A Comparison Between Lipase-Catalyzed Esterification of Oleic Acid with Glycerol in Monolayer and Microemulsion Systems

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Lipase-catalyzed synthesis—esterification of oleic acid with glycerol—was carried out in L2 microemulsions and in monolayers. The microemulsions were based on isooctane as a nonpolar component and various water–glycerol mixtures as polar component. The substrate, oleic acid/sodium oleate, constituted the microemulsion surfactant. The lipase resides mainly in the water pools. Monolayers of oleic acid/sodium oleate were formed on subsolutions of glycerol and water, and the enzyme solution was injected under the compressed monolayers. Thus, the arrangement of the reactants at the oil–water interface of the microemulsion can be regarded as analogous to that at the air–water interface of the monolayer. The microemulsion structure was characterized by self-diffusion nuclear magnetic resonance. It was found that the higher the glycerol-to-water ratio, the lower are the water D-values. The reactions in microemulsions generally gave a low degree of oleic acid conversion. The yield increased with increasing glycerol-to-water ratio. Monoglycerides were the main product, and no triglyceride could be detected. The monolayer experiments gave a somewhat higher degree of conversion, with tri- and diglycerides being the major reaction products. The reason why triglycerides are formed in monolayer experiments but not in microemulsions is believed to be due to an unfavorable partitioning of the diglyceride in the microemulsion systems. Once formed, the diglyceride will partition into the hydrocarbon domain and become inaccessible for reaction with the enzyme-O-acyl intermediate at the oil–water interface. In addition, the interfaces in the two systems are different. The monolayer interface is static, whereas the microemulsion interface is highly dynamic, and this difference may also influence the product pattern.

KEY WORDS: Enzymatic esterification, glycerol, lipase, microemulsion, monoglyceride, monolayer, oleic acid, self-diffusion coefficient, sodium oleate, spin-echo NMR, triglyceride.

Enzymatically-catalyzed lipid transformations in water-in-oil microemulsions have been under study for the last decade (1–4). Lipase-catalyzed hydrolysis of triglycerides to monoglycerides or all the way to glycerol and fatty acids, depending on the specificity of the enzyme, gives high yields. Lipase-catalyzed glycerolysis of triglycerides seems to be a useful way of synthesizing monoglycerides. Enzymatic synthesis of triglycerides from glycerol and fatty acids has not been successfully carried out in microemulsions, however (5–7). A recent paper showed that the triglyceride synthesis could be performed at the air–water interface with a monolayer of fatty acids as reaction zone (8). Because reactions in microemulsions and monolayers are both interfacial processes, it seemed to be of interest to investigate why triglycerides can be synthesized in monolayers but not in microemulsions. This paper aims at elucidating this issue.

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Lipase-catalyzed condensations of fatty acid and glycerol in microemulsion systems studied so far have been performed in the presence of sodium *bis*(2-ethylhepyl) sulfosuccinate or some other amphiphile, which is not taking an active part in the enzymatic process. Hence, the interface between oil and water domains in these studies has largely consisted of surfactant molecules, and for the reaction to occur, the enzyme and the oil-soluble reactants must squeeze their way into the palisade layer. The situation is different for the monolayer experiment, in which the fatty acids align at the air–water interface, and no surfactant palisade layer prevents contact with the enzyme, which resides in the subphase.

In the present work, which aims at comparing lipase-catalyzed condensation of fatty acid and glycerol in a monolayer and in a microemulsion of the water-in-oil type, no external nonreactive surfactant is used to formulate the microemulsion. The microemulsion palisade layer will consist of the same fatty acid/fatty acid soap molecules that constitute the monolayer at the air–water interface. The effect of the unreactive surfactant on the reaction in the microemulsion is, consequently, eliminated.

MATERIALS AND METHODS

Materials. Lipozyme (activity 10,000 LU g^{-1}), a microbial lipase, was kindly provided by Novo Nordisk A/S (Bagsveard, Denmark). Hexane, isooctane, isopropanol, methanol and chloroform were purchased from Fisher Scientific Company (Fairlawn, NJ), and oleic acid and sodium oleate were from Sigma Chemical Company (St. Louis, MO).

