Comparison of Acyl Donors for Lipase-Catalyzed Production of 1,3-Dicapryloyl-2-eicosapentaenoylglycerol

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ABSTRACT: Synthesis of 1,3-dicapryloyl-2-eicosapentaenoylglycerol (CEC) catalyzed by Lipozyme IM (immobilized *Rhizomucor miehei* lipase) was performed by interesterification of trieicosapentaenoylglycerol (EEE) with caprylic acid (CA) (acidolysis) and EEE with ethyl caprylate (EtC) (interesterification). Both methods involved two steps: (i) transesterification at an optimized water content and temperature for the high yield conversion of the substrate to CEC, 1-capryloyl-2-eicosapentaenoylglycerol (CEOH) and 2-eicosapentaenoylglycerol (OHEOH), and (ii) reesterification of CEOH and OHEOH to CEC by water removal under reduced pressure. Interesterification had clear advantages over acidolysis. The reaction rates for interesterification were higher and the reaction times shorter. The final yield of CEC by interesterification was higher, and the extent of acyl migration, indicated by the tricapryloylglycerol content, was lower. The disadvantage of the higher price of EtC used for interesterification (approximately 10 times higher than the price of CA) was overcome by synthesizing it directly in the same reaction vessel prior to the interesterification step. EtC was rapidly synthesized by esterification of CA with ethanol in high yield (92% obtained in 2.5 h). The amount of water added to the reaction mixture and the reaction temperature influenced the yields of CEC, CEOH, and OHEOH in the transesterification step for both interesterification and acidolysis methods. The regioisomeric purity of CEC was 100% for both methods at temperatures of 40°C or less. The highest yield of CEC (81%) was obtained for the interesterification of EEE with EtC, formed directly in the same reaction vessel, at a CA/EEE molar ratio of 20:1 and 30°C.

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Symmetrical structured triacylglycerols (SST) of polyunsaturated fatty acids (PUFA) with medium-chain fatty acids in *sn*-1 and *sn*-3 positions ensure an efficient enteral absorption of the nutritionally valuable PUFA from the *sn*-2 position and a rapid energy supply conferred by the acids from the primary positions. Medium-chain fatty acids are catabolized quickly and are not stored in the adipose tissues of the body (1,2). PUFA in the *sn*-2 position are presumably protected against oxidation by the two saturated residues at the primary positions (3). 1,3-Dicapryloyl-2-eicosapentaenoylglycerol (CEC) belongs to this class of compounds and has a wide range of potential applications in pharmaceutical, cosmetic, and nutraceutical formulations (4).

Interesterification catalyzed by a 1,3-specific lipase is a typical approach for the synthesis of SST (5–9). A certain amount of mono- and diacylglycerols is present in the reaction mixtures, as they are essential reaction intermediates. They are undesired by-products, and optimization of the reaction parameters was performed to lower their content (7–9). In contrast, di- and monoacylglycerol formation at an optimized water content of the reaction mixture was employed in this work to reduce the content of unreacted substrate (trieicosapentaenoylglycerol, EEE) and intermediates (1-capryloyl-2,3-dieicosapentaenoylglycerol, EEC; and 1,2-dieicosapentaenoylglycerol, EEOH) in the final product of interesterification. The di- and monoacylglycerols formed were reesterified regioselectively by removing the water under reduced pressure. The principles of this method for the enzymatic synthesis of CEC by interesterification of EEE with ethyl caprylate (EtC) were established in our previous work (10). A similar approach was used here for acidolysis of EEE with caprylic acid (CA). The aim of this work is to compare acidolysis and interesterification methods for CEC synthesis in view of prospective industrial-scale application. The economic efficiency of the interesterification method was improved by preparing EtC directly in the same reaction vessel as the subsequent steps.

