Characterization of Enzymatically Synthesized Structured Lipids Containing Eicosapentaenoic, Docosahexaenoic, and Caprylic Acids

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ABSTRACT: Structured lipids (SL) containing n-3 polyunsaturated (eicosapentaenoic or docosahexaenoic) and medium-chain (caprylic) fatty acids were synthesized in gram quantities and characterized. Tricaprylin was mixed with n-3-rich polyunsaturated fatty acids in a 1:2 molar ratio and transesterified by incubating at 55°C in hexane with SP 435 lipase (10% by wt of total substrates) in a 125-mL Erlenmeyer flask as the bioreactor. After several batches of reaction, the products were pooled and hexane was evaporated. Short-path distillation was used for purification of synthesized SL. The distillation conditions were 1.1 Torr and 170°C at a feed flow rate of 3 mL/min. Up to 240 g of SL was isolated and deacidified by alkaline extraction or ethanol–water solvents. The fatty acid profile, free fatty acid value, saponification number, iodine value, peroxide value, thiobarbituric acid, and conjugated diene contents were determined. Oxidation stability, with α-tocopherol as antioxidant, and the oxidative stability index were also determined.
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KEY WORDS: Medium-chain triacylglycerol, oxidative stability index, n-3 polyunsaturated fatty acids, structured lipids, thiobarbituric acid.

Structured lipids (SL) are any lipids restructured by chemical or enzymatic processes to change their fatty acid composition and/or the stereochemical positions of fatty acids in the glycerol molecule (1). SL can be synthesized to provide specific metabolic effects, for nutritive or therapeutic purposes, or to improve physical and/or chemical characteristics of lipids. Transesterification with lipase provides a useful way to improve the properties of triacylglycerols (TAG). Through enzymatic transesterification, it is possible to incorporate a desired acyl group onto specific positions of the glycerol, whereas chemical transesterification does not possess this regiospecificity due to the random nature of the reaction. Thus, lipase-catalyzed transesterification can provide regio- or stereospecific SL for nutritional, medical, and food applications (1,2). To synthesize the SL, functional and metabolic aspects of each fatty acid (saturated, unsaturated, short-, medium-, or long-chain) should be understood. The positional distribution of fatty acid is also important for the metabolic and physical properties of SL.

Medium-chain TAG (MCT) have primarily fatty acids that contain chainlengths of 6–12 carbons. Because of their saturation, they are stable to oxidation. They have low viscosity and melting points and are generally liquid at room temperature. Their smaller molecular size and relatively high solubility in water contribute to different digestive and absorptive properties compared to long-chain TAG (LCT) (3). MCT are mainly metabolized *via* the portal vein, providing quick energy. These properties may be beneficial to hospital patients, infants, or individuals with special dietary requirements (4). However, MCT alone cannot provide essential fatty acids.

Eicosapentaenoic acid (EPA; 5,8,11,14,17-EPA) is a fatty acid that can reduce the level of very low density lipoprotein (VLDL) and low-density lipoprotein (LDL) cholesterol in humans (5). Metabolically, EPA is an antagonist of the arachidonic acid cascade. EPA competes with arachidonic acid as a substrate for cyclooxygenase and lipoxygenase to produce eicosanoids. As a result, EPA can be used for the synthesis of eicosanoids, such as series-3 prostaglandins, which may ameliorate immunodysfunction. On the other hand, arachidonic acid may form the series-2 prostaglandins which may impair immune function. Health benefits of n-3 fatty acids, such as EPA, DHA (4,7,10,13,16,19-docosahexaenoic acid) and α-linolenic acid (9,10,15-octadecatrienoic acid), have been reported, including reduced risk of cardiovascular disease, improved immune function, and reduced inflammation (6–8).

In this study, SL of n-3 and medium-chain (caprylic) fatty acids were synthesized with immobilized lipase (SP 435) in gram quantities. After purification of SL by short-path distillation, its characteristics, such as fatty acid profile, free fatty acid (FFA) value, saponification number (SN), peroxide value (PV), iodine (IV) and thiobarbituric acid (TBA) values and oxidative stability, were determined.

