Monitoring of Lipase-Catalyzed Transesterification Between Eicosapentaenoic Acid Ethyl Ester and Tricaprylin by Silver Ion High-Performance Liquid Chromatography and High-Temperature Gas Chromatography

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ABSTRACT: A high-resolution method based on silver ion high-performance liquid chromatography (HPLC) and high-temperature gas chromatography (HTGC) with on-column injection was established for the separation of triacylglycerols (TAG) which contained eicosapentaenoic acid (EPA). A solvent system based on *n*-hexane, 2-propanol, and acetonitrile in HPLC for the separation of EPA-containing TAG was developed. It was dependent upon the number of EPA molecules and their isomeric distribution on the glycerol backbone. This technique was used to monitor the incorporation of EPA onto specific positions on the glycerol backbone. For quantitative monitoring of the synthesis reaction of the TAG containing EPA, an HTGC analysis was developed. For monitoring of the synthesis of structured TAG containing EPA, transesterification of tricaprylin with EPA ethyl ester in a solvent-free system was performed. The transesterification reaction using various lipases was studied at a different molar ratios of substrates, several initial water activities (a_w), and various reaction temperatures.

Paper no. J8887 in *JAOCS 76,* 31–39 (January 1999).

KEY WORDS: Polyunsaturated fatty acid, separation of positional isomers, silver ion HPLC, solvent-free system, structured triacylglycerol, transesterification.

Recently much attention has been given to structured triacylglycerol (TAG) in which a specific fatty acid is esterified at a specified position of each position of glycerol backbone (1–3). Of specific interest are structured TAG having polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA), docosahexaenoic acid, or arachidonic acid, because of the pharmacological effects of these fatty acids (4–9).

For the production of targeted structured TAG, it must be known which types of TAG are formed and how many fatty acids are incorporated at a specific glycerol position. In particular, we were interested in the TAG containing one PUFA molecule and two medium-chain fatty acids. It has been reported that the position of PUFA on the glycerol backbone was very important for its bodily absorption and nutritional and pharmacological properties (1,3). In this study, the separation of each TAG containing 1 EPA at the *sn*-1 (or 3) or the *sn*-2 position was our focus because it was needed to investigate selectivity of lipases to produce the target product.

For determination of the positional distribution of acyl groups in TAG, several methods, such as enzymatic hydrolysis (10,11) or chemical degradation using a Grignard reagent (12,13) followed by analysis of the mono- and diacylglycerol products by chromatography techniques, have been used. Partial lipase hydrolysis of TAG may cause acyl migration, resulting in erroneous identification of the acyl positional distribution. In addition, the lipase method is not reliable for TAG that contain significant amounts of short- or medium-chain fatty acids or very long chain PUFA (12). In chemical degradation analysis, analytical error can occur readily, and it is time-consuming and labor intensive. Some papers have used ^{13}C nuclear magnetic resonance (NMR) of TAG to determine the positional distribution of fatty acids on the glycerol backbone (14,15). This technique is very simple, useful, and precise for the analysis of the positional distribution of PUFA in TAG. None of the above methods, however, tells what kinds of molecular species of TAG are present. Only the positional distributions of fatty acids are determined. To know the composition of a sample and its positional distribution of PUFA, the sample must be separated and purified into each molecular species by some chromatographic technique, and then the purified species must be analyzed by NMR or chemical or enzymatic analysis. Accordingly, an easy, simple, and accurate method to determine simultaneously both the molecular species composition of TAG and the positional distribution of fatty acids is needed.

Silver ion chromatography is a technique that utilizes the property of silver ions to form reversible polar complexes with double bonds in organic molecules, such as unsaturated lipids (16). The technique enables separation of unsaturated species according to the number, geometrical configuration, and position of the double bond. Some workers have demonstrated the

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separation of positional isomers of TAG that contained fatty acids having the same number of double bonds (16–18). However, no separation of isomeric TAG containing long-chain PUFA such as EPA was reported. Here, we report a high-resolution method based on both silver ion high-performance liquid chromatography (HPLC) and high-temperature gas chromatography (HTGC) to separate TAG which contain EPA. We applied this technique to monitor the specific incorporation of EPA onto the glycerol molecule. For the monitoring of the synthesis of structured TAG containing EPA, transesterification of tricaprylin with EPA ethyl ester (EPAEE) in a solvent-free system was performed. We investigated this reaction using various lipases at different molar ratios of substrates, initial water activity (a_w) of lipases, and reaction temperatures.

