Improved Lipase-Catalyzed Incorporation of Long-Chain Fatty Acids into Medium-Chain Triglycerides Assisted by Supercritical Carbon Dioxide Extraction[1]

Akihiro Shishikura[a,*], Kenshiro Fujimoto[b], Teruhiko Suzuki[c] and Kunio Arai[c]

[a]Process Design & Engineering Center, Idemitsu Petrochemical Co., Ltd., 1-1 Anesaki-Kaigan, Ichihara, Chiba 299-01, Japan, [b]Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, Sendai 981, Japan and [c]Department of Biochemistry and Chemical Engineering, Faculty of Engineering Science, Tohoku University, Sendai 980, Japan

Displacement of the equilibrium of the lipase-catalyzed interesterification between medium-chain triglyceride (MCT) and long-chain polyunsaturated fatty acid was accomplished by the removal of by-products with continuous supercritical carbon dioxide (SC-CO_2) extraction at 60°C and 100 kg/cm². The incorporation of eicosapentaenoic acid to MCT was appreciably improved by this method and was 1.3 times higher than that of the equilibrium state (47 wt%) that was obtained in a closed system. The immobilized *Mucor miehei* lipase was stable for more than 180 h in SC-CO_2 at 60°C and 100 kg/cm².

KEY WORDS: Eicosapentaenoic acid, extraction, interesterification, lipase, medium-chain triglyceride, oleic acid, polyunsaturated fatty acid, supercritical carbon dioxide.

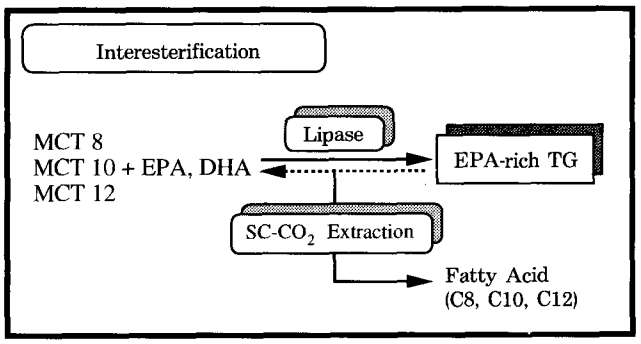

FIG. 1. Scheme of the experiment. MCT, medium-chain triglyceride; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; TG, triglyceride; SC-CO_2, supercritical carbon dioxide.

Physical and rheological properties as well as nutritional values of triglycerides (TG) are based not only on the fatty acid composition but also on the positional distribution of the acyl groups bonded to glycerol. TG with specific physical properties are required for each use, such as in bakery and confectionery goods. The structure of TG affects absorbability (1,2) and nutritional consequences (3,4). Therefore, the synthesis of structural TG will be important in the future. Currently, medium-chain triglycerides (MCT) with one of their acyl groups substituted with a long-chain fatty acid are noted for their unique physiological activity. For example, polyunsaturated fatty acids contained in the *sn*-2 position of TG with medium-chain fatty acids in the *sn*-1 and *sn*-3 positions are specifically easily absorbed (5).

Chemically catalyzed interesterification (6–9) with sodium methylate as the major catalyst has been used to improve or modify the physical properties of TG. However, it is usually difficult to introduce the desired acyl groups onto the specific positions of glycerol because the reaction occurs randomly with respect to position, although a chemical regioselective method has been reported recently (10).

On the other hand, the application of acyl exchanges catalyzed by enzymes (11–20) or microorganisms (21) has begun in the progress of biotechnology. It is known that most lipases (EC 3.1.1.3) show specificities in the hydrolysis of acyl moieties with respect to position, chainlength and degree of unsaturation of acyl groups (12,18,22,23). Therefore, lipase-catalyzed interesterification seems to be useful for modifying the composition of TG. To use lipase as a catalyst for interesterification, the reaction should take place in nonaqueous solvents to avoid hydrolysis. Over the past two decades, many enzymes have been shown to function successfully in organic solvents (24,25). Furthermore, most proteins do not denature much in supercritical (SC) (26–28) or liquid (29) carbon dioxide (CO_2), and many enzymes show and maintain their activity in SC-CO_2 (28,30–42). Nakamura and his co-workers (43,44) successfully carried out lipase-catalyzed interesterification between triolein and stearic acid in SC-CO_2 at 35 ≈ 50°C and 130 ≈ 350 kg/cm² pressure to produce a cocoa butter substitute.

