Utilization of Reaction Medium-Dependent Regiospecificity of *Candida antarctica* Lipase (Novozym 435) for the Synthesis of 1,3-Dicapryloyl-2-docosahexaenoyl (or eicosapentaenoyl) Glycerol

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ABSTRACT: A highly efficient enzymatic method for the synthesis of regioisomerically pure 1,3-dicapryloyl-2-docosahexaenoyl glycerol (CDC) in two steps was established. 2-Monoglyceride (2-MG) formation by ethanolysis of tridocosahexaenoylglycerol (DDD) with immobilized *Candida antarctica* lipase (Novozym 435) as catalyst was the key step of the synthesis. CDC was finally obtained by reesterification of 2-MG with ethylcaprylate (EtC) catalyzed by *Rhizomucor miehei* lipase (Lipozyme IM). The regiospecificity of Novozym 435 depended on the type of reaction and the initial composition of the reaction medium. It displayed strict 1,3-regiospecificity for ethanolysis at a high excess of ethanol in the reaction mixture although it displayed no regiospecificity in transesterification and esterification reactions. The highest yield of CDC (85.4%) was obtained by ethanolysis at a 4:1 weight ratio of ethanol/DDD for 6 h followed by reesterification at a 20:1 molar ratio of EtC/initial DDD for 1.5 h. The regioisomeric purity of CDC was 100%. Good results were obtained also for the synthesis of 1,3-dicapryloyl-eicosapentaenoylglycerol (CEC) by the same method: 84.2% yield and 99.8% regioisomeric purity at the same reactant ratios as above. The yield of the reesterification step and the regioisomeric purity of the product were influenced by the molar ratio of the reactants for both CDC and CEC syntheses: higher excess of EtC favored higher yields and regioisomeric purity of the products.

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KEY WORDS: 1,3-Dicapryloyl-2-docosahexaenoyl glycerol, 1,3-dicapryloyl-2-eicosapentaenoylglycerol, esterification, ethanolysis, immobilized *Candida antarctica* lipase, immobilized *Rhizomucor miehei* lipase, transesterification, regiospecificity of lipases.

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are polyunsaturated fatty acids (PUFA) involved in a wide range of biological functions. Their roles in pathological conditions, due to imbalances in their intake, have been studied intensively in recent years (1,2). 1,3-Symmetrically structured glycerides with a PUFA residue at the second position and medium-chain fatty acid residues at the outer positions, such as 1,3-dicapryloyl-2-docosahexaenoyl (or eicosapentaenoyl) glycerol (CDC or CEC), are bioactive compounds with high potential in biomedical and nutraceutical applications. In this form, the PUFA residue is protected against oxidation by the two saturated acyl residues (3) and is adsorbed better in the intestinal tract as 2-monoglyceride (2-MG) after pancreatic hydrolysis (4,5). Medium-chain fatty acids are adsorbed efficiently and are a quick source of energy without accumulation in adipose tissues.

1,3-Symmetrically structured glycerides can be obtained either by chemical or enzymatic methods. The latter have the advantages of milder reaction conditions, nontoxic reactants and catalysts, and high positional specificity of catalysts, i.e., 1,3-regiospecific lipases.

The main impediment in the enzymatic synthesis of CDC is the very low activity of 1,3-regiospecific lipases on DHA-containing triglycerides (6–8). Some studies have used oils with a high DHA content for the enzymatic synthesis of CDC-rich structured triglycerides by acidolysis with caprylic acid (CA) catalyzed by 1,3-regiospecific lipases (6,7). Naturally, part of the triglycerides of these oils contain DHA at the second position and different acyl species at the primary one. The acyl residues of the outer positions of this type of triglyceride were exchanged for CA. When the DHA residues were situated at the primary positions, they were very resistant to lipase action, and remained in place. The final product was thus a mixture of triglycerides with CDC content.

A high-yield enzymatic method for CDC production has been established in the present article. The method is based on the characteristic of immobilized *Candida antarctica* lipase (Novozym 435), an enzyme with higher activity on DHA and other PUFA, to display 1,3-regiospecificity in the transesterification of triglycerides with ethanol (ethanolysis). Tridocosahexaenoylglycerol (DDD) was transformed into 2-monodocosahexaenoylglycerol (OHDOH) by ethanolysis with Novozym 435, and then the 1,3-positions were reesterified with ethyl caprylate (EtC) catalyzed by immobilized

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Rhizomucor miehei lipase (Lipozyme IM). The same method was used for CEC synthesis from trieicosapentaenoylglycerol (EEE).

