Lipase-Catalyzed Acidolysis of Menhaden Oil with Pinolenic Acid

In-Hwan Kim[a] and Charles G. Hill, Jr.[b,*]

[a]Department of Food and Nutrition, College of Health Sciences, Korea University, Seoul, 136-703, Korea, and [b]Department of Chemical and Biological Engineering, University of Wisconsin–Madison, Madison, Wisconsin 53706

ABSTRACT: Lipase-catalyzed acidolysis of menhaden oil with a pinolenic acid (PLA) concentrate, prepared from pine nut oil, was studied in a solvent-free system. The PLA concentrate was prepared by urea complexation of the FA obtained by saponification of pine nut oil. Eight commercial lipases from different sources were screened for their ability to catalyze the acidolysis reaction. Two different types of structured lipids (SL) were synthesized. The first type, which has PLA residues as a primary FA residue at the *sn*-1,3 positions of the TAG, was synthesized using a 1,3-regiospecific lipase, namely, Lipozyme RM IM from *Rhizomucor miehei*. The second type of SL, which has PLA residues as a primary FA residue at both the *sn*-1,3 and *sn*-2 positions of the TAG, was synthesized using a nonspecific lipase, namely, Novozym 435 from *Candida antarctica*. The effects of variations in enzyme loading, temperature, and reaction time on PLA incorporation into the oil were monitored by GC analyses. The optimal temperature and enzyme loading for synthesis of the two types of SL were 50°C and 10% of the total weight of substrates for both enzymes. The optimal reaction time for the synthesis with Lipozyme RM IM was 16 h, whereas the optimal reaction time for the synthesis mediated by Novozym 435 was 36 h. Pancreatic lipase-catalyzed *sn*-2 positional analyses were also carried out on the TAG samples.

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KEY WORDS: Acidolysis, lipase, menhaden oil, pinolenic acid (PLA), pine nut oil, urea complexation.

Oils from conifer nuts contain a very unusual series of C_{18}- and C_{20}-PUFA in which the first double bond is in the Δ5 position and the next immediate double bond is at the Δ9 or Δ11 location. Hence the first two double bonds are separated by two or four methylene (CH_2) units (1). The Δ5-unsaturated polymethylene-interrupted fatty acids (Δ5-UPIFA) are characteristic components of conifer nut storage lipids (2–4). The Δ5-UPIFA that have been identified in conifer nuts include 5,9-18:2 (taxoleic); 5,11-18:2 (ephedrenic); 5,9,12-18:3 (pinolenic); 5,9,12,15-18:4 (coniferonic); 5,11-20:2 (keteleeronic); 5,11,14-20:3 (sciadonic); and 5,11,14,17-20:4 (juniperonic) acids. In these FA, all olefinic bonds are in the *cis* configuration (5–8). Dietary pine nuts are consumed as condiments in various dishes. Pine nut oil is the only commercially available conifer nut oil that is rich in pinolenic acid (PLA), a Δ5-UPIFA (9,10). Several studies have demonstrated that this oil has hypocholesterolemic activity in animals. In addition, ingestion of this oil reduces blood pressure and attenuates serum VLDL-TAG and VLDL cholesterol (11–14). Lee *et al*. (15) also have reported that concentrates of PLA may reduce serum levels of LDL by enhancing hepatic LDL uptake. Pine nut oil thus may find dietetic and pharmaceutical applications.

n-3 PUFA, such as EPA and DHA, are common to fish oils and have received significant attention by the nutrition community over the past two decades because of the health benefits they provide. Among these are improved immune function and prevention of heart disease and certain cancers (16–18). Consumption of n-3 PUFA also has been reported to provide important benefits with respect to functioning of the brain (19) and retina (20), as well as to accelerate the growth of preterm infants (21,22). Fish oils typically contain a total of 14–30% EPA and DHA. Menhaden, sardine, anchovy, herring, and cod liver are the major sources for commercial production of fish oils.

