Lipase-Catalyzed Acidolysis of Perilla Oil with Caprylic Acid to Produce Structured Lipids

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ABSTRACT: Structured lipids were synthesized by acidolysis of perilla oil and caprylic acid using two lipases, Lipozyme RM IM from *Rhizomucor miehei* and Lipozyme TL IM from *Thermomyces lanuginosa*. Effects of molar ratio, reaction time, reaction temperature, enzyme load, and solvent content on acidolysis reactions were studied. The solvent content ranged from 0.0 (solvent-free) to 85.3%. The results showed that the incorporation increased in parallel with solvent content to 49.0% with Lipozyme RM IM and to 63.8% with Lipozyme TL IM. After 24 h incubation in *n*-hexane, caprylic acids were incorporated to 48.5 mol% with Lipozyme RM IM and to 51.4 mol% with Lipozyme TL IM, respectively, whereas linolenic acid content was reduced from 61.4 to 31.5 mol% with Lipozyme RM IM and to 28.4 mol% with Lipozyme TL IM, respectively. Lipozyme TL IM showed a higher acyl migration rate than Lipozyme RM IM when acidolysis was performed in the reaction system containing *n*-hexane as a solvent, whereas the difference in acyl migration between the two lipases in the solvent-free system was negligible.

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KEY WORDS: Acidolysis, acyl migration, caprylic acid, lipase, perilla oil, structured lipids.

Perilla oil has traditionally been consumed in oriental countries such as Korea, Japan, and China. In particular, perilla oil contains a high level of α-linolenic acid, representing up to 60% of its total FA content (1,2). α-Linolenic acid, as an n-3 FA, has been known as a metabolic precursor of EPA and DHA. In animal models, α-linolenic acid has shown several beneficial effects including suppression of platelet aggregation (3–5), allergic responses (6), tumor metastasis (7), tumor genesis (8), and improved learning ability (9). Jeffery *et al*. (10) reported that α-linolenic acid from plant sources was just as potent a modulator of blood lipid levels and immune functions as n-3 FA from fish oils.

Lipase-catalyzed modification of TAG can be performed with several advantages over chemical modification to produce structured lipids (SL). Through enzymatic acidolysis, it is possible to incorporate a desired acyl group onto a specific position of the TAG, whereas chemical catalysis does not possess this regiospecificity due to the random nature of the reaction (11). As an acyl donor for SL production, medium-chain FA (MCFA) can impart desirable benefits. For example, MCFA are metabolized mainly *via* the portal vein and provide quick energy (12). Also, MCFA have several distinctive characteristics such as high oxidative stability, low viscosity and melting points, and high solubility in water. From a human nutritional point of view, SL possessing n-3 PUFA located at the *sn*-2 and MCFA at the *sn*-1 and *sn*-3 positions of TAG may be desirable (13,14). The reason is that MCFA located at the end positions are rapidly hydrolyzed by pancreatic lipase, absorbed into the intestines, and rapidly carried into the liver where they are metabolized as a quick energy source. On the other hand, the remaining 2-MAG become a source of EFA after being absorbed through the intestinal wall (15). Thus, structural and compositional modification of perilla oil TAG to contain MCFA may improve their physiological properties and oxidative stability.

In the present study, perilla oil was modified with caprylic acid (C8:0), an MCFA, using two lipases, Lipozyme RM IM (immobilized lipase from *Rhizomucor miehei*) and Lipozyme TL IM (immobilized lipase from *Thermomyces lanuginosa*). *sn*-2 Positional analysis by pancreatic lipase was performed to investigate the effect of solvent-containing vs. solvent-free reaction systems on the degree of acyl migration. The effects of reaction conditions on acidolysis were also studied.

EXPERIMENTAL PROCEDURES

Materials. Perilla seed was purchased from a local market in Korea (Buyo, Chungnam province, Korea). Lipozyme RM IM (immobilized on ion-exchange resin) and Lipozyme TL IM (silica gel-granulated) were kindly provided by Novo Nordisk Bioindustry Ltd. (Seoul, Korea). Caprylic acid (99%) and pancreatic lipase were purchased from Sigma Chemical Company (St. Louis, MO). *n*-Hexane used in this study was HPLC grade.

