Enzymatic Synthesis of Position-Specific Low-Calorie Structured Lipids[1]

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ABSTRACT: An immobilized *sn*-1,3-specific lipase from *Rhizomucor miehei* (IM 60) was used to catalyze the interesterification of tristearin ($C_{18:0}$) and tricaprin ($C_{10:0}$) to produce low-calorie structured lipids (SL). Acceptable product yields were obtained from a 1:1 mole ratio of both triacylglycerols with 10% (w/w of reactants) of IM 60 in 3 mL hexane. The SL molecular species, based on total carbon number, were 44.2% C_{41} and 40.5% C_{49}, with 3.8 and 11.5% unreacted tristearin C_{57} and tricaprin C_{27}, respectively, remaining in the product mixture. The best yield of C_{41} species (44.3%) was obtained with zero added water. Tricaprylin ($C_{8:0}$) was also successfully interesterified with tristearin in good yields at 1:1 mole ratio. Products were analyzed by reverse-phase high-performance liquid chromatography with an evaporative light-scattering detector. Reaction parameters, such as substrate mole ratio, enzyme load, time course, added water, reaction media, and enzyme reuse, were also investigated. Hydrolysis by pancreatic lipase revealed the specific fatty acids present at the *sn*-1,3 positions of SL.
JAOCS 74, 1409–1413 (1997).

KEY WORDS: Interesterification, lipase, low-calorie lipids, *Rhizomucor miehei*, structured lipids, tricaprin, tricaprylin, tristearin.

The most available intravenous lipid emulsions for use in clinical nutrition over the past 34 yr were derived from soybean and safflower oils (1). These oils are high in linoleic acid, considered an essential fatty acid, and are required to maintain the structure and function of cellular and subcellular membranes as well as to supply needed energy. However, long-chain triacylglycerol (LCT) emulsions alone are not effective as energy sources because they are metabolized slowly compared to medium-chain triacylglycerols (MCT). Because long-chain fatty acids (LCFA) are transported through the lymphatic system by incorporation into chylomicrons, it takes longer for them to get to the liver and supply the needed energy. To overcome the disadvantages of LCT emulsions in parenteral (intravenous) and enteral nutrition (oral tube feeding), MCT or medium-chain fatty acids (MCFA) have been introduced into LCT as a source of quick energy from fat (2,3). MCT are unique in that they are not carnitine-dependent for transport across the mitochondria, are not incorporated into chylomicrons, and are not readily stored in adipose tissue or accumulate in the reticuloendothelial (RES) system. Instead, they are mostly transported *via* the portal system with no hydrolysis and reesterification owing to their relatively high water solubility. Occasionally, MCT or MCFA can be transported through the lymphatic system. Use of MCT alone however, does not meet the essential fatty acid requirements and can cause gastrointestinal symptoms, such as crampy abdominal pain and diarrhea (4). At high doses, they may even be toxic, leading to a condition known as ketosis or ketonemia.

The advantages of MCT have, nevertheless, made them the basis of a new group of fats known as structured lipids (SL) (5), developed to meet the energy requirements of specific individuals (2). SL are expected to be rapidly cleared and metabolized, compared to LCT. SL can be produced from mixtures of MCT and LCT by chemical or enzymatic interesterification. Chemically, the mixtures are first hydrolyzed and allowed to reesterify in a random fashion and form modified triacylglycerol (TAG) molecular species that consist of MCFA and LCFA (1). The advantages of this tailor-made SL are two fold: (i) to obtain quick energy from MCFA, and (ii) to have LCFA serve as a source for essential fatty acids (2). On a commercial basis, SL, such as caprenin and benefat, can be synthesized through chemical hydrolysis of natural oils, followed by random esterification or through chemical interesterification. However, this method requires alkali metal or alkali metal alkylates as catalysts. Long synthetic times and high temperatures are employed, resulting in randomized TAG and unwanted side reactions that require high-energy expenditure for purification (6). Another method is through physical blending of individual MCT and LCT. Although the energy and nutritional requirements are met, the original absorption rates of individual TAG are retained, and physical mixtures are therefore not equivalent to SL. Consequently, patients with malabsorption conditions cannot effectively use them. A third method is the enzymatic approach. The use of enzymes is a viable means of producing SL because specific fatty acids at specific positions of the glycerol moiety can be produced, delivering lipids with maximum nutritional and functional properties (6). Enzymatic reactions take place

[1]Biocatalysis Symposium Paper, presented at the AOCS Annual Meeting & Expo, Seattle, Washington. May 11–14, 1997.