EXPERIMENTAL PROCEDURES

Materials. Immobilized *C. antarctica* lipase (Novozym 435) and *R. miehei* lipase (Lipozyme IM) were generous gifts from Novo Nordisk Bioindustry (Chiba, Japan). EtC and ethanol (>99%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DDD and EEE (>99%) were products of Nippon Suisan Kaisha, Ltd. (Tokyo, Japan).

CDC and CEC syntheses. DDD or EEE (0.1 mmol), ethanol (its amount varied in each experiment), and Novozym 435 (10% of the total reaction mixture) were mixed in a flask under nitrogen atmosphere at 35°C and 300 rpm agitation speed. The final reaction mixture was filtered to remove the catalyst and then the excess ethanol was evaporated under reduced pressure at 35°C. EtC (2 mmol) and Lipozyme IM (10% of the total reaction mixture) were added and mixed with a magnetic stirrer at 300 rpm. The reesterification reaction was performed under 3–5 mm Hg vacuum at 35°C. When different reaction conditions were used, they are specified in the text.

Lipozyme IM (Novozym 435)-catalyzed interesterification of DDD with EtC. In certain experiments, a single enzyme (Lipozyme IM or Novozym 435) was tested in a one-step interesterification of DDD with EtC. DDD (0.051 g, 0.05 mmol), EtC (0.861 g, 5 mmol), and Lipozyme IM (or Novozym 435) (0.101 g, 10% of the total reaction mixture) were mixed in a flask under a nitrogen atmosphere at 40°C and 300 rpm agitation speed.

Analyses. The glyceride compositions of the reaction mixtures were analyzed by high-temperature gas–liquid chromatography. The glycerides contained in the reaction mixture were separated according to their molecular weight. All the positional isomers (if formed) of each glyceride species with a specific molecular weight were included under the same name.

A gas–liquid chromatograph (GC-14; Shimadzu Corporation, Kyoto, Japan) equipped with an on-column injector (OCI-14; Shimadzu Corporation) and an Ultra ALLOY-1 (HT) capillary column (10 m length, 0.5 mm internal diameter, and 0.1 μm film thickness; Frontier Laboratories Ltd., Koriyama, Japan) was used. The oven was heated from 40°C (held 1 min) at 10°C /min to 370°C and kept at this temperature for 6 min. The on-column injector was heated from 40 to 380°C at 10°C /min and held at this temperature for 6 min. The flame-ionization detector was kept at 395°C. Dicapryloylglycerol (CCOH), a by-product in the reesterification reaction mixtures of CEC syntheses, could not be separated under the applied analytical conditions.

The regioisomeric composition of the final products was analyzed by high-performance liquid chromatography (HPLC) with a ChromSpher 5 Lipids silver ion chromatography column (250 × 4.6 mm × 1/4″, from Chrompack, Middelburg, The Netherlands). A binary solvent gradient made of solvent A (acetone) and solvent B (acetone/acetonitrile, 3:1, vol/vol) was used. The column was eluted at a flow rate of 0.75 mL/min with a linear gradient of A to B over 60 min and then with B for 20 min. The lipid species were detected with an evaporative light-scattering detector (ELSD). The retention times of CDC and CCD were confirmed according to our method reported previously (9).

RESULTS AND DISCUSSION

A well-known method for the synthesis of symmetrically structured triacylglycerols is the transesterification of a homogeneous triglyceride with an acid or its ethyl ester catalyzed by a lipase with high specificity for the *sn*-1 and *sn*-3 positions of the glycerol backbone (10). Immobilized *R. miehei* lipase (Lipozyme IM) is often used, as it has strict 1,3-regiospecificity, a broad substrate specificity, and good activity at low water concentrations in the reaction media. It was used successfully for CEC synthesis (11) by interesterification of EEE with EtC (79% CEC yield was obtained in 24 h). CDC synthesis catalyzed by Lipozyme IM under the same reaction conditions gave a very low CDC yield (24.8% at 72 h). Other 1,3-regiospecific lipases of fungal origin also have very low activities toward DHA.

Immobilized *C. antarctica* lipase (Novozym 435) has a relatively high activity on DHA (12), and its positional specificity depends on the reactants. Novozym 435 was tested for the interesterification of DDD with EtC and expected to display 1,3-specificity in this reaction. The glyceride composition of the reaction mixture at 8 h, when the CDC yield was the highest, was: 27.6% CDC, 21.7% dicapryloylglycerol (CCOH), 22.9% tricapryloylglycerol (CCC), 9.1% capryloyldocosahexaenoylglycerol (CDOH), 0.5% didocosahexaenoylglycerol (DDOH), 13.6% capryloyldidocosahexaenoylglycerol (DDC), and 4.6% DDD. The high contents of CCC and CCOH indicate that Novozym 435 did not display positional specificity in this reaction.