EXPERIMENTAL PROCEDURES

Materials. Immobilized *Candida antarctica* lipase (Novozym 435) and *Rhizomucor miehei* lipase (Lipozyme IM) were generous gifts from Novo Nordisk Bioindustry (Chiba, Japan). CA, EtC, glycerol, and ethanol (min. 99%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). EEE (min. 99%) was obtained by esterification of glycerol with the stoichiometric amount of eicosapentaenoic acid (EPA) catalyzed by Novozym 435 at 60°C and 3 mm Hg, followed by purification on a silica-gel column. EPA (min. 99%) was a product of Nippon Suisan Kaisha, Ltd. (Tokyo, Japan).

Acidolysis. EEE (0.95 g, 1 mmol), CA (1.44 g, 10 mmol), Lipozyme IM (0.27 g, 10% of the total reaction mixture without water), and water (0.052 g, 2% of the total reaction mix-

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ture) were mixed in a flask under nitrogen atmosphere at 40°C and 300 rpm agitation speed, unless otherwise stated.

Transesterification. EEE (0.95 g, 1 mmol), EtC (1.72 g, 10 mmol), Lipozyme IM (0.30 g, 10% of the total reaction mixture without water), and water (0.061 g, 2% of the total reaction mixture) were mixed in a flask under nitrogen atmosphere at 40°C and 300 rpm agitation speed, unless otherwise stated.

Reesterification. Di- and monoacylglycerols generated during CEC synthesis by acidolysis or interesterification were reesterified by water removal under reduced pressure. At a predetermined time, the reaction vessel was connected through a liquid nitrogen trap to a vacuum pump at 3–5 mm Hg.

Synthesis of CEC with EtC obtained directly in the reaction vessel. CA (1.44 g, 10 mmol), ethanol (0.46 g, 10 mmol), and Lipozyme IM [0.27 g, 10% of the sum of the amounts of CA, EEE (which is to be added in the following step), and Lipozyme IM combined] were mixed at 300 rpm agitation speed and 40°C for 1 h. The resulting water was evaporated completely in 0.5 h under 3–5 mm Hg. Extra ethanol (0.15 g, 3.33 mmol) was added, and the second esterification step was performed for 1 h. EEE (0.95 g, 1 mmol) and water (0.027 g, 1% of the amount of CA, EEE, Lipozyme IM, and water combined) were added and mixed for 8 h under nitrogen atmosphere. The reaction vessel was then connected to a vacuum pump for the reesterification of the resulting di- and monoacylglycerols (see the section on reesterification, above). Different reaction conditions are specified in the text.

Analyses. The analyses of the reaction products formed by acidolysis, transesterification, and reesterification were performed by high-temperature gas chromatography (GC). The acylglycerol species contained in the reaction mixture were separated according to their molecular weight so that all of the positional isomers (if formed) of each species with a specific molecular weight were included under the same name. Dicapryloylglycerol could not be separated under the applied analytical conditions.

A 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 (15 m length, 0.5 mm internal diameter, 0.1 mm film thickness; Frontier Laboratories Ltd., Kohriyama, Japan) was used. The oven was heated at 10°C/min from 65 to 360°C which was then held for 4 min. The on-column injector was heated from 65 to 365°C at 20°C/min and held at this temperature for 18.5 min. The detector was kept at 395°C. A chromatogram of a sample from an acidolysis reaction mixture is presented in Figure 1A.

The analyses of the products of esterification of CA with ethanol were performed on a Hewlett-Packard 5890 gas chromatograph (Avondale, PA) equipped with a DB-Wax column (30 m length, 0.25 mm internal diameter, 0.25 μm film thickness; J&W Scientific, Folsom, CA). The oven temperature was held at 100°C for 2 min, raised at 10°C/min to 250°C, and held for 2 min. The injector and detector were kept at 250°C.

The regioisomeric composition of the transesterification products, CEC and 1,2(2,3)-dicapryloyl-3(1)-eicosapentaenoylglycerol (CCE), in the reaction mixtures 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 (Middleburg, 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 with a linear gradient of A to B over 60 min at a flow rate of 0.75 mL/min. The lipid species were detected with an evaporative light-scattering detector.

