Synthesis of the Structured Lipid 1,3-Dioleoyl-2-palmitoylglycerol from Palm Oil

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ABSTRACT: Human milk fat contains 20–25% palmitic acid (16:0) and 30–35% oleic acid (18:1). More than 60% of the palmitic acid occurs at the *sn*-2 position of the glycerol backbone. Palm oil is a rich source of both palmitic and oleic acids. The structured lipid 1,3-dioleoyl-2-palmitoylglycerol (OPO) is an important ingredient in infant formula. OPO was synthesized from palm oil by a three-step method. In the first step, low-temperature fractionation was applied to palm oil FA, yielding a palmitic acid-rich fraction (87.8%) and an oleic acid-rich fraction (96%). The palmitic acid content was further increased to 98.3% by transforming palmitic acid into ethyl palmitate. In the second step, esterification of ethyl palmitate and glycerol catalyzed by lipase Novozym 435 under vacuum (40 mm Hg) was employed for the synthesis of tripalmitin. Finally, OPO was obtained by the reaction of tripalmitin with oleic acid catalyzed by Lipase IM 60. In this final step, the TAG content in the product acylglycerol mixture was 97%, and 66.1% oleic acid was incorporated into TAG. Analysis of the FA composition at the *sn*-2 position of TAG showed 90.7 mol% of palmitic acid and 9.3 mol% of oleic acid. OPO content in the product TAG was *ca.* 74 mol%. Thus, an efficient method was developed for the synthesis of OPO from palm oil.

Paper no. J10743 in *JAOCS 81,* 525–532 (June 2004).

KEY WORDS: Esterification, lipases, low-temperature crystallization, oleic acid, 1,3-oleoyl-2-palmitoylglycerol, palmitic acid, palm oil, structured lipids, tripalmitin.

Structured lipids, such as 1,3-dioleoyl-2-palmitoylglycerol (OPO), have attracted attention recently owing to their potential pharmaceutical and nutraceutical applications. Breast milk fat is one of the main sources of nutrients and energy for infants. About 50–60% of the dietary energy that an infant needs is provided by human milk fat (HMF) (1). Palmitic and oleic acids are the two most abundant FA present in all HMF and are the most interesting from the standpoint of human nutrition. Palmitic acid represents about 25% of the total milk FA and accounts for more than 10% of the infant's total energy intake. The stereospecific distribution of FA on TAG structures in HMF plays a valuable role and influences the fate of fat absorption in infants. In HMF the saturated FA, predominantly palmitic acid, are located primarily at the *sn*-2 position (>60%) of the glycerol backbone (2,3), whereas the *sn*-1,3 positions are mainly occupied by monounsaturated FA. Thus, the main component of the milk dienoic TAG is OPO.

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Earlier studies used pure tripalmitin (PPP) and oleic acid for the synthesis of the structured lipid OPO (4,5). However, large-scale applications of these methods are expensive because of the high cost of the substrates. To achieve an economically feasible process, it is important that starting substrates such as palmitic acid and oleic acid be isolated and purified from an inexpensive source. Palm oil is such a source for both palmitic and oleic acids, which constitute 44.0 and 39.2%, respectively, of the total palm oil FA content. Therefore, isolating and purifying these FA from palm oil may offer a considerable reduction in the cost of the substrates used to synthesize OPO.

The synthesis of structured TAG is usually carried out by the transesterification of different TAG or by the acidolysis reaction between a TAG and a FA. Several analytical methods, such as TLC (6), enzymatic hydrolysis (7), or chemical degradation using a Grignard reagent (8) and ^{13}C NMR (9), have been used in the past for the determination and quantification of TAG and their positional isomers. None of the above methods is capable of identifying the molecular species in the TAG, and only positional distributions of the FA are determined (10).

Silver-ion HPLC is an extremely powerful tool for the separation and quantification of positional and geometrical isomers of FA and TAG. The separation takes place according to the number, geometrical configuration, and position of double bonds in the molecule (6). RP-HPLC is an alternative to silver-ion HPLC. With the former technique, the separation of individual TAG molecules is by both chain-length and degree of unsaturation of FA. However, in many cases the order of elution of the TAG species with silver-ion HPLC is quite distinctive and easy to understand, and, depending on the degree of unsaturation, much better resolution is possible, in contrast to HPLC in the reversed-phase mode (6,11). Thus, the silver-ion technique does not replace RP-HPLC but complements it. Various detection methods have been used in the analysis of TAG separated by HPLC. Nagao *et al.* (5) used a refractive index (RI) detector to analyze structured lipids containing oleic and palmitic acids that had been separated by silver-ion HPLC. ELSD offers advantages over RI and UV detection in that no baseline drift occurs, and there are no limitations in the use of mobile phase solvents. Furthermore, ELSD is stable and sensitive when elution gradients are used, as is required to resolve TAG mixtures. The use of gradient elution is an alternative approach for reducing the retention times of higher M.W. saturated TAG and for improving the chromatographic resolution (12). In this paper, we report an efficient method for the

purification of palmitic and oleic acids from saponified palm oil; the synthesis of OPO from palmitic acid, oleic acid, and glycerol; and the separation and quantification of OPO by silver-ion HPLC with detection by ELSD.

