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

2.1.2 Chemicals

- 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
- Fixative solution
- 1 % CBB stain
- De-stain solution
- Pro-Q Emerald 300 glycoprotein stain (Invitrogen, OR)
- 100 mM iodoacetamide (IAA)
- Trypsin solution
- 30 % ACN
- 50 % ACN in 0.1% formic acid (FA)

2.2. Methods

2.2.1 Mosquitoes

An. campestris-like colonies (Thongsahuan et al. 2009) were successfully maintained for many consecutive generations in an insectary at the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Thailand, and were used in this study. The methods for rearing mosquitoes described by Choochote et al. (1983) and Kim et al. (2003) were used. The mosquitoes were reared and maintained in the insectary at 27 ± 2 °C with 70 ± 10 % relative humidity, and a photoperiod of 12:12 hr (light/dark). Adult mosquitoes were given continuous access to a 10 % sucrose solution and fed on blood from immobilized mice when required. Mosquitoes aged between three and five days after emergence and fed on sucrose solution were used in this study.

2.2.2 Ethical clearance

The protocols were approved by the Animal Ethics Committee of the Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand.

2.2.3 Salivary gland dissection

Salivary gland dissection was performed utilizing the method described by Jariyapan et al. (2010). Adult mosquitoes between three and five days of age were cold anaesthetized on ice before salivary gland dissection. Salivary glands of the mosquitoes were dissected in phosphate-buffered saline [PBS; 10 mM Na_2SO_4, 145 mM NaCl (pH 7.2)] using fine entomological needles under a stereoscopic microscope at ×4 magnification and transferred to a micro-centrifuge tube with a small volume of PBS. The samples were then kept at -80 °C until use. 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.

2.2.4 Blood feeding of *An. campestris*-like

The mosquitoes were divided into two groups (Figure 2.1), the first blood meal group and the second blood meal group. For the first blood meal, three to four day old sugar-fed mosquitoes were allowed to feed on blood from immobilized mice and those that had fed to repletion were separated from the cohort and transferred to a new cage. Artificial mating was required for this particular species and was performed on the mosquitoes fed to repletion after the first blood meal except for those used as the first blood meal group which were dissected immediately after taking the meal. A second blood meal was given after 14 days (as the sporogonic cycle of *Plasmodium* in *Anopheles* mosquitoes takes about 14 days) and the females that fed a second time were collected and dissected immediately for salivary glands as described below. Salivary glands of unfed mosquitoes from each group were collected and used to compare with the blood-fed ones. During the entire procedure, a cup filled with water was placed in

all the cages to facilitate oviposition. Triplicate experiments were performed on different cohorts and generations of mosquitoes. Salivary gland dissection was performed as the method in 2.2.3.

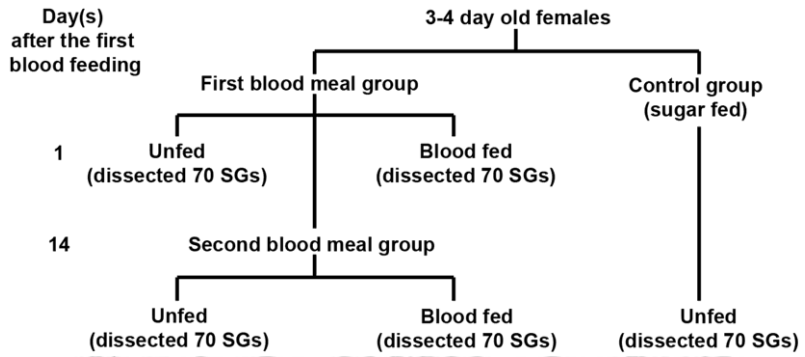

Figure 2.1 Experimental design for the first and the second blood feeding of *An. campestris*-like

2.2.5 SDS-PAGE

Salivary gland samples were thawed on ice and mixed in 1:2 (v/v) 1× SDS gel loading buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2 % SDS, 0.1 % Bromphenol blue, 10 % glycerol). Then, the samples were heated for 5 min in a boiling water bath and loaded on 15 % SDS polyacrylamide gels. Molecular weight markers (Bio-Rad Laboratories; Hercules, CA) were applied in each gel.

