Enzymatic Synthesis of Structured Lipids: Transesterification of Triolein and Caprylic Acid Ethyl Ester

Kuan-Hsiang Huang and Casimir C. Akoh*

Department of Food Science and Technology, Food Science Building, The University of Georgia, Athens, Georgia 30602-7610

ABSTRACT: Structured lipids were successfully synthesized by lipase-catalyzed transesterification (ester interchange) of caprylic acid ethyl ester and triolein. The transesterification reaction was carried out in organic solvent as reaction media. Eight commercially-available lipases (10% w/w substrates) were screened for their ability to synthesize structured lipid by incubating with 100 mg triolein and 78.0 mg caprylic acid ethyl ester in 3 mL hexane at 45°C for 24 h. The products were analyzed by reverse-phase high-performance liquid chromatography with evaporative light-scattering detector. Immobilized lipase IM60 from *Rhizomucor miehei* converted most triolein into structured lipids (41.7% dicaprylooleln, 46.0% monocaprylooleln, and 12.3% unreacted triolein). However, lipase SP435 from *Candida antarctica* had a higher activity at higher temperature. The reaction catalyzed by lipase SP435 yielded 62.0% dicaprylooleln, 33.5% monocaprylooleln, and 4.5% unreacted triolein at 55°C. Time course, incubation media, added water, and substrate concentration were also investigated in this study. The results suggest that lipase-catalyzed transesterification of long-chain triglycerides and medium-chain fatty acid ethyl ester is feasible to synthesize structured lipids.
JAOCS 73, 245–250 (1996).

KEY WORDS: Caprylic acid ethyl ester, enzymatic synthesis, lipases, organic media, structured lipids, transesterification, triolein.

To overcome the disadvantages of conventional long-chain triglyceride (LCT) emulsion in intravenous administration, medium-chain triglycerides (MCT) have been introduced into LCT lipids emulsion to meet the energy requirement of specific individuals (1). Pure MCT emulsions do not provide essential fatty acids, and do produce significant side effects including metabolic acidosis. A physical mixture of MCT and LCT can meet both nutritional and energy requirements. However, structured lipids (SL) with modified absorption rate have emerged as a preferred alternative to physical mixture (2).

SL are synthesized chemically by hydrolysis and random esterification of the mixture of LCT and MCT (1,2). 1,3-Specific lipase-catalyzed interesterified fats were distinguished from chemically-catalyzed products by the fatty acids in the 2-position (3). A novel triglyceride based on vegetable oil can be produced to mimic the fatty acid distribution of milkfat, and can be used as human milkfat substitute in infant formula (4). In addition to the synthesis of positional isomers, application of lipase in SL synthesis becomes more attractive because of the high energy cost of the conventional chemical process and the anticipated lower prices of enzymes.

Strategies for enzymatic synthesis of structured lipids include: (i) transesterification of MCT and LCT; (ii) transesterification of MCT and long-chain fatty acid (LCFA); (iii) transesterification of LCT and medium-chain fatty acid (MCFA); and (iv) transesterification of LCT and MCFA ester.

Comparison between carboxylic acids and their ethyl esters as acyl donors indicate that the ethyl ester substrates had faster reaction rate, and may lead to more diglyceride formation (5). Kuo and Parkin (6) also reported that the initial reaction rate was fatty acid glycerol esters > fatty acid methyl esters > fatty acids. However, the reported acyl donor preference for lipases varied with different laboratories, substrates, and enzymes. Schuch and Mukherjee (7) reported the rate of transesterification to be long-chain alcohol > fatty acid > triacylglycerol > methyl ester > glycerol. Bloomer *et al.* (8) reported that the acyl donor preference of lipases depended on the supports. SL containing MCFA in the 1- and 3-position and linoleic acid in the 2-position may have some beneficial effects both as an energy source and as an essential fatty acid source (9). The chemical interesterification of MCT and LCT yields randomly distributed MCFA and LCFA in all three positions. Under limited acyl migration, use of MCFA ethyl ester and LCT as substrates combined with 1,3-specific lipase can produce SL containing MCFA mainly in 1,3-position and LCFA in 2-position. We previously reported the synthesis of SL containing oleic and caprylic acid by the transesterification of triolein and caprylic acid (10). The objective of this study was to investigate the possibility of using lipases to synthesize SL containing oleic and caprylic acid by transesterification of caprylic acid ethyl ester and triolein. The effects of different biocatalysts, temperatures, molar ratios, time courses, added water, and substrate concentrations were also studied. The enzymatic synthesis of SL by a nonspecific lipase is shown in Figure 1.