EXPERIMENTAL PROCEDURES

Materials. Refined palm oil was purchased from Blessing Brothers Industrial Company (Taipei, Taiwan). Lipase from *Pseudomonas cepacia* (Lipase PS "Amano" 30, powder) was a product of Amano Enzyme Inc. (Nagoya, Japan). Lipozyme IM 60 (*Rhizomucor miehei* lipase immobilized on a macroporous anion exchange resin) and Novozym 435 (*Candida antarctica* lipase immobilized on a macroporous acrylic resin) were supplied by Novo Nordisk A/S (Bagsvaerd, Denmark) as gift samples. Microporous polypropylene powder was obtained from Akzo Nobel Faser AG (Obernburg, Germany). Silica gel 60M (230–400 mesh) was purchased from Macherey-Nagel GmbH & Co., KG (Düren, Germany). Standard TAG samples were obtained from Sigma Chemical Company (St. Louis, MO). The internal standard pentadecanoic acid (>99%) was obtained from Aldrich Chemical Co. (Milwaukee, WI). All solvents were either of HPLC or analytical reagent grade and were obtained from commercial sources.

Methods. (i) Enzyme immobilization. PS-30 lipase was immobilized onto a microporous polypropylene solid support following the method of Huang *et al.* (13). Activity of the immobilized lipase was assayed by the olive oil emulsion method (13). The immobilized Lipase PS-30 used in this study had a specific activity of 8 units/(mg immobilized lipase).

(ii) Preparation of FFA from palm oil. FFA, obtained by the saponification of palm oil, were prepared according to the method described by Haagsma *et al.* (14).

(iii) Separation of palmitic and oleic acids. The separation of palmitic and oleic acids from the FFA mixture of saponified palm oil was carried out by low-temperature solvent crystallization according to the flow chart shown in Figure 1. Typically, 400 mL of acetone was added to a glass vial containing 10 g of FFA. This mixture was stirred at 40°C until all FA were dissolved. The solution was allowed to cool to room temperature and was then stored in an ultra-low-temperature freezer chamber (MDF-192; Sanyo Gunma, Japan) at −25°C for 24 h. The content was filtered under suction using an Ace Buchner funnel (25–50 μm). The solid fraction with palmitic acid as the major FA was designated as FFA-I and was further purified by selective enzymatic esterification, giving a mixture of FA ethyl esters with 98.3% ethyl palmitate. The liquid fraction (designated as FFA-II) was further purified by successive low-temperature crystallizations, as shown in Figure 1, to yield a solid phase with 96% oleic acid.

(iv) Lipase-catalyzed selective esterification. The enrichment of palmitic acid in FFA-I was accomplished by the esterification of FFA-I with ethanol catalyzed by immobilized Lipase PS 30 in *n*-hexane. Typically, FFA-I (4 g, average M.W. = 259.4) and absolute ethanol (0.9 mL, purity 99.5%) with a molar ratio of 1:1 were put into a 50-mL screw-capped glass vessel containing 20 mL of *n*-hexane. To this was added 0.4 g of immobilized PS-30 lipase (10% based on FFA weight), and the reaction was carried out at 37°C in a thermostated water bath with stirring at 150 rpm. At various reaction times, a 100-μL sample was taken for the analysis of palmitic acid content in the unesterified FFA. To this sample, 100 μL of *n*-hexane containing 0.05 mmol tridecanoic acid was added as an internal standard. After adding 200 μL of 1 N KOH solution, the content was vortexed vigorously, and the FFA soap and its ester were separated by centrifuging (5,000 × *g*) for 5 min. FFA in the aqueous phase were extracted with 300 μL of *n*-hexane after returning it to an acidic pH (pH <2) with 200 μL of aqueous 2 N HCl. After removing the solvent by nitrogen purging, the resulting FFA were transformed into methyl esters by heating with BF_3/methanol (20%) and analyzed by GC. The degree of conversion of palmitic acid to ethyl palmitate was expressed as the initial weight of palmitic acid minus the final weight of palmitic acid divided by the initial weight of palmitic acid multiplied by 100.

(v) Lipase-catalyzed synthesis of PPP. Novozym 435 and Lipozyme IM 60 were used as biocatalysts for the synthesis of PPP from ethyl palmitate and glycerol. The reaction was performed in a solvent-free system at 60°C under vacuum in a two-necked test tube. One neck was connected to a vacuum pump, and the other was closed with a rubber septum. A typical reaction was carried out with 0.58 mmol of glycerol, 1.76 mmol of ethyl palmitate, and 50 mg of lipase (10% based on ethyl palmitate weight). After the FA ester was melted, the reaction was initiated by adding lipase, and the reactor pressure was maintained at 40 mm Hg.

(vi) Analysis of the reaction product by TLC-FID. The contents of TAG, DAG, MAG, and ethyl esters in the reaction product were measured with a thin-layer chromatograph/FID analyzer (Iatroscan MK-5; Iatron Co., Tokyo, Japan) after development in 1,2-dichloroethane/chloroform/acetic acid (92:8:0.1, by vol) (15). Sample (100 μL) was dissolved in 1 mL of chloroform and filtered through a cotton plug. The volume of the solution was reduced to 200 μL by passing N_2 gas through the solution. One microliter of this lipid solution was spotted on silica gel Chromarods-SIII (Iatron Co.) and developed in the above solvent system. The Iatroscan was operated with hydrogen and air flow rates of 160 and 2000 mL/min, respectively.

