Synthesis of Triacylglycerol from Polyunsaturated Fatty Acid by Immobilized Lipase[1]

Yoshitsugu Kosugi* and **Naoki Azuma**[2]
National Institute of Bioscience and Human Technology, Tsukuba 305, Japan

More than 95% of polyunsaturated acid (PUFA) was converted to triacylglycerol by immobilized lipase from *Candida antarctica* or *Rhizomucor miehei*. The esterification was carried out at 50–60°C with shaking and dehydration for 24 h. The substrates consisted of glycerol and free fatty acid or ethyl esters of the fatty acid at a 1:3 molar ratio. The docosahexaenoic acid (DHA) or eicosapentaenoic acid (EPA) in the substrate polymerized during the reaction, and they required 5–10% more than the stoichiometric amount to compensate for the PUFA loss. On the contrary, ethyl esters of DHA and EPA were not polymerized. Pure tridocosahexaenoyl, trieicosapentaenoly and triarachidonoyl glycerol were isolated after passing the product through a basic aluminum oxide column. Industrial feasibility of this process was discussed for the ethyl ester as substrate.

KEY WORDS: Docosahexaenoic acid, docosahexaenoyl ethyl ester, eicosapentaenoic acid, immobilized lipase, lipase, polyunsaturated fatty acid, triacylglycerol, triarachidonoyl glycerol, tridocosahexaenoyl glycerol, trieicosapentaenoyl glycerol.

There is considerable evidence of the beneficial role of long-chain polyunsaturated fatty acids (PUFA) in biochemical activities. Thomasson and Gottenbos (1) compared the essential fatty acid activity of linoleic acyl groups in triacylglycerol (TG) and methyl and ethyl esters in rats under conditions of restricted water supply and they found that acyl groups in TGs were about 50% more active than those in methyl and ethyl esters. Human absorption of fish oil fatty acids is better as TG than as ethyl ester (2). This suggests that the desirable form of fatty acids is bound into TG, the most common form in edible oils and fats.

Marine n-3 PUFAs, particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 20:6n-3), can lower plasma lipid concentration, modulate eicosanoid formation, and change cell membrane properties. PUFA reduces the incidence of cardiovascular and inflammatory diseases (3), while deficiencies of n-3 PUFA influence the fatty acid composition of the brain. In particular, the DHA content in the rat brain cell is reduced (4). Deficiency of n-3 PUFA is correlated with reduced visual performance in rhesus monkeys (5) and, together with the effects on learning ability (4), indicates the importance of n-3 PUFA for neural performance. However, volunteers who consumed large amounts of fish oils have occasionally shown severe thrombocytopenia, possibly caused by the high level of long-chain monoenes ingested (6,7). Gadoleic and cetoleic acid (20:1 n-9 and 22:1 n-11, respectively) are found in fairly large amounts in some herring and salmon oils (8,9).

Pure TG containing beneficial PUFA has medical uses, such as for intravenous infusion as well as in health foods. Highly pure trieicosapentaenoyl glycerol (trieicosapentaenoin or tri-EPA) has been used as an infusion to study thrombotic disorders in humans (10). Many attempts to prepare n-3 PUFA TG with lipase have been reported (11–18), but no one has examined the industrial feasibility of this process. The present report describes a simple method to prepare and isolate high yields of pure TG from PUFA with immobilized lipase, repeated batch reactions of the process, and some properties of the pure TG of DHA, EPA and arachidonic acid (AA).

EXPERIMENTAL PROCEDURES

Materials. EPA (98.7%), eicosapentaenoyl ethyl ester (EPA ethyl ester 98.9%) and docosahexaenoyl ethyl ester (DHA ethyl ester 90.4%) were donated by Maruha Co. (Tokyo, Japan), and the ester (95% DHA ethyl ester and 5% EPA ethyl ester, the commercial name is DHA95E) was donated by Harima Kasei Co. (Osaka, Japan). DHA (96.4%) was obtained from Idemitsu Materials Co. (Tokyo, Japan). AA (99.1%) was obtained from Kurita Water Industries Ltd. (Tokyo, Japan). α-Linolenic acid and γ-linolenic acid were the products of Serdary Research Laboratories Inc. (London, Ontario, Canada). Linoleic acid was the product of Nu-Chek-Prep Inc. (Elysian, MN). Lipase from *Rhizomucor miehei* immobilized on Duolite (Lipozyme IM 60), lipase from *Candida antarctica* immobilized on acrylic resin (sp 382), and the *Candida* lipase gene expressed on *Aspergillus oryzae* and immobilized on macropous acrylic resin (SP435) were donated by Novo Nordisk Bioindustry (Chiba, Japan).

Reaction method. The reaction mixture was placed in a glass tube. The tube was kept in an ethylene glycol bath under shaking and with dehydration or removal of the alcohol (in the case of ethyl ester as the substrate). The reaction was carried out with a vacuum pump and water trap (or ethanol trap) as shown in Figure 1. The typical procedure to prepare tridocosahexaenoyl glycerol (tri-DHA) was as follows: 0.985 g (29.98×10^{-4} mole) of DHA, 0.0829 g (9.00×10^{-4} mole) of glycerol, and 0.1 g of immobilized lipase (SP 382) were reacted with dehydration by vacuum pump and molecular sieve column. After the reaction at 60°C for 24 h, 94.6% of tri-DHA was obtained. Passage through aluminum oxide 90 gave 0.63 g (6.16×10^{-4} mole) of pure tri-DHA.