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Preparation of perilla oil. Perilla seeds (200 g) were homogenized by a grinder. Crude oil was extracted in a 2 L flask with 800 mL of *n*-hexane by a Soxhlet apparatus for 12 h and then solvent was evaporated with a rotary evaporator. Final crude oil content was 41.4% (w/w). Crude perilla oil was further purified by column chromatography. In brief, silica gel (40 g) was mixed in 100 mL *n*-hexane to make a slurry, which was packed into the column (500 × 30 mm). After the crude perilla oil was applied to the column, elution was conducted with a mixture of *n*-hexane/diethyl ether (95:5, vol/vol). The recovered fractions were pooled and analyzed by TLC. After the purification, no other spots except TAG were detected. The collected fractions were concentrated with a rotary evaporator at 30°C. The purified perilla oil (average M.W. = 872) was used as substrate for further experiments.

Enzymatic acidolysis. Acidolysis reactions with lipases were carried out with different parameters. In general, reactions were performed in a screw-capped test tube in an orbital shaking water bath at 150 rpm. To study the effect of solvent content (0.0–85.3%, vol/vol) on caprylic acid incorporation, varied amounts of a mixture of perilla oil (density = 0.93) and caprylic acid (density = 0.91) (400, 600, 1000, 1400, 1800, 2200, and 2600 mg) were mixed with appropriate amounts of *n*-hexane to make a 3 mL reaction volume. Three milliliters (2760 mg) of substrate mixture without *n*-hexane was used as a solvent-free system. Other conditions used were as follows: lipase, 10% (wt% of substrates); molar ratio, 1:6 (perilla oil/caprylic acid); reaction time, 24 h; reaction temperature, 55°C; and no added water in the reaction mixture. The hexane used was previously dried over a 4 Å molecular sieve. All experiments were performed twice. For the study of the effects of other reaction conditions such as molar ratio, reaction time, reaction temperature, and enzyme load, 200.9 mg of perilla oil and 199.1 mg of caprylic acid, representing a 1:6 molar ratio, were dissolved in 2.56 mL *n*-hexane to make a 3 mL reaction volume. Other conditions used were the same as those used for the solvent content study unless the effect of the designated parameter itself was studied.

Analysis of products. The reaction mixture was filtered through an anhydrous sodium sulfate column to remove residual enzymes and water after the acidolysis reaction. The modified TAG were isolated by TLC, with the plates being developed with petroleum ether/ethyl ether/acetic acid (80:20:0.5, by vol), and detected with 0.2% 2,7-dichlorofluorescein in methanol solution under UV light. The bands corresponding to TAG were scraped from the TLC plate and methylated with 5 mL of 6% HCl in methanol at 80°C for 2 h. The FAME were extracted with 3 mL *n*-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 (30 m × 0.32 mm i.d.; Supelco, Bellefonte, PA) and FID was used. The column was held at 100°C for 3 min and programmed to 220°C for 20 min at the rate of 10°C/min. The carrier gas was helium and the total gas flow rate was 20 mL/min. The injector and detector temperatures were 240 and 260°C, respectively. Comparing retention times with standards identified the FAME.

Hydrolysis by pancreatic lipase. Pancreatic lipase hydrolysis was conducted to determine the positional distribution of FA in TAG following the method described in our previous report (16). In brief, 3 mg of TAG was mixed with 2 mL of 1 M Tris-HCl buffer (pH 7.6), 0.5 mL of 0.05% bile salts, 0.2 mL of 2.2% $CaCl_2$, and 5 mg pancreatic lipase. The mixture was incubated in a water bath at 37°C for 2 min, vortexed vigorously, extracted with diethyl ether, and dried by anhydrous sodium sulfate. The reaction mixture was then placed on a silica gel G TLC plate (Merck Co., Darmstadt, Germany) and developed with hexane/diethyl ether/acetic acid (50:50:1, by vol). The band corresponding to 2-MAG was scraped and extracted with diethyl ether, methylated, and analyzed by GC.