MATERIALS AND METHODS

Materials. Tricaprylin (1,2,3-trioctanoylglycerol), caprylic acid (octanoic acid), and caprylic acid ethyl ester (octanoic acid ethyl ester) were obtained from Wako Pure Chemicals (Osaka, Japan). Dicaprylin (dioctanoylglycerol) was purchased from Funakoshi Co. (Tokyo, Japan). EPAEE (*cis*-5,8,11,14,17-eicosanpentaenoic acid ethyl ester) was kindly donated by Nippon Suisan Co. (Tokyo, Japan). TEPA [1,2,3-tri(*cis*-5′,8′,11′,14′,17′-eicosapentaenoyl)glycerol] was kindly donated by Bohsoh Yushi Co., Ltd. (Chiba, Japan). Lipases used were as follows: Lipozyme (*Rhizomucor miehei*, 1,3-specific), and Novozyme (*Candida antarctica*, specificity depends on reactants) were kindly donated by Novo Nordisk Bioindustry (Chiba, Japan), and Liposam (*Pseudomonas pseudoalkali*, positionally nonspecific) was donated by Showa Denko (Tokyo, Japan). All chemicals used were reagent grade and the solvents used were HPLC grade and purchased from Wako Pure Chemicals.

Adjustment of initial a_w *of lipase*. To obtain a defined initial a_w of the enzyme, it was equilibrated over a saturated salt solution in a closed desiccator for 3 d at 25°C. A saturated solution of $Mg(NO_3)_2$ (a_w = 0.52) was used unless otherwise indicated. Other saturated salt solutions used were LiCl (a_w = 0.11) and KCl (a_w = 0.86).

Transesterification reaction. Transesterification was performed with 2.35 g (5 mmol) of tricaprylin and 0.33 g (1 mmol) of EPAEE using 50 mg of 1,3-specific lipase (Lipozyme) or nonspecific lipase (Liposam) to produce reaction mixture containing various kinds of TAG with EPA. The tricaprylin and EPAEE were first mixed vigorously by a magnetic stirrer in a closed vial. The reaction was started by adding the lipase, and the reaction mixture was placed into a water bath at a predetermined temperature with stirring at 400 rpm. Unless otherwise stated, transesterification was performed from a 3:1 molar ratio of tricaprylin to EPAEE using 50 mg of lipase at 40°C.

To study the effects of initial a_w of the lipase and reaction temperature on the incorporation of EPA into tricaprylin, 3 mmol of tricaprylin and 1 mmol of EPAEE were mixed at 40°C. The reaction was started by adding the lipase that had been preequilibrated over a saturated salt solution. For monitoring the reaction at a defined a_w, the a_w of enzyme and substrates should be adjusted to the same value. However, since the equilibration process takes time, the result might be complicated by oxidation of EPAEE. Therefore, in the present investigation, only the a_w of lipase was adjusted. The investigated a_w values of lipases were 0.11, 0.52, and 0.87, and the reaction temperatures were 40 and 55°C at a_w = 0.52.

Molar ratios of tricaprylin to EPAEE from 3:1 to 2:1 and 1:1 were investigated to study the effect of molar ratio on the incorporation of EPAEE to tricaprylin at a_w = 0.52 and 40°C.

Assay methods. For time course analysis, aliquots were taken from the vial at predetermined intervals and diluted to 1% solutions in *n*-hexane/2-propanol (7:2, vol/vol) mixture. Solid materials were removed by filtration through a polytetrafluoroethylene membrane filter. The eluates were analyzed by HPLC and HTGC.

All HPLC analyses were performed using a Chrompack silver ion chromatography column (250 × 4.6 mm, Chrompack, Middleberg, Netherlands) with ultraviolet (UV) detection at 206 nm at room temperature. The solvent gradient program used is shown in Table 1.

Reaction mixtures also were analyzed by HTGC (Shimadzu GC14A: Shimadzu Co., Kyoto, Japan) equipped with an OCI-14 capillary on-column injector. The column used was a Supelco SGE HT5 aluminum-clad fused-silica capillary column (12 m long, 0.53 mm i.d., 0.15 μm film thickness, Supelco Inc., Bellefonte, PA). *n*-Eicosane was used as an internal standard. The column was programmed with an initial temperature of 80°C, held for 0.5 min, heated to 260°C at 20°C/min, then to 330°C at 10°C/min, and finally to 390°C at 5°C/min and held for 1 min. The detector was maintained at 393°C. On-column injection (19) was used for sample transfer onto the analytical column. The on-column injector was programmed with an initial temperature of 80°C, held for 0.01 min, then heated to 260°C at 40°C/min and then to 340°C at 20°C/min, and finally heated to 393°C at 10°C/min and held for 15.6 min. Helium was used as carrier gas at 15 mL/min.

