Lipase-Catalyzed Incorporation of Conjugated Linoleic Acid into Tricaprylin

In-Hwan Kim[a,*], Chil-Surk Yoon[b], Soo-Hee Cho[a], Kwang-Won Lee[c], Soo-Hyun Chung[a], and Beom-Seok Tae[d]

[a]Department of Food and Nutrition, College of Health Sciences, Korea University, Seoul, Korea, [b]Korea Food Research Institute, Sungnam, Korea, [c]Catholic Institute of Medical Science, Catholic University, Seoul, Korea, and [d]Department of Chemical Engineering, Hankyong National University, Ansung, Korea

ABSTRACT: Three commercially available immobilized lipases, Novozym 435 from *Candida antarctica,* Lipozyme IM from *Rhizomucor miehei,* and Lipase PS-C from *Pseudomonas cepacia,* were used as biocatalysts for the interesterification of conjugated linoleic acid (CLA) ethyl ester and tricaprylin. The reactions were carried out in hexane, and the products were analyzed by gas–liquid chromatography. The effects of molar ratio, enzyme load, incubation time, and temperature on CLA incorporation were investigated. Novozym 435, as compared to Lipozyme IM and Lipase PC-C, showed the highest degree of CLA incorporation into tricaprylin. By hydrolysis with pancreatic lipase, it was found that Lipozyme IM and Lipase PS-C exhibited high selectivity for the *sn*-1,3 position of the triacylglycerol early in the interesterification, with small extents of incorporation of CLA into the *sn*-2 position, probably due to acyl migration, at later reaction times. A small extent of *sn*-1,3 selectivity during interesterification by Novozym 435 was observed.

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KEY WORDS: Acyl migration, conjugated linoleic acid, immobilized lipase, interesterification, tricaprylin.

Conjugated linoleic acid (CLA) is a mixture of geometric and positional isomers of linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid) characterized by *cis* and *trans* double bonds at positions 9 and 11, 10 and 12, or 11 and 13 resulting in complex positional isomer mixtures. CLA has been identified in cooked meats such as fried ground beef and pork as well as heat-processed dairy products such as cheeses and pasteurized milk (1–4). It is also found in human milk (5). Contents of CLA in animal products are much higher than in plant oils. Among animal products, CLA contents are generally higher in ruminant tissues than in nonruminant tissues, and dairy products are recognized as major dietary sources of CLA (6).

CLA was recently reported to have potent fat-to-lean repartitioning (7) and improved feed efficiency effects in rats (8). In addition, CLA has been shown to stimulate the immune system (9) and to protect against arteriosclerosis (10) and chemically induced cancer (11). It has also been shown that CLA may have positive effects on cardiovascular risk factors in animal models (12).

*To whom correspondence should be addressed at Department of Food and Nutrition, College of Health Sciences, Korea University, Chungneung-Dong, Sungbuk-Gu, 136-703, Seoul, Korea. E-mail: K610in@chollian.net

Medium-chain triacylglycerols (MCT), another beneficial class of lipids, are defined as triacylglycerols (TAG) containing fatty acids of 6–12 carbon chain-lengths. These relatively short acyl lengths confer low viscosity and melting points compared to long-chain TAG. In addition, MCT are stable to oxidation because their acyl residues are composed of saturated fatty acids (13). Because MCT are mainly transported to the liver *via* the portal vein where they are metabolized, providing rapid energy, they may be beneficial to hospital patients, infants, or individuals with special dietary requirements (14–16). However, naturally occurring MCT are not a source of essential fatty acids for humans. Further modification of MCT is needed to add essential fatty acids such as eicosapentaenoic acid, docosahexaenoic acid, and γ-linolenic acid.

Lipases are enzymes that preferentially catalyze the hydrolysis and synthesis of TAG, depending on reaction conditions (e.g., presence of essential water). Thus, lipases can be used for modification of fats and oils, providing improvement in their properties through incorporation of desirable fatty acids into TAG molecules (17,18). This method seems more attractive than the chemical process because of the milder reaction conditions and the reduction in the number of side products (19).

