CHAPTER II

Materials and Methods

2.1 Materials and Chemicals

2.1.1 Materials

- Mini PROTEAN Tetra Electrophoresis system (Bio-Rad Laboratories, Hercules, CA)
- Boiling water bath
- Adjustable automatic pipettes and tips
- Autoclave
- Beakers
- Cylinders
- Centrifuge
- Microwave oven
- Refrigerator and Freezer (-20 °C and -80 °C)
- Thermal Cyclers
- Vortex mixer
- Classic Light Balances
- 1.5 ml centrifuge tubes
- 0.5 ml centrifuge tubes
- 0.2 ml PCR tubes
- IPG strip (pI 3-10, 7 cm)
- Ethan IPGphor III
- Shaker
- Ultimate 3000 LC system
- ESI-Ion Trap MS

- Light microscope
- Transmission electron microscope (TEM), Zeiss EM 900
- Scanning electron microscope (SEM), JEOL JSM-5910LV

2.1.2 Chemicals

- 1 % osmium tetroxide
- 2.5% glutaladehyde
- 0.1% toluidine blue
- 70% ethanol
- 1X SDS gel loading buffer
- 15 % SDS polyacrylamide gels
- Molecular weight marker
- 2D Clean Up kit
- Micro BCA protein assay kit (Pierce, Rockford, IL)
- SDS equilibration buffer
- 100 mg DTT
- 1 % CBB stain
- De-stain solution (10% methanol and 5% acetic acid)
- 100 mM iodoacetamide (IAA)
- Luria-Bertani (LB) medium
- QIAamp DNA mini Kit (Qiagen, Germany)
- QIAprep mini prep kit (Qiagen, Germany)
- 1X PCR buffer
- Taq DNA polymerase
- dNTP solution
- Ethidium bromide

2.2 Methods

2.2.1 Mosquito rearing

An. dissidens mosquitoes were reared in an insectary of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand. The mosquitoes were maintained at standard lighting conditions of 12 h light/dark, relative humidity of 70-80% and temperature of 27°C (±2°C). They were given a cotton wool pad soaked with 10% sucrose solution were provided regularly and changed every day. After emergence, all adult female mosquitoes were provided with a 10% sucrose solution until they were 4-6 days old. Then, they were fasted for 12 h prior to feeding on adult male albino rats (*Rattus norvegicus*). After that, the mosquitoes were forced into artificial mating. The gravid female mosquitoes were allowed to oviposit in a plastic cup of natural water with wet filter paper lining the inside. After hatching, one hundred first instar larvae were placed in a white plastic tray (25×35×6 cm) containing 1,500 ml of mixed with distilled water and natural water (1:1) and 15 stems of garden grass (*Axonopus compressus*). The larvae were fed on fish food. First and second instar larvae were fed twice daily. Feeding time were increased to 3-5 times daily for the third and fourth instar larvae stage. Pupae were transferred to a plastic cup containing natural water and placed in a cage. The mosquitoes were colonized continuously under laboratory conditions for several generations (Choochote and Saeung 2013).

2.2.2 Ethical clearance

The protocol for blood feeding was approved by the Animal Ethics Committee of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand (Protocol Number 05/2558).

2.2.3 Midgut and salivary gland dissection

The mosquitoes were cold anesthetized, then dissected in phosphate-buffered saline (PBS; 10 mM Na_2SO_4, 145 mM, pH 7.2) under a stereoscopic microscope.

For Light microscopy, SEM, and TEM, the midguts and salivary glands were collected at different days after emergence: early emergence (day 0), day 1, day 3, day 8,

day 12, day 16, and day 21. For bloodfeeding, the engorged-mosquitoes were carefully dissected for midgut samples at 0, 3, 6, 12, 24, 48, and 72 h after blood feeding. The dissected tissues were washed in PBS and transferred to a microcentrifuge tube with a small volume of PBS. The tissue samples were proceed immediately as described in the methods 2.2.4, 2.2.5 and 2.2.6.

For SDS-PAGE analysis of salivary glands, dissection of the various regions of the female salivary glands was performed. The medial lobes were cut at the junction of the medial lobes and the lateral lobes. The distal-lateral and proximal-lateral lobes were cut at the intermediate region separating the two lobes. The gland parts were immediately removed to separate tubes to avoid possible protein contamination between the different sections of the glands. The gland parts were placed in a small volume of PBS and stored at -80°C until use.