Lipase-catalyzed modification of TAG has several advantages over chemical modification in the production of structured lipids (SL). By using enzymatic transesterification, one can incorporate an acyl group at a specific position in the TAG. By contrast, chemical transesterification does not produce this regiospecificity because of the random nature of the reaction (23,24). Incorporation of PLA from pine nut oil in fish oil TAG would provide a unique specialty oil for specific nutritional and clinical purposes. This modification of the fish oil can be achieved *via* lipase-catalyzed acidolysis. Lipase-mediated reactions, such as transesterification of plant oils with n-3 PUFA, have been successfully used for preparation of SL (25,26). However, to our knowledge, no investigations have previously been reported in which fish oils have been modified so as to contain both PLA and n-3 PUFA in the same acylglycerol molecule.

To synthesize SL containing PLA, we prepared a PLA concentrate from pine nut oil by urea complexation. The ability of different lipases to catalyze the regiospecific acidolysis of menhaden oil with a PLA concentrate has been investigated. Two different types of SL were synthesized. The first type, which has PLA residues as the predominant FA residue at the *sn*-1,3 positions of the TAG, was synthesized using a 1,3-regiospecific lipase, namely, Lipozyme RM IM from *Rhizomucor miehei*. The second type of SL contains PLA residues as the predominant FA residue at both the *sn*-1,3 and *sn*-2 positions. These TAG were

*To whom correspondence should be addressed at Dept. of Chemical and Biological Engineering, University of Wisconsin–Madison, 1415 Engineering Dr., Madison, WI 53706. E-mail: hill@cae.wisc.edu

synthesized using a nonspecific lipase, namely, Novozym 435 from *Candida antarctica.* Pancreatic lipase-catalyzed *sn*-2 positional analyses were also performed on the SL synthesized at different reaction times. A knowledge of the positional distribution of FA residues is of interest because of the differences in the reactivity of the residues located in the *sn*-2 and *sn*-1,3 positions in the acylglycerol. Consequently, the residues contained in the two types of SL experience different histories as they pass through the gastrointestinal tract in the course of their metabolism.

EXPERIMENTAL PROCEDURES

Materials. Pine nuts were purchased from Whole Foods (Madison, WI). Immobilized lipases from *C. antarctica* (Novozym 435), *R. miehei* (Lipozyme RM IM), and *Thermomyces lanuginosa* (Lipozyme TL IM) were provided by Novo Nordisk BioChem North America, Inc. (Franklinton, NC). Other immobilized lipases from *Burkholderia cepacia*, immobilized on either ceramic particles (Lipase PS-C) or diatomaceous earth (Lipase-PS-D), as well as free lipases from *C. rugosa* (Lipase AY), *Pseudomonas fluorescens* (Lipase AK), and *B. cepacia* (Lipase PS) were obtained from Amano Enzymes (Troy, VA). FA standards, menhaden oil, and pancreatic lipase were purchased from Sigma Chemical Company (St. Louis, MO).

Preparation of FA from pine nuts. Pine nuts (200 g) were homogenized using a coffee grinder. The oil was extracted in a 2-L flask by stirring with 1 L of *n*-hexane for 6 h. The oil was then recovered by evaporation of the solvent. The resulting pine nut oil (100 g) was added to a solution of sodium hydroxide (40 g) in distilled water (100 mL) and ethanol (99%, 300 mL). The mixture was refluxed with stirring at 500 rpm for 1 h. Water (200 mL) was added to the saponified mixture, and the unsaponifiable matter was extracted into 300 mL of *n*-hexane and discarded. The aqueous layer containing the saponifiable matter was acidified by adding an aqueous solution of 6 N HCl to achieve a pH of 1.0. The resulting lower layer was removed using a separatory funnel and discarded. The upper layer containing the FA was extracted into 200 mL of *n*-hexane and washed twice with 100 mL of distilled water. The hexane layer containing the FA was then dried over anhydrous sodium sulfate. The solvent was then removed in a rotary evaporator at 40°C.

