Enzymatic Esterification of ω-3 Fatty Acid Concentrates from Seal Blubber Oil with Glycerol

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ABSTRACT: Enzymatic synthesis of acylglycerols directly from glycerol and an ω-3 fatty acid concentrate, prepared from seal blubber oil, in organic solvents was studied. Seven lipases were used as biocatalysts for esterification. Lipase LP-401-AS from *Chromobacterium viscosum* showed the highest activity for esterification. Effects of reaction parameters, namely, temperature, time course, type of solvent, water content, amount of glycerol, enzyme load and solvent volume, were followed with lipase LP-401-AS as the biocatalyst of choice. Optimal reaction conditions were established, and the maximal degree of acylglycerol synthesis reached was 94.3%. The concentrations of monoacylglycerols, diacylglycerols, and triacylglycerols were 13.8, 43.1, and 37.4%, respectively. Therefore, acylglycerols containing predominantly ω-3 fatty acid concentrates may be easily synthesized directly *via* their reaction with glycerol.
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KEY WORDS: Acylglycerol form, concentrate, enzymatic esterification, enzymes, omega-3 fatty acids, seal blubber oil.

Consumption of fish and fish oils, which are rich in ω-3 polyunsaturated fatty acids (PUFA), has been linked to improved cardiovascular and immune functions (1). Beneficial effects have been attributed to marine oils that contain all-*cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) and all-*cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) (2,3). Therefore, within the pharmaceutical industry, there has been increased demand for these fatty acids and almost exclusively in the acylglycerol form because EPA and DHA are completely absorbed only when present as acylglycerols (4). It is possible to prepare acylglycerols directly from fish oils with up to 30% EPA and DHA without splitting the fat (5,6). However, it is difficult to achieve an oil concentration of EPA and DHA above 30% owing to the presence of a large variety and combination of fatty acids in the acylglycerol molecules. Once released as individual fatty acids, fractionation is feasible to produce up to 65–80% EPA plus DHA by a number of methods (5,6). Therefore, resynthesis of acylglycerols from such highly enriched EPA and DHA fatty acid concentrates is attracting interest of lipid researchers (7,8). Li and Ward (11) have previously reported the reaction of cod liver oil concentrates with glycerol by means of enzymes. They found that lipase PS-30 from *Pseudomonas* sp. and lipase IM-60 from *Mucor miehei* resulted in the highest extent of esterification. After 24 h of enzymatic reaction at 30°C in hexane, lipases PS-30 and IM-60 gave 85.5 and 87.6% acylglycerols, respectively.

In this paper, synthesis of acylglycerols directly from glycerol and ω-3 fatty acid concentrates from seal blubber oil (SBO) is described. SBO concentrate has a different proportion of individual long-chain fatty acids from that of fish oil concentrates. Our goal was to find the optimal conditions for esterification by examining parameters such as type of enzyme, reaction temperature, time course, type of solvent, water content, amount of glycerol, enzyme load, and solvent volume.

MATERIALS AND METHODS

Chemicals and materials. Seal blubber was obtained directly from sources in Newfoundland. Seven lipases were acquired from different sources, as shown in Table 1. All solvents used in this work were of analytical grade and were supplied by Fisher Scientific Chemical Co. (Nepeon, Ontario, Canada). Molecular sieve was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Preparation of ω-3 PUFA concentrates from seal blubber. This process employed extraction of oil from blubber, saponification, extraction of free fatty acids, urea complexation, and extraction of ω-3 PUFA (9,10). The final product contained 23.8% EPA and 53.1% DHA.

Esterification reaction. Acylglycerols were synthesized from glycerol and the ω-3 fatty acid concentrates. Three grams of glycerol, 0.4 g fatty acid concentrate, water content of 5% (g/g glycerol), 3 mL isooctane, and 5000 units of lipase were placed in a 50-mL Erlenmeyer flask, which was stoppered to prevent evaporation. The vessel was agitated on an orbital shaker at 250 rpm at 50°C under nitrogen for 48 h. The reaction was stopped by the addition of 20 mL of a mixture of acetone/ethanol (1:1, vol/vol).

Estimation of degree of synthesis. The reaction mixture was titrated against a 0.1 N standardized NaOH solution. The degree of synthesis represents the percentage of initial fatty acids consumed by the reaction mixture (11).

Identification of reaction products. Reaction products were extracted from the mixture with diethyl ether and were then

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TABLE 1
Esterification Activity of Various Lipases[a]

Enzyme	Commercial code	Source	Degree of synthesis (%)
Chromobacterium viscosum	LP-401-AS	Asahi Chemical Industry Co. Ltd., Tokyo, Japan	68.5
Mucor miehei	IM-60	Novo Nordisk Inc., Bagsvaerd, Denmark	44.1
Pseudomonas sp.	PS-30	AIE[b] Co., Nagoya, Japan	46.0
Candida rugosa	AY-30	AIE Co.	13.9
Rhizopus niveus	N	AIE Co.	0.0
Aspergillus niger	AP-12	AIE Co.	0.0
R. oryzae	FAP-15	AIE Co.	39.8

[a]Esterification in hexane at 30°C.
[b]AIE: Amano International Enzyme.

separated by thin-layer chromatography (TLC) on silica gel 60 plates with fluorescein and developed in chloroform/acetone/methanol (96:3.6:0.4, vol/vol/vol). Separated bands were visualized under ultraviolet radiation at 254 nm. Bands corresponding to each lipid type were scraped from the plates, and their fatty acid compositions, after transmethylation, were analyzed by gas chromatography as described elsewhere (9).

