Two-Step Enzymatic Synthesis of Docosahexaenoic Acid-Rich Symmetrically Structured Triacylglycerols *via* 2-Monoacylglycerols

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ABSTRACT: Symmetrically structured triacylglycerols (TG) rich in docosahexaenoic acid (DHA) with caprylic acid (CA) at the outer positions were synthesized enzymatically from bonito oil in a two-step process: (i) ethanolysis of bonito oil TG to 2-monoacylglycerols (2-MG) and fatty acid ethyl esters, and (ii) reesterification of 2-MG with ethyl caprylate. Ethanolysis catalyzed by immobilized *Candida antarctica* lipase (Novozym 435) yielded 92.5% 2-MG with 43.5% DHA content in 2 h. The 2-MG formed were reesterified with ethyl caprylate by immobilized *Rhizomucor miehei* lipase (Lipozyme IM) to give structured TG with 44.9% DHA content [based on fatty acid composition with caprylic acid (CA) excluded] in 1 h. The final structured lipids comprised 85.3% TG with two CA residues and one original fatty acid residue, 13% TG with one CA residue and two original fatty acid residues, and 1.7% tricapryloylglycerol (weight percent). The amount of TG with two CA residues and one C_{22} residue (22:6 = DHA, 22:5, and 22:4) was 51 wt%. The 1,3-dicapryloyl-2-docosahexaenoylglycerol to 1,2(2,3)-dicapryloyl-3(1)-docosahexaenoylglycerol ratio (based on high-performance liquid chromatography peak area percentages) was greater than 50:1. The recovery of TG as structured lipids after silica gel column purification was approximately 71%. Ethyl esters and 2-MG formed at 2 h of ethanolysis could be used to determine the positional distribution of fatty acids in the initial TG owing to the high 1,3-regiospecificity of Novozym 435 and the reduced acyl migration in the system.

Paper no. J9837 in *JAOCS 78*, 743–748 (July 2001).

KEY WORDS: Esterification, ethanolysis, fish oil, immobilized *Candida antarctica* lipase, immobilized *Rhizomucor miehei* lipase, lipase regiospecificity, regioselective analysis of triacylglycerols, symmetrically structured triacylglycerols.

Fish oil is a well-known natural source of polyunsaturated fatty acids (PUFA), and among these are docosahexaenoic acid (DHA or 22:6) and eicosapentaenoic acid (EPA or 20:5). Various fish oils have been used for a long time as dietary supplements. There have been extensive studies on the biological and therapeutical functions of DHA and EPA (1), and, owing to the increased awareness of these functions, a large variety of health products made of or containing fish oil or fish oil-derived compounds such as ethyl esters have recently entered the nutraceuticals' market.

The fatty acid distribution on the glycerol backbone of triacylglycerols (TG) influences their absorption, distribution, and tissue uptake (2–4). PUFA are better absorbed when they are esterified at the *sn*-2 position. The type of fatty acid species at the outer positions also influences their intestinal absorption as 2-monoacylglycerols (2-MG) after regiospecific lipase hydrolysis of TG in the mouth, stomach, and small intestine. Symmetrically structured triacylglycerides (SST) have the same fatty acid groups at the *sn*-1 and *sn*-3 positions. SST with medium-chain fatty acids (C_6–C_{10}) at the outer positions and a PUFA at the *sn*-2 position have superior dietary and absorption characteristics. Medium-chain residues are easily hydrolyzed in the gastrointestinal tract, and the resulting fatty acids are rapidly absorbed and used as a high-energy source in the body (5,6). PUFA absorbed as 2-MG are the most readily absorbed among PUFA derivatives. The TG structure also influences the oxidative degradation of PUFA. SST with saturated outer residues and a PUFA at the *sn*-2 position are more resistant to oxidation (7,8).

Fish oil is an inexpensive source of PUFA-containing TG. The content of DHA and EPA and also their positional distribution in TG vary among the fish species. Fish oils with a high content of DHA and EPA at the *sn*-2 position can be used as starting materials for production of nutritionally valuable SST with medium-chain fatty acids at the primary positions.