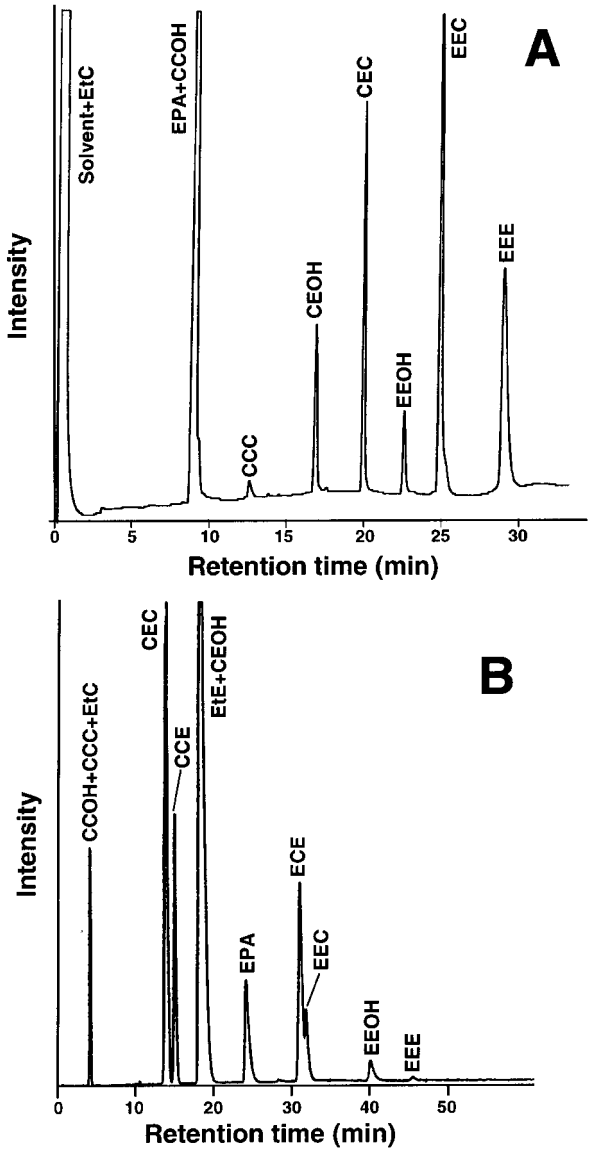

FIG. 1. Analyses of the reaction mixtures. (A) Gas–liquid chromatogram of an acidolysis reaction mixture. EtC, ethyl caprylate; EPA, eicosapentaenoic acid; CCOH, dicapryloylglycerol; OHEOH, monoeicosapentaenoylglycerol; CCC, tricapryloylglycerol; CEOH, capryloyleicosapentaenoylglycerol; CEC, dicapryloyleicosapentaenoylglycerol; EEOH, dieicosapentaenoylglycerol; EEC, capryloyldieicosapentaenoylglycerol; EEE, trieicosapentaenoylglycerol. All the positional isomers were included under the same abbreviation. (B) High-performance liquid chromatogram of a model mixture containing the regioisomeric pairs (CEC + CCE) and (ECE and EEC). EtE = ethyl eicosapentaenoate. For experimental details see the Experimental Procedures section.

Figure 1B is the chromatogram of a mixture used for setting up the conditions for HPLC analysis. The mixture was obtained by mixing the reaction mixtures of two enzymatic reactions by which CCE and 2-capryloyl-1,3-dieicosapentaenoylglycerol (ECE), and their corresponding regioisomers (CEC and EEC) were obtained selectively. One reaction mixture was the final product of the transesterification of EEE with EtC obtained directly in the reaction vessel at a CA/EEE molar ratio = 20:1 and 40°C (its acylglycerol composition is shown in the last column of Table 5). The other mixture was the reaction product of transesterification of tricapryloylglycerol (CCC) (0.47 g, 1 mmol) with ethyl eicosapentaenoate (EtE) (1.65 g, 5 mmol) catalyzed by Lipozyme IM (0.24 g, 10% of the reaction mixture) for 8 h. The composition of the latter reaction mixture determined by GC was: CCC 4.8%, 2-capryloyl-1-eicosapentaenoylglycerol 11.2%, CCE 31.1%, EEOH 4.6%, ECE 45.0%, and EEE 3.3%. The identification of the peaks in Figure 1B was performed by GC analysis. The eluate corresponding to each peak was collected and injected into a gas chromatograph programmed as described above.

