CHAPTER III
EXPERIMENTAL

3.1 Materials

Fresh rice bran was purchased from Manathanya Phanich Co., Ltd. Rice bran oil was a product from Thai Edible Oil Co., Ltd. Phenolphthalene, potassium hydrogen phthalate, dipotassium hydrogen phosphate, and standard buffer pH 4, 7, and 10 were obtained from Ajax Chemicals, USA. Ammonium sulfate was obtained from Ajax Chemicals, USA, and ground by mortar before use. Adipic acid, potassium dihydrogen phosphate, sodium chloride, isopropyl ether, 1,4-butanediol, poly(ethylene glycol) MW 200 and 6 000, celite 545, and alumina were purchased from Fluka, Switzerland. Acetone, toluene and tetrahydrofuran were bought from J.T. Baker, USA. Hexane, absolute ethanol, calcium chloride, sodium hydroxide, and fumed silica were obtained from Aldrich, USA. DEAE-cellulose, and porcine pancreatic lipase (PPL) were purchased from Sigma, USA.

3.2 Equipment

3.2.1 Fourier Transform Infrared Spectrophotometer (FTIR)

FTIR spectra were obtained from VECTOR 22 Bruker Spectrometer equipped with deuterated triglycine sulfate (DTGS) detector. An absorbance mode was used with 64 scans and 4 cm^{-1} resolution.

3.2.2 Ultraviolet-Visible Spectrophotometer (UV-Vis)

Rice bran lipase solution and its concentration were qualitatively and quantitatively analyzed by a Lambda-16 UV-Vis spectrophotometer, Perkin-Elmer.

3.2.3 Gel Permeation Chromatography (GPC)

GPC chromatograms were performed by Waters GPC 600E attached with RI (Waters 410) and UV detectors (Waters 486). Three columns, Styragel HR 0.5, HR 4E, and HR 5E were connected in series and used with HPLC grade THF as the eluent. The flow rate was maintained at 1 mL/min throughout the experiment. Refractive index detector was used for determination the molecular weight of polyester.

3.2.4 High Speed Refrigerated Centrifuge

A high speed refrigerated centrifuge (Sorval Super T-21) was used for separation the rice bran particles and rice bran lipase.

3.2.5 Lyophilizer

The rice bran lipase solution was lyophilized by Flexi-Dry FTS systems from Stone Ridge, New York, USA.

3.2.6 Nuclear Magnetic Resonance (NMR)

^{1}H-NMR spectra were obtained from ACF 200 MHz, Bruker, Switzerland. Deuterated chloroform was used as a solvent. The samples were taken with 64 scans.

3.2.7 Elemental Analysis (EA)

The percent elements were obtained from Yanako CHN Corder MT-3, MT-5 with combustion temperature 950°C. The samples were run under He (flow rate 200 mL/min) and O_2 (flow rate 20 mL/min).

3.3 Methodology

3.3.1 Preparation of Rice Bran

Fresh rice bran was excluded from broken grains, rice hull, and insects by 35 mesh size sieving machine.

3.3.2 Purification of Rice Bran Lipase

3.3.2.1 *Extraction of Lipase from Rice Bran (Step I)*

The sieved rice bran (1 000 g) was suspended in 3.0 L of 10 mM calcium chloride and stirred for 3 h in ice bath. The RBL solution was filtered to remove rice bran particles. The turbid solution was centrifuged at 4°C, 10 000 rpm for 10 minutes. The slightly turbid supernatant was obtained and designated as "crude extract".

3.3.3.2 *Fractionation with Ammonium Sulfate (Step II)*

Ammonium sulfate powder was gradually added to the constant stirring of crude extract till 60% saturation. The turbid solution was left overnight. The solution was centrifuged at 4°C, 15 000 rpm for 10 minutes to obtain brown precipitate. The precipitate was suspended in a small amount of 1 mM calcium chloride solution (~1.5 ml). Ammonium sulfate was removed by dialysis in 1.5 L of 1 mM calcium chloride overnight in ice bath. The solution was centrifuged at 4°C, 15 000 rpm for 10 minutes. The clear supernatant was designated as "crude RBL solution". Crude enzyme solution

was dried by lyophilizing to obtain RBL solid. The solid was stored at 4°C until use.

3.3.2.3 *Diethylaminoethyl(DEAE)-cellulose Column Chromatography (Step III)*

"The purified RBL" was obtained as follows. The crude enzyme solution was dialyzed in 10 mM phosphate buffer containing 1 mM calcium chloride, pH 7.0 at 4°C for 1 day. After removal of the insoluble particles by centrifugation, the supernatant was treated with poly(ethylene glycol) MW 6 000 to remove water until the volume was 5 mL. The concentrated crude enzyme was applied onto a DEAE-cellulose column (20ϕ mm x 150 mm) which was equilibrated with pH 7.0 buffer (10 mM phosphate containing 1 mM calcium chloride). The same buffer was used as a mobile phase and the column was operated with a flow rate 1 mL/min.

The collection of enzyme was done by the following steps. The sample was taken for 5 mL in tubes. Each tube was analyzed for the protein amount by measuring the absorbance at 280 nm. The activity of each fraction was measured. Similarly, the mobile phase was changed again to pH 7.0 buffer (100 mM phosphate containing 1 mM calcium chloride) after another decreasing of the absorbance at 280 nm was detected. Finally, the column was washed with pH 7.0 buffer (100 mM phosphate containing 1 M sodium chloride additionally to 1 mM calcium chloride) after all of enzyme was collected. The fractions having activity were collected together and used as "purified RBL".