Preparation of PLA concentrates by urea crystallization. Fifty grams of the FA mixture obtained by saponification of the pine nut oil was dissolved in 600 mL of ethanol. To this solution was added 150 g of urea, and this was then refluxed for 20 min. The solution was cooled to ambient temperature and held at this temperature for 30 min. The solution was then held at 4°C for 24 h to permit solutes to crystallize. The crystals were removed by filtration through a Büchner funnel. The filtrate was then vacuum-evaporated at 40°C, and 300 mL of hot 0.1 N HCl was added. The PLA concentrate was recovered by extracting the acidified solution twice with 600 mL of *n*-hexane. The hexane extract was washed twice with 100 mL of water and dried over anhydrous sodium sulfate. The solvent was removed in a rotary evaporator at 40°C to obtain the PLA concentrate, which was then stored at −40°C until used. Of the 50 g of the FA mixture used as the starting material, 13.2 g of total FA (26.4%) was recovered in the PLA concentrate prepared by urea complexation. However, of the PLA contained in the 50 g of starting material, 68.8% was recovered during the complexation process.

Acidolysis reaction. Menhaden oil (18.2 g; average M.W. 866) was mixed with 1.18 g PLA concentrate (average M.W. 280) at a mole ratio of 1:2 in a 25-mL screw-capped Erlenmeyer flask. The lipase of interest (2.5 to 20% of the weight of reactant) was then added. The mixture was stirred in an orbital shaker water bath (Model G76; New Brunswick Scientific Co. Inc., New Brunswick, NJ) at 300 rpm and temperatures from 40 to 70°C. Individual samples were removed at selected times and analyzed. All reactions were performed in duplicate.

Analysis of products. Once the reaction was complete, the enzymes were removed by filtration. The modified TAG were isolated using TLC, developed with petroleum ether/ethyl ether/acetic acid (80:20:0.5, by vol), and detected with 0.2% 2,7-dichlorofluorescein in methanol solution under UV light. The band corresponding to the TAG was scraped off the TLC plate and methylated according to AOCS standard method Ce 2-66 (27). The FAME were extracted with 3 mL *n*-hexane, dried over sodium sulfate, and concentrated under nitrogen. A gas chromatograph (Model 5890 II; Hewlett-Packard, Walnut Creek, CA) equipped with a SUPELCOWAX 10 fused-silica capillary column (30 m × 0.25 mm i.d.; Supelco, Bellefonte, PA) and FID was used in the analysis. Initially, the column was held at 180°C for 1 min and programmed to rise to 230°C at a rate of 2.0°C/min. It was then held at 230°C for 20 min. The carrier gas was helium, and the total gas flow rate was 50 mL/min. The injector and detector temperatures were 240 and 250°C, respectively. Δ5-UPIFA such as 5,9-C18:2, PLA (5,9,12-C18:3), and 5,11,14-C20:3 were identified by GC–MS in the manner reported by Lee *et al.* (15). The other FAME were identified by comparison with the retention times of the standards.

Hydrolysis by pancreatic lipase. Pancreatic hydrolysis was used to determine the positional distribution of the FA in TAG (28). Five milligrams of TAG was mixed with 2 mL of 1 M Tris-HCl buffer (pH 7.6), 0.5 mL of 0.05% bile salts, 0.2 mL of 2.2% $CaCl_2$, and 3 mg of pancreatic lipase. The mixture was incubated in a water bath at 37°C for 2 min, vortexed vigorously, extracted with diethyl ether, and dried using anhydrous sodium sulfate. The mixture was then placed on a silica gel G TLC plate (Alltech Assocciates, Inc., Deerfield, IL) and developed with hexane/diethyl ether/acetic acid (50:50:1, by vol). The band corresponding to the 2-MAG was scraped off, extracted with diethyl ether, methylated, and analyzed by GC.

