28

Synthesis of Esters Catalyzed by Lipases in Water-in-Oil Microemulsions

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1. Introduction

There are two basic advantages in using enzymes as catalysts in organic media instead of aqueous solutions. First, organic solvents favor the solubility of hydrophobic substrates and, second, the presence of such solvents shifts the thermodynamic equilibrium of condensation/hydrolysis reactions in favor of the desired product. Different approaches have been proposed to facilitate the reversal of the normal hydrolytic action of enzymes. These include various macroheterogeneous biphasic systems such as liquid–liquid systems composed of a water-immiscible organic solvent and water, nearly anhydrous systems in which the enzyme is usually suspended as a powder or in an immobilized form adsorbed onto a suitable carrier in organic solvents or gases in a supercritical state, and various homogeneous and microheterogeneous media such as mixtures of water-miscible organic solvent and water as well as different types of microemulsion system (reverse micelles). The subject of enzyme catalysis in media with low water content has been reviewed by several authors (1–6).

Water-in-oil (w/o) microemulsions are thermodynamically stable, optically isotropic colloidal dispersions of water in oil (any apolar solvent) stabilized by surfactant molecules (7). The surfactant molecules are adsorbed spontaneously at interfaces and separate the nonpolar and aqueous phases, thus decreasing the interfacial tension down to very low values (10^5 dyn/cm). The optical transparency of the system is the result of one liquid being finely dispersed into the other, forming microdroplets (reverse

micelles) with diameters typically of the order of 100 Å (*see* **Fig. 1**). The surfactant molecules used in the formation of microemulsions in apolar solvents include both natural membrane lipids and synthetic surfactants. **Table 1** provides some commonly used surfactants for enzymic studies.

The enzyme molecules can be entrapped in the water pools of the reverse micelles, avoiding direct contact with the organic solvent, potentially limiting their denaturation. The use of reverse micelles to solubilize enzymes (such as lipases) in organic solvents has attracted considerable interest in the past decade (*8,9*).

The diameters of reverse micelles strongly depend on the molar ratio of water to surfactant. This ratio is generally expressed in terms of the parameter w_0 (also mentioned as R) as the ratio of the molarity of water to the molarity of surfactant in the system $w_0=[H_2O]/[\text{surfactant}]$. For a wide variety of enzymes, a bell-shaped dependence of activity on w_0 has been observed (*8–10*). It has been proposed that an optimum of enzyme activity occurs around a value of w_0 at which the size of the droplet is equal to the size of the enzyme molecule (*9*). However, there is an ongoing discussion in the literature as to whether the water-content dependence of the enzyme activity is related to the size of the enzyme molecule (*8,11–13*).

Microemulsion systems provide an enormous interfacial area (approx 100 m^2/mL) through which the conversion of hydrophobic substrates can be catalyzed. Increasing the interfacial area is of great technological interest because this results in the increase of the number of substrate molecules available to react. Microemulsion systems offer several advantages as reaction media for bio-organic synthesis:

1. Both hydrophilic and hydrophobic substrates can be dissolved in high concentrations. The microemulsions represent a universal (i.e., an all-purpose microheterogeneous medium suitable for enzymatic reactions) (*9*).
2. The substrates can be enzymatically converted with high yields because the thermodynamic equilibrium of condensation/hydrolysis reactions can be easily shifted by adjusting the water content. It is interesting to note that the use of reverse micelles succeeded in changing the equilibrium constants of various enzymatic reactions by a factor of 10^6 (*11*).
3. The water content can be varied within a fairly broad range and the water content can be used as a tool to manipulate enzyme activity.
4. Multienzymatic reactions are feasible in reverse micelles.

