Enzymatic Modification of Triolein: Incorporation of Caproic and Butyric Acids to Produce Reduced-Calorie Structured Lipids

Lydia B. Fomuso and Casimir C. Akoh*

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

ABSTRACT: Lipase-catalyzed acidolysis of triolein with caproic and butyric acids was performed to produce reduced-calorie structured lipids (SL). The SL were obtained by incubating a 1:4:4 mole ratio of triolein, caproic acid, and butyric acid, respectively, with 10% of lipase (w/w of total substrates) in 1.5 mL hexane at 55°C for 24 h. Of nine commercially available lipases screened, IM60, which contains the lipase from *Rhizomucor miehei*, was the most effective and produced 13 mol% unreacted triolein, 49% disubstituted, and 38% monosubstituted triacylglycerols that contained short-chain fatty acids. The products were analyzed by reverse-phase high performance liquid chromatography with an evaporative light-scattering detector. Reaction parameters studied included time course, temperature, enzyme load, and substrate mole ratio. The yields obtained demonstrate that a structured lipid with long-chain and short-chain fatty acids can be synthesized by using IM60 lipase in organic medium.

JAOCS 74, 269–272 (1997).

KEY WORDS: Acidolysis, enzymatic synthesis, lipase, organic solvent, reduced-calorie fat, *Rhizomucor miehei*, structured lipid, triolein.

Structured lipids (SL) are triacylglycerols (TAG) that contain mixtures of either short-chain or medium-chain fatty acids or both, and long-chain fatty acids (LCFA), preferably esterified on the same glycerol molecule (1). They are beneficial to human nutrition because they can be tailor-made to target specific diseases and metabolic conditions (1). They can also serve as reduced- or low-calorie fats, which are presently highly desirable in the Western world. Short-chain fatty acids (SCFA) and medium-chain fatty acids (MCFA) provide a quick source of energy for infants and stressed adults (2). SCFA are volatile and more rapidly absorbed in the stomach than MCFA because of their higher water solubility, smaller molecular size, and shorter chainlength (3). They serve as a preferred energy source for the cells of the gastric mucosa (3). Unlike LCFA, which are absorbed through the lymphatic system and require chylomicron formation for transport (4), SCFA diffuse freely across the mucosal cytosol and enter venous blood while remaining in the free fatty acid form (5). On the basis of heats of combustion, SCFA provide fewer calories per unit weight than MCFA or LCFA (6). Butyrate provides 6 kcal/g and caproate about 7 kcal/g, compared to 9 kcal/g for LCFA (6). This is the basis for using SCFA in the production of reduced-calorie SL. The principle of combining SCFA and LCFA has been used by Nabisco Foods Group (East Hanover, NJ) in the chemical synthesis of Salatrim®, a reduced-calorie fat, composed of propionic, butyric and stearic acid, with useful applications in the food industry (7). In mammals, SCFA occur commonly as free fatty acids in the gastrointestinal tract and as end-products of microbial digestion of carbohydrates (8). Butyrate and caproate are released by the action of gastric lipase on bovine milk TAG (8). Medium- and short-chain fatty acids make up about 3% of the total bulk of dietary fat intake (9). Studies on oleic acid-rich diets have shown that, although a LCFA, oleic acid can be credited with reducing total cholesterol and low density lipoprotein (LDL) cholesterol levels. It also reduces the ratio of LDL to high-density lipoprotein (HDL) cholesterol (10).

In recent years, research has focused on the use of lipases in organic solvents to modify TAG. This method seems more attractive than the chemical process because of the milder reaction conditions required by the enzymatic process and the reduction in the number of side products (1). In addition, lipases can catalyze the incorporation of specific fatty acids at specific positions of TAG (1). This becomes significant when digestion of TAG is considered, because of the enhanced absorption of fatty acids at the *sn*-2 position of some TAG (11). The objective of this paper was to prepare a reduced-calorie SL, with the potential of improving nutrition, from triolein, butyric acid, and caproic acid by using lipase as the biocatalyst.

