CHAPTER II

MATERIALS AND METHODS

1. Mosquito strains

Two laboratory strains of *Ae. aegypti*, PMD and PMD-R, were used in this study. PMD and PMD-R strains were originated from Ban Pang Mai Daeng, Mae Tang District, Chiang Mai Province, Thailand and were selected in laboratory by Somboon *et al.* (2003). PMD-R is resistant to DDT and permethrin while PMD is resistant to DDT. All of mosquitoes were colonized in the insectary of the Department of Parasitology, Faculty of Medicine, Chiang Mai University. The eggs of these mosquitoes were harvested and stored in a cool place by which they can be viable for several months.

2. Rearing mosquitoes

The method for rearing of mosquitoes followed the procedure described by Limsuwan *et al.* (1987) with some modifications. The insectary was under standard conditions at 25-27°C and ~80% humidity, with 12 hours day/night cycle. The dried eggs of the mosquitoes were immersed in a plastic tray (25 x 35 x 6 cm) containing about 2 liters of tap water for hatching. After hatching, the larvae were fed with finely ground dog-biscuit. The water was changed two or three times a week in order to avoid scum formation. After pupation, the pupae were removed from the rearing tray, transferred to plastic cups containing tap water, and placed into mosquito

cages (30 x 30 x 30 cm). The emerging, adult mosquitoes were fed with 10% sucrose mixed with 10% multivitamin syrup soak in cotton wool that will be changed every other day. For this species, self mating was occurred within the cage. About 4-5 days after emergence, the female mosquitoes were given a blood-meal by exposing them to a white rat for 1-3 hours as a source of blood-meal for egg production. For oviposition, the gravid females laid eggs on filter paper soaked with water in egg collecting cups. After laying eggs, the filter paper with eggs will be air-dried for a few days and stored in a Ziploc bag until used.

3. Dose-response bioassay test for mosquito larvae

The dose-response bioassay was used to evaluate the permethrin susceptibility of mosquito larvae with and without synergists following the WHO standard method (WHO, 1981).

The synergist piperonyl butoxide (PBO), a monooxygenase inhibitor, was added into the bioassay to determine whether mixed function oxidase is involved in the resistance. The synergist Bis(4-nitrophenyl)-phosphate (BNPP), an esterase inhibitor, was added in the bioassay to determine whether esterase is involved in the resistance.

3.1 Chemicals and reagents

Insecticide: Permethrin technical grade (99.6%), Supelco (Bellefonte, PA, USA).

Synergists: Piperonyl butoxide (PBO) (90%), Sigma.

Bis(4-nitrophenyl)-phosphate (BNPP) (99%), Aldrich.

3.2 Preparation of permethrin solution

Stock and serial dilutions of permethrin were dissolved in absolute ethanol and stored at 4°C. The toxicity of permethrin was evaluated with 5-7 concentrations.

3.3 Larvicidal test procedure

For each bioassay, twenty-five early 4^{th} instar larvae were placed in a 400-ml beaker containing 249 ml of distilled water. One milliliter of permethrin (in absolute ethanol) solution was dispersed with a pipette above the water surface in each beaker. The ethanol content in each assay solution was limited to 0.4%. The experiment was performed at 25-27°C. Quadruplicate tests were carried out simultaneously with a total of 100 larvae for each concentration. In the control experiments, 0.4% ethanol was included in 250 ml of water. In parallel with this, a set of bioassay adding with either PBO or BNPP (0.3 mg/ml each) was performed (Paul *et al.,* 2006). After 24 hours, larval mortality was recorded. Moribund or dead larvae were identified as unresponsive after probing with a needle. The number of moribund and dead larvae were combined in quadruplicate and expressed as percentage mortalities of each concentration, the lethal concentration (LC_{50}) and their 95% fiducial limits, slopes and standard error calculated using the software LdP Line (LdP Line, copyright 2000 by Ehab Mostofa Bakr, Cairo, Egypt). The LC_{50} values were considered significantly different if no overlap occurs between their 95% fiducial limits.

