CHAPTER III

EXPERIMENTAL

3.1 Sources of animal material

The nudibranch *Jorunna funebris* samples were collected at Sichang islands, Chonburi, Thailand. The samples were stored at -80°C until they were used for analysis of renieramycin M/jorunnamycin A contents and preparation of crude enzyme extracts.

3.2 Determination of Renieramycin M/Jorunnamycin A contents in *J. funebris* tissues.

All *J. funebris* organisms were collected and dissected into visceral and mantle parts. A pool of each part was ground into homogeneous paste in liquid nitrogen. Each part (500 mg) was accurately weighed and pretreated with 500 µL of 10 mM KCN in 50 mM phosphate buffer pH 7.0 for 5 h. The sample suspension was macerated with 8.0 mL of methanol for 24 h. 2.0 mL of the aqueous methanolic solution was mixed with 3.0 mL of brine solution. The resulting mixture was further partitioned with 5.0 mL of ethyl acetate (EtOAc). Sodium sulfate anhydrous was added to the EtOAc part (2.0 mL) to remove excess water. The EtOAc extract was subjected to HPLC (Shimadzu SPD-M20A SIL-20A, Japan) using a LiChrospher 100 RP-18 HPLC column (4 x 125 mm, 5 µm), 20 µL of sample volume, 70% MeOH in water as a mobile phase at a flow rate of 1.0 mL/min, detection with a Photodiode Array-UV detector (Shimadzu,SDD-M10A PDA) at λ 270 nm. Retention times and UV spectra of the HPLC chromatographic peaks responsible for renieramycin M and jorunnamycin A presenting in the EtOAc extract were compared with those of the renieramycin M and jorunnamycin A reference standards.

3.3 Crude protein preparation and fractionation

3.3.1 Crude protein preparation

Each part of the crude tissues of *Jorunna funebris* was pooled and accurately weigh for 1 g. The samples were ground in liquid nitrogen and added with 4.0 mL of 50 mM Tris-HCl buffer pH 8.0 (1:4 ratio) then centrifuged at 10,000x g speed at 4 °C for 5 minutes, determined the volume of supernatant and added with ammonium sulfate to give 70% salt concentration. The resulting mixture was then stirred on ice-bath for 20 minutes and centrifuged at 15,000x g speed at 4 °C for 20 minutes. The supernatant was discarded and the pellet was collected. The obtained pellet (precipitated at the 0-70% salt concentration) was redissolved in 2.0 mL of 50 mM Tris-HCl buffer pH 8.0 and stored at -80°C until used. Desalting process of the crude protein was performed by using a PD-10 column (8.3 mL of Sephadex G-25 Medium, GE healthcare, Sweden) equilibrated by 50 mM Tris-HCl buffer pH 8.0 and the crude protein was subsequently eluted by the same buffer and collected at 3.5-5.5 mL of the elution. The solution was brought to measure total protein content.

3.3.2 Total protein assay.

Total soluble proteins were quantified by the Bradford assay (Bradford 1976) and the standard protocol for 96-well microplates (Quick start™ Bradford Protein Assay, Biorad). 5 µL of protein solution was added to 250 µL of Bio-Rad protein assay dye reagent (Bio-Rad product 500-0006). The absorbance at 595 nm of the standard protein bovine serum albumin (BSA) corresponding to its concentration (0.125–1.0 mg/mL) was plotted for the protein concentration standard curve.

3.3.3 Crude protein fractionation

The crude protein from the visceral part of *J. funebris* was partially purified by sequential ammonium sulfate precipitation. The visceral part was treated by the

process as previously mentioned. After centrifuged at 10,000x g speed at 4 °C for 5 minutes. the volume of supernatant was determined and added with ammonium sulfate to bring up the salt concentration to 40%. The initial protein pellet precipitated by a range of 0-40% ammonium sulfate concentration was discarded. The remaining supernatant was sequentially precipitated by higher concentrations (40–100%) of ammonium sulfate with 5% increment interval. The protein pellet from each precipitation was desalted by a PD-10 column (GE Healthcare) and quantified the total proteins prior to the assay of esterase activity for renieramycin M hydrolysis as previously described.