MATERIALS AND METHODS

Materials. Butyric acid, caproic acid, and triolein were obtained from Sigma Chemical Company (St. Louis, MO). Lipase G from *Penicillium cyclopium*, PS from *Pseudomonas* sp., L from *Candida lipolytica*, N from *Rhizopus niveus*, AK from *Pseudomonas* sp., and AY-30 from *C. rugosa* were

*To whom correspondence should be addressed at Department of Food Science and Technology, Food Science Building, Rm. 211, The University of Georgia, Athens, GA 30602-7610.

Copyright © 1997 by AOCS Press

kindly provided by Amano Enzyme Co. (Troy, VA). IM60 and IM20 with immobilized lipase from *Rhizomucor miehei*, and SP435 immobilized lipase from *C. antarctica* were obtained from Novo Nordisk Biochem North America Inc. (Franklinton, NC). Porcine pancreatic lipase and 1,3 distearoyl-2-oleoyl-glycerol were purchased from Sigma. All solvents used were of high-performance liquid chromatography (HPLC) grade and were obtained from Fisher Scientific (Norcross, GA).

Transesterification reaction. SL synthesis was performed in screw-capped test tubes in an orbital shaking waterbath at 200 rpm and 55°C for 24 h. The reaction mixture contained typically 50 mg triolein, 26 mg caproic acid, 20 mg butyric acid, and 9.6 mg lipase (i.e., 10% w/w of total substrates) in 1.5 mL of hexane that was previously dried over molecular sieve 4Å. All reactions were performed in duplicate.

Extraction and analytical methods. The reaction products were cooled and filtered through a sodium sulfate column to remove any moisture and enzyme particles. Products were analyzed in a Hewlett-Packard 1090 HPLC (Avondale, PA), equipped with a Sedex 45 evaporative light-scattering detector (ELSD) (Richard Scientific, Novato, CA). The ELSD was set at 40°C, a nitrogen nebulizer gas pressure of 2.1 atmospheres, 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 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. The mobile phase was comprised of acetonitrile (A) and acetone (B) with a gradient profile as follows: initial conditions (A/B) 60:40 at a flow rate of 1.3 mL/min held for 4 min, then 30:70 (A/B) at a flow rate of 1.5 mL/min held for 8.5 min, 50:50 (A/B) at 1.5 mL/min for 5.5 min, and then brought back to 60:40 (A/B) at 1.3 mL/min. Tricaprin was the internal standard.

Product identification was based on polarity, equivalent carbon number (ECN), and by use of TAG standards as previously described (12). For identification of fatty acid species at the *sn*-2 position of the TAG, samples were spotted on thin-layer chromatography (TLC) plates along with standards and developed in petroleum ether/diethyl ether/acetic acid (90:10:1, vol/vol/vol). TAG bands were visualized under ultraviolet (UV) radiation after spraying the plate with 0.2% 2,7-dichlorofluorescein in methanol. TAG bands were scraped, pooled, and eluted with ethyl ether. Pancreatic lipase analysis was carried out according to the method described by Luddy *et al.* (13). After pancreatic lipase hydrolysis, the products were extracted with ethyl ether, filtered, and dried over anhydrous sodium sulfate. Products were separated by silica gel TLC with hexane/diethyl ether/acetic acid (50:50:1, vol/vol/vol). The *sn*-2 monoacylglycerol (MAG) was scraped and propylated with 6% HCl in propanol at 75°C for 2 h. The propyl esters were extracted with hexane and 0.1 M KCl solution. To establish the accuracy of the pancreatic hydrolysis method for *sn*-2 positional analysis, a TAG standard with known structure, 1,3 distearoyl-2-oleoyl-glycerol, was similarly analyzed except that the standard was dissolved in 0.5 mL hexane prior to hydrolysis. This was done because the standard was not soluble in the assay buffer. The fatty acid composition of the MAG band was determined in a Hewlett-Packard 5890 gas chromatograph, equipped with a flame-ionization detector (FID) and operated in a splitless mode. Helium was the carrier gas, and the total gas flow rate was 24 mL/min. The oven temperature was 70°C initially and held for 4 min, and then programmed to 210°C at 10°C/min and held isothermally for 10 min. Heptadecanoic acid was the internal standard. The mol% of the fatty acid propyl esters were analyzed and integrated by an on-line computer.