Assay methods. The free fatty acid (FFA), monoacylglycerol (MG), diacylglycerol (DG) and TG contents of the reactant were determined by high-performance liquid chromatography (HPLC) with gel permeation chromatography (GPC) columns as described previously (19). The fatty acid composition was determined by gas chromatography (GC) with a capillary column. The column was a fused-silica WCOT, stationary phase CP-Sil-88 (Chrompack, Middelburg, The Netherlands), detector flame-ionization detector and carrier gas (He). Each sample (0.03–0.05 g) was saponified with 1–2 mL of 0.5 N NaOH methanolic solution at 80°C for 5 min. Methylation of the

[1]Portions of this article were presented at the annual meeting of the Japan Society for Bioscience, Biotechnology, and Agrochemistry and at the annual meeting of The Japan Oil Chemists' Society held in Kyoto, Japan, March 31, 1991, and in Hamamatsu, Japan, October 4, 1991, respectively.

*To whom correspondence should be addressed at National Institute of Bioscience and Human Technology, 1-1 Higashi, Tsukuba 305, Japan.

[2]Current address: Boso Oil & Fat Co. Ltd., 2-17-1 Hinode-chou, Funabashi, Chiba 273, Japan.

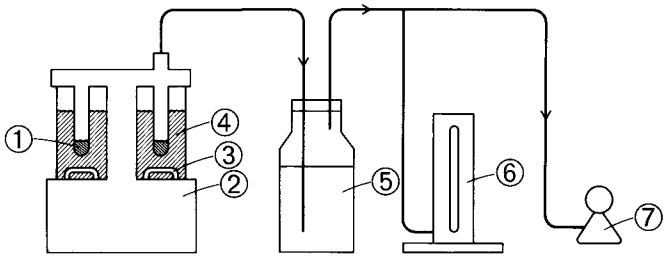

FIG. 1. Reactor with vacuum for dehydration. 1, Reaction mixture; 2, test tube shaker; 3, heater at 60°C; 4, ethylene glycol; 5, water trap, packed with molecular sieve 5A and silica gel, or ethanol trap, packed with granular activated charcoal; 6, manometer to adjust the vacuum at 5–10 Torr; 7, vacuum pump.

saponified sample was carried out with 3–5 mL of boron trifluoride-methanol complex (Merck 801663; Merck, Darmstadt, Germany) at 100°C for 1 min. Gas-chromatographic peaks were identified by comparison with fatty acid methyl ester standards (GL Sciences, Tokyo, Japan). Isomers of the fatty acids, *cis* and *trans*, were resolved into separate fractions under those conditions. The ^1H nuclear magnetic resonance (NMR) spectra were obtained at room temperature (27°C) with 20 mg of sample in 0.5 mL $CDCl_3$ on a JNM-GSX-500 instrument (500.2 MHz; JEOL, Tokyo, Japan). The sampling was done in a glove box saturated with argon. Fourier transform-infrared (FT-IR) spectra were obtained with a sample layer on a NaCl plate in a JIR-100 instrument (JEOL).

Purification of the reaction product. The reaction mixture (1–2 g) was passed through aluminum oxide 90 (Merck 1076) in a glass column with a diameter of 1.7 cm and length 8 cm. The eluent was ethyl ether that had been dried with molecular sieves. Polar impurities, composed of residual FFA, MG and DG, as well as fish odor compounds, were adsorbed on the basic aluminum oxide column and the nonpolar compounds of TG were eluted with the ethyl ether. The ethyl ether in the fraction was evaporated by bubbling dry nitrogen. Pure TG product containing PUFA was stored under argon at −40 to −60°C. Purified yields of TG from DHA and EPA were 66.2 and 60.1%, respectively.

RESULTS

EPA contains five *cis* double bonds, and the compound is chemically reactive. We anticipated that low temperatures might be preferable to synthesize TG from EPA. However, the TG yield was the same at 60 and 40°C, and the reaction rate was faster at 60°C, as shown in Table 1. Ergan and Trani (20) found that the amount of immobi-

TABLE 1

Effect of Reaction Conditions[a]

PUFA	Lipase	Amount of lipase (%)[b]	Reaction temperature (°C)	Time (h)	Product component (wt/wt %)			
					TG	DG	MG	FA
EPA	R[c]	10	40	24	41.0	60.0	0	0
				94	75.8	20.3	0	4.8
				192	82.6	17.4	0	0
EPA	R[c]	5	60	24	69.5	30.5	0.1	0
				48	81.1	18.5	0.4	0
				72	82.8	16.9	0.2	0
EPA	R[c]	10	60	7	36.6	54.7	2.4	6.3
				24	79.6	18.3	2.1	0
				48	81.6	16.7	1.8	0
EPA	C[d]	10	60	7	64.0	34.1	1.9	0
				24	80.7	17.1	2.2	0
				48	79.0	18.5	2.3	0
DHA	R[c]	10	40	24	0.0	13.1	10.1	76.9
				94	0.0	17.5	12.3	70.9
				192	0.0	12.9	9.4	80.0
DHA	C[d]	10	60	22	89.7	13.0	0.0	0.0

[a]The substrate was the stoichiometric amount of polyunsaturated fatty acids (PUFA) and glycerol necessary for synthesizing triacylglycerols (TG). Reaction was carried out with shaking and dehydration by vacuum pump. DG, diacylglycerols; MG, monoacylglycerols; FA, fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

[b]Enzyme amount (%) was expressed as 100 × weight of immobilized lipase/weight of PUFA.