Preparation of monolayers. A mixture of oleic acid and sodium oleate (70:30, w/w) or only oleic acid, dissolved in a solvent mixture consisting of hexane, methanol and chloroform (60:20:20, vol/vol/vol), was spread on a subsolution of water and glycerol (56:44, w/w) with an Agla micrometer syringe. After evaporation of the solvent, the monolayer, was compressed with a waxed glass bar up to a desired initial surface pressure. When the surface pressure had reached a steady value, the lipase was injected under the monolayer, and the sublayer was stirred gently for 30 s by a rotating magnet to give a uniform distribution of the enzyme in the subsolution. The amount of enzyme was 70 LU per gram of subsolution. The surface pressure and surface potential were measured as a function of time after 30 s of stirring, as described earlier (9).

Phase diagram. Partial phase diagrams were constructed by titrating oil/surfactant mixtures with water, glycerol or a mixture of water and glycerol until onset of turbidity. The temperature was 23 ± 0.5°C.

Preparation of microemulsions. A homogeneous mixture of oleic acid and sodium oleate (70:30, w/w) was made by heating to 50°C and then cooling to room temperature. Isooctane, containing 48% (w/w) of *t*-butanol, and the polar component [water, glycerol or a 1:1 (w/w) water/glycerol mixture] were added. The amount of enzyme was 50 LU

per gram of reaction mixture. The microemulsions were stirred during the reaction.

Reaction. The synthesis reaction taking place in microemulsions and monolayers is:

$$\text{glycerol + fatty acid} \overset{\text{lipase}}{\rightleftharpoons} \begin{cases} \text{monoglyceride} \\ \text{diglyceride + water} \\ \text{triglyceride} \end{cases} \quad [1]$$

The reactions were run at 23 ± 0.5°C.

Yield measurements. Ethanol was added after completed reaction in the microemulsion system, and the percent conversion of fatty acid into glycerides was determined by fatty acid titration with 0.1 M KOH in ethanol. The conversion of fatty acid in percent mentioned throughout this paper is defined as the consumption of fatty acid divided by the initial amount of fatty acid. A sample without lipase was used as reference. To analyze the reactions in both the microemulsion and the monolayer systems, a normal-phase high-performance liquid chromatography (HPLC) with silica column (10 mm × 2.4 mm) and ultraviolet (UV) absorbance detector of 213-nm cut-off wavelength at 0.05 absorbance units full-scale (auts) (Model SP8450; Spectra Physics, San Jose, CA) was used to separate monoglyceride, diglyceride, triglyceride and fatty acid. A mixture of isooctane and isopropanol (95:5, vol/vol) was used as the mobile phase. The flow rate of the mobile phase was kept at 1 mL/min. All analyses were made at room temperature.

Thin-layer chromatography (TLC). The reactions were monitored by TLC. The plates were run twice, first in diethyl ether up to 2 cm, and then in a combination of hexane, diethyl ether and acetic acid (70:29:1, vol/vol/vol). The products were visualized by exposure of the plates to iodine vapor.

Self-diffusion measurements. Self-diffusion coefficients were obtained by the ^1H nuclear magnetic resonance (NMR) Fourier transform pulsed-gradient spin-echo (PGSE) technique with a standard Jeol (Tokyo, Japan) FX-100 NMR spectrometer, operating at a proton frequency of 99.6 MHz and at a temperature of 23 ± 0.5°C. The PGSE measurements were performed by varying the duration of the gradient pulse at a constant measuring time of 140 ms (10).

RESULTS AND DISCUSSION

Monolayer. The change in surface potential of the monolayer, consisting of a mixture of oleic acid and sodium oleate, was measured at various initial surface pressures to elucidate the effect of surface pressure on the synthesis reaction. The reaction time was 30 min. It is evident from Figure 1 that an initial surface pressure of 18 mN/m was optimum for maximum change in the surface potential (i.e., maximum conversion). Below this value there is an increase in surface potential as the initial surface pressure increases. This can be interpreted as an increase in the two-dimensional concentration of reactant at the air-water interface. Beyond the optimum initial surface pressure (18 mN/m), the change in surface potential decreases with an increase in initial surface pressure because the oleic acid and sodium oleate molecules are so tightly packed that access of the reactant to the enzyme active site is rendered difficult.

