Enzymatic Synthesis of Structured Lipids from Single Cell Oil of High Docosahexaenoic Acid Content

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ABSTRACT: The lipase-catalyzed acidolysis of a single-cell oil (SCO) containing docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA) with caprylic acid (CA) was investigated. The targeted products were structured lipids containing CA residues at the *sn*-1 and -3 positions and a DHA or DPA residue at the *sn*-2 position of glycerol. *Rhizomucor miehei* lipase (RML) and *Pseudomonas* sp. KWI-56 lipase (PSL) were used as the biocatalysts. When PSL was used > 60 mol% of total SCO fatty acids (FA) were exchanged with CA, with DHA and DPA as well as the other saturated FA being exchanged. The content of the triacylglycerols (TG) containing two CA and one DHA or DPA (number of carbon atoms = 41, i.e., C_{41}) residue was high (36%), and the isomer with the desired configuration (unsaturated FA residue at the *sn*-2 position) represented 77–78% of C_{41}. In the case of RML, CA content reached only 23 mol% in the TG. A large amount of DHA and DPA residues remained unexchanged with RML, so that the resulting oil was rich in TG species containing two or three DHA or DPA residues (46%). TG C_{41} amounted to 22%, almost all of which had the desired configuration. This result suggested that the difference in the degree of acidolysis by the two enzymes was due to their different selectivity toward DHA and DPA, as well as the difference in their positional specificities.

Paper no. J8976 in *JAOCS 76*, 563–569 (May 1999).

KEY WORDS: Acidolysis, docosahexaenoic acid, docosapentaenoic acid, lipase, single-cell oil, structured lipid.

Structured lipids (SL) are triacylglycerols (TG) with particular fatty acids (FA) in specific positions of the glycerol hydroxyl moieties. SL with medium-chain FA at the *sn*-1 and -3 positions and long-chain FA at the *sn*-2 positions are synthesized to enhance the nutritional and pharmaceutical properties of TG. Mammalian pancreatic lipases hydrolyze the ester linkages at the *sn*-1 and -3 positions with a preference for medium-chain FA over long-chain ones (1,2). The resulting *sn*-2-monoacylglycerols are well adsorbed through the intestinal mucosa (3). Thus, these kinds of TG are expected to be effective carriers of the FA located at the *sn*-2 positions (3–5).

Based on this perspective, several efforts were reported for syntheses of SL with caprylic acid (CA) at the *sn*-1 and -3 positions and functional FA such as essential fatty acids or polyunsaturated fatty acids (PUFA) at the *sn*-2 position (6–10). These reports employed the modification of appropriate oils by acidolysis with free CA or interesterification with CA-ethyl ester. The common strategy of these studies was to exchange the FA specifically at the *sn*-1 and -3 positions of the oils for CA using 1,3-specific lipases (especially from fungi such as *Rhizopus delemar* and *Rhizomucor miehei*), leaving the FA at the *sn*-2 position unchanged. However, in the case of docosahexaenoic acid (DHA, 22:6n-3)-containing oils, the DHA residues at the *sn*-1 and -3 positions of the starting material remained unreacted due to the poor activity of the fungal lipases on DHA (8,11,12). For such oils, a possible alternative might be the use of a lipase which is active on DHA.

In the present paper, we describe the syntheses of SL with DHA and docosapentaenoic acid (DPA, 22:5n-6), both of which are poor substrates for these commonly used fungal lipases. The acidolysis reaction of DHA- and DPA-rich single-cell oil (SCO, which refers to oils produced from microorganisms) with CA was compared experimentally using two lipases with different acyl group preferences and positional specificities.

MATERIALS AND METHODS

Chemicals and enzymes. SCO produced by a marine microbe, *Schizochytrium* sp. SR21 (13,14), was a gift from Nagase Biochemical Industries (Kyoto, Japan). FA composition of the SCO was 4.2 mol% myristic acid (MA), 2.5 mol% pentadecanoic acid (PdA), 46.3 mol% palmitic acid (PA), 1.3 mol% stearic acid (SA), 10.2 mol% DPA, 35.5 mol% DHA. CA was purchased from Sigma (St. Louis, MO).