[c]Immobilized lipase from *Rhizomucor miehei.*

[d]Immobilized lipase from *Candida antarctica* (SP 382) (Novo Nordisk, Chiba, Japan).

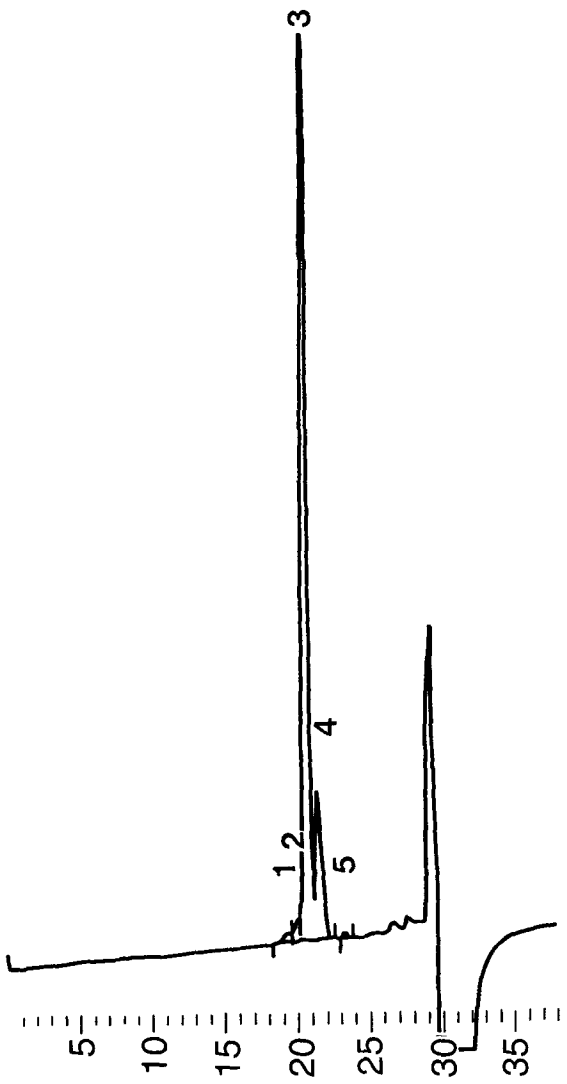

FIG. 2. High-performance liquid chromatography analysis of product from stoichiometric amounts of substrates. Reaction mixture contained 1 g docosahexaenoic acid, 0.0934 g glycerol and 0.1 g immobilized lipase (SP382) (Novo Nordisk Bioindustry, Chiba, Japan). Reaction was carried out at 60°C, for 24 h with shaking and dehydration by vacuum pump. Peaks 3, 4 and 5 were identified as triacylglycerol, diacylglycerol and monoacylglycerol, respectively, as described previously (Ref. 18).

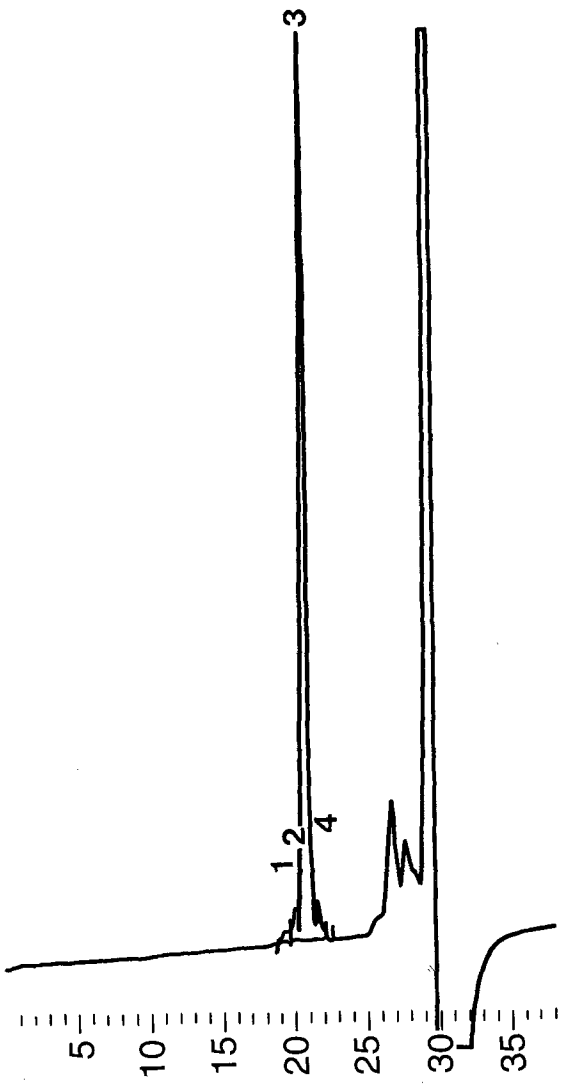

FIG. 3. High-performance liquid chromatography analysis of product when docosahexaenoic acid (DHA) amount was 10% in excess above the stoichiometric amount. Reaction mixture contained 0.985 g DHA, 0.0829 g glycerol and 0.1 g immobilized lipase (SP382). Other conditions were the same as shown in Figure 1. Peaks 3 and 4 were triacylglycerol and diacylglycerol, respectively. See Figure 2 for company source for SP382.

