Synthesis of Propylene Glycol Monoesters of Docosahexaenoic Acid and Eicosapentaenoic Acid by Lipase-Catalyzed Esterification in Organic Solvents

Kuan-Ju Liu[a] and Jei-Fu Shaw[b,c,*]

[a]Department of Marine Food Science, [b]Institute of Marine Biotechnology, National Taiwan Ocean University, Keelung, Taiwan and [c]Institute of Botany, Academia Sinica, Taipei, Taiwan

ABSTRACT: Propylene glycol monoesters (PGM) of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are potentially health-beneficial water-in-oil emulsifiers useful in the food industry. These esters were synthesized enzymatically to overcome the problems associated with chemical processes. The products were analyzed by gas chromatography. The immobilized *Mucor miehei* lipase was found to be the best enzyme for the synthesis of both propylene glycol monoesters of EPA and DHA among nine lipases tested. The anhydrous enzyme and hydrophobic organic solvents were favored for the production of both monoesters. The yields of monoesters were also affected by temperature, pH memory, fatty acid/propylene glycol ratio, and reaction time. The yields of PGMDHA and PGMEPA with 50 mM fatty acid and 225 mM propylene glycol as substrates in 1 mL solvent mixture (hexane/*t*-butyl alcohol = 9:1), catalyzed by Lipozyme IM-20 (50 mg) at 40°C for 24 h, were 47 and 49 mM, respectively. The enzyme still retained over 60% of its original activity after 10 d of batch-type operation (1 d per cycle) at 40°C for the synthesis of both PGMDHA and PGMEPA.

JAOCS 72, 1271–1274 (1995).

KEY WORDS: Emulsifier, esterification, lipase, organic solvent, polyunsaturated fatty acid.

Propylene glycol (1,2-propanediol) monoesters (PGM) are good water-in-oil emulsifiers with low hydrophilic–lipophilic balance values (1). They have been approved by the U.S. Food and Drug Administration for use in foods (2) and are most often used in cakes, cake mixes, whipped toppings, and bread (3). They can be used in combination with monoglycerides to obtain excellent cake batter behavior, resulting in increased cake volume and uniform structure. Eicosapentaenoic acid (EPA, $C_{20:5}$) and docosahexaenoic acid (DHA, $C_{22:6}$) are known to reduce both thrombotic tendency and hypertriglyceridemia (4). Therefore, PGM of EPA and DHA are potentially health-beneficial emulsifiers useful in the food industry.

Synthesis of PGM by chemical methods, e.g., esterification of propylene glycol with fatty acid in the presence of acid or alkaline catalysts, usually results in a complex mixture (5,6). Enzyme-catalyzed conversion is more efficient and selective (7).

In the present work, we found that PGM of EPA and DHA can be efficiently synthesized by esterification with lipase from *Mucor miehei* (Lipozyme IM-20; Novo Nordisk Inc., Danbury, CT) as biocatalysts in organic media. The effect of acyl donors, organic solvents, temperature, water content, kind of lipase, addition of *tert*-butyl alcohol, addition of molecular sieves, and pH memory also were investigated.

MATERIALS AND METHODS

Materials. Nine lipases were obtained from available commercial sources. Lipase from *Aspergillus niger* (Amano "AP-6"), *Mucor* sp. (Amano "MAP-10"), *Pseudomonas fluorescens* (Amano "PS"), *Rhizopus* sp. (Amano "FAP-15"), and *Rhizopus* sp. (Amano "N-conc.") were from Amano International Enzyme Co. (Nagoya, Japan). Lipases from *Candida cylindracea* Type VII and porcine pancretic lipase were purchased from Sigma Chemical Co. (St. Louis, MO). Lipase from *Chromobacterium viscosum* (LP-101-S) was purchased from Toyo Jozo Co. (Shizuoka, Japan). Immobilized lipase IM-20 from *Mucor miehei* was supplied by Novo Nordisk Inc. Pentane, *n*-hexane, *n*-heptane, cyclohexane, isooctane, chloroform, toulene, *tert*-butyl alcohol, and molecular sieve (3Å) were obtained from Merck Chemical Co. (Darmstadt, Germany). Propylene glycol was purchased from Sigma Chemical Co. EPA and DHA were purchased from Nu-Chek-Prep, Inc. (Elysian, MN).