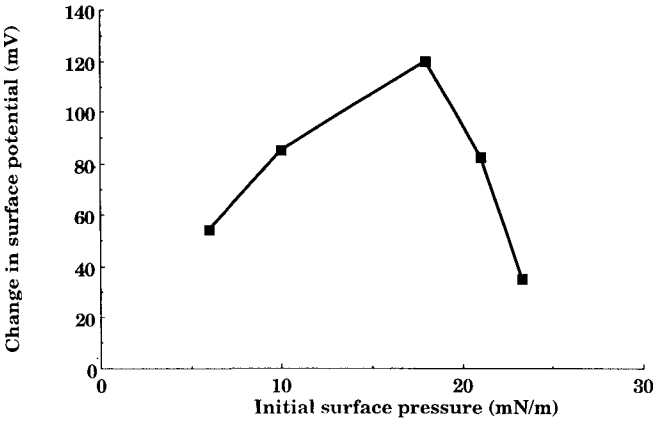


FIG. 1. Change in surface potential of the monolayer after 30 min reaction time as a function of initial surface pressure for lipase-catalyzed esterification of oleic acid with glycerol. The monolayer consisted of oleic acid and sodium oleate (70:30, w/w) with a glycerol and water (56:44, w/w) mixture as subsolution. The lipase concentration was 70 LU/gram subsolution.

An initial surface pressure of 18 mN/m was now chosen, and the reaction was monitored by change in surface pressure and surface potential. Figure 2 shows that the surface pressure increases by 5 mN/m, and the surface potential by about 120 mV. The reaction is completed after 10–15 min because there is no further change in the surface potential (9). The monolayer from the experiment of Figure 2 was analyzed to confirm the synthesis reaction. After 30 min reaction time, the monolayer was removed and dissolved in a mixture of isooctane and isopropanol (50:50, vol/vol). HPLC analysis showed the presence of triolein [23% (w/w)], diolein [19% (w/w)] and oleic acid [58% (w/w)] in the monolayer (Table 1). TLC of the extracted monolayer also confirmed the presence of these products. The degree of conversion of oleic acid was 41%.

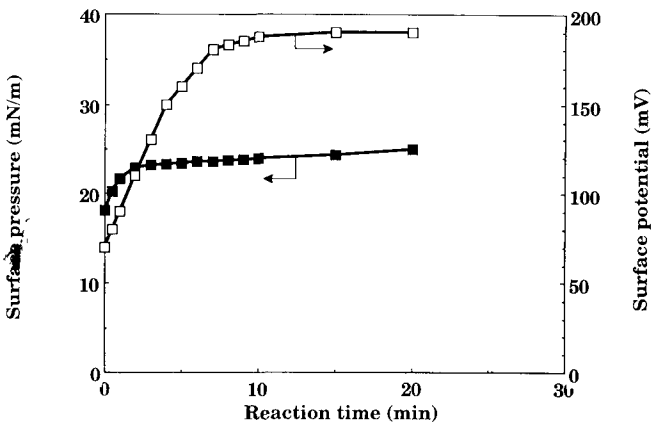


FIG. 2. Change in surface pressure and surface potential as a function of reaction time for the lipase-catalyzed esterification reaction in monolayer. The initial surface pressure was 18 mN/m. The monolayer consisted of oleic acid and sodium oleate (70:30, w/w) with a glycerol and water (56:44, w/w) mixture as subsolution. The lipase concentration was 70 LU subsolution. ■, Surface pressure; □, surface potential.

TABLE 1

HPLC Analysis (w/w) of Products Formed After 30 min Reaction in Monolayer and Microemulsion System[a]

System	Monoglyceride (%)	Diglyceride (%)	Triglyceride (%)
Monolayer based on oleic acid and sodium oleate	0	19	23
Monolayer based on oleic acid	0	10	31
Microemulsion 5[a]	31	4	0

[a]The microemulsion composition is shown in Table 2.

The same reaction as described above was repeated without sodium oleate. The monolayer consisted of only oleic acid. After 30 min, the monolayer was analyzed by HPLC. As shown in Table 1, the amount of triolein was higher as compared to the reaction with both oleic acid and sodium oleate present in the monolayer. The conversion of oleic acid was the same (around 40%), however.