lized lipase did not affect the rate of TG formation from oleic acid and glycerol at 60°C in the early stage of the reaction. They considered that the enzymatic reaction is not the limiting step of the overall synthesis when using the immobilized lipase of *R. miehei* as a catalyst. However, we found that TG formation is a little faster when the immobilized lipase amount was doubled. The formation rate from EPA and glycerol with the immobilized lipase of *C. antarctica* was much greater than that with the immobilized lipase of *R. miehei* (Table 1). The immobilized lipase from *C. antarctica* could synthesize TG from DHA. The immobilized lipase from *R. miehei* did not catalyze the reaction of tri-DHA formation, but it catalyzed the reactions of mono-DHA and di-DHA formation (Table 1).

Figure 2 shows the molecular weight fractionation analysis of the product from DHA reacted at 60°C for 24 h. The peak area percentage of TG (no. 3), DG (no. 4) and MG (no. 5) were 84.5, 12.4 and 0.4%, respectively. The peaks of no. 1 and 2 had area percentages of 1.2 and 1.6%, respectively. These peaks are the polymerization products from DHA with molecular weights of more than 1000. These peaks were also observed in the product of TG synthesis from EPA and even occurred in the reaction at 40°C. Conditions to decrease the amount of DG were examined. When the amount of glycerol was decreased by

5–10%, the DG content decreased from 12.4 to 1–2%, and the TG yield from DHA increased from 84.5 to 94–96% (Fig. 3 and Table 2). The polymerization products of peak 1 (1.1%) and peak 2 (2.8%) did not increase significantly (Fig. 3). On the contrary, DHA ethyl ester was not polymerized, and a maximum TG yield was obtained at the stoichiometric amount of substrate concentration (Table 2). The residual ethyl ester was difficult to eliminate by aluminum oxide or silica gel adsorption. The ethyl ester was eliminated when more than 10% excess of glycerol over the stoichiometric amount was used (Table 2). Table 3 shows that TG synthesis from various fatty acids or fatty acids esters was possible with the immobilized lipase from *R. miehei* and *C. antarctica*. Table 4 shows that tri-

TABLE 2

Effect of Glycerol Concentration on Conversion of DHA[a] or DHA Ethyl Ester (EE)[b] to TG

Glycerol		Product component (area %)					
Amount (10^{-3} mole)	Ratio (%)[c]	Polymer	TG	DG	MG	FA	EE[d]
1.014	100.0	2.7	84.5	12.4	0.4	0.0	
0.965	95.2	2.6	94.7	2.4	0.3	0.0	
0.939	92.6	2.9	95.8	1.3	0.0	0.0	
0.913	90.0	3.4	95.4	1.2	0.0	0.0	
0.888	87.5	2.9	95.4	1.8	0.0	0.0	
0.863	85.1	3.1	95.2	1.7	0.0	0.0	
0.374	80.0	0.0	87.7	0.0	0.5		11.3
0.421	90.0	0.0	93.6	0.8	0.0		4.9
0.467	100.0	0.0	94.9	3.7	0.7		0.7
0.514	110.0	0.0	89.8	9.6	0.5		0.0
0.561	120.0	0.0	78.3	20.3	1.3		0.0

[a]Reaction mixtures contained 3.044×10^{-3} mole (1.0 g) of DHA, 0.1 g of immobilized lipase (SP382) and various amounts of glycerol. Reaction was carried out at 60°C for 24 h with shaking and dehydration by vacuum pump. See Table 1 for abbreviations and company source.
[b]Reaction mixtures contained 1.402×10^{-3} mole (0.5 g) DHA EE (DHA95E) (Harima Kasei Co., Osaka, Japan), 0.05 g of immobilized lipase (SP435) (Novo Nordisk Bioindustry, Chiba, Japan) and various amounts of glycerol. Reaction was carried out at 50°C for 24 h with shaking and trapping ethanol by charcoal and vacuum pump. The shaking of the EE substrate should be more vigorous than fatty acid substrate because ester was more difficult to mix with glycerol.
[c]The ratio = 100 × glycerol amount in the reactant (mole)/stoichiometric amount of glycerol necessary for synthesizing TG (mole.
[d]DHA EE.

