Lipase-Catalyzed Esterification of Glycerol and n-3 Polyunsaturated Fatty Acid Concentrate in Organic Solvent

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Enzymatic synthesis of glycerides from glycerol and n-3 polyunsaturated fatty acid in organic solvent was studied. Optimal conditions for glyceride synthesis by lipases were established. Of the commercially available lipases that were investigated, lipase PS-30 from *Pseudomonas* sp. and lipase IM-60 from *Mucor miehei* resulted in the highest extent of esterification. Isooctane and hexane were particularly useful organic solvents in glyceride synthesis. The water content in the reaction mixture was of primary importance. For lipase PS-30 and lipase IM-60, optimal water contents were 5 and 1%, respectively. Lipases PS-30 and IM-60 manifested contrasting positional specificities in glyceride synthesis. Glycerides containing predominantly eicosapentaenoic acid and docosahexaenoic acid can be easily synthesized.

KEY WORDS: Esterification, lipase, organic solvent, polyunsaturated fatty acid.

Modification of fats and oils with lipases as biocatalysts in the presence or absence of organic solvents is attracting much interest from lipid researchers (1,2). Lipases are known to catalyze mild esterification reactions with the formation of specific compounds that are easy to isolate without molecular distillation. There appears to be great potential for using lipases for the synthesis of mono- and diglycerides of food, health and pharmaceutical interests. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) have been shown to have positive preventive effects on people with different cardiovascular disorders (3,4). Recently, Akoh and Hearnsberger (5) demonstrated that diets high in n-3 polyunsaturated fatty acids (PUFA), mainly EPA and DHA, have comparable effects in altering platelet lipid composition and blood-clotting factors, and may reduce the incidence of thrombosis.

Lawson and Hughes (6) have pointed out that EPA and DHA are completely absorbed only as glycerides, whereas free fatty acid forms are less acceptable in foods. Therefore, glycerides are considered to be the most desirable form.

Use of appropriate organic solvents is beneficial for construction of homogeneous reaction systems containing lipophilic and water-insoluble substrates and to facilitate continuous reactor processes. For the synthesis of useful compounds by means of hydrolytic enzymes, such as lipases and esterases, it is essential to reduce the water content in the reaction mixture by replacing water with the appropriate organic solvents.

Recently, Osada and co-workers (7) have employed the lipase from *Chromobacterium viscosum* for the direct esterification of glycerol with individual free fatty acids, including EPA and DHA (19.3% of EPA and 94.9% of DHA were incorporated into the glycerol moiety). More recently, Akoh and co-workers (8) described synthesis of monoglycerides in organic solvent by lipase G. This enzyme successfully catalyzed the esterification of glycerol with oleic acid or EPA in hexane. Esterification at 40°C for 24 h resulted in 86.3 and 64.3 mol% incorporation of oleic acid and EPA, respectively (8).

In this paper we describe the synthesis of glycerides from glycerol and n-3 fatty acid concentrate in organic solvent by lipases. Our goal was to optimize the reaction in favor of synthesis of glycerides by studying the effects of various kinds of lipase, organic solvents, water content, glycerol content and temperature.

MATERIALS AND METHODS

Chemicals and materials. Cod liver oil was obtained from R.P. Scherer (Windsor, Ontario, Canada). Eight types of lipases were obtained from different sources, as shown in Table 1. Lipases 1–4 are random, whereas the others have 1,3-positional specificity. All solvents used in this work were of analytical grade and were obtained from Aldrich Chemical Co. (Milwaukee, WI). Molecular sieve material was obtained from BDH Inc. (Toronto, Ontario, Canada).

Preparation of highly polyunsaturated n-3 fatty acids concentrate from cod liver oil (9). This process employed saponification, extraction of fatty acids, formation of urea inclusion compounds and extraction of n-3 fatty acids. This product contained 81.4% of EPA and DHA.

TABLE 1

Esterification Activity of Various Kinds of Lipase

Enzyme origin	Commercial code	Source	Specificity	Degree of synthesis (%)
1. *Candida cylindracea*	CCL	Sigma Chemical Co. (St. Louis, MO)	Random	0.0
2. *Geotrichum candidum*	GC	Amano International Enzyme Co. (Nagoya, Japan)	Random	5.6
3. *Pseudomonas* sp.	CES	Amano International Enzyme Co.	Random	42.0
4. *Pseudomonas* sp.	PS-30	Amano International Enzyme Co.	Random	85.5
5. *Penicillium* sp.	G	Amano International Enzyme Co.	1,3-Specific	43.4
6. Porcine pancreas	PPL	Sigma Chemical Co.	1,3-Specific	41.5
7. *Mucor* sp.	MAP-10	Amano International Enzyme Co.	1,3-Specific	68.9
8. *Mucor miehei*	IM-60	Novo Nordisk Inc. (Danbury, CT)	1,3-Specific	87.6