Adschiri *et al.* (45) improved the equilibrium of lipase-catalyzed interesterification between tricaprylin and methyl oleate in SC-CO_2 by removal of the by-product, methyl caprylate, through SC-CO_2 extraction with a reflux column.

In this paper, we describe lipase-catalyzed interesterification, while taking advantage of SC-CO_2 extraction, between MCT and free long-chain fatty acids (Fig. 1).

MATERIALS AND METHODS

Materials. Three types of uni-acyl TG, with acyl carbon numbers of 8 (MCT 8), 10 (MCT 10) and 12 (MCT 12), were used as MCT. Oleic acid and eicosapentaenoic acid (EPA) were used as long-chain fatty acids. All substrate MCT and fatty acids were provided by Nippon Oil & Fats Co. (Tokyo, Japan). Five types of lipases were used as the enzymatic catalysts (Table 1). Celite No. 535 and glycerol (98%) were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Enzymatic reactions in n-*hexane.* The reactions were carried out at 50°C with shaking at 120 strokes per min. The reaction mixture was composed of a celite-adsorbed lipase (lipase, 0.3 g; celite, 0.5 g), 2.4 mmol of MCT and 5.3 mmol of oleic acid in water saturated with *n*-hexane (8 mL) or in *n*-hexane (8 mL) supplemented with 0.6 g of glycerol as an enzyme activator (13,14).

Enzymatic reactions in SC-CO_2. A flow diagram of the experimental apparatus is shown in Figure 2. The apparatus is principally similar to that previously used (46,47), except for the reactor, which has two windows to allow observation. The volume of the reactor is variable from 210 to 800 mL by exchanging a glass cell set in the reactor

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*To whom correspondence should be addressed.

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TABLE 1

Sources and Properties of Lipases

Origin (specificity)	Commercial name	Specific activity	Purchased from (or provided by)
Candida cylindracea (1,2,3)	Lipase OF	360 U/mg	Meito Sangyo Co. (Nagoya, Japan)
Chromobacterium viscosum (1,3)	Lipase TOYO	120 U/mg	Toyo Brew. Co. (Siguoka, Japan)
Mucor miehei (1,3)	Lipase Novo	210 U/mg	Novo Ind. Jpn. (Tokyo, Japan)
M. miehei (1,3)	LIPOZYME™	25 BIU/g	Novo Ind. Jpn. (Tokyo, Japan)
Rhizopus delemar (1,3)	Talipase	15 IU/mg	Tanabe Pham. Co. (Osaka, Japan)

and adjusted to 210 mL otherwise. The sampling line of the gas phase and the pressure synchrony line were located on the lid of the reactor. The sampling line for the liquid phase and the SC-CO_2 supply line were placed at the bottom of the reactor.

The reactions were carried out in the range of 40 to 60°C and 0 to 300 kg/cm^2 pressure with stirring (250 rpm). The reaction mixture, which was composed of 24 mmol TG, 39 mmol free fatty acid (FFA) (oleic acid or EPA), 0 to 5 g of glycerol and 1.5 g of immobilized lipase (LIPOZYME™; Novo Nordisk, Tokyo, Japan), was loaded into the reactor. The temperature of the reactor was regulated by a temperature-controlled water bath. The temperature of the reactor became constant within 15 to 20 min. The progress of the reactions was followed by the analysis of liquid-phase samples withdrawn from the reactor at regular time intervals. After a definite reaction time, the pressure was diminished, and oils and enzymes were recovered separately. Reactions were carried out in a closed system, in which CO_2 was not introduced during the reaction, to simplify the reaction system and make it easy to elucidate reaction kinetics.

Enzymatic reaction with continuous SC-CO_2 extraction. The reaction mixture, which was composed of 42 mmol of MCT 8 (tricaprylin), 230 mmol of oleic acid (or EPA), 2.7 g of immobilized lipase (LIPOZYME™) and 2.2 g of glycerol, was loaded into the reactor. Reactions were carried out at 60°C and 100 atm with stirring (250 rpm/min). CO_2 continuously flowed at 4 NL/min (liter at atmospheric pressure/min) during the reaction to extract the liberated medium-chain fatty acids. The pressure of the reactor was increased immediately, and its temperature was regulated by a water bath. The start of the reaction (0 h) was when the enzyme was mixed with the substrate. Reactions were followed by analysis of liquid-phase samples withdrawn from the reactor at regular time intervals.