RESULTS AND DISCUSSION

Optimization of reaction parameters. The effect of molar ratios from 1:1 to 1:10 (perilla oil/caprylic acid) was studied (Fig. 1). With Lipozyme TL IM and Lipozyme RM IM, incorporation of caprylic acid increased up to a 1:6 molar ratio. At a 1:6 molar ratio, incorporated caprylic acid was 51.4 mol% (Lipozyme TL IM) and 48.5 mol% (Lipozyme RM IM). Over a 1:6 ratio there was little or no increase in incorporation. Overall, the highest caprylic acid incorporation was obtained at a molar ratio of 1:10 with Lipozyme TL IM (53.6 mol%).

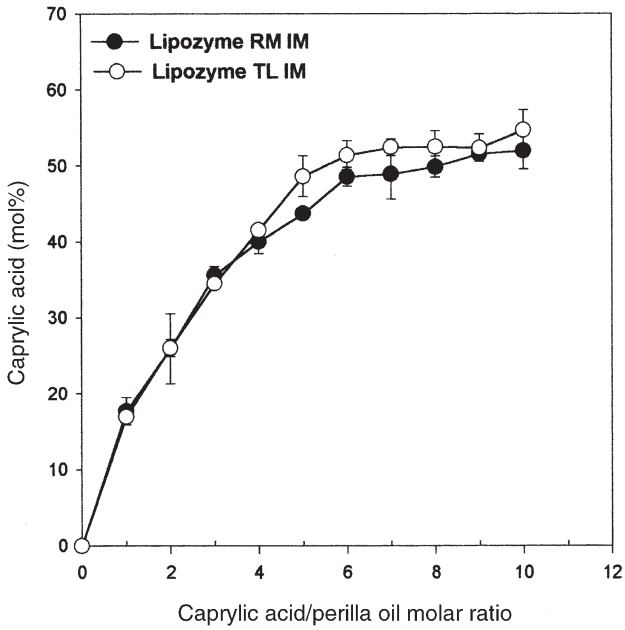

FIG. 1. Effect of molar ratio of substrates on the incorporation of caprylic acid into perilla oil. Molar ratios of perilla oil to caprylic acid were varied from 1:1 to 1:10, with the total amount of substrate being 400 mg in 3 mL reaction mixture. Each reaction mixture was incubated at 55°C for 24 h in an orbital shaking water bath at 150 rpm. Enzyme amount was 10% by weight of substrates. Lipozyme RM IM (*Rhizomucor miehei*) and Lipozyme TL IM (*Thermomyces lanuginosa*) were supplied by Novo Nordisk Bioindustry Ltd. (Seoul, Korea). Vertical bars represent SD.

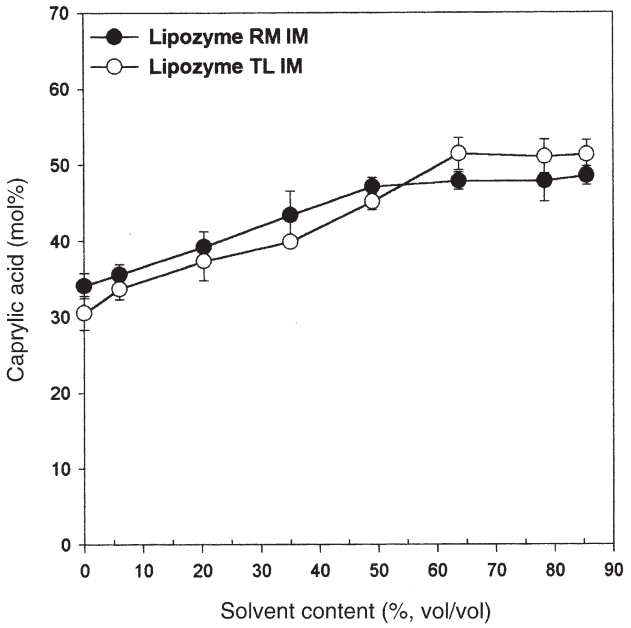

FIG. 2. Effect of solvent content on the incorporation of caprylic acid into perilla oil. Molar ratio of perilla oil to caprylic acid was 1:6. Each reaction mixture was incubated at 55°C for 24 h in an orbital shaking water bath at 150 rpm. Enzyme amount was 10% by weight of substrates. Solvent content in reaction mixture ranged from 0.0 (1386.0 mg; 1.49 mL perilla oil, 1374.0 mg; 1.51 mL caprylic acid, solvent-free system, total reaction mixture; 3 mL) to 85.3% (200.9 mg; 0.22 mL perilla oil, 199.1 mg; 0.22 mL caprylic acid, 2.56 mL *n*-hexane, total reaction mixture; 3 mL). Vertical bars represent SD.

