Glyceride Synthesis in a Solvent-Free System

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ABSTRACT: Synthesis of partial glycerides in a solvent-free system has been investigated with various acyl donors and glycerol as substrates and a 1,3-specific immobilized lipase to catalyze the reaction. Capric acid was the most efficient acyl donor, compared with ethyl caprate and tricaprin. However, to obtain a high yield of dicaprin and a low amount of tricaprin, ethyl caprate was the acyl donor of choice. The composition of the product mixture was determined by the ratio of ethyl caprate to glycerol; a molar ratio of 3:1 was optimum for dicaprin synthesis. The water content in glycerol did not influence the final yield of dicaprin, but initial production of capric acid increased with increasing water content. The reaction was found to be controlled entirely by external mass transfer. The yield of diglyceride could be increased from 70 to 90% by lowering the reaction temperature, so that the diglyceride precipitated during the reaction.

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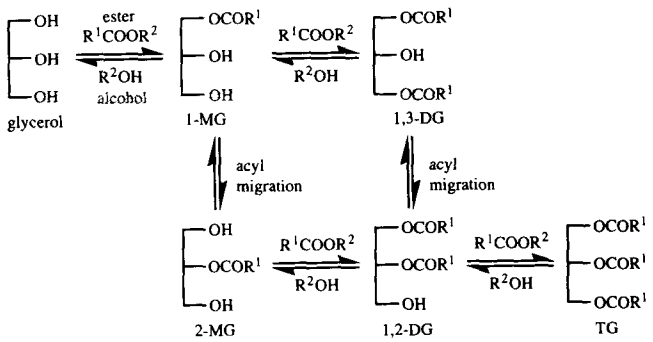
KEY WORDS: Acyl donors, glyceride synthesis, lipase, solvent-free.

Partial glycerides, in particular monoglycerides, are commonly used as food emulsifiers (1) because of their excellent emulsifying properties, low odor, lack of taste, biodegradability, and safety. They are also common in the pharmaceutical and cosmetic industries. Diglycerides have thus far found limited use in industry, but potential applications are found in the food industry where they can be used to improve the texture of margarine (2). Further, diglycerides can be used as starting materials for prodrugs (3–5) and in organic synthesis of lipids (6,7). At present, partial glycerides are produced by chemical glycerolysis, which gives rather low yields and products of poor quality which require purification before use (8). An alternative method would be to use lipases as the catalyst for glyceride synthesis. This has been a growing area of research during the last decades, and some industrial processes that employ lipases for lipid transformations are now in operation.

The biological reaction catalyzed by lipases is hydrolysis of triglycerides, but if the amount of water present is reduced, the reverse reaction can take place (9). Lipases work well under these unnatural conditions and have been used for various types of ester synthesis: resolution of racemic alcohols (10,11) and synthesis of fatty acid esters (12–14) and glycerides (15–23). The reactions that take place during glyceride synthesis are shown in Scheme 1. The acyl donor, fatty acid, or fatty acid ester in Scheme 1 can also be replaced by triglyceride, and the process is then called glycerolysis. Lipases can be either nonspecific or 1,3-specific towards the hydroxyl groups on glycerol, i.e., the lipase catalyzes reactions on all hydroxyl groups of glycerol, or on only the primary hydroxyl groups, respectively. By choosing a 1,3-specific lipase for glyceride synthesis, the expected products are 1-monoglyceride and 1,3-diglyceride. However, spontaneous acyl migration, i.e., the intramolecular transfer of a fatty acid moiety from one hydroxyl to an adjacent one, may occur and give rise to 2-monoglyceride and 1,2-diglyceride, respectively. These compounds can then act as acyl acceptors in the lipase-catalyzed reaction (Scheme 1).

