Synthesis of 1,3-Dicapryloyl-2-docosahexaenoylglycerol by a Combination of Nonselective and *sn*-1,3-Selective Lipase Reactions

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ABSTRACT: A two-step consecutive synthetic method for the production of symmetrical, structured lipids by a combination of nonselective and *sn*-1,3 regioselective ester exchange reactions was investigated. In the first step, TAG with unspecifically substituted DHA were obtained by reacting tricapryloylglycerol (CCC) with ethyl docosahexanoate (EtDHA) using the lipase QLM (from *Alcaligenes* sp.), followed by removing the ethyl ester and CCC by molecular distillation. In the second step, *sn*-1,3 regioselective ester exchange was achieved by reacting the resulting TAG with ethyl caprylate (EtC) using the immobilized lipase Novozyme 435 (*Candida antarctica* lipase), followed by distillation of the ethyl ester and CCC to give *sn*-1,3-dicapryloyl-*sn*-2-docosahexaenoylglycerol (CDC). The acylglycerol composition of CDC was analyzed by GLC, which showed that the content of dicapryloyldocosahexaenoylglycerols (2CD) in the product was 76.4%, and that the ratio of CDC to *sn*-1,2-dicapryloyl-*sn*-3-docosahexaenoylglycerol contained in 2CD was 82.7:17.3 (%). The distillates CCC, EtDHA, and EtC could be recycled repeatedly to produce CDC as the substrate for the consecutive ester exchange reaction. In addition, separation of CCC and EtDHA was unnecessary for reuse. The present method is considered to meet the requirements for industrial utilization, in which simplicity in scaleup, high yields, compact reaction system, and minimal formation of by-products are important factors.

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KEY WORDS: 1,3-Dicapryloyl-2-docosahexaenoylglycerol, DHA, esterification, lipase, structured lipid

The nutritional benefits of PUFA such as EPA and DHA have been attracting attention recently (1). Among the structured lipids containing these FA, ones in which a PUFA is located at the *sn*-2 position and medium chain-FA (MCFA) at the *sn*-1,3 positions are considered especially noteworthy because of their favorable absorption profile in the body (2).

As for the production of structured lipids, several methods using lipase, which can react under mild conditions, have been investigated. For example, Akoh and Moussata (3) reported on a large-scale production of structured lipids containing PUFA by using fish oil as the starting material. But this raw material contained FA other than PUFA, and these authors did not examine the disposal and reuse of FA removed by distillation. Although the desired structured lipids were produced in relatively large scale, application of the method to the industrial production of symmetrically substituted lipids is considered unlikely for this reason.

To synthesize structured lipids in which the PUFA-binding position is specified, *sn*-1,3 regioselective reaction of TAG with a FA or its ester has been most commonly used. Shimada *et al.* (4) have focused their attention on the fact that tuna oil contains DHA most abundantly at the *sn*-2 position, and they obtained *sn*-1,3-dicapryloyl-*sn*-2-docosahexaenoylglycerol (CDC) by the *sn*-1,3-regioselective acidolysis reaction of tuna oil with caprylic acid using a lipase. This method is suitable for industrial production but unsatisfactory in regard to purity of the PUFA.

On the other hand, high-purity PUFA are commercially available in an acid or ester form. Irimescu *et al.* (5) synthesized TAG with high-purity PUFA chains by using PUFA ethyl ester as the starting material. To synthesize structured lipids with a DHA chain, these authors have proposed the following new synthetic process (6,7). In the direct *sn*-1,3 regioselective ester exchange reaction of tridocosahexaenoylglycerol (DDD) with ethyl caprylate (EtC), separation of the target product from *sn*-1-capryloyl-*sn*-2,3-didocosahexaenoylglycerol (CDD) is difficult. Besides, a large amount of EtC, relative to DDD, is required. To avoid these problems, they converted DDD to *sn*-2-docosahexaenoyl-monoglycerol by *sn*-1,3 regioselective ethanolysis, then they esterified the resulting MAG with EtC specifically at the *sn*-1,3 positions to give highly pure CDC. To produce CDC using very expensive starting materials such as DHA, its efficient utilization is an important factor for cost reduction.

We have recently reported the production of ester-exchanged edible oils using powdered lipase (8). This lipase reaction occurred selectively at the *sn*-1,3 position, but the specificity was not high.

On the basis of these background data, the present study was undertaken to investigate a new synthetic process to produce CDC with efficient DHA utilization by a combination of nonselective and *sn*-1,3 regioselective ester exchange reactions.

