Production of Medium-Chain Glycerides by Immobilized Lipase in a Solvent-Free System

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Enzymatic synthesis of medium-chain glycerides (MCGs) was studied by using capric acid (decanoic acid) and glycerol as substrates for immobilized lipase (Lipozyme™) without any solvents or surfactants. Quantitative analysis of the reaction mixture was conducted by using high-performance liquid chromatography (HPLC), which enabled the exact tracing of the capric glyceride synthesis. Oleic acid was also used for comparison. The esterification activity of Lipozyme was determined at 40°C in an open batch reactor; the activities were 400 and 200 units/g for the capric glyceride and oleic glyceride synthesis, respectively. Maximum initial reaction rate was obtained at 50°C for capric and 60°C for oleic glyceride synthesis. The time course of the capric glyceride synthesis was compared in terms of different molar ratios, from which we infer that this enzyme is 1,3-specific, but not absolute, in this esterification reaction. The final conversion was greatly influenced by the methods used to remove water, among which the cold trap method resulted in a noticeable improvement.

KEY WORDS: Esterification, immobilized lipase, Lipozyme, MCT, medium-chain glyceride, regiospecificity, reversed phase HPLC.

Enzyme catalysis in organic solvents has been studied extensively for fat hydrolysis (1,2), interesterification (3–5), and syntheses of esters (5–7), peptides (8,9) and optically active compounds (10). Free or immobilized enzymes were used in two-phase (4,7,9,10), reversed phase (2,3,5,8) and reversed micelle systems (1,6), where the organic substrates are dissolved in the solvent phase. However, commercial scale-ups of such new enzymatic catalyses have seldom been reported. One reason for this is that toxic and expensive solvents and/or surfactants are being employed that ought to be avoided, especially if the products are intended for use as foods or food ingredients.

Recently, several workers have reported enzymatic catalyses in solvent-free systems in an attempt to make the processes commercially feasible. These efforts not only excluded the toxicity problem of the solvents and surfactants, but also reduced many steps in the purification process. (11–14). Although enzymes used in a solvent-free system have several advantages, the application is quite limited because most organic substrates have high melting points and the enzyme is not stable at a higher temperature. The medium-chain glycerides (MCGs) studied here are mono-, di- and triglycerides of caprylic acid (octanoic acid) and capric acid (decanoic acid). Medium-chain monoglyceride is used as a solvent for aromatics, steroids, dyes and perfume bases in the cosmetics, toiletries and pharmaceutical field. A mixture of medium-chain monoglyceride and diglyceride was found to be an effective solvent for dissolving cholesterol gallstones in humans. Medium-chain triglyceride (MCT) has been used as a nutritional supplement for patients suffering from malabsorption caused by intestinal resection or diseases, and as a component for infant feeding formula (15–20). It is also used as a solvent or a carrier of lipophilic nutrients or drugs such as vitamin K (21) and phospholipid (22), and as a base material for edible films or edible lubricants for foods and food processing. The commercial manufacturing process of such MCGs is a direct esterification of medium-chain fatty acids and glycerol at high temperature and high pressure, followed by alkali washing, steam refining, molecular distillation, ultrafiltration and activated carbon treatment for the purification of the product (23). In order to study the feasibility of the enzymatic MCGs manufacturing process, we examined the characteristics of an MCG's synthesis reaction by using glycerol, capric acid and immobilized lipase in a solvent-free system. In this study, only capric acid was used for a quantitative tracing of the reaction.

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EXPERIMENTAL PROCEDURES

Materials. A commercial product called Lipozyme™, IM-20, (donated by Novo Industri A/S, Copenhagen, Denmark) was used. This is a fungal lipase (EC 3.1.1.3) from *Mucor miehei* immobilized on a macroporous anion exchange resin. Capric acid, molecular sieves 5A, and authentic glycerides for high-performance liquid chromatography (HPLC) analysis such as 1(3)-monocaprin, 1,2-dicaprin, 1,3-dicaprin and tricaprin were purchased from Sigma Chemical Co. (St. Louis, MO). Oleic acid was obtained from Wako Pure Chemicals (Osaka, Japan). Glycerol was purchased from Mallinckrodt, Inc. (Paris, KY).