TABLE 3

Substrate Specificity of Immobilized Lipase for TG Synthesis[a]

	Immobilized lipase			
	Rhizomucor miehei (Lipozyme IM 60)		*Candida antarctica* (SP382)	
	Reaction time (h)			
	24	48	24	48
Substrate (FA and glycerol)				
10:0 Decanoic	79.7	98.4	97.4	98.4
14:0 Myristic	99.2	100.0	100.0	100.0
16:0 Palmitic	97.9	98.2	99.0	100.0
18:1 Oleic	87.1	99.0	77.0	97.5
18:2 Linoleic	81.0	93.2	85.4	91.1
18:3 α-Linolenic n-3	92.1	95.0	86.6	88.4
18:3 γ-Linolenic	66.9	83.4	94.0	94.9
20:4 Arachidonic	82.5	93.7	97.0	95.0
20:5 EPA n-3	80.7	79.2	79.0	81.6
20:6 DHA n-3	0.0	0.0	84.0	—
Substrate (ester and glycerol)				
EPA ethyl ester	1.5	3.1	78.5[b]	—
DHA ethyl ester	0.5	1.3	78.1[c]	—
Ethyl oleate	—	—	81.6	—
Methyl oleate	72.8	82.8	83.7	—

[a]TG yields are shown. The reaction mixture contained stoichiometric amounts of substrates necessary for synthesis of TG and immobilized lipase. The amount of immobilized lipase was 10% (w/w) of fatty acid or ester. The reaction conditions were the same as shown in footnote *a* in Table 2. See Table 1 for abbreviations.
[b]Glycerol was 8% in excess of the conditions above.
[c]Glycerol was 4% in excess.

TABLE 4

Continual Tri-DHA Synthesis with Ethanol Trap and Vacuum Pump[a]

	TG content (%)			
	Reaction time			
Batch number	2 h	4 h	7 h	23 h
1	16.8	62.6	89.2	97.5
2	22.1	71.4	92.3	97.8
3	18.4	56.8	89.8	97.2
4	15.0	64.9	87.2	96.7
5	11.4	56.2	84.8	96.0

[a]Reaction mixture contained 1.402×10^{-3} mole (0.5 g) DHA ethyl ester (DHA95E) (Harima Kasei Co., Japan), 0.05 g of immobilized lipase (SP435) and 4.67×10^{-4} mole (0.043 g) of glycerol. Reaction was carried out at 50°C for 23 h with vigorous shaking and trapping ethanol by charcoal and vacuum pump. After the reaction, immobilized enzyme was collected by filtration, washed with hexane and reacted repeatedly. See Table 1 for abbreviations and company source.

from DHA ethyl ester with the immobilized lipase could be synthesized repeatedly. After the fifth batch, the product contained 96% TG, 3.0% DG and 0.6% MG. Residual DHA ethyl ester was not observed in the product, probably because several samplings from the top of the reaction mixture caused an excess amount of glycerol concentrated in the bottom of the reaction mixture. The half life of the immobilized lipase under these reaction conditions was about 120 h, if calculated from the change of the initial TG production rate.

For the purification of TG from PUFA, filtration through the aluminum oxide was used. Purified TG from DHA, EPA and AA contained more than 99% TG (HPLC analysis). The composition of the fatty acid in the synthesized TG was the same as the fatty acid substrate (GC analysis). The isolated TGs from DHA, EPA and AA were colorless, transparent, viscous liquids, and did not solidify at −60°C. The spectral data were assigned according to the literature (21,22). Table 5 shows that the observed integral intensity of ^{1}H NMR peaks of TG from the PUFAs corresponded to theoretically deduced proton numbers. The relative errors were within 3%, except for the peaks of 17-CH_2 and 18,19-CH_2 in tri-AA. The larger integral intensity of the peaks in the tri-AA may be caused

TABLE 5

Assignment of ^{1}H Nuclear Magnetic Resonance Spectra in $CDCl_3$

Chemical shift (ppm)	Signal[a]	Peak areas		Relative error[b] (%)	Functional group	Symbol[e]
		Observed[c]	Predicted[d]			
			Triodocosahexaenoyl glycerol			
5.38	*m*	36.044	37	2.58	Olefinic H, OCH-	*l*
4.29	*dd*	4.000	4	0.00	OCH_2	*j*
4.16	*dd*				OCH_2	*i*
2.84	*m*	29.597	30	1.34	6-,9-,12-,15-,18-CH_2	*h*
2.38	*m*	11.890	12	0.92	2-,3-CH_2	*g*
2.07	*p*	6.146	6	−2.43	21-CH_2	*e*
0.97	*t*	8.979	9	0.23	22-CH_3	*a*
Total		96.66	98	1.34		
			Trieicosapentaenoyl glycerol			
5.37	*m*	30.358	31	2.07	Olefinic H, OCH	*l,k*
4.29	*dd*	4.000	4	0.00	OCH_2	*j*
4.16	*dd*				OCH_2	*i*
2.84	*m*	23.441	24	2.32	7-,10-,13-,16-CH_2	*l*
2.33	*m*	5.886	6	1.90	2-CH_2	*g*
2.08	*m*	11.888	12	0.93	4-,19-CH_2	*e*
1.69	*m*	5.902	6	1.63	3-CH_3	*d*
0.97	*t*	8.718	9	3.13	20-CH_3	*a*
Total		90.19	92	1.97		
			Triarachidonoyl glycerol			
5.37	*m*	24.616	25	1.53	Olefinic H	*l*
5.27	*m*				OCH	*k*
4.29	*dd*	4.000	4	0.00	OCH_2	*j*
4.15	*dd*				OCH_2	*i*
2.81	*m*	17.670	18	1.83	7-,10-,13-CH_2	*h*
2.33	*m*	5.891	6	1.82	2-CH_2	*g*
2.10	*m*	11.909	12	0.26	4-CH_2[f]	*f*
2.05	*q*				16-CH_2[f]	*e*
1.70	*m*	6.193	6	−3.21	3-CH_2	*d*
1.36	*m*	19.433	18	−7.96	17-CH_2[f]	*c*
1.30	*m*				18-,19-CH_2[f]	*b*
0.89	*t*	9.000	9	0.00	20-CH_3	*a*
Total		98.77	98	−0.79		