EXPERIMENTAL PROCEDURES

Materials. Tricaprylin (1,2,3-trioctanoyl glycerol, 97–98%), porcine pancreatic lipase (type II, crude), TEP (1,1,3,3,-tetramethoxy propane), BHT (butylated hydroxytoluene), and α-

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tocopherol were obtained from Sigma Chemical Company (St. Louis, MO). n-3 Fatty acids (EPAX 6000) and fish oil TAG (EPAX 5500) were provided by Pronova Biocare (Sandefjord, Norway). Hanus solution (Labchem, Inc., Pittsburgh, PA) and 4,6-dihydroxy pyrimidine-2-thiol (Aldrich Chemical Company, Milwaukee, WI) were purchased. TCA (trichloroacetic acid), chloroform, and potassium iodide were purchased from J.T. Baker, Inc. (Phillipsburg, NJ). Isooctane, ethanol, and hexane were obtained from Fisher Scientific (Fair Lawn, NJ). Sodium thiosulfate was obtained from EM Science (Gibbstown, NJ). Immobilized enzyme, SP 435, was provided by Novo Nordisk Biochem North America, Inc. (Franklinton, NC).

Synthesis of SL. SL with EPA, DHA, and medium-chain (caprylic) fatty acid were synthesized in gram quantities by mixing 5 g tricaprylin with EPA-rich fish oil fatty acids, EPAX 6000 (6.5 g), in a 1:2 molar ratio in 90 mL hexane and transesterified by incubation at 55°C in a shaking water bath (200 rpm) for 24 h with SP 435 lipase (10% by weight of total substrates) as the biocatalyst in 125-mL Erlenmeyer flasks as bioreactors. After 48 batches of reaction, the products were pooled, and the hexane was evaporated in a Büchi rotary evaporator (Postfach, Switzerland). Short-path distillation was then used for the purification of synthesized SL. Distillation conditions were 1.1 Torr and 170°C at a feed flow rate of 3 mL/min. After distillation, approximately 240 g of purified SL was obtained.

Deacidification by ethanol extraction. Ethanol–water (80, 75, and 70% by vol) solvents were prepared and saturated with hexane (9). Distilled SL was mixed with hexane (1:2, w/w) to form miscella. This SL miscella in hexane was mixed with prepared 80, 75, and 70% ethanol at a ratio of 2:1 (miscella/solvent, vol/vol). A separatory funnel was used for phase separation. The separatory funnel was shaken for 5 min and left for 20 min. The upper hexane miscella phase was separated and evaporated under nitrogen.

Deacidification by alkaline extraction. Deacidification by alkaline extraction was modified from the method described by Shimada *et al.* (10). SL (5 g), purified by short-path distillation, was mixed with hexane (150 mL), phenolphthalein solution, and 80 mL of 0.5 N KOH solution in 20% ethanol. The separatory funnel was shaken, and the upper phase was collected. Then, 30 mL of 0.5 N KOH in 20% ethanol and 60 mL of saturated NaCl solution were mixed, and the hexane phase was collected. The hexane phase, containing SL, was passed through an anhydrous sodium sulfate column, and hexane was evaporated to obtain the deacidified SL (3.6 g). The deacidification steps were repeated to obtain sufficient purified SL for further studies. The deacidification procedure is illustrated in Figure 1.

Chemical properties of SL. FFA (AOCS, Ca 5a-40), PV (AOAC, 965.33), IV (AOAC, 920.158), and SN (AOAC, 920.160) were determined (11,12).

TBA. A modified extraction 2-TBA method was used (13). The TBA test expresses lipid oxidation in milligrams malonaldehyde (secondary oxidation product of polyunsaturated fatty acids) per kilogram of sample. SL (1 ± 0.002 g) was mixed with 0.01, 0.02, and 0.04 g of α-tocopherol in a 25-mL flask and left at room temperature without protection from light for 12, 24, and 48 h. Each sample was then purged with nitrogen and stored at –90°C until assay. One milliliter of 6% BHT in ethanol and 25 mL of 5% TCA solution in distilled water were added to each sample and blended. A 2-mL aliquot of the blended sample was mixed with 0.02 M TBA solution (3 mL) in screw-capped test tubes. After vortexing (30 s), the tubes were incubated in boiling water for 30 min and cooled, and the absorbance against a blank was measured at 535 nm. A 5-μL aliquot of TEP (0.92 g/mL) was diluted to 5 mL with 5% TCA solution, then diluted to prepare various concentrations that ranged from 9.2×10^{-8} g/mL to 3.68×10^{-6} g/mL. Each dilution was incubated, and the absorbance against a blank was measured after cooling. A standard curve was plotted from absorbance values vs. amount of TEP, which was converted from concentration.

