CHAPTER III
EXPERIMENTAL

3.1 Materials

Bis(2-ethylhexyl) hydrogen phosphate (HDEHP) was obtained from Aldrich (WI., U.S.A.) with the purity of 97%. 2,2,4-Trimethylpentane (isooctane; Lab Scan, Thailand) was used as organic solvent. Fresh rice bran was purchased from Chachengsao Rice Mill, Thailand. Dialysis bag was kindly supplied by Department of Microbiology and Biochemical Technology Chulalongkorn University. Diethyl ether, calcium chloride, ammonium sulphate, and cupric (II) acetate with high purity (> 99%) were purchased from Carlo Erba (Milano, Italy). All substrates used in this work; caprylic acid (> 99% purity), oleic acid (~65% purity), and hexanol (> 98% purity) were purchased from Sigma (UK). Phosphate buffer salts, sodium chloride, sodium hydroxide, and pyridine were obtained from Riedel-deHaën (Germany), Ajax Chemical (Auburn, Australia) and Merck (Doserset, UK.), and Lab Scans (Thailand), respectively. Distilled water was used throughout this work.

3.2 Methodology

3.2.1 Rice bran Lipase Preparation Procedure

In this study the rice bran lipase preparation procedure was modified from Phraephrewngarm (1999), Prabhu *et al.* (1999), and Sirisansaneeyakul *et al.* (1997). The procedure consisted of three steps: defatting, extraction, and purification. Details of each step are described as follows:

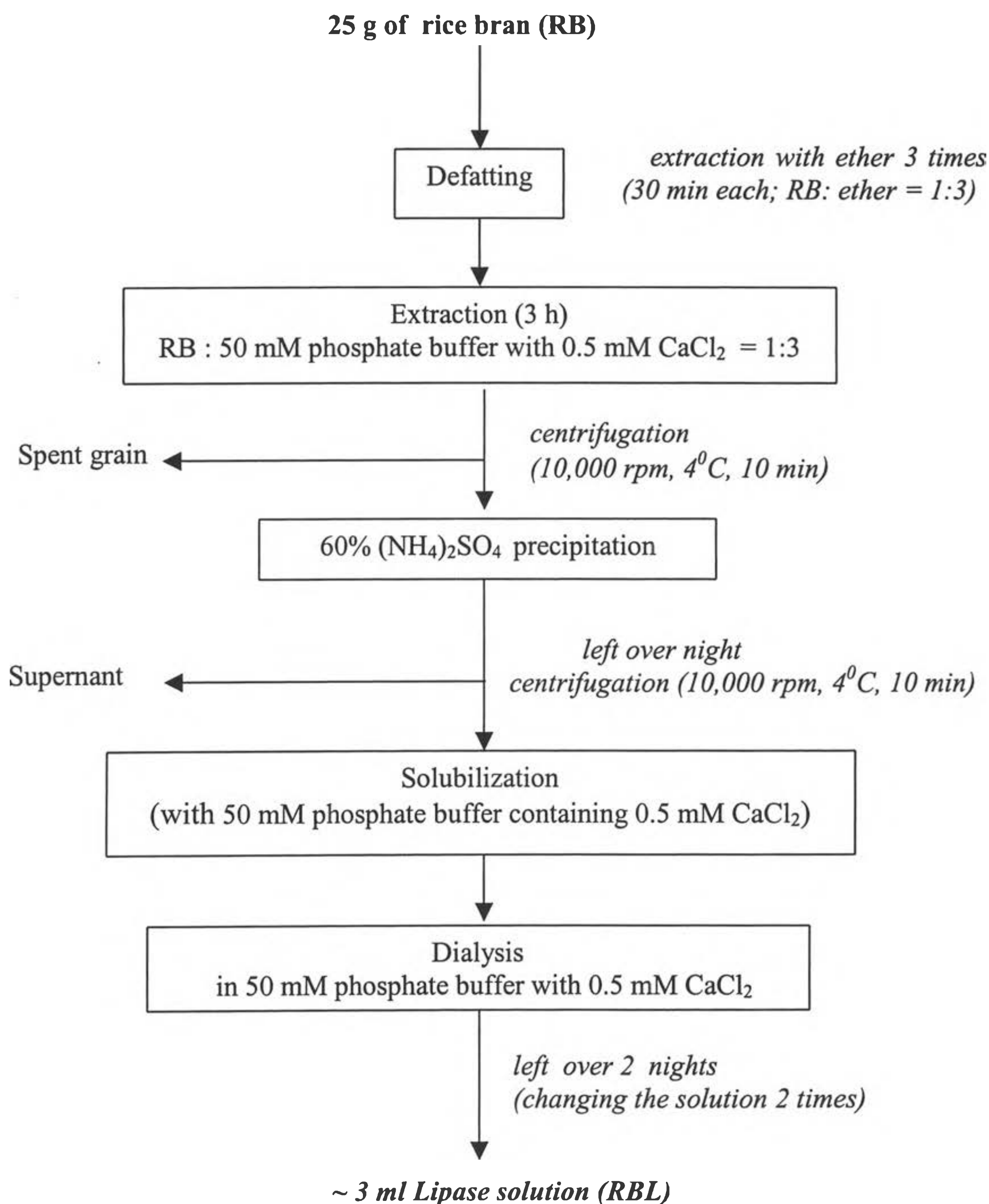
3.2.1.1 *Defatting of rice bran*

Rice bran lipase was obtained by first defatting the rice bran to remove the lipid component. For defatting, 25 g of rice bran was stirred in an ice bath with 75 ml of diethyl ether for 30 min each in three batches. The defatted rice bran was left about 5 h for removing all diethyl ether by evaporation.