For the liquid chromatography–mass spectroscopy–atmospheric-pressure chemical ionization (LC–MS–APCI) assay, VG platform MS (VG Organic, Beverly, MA) was used for

TABLE 1
The Mobile Phase Gradient Program Used for Silver Ion High-Performance Liquid Chromatography

Time (min)	A (%)[a]	B (%)[b]	Flow rate (mL/min)
0	100	0	0.65
3	100	0	0.65
25	0	100	0.65
33	0	100	0.65
37	100	0	0.65

[a]Solvent A: *n*-hexane/2-propanol/acetonitrile = 350:100:3.5.
[b]Solvent B: *n*-hexane/2-propanol/acetonitrile = 350:100:10.

identifying the components of the reaction mixture. The same HPLC conditions cited above were used. Tuning parameters were: ion source was APCI; corona, 3.2 kV; cone, 30 V; source temperature, 100°C; APCI probe temperature, 400°C; (MS) ion energy, 1.5 V; LM resolution, 12.0; HM resolution. 12.0, multiplier, 650 V; (pressures) analyzer vacuum, 2.2×10^{-5}; backing 5.3×10^{-1}. Function type, scan; cycle time, 4.1 s; scan duration, 3.95 s; interscan delay, 0.5 s; data type, compressed centroid. Scan range was 100–1500 (*m*/*z*). Nitrogen was used as a source gas. The sheath gas flow was 50 L/h and drying gas flow was 300 L/h, respectively.

RESULTS AND DISCUSSION

Silver ion HPLC for the separation of the reaction mixture. Twelve-hour reaction mixtures were analyzed by the silver ion HPLC method. In applying the solvent gradient system (Table 1), the separation of TAG containing EPA depended on the number of EPA molecules and their isomeric distribution. Figure 1A and 1B shows the chromatograms of a reaction mixture produced from a 1,3-specific lipase (Lipozyme) and a nonspecific lipase (Liposam), respectively. Tricaprylin and caprylic acid ethyl ester appeared together at Peak 1, and dicaprylin and caprylic acid eluted together at Peak 2. Peaks 5 and 10 corresponded to EPAEE and TEPA, respectively. The molecular weight of each peak was identified by HPLC–APCI–MS and shown in Table 2. The compounds which were eluted as peaks 3 and 4 had the same molecular weight (MW ≈ 629), thereby the two compounds were considered isomers of TAG-A. This identification was further confirmed by the observation of fragment ions (M – EPA acyl, 327; and M – capryl, 485) as well as the molecular ion (Fig. 1). The identification of Peaks 3 and 4 as TAG-A3 and TAG-A1(2), respectively, was made based on the selectivities of the enzymes. Only Peak 4 appeared in the 1,3-specific lipase reactions, whereas Peaks 3 and 4 were obtained in the nonspecific lipase reaction. Therefore, it was concluded that Peak 4 was the peak of TAG-A1(and/or 2) containing EPA at *sn*-1 (or 3), and Peak 3 was the *sn*-2 positional isomer (TAG-A3). Peak 8 also was identified as TAG-B (MW ≈ 787) containing 2 EPA molecules. The positional isomers of TAG-B were not separated.

The solvent gradient system based on *n*-hexane, 2-propanol, and acetonitrile was very efficient and reproducible for the analysis of the distribution of each TAG containing 1

TABLE 2
Peak Identification for the Transesterification Between Tricaprylin and EPAEE by Silver Ion High-Performance Liquid Chromatography[a]

Peak no.	Possible isomers	MW	No. of double bonds
1	Tricaprylin, caprylic acid ethyl ester		
2	Caprylic acid, dicaprylin		
3	Dioctanoyleicosapentaenoin (TAG-A) — C_8, EPA, C_8 (TAG-A3)	629	5
4	Dioctanoyleicosapentaenoin (TAG-A) — EPA, C_8, C_8 (TAG-A1); C_8, C_8, EPA (TAG-A2)	629	5
5	Eicosapentaenoic acid ethyl ester (EPAEE)	331	5
6	Eicosapentaenoyloctanoyl diacylglycerol (DAG)	503	5
7	Eicosapentaenoic acid (EPA)	303	5
8	Dieicosapentaenoyloctanoin (TAG-B) — EPA, C_8, EPA; EPA, EPA, C_8; C_8, EPA, EPA	787	10
9	Dieicosapentaenoyl diacylglycerol — EPA, EPA, OH; EPA, OH, EPA; OH, EPA, EPA	661	10
10	Trieicosapentaenoin — EPA, EPA, EPA	945	15

[a]Abbreviations: TAG, triacylglycerols; DAG, diacylglycerols.