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under mild conditions, with no side reactions. Products of an enzymatic process are easy to purify and may also be considered natural, a property that consumers desire.

The objective of this research was to produce low-calorie SL with specific fatty acids at the *sn*-1,3 positions by interesterifying tristearin ($C_{18:0}$) with tricaprin ($C_{10:0}$) or tricaprylin ($C_{8:0}$) with *sn*-1,3-specific immobilized lipase IM 60 from *Rhizomucor miehei*. Free fatty acid counterparts of these MCT were evaluated as acyl donors. Reaction parameters that affect product yield and composition were also investigated.

MATERIALS AND METHODS

Materials. Tristearin (1,2,3-trioctadecanoylglycerol), tricaprin (1,2,3-tridecanoylglycerol), tricaprylin (1,2,3-trioctanoylglycerol), and porcine pancreatic lipase (Type II, crude) were obtained from Sigma Chemical Co. (St. Louis, MO). Immobilized *sn*-1,3-specific lipase IM 60 from *R. miehei* was obtained from Novo Nordisk Biochem North America Inc. (Franklinton, NC). *n*-Hexane and all other organic solvents were purchased from Fisher Scientific (Norcross, GA).

Enzymatic interesterification. Typical interesterification consisted of adding 50 mg tristearin, 31.1 mg tricaprin, 8.1 mg lipase (10% w/w of reactants), and 3 mL hexane to screw-capped test tubes. The reaction mixture was incubated in a gyratory shaking water bath at 55°C for 24 h at 200 rpm. All reactions were in duplicate.

Extraction and analysis. After incubation, reaction mixtures were filtered through an anhydrous sodium sulfate column to remove the enzyme and any residual water. A 50-µL aliquot of product mixture was redissolved in 950 µL acetone/acetonitrile (50:50, vol/vol), and 50 µL trilaurin ($C_{12:0}$) was added as internal standard. Products were analyzed with a Hewlett-Packard (Avondale, PA) 1090 Win high-performance liquid chromatograph (HPLC), equipped with a Sedex 45 evaporative light-scattering detector (ELSD) (Richard Scientific, Novato, CA). The ELSD was set to 45°C, a nebulizer gas pressure of 2.1, and a gain of 5 for the nonaqueous reverse-phase system. A Hewlett-Packard 35900 digital A/D analog interface connected the ELSD electronically to the on-line computer. TAG and SL molecular species were analyzed by nonaqueous reverse-phase HPLC on a Beckman/Altex (San Ramon, CA) Ultrasphere ODS 5 µm, (4.6 mm × 25 cm) column. Separation was obtained with acetonitrile (solvent A) and acetone (solvent B) as eluent, with the following gradient profile: initial condition 50:50 (A/B) at a flow rate of 1.8 mL/min; brought to 5:95 (A/B) at 2 mL/min for 12.5 min, and returned to original conditions (7). Total run time was 18 min.

Products were further 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, vol/vol/vol). The bands were visualized under ultraviolet light after spraying with 0.2% 2,7-dichlorofluorescein in ethanol. Bands corresponding to TAG were scraped and methylated in 3 mL methanolic HCL at 75°C for 2 h. The fatty acid methyl esters (FAME) were extracted with 2 mL hexane and 1 mL of 0.1 M KCL solution, centrifuged at 1000 rpm for 3 min, and concentrated under nitrogen.

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). The column was held at 120°C for 3 min and programmed to 215°C for 10 min at a rate of 10°C/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. Heptadecanoic acid methyl ester was the internal standard and was used to determine mol% of FAME with the aid of an on-line computer.

A modified version of Luddy *et al.* (8) was used to perform pancreatic lipase hydrolysis to determine the fatty acids at the *sn*-2 position of the SL products. At least 2 mg SL product was mixed with 1 mL of 0.4 M Tris-HCL buffer (pH 7.6), 0.25 mL of 0.05% bile salts, 0.1 mL of 2.2% $CaCl_2$, and 8 mg pancreatic lipase. The mixture was incubated in a 37°C water bath for 30 s and vortexed vigorously. This was repeated two times before centrifugation at 1000 rpm for 3 min and extraction with 3 mL ethyl ether (two times). The extract was filtered through a sodium sulfate column, and the sample was concentrated to a small volume with nitrogen. The residual *sn*-2 monoacylglycerol (MAG) was separated by TLC on a silica gel G plate, developed with hexane/diethyl ether/acetic acid (50:50:1, vol/vol/vol). Bands corresponding to the *sn*-2 MAG were scraped, methylated, and analyzed by gas chromatography (GC) as described above.