Some research groups have used fish oil (9) or DHA-rich oils such as single-cell oil (10) for the production of SST by acidolysis with caprylic acid (CA) catalyzed by 1,3-regiospecific lipases. DHA residues situated at the outer positions could not be exchanged (owing to the low specificity of 1,3-regiospecific lipases for DHA), resulting in a limited yield of SST. The same problem was encountered in a study in which SST were obtained in two steps (11). 2-MG were obtained by ethanolysis of TG in an organic solvent with a 1,3-regiospecific lipase and then reesterified with oleic acid. The yields of ethanolysis were less than 40% for fish oils with 8% DHA content or more. A large amount of 1-MG was formed, probably owing to acyl migration.

This work describes a fast and straightforward two-step method for the synthesis of SST, with CA residues at the outer positions, from DHA- and EPA-rich bonito oil. Fish oil (bonito oil) TG were subjected to ethanolysis with immobilized *Candida antarctica* lipase (Novozym 435) to yield 2-MG, which were subsequently reesterified with ethyl caprylate (EtC) by immobilized *Rhizomucor miehei* lipase (Lipozyme IM) to form SST. The positional distribution of fatty acids in the initial oil could be determined from the fatty

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acid composition of 2-MG or fatty acid ethyl esters (EE) formed in the first step.

EXPERIMENTAL PROCEDURES

Materials. Immobilized *C. antarctica* lipase (Novozym 435) and *R. miehei* lipase (Lipozyme IM) were generous gifts from Novo Nordisk Bioindustry Co. Ltd. (Chiba, Japan). Ethyl caprylate (EtC), tricapryloylglycerol (CCC), and ethanol (min. 99%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Refined bonito oil (100% TG) was a product of Nippon Suisan Kaisha, Ltd. (Tokyo, Japan).

2-MG synthesis. Bonito oil (1 g, 1.11 mmol, mean molecular weight = 901.7, 0.002% water), ethanol (3 g, 65.2 mmol, 0.23% water), and Novozym 435 (0.4 g, 1.57% water) were mixed with a magnetic stirrer at 300 rpm agitation speed for 2 h at 35°C.

Synthesis of SST. 2-MG were synthesized from bonito oil as described above, and the final reaction mixture was filtered to remove the catalyst. The excess of ethanol was evaporated under low pressure at 35°C. EtC (3 g, 17.44 mmol, 0.003% water) and Lipozyme IM (0.4 g, 4.23% water) were added to the final ethanolysis reaction mixture (containing partial acylglycerols and EE) free of catalyst (Novozym 435) and ethanol. The reaction vessel was connected to a vacuum pump at 3–5 mm Hg through a nitrogen trap for the removal of the ethanol produced during the reesterification of partial acylglycerols. The reesterification reaction was performed for 1 h at 300 rpm and 35°C.

Thin-layer chromatography (TLC)/flame-ionization detector (FID) analysis of ethanolysis reaction mixture. Samples were withdrawn from the reaction mixture at 30-min intervals, dissolved into diethyl ether, and filtered to remove the catalyst. The resulting solution was analyzed with a thin-layer chromatograph equipped with a flame-ionization detector (Iatroscan MK-5; Iatron Laboratories, Tokyo, Japan) using Chromarod S III quartz rods. The rods loaded with the samples were eluted for 10 cm with hexane/diethyl ether (9:1) and then the upper 8 cm were burned for the quantification of EE and TG. Diacylglycerols (DG) and MG (which remained close to the rod origin after the first development) were separated by a second development with benzene/chloroform/acetic acid (100:5:1). No free fatty acids were detected. The results were expressed as percentage of peak areas.

Separation of EE and 2-MG from ethanolysis reaction mixture. Following removal of the catalyst by filtration, the ethanol was evaporated from 200 μL of the final ethanolysis mixture at 35°C under reduced pressure. The sample was taken up with 1 mL hexane/diethyl ether (9:1) and applied to a 500-mg silica gel separation cartridge (Sep-Pak 500 Silica Cartridge; Waters Corp., Milford, MA). EE were eluted completely with another 4 mL of the above solvent.

