Synthesis of Structured Lipids by Transesterification of Trilinolein Catalyzed by Lipozyme IM60

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Structured lipids (SL) containing caprylic, stearic, and linoleic acids were synthesized by enzymatic transesterification using Lipozyme IM60. Pure trilinolein and free fatty acids were used as substrates. Incorporation of stearic acid was higher than that of caprylic acid in all parameters. Highest incorporations of both acids were achieved at 32 h, mole ratio of 1:4:4 (trilinolein/caprylic/stearic acids), water content of 1% (wt %), temperature of 55 °C, and 10% (wt %) enzyme load. The maximal incorporations of caprylic and stearic acids were 23.73 and 62.46 mol %, respectively. Reaction time, water content, and enzyme load had major influences on the reaction, whereas substrate mole ratio and temperature showed less influence. Lipozyme showed good stability over six reuses. Differential scanning calorimetric analysis of SL gave a melting profile with a very low melting peak of $0-3.3$ °C and a solid fat content of 25.21% at 0 °C. The melting profile and solid fat content of SL were compared with those of fats extracted from commercially available solid and liquid margarine products. The data suggest that enzymatically produced SL could be used in liquid margarine products.

INTRODUCTION

Recent advances in lipid research emphasize the role of essential fatty acids in maintaining good health and prevention of diseases (*1*). Essential fatty acids are involved in biochemical pathways that regulate body functions at the cellular level (*2, 3*). The types of fatty acids in a triacylglycerol (TAG) molecule play an important role in the nutritional and functional properties of fats and oils. Nutritional dietary management is traditionally carried out by physically mixing medium-chain and long-chain TAG from different oils and fats to meet particular requirements (*4, 5*). Recently, chemically and structurally more defined lipids have been developed using an enzymatic route, with position-specific lipases (*6*). Apart from the fatty acid composition of a particular TAG, the positional distribution among *sn*-1, -2, and -3 in the glycerol backbone further determines their absorption and nutritional quality (*7, 8*). Fatty acids at *sn*-1, and -3 positions are hydrolyzed by pancreatic lipase into free fatty acids. The pancreatic lipase is specific for the *sn*-1 and -3 positions, leaving the fatty acids at the *sn*-2 position intact. Much attention is focused on the fatty acid present at the *sn*-2 position, which is probably absorbed as 2-monoacylglycerols (*9, 10*) and serves as a template for reesterification by intestinal cells. Cell membrane is mainly made of lipids and lipoproteins, and its function is strongly influenced by the nature of the lipids present and their compositions (*11*). Lipids with a high ratio of saturated fatty acids make the cell membrane more rigid (*12*).

Linoleic acid was selected as an unsaturated fatty acid and targeted to the *sn*-2 position of the synthesized

structured lipids (SL). Linoleic acid has the ability to reduce plasma cholesterol and inhibit arterial thrombus formation (*13*). Dietary deficiency of linoleic acid and linolenic acid during development has been shown to result in reduced levels of 20:4*n*−6 and 22:6*n*−3 fatty acids, respectively, in the developing central nervous system, and this has been associated with altered learning behavior and visual function (*14*). Furthermore, *n*−6 fatty acids cannot be synthesized by humans and other mammals and must be supplied from external sources.

SL can be produced by random transestrification of fatty acids from mixed TAG containing combinations of short-, medium-, and long-chain fatty acids on a single glycerol backbone or by using pure compounds with known composition and molecular species. Lipids can be modified or synthesized by transesterification or direct synthesis using lipases in low-water media, especially in hexane or in solvent-free systems. Lipids with specific compositions and functionality have been synthesized enzymatically (*15−18*).

In this study, caprylic and stearic acids were used to transesterify trilinolein at the *sn*-1 and *sn*-3 positions with Lipozyme IM60. Factors that govern the reaction were investigated. The final product was characterized for its melting behavior and *sn*-2 positional composition.