Analytical methods. The capric glycerides in the reaction mixture were analyzed by using a Waters Associates (Milford, MA) high-performance liquid chromatograph equipped with a model 6000 A solvent delivery system, R 401 RI detector, M 680 automated gradient controller and M 740 data handling system. The column was a Waters Radial-Pak μ bondapak C_{18} (8 mm i.d. × 10 cm L, 10 μm) and used with a Z-module (radial compression separation system). The mobile phase was acetonitrile-acetic acid (94:6, v/v). The flow gradient was applied as follows: 1 mL/min for 7 min, increased up to 2 mL/min linearly in 1 min, held at 2 mL/min for 8 min, decreased back to 1 mL/min linearly in 1 min, and held at 1 mL/min for another 3 min before the next injection. Acetone was used as a sample-dissolving solvent, and injection volume was 10 μL. A quick measurement of residual fatty acid concentration in the reaction mixture was done by the cupric-acetate method (24).

Determination of esterification activity of Lipozyme. Fifty mmoles of fatty acid and 16.7 mmoles of glycerol were mixed vigorously by a magnetic stirrer (400 rpm) in an open glass vial (30 mm i.d. × 85 mm L) which was silanized before use (25). The reaction was started by adding 0.1 g of Lipozyme to the reaction mixture, which had been thermally equilibrated to 40°C in a water bath.

Moisture produced was allowed to evaporate spontaneously during the reaction. Aliquots (40 μL) were taken from the vial at predetermined intervals and diluted in 3.96 mL of isooctane, in which residual fatty acid was checked by the cupric-acetate method. The initial reaction rate was calculated from the slope drawn at the beginning of the progress curve of residual fatty acid concentration. One esterification unit of Lipozyme was defined as 1 μmole of fatty acid consumed per minute under the experimental conditions.

Batch reactor. The batch esterification reaction was carried out under the above-mentioned conditions at various temperatures by using capric acid or oleic acid. The batch reactions with different molar ratios of capric acid to glycerol (1:1, 3:1 and 9:1) were conducted at 40°C.

Loop reactor. In order to overcome the breakage of enzyme matrix in a batch reactor, we prepared a loop reactor. Three grams of Lipozyme were packed in a vertical column (18 mm i.d. × 15 cm, water-jacketed) and 900 mmoles of capric acid and 300 mmoles of glycerol were mixed vigorously in an open reservoir. The reaction mixture was then recirculated by a gear pump (Micropump Co., Concord, CA) at 30 mL/min through the column and back to the reservoir. All of the lines, pump, column and the reservoir were water-jacketed and insulated. Moisture produced was allowed to evaporate from the reservoir.

Water-removing methods. The performance of a cap-sealed reactor (no removal of water) and a cap-opened reactor (spontaneous evaporation of water) were compared. In order to remove water rapidly, several methods were employed for a batch reaction (200 mmoles of fatty acid and 66.7 mmoles of glycerol with 0.4 g of Lipozyme in a 150-mL glass vial) solely or in combination with others. The following methods were used: vacuum (5 cm Hg absolute), bubbling air into the reactor, air recirculation through a molecular sieve column and cold trap (−5°C).