[a]Signals of *dd*, *t*, *q*, *p* and *m* are double doublet, triplet, quartet, quintet and multiplet.
[b](Theoretical predicted proton number − observed relative area) × 100/theoretical predicted proton number.
[c]Observed area of peaks was expressed relative to integral value when the peaks (*i* + *j*) of OCH_2 were defined as 4.000.
[d]Theoretically predicted proton number.
[e]Symbols are the position of the functional group as shown in Figure 4.
[f]Their assignments were done by ^{1}H-^{1}H cosy measurement.

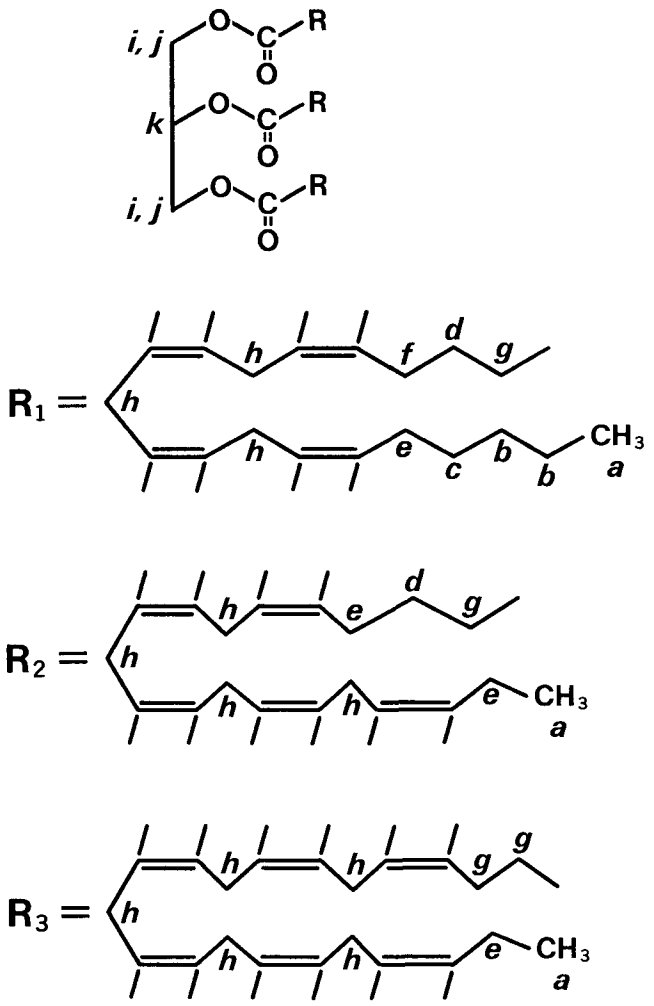

FIG. 4. Chemical structure of triarachidonoyl glycerol, trieicosapentaenoyl glycerol and tridocosahexaenoyl glycerol. R_1, R_2 and R_3 were designated arachidonyl, eicosapentaenoyl and docosahexaenoyl residue, respectively. Symbols *i–k* are positions of Functional group column in Table 5.

TABLE 6

FT-IR Spectra of TG from PUFA

Functional group[a]	Position (cm^{-1})		
	tri-AA	tri-EPA	tri-DHA
νCH=	3012	3012	3014
$\nu_{as}CH_3$	2956	2964	2964
$\nu_{as}CH_2$	2927	2933	2933
$\nu_s CH_3$	2872	2873	2873
νC=O ester	1745	1745	1745
νC=C (*cis*)	1655	1653	1653
$\delta_{as}CH_3$	1456	1456	1441
$\delta_s CH_3$	1392	1392	1392
νC-O ester	1144	1144	1147

[a]Symbols ν, δ, as and g represent stretching, deformation, assymmetric and symmetric vibrations, respectively; FT-IR, Fourier transform infrared. See Table 1 for other abbreviations.

by contaminated residual ethyl ether. Table 6 shows that the absorption peak positions in FT-IR spectra of the three TGs were nearly same. The stronger three peaks in tri-DHA spectra were 1745 (the strongest), 3014 and 1147, in tri-EPA they were 1745, 3012 and 1144, and in tri-AA they were 1745, 1144 and 2927 cm^{-1}. The above data for TGs from DHA, EPA and AA coincide with the theoretically possible values for tri-DHA, tri-EPA and tri-AA, respectively.