(vii) Purification of TAG from the reaction mixture by silica gel column chromatography. Two grams of reaction mixture was dissolved in 12 mL of hexane and then applied to the head of a column (32 × 2.4 cm i.d.) of silica gel (60 g, 60–200 mesh; Merck, Darmstadt, Germany). Unreacted ethyl palmitate was eluted first with a mixture of *n*-hexane/ethyl acetate (200 mL of 99:1, vol/vol), followed by the elution of TAG with *n*-hexane/ethyl acetate (500 mL of 97:3,vol/vol) at a flow rate of about 2 mL/min; eluents were collected in 20-mL fractions. The purity of each fraction was analyzed on a silica TLC plate (6 × 2 cm, 250 μm) using authentic standards. Plates were developed with a solvent system composed of *n*-hexane/ethyl acetate/acetic acid (90:10:0.5, by vol) as developing solvent. After air-drying, the spots were visualized by

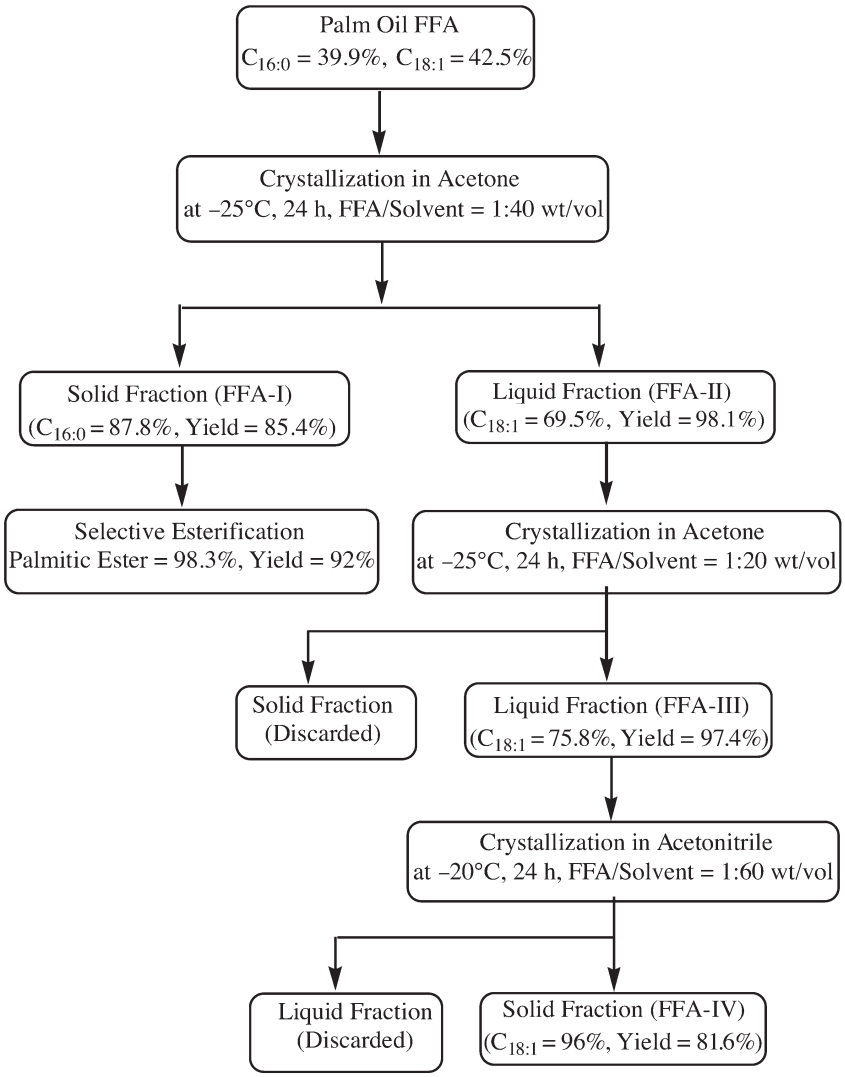

FIG. 1. Flow chart for low-temperature and enzymatic fractionation of mixed FA of palm oil.

spraying with methanolic sulfuric acid (10%), followed by heating at 150°C. Fractions containing only TAG were combined, and the solvent was removed with a rotary evaporator. PPP with a purity greater than 99% and a corresponding recovery of 92.5% was obtained by silica gel chromatographic purification.

(viii) Acidolysis of PPP. PPP (80.7 mg, 0.1 mmol), oleic acid (225.6 mg, 0.8 mmol), 20 mg of Lipase IM 60, and 3 mL of hexane were placed in a 10-mL glass vial, which was then sealed. The mixture was incubated for 24 h in a thermostated water bath at 50°C and magnetically stirred at 150 rpm. Aliquots of the mixture (100 μL) were withdrawn at definite intervals for the analysis of acylglycerides.

(ix) Analysis of the acidolysis reaction product. The extent of the reaction was determined by taking samples at regular time intervals and analyzing them by TLC-FID. The contents of TAG, DAG, MAG, and FFA in the reaction mixture were analyzed by a TLC-FID analyzer after development in 1,2-dichloroethane/chloroform/acetic acid (92:8:0.1, by vol) (15).

(x) Purification of the acidolysis reaction product. At the end of incubation, the enzyme was removed from the reaction product by passing it through a bed of anhydrous sodium sulfate. The FA composition of the TAG product was determined by GC analysis after separation by TLC. Twenty milligrams of reaction product was dissolved in 0.2 mL of hexane, and TAG were separated by TLC on precoated silica gel G 60 F 254 plates (1-mm thick; Merck), developed with *n*-hexane/diethyl ether/acetic acid (90:10:0.1, by vol). The bands were visualized under UV light after spraying with 0.2% 2,7-dichlorofluorescein in ethanol. Bands corresponding to TAG were scraped off and methylated with 6% methanolic HCl at 75°C for 2 h (16). FAME were separated by the addition of 2 mL of hexane and 1 mL of 0.1 M KCl, centrifuged, and the top hexane layer recovered and its FA composition determined by GC.