*To whom correspondence should be addressed.

Copyright © 1996 by AOCS Press

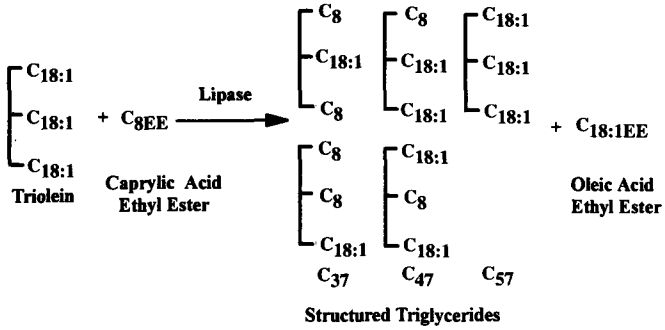

FIG. 1. Scheme for lipase-catalyzed transesterification of triolein and caprylic acid ethyl ester (EE). C_{37} = Dicaprylooleln; C_{47} = monocaprylooleln; C_{57} = triolein.

MATERIALS AND METHODS

Materials. Caprylic acid ethyl ester (99%) and triolein (99% pure) were purchased from Sigma Chemical Company (St. Louis, MO). Immobilized lipase IM60, IM20, SP435, and SP382 were kindly provided by Novo Nordisk Bioindustrial Inc. (Danbury, CT). Lipases G, PS, and AK were gifts from Amano Enzyme Co., Ltd. (Troy, VA). Pancreatic lipase was a gift from Biocatalysts Ltd. (Mid Glamorgan, United Kingdom). Sodium sulfate and all the organic solvents were purchased from Fisher Scientific (Norcross, GA).

Enzymatic transesterification. For a typical transesterification (ester interchange) reaction, the reaction mixture contained 100 mg triolein, 78.0 mg caprylic acid ethyl ester, 3 mL hexane, and 17.8 mg lipase. The reaction mixture was incubated at 45°C in an orbital shaking water bath at 200 rpm for 24 h. All reactions were in duplicate.

Analysis of SL. The reaction mixture was filtered through a sodium sulfate column to remove enzymes and any residual water, then analyzed with a Hewlett-Packard (Avondale, PA) 1090 high-performance liquid chromatography (HPLC) equipped with a Sedex 45 evaporative light-scattering detector (ELSD) (Richard Scientific, Novato, CA). The ELSD was set at 40°C, a nebulizer gas pressure of 2.1 atmosphere, and a gain of 5 for nonaqueous reverse-phase system. A Hewlett-Packard 35900 digital A/D analog interface connected the ELSD electronically to the on-line computer. Triglyceride species were separated 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 an eluent, and the following gradient profile: initial condition 50:50 A/B, hold 4 min, at a flow rate of 1.8 mL/min; 5:95, A/B, hold 8.5 min at a flow rate of 2.0 mL/min; return to original condition. Sample concentration was 50 μg/20 mL injection. Tricaprylin was the internal standard.

RESULTS AND DISCUSSION

Enzyme screening. Eight commercial enzymes [immobilized lipase IM60, IM20 (*Rhizomucor miehei*), SP435, SP382 (*Candida antarctica*), lipase G (*Penicillium cylcopium*), lipase AK and PS (*Pseudomonas* sp.), pancreatic lipase] were screened for their ability to catalyze the transesterification of triolein and caprylic acid ethyl ester at 45°C in hexane. Figure 2 shows the triglyceride species of the products. We previously reported the lipase-catalyzed transesterification of caprylic acid and triolein in hexane (10). The synthesis of monocaprylooleln (C_{37}) was up by 3.9 and 11.4% for *R. miehei* lipases IM60 and IM20, respectively, when caprylic acid ethyl ester was used instead of caprylic acid (10) (Fig. 2). The C_{37} increased from 0 and 0.5% to 31.1 and 41.5%, respectively, with *C. antarctica* lipases SP435 and SP382. *Pseudomonas* lipase PS, which showed no activity with caprylic acid as acyl donor, was found to incorporate caprylic acid and yielded 12.6% C_{37} and 45% C_{47} with caprylic acid ethyl ester as acyl donor.