Rhizomucor miehei lipase (RML, *sn*-1,3-regiospecific, with low activity toward DHA and DPA) immobilized on an ion-exchange resin (commercially available as Lipozyme) was a gift from Novo Nordisk Bioindustry (Chiba, Japan). *Pseudomonas* sp. KWI-56 lipase (PSL, nonregiospecific, active toward DHA and DPA) from Kurita Water Industries (Tokyo, Japan) was used after immobilization on calcium carbonate powder (15).

Acidolysis reaction. One g (1.1 mmol) of SCO, 1 to 4 g (6.9 to 27.8 mmol) of CA, and either 540 mg of PSL or 100

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mg of RML were incubated at 30°C with mixing by a magnetic stirrer at 300 rpm for 6–7 d. The initial reaction mixture contained 0.2–0.3% (w/w) water, which was analyzed with a Karl Fischer moisture meter (Model MKS1, Kyoto Electric, Kyoto, Japan)

FA composition analysis. At 4, 8, 24, and then at 24-h intervals, 20-μL samples were withdrawn from the reaction mixture, mixed with 0.4 mL of *n*-hexane, and the enzyme was removed by centrifugation. The remaining amount of FA was removed by washing twice with 0.28 mL of 0.5 N KOH in 20% ethanol solution. The resulting glyceride solution was concentrated by a nitrogen stream and applied to a thin-layer chromatography (TLC) plate (silica gel 60, Merck, Darmstadt, Germany). The plate was developed with *n*-hexane/diethyl ether/acetic acid = 70:30:1 (vol/vol/vol), and the lipids were visualized by spraying with 0.1% 2′,7′-dichlorofluorescein solution in ethanol. The TG fraction was scraped off and extracted with chloroform/methanol = 2:1 (vol/vol). The FA residues of the recovered TG were converted into FA methyl esters (16).

The FA methyl esters were analyzed with a gas chromatograph (Model GC 8A; Shimadzu, Kyoto, Japan) equipped with a capillary column (Type HP-INNOWAX, cross-linked polyethyleneglycol, 0.32 mm × 30 m, Hewlett Packard, Palo Alto, CA). Helium was used as carrier gas at 10 mL/min. The column was kept at 150°C for 3 min, heated at a rate of 16°C/min to 230°C, and then kept at 230°C for 30 min. Detection was done by a flame-ionization detector (FID) with hydrogen and air at 50 mL/min and 470 mL/min, respectively. The FA composition was determined from the peak areas of the esters, multiplied by their relative sensitivities, calculated with the effective carbon number method (17). In this study, only CA, MA, PdA, PA, SA, DPA, and DHA were taken into consideration. The peaks of the other components (not identified) were so small (less than 0.1% of total area) that they were ignored.

Purification of the products. Acidolysis was performed for 144 h at substrate molar ratios (CA/SCO) of 12.4 for RML and 18.8 for PSL as described above. At the end of the reaction, 40 mL of *n*-hexane was added to the whole reaction mixture. The enzyme was removed by centrifugation. The solution was washed twice with 30 mL 0.5 N KOH in 20% ethanol to remove FA, then succesively with 30 mL water and 30 mL saturated NaCl solution. The resultant FA-free glyceride solution was dried with anhydrous Na_2SO_4, and the solvent was removed by a rotary evaporator. The residue was dissolved in 10 mL *n*-hexane, applied on a silica gel column (1.6 × 20 cm), and eluted with about 400 mL *n*-hexane/diethyl ether (9:1, vol/vol). The eluate was collected as 20-mL fractions. After checking the content of each fraction by TLC, TG fractions were pooled and used for subsequent analyses.

High-temperature gas chromatography (HTGC). The molecular species of the purified TG were analyzed with a gas chromatograph (Model GC14; Shimazu) equipped with an on-column injector (Model OCI-14, Shimazu) and a capillary column (Type HT5, 0.53 mm × 6 m, SGE, Australia).

