Production of Structured Lipids by Lipase-Catalyzed Acidolysis in Supercritical Carbon Dioxide: Effect on Acyl Migration

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ABSTRACT: Structured lipids were synthesized by the acidolysis of corn oil by caprylic acid in supercritical carbon dioxide ($SCCO_2$) with Lipozyme RM IM from *Rhizomucor miehei*. The effects of pressure and temperature on the reaction were studied. To compare the degrees of acyl migration in the $SCCO_2$ and solvent-free reaction systems, the effects of reaction time on the degree of acyl migration were also studied. The highest mole percentage incorporation of caprylic acid (62.2 mol%) occurred at 24.13 MPa in $SCCO_2$. The overall incorporation of caprylic acid in the $SCCO_2$ system remained higher than that in the solvent-free system at every temperature tested. This trend was observed more clearly at lower temperatures (35–55°C) than at higher temperatures (65–75°C). Acyl migration with both reaction systems was low, with a negligible difference between them up to 12 h, after which the degree of acyl migration in the solvent-free system increased rapidly with time up to 24 h compared with the $SCCO_2$ system.

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KEY WORDS: Acidolysis, acyl migration, caprylic acid, corn oil, *Rhizomucor miehei*, structured lipids, supercritical carbon dioxide.

Lipase-catalyzed modification of TAG can be performed, and this has 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 reaction does not possess this regiospecificity due to the random nature of the reaction (1–3).

Supercritical fluids (SCF) have been considered as attractive alternatives to ordinary organic solvents. Among the SCF, supercritical carbon dioxide ($SCCO_2$) has been used the most frequently because of its relatively mild critical temperature (31.1°C) and pressure (7.38 MPa) (4). Moreover, $SCCO_2$ is nontoxic, nonflammable, less expensive than conventional organic solvents, and easily removed from the reaction product, so it is very attractive not only as a nonaqueous reaction medium but also as an extraction solvent, especially in the food and pharmaceutical industries (5–8).

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A number of investigations on enzymatic reactions have been carried out in $SCCO_2$ because of its unique solvent properties, such as low viscosity and high diffusivity, in comparison with ordinary organic solvents. $SCCO_2$ has been used, for example, as a medium for reactions catalyzed by polyphenol oxidase (9) and cholesterol oxidase (10). Chi *et al.* (11) studied lipase-catalyzed TAG interesterification in $SCCO_2$. Van Eijs *et al.* (12) successfully used $SCCO_2$ to produce isoamyl acetate and nonyl acetate from ethyl acetate and the corresponding alcohols. Glowacz *et al.* (13) studied the hydrolysis of triolein and its partial glycerides by porcine pancreatic lipase in $SCCO_2$ in a batch reactor system. Marty *et al.* (14) demonstrated oleic acid esterification with ethanol by using an immobilized lipase from *Mucor miehei* with both $SCCO_2$ and *n*-hexane as solvents.

In spite of the many investigations, lipase reactions in $SCCO_2$ are not completely understood and not optimized well because insufficient information exists on $SCCO_2$ as an enzymatic reaction medium compared with ordinary solvents.

With $SCCO_2$ as a solvent for enzymatic reactions, both pressure and temperature are important process parameters. In this study, we investigated the effects of pressure and temperature on the lipase-catalyzed acidolysis reaction of corn oil with caprylic acid in $SCCO_2$ as a reaction medium. *sn*-2 Positional analysis by pancreatic lipase was also performed to compare the effect of an $SCCO_2$ vs. solvent-free reaction system on the degree of acyl migration.

EXPERIMENTAL PROCEDURES

Materials. Refined, bleached, and deodorized corn oil was purchased from a local market (Seoul, Korea). Lipozyme RM IM (immobilized on an ion-exchange resin) from *Rhizomucor miehei* was purchased from Novo Nordisk Bioindustry Ltd. (Seoul, Korea). Caprylic acid (99%), 4-Å molecular seive, and pancreatic lipase were purchased from Sigma Chemical Company (St. Louis, MO). Carbon dioxide with a purity of 99.999% was obtained from Shinyang Gas Products, Inc. (Seoul, Korea). Other chemicals used in this study were purchased from Sigma Chemical Company and were of analytical grade unless mentioned otherwise.