MATERIALS AND METHODS

Materials. Trilinolein [1,2,3-tri[(*cis,cis*)-9,12-octadecadienoyl]glycerol], caprylic and stearic acids of 99% purity were obtained from Sigma Chemical Co. (St. Louis, MO). Gallium of 99.9999% purity was purchased from Aldrich Chemical Co. (Milwaukee, WI). Immobilized *sn*-1,3-specific lipase, Lipozyme IM60, 7.1 BAUN/g (Batch Acidolysis Units Novo) from *Rhizomucor miehei* was obtained from Novo Nordisk Biochem North America, Inc. (Franklinton, NC). *n*-Hexane and

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all other chemicals were purchased from Fisher Scientific (Norcross, GA).

Enzymatic Transesterification. Transesterification reaction was carried out in screw-capped test tubes containing 87.94 mg of trilinolein (0.1 mM), 57.68 mg of caprylic acid (0.4 mM), 113.80 mg of stearic acid (0.4 mM), 25.94 mg (0.184 BAUN, 10% w/w of reactants) of lipase, and 3 mL of hexane. The reaction mixture was incubated in a gyratory shaking water bath at 55 °C for 48 h at 200 rpm unless otherwise stated. All reactions were conducted in duplicate.

Extraction and TLC Analysis. After a predetermined time of incubation, the reaction was stopped by filtering the reaction mixture through an anhydrous sodium sulfate column (~3 cm) to remove the enzyme and any residual water. A 50-μL aliquot of product mixture was analyzed by thin-layer chromatography (TLC) on precoated silica gel G plates (Fisher Scientific), developed with petroleum ether/diethyl ether/acetic acid (90:10:1, v/v/v). The bands were visualized under ultraviolet light after spraying with 0.2% 2,7-dichlorofluorescein in ethanol. Bands corresponding to SL triacylglycerol including unreacted trilinolein were scraped and methylated with 3 mL of methanolic HCl at 75 °C for 2 h. The fatty acid methyl esters (FAME) were extracted with 2 mL of hexane and 1 mL of 0.1 M KCl solution and centrifuged at 1000 rpm for 10 min. The top hexane layer was separated into 5-mL capacity vials and concentrated under nitrogen. The residue was redissolved in 50 μL of hexane.

Fatty Acid Composition Analysis. The fatty acid composition and identification were obtained with a Hewlett-Packard 5890 series II gas chromatograph, equipped with a flame ionization detector (FID) and a fused silica capillary column (DB-225, 30 m × 0.25 mm i.d.; J&W Scientific, Folsom, CA) using pure standard FAME. The column was held at 80 °C for 3 min, programmed to 215 °C at a rate of 16 °C/min, and held for a final 5 min. Helium was the carrier gas, and the total flow rate was 23 mL/min. Injector and detector temperatures were set at 250 and 260 °C, respectively. One microliter of sample was injected for analysis. Heptadecanoic acid was the internal standard and was used to determine the mole percent of FAME with the aid of an on-line computer.

Large-Scale Synthesis. Gram scale (59 g) synthesis was performed with 20 g of trilinolein, 13.11 g of caprylic acid, 25.88 g of stearic acid, and 3.54 g of enzyme (6%) for 48 h at 55 °C. After the reaction, the free fatty acids and modified TAG were extracted and purified twice by an alcoholic alkaline extraction method as previously described (*19*). The purified products were analyzed for fatty acid composition and melting profiles.

Positional Analysis. The fatty acid distribution at the *sn*-2 position in TAG species was determined according to the method of Luddy et al. (*20*).

Differential Scanning Calorimetry (DSC). The melting profile of transesterified and purified product was determined by DSC on a Perkin-Elmer (Norwalk, CT) model DSC7. A sample of 7−9 mg of TAG was hermetically sealed in a 30-μL capacity aluminum pan, with an empty pan serving as a reference. DSC procedure Cj 1-94 as described in the AOCS recommended methods was followed (*21*). Samples were initially rapidly heated (200 °C/ min) from room temperature to 80 °C and held at this temperature for 10 min to destroy crystal memory; cooled to −30 °C at 10 °C/min and held for 30 min, an finally heated to 80 °C at 20 °C/min to determine the melting profile. Normal standardization of the instrument was performed with gallium (mp = 29.78 °C) as reference standard. Dry ice was used as coolant.