2.2.6 Two-dimensional gel electrophoresis

2-DE was performed using the 2D system (GE Healthcare, UK). The protein concentration was determined by the Bradford method using Bio-Rad protein assay (Bio-Rad Laboratories; Hercules, CA). In each female sample, 70 pairs of female salivary glands (~90 μg of total proteins) were used. For the male sample, 200 pairs of salivary glands (~20 μg of total proteins) were used. The salivary glands 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 (pI3–10, 7 cm, GE Healthcare, UK) to perform the first dimension isoelectric focusing (IEF) separation. Following 13 hr rehydration, the strips were

focused using Ettan IPGphor III (GE Healthcare, UK) according to the manufacturer's instruction. The focused IPG strips were then incubated in 10 ml SDS equilibration buffer (6 M urea, 2 % SDS, 0.05 M Tris, pH8.8, 30 % glycerol, 0.002 % Bromophenol blue) containing 100 mg DTT for 15 min and for a further 15 mins in 10 ml equilibration buffer containing 250 mg iodoacetamide. The equilibrated strips were applied to the surface of vertical 15 % SDS-polyacrylamide gels and proteins separated in the second dimension using the Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad Laboratories; Hercules, CA). Protein molecular weight markers (Bio-Rad Laboratories; Hercules, CA) were applied in each gel.

2.2.7 Coomassie Brilliant Blue (CBB) staining, glycoprotein staining, and gel image analysis

Following the electrophoresis, the gels were CBB stained. First, 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 hr, and finally destained in 10 % methanol and 5 % acetic acid until dark protein bands were visible. The gels were scanned with the Image scanner III (GE Healthcare, UK). For glycoproteins, the gels were stained with Pro-Q Emerald 300 glycoprotein stain (Invitrogen, OR) according to the manufacturer's instruction. A bioinformatics program (Image Master 2D Platinum, GE Healthcare, UK) was used to detect the number of spots in each gel, measure the molecular weight and the isoelectric point of each spot, and determine volume of each spot.

2.2.8 Protein quantification and statistical analysis

A Micro BCA protein assay kit (Pierce, Rockford, IL) was used for the quantification of proteins according to the manufacturer's instruction. The mean and standard error of the mean (SEM) were calculated for the total proteins of each salivary gland pair (n=25). Each 2-DE sample was subjected to triplicate runs. Quantification of the average spot density (ASD) for each protein on 2-DE gels was carried out using the Image Master 2D Platinum software (GE Healthcare, UK). Statistical analysis (Student's t-test, $p \leq 0.05$) was performed using SPSS 17.0 software (SPSS, Chicago, IL, USA) to compare of the average density of each protein spot during each feeding. Heat

shock cognate (HSC) 70 was used as an internal control protein, based on previous work showing constitutive expression in *Ae. aegypti* salivary glands in response to heat shock (Gross et al. 2009) and no change in expression in response to blood feeding (Wasinpiyamongkol et al. 2010), and in *An. barbirostris* salivary glands no response to ageing (Jariyapan et al. 2012).

2.2.9 In-gel digestion

Protein spots of interest were excised from the 2-DE gels using sterile surgical blades with aseptic technique. 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 (BIOTEC), National Science and Technology Development Agency (NSTDA), Thailand (Jaresitthikunchai et al. 2009). The gel plugs were dehydrated with 100% acetonitrile (ACN), reduced with 10 mM DTT in 10 mM ammonium bicarbonate at room temperature for 1 hr and alkylated at room temperature for 1 hr in the dark in the presence of 100 mM iodoacetamide (IAA) in 10 mM ammonium bicarbonate. After alkylation, the gel pieces were dehydrated twice with 100 % ACN for 5 mins. To perform in-gel digestion of proteins, 10 µl of trypsin solution (10 ng/µl trypsin in 50 % ACN/10 mM ammonium bicarbonate) was added to the gels followed by incubation at room temperature for 20 mins, and then 20 µl of 30 % ACN was added to keep the gels immersed throughout digestion. The gels were incubated at 37 °C for a few hours or overnight. To extract peptide digestion products, 30 µl of 50 % ACN in 0.1 % formic acid (FA) was added into the gels, and then the gels were incubated at room temperature for 10 mins in a shaker. The extracted peptides were collected and pooled together in a new tube. The extracted peptide pools were dried by vacuum centrifuge and kept at -80 °C for further mass spectrometric analysis.

