CHAPTER III
EXPERIMENTAL

3.1 Chemicals

97% purity di-2-ethylhexyl phosphoric acid (HDEHP) or bis (2-ethylhexyl) hydrogen phosphoric acid was obtained from Aldrich (Milwaukee, WI) and was used without further purification to form microemulsions. Protein α-chymotrypsin from bovine pancreas and substrate *N*-glutaryl-L-phenyl-*p*-nitroanilide (GPNA) were obtained from Sigma (Poole, UK). Three cosurfactants were tributyl phosphate (TBP), reagent grade was obtained from Carlo ERBA Reagent (Milano, Italy), 2-ethylhexanol and 1-heptanol were obtained from Fluka (Switzerland). Isooctane as an organic phase was obtained from Labscan (Bangkok, Thailand). Sodium chloride (NaCl), potassium chloride (KCl), sodium hydroxide (NaOH), calcium chloride ($CaCl_2$) and *p*-nitroaniline were obtained from Fluka (Switzerland). Two buffers and Trizma hydrochloride were obtained from Aldrich (Milwaukee, WI) and Tris (hydroxyl methyl) aminomethane was obtained from Carlo ERBA Reagent (Milano, Italy). Deionized water used to prepare aqueous solution was obtained from Nontri (Bangkok, Thailand).

3.2 Experimental Methods

3.2.1 Preparation of NaDEHP Reverse Micellar Phase

The NaDEHP reverse micelle system was prepared by mixing 10 ml of 0.1 M HDEHP/0.1 M TBP/isooctane solution with 10 ml of 0.1 M NaOH aqueous solution in a 6-dam vial (24 ml). The pH of the aqueous phase was adjusted by using NaOH or HCl solution. The vial was shaken vigorously and kept overnight in a water bath at temperature of 25°C for equilibrium and phase separation. After phase separation, NaDEHP reverse micelles were formed in the organic upper phase.

3.2.1.1 Effect of salt concentration on the phase transition with and without cosurfactant

The reverse micellar system was prepared by mixing 10 ml of 0.1 M HDEHP/0.1 M TBP/isooctane solution with 10 ml of 0.1 M NaOH at various salt concentrations ranging from 0.1-4 M for the case in the absence of cosurfactant. In the presence of cosurfactant, the reverse micelle was prepared by mixing 10 ml of 0.1 M HDEHP/0.1 M TBP or 0.1 M 2-ethyl-1-hexanol or 1-heptanol as a cosurfactant /isooctane with equal volume of 0.1 M NaOH at various NaCl concentrations (0.1-3 M).

3.2.1.2 Effect of salt concentration on water content of the reverse micelle

The reverse micellar system was prepared at various salt concentrations (0.1-3 M) for all three types of cosurfactant in a similar manner as described earlier. After equilibrium and phase separation, water content (ω_o) of reverse micelle was measured by coulometer (Metrohm, 737KF).

3.2.2 Forward extraction

The forward extraction was performed by mixing 8 ml of 0.5 mg/ml α-chymotrypsin/0.1 M NaCl/0.025 M Tris-HCl aqueous solution with 8 ml of reverse micelle solution in a vial. The vial was shaken vigorously for 5 minutes and then centrifuged for 2 minutes for phase separation. The α-chymotrypsin was extracted to the upper phase. The concentration of the α-chymotrypsin in the aqueous solutions was determined by UV-Visible spectrophotometer at 281 nm. Amount of α-chymotrypsin being extracted was then calculated by using method of difference.

3.2.3 Backward extraction

The backward extraction was carried out by mixing 6 ml of 0.1 M $CaCl_2$ aqueous solution with 6 ml of the α-chymotrypsin loaded in a W/O microemulsion solution in a 6-dam vial (24 ml). The vial was shaken vigorously for 5 minutes and then, the sample was centrifuged for 2 minutes to obtain phase

separation. The α-chymotrypsin was then transferred back to aqueous phase. Amount of α-chymotrypsin being extracted was then calculated by using method of difference.

3.2.4 Activity test of α-Chymotrypsin After Backward Transfer

The activity of α-chymotrypsin after backward extraction was measured from the hydrolysis reaction as shown below:

α-chymotrypsin + GPNA *p*-nitroaniline (yellow)

3.2.4.1 Preparation of GPNA

The substrate *N*-glutaryl-L-phenyl-*p*-nitroanilide (GPNA) solution was prepared by dissolving 20 mg of GPNA in 1 ml of methanol, and then 50 ml of 0.005 M Tris-HCl buffer (pH 7.5) was added.

3.2.4.2 Activity test

The enzymatic activity test of α-chymotrypsin was conducted by mixing 0.5 ml of α-chymotrypsin with 2.5 ml of GPNA as a substrate, *p*-nitroaniline (yellow color) was then released as a product from the hydrolysis reaction. The reaction was allowed to proceed for 10 minutes, then 0.5 ml of 30% acetic acid was added to stop the reaction. The concentration of the *p*-nitroaniline product was measured by UV-Visible spectrophotometer at 410 nm.

3.3 Analytical and Characterization Techniques

3.3.1 Analytical Equipment

UV-Visible spectrophotometer (Cecil Instrument Limited, CE 2040, Cambridge, England) was used to determine protein concentration by measuring its absorbance at 281 nm. Centrifuge (Coolworking system, 4236 CWS, Milano, Italy) was used for phase separation and Vortex (Scientific industries, Genie-2 G560E, New York, U.S.A.) was used for mixing the microemulsion, oil and aqueous phases in the forward extraction and the backward extraction steps. Temperature controlled

water bath (Heto, DT2, Scandinavia) was used at a constant 25 °C for all experiments. Dynamic light scattering (Cohenrent, California, U.S.A.) with Malvern application software that consists of stepper motor controller (PCS7), temperature controller (PCS8) & photo multiplier supplies, and multi-8 (series 7032) were used for estimating the micelle size. Coulometer (Metrohm, 737 KF, Switzerland) was used for determining the amount of water in microemulsion solution. pH meter (Schott, CG842, Germany) was used for controlling the pH of microemulsion and aqueous phases.

3.3.2 Characterization

The size of the reverse micelles before and after forward extraction was estimated using dynamic light scattering at the constant angle 90° and constant temperature of 25 °C. The refractive index and the viscosity of isooctane at 25 °C are 1.3890 and 0.467 cP, respectively. The concentration of α-chymotrypsin and enzymatic activity tests were determined by using UV-Visible spectrophotometer to follow a decrease in the absorbance corresponding to the substrate disappearance. Amount of water of microemulsion in the upper phase was determined using coulometer. Microemulsion solution 50.0 μl was titrated with hydramal coulometer solution and the experiment was repeated three times.