Enzymatic Synthesis of Structured Lipid Containing Arachidonic and Palmitic Acids

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ABSTRACT: Human milk fat contains 20–25% palmitic acid, and about 70% of the fatty acid is esterified to the 2-position of triglycerides. It was also reported that arachidonic acid (AA) accelerated the growth of preterm infants. Thus, we attempted the synthesis of 1,3-arachidonoyl-2-palmitoyl-glycerol by acidolysis of tripalmitin with AA using 1,3-specific *Rhizopus delemar* lipase. When a mixture of 10 g tripalmitin/AA (1:5, w/w) and 0.7 g immobilized *Rhizopus* lipase was incubated at 40°C for 24 h with stirring, the AA content in glycerides reached 59 mol%. The immobilized lipase could be used five times without a decrease in the extent of acidolysis. Glycerides were extracted from the reaction mixture with *n*-hexane, and regiospecific analysis was performed. As a result, the AA contents at the 1,3- and 2-positions were 56.9 and 3.2 mol%, respectively. It was therefore confirmed that the fatty acids at the 1,3-positions of triglyceride were exchanged for AA. High-performance liquid chromatography showed that the contents of triarachidonin, 1,3-arachidonoyl-2-palmitoyl-glycerol, and 1(3)-arachidonoyl-2,3(1)-palmitoyl-glycerol were 7.3, 75.9, and 12.4 wt%, respectively.

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KEY WORDS: Acidolysis, arachidonic acid, immobilized enzyme, lipase, palmitic acid, *Rhizopus delemar*, structured lipid.

Arachidonic acid (AA, 20:4n-6) is a rare fatty acid that has potential pharmaceutical value and is a precursor of hormones (prostaglandins, leukotrienes, and thromboxanes) involved in the AA cascade (1,2). Arachidonylethanolamide (anandamide) (3) and 2-arachidonylglycerol (4) were recently reported to bind to the receptor of cannabinoid, a psychotropic agent, and are expected to act as analgesics. Furthermore, AA is contained in human milk and accelerates the growth of preterm infants, as does docosahexaenoic acid (22:6n-3) (5,6). Human milk fat contains 20–25% palmitic acid (PA, 16:0), and about 70% of the fatty acid is esterified to the 2-position of the triglycerides (7,8). In addition, the main component of the milk dienoic triglycerides is 1,3-oleoyl-2-palmitoyl-glycerol. Gastric and pancreatic lipases hydrolyze dietary fat to free fatty acids and 2-monoglycerides, and the absorption efficiency of free PA is relatively low compared with that of free unsaturated fatty acids (9). Thus, fat absorption is higher in infants fed fats with PA at the 2-position of triglycerides than the 1,3-position (10). It has been hypothesized from these facts that the high absorption efficiency of human milk fat is the result of specific positioning of PA at the 2-position of the triglyceride moiety (10,11). Thus, we attempted to synthesize 1,3-arachidonoyl-2-palmitoyl-glycerol for possible use as a component of infant formula.

Structured lipids have been efficiently produced by exchanging fatty acids at the 1,3-position of triglycerides with desired fatty acids using immobilized 1,3-specific lipase (12–18). The system containing organic solvent is effective in the reaction, especially when the substrate is in a solid state. However, when the substrate is in a liquid state at the reaction temperature, the reaction efficiently proceeds even in a solvent-free system. This paper deals with the synthesis of 1,3-arachidonoyl-2-palmitoyl-glycerol in a solvent-free system by acidolysis of tripalmitin with AA using immobilized *Rhizopus delemar* lipase.

MATERIALS AND METHODS

Materials. Tripalmitin (PA content, 90 mol%; stearic acid content, 8 mol%) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). AA (purity, 91 mol%) is a product of Suntory Co. (Osaka, Japan).

Preparation of immobilized lipase. Rhizopus delemar lipase (Ta-lipase) was a gift from Tanabe Seiyaku Co. Ltd. (Osaka, Japan). The lipase was immobilized on a ceramic carrier (SM-10; NGK Insulators, Aichi, Japan) as described in our previous paper (14). After the ceramic carrier (20 g) was suspended in 80 mL of 10% lipase solution, 240 mL of cold acetone (–80°C) was gradually added with stirring, and the precipitate was dried *in vacuo*. Approximately 90% of the lipase was immobilized on the carrier by this procedure.