Temperature. The optimum temperature of enzymes depend on the source, immobilization, chemical modification of enzymes, and pH of reaction mixture (11). We further tested the optimum temperature for *R. miehei* lipase, IM60, and *C. antarctica* lipase, SP435. The temperature range tested was 25–65°C (Fig. 3). IM60 had very constant activity at a temperature range from 25 to 65°C, but slightly higher activity at higher temperature. The C_{37} was increased from 43.3 to 49.5% when temperature increased from 25 to 65°C. Caprylic acid was not incorporated into the triglyceride at 25°C with *C. antarctica* lipase, SP435. At 55°C, C_{37} was increased to 62.0%, and unreacted triolein (C_{57}) was decreased to 4.5%. The temperature preference was similar to results previously reported with caprylic acid as acyl donor; however, much higher caprylic acid incorporation was observed with ethyl ester as acyl donor.

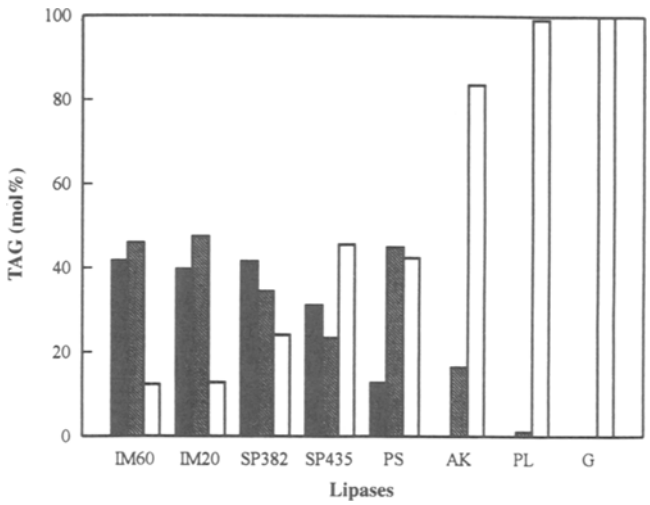

FIG. 2. Synthesis of structured triglycerides (TAG) with different lipases. The reaction mixture contained 100 mg triolein, 68.8 mg caprylic acid, 17.8 mg lipase, and 3 mL hexane. The reaction mixture was incubated at 45°C in an orbital shaking water bath at 200 rpm. Black bar, C_{37}; hatched bar, C_{47}; white bar, C_{57}. Lipases IM60, IM20, SP382, and SP435 from Novo Nordisk (Danbury, CT); lipases PS, AK, and G from Amano (Troy, VA).