The objective of this study was to investigate the possibility of synthesizing TAG containing CLA and caprylic acid by the interesterification of CLA ethyl ester and tricaprylin using lipases. The effects of molar ratio, enzyme load, incubation time, and temperature on the reaction were studied. In addition, fatty acid profiles at the *sn*-2 position of modified TAG were also investigated.

MATERIALS AND METHODS

Materials. Lipozyme IM (*Rhizomucor miehei* lipase immobilized on an ion exchange resin) and Novozym 435 (*Candida antarctica* lipase immobilized on a macroporous acrylic resin) were kindly provided by Novo Nordisk Bioindustry Ltd. (Seoul, Korea). Lipase PS-C (*Pseudomonas cepacia* lipase immobilized on chemically modified ceramic particles) was the kind gift of Amano Pharmaceutical Co., Ltd. (Nagoya, Japan). Tricaprylin (99%), pancreatic lipase, and a molecular sieve (4 Å) were purchased from Sigma Chemical Company (St. Louis, MO). CLA ethyl ester (99%) was pur-

chased from Nu-Chek-Prep, Inc. (Elysian, MN). Hexane was high-performance liquid chromatography grade.

Interesterification. Enzymatic interesterification was performed in screw-capped test tubes in an orbital shaking water bath at 200 rpm at 55°C. A typical reaction contained 33.7 mg tricaprylin, 66.3 mg CLA ethyl ester, and 10.0 mg lipase in 3 mL hexane. Hexane was previously dried over a molecular sieve of 4 Å. All reactions were duplicated.

Analysis of products. The reaction mixture was filtered through an anhydrous sodium sulfate column to remove enzymes and water. The TAG were isolated by preparative thin-layer chromatography (TLC) with petroleum ether/ethyl ether/acetic acid (90:10:1, vol/vol/vol) as developing solvent and detected with 0.2% 2,7-dichlorofluorescein in ethanol. The bands corresponding to TAG were scraped from the TLC plate and ethylated with 10 mL of 2% H_2SO_4 in anhydrous ethanol at 80°C for 1 h. The fatty acid ethyl esters were extracted with 3 mL hexane, dried over sodium sulfate, and concentrated under nitrogen. A gas chromatograph (Varian 3800; Varian Inc., Walnut Creek, CA) equipped with a Supelcowax 10 fused-silica capillary column (60 m × 0.32 mm i.d.; Supelco, Bellefonte, PA) and flame-ionization detector was used. The column was held at 190°C for 1 min and programmed to rise to 240°C at the rate of 1.5°C/min and held for 10 min. The carrier gas was helium, and the total gas flow rate was 24 mL/min. The injector and detector temperatures were 240 and 280°C, respectively. The fatty acid ethyl esters were identified by comparing retention times with standards.

Hydrolysis by pancreatic lipase. Determination of the *sn*-2 positional distribution of fatty acids in TAG species obtained after TLC was conducted by the method of Luddy *et al.* (20). One milligram of TAG was mixed with 1 mL of 1 M Tris-HCl buffer (pH 7.6), 0.25 mL of 0.05% bile salts, 0.1 mL of 2.2% $CaCl_2$, and 1 mg of pancreatic lipase. The mixture was incubated in a water bath at 37°C for 3 min, vortexed vigorously, extracted with 5 mL diethyl ether, and dried by anhydrous sodium sulfate. TLC analysis was on silica gel G (Merck Co., Darmstadt, Germany), and the developing solvent system was hexane/diethyl ether/acetic acid (50:50:1, vol/vol/vol). The band corresponding to 2-monoglycerides was scraped and extracted with diethyl ether, ethylated, and analyzed by gas chromatography.