(xi) Regiospecific analysis of TAG. The positional distribution of FA in TAG was determined by the method of Liu *et al.* (17). Typically, 100 mg of TAG was dissolved in 0.2 mL of DMSO and mixed with 3 mL of Tris-HCl buffer (pH 7.6), 0.1

mL of 0.2% (wt/vol) of bile salts, 0.2 mL of 22% (wt/vol) calcium chloride, and 100 mg pancreatic lipase in a 10-mL test tube. The mixture was incubated in a water bath at 37°C for 5 min. Thereafter, the reaction mixture was extracted with diethyl ether and 2-MAG was separated by a silica gel G TLC plate (Merck) after developing in *n*-hexane/ethyl acetate/acetic acid (50:50:1, by vol). The band corresponding to 2-MAG was scraped off and extracted with ethyl acetate, methylated, and then analyzed by GC.

(xii) GC analysis of the FA composition. FFA were converted into their corresponding FAME by heating with BF_3/methanol (20%). TAG and MAG were methylated with 6% methanolic HCl at 75°C for 2 h. The FA composition was analyzed by a model 8700F China Chromatographic gas chromatograph (Taipei, Taiwan) equipped with an FID. The column used was an SP-2330 (30 × 0.25 mm i.d.; Supelco, Bellefonte, PA). The injector and detector temperatures were set at 250 and 270°C, respectively. The column was held at 180°C for 10 min, increased to 235°C at a constant rate of 15°C/min, and then held at 235°C for 3 min. One microliter of sample was injected at a split ratio of 1:50.

(xiii) Separation and quantification of TAG of the acidolysis product by silver-ion HPLC-ELSD. A JASCO HPLC system with a ChromSpher 5 Lipids silver-ion chromatography column (250 × 4.6 mm × 1/4″; Chrompack, Middelburg, The Netherlands), and an ELSD 2000 (Alltech, Deerfield, IL) was used for the TAG class composition analysis. The separation of TAG was carried out using a binary solvent gradient program of A (acetone) and B (dichloromethane). The gradient program of these two mobile phases is shown in Table 1. The mobile-phase flow rate was 0.8 mL/min. The column was held at ambient temperature. ELSD was used at a 50°C drift tube temperature. Air at a flow rate of 1.6 mL/min was used to evaporate the solvent in the heated chamber. The lipid samples were dissolved in dichloromethane (20 mg/mL), and 20 μL of solution was injected.

(ix) Standard calibration curves for each individual TAG. Lipid standards such as PPP, 1,3-dipalmitoyl-2-oleoylglycerol (POP), 1,2-dipalmitoyl-3-oleoylglycerol (PPO), 1,3-dioleoyl-2-palmitoylglycerol (OPO), 1,2-dioleoyl-3-palmitoylglycerol (OOP), and triolein (OOO) with purity greater than 99% (Sigma Chemical) were dissolved in dichloromethane at different concentrations and analyzed by HPLC-ELSD under the previously mentioned conditions. Relationships between log peak area and log sample concentration were plotted to obtain standard calibration curves for each of the TAG standards.

TABLE 1
Gradient Program of Mobile Phase in the HPLC analysis of TAG

Time (min)	Acetone (%)	Dichloromethane (%)
0	2	98
8	2	98
10	16	84
22	28	72
28	98	2
32	2	98
35	2	98

RESULTS AND DISCUSSION

Separation and purification of palmitic and oleic acids from palm oil. Table 2 shows the FA compositions of palm oil before and after purification from saponified palm oil. The results show that the crystallization of FA in acetone at −25°C yielded two distinct fractions: a solid fraction containing mainly saturated FA (mostly palmitic acid, ~88 wt%) and a liquid fraction containing mainly unsaturated FA (mostly oleic acid). Stearic acid, which accounted for *ca.* 8.7%, was the other major acid in the solid fraction.

Further enrichment of palmitic acid by the removal of stearic acid *via* solvent crystallization was difficult because of their similarity in structure, polarity, and solubility. On the other hand, removal of the remaining saturated acids in the liquid fraction was carried out in the second stage by low-temperature solvent crystallization. The resulting crystals of saturated FA were removed by filtration. As shown in Table 2, this step removed 78% of the saturated FA, raising the oleic acid content from 69.5 to 75.8 wt% with a yield of 97.4%. The liquid fraction from the second-stage crystallization still contained 21.3% linoleic acid. Previous reports from this laboratory suggest the use of acetonitrile for the enrichment of PUFA (18,19). When crystallization was performed at –20°C and an acetonitrile-to-FFA ratio of 60 mL/g, after 24 h the oleic acid content was raised from 75.83 to 96 wt%, with a yield of 81.6%. The linoleic acid content was reduced from 21.3 to 1.0 wt%.

Lipase-catalyzed selective esterification of FFA-I and alcohol. Our preliminary results on the selective esterification of different FA with alcohols showed that lipase from *P. cepacia* exhibited substantial selectivity for palmitic acid over stearic acid (data not shown). The palmitic acid content in the solid fraction of the first solvent crystallization (FFA-I) was further enriched by the selective esterification of FA with ethanol catalyzed by immobilized *P. cepacia* lipase. Figure 2 shows a time-course plot of the formation of ethyl palmitate and the degree of esterification. As expected, *P. cepacia* showed high selectivity for palmitic acid and converted it into ethyl esters, leaving more stearic acid in the residual FA. After 12 h of reaction, the degree of esterification was 89.5%, and the product contained 98.3 wt% ethyl palmitate. It is important to use a stoichiometric amount of ethanol to achieve a high degree of esterification.