RESULTS AND DISCUSSION

The extent of esterification of glycerol and the ω-3 fatty acid concentrate, prepared from SBO, in hexane at 30°C by individual lipases at 5000 units is given in Table 1. Lipase LP-401-AS yielded the highest degree of synthesis, up to 68.5%. Thus, this lipase was chosen for subsequent experiments to determine optimal esterification conditions. However, Li and Ward (11) reported that lipases PS-30 and IM-60 exhibited the best esterification activity when fish oil concentrates were reacted with glycerol.

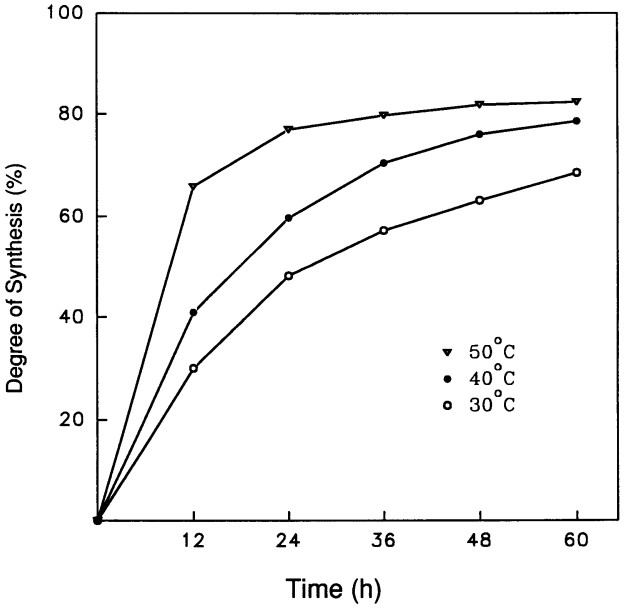

FIG. 1. Effect of temperature on esterification with lipase LP-401-AS. The reaction mixture contained 3.0 g glycerol, 0.4 g of ω-3 fatty acid concentrate, 3.0 mL *n*-hexane, 5% water (g/g glycerol), 5000 U of lipase LP-401-AS.

Figure 1 illustrates the effect of temperature on esterification catalyzed by lipase LP-401-AS. The degree of synthesis increased with increasing reaction temperature from 30 to 50°C. A time course study showed that the degree of synthesis at 50°C remained constant after 48 h of reaction. Therefore, a 48-h reaction period was chosen as the end point for esterification at 50°C.

To carry out bioconversion of lipophilic compounds effectively, it is essential to introduce organic solvents into the reaction medium. The use of organic solvents can improve the solubility in water of substrates or other reaction components with hydrophobic nature (11). Moreover, organic solvents produce various physicochemical effects on enzymes and result in alteration of substrate specificity and the affinity for enzymes (12). To select the most suitable solvent for esterification by lipase LP-401-AS, the effect of the presence of various organic solvents in the reaction medium was examined (Table 2). Thus, isooctane proved to be the best solvent and afforded results similar to these reported by Li and Ward (11).

In the enzymatic esterification, the content of water in the mixture affects the reaction rate. There are two reasons for this: first, a trace amount of water is necessary for the function of the enzyme and maintenance of its three-dimensional structure (12); second, water is also a product of the esterification reaction. Presence of large amounts of water favors the hydrolysis reaction. The effect of initial water content on esterification by lipase LP-401-AS was investigated (Table 3). As expected, the added water increased the degree of synthesis, and when at 5% (g/g glycerol) in the reaction mixture, lipase LP-401-AS showed maximal esterification of up to 85.0%. A further increase in water content decreased the degree of synthesis. To remove water that is formed during the esterification reaction, 0.5 g (25% excess compared to

TABLE 2
Effect of Organic Solvents on Esterification by Lipase LP-401-AS[a]

Organic solvent	Degree of synthesis (%)
Isooctane	85.0
Hexane	81.8
Toluene	43.9
Ethyl acetate	56.1

[a]See Table 1 for company source.

TABLE 3
Effect of Water Content on Esterification by Lipase LP-401-AS[a]

Water content (g/100 g glycerol)	Degree of synthesis (%)
0.0	56.0
2.5	75.5
5.0	85.0
7.5	78.3
5.0 (0.5 g molecular sieve)	88.9
5.0 (1.0 g molecular sieve)	81.0

[a]See Table 1 for company source.