EXPERIMENTAL PROCEDURES

Substrates and lipases. Tricapryloylglycerol (CCC) of 99% purity from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and EtC of 99% purity from Inoue Perfumery Mfg. Co., Ltd. (Tokyo, Japan) were used for this study. Ethyl docosahexanoate (EtDHA) of 92% purity from Nippon Chemical Reagents, Ltd.

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(Hakodate, Japan) was used. As for the lipase enzymes, Lipase QLM (*Alcaligenes* sp.) from Meito Sangyo Co., Ltd. (Nagoya, Japan) and the immobilized lipase Novozyme 435 (*Candida antarctica*) from Novozymes Co., Ltd. (Bagsvaerd, Denmark) were used. Other reagents used in this study were commercially available and of SP grade.

Analysis of the compositions of TAG. The composition of TAG was determined by GLC using a DB-1ht (0.32 mm × 0.1 μm × 5 m) column (Agilent Technologies, Palo Alto, CA), under the following analytical conditions: The injection temperature was 370°C, the detector temperature was 370°C, the column temperature was raised from 40 to 370°C at a rate of 15°C/min, the split ratio was 90:1, and helium was used as the carrier gas with a constant flow rate of 7.0 mL/min. The composition of TAG was determined by calculating the corresponding percentage of the peak area.

Analysis of the relative ratio of CDC to sn-*1,2-dicapryloyl*-sn-*3-docosahexaenoylglycerol (CCD).* According to the method reported by Adlof (9), the HPLC determination of the relative ratio of CDC to CCD was carried out using a ChromSpher Lipids™ 250 × 4.6 mm column (Varian, Palo Alto, CA), under the following conditions: UV detector at 206 nm, and a binary solvent A (*n*-hexane/2-propanol/acetonitrile = 350:100:2, by vol) and solvent B (*n*-hexane/2-propanol/acetonitrile = 350:100:10, by vol). The column was eluted with a linear gradient of A to B over 22 min at a flow rate of 0.7 mL/min. The relative ratio (CDC/CCD) was determined by calculating the corresponding percentage of the peak area.

Synthesis of CDC. The first lipase reaction was carried out according to a previously reported method (8). To a mixture of CCC (500 g) and EtDHA (500 g), lipase QLM (20 g) was added, and the mixture was allowed to react for 90 h at 50°C with stirring. The reaction was carried out in a nitrogen atmosphere. After the reaction, the enzyme was removed by filtration to give the filtrate (960 g) of the reaction mixture. By using a molecular distillation apparatus (MS150; Nippon Sharyo Co. Ltd., Nagoya, Japan), EtC was distilled off from the reaction mixture 50°C and a pressure of 100 Pa. EtDHA was then distilled off at 180°C and a pressure of 20 Pa to yield 350 g TAG. The second lipase reaction was carried out using the 350 g TAG thus obtained, 1700 g EtC, and 41 g Novozyme 435 in a nitrogen atmosphere for 40 h at 40°C with stirring. The enzyme was removed by filtration to yield 1900 g filtrate of the reaction mixture. After that, EtC, EtDHA, and CCC were removed from the reaction mixture by molecular distillation in a similar manner to yield 122 g CDC.

RESULTS AND DISCUSSION

A flow chart of the reaction process of the present method is presented in Figure 1. In the first lipase reaction, the nonselective ester exchange was carried out between CCC and EtDHA using lipase QLM. In the second lipase reaction, *sn*-1,3 regioselective ester exchange was achieved between the first reaction product and EtC using Novozyme 435 to obtain the expected CDC. In these processes, CCC, EtDHA, and EtC were recovered by molecular distillation, and they could be recycled as