The biotechnological relevance of enzymes in reverse micelles for the transformation of various substrates has been demonstrated by several authors. Some examples of enzymatic reactions in microemulsions are reverse hydrolytic reactions such as peptide synthesis (*14*), esterifications and transesterifications (*6,15*), and oxidation and reduction of steroids (*16,17*). A particular case of enzymatic studies in microemulsions is that of lipases, which act almost exclusively near interfaces in a classical heterogeneous procedure.
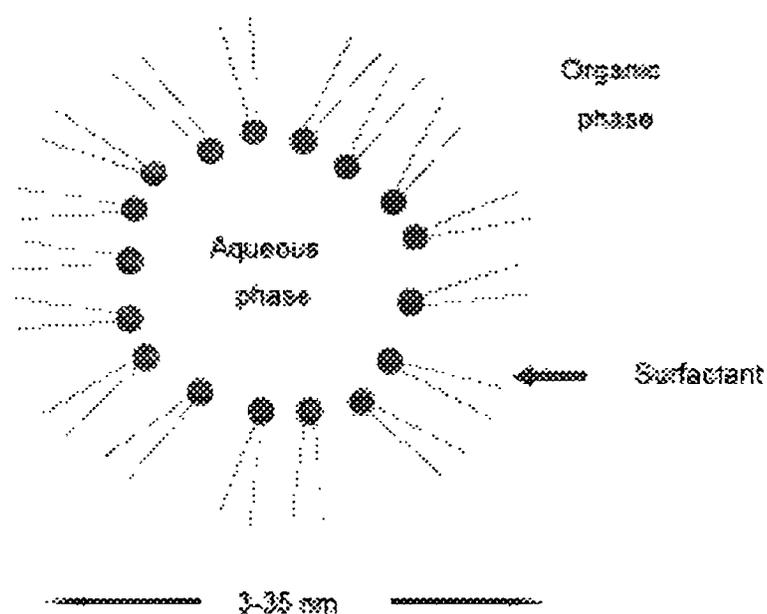
Fig. 1. Schematic presentation of reverse micelle.

Table 1
Commonly Used Surfactants for the Formation of w/o Microemulsions

Surfactants	Solvent systems
Bis-(2-ethylhexyl) sulfosuccinate sodium salt (AOT)	n-Hydrocarbons (C_6-C_{16})
	Iso-octane
	Cyclohexane
Cetyltrimethyl ammonium bromide (CTAB)	Heptane : chloroform
	n-Hydrocarbon : primary alcohols
Polyethylene glycol monododecylethers,	
$C_{12}E_n$ (n=3 5)	n-Hydrocarbons (C_6 C_{12})
Phoshatidylcholine	n-Hydrocarbons (C_6 C_{12})
Phosphatidylethanolamine	

One of the most important problems that must be solved for the employment of a microemulsion system in industrial processes is the recovery of the products from the reaction mixture and the regeneration of the enzyme. The presence of surfactant leads to a poor separation by normal techniques such as extraction and distillation because of the problems of emulsion-forming and foaming caused by the surfactant. Larsson et al. *(18)* proposed a simple technique for enzyme reuse and product recovery, taking advantage of the phase

behavior of the surfactant system to separate the microemulsion constituents into an oil-rich phase and a water-rich phase that contain almost all of the surfactant. By changing the temperature, a biphasic system was formed, where an oil-rich phase containing the product coexists with a water-rich phase containing the surfactant and the enzyme. The oil-rich phase may be replaced by a new solution of substrate in oil; then, the temperature is brought back to the level where the monophasic microemulsion is stable, and the reaction is repeated. A similar separation procedure (*see* Fig. 2), have been proposed by our group consisted of (1) a liquid–liquid extraction of the enzyme from the micellar phase into a new aqueous phase and (2) the equilibration of the remaining organic phase with an additional aqueous volume in order to separate the product from the surfactant. By this procedure, a significant amount of active lipase was extracted and the separation of the product from the surfactant system was almost complete *(19)*.

We have successfully used water-in-oil microemulsion system as a reaction media for the lipase-catalyzed esterification of both hydrophilic *(20,21)* and hydrophobic substrates *(6,21)*. The results of these experiments demonstrate that the present method should have quite general applicability for lipase-catalyzed synthetic reactions.

2. Materials

1. Lipase from *Penicillium simplicissimum* (from GBF, Germany), with a specific activity of 142 units/mg of protein (determined using triolein as the substrate) in 50 mM acetate buffer, pH 5.5.
2. Lipase from *Rhizopus delemar* (Fluka, Basel, Switzerland). The crude enzyme preparation was solubilized in a pH 5.5 buffer containing 20 mM acetate, 20 mM NaCl, 20 mM CaCl$_2$, and 1 mM NaN$_3$. The enzyme was precipitated by 70% (NH$_4$)$_2$SO$_4$ solution. After centrifugation, it was resolubilized in the same buffer and applied on a Sephadex G-100 gel filtration column and equilibrated with the above-mentioned buffer. The partially purified enzyme had a specific activity of 930 units/mg of protein, using tributyrin as the substrate.
3. Lipolase™ (Novo Nordisk A/S, Denmark), in the form of a straw-colored aqueous solution and was dialyzed before used at 4°C, against 25 mM Tris-HCl, pH 8.0. The enzyme exhibited a specific activity of 1350 units/mg of protein using p-nitrophenyl palmitate as the substrate.
4. Bis-(2-ethylhexyl)sulfosuccinate sodium salt (AOT) 99% pure (Sigma).
5. Isooctane, aliphatic alcohols, diols, glycerol, and fatty acids (Sigma).