RESULTS AND DISCUSSION

Time Course of Reaction. The time course of synthesis was conducted at a substrate ratio of 1:4:4 of trilinolein/caprylic/stearic acids from 0 to 72 h in order to optimize the yield of desired products. Figure 1 shows the incorporation level of caprylic and stearic acids into trilinolein. Very low incorporations of both acids were observed for the initial 6 h. However, the incorporation
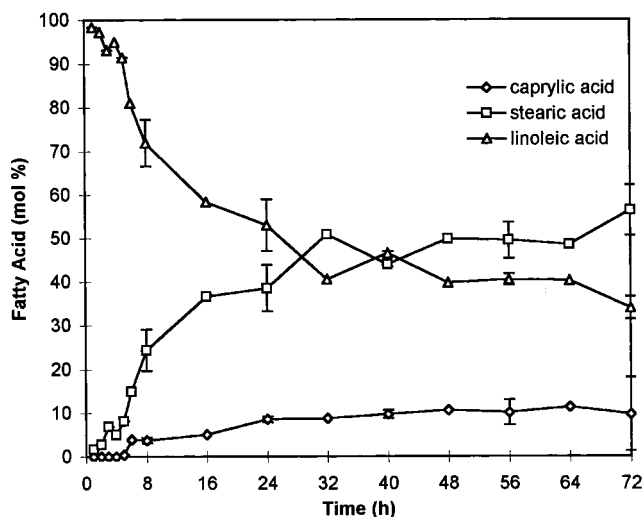

Figure 1. Time course of formation of transesterification products of trilinolein with caprylic and stearic acids. Substrate mole ratio was maintained at 1:4:4. Reaction was carried out with 10% Lipozyme (reactant-based weight percent, w/w) at 55 °C with 200 rpm shaking and no additional water.

of stearic acid increased rapidly with further increase in time, reaching a maximal level of 50.78% at 32 h. Prolonging the time did not increase the incorporation level of stearic acid, suggesting that equilibrium was reached at 32 h. Unlike stearic acid, the initial rate of incorporation of caprylic acid was very slow and continued to increase up to 11.33% at 64 h. Further increase in time did not improve the incorporation level. Incorporation of stearic acid remained higher compared to that of caprylic acid from initial time to 72 h. The chain length, relative polarity of stearic acid, and/or selectivity of IM60 lipase toward stearic acid could be the reason for the higher incorporation. Lipozyme is more active toward long-chain fatty acids than medium-chain fatty acids (*22*). On the basis of these observations, further experiments were carried out keeping the reaction time at 48 h.

Substrate Mole Ratio. The substrate mole ratio influences the equilibrium and can affect the incorporation level of fatty acids in transesterification. In many transestrification reactions involving TAG and fatty acids, an optimum incorporation was noticed upon increase in fatty acid ratio (*18, 23*). Figure 2 shows the effect of substrate mole ratio of trilinolein to caprylic and stearic acid from 1:1:1 to 1:5:5. At an equal substrate ratio of 1:1:1, the incorporations of caprylic and stearic acids were at 3.06 and 25.02 mol %, respectively. Higher mole percent incorporation of stearic acid against caprylic acid indicates fatty acid specificity, higher activity, and selectivity of Lipozyme IM60. With an increase in the substrate ratio to 1:5:5, the incorporation of both fatty acids gradually increased and reached optimal levels of 13.75 and 44.87 mol % for caprylic and stearic acids, respectively, at a 1:4:4 ratio. A further increase in ratio to 1:5:5 decreased the incorporation of both fatty acids. This could be due to the effect of higher substrate concentration toward the equilibrium constant and possible inhibition of lipase activity. Nevertheless, the mole percent incorporation of caprylic acid remained low at all ratios compared to that of stearic acid. Such variations are commonly observed in SL synthesis (*23*).

Water Content. The amount of water present in a reaction system plays a vital role in enzymatic synthe-

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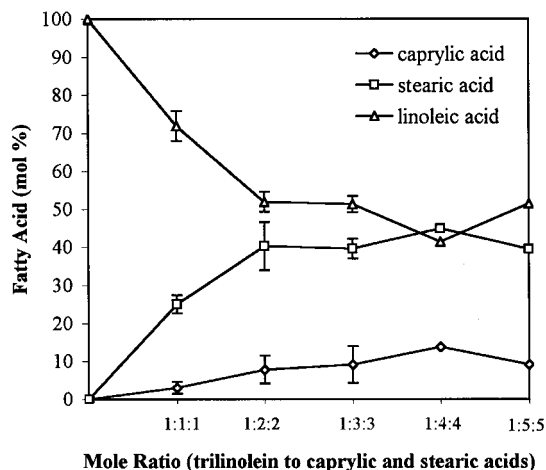*J. Agric. Food Chem.,* Vol. 49, No. 4, 2001 **2073**