3.3.4 Assay of esterase activity for renieramycin M hydrolysis

Assay of renieramycin M hydrolysis by esterase(s) in the crude protein/enzyme extract was achieved by using HPLC-UV for detection of substrates and products. A typical assay was carried out in 200-µL reaction volume with 50 mM Tris-HCl buffer pH 8.0, 20 µL of renieramycin M (0.05 mM renieramycin M solution in 10% DMSO in MeOH as final concentration) and 0.4 mg protein of the crude enzyme extract in 50 mM Tris-HCl buffer, then incubated at 25 °C for 90 minutes. Then 200 µL of EtOAc was added to stop the reaction and to extract the products from the aqueous phase. 100 µL of the EtOAc part was collected and evaporated to dryness. 100 µL of MeOH was added to dissolve the dried extract and subsequently dispensed into 1.5-mL autosampler HPLC vials for HPLC analysis. HPLC-UV analysis condition included a mobile phase composed of 7:3 MeOH-water, a flow rate of 1.0 mL/min, an injection volume of 20 µL. Absorption of jorunnamycin A was monitored at 270 nm and calculated by using renieramycin M standard curve (31.5 ng - 500 ng) to give the amount of jorunnamycin A equivalent to renieramycin M. The enzyme unit (U) was calculated as production of the amount of JA (µmol) per minute and the activity was calculated by the enzyme units divided by the amount of protein (mg) used in the reaction mixture. All experiments were performed in duplicate and repeated three times

3.3.5 Liquid chromatography-Mass spectrometry (LC-HRMS) analysis

HPLC-MS analysis was carried out using a LiChrospher 100 RP-18 HPLC column (4 x 125 mm, 5 µm) by LC/MS mass spectrometer (1200 Infinity series and 6540 UHD Accurate-Mass Q-TOF, Agilent Technologies) at a flow rate of 0.5 mL/min. 70% MeOH in water was used as a mobile phase. The MS spectra were analyzed by the software, Agilent Mass Hunter Qualitative Analysis B.06.00. The reaction product (jorunnamycin A) and the substrate (renieramycin M) were identified by their molecular weight according to their accurate mass measurement.

3.3.6 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) for the crude protein extracts

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. 30 µg of ammonium sulfate precipitated proteins in Tris-HCl buffer solution was mixed with 5 µL of loading dye (50 mM Tris-HCl, 40 mM EDTA, 10% glycerol, 4% SDS, 0.02% bromophenol blue, 3% 2-mercaptoethanol) and boiled for 5 minutes at 100 °C. The sample solution was then loaded into each well using 12% polyacrylamide separating gel prepared with 8.5x10 cm cassette (Biorad), 120 Volt, 3 hours in running buffer (0.25 M Tris base, 2 M glycine, 1% SDS, pH 8.3). After removing the polyacrylamide gel from the electrophoresis device, the gel was stained overnight with 0.1% coomassie-blue R-250 in fixative solution (40% methanol, 10% acetic acid) and rinsed with water 3 times. The protein bands were detected by Gel Doc image analysis system (Chemi Doc XRS, Bio-Rad, U.S.A.).

3.4 Optimization of esterase activity from fractionated crude enzyme extract

3.4.1 Crude protein preparation

The crude protein from the visceral part of *J. funebris* was treated with the process as mentioned above. After being centrifuged at 10,000x g speed at 4 °C for 5 minutes, the supernatant was determined for its volume and added with ammonium

sulfate to give 55% ammonium sulfate concentration. The pellet was discarded whereas the supernatant volume was determined for calculating amount of ammonium sulfate added to bring up the salt concentration to 75%. The protein pellet from the precipitation of 55-75% ammonium sulfate concentrations was desalted by a PD-10 column prior to the esterase activity assay, as previously described.

3.4.2 Modification of parameters for optimization study

3.4.2.1 Effect of time and substrate concentration

The assay for esterase activity was carried out in the same condition as previously described (50 mM Tris-HCl buffer, pH 8.0). Three concentrations of renieramycin M substrates in 10% DMSO/MeOH were used (0.025 mM, 0.05 mM, and 0.1 mM). 20 µL of various renieramycin M substrate concentrations were added into the 0.4 mg protein of crude enzyme extract in Tris-HCl buffer solution and incubated at 25 °C for 30, 60, 90, and 120 minutes and proceeded with the method in section 3.3.4. HPLC-UV analysis using the previously described condition was performed to detect the product and the substrate. The absorption of jorunnamycin A product was detected at 270 nm. The peak of jorunnamycin A was identified with UV spectral pattern. All experiments were performed in duplicate and repeated three times.