Partial glycerides have been produced in a number of ways with lipases. Triglycerides in organic solvent have been hydrolyzed or alcoholized by 1,3-specific lipases to yield 2-monoglycerides (24–26) or 1,2-diglycerides (27). Synthesis, starting from glycerol and an acyl donor in the presence of solvent, can be accomplished (19–23,28), and yields are then highly improved by precipitation of the partial glycerides during the reaction (20,22), or by adsorption of monoglyceride on silica (21). The choice of solvent has a strong influence on the reaction equilibrium (28). Free fatty acid is the most common acyl donor, but fatty acid esters (23,27), vinyl esters (27), and triglycerides (22,29) can also be used as acyl donors. The vinyl esters have the advantage of an irreversible reaction, but the by-product acetaldehyde may inactivate the enzyme (30).



SCHEME 1

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These reactions can also be conducted without solvent, which is an advantage if the products are intended for use in foods. Glycerolysis of triglycerides has been employed for preparation of both mono- and diglycerides (31,32). *De novo* synthesis from fatty acid and glycerol has also been studied in solvent-free systems (17,18).

The purpose of this study is to investigate how the reaction and product composition are influenced by the various reaction parameters and the type of acyl donor, with the aim of achieving a high yield of diglyceride. A solvent-free system was chosen because the products have a potential use in foods. We have used acyl donors, such as ethyl ester, free fatty acid, and triglyceride, and glycerol as acyl acceptor. To be able to obtain a reaction system that is liquid for all donor types studied, we chose to work with capric acid derivatives. The reaction was catalyzed by an immobilized 1,3-specific lipase from *Rhizopus arrhizus*.

MATERIALS AND METHODS

Chemicals. Lipase (triacylglycerol acyl hydrolase, EC 3.1.1.3) from *R. arrhizus* was a generous gift from Gist-Brocades (Delft, Netherlands). EP100 (porous polypropylene particles, 200–400 μm) was a gift from AKZO (Obernburg, Germany). Ethyl caprate, capric acid, monocaprin, tricaprin, and glycerol (99%) were purchased from Sigma (St. Louis, MO). Derivatization reagent MSHFBA (*N*-methyl-*N*-trimethylsilyl-heptafluoro-butyramide) was obtained from Macherey & Nagel (Düren, Germany). 1,3-Dicaprin was prepared in our laboratory and was 99% pure by gas chromatography (GC). Ethyl palmitate was prepared in our laboratory and was >99% pure by GC. Other chemicals were of analytical grade.

Immobilization of lipase. One gram lipase powder was dissolved in 20 mL phosphate buffer pH 6.0, 20 mM. The solution was added to 1.0 g EP100, pre-wet with 3 mL ethanol, and incubated overnight on an end-to-end incubator at room temperature. The immobilized enzyme preparation was collected by filtration and washed several times with water and phosphate buffer pH 7.0, 20 mM and dried under vacuum overnight. The immobilized enzyme preparation is referred to in the following as the lipase preparation.

Synthesis reaction. Ethyl caprate (3.0 g, 15.0 mmol) and an appropriate amount of glycerol, containing 4% water, to give the desired molar ratio of ester to glycerol, were mixed in an uncapped reaction vessel to allow free evaporation of ethanol produced during reaction. The substrates were incubated at 40°C in an incubator. The two phases were mixed by magnetic stirring. The reaction was started by adding the lipase preparation (150 mg). At intervals, samples (~20 μL) were withdrawn for analysis. Capric acid (2.58 g, 15 mmol) and tricaprin (2.77 g, 5 mmol) were used as substrates in the same manner.

Analysis. The samples were immediately diluted with 1 mL methyl *tert*-butyl ether, and the enzyme particles were removed; 5–10 μL of this solution was taken out for analysis by gas chromatography. The samples were dried under nitrogen and then derivatized by addition of 10 μL MSHFBA. The derivatization reagent converts all hydroxyl groups of the different glyceride species to the corresponding trimethylsilyl ethers. After addition of MSHFBA, the samples were kept for 15 min at room temperature and were then diluted with 1 mL hexane. Dry ethanol (15 μL) was added to react with any excess MSHFBA. Analysis was carried out on a Varian 3400 gas chromatograph (Palo Alto, CA), equipped with a septum-equipped programmable injector, an 8035 autosampler and a flame ionization detector. An eight-meter DB-1 column (0.32-mm diameter, 0.15-μm film thickness) from J&W (Folsom, CA) was fitted to the high-performance insert of the injector. Helium was used as carrier gas at constant pressure (75 kPa). The injector was programmed from 70 to 300°C at 40°C/min. The column was held at 70°C for 0.3 min; then the temperature was increased to 300°C at 13°C/min. The detector temperature was 350°C. With this method, all glyceride species, i.e., ester, fatty acid, monoglyceride, diglyceride, and triglyceride, were detected. The different positional isomers, 1(3)- and 2-monocaprin and 1,3- and 1,2(2,3)-dicaprin, could be separated. Response factors were determined by using an equimolar standard mixture of ethyl ester, fatty acid, 1-monoglyceride, 1,3-diglyceride, and triglyceride.