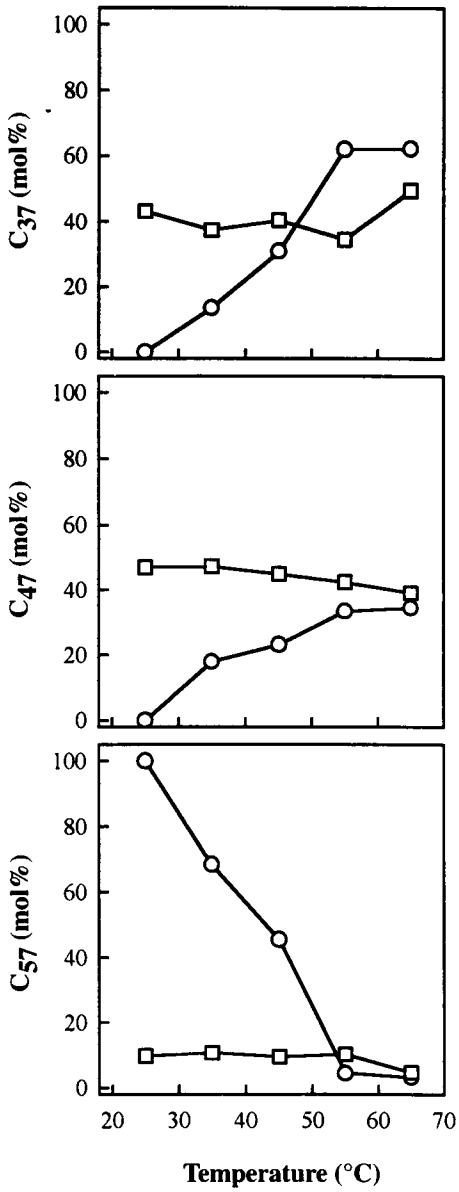

FIG. 3. Effect of temperature on the lipase-catalyzed transesterification of triolein and caprylic acid ethyl ester with IM60 (□) from *Rhizomucor miehei* or SP435 (○) from *Candida antarctica*. The reaction mixture was incubated at 25–65°C in an orbital shaking water bath for 24 h (see Fig. 2 for composition of reaction mixutre and lipase sources).

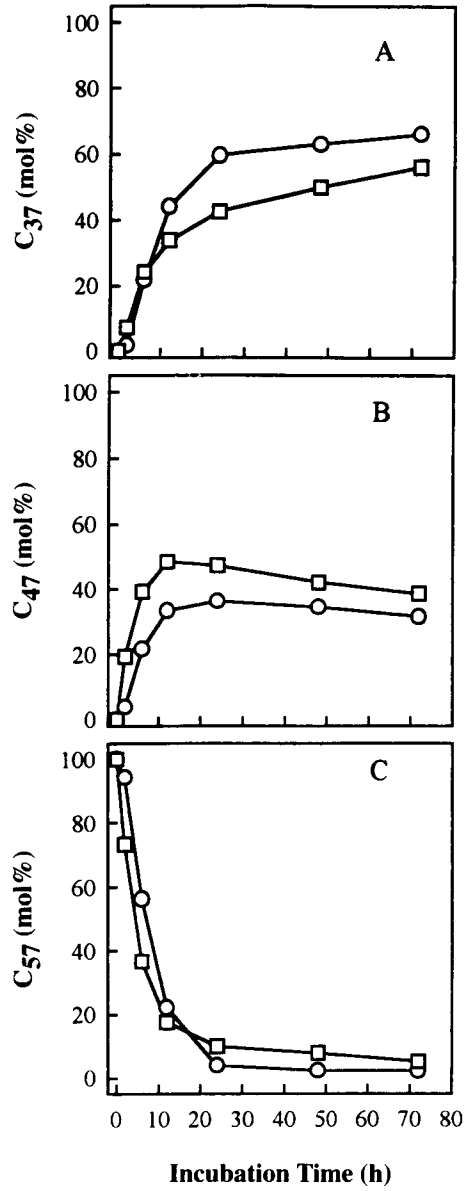

FIG. 4. Time course of lipase-catalyzed transesterification of triolein and caprylic acid with different caprylic acid ethyl ester to triolein ratio (mol/mol). See Figure 2 for reaction condition, reaction mixture composition, and lipase source. SP435, ○; IM60, □.

Incubation time. To determine the incubation time required to reach equilibrium, the tranesterification products were analyzed at 0, 2, 6, 12, 24, 48, and 72 h. The synthesis of C_{37} was very fast in the first 24 h with both enzymes (Fig. 4A). The molar percentage of C_{37} slowly but steadily increased in the next 48 h. There was a higher level of C_{47} with IM60 than with SP435 (Fig. 4B), and higher C_{37} in first 6 h, but lower C_{37} after 6 h (Fig. 4A). This indicated a higher conversion of C_{57} to C_{47} but lower conversion of C_{47} to C_{37} with IM60. IM60 might be more positional-specific than SP435, and thus restricted the incorporation of caprylic acid into 2-position. 1,3-Dicaprylooleln was probably the major C_{37} molecular species with IM60 as biocatalyst. In addition to 1.3-dicaprylooleln, 1,2- and 2,3-dicaprylooleln were also responsible for the increase in the C_{37} molecular species when SP435 catalyzed the incorporation of caprylic acid into the 2-position. It may also be due to nonspecific activity or acyl migration during the initial triglyceride hydrolysis which preceded reesterification. Incorporation of caprylic acid into 2-position by SP435 suggested that tricaprylin might be present in the products. However, we did not detect tricaprylin in the products. Bloomer *et al.* (8) reported lipase-catalyzed synthesis of cocoa butter equivalents with ethyl stearate and palm oil midfraction. Trisaturated glycerides formed were 10.1 to 13.5%,

depending on the lipase used. We did not detect tricaprylin, probably due to short reaction time and lower ethyl ester to triglyceride ratio employed. In addition, we did not use water-saturated solvent in our reactions.