Enzymatic acidolysis in $SCCO_2$. Figure 1 presents the experimental equipment used to conduct all experiments in

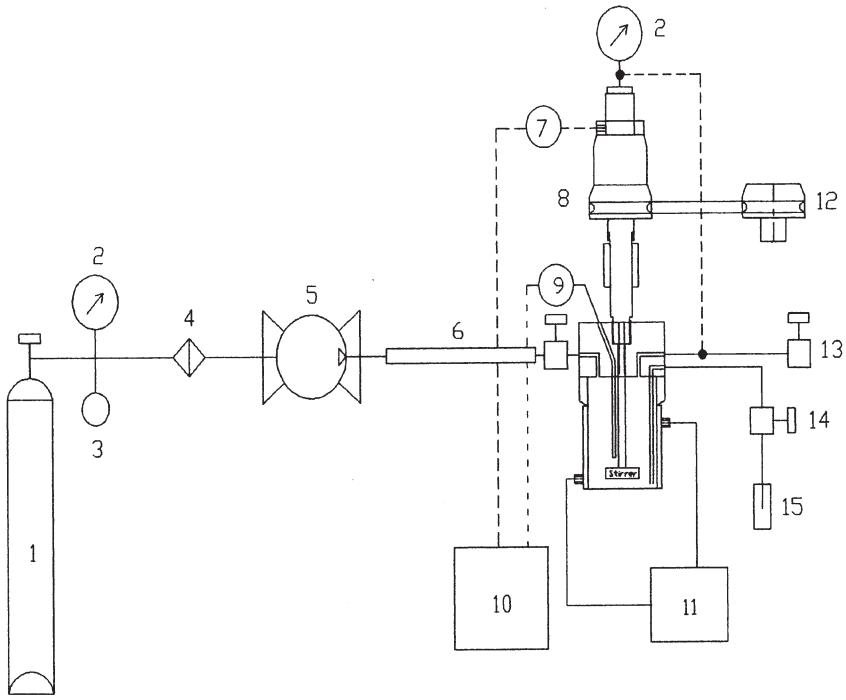

FIG. 1. Schematic diagram of the supercritical carbon dioxide ($SCCO_2$) reaction system for an enzymatic reaction. (1) CO_2 tank; (2) pressure gauge; (3) rupture disk; (4) filter; (5) pump; (6) molecular sieve trap; (7) rpm reader; (8) magnetic device; (9) thermocouple; (10) controller; (11) circulator; (12) motor; (13) depressurization valve; (14) sampling valve; (15) sampling vial.

$SCCO_2$. The reactor was a high-pressure vessel with a 300-mL volume into which 6 g of immobilized lipase (10% by weight of substrates), 22.7 g of corn oil, 37.3 g of caprylic acid, and 0.6 g of water (1% by weight of substrates) were loaded. Prior to starting the reaction, CO_2 gas, dried using a molecular sieve, was flushed through the reactor, and the reactor was then heated to the desired temperature with a water circulator. Dried CO_2 was pumped into the reactor with a gas booster pump to the desired pressure. The reaction was started by agitating with the magnetic device and was continued for 6 or 24 h. A 0.1-g sample of reaction mixture was removed through the sampling valve for analysis at various time intervals. To compare the effectiveness of $SCCO_2$ on the reaction, a solvent-free reaction was carried out at 0.1 MPa of nitrogen. Other reaction conditions were the same in $SCCO_2$.

Analysis of products. The analysis of reaction products was performed according to a previous method (15). In brief, the reaction mixture collected from the reactor was dissolved in 5 mL *n*-hexane and filtered through an anhydrous sodium sulfate column to remove the water. The modified TAG were isolated by TLC on silica gel G (Merck Co., Darmstadt, Germany), developed with petroleum ether/ethyl ether/acetic acid (80:20:0.5, by vol), and detected with 0.2% 2,7-dichlorofluorescein in a methanol solution under UV light. The band corresponding to TAG was scraped off 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 10.0°C/min. The carrier gas was helium, and the total gas flow rate was 50 mL/min. The injector and detector temperatures were 240 and 260°C, respectively. FAME were identified by comparing their retention times with those of standards

Determination of the positional distribution of FA in TAG. Pancreatic lipase hydrolysis was conducted to determine the positional distribution of FA in TAG following the method described in our previous report (15). 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 of pancreatic lipase. The mixture was incubated in a water bath at 37°C for 2 min, mixed vigorously, extracted with diethyl ether, and dried with anhydrous sodium sulfate. The reaction mixture was then chromatographed on a silica gel G TLC plate (Merck Co.) developed with hexane/diethyl ether/acetic acid (50:50:1, by vol). The band corresponding to 2-MAG was scraped off and extracted with diethyl ether, methylated, and analyzed by GC.

