Synthesis of Mono- and Diglycerides in Water-in-Oil Microemulsions

C.P. Singh, D.O. Shah* and **Krister Holmberg**[1]

Center for Surface Science and Engineering, Departments of Chemical Engineering and Anesthesiology, University of Florida, Gainesville, Florida 32611

Enzyme-catalyzed esterification was carried out in single-phase, oil-continuous microemulsions. The lipozyme was solubilized, along with glycerol and water, in the aqueous core of water/diethylhexyl sodium sulfosuccinate/hydrocarbon microemulsion system. Upon addition of fatty acid, mono- and diglycerides were formed, due to the esterification reaction taking place at the interface of the droplets in the microemulsion. The initial rate of conversion of oleic acid increases with oil chainlength of the continuous phase whereas final conversion is maximum for hexane. The conversion of stearic acid is 30% whereas conversion of oleic acid is 70%. The percent conversion of various fatty acids in the same continuous medium increases with fatty acid chainlength. The oleic acid/glycerol ratio is an important parameter for optimum conversion of oleic acid into glycerides. The yield can be increased by subsequent addition of glycerol after equilibrium is reached. High-performance liquid chromatography analysis of samples from microemulsions shows the presence of mono- and diglycerides. Possible mechanisms for the abovementioned effects are discussed.

KEY WORDS: AOT, diglyceride, esterification, fatty acid, glycerol, hydrolysis, lipase, microemulsion, monoglyceride, reversed micelle.

A microemulsion may be defined as a thermodynamically stable isotropic dispersion of two immiscible liquids that consists of microdomains of one or both liquids stabilized by an interfacial film of surface-active molecules (1,2). Microstructural studies of microemulsions have been given considerable attention because of their interesting physicochemical properties and various applications of commercial importance (3–7). Much of this effort has been directed toward elucidating the type of fluid microstructures present in microemulsions and the changes that take place as the relative amounts of oil and water vary, particularly for microemulsions that progressively invert from oil-continuous to water-continuous type. The structure of microemulsions has been investigated by a variety of techniques, such as electrical conductivity, nuclear magnetic resonance spectroscopy, self-diffusion measurements, quasi-elastic light scattering, small angle neutron and x-ray scattering and optical spectroscopy (8–17). A microemulsion that contains a relatively low fraction of oil confined within small isolated droplets dispersed in water is known as oil-in-water (o/w) microemulsion, while the reverse type (small amount of water dispersed in large amount of oil) is a water-in-oil (w/o) microemulsion. Upon continuously increasing the water-to-oil ratio in a w/o microemulsion, phase inversion ultimately occurs. During such an inversion, an intermediate transparent, isotropic bicontinuous structure may form, involving both oil and water-continuous domains separated by interfacial surfactant layers. On the other hand, in certain systems, a sharp transition from well-defined w/o to o/w structure (without the intermediate formation of a bicontinuous phase) is believed to occur.

*To whom correspondence should be addressed.

[1]Current address: Ytkemiska Institutet, Institute for Surface Chemistry, Box 5607, S-114 86 Stockholm, Sweden.

The development of molecular enzymology has come about mainly through studies of free enzymes. Experiments can be designed to elucidate the structure of the catalytic site and the physicochemical conditions for its optimal activity. The use of microemulsions with low water content as a medium for enzymic reactions has been reported by many researchers (18–24). The majority of work reported in the literature is primarily concerned with lipases. It has been demonstrated that the enzyme retains a high intrinsic activity in these highly ordered nonpolar solutions (i.e., w/o microemulsions). Immobilized enzymes can also be used in a microemulsion medium without too much impairment of the reaction rate. The anionic double-tailed surfactant sodium di-[2-ethylhexyl] sulfosuccinate (AOT) is most frequently used to form microemulsions. Unlike most surfactants, AOT does not require additional amphiphiles as cosurfactants for the formation of reverse micelles because of its wedge-shaped molecular geometry. All esterification reactions in microemulsions reported to date in the literature are based on the expensive and pure lipase from *Rhizopus delemar* and *Candida cylindracea* (22,25,26). We have designed experiments for the commercially used lipase called Lipozyme (10,000 LU/g). We have investigated many physicochemical conditions that can effect this synthesis reaction, such as oil chainlength of continuous phase, glycerol/water ratio, chainlength of fatty acid, oleic acid/glycerol ratio, water/enzyme ratio and unsaturation of fatty acid.