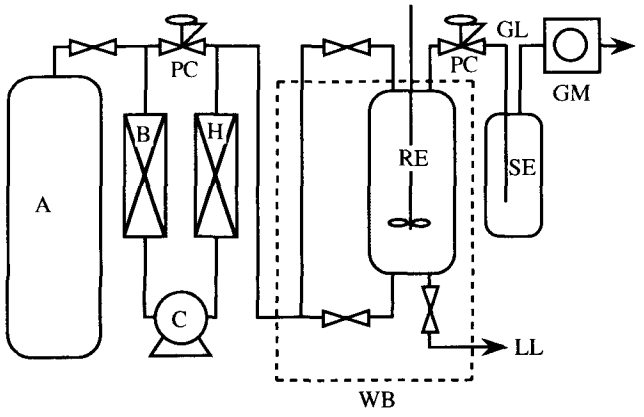

FIG. 2. A flow diagram of the experimental apparatus. A, CO_2 cylinder; B, cooler; C, compressor; H, heater; PC, pressure control valve; RE, reactor; WB, water bath; SE, separator; GM, gas meter; GL, SC-CO_2-phase sampling line and LL; liquid-phase sampling line. See Figure 1 for abbreviations.

Enzyme stability. The stability of the immobilized lipase (LIPOZYME™) recovered from the reaction mixture under various supercritical conditions was assessed by interesterification between MCT 8 and oleic acid in 8 mL of water-saturated *n*-hexane at 50°C for 10 h as described for the reactions in *n*-hexane. The recovered enzyme (80 mg) was subjected to incubation after washing three times with 100 mL *n*-hexane.

Analysis of the reaction products. The lipid composition in the reaction mixtures was determined by thin-layer chromatography with a flame-ionization detector (Iatroscan TH-10 analyzer; Iatron Co., Tokyo, Japan) on silica rods with *n*-hexane/diethyl ether/formic acid (65:35:1, vol/vol/vol) as the mobile phase.

The fatty acid composition of TG and FFA was determined by gas chromatography. The methyl esters, prepared with BF_3 as a catalyst (16), were analyzed by a JEOL JEC-20K apparatus (Japan Electric Co., Tokyo, Japan) equipped with a glass column (3 mm × 2 m) containing 5% Silar 10C on Chromosorb W HP (100:120 mesh; GL Science Co., Tokyo, Japan). The column temperature was programmed from 140 to 200°C at 3°C/min.

The TG composition was determined by gas chromatography in the same apparatus equipped with a glass column (3 mm × 0.5 m) containing 1% OV-1 on Chromosorb W (80:100 mesh; GL Science Co.). The column temperature was programmed from 160 to 330°C at 3°C/min.

RESULTS AND DISCUSSION

Enzymatic reactions as shown in Figure 1 were carried out in the liquid phase, which coexists with the SC-CO_2 phase in a reactor. The solubilities of fatty acids in SC-CO_2 are greatly dependent on their vapor pressures, which are exclusively correlated to the carbon chainlength of the fatty acids (48). Therefore, medium-chain fatty acids liberated from MCT are expected to be preferentially dissolved in the SC-CO_2 phase because the solubility of medium-chain fatty acids in SC-CO_2 is much higher than those of long-chain fatty acids and TG. Thus, the reaction equilibrium of

$$\text{MCT} + \text{long-chain fatty acids} \rightleftharpoons \text{TG containing long-chain fatty acids} + \text{medium-chain fatty acids} \quad [1]$$

will be shifted in the direction of the introduction of

long-chain fatty acids into TG. The equilibrium of this reaction will be further displaced by the continuous SC-CO_2 extraction to improve efficiency. The following experiments were carried out to determine the optimal conditions for both lipase reaction and SC-CO_2 extraction to promote the introduction of long-chain fatty acids into MCT.

Selection of enzyme species. Because one of our objectives of lipase-catalyzed interesterification is to prepare TG containing EPA and docosahexaenoic acid (DHA), we preliminarily compared the activity of lipases listed in Table 1 for the acidolysis between MCT and oleic acid or EPA in water-saturated *n*-hexane.