The acidolysis products were analyzed by HPLC as described above after the excess of CA has been removed by passing the sample dissolved in hexane/diethylether (1:1, vol/vol) through a short column filled with silica gel NH (Chromatorex® chromatography silica gel NH, size = 100–200 mesh; Fuji Silysia Chemical Ltd., Kasugai, Aichi, Japan).

Water contents of substrates and Lipozyme IM were measured with a Karl Fischer moisture meter (MKS-1; Kyoto Electronics, Kyoto, Japan).

RESULTS

Enzymatic synthesis of SST by interesterification of a homogeneous triacylglycerol with a 1,3-regiospecific lipase can be performed either with a fatty acid (acidolysis) or its ethyl ester (transesterification) as acyl donors. The acid is usually preferred as it is much cheaper. In the case of CEC synthesis, CA is approximately 10 times cheaper than its ethyl ester. Although good results were obtained for the transesterification of EEE with EtC (10), acidolysis should not be ruled out, as it would be more advantageous economically. A comparison of the two methods would be useful for choosing the system for the industrial-scale production of CEC.

Acidolysis. The acidolysis reaction was performed in two steps: (i) acidolysis of EEE at an optimized water content for the high yield conversion of the substrate to CEC, 1-capryloyl-2-eicosapentaenoylglycerol (CEOH), and 2-eicosapentaenoylglycerol (OHEOH), and (ii) reesterification of CEOH and OHEOH to CEC by water removal under reduced pressure. Lipozyme IM (immobilized *R. miehei* lipase) was used as catalyst for both the steps.

The amount of water added to the reaction mixture influenced the acylglycerol composition of the final reaction product (Table 1). When no water was added to the reaction mixture, the combined content of the unreacted substrate (EEE) and intermediates EEC and EEOH was approximately 59% at 24 h. Water (2%) that was added to the reaction mixture raised the reaction rates, and the above content decreased to approximately 20%. Further addition of water did not bring any improvement of the final yield.

At reaction temperatures higher than 40°C, the final yield of the product and the desired intermediates (CEOH and OHEOH) decreased and the content of CCC increased (Table 2). Higher temperatures favored acyl migration which affected not only the final yield of the product (higher amounts of CCC formed) but also its regioisomeric purity. While no CCE isomer was detected in the reaction product at 40°C, the "CEC" product at 50°C was composed of 98.2% CEC and 1.8% CCE. The content of CCE rose to 6.1% at 60°C.

The time course of the reaction performed with a CA/EEE molar ratio of 10:1 and 2% added water at 40°C is presented in Figure 2. The content of CEC, CEOH, and OHEOH combined accounted for approximately 76% of the acylglycerols in the reaction product. Theoretically, if no acyl migration had occurred, all CEOH and OHEOH formed in the first step (22.8 and 2.2%, respectively) would have been reesterified to CEC in the second step. OHEOH was not detected after 6 h of reesterification while a part of the CEOH remained unesterified even after 19 h of reesterification. The content of CEC slightly increased from 64.3% at 1 h of reesterification. From these data, it can be inferred that CEOH formed in the first step was actually a mixture of 1,2- and 1,3-positional isomers. As Lipozyme IM is a 1,3-regiospecific lipase and cannot catalyze the esterification of the hydroxyl group in the *sn*-2 position of glycerol, the 1,2-diacylglycerol was probably esterified quickly to CEC in the second step, whereas the 1,3-diacylglycerol remained in the system. The content of CCC, which is a measure of acyl migration, increased continuously with the reaction time. CCC is formed due to acyl migration of the di- and monoacylglycerols formed in the reaction. Two