Generally, in the acidolysis reaction, a high concentration of free acid as acyl donor results in decreased enzyme activity (17) and difficulties in separating the TAG from FFA during subsequent refining. In the study by Lee *et al.* (18), deactivation of the lipase by an excess amount of acid (as an acyl donor) was also observed.

Other optimal reaction conditions were determined to be 55°C reaction temperature, 24 h incubation time, and 10% enzyme load (wt% of substrates) (data not shown).

Effect of solvent content. The effect of solvent content in the reaction mixture on the incorporation is shown in Figure 2. The solvent content used ranged from 0.0 to 85.3% (vol/vol). The results showed that the incorporation increased in parallel with solvent content to 49.0% with Lipozyme RM IM and 63.8% with Lipozyme TL IM. It seemed that a certain amount of solvent in the reaction system was needed to promote the incorporation reaction. Suggested mechanisms for this effect include decreasing the viscosity of the oils, increasing the solubility of the oils, and shifting the thermodynamic equilibrium to synthesis rather than hydrolysis (19,20).

The degree of modification in SL. Acidolysis reactions catalyzed by *sn*-1,3 specific Lipozyme RM IM and Lipozyme TL IM lipases were carried out with a 1:6 molar ratio mixture of perilla oil and caprylic acid in an *n*-hexane or solvent-free system for 24 h. As expected, the FA composition of perilla oil was significantly changed after modification (Table 1). Originally, linolenic acid was the predominant FA in perilla oil, composing over 60 mol%. After the acidolysis reaction, however, caprylic acid as well as linolenic acid became the major FA in SL. After 24 h incubation in *n*-hexane, incorporated caprylic acid was 48.5 and 51.4 mol% when catalyzed by Lipozyme RM IM and Lipozyme TL IM, respectively. Meanwhile, in the solvent-free system, incorporated caprylic acid was 34.2 and 30.6 mol%, respectively. These results demonstrate that a reaction mixture with *n*-hexane as a solvent was more efficient than a solvent-free system for caprylic acid incorporation. This phenomenon can be explained by the fact that the substrates are readily dissolved in the reaction mixture in the presence of solvent, providing increased contact between substrate and lipase. Between the two different lipases examined, Lipozyme TL IM was more effective at the incorporation of caprylic acid than Lipozyme RM IM in both reaction systems.

TABLE 1
FA Composition (mol% and SD) of Perilla Oil Before and After Enzymatic Acidolysis With and Without Solvent[a]

		After modification			
	Before	Lipozyme RM IM[b]		Lipozyme TL IM[b]	
FA	modification	*n*-Hexane	Solvent-free	*n*-Hexane	Solvent-free
8:0	ND[c]	48.5 ± 1.2	34.2 ± 1.7	51.4 ± 1.9	30.6 ± 2.2
16:0	7.1 ± 0.2	2.6 ± 0.6	3.1 ± 0.4	4.2 ± 1.6	4.1 ± 1.1
18:0	1.9 ± 0.1	1.5 ± 0.1	0.9 ± 0.1	0.7 ± 0.2	1.1 ± 0.3
18:1	15.4 ± 0.4	8.1 ± 0.3	11.0 ± 2.1	8.0 ± 2.2	11.1 ± 1.1
18:2	14.2 ± 0.2	7.8 ± 0.2	10.5 ± 1.2	7.4 ± 0.9	10.6 ± 0.6
18:3	61.4 ± 2.7	31.5 ± 1.6	40.3 ± 2.0	28.3 ± 1.7	42.5 ± 2.4

[a]Molar ratio of perilla oil to caprylic acid, 1:6, enzyme amount, 10% (wt% of substrates); temperature, 55°C; reaction time, 24 h; shaking speed, 150 rpm. *n*-Hexane in column represents the acidolysis reaction (200.9 mg perilla oil and 199.1 mg caprylic acid dissolved in 2.56 mL *n*-hexane to make a 3-mL reaction system) containing *n*-hexane as solvent. "Solvent-free" represents the reaction without *n*-hexane, composed of 1386 mg of perilla oil, 1374 mg of caprylic acid, and 276 mg of each enzyme.
[b]Lipozyme RM IM (*Rhizomucor miehei*) and Lipozyme TL IM (*Thermomyces lanuginosa*) were obtained from Novo Nordisk Bioindustry Ltd. (Seoul, Korea).
[c]ND, not detected.