RESULTS AND DISCUSSION

Reaction progress. The reaction was initiated by addition of lipase preparation to ethyl ester and glycerol, which constituted a two-phase system. Stirring was vigorous to disperse the two phases in one another. The enzyme particles had a tendency to adhere to the walls of the vessel; one possible explanation for this phenomenon could be that the particles were covered with glycerol. When a small amount of partial glycerides had been produced, a one-phase system was formed. 1-Monocaprin was first synthesized, and then further esterified to yield 1,3-dicaprin (Fig. 1). In addition, a small amount of ethyl caprate was hydrolyzed to capric acid. Furthermore, 1,2-dicaprin and tricaprin were also formed, but in small amounts. Because the enzyme is 1,3-specific, the origin of tricaprin did not result only from enzymatic reaction: 1-monocaprin and 1,3-dicaprin undergo acyl migration, and 2-monocaprin and 1,2-dicaprin are formed, respectively. These compounds can be used subsequently as acyl acceptors by the enzyme, and 1,2-dicaprin and tricaprin are formed (Scheme 1). The acyl migration is a spontaneous reaction, which can be catalyzed by acids, bases, and heat (33,34). The enzyme support material can also affect acyl migration if it has an acidic or basic surface, such as silica, celite, and ion exchange resins. We used polypropylene as support material, and this material does not affect acyl migration (34). Only small amounts of 2-monocaprin could be detected, suggesting that this compound is quickly esterified to 1,2-dicaprin or formed in small amounts. At equilibrium, a mixture of pure monoglycerides consists of ~10% 2-monoglyceride and 90% 1-monoglyceride; thus the driving force for formation of 2-

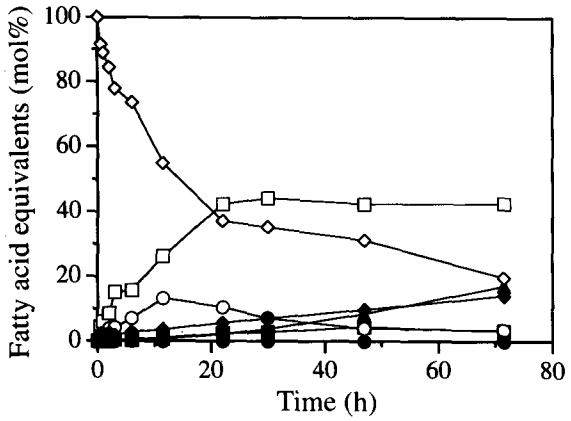

FIG. 1. Reaction progress of lipase-catalyzed glyceride synthesis. The reaction mixture consisted of 15 mmol ethyl caprate and 5 mmol glycerol, containing 4% water, in an open reactor. The temperature was 40°C, and 150 mg lipase preparation was used to catalyze the reaction. Ethyl caprate (◇). capric acid (◆), 2-monocaprin (●), 1-monocaprin (○), 1,2-dicaprin (■), 1,3-dicaprin (□), and tricaprin (▲).