3.2.1.2 *Extraction and purification rice bran lipase*

The defatted rice bran was stirred with three times its weight of 50 mM phosphate buffer, pH 7.4, containing 0.5 mM calcium chloride at 4°C for 3 h. The rice bran lipase solution was filtered to remove rice bran particle, after which the filtered solution was centrifuged at 4°C, 10,000 rpm for 10 min using refrigerated centrifuge (Sorval, super T21). The supernatant was collected to yield the crude lipase extract. Then, the filtered solution was purified by gradually adding ammonium sulphate powder to make the concentration to 60% saturation and turbid solution was left overnight. The solution was centrifuged at 4°C, 10,000 rpm for 10 min to obtain brown slurry. A small amount of 50 mM phosphate buffer, pH 7.4, containing 0.5 mM $CaCl_2$ was added to dissolve the slurry before removing ammonium sulphate by dialysis in the same solution (50 mM phosphate buffer, containing 0.5 mM $CaCl_2$) over 2 nights in an ice bath.

The rice bran lipase preparation procedure is shown in Figure 3.1.



* Keep temperature at 4^0C in every step

Figure 3.1 The schematic diagram for the preparation of rice bran lipase.

3.2.2 Preparation of NaDEHP Reverse Micelles

Two solutions were prepared to form microemulsion. The first was the organic phase containing HDEHP and cosurfactant (hexanol) in isooctane and the second was sodium hydroxide and sodium chloride in the aqueous buffer solution (250 mM phosphate, pH 7.4). After their preparation, equal volumes of the two solutions were mixed in a screw cap vial at equal molar concentration of HDEHP in the organic phase and NaOH in the aqueous phase, typically 100 mM. A clear solution was obtained after vortex mixing for a few seconds, indicating the formation of microemulsion.

For esterification reactions, lipase was first added into the prepared reverse micelles by injecting lipase solution to the aqueous phase of the NaDEHP microemulsion followed by gentle shaking for 30 seconds. The enzyme would be transferred to the organic solution (upper phase) and be encapsulated in the reverse micelle due to the electrostatic interaction between lipase surface and surfactant head group. After that, the mixture of fatty acid and alcohol was added to the organic phase to begin the reaction; the upper phase was stirred for a few seconds. All reactions were conducted at 35°C in a water bath. The water to surfactant ratio of reverse micelle (W_0) can be adjusted by the addition of the required amount of salt (NaCl).

3.2.3 Measurement of Lipase Activity

The reactions were carried out in screw-cap vials placed in a temperature-controlled water bath. Aliquots of the reaction mixture were withdrawn at a selected time interval. The reaction rate was determined by tracing the depletion of fatty acid substrate in the upper phase using the spectrophotometric assay developed by Lowry and Tinsley (1976) 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 3000 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 (Perkin-Elmer, Lamda 10) and was 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 were generated and the reaction rate was

determined from the linear initial slope. The activity of lipase could then be calculated.

3.2.4 Analysis of Products

The ester product formed during the reaction was isolated by taking a small amount of upper phase and placing the sample in ZnSe cell for FTIR analysis using Infrared Spectrophotometer (Bruker, VECTOR 22).

3.2.5 Determination of Water Content

Appropriate amount of upper phase (40 μl) was injected to a coulometer (Metrohm, KF 737) to determine the content of water in the microemulsion system. After that, a molar ratio of water to surfactant (W_0) of system was calculated by the following equation.

$$W_0 = \frac{(W/100)(Wt) * 1000}{(Mw)(V) * [NaDEHP]} \quad (3.1)$$

Where:

W_0	= water to surfactant ratio
W	= average weight of water content (wt%)
Wt	= average weight of total sample (g)
Mw	= molecular weight of water (18)
V	= volume of injected sample (ml)
[NADEHP]	= molar concentration of NaDEHP in organic phase (M)

3.2.6 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° 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.

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

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°)

n = the refractive index of solution at 35^oC

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

$$Rh = \frac{KT}{6\pi\mu\ Dz} \quad (3.3)$$

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.

3.2.7 Determination of Lipase Concentration

The aqueous phase (lower phase) was withdrawn to determine the lipase concentration spectrophotometrically by measuring the absorbance at 276 nm. The concentration of lipase can be calculated by the following equation.

$$\text{enzyme concentration} = \frac{A_{276}}{E}\ \text{mg/ml} \quad (3.4)$$

Where:

A_{276} = absorbance of RBL at 276 nm

E = molar extinction coefficient of RBL at 276 nm (15.25)

After that, the concentration of lipase in micellar solution can be determined by subtracting the initial amount with the remaining amount of lipase in the aqueous phase.