MATERIALS AND METHODS

Lipozyme, a fungal lipase produced by Novo Nordisk (Bagsveard, Denmark) was made available by Kraft Research Inc. (Glenview, IL). Hexane, isooctane, isopropanol [all high-performance liquid chromatography (HPLC)-grade] and buffer solutions were bought from Fisher Scientific Company (Fairlawn, NJ). Fatty acids (C_8–C_{20}) (95% pure), lipids (95% pure) and AOT (99% pure) were bought from Sigma Chemical Company (St. Louis, MO). All chemicals were used as supplied. Deionized double-distilled water was used in all chemical procedures. All experiments were repeated three times, and results were found to lie within a range of 2%.

Microemulsion reaction. Microemulsions were prepared by mixing the alkanes, surfactant, glycerol, Lipozyme and water in the required proportions under constant stirring, which yielded a transparent microemulsion. Subsequently, a desired amount of fatty acid was added to the microemulsions. The addition of fatty acid to the microemulsion initiates the esterification reaction. The extent of reaction was monitored by NaOH titration in nonaqueous medium with phenolphthalein as indicator. The quantitative analysis of products was carried out by normal-phase HPLC and thin-layer chromatography (TLC). The synthesis reaction taking place in the microemulsion is:

$$\text{glycerol} + \text{fatty acid} \xrightarrow{\text{lipase}} \begin{cases} \text{monoglyceride} \\ \text{diglyceride} \\ \text{triglyceride} \end{cases} + \text{water}$$

Monitoring the reaction using HPLC and TLC. Normal-

phase HPLC with an ultraviolet absorbance detector having a cut-off wavelength of 213 nm and 0.05 absorbance units full-scale was used to separate monoglyceride, diglyceride, triglyceride and fatty acid in order to monitor the reaction. The silica column (10 mm × 2.4 mm) and the mixture of isooctane and isopropanol (94:6, vol/vol) as a mobile phase were used at room temperature. The flow rate of mobile phase was kept at 1 mL/min (27). The absorbance detector of the HPLC was from Spectra Physics (San Jose, CA) (Model SP8450). The integrator (Model SP8880; Spectra Physics) plots raw signal from the detector and determines the presence of peaks, retention times and quantities of various species. The standard samples of triolein, diolein, monooleic and oleic acid were dissolved in the eluent separately (0.001 g/mL) and were filtered through 0.2 μm. The autosampler (Model SP8880; Spectra Physics) was used to inject 20 μL of sample each time. At first, residence times of triolein, oleic acid, diolein and monoolein were identified individually. Then, a mixture of above lipids and oleic acid were run three times to determine the reproducibility of the technique. The microemulsion sample containing AOT was diluted ten times with the eluent before injecting into the column. Slight shifts in the residence times of various species were observed due to adsorption of AOT in the column. The standard and microemulsion sample were injected several times to confirm reproducibility of residence times and peak areas. The external standard method was used on the integrator to find the actual weight percent of products in the microemulsion sample. With external standards, each peak of interest in the analysis sample was compared with the same peak in the calibration sample. Because the amount of each component in the calibration sample is known, a ratio between that component and analysis sample can be calculated, giving the amount of the component in the analysis sample.

TLC plates were run twice in diethyl ether up to 2 cm, and then in a hexane/diethyl ether/acetic acid mixture (70:30:1, vol/vol/vol).