The reaction seems to reach equilibrium in both cases, but the presence of sodium oleate decreases the formation of triolein. This could be an indication that the interaction of the lipase with the monolayer is weaker in the system that contains sodium oleate. It has been previously observed that adsorption of lipase to a monolayer is weaker when the interface is more negatively charged (Skagerlind, P., M. Jansson, B. Bergenstohl and K. Hult, unpublished data).

When the enzyme was added to an aqueous subsolution without glycerol, the surface pressure increased by 3 mN/m and the surface potential increased by 5 mV (data not shown). The increase in surface pressure without glycerol in the subsolution can be seen as an indication of the lipase being surface-active (Skagerlind, P., M. Jansson, B. Bergenstohl and K. Hult, unpublished data).

Microemulsion phase diagrams. Figure 3 shows schematic pseudo-ternary phase diagrams for systems comprising polar component/isooctane + *t*-butanol/oleic acid plus sodium oleate, in which the polar component is either glycerol, water or a 1:1 (w/w) glycerol/water mixture. The isotropic L2 phase is relatively large in all systems. In the water and water-glycerol systems, the microemulsion phase extends further toward the corner of the polar component (and also further toward the opposite base line) than in the glycerol system. Without the cosurfactant, *t*-butanol, no L2 phase was obtained for any water-glycerol mixture combined with the hydrocarbons heptane, isooctane and nonane. *t*-Butanol was chosen as cosurfactant because, as a tertiary alcohol, it is not a substrate for normal lipases.

Reactions in microemulsions and self-diffusion NMR measurements. Lipase-catalyzed reactions were carried out with six different microemulsion samples, all within the isotropic L2 region (the compositions are given in Table 2). As shown, five out of the six samples have the same water content of 9.1% (w/w), with the glycerol-to-water ratio varying from less than 1:2 (w/w) to more than 3:1 (w/w). The remaining sample is low both in glycerol and in water content. Figure 4 shows the conversion of fatty acid as a function of reaction time for the six different starting compositions. Some obvious conclusions can be drawn: (i) The degree of conversion of fatty acid is generally low compared to the monolayer experiments. (ii) A higher glycerol-to-water ratio gives an increase in the degree of conversion. This is expected behavior because water is a product of the synthesis reaction. However, we have seen that a high glycerol-to-water ratio leads to poor lipase activity, possibly due to dehydration of the enzyme (11). Most likely there exists an optimum ratio between the two polar components. (iii) Some reac-

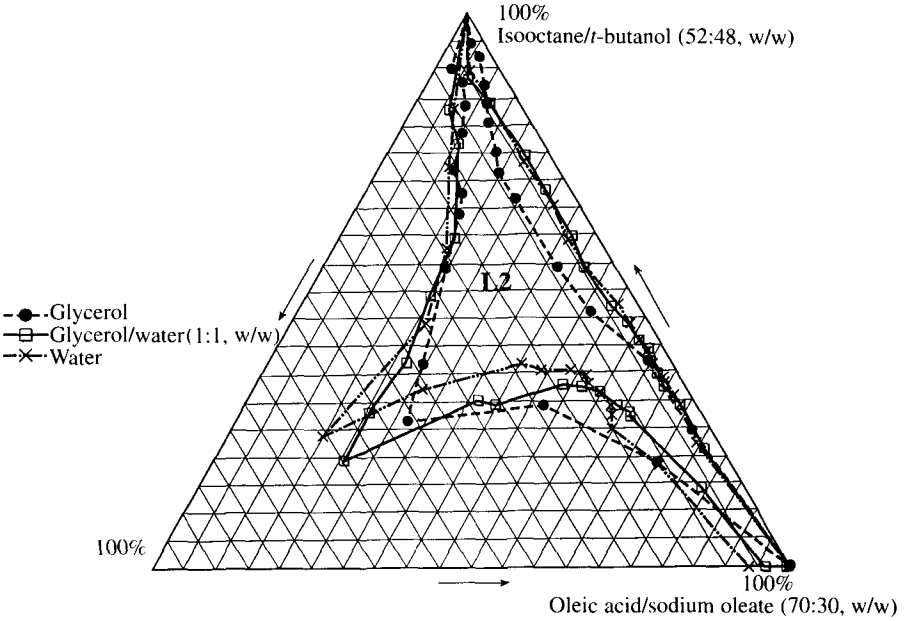


FIG. 3. Technical partial phase diagram for the water, glycerol or a 1:1 (w/w) glycerol-water mixture systems. The other components are isooctane + *t*-butanol (52:48, w/w) and oleic acid + sodium oleate (70:30, w/w). ●, Glycerol; □, glycerol/water (1:1, w/w); X, water.

