2 and 4 were their respective hydrolysates. The Rf values of DGDG and TGDG coincided with the results previously reported by Ito et al. (1974). Their structures have already been identified as 1,2-diacyl-3-O-α-D-galactopyranosyl-6'-O-β-D-galactopyranosyl-sn-glycerol and 1,2-diacyl-3-O-α-D-galactopyranosyl-6'-O-β-D-galactopyranosyl-sn-glycerol, respectively (Ito et al., 1974). After lipase treat-
Emulsifying Properties of Glyceroglycolipids

FFA

DGDG

DGMG

TGDG

TGMG

Fig. 1. TLC analysis of hydrolyzate of DGDG and TGDG by lipase (Rhizopus arrhizus). Hydrolyzation of DGDG or TGDG and TLC analysis described in Materials and Methods was done. Lane 1, DGDG; lane 2, reaction mixture containing DGDG; lane 3, TGDG; lane 4, reaction mixture containing TGDG.

Table 1. Fatty acid compositions of DGDG, TGDG, DGMG and TGMG in pumpkin (% w/w).

| Fatty acid | DGDG | TGDG | DGMG | TGMG |
|-----------|------|------|------|------|
| C16:0     | 12.2 | 11.8 | 1.9  | 1.5  |
| C18:0     | 1.4  | 0.9  | —    | —    |
| C18:1     | 5.9  | 6.0  | 4.5  | 0.7  |
| C18:2     | 29.0 | 30.9 | 28.9 | 28.1 |
| C18:3     | 51.5 | 50.4 | 64.4 | 69.7 |

C16:0, palmitic acid; C18:0, stearic acid; C18:1, oleic acid; C18:2, linoleic acid; C18:3, linolenic acid.

Fig. 2. Structures of DGMG and TGMG. R represents acyl residue.

The foaming power and the emulsifying property As both DGMG and TGMG have hydrophobic part (fatty acid) and a hydrophilic one (digalactoside or trigalactoside), they seemed to have both foaming power and emulsifying activities.

Surface and interfacial tension Figure 3 displays the curves showing the reduction in surface tension at the air/water interface to concentration of DGMG and TGMG with LPC as the control. LPC, the major component of lysolecithin.
in, is a hydrolyzate of phosphatidylcholin by phospholipase A₂ like DGMG and TGMG. The surface tensions decreased with the DGMG and TGMG contents. The surface tensions at 0.15% DGMG, 0.15% TGMG and 0.15% LPC were 36.9, 39.1 and 38.6 mN/m, respectively.

Figure 4 displays the curves showing the reduction in interfacial tension at the water/kerosene interface to concentration of DGMG, TGMG and LPC at 6 h after preparation. The interfacial tension at the water/kerosene interface without an emulsifier was 42.1 mN/m at 25°C. The interfacial tensions at the water/kerosene interface decreased with the DGMG and TGMG contents. The interfacial tensions at 0.04% DGMG, 0.04% TGMG and 0.04% LPC were 2.8, 3.1 and 3.4 mN/m, respectively. Moreover, we also measured the interfacial tensions at the water/kerosene interface for 0.04% SE and lysolecithin. The interfacial tensions for 0.04% SE and lysolecithin were 5.4 and 4.6 mN/m, respectively.

From these results, reduction of the surface and interfacial tensions of DGMG and TGMG were at least equivalent to LPC, SE and lysolecithin.

**Foamability and foaming stability** The effects of the DGMG and TGMG concentrations on the height of the foams formed immediately after preparation are shown in Fig. 5. The height of the formed foams increased with DGMG and TGMG contents and reached a constant above 0.025%. The height of the foams formed at 0.1% DGMG and TGMG solutions were 16.7 mm and 20.2 mm, respectively.

The changes in the height of the formed foams after preparation by the aqueous DGMG and TGMG solutions were measured as a function of time. The height of the foams formed on the aqueous DGMG and TGMG solutions significantly decreased during 60 min after preparation, and then gradually decreased with aging time.

To estimate the foaming stabilities of DGMG and TGMG, the commercial foaming agents as comparative samples were measured by the same procedure. As shown in Fig. 6, the height of the foams formed for 0.1% DGMG and TGMG solutions were higher than those of lysolecithin and SE at 1
Emulsifying Properties of Glyceroglycolipids

Fig. 6. The height of foam formed using various 0.1% foaming emulsifiers. The height of foam formed was measured using the methods described in Materials and Methods during aging for 1 (●) and 60 (△) min. Each value represents mean±SD, n=3. *Significantly different from TGMG (p<0.05). **Significantly different from TGMG (p<0.01).

Fig. 7. Effects of concentration of emulsifiers on the creaming stabilities in emulsions. The creaming stabilities represented in terms of ratio of separated water phase to total solution during aging for 1 (●), 3 (△) and 24 (□) h. Each value represents mean±SD, n=3.