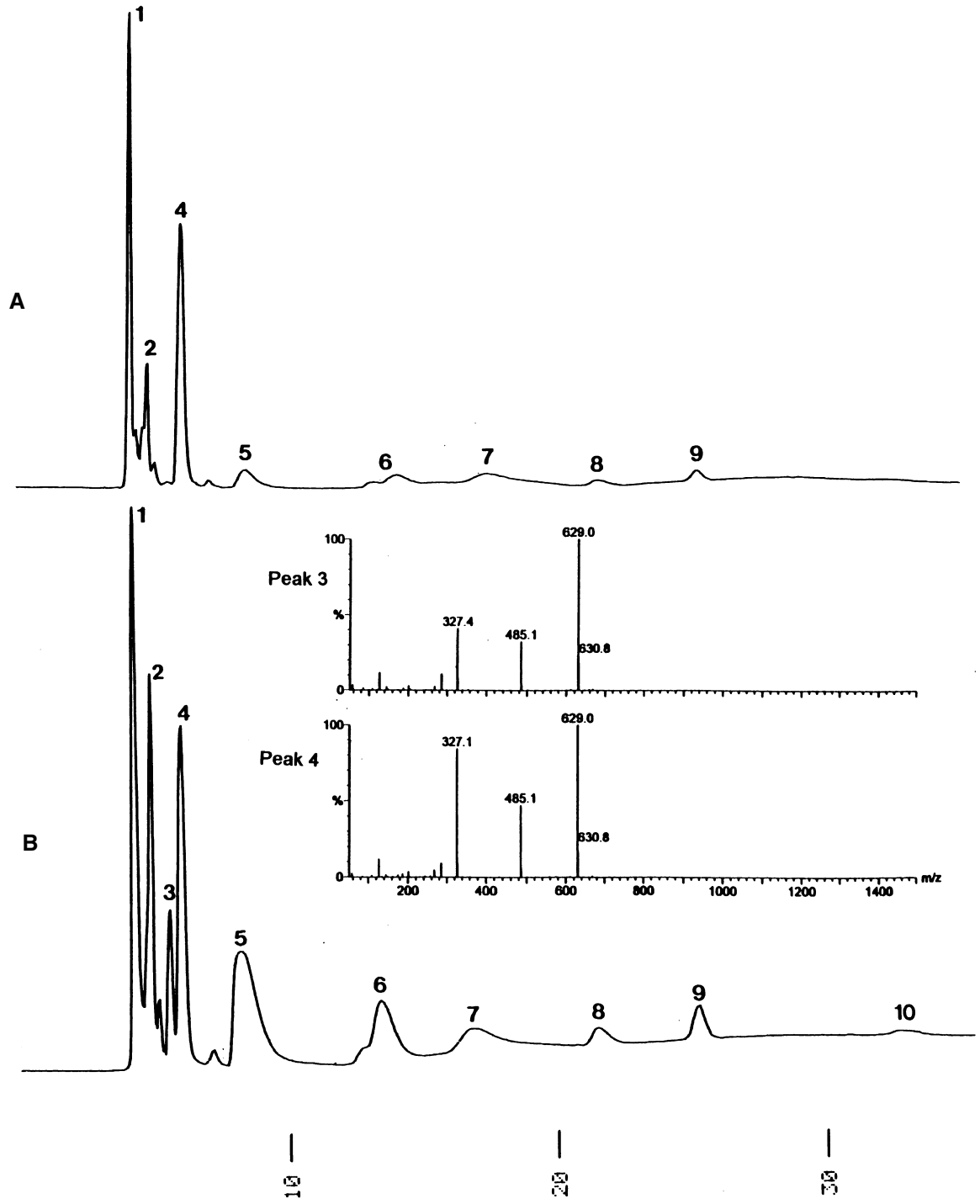

FIG. 1. Silver ion high-performance liquid chromatography (HPLC) chromatogram of reaction mixture by various lipases. (A) Reaction mixture from tricaprylin and eicosapentaenoic acid ethyl ester (EPAEE) by Lipozyme (Novo Nordisk, Chiba, Japan); (B) reaction mixture from tricaprylin and EPAEE by Liposam (Showa Denka, Tokyo, Japan). Peak 1, tricaprylin and caprylic acid ethyl ester; 2, caprylic acid and dicaprylin; 3, triacylglycerol (TAG)-A3; 4, TAG-A1 (and/or TAG-A2); 5, EPAEE; 6, diacylglycerol (DAG); 7, TAG-B; 8, 1,2,3-tri(*cis*-5′,8′,11′,14′,17′-eicosanpentaenoyl)glycerol (TEPA). Molecular ion and fragmentation of peaks 3 and 4 analyzed by HPLC–atmospheric-pressure chemical ionization (APCI)–mass spectrometry (MS) are also shown.