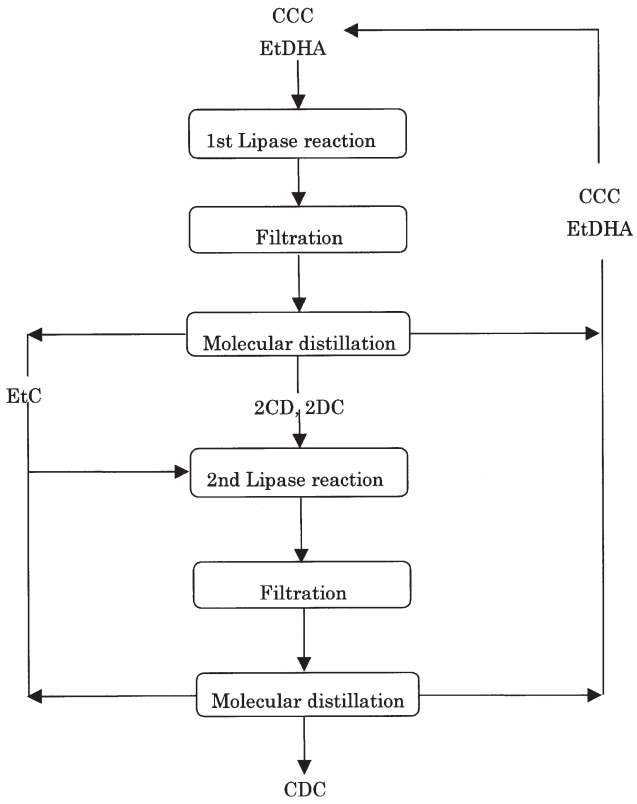

FIG. 1. Schematic diagram of the reaction process showing a combination of different enzyme reactions. CCC, tricapryloylglycerol; EtDHA, ethyl docosahexaenoate; 2CD, dicapryloyl-docosahexaenoylglycerol, 2DC, didocosahexaenoyl-capryloylglycerol; EtC, ethyl caprylate; CDC, *sn*-1,3-dicapryloyl-*sn*-2-docosahexaenoylglycerol.

the substrates for the first and second lipase reactions, respectively. In recycling these distillates, EtC could be separated from CCC and EtDHA by molecular distillation because of its lower M.W. compared to the latter two. In addition, CCC and EtDHA did not need to be separated; they were reused together as the substrates for the first lipase reaction.

The first lipase reaction. The time course of the change in acylglycerol composition is shown in Figure 2. The final concentration of TAG with a DHA chain was more than 50%. DDD was not produced in this reaction. In addition, although DAG were produced by the enzymatic reaction, they accounted for as little as 1.5%.

The relative ratio of CDC to CCD + DCC (*sn*-1-DHA or *sn*-3-DHA) contained in dicapryloyl-docosahexaenoylglycerol (2CD) was determined by HPLC to be 34.9:65.1 (%) after a 90-h reaction, indicating that the ester exchange occurred nonregioselectively. That is, in the TAG produced in this reaction, the DHA chain was added nonselectively at the *sn*-2 and *sn*-1,3 positions.

After the first lipase reaction, EtC, EtDHA, and CCC were removed using a thin-film molecular distillation apparatus. The thin-film molecular distillation method was chosen to shorten the heating time and thereby minimize thermal deterioration. The GLC analytical results of the distillate composition are shown in Table 1. The concentration of acylglycerol with a

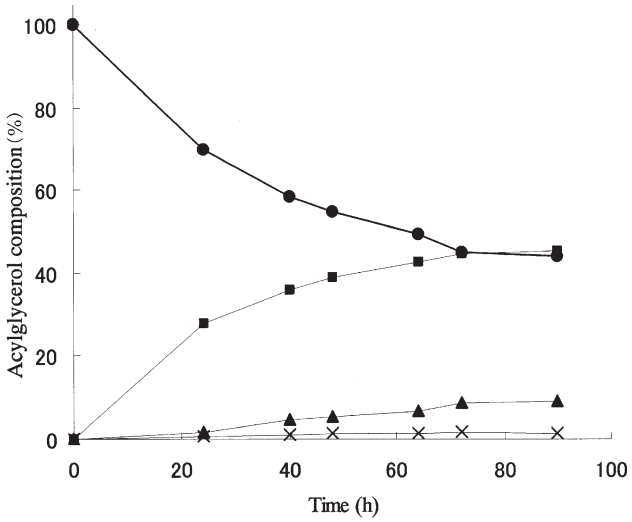

FIG. 2. Time course of acylglycerol composition during the first enzyme reaction. The reaction was performed with 2% of lipase QLM (Meito Sangyo Co., Nagoya, Japan) at 50°C. Acylglycerol content [CCC (-●-), 2CD (-■-), 2DC (-▲-), DAG (-×-)]. For abbreviations see Figure 1.