In the case of the MCT 12–oleic acid system (Fig. 3), the *Candida cylindracea* lipase (Meito Sangyo Co., Tokyo, Japan), which is known to be nonspecific to positions on glycerol (22,23), showed the highest activity among all tested lipases, as expected from the indicated activity.

In the MCT 12–EPA system (Fig. 4), the activity of the *Mucor miehei* lipase was much greater than that of the others. Incorporation of EPA into MCT by *M. miehei* lipase was linear with time up to 6 h, and thereafter, the rate of incorporation gradually decreased and reached a maximum of around 50 wt%. This value is 1.2 times higher than the theoretical value (about 75%) that corresponds to the complete random distribution of EPA and lauric acid in the *sn*-1 and *sn*-2 positions of TG. Therefore, *M. miehei* lipase is well-suited for this study, and subsequent experiments were carried out with *M. miehei* lipase immobilized on an ion exchange resin (LIPOZYME™).

Effect of glycerol on enzyme activity. Figure 5 shows the effect of added glycerol on the incorporation of oleic acid to TG after incubation for 10 h at 60°C and 100 kg/cm^2. The enzymatic reaction proceeded slowly when the system did not contain glycerol. When 1 or 2 g of glycerol was added, the reaction rates were improved almost proportionally to the amount of added glycerol as suggested by Tanaka *et al.* (13,14). The lipase-catalyzed interesterification consists of hydrolysis of an acyl moiety followed by reesterification of a fatty acid (49). Water activates the former reaction, but glycerol is suggested to activate the latter reaction. A trace amount of water is essential for lipase-catalyzed reactions (50). In the present study, no water was supplemented into the reaction mixture. Water contained in the LIPOZYME™ (10 wt%) should participate in the reaction. It appears that glycerol also participated in the reaction as a substrate, because a considerable amount of 1, 3-diglyceride was formed after incubation.

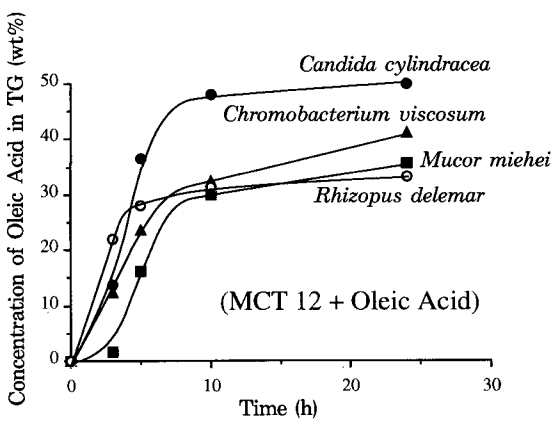

FIG. 3. Time course of interesterifications between MCT 12 and oleic acid in *n*-hexane. TG, triglyceride; see Figure 1 for other abbreviations.

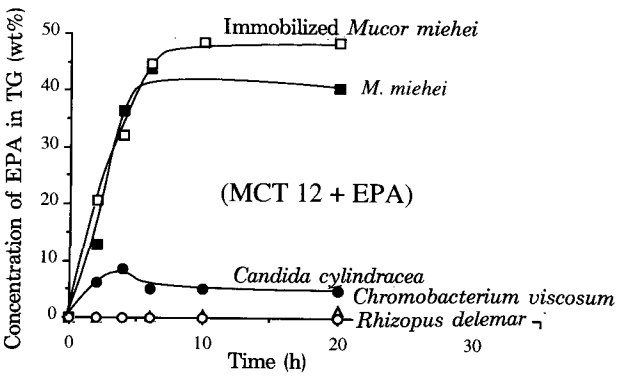

FIG. 4. Time course of interesterifications between MCT 12 and EPA in *n*-hexane. See Figure 1 for abbreviations.

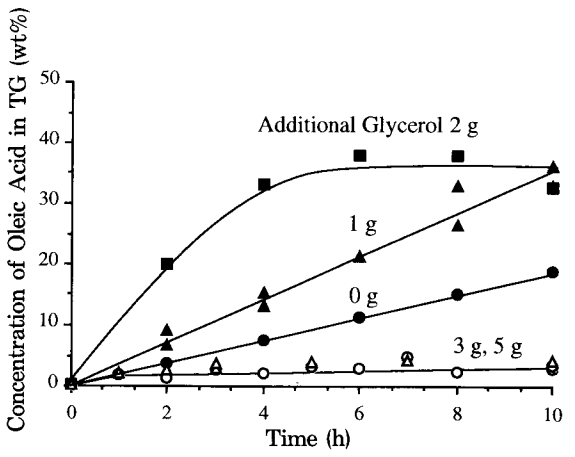

FIG. 5. Time course of interesterifications between MCT 12 and oleic acid in SC-CO_2 at 60°C and 100 kg/cm^2. See Figure 1 for abbreviations.