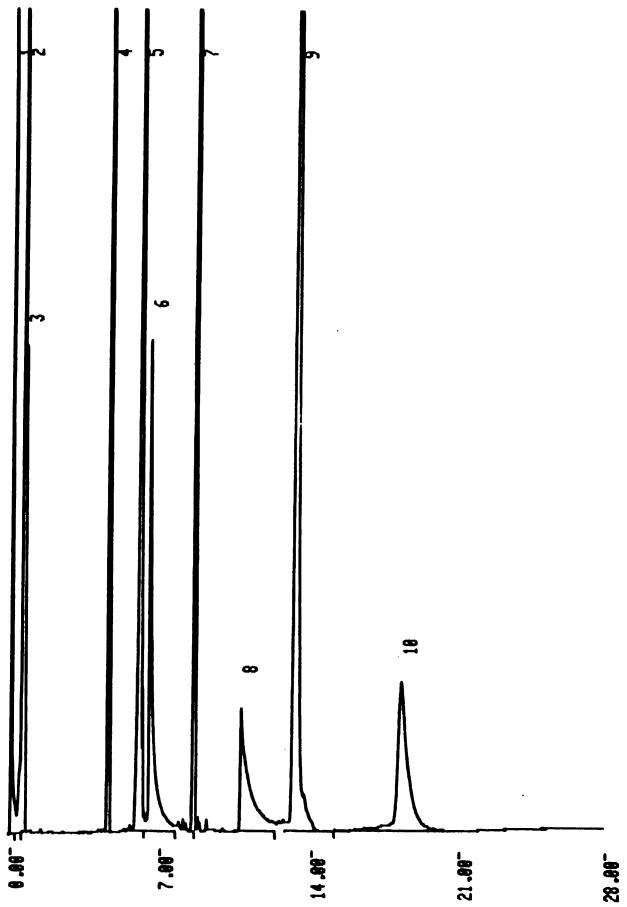

FIG. 2. Gas chromatogram of reaction mixture from tricaprylin and EPAEE by Novozyme. Peak 1, solvent; 2, caprylic acid ethyl ester; 3, caprylic acid; 4, internal standard (*n*-eicosane); 5, EPAEE; 6, dicaprylin; 7, tricaprylin; 8, DAG; 9, TAG-A; 10, TAG-B. See Figure 1 for abbreviations and manufacturer.

EPA molecule at the *sn*-1 (or 3) and the *sn*-2 positions. By using this method, time course distributions of each isomer of TAG-A that contained 1 EPA molecule were monitored.

GC separation of the reaction mixture. On-column injection is the best technique for transferring a TAG onto a column. Figure 2 shows the GC chromatogram of separation of the reaction mixture from the transesterification between tricaprylin and EPAEE using Novozyme as the catalyst. Peaks 1, 2, 3, 5, 6, and 7 were confirmed with standards. Peaks 8, 9, and 10 were identified by comparison with the purified samples obtained from the eluent of the silver ion HPLC. By using this GC analysis, time course changes in reaction composition were monitored.

Determination of both the selectivity and synthetic activity of lipases was important for the synthesis of each target product, TAG-A1(2) and TAG-A3. Therefore, we monitored incorporation of EPA into tricaprylin to select the suitable lipase to produce each target product using both HTGC and HPLC at different molar ratios of substrates, several initial a_w, and various reaction temperatures.

Effect of a_w *of lipase on the incorporation of EPAEE into tricaprylin.* Figure 3 shows the time course change in the contents of the TAG-A and TAG-B at different initial a_w of each lipase. For Novozyme and Liposam, the transesterification was the highest at an initial a_w of 0.52. The initial reactivity was also high at an initial a_w of 0.87, but after 9 h reaction, the content of TAG-A decreased slightly and the formation of the hydrolyzed product, diacylglycerol (DAG), was observed (data not shown). In the case of Lipozyme, TAG-A syntheses at a_w 0.11 and 0.52 were slightly higher than that at a_w 0.87. Therefore, the following reactions were performed at a_w 0.52.

Table 3 shows the time course in the change of ratio of regio isomers of TAG-A as the reaction progressed using the

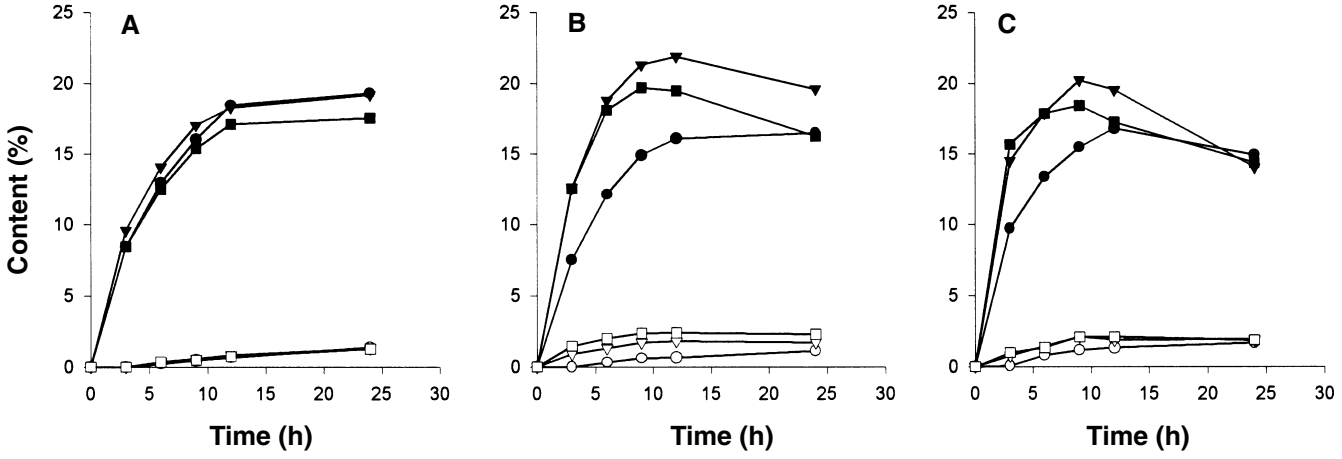

FIG. 3. Time course change of synthesis of TAG-A and TAG-B at different initial water activity (a_w) of lipases. (A) Lipozyme, (B) Novozyme (Novo Nordisk, Chiba, Japan), (C) Liposam. -●-,TAG-A (a_w = 0.11); -○-, TAG-B (a_w = 0.11); -▼-, TAG-A (a_w = 0.52); -▽-, TAG-B (a_w = 0.52); -■-, TAG-A (a_w = 0.87); -□-, TAG-B (a_w = 0.87). Reaction conditions: molar ratio of tricaprylin to EPAEE = 3:1, 400 rpm, 40°C, and lipase content = 50 mg. See Figure 1 for abbreviations and other manufacturers.