Goh and co-workers (12) reported a solvent-dependent 1,3-specificity of fungal lipases. When hexane was used, oleic acid was liberated from 2-position of cocoa butter in a transesterification reaction with lipases from *R. miehei* and *Humicola lanuginosa*. However, both lipases were 1.3-specific in the reaction when diethyl ether was used as a solvent. The loss of positional specificity of lipases are attributable to acyl migration, which is increased during prolonged reactions (13).

Molar ratio. To study the effect of molar ratio, 17.8 mg IM60 was added in all molar ratio incubations rather than 10% (w/w substrate). Figure 5 shows the mol% of C_{37}, C_{47}, and unreacted C_{57} in the products after transesterification of caprylic acid ethyl ester and triolein at molar ratio of 2 to 10 (caprylic acid ethyl ester: triolein) catalyzed by lipase IM60 and SP435. We previously reported an inhibition effect with caprylic acid at high caprylic acid to triolein ratio (10), but that was not the case with ethyl ester as acyl donor. However, there was no increase in C_{37} synthesis when the molar ratio increased from 8 to 10.

Water. Controlling water activity is very important in lipid modification. Monolayer of water on the surface of enzyme is required to maintain the three-dimensional structure of enzyme, but too much water can cause the hydrolysis of triglyceride (13). In a packed-bed reactor, the presence of dissolved water in the feedstream caused the formation of minor amount of the hydrolysis by-products, diglyceride, and free fatty acid (14). Figure 6 gives the mol% of C_{37}, C_{47}, and C_{57} at different amounts of added water up to 62% (wt% enzyme) with IM60 and SP435. Water can accelerate acyl migration and make *sn*-2 position available. With ethyl ester as acyl donor, added water had little effect on improving caprylic acid incorporation compared with free acid for *R. miehei* lipase catalysis. Added water had inhibitory effect on *C. antarctica* lipase. It may well be that water accelerates the hydrolysis of caprylic acid ethyl ester to free caprylic acid which, in turn, inhibits the SP435 enzyme. There was a zero incorporation at 11.2% (w/w enzyme) added water. The result was consistent with our previous report on the incorporation of n-3 PUFA into vegetable oils (15).

Substrate concentration. The use of organic media had some advantages over aqueous media, such as increased solubility of nonpolar substrate, shifting of thermodynamic equilibrium to favor synthesis, ease of product recovery, prevention of enzyme desorption from support material (8). Bloomer *et al.* (5) reported that 1–1.5 g/g substrate was the optimum heptane level for enzymatic transesterification of stearic acid ethyl ester and palm oil mid fraction at 40°C and the reaction rate was highest at maximum heptane level of 0.5 g/g substrates at 60°C. Trisaturated glyceride was increased when more solvent was added. Macrae (14) reported that the variation in the solvent concentration had little effect on either the reaction rate or by-product formation for the enzymatic transesterification of olive oil and methyl palmitate. Figure 7 shows the effect of caprylic acid ethyl ester concentration on the synthesis of structured lipids. For both enzymes, caprylic acid ethyl ester concentration range from 0.288 to 1.152 M had little effect on $C_{37}/C_{47}/C_{57}$ ratio change. Higher or lower caprylic acid ethyl ester concentration resulted in lower C_{37} yield.