3.3.3 Rice Bran Lipase Immobilization

3.3.3.1 *Rice Bran Lipase Immobilization via Physisorption*

Fumed silica, alumina or celite was chosen as a carrier. The crude RBL solution (30, 40, and 50 mL) or purified RBL solution (40 mL) was mixed with the carrier (1 g) in a 250 mL flask. The suspension was

shaken at room temperature for 1 h. The immobilized RBL was centrifuged at 4°C, 15 000 rpm for 5 minutes to exclude excess RBL solution. After washing with 50 mM phosphate buffer, pH 7.0, the immobilized RBL was dried under vacuum at room temperature and stored at 4°C until use.

3.3.3.2 *Rice Bran Lipase Immobilization via Covalent Bonding*

To activate fumed silica surface, fumed silica (4 g) was refuxed in 200 mL of 3-aminopropyltriethoxysilane in toluene (5% v/v) for overnight. After filtration, the activated silica (Si-APTES) was washed with toluene, acetone, and distilled water, respectively, and dried at 50°C for overnight. The obtained silica (3 g) was added to 60 mL of glutalraldehyde (5% v/v) and the reaction mixture was stirred at room temperature for 2 h. After washing with distilled water, the activated silica-glutaraldehyde (Si-APTES-Glu) and 40 mL of crude RBL or purified RBL were mixed in 250 mL flask. The mixture was shaken at room temperature for 1 h. The immobilized RBL was centrifuged at 4°C, 15 000 rpm for 5 minutes to exclude excess RBL solution. The immobilized RBL was washed with 50 mM phosphate buffer, pH 7.0, dried under vacuum at room temperature and stored at 4°C until use.

3.3.4 Hydrolytic Activity of Rice Bran Lipase

3.3.4.1 *Hydrolytic Activity of Rice Bran Lipase Solution*

RBL activity was measured by titration technique. A rice bran oil solution was prepared in a 250 mL flask from the mixture of rice bran oil (1 mL), 10 mM calcium chloride (1 mL), 0.05 M phosphate buffer pH 7.0 (4 mL), and distilled water (2 mL). The solution was heated to 35°C in shaking bath for 15 minutes and the prepared RBL solution (2 mL) was added. The mixture was shaken at 35°C for 1 h, followed by adding acetone:ethanol (1:1) (20 mL) to terminate the hydrolytic reaction. The solution was centrifuged at 10 000 rpm for 10 minutes. The fatty acid was determined by titrating the

aqueous phase with 0.05 N sodium hydroxide using phenolphthalene as an indicator. The changing of pH was also confirmed by a pH meter. The procedures were repeated for the control batch without the addition of the RBL solution. The specific activity was evaluated from the average values of the fatty acid in micromole (μmole) unit per 1 minute per mg protein, as shown in equation (3.1);

$$\text{Specific activity (mU/mg)} = \frac{\text{activity (mU/mL)}}{\text{amount of protein (mg/mL)}} \qquad (3.1)$$

The amount of protein was determined by equation (3.2);

$$\text{amount of protein (mg/mL)} = \frac{A_{RBL}}{E_{280}^{1\%}} \qquad (3.2)$$

where A_{RBL} is absorbance at 280 nm of rice bran lipase solution

$E_{280}^{1\%}$ is molar extinction coefficient of rice bran lipase

3.3.4.2 *Hydrolytic Activity of Rice Bran Lipase Solid*

Rice bran lipase solution was prepared by dissolving 200 mg rice bran lipase solid in 2 mL distilled water. The activity was measured as in 3.3.4.1.

3.3.4.3 *Hydrolytic Activity of Immobilized Rice Bran Lipase*

Immobilized rice bran lipase (100 mg) was added into rice bran oil solution and the activity was measured as in 3.3.4.2.

3.3.5 Percent Immobilization of Rice Bran Lipase onto Carrier

Percent immobilization was determined by calculating the loss of rice bran lipase activity as the equation shown in (3.3);

$$\% \text{ Immobilization} = \frac{A_0 - A_1}{A_0} \quad (3.3)$$

where A_0 is rice bran lipase activity before immobilization

A_1 is rice bran lipase activity after immobilization

3.3.6 Thermal Stability of Rice Bran Lipase Solid in Toluene

Rice bran lipase solid (1g) was added into toluene (25 mL) which was previously heated at varied temperatures (30°, 40°, 60°, 80°, and 100°C). The mixture was stirred for 24 h. After filtration, rice bran lipase solid was dried under vacuum at room temperature. The remaining activity was measured as in 3.3.4.1.

3.3.7 Rice Bran Lipase-Catalysed Polyesterification

3.3.7.1 *Rice Bran Lipase-Catalysed Polyesterification*

Two RBL catalytic systems of polymerization were studied. For the first system, an equimolar amount of adipic acid (1.46 g, 10 mmole) and 1,4-butanediol (0.9 g, 10 mmol) were used as starting materials. Adipic acid was added into toluene (50 mL) followed by RBL (1.18 g, 50% total weight of starting materials) and 1,4-butanediol. The mixture was suspended in the solvent at varied temperatures (30°, 40°, 60°, 80°, and 100°C). When the reaction time has reached 1, 3, 5, and 7 days, the suspension was filtered to separate RBL from the solution mixture and washed thoroughly with toluene. The collected solution was removed out the solvent to obtain crude product. The crude product was extracted by hexane. The hexane solution was evaporated to get the purified product. The product was characterized by FTIR, ^{1}H-NMR and GPC.

3.3.7.2 Rice Bran Lipase-Catalysed Polyesterification via Polyol

An equimolar amount of adipic acid (1.46 g, 10 mmole) and poly (ethylene glycol (2.00 g, 10 mmol) was used as starting material and rice bran lipase (1.73 g, 50% total weight of starting materials) was applied as a