Lipase-catalyzed synthesis of PPP. Figure 3 illustrates a time-course plot of the PPP content in the acylglycerol fraction and the extent of esterification catalyzed by Lipase IM 60 and Novozym 435. Novozym 435 showed a higher degree of esterification and a higher TAG content in the acylglycerols after 36 h of reaction and was therefore selected for the synthesis of PPP from ethyl palmitate and glycerol. Table 3 shows time-course variations in the acylglycerol composition (wt%) of the reaction product and the degree of esterification. At the early stage of the reaction, the contents of MAG and DAG were higher than that of TAG. As the reaction proceeded, the conversion of these partial glycerides led to a gradual increase in TAG. About 88% conversion with 91 mol% of PPP, along

TABLE 2
FA Compositions (wt%)[a] of Palm Oil Before and After Various Stages of Solvent Crystallization

		Stage #1[b]		Stage #2[c]	Stage #3[d]
FA	Initial	Solid fraction (FFA-I)	Liquid fraction (FFA-II)	Liquid fraction (FFA-III)	Liquid fraction (FFA-IV)
14:0	0.43 ± 0.3	0.72 ± 0.09	0.32 ± 0.08	0.34 ± 0.02	0.49 ± 0.02
16:0	39.98 ± 0.1	87.82 ± 0.21 (85.35%)[e]	9.69 ± 0.11	2.22 ± 0.08	2.46 ± 0.03
16:1	0.32 ± 0.5	ND[f]	0.46 ± 0.08	ND	ND
18:0	4.67 ± 0.3	8.72 ± 0.1	0.87 ± 0.16	0.29 ± 0.03	ND
18:1	42.49 ± 0.1	2.03 ± 0.12	69.52 ± 0.09 (98.1%)[g]	75.83 ± 0.11 (97.41%)[g]	96.04 ± 0.12 (81.61%)[g]
18:2	12.1 ± 0.3	0.71 ± 0.08	19.14 ± 0.11	21.32 ± 0.08	1.01 ± 0.04

[a]Mean absolute deviation of three independent determinations.
[b]Solvent = acetone, solvent/FFA = 40 mL/g, temperature = –25°C, storage time = 24 h.
[c]Solvent = acetone, solvent/FFA = 20 mL/g, temperature = –25°C, storage time = 24 h.
[d]Solvent = acetonitrile, solvent/FFA = 60 mL/g, temperature = –20°C, storage time = 24 h.
[e]Yield of palmitic acid (16:0) in the solid fraction.
[f]ND, not detected.
[g]Yield of oleic acid (18:1) in the liquid fractions.

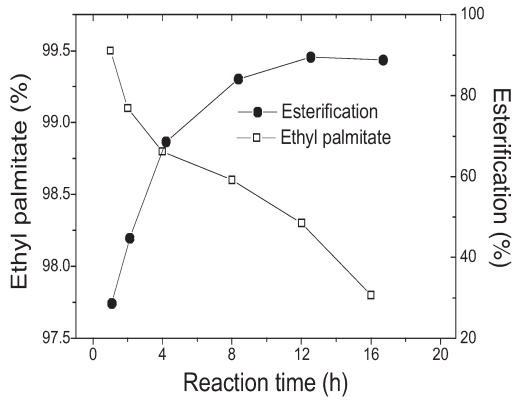

FIG. 2. Time-course plot of the formation of ethyl palmitate and the degree of esterification. Reaction conditions: Lipase PS 30 (Amano Enzyme Inc., Nagoya, Japan) 0.4 g (10% based on FA weight), molar ratio of FA to ethyl alcohol = 1:1 (4 g FFA-I), 20 mL of *n*-hexane, 37°C, stirrer speed of 150 rpm.

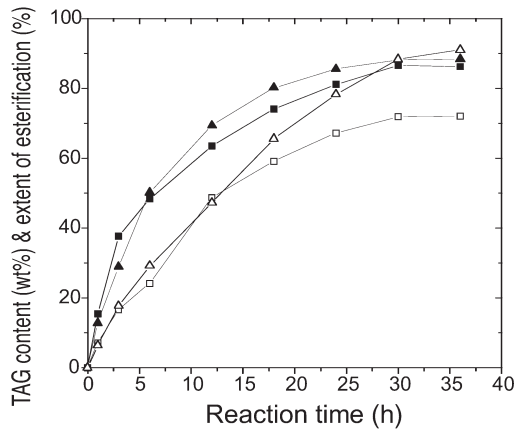

FIG. 3. Time-course plot of tripalmitin (PPP) formation and the degree of esterification. Reaction conditions: molar ratio of glycerol to ethyl palmitate = 1:3 (0.58 mmol glycerol), lipase load of 50 mg (10% based on ethyl palmitate weight), 60°C, reactor pressure of 40 mm Hg, speed of 300 rpm. (▲,■) Degree of esterification; (△,□) PPP content in acylglycerols; (▲,△) Novozym 435; (■,□) Lipase IM 60 (both from Novo Nordisk, Bagsvaerd, Denmark).

with a minor amount of partial glycerides, was accomplished after 36 h of reaction. PPP with a purity greater than 99% (and a corresponding recovery of 92.5%) was obtained by silica gel chromatographic purification.

Acidolysis of PPP. Figure 4 shows the effects of various parameters on the time course of oleic acid incorporation in the acidolysis of PPP and oleic acid. When the reaction mixture contained 0.1 mmol of PPP, 0.8 mmol of oleic acid, 20 mg of Lipase IM 60 (6.5% based on total substrate weight), and 3 mL of *n*-hexane, the reaction product, after 24 h of reaction at 50°C, contained 97% TAG with an oleic acid content of 66.1 mol%.