RESULTS AND DISCUSSION

A concentrate of PLA was obtained by urea complexation of the FA of pine nut oil. The FA compositions of the original pine nut oil and the PLA concentrate produced therefrom are given in Table 1. The major FA present in the starting pine nut oil were C16:0, C18:1, C18:2, and C18:3 (Δ5, PLA). As a consequence of the treatment with ethanol and urea, the C16:0 and C18:1 FA were almost eliminated from the FA mixture whereas the PLA

TABLE 1
FA Compositions (mol%) of Pine Nut Oil and the PLA Concentrate Obtained by Urea Complexation[a]

FA	Pine nut oil	PLA concentrate
C16:0	5.3	ND
C18:1n-9	26.8	1.1
C18:2 Δ5	2.2	1.7
C18:2n-6	46.2	51.0
C18:3 Δ5	16.8	43.8
C18:3n-3	0.2	0.3
C20:1n-9	1.1	ND
C20:2n-6	0.5	0.2
C20:3 Δ5	0.9	1.9

[a]Results are the average of duplicate determinations from different experiments. ND, not detected; PLA, pinolenic acid

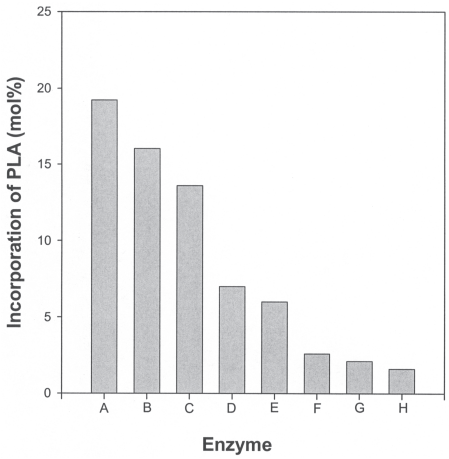

FIG. 1. Extent of incorporation of pinolenic acid (PLA) in menhaden oil by various enzymes. Enzymes: A, Novozym 435 (an immobilized lipase from *Candida antarctica*); B, Lipozyme TL IM (an immobilized lipase from *Thermomyces lanuginosa*); C, Lipozyme RM IM (an immobilized lipase from *Rhizomucor miehei*); D, Lipase PS-D (an immobilized lipase from *Burkholderia cepacia*); E, Lipase PS-C (an immobilized lipase from *B. cepacia*); F, Lipase AY (from *C. rugosa*); G, Lipase AK (from *Pseudomonas fluorescens*); H, Lipase PS (from *B. cepacia*). Reaction conditions: a mixture consisting of 1.18 g PLA concentrate, 1.82 g menhaden oil, and 0.3 g enzyme was incubated for 24 h in an orbital shaker operating at 300 rpm and 50°C.

was enriched from 16.8 to 43.8 mol%. This PLA concentrate was used as an acyl donor for synthesis of SL by lipase-catalyzed acidolysis of menhaden oil.

Eight commercial lipases from different sources were screened for their ability to incorporate PLA into menhaden oil after 24 h of reaction in a solvent-free system and an enzyme loading of 10 wt% based on the total weight of substrates. Although the extent of incorporation of PLA residues in the TAG depends on both the enzyme loading and the time of reaction, inspection of Figure 1 indicates that 24 h is appropriate for discriminating between the activities of the various enzymes. Novozym 435 from *C. antarctica*, which is considered a nonspecific lipase, gave the highest degree of incorporation, namely, 19.2 mol%. The degrees of incorporation of PLA into menhaden oil for Lipozyme TL IM from *T. lanuginosa* and Lipozyme RM IM from *R. miehei* were 16.1 and 13.6 mol%, respectively. Other lipases examined in this study were less effective.

The FA compositions at the *sn*-2 positions of the original menhaden oil and the oils modified using three different lipases

TABLE 2
Positional Distribution (mol%) of FA in Unmodified Menhaden Oil and PLA-Enriched Modified Menhaden Oils[a]