RESULTS AND DISCUSSION

Solubilization of glycerol/water in AOT and hexane. Mixtures of glycerol and water in various proportions were titrated separately in 1 mL of 0.25 M solutions of AOT in hexane to study the effect of glycerol/water ratio on the solubilization capacity of these microemulsion systems. The maximum solubilization was taken as the point where onset of turbidity took place. The solubilization capacities for various mole fractions of glycerol in water are plotted in Figure 1. The result shows a maximum at a mole fraction of 0.5 (Fig. 1). Before this maximum, glycerol molecules get solubilized near the hydrocarbon chain of AOT by pushing the water molecules inside the aqueous core, and at this maximum, the hydrocarbon chain of AOT becomes fully saturated with glycerol molecules. Upon further increasing the mole fraction, glycerol molecules start to solubilize in the aqueous core, which causes a decrease in the ionization of AOT molecules and decreases the total solubility. The decrease of repulsive force between the ionic groups causes a decrease in the solubilization of the aqueous phase. On the other hand, the synthesis reaction was found to be optimum at 0.64 mole fraction of glycerol because more water shifts the equilibrium toward hydrolysis.

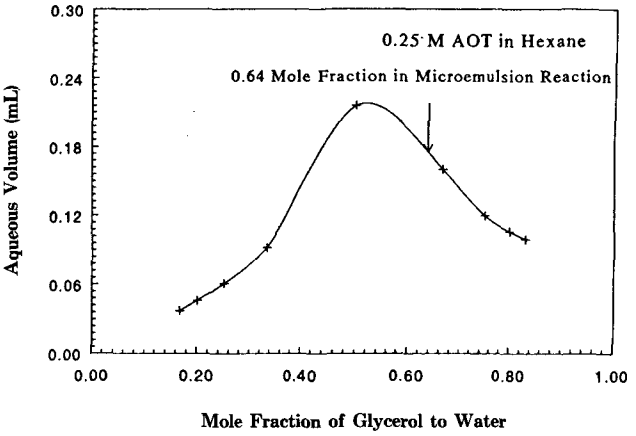

FIG. 1. Effect of mole fraction of glycerol to water on solubilization in AOT microemulsion. AOT, sodium di-[2-ethylhexyl]sulfosuccinate.

Effect of oil chainlength on the reaction kinetics. In this experiment, various alkanes (C_6–C_{16}) were taken as the oil phase for making the microemulsions, and percent conversion of oleic acid was monitored by NaOH titration. The oil chainlength has an interesting effect on the reaction. The conversion increases with chainlength (from hexane to hexadecane) after one hour, but for equilibrium conversion, the reverse is true as shown by data after 9 h of reaction (Fig. 2). There are two rate-controlling steps in this reaction system. First, the inter-droplet interaction, which is maximum for the hexadecane microemulsion system (17), and second, the diffusion of products from the interface to the bulk, which is maximum in the hexane system. From the consideration of geometric packing at the interface, longer oil chainlength creates more difficulty in penetrating the surfactant layer. The magnitude of attraction between droplets affects the difference between the composition of interface and continuous phase. The greater difficulty in penetrating the interface by larger oil molecules would increase this difference, thus increasing the magnitude of interaction. The

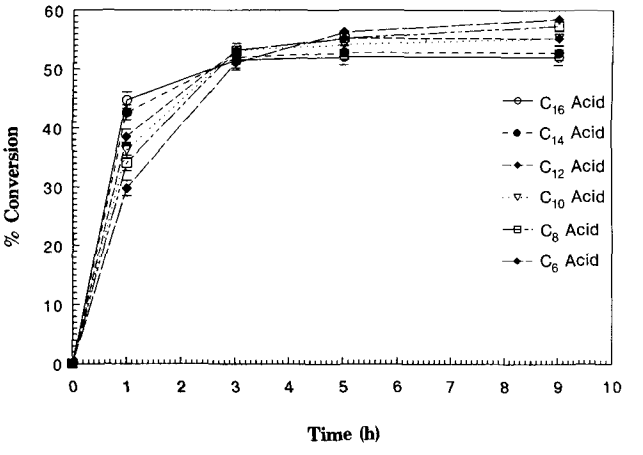

FIG. 2. Effect of oil chainlength of continuous phase on percent conversion of oleic acid.