RESULTS AND DISCUSSION

The FA composition of corn oil was significantly changed after modification (Table 1). Originally, linoleic acid was the predominant FA in corn oil, constituting over 50 mol%. After

TABLE 1
FA Composition (mol% and SD) of Corn Oil Before and After Enzymatic Acidolysis with a Supercritical Carbon Dioxide ($SCCO_2$) and a Solvent-free System[a]

FA	Before modification	After modification	
		$SCCO_2$	Solvent-free
8:0	ND[b]	62.2 ± 0.8	58.7 ± 0.5
16:0	13.6 ± 0.3	2.6 ± 0.2	3.1 ± 0.1
18:0	2.0 ± 0.1	0.4 ± 0.0	0.4 ± 0.1
18:1	30.6 ± 0.8	11.0 ± 0.4	10.8 ± 0.2
18:2	52.9 ± 1.4	23.4 ± 0.2	26.5 ± 0.4
18:3	0.9 ± 0.1	0.4 ± 0.1	0.5 ± 0.1

[a]The reaction was carried out in a 300-mL high-pressure reactor containing 22.7 g of corn oil, 37.3 g of caprylic acid, 10% (by weight of substrates) of Lipozyme RM IM from *Rhizomucor miehei* (Novo Nordisk Bioindustry, Seoul, Korea), and 0.6 g of water (1% of substrate). Temperature, pressure, reaction time, and stirring speed in $SCCO_2$ were 55°C, 24.13 MPa, 6 h, and 200 rpm, respectively. "Solvent-free" represents the reaction with nitrogen at 0.1 MPa; other conditions are the same as in $SCCO_2$.
[b]ND, not detected.

acidolysis, however, caprylic and linoleic acids became the major FA in the SL. After 6 h of reaction in $SCCO_2$, an average of 62.2 mol% of caprylic acid was incorporated into the corn oil. Meanwhile, in the solvent-free system, 58.7 mol% of caprylic acid was incorporated. The results demonstrated that acidolysis of corn oil with caprylic acid in $SCCO_2$ was more efficient than in a solvent-free system.

Pressure effect. Unlike the case of conventional organic solvents, biocatalysis in SCF was carried out above atmospheric pressure; therefore, the intrinsic effect of pressure on the enzyme was an important parameter. Lipase-catalyzed acidolysis between corn oil and caprylic acid as the acyl donor was carried out in the pressure range of 10.34 to 37.92 MPa for 6 h at 55°C with stirring (200 rpm) in $SCCO_2$ (Fig. 2). The highest incorporation of caprylic acid (62.2 mol%) occurred at 24.13 MPa in $SCCO_2$. However, no significant increase in the amount of caprylic acid incorporated into the corn oil was observed above 24.13 MPa at an early stage of reaction (0.5–2 h). A slight decrease in caprylic acid incorporation was observed at pressures above 24.13 MPa between 4 and 6 h of reaction. Erickson *et al.* (16) reported the effect of $SCCO_2$ pressure in the lipase-catalyzed transesterification between trilaurin and palmitic acid, which showed a decrease in the reaction rate with an increase in pressure. In contrast, Nakaya *et al.* (17) reported an increase in the transesterification rate of triolein and stearic acid catalyzed by Lipozyme IM in $SCCO_2$ when the pressure was increased stepwise from 5 to 20 MPa. Thus, the effect of pressure on the enzymatic reaction in $SCCO_2$ has not been uniformly described and appears complicated.

Temperature effect. The thermostability of enzymes is a major consideration in their industrial use, mostly because of the potential for minimizing thermal degradation. Temperature is also related to the mass-transfer limitations in the reactor. A higher temperature reduces the viscosity of the lipid mixture and certainly increases the substrate and product transfer on the surface or inside the enzyme particles. Also, the reaction temperature can affect the density of CO_2 in the $SCCO_2$ reaction system as well as parameters such as enzyme stability, affinity of the enzyme for the substrate, and the preponderance of competing reactions (18,19).