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Esterification reaction. Reaction mixtures for glyceride synthesis from glycerol and n-3 fatty acid concentrate consisted of: glycerol, 2 g (about 20 mmol); n-3 fatty acid concentrate, 0.4 g (about 1.2 mmol); water, 150 μL; and *n*-hexane, 3 mL. Reaction mixtures were placed in 50-mL Erlenmeyer flasks with silicone-capped stoppers to prevent evaporation of the reactants. The reaction was started by the addition of 5,000 u lipase in the form of dry powder. The suspension that resulted was agitated on an orbital shaker at 200 rpm at 30°C. At various times during incubation, 0.2 mL of the reaction mixture was withdrawn and analyzed by thin-layer chromatography (TLC) and gas chromatography (GC).

Estimation of degree of synthesis. The reaction was stopped by addition of 20 mL of acetone/ethanol mixture (1:1, vol/vol), and free fatty acid was titrated with 0.1N NaOH. The degree of synthesis (%) represents the percent of initial fatty acids consumed in the reaction mixture.

Identification of reaction products. Reaction products were extracted from each reaction mixture with diethyl ether and identified by TLC. A boric acid-impregnated silica gel plate was prepared and developed in chloroform/acetone/methanol (95:4.5:0.5, vol/vol/vol). Spots of each lipid were visualized by spraying 12.5% (wt/vol) ethanol solution of phosphomolybdic acid (Sigma Chemical Co., St. Louis, MO) and heating (10). Fractions corresponding to each lipid type were scraped from the plates and derivatized for GC analysis.

GC. Each lipid class separated by TLC was methylated according to the method of Holub and Skeaff (11). The methyl esters of fatty acids were dissolved in *n*-hexane for analysis. Analysis was performed in a Shimadzu (Tokyo, Japan) Gas Chromatograph GC-14A with CR 601 data integrator. The gas–liquid chromatograph was fitted with a megabore column DB-225 and an ionization detector. Helium was chosen as the carrier gas at a flow rate of 38 mL/min. The injection port and flame-ionization detector temperatures were both 250°C, and the column temperature was 210°C. The peaks of fatty acid esters were identified and calibrated with standard fatty acid. Pentadecanoic acid (15:0) was used as an internal standard.

RESULTS AND DISCUSSION

Esterification activity of various kinds of lipases. Lipases from different sources exhibit characteristic specific activities. The esterification of glycerol and n-3 fatty acid concentrate in organic solvent by the eight lipases at the same enzyme unit level are compared in Table 1. Lipases MAP-10, IM-60 (1,3-positional specificity), CES and PS-30 (no positional specificity) showed degrees of synthesis to 68.9, 87.6, 42.0 and 85.5%, respectively, after a 24-h enzymatic reaction. The results demonstrated that there is no clear correlation between positional specificity and degree of synthesis. Both 2-monoglyceride and 1,2-diglyceride are chemically unstable and undergo acyl migration to give 1-monoglyceride (1-MG) and 1,3-diglyceride (1,3-DG), respectively. To prevent such nonenzymatic acyl migration, glyceride synthetic reactions were carried out on the thin-layer plate for a short time, and the plate was developed immediately. Figure 1 shows the reaction products of glyceride synthetic reaction by four lipases. Lipase MAP-10 and IM-60 produced only 1-MG and 1,3-DG while 1-MG, 2-MG, 1,2-DG and 1,3-DG were