RESULTS AND DISCUSSION

Lipase screening. Nine commercially available lipases from different sources were screened by incubating 7.3 mg of lipase with a 1:2:2 mole ratio of triolein, caproic acid, and butyric acid at 55°C for 24 h. The enzymes screened were lipase G, PS, L, N, AK, AY-30 (Amano Enzyme Co.), and IM20, IM60, and SP435 (Novo Nordisk Biochem North America Inc.). IM20, IM60, SP435 and, to a limited extent, lipase AK incorporated short-chain fatty acids into triolein. Lipase G from *Penicillium* sp. has been shown to attach LCFA acids to glycerol to form MAG (14), but we now found that it was not efficient in incorporating butyric and caproic acids into triolein. IM60 lipase from *R. miehei* produced 32% disubstituted (SLS) and 45% monosubstituted (LLS) TAG. IM60 lipase was used further in this study because of its relative activity at the conditions described above and specificity for the *sn*-1 and *sn*-3 positions of TAG.

Mole ratio. A substrate mole ratio study was done by varying the mole ratio of triolein to butyric and caproic acids, respectively, from 1:1:1 to 1:12:12 (Fig. 1). The enzyme amount was kept constant at 7.3 mg instead of being kept at 10% by weight of total substrates. The mol% of SL formed increased with increasing mole ratio, up to a mole ratio of 1:4:4. A yield of 49% SLS and 38% LLS structured TAG was obtained at a mole ratio of 1:4:4. Hydrolysis was not a significant event in these reactions. Less than 5% of diacylglycerol was detected at this level (small scale) of assay. All yield calculations were based on the amount of SL formed and the unreacted triolein (LLL). This mole ratio was used for the remainder of the study. Beyond 1:4:4 ratio, an increase in unreacted triolein was observed. Studies by Nishio and Kamimura (15) have shown some inactivation of *P. fragi* 22.39 B lipase by SCFA (C_2–C_6). This could also be true with the increase of caproic and butyric acid in our reaction medium. This increase was attributed to acidification of the microaqueous environment of the lipase (16).

Temperature effect. Adequate temperature control is important for the reproducible assay of enzyme-catalyzed reactions. Temperature changes can affect parameters, such as enzyme stability, affinity of enzyme for substrate, and preponderance of competing reactions (17). The effect of temperature on the transesterification of triolein with butyric

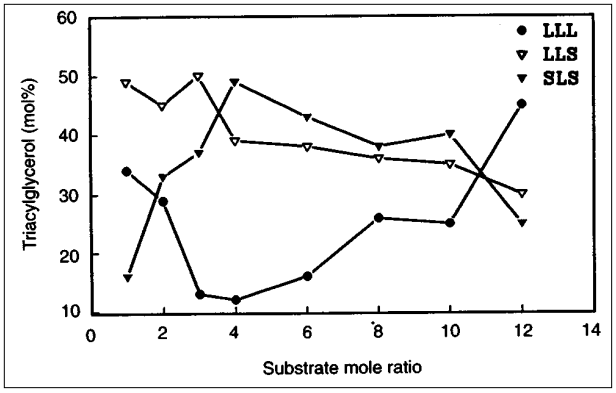

FIG. 1. Effect of mole ratio of substrates on structured lipids synthesis with IM60 (Novo Nordisk Biochem North America Inc., Franklinton, NC) as biocatalyst. The letter S designates short-chain fatty acids ($C_{6:0}$, $C_{4:0}$) and L designates long-chain fatty acid (18:1n-9). LLL = triolein (unreacted), LLS = monosubstituted structured lipid, and SLS = disubstituted structured lipid. Mole ratios of triolein to caproic and butyric acids were varied from 1:1:1 to 1:12:12, respectively. The reaction mixture was incubated in 1.5 mL hexane at 55°C for 24 h.