Helium was used as carrier gas at 15 mL/min. The column was kept at 80°C for 0.5 min, heated at a rate of 20°C/min to 260°C, then at 10°C/min to 330°C, and finally at 5°C/min to 390°C and kept at this temperature for 1 min. The injector temperature was held at 80°C for 0.01 min, raised at 40°C/min to 260°C, then at 20°C/min to 340°C, and at 10°C/min to 393°C and kept at this temperature for 15.6 min. The FID temperature was kept at 393°C with air and hydrogen at 470 mL/min and 50 mL/min, respectively. Nitrogen was used as make-up gas for the FID at 7.5 mL/min.

Under these analytical conditions, TG species were separated depending on their carbon numbers. The peaks were identified by comparing their retention time to those of authentic TG. Composition of TG species was calculated from the peak areas.

High-performance liquid chromatography (HPLC). A silver-ion HPLC column (Chromspher 5 lipid, silver-modified cation-exchange ligand-covered spherical silica, 4.6 × 150 mm, Chrompack, Middleburg, The Netherlands) was used (18,19). Approximately 5 μL of a 1% solution of the purified TG in *n*-hexane/2-propanol = 7:2 was injected into the column previously equilibrated with *n*-hexane/2-propanol/acetonitrile = 350:100:2.75 (vol/vol/vol: solvent A). The column was eluted with solvent A at a flow rate of 0.65 mL/min for 3 min, then with a linear gradient from solvent A to *n*-hexane/2-propanol/acetonitrile = 350:100:10 (vol/vol/vol: solvent B) in 10 min, and finally with solvent B for 25 min. The lipids were detected spectrophotometrically at 206 nm.

By this detection method, the sensitivity of each TG species depended mainly on the number of double bonds in its structure. For this reason, the detector showed very weak responses for all the saturated TG species. We employed this analytical method to estimate the ratio of the desired structured TG to their positional isomers that contained the same number of double bonds, and thus, the detector's response was assumed to have the same value for each isomer. The ratio of the positional isomers was estimated from the corresponding peak areas.

RESULTS

Incorporation of CA by two different lipases. Figure 1A shows the time course of the CA incorporation by RML at different ratios of CA/SCO. For the molar ratio CA/SCO = 12.4, the incorporation stopped at the CA content in TG of about 23 mol%. Since the incorporation was not improved by raising the excess of CA (CA/SCO = 18.8 or 25.0), it is likely that the amount of CA in the reaction mixture was enough to ensure the progress of the reaction. Contrary to the case of RML, PSL showed higher incorporation (Fig. 1B). The degree of the incorporation increased with the increase in the molar ratio of CA. At the molar ratio (CA/SCO) of 18.8, the content of CA in the TG reached about 65 mol% at 168 h.

The lower initial reaction rates for higher molar excess were possibly due to the dilution of enzyme in the reaction mixture (the same amount of enzyme was used for each set of experiments). The possibility of enzyme inactivation was