Reaction. Because the freshly prepared immobilized lipase did not show any acidolysis activity (14,19), it was activated as follows: A mixture of 10 g AA/tripalmitin (5:1, w/w), 0.2 g water, and 0.7 g immobilized lipase was incubated at 40°C for 24 h in a screw-capped vessel with shaking at 140 oscilla-

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tions/min. The activated enzyme was transferred into a fresh substrate mixture of AA/tripalmitin without water, and the acidolysis was then conducted under the same conditions as those for the pretreatment.

Analysis. Glycerides were extracted with 100 mL *n*-hexane after adding 70 mL of 0.5 N KOH (20% ethanol solution) in 10 g reaction mixture. Fatty acids in glycerides were methylated in methanol using sodium methylate as a methylating reagent. The methylesters were analyzed by a Hewlett-Packard (Avondale, PA) 5890 gas chromatograph equipped with a DB-23 capillary column (0.25 mm × 30 m; J&W Scientific, Folsom, CA) as described previously (20). Thin-layer chromatography (TLC) of glycerides was performed according to our previous paper (21). The glycerides were applied to a silica gel 60 plate (Merck, Darmstadt, Germany), and then developed with a mixture of chloroform/acetone/acetic acid (96:4:1, vol/vol/vol). The contents of mono-, di-, and triglycerides were measured by a TLC/flame-ionization detector (FID) analyzer (Iatroscan MK-5; Iatron Co., Tokyo, Japan) after development with a mixture of benzene/chloroform/acetic acid (50:20:0.7, vol/vol/vol). Regiospecific analysis of triglycerides was carried out by Grignard degradation with allyl magnesium bromide, and followed by isolation and analysis of the 1,3-diglyceride fraction (22). Triglycerides were analyzed on two octadecyl silica (ODS) columns (4.6 × 150 mm; Cosmosil 5C18-AR, Nacalai Tesque Inc., Kyoto, Japan), connected with a high-performance liquid chromatography (HPLC) system (LC-9A; Shimadzu Co., Kyoto, Japan). The sample was eluted with a mixture of acetone/acetonitrile (2:1, vol/vol) at a flow rate of 0.4 mL/min at 40°C, and detected with a refractometer.

RESULTS

Factors affecting acidolysis of tripalmitin with AA. In general, lipases act well on liquid-state substrates, but not on solid-state ones. Because a mixture of AA/tripalmitin (3:1, w/w) was liquid at 40°C, the reaction temperature was fixed at 40°C. To investigate the effect of the amount of AA, acidolysis was conducted at various ratios of AA to tripalmitin (Fig. 1). When tripalmitin was acidolyzed with more than three times excess AA for 6 h using 4% immobilized lipase, the AA incorporated in glycerides was almost the same in its content (25–30%). However, the AA content in glycerides after 24- and 48-h reactions depended on the amount of AA, and reached a constant value at a weight ratio of 5:1.

Acidolysis of tripalmitin with AA was performed using 2–10% immobilized *Rhizopus* lipase as a catalyst (Table 1). The first reaction with 4% enzyme achieved 57 mol% AA incorporation in glycerides, and larger amounts of the lipase did not increase the AA incorporation. However, when the acidolysis was repeated by transferring the enzyme to a fresh substrate mixture, the decrease in the acidolysis extent depended on the amount of immobilized lipase. Repeated acidolyses with 7 and 10% lipase did not decrease the AA content in glycerides during cycles 3 and 5, respectively. The decrease of the acidolysis extent may be due to the inactivation of enzyme at 40°C.

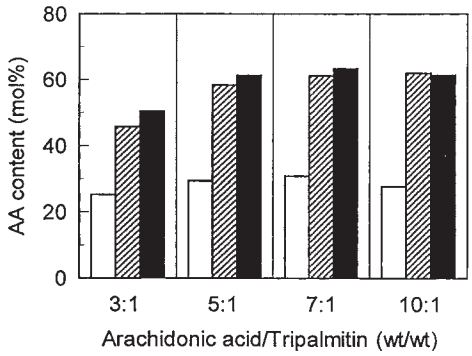

FIG. 1. Effect of amount of arachidonic acid (AA) on acidolysis of tripalmitin with AA. The acidolysis was performed at 40°C in a mixture of 10 g of tripalmitin/AA and 0.4 g immobilized *Rhizopus delemar* lipase which had been activated by incubating in the same mixture containing 2% water. Open boxes, 6-h reaction; hatched boxes, 24-h reaction; closed boxes, 48-h reaction.