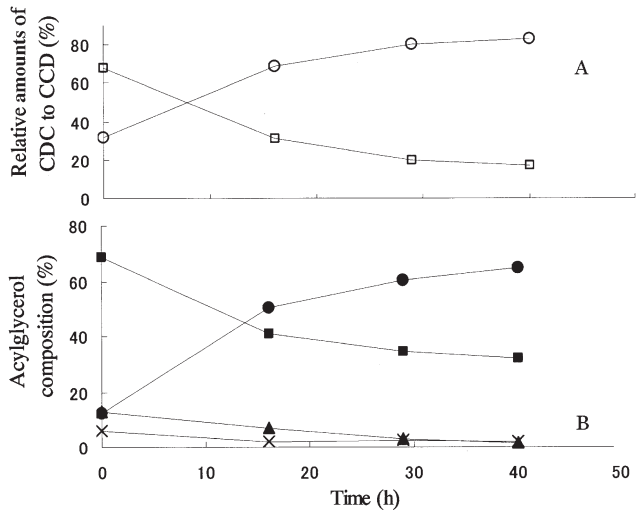

FIG. 3. Time course of acylglycerol composition and relative ratio of CDC and *sn*-1,2-dicapryloyl-*sn*-3-docosahexaenoylglycerol (CCD) during the second enzyme reaction. The reaction was performed with 2% of Novozyme 435 (Novo Nordisk, Bagsvaerd, Denmark) at 40°C. (A) Relative ratio of CDC and CCD [CDC (-○-), CCD (-□-)]. (B) Acylglycerol content [CCC (-●-), 2CD (-■-), 2DC (-▲-), DAG (-×-)]. For other abbreviations see Figure 1.

DHA chain was increased to 80% in the residue remaining after molecular distillation. Less formation of concomitant CCC is preferable, but this product, if present in small amounts, does not affect the second lipase reaction because it is also produced in this reaction. Moreover, the distillate could be fractionated to distillate 1 (EtC) and distillate 2 (CCC and EtDHA) by changing the distillation condition.

The second lipase reaction. The time courses of the change in acylglycerol composition and the relative amounts of CDC and CCD are shown in Figure 3. By progression of the reaction, CCC increased as 2CD and didocosahexaenoyl-capryloylglycerol (2DC) decreased, and most of the DHA-containing TAG corresponded to 2CD at the final time point. Although CCC can be removed by distillation, 2DC cannot be separated by distillation due to the high b.p.; therefore, less formation of this product is an advantage of the present method. Moreover, DAG decreased to 0.2% with the progression of the reaction. The relative ratio of CDC to CCD contained in 2CD increased with the progression of the reaction, the final CDC/CCD ratio being 83.9:16.9 (%), indicating that the reaction occurred *sn*-1,3 regioselectively. The relative content of CDC could be further increased when the substrate concentration of EtC was increased, but the obtainable quantity of CDC was considerably decreased.

Removal of EtC, EtDHA and CCC by distillation was carried out again by the thin-film molecular distillation method. The distillate composition was analyzed by GLC, and the results are shown in Table 2. As described in Table 2, 2CD was concentrated up to 76.4%, and the relative CDC/CCD ratio in 2CD was 82.7:17.3 (%) after the second molecular distillation. The GLC and HPLC chromatograms of the second distillation residue are shown in Figures 4A and 4B, respectively. The respective peaks were identified by comparison with reference reagents and also by other analytical methods (10).

Repeated use. CDC could be produced by recycling the recovered EtC, EtDHA, CCC, and the lipase enzymes that were used in the first and second ester exchange reactions according to the same flow of the synthetic process depicted in Figure 1. The reused amounts of EtC, EtDHA, and CCC were 2,000, 350, and 200 g, respectively, yielding 127 g of CDC. The GLC analysis showed that 2CD constituted 83.4% of the product, and the relative CDC/CCD ratio was 83.1:16.9 (%).

TABLE 1
Composition of the First Molecular Distillate (%)

	EtC	EtDHA	CCC	2CD	2DC	DAG	Other
Distillate 1[a]	99.0	—	—	—	—	—	1.0
Distillate 2[b]	6.6	53.8	31.0	0.5	—	4.3	3.8
Residue	1.2	1.5	8.5	65.7	15.9	4.7	2.5

[a]Distillate 1 was distilled off at 50°C and a pressure of 100 Pa.
[b]Distillate 2 was distilled off at 180°C and a pressure of 20 Pa. EtC, ethyl caprylate; EtDHA, ethyl docosahexaenoate; CCC, tricapryloylglycerol; 2CD, dicapryloyl-docosahexaenoylglycerol; 2DC, didocosahexaenoyl capryloylglycerol.

TABLE 2
Composition of the Second Molecular Distillate (%)

	EtC	EtDHA	CCC	2CD	2DC	DAG	Other
Distillate 1[a]	99.3	0.1	—	—	—	—	0.6
Distillate 2[b]	8.1	51.0	29.4	0.6	—	0.1	10.8
Residue	0.6	0.7	6.7	76.4	10.2	3.7	1.7

[a]Distillate 1 was distilled off at 50°C and a pressure of 100 Pa.
[b]Distillate 2 was distilled off at 180°C and a pressure of 20 Pa. For abbreviations see Table 1.

