CHAPTER III
EXPERIMENTAL

3.1 Chemicals

Bis(2-ethylhexyl) hydrogen phosphate (HDEHP) was obtained from Aldrich (WI., U.S.A.) with the purity of 97%. 2,2,4-trimethylpentne (isooctane; Fisher Chemical, UK.) was used as organic solvent. *Candida antarctica* lipase B and *Thermomyces lanuginosa* lipase were kindly supplied from Novozymes, Denmark. All substrates in this work; caprylic acid (> 99% purity), oleic acid (~65% purity), and hexanol (> 98% purity) were purchased from Sigma, UK. Phosphate buffer salts was purchased from Fluka (Switzerland). Sodium chloride, sodium hydroxide, and pyridine were obtained from BDH (England), Merck (Doserset, UK.), and Lab Scans (Thailand), respectively. Cupric (II) acetate with high purity (> 99%) were purchased from Carlo Erba Reagent Company. Distillated water was used throughout this work.

3.2 Equipment

1. Fourier transform infrared spectrophotometer (FT-IT)-Bruker, Equinox 55/FRA 1065 (U.S.A.)
2. Ultraviolet-Visible spectrophotometer (UV-VIS)-UV Probe version1.1, Shimadzu Corporation, Japan
3. Coherent dynamic light scattering (DLS) with Malvern software application
4. Coulometer (Metrohm 737 KF, Switzerland)
5. pH meter (Schott CG842, GmbH)
6. High speeds and low temperature centrifuge (Sorvall super T21, U.S.A.)
7. Temperature controlled water bath (Heto DT2, Scadinavia)

3.3 Methodology

3.3.1 Preparation of Microemulsion

Two solutions were prepared to form microemulsions. The first solution was organic phase of HDEHP in isooctane and the second was aqueous solution of sodium chloride, sodium hydroxide and 0.04 mg/ml of lipases in phosphate buffer solution (pH ~7.6). After that, equal volume of the two solutions was mixed at equal molar concentration of HDEHP in isooctane and NaOH in water in a screw-cap vial. Then, with gentle agitation for 0.5 min, the solution was separated into two phases and its appearance changed from cloudy to clear, indicating the formation of microemulsion.

For esterification reactions, fatty acids (caprylic acid and oleic acid) and alcohol (hexanol) were added directly to organic phase (isooctane). Lipase was added to buffer solution to form aqueous phase. Both solutions were mixed in a capped vial to begin the reaction. All reactions are conducted at 30°C.

3.3.2 Measurement of Lipase Activity

The reactions are carried out in screw-cap vials placed in a thermostat bath. Aliquots of the reaction mixture are withdrawn at selected time. The reaction rate is determined by tracing the depletion of fatty acid substrate in the upper phase as follows: 0.1 ml was added to a screw-cap vial containing 4.9 ml of isooctane and 1 ml of cupric acetate-pyridine (5% w/v, pH 6.0). After centrifugation at 1800 rpm for 1 min, free fatty acid was determined in the upper organic phase. The absorbance was measured at 713 nm and used to construct calibration curve of each fatty acid as a function of concentration. After calibrating, curves of fatty acid depletion as a function of time are generated and the reaction rate is determined from the linear initial slope. The activity of lipase can then be calculated.

3.3.3 Identification of Products

The ester produced from the reaction was identified by taking a small amount of the upper phase and placed in ATR cell for FTIR analysis using VECTOR 22 Bruker Infrared Spectrometer.

3.3.4 Determination of Water Content

The water content of micellar solutions can be determined by injecting appropriate amount of the upper phase (usually 0.05 ml) to a coulometer (Metrohm, KF 737). Each sample is repeated at least 3 times to find the average value of water content and average total weight of sample. From these determinations it is possible to calculate the parameter (Wo) of the system by the following equation:

$$Wo = \frac{(W)(Wt)(1000)}{(Mw^{w})(V)[NaDEHP]} \qquad (3.1)$$

Where:

Wo	=	water to surfactant molar ratio
W	=	average weight of water content (wt%)
Wt	=	average weight of total sample (g)
Mw^{w}	=	molecular weight of water (18)
V	=	volume of injected sample (ml)
[NADEHP]	=	molar concentration of NaDEHP in organic phase (M)

3.3.5 Measurement of Reverse Micelle Size

The apparent dynamic radius of reverse micelle (Rh) was determined by dynamic light scattering method (Malvern) with the following settings: 514.3 nm, 90^{o} fixed angle, setting a pinhole 150, and using monomodel mode. Rh was defined as the distance from the center of water pool to the outside edge of surfactant layer. The correlator determined the electric field autocorrelation function, $g(\tau)$, from the measurements of scattered intensity according to Equation (3.2). From this function the translational average diffusion coefficient, Dz, was determined.

$$|g(\tau)| = \exp(-D_z Q^2 \tau) \quad (3.2)$$

Where:

$Q = (4\pi n/\lambda)\sin(\theta/2)$ = magnitude of scattering vector

λ = the wavelength of incident light (514.3 nm)

θ = the scattering angle (90^o)

n = the refractive index of solution at 35^oC

Rh can then be calculated from D_z by the Stokes-Einstein equation:

$$R_h = \frac{kT}{6\pi\mu D_z} \quad (3.3)$$

Where:

k = the Boltzman constant

T = the absolute temperature

μ = the solution viscosity (35^oC)

This equation applies only for spherical particles, of which description holds true for reverse micelle.

3.3.6 Determination of Lipase Concentration

The aqueous phase (lower phase) is withdrawn to determine the lipase concentration by measuring the absorbance at 280 nm using UV-VIS spectrophotometer. After that, the concentration of lipase in micellar solution can be determined by the remaining lipase in the aqueous phase.

3.3.7 Determination of Fatty Acid Concentration

Fatty acid concentration was determined using the ultraviolet-visible spectrophotometer (UV-VIS)-UV Probe version1.1 as follows: 0.1 ml of organic solution (upper phase) was added to screw-cap vial containing 4.9 ml of isooctane and 1 ml of cupric acetate-pyridine (5% w/v, pH 6.0). After centrifugation at 1800 rpm for 1 min, free fatty acid was determined in the upper organic phase. The absorbance was measured at 715 nm using UV-VIS spectrophotometer.

Investigation of other components in the reverse micelle (upper phase) and the aqueous phase was also verified by UV-VIS spectrophotometer. The absorbance of lipase, phosphate buffer and hexanol did not interfere with the absorbance of fatty acid except NaDEHP, which absorbs light near fatty acid range (713nm). Therefore, calculation of the actual rate of reaction, the absorbance of microemulsion without fatty acid was used to subtract from absorbance measured during the reaction.