TABLE 3
Time Course in the Change of Ratio of Regio Isomers of TAG-A Using Three Lipases at Different Initial Water Activity (a_w)[a]

	Novozyme					
	a_w = 0.11		a_w = 0.52		a_w = 0.87	
Time (h)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)
3	99.5	0.5	99.3	0.7	94.5	5.5
6	98.5	1.5	97.7	2.3	92.8	7.2
9	95.9	4.1	94.9	5.1	90.3	9.7
12	95.2	4.8	94.7	5.3	87.6	12.4
24	90.5	9.5	89.5	10.5	84.8	15.2
	Lipozyme					
	a_w = 0.11		a_w = 0.52		a_w = 0.87	
Time (h)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)
3	>99	<1	>99	<1	>99	<1
6	>99	<1	>99	<1	>99	<1
9	>99	<1	>99	<1	>99	<1
12	>99	<1	>99	<1	>98	<2
24	>99	<1	>99	<1	>98	<2
	Liposam					
	a_w = 0.11		a_w = 0.52		a_w = 0.87	
Time (h)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)
3	84.7	15.3	77.4	22.6	71.5	28.5
6	80.9	19.1	71.4	28.6	68.6	31.4
9	77.1	22.9	67.2	32.8	66.6	33.4
12	71.9	28.1	66.4	33.6	65.4	34.6
24	68.5	31.5	65.0	35.0	65.0	35.0

[a]Molar ratio of tricaprylin/EPAEE = 3:1; lipase content, 50 mg; stirring speed, 400 rpm; temperature, 40°C. Manufacturers: Lipozyme and Novozyme (novo Nordisk, Chiba, Japan); Liposam (Showa Denko, Tokyo, Japan). For abbreviations see Table 2.

three lipases at different initial a_w. Lipozyme had a specificity for the *sn*-1(or 3) position for incorporation of the EPA molecule into tricaprylin. During the reaction, the ratio of the TAG-A1 and/or TAG-A2 to TAG-A3 was nearly constant at different a_w, therefore, only TAG-A1 (and/or TAG-A2) (≥98%) could be obtained. Novozyme has much higher specificity for the *sn*-1(or 3) position under the same conditions, but its selectivity decreased slightly at higher a_w. Therefore, TAG-A3 increased as the initial a_w increased. On the other hand, Liposam showed nonselectivity for the incorporation of EPAEE into tricaprylin. As the initial a_w of Liposam was increased, the formation of TAG-A3 increased. Since the content of TAG-A1 (and/or TAG-A2) decreased slightly after 9 h, it was unlikely that EPA had been incorporated into tricaprylin after 9 h (Fig. 3). From the results of this time course change in ratio of positional isomers under different a_w conditions during 9 h, it was inferred that water influenced the selectivity of Novozyme and Liposam in these particular reaction conditions, but did not influence selectivity of Lipozyme.

Effect of molar ratio of substrates on the incorporation of EPAEE into tricaprylin. As the mole ratio of tricaprylin to EPAEE decreased from 3:1 to 1:1, the hydrolysis of the synthesized TAG-A occurred earlier (Fig. 4). One molecule of EPA was incorporated into tricaprylin relatively easily, but an additional EPA molecule was much slower. This may be because TAG-A and EPA molecules are relatively bulky compared to tricaprylin. Therefore, the steric hindrance by these molecules seemed to decrease the reactivity of the lipase. The newly formed TAG-A have to be hydrolyzed for incorporation of a second EPA molecule. However, since an additional EPA incorporation to TAG-A was slow, only minor amounts of TAG-B were synthesized. At the same time, the amount of hydrolyzed product, DAG, increased (data not shown). In the case of Novozyme, as the molar ratio of tricaprylin to EPAEE decreased from 3:1 to 1:1, the formation of TAG-A3 increased. As a result, the selectivity of Novozyme decreased (Table 4). But Lipozyme showed high selectivity (>98%) for the *sn*-1 (or 3) position, and this selectivity was not affected by increase in EPAEE content studied.