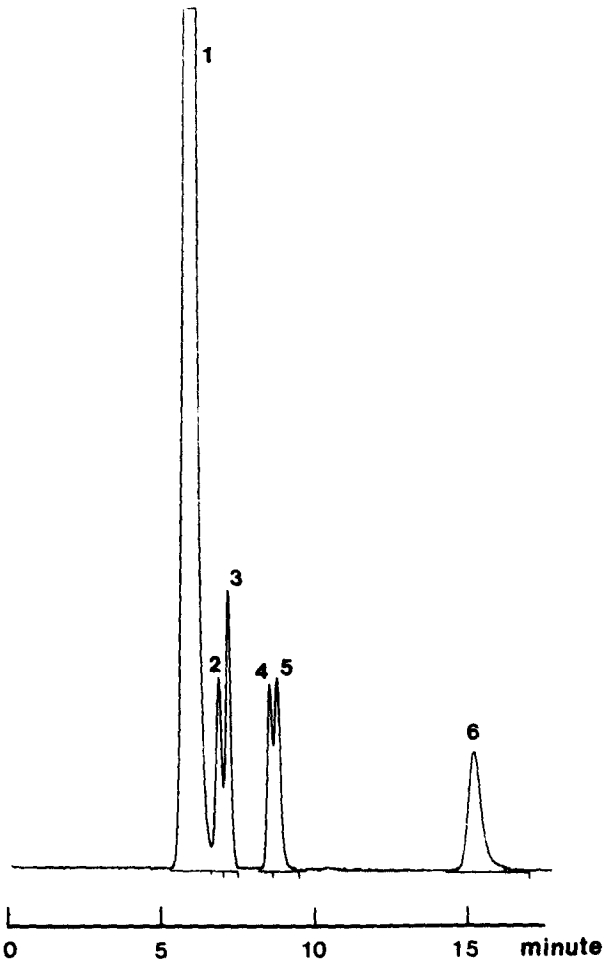

FIG. 1. HPLC chromatogram of capric acid and capric glycerides in a Waters μ bondapak C_{18} column (8 mm i.d. × 10 cm L, 10 μm), a RI detector was used; mobile phase, acetonitrile-acetic acid (94:6, v/v); flow gradient was used (see text); dosage, 0.75 μmole of capric acid, 0.3 μmole of capric glycerides in 10 μL acetone. [1] acetone, [2] 1-monocaprin, [3] capric acid, [4] 1,3-dicaprin, [5] 1,2-dicaprin, [6] tricaprin.

RESULTS AND DISCUSSION

Esterification activity. The esterification activity of Lipozyme for capric glycerides in a solvent-free system was found to be about 400 units per gram (40°C). For the case of oleic glycerides, the activity was about 200 units per gram (40°C). By comparison, the interesterification activity of this enzyme is reportedly 31 BIU (Batch Interesterification Unit) per gram, where 1 BIU is defined as 1 μmole of incorporated palmitic acid into triolein per minute at 40°C in petroleum ether (26). The hydrolytic activity was about 100 units per gram, where 1 unit is defined as 1 μmole of free fatty acid liberated from olive oil per minute at 37°C in an emulsion system [5% (v/v) of olive oil emulsified by 5% (w/v) of gum arabic at pH 7.0].

HPLC analysis. Figure 1 represents the chromatogram of capric acid and its glycerides. The retention time of each component was as follows (mean value ± standard deviation based on 10 samples): 1-monocaprin (6.15 min ± 0.02), capric acid (6.60 min ± 0.03), 1,3-dicaprin (8.93 min ± 0.03), 1,2-dicaprin (9.46 min ± 0.03) and tricaprin (15.69 min ± 0.10). But the retention time of 2-monocaprin could not be identified because the authentic sample was not available. From the chromatogram of a reaction mixture (not shown), a peak of 1,2-dicaprin was identified, although the peak area was comparatively small (less than 5% of 1,3-dicaprin). A small amount of tricaprin was also detected at the end of the reaction. These observations support the view that Lipozyme has 1,3-specificity, but the specificity is not so strict in this esterification reaction. In other words, the esterification reaction at the 2-position of glycerol may take place, but the rate is much slower than that of the 1- and/or 3-position. It is notable that triolein was produced from oleic acid and glycerol in a yield of 80–90% based on oleic acid by using Lipozyme (14). McNeill *et al.* (27) also suggested that this enzyme does not possess absolute regiospecificity in the glycerolysis of beef tallow.

Effect of varying molar ratio. The esterification reactions were performed with molar ratios of 1:1, 3:1 and 9:1 of capric acid to glycerol in open batch reactors at 40°C. The product composition was analyzed by HPLC. For a ratio of 1:1 (glycerol-excess condition), the conversion defined as the percentage of consumed capric acid was close to 90% at the end of the reaction (Fig. 2). Monocaprin was produced at about twice the concentration of dicaprin, but slowly decreased after 60 hr. In the meantime, dicaprin increased gradually throughout the reaction and

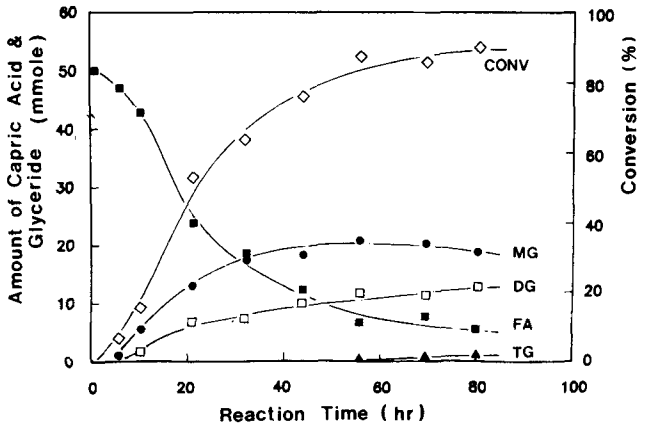

FIG. 2. Time course for the synthetic reaction of capric glycerides (molar ratio = 1:1). Reaction mixture contained 50 mmoles of capric acid, 50 mmoles of glycerol and 0.1 g of Lipozyme in an open batch reactor at 40°C. (■) FA, capric acid; (●) MG, monocaprin; (□) DG, dicaprin; (▲) TG, tricaprin; (◇) CONV, conversion.