For recovery of 2-MG 200 mL of the final ethanolysis mixture (from which the catalyst had been removed by filtration) was applied to a 0.5-mm silica gel TLC plate ($60F_{254}$; Merck, Darmstadt, Germany) treated with boric acid. The plate was developed with chloroform/acetone (98:2) and dried at room temperature. The band corresponding to 2-MG was scraped off and used for derivatization to fatty acid methyl esters (FAME).

Purification of TG from the final reesterification reaction mixture. A 500-mg portion of the reaction mixture was applied to an NH-silica gel column (Chromatorex® chromatography silica gel NH, 100–200 mesh; Fuji Silysia Chemical Ltd., Kasugai, Japan). EE were eluted with hexane/diethyl ether (98:2), and then TG were eluted with hexane/diethyl ether (90:10).

Gas–liquid chromatography (GC) analyses. Fatty acid composition of initial TG and 2-MG was determined by GC analysis after derivatization to FAME. Acylglycerols (10–20 mg) were reacted with 0.5 N NaOH in methanol (1.5 mL) for 5 min at 80°C, and then 14% BF_3 in methanol (2 mL) was added and reacted for a further 30 min. Hexane (1 mL) and a saturated NaCl solution (5 mL) were added, and the mixture was shaken well. The upper hexane layer containing FAME was separated. FAME, and EE resulting from ethanolysis, were analyzed on a Hewlett-Packard (Avondale, PA) model 6890 chromatograph equipped with a DB-WAX column (30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness; J&W Scientific, Folsom, CA). The carrier gas was He (1 mL min^{-1} flow rate) at a 100:1 split ratio. Separation was carried out with temperature programming from 170 to 230°C at 2°C min^{-1} and hold for 10 min. The peaks were identified by comparison with standards.

TG species of bonito oil and SST were separated according to their carbon number by high-temperature GC. A chromatograph (GC-14; Shimadzu Corporation, Kyoto, Japan) equipped with an on-column injector (OCI-14; Shimadzu Corporation, Kyoto, Japan) and an Ultra Alloy-1 (HT) capillary column (10 m length, 0.5 mm internal diameter, 0.1 μm film thickness; Frontier Laboratories Ltd., Kohriyama, Japan) was used. The oven was heated from 40°C (held for 1 min) at 10°C min^{-1} to 370°C (held for 6 min). The on-column injector was heated from 40 to 380°C at 10°C min^{-1} and held at this temperature for 6 min. The detector was kept at 395°C.

High-performance liquid chromatography (HPLC) analyses. HPLC analyses of bonito oil and SST were carried out on a ChromSpher 5 Lipids silver-ion chromatography column (250 × 4.6 mm × 1/4″; Chrompack, Middleburg, The Netherlands). The TG species were detected with an evaporative light-scattering detector (S.E.D.E.R.E., Alfortville, France). The TG separation was carried out with a ternary solvent gradient system (12): (A) 1,2-dichloroethane/dichloromethane (1:1, vol/vol); (B) acetone; and (C) acetone/acetonitrile (3:1, vol/vol). Linear gradients were generated from 100% A to 50% A/50% B over 10 min, then to 70% B/30% C over 20 min, and, finally, to 100% C over a further 30 min. The column was maintained at ambient temperature. The flow rate was 0.75 mL min^{-1}. Samples were dissolved in 1,2-dichloroethane/dichloromethane (1:1, vol/vol).

TG species of bonito oil were fractionated with the above gradient program (12). The fractions were collected manually, and a standard solution of trilaurin in *n*-hexane (1 mg/

mL) was added to each fraction as an internal standard. The solvent was evaporated under a nitrogen stream, and the TG were derivatized to FAME as described above. Quantification was performed by GC analysis of FAME.

Synthesis of 1,3-dicapryloyl-2-docosahexaenoylglycerol (CDC) and 1,3-dicapryloyl-2-eicosapentaenoylglycerol (CEC) standards. CDC and CEC (>90% isomeric purity) were prepared as described in our previous work (13).