RESULTS AND DISCUSSION

Molar ratio. The CLA ethyl ester isomers mixture employed here was more than 99% pure. The composition of isomers in the mixture was 41% of 9,11-*cis/trans* or *trans/cis*-CLA, 44% of 10,12-*trans/cis* or *cis/trans*-CLA, 10% of 10,12-*cis/cis*-CLA, and 5% of other CLA composed of 9,11-*trans/trans*, 10,12-*trans/trans,* and 9,11-*cis/cis*-CLA. However, because the three lipases in this study did not show any specificity toward the isomers of CLA, results were not speciated with respect to CLA isomers.

The molar ratio of tricaprylin to CLA ethyl ester varied from 1:1 to 1:5 (Fig. 1). The enzyme amount was kept constant at

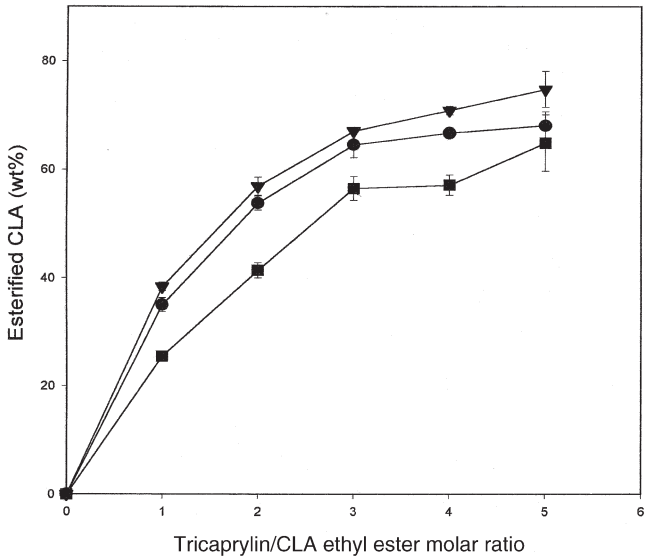

FIG. 1. Effect of molar ratio of substrates on the incorporation of conjugated linoleic acid (CLA) into tricaprylin. Molar ratios of tricaprylin to CLA ethyl ester were varied from 1:1 to 1:5, with the total amount of substrate being 100 mg. The reaction mixture was incubated in 3 mL hexane at 55°C for 24 h in an orbital shaking water bath at 200 rpm. Enzyme amount was 10% by weight of reactants. Novozym 435 (*Candida antarctica:* ▼), Lipozyme IM (*Rhizomucor miehei:* ●) were supplied by Novo Nordisk Bioindustry Ltd. (Seoul, Korea). Lipase PS-C (*Pseudomonas cepacia:* ■) was supplied by Amano Pharmaceutical Co. Ltd. (Nagoya, Japan). Vertical bars represent standard deviations.

10% by weight of total reactants. Incubation was carried out for 24 h. With Novozym 435 and Lipozyme IM, CLA incorporation into tricaprylin increased rapidly with increasing molar ratio up to 1:3. Over a 1:3 ratio, a significant increase in CLA incorporation for both enzymes (Lipozyme IM and Novozym 435) was not observed. Lipozyme IM is considered an *sn*-1,3 selective lipase. The maximal incorporation of CLA into tricaprylin was approximately 68.1%. We conclude that an equilibrium state of reaction was reached in the reaction system when a 1:3 (tricaprylin/CLA) molar ratio was used for 24 h with Lipozyme IM. Incorporation greater than 66.7% (at the 1:5 substrate molar ratio) was probably the result of acyl migration during reaction. Novozym 435 (which is considered a nonselective lipase), however, showed 74.8% incorporation at 1:5. Among the lipases used in this study, Lipase PS-C had the lowest activity in incorporating CLA into tricaprylin. The degrees of incorporation of CLA into tricaprylin were 67.1% with Novozym 435, 64.6% with Lipozyme IM, and 56.5% with Lipase PS-C at a molar ratio of 1:3.