TABLE 4
Effect of Glycerol Content on Esterification by Lipase LP-401-AS[a]

Amount of glycerol (g)	Degree of synthesis (%)
1	57.3
2	73.9
3	85.0
4	86.8
5	87.0

[a]See Table 1 for company source.

TABLE 5
Effect of Enzyme Content on Esterification by Lipase LP-401-AS[a]

Amount of enzyme (mg)	Degree of synthesis (%)
20	69.1
30	73.8
40	84.1
50	85.0
70	86.2

[a]Lipase LP-401-AS activity: 100,000 unit/g. See Table 1 for company source.

TABLE 6
Effect of Solvent Volume on Esterification by Lipase LP-401-AS[a]

Solvent volume (mL)	Degree of synthesis (%)
1	89.9
2	87.7
3	85.0
4	77.8
6	69.4

[a]See Table 1 for company source.

oil, w/w) or 1 g (150% excess compared to oil, w/w) of molecular sieve (a dehydrating agent) was added. When 0.5 g of molecular sieve was added, the degree of synthesis reached 88.9%. In contrast, addition of 1 g of molecular sieve decreased the degree of synthesis to 81.0%. Obviously, presence of excessive amounts of molecular sieve not only eliminates water formed during the reaction but also decreases the amount of required water for the reaction to proceed for a maximal yield.

The effect of quantity of glycerol in the reaction mixture on acylglycerol synthesis by lipase LP-401-AS was also investigated (Table 4). The degree of synthesis increased when large amounts of glycerol were present. Depending on the desired yield, a glycerol content of 4 g (glycerol/oil, 10:1) was sufficient.

Table 5 shows the effect of enzyme load on esterification. At 20 mg of lipase LP-401-AS, only 69.1% synthesis occurred; beyond a 40 mg addition level, there was no significant increase in product yield. Thus, it seems that there is no obvious advantage in using more than 40 mg of lipase LP-401-AS.

TABLE 7
Fatty Acid Composition of ω-3 Fatty Acid Concentrate and Components Separated After Esterification by Lipase LP-401-AS[a]

	ω-3 Fatty acid concentrate	Lipid component (%)			
Fatty acid	(%)	MAG (13.8)[b]	DAG (43.1)	TAG (37.4)	FFA (5.7)
14:0	1.14	1.07	1.05	1.06	1.36
14:1	0.71	0.77	0.81	1.18	0.65
16:1	0.89	0.87	1.04	1.31	0.52
17:0	1.42	1.51	1.61	2.15	n.d.
17:1	1.22	1.46	1.48	2.16	0.63
Unidentified	1.56	1.84	1.70	2.29	0.71
18:2	0.30	0.33	0.36	0.45	n.d.
18:3	0.95	1.05	1.07	1.34	n.d.
18:4	5.47	5.71	6.19	7.94	2.59
20:3	0.34	n.d.[c]	0.37	0.41	n.d.
20:4	0.91	0.90	0.87	0.69	1.39
Unidentified	1.63	1.28	1.58	1.64	1.44
20:5	23.82	26.75	25.25	20.78	28.16
22:4	2.32	2.32	2.53	2.93	3.87
22:5	4.19	4.10-	4.38	3.83	9.79
22:6	53.11	50.02	49.71	49.83	48.89

[a]Esterification in 1 mL isooctane with 0.5 g molecular sieve. See Table 1 for company source.
[b]MAG, monoacylglycerol; DAG, diacylglycerol; TAG, triacylglycerol; FFA, free fatty acid.
[c]n.d., not detected.

During esterification by lipase LP-401-AS, evaporation of isooctane increased the degree of synthesis. The effect of solvent volume on esterification was investigated (Table 6). Increasing solvent volume decreased the acylglycerol synthesis because presence of a large volume of solvent decreased the concentration of the reactants. With a volume of 1 mL isooctane in the reaction mixture, a degree of synthesis of 89.9% was reached. A maximal yield of 94.3% was obtained after the addition of 0.5 g of molecular sieve. The products were further separated by TLC. Surprisingly, the relative content of diacylglycerols (43.1%) and triacylglycerols (37.4%) was much higher than that of monoacylglycerols (13.8%). The fatty acid composition of the isolated bands was analyzed by gas chromatography (Table 7); no substantial change in EPA and DHA contents was noted as compared to those of the initial ω-3 fatty acid concentrates.

From the foregoing results, we concluded that acylglycerols containing highly concentrated EPA and DHA can be easily synthesized directly from glycerol and ω-3 fatty acid concentrates from SBO. Lipase LP-401-AS showed the best esterification activity among enzymes examined. A maximal yield of 94.3% was obtained under optimal reaction conditions; diacylglycerols and triacylglycerols were the major products formed. However, in experiments of Li and Ward (11), the experiments, lipase IM-60 and lipase PS-30 were the best biocatalysts; monoacylglycerols and diacylglycerols were also the major reaction products. Thus, existing differences between the present results and those of Li and Ward (11) may arise from differences in the proportion of individual long-chain ω-3 fatty acids in the concentrate from SBO.

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