The temperature range tested was 35–75°C (Table 2). We found that 55°C was suitable for the reaction in both reaction systems. In the initial 2 h of reaction, the incorporation of caprylic acid in the $SCCO_2$ system increased significantly as the temperature increased. In $SCCO_2$, the increase in temperature resulted

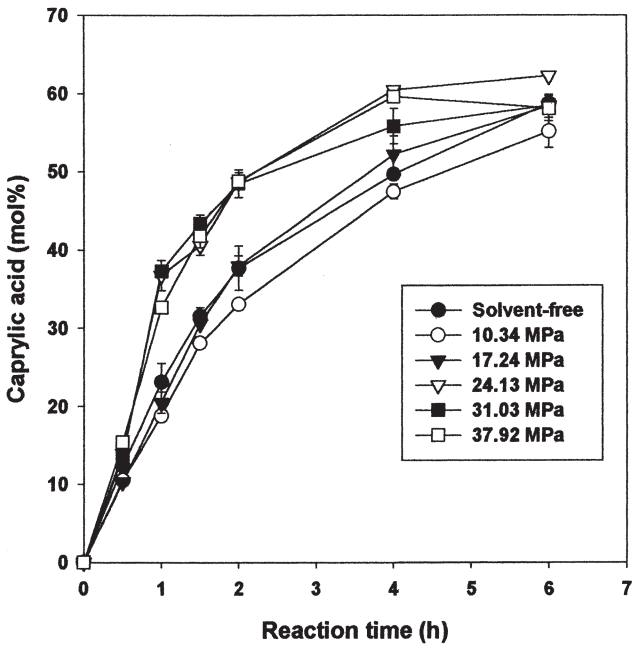

FIG. 2. Effect of pressure on the incorporation of caprylic acid into corn oil in $SCCO_2$. The reaction was carried out in a 300-mL high-pressure reactor containing 22.7 g of corn oil, 37.3 g of caprylic acid, 10% (by weight of substrates) of Lipozyme RM IM from *Rhizomucor miehei*, and 0.6 g of water (1% of substrate). The temperature, reaction time, and stirring speed with $SCCO_2$ were 55°C, 6 h, and 200 rpm, respectively. "Solvent-free" represents the reaction with nitrogen at 0.1 MPa; other conditions were the same as in the $SCCO_2$ system. The vertical bars represent SD. For abbreviation see Figure 1.

TABLE 2
Effect of Temperature on the Incorporation (mol% and SD) of Caprylic Acid into Corn Oil with an $SCCO_2$ and a Solvent-free System[a]

		Reaction time (h)					
	Temperature (°C)	0.5	1	1.5	2.0	4.0	6.0
$SCCO_2$	35	5.4 ± 0.4	20.3 ± 1.3	26.5 ± 1.0	36.2 ± 0.1	55.4 ± 0.7	58.8 ± 0.9
	45	8.9 ± 0.2	28.9 ± 1.7	36.7 ± 0.8	42.4 ± 1.0	54.2 ± 0.0	61.7 ± 0.5
	55	14.0 ± 0.6	32.7 ± 1.8	40.6 ± 1.2	48.7 ± 0.2	60.4 ± 0.3	62.2 ± 0.2
	65	16.3 ± 0.6	35.4 ± 1.6	44.0 ± 0.2	51.4 ± 0.7	55.7 ± 1.1	60.5 ± 0.8
	75	17.7 ± 1.8	32.1 ± 2.1	37.9 ± 0.3	46.6 ± 0.5	55.2 ± 0.7	59.4 ± 1.0
Solvent-free	35	5.1 ± 0.4	9.0 ± 0.4	18.3 ± 1.5	24.4 ± 0.1	31.8 ± 0.2	50.2 ± 0.7
	45	6.5 ± 0.1	15.5 ± 0.5	23.4 ± 2.1	30.5 ± 1.7	50.8 ± 0.2	54.2 ± 1.0
	55	12.4 ± 2.4	23.1 ± 2.4	31.6 ± 1.1	37.7 ± 2.8	49.7 ± 0.1	58.7 ± 0.2
	65	9.5 ± 0.0	19.2 ± 1.9	30.0 ± 1.5	39.8 ± 0.4	46.9 ± 0.5	57.4 ± 0.6
	75	11.1 ± 0.6	20.4 ± 1.4	32.4 ± 0.8	40.1 ± 1.6	47.1 ± 1.7	54.5 ± 0.5

[a]The reaction was carried out in a 300-mL high-pressure reactor containing 22.7 g of corn oil, 37.3 g caprylic acid, 10% (by weight of substrates) of Lipozyme RM IM from *R. miehei* (Novo Nordisk Bioindustry, Seoul, Korea), and 0.6 g of water (1% of substrate). The pressure and stirring speed in $SCCO_2$ were 24.13 MPa and 200 rpm, respectively. "Solvent-free" represents the reaction with nitrogen at 0.1 MPa; other conditions are the same as in $SCCO_2$. For abbreviation see Table 1.

in decreases in the density and viscosity of the reaction medium. The lower density of the $SCCO_2$ and the higher diffusivity resulted in an increase in the mass-transfer rate of substrates and products with the immobilized enzyme (20). A similar trend was observed for the solvent-free system, although the extent of caprylic acid incorporation was lower than for the $SCCO_2$ system. The overall incorporation of caprylic acid in the $SCCO_2$ system remained higher than in the solvent-free system. Overall, the reaction rate under $SCCO_2$-conditions was higher than that under solvent-free conditions, indicating that $SCCO_2$ is a potential solvent for an acidolysis reaction.