Enzyme load. The effects of enzyme load on the incorporation of CLA into tricaprylin are shown in Figure 2. With Lipozyme IM and Novozym 435, the extents of CLA incorporation were increased by increasing the amount of enzymes in the reaction mixtures, but a significant increase was not observed when both immobilized enzymes (Lipozyme IM and Novozym 435) were present at greater than 5 mg (5%). Overall, Novozym 435 showed higher CLA incorporation than Lipozyme IM did. CLA incorporation with Lipase PS-C, on

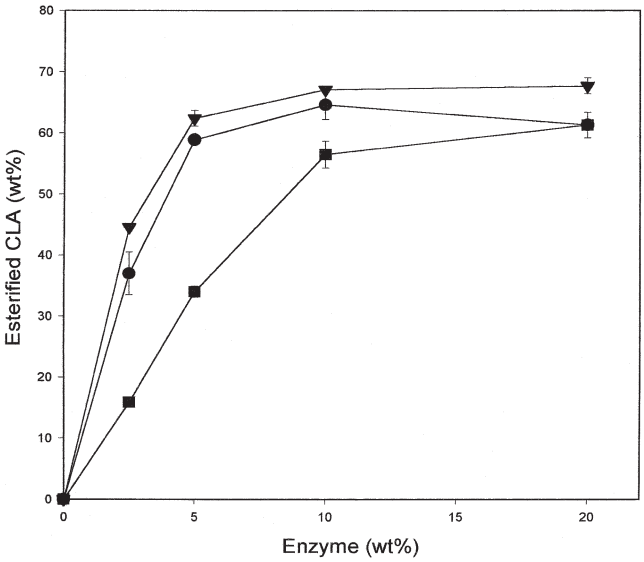

FIG. 2. Effect of enzyme load on the incorporation of CLA into tricaprylin. Molar ratio of tricaprylin to CLA ethyl ester was 1:3 (33.7 mg tricaprylin, 66.3 mg CLA ester). Amount of enzyme was based on weight percentage of reactants. The reaction conditions, symbols, and abbreviation are the same as in Figure 1.

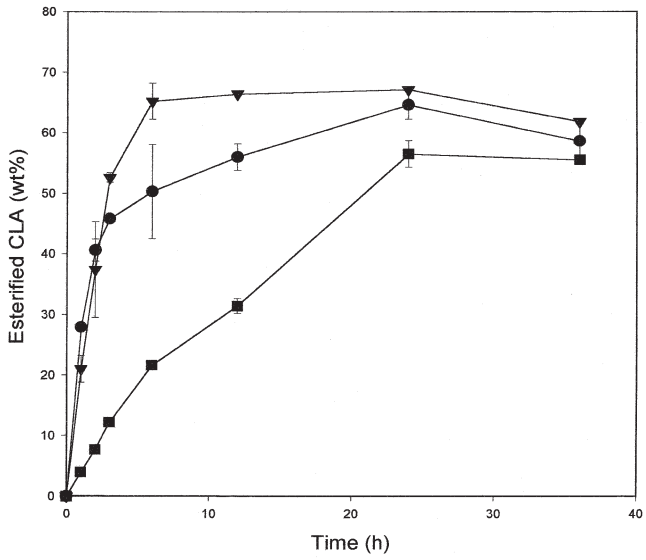

FIG. 3. Time course of incorporation of CLA into tricaprylin by three immobilized lipases. Molar ratio of tricaprylin to CLA ethyl ester was 1:3. Amounts of substrate were as indicated in Figure 1. Samples were analyzed at 1, 2, 3, 6, 12, 24, and 36 h in duplicate. See Figure 1 for reaction conditions, symbols, and abbreviation.

the other hand, increased as the amount of the enzyme was increased to 10%. When the amount of the enzyme was increased further, the degree of CLA incorporation remained constant. Therefore, optimal enzyme loads were 5% for Novozym 435 and Lipozyme IM and 10% for Lipase PS-C.