3. Method
3.1. Esterification of Aliphatic Alcohols with Fatty Acids

1. Prepare a stock isooctane solution containing 100 mM AOT, 100 mM of alcoholic substrate (e.g., butanol, hexanol, octanol, geraniol, cholesterol), and 50 mM of fatty acid (e.g., lauric, myristic, palmitic, etc.).
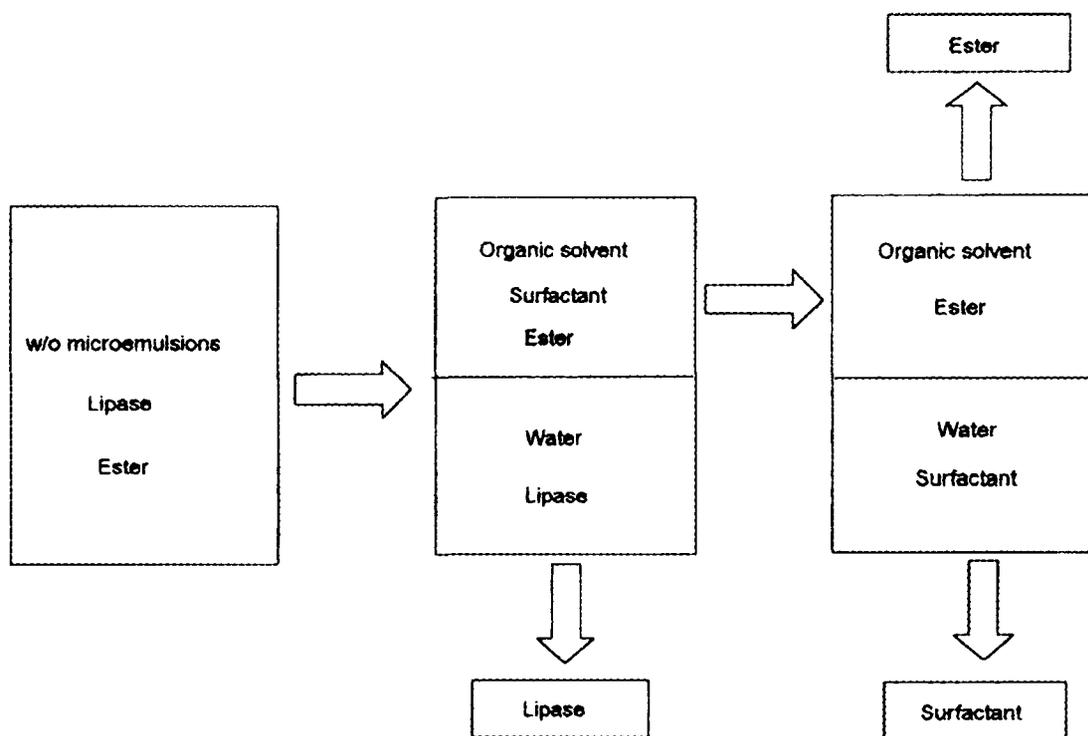
Fig. 2. Separation procedure of the enzyme and product recovery after completing an esterification reaction in a microemulsion system.

2. In a screw-cup reaction vessel, put 2 mL of the iso-octane solution and add 25 µL of 20 m*M* acetate (pH 5.5) buffer solution. (*See* **Note 1.**)

3. Vortex the mixture for 30–60 s until a clear solution (w/o microemulsion) formed.

4. Place the tube in a thermostable water bath, at 30°C for 10 min.

5. Add 10 µL of aqueous lipase solution (*Penicillium simplicissimum* or *Rhizopus delemar*) and mix the reaction mixture in vortex for 30 s. (*See* **Notes 2 and 3.**)

6. At various time intervals (total period 1–2 h), take aliquots of 1–2 µL and analyze them by gas chromatography (GC).

3.2. Esterification of Polar Diols with Fatty Acids

1. Prepare a stock iso-octane solution containing 100 m*M* AOT, 100 m*M* of a diol substrate (ethylene glycol, propane-1,3-diol, butane-1,4-diol, or pentane-1,4-diol), and 50 m*M* fatty acid (e.g., lauric, myristic, palmitic, etc.). (*See* **Note 4.**)

2. In a screw-cup reaction vessel, put 2 mL of the iso-octane solution and add 25 µL of a 25-m*M* Tris-HCl buffer, pH 8.0.