Figure 2. Effect of mole ratio of trilinolein and caprylic and stearic acids on transesterification products. Reaction was performed at 55 °C for 24 h with 10% Lipozyme (reactant-based weight percent, w/w) with no additional water at 200 rpm.
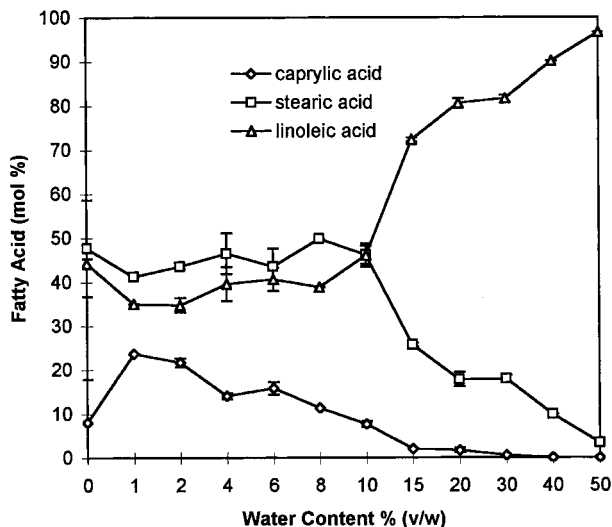

Figure 3. Effect of water content on transesterification products of trilinolein with caprylic and stearic acids at susbtrate mole ratio of 1:4:4. Reaction was performed at 55 °C for 48 h with 10% Lipozyme (reactant-based weight percent, w/w) and shaking at 200 rpm.

sis, especially when hydrolytic enzymes are used for synthetic purposes. Enzymes basically evolved to work in water-rich environments, but the amount of water required for expression of activity is very low. Water activity rather than water content is a crucial factor in controlling the activity and stability of enzymes. Reduction of water activity by reducing total water or by means of additives is the practical way to use enzymes for synthetic purposes (*24, 25*). Water immiscible organic solvents such as hexane are extensively used in fat and oil modification. Use of solvent solubilizes the hydrophobic substrates and keeps the water content low. Water influences the transesterification reaction in two ways. First, it directly affects enzyme functionality, influencing its structural mobility by hydration. Second, water can be a substrate in the reaction itself. Figure 3 shows the effect of water on the transesterification of trilinolein. Lipase could catalyze the reaction with no additional water. The absorbed moisture present in the enzyme particles seems to be sufficient for such transformation. Addition of water from 1 to 50% (v/w)
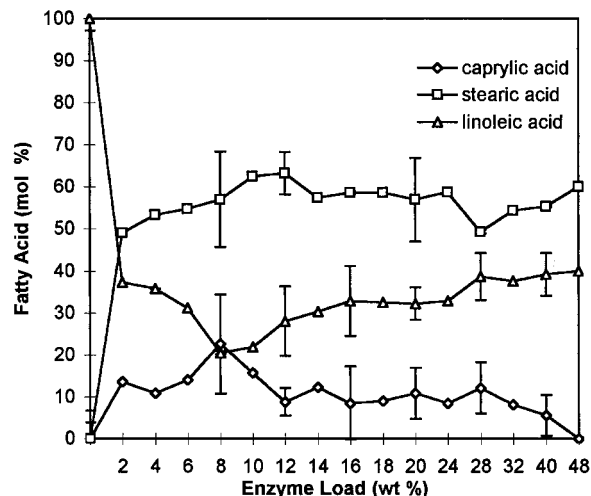

Figure 4. Effect of enzyme load on transesterification products of trilinolein with caprylic and stearic acids. Water content was maintained at 1% (v/w) level based on reactant weight. Reaction was performed at 55 °C for 48 h with substrate ratio of 1:4:4 and shaking at 200 rpm.