larger the interaction, the greater is the likelihood for droplets containing enzyme to collide with other droplets containing glycerol or enzyme. Such collisions cause exchange of glycerol to enzyme-containing droplets. When a glycerol- and enzyme-containing droplet comes in contact with oleic acid, a reaction can take place. So, the distribution of glycerol into an enzyme-containing droplet plays an important role in the beginning, which causes a difference of 15% conversion between hexane to hexadecane systems. Diffusion of products from the interface to the bulk phase plays a vital role in controlling the reaction. Hence, the equilibrium conversion of oleic acid into products was 58% in hexane (low viscosity), and 50% in hexadecane (high viscosity).

Effect of fatty acid chainlength on the reaction. For this experiment, we used microemulsions with hexane as the oil phase. The glycerol/fatty acid ratio was kept constant at 10:1 to be able to dissolve the long- chain fatty acids in the oil phase. The results (Fig. 3) show that the conversion of fatty acids into glycerides increases with increased fatty acid chainlength. Maximum conversion is shown by arachidic (C_{20}) acid. The product of C_{20} acid is more nonpolar than the products of other fatty acids (due to its longer hydrocarbon chain). So, the product partitions preferentially into the oil phase after it forms, allowing more C_{20} acid to come to the interface and react at the same site. Here, the dominant factor controlling the reaction is the partitioning of products into the oil phase, which is maximum for C_{20} acid and minimum for C_{10} acid.

Effect of oleic acid/glycerol ratio on the reaction. In this experiment, we used microemulsions with hexane as the oil phase, while the oleic acid/glycerol ratio was varied. This shows a maximum equilibrium percent conversion at 1:1 molar ratio of oleic acid to glycerol (Fig. 4). It seems that, for molar ratios greater than 1, an increase in oleic acid at the interface has a significant effect in decreasing the diameter of the microemulsion droplets, which ultimately decreases the conversion by exposing part of the enzyme into the oil phase. As the reaction proceeds, the volume of aqueous phase decreases because the molar volume of glycerol is 62.46 mL/mole whereas the molar volume of water is 18 mL/mole (Table 1). Hence microemulsion droplet size decreases with time during the esterification reaction.

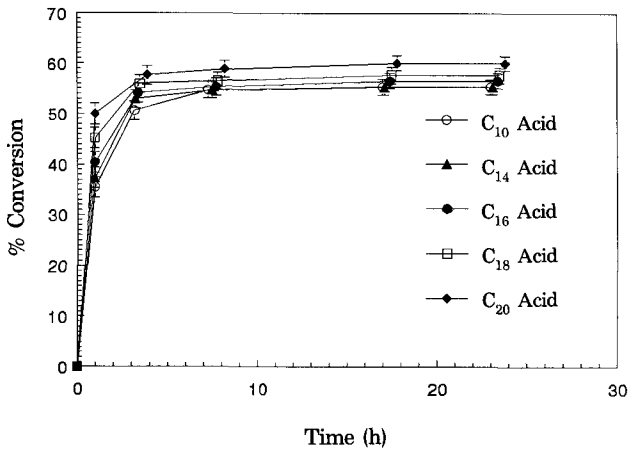

FIG. 3. Effect of fatty acid chainlength on its percent conversion with hexane as continuous phase.

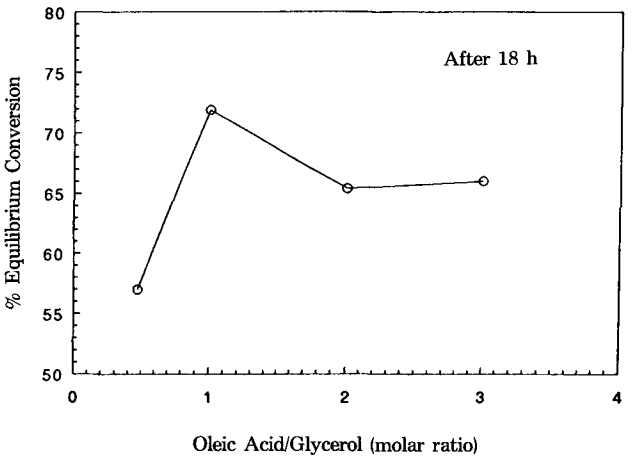

FIG. 4. Effect of oleic acid/glycerol ratio on the equilibrium percent conversion after 18 h.