3. Vortex the mixture until a clear solution (w/o microemulsion) forms.

4. Place the tube in a thermostable water bath, at 25°C for 10 min.

5. Add 10 µL of aqueous lipase solution (Lipolase) and mix the reaction mixture in vortex for 30–60 s.

6. At various time intervals (total period 1–2 h), take aliquots of 1–2 µL and analyze them by GC. (*See* **Note 5.**)

3.3. Ester Recovery

1. After completing the esterification reaction, add on the micellar solution (microemulsion) an equivalent volume of a 25-mM Tris-HCl (pH 9.0) buffer solution containing different 100 mM NaCl. (*See* **Note 6.**)
2. Mix the two-phase system on a rotary shaker for 5 min at 350 rpm.
3. Separate the two phases by centrifugation for 10 min at 5000g.
4. Add on the upper organic phase (approx 5 mL) containing the product, the surfactant as well as some unreacted substrates), 3.5 mL fresh aqueous solution containing 5 mM Tris-HCl at pH 7.3. (*see* **Note 7**).
6. Incubate the mixture at 35°C for 30–60 min in order to separate the two phases (aqueous and organic).
7. Determine the ester and AOT concentrations in the oil top phase with GC.
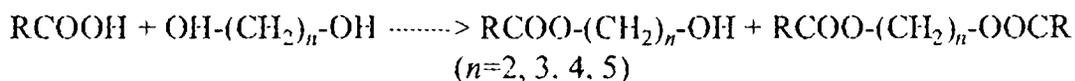
3.4. Gas Chromatographic Analysis

Analysis of products is achieved by employing gas chromatography. Aliquots of the reaction mixture are withdrawn at selected time intervals and diluted four times with chloroform before analyzed. For the determination of the concentration of the fatty acid esters as well as AOT, a Perkin-Elmer 8500 chromatographer is used, equipped with a 2-m glass packed column, loaded with GP 5%-DEGS-PS (Supelco). The carrier gas is nitrogen at a flow rate of 15 mL/min, the oven temperature is kept at 180°C, and a flame-ionization detector (FID) detector is used. For the determination of diols monoesters and diesters a 3-ft glass packed column, loaded with 1%-Dexsil 300 (Supelco) and an FID detector are used. Nitrogen is used as the carrier gas at a flow rate of 20 mL/min, with detector port temperature at 300°C. The oven temperature is kept constant for 2 min at 185°C, linearly increased (10°C/min) up to 300°C.

4. Notes

1. The final w_o value (w_o = [H$_2$O]/[AOT]), which affects the lipase catalytic behavior, depends on the water content of the system. The water content was adjusted by the addition of the required amount of buffer. The water content of the system as indicated in method (25 μL of buffer + 10 μL of lipase solution per 2 mL of microemulsion) corresponds to a w_0 =10.
2. The bioconversions were started by adding the lipase solution to the reaction mixture.
3. *Penicillium simplicissimum* lipase shows higher reaction rates in the esterification of long chain alcohols as well as secondary alcohols. *Rhizopus delemar* lipase shows a preference for the esterification of short-chain primary alcohols, whereas the secondary alcohols had a low rate of esterification and the tertiary ones could not be converted *(6)*.

4. The esterification of various hydrophilic diols with fatty acids catalyzed by Lipolase in w/o microemulsions leads to synthesis of a monoester and a diester, which are highly valuable products for industrial purposes *(20)*. The desired reaction was:

$$RCOOH + OH\text{-}(CH_2)_n\text{-}OH \longrightarrow RCOO\text{-}(CH_2)_n\text{-}OH + RCOO\text{-}(CH_2)_n\text{-}OOCR$$
$$(n=2, 3. 4. 5)$$

5. The maximum monoester formation is observed after 1–2 h incubation, when the overall conversion of fatty acid was 40–45%. Moreover, the product distribution can be composed almost quantitatively of the corresponding monoester if the reaction is stopped when the fatty acid conversion is lower than 25% *(20)*.

6. It is observed that when the pH value of aqueous phase is increased, an increase in lipase recovery yield is obtained. In the case of *R. delamar* lipase, the recovery yield is about 90% when the pH of the aqueous phase is 8.0–9.0 and 100 mM salt is used. This observation can be attributed to the electrostatic repulsive interactions between the negatively charged protein and surfactant molecules, at pH values of the aqueous phase beyond the isoelectric point of the protein. As a result of such interactions, the protein exhibits a decreased binding tendency to the surfactant molecules under alkaline conditions *(22)*.

7. The separation of a hydrophobic ester from the surfactant system was based on the phase behavior of such systems (i.e., the ability of the surfactant molecules to provoke, under certain conditions, the separation of the homogeneous system to an oil-rich phase and an aqueous phase). This ability depends on the incubation temperature of the system and also on the buffer-to-oil ratio *(19)*.

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