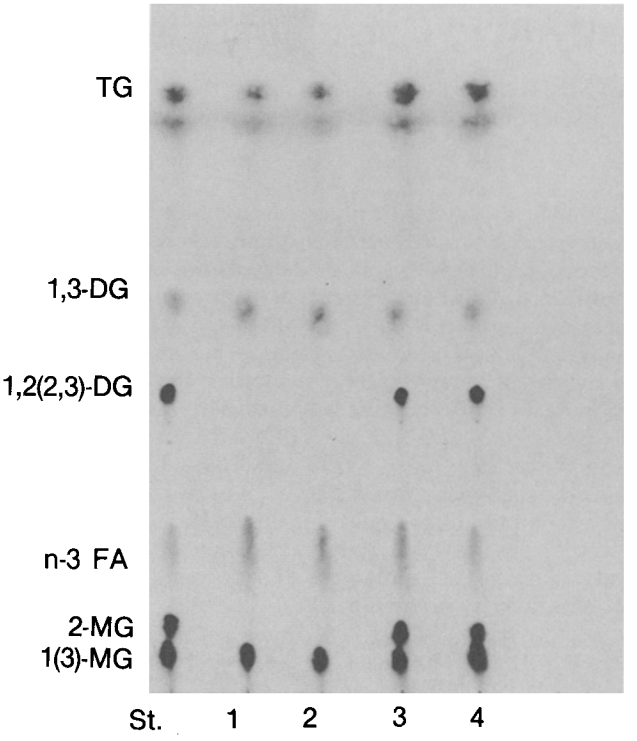

FIG. 1. Thin-layer chromatogram of the reaction products from glycerol and n-3 fatty acid. Lane 1, lipase MAP-10; Lane 2, 2-lipase IM-60; Lane 3, lipase CES; Lane 4, lipase PS-30. MG, monoglyceride; DG, diglyceride; TG, triglyceride; FA, fatty acid; St., standard.

synthesized by lipase CES and PS-30. From the above results, it was concluded that lipase IM-60 was different from lipase PS-30 with respect to the positional specificity in glyceride synthesis.

Effects of organic solvent on the glyceride synthetic reaction. To carry out bioconversions of lipophilic compounds effectively, it is essential to introduce organic solvents into the reaction systems. The use of organic solvents can improve the poor solubility in water of substrates or other reaction components of a hydrophobic nature. Organic solvents produce various physicochemical effects on enzyme molecules, and the effects differ depending upon the kinds of organic solvents and enzymes used. Conformational changes in enzymes, when suspended in organic solvents, have been reported to result in alteration of substrate specificity and affinity of substrates for enzymes (1). To select the most suitable solvent for glyceride synthetic reaction systems, the effect of organic solvents on the catalytic activity of lipase for the esterification has been examined (Table 2). For ester synthesis by lipase PS-30, a higher extent of esterification was observed in isooctane and hexane as compared to other solvents. With lipase IM-60, high activities (85.2–92.2% synthesis) were observed in heptane, hexane, pentane and isooctane. More polar solvents, such as benzene and acetone, were found to be unsuitable for the synthetic reaction.

Effects of initial water content on glyceride synthesis by lipase. Lipase-catalyzed reactions are reversible and governed by the water content of the reaction mixture. In the esterification reaction, the content of water in the reaction mixture affects the reaction because water is one

TABLE 2

Activity of the Lipase in Organic Solvent-Aqueous Phase Reactions

Organic solvent	Degree of synthesis (%)	
	Lipase PS-30	Lipase IM-60
Pentane	41.1	89.2
Hexane	85.5	87.6
Heptane	72.1	85.2
Isooctane	89.4	92.2
Decane	65.8	64.2
Benzene	22.1	11.2
Acetone	34.2	0
Chloroform	24.9	34.2

of the reaction products. A small amount of water is needed to maintain enzyme activity. However, at higher initial water contents, the degree of synthesis gradually declined. We thus investigated the effects of the initial water content on glyceride synthesis by lipases.

Time courses of the glyceride synthesis by lipase PS-30 and lipase IM-60 in hexane containing different initial moisture contents are illustrated in Figures 2 and 3. With a moisture content of 5% (vol/vol) in the reaction mixture, lipase PS-30 showed maximum glyceride synthesis of about 82%. As expected, the percent conversion decreased as the initial water content increased. The elimination of water from the reaction mixture derived from the esterification was attempted by the addition of molecular sieve (4A) pellets as dehydrating agents to obtain a higher conversion rate. As shown in Figure 2, the conversion reached 86% by the addition of 1.0 g of molecular sieve. Lipase IM-60 was an immobilized lipase of *Mucor miehei* containing about 5% water. With a moisture content of 1% (vol/vol) in the reaction mixture, a maximum synthesis degree of 92% was obtained.

Effects of glycerol content on glyceride synthesis by lipase. The effects of glycerol content in the reaction mixture on the esterification by two lipases were investigated (Table 3). Glyceride synthesis increased with increasing glycerol content. For lipase PS-30 and lipase IM-60, glycerol contents of 2–5 g were optimal. For lipase IM-60, the reaction results in degrees of glyceride synthesis of 89.1–91.4%.

Effects of temperature on esterification by lipase. The optimum temperature for glyceride synthetic reaction by lipase PS-30 was 30°C (Table 4). In contrast, the degree of esterification increased with temperature in the range 0–50°C with lipase IM-60. The higher temperature optimum for the latter enzyme was probably partly due to the fact that immobilization conferred greater thermostability on this enzyme.