For 2-DE analysis of midgut proteins, sixty midguts of the mosquito aged 3-5 days after emergence were collected and extracted by mechanically homogenized mixtures were with a 1X PBS. The samples were centrifuged at 16,000 g for 20 min at 4°C and kept at -80ºC until use. The experiment was performed three times on different cohorts of mosquitoes, and 2D gel analysis performed at all sampling points for each of these biological replicates.

For 2-DE analysis of the salivary gland proteins, eighty pairs of the female salivary glands were used in each experiment. The mosquitoes were divided into two groups. The first was consist of the sugar-fed female mosquitoes collected on different day-points: day 0, 1, 2, 3, 8, 12, 16, 21 after emergence. The second group, 12 to 14 days old sugar-fed mosquitoes were allowed to feed on blood from immobilized rats and those that had fed to repletion were dissected immediately to remove the salivary glands as described above. Sugar-fed mosquitoes from the same cohort were used as a control. Both development and blood feeding experiments were each performed three times on different cohorts of mosquitoes, and 2D gel analysis performed as describe above.

2.2.4 Light microscopy

Midguts and salivary glands of female mosquitoes were dissected in PBS and allowed to settle onto slides without drying. Photographs of the glands were taken using a digital camera attached to a light microscope.

2.2.5 Scanning electron microscopy (SEM)

The tissues were fixed for 3 days with a solution of 2.5% glutaraldehyde mixed in phosphate buffer solution at a pH of 7.4 at 4°C. The tissues were then rinsed twice with phosphate buffer solution at 10-min intervals and later postfixed in a solution of 1 % osmium tetroxide for 2 h. Post fixation was followed by rinsing twice with phosphate buffer solution and dehydrating with serial alcohol, by following increasing concentrations of alcohol: 30, 50, 70, 80, 90, and 95%. The specimens were then placed in absolute alcohol for two 12-h periods and finally subjected to critical point drying. The specimens were attached with double-stick tape to aluminum stubs, and coated with gold in a sputter-coating apparatus before being viewed with a scanning electron microscope (JEOL JSM-5910LV, JEOL Ltd., Japan). To observe the interface between the midgut surface and the blood meal, some fixed samples were fractured before being coated with gold, while others were gently opened and the contents were washed out with phosphate buffer saline before the fixation.

2.2.6 Transmission electron microscopy (TEM)

The tissues were fixed for 3 days with a solution of 2.5% glutaraldehyde mixed in phosphate buffer solution at a pH of 7.4 at 4°C. The specimens were then washed twice in buffer and post-fixed for one h with 1% osmium tetroxide. The specimens were dehydrated in a crescent series of graded ethanol and were incubated overnight in an epoxy resin (PolyBed 812)/acetone solution (1:1). Embedded inpure resin and polymerized were incubated for 48 h at 60°C. Ultra-thin sections were stained with uranylacetate and lead citrate and observed in a Zeiss EM10C transmission electron microscope, operated at 60 kV.

2.2.7 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in 15% polyacrylamide gels using the method of Laemmli (1970). Electrophoresis was carried out under constant current (30 mA) for 1-2 h using Bio-rad apparatus.

2.2.8 Two-dimensional polyacrylamide gel electrophoresis (2-DE)

The 2-DE was performed using the 2D system (GE Healthcare, UK). A QUANT-IT Protein Assay Kit (Invitrogen, USA) were used for the quantification of proteins. The tissues were extracted and desalted using a 2-D Clean-Up kit (GE Healthcare, UK). Each pellet sample was solubilized in a 125 µl sample solubilization solution (8 M urea, 50 mM DTT, 4% CHAPS, 0.2% 3/10 Bio-lyte Ampholyte, 0.002% Bromophenol Blue) and then loaded on an IPG strip (pI 3-10, 7 cm, GE Healthcare, UK) for performing the first dimension isoelectric focusing (IEF) separation. Following 13 h rehydration, the strips were focused using Ettan IPGphor III (GE Healthcare, UK) according to the manufacturer's instruction. Then, the focused IPG strips were incubated in 10 ml of SDS equilibration buffer (6 M urea, 2% SDS, 0.05 M Tris, pH 8.8, 30% glycerol, 0.002% Bromophenol blue) containing 100 mg of DTT for 15 min, and a further 15 min in 10 ml of equilibration buffer containing 250 mg of iodoacetamide. The equilibrated strips were applied to the surface of vertical 15% SDS-polyacrylamide gels, and proteins were separated in the second dimension using the Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad, USA). Protein molecular weight markers (Bio-Rad, USA) were applied in each gel.