Novozym 435 was used in some previous studies for the ethanolysis of fish oil and glycerides rich in DHA and eicosapentaenoic acid (EPA) (13,14). Complete (100%) conversion of glycerides to ethyl esters was obtained in 22 h at an approximately 1.7:1 molar ratio of ethanol/acyl equivalents at 20°C (13). A similar approach was used for DDD as a substrate at a higher ethanol/DHA equivalents molar ratio (22:1), which was expressed as ethanol/DDD weight ratio in this article (3:1 in this case). Surprisingly, a 93% yield of OHDOH was obtained in 4 h at 35°C (Table 1). We supposed that the monoglycerides were mainly 2-MG, and tried to reesterify them with EtC to form CDC. Novozym 435 could not be used to catalyze the reesterification as it displayed low positional specificity in this reaction (the results are discussed later). Lipozyme IM gave good results for the reesterification of partial glycerides in CEC synthesis (11), and, therefore, it was used to catalyze the reesterification of MG in this study also. Prior to the reesterification step, Novozym 435 was removed by filtration and the excess ethanol was evaporated under vac-

TABLE 1
Glyceride Composition (mol%) During CDC Synthesis

Glyceride species[a]	Ethanolysis step[b]				Reesterification step[c]		
	1 h	2 h	3 h	4 h	1.5 h	2.5 h	3.5 h
CCOH					9.2	8.1	6.6
OHDOH	14.7	51.7	81.6	92.7	0.0	0.0	0.0
CCC					1.5	3.0	4.3
CDOH					1.5	1.4	1.4
CDC					79.4	79.0	79.5
DDOH	13.7	10.9	7.5	5.3	0.0	0.0	0.0
DDC					8.4	8.5	8.2
DDD	71.6	37.4	10.9	2.0	0.0	0.0	0.0

[a]CCOH, dicapryloylglycerol; OHDOH, monodocosahexaenoylglycerol; CCC, tricapryloylglycerol; CDOH, capryloyldocosahexaenoylglycerol; CDC, dicapryloyldocosahexaenoylglycerol; DDOH, didocosahexaenoylglycerol; DDC, caprylоyldidocosahexaenoylglycerol; DDD, tridocosahexaenoylglycerol. All the positional isomers (if formed) were included under the same abbreviation.
[b]Ethanolysis step was performed at ethanol/DDD ratio = 3:1 (w/w) with Novozym 435 (Novo Nordisk Bioindustry, Chiba, Japan) as catalyst.
[c]Reesterification step was performed at ethylcaprylate (EtC)/initial DDD molar ratio = 20:1 with Lipozyme IM (Novo Nordisk Bioindustry) as catalyst.

uum. EtC was used as the acyl donor for reesterification rather than the free acid (CA) because the latter fosters acyl migration in partial glycerides (11). The ethanol resulting from the esterification reaction was evaporated under reduced pressure to push the reaction equilibrium toward the synthetic side. Although glycerol (if formed) could not be detected under the applied analytical conditions for ethanolysis, it would be esterified to 1,3-dicapryloylglycerol in the second step and quantified in the reesterification reaction mixtures.

The maximal yield of CDC was attained at 1.5 h (Table 1). OHDOH and DDOH disappeared completely by this time. The regioisomeric composition of the product (CDC + CCD) checked by silver-ion HPLC was 100% CDC. The amounts of CCOH (most probably the 1,3-isomer) and CCC formed in the second step might be the result of the acyl migration process combined with the action of the lipases (Novozym in the first step and Lipozyme in the second) on the *sn*-2 position of glycerol. The amount of CCOH and CCC combined (approximately 11%) remained unchanged from 1.5 h on, but the CCC/CCOH molar ratio increased. The small amount of CDOH (which remained in the reaction mixture at an almost unchanged value from 1.5 h) was probably the 1,3-isomer resulting from the esterification of the 1-MG of DHA formed in the first step with a CA residue.