Time course. Time course is important in monitoring enzymatic reactions in order to determine the optimal rate necessary to obtain good yields and to minimize the overall production cost for the process. To determine the time to reach equilibrium, the reaction products were analyzed at 0, 1, 2, 3, 6, 12, 24, and 36 h. Figure 3 shows that the incorporation of CLA was very fast in the first 6 h for Novozym 435. However, no significant increase in incorporating CLA into tricaprylin was observed from 6 to 24 h with this enzyme. The degree of CLA incorporation by Lipozyme IM increased to 45.8% at 3 h. After 24 h of incubation, only a moderate further increase (18.8%) of the CLA incorporation was observed. Novozym 435 gave higher CLA incorporation into tricaprylin compared to Lipozyme IM and Lipase PS-C. After 24 h, the degree of incorporation of CLA was 67.1% with Novozym 435 and 64.6% with Lipozyme IM, respectively. Maximal incorporation of CLA was obtained within 10 h with Novozym 435 and 24 h with Lipozyme IM and Lipase PS-C under the study conditions employed. Lipase PS-C showed a low initial reaction rate, which increased up to 24 h. This may indicate that the protein content of prepared immobilized Lipase PS-C is lower than that of both Lipozyme IM and Novozym 435, or the esterification activity of *Pseudomonas cepacia* lipase may be lower than that of Lipozyme IM or Novozym 435.

Temperature. Reaction temperature can affect parameters such as enzyme stability, affinity of enzyme for substrate, and preponderance of competing reactions (21). Thermostability of enzymes is a major factor considered in their industrial use, mostly because of the potential for minimizing thermal degradation. The effect of temperature on the interesterification of tricaprylin with CLA ethyl ester as acyl donor was studied. Molar ratio of substrates, amount of enzyme, and reaction time were kept constant at 1:3, 10% of the substrate, and 24 h, respectively. The temperature range tested was between 35 and 75°C (Fig. 4). The CLA incorporation by Novozym 435 increased slightly from 59.4 to 67.1% when temperature increased from 35 to 55°C. A similar trend was observed for Lipozyme IM, although the extent of CLA incorporation was

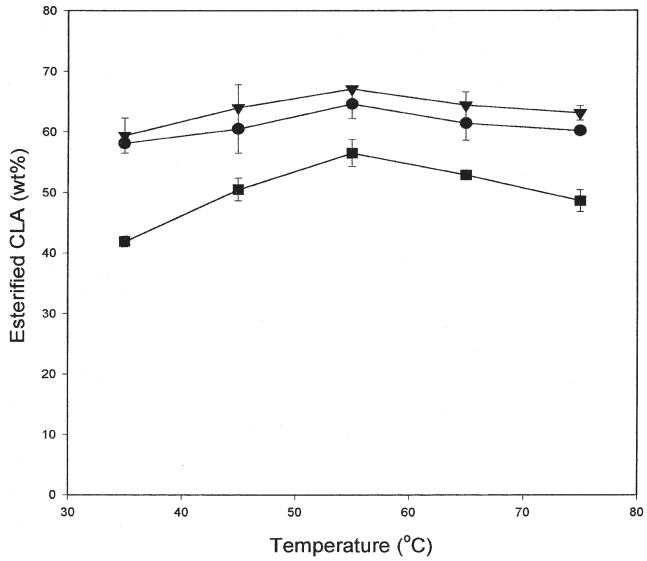

FIG. 4. Effect of temperature on the incorporation of CLA into tricaprylin. Molar ratio of tricaprylin to CLA ethyl ester was 1:3. Amounts of substrate were as indicated in Figure 2. Temperatures ranged from 35–75°C. See Figure 1 for reaction conditions, symbols, and abbreviation.