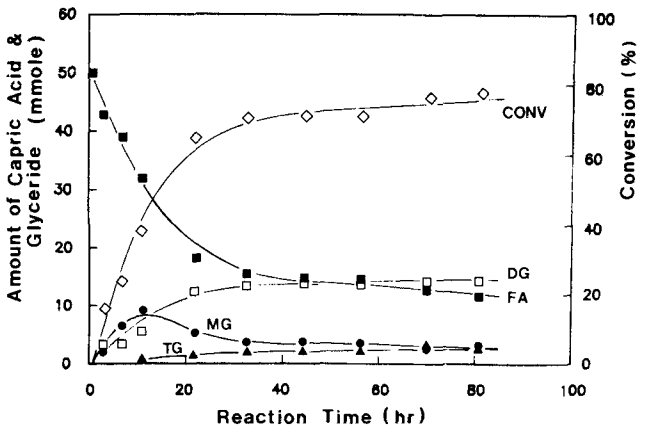

FIG. 3. Time course for the synthetic reaction of capric glycerides (molar ratio = 3:1). Reaction mixture contained 50 mmoles of capric acid, 16.7 mmoles of glycerol and 0.1 g of Lipozyme in an open batch reactor at 40°C. Symbols are the same as in Figure 2.

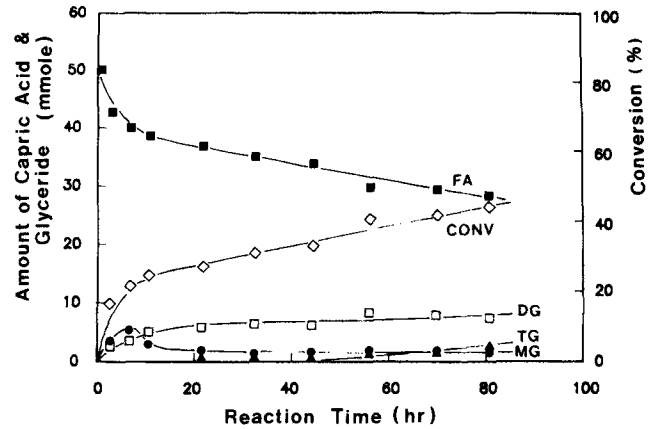

FIG. 4. Time course for the synthetic reaction of capric glycerides (molar ratio = 9:1). Reaction mixture contained 50 mmoles of capric acid, 5.6 mmoles of glycerol and 0.1 g of Lipozyme in an open batch reactor, at 40°C. Symbols are the same as in Figure 2.

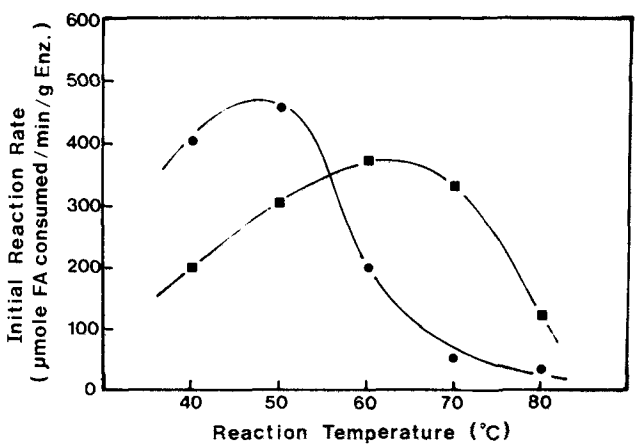

FIG. 5. Effect of temperature on the initial reaction rate of Lipozyme. Reaction mixture contained 50 mmoles of fatty acid, 16.7 mmoles of glycerol and 0.1 g of Lipozyme in an open batch reactor. (●), capric acid; (■), oleic acid.