TABLE 1

Volume Change of Aqueous Phase with Formation of Glycerides

Product	Net loss of aqueous volume (per mole of product)
Monoolein	62.46 − 1 × 18 = 44.46 mL
Diolein	62.46 − 2 × 18 = 26.46 mL
Triolein	62.46 − 3 × 18 = 8.46 mL

Effect of subsequent addition of glycerol or water after equilibrium. Brozozowski (28) showed that the active site of the enzyme is close to the surface but not completely exposed to the solvent. It is buried under the head of a long loop (lid), folded onto the triad and stabilized by extensive hydrophobic and electrostatic interactions. The enzyme could be working in two stages: The lid is removed or displaced, possibly through interfacial activation; then the ester bond is subsequently hydrolyzed or synthesized. The lid also serves as a device to inhibit the proteolytic activity of the triad, thereby protecting the enzyme itself. The size of the droplet decreases as synthesis proceeds due to consumption of glycerol. At a certain point, the droplet size has become too small to accommodate the enzyme, which may cause the lid to close. Addition of extra glycerol may reactivate the enzyme because it may move out of the oil phase into the interface, and again, the lid may open. Figure 5 shows an increase in percent conversion of oleic acid from 58 to 70% after the addition of glycerol (glycerol-to-oleic acid ratio after addition of glycerol was 2.1:1). It is not due to the increase in substrate (glycerol) concentration because the calculated glycerol-to-oleic acid ratio after equilibrium (before the addition of glycerol) was 1.7:1, and Figure 4 indicates that this ratio of glycerol to water is sufficient for the synthesis reaction. Addition of oleic acid will also enlarge the droplets, thus reactivating the enzyme. However, addition of water decreases the percent conversion (Fig. 6) because the enzyme starts to catalyze hydrolysis of mono- and diglycerides formed during the synthesis reaction. This proves that the enzyme is indeed active, even after equilibrium has been achieved.

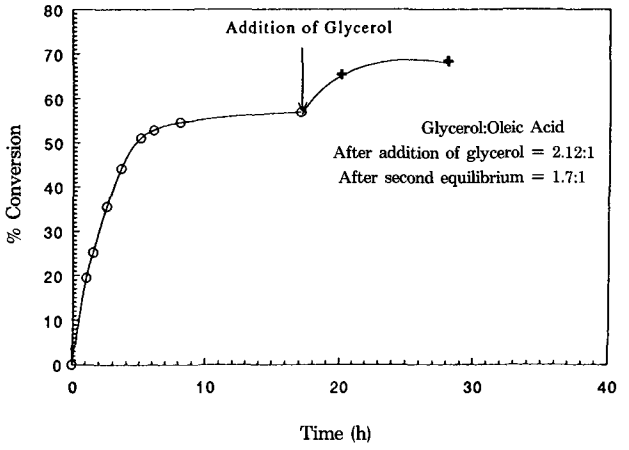

FIG. 5. Effect of addition of glycerol on percent conversion after first equilibrium.

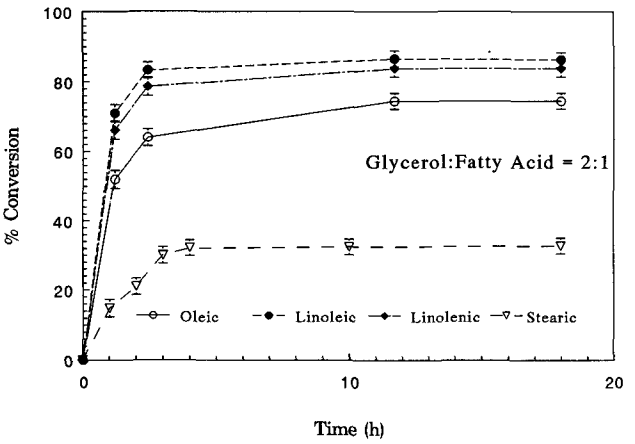

FIG. 7. Effect of unsaturation of fatty acid on percent conversion.