Quantification of TAG by HPLC. The distribution of oleic acid in TAG positions (*sn*-1,3 and *sn*-2) was determined by silver-ion complexation HPLC-ELSD. The standard lipids PPP, POP, PPO, OPO, OOP, and OOO at different concentrations were injected separately into the high-performance liquid chromatograph. The standard calibration equations for the six TAG were as follows (X = log mg/L, and Y = log peak area):

$$\text{PPP: } Y = 1.02956 + 1.25793 * X \ (R^2 = 0.99787) \quad [1]$$

$$\text{POP: } Y = 0.45319 + 1.26938 * X \ (R^2 = 0.99759) \quad [2]$$

TABLE 3
Time-Course Variations in Composition of Products (%) During the Synthesis of Tripalmitin Under Vacuum with Novozym 435[a]

		Composition of the acylglycerols (wt%)		
Time (h)	Degree of esterification	TAG	DAG	MAG
1	12.8 ± 2.2	6.37 ± 0.5	26.26 ± 0.7	67.37 ± 1.0
3	28.8 ± 2.0	17.53 ± 0.7	30.00 ± 0.5	52.47 ± 0.8
6	50.1 ± 1.6	34.88 ± 0.6	37.40 ± 0.5	27.72 ± 0.5
12	69.3 ± 1.6	54.88 ± 0.5	28.62 ± 0.5	16.51 ± 0.6
18	80.1 ± 1.4	65.50 ± 0.7	22.36 ± 0.5	12.14 ± 0.2
24	85.5 ± 1.7	78.24 ± 1.1	14.32 ± 0.3	7.44 ± 0.3
30	88.1 ± 1.6	89.0 ± 1.1	7.56 ± 0.3	3.48 ± 0.2
36	88.3 ± 1.1	90.9 ± 0.8	6.21 ± 0.2	2.89 ± 0.1

[a]Mean absolute deviation of three independent determinations. Reaction conditions: 50 mg (10 wt% of ethyl palmitate) Novozym 435 (Novo Nordisk A/S, Bagsvaerd, Denmark), 0.58 mmol glycerol, and 1.76 mmol ethyl palmitate were incubated at 60°C under 40 mm Hg with a stirrer speed of 300 rpm for 36 h.