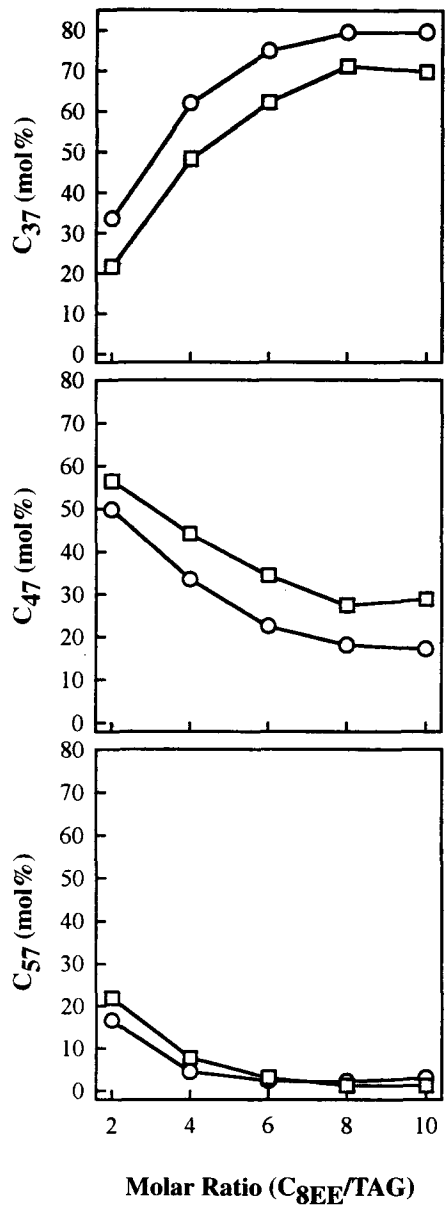

FIG. 5. Effect of caprylic acid ethyl ester (EE) to triolein ratio on lipase-catalyzed transesterification of triolein and caprylic acid ethyl ester. The reaction mixture contained 100 mg triolein with varied amount of caprylic acid ethyl ester [caprylic acid ethyl ester/triolein = 2, 4, 6, 8, 10 (mol/mol)], 17.8 mg lipase, and 3 mL hexane. See Figure 2 for reaction condition and lipase source; see Figure 1 for abbreviation. SP435, ○; IM60, □.

The results reported in the present investigation demonstrate the potentials of lipase-catalyzed transesterification of LCT and MCFA ethyl ester to synthesize SL. Structured

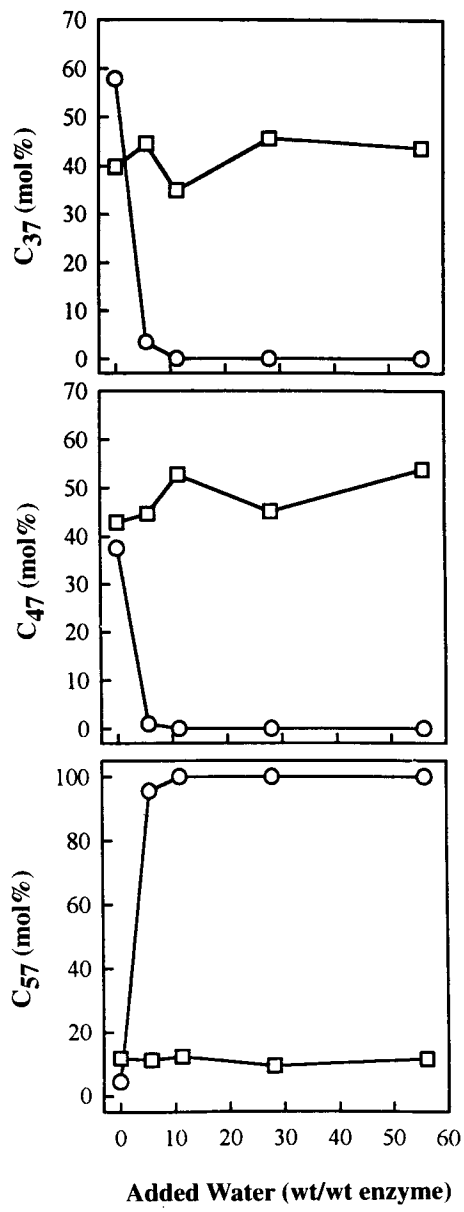

FIG. 6. Effect of added water on lipase-catalyzed transesterification of triolein and caprylic acid ethyl ester with IM60 (□) or SP435 (○) as biocatalyst. See Figure 2 for reaction condition and lipase source.

