# 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 (Milwaulkee, U.S.A.) and was used without further purification to form microemulsions. Protein α-chymotrypsin from bovine pancreas and substrate *N*-glutaryl-L-phynyl-*p*-nitroamilide (GPNA) were obtained from Sigma (St. Louis, U.S.A.). Two cosurfactants used in this study were tributylphosphate (TBP) and 3-[(3-cholamidyl-propyl)dimethylammonio]-1-propanesulfonate (CHAPS). TBP reagent grade was obtained from Carlo ERBA (Milano, Italy) and 98% purity CHAPS was obtained from Fluka (Buchs, Switzerland). Isooctane, sodium chloride (NaCl), and sodium hydroxide (NaOH) were obtained from Labscan (Bangkok, Thailand). Calcium chloride ($CaCl_2$) was obtained from Carlo ERBA Reagent (Milano, Italy). *p*-nitroaniline was obtained from Fluka (Buchs, Switzerland). Trizma hydrochloride was obtained from Aldrich (Milwaulkee, WI) and Tris (hydroxyl methyl) aminomethane was obtained from Carlo ERBA (Milano, Italy). Distrilled water used for aqueous solution preparation was obtained from Nontri (Bangkok, Thailand).

## 3.2 Experimental Methods

3.2.1 Microemulsion Formation of NaDEHP System

*3.2.1.1 Microemulsion formation of NaDEHP system in the absence of cosurfactant*

The NaDEHP microemulsion systems were prepared by mixing 10 ml isooctane solution of 0.1 M HDEHP with 10 ml aqueous solution of NaCl (0.0-4.0 M) and 0.1 M NaOH in a 8-dam vial. After mixing, pH of the aqueous phase was adjusted to 7.4 by using NaOH or HCl solution. The vial was shaken vigorously for 5 minutes and kept overnight in a water bath at temperature of 25°C for equilibrium

and phase separation. After phase separation, the series of microemulsion in the absence of cosurfactant were observed.

*3.2.1.2 Microemulsion formation of NaDEHP system using TBP as a cosurfactant*

The NaDEHP microemulsion system was prepared by mixing 10 ml isooctane solution of 0.1 M HDEHP and 0.1 M TBP with 10 ml aqueous solution of NaCl (0.0-0.4 M) and 0.1 M NaOH in a 8-dam vial. After mixing, pH of the aqueous phase was adjusted to 7.4 by using NaOH or HCl solution. The vial was shaken vigorously for 5 minutes and kept overnight in a water bath at temperature of 25°C for equilibrium and phase separation. After phase separation, the series of microemulsion using TBP as a cosurfactant were observed.

*3.2.1.3 Microemulsion formation of NaDEHP system using CHAPS as a cosurfactant*

The NaDEHP microemulsion system was performed by adding 10 ml of stock solution of NaCl/0.1 M NaOH/aqueous solution to a 8-dam vial containing solid CHAPS, stirring until the solution was clear and then mixing with 10 ml of 0.1 M HDEHP/isooctane solution. The NaCl concentration was varied from 0.0 to 4.0 M. After mixing, the pH of the aqueous phase was adjusted to 7.4 by using NaOH or HCl solution. The vial was shaken vigorously for 5 minutes and kept for 5 days in a water bath at temperature of 25°C for equilibrium and phase separation. After phase separation, the series of microemulsion using CHAPS as a cosurfactant were observed.

In order to determine the effect of CHAPS as a cosurfactant on the microemulsion formation the microemulsion system of NaDEHP/isooctane/brine with CHAPS cosurfactant was performed in the same manner as previously described in section *3.2.1.3*, but the NaCl concentration was fixed at 0.2 M and the CHAPS concentration was varied from 0.0 to 50.0 mM.

*3.2.1.4 Effect of salt concentration on water content and microemulsion size*

The microemulsion system was prepared at various salt concentrations in a similar manner as described earlier. After equilibrium and phase separation, water content ($\omega_o$) and microemulsion size were measured by coulometer (Metrohm, 737

KF) and dynamic light scattering (Cohenrent) with Malvern application software, respectively.

*3.2.1.5 Effect of CHAPS concentration on water content and microemulsion size*

As described in section *3.2.1.3*, to investigate the effect of CHAPS on water content the microemulsion system was prepared at various CHAPS concentrations. After equilibrium and phase separation, water content ($\omega_o$) and size of microemulsion were measured by coulometer (Metrohm, 737 KF) and dynamic light scattering (Cohenrent) with Malvern application software, respectively.

3.2.2 Forward Extraction

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 the upper phase of microemulsion in a 6-dam vial (24 ml). The vial was shaken vigorously for 5 minutes, centrifuged at 2000 rpm for 2 minutes for phase separation and kept in water bath at temperature 25°C for 1 day. Then α-chymotrypsin was extracted to the upper phase. The concentration of the α-chymotrypsin left 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 (Hu and Gulari, 1996). For spectrophotometric measurements, it was necessary to have a blank with the same environment as the sample, therefore; the controlled vial was always prepared in this work exactly in the same manner as the extracted vial but the only difference was the use of buffer solution in the absence of protein.

3.2.3 Backward Extraction

Backward extraction was carried out by mixing 6 ml of 0.1 M $CaCl_2$/0.025 M Tris-HCl aqueous solution with 6 ml of the α-chymotrypsin loaded in an upper phase solution in a 6-dam vial (24 ml). The vial was shaken vigorously for 5 minutes, centrifuged for 2 minutes to obtain phase separation and kept in water bath at temperature 25°C for 6 hours. The α-chymotrypsin was then transferred back to aqueous phase. Amount of α-chymotrypsin being extracted was determined by UV-

Visible spectrophotometer at 281 nm. The controlled vial was prepared and used for comparison as described in the previous section.

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) (3.1)

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 0.05 M Tris-HCl buffer (pH 7.4) was added. The enzymatic activity test of α-chymotrypsin was conducted by mixing 1.0 ml of α-chymotrypsin with 5.0 ml of GPNA as a substrate in a 2-dam vial. After vial was shaken vigorously for 30 second, *p*-nitroaniline (yellow color) was 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 365 nm (scanning mode) and compared with the controlled vial.

## 3.3 Analytical and Characterization Techniques

3.3.1 Analytical Equipment

UV-Visible spectrophotometer (Perkin Elmer, Lamda 10) 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 to control temperature 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), temparature 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 Characterizations

The concentration of α-chymotrypsin and the enzymatic activity tests, which follow the increase in the absorbance corresponding to the product generation, were determined by using UV-Visible spectrophotometer. The microemulsion size along the extraction was estimated by 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 amount of water in the upper phase of microemulsion was determined using coulometer. Microemulsion solution of 20.0 μl was titrated with hydramal coulometer solution and the measurement was repeated five times.