The effects of the substrate ratio were studied briefly for both steps. The excess ethanol used in the ethanolysis step acted as a substrate and a solvent in this reaction. In the beginning, the reaction mixture was heterogeneous with two immiscible liquid phases (i.e., ethanol and DDD) in which the catalyst was suspended. As the reaction proceeded, the two liquid phases became completely miscible. This phenomenon was responsible for the reaction time-lag, which was more marked at low substrate ratio (Fig. 1). Higher ethanol/DDD ratios improved the final yield of MG. The reaction at an ethanol/DDD ratio of 3:1 (w/w) (67:1 in molar ratio) was the fastest, with an almost 93% yield obtained in 4 h. The MG yield increased

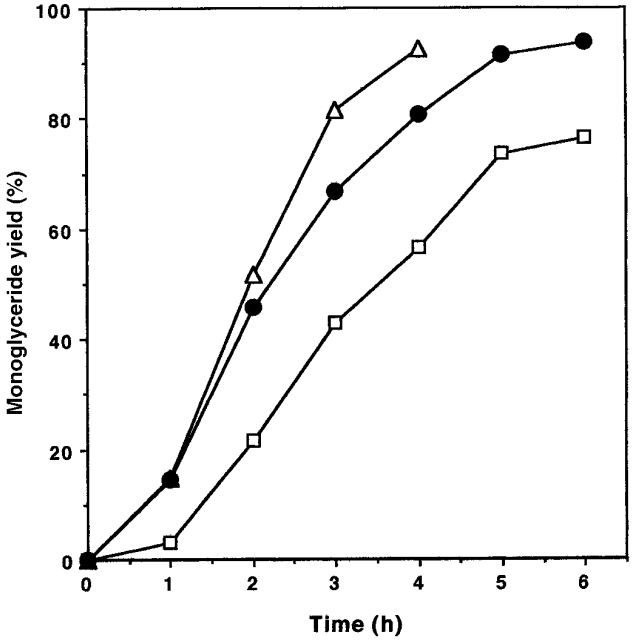

FIG. 1. Effect of the excess of ethanol on ethanolysis of tridocosahexaenoylglycerol (DDD) with Novozym 435 (Novo Nordisk Bioindustry, Chiba, Japan). Ethanol/DDD ratio (w/w) shown as 4:1 (●), 3:1 (△), and 2:1 (□).

slightly for the reaction at an ethanol/DDD ratio of 4:1 (w/w) (89:1 in molar ratio), but it required a longer time (6 h). Further increase of the reactant ratio resulted in longer reaction times with lower reaction yields affected by acyl migration. The reaction at a 2:1 ratio was slow and the MG yield was only 76% at 6 h. Reactant ratios close to the stoichiometric ratio (ethanol/acyl equivalents molar ratio of 1:1) influence not only the reaction rates, but most likely the positional specificity of the enzyme. This hypothesis is supported by the results of a work using Novozym as catalyst for the ethanolysis of fish oil glycerides at a 1.7:1 molar ratio of ethanol/acyl equivalents (13). That complete conversion to ethyl esters was obtained in relatively short reaction times (22 h) at low temperature

TABLE 2
Effect of CA-Et/DDD Molar Ratio on Lipozyme IM-Catalyzed Reesterification Step of CDC Synthesis

Glyceride species[a]	Glyceride composition (mol%)[b]	
	10:1 (molar ratio)	20:1 (molar ratio)
CCOH	17.4	11.8
OHDOH	0.0	0.0
CCC	3.8	0.0
CDOH	4.1	0.8
CDC	73.8	85.4
DDOH	0.0	0.0
DDC	0.9	2.0
DDD	0.0	0.0

[a]For abbreviations and manufacturer see Table 1.
[b]Glyceride composition of the reesterification reaction mixture at 1.5 h. The ethanolysis step was performed with Novozym 435 as catalyst for 6 h at EtOH/DDD = 4:1 (w/w) for both experiments.

(20°C), where spontaneous acyl migration is not significant, suggests that the lipase acted in a nonpositionally specific manner at these reaction conditions.

The effect of the molar ratio of EtC/DDD in the reesterification step of the stepwise synthesis of CDC catalyzed by Lipozyme IM was investigated at 10:1 and 20:1 ratios (Table 2). The ethanolysis step was performed with Novozym 435 at an ethanol/DDD molar ratio of 4:1 (the results shown in Fig. 1). The regioisomeric purity of product CDC was 100% for both experiments. The CDC yield was significantly lower for the reaction at a 10:1 molar ratio due to the higher amounts of CCC and CCOH formed. Actually, no CCC was detected at 1.5 h for the reaction at a 20:1 molar ratio of reactants. This difference might be the result of either different acyl migration conditions or modified positional specificity of Lipozyme. The short reaction times and the relatively low reaction temperature rule out acyl migration as the main cause for this difference. There is a high probability that the positional specificity of Lipozyme is also affected by the reaction conditions. Similar results obtained for CEC synthesis with the same method back up this hypothesis (the results are discussed later).