Methods. For standard reactions, the commercial lipase powder (0.05 g) was added to a reaction mixture (1 mL) containing 50 mM fatty acid and 225 mM propylene glycol in the mixture solvent of *n*-hexane and *tert*-butyl alcohol (9:1, vol/vol). The reaction mixture was incubated in an orbital shaker with a speed of 250 rpm at 40°C. At various time intervals, 8 μL of the reaction mixture was withdrawn and analyzed by gas chromatography (Hitachi model G-3000; Hitachi, Tokyo, Japan). An Rtx®-65 TG fused-silica capillary column of 30 m × 0.25 mm i.d. (Restek Corporation, Belle-

*To whom correspondence should be addressed at Institute of Botany, Academia Sinica, Nankang, Taipei, Taiwan 11529.

fonte, PA) was used. Hydrogen gas was chosen as the carrier gas at a flow rate of 1.4 kg/cm^2. The injection port and flame-ionization detector temperatures program was: 200°C, (20°C/min) to 215°C, holding 1 min, (13°C/min) to 350°C, holding 7 min. The product compositions (monoesters and diesters) were quantitated by an integrator with stearic acid as internal standard.

To study the effect of water content on the lipase-catalyzed synthesis, the enzyme was lyophilized by a Savant Speed Vac Concentrator (Savant Instruments, Inc., Farmingdale, NY) under 50 millitorr for 24 h. Water was removed from organic media by 3Å molecular sieve (Merck). To study the pH effect on synthesis, the lipase was dissolved in 10 mM mixed Good's buffer solution of different pH (10 mM each of BICINE, CAPS, sodium acetate, and BIS-TRIS propane were mixed and adjusted to various pH values by either concentrated HCl or NaOH) and then lyophilized as described earlier.

Measurement of lipase. The lipase hydrolytic activities were measured according to the method described by R'ua *et al.* (8).

RESULTS AND DISCUSSION

Among nine commercial lipases tested, *M. miehei* lipase IM-20 showed the best catalytic efficiency and specificity for enzymatic synthesis of PGM of EPA (PGMEPA) and DHA (PGMDHA) (Table 1). It is interesting that Amano PS lipase, which was the best enzyme for the synthesis of propylene glycol esters of C_{12}–C_{18} fatty acids (7), apparently showed no significant activity for PGMEPA or PGMDHA synthesis. This is due to the different substrate specificity of the enzyme. The Lipozyme IM-20-catalyzed esterification of propylene glycol with EPA or DHA was efficient. The yields of PGMEPA and PGMDHA were 45 and 46.4 mM, respectively, from 50 mM of EPA and DHA reacting with 225 mM propylene glycol, catalyzed by 50 mg lipase in 1 mL of mixed organic solvent (hexane and *t*-butyl alcohol = 9:1) at 40°C (Table 1). The specific lipase hydrolysis activity of each enzyme also is listed in Table 1.

The yield of monoesters was affected by acyl donors, reaction temperature, fatty acid/propylene glycol ratio, organic solvents, water content, and pH memory. As shown in Table 2, the yields of PGMEPA and PGMDHA catalyzed by Lipozyme IM-20 increased as the temperature increased up to 50°C. However, some other by-products appeared when the reaction was carried out at 50°C. This could be due to the oxidation of the n-3 polyunsaturated fatty acid (EPA and DHA). Therefore, it is more desirable to carry out the esterification reaction at 40°C.

The effects of the propylene glycol/fatty acid ratio in the reaction mixture on the Lipozyme IM-20-catalyzed esterification are shown in Table 3. The yields of both PGMEPA and PGMDHA increased as the ratio of propylene glycol/fatty acid ratio increased up to 5. Higher ratios decreased the yields of both monoesters.

Organic solvents greatly affected the yield of monoesters. Table 4 showed the effect of *t*-butyl alcohol on the synthesis of PGMEPA. It appeared that the yield of PGMEPA was much better in mixed solvent (hexane/*t*-butyl alcohol = 9:1) than in pure hexane. The lipase-catalyzed synthesis proceeds as follows: propylene glycol → propylene glycol monoester → propylene glycol diester. The effect of *t*-butyl alcohol could be in the inhibition of the second step reaction, leading to the accumulation of monoesters. The composition of the mixed organic solvent also affects the yields of both monoesters (PGMEPA and PGMDHA). As shown in Table 5, among the organic solvents used to mix with *t*-butyl alcohol, *n*-hexane was the best for PGMDHA production. In contrast,