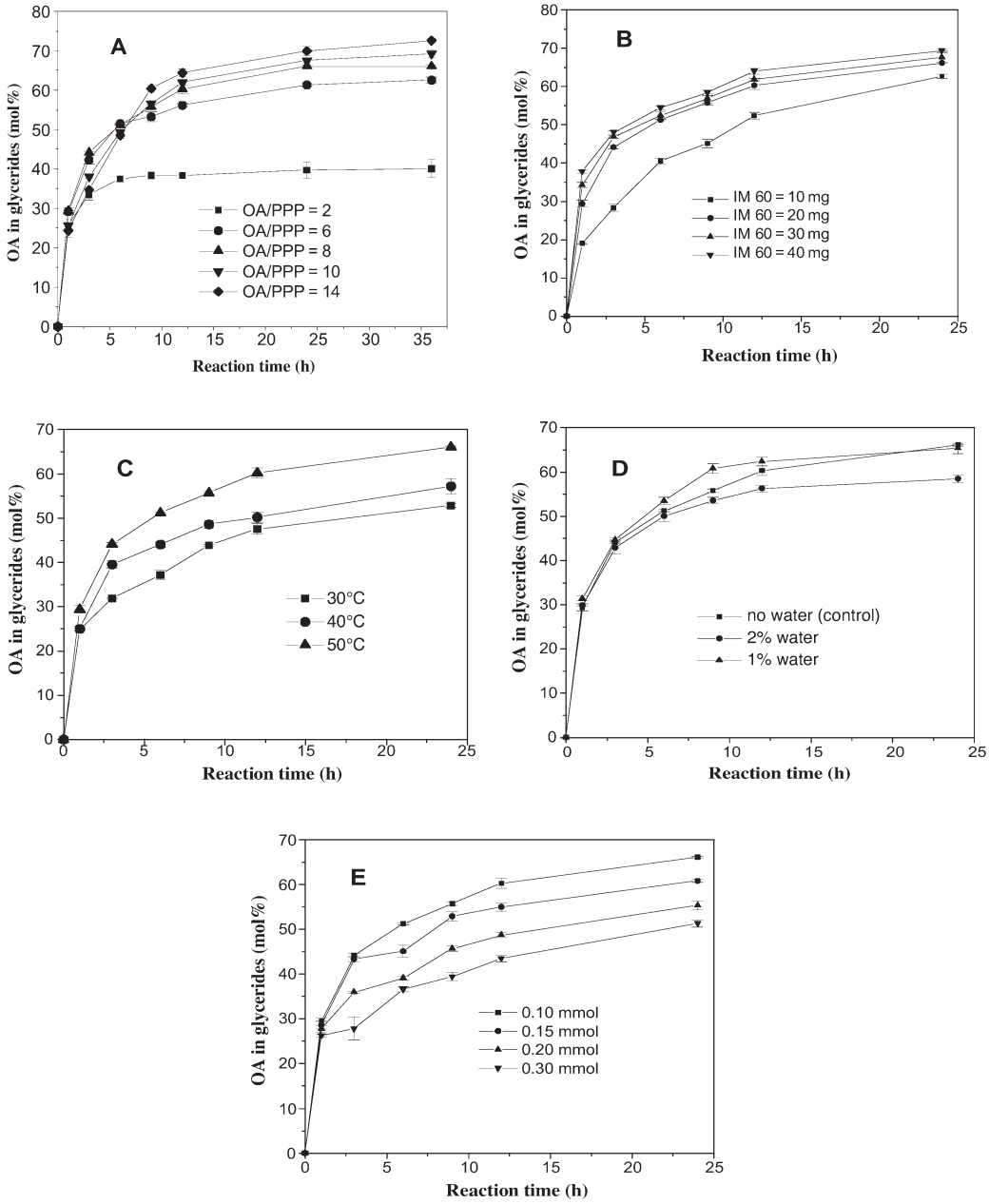

FIG. 4. Effects of various parameters on the incorporation of oleic acid (OA) in the acidolysis of PPP with lipase IM 60. (A) Effect of the molar ratio of OA/PPP. Reaction conditions: 0.1 mmol PPP in 3 mL hexane, 20 mg of lipase (6.5% substrate weight), 50°C, and 150 rpm magnetic stirring. (B) Effect of enzyme load. Molar ratio of OA/PPP = 8:1; other conditions are the same as in A except for the enzyme load. (C) Effect of temperature. Molar ratio of OA/PPP = 8:1; other conditions are the same as in A except for the temperature. (D) Effect of water content. Molar ratio of OA/PPP = 8:1; other conditions are the same as in A except for the water content. (E) Effect of PPP concentration. Molar ratio of OA/PPP = 8:1; other conditions are same as in A except for the PPP concentration. For enzyme supplier, see Figure 2. For other abbreviation, see Figure 3.

PPO: $Y = 0.6207 + 1.44453 * X$ ($R^2 = 0.99578$) [3]

OPO: $Y = 0.54614 + 1.33721 * X$ ($R^2 = 0.99948$) [4]

OOP: $Y = 1.16442 + 1.12684 * X$ ($R^2 = 0.99892$) [5]

OOO: $Y = 1.14749 + 1.17615 * X$ ($R^2 = 0.99775$) [6]

The separation of TAG isomers was based on the number and position of the double bonds and the chain length of the FA in the molecule. The separation of regioisomeric pairs such as POP, PPO, OPO, and OOP were the most important in quantifying individual structured TAG in the reaction product. Good separation of TAG was obtained on a ChromSpher 5 Lipids silver-ion chromatography column using a binary solvent gradient program of A (acetone) and B (dichloromethane) as eluents. Figure 5A shows a chromatogram in which all six TAG

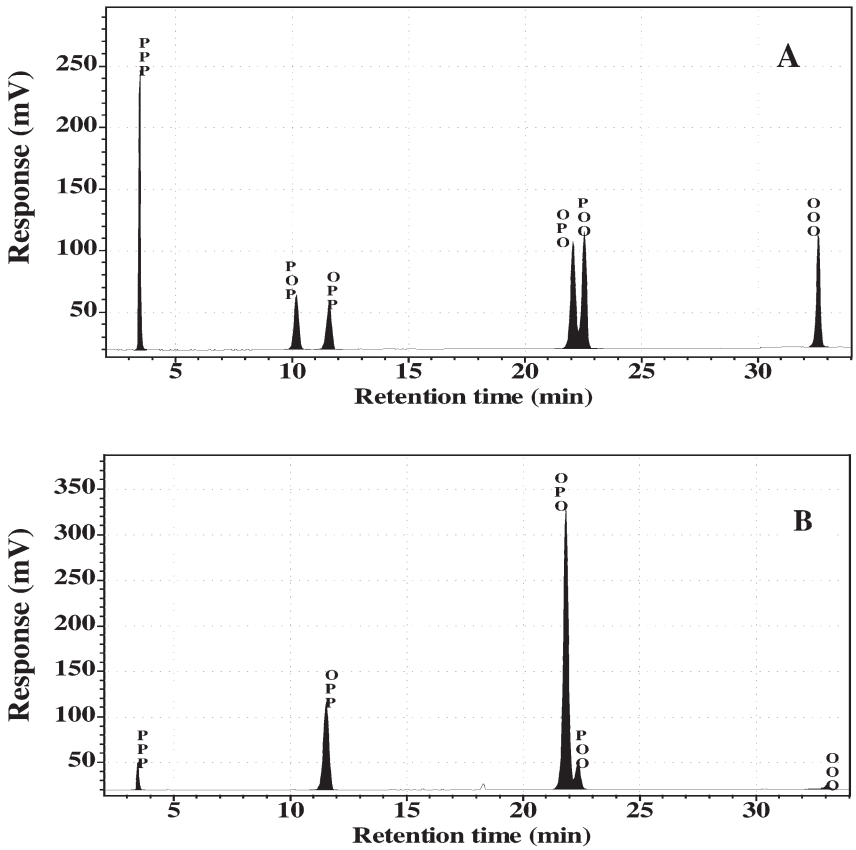

FIG. 5. HPLC separation of (A) a standard TAG lipid mixture, PPP; POP (1,3-dipalmitoyl-2-oleoylglycerol); PPO (1,2-dipalmitoyl-3-oleoylglycerol); OPO (1,3-dioleoyl-2-palmitoylglycerol); OOP (1,2-dioleoyl-3-palmitoylglycerol); and OOO (triolein). (B) Structured lipids obtained after the acidolysis of PPP. Reaction conditions: 0.1 mmol of PPP, molar ratio of PPP/OA = 1:8, 3 mL of hexane, 20 mg of Lipase IM 60 (6.5% based on substrate weight), 50°C, 150 rpm magnetic stirring for 24 h. For enzyme supplier, see Figure 2. For other abbreviation, see Figure 3.

components from a standard lipid mixture were baseline resolved. Linear responses for all six TAG were obtained in the concentration range of 0.12 to 10 μg per injection. Contents of each individual TAG were quantified using the corresponding calibration curves.

