Lipase G-Catalyzed Synthesis of Monoglycerides in Organic Solvent and Analysis by HPLC

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Monoglycerides were synthesized with Lipase G from *Penicillium* sp. as biocatalyst. This enzyme successfully catalyzed the esterification of glycerol with oleic acid (18:1 n-9) or eicosapentaenoic acid, EPA (20:5 n-3) in hexane. Esterification at 40°C for 24 hr resulted in 86.3 mol% and 64.3 mol% incorporation of 18:1 n-9 and 20:5 n-3, respectively. Lipase GC from *Geotrichum candidum* was not effective in esterifying the fatty acids under the present experimental conditions. Lipase G was able to incorporate 98.5 mol% and 98.1 mol% of 18:1 n-9 onto glycerol in 24 hr or less at 25°C and 37°C, respectively, in the absence of molecular sieve 4A. The product formed was analyzed by high performance liquid chromatography (HPLC) with a combined evaporative light scattering mass detector (ELSD) and ultraviolet detection at 206 nm. The result of this study demonstrates that Lipase G is capable of incorporating long-chain fatty acids of potential health benefit onto glycerol.

KEY WORDS: Esterification, HPLC analysis, monoglyceride containing eicosapentaenoic acid, monoolein.

Monoglycerides are widely used in food and pharmaceutical industries to promote the formation and stability of emulsions. Conventional synthesis by chemical catalysts requires temperatures in excess of 200°C, leading to dark-colored products and unwanted side reactions (1). Modification of fats and oils with lipases as biocatalysts in the presence or absence of organic solvents has attracted recent attention of lipid biotechnologists (2,3). Lipases are known to catalyze mild esterification reactions with the formation of specific compounds that are easy to isolate without molecular distillation. Esterification of glycerol to produce primarily monoglycerides with immobilized Lipase G has been reported (4). Other ways of producing monoglycerides involve hydrolysis of a triglyceride with 1, 3-specific lipase, forming monoglyceride with the fatty acid at position 2 (5); and glycerolysis of a triglyceride in the presence of solvents (6) or absence of solvents (7,8), resulting in a mixture of mono-, di- and triglycerides.

The potential for using lipases for the synthesis of partial glycerides of food, health and pharmaceutical interest appears to be great. The nature of the fatty acid and the position of attachment (*i.e.*, 1-, 3- or 2-position) to the glycerol are important for medical applications. Eicosapentaenoic acid (EPA, 20:5 n-3) found in marine fish and fish oils (9) and oleic acid (18:1 n-9) (10) found in vegetable oils have been suggested to have preventive effects on cardiovascular disorders. It has been proposed that large quantities of n-3 polyunsaturated fatty acids (PUFAs), mainly EPA, in their diets protect Eskimos against thrombotic cardiovascular disorders (9). Indeed, their intake of monoenes and n-3 PUFAs is high and that of linoleic and arachidonic acid is low. Recently, Akoh and Hearnsberger (11) demonstrated that diets high in 18:1 n-9 and n-3 PUFAs have comparable effects in altering platelet lipid composition and blood-clotting factors, and may reduce the incidence of thrombosis.

We report here the synthesis of monoglycerides containing oleic acid and n-3 PUFA such as EPA by Lipase G in organic solvent. A high-performance liquid chromatography (HPLC) method for separating the reactants and the products is also reported.

MATERIALS AND METHODS

Materials. Lipase G, an unspecific lipase from *Penicillium* sp. (51,000 U/g), and Lipase GC from *Geotrichum candidum* (22,000 U/g), were kindly provided by Amano International Enzyme Co. (Troy, VA). Molecular sieve 4A and glycerol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Oleic acid, 99% pure, 1-monoolein, 1, 3- and 1, 2-diolein, and triolein were purchased from Sigma Chemical Co. (St. Louis, MO). EPA-45, 45% pure, was supplied by Callanish Ltd. (Scotland, U.K.). Silica gel 60 was purchased from E. Merck (Darmstadt, Germany). All solvents were of HPLC grade and obtained from Fisher Scientific (Norcross, GA).