Conjugated diene (CD) value. Two grams of SL were placed into a 25-mL flask and exposed to standard room light at room temperature for 12, 24, and 48 h. During lipid oxidation, CD formation can be measured at 233 nm (AOCS, Ti 1a-64) (10). To measure CD, 13 μL of sample (approximately 0.01 g) was mixed thoroughly with 10 mL isooctane and diluted 10 times with isooctane. Absorbance was then measured against a blank with a Beckman DU-64 spectrophotometer (Beckman Instruments, Fullerton, CA). The observed absorbance was between 0.2 and 0.8.

Oxidative stability index (OSI). An Oxidative Stability Instrument (Omnion, Rockland, MA) was used to measure induction times. Five grams of tricaprylin, EPAX 5500 (fish oil TAG; Pronova Biocare, Sandefjord, Norway), and SL, which was deacidified by alkali-liquid extraction, were placed into the disposable borosilicate glass reaction tubes with disposable pipets. The heating temperature was 80°C. The polycarbonate conductivity tubes were filled with deionized water, and the probes were connected. After temperature reached 80°C, air was bubbled in. The air flow was set to 2.5 mL/s. OSI time was determined with an on-line computer, which monitored the conductivity vs. time and plotted the induction period automatically. OSI values at 80°C were converted to active oxygen method (AOM) and OSI values at 97.8°C (14).

sn-*2 Fatty acids in SL by pancreatic hydrolysis.* One mL of 1 M Tris-HCl buffer (pH 7.6), 0.25 mL of bile salt solution (0.05%), 0.1 mL of 2.2% $CaCl_2$ solution, and 10 mg of pancreatic lipase were mixed and incubated at 37°C for 2 min. Extraction of the *sn*-2 monoacylglycerol and methylation for fatty acid analysis were described in our previous paper (15).

Gas chromatography (GC) analysis. For fatty acid composition, a Hewlett-Packard 5890 Series II gas chromatograph, equipped with flame-ionization detector (Hewlett-Packard, Avondale, PA), was used. The column and analysis condition were described previously (15).

Statistics. The Statistical Analysis System (SAS, Cary, NC) was used to perform statistical computations (16). Data were expressed as means ± standard deviations. Duncan's