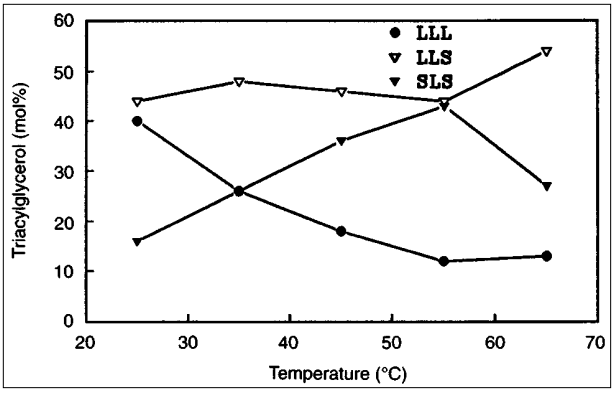

FIG. 2. Effect of temperature on incorporation of caproic and butyric acids into triolein. See Figure 1 for abbreviations and conditions.

and caproic acids was studied. Mole ratio, incubation time, and solvent were kept constant at 1:4:4 (triolein, butyric acid, and caproic acid, respectively), 24 h, and 1.5 mL of hexane, respectively. Reaction temperatures were varied from 25 to 65°C. We found that 55°C was most suitable for the reaction (Fig. 2). At 55°C, 12.4% LLL remained unreacted, and 44% of LLS and 43% of SLS TAG were produced. As temperature increased, there were more disubstituted TAG, but a decrease was observed above 55°C.

Time course. Time-course studies (Fig. 3) indicated that incorporation of caproic and butyric acids increased steadily with increasing incubation but later dropped after 48 h of incubation. The mol% of SLS TAG increased to 40% at 24 h. After 48 h of incubation, only a moderate further increase (15%) of the disubstituted SL product (SLS) was observed. These results indicate that incorporation was more rapid in the first 24 h than in the second 24 h. As the concentration of LLL in the reaction mixture dropped, the concentration of disubstituted TAG (SLS) increased. For the purposes of this study, a 24-h reaction time was selected for further experiments.

Enzyme load. Previous studies in our laboratory have shown that 10% lipase by weight was suitable to catalyze the synthesis of a SL that contained oleic acid and caprylic acid (12). Figure 4 shows that the SL yield increased with enzyme concentration up to 10% by weight of reactants. Thereafter, there were no significant increases in the SL yield, indicating that a lipase content of 10% was suitable in this study.

Reaction media. When lipases are placed in organic solvents, they can exhibit a number of novel properties, such as altered stereoselectivity, enhanced stability, and altered mode of catalysis (18). Compounds that are soluble in organic solvents become potential substrates for lipases (18). Previous reports suggest that the interaction between organic solvents

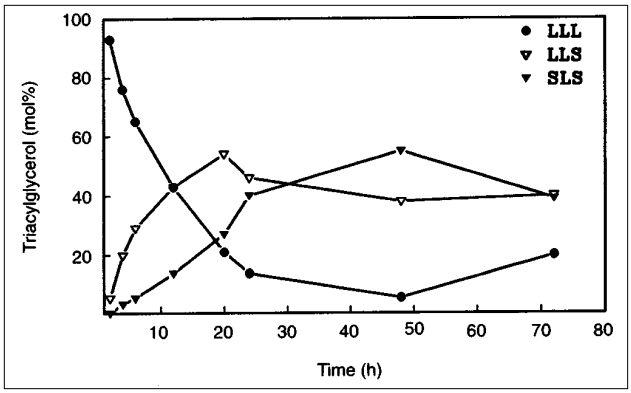

FIG. 3. Time course of caproic and butyric acids incorporation into triolein catalyzed by IM60 lipase. Samples were analyzed at 2, 4, 6, 12, 24, 48, and 72 h in duplicate. See Figure 1 for abbreviations and conditions.

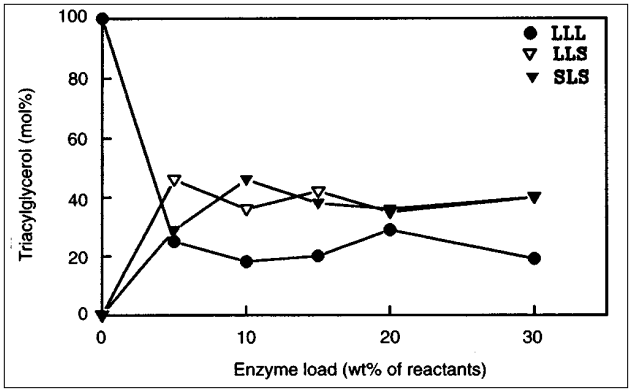

FIG. 4. Effect of enzyme load on incorporation of butyric and caproic acids into triolein. Amount of enzyme varied from 0 to 30%, total weight of substrates. See Figure 1 for abbreviations and conditions.