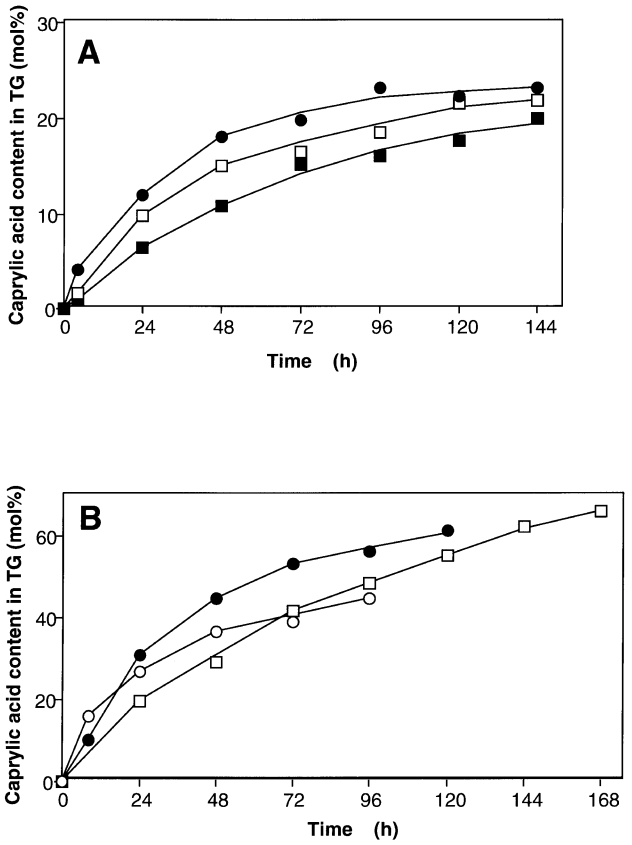

FIG. 1. Time course of caprylic acid (CA) incorporation into triglycerides (TG) during acidolysis by *Rhizomucor miehei* lipase (RML) (A) and *Pseudomonas* sp. lipase (PSL) (B). The initial molar ratios [CA/single-cell oil (SCO)] were 6.3 (○), 12.4 (●), 18.8 (□), and 25.0 (■). The enzyme amounted to 100 mg (A) and 540 mg (B) per 1 g (1.1 mmol) of SCO.

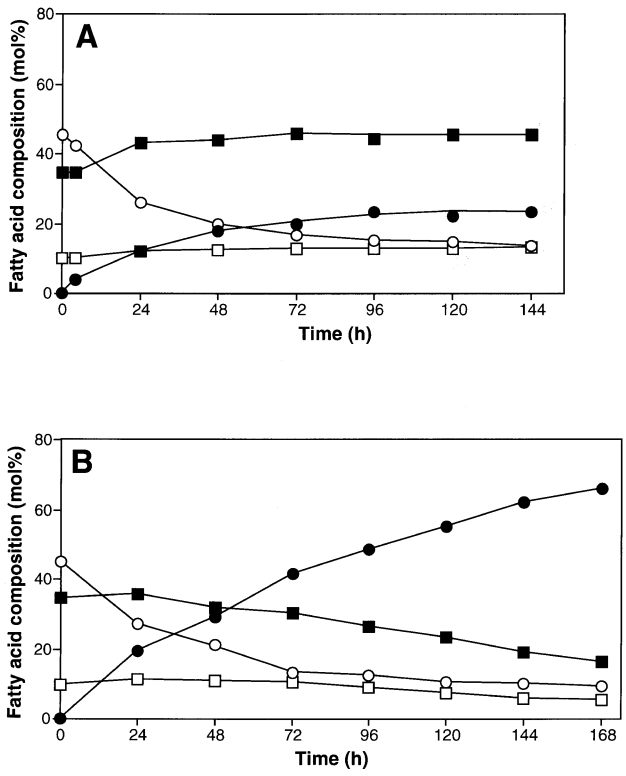

FIG. 2. Changes in fatty acid composition of TG during acidolysis. The contents of CA (●), palmitic acid (○), docosapentaenoic acid (□), and docosahexaenoic acid (■) are shown for the reactions catalyzed by RML (A) and PSL (B) at the initial molar ratio (CA/SCO) of 12.4. and 18.8, respectively. For abbreviations see Figure 1.

excluded, because the enzyme could be recovered from the mixture at the end of a reaction and reused with almost the same reaction rate for a subsequent reaction with fresh reaction mixture (data not shown).

Figure 2 shows the time course of the FA composition of the TG fraction during acidolysis by RML (CA/SCO = 12.4) and PSL (CA/SCO = 18.8). RML effectively exchanged PA, which was the most abundant FA in the original oil. However, the enzyme replaced neither DPA nor DHA because of its poor activity on these PUFA. The contents of these PUFA increased slightly possibly owing to the selective hydrolysis of the other FA residues, which occurred to some extent. On the other hand, PSL exchanged the PUFA as well as PA, but the reaction rates for these PUFA were lower than that for PA. Therefore, it is suggested that the difference in the degree of acidolysis by the two enzymes is due to the difference in their activities on these PUFA.