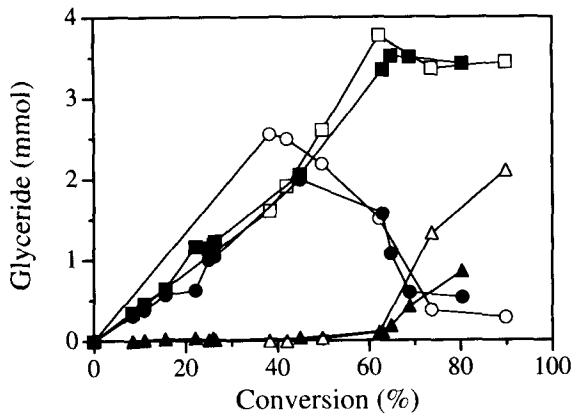

FIG. 2. Comparison of synthesis of capric glycerides, starting from ethyl caprate (filled symbols) or capric acid (open symbols). The reaction mixture consisted of 5 mmol glycerol (4% water) and 15 mmol ethyl caprate or capric acid. The reactions were conducted in open reactors at 40°C, and 150 mg lipase preparation was used. Monocaprin (●, ○), dicaprin (■, □), and tricaprin (▲, △).

monocaprin in this system is small. An equilibrium that corresponds to the selectivity of the enzyme, which would theoretically involve only ester, fatty acid, 1-monoglyceride, and 1,3-diglyceride, was not reached due to acyl migration. To obtain a desired product mixture, the reaction must be stopped at the proper conversion.

Acyl donor. Ethyl caprate, capric acid, and tricaprin were compared as substrates in an open reaction system. Capric acid was the most efficient acyl donor, followed by tricaprin, whereas ethyl caprate gave an initial reaction rate approximately six times lower than that of the acid (Table 1). The dicaprin content at the same conversion was approximately equal for ester and acid as substrates (Fig. 2), but enhanced tricaprin production occurred when acid was used as substrate. This implies that acyl migration is slower when ester is used as substrate (35). The higher reaction rate might be a K_m effect; it has been shown that this lipase has a higher value of K_m for ester than for acid (Wehtje, E., and P. Adlercreutz, submitted for publication). The removal of water from the reaction mixture can be expected to be more difficult than removal of ethanol because ethanol is more volatile than water. However, this may have been counteracted by the K_m effect. Moreover, ethanol has better solubility in the organic phase than water, and ethanol may have a harmful effect on the enzyme. It has been shown previously that increasing ethanol concentration decreases the activity of the *R. arrhizus* lipase (26). Because the concentration of ethanol will be higher than the concentration of water in the organic phase, ethanol may have a stronger effect on the equilibrium in the organic phase, reducing the driving force for glyceride synthesis.

TABLE 1
Acyl Donors for Glyceride Synthesis[a]

Acyl donor	Donor/glycerol molar ratio	Initial rate (μmol donor/h · mg)	Maximum dicaprin yield (mmol)
Ethyl caprate	3:1	9.6	3.6
Capric acid	3:1	63	3.8
Tricaprin	2:1	27	3.6

[a]Experiments were conducted as described in the Materials and Methods section.
[b]Theoretical maximum yield was 5 mmol for ethyl caprate and capric acid as substrates, and 7.5 mmol for tricaprin as substrate.

The reactions with ester and capric acid as substrates were not allowed to reach equilibrium because higher amounts of triglyceride would then be present. Despite the fact that the initial reaction rate was lower with tricaprin than with capric acid, the reaction with tricaprin as substrate reached equilibrium after only 4 h. The lower initial reaction rate was compensated by the fact that the fatty acid moieties were already esterified with glycerol, and only one third of the fatty acid moieties would have to take part in any reaction to give a theoretically complete conversion to dicaprin. After equilibrium between the glyceride classes had been reached, acyl migration among the diglycerides continued and reached an equilibrium after 72 h, the composition of the dicaprin was then 32% 1,2-dicaprin and 68% 1,3-dicaprin.

For efficient preparation of dicaprin, capric acid was a better substrate for the enzyme and can be useful for preparation of dicaprin if the presence of triglyceride in the product does not pose a disadvantage. The yield of dicaprin with tricaprin as substrate could probably be increased by changing the reaction temperature, so that the product would precipitate (32). If pure diglycerides are desired, ethyl ester is preferred, because the tricaprin content is less than when starting from capric acid. In addition, precipitation of glycerides from remaining ester is easier to accomplish than a similar separation from fatty acid.