TABLE 2

Compositions (w/w) and Self-Diffusion Coefficients for Water (D_w) for the Microemulsions Used

System	Isooctane + *t*-butanol (52:48) (%)	Oleic acid + sodium oleate (70:30) (%)	Glycerol (%)	Water (%)	D_w ($m^2s^{-1} \times 10^9$)	D_w/D^o_w[a]
Microemulsion 1	77.3	9.7	3.9	9.1	0.38	0.2
Microemulsion 2	59.1	22.7	9.1	9.1	0.16	0.08
Microemulsion 3	50.0	29.2	11.7	9.1	0.12	0.06
Microemulsion 4	31.8	42.2	16.9	9.1	0.04	0.02
Microemulsion 5	31.8	27.3	31.8	9.1	0.04	0.02
Microemulsion 6	24.0	67.3	4.8	3.9	0.07	0.04

[a]D^o_w is the self-diffusion coefficient of neat water ($2.1 \cdot 10^{-9}$ m^2 s^{-1}).

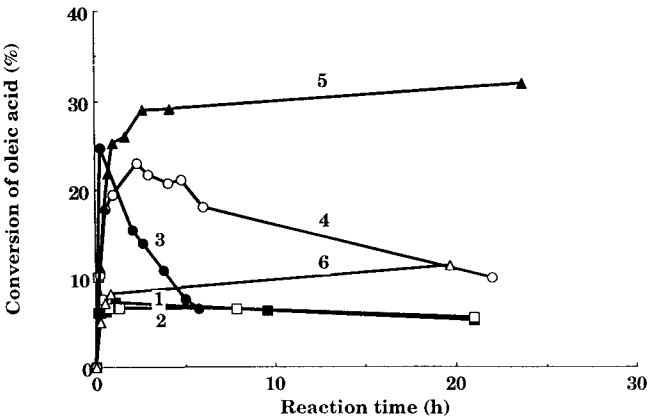


FIG. 4. Conversion of oleic acid in different microemulsions as a function of reaction times for lipase-catalyzed esterification of oleic acid with glycerol. The numbers in the figure refer to the microemulsion compositions in Table 2. The lipase concentration was 50 LU/microemulsion. ■, Microemulsion composition 1; □, microemulsion composition 2; ●, microemulsion composition 3; ○, microemulsion composition 4; ▲, microemulsion composition 5; △, microemulsion composition 6.

tions exhibit a pronounced maximum in the degree of conversion with time. Most likely, this behavior is due to an uneven partitioning between oil and water domains of the various species involved. Lipase-catalyzed lipid transformations that proceed *via* a maximum in yield at a certain time have been experienced before (11).

Self-diffusion NMR measurements of water were performed on the six starting compositions used for the enzymatic reactions (the results are included in Table 2). Table 2 shows that the D-value of water in the series of samples with constant water content (samples 1–5) decreases with increasing glycerol-to-water ratio. Composition 6, which has the smallest content of both water and glycerol, does not give the lowest value of D_w/D^o_w. It seems that an increased glycerol content favors closed water/oil structures. From the values of D_w/D^o_w, it is obvious that all samples, possibly with the exception of composition 1, can be regarded as true water/oil structures.

To investigate if the microstructure of the reaction medium undergoes major changes during the synthesis, the reaction of composition 4 was monitored by self-diffusion NMR. Samples were taken after 0.07, 4 and 24 h, and the D_w-values were recorded. The results shown in Table 3 indicate that there are no major changes occurring in the microstructure of the microemulsion during the course of the reaction. A small decrease in diffusion rate for water was observed, however, after four hours of reaction time. One may speculate that this corresponds to the maximum in conversion found after approximately four hours of reaction time (Fig. 4). However, we have no other evidence that low D_w-values favor the esterification reaction.