Effect of temperature on the incorporation of EPAEE into tricaprylin. The time course changes of isomer ratio at 40 and 55°C are shown in Table 5. For both Novozyme and Liposam, the relative amount of TAG-A3 increased at 55°C (Fig. 5). In case of Lipozyme, the content of TAG-A increased (until 9

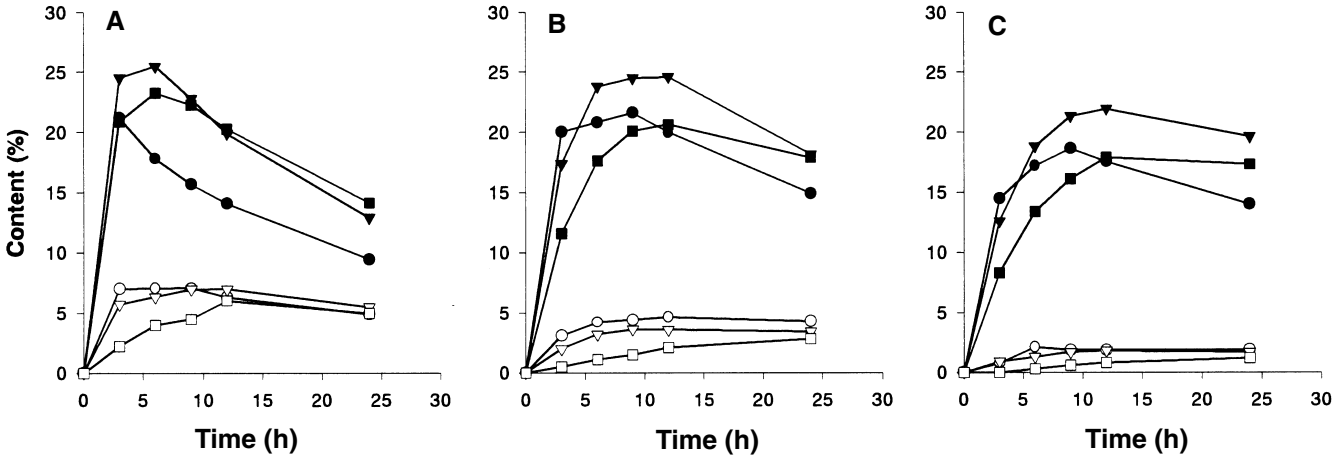

FIG. 4. Time course change of synthesis of TAG-A and TAG-B at different molar ratios of substrates. Molar ratio of tricaprylin to EPAEE: (A) 1:1, (B) 2:1, (C) 3:1. -●-,TAG-A (Liposam); -○-, TAG-B (Liposam); -▼-, TAG-A (Novozyme); -▽-, TAG-B (Novozyme); -■-, TAG-A (Lipozyme); -□-, TAG-B (Lipozyme). Reaction conditions: 400 rpm, 40°C, initial a_w of lipase = 0.52, and lipase content = 50 mg. See Figures 1 and 3 for abbreviations and manufacturers.

TABLE 4
Time Course in the Change of Ratio of Regio Isomers of TAG-A Using Three Lipases at Different Molar Ratio of Tricaprylin/EPAEE[a]

	Novozyme					
	Molar ratio = 1:1		Molar ratio = 2:1		Molar ratio = 3:1	
Time (h)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)
3	91.8	8.2	94.1	5.9	99.3	0.7
6	89.6	10.4	93.7	6.3	97.7	2.3
9	87.0	13.0	91.9	8.1	94.9	5.1
12	82.1	17.9	90.8	9.2	94.7	5.3
24	70.9	29.1	83.5	16.5	89.5	10.5
	Lipozyme					
	Molar ratio = 1:1		Molar ratio = 2:1		Molar ratio = 3:1	
Time (h)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)
3	>99	<1	>99	<1	>99	<1
6	>99	<1	>99	<1	>99	<1
9	>98	<2	>99	<1	>99	<1
12	>98	<2	>98	<2	>99	<1
24	>98	<2	>98	<2	>98	<2
	Liposam					
	Molar ratio = 1:1		Molar ratio = 2:1		Molar ratio = 3:1	
Time (h)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)
3	69.4	30.6	72.6	27.4	77.4	22.6
6	65.0	35.0	67.4	32.6	71.4	28.6
9	63.9	36.1	65.7	34.3	67.2	32.8
12	63.8	36.2	65.1	34.9	66.4	33.6
24	63.3	36.7	64.8	35.2	65.0	35.0

[a]Lipase content, 50 mg; stirring speed, 400 rpm; temperature, 40°C, initial water activity of lipase, 0.52. For manufacturers see Table 3. For abbreviations see Table 2.