Regiospecific analysis of TG. Regiospecific analysis of bonito oil TG was performed after degradation to partial acylglycerols with allyl magnesium bromide (14). The resulting mixture of partial acyglycerols was separated by preparative TLC, and the 2-MG band was scraped off. The 2-MG fraction was converted to FAME and analyzed by GC.

Water content analysis. Water contents of substrates and enzyme preparations were measured with a Karl Fischer moisture meter (MKS-1; Kyoto Electronics, Kyoto, Japan).

RESULTS AND DISCUSSION

Characterization of the original oil. Enzymatic synthesis of SST consists in substitution of the two outer residues in the original TG with residues of a chosen fatty acid so that the composition and properties of the final SST are theoretically determined by the fatty acid composition of the midposition of the intial TG. Therefore, analysis of the distribution of fatty acid residues in the TG, in addition to analysis of the total fatty acid composition, is required when a natural oil is chosen as starting material for synthesis of the SST of interest. These analytical results would help to determine the behavior of the catalyst with different types of TG and also to predict the composition of the final product.

Bonito oil was used in this work as a source of TG with a high content of DHA and EPA at the midposition for the synthesis of SST with CA residues at the primary positions.

Approximately 40% of the total fatty acids contained in TG species of bonito oil had two or more double bonds (Table 1). The content of DHA and EPA combined was approximately 30%. Information about their distribution in the TG was obtained by HPLC analysis with a silver-ion-treated column and a solvent system that enabled separation of TG according to the total number of double bonds in their molecules (12). Seven fractions were collected (Fig. 1A). An internal standard (trilaurin) was added to each fraction for quantification, and the TG were converted to FAME. The identification and quantification was performed by GC analysis of the resulting FAME. The average number of double bonds of the TG or the double bond index for each fraction (12) was calculated (Table 1).

DHA and EPA were concentrated in fractions 5, 6, and 7 with double bond indices of 5.97, 10.38 and 13.77, respectively. The double bond indices of fractions 6 and 7 as well as their fatty acid compositions indicate that their TG have at least one DHA or EPA residue per molecule. Fraction 5 contained 86.2 mol% TG with one DHA or EPA residue (calculated from the fatty acid composition). Fractions 5 and 6 were the most important quantitatively (53.2 and 26.5 mol%, respectively). From the fatty acid composition and relative proportions in the initial oil of fractions 5, 6, and 7, it was calculated that 75% of the TG molecules of bonito oil contained at least one DHA or EPA residue. The regiospecific analysis of bonito oil by Grignard degradation indicated that 46.4% of the total TG contained DHA or EPA at the midposition (Table 2). The rest of the molecules containing DHA or/and EPA (28.6%) had these fatty acid residues placed at the outer posi-

TABLE 1
Fatty Acid Compositions (mol%), Proportions, and Double Bond Indices of Triacylglycerol (TG) Fractions Obtained by Fractionation of Bonito Oil TG by Silver-Ion High-Performance Liquid Chromatography

		Fraction						
Fatty acid	Total	1	2	3	4	5	6	7
14:0	5.12	15.81	10.08	5.90	5.58	5.22	3.12	1.77
15:0	1.57	5.07	3.47	5.23	4.28	3.66	0.00	0.00
16:0	24.79	52.08	39.81	22.66	18.05	25.08	13.17	3.32
16:1	6.18	3.95	8.82	13.17	14.01	5.64	2.92	0.00
17:0	1.47	9.22	2.31	0.00	1.54	1.88	0.00	0.00
17:1	0.95	0.00	1.89	3.10	3.68	2.19	1.70	1.20
18:0	5.99	12.36	7.56	4.24	4.75	5.96	6.71	2.60
18:1	15.25	1.52	24.79	41.99	39.79	16.82	7.27	1.21
18:2n-6	1.43	0.00	0.00	1.54	8.31	2.82	4.42	2.22
18:3n-3	0.57	0.00	0.00	0.00	0.00	0.00	3.60	6.56
18:4	1.08	0.00	0.00	0.00	0.00	0.00	2.76	13.48
20:1	0.71	0.00	1.26	2.18	0.00	0.00	0.00	0.00
20:4n-6	1.63	0.00	0.00	0.00	0.00	1.99	3.41	3.00
20:5	6.31	0.00	0.00	0.00	0.00	5.54	10.74	15.58
22:4	1.50	0.00	0.00	0.00	0.00	0.00	2.50	1.92
22:5	0.84	0.00	0.00	0.00	0.00	0.00	0.00	1.99
22:6	24.61	0.00	0.00	0.00	0.00	23.20	37.67	45.15
Amount (mol%)		2.2	7.5	5.2	2.8	53.2	26.5	2.6
Double bond index[a]	6.84	0.16	1.10	1.91	2.22	5.97	10.38	13.77

