CHAPTER 3

MATERIALS AND METHODS

3.1 Mosquito collection and isoline colonization

Wild-caught, fully engorged females of the *An. barbirostris* complex were collected during June 2008 to June 2009 in 8 provinces in Thailand (Fig. 1 and Table 1), where different populations of the *An. barbirostris* complex were reported (Saeung et al. 2007, 2008; Suwannamit et al. 2009). Collection of wild-caught females was performed using both human-baited and animal-baited traps. The live females were transported to the laboratory of the Department of Parasitology, Faculty of Medicine, Chiang Mai University, Chiang Mai, Thailand, for isoline colonization using the techniques described by Choochote et al (1983) and Kim et al (2003).

3.2 Mitotic karyotype

Metaphase chromosomes were prepared from the early fourth-instar larval brains of F_1 and/or F_2 progenies of each isoline using the method of Baimai et al (1995) and Saeung et al (2007, 2008).

3.3 Identification of mosquito species using rDNA ITS2 region as a molecular marker

Genomic DNA was extracted from a pool of five larvae from each isoline using a DNeasy Tissue Kit (Qiagen) according to the manufacturer's instructions. The rDNA ITS2 region was amplified by polymerase chain reaction (PCR) using the following primers: ITS2A, 5'-TGTGAACTGCAGGACACAT-3' and ITS2B, 5'-TATGCTTAAATTCAGGGGGT-3' for rDNA ITS2 (Beebe and Saul 1995). PCR and sequencing reactions were carried out as methods described by Saeung et al (2007, 2008) and Suwannamit et al (2009). To identify the mosquito species, the obtained sequence data were compared with the nucleotide sequences of the five sibling species deposited in the GenBank database (Saeung et al. 2007, 2008; Suwannamit et al. 2009).

3.4 Salivary gland dissection

The mosquitoes were cold anaesthetized on ice before salivary gland dissection. Salivary glands of the mosquitoes were dissected in RNase-free phosphate-buffered saline [PBS; 10 mM Na_2SO_4, 145 mM NaCl (pH 7.2)] using fine entomological needles under a stereoscopic microscope at 4X magnification and transferred to a microcentrifuge tube with a small volume of PBS. Dissection of various regions of the female salivary glands was also performed. The medial lobes were cut at the junction of the medial and lateral lobes. The distal-lateral and proximal-lateral lobes were cut at the intermediate region separating the two lobes. The gland parts were immediately transferred to a new the tubes in order 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^{o}C until use.

3.5 Sodium dodecyl sulphate polyacrylamide gel eletrophoresis (SDS-PAGE)

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

3.6 Two-dimensional gel electrophoresis (2-DE)

Two-dimensional gel electrophoresis 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 ($\approx$ 90 μg of total proteins) were used. For the male sample, 200 pairs of salivary glands ($\approx$ 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 (pI 3–10, 7 cm, GE Healthcare, UK) to perform 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. The focused IPG strips were then incubated in 10 ml SDS equilibration buffer (6 M urea, 2 % SDS, 0.05 M Tris, pH 8.8, 30 % glycerol, 0.002 %

Bromophenol blue) containing 100 mg DTT for 15 min and for a further 15 min 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.

3.7 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 h, and finally de-stained in 10 % methanol and 5 % acetic acid until dark protein bands were visible. The gels were scanned with the Imagescanner 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.

3.8 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).

3.9 In-gel digestion

Protein bands or spots of interest were excised from the SDS-polyacrylamide gels or 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 h and alkylated at room temperature for 1 h 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 min. 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 min, 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 min in a shaker. Peptides extracted were collected and pooled together in a new tube. The pool extracted peptides were dried by vacuum centrifuge and kept at -80°C for further mass spectrometric analysis.

3.10 NanoLC-MS analysis and protein identification

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 x 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 min. Mascot from Matrix Science Ltd. (London, UK) was used to search 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 $\pm$ 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).