TABLE 1
Propylene Glycol Monoesters Formation by Lipases from Different Sources

Source	Trade name or brand	Yields (%) PGMEPA[a]	Yields (%) PGMDHA[b]	Hydrolysis[c] activity (unit/g)
Aspergillus niger	Amano AP-6[d]	0.27	0.03	180.8
Candida cylindracea type VII	Sigma[e]	1.60	0.06	131.0
Chromobacterium viscosum LP-101-S	Toyo Jozo[f]	0.26	0.04	227.7
Mucor sp.	Amano MAP-10[d]	0.00	0.06	50.7
Mucor miehei	Novo IM-20[g]	89.47	92.82	0.1
Pseudomonas fluorescens	Amano PS[d]	0.00	1.15	320.0
Rhizopus sp.	Amano FAP-15[d]	0.00	0.00	28.1
Rhizopus sp.	Amano N-conc.[d]	0.00	0.00	16.0
Porcine pancrease lipase	Sigma[e]	0.00	2.89	22.9

[a]PGMEPA: propylene glycol of eicosapentaenoic acid.
[b]PGMDHA: propylene glycol of docosahexaenoic acid.
[c]Hydrolysis activity was measured by the hydrolysis of *p*-nitrophenyl butyrate as substrate. One unit of enzyme was defined as the amount of enzyme which produced 1 μmol of *p*-nitrophenol per min.
[d]Amano International Enzyme Co. (Nagoya, Japan).
[e]Sigma Chemical Co. (St. Louis, MO).
[f]Toyo Jozo Co. (Shizuoka, Japan).
[g]Novo Nordisk Inc. (Danbury, CT).

TABLE 2
Kinetics of Lipozyme IM-20-Catalyzed Esterification of Propylene Glycol with EPA and DHA at Various Temperature[a]

Reaction temperature (°C)	Formation of propylene glycol monoester (%)					
	PGMEPA			PGMDHA		
	3 h	12 h	24 h	3 h	12 h	24 h
10	30.1	43.6	70.3	5.5	31.3	46.6
20	46.2	56.6	68.7	9.3	39.8	48.7
30	47.8	58.8	68.3	11.8	30.2	49.2
40	48.9	62.8	89.5	22.4	65.7	93.2
50	49.4	80.1	96.1	25.3	75.2	94.4

[a]EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid. See Table 1 for other abbreviations and company source.

TABLE 3
Effect of Propylene Glycol/Fatty Acid Ratio on the Synthesis of Propylene Glycol Monoester by Lipozyme IM-20[a]

Ratio (propylene glycol/fatty acid)	Yields (%)	
	PGMEPA	PGMDHA
1	26	15
2	39	39
5	90	93
10	55	0

[a]See Table 1 for abbreviations and company source.

TABLE 4
Effect of *tert*-Butyl Alcohol on the Synthesis of PGMEPA by Lipozyme IM-20[a]

System	Time (h)	Yields (%)	
		Monoester	Diester
n-Hexane	1	50.5	0.5
	3	58.9	1.8
	6	64.4	5.1
	11	66.6	6.8
	24	69.4	9.2
	48	76.1	13.0
n-Hexane/*tert*-butyl alcohol (9:1, vol/vol)	1	37.2	0.1
	3	37.2	0.4
	6	81.1	0.8
	11	87.5	1.8
	24	89.5	3.3
	48	94.0	3.6

[a]See Table 1 for abbreviation and company source.

n-hexane, *n*-heptane, cyclohexane, isooctane, and toluene were equally well-suited for PGMEPA production.

The effect of water content on the synthesis of PGMEPA and PGMDHA is shown in Figure 1. Apparently, lipase IM-20 performs best under anhydrous condition for the esterification of propylene glycol with either EPA or DHA. This is consistent with our previous observation that *Pseudomonas* lipase performed best under lyophilized conditions for the esterification of propylene glycol with stearic acid (7). The inclusion of water-adsorbant molecular sieve greatly improved the yields of both PGMEPA and PGMDHA. It is possible that diol monoesters are easily hydrolyzed by lipase, even in the presence of small amounts of water. Okumura *et al.* (9) found that the yield of propylene glycol oleate was poor by lipase-catalyzed reaction in phosphate buffer (high water content), and Berger *et al.* (10) also reported that high yields of diol esters can be obtained from lipase-catalyzed esterification of

TABLE 5
Effect of the Mixture of Organic Solvents on the Lipozyme IM-20-Catalyzed Esterification of Propylene Glycol with EPA and DHA at 40°C[a]