and enzyme-bound water controls the activity of an enzyme and that a physical disruption of the enzyme bound water results in enzyme deactivation (19). Nonpolar solvents, such as

TABLE 1
Effect of Organic Solvents as Reaction Media on Lipase-Catalyzed Incorporation of Butyric and Caproic Acids into Triolein with IM60 Lipase as Biocatalyst

Solvent[a]	Water content[b] (ppm)	Log P value[c]	Mol%[d] LLL	LLS	SLS
Petroleum ether	124	—	5.6	62.1	32.3
Isooctane	40	4.51	4.7	42.1	53.2
n-Hexane	35.5	3.50	5.9	45.1	49.0
Toluene	119	2.50	84.7	10.0	5.3
Benzene	156	2.00	95.6	4.4	N/A[e]
Acetone	—	–0.23	100	N/A	N/A
Acetonitrile	524.7	–0.33	100	N/A	N/A

[a]Solvents were dried over molecular sieve 4 Å.
[b]The water content of the solvents was measured with a 684 KF coulometer, equipped with a 649 stirrer (Brinkmann Instrument, Inc., Westbury, NY).
[c]Sources: Reference 21 and Reference 22 isooctane.
[d]L designates long-chain fatty acid (18:1n-9), and S short-chain fatty acid ($C_{6:0}$, $C_{4:0}$); LLL, triolein (unreacted); LLS, monosubstituted structured lipid; and SLS, disubstituted structured lipid.
[e]N/A indicates no structured lipid formation.

toluene and hexane, are incapable of containing large amounts of soluble water, and are therefore unable to strip away substantial amounts of water from enzymes (20). We found that nonpolar solvents, such as hexane and isooctane, gave higher yields of SL than polar solvents, such as acetone and acetonitrile. Hexane and isooctane gave disubstituted TAG yields of 49 and 53%, respectively (Table 1). With petroleum ether, the amount of unreacted LLL was not much different from the unreacted amount when the reaction was performed in hexane and isooctane. The amount of disubstituted TAG was 32% in petroleum ether.

Pancreatic lipase study. IM60 lipase from *R. miehei* preferentially hydrolyzes the fatty acids at the *sn*-1 and 3 positions of TAG. This characteristic is especially desirable when the nutritional benefits of TAG are considered, largely because specialty oils with desired fatty acids at specific positions can be prepared with *sn*-1,3-specific lipases. In this study, a pancreatic lipase hydrolysis was performed to determine the fatty acid composition of the *sn*-2 position of the SL. Results from the pancreatic lipase hydrolysis of a TAG standard with known structure, 1,3 distearoyl-2-oleoyl-glycerol confirmed that only oleic acid was present at the *sn*-2 position of this molecule. Analysis of our SL products indicated that only oleic acid could be found at the *sn*-2 position. No butyric or caproic acid was found at the *sn*-2 position. This indicates that under the conditions of our assay, IM60 lipase from *R. miehei* retained its *sn*-1,3 selectivity. The SL prepared in this study are potentially useful as reduced-calorie oils similar to Salatrim®; they contain desirable functional fatty acids in the same molecule and may provide rapid delivery of energy.

ACKNOWLEDGMENTS

Contributed by the Agricultural Experiment Station, College of Agricultural and Environmental Sciences, The University of Georgia. Research supported by Food Science Research and in part by an award from the North American Branch of the International Life Sciences Institute 2521-RC294-175.