[a]Double bond index = average number of double bonds in each TG molecule.

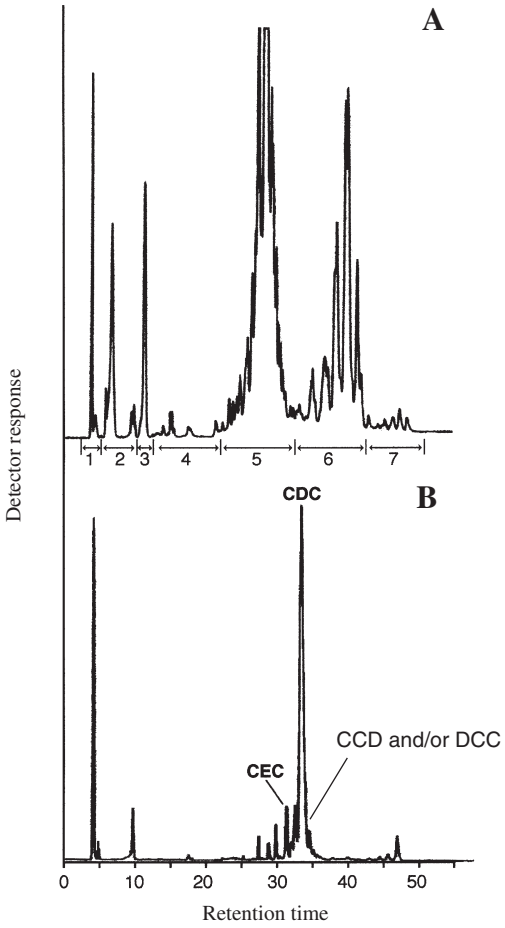

FIG. 1. Silver-ion high-performance liquid chromatography separation of (A) bonito oil triacylglycerols (TG) (fraction numbers are the same as in Table 1) and (B) structured lipids derived therefrom. CEC, 1,3-dicapryloyl-2-eicosapentaenoylglycerol; CDC, 1,3-dicapryloyl-2-docosahexaenoylglycerol.

TABLE 2
Fatty Acid Composition (mol%) of Bonito Oil, 2-Monoacylglycerols (2-MG) and Fatty Acid Ethyl Esters (EE) Formed in Ethanolysis Step, and Final Structured Triacylglycerols (ST)

Fatty acid	TG[a] original	TG[b] calc.	2-MG[c] Grignard	2-MG[d]	EE[d]	ST[e]
14:0	5.06	5.53	6.68	6.95	4.83	5.64
15:0	1.55	1.65	1.72	1.68	1.64	1.43
16:0	24.53	24.97	19.93	19.09	27.91	17.65
16:1	6.11	5.71	5.50	4.84	6.14	4.68
16:2	0.35	0.37	0.45	0.52	0.29	0.51
17:0	1.45	1.41	0.70	0.67	1.78	0.75
17:1	0.94	0.83	0.36	0.48	1.01	0.58
18:0	5.93	5.33	1.40	2.39	6.79	2.99
18:1	15.10	14.71	7.59	7.17	18.48	8.44
18:2n-6	1.41	1.37	1.10	1.02	1.55	1.10
18:3n-3	0.56	0.54	0.43	0.42	0.60	0.43
18:4	1.07	1.12	1.77	1.29	1.03	1.09
20.0	0.36	0.36	0.00	0.09	0.50	0.13
20:1	0.70	0.70	0.25	0.26	0.93	0.33
20:4n-6	1.62	1.62	2.24	1.52	1.66	1.40
20:4n-3	0.11	0.10	0.00	0.00	0.15	0.06
20:5	6.24	6.16	7.00	4.36	7.05	4.07
21:5	0.21	0.23	0.00	0.19	0.25	0.26
22:4	1.49	1.50	2.18	2.49	1.01	2.58
22:5	0.83	0.88	1.26	1.03	0.80	0.98
22:6	24.36	24.91	39.44	43.54	15.59	44.89