	Unmodified menhaden oil		Oil modified with Lipozyme RM IM		Oil modified with Lipozyme TL IM		Oil modified with Novozym 435	
FA	*sn*-1,2,3	*sn*-2	*sn*-1,2,3	*sn*-2	*sn*-1,2,3	*sn*-2	*sn*-1,2,3	*sn*-2
C14:0	10.0	13.5	7.0	12.9	6.4	10.5	6.2	8.0
C16:0	20.8	24.7	12.9	24.7	12.6	21.2	12.3	17.3
C16:1n-7	11.1	10.6	6.8	10.3	6.5	9.0	6.8	7.9
C16:2n-4	2.6	2.8	1.7	2.5	1.6	2.1	1.6	1.8
C17:0	0.6	0.3	0.3	0.3	0.3	0.4	0.3	0.4
C16:3n-4	2.6	3.9	2.2	3.3	2.0	3.2	1.5	1.8
C18:0	3.4	1.0	1.4	1.2	1.6	1.8	1.7	2.0
C18:1n-9	10.4	3.9	5.6	4.0	5.6	5.0	5.9	5.1
C18:1n-7	3.2	1.3	1.6	1.3	1.6	1.5	1.7	1.6
C18:2 Δ5	ND	ND	0.6	ND	0.7	0.3	0.8	0.7
C18:2n-6	1.5	1.1	17.5	3.8	19.2	9.5	20.9	14.5
C18:3 Δ5	ND	ND	13.6	2.7	16.1	6.6	19.2	14.2
C18:3n-3	1.6	1.2	1.1	1.1	1.0	1.1	1.1	1.0
C18:4n-3	3.7	4.2	3.3	3.8	3.0	1.9	2.1	2.3
C20:1n-9	1.7	0.6	0.8	0.6	0.8	0.8	0.8	0.9
C20:2n-6	0.2	0.1	0.2	ND	0.2	ND	0.2	0.2
C20:3 Δ5	ND	ND	0.5	ND	0.6	0.2	0.7	0.6
C20:4n-6	0.8	1.0	0.7	0.9	0.6	0.8	0.5	0.6
C20:4n-3	1.9	0.6	1.5	0.6	1.2	0.8	1.0	0.7
C20:5n-3	10.4	10.3	8.5	9.2	6.8	8.3	6.0	6.0
C22:5n-3	2.0	3.1	1.6	2.8	1.4	2.3	1.2	1.7
C22:6n-3	11.5	15.8	10.6	14.0	10.2	12.7	7.5	10.7

[a]The reaction conditions and enzyme sources were the same as those used in the lipase screening experiments of Figure 1. ND, not detected; for other abbreviation see Table 1.

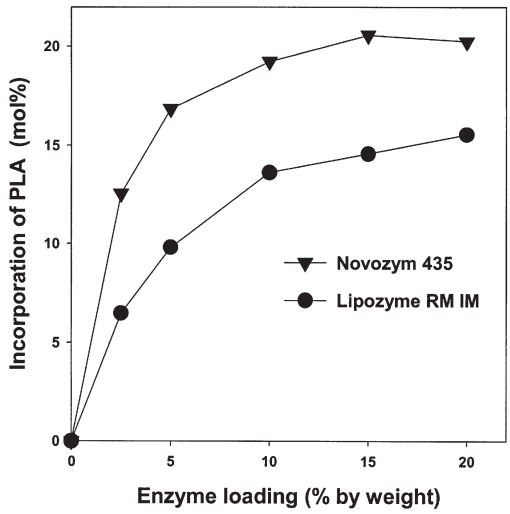

FIG. 2. Effect of enzyme loading on incorporation of PLA into the TAG of menhaden oil. The amount of enzyme used is expressed as a weight percentage of the total amount of reactants. Other conditions were the same as those for the trials in Figure 1.

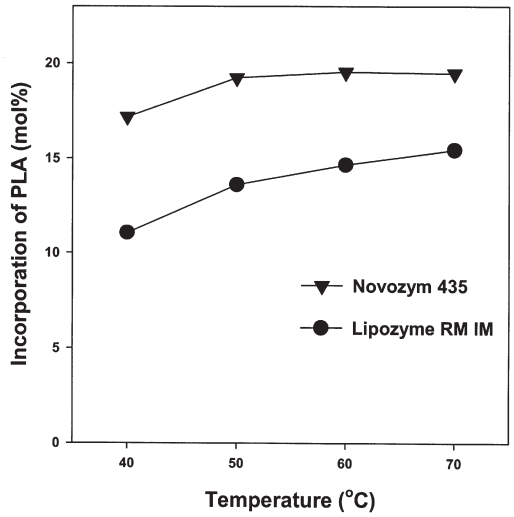

FIG. 3. Effect of reaction temperature on incorporation of PLA into the TAG of menhaden oil. Temperatures ranged from 40 to 70°C. Other conditions were the same as those for the trials in Figure 1.