System	Yields (%)	
	PGMEPA	PGMDHA
Pentane/*tert*-butyl alcohol (9:1, vol/vol)	74.9	56.4
n-Hexane/*tert*-butyl alcohol (9:1, vol/vol)	89.5	93.2
n-Heptane/*tert*-butyl alcohol (9:1, vol/vol)	89.6	57.6
Cyclohexane/*tert*-butyl alcohol (9:1, vol/vol)	85.0	51.7
Isooctane/*tert*-butyl alcohol (9:1, vol/vol)	89.5	58.9
Chloroform/*tert*-butyl alcohol (9:1, vol/vol)	50.3	0.0
Toluene/*tert*-butyl alcohol (9:1, vol/vol)	90.6	37.6

[a]See Tables 1 and 2 for abbreviations. See Table 1 for company source.

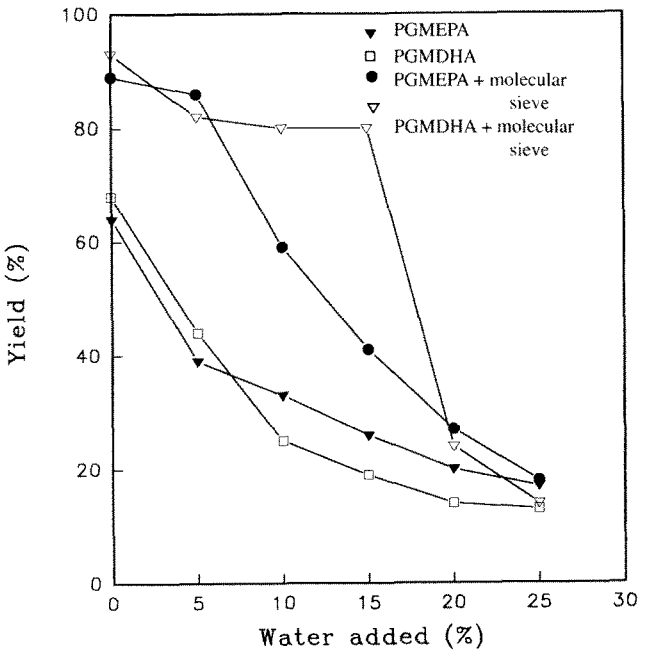

FIG. 1. Effect of water on the synthesis of propylene glycol of eicosapentaenoic acid (PGMEPA) and propylene glycol of docosahexaenoic acid (PGMDHA) by Lipozyme IM-20 (Novo Nordisk, Danbury, CT). The lipase (0.05 g) was added to a reaction mixture (1 mL) containing 50 mM fatty acid and 225 mM propylene glycol in a mixture of *n*-hexane and *tert*-butyl alcohol (9:1, vol/vol) with various amounts of added water at 40°C for 24 h.

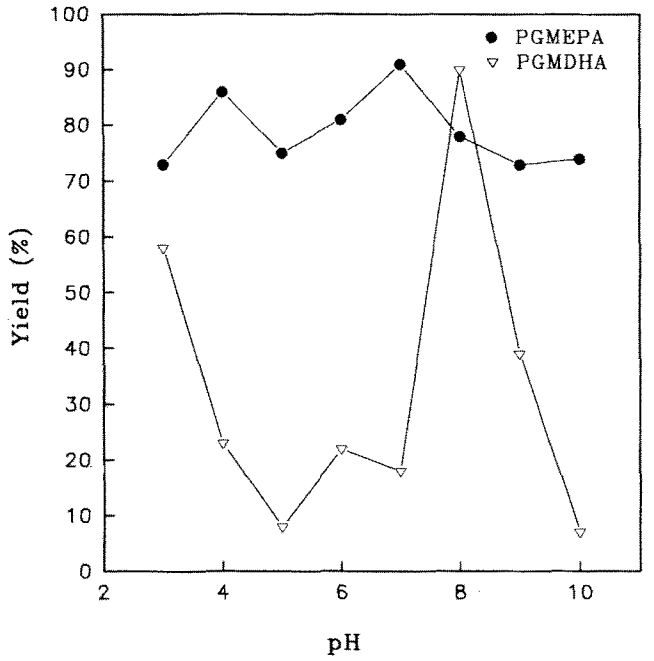

FIG. 2. Dependence of PGMEPA and PGMDHA formation on the pH of the aqueous solution from which the Lipozyme IM-20 was lyophilized. Experimental conditions: 0.05 g of the lipase was added to a reaction mixture (1 mL) containing 50 mM fatty acid and 225 mM propylene glycol in a mixture of *n*-hexane and *tert*-butyl alcohol (9:1, vol/vol); the suspension was shaken at 40°C and 250 rpm for 24 h. See Figure 1 for abbreviations and company source.