Esterification method. In a typical synthesis, 500 mg of glycerol was combined with 500 mg oleic acid, eicosapentaenoic acid (EPA) or methyl oleate and 10% (w/w) of powdered Lipase G or Lipase GC. To the reaction mixture was added 5% (w/w) water and 2 mL hexane, and the mixture was then incubated in a shaking water bath at 40°C for 24 hr at 180 rpm. The mole ratio of glycerol:fatty acid was 1:0.33. Molecular sieve 4A was added after 1 hr incubation to remove water formed during the reaction.

Time course. Time course studies were carried out at 25° and 37°C with oleic acid and Lipase G. Oleic acid (300 mg), glycerol (1.35 g) and Lipase G (165 mg) [*i.e.*, 10%, w/w, of reactants] were combined in a test tube. The mole ratio of glycerol:oleic acid was 12.3:1. To the reaction mixture was added 100 μL of water (6% w/w) and 2 mL hexane. The mixture was incubated at 25°C for 96 hr with magnetic stirring at approximately 400 rpm, or at 37°C for 96 hr in an air oven with magnetic stirring. In both cases, molecular sieve 4A was added after 24 hr of incubation. Aliquots of the reaction mixture (400 μL) were taken at time zero and every 24 hr for analysis.

Extraction and analysis. The reaction products were cooled and extracted twice with petroleum ether, and the lipase was separated by centrifugation at 2000 rpm for 5 min. The extract was divided into two portions for thin-layer chromatography (TLC)/gas chromatography (GC) and HPLC analyses. Progress of the reaction was monitored by thin-layer chromatography on precoated silica gel 60 activated by heating at 110°C for 1 hr. For TLC, the samples were redissolved in 50 μL chloroform:methanol (2:1 v/v) and spotted on the plate along with authentic

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standards of monoolein, diolein, oleic acid, EPA and methyl oleate. The plates were developed with hexane: diethyl ether:formic acid (80:20:2 v/v/v), and bands were visualized by examination under ultraviolet light after spraying with 2, 7-dichlorofluorescein (0.2% in ethanol). The bands corresponding to monoglycerides were scraped into a test tube with a Teflon-lined cap to which 10 μg of heptadecanoic acid (17:0) internal standard had been added. Three milliliters of 6% HCl in methanol (v/v) was added and the mixture was incubated under N_2 at 70–80°C for 2 hr. Fatty acid methyl esters were extracted with hexane, dried over anhydrous sodium sulfate and analyzed by gas-liquid chromatography (GLC). An HP 5890 Series II gas chromatograph (Hewlett-Packard, Avondale, PA) was used. A DB 225 fused silica capillary column 30 m × 0.25 mm i.d. (J&W Scientific, Folsom, CA) was used and operated isothermally at 200°C. Injector and detector temperatures were set at 250°C and He was the carrier gas. The relative content of fatty acids as mol% was quantitated by an on-line computer with 17:0 as internal standard. Percent incorporation was calculated from the initial amount of 18:1 n-9 or 20:5 n-3 present. HPLC was used to follow the reaction and to detect the unreacted fatty acids as well as to monitor monoglycerides formed. About 200 μL of each sample was evaporated to dryness and redissolved in 1 mL acetone, and 20 μL was injected into a Beckman System Gold Module HPLC System (Beckman Instruments, Fullerton, CA), fitted with a C-18 reverse-phase pre-column and a 25 cm × 4.6 mm analytical column. Both columns were packed with 5μ Ultrasphere - ODS (Altex, San Ramon, CA). Component monoglycerides and reactants were quantitated on-line at 206 nm by means of a Beckman 167 UV-visible Scanning Detector (Beckman) connected in series with a Varex Evaporative Light Scattering Mass Detector, ELSD IIA (Varex Corporation, Burtonsville, MD). The mobile phase was acetone:acetonitrile (50:50 v/v) run isocratically. The ELSD mass detector heater temperature was 120°C, N_2 nebulizer gas flow was 60 mm and 21 psi. Flow rate was 1.5 mL/min. Authentic free fatty acids, mono-, di- and triolein standards were prepared and analyzed by HPLC as described above for identification of the products and to optimize separation conditions. In this process, resolution of monoolein from oleic acid was of primary consideration. Products purified by TLC were also chromatographed and detected by both ultraviolet (UV) and mass detector.