The ratio of ester to glycerol. Experiments were conducted at different molar ratios of ethyl caprate to glycerol in open vessels at 40°C to determine the product composition at the selected end point of reaction. The values in Figure 3 were

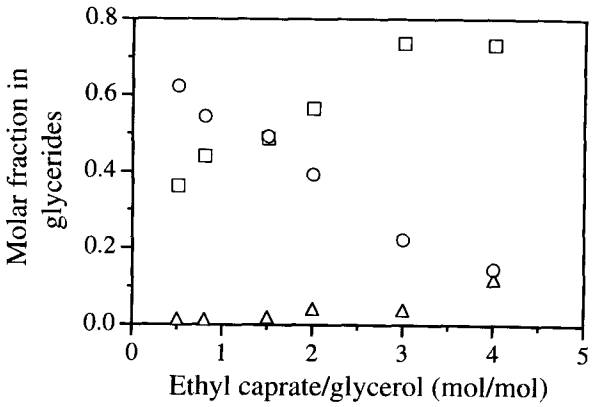

FIG. 3. The composition of the glyceride mixture at varying ratios of ethyl caprate to glycerol. The reaction mixture consisted of 15 mmol ethyl caprate and varying amounts of glycerol (4% water). The reactions were conducted in open reactors at 40°C, and 150 mg lipase preparation was used. The values represented in the figure were taken after ~100 h. Monocaprin (○), dicaprin (□), and tricaprin (△).

obtained when the highest yield of dicaprin was reached (this occurs after ~100 h) and are calculated on the glyceride species; the ester and fatty acid are left out because the excess of ester varies. If the reaction is still proceeding at this moment, mainly conversion of dicaprin and monocaprin to tricaprin is taking place. As can be observed from Figure 3, monocaprin is the dominating product at a low ratio of ester to glycerol. The fraction of monocaprin is decreased when the ratio of ester to glycerol increases. The fraction of both dicaprin and tricaprin is increased when the ratio of ester to glycerol is increased to 3:1; subsequently, the fraction of dicaprin decreased slightly. Accordingly, the product mixture obtained is determined by the molar ratio of the substrates. The molar ratios between mono-, di- and tricaprin obtained with Lipozyme as a catalyst for synthesis of glycerides from glycerol and capric acid (18) were close to the ratios obtained here. Lipozyme has also been used for synthesis of triolein in 80% yield (15), starting from oleic acid and glycerol. Despite the use of the same enzyme, these two groups achieved different results. The synthesis of triolein took place at 60°C, and the synthesis of capric glycerides at 40°C. The high yield of triolein can be explained only by extensive acyl migration due to the anionic resin on which the 1,3-specific *Mucor miehei* lipase is immobilized. Acyl migration in 1,2-diolein was the rate-limiting step in the triolein synthesis (16). Obviously, the acyl migration rate must have been much higher at 60°C than at 40°C because little triglyceride was formed at this temperature.

Water content in glycerol. To investigate the effect of water content on the reaction rate and overall product composition, water was added to glycerol to different concentrations; 4, 10, and 25% (w/w). It had previously been found that increasing water content to 4% increased initial reaction rates in glycerolysis of beef tallow (31), but above this level, only hydrolysis was favored. Furthermore, the equilibrium was less in favor of monoglycerides when the water content of glycerol was above 8.5%. These reactions were conducted in closed reactors to maintain the water concentration at a constant level. Here, the reactions were conducted in open reactors, and in terms of overall reaction performance, i.e., the yield of diglyceride, only small effects due to the different water contents could be detected (Table 2). Table 2 shows that increasing water content increases the initial reaction rate calculated as consumption of ester. However, the main initial reaction is hydrolysis of the ester to yield fatty acid, which is an unproductive reaction in terms of glyceride synthesis. The fatty acid initially produced acted as an acyl donor, and after 24 h, no significant difference in fatty acid content remained. It can therefore be concluded that the fatty acid is a better substrate than the ester (Table 1). An increased level of tricaprin was found after prolonged reaction in the reactions with 10 and 25% water addition to glycerol; a plausible explanation for this could be enhanced acyl migration in these reactions.