TABLE 1
Analysis of Fatty Acids at the *sn*-2 Position of Triacylglycerols (TAG) Synthesized for 24 h by Interesterification of Tricaprylin and Conjugated Linoleic Acid (CLA) Ethyl Ester Catalyzed by Three Lipases

	Fatty acid at the *sn*-2 position (wt%)	
Enzyme[a]	Caprylic acid	CLA
Novozym 435	38.5 ± 1.7	61.5 ± 2.8
Lipozyme IM	91.5 ± 5.8	8.5 ± 2.1
Lipase PS-C	95.7 ± 7.9	4.3 ± 0.8

[a]Novozym 435 *(Candida antarctica)* and Lipozyme IM *(Rhizomucor miehei)* from Novo Nordisk Bioindustry Ltd. (Seoul, Korea); Lipase PS-C *(Pseudomonas cepacia)* from Amano Pharmaceutical Co. Ltd. (Nagoya, Japan).

lower than that for Novozym 435. On the other hand, a slight decrease of CLA incorporation was observed in both enzymes above 55°C. With Lipase PS-C, substantial increase in CLA incorporation was observed between 35 and 55°C, whereas a significant decrease was observed above 55°C. Thus, Lipase PS-C was more temperature sensitive in CLA incorporation than Novozym 435 and Lipozyme IM.

Determination of the degree of interesterification of the sn*-2 position of TAG.* Pancreatic lipase digestion was performed to determine the fatty acid profile at the *sn*-2 position of TAG. Jandacek *et al*. (22) reported that the fatty acid at the *sn*-2 position of TAG improved absorption. Thus, incorporation of a desirable fatty acid, possessing nutritional or pharmaceutical properties, at the *sn*-2 position of modified TAG would seem to be desirable. The degree of CLA incorporation at the *sn*-2 position of the TAG obtained by interesterification for 24 h is given in Table 1. Substantially more of the total CLA (61.5%) was located at the *sn*-2 position of TAG synthesized by Novozym 435 than ones synthesized by Lipozyme IM (8.5%) or Lipase PS-C (4.3%). The incorporation of lauric acid into triolein has been reported by Miura *et al*. (23), who showed that the use of Lipozyme IM resulted in a 3.8% incorporation at the *sn*-2 position of the TAG. Despite the specificity of a 1,3-specific lipase, incorporation of fatty acids into acylglycerols at the *sn*-2 position can occur, due to acyl migration during interesterification or purification (24,25). Our data using Lipozyme IM and Lipase PS-C confirmed these results. Therefore, we investigated the effect of reaction time with the above three enzymes on acyl migration (Table 2). Incorporation of CLA at the *sn*-2 position was not observed with Lipozyme IM prior to 3 h of incubation, and with Lipase PS-C prior to 6 h. These results suggest that Lipozyme IM and Lipase PS-C have high selectivity for the *sn*-1,3 position of the TAG, and that small extents of incorporation of CLA into *sn*-2 position were due to acyl migration at later incubation time. In addition, a weak selectivity for the *sn*-1,3 position of TAG by Novozym 435 was observed early in the reaction.

TABLE 2
Analysis of Fatty Acids at the *sn*-2 Position of TAG Synthesized with Different Times by Interesterification of Tricaprylin and CLA Ethyl Ester Catalyzed by Three Lipases

		Fatty acid at the *sn*-2 position (wt%)	
Enzyme[a]	Time (h)	Caprylic acid	CLA
Novozym 435	1	85.3 ± 2.3	10.7 ± 1.4
	2	82.0 ± 4.6	18.0 ± 0.8
	3	65.7 ± 3.1	30.3 ± 2.4
	6	40.8 ± 1.8	59.2 ± 3.7
	9	39.0 ± 1.6	61.0 ± 2.5
	12	39.4 ± 4.6	60.6 ± 3.3
Lipozyme IM	1	100.0 ± 0.0	—
	2	100.0 ± 0.0	—
	3	100.0 ± 0.0	—
	6	99.2 ± 2.2	0.8 ± 0.3
	9	98.4 ± 3.5	1.6 ± 0.1
	12	93.6 ± 1.8	6.4 ± 0.5
Lipase PS-C	1	100.0 ± 0.0	—
	2	100.0 ± 0.0	—
	3	100.0 ± 0.0	—
	6	100.0 ± 0.0	—
	9	99.6 ± 0.2	0.4 ± 0.2
	12	98.4 ± 0.5	1.6 ± 0.1

[a]For sources and abbreviations see Table 1.

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