Oleic acid was not incorporated into TG when more than 3 g of glycerol was added. This was presumed to be due to interference with the contact between the enzyme and the substrates and also due to dispersion of the enzyme in the reaction mixture because the immobilized lipase was coated when an excess amount of glycerol, which was insoluble in the substrates and SC-CO_2, was added to the reaction mixture. About 95% TG was recovered in these ranges of glycerol concentration, and the TG recovery was hardly influenced by the additional glycerol.

Effect of pressure on interesterification between MCT and oleic acid. Interesterification between MCT and oleic acid was carried out under various conditions for 10 h at 60°C in the presence of 2 g of glycerol (Fig. 6). At up to 100 kg/cm^2, the pressure did not show any effect on the incorporation of oleic acid into MCT 10 and 12. However, when the pressure increased over 100 kg/cm^2, the incorporation ratio in the three systems commonly decreased with the increase in pressure. Based on these results, the increase in pressure above 100 kg/cm^2 retards the interesterification by lipase in these reaction systems.

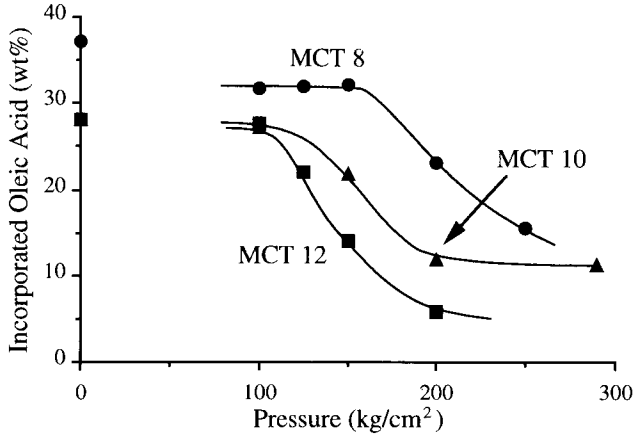

FIG. 6. Effect of pressure on the incorporation of oleic acid into MCT. Interesterification was catalyzed by immobilized *Mucor miehei* lipase with 2 g of glycerol at 60°C for 10 h. See Figure 1 for abbreviation.

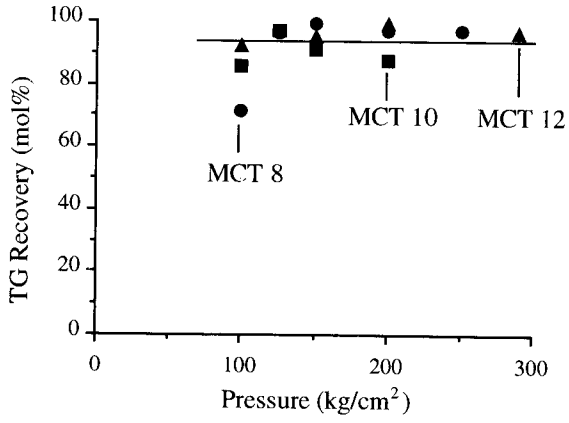

FIG. 7. Effect of pressure on TG recovery after incubation at 60°C for 10 h. See Figure 1 for abbreviations.

Two major factors are suggested to be responsible for the poor incorporation of oleic acid in the higher pressure range. One is the decrease in the partition rate of the substrates in the liquid phase. Another factor is the increase in the reaction volume in the reactor. With increased pressure, the solubility of the substrates in the SC-CO_2 phase increased drastically. This causes dilution of the substrate and, as a result, induces retardation of interesterification. Dependence of the lipase-catalyzed reaction efficiency on the substrate concentration was also confirmed by the model experiments in water-saturated *n*-hexane (data not shown). Deactivation of the enzyme by high-pressure SC-CO_2 was not observed in this case. This will be discussed later.

The recovery of TG after incubation is shown in Figure 7. In most cases, TG recovery in an atmosphere of SC-CO_2 was higher than 85 mol%, and these values were much higher than those of most conventional methods involving reaction in water-saturated *n*-hexane (55 ≈ 75 wt%) (13,14). No supplementation of excess water is suggested to contribute to the high recovery.