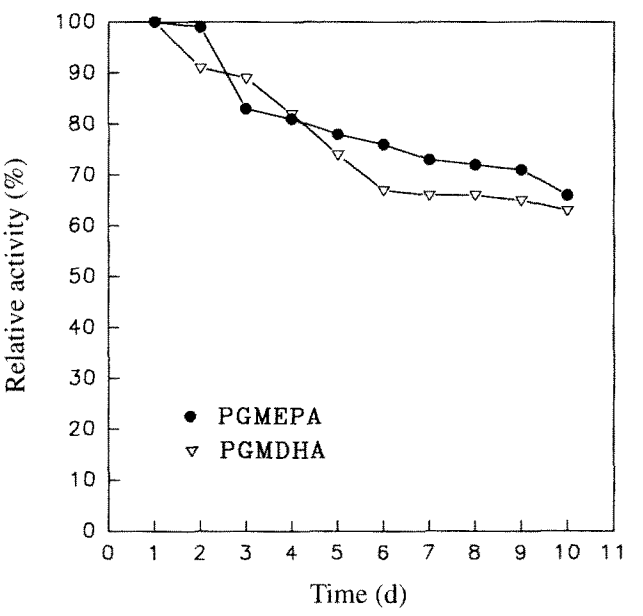

FIG. 3. Operational stability of Lipozyme IM-20-immobilized lipase. The reaction mixture was refreshed every day, and the relative activity was assayed immediately after each change. The reaction conditions are the same as described in Figure 2. See Figure 1 for abbreviations and company source.

silica gel-adsorbed diols with vinyl esters in anhydrous nonpolar organic solvent.

The yields of both monoesters also were affected by the pH of the aqueous solution from which the lipase was lyophilized, a phenomenon named "pH memory" by Klibanov (11). As shown in Figure 2, the optimum pH was 8 for the lyophilized lipase-catalyzed synthesis of PGMDHA. In contrast, the yield of PGMEPA was less sensitive to pH changes. It is possible that small changes in enzyme conformation, resulting from pH changes, affect enzyme binding more for the longer-chain substrate DHA ($C_{22:6}$) than for the shorter-chain substrate EPA ($C_{20:5}$).

The operational stabilities of Lipozyme IM-20 in catalyzing the esterification of propylene glycol with EPA and DHA are shown in Figure 3. In both cases, the enzyme still retained over 60% of its original activity after 10 d of batch-type operation (1 d per cycle) at 40°C for the synthesis of both PGMDHA and PGMEPA.

REFERENCES

1. Stutz, R.L., A.J. Del Vecchio and R.J. Tenney, *Food Prod. Develop. 7*:52 (1973).
2. Artman, N.R., *J. Am. Oil. Chem. Soc. 52*:49 (1975).
3. Nash, N.H., and L.M. Brinkman, *Ibid. 49*:457 (1972).
4. Hansen, J.B., J.O. Olsen, L. Wilsgård, V. Lyngmo and B. Sevensson, *Eur. J. Clin. Nutr. 47*:497 (1993).
5. Lauridsen, J.B., *J. Am. Oil Chem. Soc. 53*:400 (1976).
6. Markley, K.S., in *Fatty Acids, Their Chemistry, Properties, Production, and Uses*, edited by K.S. Markley, 2nd edn., Part 2, Interscience Publishers, Inc., New York, 1961, pp. 757–984.
7. Shaw, J.F., and S. Lo, *J. Am. Oil Chem. Soc. 71*:715 (1994).
8. R'ua, M.L., T.D. Maurino, V.M. Fern'andez, C. Otero and A. Ballesteros, *Biochim. Biophys. Acta 1156*:181 (1993).
9. Okumura, S., M. Iwai and Y. Tsujisaka, *Ibid. 575*:156 (1979).
10. Berger, M., K. Laumen and M.P. Schneider, *Biotechnol. Lett. 14*:553 (1992).
11. Klibanov, A.M., *Trends Biochem. Sci. 14*:141 (1989).

[Received July 23, 1995; accepted July 29, 1995]