4. HPLC validation methods

Validation of a method is the process defining an analytical requirement to generate reliable and accurate data analysis (EURACHEM Guide, 1998). The parameters for permethrin, PBOH, PBCHO and PBCOOH analytical method validation are linearity, accuracy, precision, limit of detection (LOD) and limit of quantification (LOQ).

4.1 Linearity

The linearity is the relationship between sample peak area ratios and spiked known concentrations of samples. Concentrations of standards should be chosen on the basis of the concentration range expected in sample. For the linearity, a standard curve was defined by at least 5-6 concentrations. Calibration curve were generated and the coefficient of determination (R^2) were performed. The respective correlation coefficient as proved excellent linearity should be ≥ 0.99.

4.2 Limit of detection (LOD)

Limit of detection is the lowest concentration in the sample that can be detected, might not be necessarily quantitated, under the stated experimental condition. The LOD value was estimated from 3 times of standard deviation of the blank.

4.3 Limit of quantification (LOQ)

Limit of quantification is the lowest concentration that can be detected with acceptable precision and accuracy under the stated experimental condition. The LOQ value was estimated from 10 times of standard deviation of the blank.

4.4 Accuracy and precision

Accuracy is a measure of the closeness of the test results obtained by a method to the true value. Accuracy was measured using a minimum of 5-7 determinations per concentration. The accuracy was calculated from the test results as a percentage of recovery of the assay and it should be in range 80 – 120 %.

Precision is the closeness of individual measures of test result when the method is applied to multiple sampling. Precision was measured using a minimum of 5-7 determinations per concentration. The precision was expressed as the relative standard deviation and should not exceed 20% (EURACHEM Guide, 1998).

$$\%RSD = \frac{\text{standard deviation} \times 100\%}{\text{Mean}}$$

5. In vitro permethrin metabolism assay

5.1 Enzymes preparation

The 4th instar larvae were snap-frozen in liquid nitrogen and stored at -70°C until used. All procedures for enzymes preparation were performed in ice-cold condition. The larvae (10 g) were homogenized with a motor-driven Teflon pestle and glass mortar in 20 ml of homogenizing buffer [100 mM sodium phosphate buffer pH 7.4, 0.25 M sucrose, 1mM EDTA, 100 mM phenylmethylsulphonyl fluoride (PMSF) as a protease inhibitor and 1 mM dithiothreitol (DTT) as a reducing agent]. The homogenate was centrifuged at 12,000 g, 4°C for 20 min. The supernatant was centrifuged again at 100,000 g, 4°C, 60 min to obtain cytosol and microsomes. The microsomal pellet fractions were washed with 10 ml homogenizing buffer by centrifugation at 100,000 g, 4°C for 60 min. The microsomal pellet was resuspended with 1 ml of homogenizing buffer and purified with PD-10 desalting column for cleaning-up the sample, then using in the next experiments. A schematic diagram of the enzymes preparation was shown in Figure 8.