Degree of acyl migration. To compare the degree of acyl migration in the $SCCO_2$ and solvent-free reaction systems, pancreatic lipase digestion was performed to determine caprylic acid at the *sn*-2 position of the TAG species.

Acyl migration leads to nonspecific by-products. It occurs during lipase-catalyzed acidolysis and is affected by factors such as water content, reaction time, reaction temperature, enzyme load, reactor type, and reaction system (21). However, to our knowledge, few studies has been conducted on the degree of acyl migration for lipase-catalyzed acidolysis in $SCCO_2$. Therefore, we investigated the effect of reaction time in two reaction systems, $SCCO_2$ and solvent-free, on the degree of acyl migration. Incorporation was defined as the mole percentage of caprylic acid in the TAG, and acyl migration was defined as the mole percentage of caprylic acid at the *sn*-2 position of the TAG (Fig. 3). The incorporation of caprylic acid increased rapidly in the first 6 h in both reaction systems. In the next 18 h, there was a slow but steady increase in incorporation. The degree of caprylic acid incorporation was 68.3 mol% in the solvent-free system and 67.5 mol% in the $SCCO_2$ system, respectively. Mu *et al*. (22) reported that longer reaction times resulted in higher incorporation of medium-chain FA but also led to increased acyl migration in a laboratory-scale continuous reactor. Since the lipase (Lipozyme RM IM) used in this study was indicated by the manufacturer to be 1,3-specific, the presence of caprylic acid at the *sn*-2 position of the modified corn oil implied that acyl migration had occurred during the lipase-catalyzed acidolysis. About 1.5 to 2.3 mol% of caprylic acid was found at the *sn*-2 position of TAG produced in both reaction systems up to 6 h. However, the incorporation of caprylic acid at the *sn*-2 position of TAG species produced in the solvent-free system increased rapidly with a further increase in time, reaching a maximal level of 18.0 mol% at 24 h. On the other hand, the degree of acyl migration in the reaction system

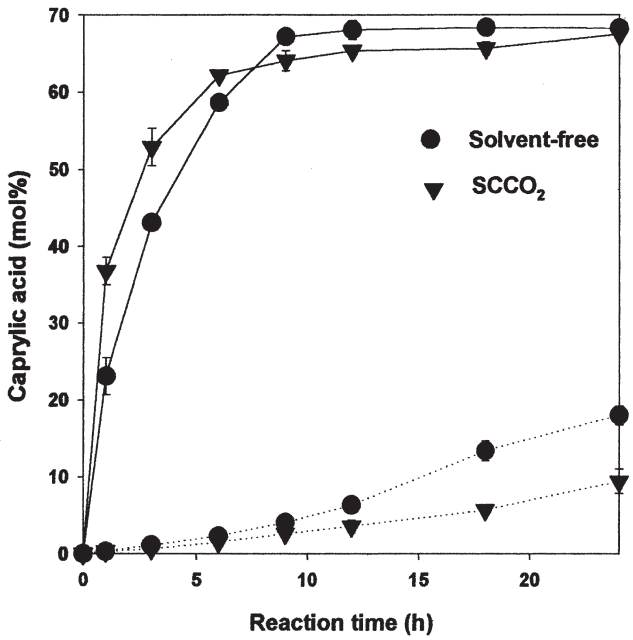

FIG. 3. Effect of reaction time on the acyl migration of caprylic acid into *sn*-2 position of TAG synthesized in an $SCCO_2$ and a solvent-free system. For reaction conditions see Table 1. The solid line represents incorporation, and the dotted line represents migration. Vertical bars represent SD.

with $SCCO_2$ was lower than that in the solvent-free reaction system, and only 9.4 mol% of caprylic acid was found at the *sn*-2 position of TAG produced at 24 h. These results suggested that the reaction system containing $SCCO_2$ could possibly reduce acyl migration. In lipase-catalyzed acidolysis, an increase in acyl migration has been observed concurrent with an increased amount of water from the reaction system (23). Unlike hydrophobic solvents, however, $SCCO_2$ may dissolve more than 0.1% of water (24). Thus, $SCCO_2$ can remove the water from the reaction system, resulting in reduced acyl migration.

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