TABLE 1
Effect of Water on Acidolysis

Acylglycerol species[a]	Acylglycerol composition (mol%)[b]			
	0% H_2O[c]	2% H_2O[d]	3% H_2O[d]	10% H_2O[d]
OHEOH	0.0	2.2	2.9	6.8
CCC	4.3	3.8	2.1	1.2
CEOH	13.1	22.8	22.3	30.0
CEC	23.7	51.2	45.8	34.7
EEOH	2.0	2.5	2.0	3.1
EEC	38.7	14.9	21.9	20.4
EEE	18.2	2.6	3.0	3.8

[a]OHEOH, monoeicosapentaenoylglycerol; CCC, tricapryloylglycerol; CEOH, capryloyleicosapentaenoylglycerol; CEC, dicapryloyleicosapentaenoylglycerol; EEOH, dieicosapentaenoylglycerol; EEC, capryloyldieicosapentaenoylglycerol; EEE, trieicosapentaenoylglycerol. All the positional isomers were included under the same abbreviation. For example, COHE and OHCE were included in CEOH.

[b]Results of 24-h reaction. The reactions were performed at a caprylic acid (CA)/EEE molar ratio of 10:1 and at 40°C. The water contents of the starting materials were as follows: Lipozyme IM (Novo Nordisk Bioindustry, Chiba, Japan), 4.23%; CA, 0.03%; and EEE, 0.004%.

[c]No water was added to the reaction mixture.

[d]The amount of water added to the reaction mixture is expressed as a percentage of the total reaction mixture.

TABLE 2
Effect of Temperature on Acidolysis

Acylglycerol species	Acylglycerol composition (mol%)[a] 40°C	50°C	60°C
OHEOH	2.2	1.2	1.7
CCC	3.8	4.4	11.1
CEOH	22.8	18.9	11.7
CEC	51.2	51.9	49.9
EEOH	2.5	0	0.8
EEC	14.9	21.7	23.2
EEE	2.6	1.9	1.6

[a]Results of 24-h reaction. The experiments were performed at an ethyl caprylate (EtC)/EEE molar ratio of 10:1, with 2% water added to the reaction mixture. For other abbreviations see Table 1.

routes for CCC formation from CEOH can be imagined. One is the acyl migration of CEOH to COHE and then interesterification to COHC followed by migration to CCOH and finally reesterification to CCC. The other route is the acyl migration of CEOH to COHE followed by another migration step to OHCE and then esterification to CCE and interesterification to CCC. For both routes, the acyl migration steps are rate-determining. At 40°C, the rates of acyl migration are much slower than for interesterification so that COHE is most probably interesterified quickly to COHC (former route) before the migration to (OH)CE takes place (latter route). This hypothesis was supported by the HPLC analysis of the final product that showed no detectable amount of CCE isomer resulting from the process. An interesting study on acyl migration and trisaturated impurities formation during interesterification was performed by Bloomer *et al.* (9).

Transesterification. For the comparison of the two methods (i.e., acidolysis and transesterification), the transesterification of EEE with EtC was performed at the same molar ratio of the reactants and reaction conditions as the acidolysis (Fig. 3). The transesterification was much faster than the acidolysis. The yield of CEC, CEOH, and OHEOH combined reached a plateau after 8 h at 78% which is slightly higher than the corresponding yield for acidolysis after 24 h (approximately 76%, Fig. 2). The content of partial glycerides (OHEOH and CEOH) in the final reaction product was much higher for transesterification (49.4%) than for acidolysis (25%).

Transesterification of EEE with EtC obtained directly in the reaction vessel. As the interesterification is faster than acidolysis, it would be preferred for CEC synthesis if the price of EtC was not much higher. This drawback can be overcome if EtC is synthesized directly in the reaction vessel and used subsequently for transesterification and reesterification steps.