RESULTS AND DISCUSSION

Monoglyceride synthesis with Lipase G from *Penicillium* sp. and Lipase GC from *Geotrichum candidum* as biocatalysts in hexane was investigated. Esterification of glycerol with oleic acid (18:1 n-9, 99% pure) and eicosapentaenoic acid (20:5 n-3, 45% pure) were investigated at 40°C. Table 1 shows the fatty acid composition of the starting acids and that of the corresponding monoglyceride product isolated by thin-layer chromatography (TLC). The mol% of 18:1 n-9 in the starting oleic acid was 99.13% as determined by gas liquid chromatography (GLC). As shown in Table 1, the mol% of 18:1 n-9 in the monoglyceride was 85.54. The EPA-45 starting material contained 47.6 mol% 20:5 n-3, while the monoglyceride product contained 30.64 mol% 20:5 n-3. The results indicate a high level of esterification of long-chain fatty acids. Overall, most of the fatty acids present in the starting material were incorporated into the glycerol by Lipase G except for minor differences. Water was added at 10% w/w of the reactants. To drive the reaction toward ester formation, molecular sieve 4A was added after 1 hr of incubation, and the reaction was continued for 24 hr. Ergan *et al.* (12) reported that molecular sieve is effective in removing the water formed during esterification. Stoichiometric amounts of glycerol:oleic acid or glycerol:EPA-45 resulted in 86.3 mol% and 64.3 mol% incorporation of 18:1 n-9 and 20:5 n-3, respectively, onto glycerol. The proportion of monoglyceride formed was approximately 72% for oleic acid and 65% for EPA. Our result is consistent with esterification of glycerol with oleic acid catalyzed by immobilized Lipase G reported by Schuch and Mukherjee (4). However, in the present report, powdered Lipasae G was used and monoglyceride was the predominant product.

TABLE 1

Fatty Acid Composition of Monoglycerides Synthesized with Lipase G at 40°C

	Mol%		Mol%	
Fatty acids	Oleic acid	MG[a] w/oleic acid	EPA-45	MG w/EPA-45
14:0			1.09	3.87
14:1			0.19	0.71
15:0			n.d.	2.23
16:0	0.25	3.83	0.58	16.16
16:1 n-7	0.13	0.55	17.71	14.06
18:0	0.03	5.69	9.54	10.01
18:1 n-9	99.13	85.54	2.03	9.47
18:1 n-7			0.71	1.25
18:2 n-6	0.22	0.49	2.4	3.99
18:3 n-6			0.29	n.d.
18:3 n-3	0.01	n.d.[b]	1.33	0.87
18:4 n-3			4.39	3.10
20:1 n-9	0.03	n.d.		
20:4 n-6			0.90	n.d.
20:5 n-3			47.65	30.64
22:0	0.20	n.d.		
22:2 n-6			1.25	n.d.
22:5 n-3			3.33	1.69
22:6 n-6			6.60	1.00

[a]MG = monoglyceride. [b]n.d. = not detected.

Table 2 compares the use of Lipase G from *Penicillium* sp. with Lipase GC from *Geotrichum candidum* in the synthesis of EPA-containing and oleic acid-containing monoglycerides. Lipase G was able to catalyze esterification of glycerol with oleic acid, resulting in 86.3 mol% incorporation of 18:1 n-9. About 64.3 mol% incorporation of 20:5 n-3 was observed with Lipase G as the biocatalyst. In either case, Lipase GC was not active at the reaction conditions employed. It may well be that this enzyme is not active in hexane, or in systems with the degrees of water present here.

Figure 1 represents the HPLC separation with ELSD mass detection of authentic standards of possible products and reactants in the lipase-catalyzed synthesis of monoglycerides. At the flow rate of 1.5 mL/min, monoolein and oleic acid were clearly separated with retention times of 2.8 and 3.1 min, respectively. The retention times for

TABLE 2

Monoglyceride Synthesis with Lipase G and Lipase GC at 10% Initial Water Concentration[a]

Acid	Enzyme	mol% starting acid	mol% incorporation
Oleic acid	Lipase G	99.1	86.3 as (18:1 n-9)
	Lipase GC	99.1	0.0
EPA-45	Lipase G	47.7	64.3 as (20:5 n-3)
	Lipase GC	47.7	0.0

[a]Incubated in a shaking water bath at 40°C in hexane at 180 rpm for 24 hr. Molecular sieve 4A added after 1 hr.