Preparation of the acidolyzed TGs. According to the results shown in Figures 1 and 2, we chose the reaction period of 144 h and substrate molar ratios (CA/SCO) of 12.4 for RML and 18.8 for PSL, for which high incorporations were achieved. The TG were purified by washing the reaction mixture with alkaline solution and subsequent silica gel column chromatography. From the reaction mixtures of RML and PSL (which contained initially 1g of SCO), 0.65 and 0.42 g of TG were recovered, respectively. The recovered RML-acidolyzed oil contained 23.2 mol% CA, 2.2 mol% MA, 0.9 mol% PdA, 13.8 mol% PA, 0.8 mol% SA, 13.1 mol% of DPA, and 45.8 mol% DHA, whereas the PSL-acidolyzed oil contained 64.8 mol% CA, 1.2 mol% MA, 0.8 mol% PdA, 9.3 mol% PA, 0.8 mol% SA, 5.7 mol% DPA, and 17.4 mol% DHA. Considering the changes in the average molecular weight of the TG before and after the reaction, the recovery yields of the TG were approximately 70 and 60% for RML and PSL, respectively.

Composition of molecular species of TG. The acidolyzed oils and the original SCO were analyzed by HTGC as shown in Figure 3. C_{57}, i.e., TG with 57 carbons, with two PA residues and one DPA or DHA residue was the largest fraction in the original SCO (Fig. 3A). Another major component was C_{63}, with one PA and two of the PUFA residues. In addition, there was a small amount of C_{69} with three PUFA residues.

The C_{57} peak disappeared almost completely in both the RML- and PSL-acidolyzed oils (Figs. 3B and 3C). Instead, several new peaks such as C_{27} (TG with three CA residues), C_{35} (two CA and one PA), C_{41} (two CA and one PUFA), C_{49} (one CA, one PA, and one PUFA), and C_{55} (one CA and two PUFA) were generated.

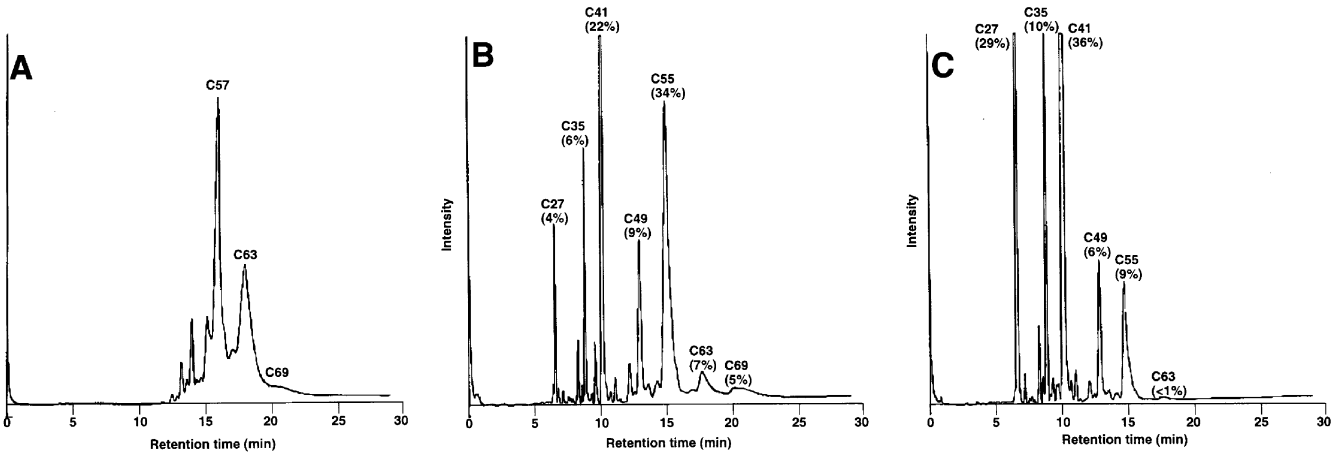

FIG. 3. High-temperature gas chromatograms of the products. TG in the initial SCO (A) and in the oils acidolyzed by RML (B) and PSL (C) were analyzed by high-temperature gas chromatography. The number of carbon atoms for the major peaks is indicated after the symbol C. For (B) and (C), the contents of TG species (% peak area) are shown in parentheses. For abbreviations see Figure 1.