are presented in Table 2. No Δ5-UPIFA were present in the menhaden oil used in this study. Although Lipozyme TL IM produced slightly greater incorporation of PLA than Lipozyme RM IM, the trial with Lipozyme TL IM also produced a higher degree of acyl migration than that with Lipozyme RM IM. This result is consistent with that of Kim *et al*. (29), who reported that when SL were synthesized by acidolysis of perilla oil with caprylic acid, systems mediated by Lipozyme TL IM exhibited greater acyl migration than systems mediated by Lipozyme RM IM. Consequently, Lipozyme RM IM was selected as the more suitable lipase for use in production of the first type of SL, which has PLA as the primary FA at the *sn*-1,3 positions of the TAG.

The effect of enzyme loading on the incorporation of PLA into menhaden oil is shown in Figure 2. For reactions catalyzed by Lipozyme RM IM and Novozym 435, the extents of incorporation of PLA increased as the amount of enzyme in the reaction mixture increased, but significant increases were not observed once either enzyme was present at a level ≥10% of the total weight of substrates. The optimal enzyme loadings were 10% for both enzymes. Overall, Novozym 435 produced greater incorporation of PLA than Lipozyme RM IM.

The optimal temperature for use of an enzyme depends on its source, the nature of immobilization or chemical modification, the solvent, and the pH of the reaction medium (30). The effect of temperature on the acidolysis of menhaden oil with the PLA concentrate as the acyl donor is shown in Figure 3. For these trials the enzyme loading and the reaction time were held constant at 10% of the total weight of substrate and 24 h, respectively. The temperature range tested was between 40 and 70°C. For the trials with Novozym 435, the incorporation of PLA increased from 17.2 to 19.2 mol% when the temperature was increased from 40 to 50°C. However, the incorporation of PLA catalyzed by Novozym 435 remained constant when the temperature was further increased to 70 °C. For the trials involving Lipozyme RM IM, the incorporation of PLA increased significantly as the temperature increased from 40 to 50°C. However, incorporation of PLA continued to increase as the temperature rose from 50 to 70°C, even though the extent of additional incorporation was small. Xu *et al*. (31) have reported that reaction temperature is the most important factor in acyl migration. These researchers also reported that for Lipozyme RM IM the effect of temperature is approximately linear for temperatures from 50 to 70°C. Xu *et al*. also have suggested that selectivity can be increased by decreasing the reaction temperature if less acyl migration is desired. On the other hand, thermal degradation of PUFA may occur faster at temperatures higher than 60°C (32). Thus, 50°C was chosen as an appropriate temperature for use with both enzymes.

Monitoring the time course of enzymatic reactions is important for determining the optimal conditions for obtaining good yields and minimizing overall process costs. The time course of the reaction was studied for each lipase. Samples taken at selected times were analyzed to determine both the PLA and n-3 PUFA at all three locations on the glycerol backbone (*sn*-1,2,3) and at the *sn*-2 position. The data for the trial with Lipozyme RM IM are illustrated in Figure 4. n-3 PUFA is defined as the sum of EPA, docosapentaenoic acid, and DHA. During the initial 8 h of reaction, significant incorporation of PLA (8.9 mol%) was obtained. During the next 16 h, there was a slow but steady increase in the extent of incorporation of PLA, such that 13.6 mol% PLA was incorporated in the TAG after a total of 24 h of reaction. However, for times greater than 24 h, further increases in incorporation of PLA were minimal for Lipozyme RM IM. The type of SL that has PLA as the primary FA at the *sn*-1,3 positions of the TAG can be synthesized using a 1,3-regiospecific lipase, for example, Lipozyme RM IM from *R. miehei.* In principle, SL of this type could be synthesized without any decrease in the n-3 PUFA at the *sn*-2 position if acyl migration does not occur. How-