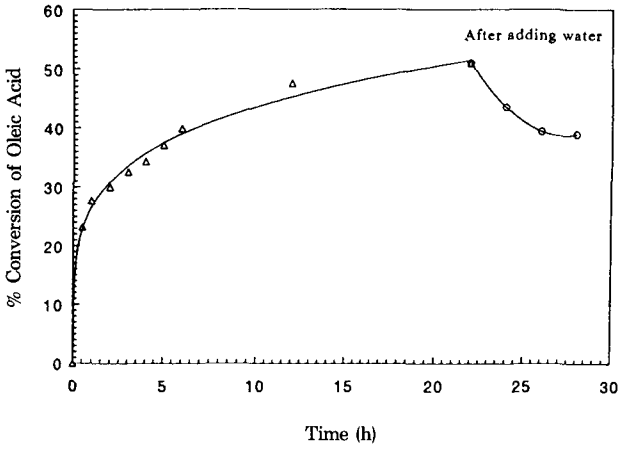

FIG. 6. Effect of addition of water on percent conversion after first equilibrium.

Effect of unsaturation of fatty acid. The structure of the fatty acid had a profound effect on the percent conversion of fatty acid to mono- and diglycerides in microemulsions (Fig. 7). A maximum conversion of 84% was found for linoleic acid, 80% for linolenic acid, 71% for oleic acid and 33% for stearic acid. This is believed to be due to the fluidity of the interface caused by unsaturation in the fatty acid. An increase in fluidity causes an increase in the rate of the reaction. Secondly, as the unsaturation in fatty acid increases, its solubility in hexane also increases. This increase in solubility from stearic acid to linoleic acid gives a driving force to the products at the interface to move into the oil phase quickly. This results in an increase in percent conversion. The small difference in the percent conversion for linoleic acid and linolenic acid is within experimental error. Although linolenic acid is more unsaturated than linoleic acid, the difference in the partitioning of their products is low.

HPLC of glycerol and oleic acid microemulsion reaction. Samples from microemulsion systems were mixed with equal volumes of ethanol for HPLC analysis by the external standard method. These studies indicate that monoolein (42%) and diolein (21%) are the only products formed during the enzymic reaction of oleic acid and glycerol in microemulsion. The conversion of oleic acid is 63% after the reaction reaches equilibrium in 18 h. This is a much higher conversion than that reported by Ergan and Andre (29), who found 22% diolein, 11% monoolein and 5% triolein after a 60-h reaction in bulk aqueous media. Diglyceride probably diffuses from the interface to the oil phase due to its high partitioning in the oil phase; and hence, no triolein formation is observed. A similar esterification reaction was carried out in bulk phase by Schuch and Mukherjee (30). They observed formation of triolein. It means that the microemulsion system allows diglyceride to diffuse into the oil phase. Therefore, one can use microemulsions for selective synthesis of monoolein and diolein (Table 2).

TABLE 2

Equilibrium Composition High-Performance Liquid Chromatography for Glyceride Synthesis in Microemulsion

	High-performance liquid chromatography (% by wt)	NaOH titration (% by wt)
Monoolein	42	—
Diolein	21	—
Triolein	0	—
Oleic acid	37	43

The described experiments have shown the following points: (i) The enzymic reaction of oleic acid and glycerol in microemulsions produces mainly monoglyceride and diglyceride; (ii) The percent conversion in microemulsion systems can be increased by further addition of glycerol; (iii) The enzyme remains active in w/o microemulsions, as seen by the increase in the percent conversion of oleic acid after equilibrium on further addition of glycerol; and (iv) The synthesis reaction is sensitive to the nature of the continuous phase, chainlength of fatty acid and glycerol/water ratio.

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