DISCUSSION

Adlof and Emken (23) chemically synthesized linolenic and linoleic acid-rich TG and purified the products. Their synthesized TGs were purified with Ag^+/a^+ resin. Impurities, fatty acid methyl ester and TG were eluted in separate fractions. The isolated TG was analyzed by ultraviolet and IR spectroscopy and was found to contain no conjugated or *trans* isomers. They analyzed TG by thin-layer chromatography and GC and found that the TG contained no DG or other reaction components. Hamazaki *et al.* (10) synthesized tri-EPA for infusion into two male healthy volunteers. The tri-EPA was synthesized by chemical condensation of glycerol and 90% pure EPA. Partial acylglycerol and impurities were removed in a silica gel column (10).

Production of n-3 PUFA-enriched acylglycerol from fish oil with lipase has been reported, but the products are mixed TG (11-15) and have no evidence of eliminating monoenoic acid content. Osada *et al.* (16) attempted to synthesize pure acylglycerol and reported that lipase from *Chromobacterium viscosum* incorporated PUFA into acylglycerol at levels of 85–95%. However, TG contents were only 26–38%. Tanaka *et al.* (17) employed a two-step esterification process to produce PUFA TG. They used double the stoichiometric amount of PUFA necessary for converting all DG into TG. However, there was no explanation for the excess amount of PUFA in the final product. They did not isolate TG of PUFA. Haraldsson *et al.* (18) reported a similar reaction to this work as a preliminary communication. They synthesized and purified tri-EPA and tri-GDHA with immobilized *C. antarctica* lipase. Their reaction temperature is 65°C, and reaction time is 72–96 h. They monitored the incorporation of the PUFA or PUFA ester by ^1H NMR spectroscopy. They did not observe polymerization products in spite of their reaction conditions of high temperature and long reaction time. They reported that the incorporation rate of PUFA ethyl ester into tributyrin was slower than the direct esterification rate. The slower rate may be improved by using excess amounts (1–2%) of the fatty acid substrates. However, our results clearly show that excess amounts of fatty acid ester decreased TG content (Table 2). We found merits in using fatty acid ethyl esters, which are more stable than free fatty acids, and we used them for the experiment to study the industrial feasibility of this process (Table 4).

We have reported earlier the enzymatic refining of high-FFA rice bran oil (19,24–27). FFA, MG and DG in the oil were esterified with glycerol to produce TG by means of immobilized lipase from *R. miehei*. The substrate was the stoichiometric amount of the FFA oil and glycerol required for synthesizing TG. The reaction was continued for more than one month by a reactor with two circulation loops, each being connected to a fixed-bed reactor and a dehydrator. This technology was applied to synthesize TG from PUFA (28–32). The reaction temperature and time in this work were 50–60°C and 23–48 h, respectively, because

temperatures above 50°C could not significantly improve the reaction rate. Lower temperature and shorter time are better to prevent thermal decomposition of the PUFA. Free DHA and EPA were polymerized during the reaction. The amount of DHA or EPA in the substrate was 5–10% higher than the stoichiometric amount required to compensate for the loss during the reaction. On the contrary, DHA ethyl ester and EPA ethyl ester were not polymerized during the reaction. The maximum yield of TG was obtained with stoichiometric amounts of the ethyl ester and glycerol necessary for synthesizing TG. Excess amounts of PUFA ester substrate caused an increase of DG (Table 2). For DHA and ethyl esters of DHA and EPA, the immobilized lipase from *C. antarctica* was used because the immobilized lipase from *R. miehei* did not synthesize TG from these substrates.

DHA and ethyl ester and EPA ethyl ester were more stable than free DHA and EPA. Residual ethyl ester can be reduced by using an excess amount of glycerol (Table 2). Because this experiment was performed on a small scale, a repeated batch reaction was carried out with vigorous shaking and an ethanol trap and a vacuum pump under reduced pressure. The immobilized lipase was washed with hexane and used repeatedly. The results in Table 4 show that about 95% tri-DHA was obtained for a day with 10% immobilized enzyme. Thus the productivity (Po) was 9.5 (kg TG day^{-1} kg $SP435^{-1}$). The cost of the enzyme to produce 1 kg of tri-DHA can be calculated from the productivity (Po), the life span of the immobilized lipase (5 d from Table 4) and the enzyme price (100,000 yen/kg of SP435 in Japan) as follows: enzyme cost (yen/kg of tri-DHA) is 2.105. The enzyme cost was less than 0.3% of the substrate price because the price of DHA ethyl ester in Japan is about 1,000 yen/g. The enzyme span of 5 d can be prolonged by using more time, repeated use, or other type of reactor, as previously reported (19,27). The enzymatic process is carried out at ambient pressure and temperature, so the cost of energy and capital investment required for setting up the process will not be too high. Large markets for homogeneous TG will create the need for industrial usage of this process. The market will be realized in the near future because many studies of the dietary effects of PUFA and oxidative stability of PUFAs are now reported.

TG of EPA, DHA or AA were purified by passing through a basic aluminum oxide column. The eluent contained only TG in ethyl ester. The TG was isolated easily by evaporating ethyl ether from the eluent. Isolated tri-AA, tri-EPA and tri-DHA were colorless, transparent, viscous liquids, and did not solidify at −60°C. Isolated samples gave one peak on a GPC column and were free of DG, MG, FFA and polymerization products. Isolated samples, when checked by capillary-column GC, were not contaminated by *trans* isomers or volatile by-products. Isolated samples gave reasonable spectral data and did not display conjugation or migration during the process of the enzymatic synthesis. Pure TGs from PUFA could be used for fundamental metabolic studies, such as synthesis of deuterium-labeled compounds as well as for medical purposes, i.e., for intravenous infusion to prevent thrombotic disorders (10). The dietary form of TG from PUFA will be mixed in an aqueous solution because Miyashita *et al.* (33) reported that oxidative stability of PUFA (33) in aqueous solution increases with increasing degree of unsaturation.