Time course of acidolysis of tripalmitin with AA. On the basis of the above results, the reaction conditions for the acidolysis were determined as follows: A mixture of 10 g AA/tripalmitin (5:1, w/w) and 0.7 g immobilized lipase was incubated at 40°C with stirring. Figure 2 shows a typical time course of the acidolysis under these conditions. The contents of PA in glycerides rapidly decreased until 10 h, and then gradually decreased thereafter. The increase in AA content in glycerides corresponded completely with the decrease in these fatty acid contents. The AA contents after 24- and 34-h reactions reached 59 and 63 mol%, respectively.

TABLE 1
Effect of Enzyme Amount on Acidolysis of Tripalmitin with Arachidonic Acid[a]

Enzyme amount (%)	Cycle number	Fatty acid composition (mol%)[b] 16:0	18:0	18:3	20:3	20:4
2	1	75.5	6.2	0.3	ND	16.0
	2	78.6	6.2	0.2	ND	13.1
4	1	35.3	3.0	1.5	0.8	56.8
	2	47.2	3.8	1.1	0.6	45.8
	3	64.5	4.9	0.5	0.3	27.5
7	1	33.1	2.9	1.7	0.8	58.9
	3	32.3	2.9	1.7	0.9	59.5
	4	56.8	4.6	1.0	0.5	35.7
	5	73.8	5.6	0.3	ND	18.3
10	1	35.2	3.1	1.4	0.8	57.0
	3	32.6	2.9	1.9	0.9	59.8
	5	32.8	2.9	2.0	0.9	59.0
	6	47.2	3.8	1.1	0.6	44.8
	7	70.9	6.7	0.3	ND	20.2

[a]Acidolysis was performed at 40°C for 24 h in a mixture of 10 g arachidonic acid (AA)/tripalmitin (5:1, w/w), and 0.2–1.0 g of immobilized *Rhizopus delemar* lipase which had been activated by incubating in the same mixture containing 2% water. The reaction was repeated by transferring the lipase to a fresh substrate mixture without adding water.
[b]Fatty acid composition of glycerides.

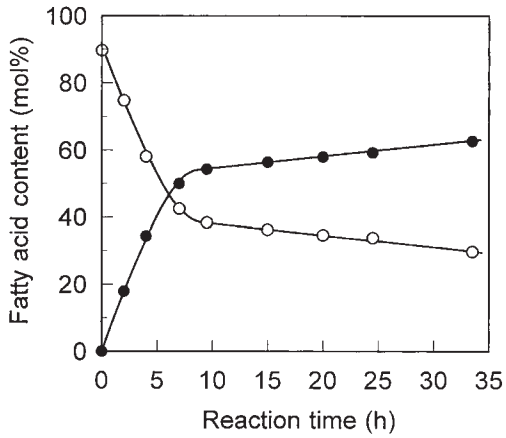

FIG. 2. Time course of acidolysis of tripalmitin with AA. A mixture of 30 g AA/tripalmitin (5:1, w/w) and 2.1 g immobilized *Rhizopus* lipase was incubated at 40°C with shaking. ○, palmitic acid content in glycerides; ●, AA content. See Figure 1 for abbreviations.

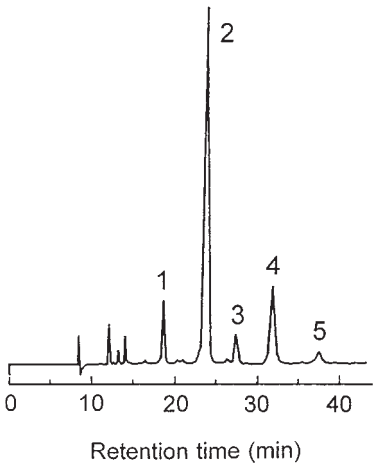

FIG. 3. Triglyceride components of transesterified oil. High-performance liquid chromatographic analysis was carried out as described in Materials and Methods section. As shown in Table 3, the triglyceride structures of peaks 1 to 5 were estimated as follows: peak 1, triarachidonin; 1,3-arachidonoyl-2-palmitoyl-glycerol; 1,3-arachidonoyl-2-stearoyl-glycerol; 1(3)-arachidonoyl-2-palmitoyl-3(1)-palmitoyl-glycerol; and triglycerides with arachidonic, palmitic, and stearic acids, respectively.