influenced the reaction pattern to a great degree. External addition of water increased the incorporation of both caprylic and stearic acids, but their optimal incorporation was observed at different water contents. Maximum incorporation of 23.73 mol % was achieved for caprylic acid with the addition of 1% water. Increase in water content to 30% decreased the caprylic acid incorporation to zero. Nonetheless, stearic acid incorporation slightly increased (from 47.70 to 49.87 mol %) with increasing water content from 0 to 8%. Beyond this point, the incorporation decreased to 4 mol % at 50% water content. The optimum incorporation of stearic acid was observed at 8% water with 49.87 mol % incorporation. The variations could be due to chain length and polarity differences of these two acids. In addition, water can also influence the substrate selectivity of lipases (*26*). These effects may also be due to the role of water in lipase activation, acidolysis, and the initial sequential hydrolysis and esterification mechanism (*27*). Maintenance of extremely low water content contributes to lower enzyme activity.

Enzyme Load. The amount of enzyme can influence the reaction rate; however, it cannot influence the equilibrium constant itself. Excessive use of enzyme can reduce the reaction time, but the final proportion of reactants will remain the same at equilibrium. This effect was studied in our system by using 2–48 wt % enzyme load based on reactant weight as shown in Figure 4. Optimal incorporation of caprylic acid (22.55 mol %) was obtained at 8 wt % enzyme load. Increase in enzyme load to 48 wt % decreased the incorporation of caprylic acid to 0 mol %. Stearic acid was incorporated to optimal level (63.22 mol %) at 10–12% enzyme load. A lower or higher enzyme load negatively influenced the incorporation. A 10% enzyme load was used to carry out further experiments.

Effect of Temperature. Temperature can influence the equilibrium constant for a given set of reactants. To study these effects, reactions were carried out at 40–70 °C with 1% water content in 3 mL of *n*-hexane. Figure 5 shows the influence of temperature on the incorporation of caprylic and stearic acids. Variations in incorporation of caprylic acid were observed from lower to higher temperature. Highest incorporation
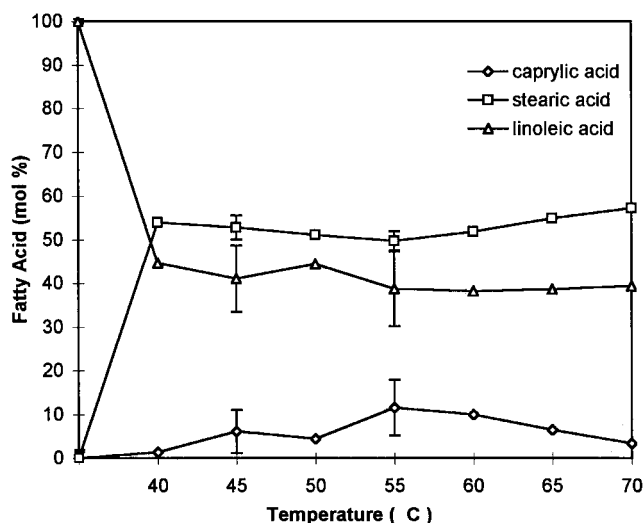
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Figure 5. Effect of temperature on transesterification products of trilinolein with caprylic and stearic acids. Substrate mole ratio was maintained at 1:4:4. Reaction was performed for 48 h with 10% Lipozyme (reactant based weight percent, w/w) and 1% water (reactant-based weight percent, v/w) and shaking at 200 rpm.

(11.50 mol %) was observed at 55 °C, and further increase in temperature reduced the incorporation of caprylic acid. The reduction of caprylic acid at high temperature could be due to a temperature effect on equilibrium rather than enzyme instability because Lipozyme IM60 was able to incorporate the stearic acid at these temperatures. Temperature did not affect the incorporation of stearic acid throughout the temperature range tested from 40 to 70 °C, although a slight increase at 70 °C was observed. The higher selectivity of IM60 toward stearic acid could be the possible reason for sustained incorporation of stearic acid at all temperatures.