In the RML-treated oil, C_{41} compound, which included the desired SL (all the positional isomers containing two CA and one DHA or DPA), amounted to 22% of the total TG (% area). However, the largest peak in the RML-treated oil was C_{55} (34%), which was an intermediate generated by the replacement of a single PA residue of C_{63} with CA. In addition, certain amounts of C_{63} (7%) and C_{69} (5%) were still present. The accumulation of C_{55} or preservation of C_{63} and C_{69} was explained by the fact that these TG molecules could not be (further) converted by RML due to its poor activity for the PUFA. A part of the unreacted C_{63} might have the PA group located at the *sn*-2 position, from which it could not be exchanged by RML, which is a 1,3-specific enzyme.

In contrast, PSL generated a larger amount of C_{41} (the most abundant peak, 36%) than RML. A large quantity of C_{27} (29%) was obtained, indicating that PSL was not as 1,3-specific as RML and that it could introduce CA into the *sn*-2 positions as well. The content of the intermediate C_{55} (9%) was much smaller than that for RML, and C_{63} and C_{69} were almost negligible (less than 1%). Thus, it is likely that the contents of the PUFA in the resultant oil (Fig. 2) were reduced mainly as a result of the acidolysis of C_{55}, C_{63} and C_{69} containing two or three PUFA residues.

Positional isomer composition of resultant SL. Figure 4 shows the HPLC elution profiles of the original SCO and the oils acidolyzed by RML and PSL. The peaks were identified by their molecular weight and molecular fragmentation with a HPLC-mass spectrometer (VG platform, Beverly, MA). A preliminary study enabled the separation of the positional isomers with two saturated FA and one PUFA residues (18,19). In each regioisomeric pair of such compounds, one isomer with the PUFA residue at the *sn*-2 position eluted faster than the one with the PUFA residue at the *sn*-1 or -3 position, according to Jeffrey's study (20). Additional proof helped us distinguish some pairs of isomers as follows. First, the pair of isomers with two CA and one DHA (i.e., CCDh and CDhC, where C = capryloyl; Dh = docosahexaenoyl) were identified by comparing their retention times to that of an authentic CCDh. The authentic CCDh was prepared by interesterification of CCC and DHA-ethylester with a 1,3-specific lipase (19). Second, it was reported that PDhP (where P = palmitoyl) was dominant in the SCO produced by *Schizochytrium* sp. SR21 (13). Thereby, of the two peaks with the same molecular weight corresponding to either PPDh or PDhP, the larger one was allocated to PDhP and the smaller one to PPDh.

The C_{41} fraction of the RML-reacted oil contained mainly the desired SL (i.e., CDpC and CDhC, where Dp = docosapentaenoyl). Their positional isomers, in this case by-products (i.e., CCDp and CCDh), were almost negligible (Fig. 4B), and the content of the desired SL was estimated to be approximately 22%.

PSL formed the desired SL and certain amounts of their isomeric by-products (Fig. 4C). In fact, the C_{41} compounds, which amounted to 36% in Figure 3C, corresponded to the four peaks (CDpC, CCDp, CDhC and CCDh) in Figure 4C. From the peak areas in Figure 4C, the ratios of CDpC/CCDp and CDhC/CCDh were estimated at 78:22 and 77:23, respectively. Thus, the content of the desired SL (CDpC plus CDhC) was estimated to be approximately 28% in the PSL-acidolyzed oil. These figures indicate that larger amounts of the desired SL could be synthesized by PSL than by RML.

DISCUSSION

Tanaka *et al.* (21) used synthetic TG to demonstrate that oleic acid (OA) residues coexisting with one or two DHA residues in TG were more resistant to hydrolysis with *Candida cylindracea* lipase than OA residues in homogeneous TG. Their explanation for this phenomenon (so-called TG specificity) was that the lipase might not be able to access OA residues owing to the steric hindrance of the coexisting DHA residues