Components in transesterified oil. Acidolysis of tripalmitin with AA was performed for 24 h under the conditions determined, and the glycerides were extracted with *n*-hexane. The glycerides contained 60 mol% of AA. The contents of triglycerides and diglycerides were 95.4 and 4.6 wt%, respectively. Monoglycerides were not present in the glyceride fraction.

Table 2 shows the fatty acid compositions of the 1(3)- and 2-positions in the transesterified oil. The AA contents at the 1(3)- and 2-positions were 56.9 and 3.2 mol%, respectively, and palmitic acid was esterified mainly at the 2-position. These results suggested that fatty acid at the 1(3)-position in triglyceride was exchanged for AA by the acidolysis with immobilized *Rhizopus* lipase.

Triglycerides in the transesterified oil were analyzed by HPLC (Fig. 3). The components eluted between 12 and 14 min were diglycerides as shown by TLC, and the components eluted after 18 min were triglycerides. To identify the triglyceride components fractionated by HPLC, the peaks numbered in Figure 3 were collected and their fatty acid compositions were analyzed (Table 3). On the basis of the molar ratio of fatty acids, the structures of peaks 1 to 5 were estimated as follows: peak 1, triarachidonin (AAA); peak 2, 1,3-arachidonoyl-2-palmitoyl-glycerol (APA); peak 3, 1,3-arachidonoyl-2-stearoyl-glycerol; peak 4, 1(3)-arachidonoyl-2-palmitoyl-3(1)-palmitoyl-glycerol; peak 5, triglyceride with AA, stearic acid and PA. The relative areas for peaks 1 to 5 were 7.3, 75.9, 3.1, 12.4, and 1.3%, respectively.

TABLE 2
Fatty Acid Composition of 1(3)- and 2-Positions in Transesterified Oil[a]

	Fatty acid composition (mol%)					
Position	16:0	18:0	18:2	18:3	20:3	20:4
1,2,3 (Total)[b]	31.7	2.8	0.5	2.1	0.9	60.1
1,3	3.0	0.9	0.5	2.0	0.9	56.9
2	28.7	1.9	ND[c]	0.1	ND	3.2

[a]The fatty acids located at the 1(3)-position of triglyceride were analyzed using 1,3-diglycerides obtained by Grignard degradation. The content of a fatty acid located at the 2-position was calculated by subtracting the content at the 1(3)-position from that of undigested triglycerides.
[b]Fatty acid composition of the transesterified oil.
[c]Not detected.

DISCUSSION

We have reported how APA was efficiently produced by acidolysis of tripalmitin with AA using immobilized *Rhizopus* lipase in a solvent-free system. The absorption of AA in the structured lipid couples to hydrolysis of the lipid by pancreatic lipase. Pancreatic lipase is a 1,3-specific lipase. However, it acts on polyunsaturated fatty acids weakly. Hence, 0.5 g of structured lipid was stirred at 35°C in 5 mL of 50 mM acetate buffer (pH 5.6) with porcine pancreatic lipase. As a result, the lipid was hydrolyzed, although the hydrolysis rates were 24% of the hydrolysis rate of olive oil (data not shown). The release of AA by hydrolysis with the lipase suggests that AA can be absorbed into the intestinal mucosa.