RESULTS AND DISCUSSION

Mole ratio. Figure 1 shows the mol% of SL molecular species C_{41}, C_{49}, and unreacted tristearin (C_{57}) in the reaction product after interesterification of tristearin with tricaprin catalyzed by IM 60 lipase. In general, as mole ratio increased (1:1 to 1:5, tristearin/tricaprin), yields for C_{41} and C_{49} dropped (except for C_{41} at 1:2 mole ratio), possibly indicat-

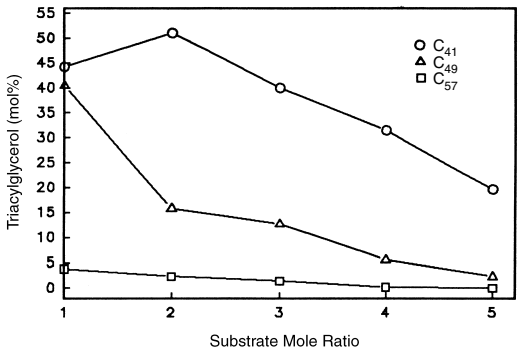

FIG. 1. Effect of substrate mole ratio on the interesterification of tristearin and tricaprin with IM 60 lipase (Novo Nordisk Biochem North America Inc., Franklinton, NC) from *Rhizomucor miehei*. Mole ratios were varied from 1:1 to 1:5 (tristearin/tricaprin). Reaction mixtures were incubated in 3 mL hexane at 55°C for 24 h.

ing inhibitory effects by tricaprin at high substrate mole ratios. The drop in yield was more noticeable for C_{49} than for C_{41}. Similar observations were reported previously (7) in a reaction involving lipase SP 435-catalyzed synthesis of SL from caprylic acid and triolein.

However, the same was not observed when free capric acid was used (data not shown). On the contrary, SL synthesis was enhanced as substrate mole ratio increased from 1 to 5 (tristearin/capric acid), with yields as high as 48.6 and 48.5% of C_{41} and C_{49}, respectively. We have previously shown that *R. miehei* lipase (IM 60) has a preference for free fatty acids, compared to *Candida antarctica* lipase, SP435 (7). Table 1 shows the SL product yields when using different medium-chain TAG-tricaprin-, tricaprylin-, and free fatty acids, to transesterify tristearin at a 1:1 mole ratio. Comparable yields (41 and 43.5%) for the disubstituted SL (C_{37} and C_{41}, respectively) molecular species were obtained with the medium-chain TAG substrates. Only 2.3% of tristearin was left unreacted when $T_{10:0}$ was the substrate, indicating excellent conversion to desired products. Free caprylic acid as acyl donor at a 1:1 mole ratio gave better product yield than free capric acid. Our results indicate that both capric and caprylic acids, as well as their TAG, can be used to produce high yields of desired SL with IM 60 lipase as biocatalyst. However, caprylic acid and tricaprylin cost less compared to their $C_{10:0}$ counterparts.

Enzyme load. Enzyme load was investigated with lipase concentrations ranging from 2–30% (w/w of reactants). Levels of 2–10% added lipase gave high yields of desired products, with 10% yielding up to 41% C_{41}, 43.7% C_{49}, and 4.1% C_{57} (Fig. 2). No major improvements were observed at greater enzyme loads.

Time course. Products were analyzed after 1, 2 ,3, 4, 5, 6, 12, 18, 24, 36, 48, and 72 h (Fig. 3) to follow the interesterification ability of IM 60 lipase with time. For the first 5 h, product formation was low. However, yields increased greatly for both C_{41} and C_{49} after 6 h (33.4 and 29.4%, respectively). The reaction seemed to have reached equilibrium at 24 h, after which there was no point in prolonging the reaction. All further experiments were conducted for 24 h.

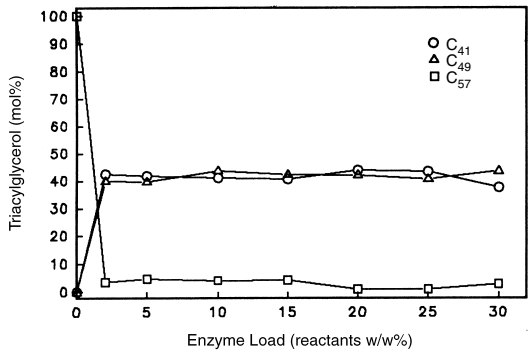

FIG. 2. Effect of enzyme load on structured lipid production from tricaprin and tristearin. The amount of enzyme ranged from 0 to 30% (w/w of reactants).

