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%. Lipase from *R. delemar* purchased from Fluka (Switzerland) and was used without further purification. 2,2,4-trimethylpentane (isooctane; Lab Scan, Thailand) was used as organic solvent. All fatty acids (Sigma, UK.), alcohols (Fluka, Switzerland), acetate buffer salts (Riedel-deHaën, Germany), and assay reagents used were of high purity (> 99%) excepted 1-hexanol was used at minimum 98% purity. Sodium hydroxide was obtained from Merck (Dorset, UK.). Sodium chloride was purchased from AJAX Chemical (Auburn, Australia). Cupric (II) acetate was obtained from CARLO ERBA Reagent (Milano, Italy). Pyridine was purchased from Lab Scan (Bangkok, Thailand). Distillated water was used throughout this work.

3.2 Equipment

1. Fourier transform infrared spectrophotometer (FTIR)-Bruker, Equinox 55/FRA 1065 (U.S.A.)
2. Ultraviolet-Visible spectrophotometer (UV-VIS)-Cecil instrument, CE 2040 (Cambridge, England)
3. Coherent dynamic light scattering (DLS) with Malvern software application (C. A., U. S. A.)
4. Coulometer (Metrohm 737 KF, Switzerland)
5. pH meter (Schott CG842, GmbH)
6. High speeds and low temperature centrifuge (Sorvall superT21, U.S.A.)
7. Temperature controlled water bath (Heto DT2, Scadinavia)

3.3 Methodology

3.1 Preparation of Microemulsions

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 and sodium hydroxide in acetate buffer solution (pH 6.0). After that, equal volume of the two solutions were 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 and long chain alcohol (hexanol) were added directly to organic phase (isooctane). While lipase and short chain alcohol were added to buffer solution to form aqueous phase. Both solutions were mixed in a capped vial to begin the reaction. All reactions were conducted at 30 ^{o}C.

3.2 Measurement of Lipase Activity

The reactions were carried out in screw cap vials placed in a thermostatted bath. Aliqouts of the reaction mixture were withdrawn at selected time. The reaction rate was determined by tracing the depletion of fatty acid substrate in the upper phase as follows: 0.1 ml 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 acids were 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 (see in Apendix F). After calibrating, curves of fatty acid depletion as a function of time were generated and the reaction rate was determined from the linear initial slope. Then the activity of lipase was calculated.

3.3 Analyzed of Products

The produced ester were isolated from a small amount of upper phase and placed in ATR cell for FTIR analysis using VECTOR 22 Bruker Infrared Spectrometer.

3.4 Determination of Water Content

Appropriate amount of the upper phase (usually 0.05 ml) was injected to Coulometer (Metrohm, KF 737) to determine the content of water in microemulsion system. Each sample was repeated at least 3 times to find the average value of water content and average total weight of sample. After that, molar ratio of water to surfactant (Wo) of the system was calculated by the following equation (Appendix H):

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

Where:

Wo = water to surfactant ratio

W = average weight of water content (wt %)

W_t = 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)

3.5 Measurment of Reverse Micelle Size

The hydrodynamic radius (or Stokes radii) of reverse micelle (R_h) was determined by DLS (514.3 nm, 90^o fixed angle, set pin hole 150, and using monomodel mode) with R_h being defined as the distance from the center of water pool to the outside edge of the surfactant layer. The correlator determined the electric field autocorrelation function, $g(\tau)$, from measurements of scattered intensity. From this function the translational average diffusion coefficient, D_z, was determined.

$$|g(\tau)| = \exp(-DzQ^2\tau) \qquad (3.2)$$

where:

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

λ = the wavelength of incident light (514.3 nm)

θ = the scattering angle (90^{o})

n = the refractive index of solution ($30^{o}C$)

This equation is valid for monodisperse populations of colloidal aggregates present in dilute concentrations. Assume that our system is valid in concentrations of water in microemulsion system. R_h can be calculated from D_z by the Stokes-Einstein equation:

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

where:

k = the Boltzman constant

T = the absolute temperature

μ = the solution viscosity ($30^{o}C$)

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