Fig. 8. Effects of concentration of emulsifiers on the oil phase separation by centrifuging the emulsions. The oil phase separation by centrifuging the emulsions prepared with DGMG (●), TGMG (△), SE (△) and lysolecithin (●) were measured using the methods described in Materials and Methods. Each value represents mean±SD, n=3.

and 60 min after preparation. Furthermore, the height of foams formed for 0.1% TGMG solutions were higher than those of PGE10 that is known to have the highest foamability of commercial emulsifiers.

Types of emulsions The types of emulsions prepared with DGMG and TGMG were determined from the solvent dilution method by water or kerosene (Shimbo et al., 1993). As a result, both emulsions were O/W types.

Creaming stability of emulsion As DGMG and TGMG had the emulsifying activities and were thought to be nonionic and hydrophilic emulsifiers, SE (nonionic and hydrophilic one), and lysolecithin (ionic and hydrophilic one) were used as controls for a comparison of their properties. Changes in the creaming stabilities of the water/kerosene (1:1, w/w) emulsions with DGMG and TGMG contents are presented in terms of the ratio of the separated water phase to total solution as a function of time. The effects of concentration on the water phase separation in the emulsions prepared with DGMG, TGMG, SE and lysolecithin are shown in Fig. 7. The water phase separation of the emulsions prepared with DGMG, TGMG, SE and lysolecithin significantly decreased up to a 0.05% content, and then decreased slightly above that level.

We also measured the creaming stabilities of emulsions prepared with DGDG and TGDG. As a result, both emulsions were O/W types. However, their creaming stabilities were inferior to those of DGMG and TGMG (data not shown).

Oil phase separation of emulsion The ratio of oil phase separation by centrifuging the emulsions prepared with DGMG, TGMG, SE and lysolecithin on various concentrations are shown in Fig. 8. The oil phase separation of the emulsions decreased with the DGMG and TGMG contents. The emulsions prepared with more than 0.025% DGMG and those with more than 0.05% TGMG did not form the oil phase separation. The oil phase separation of the DGMG and TGMG emulsions were more stable than those of the SE and lysolecithin emulsions.

Discussion

Recently, the demand for natural materials for food manufacture is increasing, because of customer’s increasing consciousness for safety in food. As for emulsifiers, natural ones such as lecithin and saponin are preferred to artificial ones such as SE and monooacylglycerol. However, lecithin is rather hydrophobic and its emulsifying activity is not as high as artificial emulsifiers. Also, the utilization of saponin is not sufficient because it has been only slightly investigated. Accordingly, natural emulsifiers having high emulsifying activity and high hydrophilicity are needed for food manufacture.

For the purpose of obtaining a new natural emulsifier, we extracted and purified DGDG and TGDG from pumpkin,
and modified them to DGMG and TGMG and then investigated their characteristics.

As for the structures of the hydrolyzates from DGDG and TGDG, we ascertained that they were DGMG and TGMG by TLC analysis and acid hydrolysis, respectively. Also, their fatty acid compositions were confirmed by GC analysis. However, in the NMR analysis, we found that there are at least two kinds of compounds, which seemed to be 2-acyl- and 1-acyl-digalactosyl glycerol in DGMG, and 2-acyl- and 1-acyl-trigalactosyl glycerol in TGMG. This seems to be due to the spontaneous transfer of the acyl substituent from the sn-2 position to the sn-1 position (Murakami et al., 1994). We are now providing further purification and determining their precise structures. We planned to estimate the emulsifying abilities and stabilities of DGMG and TGMG with a single fatty acid composition. These results will be published in another paper.

These new emulsifiers had higher foaming abilities than known emulsifiers for food such as SE or lysolecithin, and had foamabilities as same as PGE10 whose foamabilities are the highest of all emulsifiers used in food manufacturing. In general, the surface tension at the air-liquid interface is an important physical property of foam, and it is known that the solution with a lower surface tension has higher foamability. Also, the reductions in surface tensions by DGMG and TGMG were equivalent to that by LPC, which was used as a control for measuring the reduction in surface tension. These surface tension results of aqueous DGMG and TGMG solutions were coincident with their high foamabilities.

We also investigated the emulsifying abilities and stabilities of the DGMG and TGMG emulsions. In general, the emulsifying abilities of the emulsifiers are estimated from the interfacial tension at the liquid-liquid interface, the droplet sizes and the distributions of their emulsions immediately after preparation. The reduction in the interfacial tensions at the water/kerosene interface of DGMG and TGMG were found to be equivalent to those by LPC, SE and lysolecithin. Moreover, the medium droplet sizes and the droplet size distributions of the emulsions with 0.2% DGMG and TGMG immediately after preparation were equivalent to those by SE and lysolecithin (data not shown).