EtC was synthesized easily by Lipozyme IM (Table 3). The reaction was very fast with an equilbrium reached after 1 h at 83.5% EtC yield. The yield was increased by the removal of the water formed under vacuum, followed by addition of fresh ethanol in slight excess. The yield of esterification decreased a few percent after the water removal due to the simultaneous removal of the unreacted ethanol. Actually, the ethanol is removed faster than water as it has a lower boiling point and the reaction equilibrium was shifted toward the hydrolytic side. After the ad-

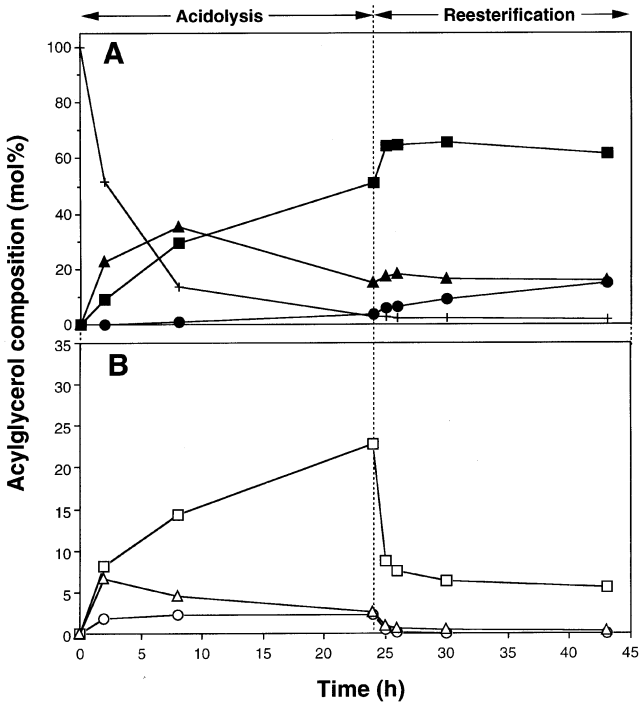

FIG. 2. Acylglycerol composition during CEC synthesis by acidolysis method (A) triacylglycerols: CCC (●), CEC (■), EEC (▲), EEE (+). (B) Mono- and diacylglycerols: OHEOH (○), CEOH (□), EEOH (△). All the positional isomers were included under the same name. For abbreviations see Figure 1.

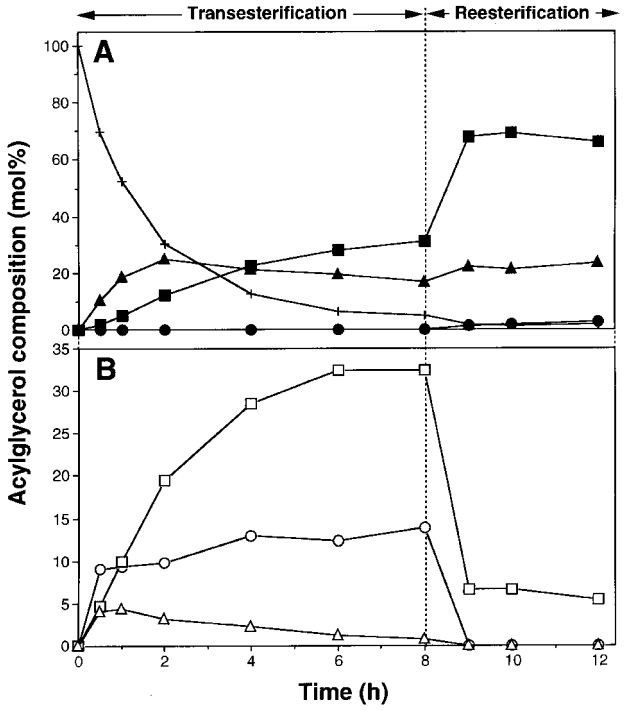

FIG. 3. Acylglycerol composition of reaction mixture during synthesis of CEC by transesterification with EtC obtained directly in the reaction vessel. Transesterification was performed following EtC formation in the reaction vessel (see Table 3). For symbols see Figure 2, and for abbreviations see Figure 1.