The positional specificity of Novozym for the reesterification step was investigated in an experiment at the optimal reaction conditions found previously (weight ratio of ethanol/DDD at 4:1 for ethanolysis and molar ratio of EtC/initial DDD at 20:1 for reesterification). The ethanolysis step was performed for 6 h (based on the results in Fig. 1), then the ethanol was completely evaporated at 35°C. EtC in excess was added and the vessel was connected to 3–5 mm Hg vacuum. The glyceride composition of the reaction mixture after 2 h of reesterification was: 21.6% CDC, 34.9% CCOH, 27.5% CCC, 7.8% CDOH, and 8.21% DDC. The DHA residue at the *sn*-2 position of glycerides was exchanged partially with CA, and therefore large amounts of CCC and CCOH were formed. The HPLC analysis of the CDC regioisomers showed that the formed product was 87.5% CDC and 12.5% CCD. These results indicate that Novozym displays very low 1,3-positional specificity in this reaction and justify the change of the catalyst for the reesterification step.

The same method of ethanolysis with Novozym 435 followed by reesterification with Lipozyme IM was applied to CEC synthesis. The ethanolysis step was faster and the MG yield higher for EEE than for DDD owing to the higher specificity of the enzyme for EPA than for DHA (Table 3 vs. Table 1). The molar ratio of EtC/initial EEE in the Lipozyme IM-catalyzed reesterification step of the CEC stepwise synthesis influenced not only the reaction yield but also the regioisomeric purity of the product (CEC + CCE). The product at a 10:1 molar ratio consisted of 97.3% CEC and 2.7% CCE while the one at a 20:1 ratio was made of 99.8% CEC and 0.2% CCE. Since the ethanolysis step was performed at exactly the same conditions, these differences are the results of the phenomena occurring in the reesterification step. The difference in the regioisomeric purity of the products obtained at different reactant molar ratios also strongly supports the hypothesis of variable positional specificity of Lipozyme. Lipozyme was used previously for the reesterification of partial glycerides resulting from the interesterification of EEE with EtC (15). At 1:10 and 1:20 EtC/initial EEE molar ratios, it produced CEC of 100% regioisomeric purity for both ratios although the reaction times were much longer and the reaction temperature higher (over 10 h at 40°C). It is thus inferred that Lipozyme also might change its positional specificity depending on the properties of the reaction media. In this case, it might have become partially able to work on the second position of glycerol. This effect is more evident for EPA since Lipozyme has higher specificity for it than for DHA. However, more studies are necessary to clearly elucidate these phenomena.

The ethyldocosahexaenoate or ethyleicosapentaenoate formed in the ethanolysis step can be separated after completion of the reesterification step by either molecular distillation or liquid chromatography and reused as a substrate for DDD and EEE preparation.

The present approach is a breakthrough for synthesis of pure structured glycerides of DHA as it has solved the problem of finding a lipase that displays 1,3-regiospecificity and also reasonable activity on DHA residues. The reaction times are much shorter than in preceding enzymatic methods, and simple intermediary operations are necessary. The 2-MG in the final ethanolysis reaction mixture need not be purified before their use in the reesterification step. Thereby, spontaneous acyl migration due to long handling time is minimized. The ethanolysis step itself can become a powerful method for 2-MG synthesis from various sources.

Thus, the optimum procedure for CDC and CEC production starting from DDD and EEE is the two-step synthesis, with 2-MG as intermediates, newly established in this

TABLE 3
Glyceride Composition (mol%) During CEC Synthesis

Glyceride species[a]	Ethanolysis step[b] 1 h	Ethanolysis step[b] 1.5 h	Reesterification step (1.5 h)[c] 10:1[d] (EtC/EEE molar ratio)	Reesterification step (1.5 h)[c] 20:1[d] (EtC/EEE molar ratio)
OHEOH	91.7	98.5	0.0	0.0
CCC			3.7	2.4
CEOH			13.7	7.8
CEC			74.7	84.2
EEOH	4.0	1.1	0.0	0.0
EEC			7.9	5.6
EEE	4.3	0.4	0.00	0.0

[a]OHEOH, monoeicosapentaenoylglycerol; CCC, tricapryloylglycerol; CEOH, capryloyleicosapentaenoylglycerol; CEC,dicapryloyleicosapentaenoylglycerol; EEOH, dieicosapentaenoylglycerol; EEC, capryloyldieicosapentaenoylglycerol; EEE, trieicosapentaenoylglycerol. All the positional isomers (if formed) were included under the same abbreviation.
[b]Ethanolysis was performed at EtOH/EEE = 4:1 (w/w) with Novozym 435 as catalyst.
[c]Reesterification of partial glycerides was performed with Lipozyme IM as catalyst.
[d]Glyceride composition of the reesterification reaction mixture at 1.5 h after 1.5 h ethanolysis.

paper.

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