On the other hand, the emulsion stability of the O/W type had been found to be related to emulsion breakdown, namely, the phenomenon from creaming and flocculation to coalescence (Bergenstahl & Claesson, 1990). Creaming is a separation caused by the upwards motion of emulsion droplets that have a lower density than the surrounding medium. Flocculation is an aggregation of droplets. Coalescence means that two droplets, when colliding, lose their identity and form a single larger one. These phenomena are influenced by the physical strength of the interfacial film, the electrostatic repulsive force between electrical double-layers of identical sign, and so on (Friberg et al., 1990). To estimate the physical strength of the interfacial film, the ratio of the oil phase separation by centrifuging the emulsions are measured. The results showed that the oil phase separation of the DGMG and TGMG emulsions were more stable than those of the SE and lysolecithin emulsions.

Consequently, we considered that the emulsifying abilities of DGMG and TGMG were equivalent to those of the commercial emulsifiers. However, we found that the interfacial film of the DGMG and TGMG emulsions were stronger than those of the commercial emulsifiers. It was also suggested that the physical strength of the interfacial film of the DGMG and TGMG emulsions were correlated to the higher foaming abilities of DGMG and TGMG.

In this study, it was suggested that DGMG and TGMG were good foaming agents and emulsifiers for food. Furthermore, as DGMG and TGMG are derived from natural matter such as pumpkin, the foods foamed and emulsified with DGMG and TGMG are safe.

References
Aoi, N. (1990). Soy lysolecithin. Yukagaku, 39, 10-15 (in Japanese).
Bergenstahl, B.A. and Claesson, P.M. (1990). Surface forces in emulsions. In “Food Emulsions, Second Edition, Revised and Expanded,” ed. by K. Larsson and S.E. Friberg. Marcel Dekker Inc., New York and Basel, pp. 74-81.
Chapman, D.J., De-Felice, J. and Barber, J. (1983). Influence of winter and summer growth conditions on leaf membrane lipids of Pismus Sativum L. Planta, 157, 218-223.
Chen, E., Gohtani, S. and Yamano, Y. (1993). Measurement of phase separation in O/W emulsion by image-analysis method. J. Jpn. Oil Chem. Soc., 42, 399-403.
Friberg, S.E., Goubran, R.F. and Kayali, I.H. (1990). Emulsion stability. In “Food Emulsions, Second Edition, Revised and Expanded,” ed. by K. Larsson and S.E. Friberg. Marcel Dekker Inc., New York and Basel, pp. 1-40.
Ito, S., Okada, S. and Fujino, Y. (1974). Glyceroglycolipids in pumpkin. Nippon Nogeikagaku Kaishi, 48, 431-436 (in Japanese).
Kates, M. (1990). In “Handbook of Lipid Research, vol. 6,” ed. by Plenum. New York, pp. 1-122, 235-320.
Katsuoka, M., Ogura, C., Etoh, H., Sakata, K. and Ina, K. (1990). Galactosyl- and sulfoquinovosylldiacyl glycerols isolated from the brown alga, Undaria pinnatifida and Costaria costata as repellents of the blue mussel, Mytilus edulis. Agric. Biol. Chem., 54, 3034-3044.
Metcalf, L.D. and Schmitz, A.A. (1961). The rapid preparation of fatty acid esters for gas chromatographic analysis. Anal. Chem., 33, 363-364.
Murakami, A., Nakamura, Y., Koshimizu, K. and Ohigashi, H. (1995). Glyceroglycolipids from Citrus hystrix, a traditional herb in Thailand, potently inhibit the tumor-promoting activity of 12-O-tetradecanoylphorbol 13-acetate in mouse skin. J. Agric. Food Chem., 43, 2779-2783.
Murakami, N., Morimoto, T., Imamura, H., Nagatsu, A. and Saka-kihara, J. (1994). Enzymatic transformation of glyceroglycolipids into sn-1 and sn-2 glyceroglycolipids by use of Rhizopus arrhizus lipase. Tetrahedron, 50, 1993-2002.
Sakata, K. and Ina, K. (1983). Digalactosyldiacylglycerols isolated from a brown alga as effective phagostimulants for a young abalone. Agric. Biol. Chem., 47, 2957-2960.
Shimbo, K., Gohtani, S., Yamato, Y. and Ina, K. (1993). Emulsifying properties of enzymatically modified phospholipids. Nippon Shoku- hin Kogyo Gakkaishi, 40, 755-763 (in Japanese).
Vorbeck, M.L. and Marinetti, G.V. (1965). Separation of glycosyl diglycerides from phosphatides using silicic acid column chromatography. J. Lipid Res., 6, 3-6.
Yamano, Y., Gohtani, S. and Nakayama, S. (1984). Foaming power and emulsifying properties of Gypsophila paniculata L. saponin. Nippon Nogeikagaku Kaishi, 58, 161-168 (in Japanese).