2.2.9 Coomassie Brilliant Blue (CBB) staining

Following electrophoresis, gels were CBB-stained. Firstly, the gels were fixed in 50% methanol and 10% acetic acid for 30 min, then stained with 1% CBB in 10% methanol and 5% acetic acid for 2 h, and finally de-stained in 10% methanol and 5% acetic acid until dark protein bands or spots are visible.

2.2.10 In-gel digestion and Nano Liquid chromatography-mass spectrometry (nano LC-MS) analysis

Protein spots of interest were excised from the two-dimensional electrophoresis gels using sterile surgical blades ensuring that techniques were aseptic. The gel pieces were subjected to in-gel digestion using an in-house method developed by Proteomics Laboratory, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand (Jaresitthikunchai et al. 2009).

The digested proteins were injected into an Ultimate 3000 LC System (Dionex, USA) coupled to an ESI-Ion Trap MS (HCT Ultra PTM Discovery System, Bruker, Germany), with electrospray at a flow rate of 300 nl/m to a nanocolumn (Acclaim PepMap 100 C18, 3 µm, 100A, 75 µm id x 150 mm). A solvent gradient (solvent A: 0.1% formic acid in water; solvent B: 80% 0.1% formic acid in 80% acetonitrile) was run for 40 m. Mascot from Matrix Science Ltd. (London, U.K.) was used for searching all of the tandem mass spectra (Perkins et al. 1999). The data were sent to the National Center for Biotechnology nonredundant (NCBInr) protein database. The search was performed taking other Metazoa as taxonomy. The other search parameters were enzyme of specificity strict trypsin; one missed cleavage; fixed modifications of carbamidomethyl (C); oxidation (Met); peptide tolerance of 100 ppm; Fragment Mass Tolerance of ± 0.5 Da; peptide change of 1+; and monoisotopic. Protein identification was made on the basis of statistically significant Mowse score ($P < 0.05$ or ≥ 30).

2.2.11 Protein quantification and statistical analysis

Three independent biological replicated 2-DE gel images were analyzed using the Image Master 2D Platinum 7.0 software (GE Healthcare, UK). This program was used to measure all protein spot densities. Heat shock cognate (HSC) 70 was used as an internal control protein to normalize quantitation. Previous work showed no change in HSC70 expression in *Ae. aegypti* salivary glands in response to heat shock (Gross et al. 2009), blood feeding (Wasinpiyamongkol et al. 2010), and in *An. barbirostris* species A2 salivary glands in response to ageing (Jariyapan et al. 2012). The choice of HSC70 in the current study was further validated by analysis of three-dimensional images of HSC70

peaks (Appendix A), showing stable levels of expression in *An. dissidens* on various different days of development. Normalisation was performed using the Image Master 2D Platinum 7.0 software using a spot volume of 0.02 for HSC70.

For developmental experiments, the mean and standard deviations were calculated using SPSS version 22.0 software. The analysis of variance (One Way ANOVA) was used to detect significant differences of the densities of the same spot in triplicate gels. Post Hoc analysis (Duncan's Multiple Range test) was used to test the statistical difference of the means of the expression level between all age groups, and the use of different letters (a, b, c, d, e, f) indicates significantly different levels of protein expression ($p < 0.05$), i.e. groups labelled with the letter "a" are not significantly different from each other, but are different to groups labelled with b, c, d, e, or f, and so on. Any given letter is only relevant within that protein (i.e. "a" in SN1 has nothing to do with "a" in SN2). In the blood feeding experiment, the protein expression level was assessed using a Student's t-test, $p < 0.05$.

2.2.12 Bacterial isolation and identification from *An. dissidens* midguts

All necessary materials for handling and dissection of mosquitoes were autoclaved at 121ºC for 20 min. Some materials, such as stereomicroscopic and pipettes, were sanitized with 70% ethanol. Bacterial isolation assays were carried out using 5 midguts for each of the sugar fed females on 5 days old. Culture dependent and culture independent methods described by Rani et al. (2009) were used.