[a]See Table 1 for abbreviation.
[b]Fatty acid composition of bonito oil was calculated from the compositions of 2-MG and EE at 2 h of ethanolysis using the following equation for each fatty acid: (2-MG + 2 × EE)/3.
[c]Fatty acid composition of 2-MG from Grignard degradation of original TG.
[d]Fatty acid composition of 2-MG and EE at 2 h of ethanolysis.
[e]Fatty acid composition of ST with caprylic acid excluded.

tions of the glycerol backbone. Only the fatty acid residues at the midposition remain unchanged after SST synthesis. DHA and EPA residues at the primary positions are removed in the process so that their presence in the initial oil does not improve the characteristics of the final product.

SST production was performed in two steps: 2-MG synthesis by ethanolysis followed by reesterification of the primary hydroxyl groups with CA residues.

2-MG synthesis step. Novozym 435 was used in this work to catalyze the 1,3-regiospecific ethanolysis of bonito oil TG for the production of 2-MG rich in DHA and EPA. Ethanolysis of trieicosapentaenoylglycerol (EEE) and tridocosahexaenoylglycerol (DDD) was employed previously as an intermediary step for the synthesis of CEC and CDC (15). The molar ratio of the reactants influenced the regiospecificity of Novozym 435 and also the reaction rate and yield. The ethanolysis of DDD performed at an ethanol/DDD weight ratio of 3:1 and 35°C had the highest rate and the highest final yield, and, therefore, these conditions were chosen for ethanolysis of bonito oil.

The ethanolysis reaction was very fast (Fig. 2). The glyceride composition at 2 h was 92.5% 2-MG and 7.5% 1,2(2,3)-DG (area %). The TLC analysis of the ethanolysis product showed that the formed MG were 100% 2-MG. No spot of 1-MG or 1,3-DG was detected. The reaction mixture composition (area %) at 2 h determined by TLC-FID was 69.5% EE, 2.3% 1,2(2,3)-DG, and 28.2% 2-MG. The FID response for EE is higher than for partial acylglycerols so that their actual weight percentage might be lower. No free fatty acids were detected in the reaction mixture.

The fatty acid composition of 2-MG resulting from ethanolysis was in good agreement with the composition of 2-MG obtained by Grignard degradation of the initial oil, except for the EPA and DHA contents (Table 2). Similar differences between the results obtained by enzymatic hydrolysis and by Grignard degradation applied to fish oil were also mentioned in another work (16). The composition of EE resulting from ethanolysis was also determined. These EE are theoretically removed only from the primary positions of TG, allowing the fatty acid composition of the initial TG to be recalculated using the fatty acid compositions of 2-MG and EE resulting from ethanolysis. The result of these calculations was very close to the initial composition determined directly after derivatization of the initial TG to FAME, and thus the above hypothesis was confirmed. The analytical procedure using Grignard degradation might cause partial damage of PUFA, resulting in the observed difference in the 2-MG composition determined by the two methods.