We previously reported that immobilized *Rhizopus* lipase was effective for the production of structured lipids with caprylic acid and a functional fatty acid at the 1,3- and 2-positions, respectively (14–17). When acidolysis of tuna oil with caprylic acid was conducted using 4% immobilized enzyme, the lipase could be used 15 times without a decrease in the acidolysis extent (14). Furthermore, the enzyme maintained this acidolysis extent for 35 to 50 cycles in acidolyses of safflower and linseed oils with caprylic acid (15). On the other hand, the enzyme was usable only six times in the present study, although 10% lipase was used as a catalyst. The reactions reported previously were conducted at 30°C, but the reaction presented here was done at 40°C. The results suggest

TABLE 3
Fatty Acid Composition of Triglycerides Fractionated by High-Performance Liquid Chromatography

Triglyceride[a]	Retention time (min)	Fatty acid composition (mol%) 16:0	18:0	18:3	20:4	Triglyceride structure[b]
Peak 1	18.9	1.6	0.3	2.6	94.4	AAA
Peak 2	24.0	33.7	ND	1.8	64.5	APA
Peak 3	27.7	5.1	29.8	1.9	63.2	ASA
Peak 4	32.1	66.3	ND	1.0	32.7	APP
Peak 5	37.5	35.8	31.0	0.7	31.4	APS

[a]Peaks 1 to 5 correspond to the components numbered in Figure 3.
[b]AAA, triarachidonin; APA, 1,3-arachidonoyl-2-palmitoyl-glycerol; ASA, 1,3-arachidonoyl-2-stearoyl-glycerol; APP, 1(3)-arachidonoyl-2 palmitoyl-3(1)-palmitoyl-glycerol; APS, triglycerides with arachidonic, palmitic, and stearic acids.

that the immobilized lipase was gradually inactivated by use at 40°C for a long period.

Acidolysis of tripalmitin with AA produced 7 wt% of AAA as a by-product (Fig. 3). Because the positional specificity of *Rhizopus* lipase is very strict (15,16), it was strongly suggested that AAA was not generated by exchanging fatty acid at the 2-position for AA. The presence of 4.6 wt% of diglycerides showed that hydrolysis occurred concomitantly with the acidolysis. In addition, concomitant esterification could be expected to occur during the reaction. Therefore, AAA may be generated *via* the following process: Diglyceride with AA at the 1(3)-position and PA at the 2-position is generated by hydrolysis, and the PA migrates spontaneously to the 3(1)-position. PA at the 3(1)-position is acidolyzed with AA, and diarachidonin is generated. After AA at the 1(3)-position migrated to the 2-position, AAA is synthesized by esterification of free AA to the 1(3)-position. It was confirmed that diarachidonin was present in the reaction mixture, because the peak eluted at 12 min by HPLC analysis contained 95 mol% of AA (Fig. 3). This fact strongly supported the hypothesis.