TABLE 3
EtC Yield During Esterification of CA with Ethanol

	1st esterification step		Water removal	2nd esterification step	
Time (h)	0.5	1	0.5[a]	0.5[b]	1[b]
EtC yield (mol%)	83.1	83.5	79.6	91.8	92.2

[a]Elapsed time from the start of water removal under vacuum.
[b]Elapsed time from the second addition of ethanol to the reaction mixture.
For abbreviations see Tables 1 and 2.

dition of extra ethanol, the reaction equilibrium was reestablished at 1 h at approximately 92% EtC yield. The interesterification reaction was started by the addition of EEE and extra water at this moment (Fig. 3). The yield of CEC, CEOH, and OHEOH combined (approximately 77%) after 8 h of transesterification was almost as high as for the reaction that was started directly with EtC (78%). The reesterification of di- and monoacylglycerols in the reaction mixture was very fast with the maximal yield obtained at 2 h (approximately 69%, Fig. 3). OHEOH disappeared after 1 h of reaction, but again, a part of the CEOH remained unesterified even after 4 h of water removal. The content of CCC in the final reaction product was much smaller (Fig. 3) than for the acidolysis method (Fig. 2). Longer reaction times necessary for acidolysis accompanied by the catalytic effect of the free acid in the acyl migration mechanism could account for this difference.

The effect of water amount (added to the reaction mixture after the EtC formation step) influenced the reaction yields of the transesterification step (Table 4). When no water was added to the reaction mixture, the extra ethanol added at the same time with EEE reacted with the remaining CA and the resulting water was used by the enzyme for the transesterification reaction. The ethanol that remained in the reaction mixture in small excess could also react as an acyl acceptor in the transesterification of the acylglycerols present and promote the formation of di- and monoacylglycerols (alcoholysis). The yield of the desired product and intermediates increased slightly when 1% water was added before the interesterification step. Larger contents of water reduced the final yields.

The effect of temperature was studied at a CA/EEE molar ratio of 20:1 (Table 5). The yield of the transesterification method increased more than 10% due to the combined effect of the higher molar ratio of the reactants and lower reaction temperature (30°C). The reaction at 30°C had the advantage of reduced acyl migration, which resulted in an increase in the final yield although the reaction times were longer for both transesterification and reesterification step.

The isomeric purity of CEC obtained in all the transesterification experiments was 100%.

TABLE 4
Effect of Water on Transesterification with EtC Formed Directly in the Reaction Vessel

Acylglycerol species	Acylglycerol composition (mol%)[a]			
	0% H_2O[b]	2% H_2O	3% H_2O	10% H_2O[c]
OHEOH	11.7	13.9	7.5	24.0
CCC	0	0	0	0
CEOH	34.0	32.5	34.8	12.2
CEC	27.8	31.1	22.6	2.9
EEOH	1.4	0.7	7.7	5.4
EEC	18.6	16.8	20.2	11.7
EEE	6.5	5.0	7.2	43.8

[a]Reaction mixture compositions at 8 h of transesterification. The reactions were performed at a CA/EEE molar ratio of 10:1 and 40°C. The water contents of the starting materials were as follows: Lipozyme IM, 4.23%; CA, 0.03%; EtOH, 0.23%; and EEE, 0.004%. For abbreviations and manufacturer see Tables 1 and 2.
[b]After 1 h of esterification (EtC formation), all the water in the reaction mixture was removed at low pressure and then extra ethanol and EEE were added.
[c]Water was not removed after 1 h of EtC formation. EEE and the extra ethanol were added to the esterification mixture at 1 h. No extra water was added. The water percentage was calculated for 90% yield of EtC formation.

DISCUSSION

Often, di- and monoacylglycerols formed during the transesterification process are regarded as undesired by-products (7–9), but they were key intermediates for the achievement of high final yields of the product in this work. They could be easily reesterified after the transesterification step by the removal of water from the reaction mixture. The excess of acyl donor (CA or EtC), the lower activity of Lipozyme IM on

TABLE 5
Effect of Temperature on Transesterification With EtC Formed Directly in the Reaction Mixture