a trace amount of tricaprin was produced at the end of the reaction. About 40% of glycerol remained to the last, as calculated from the total amount of glycerides. When the ratio was changed to 3:1 (stoichiometric ratio), monocaprin showed a maximum peak at an early stage of the reaction and decreased down to one-fifth of the concentration of dicaprin at the end of the reaction (Fig. 3). Tricaprin was produced earlier and the final conversion was about 80%. Glycerol was almost completely consumed at 20 hr. It should be noted that the time course of this reaction looked similar to the typical pattern of a consecutive reaction, in which the last reaction step is rate-limiting (28). When the ratio was increased to 9:1 (capric acid-excess condition), the progress curves of the glycerides were similar to the case of 3:1. Final conversion, however, fell down to 40%, probably because the glycerol was exhausted at an early stage of the reaction (Fig. 4). We believe that evaluation of kinetic constants of each reaction step would be possible through the dynamic analysis of these data and this deserves further studies.

Temperature effect. The initial reaction rate and final conversion were measured at various temperatures in an open batch reactor. The maximum initial reaction rate was obtained at 50°C and 60°C for capric and oleic glyceride synthesis, respectively (Fig. 5). The final conversion, however, may depend to some extent on the degree to which the enzyme matrix was mechanically broken. In order to check the inactivation of enzyme, we separated the broken enzyme matrices by centrifugation after a 70-hr operation, put them into a new substrate mixture and measured the residual activity. In the case of the capric glyceride synthesis, the enzyme matrices were easily broken above 50°C, and residual activity was not detected after a 70-hr operation at that temperature. But in the case of oleic glyceride synthesis, the enzyme matrices were somewhat less broken and residual activity was about 80% of initial value after a 70-hr operation even at 60°C. These observations suggest that the stability in a batch reactor is largely dependent on the matrix susceptibility to mechanical abrasion as well as to the

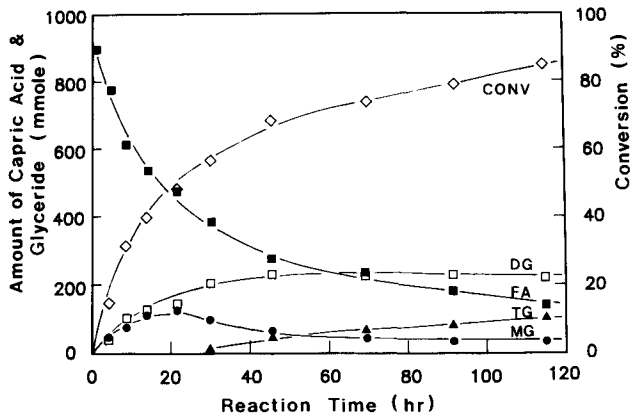

FIG. 6. Time course for the synthetic reaction of capric glyceride in a loop reactor. Reaction mixture contained 900 mmoles of capric acid, 300 mmoles of glycerol and 3.0 g of Lipozyme, at 50°C. Symbols are the same as in Figure 2.

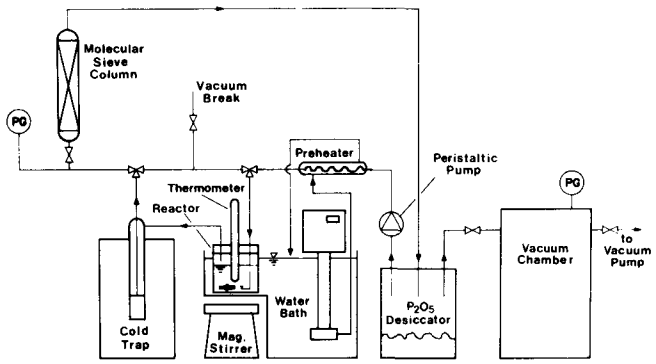

FIG. 7. Schematic drawing of water-removing system. Air was recirculated by a peristaltic pump through a preheater and bubbled into the reactor. The effluent flew through a cold trap (−5°C), molecular sieve column and back to the P_2O_5 desiccator. All the lines were kept under vacuum (5 cm Hg absolute).