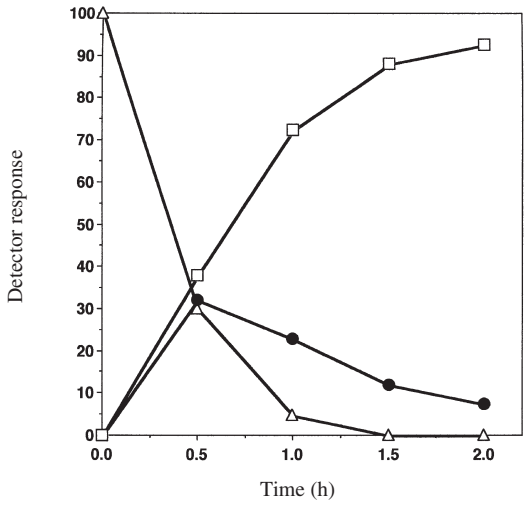

FIG. 2. Acylglycerol composition during ethanolysis: TG (△), 1,2(2,3)-diacylglycerols (●), 2-monoacylglycerols (□). See Figure 1 for abbreviation.

The results of fatty acid composition analysis (Table 2) of the ethanolysis reaction products (2-MG and EE) demonstrate that Novozym 435 displays very strict 1,3-regiospecificity in this reaction under the reaction conditions employed. Bonito oil is a very complex mixture of TG containing a large variety of fatty acid species with a complex positional distribution, and therefore it is an appropriate substrate to test the fatty acid specificity of Novozym 435 in ethanolysis. The fatty acid composition of 2-MG and EE formed after 2 h of ethanolysis indicated no distinguishable preference of Novozym 435 for any of the fatty acid species present in TG. The high conversion of the original TG to 2-MG at 2 h might have alleviated the effect of the enzyme's fatty acid specificity. Acyl migration reactions were limited by the low water content of the ethanolysis reaction mixture (only the water contained in the enzyme preparation and reactants), the absence of fatty acids, and the low reaction temperature. Therefore, ethanolysis did not proceed to the removal of fatty acid residues at the midposition of TG, which usually affects analytical methods based on TG hydrolysis with 1,3-specific lipases. Reduced acyl migration, strict 1,3-regiospecificity, and good activity of Novozym 435 on PUFA enable the use of the EE resulting from ethanolysis for the determination of fatty acid composition at the midposition of TG.

2-MG reesterification step. SST were obtained by reesterification with EtC of 2-MG formed in the first step. The ethanolysis reaction mixture was used directly in the second step after the catalyst had been filtered out and the excess of ethanol had been removed. Novozym 435 showed no regiospecificity in this reaction (15) so that it had to be replaced with a 1,3-regiospecific lipase that could work at the low water concentrations of the reaction medium. Lipozyme IM gave good results for a similar reaction (15), and therefore it was used for this work also. The amount of excess EtC used in the reesterification step was chosen on the basis of previous results that showed that higher EtC/partial glyceride ratios improved the final reaction yield (15). The reaction equilibrium was pushed to high yields by removing the resulting ethanol under reduced pressure.

The reaction was completed after 1 h as shown by the disappearance of the 2-MG and 1,2-DG spots in TLC analysis. The separation of the final purified SST by high-temperature GC was much better than that of the initial bonito oil TG (Fig. 3). The final SST had lower molecular weight and therefore higher volatility, resulting in shorter retention times and better resolution. The area percentages of C_{38} (TG with two CA residues and one 22:6, 22:5, or 22:4 residue) and C_{36} (TG with two CA residues and one 20:5, 20:4, 20:1, or 20:0 residue) were 51.0 and 4.5%, respectively. The percentage of CCC was 1.7%. The last peak in Figure 3B was identified as C_{52} (TG with one CA residue and two 22:6, 22:5, or 22:4 residues), and therefore it was inferred that all the peaks following the C_{38} peak have one CA residue and two original fatty acid residues and that none of the original TG were left unchanged. The percentage of TG heavier than C_{38} (TG with one CA residue and two original fatty acid residues) in the product was 13.0%. Such TG resulted probably from 1,2-DG remaining after ethanolysis that were reesterified with caprylic acid. Similarly, the peaks between the C_{24} (CCC) and C_{38} peaks were assigned to TG with two CA residues and one original fatty acid residue. These TG amounted to 85.3% of the final structured TG. The fatty acid composition of the product without CA was slightly different from that of the starting 2-MG due to the TG with two remaining original fatty acid residues (Table 2). Silver-ion HPLC of the product (Fig. 1B) showed a less complicated composition than that of the initial oil (Fig. 1A). CCD (both CCD + DCC enantiomers included) and CDC regioisomers can be baseline separated under the analytical conditions used. The main peak in Figure 1B was iden-