Effect of temperature on interesterification. The effect of reaction temperature on the interesterification was studied with the MCT 10–oleic acid system at various pressures (Fig. 8). The reactions were carried out for 10 h at 40, 50 and 60°C.

In the pressure range up to 120 kg/cm², the interesterification proceeded more efficiently at higher temperatures, owing to increasing enzyme activity. In these pressure ranges, the volume of the liquid phase is almost independent of temperature. However, in the pressure range above 150 kg/cm², the incorporation of oleic acid into TG decreased linearly because the solubility of the substrates in SC-CO_2 decreased with rising temperature due to the decrease in the density of SC-CO_2 in these pressure ranges. This decrease in incorporation rate at 60°C was attributable to the change in substrate composition in the liquid phase (data not shown) because the partition rate of the substrates in the liquid phase decreased enormously in the pressure range above 150 kg/cm² at 60°C. For pressures above 200 kg/cm², the incorporation of oleic acid into TG remained almost constant (15%) because the composition and concentration of substrates were close to constant values, although the contents of the reactor approached homogeneity with increased pressure.

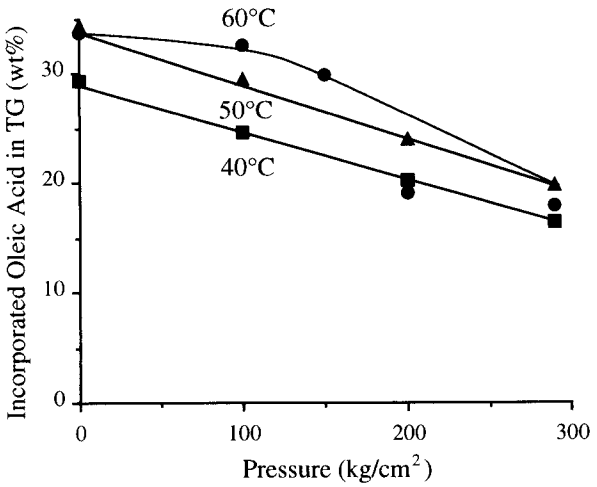

FIG. 8. Effect of temperature on the interesterification between MCT 10 and oleic acid in SC-CO_2 catalyzed by immobilized *Mucor miehei* lipase. See Figure 1 for abbreviations.

Consequently, the interesterification proceeded most efficiently at 60°C, which is similar to the optimum temperature for reactions in buffers (51,52).

Interesterification between EPA and each MCT in SC-CO_2. Incorporation of EPA into MCT was also attempted in SC-CO_2, similarly to that of oleic acid. Figure 9 shows the time course of the interesterification between EPA and three types of MCT in SC-CO_2. The reactions were carried out for 20 h at 60°C and 100 kg/cm² in the closed system. The results were similar to those of reactions in *n*-hexane previously described (Fig. 4). The molar ratios of EPA incorporated into TG did not differ much among the three types of MCT. In the case of oleic acid, two out of three acyl groups of TG were interesterified with oleic acid; however, only one acyl group of MCT could be exchanged with EPA in these reactions. Almost the same result was obtained in the reaction between EPA and

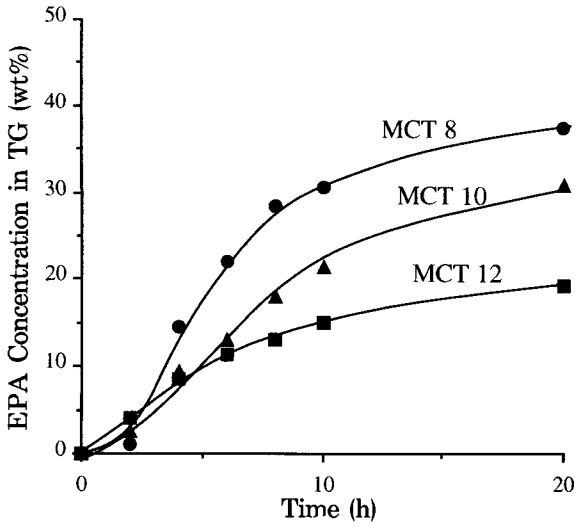

FIG. 9. Time course of the interesterification between EPA and three types of MCT in SC-CO_2 at 60°C and 100 kg/cm^2 for 20 h. See Figure 1 for abbreviations.