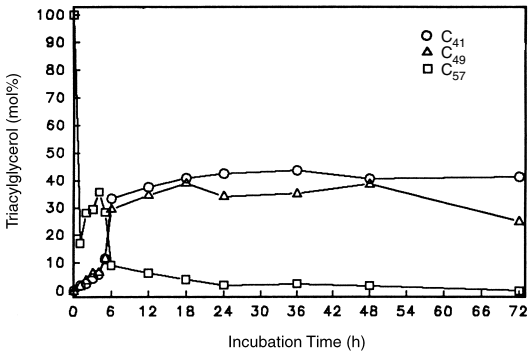

FIG. 3. Time course of IM 60 (Novo Nordisk Biochem North America Inc., Franklinton, NC) lipase-catalyzed interesterification of tristearin and tricaprin. Samples were analyzed at times indicated and in duplicate. See Figure 1 for company source.

TABLE 1
Yields of Structured Lipid Molecular Species Produced by Transesterification of Different Substrates with Tristearin[a]

Substrate type	Molecular species (mol%)				
	C_{37}	C_{41}	C_{47}	C_{49}	C_{57}
TAG as acyl donor					
$T_{8:0}$	41.0		44.9		9.7
$T_{10:0}$		43.5		37.8	2.3
FFA as acyl donor					
$C_{8:0}$	20.0		52.0		27.9
$C_{10:0}$		3.7		56.4	39.8

[a]Reactions were performed with a 1:1 mole ratio of substrates. Incubations were at 55°C, 200 rpm for 24 h with IM 60 lipase (Novo Nordisk Biochem North America Inc., Franklinton, NC) as the biocatalyst. TAG = triacylglycerol, FFA = free fatty acids, $T_{8:0}$ = tricaprilyn, $T_{10:0}$ = tricaprin, C_{37} and C_{41} = disubstituted products with caprylic and capric acids, respectively, C_{47} and C_{49} = monosubstituted products with caprylic and capric acids, respectively, and C_{57} = unreacted tristearin. The balance of the reported mol% belongs to the medium-chain fatty acid or TAG.

Effect of added water. The effect of added water on IM 60 lipase interesterification ability and yields of C_{41} and C_{49} SL molecular species is shown in Figure 4. Although it is known that a minuscule amount of water is required by enzymes to maintain their three-dimensional structure and catalytic ability, our highest product formation was obtained under near-anhydrous conditions. As the amount of water increased from 0 to 10% (w/w of reactants), inhibitory effects on IM 60 lipase become more noticeable, especially for C_{49} product formation. Between 15–25% added water, an increase in yields, although small, was observed for both SL, possibly indicating water's ability to accelerate acyl migration, thus making the *sn*-2 or *sn*-3 position available for capric acid incorporation. From our experience, this lipase seemed to perform best between 10–20% added water. However, the best yield for C_{41} species (44.3%) was obtained with zero added water.

Effect of reaction media. Organic solvents of varying log *P* values were chosen to study the effect of reaction media on the interesterification of tristearin and tricaprin (Table 2). Log *P* value, the partition coefficient between water and octanol, is often used as an indicator of solvent polarity (9). It has been postulated that solvents with log $P > 4$ (nonpolar solvents) allow high biocatalytic activity. Our results show that sol-

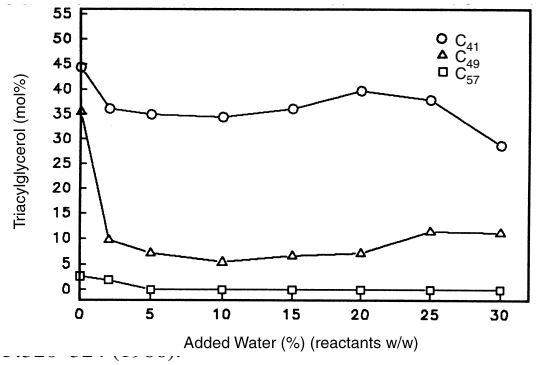

FIG. 4. Effect of added water on lipase-catalyzed interesterification of tristearin and tricaprin with IM 60 as biocatalyst. Amount of water added varied from 0 to 30% (w/w of reactants). See Figure 1 for company source.