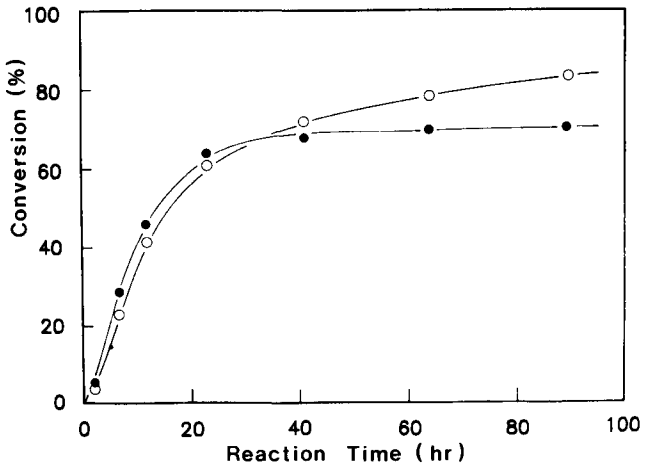

FIG. 8. Effect of water removal on the synthetic reaction of capric glycerides. Reaction mixture contained 200 mmoles of capric acid, 66.7 mmoles of glycerol and 0.4 g of Lipozyme at 40°C. (●), open reactor; (○), water removal with cold trap.

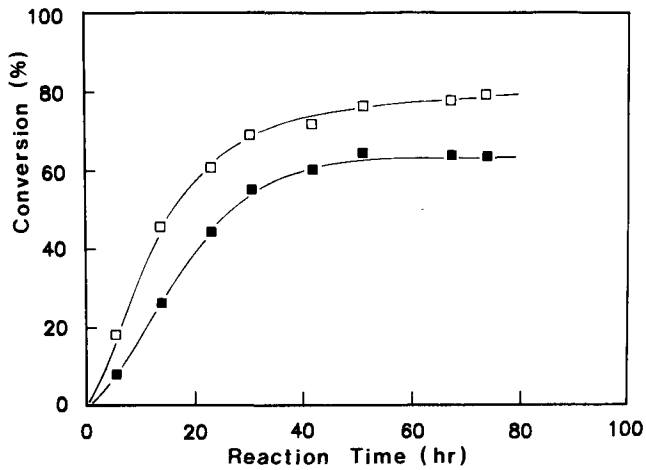

FIG. 9. Effect of water removal on the synthetic reaction of oleic glycerides. Reaction mixture contained 200 mmoles of oleic acid, 66.7 mmoles of glycerol and 0.4 g of Lipozyme, at 50°C. (■), open reactor; (□), water removal with cold trap.

physical properties of the substrate. Thus, we examined stability in a loop reactor where the matrix is not subjected to mechanical stress. The time course in a loop reactor was about the same as in the batch reactor (Fig. 6), except that the conversion and tricaprin concentration kept increasing even after the conversion was close to 85%. It must be noted also that nearly 90% of the initial activity of Lipozyme was retained even after 10 batches of capric glyceride synthesis at 50°C. Further research will be conducted to examine the stability and productivity as a function of the operating temperature with this loop reactor.

Water-removing effect on the esterification reaction. In order to shift the equilibrium toward synthesis in the esterification reaction, various water-removing methods have been employed by several workers. Positive results were obtained by recirculating the product mixture (14,29) or headspace gas (30) through the molecular sieve column. Vacuum and dry air-bubbling were applied for forced dehydration (14). When we conducted capric glyceride synthesis in a closed batch reactor, the final conversion was only 50% at 40°C, while the open reactor gave a final conversion of 80% under the same conditions. Certainly this difference is due to the law of mass action. When we applied vacuum alone in a batch reactor, there was no significant difference compared to the open system. When headspace gas was recirculated through the molecular sieve column and bubbled into the reactor under vacuum, there was also no noticeable effect during the reaction. However, near the end of the reaction the conversion was increased slightly. These results suggested that the water absorption rate of molecular sieves might not be fast enough and prompted us to employ a more powerful water-removing method. We added a cold trap at the exhaust line of the reactor in an attempt to remove water more rapidly (Fig. 7). This system gave a considerably higher final conversion. The progress curves of the capric glyceride synthesis were generally the same during the reaction. However, the final conversion was about 15% higher than in the open system (Fig. 8). For oleic glyceride synthesis, a 20% increase in final conversion was observed (Fig. 9). It is of interest, however, that two curves were

quite different from the beginning of the reaction. Although it seems likely that this difference may be related to the water solubilities of each fatty acid, further investigations are required to explain these differences.

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