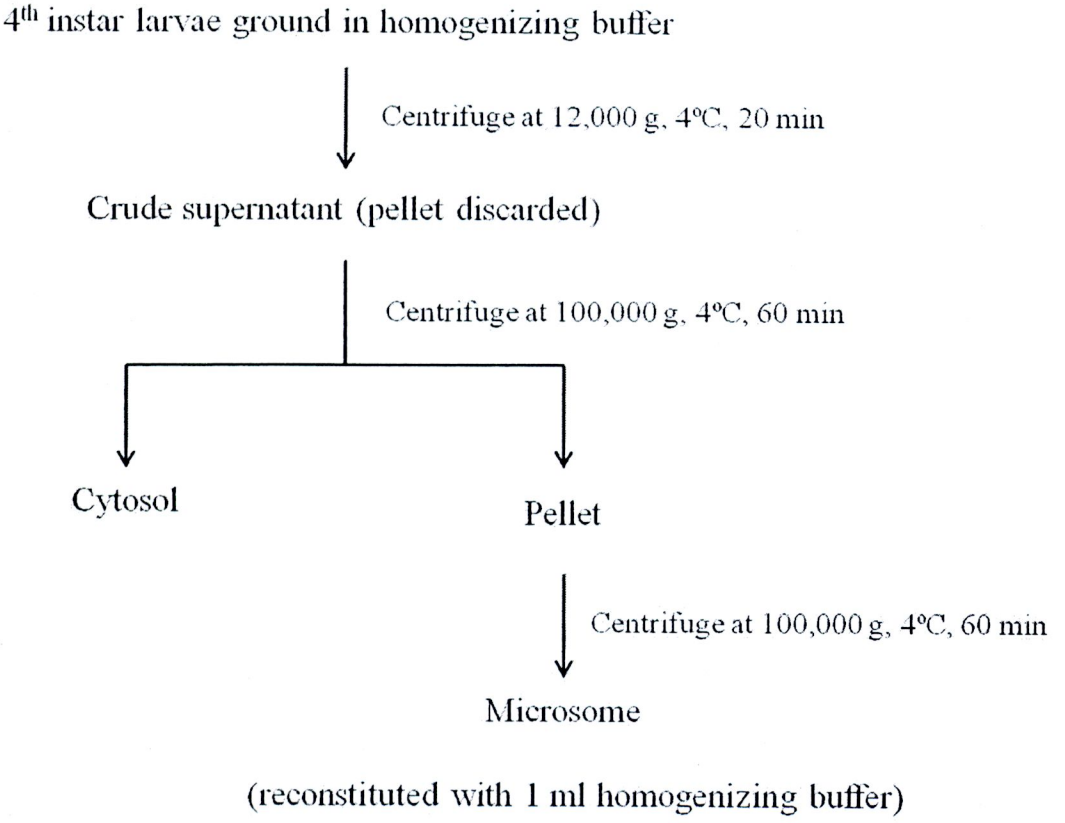

Figure 8 A schematic diagram of the enzymes preparation.

5.2 Determination of protein concentration

To obtain specific activities of enzymes, protein concentrations for all preparations were determined with a commercially Bio-Rad protein reagent (Life Science Research) with bovine serum albumin (BSA) as the standard protein. The concentrated reagent was diluted 1:4 in distilled water and filtered through Whatmans No 1 filter paper to remove the insoluble dye before use. The assay was started by adding 300 µl of diluted Bio-Rad protein reagent to 10 µl of enzyme solution in a microtitre plate at room temperature. Blank contained 10 µl of distilled water and 300 µl of Bio-Rad solution. The end point absorbance was measured at 595 nm after 5 min incubation in a microtitre plate reader. Protein values were calculated from a standard curve of known concentrations of BSA (0-0.5 mg/ml).

5.3 Study of in vitro permethrin metabolism

Chemicals and reagents

Insecticide: Permethrin technical grade (99.6%), Supelco (Bellefonte, PA, USA).

Synergists: Piperonyl butoxide (PBO) (90%), Sigma.

Bis(4-nitrophenyl)-phosphate (BNPP) (99%), Aldrich.

Internal control: Pyrene (≥ 99.0%), Sigma.

The method for study of permethrin metabolism followed Choi *et al.,* (2002), Durham *et al.,* (2002), Enayati *et al.,* (2006) and Nakamura *et al.,* (2007) with some modifications.