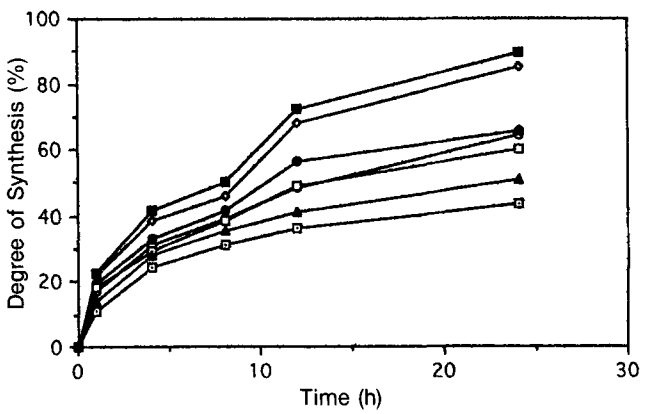

FIG. 2. Effects of water content on glyceride synthesis by lipase PS-30. The reaction mixture contained 2.0 g glycerol, 0.4 g of n-3 fatty acid concentrate, 3.0 mL *n*-hexane and various amounts of water. The amount of enzyme used was 5,000 u. The reaction was carried out at 30°C. Square with centered dot, 0% H_2O; open circle, 1% H_2O; closed circle, 2.5% H_2O; open diamond, 5.0% H_2O; closed square, 5.0% H_2 + molecular sieve; open square, 10% H_2O; and closed triangle, 20% H_2O.

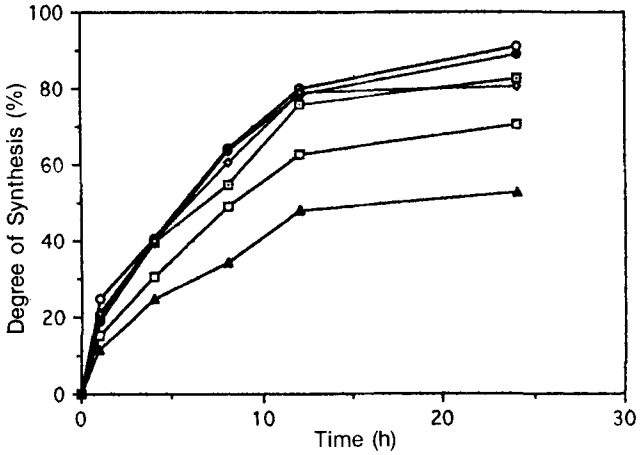

FIG. 3. Effects of water content on glyceride synthesis by lipase IM-60. The reaction mixture contained 2.0 g glycerol, 0.4 g of n-3 fatty acid concentrate, 3.0 mL *n*-hexane and various amounts of water. The amount of enzyme used was 5,000 u. The reaction was carried out at 30°C. Key as in Figure 2.

TABLE 3

Effects of Glycerol Content on Esterification by Lipases

Glycerol (g)	Degree of synthesis (%)	
	Lipase PS-30	Lipase IM-60
0.25	14.5	29.2
0.50	58.5	65.8
1.0	82.5	87.5
2.0	89.2	90.2
5.0	89.1	91.4

TABLE 4

Effects of Temperature on Esterification by Lipases

Temperature (°C)	Degree of synthesis (%)	
	Lipase PS-30	Lipase IM-60
0	17.1	10.4
10	24.3	20.2
20	56.5	61.3
30	85.5	87.6
40	74.3	90.7
50	25.6	92.4

TABLE 5

Fatty Acid Composition of Glyceride and Free Fatty Acid Components Separated After Esterifications by Lipase PS-30 and Lipase IM-60

Lipase	Lipid component (%)	Fatty acid component (%)[a]									
		14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4	20:5	22:6
PS-30	MG (23.8)	ND[b]	ND	2.34	ND	1.56	4.43	3.76	1.68	33.45	42.5
	DG (40.6)	ND	ND	2.06	ND	2.36	3.98	3.38	1.84	33.77	44.7
	TG (18.1)	ND	0.58	1.76	ND	1.61	3.62	3.48	1.65	36.47	40.4
	FFA (17.5)	2.38	1.58	2.06	ND	0.97	4.22	3.37	1.75	32.03	34.0
IM-60	MG (34.5)	2.29	1.13	3.05	ND	5.65	5.46	4.10	2.0	35.90	29.43
	DG (40.5)	2.34	1.17	3.10	ND	11.97	5.38	3.85	1.83	31.49	22.89
	TG (12.6)	1.68	3.02	4.02	ND	3.76	6.92	5.40	ND	40.44	20.45
	FFA (12.4)	2.14	ND	5.4	ND	14.80	5.18	3.64	ND	23.64	21.94

[a]Low levels of a number of other fatty acids, including 16:2, 16:3, 16:4 and 18:3 (n-6), were detected but not quantified. MG, monoglyceride; DG, diglyceride; FFA, free fatty acids; TG, triglyceride.
[b]ND, not detected.