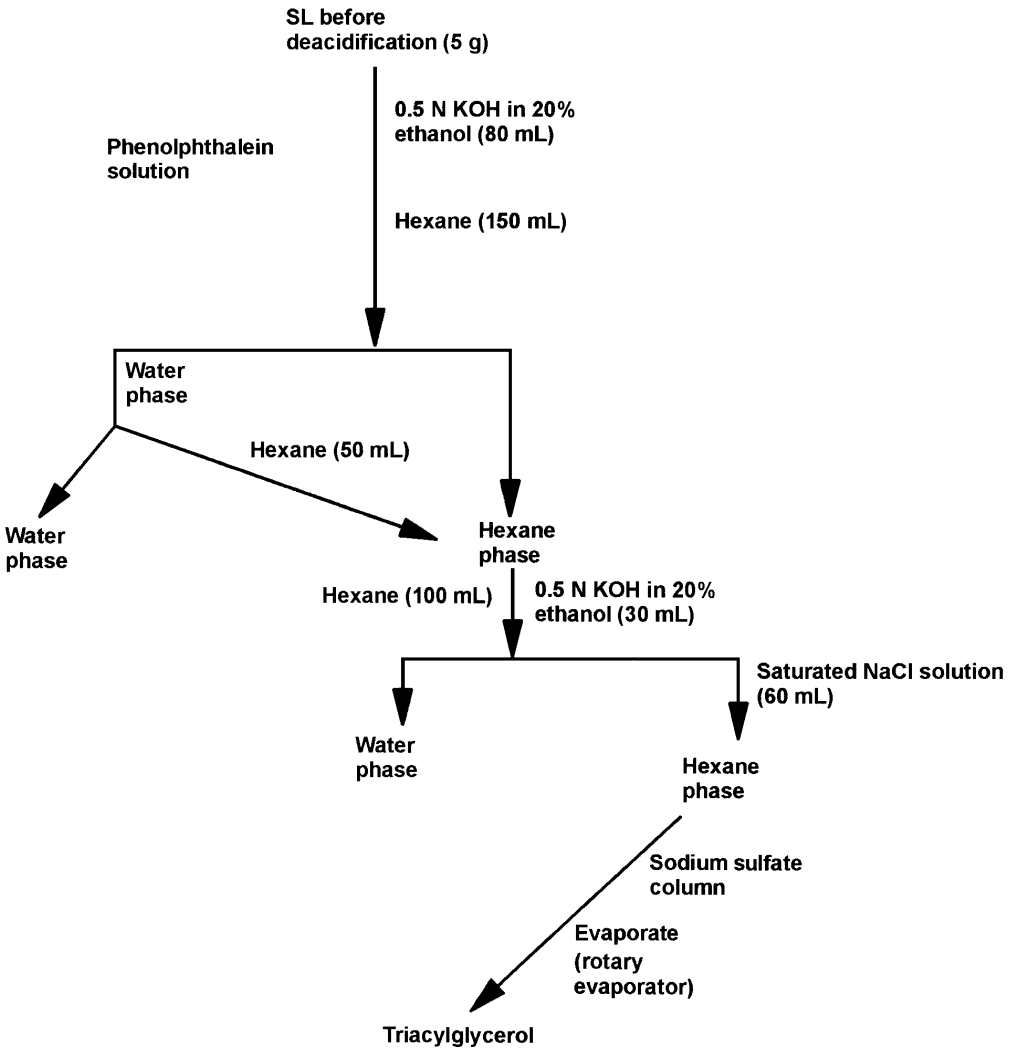

FIG. 1. Scheme for deacidification by alkaline extraction.

multiple range test was performed to determine significance of difference. Significance was determined at $P < 0.05$.

RESULTS AND DISCUSSION

Synthesis and fatty acid analysis of SL. n-3 Free acids (EPAX 6000), which were used as a substrate for providing n-3 acyl moieties, contained 33.8% EPA, 26% DHA, and 73% of total n-3 polyunsaturated fatty acids (specified by manufacturer). SL, purified by short-path distillation (approximately 240 g, total yield = 43.5% based on the total weight of substrates), contained 46.9 mol% caprylic acid, 23.2 mol% EPA, and 21.7 mol% DHA as major fatty acids. The fatty acid composition of SL is described in Table 1.

Deacidification. Table 2 shows the FFA values after deacidification by alkaline extraction or 80, 75, and 70% ethanol–water solvents. Refining with alkaline extraction was the most effective in reducing FFA among these methods. FFA was reduced to 1%. In this way, 14.6 g of refined SL (73% yield) was obtained from 20 g of unrefined sample after alkaline extraction, leading to 27% weight loss. Deacidification of miscella by ethanol–water solvents was not effective in removing FFA in this study. Only a 2% drop in FFA value was obtained with 80% ethanol–water solvent, compared to SL before refining. Higher ethanol contents (85, 90, and 95%)

TABLE 1
Fatty Acid Composition (mol%) of Structured Lipids and Their *sn*-2 Positions

Fatty acid	SL[a]	*sn*-2 Position
8:0	46.9	64.3
16:0	0.6	n.d.[b]
16:1n-7	1.3	3
18:0	2.1	n.d.
18:1n-9	0.5	n.d.
18:2n-6	0.5	n.d.
18:3n-3	0.6	n.d.
20:0	2.6	n.d.
20:5n-3	23.2	17.8
22:6n-3	21.7	15.0

[a]Structured lipids of caprylic and n-3 (eicosapentaenoic and docosahexaenoic) acids.
[b]Nondetectable.