TABLE 2
***sn*-2 Positional FA Composition (mol% and SD) of Perilla Oil Before and After Enzymatic Acidolysis[a]**

FA	Unmodified perilla oil	Lipozyme RM IM[b] modified perilla oil		Lipozyme TL IM[b] modified perilla oil	
		n-Hexane[a]	Solvent-free[a]	*n*-Hexane	Solvent-free
8:0	ND[c]	7.1 ± 0.3	2.8 ± 0.3	10.7 ± 1.2	2.6 ± 0.2
	(migration rate)[d]	(14.6%)[d]	(8.3%)[d]	(20.9%)[d]	(8.5%)[d]
16:0	0.5 ± 0.1	1.3 ± 0.1	1.0 ± 0.3	1.7 ± 0.2	1.0 ± 0.1
18:0	ND	0.4 ± 0.0	0.2 ± 0.1	0.5 ± 0.1	0.3 ± 0.1
18:1	17.4 ± 0.5	16.2 ± 0.2	16.8 ± 0.4	16.1 ± 0.6	18.0 ± 0.7
18:2	17.3 ± 0.3	16.1 ± 0.2	16.9 ± 0.5	15.5 ± 0.6	17.0 ± 0.5
18:3	64.8 ± 1.9	58.9 ± 3.6	62.3 ± 2.8	55.5 ± 1.4	61.1 ± 1.2

[a]For reaction conditions, see Table 1.
[b]For sources and abbreviations, see Table 1.
[c]ND, not detected.
[d]Note percent of caprylic acid in the *sn*-2 position/total mol% of caprylic acid in TAG × 100.

FA profile at the sn-2 position. Pancreatic lipase digestion was performed to determine the FA profile at the *sn*-2 position of the TAG species. FA at the *sn*-2 position of TAG are known to be easily absorbed, regardless of the type of FA esterified at that position (21,22). Table 2 shows the FA compositions at the *sn*-2 position of the original perilla oil and the modified oils in two reaction systems: *n*-hexane (with solvent) and solvent-free. Although the lipases used in this study have been shown to be 1,3-specific lipases, the presence of caprylic acid at the *sn*-2 position of the modified perilla oil implied that acyl migration occurred during lipase-catalyzed acidolysis or pancreatic lipase hydrolysis. About 7.1 to 10.7 mol% of caprylic acid was found at the *sn*-2 positions of TAG modified in the presence of *n*-hexane. However, only 2.6 to 2.8 mol% of caprylic acid was found in *sn*-2 positions of TAG produced in solvent-free systems. These results suggested that reaction systems containing nonpolar organic solvents, such as *n*-hexane, could possibly facilitate acyl migration. We assumed that higher incorporation of caprylic acid in the reaction with *n*-hexane led to the increased chance of acyl migration (Table 1). To prove this assumption, acyl migration rate, expressed as the mol% of caprylic acid in the *sn*-2 position divided by total mol% of caprylic acid in TAG, was calculated. Both lipases showed higher migration rates in the reaction with *n*-hexane than the solvent-free systems (Table 2). Therefore, the inclusion of *n*-hexane as a solvent in the reaction system seemed to facilitate acyl migration as well as acidolysis.

When the two lipases were compared, Lipozyme TL IM showed a higher degree of acyl migration than Lipozyme RM IM in the reaction system containing *n*-hexane. In the solvent-free systems, however, the difference between the two lipases was negligible (Table 2). This result can be supported in part by the results from Goh *et al.* (23). In their study of the transesterification of cocoa butter by fungal lipases, acyl migration was higher in *n*-hexane than in diethyl ether. Undoubtedly, properties of each lipase and carrier can also influence the degree of acyl migration (24).

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