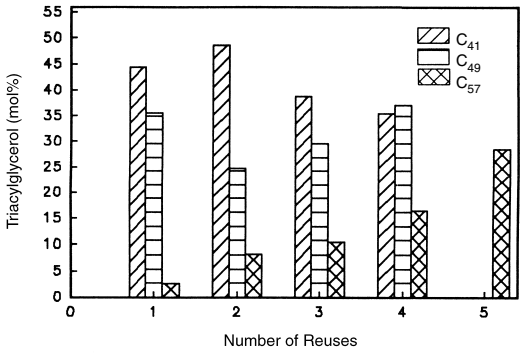

FIG. 5. Effect of enzyme reuse on lipase-catalyzed interesterification of tristearin and tricaprin with IM 60 as biocatalyst. A total of five runs was performed. See Figure 1 for company source.

vents with log $P > 3$ were the most suitable media, with heptane (log $P = 4$, 41.6 and 44.2%), hexane (log $P = 3.5$, 43.5 and 37.8%), and isooctane (log $P = 4.5$, 40.8 and 38.7%), giving the best yields for C_{41} and C_{49}, respectively. Petroleum ether (no log P value reported) also gave high yields (39.0 and 47.5% for C_{41} and C_{49}, respectively). Polar solvents, such as chloroform (log $P = 2$), ethyl ether (log $P = 0.85$), and toluene (log $P = 2.5$), gave low or no product formation. The poor yield obtained in the reaction with no solvent indicates that a mutual solvent that will allow mobility of the substrates to the enzyme active site is necessary. Tristearin is solid at room temperature and does not melt completely at 55°C used in this reaction and therefore must be solubilized in a solvent. Higher temperatures than used in this report may seem like a good alternative, but one must weigh the advantage against possible loss of the medium-chain fatty acids owing to volatility and poor product yields.

Enzyme reuse. One of the advantages of enzyme reuse is that it allows for enzymes to be recovered. For this study, the enzyme was isolated from reaction products after each batch, washed with distilled water (three times), and dried under vacuum until next use. Rinsing with hexane after water wash did not improve enzyme activity. Up to five runs were performed. Figure 5 shows the experimental results. Acceptable yields were obtained for the first four runs. After run 5, no SL formation was detected.

Pancreatic lipase study. Several studies have shown that the nature of the fatty acids and their positions in a glycerol molecule can affect their metabolism, availability as an energy source, and as an essential fatty acid source (6,10,11). Jandacek *et al.* (12) reported that a TAG with octanoic acid at the *sn*-1 and 3 positions and long-chain fatty acids (LCFA) at the *sn*-2 position were hydrolyzed and absorbed more efficiently than TAG composed of only LCFA. Christensen *et al.* (13) reported that a defined TAG molecule with MCFA at the *sn*-1,3 positions could be a better source of fat. They observed increased absorption of essential fatty acids at the *sn*-2 position of glycerol. Pancreatic lipase hydrolysis was performed to determine the fatty acid composition at the *sn*-2 position (Table 3). Values show that IM 60 lipase incorporated 21.2% C_{10} and 78.8% C_{18} at the *sn*-2 position. IM 60 was able to produce SL that contained specific fatty acids at the *sn*-1,3 positions.

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TABLE 2
Effect of Reaction Media on Lipase-Catalyzed Interesterification of Tristearin and Tricaprin with IM 60 as Biocatalyst

Solvent[a]	Log P^b	Molecular species (mol %)		
		C_{41}	C_{49}	C_{57}
No solvent	—	23.0	3.2	16.3
Petroleum ether	—	39.0	47.5	4.0
Isooctane	4.5	40.8	38.7	6.7
Heptane	4.0	41.6	44.2	3.0
Hexane	3.5	43.5	37.8	2.3
Cyclohexane	3.2	38.4	35.2	6.3
Pentane	3.0	36.9	44.9	10.7
Toluene	2.5	30.1	17.4	11.7
Chloroform	2.0	—	—	46.9
Ethyl ether	0.85	—	—	59.9

[a]Solvents dried over molecular sieve 4 Å .
[b]Source: Reference 9. See Table 1 for abbreviations and company source.

TABLE 3
***sn*-2 Positional Analysis of Structured Lipid Products by Lipase-Catalyzed Interesterification of Tristearin and Tricaprin with IM 60 Lipase as Biocatalyst**

Fatty acid	Gas chromatography (mol%)[a]	Mol% of fatty acid at *sn*-2 position[b]
$C_{10:0}$	35.2	21.2
$C_{18:0}$	64.8	78.8

[a]Fatty acid composition of structured lipids after thin-layer chromatography separation of products from reactants.
[b]After pancreatic lipase hydrolysis of structured lipid products. See Table 1 for company source.

ACKNOWLEDGMENTS

Information was contributed by the Agricultural Experiment Station, College of Agricultural and Environmental Sciences, The Univer-

tanoic Acid in the 1- and 3-Positions and Long-Chain Fatty Acid in the 2-Position, *Ibid. 45*:940–945 (1987).
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[Received April 15, 1997; accepted July 25, 1997]