Permethrin metabolism experiments were conducted in vitro using three enzyme fractions prepared as mentioned above, i.e. crude supernatant, cytosol and microsomes. The 1-ml of individual reactions contained with 0.8 mg of crude

supernatant, cytosolic or microsomal protein in 50 mM sodium phosphate buffer pH 7.4, and 20 µl of 5 mM permethrin (final concentration 0.1 mM) was added as a substrate (Table 5). To evaluate the role of cytochrome P450 detoxifying permethrin, the reaction was performed in the presence of 25 µl of NADPH regenerating system solution A and 10 µl of NADPH regenerating system solution B, as an electron donor for cytochrome P450. Solution A of the NADPH regenerating system contained 26.0 mM $NADP^+$ and 66 mM of glucose-6-phosphate (G6P) in 50 mM phosphate buffer pH 7.4. Solution B of the NADPH regenerating system contained 40 U/ml of glucose-6-phosphate dehydrogenase (G6PD) in 50 mM phosphate buffer pH 7.4. The reaction mixtures were incubated at 30°C in a water bath for 2 hours. After incubation, pyrene was added to the reaction mixture as an internal control to determine the efficiency of extraction. For extraction, 1.5 ml of chloroform was added in the solution, shaken for 5 min and centrifuged at 2,500 rpm for 15 min. The entire extraction process was repeated 3 times. After extraction, the solution (4.5 ml) was evaporated to dryness by N_2. Finally, the residue was reconstituted with 200 µl of acetonitrile, then 10 µl of the suspension injected for analysis by high-performance liquid chromatography (HPLC) (see section 5.5). Standard solutions (permethrin 0.5 mM, PBOH 1 mM, PBCHO 0.4 mM, PBCOOH 0.3 mM, and pyrene 0.4 mM) were set as a control in each experiment. The concentration of each metabolite was analyzed based on the standard constructed at 230 nm.

To determine the inhibition activity of PBO on cytochrome P450, permethrin and its metabolized products were measured in the reaction added with PBO (final concentration 0.1 mM).

To determine the inhibition activity of BNPP on esterase enzymes, permethrin and its metabolized products were measured in the reaction added with BNPP (final concentration 0.1 mM).

5.4 Assay methods for PBOH and PBCHO oxidase activities

Substrates: 3-phenoxybenxyl alcohol (PBOH) (97.5%) from Fluka.

3-phenoxybenzylaldehyde (PBCHO) (98%) from Sigma

Synergists: Piperonyl butoxide (PBO) (90%), from Sigma.

Bis(4-nitrophenyl)-phosphate (BNPP) (99%), from Aldrich.

Internal control: Pyrene ($\geq$ 99.0%) from Sigma.

PBOH and PBCHO were used as substrates instead of permethrin in order to determine the role of alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) in metabolic pathway.

The 1-ml of individual reactions consisted of 0.8 mg of microsomal protein in 50 mM sodium phosphate buffer pH 7.4, and 50 µl of 5 mM PBOH (final concentration 0.25 mM) or 10 µl of 50 mM PBCHO (final concentration 0.5 mM) as the substrate. The reaction was performed in the presence of NAD^+ as the electron donor for these enzymes (final concentration 2.5 mM). The reaction mixtures were incubated at 30°C in a water bath for 2 hours. Next steps of experiment were the same as those above mentioned in using permethrin as the substrate.

To determine the role of enzyme inhibitors in the metabolic pathway, two enzyme inhibitors, PBO and BNPP, were used (final concentration 0.1 mM each).

Each of them was added in each reaction, and analyzed with HPLC compared with the standards as mentioned above.

5.5 High Performance Liquid Chromatography method (HPLC)

Permethrin and its metabolites were determined in a Shimadzu HPLC system (LC-20A, Japan) (Kyoto, Japan) consisting of a pump liquid chromatograph (LC-20AB), a degasser (DGU-20A3), an auto sampler (SIL-20A), a column oven (CTO-10AS vp) and a diode array detector (SPD-M20A). They were separated on Novapak®C18 mobile phase column (reverse phase C18, 4 μm, 150 x 3.9 mm) and detected using a Shimadzu PDA (photo diode array) detector. For the assay of cytochrome P450 activity, two solvents (solvent A: distilled water adjusted to pH 3.5 with 1N acetic acid and solvent B: acetonitrile) were used for gradient elution (flow rate: 1 ml/min). The chromatographic analysis was conducted at 35°C, operated at a flow rate of 1 ml/min and at a wavelength of 230 nm. Elution times of permethrin, PBOH, PBCHO, PBCOOH, pyrene (an internal standard) and PBO were approximately at 13.1, 4.6, 8.8, 5.8, 12.3, 11.8 min, respectively. The amounts of metabolites formed were determined from the peak areas.