	Acylglycerol composition (mol%)[a]							
	30°C[b]				40°C			
	Transesterification		Reesterification		Transesterification		Reesterification	
Acylglycerol species	20 h	22 h	2 h	3 h	14 h	16 h	1 h	2 h
OHEOH	14.3	12.6	0	0	8.7	5.4	0	0
CCC	0	0	0.9	1.7	0	1.2	2.3	3.7
CEOH	47.1	47.9	5.8	3.2	44.8	47.8	9.0	7.4
CEC	23.1	24.8	79.1	81.1	32.2	32.8	75.5	77.0
EEOH	3.5	3.4	0	0	2.6	2.1	0	0
EEC	9.4	9.2	13.8	14.0	10.0	9.5	13.2	11.9
EEE	2.6	2.1	0.4	0	1.7	1.4	0	0

[a]Reactions performed at CA/EEE molar ratio of 20:1. For abbreviations see Tables 1 and 2.
[b]The EtC synthesis step was performed at 40°C (Table 3); then the temperature was lowered to 30°C for the transesterification and reesterification steps.

EPA, and its strict 1,3-positional specificity ensured that only the 1,2-diacylglycerols and 2-monoacylglycerols are reesterified with caprylic acid, maintaining the high isomeric purity of the product.

An evident difference between transesterification and acidolysis is the amount of di- and monoacylglycerols formed during the transesterification step. Approximately 50% of the acylglycerols in the reaction product at 8 h were di- and monoacylglycerols for interesterification, whereas their content was only 27% for acidolysis at 24 h (Fig. 2). In the chemical equilibrium of the ester hydrolysis reactions, the excess of free acid inhibits the hydrolytic reactions by pushing the equilibrium toward the synthetic side. This leads to higher yield of the product (CEC) by acidolysis (51% at 24 h) than by transesterification (28% at 8 h) at reaction times close to equilibrium. The excess of ethyl ester acts in an opposite way by promoting the hydrolytic side reactions. The rates of hydrolytic reactions are also raised, and equilibrium is reached faster. This effect makes the transesterification method faster than acidolysis. Almost the same conversion of the substrate to CEC, CEOH, and OHEOH was obtained three times faster for transesterification than for acidolysis.

Shorter reaction times and higher reaction yields make transesterification a better synthetic approach than acidolysis. Also, EtC can be removed easier than CA from the final reaction mixture by distillation and recycled. The disadvantage of the higher cost of EtC was solved by synthesizing it in high yield directly in the same reaction vessel prior to the transesterification step. This additional step is very fast and does not require any downstream separation or purification as it uses the same catalyst as the following transesterification and reesterification steps.

CEC obtained by this method is to be used in drug formulations. Therefore, its purity after the downstream purification should be close to 100%. The downstream purification of the product was taken into consideration when the optimal reaction conditions were determined. EtC, CCC, and EtE can be removed by fractional distillation. EtC and EtE can then be reused for CEC and EEE synthesis, respectively. In particular, the excess of EtC can be evaporated easily from the final reaction mixture at 88–90°C and 5 mm Hg. The remaining di- and monoacylglycerols can be removed by adsorption on a silica-gel column. The rest of the impurities (EEC and EEE) are difficult to remove by molecular distillation and must be separated by reversed-phase column chromatography. Lower contents of EEC and EEE in the reaction mixture would improve the effectiveness of the last purification step and the yield of the whole process. This can be achieved either by using high excess of acyl donor (as in this study) or by repeating the interesterification step after separating the acylglycerols from the reaction mixture (11). The last method has the advantage of higher reaction rates if a lower excess of EtC is used, but it is laborious and the losses occurring during the separation steps are higher. Shimada *et al.* (11) acidolyzed tuna oil with a 12-fold molar excess of CA in three cycles of 2-d reaction catalyzed by *Rhizopus delemar* lipase (11). After each cycle, the acylglycerols were recovered by extraction with hexane after the neutralization of the excess of fatty acid with a solution of alkali and then reacted with fresh CA.

The interesterification at higher excess of EtC is slower, but the gains in the reaction yield are substantial. The effect of EtC/EEE molar ratio on transesterification of EEE with EtC was studied in our previous work (10). At a 10:1 EtC/EEE molar ratio, the initial rate of transesterification was 1:1.06 of the rate at 5/1 molar ratio, but the final yields of CEC were 64.8 and 48.7%, respectively. All these considerations justify the use of higher molar rates of reactants in the production of CEC.

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