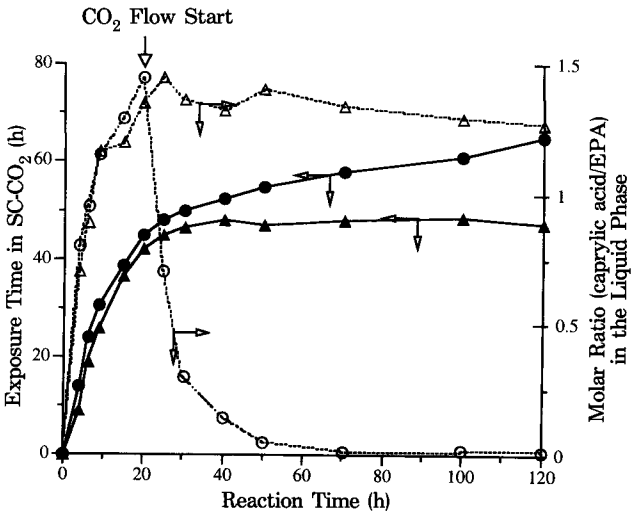

FIG. 10. Time course of the interesterification between MCT 8 (tricaprylin) and EPA at 60°C in an atmosphere of SC-CO_2 at 100 kg/cm^2; ● and ○; with SC-CO_2 extraction; ▲ and △, without SC-CO_2 extraction. See Figure 1 for abbreviations.

MCT 12 in *n*-hexane. These results suggest that the highly folded structure of EPA causes steric hindrance to the reaction.

Based on these studies, the lipase-catalyzed interesterification in the liquid phase under SC-CO_2 conditions was concluded to be remarkably influenced by both pressure and temperature, mainly *via* the changes in substrate concentration in the liquid phase. These results suggest that these enzymatic reactions can be regulated by the modulation of pressure and temperature. For interesterification between MCT and oleic acid, the pressure was preferably not above 150 kg/cm^2. Because liberated medium-chain fatty acids are extractable with SC-CO_2 in these pressure ranges, the interesterification efficiency can be improved by combination with SC-CO_2 extraction, which displaces the equilibrium in the direction of the introduction of long-chain fatty acids into MCT.

Lipase-catalyzed interesterification with continuous SC-CO_2 extraction. To improve the incorporation ratio of oleic acid into TG, we tried a novel lipase-catalyzed interesterification procedure in combination with SC-CO_2 extraction.

Figure 10 shows the time course of the interesterification between MCT 8 and EPA at 60°C and 100 kg/cm^2. Because the solubility of MCT 8 in SC-CO_2 is higher than that of EPA under these conditions, the reaction was started with a closed system to avoid the extraction of unreacted MCT with SC-CO_2. SC-CO_2 extraction was started at 20 h, and the reaction was continued for more than 120 h. The incorporation of EPA into TG rapidly increased with time up to 8 h and gradually approached an equilibrium state of 47 wt% (29.7 mol%). This equilibrium value was lower than the expected value (80.7 wt%), which can be obtained theoretically by the incorporation of EPA completely into both the *sn*-1 and *sn*-3 positions of TG. This was related to the higher reactivity of the generated caprylic acid than of EPA.

In the liquid phase, the generated caprylic acid was rapidly increased with time up to 30 h. The molar ratio of caprylic acid in FFA in the liquid phase was 1.4 times higher than that of EPA. As the SC-CO_2 extraction progressed, the molar ratio of caprylic acid in FFA in the liquid phase was gradually decreased to 1:10 that of EPA at 50 h, while the incorporation of EPA into TG was slightly increased. However, because the fatty acid composition of the extract was 73 wt% caprylic acid and 26 wt% EPA, a certain amount of EPA was extracted with the caprylic acid by SC-CO_2. Therefore, this suggests that it is necessary to improve the separation efficiency of SC-CO_2 extraction for caprylic acid, for example, by utilizing a rectification column (45,48).