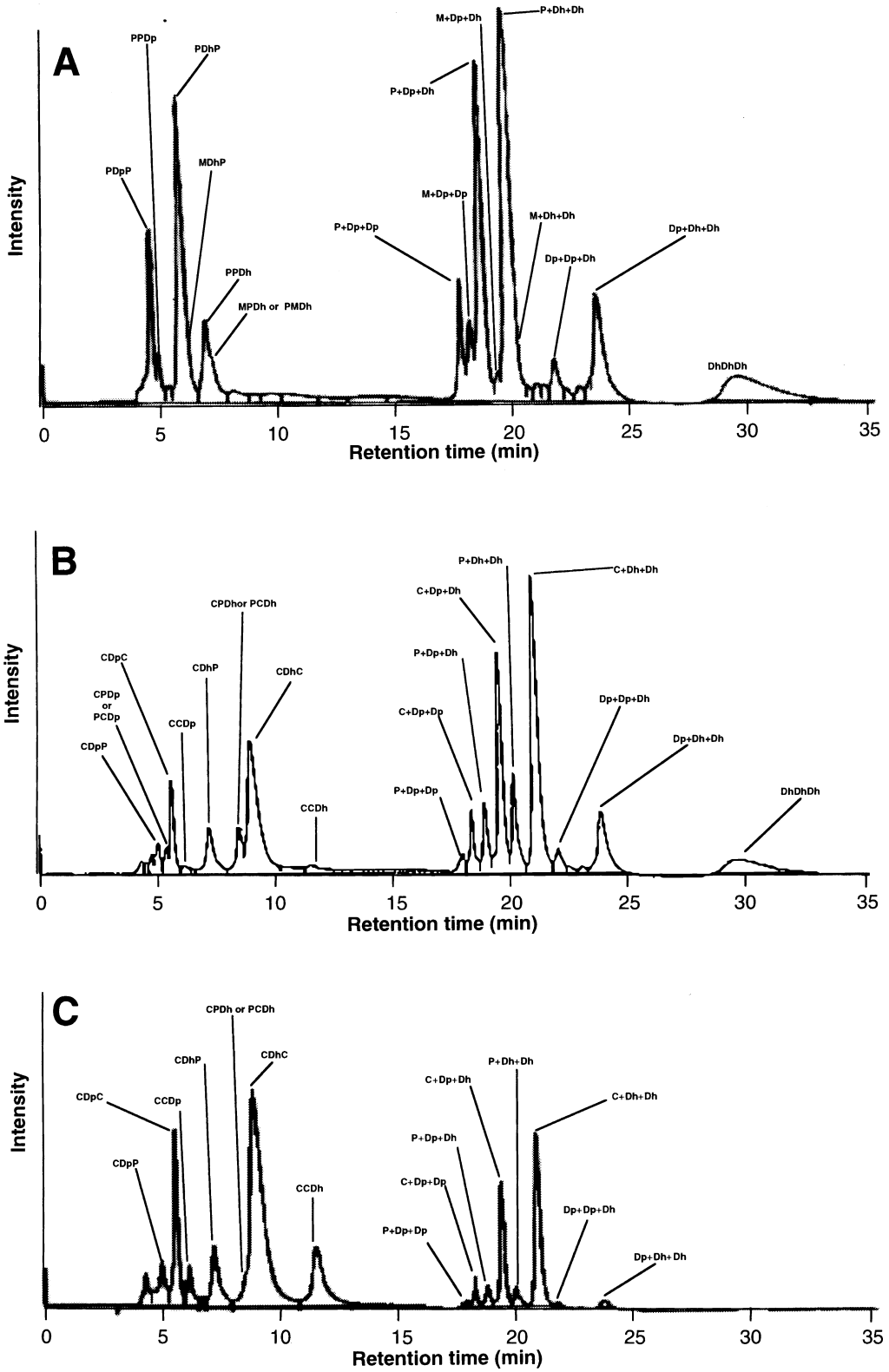

FIG. 4. Analyses of the TG by high-performance liquid chromatography. The elution profiles of SCO (A) and of the oils acidolyzed by RML (B) and PSL (C) are shown with the identified peaks as follows. Acyl groups are abbreviated as C, M, P, Dp, and Dh for capryloyl, myristoyl, palmitoyl, docosapentaenoyl, and docosahexaenoyl groups, respectively. TG species are represented using triplets of the abbreviated forms of the corresponding acyl groups with or without "+". Directly connected (without "+") triplets indicate the species of which the positional isomerism is taken into consideration. The fatty acid residues at the *sn*-2 position of such species are indicated at the middle of the triplets. Triplets connected with "+" indicate the species of which the positional isomerism was not referred. These species may comprise two or more different positional isomers. Note that no optical isomerism was considered. Also note that TG molecules comprising only saturated FA were eluted as one peak at the retention time of approximately 4 min, showing very weak sensitivity to the ultraviolet detector. For abbreviations see Figure 1.