TABLE 3

Self-Diffusion Coefficient for Water (D_w), for Microemulsion 4 During the Course of the Reaction

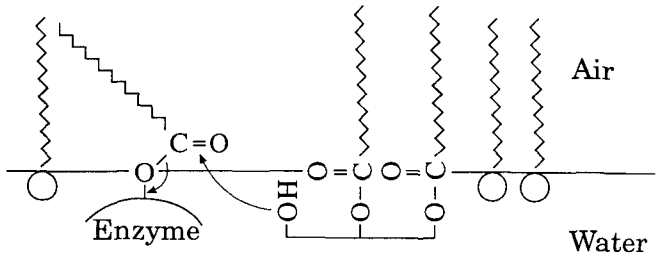
Reaction time (h)	D_w/D^o_w[a]
0.07	0.022
4	0.018
24	0.021

[a]D^o_w is the self-diffusion coefficient of neat water ($2.1 \cdot 10^{-9}$ m^2 s^{-1}).

The low degree of conversion for all microemulsion reactions is probably due to the relatively high water content of the systems. A much higher glycerol-to-water ratio should be favorable from an equilibrium point of view. However, we have previously seen that lipases exhibit low activity in such systems (11). An interesting observation is that the reactions in monolayers give a slightly higher oleic acid conversion (40–41%) than the microemulsion reactions, in spite of the fact that in the monolayer experiments the water content is almost infinitely large as compared with the amount of ester formed. Evidently, the special arrangement of the reactants at the water–air interface sets aside the normal esterification equilibrium.

Table 1 shows the results from the HPLC analysis after 30 min reaction time in the microemulsion system. Monoolein, but no triolein and almost no diolein, is produced. This is different from the monolayer experiment in which 23% (w/w) triolein and 18% (w/w) diolein, but no monoolein, were produced. Evidently, there is a fundamental difference between microemulsions and monolayers as reaction media for triglyceride synthesis, and this difference is not caused by the microemulsion surfactant (as no surfactant besides the substrate was used in these experiments). We believe that the main reason why triglycerides do not form in the microemulsion mileau is that the diglyceride is too lipophilic and has too low a surface activity to stay at the interface. Once formed, the diglyceride will partition into the hydrocarbon domain. The results obtained in this work seem to tell us that the disappearance of the diglyceride from the interface is a

The monolayer situation:



The microemulsion situation:

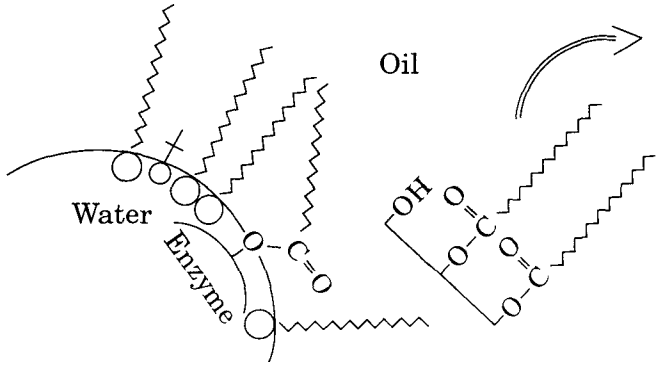


FIG. 5. A schematic picture of the two different systems. In the monolayer system, the diglyceride has no other choice than staying at the interface, whereas in the microemulsion system the diglyceride will partition into the hydrocarbon domain.

fast process compared with the lipase-catalyzed esterification to form triglyceride. Also the monoglyceride formed is a lipophilic species and will mainly partition into the hydrocarbon domain. This explains the low yield of diglycerides in the microemulsion experiments. The monolayer situation is different. The diglyceride formed has no other choice than staying at the interface. The suggested difference in interfacial behavior is shown schematically in Figure 5. In addition, the static nature of the water–air interface must constitute an ideal reaction zone for triglyceride synthesis. Formation of three consecutive ester bonds to glycerol at this interface must be a much less complicated event than the corresponding process at the highly dynamic interfaces of the microemulsions.

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[Received March 23, 1994; accepted August 31, 1994]