TABLE 2
% FFA (free fatty acid) Values Before and After Deacidification by Alkaline Extraction or Various Concentrations of Ethanol–Water Solvents

Refining Methods	%FFA
Before refining	10.2
70% Ethanol–water	9.3
75% Ethanol–water	8.6
80% Ethanol–water	8.1
Alkaline extraction	1.0

tended to form one phase, in which extraction of miscella was impossible. Deacidification of oil by alkaline extraction, as used in this study, can serve as an alternative process to reduce FFA from high-acidity oils when the procedure is conducted at normal atmospheric pressure and temperature.

Chemical properties of SL. Table 3 shows selected chemical properties of SL, compared to other oils (17,18). IV is a measurement of the unsaturation of a lipid and thus of the sample's content of double bonds. The result is defined as the number of grams of iodine absorbed by 100 g of sample. The IV of this SL was 129.2. Generally, menhaden oil and cod liver oil are reported to contain >22% EPA and DHA. When compared with menhaden oil (IV, 150–165) and cod liver oil (IV, 159–166), SL has a lower IV due to the saturated caprylic acid. The IV of a rich source of medium-chain fatty acid, palm kernel oil, is 16–20. SN is defined as the number of milligrams of potassium hydroxide to saponify 1 g of sample. SN is an estimate of the mean molecular weight of the constituent acids in oils. This SL has a SN of 292.9. SN of coconut oil, menhaden oil, and cod liver were 248–265, 189–193, and 180–190, respectively (17,18).

PV, CD, TBA, and OSI. PV determination is one method for measuring oxidative deterioration and is defined in units of milli-equivalents of peroxide per kg of sample. Generally, a fresh fish oil sample has a PV in the range of 0 to 2 (17). This SL had a PV of 2.1, and we can assume that oxidation of SL has not occurred to any appreciable extent.

Spectrophotometric determination of conjugated dienoic acid determines the diene conjugation of unsaturated linkages present, which is expressed as a percentage of conjugated dienoic acid. Formation of conjugated dienoic acid increased with time of exposure, as expected. It seems that acceleration of oxidation occurred after 24 h because the change in absorbance was increased during the 24 to 48 h period. The CD values after 0, 12, 24, and 48 h were 1.7, 1.7, 1.8, and 2.2%, respectively. Thus, this SL was more susceptible to oxidation after 24 h in the absence of an antioxidant.

Figure 2 shows the antioxidant effect of α-tocopherol on SL. The TBA value increased with time, as expected: TBA of fresh SL was 0.07, and this value increased to 0.77, 1.01, and 1.4 after 12, 24, and 48 h, respectively, without antioxidant present. However, after 48 h, the presence of 1, 2, and 4% α-tocopherol reduced the oxidation of SL significantly (Fig. 2); 4% α-tocopherol, the highest concentration examined, was the most effective in reducing oxidation.

An OSI value for tricaprylin could not be obtained in this study because of complete saturation of its fatty acids, indicating that tricaprylin was quite stable to oxidation (Fig. 3). The OSI value of SL purified by alkaline extraction was 2.55 h at 80°C, which was longer than the OSI value for EPAX 5500 (fish oil). However, approximately 1% FFA still remained in this SL and should be considered, because FFA can easily be converted to volatile organic acids, leading to an increase in conductivity. Because the FFA form, rather than the TAG form, of n-3 polyunsaturated fatty acids causes more rapid oxidation and because it is difficult to obtain its OSI value, we used fish oil (EPAX 5500, the TAG form of n-3-rich fatty acids) for OSI comparison with SL. From the OSI results, we can assume that caprylic acid, the saturated fatty acid moiety in this SL, tends to protect SL against oxidation and increases the induction periods. The high unsaturation in EPAX 5500 contributed to its lower OSI values. The usual operating temperature of OSI is 110°C, but OSI can be run at a lower temperature (80°C) for highly unstable oils, such as fish oil (14). The automatic conversion of OSI value at 80°C to AOM and OSI at 97.8°C was used for comparative purposes (Fig. 3).