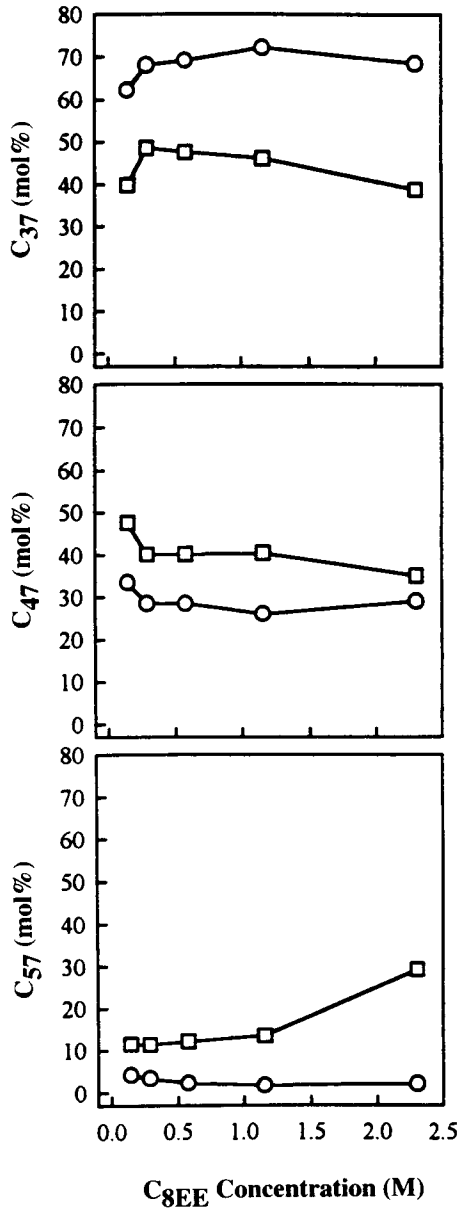

FIG. 7. Effect of substrate concentration on the lipase-catalyzed transesterification of triolein and caprylic acid ethyl ester with 10% (w/w substrates) IM60 (□) or SP435 (○) as biocatalyst. See Figure 2 for reaction condition and lipase source.

triglycerides containing MCFA at 1,3-position and LCFA at 2-position can provide both essential fatty acid and quick energy. Such products can easily be synthesized with 1,3-specific lipase, but not with chemical catalysts. The use of MCFA ethyl ester as acyl donor had some advantages, such as high reaction rate, no inhibition at high substrate molar ratio and high substrate concentration, as opposed to use of free acid form (10). Enzymatic transesterification of vegetable oils and MCFA ethyl ester offers considerable commercial potential for the synthesis of SL. Obviously, such products will compete favorably with oils seeds containing MCFA that may be produced through genetic engineering and SL produced *via* chemical catalysis.

ACKNOWLEDGMENTS

Contributed to by the Agricultural Experiment Station, College of Agricultural and Environmental Sciences, The University of Georgia. Research supported by Food Science Research 2526GC 29400 and USDA Hatch Project GEO 00695.

REFERENCES

1. Heird, W.C., S.M. Grundy, and V.S. Hubbard, *Am. J. Clin. Nutr. 43*:320 (1986).
2. Kennedy, J.P., *Food Technol. 45*:76 (1991).
3. Roy, S.S., and D.K. Bhattacharyya, *J. Am. Oil Chem. Soc. 70*:923 (1993).
4. Quinlan, P., and S. Moore, *INFORM 4*:580 (1993).

5. Bloomer, S., P. Adlercreutz, and B. Mattiasson, *J. Am. Oil Chem. Soc. 69*:966 (1992).
6. Kuo, S.-J., and K.L. Parkin, *Ibid. 70*:393 (1993).
7. Schuch, T., and K.M. Mukherjee, *J. Agric. Food Chem. 35*:1005 (1987).
8. Bloomer, S., P.A. Adlercreutz, and B. Mattiasson, *J. Am. Oil Chem. Soc. 67*:519 (1990).
9. Ikeda, I., Y. Tomari, S. Michihiro, W. Soichiro, and N. Junichi, *Lipids 26*:369 (1991).
10. Akoh, C.C., and K.-H. Huang, *J. Food Lipids 2*:219 (1995).
11. Dordick, J.S., *Enzyme Microb. Technol. 11*:94 (1989).
12. Goh, S.H., S.K. Yeong, and C.W. Wang, *J. Am. Oil Chem. Soc. 70*:567 (1993).
13. Zaks, A., and A.M. Klibanov, *J. Biol. Chem. 263*:3194 (1988).
14. Macrae, A.R., *Biochem. Soc. Trans. 17*:1146 (1989).
15. Huang, K.-H., and Akoh, C.C., *J. Am. Oil Chem. Soc. 71*:1277 (1994).

[Received June 21, 1995; accepted October 18, 1995]