2.2.10 NanoLC-MS analysis

The protein digest was injected into an Ultimate 3000 LC System (Dionex, Sunnyvale, CA) coupled to an ESI-Ion Trap MS (HCT Ultra PTM Discovery System, Bruker, Germany) with electrospray at a flow rate of 300 nl/min to a nanocolumn (Acclaim PepMap 100 C18, 3 µm, 100A, 75 µm id×150 mm). A solvent gradient

(solvent A, 0.1 % formic acid in water; solvent B, 80 % of 0.1 % formic acid in 80 % acetonitrile) was run for 40 mins.

2.2.11 Data analysis and protein identification

Mass-Lynx was employed to generate peak lists (pkl files) from the raw data using the following parameters: (a) smooth windows (channels): 4.00, number of smooths: 2, smooth mode: Savitzky Golay; (b) percentage of peak height to calculate the centroid spectra, 80%; and (c) no baseline subtract was allowed. Mascot from Matrix Science Ltd. (London, U.K.) was used to search all of the tandem mass spectra (Perkins et al. 1999). The data was 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 Mowse score ≥30. All accession numbers of the best hit protein presented in this study is available online at http://www. ncbi.nlm.nih.gov.

2.2.12 Library construction

An. campestris-like Form E salivary gland mRNA will be isolated from 50 adult female salivary-gland pairs using the Micro-FastTrack™ mRNA isolation kit (Invitrogen, USA). The polymerase chain reaction (PCR)-based cDNA library was made following instructions for the SMART cDNA library construction kit (Clontech, USA). Salivary gland polyA$^+$ RNA were used for reverse transcription to cDNA using PowerScript reverse transcriptase (Clontech, USA), the SMART IV oligonucleotide, and CDS III/3' primer (Clontech, USA). The reaction were carried out at 42 ºC for 1 hr. Second-strand synthesis were performed by a long-distance (LD), PCR-based protocol using the 5' PCR primer and CDS III/3' primer as the sense and anti-sense primer, respectively. These two primers also create *Sfi1 A* and *B* restriction enzyme sites at the end of nascent cDNA. Advantage™ Taq polymerase mix (Clontech, USA) was used to carry out the LD-PCR reaction on a GeneAmp® PCR system 9700 (Perkin-Elmer Corp., USA). The PCR conditions were: 95 ºC for 20 sec; 24 cycles in 95 ºC for 5 sec; and 68

ºC for 6 mins. A small portion of the cDNA was analyzed on a 1.1 % agarose/EtBr (0.1µg/ml) gel to check for the quality and range of the synthesized cDNA. Double-stranded cDNA were treated immediately with proteinase K (0.8 µg/ml) at 45 ºC for 20 mins. Proteinase K was removed using a Microcon YM-100 mini-column (100,000 MWCO; Millipore, USA) following the manufacturer's recommendations.

The clean, double-stranded cDNA were then digested with S*fiI* restriction enzyme at 50 °C for 2 hr, followed by size fractionation on a ChromaSpin-400 drip column (Clontech, USA). The profiles of the fractions were checked on a 1.1 % agarose/EtBr (0.1 µg/ml): Fractions containing cDNA of more than 400 bp were pooled and concentrated by the mini-column, as described above. The cDNAs were then ligated into a λ TriplEx2 vector (Clontech, USA), and resulting ligation mixture were packaged using GigaPack® III Gold packaging extract (Stratagene, USA) according to the manufacturer's instruction. The cDNA packaged library of *An. campestris*-like salivary gland was plated by infecting log-phase XL1-Blue *Escherichia coli* cells (Clontech, USA). The percentage of recombinant clones was determined by performing a blue-white selection screening on LB/$MgSO_4$ plates containing X-gal/IPTG at an average of 250 plaques per 150-mm Petri dish. Recombinant (white) plaques were selected randomly and transferred to 96-well Microtest™ U-bottom plates (BD BioSciences, USA) containing 100 µl of SM buffer (0.1 M NaCl, 0.01 M $MgSO_4$ 7 H_2O, 0.035 M Tris-HCl, pH 7.5, 0.01% gelatin) per well. The plates were covered and placed on a gyrating shaker for 30 min at room temperature. The phage suspension was either used immediately for PCR or stored at 4ºC for future use.