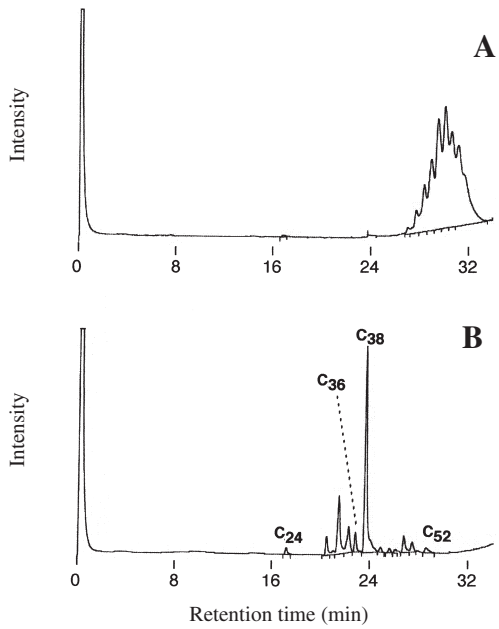

FIG. 3. Gas chromatographic separation of (A) bonito oil TG and (B) structured TG derived therefrom. See Figure 1 for abbreviation.

tified as CDC with a chemically synthesized standard. The CCD isomer, if formed, would be eluted approximately 90 s after the CDC isomer. The area of the peak following the CDC peak, which might be the CCD isomer, is less than 2% of that of the CDC peak. The total TG recovery or yield of the whole process after silica gel purification of the structured lipids was 71%.

In a scaled-up reaction, the excess EtC remaining in the final reaction mixture can be evaporated easily at 5 mm Hg and 88–99°C and reused in the reesterification step. The rest of the EE can be removed by molecular distillation before silica gel column purification (the last purification step), which is used for removal of 1,3-DG and traces of free fatty acids formed as by-products in the reesterification step.

The whole synthetic procedure was very fast (approximately 3 h) compared to 120 h for acidolysis with Lipozyme IM (10) or immobilized *Rhizopus delemar* lipase (9). A similar method based on 2-MG synthesis by ethanolysis of TG with Lipozyme IM in an organic solvent required 24 h for 88% 2-MG yield from tripalmitin in methyl -*t*-butyl ether and 2 h for 2-MG esterification in *n*-hexane (11). The method had limited success for fish oil TG as starting material. The yield of 2-MG was only 28% for fish oil TG containing 34% DHA and was affected by extensive acyl migration to form 1-MG.

A chemoenzymatic approach for synthesis of 2-eicosapentaenoyl-1,3-distearoylglycerol (SES) and 2-docosahexaenoyl-1,3-distearoylglycerol (SDS) was presented in a recent paper (17). 1,3-Distearoylglycerol was synthesized enzymatically in diethyl ether with 74% yield in 48 h and then esterified with DHA or EPA chemically in dichloromethane in 24 h with 91 and 94% yield, respectively. Reaction yields comparable to ours are afforded by this method, but the reaction times are much longer. Another drawback of the method is the use of large quantities of organic solvents as reaction media and the use of toxic chemical catalysts for the esterification step [1-(3-dimethylaminopropyl)-3-ethylcarbodiimide and 4-dimethylaminopyridine].

Thus, the procedure used in this work for structured lipid synthesis from DHA-rich fish oils has clear advantages over the previously reported methods. It is faster and the TG conversion to SST is higher. The product is obtained without the use of toxic solvents or catalysts. The excess of ethanol (used for TG ethanolysis) and EtC (used for 2-MG reesterification) is easy to remove and recycle. In addition, EtC can be obtained from CA and ethanol in high yield very rapidly prior to the reesterification of 2-MG using Lipozyme IM (18). This will reduce the cost of the whole process, as EtC is considerably more expensive than CA.

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[Received December 11, 2000; accepted March 29, 2001]