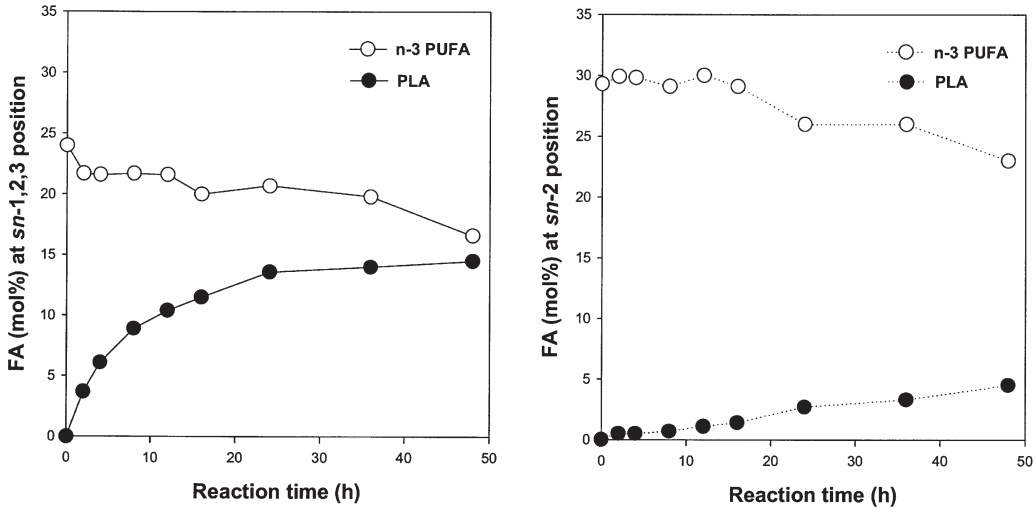

FIG. 4. Effect of reaction time on the incorporation (*sn*-1,2,3) and migration (*sn*-2) of FA residues for the reaction catalyzed by Lipozyme RM IM (10% of the total weight of substrates). Samples were analyzed at 0, 2, 4, 6, 8, 12, 16, 24, 36, and 48 h. Other conditions were the same as those for the trials in Figure 1. n-3 PUFA is defined as the sum of EPA (C20:5n-3), docosapentaenoic acid (C22:5n-3), and DHA (C22:6n-3).

ever, acyl migration is one of the major problems that occur during acidolysis in batch reactors, even when highly 1,3-regiospecific lipases are used (33). Migration is affected by factors such as water content, reaction time, reaction temperature, enzyme loading, and the reaction medium (34). Even though longer reaction times for acidolysis yield higher overall incorporation of PLA in menhaden oil, the acidolysis reaction is accompanied by a parallel increase in migration of PLA residues from the *sn*-1,3 positions to the *sn*-2 position.

For the reaction mediated by Lipozyme RM IM, we did not observe a significant decrease in the mole percentage of n-3 PUFA at the *sn*-2 position of the TAG during the first 16 h of incubation. However, in the subsequent 8 h, there was a significant decrease in the mole percentage of n-3 PUFA at the *sn*-2 position. This decrease is accompanied by higher degrees of acyl migration after 24 h. Because acyl migration results in decreased n-3 PUFA content at the *sn*-2 position of the TAG, it is desirable to minimize acyl migration during acidolysis reactions. Hence we selected 16 h as the optimal reaction time for synthesis of the first type of SL in the presence of Lipozyme RM IM from *R. miehei*.