Enzyme Reuse. The stability of Lipozyme IM60 was studied under optimal assay condition by reusing it six times. Each time, the enzyme was isolated from reaction products by filtration. Isolated enzyme was carefully washed with hexane and distilled water (three times) and dried under vacuum for 24 h at room temperature before each reuse. The stability of Lipozyme IM60 was found to be good even after six reuses, indicating potential for industrial application. The results of this observation are shown in Figure 6. Incorporation of caprylic acid was slightly reduced from the first use (13.28%) to the seventh use (7.60%). On the contrary, the incorporation of stearic acid was slightly increased from 44.61 to 50.07%. The high stability of the enzyme may be due to its low water content, which controls enzyme inactivation due to water-induced denaturation at high water content and temperature. Immobilized lipases are stable at high temperatures up to 75 °C.

Positional Analysis and Fatty Acid Composition of SL. The composition of SL from gram-scale synthesis is shown in Table 1. A high percentage of linoleic acid (55.51%) was obtained for SL with the remaining being stearic (36.93%) and caprylic (7.54%) acids. *sn*-2 positional analysis indicated 81.10% linoleic acid and 18.89% stearic acid. Although Lipozyme IM60 is selective for the *sn*-1 and *sn*-3 positions, incorporation of stearic acid at the *sn*-2 position may be due to acyl migration from *sn*-1 or *sn*-3 to the *sn*-2 position. Such an observation was discussed by Xu et al. (*28*).
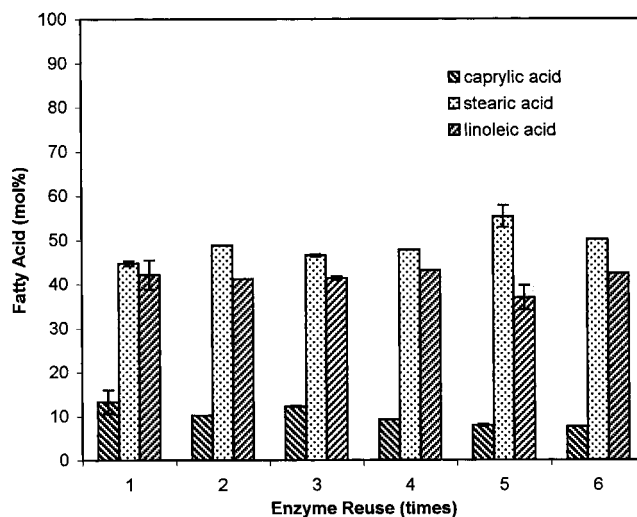

Figure 6. Composition of products synthesized by reused Lipozyme IM60 at substrate mole ratio of 1:4:4, shaking at 200 rpm, 55 °C for 48 h with 10% Lipozyme (reactant-based weight percent, w/w) and 1% additional water.
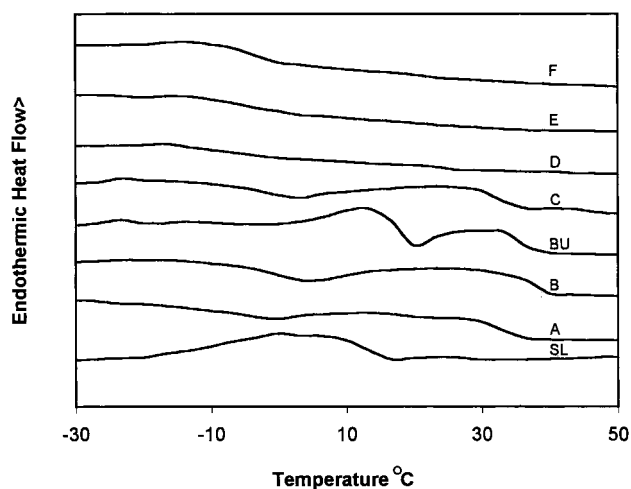

Figure 7. DSC of SL synthesized with substrate mole ratio of 1:4:4 of trilinolein to caprylic and stearic acids and fats extracted from commercial margarine samples. See DSC conditions in the text. SL, structured lipids; BU, butter fat; A, B, and C, margarine fat extracts; D, E, and F, liquid margarine fat extracts.