When the TAG fraction of the acidolysis product at a reaction time of 24 h was analyzed, only five peaks were observed, corresponding to PPP, PPO, OPO, OOP, and OOO (Fig. 5B). At short reaction times (<8 h), in which the incorporation of oleic acid in TAG was less than 35%, only PPP, PPO, and OPO were observed (data not shown). The absence of oleic acid at the *sn*-2 position of TAG implied that the *R. miehei* lipase exhibited strict *sn*-1,3 specificity and that there was no acyl exchange involved at the *sn*-2 position with a short reaction time. However, at longer reaction times with a higher incorporation of oleic acid, small quantities of OOP and OOO were also detected, as shown in Figure 5B. Pancreatic lipase digestion of the TAG fraction of the acidolysis product showed that the palmitic and oleic acid contents at the *sn*-2 position were 90.7 and 9.3 mol%, respectively. The minor unknown peaks at retention times of 18.23 and 30.89 min were attributed to impurities present in the substrate oleic acid (purity 96.7%). The OPO content in TAG produced by the acidolysis reaction was 74 ± 1.2 mol%, as illustrated in Figure 5B.

ACKNOWLEDGMENTS

Financial support from the National Science Council of Taiwan under grant NSC90-2214-E011-004 is gratefully acknowledged.

REFERENCES

1. Jensen, R.G., and G.L. Jensen, Specialty Lipids for Infant Nutrition. 1. Milks and Formulas, *J. Pediatr. Gastroenterol. Nutr. 15*:232–245 (1992).
2. Martin, J.-C., P. Bougnoux, J.-M. Antoine, M. Lanson, and C. Couet, Triacylglycerol Structure of Human Colostrum and Mature Milk, *Lipids 28*:637–643 (1993).
3. Jensen, C., N.R. Buist, and T. Wilson, Absorption of Individual Fatty Acids from Long-Chain or Medium-Chain Triglycerides in Very Small Infants, *Am. J. Clin. Nutr. 43*:745–751 (1986).
4. Schmid, U., U.T. Bornscheuer, M.M. Soumanou, G.P. McNeil, and R.D. Schmid, Highly Selective Synthesis of 1,3-Oleoyl-2-

palmitoylglycerol by Lipase Catalysis, *Biotechnol. Bioeng. 64–66*:678–684 (1999).
5. Nagao, T., Y. Shimada, A. Sugihara, A. Murata, S. Komemushi, and Y. Tominaga, Use of Thermostable *Fusarium heterosporum* Lipase for Production of Structured Lipid Containing Oleic and Palmitic Acids in Organic Solvent-Free System, *J. Am. Oil Chem. Soc. 78:*167–172 (2001).
6. Dobson, G., W.W. Christie, and B. Nikolova-Damyanova, Silver Ion Chromatography of Lipids and Fatty Acids, *J. Chromatogr. B 671*:197–222 (1995).
7. Foglia, T.A., E.J. Conkerton, and P.E. Sonnet, Regioselective Analysis of Triacylglycerols by Lipase Hydrolysis, *J. Am. Oil Chem. Soc. 72:*1275–1279 (1995).
8. Becker, C.C., A. Rosenquist, and G. Holmer, Regiospecific Analysis of Triacylglycerols Using Allyl Magnesium Bromide, *Lipids 28*:147–149 (1993).
9. Bergana, M.M., and T.W. Lee, Structure Determination of Long-Chain Polyunsaturated Triacylglycerols by High-Resolution ^{13}C Nuclear Magnetic Resonance, *J. Am. Oil Chem. Soc. 73*:551–556 (1996).
10. Han, J.J., Y. Iwasaki, and T. Yamane, Monitoring of Lipase-Catalyzed Transeterification Between Eicosapentaenoic Acid Ethyl Ester and Tricaprylin by Silver Ion High-Performance Liquid Chromatography and High-Temperature Gas Chromatography, *Ibid. 76*:31–39 (1999).
11. Nikolova-Damyanova, B., Silver Ion Chromatography and Lipids, in *Advances in Lipid Methodology—One*, edited by W.W. Christie, The Oily Press, Dundee, United Kingdom, 1992, pp. 181–237.
12. Hierro, M.T.G., A.I. Najera, and G. Santa-Maria, Analysis of Triglycerides by Reversed-Phase HPLC with Gradient Elution Using a Light-Scattering Detector, *Rev. Esp. Cienc. Tecnol. 32*:635–651 (1992).
13. Huang, F.C., Y.H. Ju, and C.W. Huang, Enrichment of γ-Linolenic Acid from Borage Oil *via* Lipase-Catalyzed Reactions, *J. Am. Oil Chem. Soc. 74*:977–981 (1997).
14. Haagsma, N., C.M. van Gent, J.B. Luten, R.W. de Jong, and E. van Doorn, Preparation of an ω-3 Fatty Acid Concentrate from Cod Liver Oil*, Ibid. 59*:117–118 (1982).
15. Christie, W.W., and M.L. Hunter, Separation of Neutral Lipids on Chromarods, *J. Chromatogr. 171*:517–518 (1979).
16. Akoh, C.C., and C.O Moussata, Lipase-Catalyzed Modification of Borage Oil: Incorporation of Capric and Eicosapentaenoic Acids to Form Structured Lipids, *J. Am. Oil Chem. Soc. 75*:697–701 (1998).
17. Liu, J.W., E.G. Bobik, Jr., and Y.S. Huang, *In vitro* Hydrolysis of Fungal Oils: Distribution of Arachidonic Acid-Containing Triacylglycerol Molecular Species, *Ibid. 75*:507–510 (1998).
18. Chen, T.C., and Y.H. Ju, Polyunsaturated Fatty Acid Concentrates from Borage and Linseed Oil Fatty Acids*, Ibid. 78:*485–488 (2001).
19. Chen, T.C., and Y.H. Ju, Concentration of n-3 PUFA in Saponified Menhaden Oil by Fractional Solvent Crystallization, *J. Chin. Inst. Chem. Engr. 33:*143–147 (2002).

[Received October 23, 2003; accepted April 12, 2004]