Open and closed reaction vessels. To obtain a high conversion of the acyl donors, it is essential that the equilibrium be displaced by efficient removal of the by-products (11,15, 18) or precipitation of products (17,31). The effect of the by-product, water or ethanol, on the reaction progress was investigated by utilizing closed or open reactors and ethyl caprate and capric acid as substrates. Vacuum could also be applied to remove the by-product more effectively. Figure 4 shows that the reaction reached equilibrium at low substrate conversion in closed reactors. The conversion for the ester was 1.5 mmol (10%, 0.15 M ethanol), and for the acid 5 mmol (33%). From these results, it can be concluded that ethanol had a much stronger effect on the reaction than water, as mentioned above. When the reactors were opened, the reactions started again and proceeded as if the reactor had been open from the start, although at a lower rate for the ester substrate. This suggests that some irreversible damage to the enzyme by the high

TABLE 2
Influence of Water Content in Glycerol[a]

Water content (% w/w)	Initial rate (μmol ester/h · mg)	Initial rate (μmol acid/h · mg)	Dicaprin yield (mmol)	Tricaprin yield (mmol)
4	6.7	0.4	3.6	0.4
10	12.0	3.3	3.4	0.7
25	20.0	14.2	3.4	0.7

[a]Glycerol (5 mmol), containing different amounts of water, was esterified with ethyl caprate (15 mmol) at 40°C in open containers. The reaction was catalyzed by 150 mg lipase preparation. The yields reported in the table were obtained after 72 h.

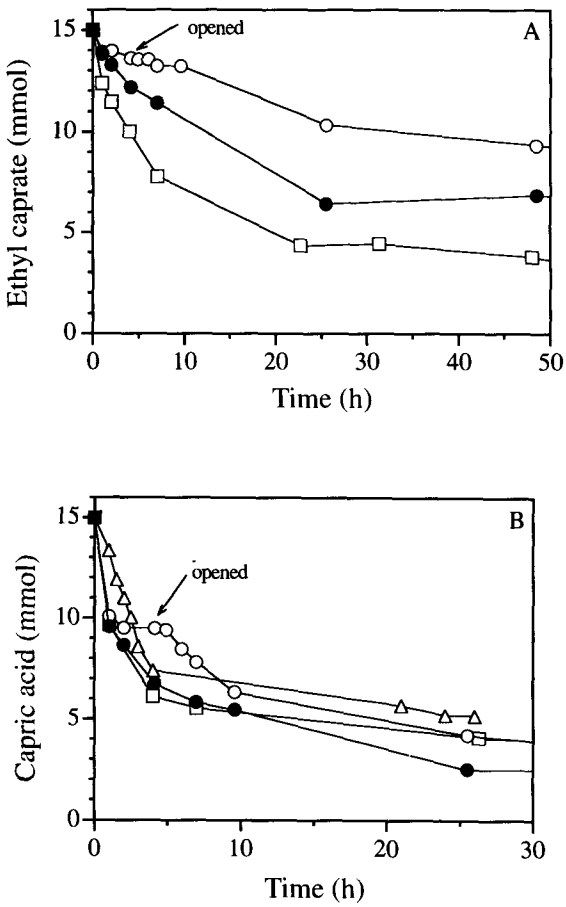

FIG. 4. Synthesis of glycerides from (A) ethyl caprate and (B) capric acid, utilizing different means of by-product removal. The reaction mixture consisted of 5 mmol glycerol (4% water) and 15 mmol ethyl caprate or capric acid. The reactions were conducted at 40°C, and 150 mg lipase preparation was used. Open reactor (●), closed reactor and opened after 4.9 h (○), vacuum achieved by water pump (□), and vacuum achieved by vacuum pump (Δ).

concentration of ethanol had occurred, or that the accumulated ethanol could not be removed by free evaporation.