3.4.2.2 Effect of temperature

A typical assay was carried out in 200-µL reaction volume with 50 mM Tris-HCl buffer, pH 8.0, 20 µL renieramycin M substrates (0.05 mM 10% DMSO in MeOH) and 0.4 mg protein of the crude enzyme extract in Tris-HCl buffer solution, incubated for 90 minutes. For this investigation, the reaction temperatures were performed at 20, 25, 30, 35, 40, 45, 50, 55, and 60 °C. All experiments were performed in duplicate and repeated three times.

3.4.2.3 Effect of pH

The assay performed according to the method as previously described. 200-µL reaction volume with 50 mM Tris-HCl buffer, 20 µL of renieramycin M substrates (0.05 mM 10% DMSO in MeOH) and 0.4 mg protein of the crude enzyme extract in Tris-HCl buffer solution, was incubated at 25 °C for 90 minutes. The pH value of the Tris-HCl buffer in the reactions was ranged from 7 to 11.5 with 0.5 interval. All experiments were performed in duplicate and repeated three times.

3.4.2.4 Effect of buffer types

The assay was followed by the method as previously explained. 200-µL reaction volume with 50 mM buffer pH 8.0, 20 µL renieramycin M substrates (0.05 mM 10%DMSO in MeOH) and 0.4 mg protein of the crude enzyme extract in Tris-HCl buffer solution, was incubated at 25 °C for 90 minutes. Three buffers (TrisHCl, Tricine, and phosphate buffer) were used for the experiment. All experiments were performed in duplicate and repeated three times.

Table 3. Enzyme assay conditions used in the reaction for optimization study.

Experiment	Condition parameters used in enzyme assay		
	Temperature (°C)	pH	Buffer
Typical assay	25	8.0	Tris-HCl
Effect of temperature	20-60*	8.0	Tris-HCl
Effect of pH	25	7.0-11.5*	Tris-HCl
Effect of buffer types	25	8.0	TrisHCl*, Tricine*, phosphate buffer*

*varied parameter

3.4.2.5 Stability test

Stability of the crude enzyme was investigated by detection of the remaining activity after being prepared and stored in various temperatures. The crude enzymes

solution in 1.5-mL microcentrifuge tubes preparing as previously described (section 3.4.1) were stored at 3 different temperatures including 4 °C, -20 °C and -80 °C. The activity of the stored crude enzymes from each temperature was tested every 2 months (0, 2, 4 months). The assay of activity was followed by the method previously described. All experiments were carried out in duplicate and repeated three times.

3.5 Substrate specificity

3.5.1 Renieramycin series hydrolysis assay

The assay of renieramycin series hydrolysis was carried out under the same condition as described previously in renieramycin M hydrolysis assay (section 3.3.4). For this experiment, the renireramycin series compounds, such as renieramycin N (RN), renieramycin O (RO) and renieramycin Q (RQ), were used as substrates. The EtOAc extract of the reaction was evaporated. 100 µL of MeOH was added and dispensed into HPLC vials. The condition for HPLC analysis included a mobile phase containing 7:3 MeOH-water, a flow rate of 1.0 mL/min, and an injection volume of 20 µL. UV absorption of *bis*-tetrahydroisoquinoline alkaloids at 270 nm was used to determine the product. All experiments were performed in duplicate and repeated three times.

3.5.2 Renieramycin M derivatives hydrolysis assay

The assay of renieramycin M derivative hydrolysis was performed under the same condition as described previously in renieramycin M hydrolysis assay (section 3.3.4). For this experiment, the renireramycin M derivatives compounds were used as substrates. HPLC-UV analysis was used for the detection UV absorption of *bis*-tetrahydroisoquinoline alkaloids at 270 nm was determined. All experiments were performed in duplicate and repeated three times.

3.5.3 Lipase hydrolysis assay

The lipase hydrolysis assay was modified from the method mentioned above (section 3.3.4). A typical assay was carried out in 200-µL reaction volume with 50 mM Tris-HCl buffer pH 8.0, 20 µL of renieramycin M (0.05 mM renieramycin M solution in 10% DMSO in MeOH as final concentration) and the crude enzyme extract (2 mg/mL) or lipase (2 mg/mL) incubated for 120 minutes at room temperature. The absorption of jorunnamycin A product was detected at 270 nm. The peak of jorunnamycin A was identified with UV spectral pattern. All experiments were performed in duplicate and repeated three times.