sn-*2 Analysis of SL.* Caprylic acid (64.3 mol%), EPA (17.8 mol%), and DHA (15 mol%) were the major fatty acids incorporated at the *sn*-2 position (Table 1). Because we used a

TABLE 3
Saponification Number and Iodine Values of Structured Lipids Compared to Selected Oils[a]

Oil	Saponification number	Iodine value
SL	292.9	129.2
Soybean oil	188–195	120–141
Coconut oil	248–265	7.5–10
Palm kernel oil	230–254	16–20
Cod liver oil	185–187	159–166
Menhaden oil	192–199	150–165

[a]The saponification numbers and iodine values for other oils are literature values (Refs. 17,18).

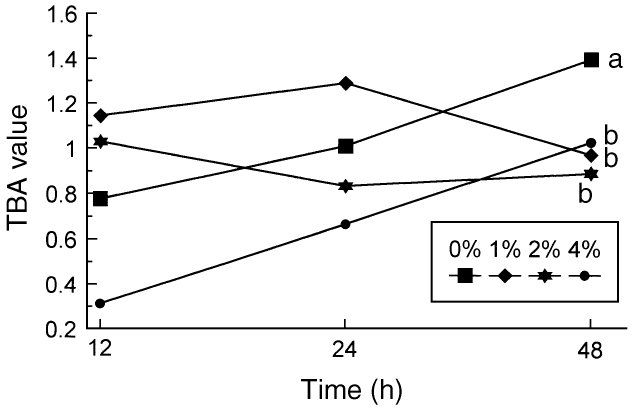

FIG. 2. Thiobarbituric acid (TBA) number of structured lipid without or with α-tocopherol (1, 2, 4%) after 12, 24, and 48 h. Values with the same letters are not significantly different ($P < 0.05$).

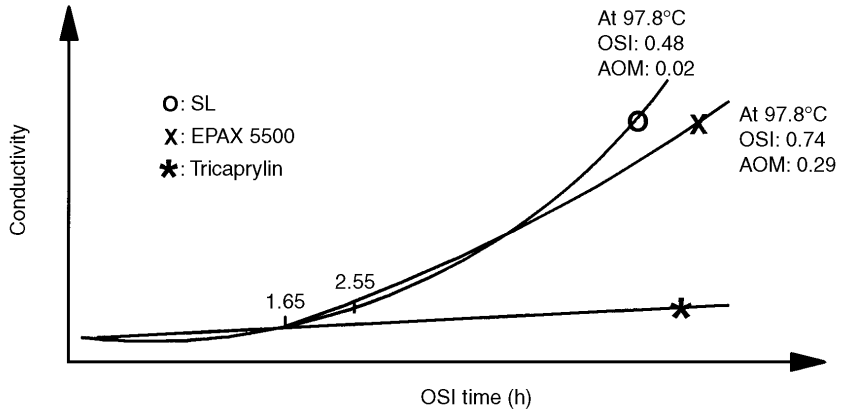

FIG. 3. Graphical determination of the induction period of SL, EPAX 5500 (fish oil TAG), and tricaprylin by a slope/change algorithm method. OSI values were converted to AOM values by the automated Omnion instrument. SL = structured lipids, OSI = oxidative stability index, AOM = active oxygen method, TAG = triacylglycerols.

nonspecific lipase (SP 435), some of the caprylic acid at the *sn*-2 position as well as at the *sn*-1, 3 positions on tricaprylin was replaced by EPA and DHA, the major fatty acids in EPAX 6000. In our previous reports, 34.8 mol% incorporation of EPA at the *sn*-2 position was observed with tricaprylin and 97% pure EPA ethyl ester (1:2 molar ratio) (15). The total n-3 incorporation at the *sn*-2 position in the current report (32.8%), in which the same enzyme was used, is comparable to our previous report (15). Because the regiospecific positions of fatty acids in TAG molecules are important for the metabolic and physical properties of lipids, the positional distribution of fatty acids, especially at the *sn*-2 position, should be considered in designing SL molecules. This SL will be used to study the nutritional and immunological effects of feeding SL in mice.

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