To increase the efficiency of the removal of the by-products, vacuum was applied to the reactors by means of a water pump or a vacuum pump, both giving a pressure of ~15 mm Hg. Esterification of glycerol with ethyl caprate was more efficient when vacuum was applied (Fig. 4A). The difference was less for capric acid as acyl donor (Fig. 4B), which agrees with previous studies (15,18). It was expected that the vacuum pump would be more efficient in removing water, because the water pump produces a water-saturated atmosphere in the reactor, and the vacuum pump creates a dry atmosphere. It seems that the lower water pressure in the reactor had no significant effect on the reaction rate. The high levels of remaining substrate are due to excess of substrate to glycerol, calculated on the primary hydroxyl groups of glycerol. Complete conversion of glycerol to diglyceride would leave 5 mmol acyl donor unreacted. In the present reactions, the remaining acyl donor is <5 mmol in most cases, due to acyl migration and formation of tricaprin.

To achieve high conversion, it is essential that the by-products ethanol and water be allowed to evaporate from the reaction mixture; otherwise, the reaction reaches equilibrium at low conversion. The results show that a water pump or vacuum pump was more efficient than free evaporation of the ethanol, whereas the effect of reduced pressure was negligible for removal of water.

Mass transfer limitations. The aim of this investigation is to produce partial glycerides; tricaprin is thus an unwanted by-product that has to be minimized. One way to decrease the formation of tricaprin is to enhance the rate of the enzymatic reaction so that the ratio of the enzymatic reaction rate to the acyl migration rate is increased.

The loading of lipase on polypropylene was increased by up to three times. The specific activity of the enzyme is not changed at this high loading in an esterification reaction in hexane (T. Werkhoven, personal communication). However, the enzymatic reaction rate in glycerol synthesis was not changed to any significant extent by this procedure. This implies that the reaction is entirely controlled by external mass transfer.

To study the effects of external mass transfer limitations, varying amounts of lipase preparation were utilized to catalyze the esterification of glycerol with ethyl caprate. Theoretically, if no external mass transfer limitations are at hand, the specific enzyme activity will remain unchanged as the amount of enzyme preparation is increased. However, in the present situation the specific enzyme activity decreased as the amount of lipase preparation was increased (Fig. 5). The particles of the enzyme preparation tended to clump together or adhere to the walls of the reactor, presumably together with the glycerol phase. The transfer of ethyl caprate to the lipase is poor because transfer of a nonpolar substrate through a polar phase is slow. Furthermore, transfer of the products (partial glycerides) back to the organic phase is also slow. These external mass transfer limitations could possibly be re-

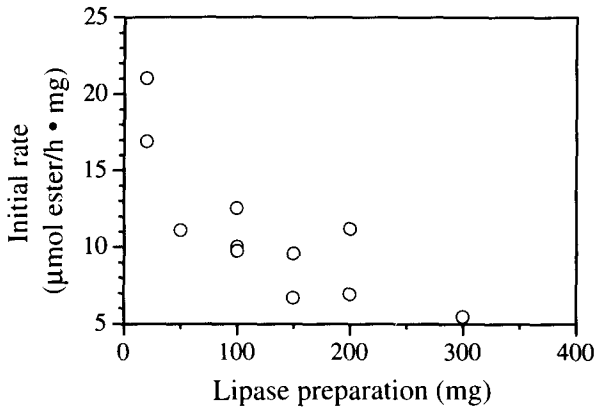

FIG. 5. The specific reaction rate at varying amounts of lipase preparation. The reaction mixture consisted of 15 mmol ethyl caprate and 5 mmol glycerol (4% water). The reactions were conducted in open reactors at 40°C, and 20–300 mg lipase preparation was used.

duced by better mixing of the two phases with the lipase preparation.

Reaction temperature. The reaction temperature has a profound effect on the reaction performance; both initial reaction rate and equilibrium composition, as well as enzyme stability, are influenced by the reaction temperature. Furthermore, the melting points of the various components of the reaction mixture also influence the reaction. It is usually considered an advantage if the products precipitate during the reaction because they are then removed from the equilibrium and the reaction is displaced in favor of more products. This has been used advantageously in preparation of mono- and diglycerides in systems with and without solvent present (17,22,31).