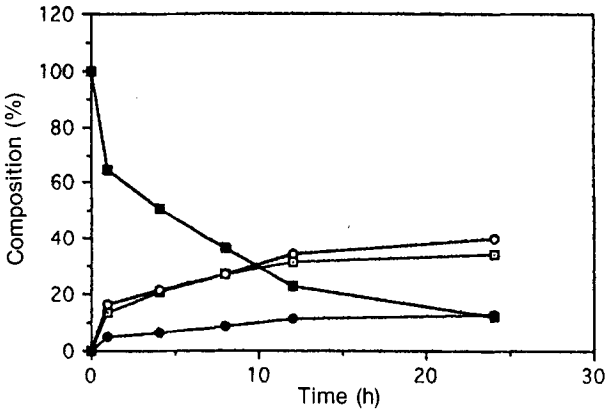

FIG. 4. Composition of glycerides synthesized by lipase PS-30. The reaction mixture contained 2.0 g glycerol, 0.4 g of n-3 fatty acid concentrate, 3.0 mL *n*-hexane and various amounts of water. The amount of enzyme used was 5,000 u. The reaction was carried out at 30°C. Square with centered dot, Monoglyceride; open circle, diglyceride; closed circle, triglyceride; closed box, n-3 free fatty acid.

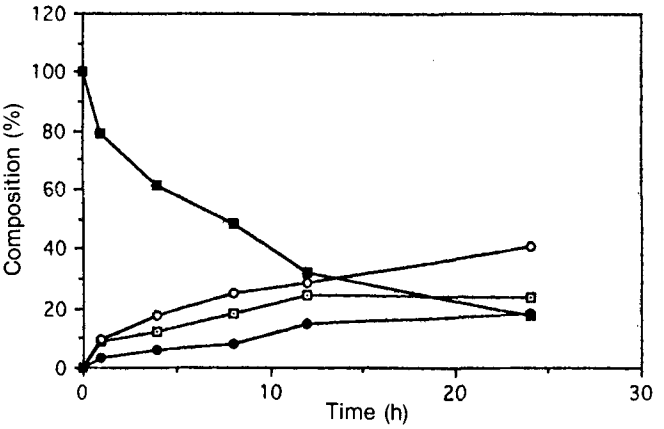

FIG. 5. Composition of glycerides synthesized by lipase IM-60. The reaction mixture contained 2.0 g glycerol, 0.4 g of n-3 fatty acid concentrate, 3.0 mL *n*-hexane and various amounts of water. The amount of enzyme used was 5,000 u. The reaction was carried out at 30°C.

Composition of glycerides synthesized by lipases. The detailed changes in composition of lipid in the *n*-hexane mixture during the course of esterification reaction by lipases are given in Figures 4 and 5. Initially, almost equimolar mono- and diglycerides were produced as n-3 fatty acid decreased. Formation of triglyceride was much less than that of mono- and diglyceride. Because lipases PS-30 and IM-60 are 1,3-specific, production of triglyceride is likely to have resulted from acyl migration to the 2-position in 1- or 3-MG or 1,3-DG followed by further enzymatic esterification. For lipase PS-30, similar amounts of mono- and diglyceride were observed during the first 12 h of the enzyme reaction. At 24 h, as shown in Table 5, the concentration of monoglyceride, diglyceride and triglyceride reached 23.8, 40.6 and 18.1%, respectively, containing n-3 fatty acid (mainly EPA and DHA) at 75.95, 78.47 and 76.87%, respectively. For lipase IM-60, at 24 h, the concentration of monoglyceride, diglyceride and triglyceride reached 34.5, 40.5 and 12.6%, respectively, containing n-3 fatty acid at 65.33, 60.92 and 60.89%, respectively.

From the above results it can be concluded that glycerides containing highly concentrated EPA and DHA may be easily synthesized. Monoglyceride and diglyceride are the major products formed, and lipase PS-30 was different from lipase IM-60 with respect to the positional specificity in glyceride synthesis.

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