REFERENCES

1. Akoh, C.C., Structured Lipids—Enzymatic Approach, *INFORM 6*:1055–1061 (1995).
2. Heird, W.G., S.M. Grundy, and V.S. Hubbard, Structured Lipids and Their Uses in Clinical Nutrition, *Am. J. Clin. Nutr. 43*:320–324 (1986).
3. Hayes, J.R., D.H. Pence, S. Scheinbach, P.R. D'Amelia, L.P. Klemann, N.H. Wilson, and J.W. Finley, Review of Triacylglycerol Digestion, Absorption and Metabolism with Respect to Salatrim Triacylglycerols, *J. Agric. Food Chem. 42*:474–483 (1994).
4. Babayan, V.K., Medium-Chain Triglycerides, in *Dietary Fat Requirements in Health and Development,* edited by Joyce Beare-Rogers. American Oil Chemists' Society, Champaign, 1988, pp. 73–86.
5. Bugaut, M., Occurrence, Absorption and Metabolism of Short-Chain Fatty Acids in the Digestive Tract of Mammals, *Comp. Biochem. Physiol. 86B(3)*:439–472 (1987).
6. Lide, D.R. (ed.), *CRC Handbook of Chemistry and Physics*, 73rd edn., CRC Press, Boca Raton, 1992–1993, pp. 5–84; 5–86.
7. Finley, J.W., L.P. Klemann, G.A. Leveille, M.S. Otterburn, and C.G. Walchak, Caloric Availability of Salatrim in Rats and Humans, *J. Agric. Food. Chem. 42*:495–499 (1994).
8. Wolin, M.J., Fermentation in the Rumen and Human Large Intestine, *Science 213*:1463–1468 (1981).
9. Hashim, A., and V.K. Babayan, Studies in Man of Partially Absorbed Dietary Fats, *Am. J. Clin. Nutr. 31*:S273–S276 (1978).
10. Bonanome, A., and G.M. Grundy, Effect of Dietary Stearic Acid on Plasma Cholesterol and Lipoprotein Levels, *N. Engl. J. Med. 318*:1244–1248 (1988).
11. Quinlan, P., and S. Moore, Modification of Triglycerides by Lipases: Process Technology and Its Application to the Production of Nutritionally Improved Fats, *INFORM 4*:580 (1993).
12. Akoh, C.C., and K.H. Huang, Enzymatic Synthesis of Structure Lipids: Transesterification of Triolein and Caprylic Acid, *J. Food Lipids 2*:219–230 (1995).
13. Luddy, F.E., R.A. Barford, S.F. Herb, P. Magidman, and R.W. Riemenschneider, Pancreatic Lipase Hydrolysis of Triglycerides by a Semi-Micro Technique, *J. Am. Oil Chem. Soc. 41*:693–696 (1964).
14. Akoh, C.C., C. Cooper, and C.V. Nwosu, Lipase G-Catalyzed Synthesis of Monoglycerides in Organic Solvent and Analysis by HPLC, *Ibid. 69*:257–260 (1992).
15. Nishio, T., and M. Kamimura, Ester Synthesis by Crude Lipase Preparation from *Pseudomonas fragi* 22.39 B in *n*-Hexane, *Agric. Biol. Chem. 52*:2933–2935 (1988).
16. Kuo, S.J., and K.L. Parkin, Substrate Preference for Lipase-Mediated Acyl-Exchange Reactions with Butteroil Are Concentration-Dependent, *J. Am. Oil Chem. Soc. 70*:393–399 (1993).
17. Whitaker, J.R., *Principles of Enzymology for the Food Sciences*, Marcel Dekker, New York, 1972, p. 320.
18. Zaks, A., and A.J. Rusell, Enzymes in Organic Solvents: Properties and Applications, *J. Biotechnol. 8*:259–270 (1988).
19. Zaks, A., and A.M. Klibanov, The Effect of Water on Enzyme Action in Organic Media, *J. Biol. Chem. 263*:8017–8021 (1988).
20. Gorman, L.S., and J.S. Dordick, Organic Solvents Strip Water off Enzymes, *Biotechnol. Bioeng. 39*:392–397 (1992).
21. Laane, C., S. Boeren, K. Vos, and C. Veeger, Rules for Optimization of Biocatalysis in Organic Solvents, *Biotechnol. Bioeng. 30*:81–87 (1987).
22. Manjòn, A., J.L. Iborra, and A. Arocas, Short-Chain Flavor Ester Synthesis by Immobilized Lipase in Organic Media, *Biotechnol. Lett. 13*:339–344 (1991).

[Received March 18, 1996; accepted November 18, 1996]