Glyceride synthesis was conducted at temperatures between 15 and 50°C with ethyl caprate as acyl donor (Table 3). Ethyl caprate had the advantage over capric acid that it is liquid at these temperatures. The initial reaction rate increased as the temperature was raised from 15 to 40°C, and then decreased. The decrease was presumably due to denaturation of the lipase at this high temperature; at 60°C, the lipase showed no activity. At 15°C, the reaction mixture started to solidify after 12–15 h reaction due to crystallization of mono- and dicaprin, but the reaction continued. Obviously, enough reactants were still available to the lipase, possibly as small pools of liquid material mixed with crystalline material. Monocaprin and dicaprin have similar melting points, suggesting that they would precipitate at the same temperature. However, they may have different solubilities in ethyl caprate, accounting for the enrichment of dicaprin and not monocaprin. The highest yield was obtained at 15°C, whereas the yields in the reactions that remained in a liquid state throughout the reaction were similar (Table 3); there is no explanation for the low yield at 25°C. Tricaprin was formed at all temperatures and to a greater extent as the temperature rose, which can be explained by increasing acyl migration caused by the higher temperature (34,36).

Synthesis with precipitation of products was also studied with ethyl palmitate as the acyl donor at 40 and 50°C (Fig. 6). Similar to the reaction with ethyl caprate at 15°C, the reaction mixture slowly solidified, while the reaction proceeded. The yields of dipalmitin (4.6 and 4.4 mmol, respectively) were similar to the yield of dicaprin (4.4 mmol) at 15°C. More tripalmitin was produced at 50 than at 40°C, indicating

TABLE 3
Influence of Reaction Temperature on Final Diglyceride Yield[a]

Temperature (°C)	Initial rate (μmol donor/h · mg)	Dicaprin yield (mmol)	Tricaprin yield (mmol)
15	2.3	4.4	0.04
25	3.4	2.9	0.4
40	9.6	3.6	0.4
50	5.9	3.9	1.2

[a]Glycerol (5 mmol), containing 4% water (w/w), was esterified with ethyl caprate (15 mmol) at 40°C in open containers. The reaction was catalyzed by 150 mg lipase preparation. The highest yield of dicaprin and the concurrent yield of tricaprin are reported in the table.

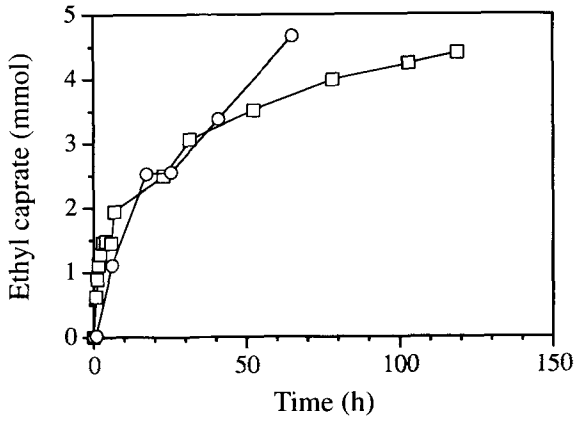

FIG. 6. Synthesis of glycerides from ethyl palmitate and glycerol at different temperatures. The reaction mixture consisted of 15 mmol ethyl palmitate and 5 mmol glycerol (4% water). The reactions were conducted in open reactors, and 200 mg lipase preparation was used. Reaction temperature 40°C (○) and 50°C (□).

that more acyl migration took place at the higher temperature. Presumably, acyl migration took place in the melted parts of the reaction mixture, because acyl migration is slow in the solid state (37). It can thus be concluded that a useful means of increasing the yield of diglycerides is to operate at such a temperature that the glycerides precipitate during the reaction. A drawback of this method, when producing glycerides with high melting points, is that the enzyme might be inactivated when the product mixture is melted prior to removal of the enzyme preparation and glycerol, and thus the enzyme cannot be reused. By using a thermostable enzyme, this drawback can be overcome.

ACKNOWLEDGMENTS

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