REFERENCES

1. Thomasson, H.J., and J.J. Gottenbos, *Proc. Soc. Exp. Biol. Med. 111*:261 (1962).
2. Lawson, L.D., and B.G. Hughes, *Biochem. Biophys. Res. Commun. 152*:328 (1988).
3. Budowski, P., *Wld. Rev. Nutr. Diet. 57*:217 (1988).
4. Bourre, J.M., M. Francois, A. Youyou, O. Dumont, M. Piciotti, G. Pascal and G. Durand, *J. Neurochem. 119*:1880 (1989).
5. Neuringer, M., W.E. Conner, D. Lin, L. Barstad and S. Luck, *Proc. Natl. Acad. Sci. USA 83*:4021 (1986).
6. Goodnight, S.H., Jr., W.S. Harris and W.E. Coner, *Blood 58*:880 (1981).
7. Sinclair, H.M., *Drugs Affecting Lipid Metabolism*, Elsevier, North-Holland, Amsterdam, 1982, pp. 363–370.
8. Ackman, R.G., *Nutritional Evaluation of Long-Chain Fatty Acids In Fish Oil*, Academic Press, London, 1982, pp. 25–88.
9. Sasaki, S., T. Ota and T. Takagi, *Nippon Suisan Gakkaishi 55*:1655 (1989).
10. Hamazaki, T., S. Fischer, H. Schwer, C.O. Meese, M. Urakase, A. Yokoyama and S. Yano, *Biochem. Biophys. Res. Commun. 151*:1386 (1988).
11. Haraldsson, G.G., and P.A. Hoskuldsson, *Tetrahedron Lett. 30*:1671 (1989).
12. Osada, K., M. Nakamura, M. Nonaka and M. Hatano, *Jpn. Oil Chem. Soc. 41*:39 (1992).
13. Yamane, T., T. Suzuki, Y. Sahashi, L. Vikersveen and T. Hoshino, *J. Am. Oil Chem. Soc. 69*:1104 (1992).
14. Tanaka, Y., J. Hirano and T. Funafa, *Ibid. 69*:1210 (1992).
15. Adachi, S., K. Okumara, Y. Ota and M. Mankura, *J. Ferment. Bioeng. 75*:259 (1993).
16. Osada, K., K. Takahashi and M. Hatano, *J. Am. Oil Chem. Soc. 67*:921 (1990).
17. Tanaka, Y., T. Funada and J. Hirano, *J. Jpn. Oil Chem. Soc. 41*:563 (1992).
18. Haraldsson, G.G., B.O. Gudmundsson and O. Almarsson, *Tetrahedron Lett. 34*:5791 (1993).
19. Kosugi, Y., T. Kunieda and A. Naoki, *J. Am. Oil Chem. Soc. 71*:445 (1994).
20. Ergan, F., and M. Trani, *Biotechnol. Lett. 13*:19 (1991).
21. Wright, S.W., E.Y. Kuo and E.J. Corey, *J. Org. Chem. 52*:4399 (1987).
22. Corey, E.J., and S.W. Wright, *Ibid. 53*:5980 (1988).
23. Adlof, R.O., and E.A. Emken, *J. Am. Oil Chem. Soc. 61*:83 (1984).
24. Kosugi, Y., and N. Azuma, *Proceedings of 29th Annual Meeting of J. Oil Chem. Soc.*, October 16, 1990, Sendai, 1990, p. 165.
25. Kosugi, Y., and N. Azuma, *Proceedings of Annual Meeting of the Society of Fermentation Technology*, November 7, 1991, Hiroshima, 1991, p. 201.
26. Kosugi, Y., and N. Azuma, *Abstracts of 9th International Biotechnology Symposium and Exposition*, Crystal City, Virginia, 1992, No. 430.
27. Kosugi, Y., and N. Azuma, *Appl. Microbiol. Biotechnol. 41*:407 (1994).
28. Kosugi, Y., C. Lopez and N. Azuma, *Nippon Nogeikagaku Kaishi. 65*:438 (1991).
29. Kosugi, Y., and N. Azuma, *Proceedings of 30th Annual Meeting of J. Oil Chem. Soc.*, October 4, 1991, Hamamatsu, 1991, p. 60.
30. Kosugi, Y., C. Lopez and N. Azuma, *Abstracts of 9th International Biotechnology Symposium and Exposition*, Crystal City, Virginia, 1992, No. 398.
31. Kosugi, Y., M. Shiraki and N. Azuma, Japan Kokai, Hei5-331105 (1993).
32. Kosugi, Y., M. Shiraki and N. Azuma, Japan Kokai, Hei6-72959 (1994).
33. Miyashita, K., E. Nara and T. Ota. *Biosci. Biotech. Biochem. 57*:1638 (1993).

[Received May 12, 1994; accepted September 13, 1994]