The mole percentages of PLA and n-3 PUFA at both the *sn*-1,2,3 and *sn*-2 positions of menhaden oil produced in the trials involving Novozym 435 are plotted in Figure 5. Incorporation of PLA was time dependent and attained a maximum of 19.4 mol%

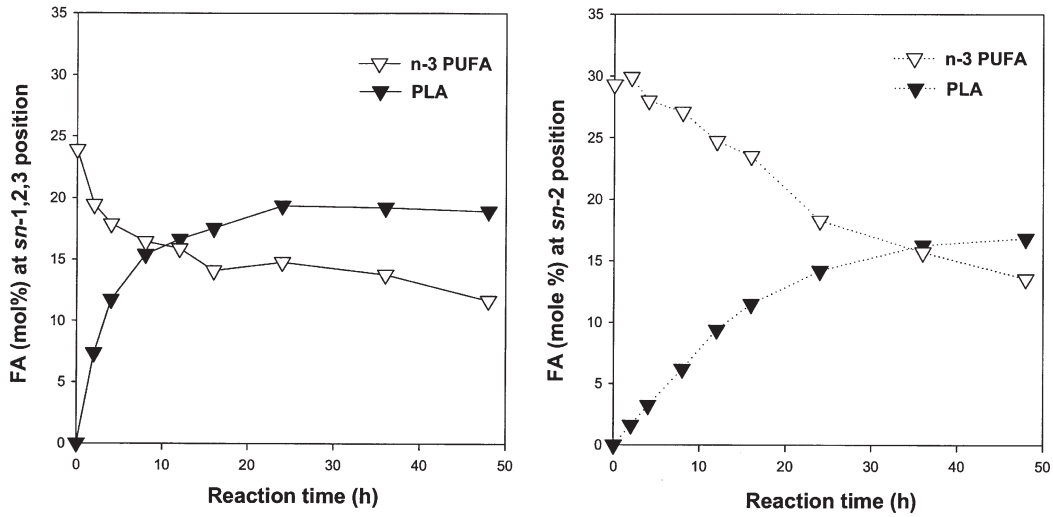

FIG. 5. Effect of reaction time on the incorporation (*sn*-1,2,3) and migration (*sn*-2) of FA residues for the reaction catalyzed by Novozym 435 (10% of the total weight of substrates). Reaction conditions and abbreviations are the same as those for Figure 4.

after incubation for 24 h, after which no further increase was observed. However, significant increases in incorporation of PLA at the *sn*-2 position were observed for times up to 36 h.

Another goal of this study was to produce SL containing PLA residues as the primary species at both the *sn*-1,3 and *sn*-2 positions *via* lipase-catalyzed acidolysis of menhaden oil with the PLA concentrate using a nonspecific lipase as the biocatalyst. For synthesis of the second type of SL with Novozym 435 from *C. antarctica*, we determined that 36 h was the optimal reaction time. This enzyme exhibited some 1,3-regiospecificity for times up to 24 h, although the level of 1,3-regiospecificity was not high compared with that of Lipozyme RM IM. For reaction times in excess of 24 h, a small degree of 1,3-regiospecificity was observed. Novozym 435 or Chirazyme® L-2 from *C. antarctica* (which is considered a positionally nonspecific lipase) has been widely used as a biocatalyst for synthesis of SL (35–37). However, this enzyme can exhibit nonspecific or 1,3-regiospecificity, depending on reactants and reaction conditions (38). Yang *et al.* (39) have reported that Chirazyme L-2 from *C. antarctica* displayed 1,3- regiospecificity in the early stage (up to 8 h) of reaction when a low-calorie SL was synthesized by acidolysis of triacetin with stearic acid. Kim *et al*. (40) also have reported that early in the acidolysis of tricaprylin by CLA, Novozym 435 from *C. antarctica* exhibited a weak selectivity for the *sn*-1,3 position.

The results of enzyme-catalyzed incorporation of PLA into menhaden oil using various lipases in solvent-free systems lead one to conclude that Novozym 435 can incorporate PLA at both the *sn*-1,3 and *sn*-2 positions of the glycerol backbone, whereas Lipozyme RM IM shows a high selectivity for the *sn*-1,3 positions. Novozym 435 also exhibited some 1,3-regiospecificity in the early stages of the reaction. However, for longer reaction times, the 1,3-regiospecificity of this enzyme decreased significantly.

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