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. KW1-56 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., C41) residue was high (36%), and the isomer with the desired configuration (unsaturated FA residue at the sn-2 position) represented 77–78% of C41. 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 C41 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.


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 long-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. KW1-56 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
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 polyethylene glycol, 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, 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 successively with 30 mL water and 30 mL saturated NaCl solution. The resultant FA-free glyceride solution was dried with anhydrous Na₂SO₄, 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 detector 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 molar 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