2.2.13 PCR screening inserts cDNA library and sequencing

Transfected plaques were randomly selected and transferred into 96-well plates with 100 ml of SM Buffer (NaCl, $MgSO_4$, 1 M Tris-HCl pH 7.5, 2 % gelatin) per well. Ten clones of 96-well plates of phage were subjected to PCR using PCR Master Mix following Table 2.1. The vector specific primers SSF (5'-ACCATGATTACGCCAAGCTC -3') and SSR (5'- GTAAAACGACGGCCAGTGAA -3') were designed by Primer3 program and used for screening insert cDNA library. Amplification conditions were as follows: 95 °C for 5 mins, 35 cycles of 95 °C for 30

sec, 57 °C for 30 sec, and 72 °C for 1 min. The final elongation step lasted for 10 min at 72 °C and hold at 4 °C infinity. Product size when used for screening with no insert was approximately 210 bp, but product size with insert was greater than 221 bp of vector sequence. PCR products were cleaned using Thermo Scientific: GeneJET PCR Purification kit (Thermo Fisher Scientific, Pittsburgh PA, USA). Vector-specific primers (SSF 5'- ACCATGATTACGCCAAGCTC -3') and cleaned PCR products were prepared for sequencing by Source Bioscience, Nottingham, UK.

Table 2.1 PCR Master Mix reagents for PCR screening insert cDNA library.

Reagent	volume for a 25 µl reaction (µL)	Master Mix 24 µL/tube, then add DNA (for 4 tubes)
Molecular grade dH_2O	16.5 µl	66 µl
HotStar HiFidelity PCR Buffer, 5X	5 µl	20 µl
primer #1; 10 µM	1 µl	4 µl
primer #2; 10 µM	1 µl	4 µl
HotStar HiFidelity DNA polymerase (2.5 units/µl)	0.5 µl	2 µl
DNA template 100 ng/µl	1 µl	-
Total volume	25 µl	96 µl

2.2.14 Bioinformatic tools and procedures used

Expressed sequence tags (EST) were trimmed of primer and vector sequences. The BALST tool was used to identify similar sequences in various databases (Altschul et al. 1990) and ClustalW tool was used to align multiple sequences (Thompson et al. 1997). Phylogenictic analysis and statistical neighbor-joining (NJ) bootstrap tests of the phylogenies were done with the Mega6 software (Tamura et al. 2013). For functional annotation of the transcripts, the blastX program (Altschul et al. 1997) was used to compare nucleotide sequences with the nonredundant (NR) protein database of the National Center for Biotechnology Information (NCBI) and the gene ontology database (Ashburner et al. 2000). The transcripts were also compared with other subsets of mitochondrial and rRNA nucleotide sequences and several organism proteomes downloaded from the NCBI. All blast comparisons were corned out with the complexity filter off, but 20 base segments of polymononucleotides were masked. All six frame translations were used in the case of blastX. To identify possible transcripts coding for secreted proteins, segments of the three-frame translations of all ESTs starting with a methionine found in the first 100 predicted amino acids (aa), or the predicted protein translation in the case of complete coding sequences, were submitted to the SignalP server (Nielsen et al. 1997) to help identify translation products that could be secreted. *O*-glycosylation sites on the proteins were predicted by the NetOGlyc program (http://www.cbs.dtu.dk/services/NetOGlyc/) (Hansen et al. 1998). Functional annotation of the transcripts was based on all the comparisons above. Following inspection of all these results, transcripts were classified as Secretory (S), Housekeeping (H), or of Unknown (U) function, with further subdivitions based on function and/or protein families.