TABLE 5
Time Course in the Changes of Ratio of Regio Isomers of TAG-A Using Three Lipases at Different Reaction Temperature

	Novozyme			
	40°C		55°C	
Time (h)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)
3	99.3	0.7	95.1	4.9
6	97.7	2.3	93.6	6.4
9	94.9	5.1	91.2	8.8
12	94.7	5.3	89.1	10.9
24	89.5	10.5	81.9	18.1
	Lipozyme			
	40°C		55°C	
Time (h)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)
3	>99	<1	>99	>1
6	>99	<1	>99	>1
9	>99	<1	>98	>2
12	>99	<1	97.2	2.8
24	>98	<2	96.3	3.7
	Liposam			
	40°C		55°C	
Time (h)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)	TAG-A1 + TAG-A2 (%)	TAG-A3 (%)
3	77.4	22.6	72.7	27.3
6	71.4	28.6	69.5	30.5
9	67.2	32.8	66.8	33.2
12	66.4	33.6	65.2	34.8
24	65.0	35.0	63.1	36.9

[a]Molar ratio of tricaprylin/EPAEE, 3:1; lipase content, 50 mg; stirring speed, 400 rpm; initial a_w of lipase, 0.52. For abbreviations see Table 2. For manufacturers see Table 3.

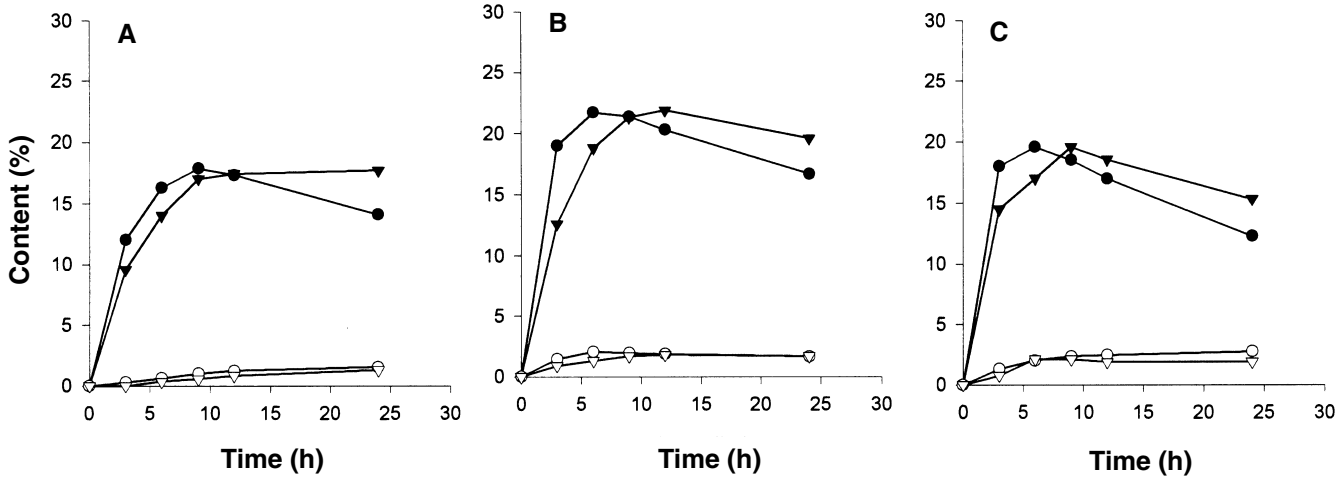

FIG. 5. Time course change of synthesis of TAG-A and TAG-B at different reaction temperatures. (A) Lipozyme, (B) Novozyme, (C) Liposam. -●-, TAG-A (55°C); -○-, TAG-B (55°C); -▼-, TAG-A (44°C); -▽-, TAG-B (44°C). Reaction conditions: 400 rpm, initial a_w of lipase = 0.52, and lipase content = 50 mg. See Figures 1 and 3 for abbreviations and manufacturers.

h), but TAG-A3 was hardly formed. However, after 9 h, the ratio of TAG-A3 to TAG-A1 (and/or TAG-A2) increased slightly at 55°C. The selectivity of the lipases seems to be decreased slightly at higher temperature.

In this study, we established the chromatographic separation of TAG containing EPA using HTGC and silver ion HPLC. The HTGC was useful for quantitative monitoring of the reaction. The silver ion HPLC using a solvent gradient system based on *n*-hexane, 2-propanol, and acetonitrile was very efficient and reproducible for the analysis of the distribution of each TAG containing 1 EPA molecule at the *sn*-1 (or 3) or the *sn*-2 position. Additionally, the HPLC analysis could be used to identify the isomeric purity of each target product that was produced by various methods (data not shown).

From the results of incorporation of EPA into tricaprylin using various lipases at different molar ratios of substrates, initial a_w of lipase, and reaction temperatures, it was concluded that Liposam could incorporate EPA at both the *sn*-1(or 3) and the *sn*-2 positions of the glycerol backbone, whereas Lipozyme showed a high selectivity for the *sn*-1 (or 3) position. Novozyme also had a higher selectivity for the *sn*-1 (or 3) position. However, longer reaction times, higher amounts of EPAEE, or higher initial a_w of the lipase resulted in a slight increase in *sn*-2 positional isomers.

This separation technique will enable us to further expand our capability to incorporate other PUFA into medium-chain TAG and screen lipases for the selective incorporation of PUFA. We are in the process of optimizing production of structured TAG that contain EPA or other PUFA.

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[Received May 26, 1998; accepted September 17, 1998]