in TG. This might be extrapolated for acidolysis of TG. Thereby, it is likely that TG without DHA can be modified more easily than TG containing DHA. In accordance with this hypothesis, Shimada *et al.* (7,8) reported that acidolysis of safflower oil and linseed oil (which do not contain DHA) with CA reached steady state three times faster than acidolysis of tuna oil (which contains approximately 20 mol% DHA).

The reaction employed here was a transesterification where the FA residues of the TG were exchanged for CA. This is a reversible reaction where the free FA released from the TG during the reaction compete with CA in being (re)introduced to TG. To elevate the incorporation of CA, excess CA is necessary. In the strategy employed by Shimada *et al.* (8) for synthesis of SL, tuna oil was acidolyzed with excess CA in three cycles of 2-d reaction. After each cycle, the TG fraction was recovered and reacted with fresh CA in the next cycle. The initial reaction mixture of each cycle contained an approximately 12-fold molar excess of CA, calculated on the average molecular weight of tuna oil. In a simple estimation, the total molar excess of CA to the initial TG was approximately 36-fold and the reaction period was 6 d for the whole process.

Our reaction was the incorporation of CA into an oil with a much higher content of DHA (35 mol%) and DPA (10 mol%), consisting mainly of TG with one or more PUFA residues (i.e., C_{57}, C_{63}, and C_{69} totaling over 90%) in a single batch reaction. This might be the reason why the reaction periods were long (6 d) and high initial molar ratios of CA to SCO (12.4 for RML and 18.8 for PSL) were necessary in our study.

The strategy for the synthesis of the SL by acidolysis is to exchange the FA residues at *sn*-1 and -3 positions and leave the ones at the *sn*-2 position unreacted by employing 1,3-specific lipases. The strategy was previously employed in *R. delemar* lipase-catalyzed acidolysis for the modification of various oils containing essential FA (safflower oil and linseed oil), γ-linoleic acid (borage oil), arachidonic acid (SCO from *Mortiellera alpina*), and DHA (tuna oil) (7,8). Most of the FA species at *sn*-1 and -3 positions could be replaced completely. However, in the case of the tuna oil, the DHA residues at the *sn*-1 and -3 positions could not be exchanged because of the very low activity of the *R. delemar* lipase for this FA species. A similar result was obtained in our present work for RML.

On the assumption that a mixture of TG species comprising all possible combinations of PA and DHA (i.e., PPP, PPDh, PDhP, PDhDh, DhPDh, and DhDhDh) is used, the species containing a DHA residue at the *sn*-2 positions of TG (i.e., PDhP, PDhDh, and DhDhDh) are potent substrates which theoretically can be converted into the desired SL (i.e., CDhC). However, RML can convert only PDhP into the desired SL because of its low reactivity for DHA. PDhDh is transformed into CDhDh which is hardly converted further, and DhDhDh remains unreacted. These considerations can explain well the chromatographic profiles of the RML-treated oil (Figs. 3B and 4B).

On the other hand, PSL is capable of converting all the components (PDhP, PDhDh, and DhDhDh) into the desired product. The disadvantage of using PSL is that the PUFA residues at the *sn*-2 position are also exchanged, wasting the potential substrates to some extent. However, as demonstrated, the yields of the desired products were higher for PSL than for RML. Thus, the use of PSL is promising for the syntheses of DHA- or DPA-containing SL, when starting materials are oils having high contents of TG species with two or three DHA or DPA residues, such as the SCO used in this study.

ACKNOWLEDGMENTS

The authors are grateful to Nagase Biochemical Industries for providing us with the SCO. We also thank Novo Nordisk Bioindustry and Kurita Water Industries for the gifts of the lipases used in this work.

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[Received August 14, 1998; accepted January 7, 1999]