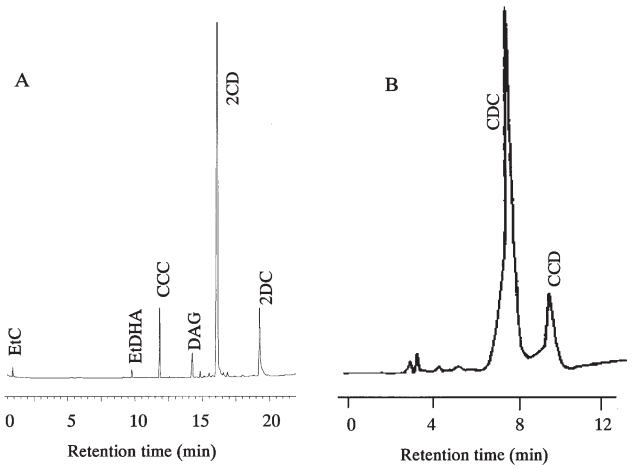

FIG. 4. (A) Gas–liquid chromatogram of the structured lipid CDC synthesized using a combination of different enzyme reaction systems. (B) HPLC of the structured lipid CDC synthesized using a combination of different enzyme reaction systems. For abbreviations see Figures 1 and 3.

In the HPLC chromatogram, no deterioration of DHA was observed.

Considering the heat history, it is desirable to use all new materials including the enzyme after a certain number of reuses even if sufficient enzyme activity is still maintained, although no deterioration of enzyme activity was observed in the second use. Furthermore, for industrialization it is considered necessary to confirm the safety of CDC when it is produced from recycled substrates.

Application of the synthetic process reported here is not limited to the production of CDC but can be extended in general to the production of symmetrical TAG having a functional FA chain at the *sn*-2 position. Therefore, it can be considered to be an efficient method for the production of structured lipids with various functions.

REFERENCES

1. Connor, W.E., Importance of n-3 Fatty Acids in Health and Disease, *Am. J. Clin. Nutr. 71*:171s–175s (2000).
2. Christensen, M.S., C.E. Høy, C.C. Becker, and T.G. Redgrave, Intestinal Adsorption and Lymphatic Transport of Eicosapentaenoic (EPA), Docosahexaenoic (DHA), and Decanoic Acids: Dependence on Intramolecular Triacylglycerol Structure, *Am. J. Clin. Nutr. 61*:56–61 (1995).
3. Akoh, C.C., and C.O. Moussata, Characterization and Oxidative Stability of Enzymatically Produced Fish and Canola Oil-Based Structured Lipids, *J. Am. Oil Chem. Soc. 78*:25–30 (2001).
4. Shimada, Y., A. Sugihara, K. Maruyama, T. Nagano, 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).
5. Irimescu, R., K. Hata, Y. Iwasaki, and T. Yamane, Comparison of Acyl Donors for Lipase-Catalyzed Production of 1,3-Dicapryloyl-2-eicosapentaenoylglycerol, *J. Am. Oil Chem. Soc. 78*:65–70 (2001).
6. Irimescu, R., K. Furihata, K. Hata, Y. Iwasaki, and T. Yamane, Utilization of Reaction Medium-Dependent Regiospecificity of *Candida antarctica* Lipase (Novozym 435) for the Synthesis of 1,3-Dicapryloyl-2-docosahexaenoyl (or eicosapentaenoyl) Glycerol, *Ibid. 78*:285–289 (2001).
7. Irimescu, R., K. Furihata, K. Hata, Y. Iwasaki, and T. Yamane, Two-Step Enzymatic Synthesis of Docosahexaenoic Acid-Rich Symmetrically Structured Triacylglycerols *via* 2-Monoacylglycerols, *Ibid. 78*:743–748 (2001).
8. Negishi, S., S. Shirasawa, Y. Arai, J. Suzuki, and S. Mukataka, Activation of Powdered Lipase by Cluster Water and the Use of Lipase Powders for Commercial Esterification of Food Oils, *Enzyme Microb. Technol 32*:66–70 (2003).
9. Adlof, R.O., Analysis of Triacylglycerol Positional Isomers by Silver Ion High Performance Liquid Chromatography, *J. High Resolut. Chromatogr. 18*:105–107 (1995).
10. Negishi, S., Y. Arai, S. Shirasawa, S. Arimoto, T. Nagasawa, H. Kouzui, and K. Tsuchiya, Analysis of Regiospecific Distribution of FA of TAG Using the Lipase-Catalyzed Ester-Exchange, *J. Am. Oil Chem. Soc. 80*:353–356 (2003).

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