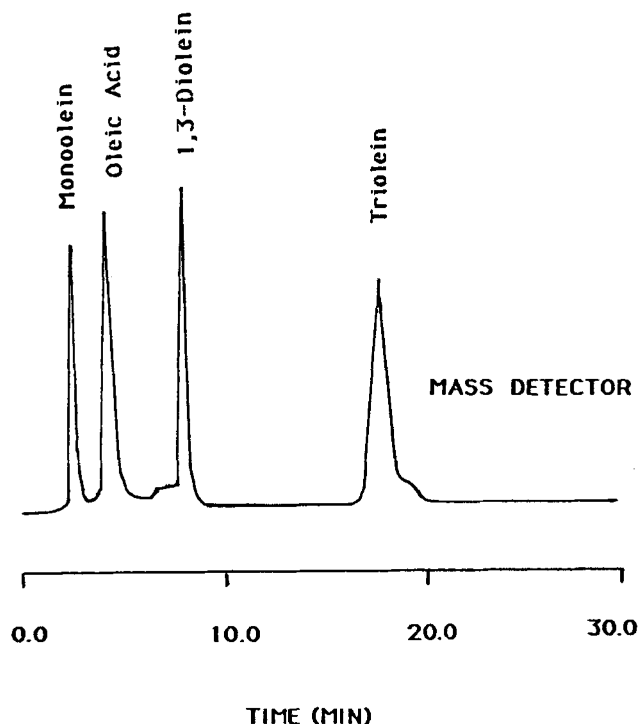

FIG. 1. HPLC chromatogram of authentic standards detected with a Varex ELSD IIA mass detector. Column: ODS C-18 reversed phase 25 cm × 4.6 mm. Mobile phase: acetone: acetonitrile, 50:50 v/v. Flow rate: 1.5 mL/min. Injected 20 µL of standards mixture. Heater temperature: 120°C, nebulizer (N_2) gas flow: 60 mm and 21 psi. Mode: isocratic.

1, 3-diolein and triolein were 8.3 and 17.8 min, respectively. The 1, 2-diolein isomer (not shown) had a retention time of 8.7 min in the C-18 reversed phase column. The mass detector gave a flat baseline, and no impurities were detected compared to UV detection at 206 nm (data not shown). A mass detector is particularly useful in detecting simple and complex lipids and compounds without a chromophore, provided the material under investigation is nonvolatile (13). The use of a mass detector in the analysis of the lipase-catalyzed synthesis of monoglycerides has not been reported. Ergan and Andre (14) reported the use of a UV detector at 206 nm to monitor lipase reactions at a flow gradient that was increased to 4 mL/min. This high flow rate obviously may be detrimental to the pump after prolonged use. Our results were obtained from an isocratic run with the same mobile phase of acetone: acetonitrile (50:50 v/v) but at a slower flow rate of 1.5 mL/min. Our run was at room temperature while Ergan and Andre (14) analyzed at a column temperature of 35°C.

Figure 2 shows a comparison of the use of a mass detector and a UV detector at 206 nm in the analysis of the HPLC separation of purified monoolein product from lipase G-catalyzed esterification of glycerol with oleic acid. The mass detector showed a much cleaner product, while the UV detector showed the presence of the solvent peak and some minor contaminants. The purity of monoolein by mass detector was 100%, while that by UV detector was 98%. In the purified product, monoolein is the predominant compound, indicating that Lipase G was able to incorporate 18:1 n-9 onto the glycerol, provided the reaction was run for only 24 hr. We found (data not shown) in time course studies that some diolein was formed when the reaction was continued for more than 24 hr. Schuch and Mukherjee (4) reported the formation of some diolein and triolein by immobilized lipase G when the reaction was carried on for 3.3 hr. Osada *et al.* (15) reported better than 89% incorporation of 18:1, 20:5 and 22:6 with lipase from *Chromabacterium viscosum* with stirring at 25°C for 24 hr. In that study (15) the interest was di- and triglyceride synthesis and not monoglycerides.