At the end of the reaction, 62 wt% (43.7 mol%) of EPA could be incorporated into TG. This value was 32% higher than the 47 wt% equilibrium value in the closed system and was not due to the preferential extraction of unreacted MCT with SC-CO_2 because the incubation of the reaction mixture (enzyme-free preincubation for 100 h) with SC-CO_2 extraction under the same conditions resulted in only a little increase in the EPA incorporation into TG. When the reaction was scaled up to twice that of this experiment with the same flow rate, the incorporation rate of EPA into the equilibrium reaction mixture was decreased to less than one-third that of this experiment. This indicates that the interesterification is highly dependent on the rate of the caprylic acid extraction with SC-CO_2. The mixture of TG, reacted for 120 h, contained 27 mol% of 2-caprylil-1,3-eicosapentaenoyl-glycerol. However, only one acyl group of MCT 8 was exchanged with EPA in the reaction without SC-CO_2 extraction.

These results suggested that the equilibrium state of the enzymatic interesterification could be shifted by selective extraction of the by-product with SC-CO_2. TG recovery of 85 ≈ 92 mol% by this method was appreciably improved when compared with that of conventional enzymatic methods (55 ≈ 75 wt%) (13,14).

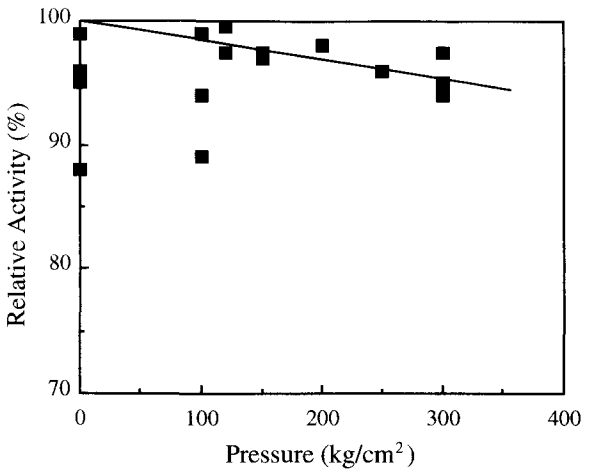

FIG. 11. Effect of pressure on the enzyme stability in SC-CO_2 at 60°C. See Figure 1 for abbreviations.

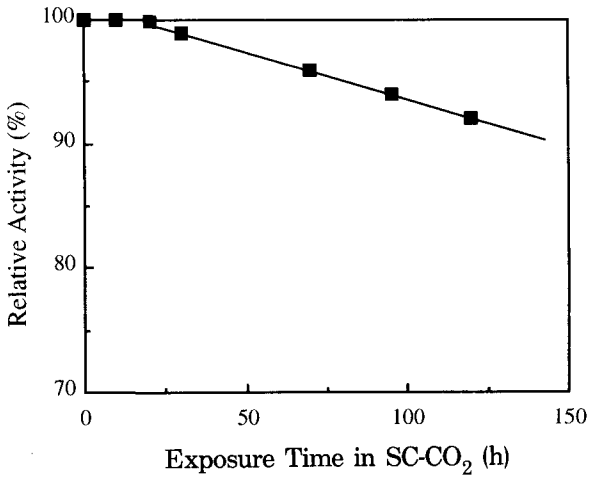

FIG. 12. Effect of exposure time in an atmosphere of SC-CO_2 on the enzyme stability at 60°C and 100 kg/cm². See Figure 1 for abbreviations.

Enzyme stability. Figure 11 shows the comparison of the activity of immobilized lipase recovered after the reaction at 60°C for 10 h under various SC conditions. The incubation of lipase in an atmosphere of SC-CO_2 at up to 300 kg/cm² at 60°C for 10 h did not seriously damage the activity. However, the incubation in SC-CO_2 at 100 kg/cm² and 60°C for more than 70 h slightly impaired the activity, although this inactivation may be mostly due to the long heating time (Fig. 12). Occurrence of some structural changes in lysozyme preparations after heating in SC-CO_2 for a long period was reported by Weder (26,27). Judging from these experimental results, immobilized *M. miehei* lipase is fairly stable under SC-CO_2 conditions.

In the future, to improve the separation efficiency of SC-CO_2 and to reduce the loss of extraction, we will investigate the lipase-catalyzed interesterification procedure in combination with SC-CO_2 extraction equipped with a rectification column. In this study, the reaction rate was not exactly analyzed on the basis of reaction engineering. The exact feature of the kinetics is a future subject to be elucidated. The physical properties and solubility data should also be elucidated in the analysis of the reaction rate. In addition, determination of a high-activity and high-stability lipase under SC conditions is most important.

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