1) Culture dependent method

To isolate bacteria from midguts, mosquitoes were surface sterilized by rinsing in solutions (1% sodium hypochlorite, PBS and 70% ethanol) for 1 min. The insects were rinsed 3 times in sterile PBS for 1 min. Aliquots of 100 µl from the last PBS washes were plated in Luria-Bertani (LB) agar as control groups of the insect surface sterilization process. Mosquitoes were dissected under a stereomicroscope, in a double cavity glass slide containing sterile PBS. The midgut was carefully removed from each insect, rinsed in sterile PBS and transferred into a 1.5 ml tube containing 100 µl of PBS. Tube content was homogenized and serially diluted (10^{-1} through 10^{-7}) and an

aliquot of 100 µl of each one was transferred to Petri dishes containing LB agar. The plates were incubated at 28ºC for 24-48 h in an incubator. Bacterial isolates were maintained at -70ºC in a 15% glycerol solution for further identification. The described procedure was also employed for microbial enumeration that was done in triplicates by counting the CFU, using one midgut for each plate. The Bacterial isolates were first screened based on (a) colony characteristics (color, size, shape, opacity, margin, elevation and viscosity); (b) morphology, arrangement of isolates, and Gram's staining.

Bacterial isolates were identified by 16S rRNA gene sequencing. Briefly, each colony was picked to perform DNA extraction using with the QIAamp DNA mini Kit (Qiagen, Germany) according to the manufacturer's instructions. PCR products obtained using universal bacterial primers, 27F (Lene et al. 1985): (5'-AGAGTTTGATCMTGGCTCAG-3', M = C:A) and 1492R (Delong 1992): (5'-TACGGYTACCTTAGTTACGACTT-3', Y = C:T), target on the 16S rRNA gene. PCR reactions were performed with, template DNA solution (2µl/100 ng); 27F primer (1 µl/6 pmol); 1492R primer (1 µl/6 pmol); 25mM $MgCl_2$ (1.5 µl), and 17.5 µl of ultra pure water. Cycling parameters for PCR reactions included initial denaturation step at 95ºC for 5 min, followed by 35 cycles of a denaturation step at 95ºC for 1 min, a primer annealing step at 50ºC for 1 min, an extension step at 72ºC for 3 min and a final step of 4 min at 72ºC. The 16S rDNA amplification generated a product of approximately 1,500 bp. The presence and yield of PCR product were determined on 1% agarose gel electrophoresis at 200 V for 30 min in 1X Tris-acetate-EDTA (TAE) buffer and stained with ethidium bromide. The PCR products were purified using QIAprep mini prep kit (Qiagen, Germany) and sent for sequencing at Macrogen, Korea. The sequences obtained were initially compared to the ones deposited at the GenBank, using BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

2) Culture independent method

The dissected midguts were carried out under a stereomicroscope on a sterile slide. To avoid cross-contamination among tissues, the midgut was carefully dissected. DNA extraction and PCR amplification were performed as described in the culture dependent method.

Bacterial 16S rRNA gene from total DNA was amplified by PCR in a reaction mixture (50 µl) containing (as final concentration) 1X PCR buffer, with 2 mM $MgCl_2$, 200 µM of each dNTPs, DNA (50 ng), 2 µM each of primers (27F, 1492R) and 2.5 units of Taq DNA polymerase. The reaction mixture was incubated at 94°C for 5 min for initial denaturation, followed by 30 cycles of 95°C for 30 sec, 55°C for 90 sec, 72°C for 2.30 min and a final extension at 72°C for 10 min. The 16S rDNA amplification generated a product of approximately 1500 bp, which examined by electrophoresis on 1% agarose gels containing ethidium bromide. The PCR products were purified using QIAquick gel extraction kit (Qiagen, Germany) and partially sequenced using universal primers. After purified, 16S rRNA fragments were cloned into pCR®8/GW/TOPO® vector using pCR®8/GW/TOPO® TA cloning kit (Invitrogen, USA). The 16S rRNA gene inserts in the plasmids were purified using QIAprep mini prep kit (Qiagen, Germany) and sent for sequencing. Sequence analysis were performed as described in the culture dependent method.