Table 3 shows the time course of monoolein synthesis by Lipase G at 25 and 37°C, respectively. At 25°C, 98.5 mol% incorporation of 18:1 n-9 was achieved in 24 hr

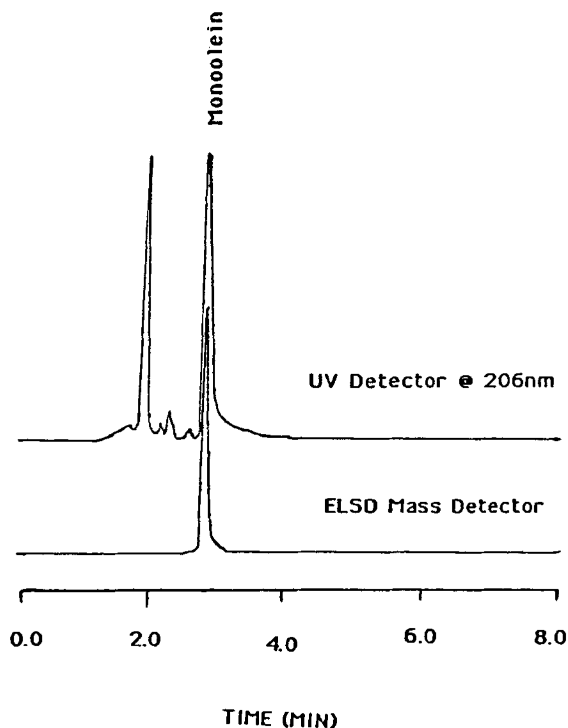

FIG. 2. Comparison of mass detector and UV detector in the analysis of the HPLC separation of TLC-purified monoolein synthesized with Lipase G. See Figure 1 for conditions. UV detector was at 206 nm.

TABLE 3

Time Course of Monoglyceride Synthesis from Oleic Acid and Glycerol at 25°C and 37°C[a]

Time (hr)	Mol% incorporation	
	25°C	37°C
0	0.0	0.0
24	98.5	98.1
48	89.5	97.5
72	94.3	98.4
96	94.5	98.4

[a]Molecular sieve 4A added after 24-hr incubation. Water was initially added to the mixture at 6% w/w of the reactants. Magnetic stirring was at 400 rpm. All reactions were in 2 mL hexane.

or less. Note that molecular sieve 4A was added only after 24 hr, indicating that, with 6% w/w water, esterification was not inhibited. The introduction of the molecular sieve produced a net reduction in the amount of product midway through the reaction at both 25° and 37°C. At 37°C, 98.4 mol% incorporation of 18:1 n-9 was achieved only after 72 hr, although the result at 24 hr (98.1 mol%) could be considered an acceptable level of incorporation. After the net reduction at both temperatures, the level of incorporation increased again and plateaued at 72 hr. In Table 2, the mol% incorporation of oleic acid was lower compared to the value in Table 3, possibly due to the hydrolysis of the product formed by the water or due to the early introduction of the molecular sieve. Therefore, to achieve acceptable incorporation, the esterification reaction at 25°C must be stopped after 24-hr incubation without molecular sieve addition. McNeill *et al.* (7,8) reported that in the glycerolysis of fats to produce monoglycerides, the yield is greatly dependent on the reaction temperature. According to McNeill *et al.* (7,8), only 30% monoglyceride was obtained at 48–50°C, whereas 70% monoglyceride was obtained at lower temperatures of 38° to 46°C. Even though the optimum temperature of Lipase G is about 40°C, we found that it is possible to carry out esterification at lower temperatures.

We demonstrated that powdered Lipase G is capable of esterifying glycerol with long-chain fatty acids to produce oleic acid-enriched and EPA-enriched monoglycerides, both of which are of potential benefit to cardiovascular disease patients. More work is needed to ascertain the effect of immobilization, water, temperature, enzyme load, substrate ratios and specificities to better define the mechanism of lipase-catalyzed esterification in mono- and diglyceride synthesis.

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