Table 1. Composition of Structured Lipid

	SL composition	
fatty acid	fatty acid composition (mol %)	*sn*-2 positional fatty acid composition (mol %)
caprylic acid	7.54	−
stearic acid	36.93	18.89
linoleic acid	55.51	81.10

DSC. Plastic fats resembling margarine fats with improved spreadability can be produced by enzymatic transesterification (*29*). SL from gram-scale synthesis was analyzed for melting profile and solid fat content and compared with commercially available margarine fat extracts. Figure 7 shows a broad melting range for all of the samples. Onset melting temperature for SL was at −27.32 °C with a melting peak of 0−3.3 °C. Butter (BU) and margarine fat samples A−C showed higher melting peaks compared to SL. Unlike other samples, BU and margarine samples A and C had two melting peaks. SL showed a single but wider peak with melting profile, which is closer to liquid margarine fat

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Table 2. Solid Fat Content of Structured Lipid and Fats Extracted from Commercial Samples[a]

temp (°C)	% solid fat content							
			margarine fat			liquid margarine fat		
	SL	BU	A	B	C	D	E	F
−30	100	100	100	100	100	100	100	100
−20	83.63	83.60	78.59	79.12	79.92	72.35	73.81	75.37
−10	57.03	66.43	58.74	59.24	60.92	46.47	47.60	49.05
0	25.21	50.93	44.68	45.80	48.58	29.78	31.03	32.99
10	5.89	27.46	27.87	33.37	33.81	15.33	17.66	19.82
20	0.29	16.38	12.04	16.96	16.82	5.91	9.26	11.16
30	0.23	4.73	1.62	2.97	4.78	1.67	4.49	5.92
40	0.21	1.04	0.81	0.79	0.57	0.45	1.37	2.31
50	0	0.01	0.82	0	0.56	0.23	0.66	0.37

[a] Abbreviations: SL, structured lipid; BU, butter fat; A, B, and C, margarine fat extracts; D, E, and F, liquid margarine fat extracts.

Table 3. Melting Profile of Structured Lipid and Commercial Margarine Fat Extracts[a]

sample	peak	melting onset temp (°C)	melting peak temp (°C)
SL	I	−27.32	0 to 3.3
BU	I	−00.04	9.63
	II	18.29	28.69
A	I	00.33	14.76
	II	23.83	30.98
B	I	3.35	23.47
C	I	−29.71	−10.31
	II	3.28	25.31
D	I	−30.15	−21.42
E	I	−30.12	−26.00
F	I	−30.60	−18.38

[a] Abbreviations: SL, structured lipid; BU, butter fat; A, B, and C = margarine fat extracts; D, E, and F = liquid margarine fat extracts.

extracts D−F. The solid fat content of SL at onset melting temperature was 83.63%, which was further decreased to 25.21 at peak melting point of 0–3.3 °C. The presence of unsaturated linoleic acid and short-chain caprylic acid seemed to be responsible for lower melting points and a wider peak for the SL sample. The solid fat content of SL was comparable with that of commercial fats as shown in Table 2. At −20 °C the solid fat content of SL was higher than that of all commercial samples. Increase in temperature to −10 °C lowered the solid fat content of SL to 57.03%, which is lower than that of butter fat and margarine samples A−C but higher than that of liquid margarine fats D−F. At 0 °C the solid fat content of SL was 25.21%, which is much closer to that of liquid margarine than that of margarine or butter fat. At a 10−50 °C range the solid fat content of SL was lower than that of all other samples, which implies the liquid nature of SL. At 30 °C, the most probable temperature for product formulation and storage, the solid fat content of SL was <1%, which is very much closer to that of margarine fat A (1.62%) and liquid margarine D (1.67%).

Onset and melting peak temperatures for SL are compared with those of commercial margarine fats in Table 3. The onset melting temperatures of margarine fat extract C first peak (−29.71 °C) and liquid margarine fat extracts D (−30.15 °C), E (−30.12 °C), and F (−30.60 °C) were comparable to the SL onset melting temperature (−27.32 °C). However, the melting peak of SL (0–3.3 °C) was higher than that of liquid margarine fat extracts D (−21.42 °C), E (−26 °C), and F (−18.38 °C) and lower than that of all other samples except for the first peak of C (−10.31 °C). The melting peak of SL was

higher than that of liquid margarine fat samples but lower than that of butter and margarine fats, except for the first peak of sample C. The closeness of onset melting temperature and peak melting points of SL toward liquid margarine is of interest. These findings suggest the possibility and suitability of using this type of SL in liquid and pourable margarine products.

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