REFERENCES

1. Borgeat, P., M. Nadeau, H. Salari, P. Poubelle, and B.F. deLaclos, Leukotrienes: Biosynthesis, Metabolism, and Analysis, in *Advances in Lipid Research*, edited by R. Paoletti and D. Kritchevsky, Academic Press, New York, 1985, Vol. 21, pp. 47–77.
2. Minno, G.D., A.M. Cerbone, and A. Postiglione, Lipids in Platelet Function: Platelet and Vascular Prostaglandins in Thromboembolic Disease, in *Advances in Lipid Research*, edited by R. Paoletti, and D. Kritchevsky, Academic Press, New York, 1987, Vol. 22, pp. 63–82.
3. Devane, W.A., L. Hanus, A. Breuer, R.G. Pertwee, L.A. Stevenson, G. Griffin, D. Gibson, A. Mandelbaum, A. Etinger, and R. Mechoulam, Isolation and Structure of Brain Constituent That Binds to the Cannabinoid Receptor, *Science 258*:1946–1949 (1992).
4. Stella, N., P. Schweltzer, and D. Plomelli, A Second Endogenous Cannabinoid That Modulates Long-Term Potentiation, *Nature 388*:773–778 (1997).
5. Carlson, S.E., S.H. Werkman, J.M. Peeples, R.J. Cooke, and E.A. Tolley, Arachidonic Acid Status Correlates with First Year Growth in Preterm Infants, *Proc. Natl. Acad. Sci. USA 90*:1073–1077 (1993).
6. Lanting, C.I., V. Fidler, M. Huisman, B.C.L. Touwen, and E.R. Boersma, Neurological Differences Between 9-Year-Old Children Fed Breast-Milk as Babies, *Lancet 344*:1319–1322 (1994).
7. Breckenridge, W.C., L. Marai, and A. Kuksis, Triglyceride Structure of Human Milk Fat, *Can. J. Biochim. 47*:761–769 (1969).
8. Martin, J.-C., P. Bougnoux, J.-M. Antoine, M. Lanson, and C. Couet, Triacylglycerol Structure of Human Colostrum and Mature Milk, *Lipids 28*:637–643 (1993).
9. Jensen, C., N.R. Buist, and T. Wilson, Absorption of Individual Fatty Acids from Long Chain or Medium Chain Triglycerides in Very Small Infants, *Am. J. Clin. Nutr. 43*:745–751 (1986).
10. Innis, S.M., R. Dyer, and C.M. Nelson, Evidence That Palmitic Acid Is Absorbed as *sn*-2 Monoacyglycerol from Human Milk by Breast-Fed Infants, *Lipids 29*:541–545 (1994).
11. Fomon, S.J., E.F. Ziegler, L.N. Thomas, R.L. Jensen, and L.J. Filer Jr., Excretion of Fat by Normal Full-Term Infants Fed Various Milks and Formulas, *Am. J. Clin. Nutr. 23*:1299–1313 (1970).
12. Akoh, C.C., Structured Lipids—Enzymatic Approach, *INFORM 6*:1055–1061 (1995).
13. Akoh, C.C., and C.O. Moussata, Lipase-Catalyzed Modification of Borage Oil: Incorporation of Caprylic and Eicosapentaenoic Acids to Form Structured Lipids, *J. Am Oil Chem. Soc. 75*:697–701 (1998).
14. Shimada, Y., A. Sugihara, K. Maruyama, T. Nagao, S. Nakayama, H. Nakano, and Y. Tominaga, Production of Structured Lipid Containing Docosahexaenoic and Caprylic Acids Using Immobilized *Rhizopus delemar* Lipase, *J. Ferment. Bioeng. 81*:299–303 (1996).
15. Shimada, Y., A. Sugihara, H. Nakano, T. Yokota, T. Nagao, S. Komemushi, and Y. Tominaga, Production of Structured Lipids Containing Essential Fatty Acids by Immobilized *Rhizopus delemar* Lipase, *J. Am. Oil Chem. Soc. 73*:1415–1420 (1996).
16. Shimada, Y., A. Sugihara, H. Nakano, T. Nagao, M. Suenaga, S. Nakai, and Y. Tominaga, Fatty Acid Specificity of *Rhizopus delemar* Lipase in Acidolysis, *J. Ferment. Bioeng. 83*:321–327 (1997).
17. Shimada, Y., M. Suenaga, A. Sugihara, S. Nakai, and Y. Tominaga, Continuous Production of Structured Lipid Containing γ-Linolenic and Caprylic Acids by Immobilized *Rhizopus delemar* Lipase, *Ibid. 76*:189–193 (1999).
18. Lee, K., and C.C. Akoh, Solvent-Free Enzymatic Synthesis of Structured Lipids from Peanut Oil and Caprylic Acid in a Stirred Tank Batch Reactor, *Ibid. 75*:1533–1537 (1998).
19. Shimada, Y., A. Sugihara, S. Yodono, T. Nagao, K. Maruyama, H. Nakano, S. Komemushi, and Y. Tominaga, Enrichment of

Ethyl Docosahexaenoate by Selective Alcoholysis with Immobilized *Rhizopus delemar* Lipase, *J. Ferment. Bioeng. 84*:138–143 (1997).
20. Shimada Y., K. Maruyama, S. Okazaki, M. Nakamura, A. Sugihara, and Y. Tominaga, Enrichment of Polyunsaturated Fatty Acids with *Geotrichum candidum* Lipase, *J. Am. Oil Chem. Soc. 71*:951–954 (1994).
21. Sugihara, A., M. Ueshima, Y. Shimada, S. Tsunasawa, and Y. Tominaga, Purification and Characterization of a Novel Thermostable Lipase from *Pseudomonas cepacia*, *J. Biochem. 112*:598–603 (1992).
22. Becker, C.C., A. Rosenquist, and G. Holmer, Regiospecific Analysis of Triglycerols Using Allyl Magnesium Bromide, *Lipids 28*:147–149 (1993).

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