Table 5 Reaction mixture for in vitro permethrin metabolism

Reagents	Stock concentration	Reaction concentration	Volume (µl)
Sodium phosphate buffer (pH 7.2)	50 mM	50 mM	680
Permethrin	5 mM	0.1 mM	20
NADPH	26 mM $NADP^+$: 66 mM G6P		25
G6PD	500 U/ml	40 U/ml	10
NAD^+	100 mM	2.5 mM	7.5
PBO	3 mM	0.1 mM	34
Microsomal fraction		0.8 mg	~ 200
Total volume			**1000**

6. Determination of P450s levels

The assay for detection the total amount of heam containing protein (the bulk of which is cytochrome P450) was determined by indirect assay with tetramethyl benzidine (TMBZ) (Brogdon *et al.*, 1997, Penilla *et al.*, 2007). TMBZ substrate was oxidized and produced a blue color upon reaction with heam peroxidase (Figure 9).

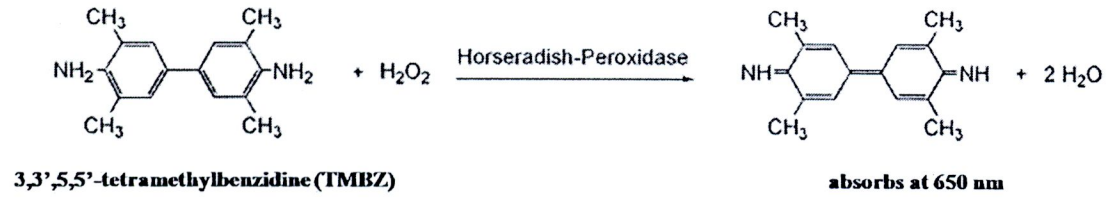

Figure 9 Peroxidase reaction (http://www.biosynth.com).

The 4th larvae were homogenized with homogenizing buffer (as mentioned in enzyme preparation section 5.1) to prepare microsomes. Twenty microliters of microsomes were filled into the wells of a 96-well microtitre plate, then 80 µl of 0.0625 M potassium phosphate buffer pH 7.2 and 200 µl of TMBZ solution (0.01 g of 3,3',5,5'-tetramethylbenzidine in 5 ml of absolute methanol mixed with 15 ml of 0.25 M sodium acetate buffer pH 5.0, preparing fresh daily) were added. Twenty-five microliters of 3% H_2O_2 were added, and incubated for 2 hours at room temperature. Two controls per plate were prepared with 20 µl of homogenizing buffer, 200 µl of the TMBZ solution and 25 µl of 3% H_2O_2. The plate was read at 650 nm in a microtitre plate reader and the values were compared with known concentrations of cytochrome C from horse heart type VI (Sigma). The standard curve was designed to show the linearity of the heme peroxidase assay with hemoprotein concentration

(Table 6). The values were reported as equivalent units of cytochrome P450/mg protein.

6.2 Preparation standard cytochrome C

Standard cytochrome C from horse heart type VI (Sigma) ($\geq$ 95%, MW = 12384)

Stock solution: standard cytochrome C 25 mg was dissolved with 2.5 ml sodium acetate buffer pH 5.0.

Standard cytochrome C working solutions: Five concentrations (0.25-2 nmole/ml) were diluted from stock solution.

Table 6 Cytochrome C standard assay

Working solution (nmole/ml)	Final concentration (nmole/20 µl)
2	0.04
1	0.02
0.75	0.015
0.5	0.01
0.25	0.005