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APPENDIX

Reagent preparations

1. Sodium Phosphate buffer (pH 7.4)

100 mM Na_2HPO_4

100 mM NaH_2PO_4

Adjust to 1L with distilled water

2. Homogenizing buffer (pH 7.4)

0.25 M sucrose	85.55 g
1 mM EDTA	0.3722 g
1% polyvinyl pyrolidone (PVP)	1 g

Dissolved and Adjust to 1L with sodium phosphate buffer pH 7.4

3. NADPH regenerating system

NADPH solution (26 mM $NADP^+$: 66 mM G6P)

26.0 mM $NADP^+$: $NADP^+$ 0.0096 g in 500 µl phosphate buffer pH7.4

66.0 mM G6P : G6P 0.0093 g in 500 µl phosphate buffer pH7.4

G6PD (40 U/ml):

24 µl of G6PD in 976 µl phosphate buffer pH7.4

4. NAD^+ (20 mg/vial) (MW=663.4)

Stock 100 mM : NAD^+ 20 mg was dissolved with 300 µl of distilled water.

5. Piperonyl butoxide (PBO) (D=1.059 g/ml)

PBO (3 mM) : PBO 5.25 µl dissolved with 5 ml of ethanol

6. Bis(4-nitrophenyl)-phosphate (BNPP) (MW=340.18)

BNPP (1 mM) : BNPP 0.0017 g dissolved with 5 ml of distilled water

7. Phenoxybenzyl alcohol (PBOH) (stock conc. 10^6 ng/ml) (D=1.151 g/ml)

PBOH 8.77 µl dissolved with 10 ml of acetonitrile

8. Phenoxybenzaldehyde (PBCHO) (stock conc. 10^6 ng/ml) Sigma (D=1.153 g/ml)

PBCHO 8.85 µl dissolved with 10 ml of ethanol

9. Phenoxybenzoic acid (PBCOOH) (stock conc. 10^6 ng/ml)

PBCOOH 0.01 g adjusted with acetonitrile for 10 ml

10. Permethrin (stock conc. 5 mM) (MW=391.3)

Permethrin 0.02 g dissolved with 10 ml of ethanol

PD-10 desalting column purification

PD-10 desalting columns are used for desalting, buffer exchange and sample clean up. The method followed the procedure described by a commercially Amersham Biosciences instruction (Figure 1). The columns were equilibrated with 25 ml elution buffer. The sample was added of the total volume 2.5 ml, then eluted with 3.5 ml buffer and collected the flow-through.

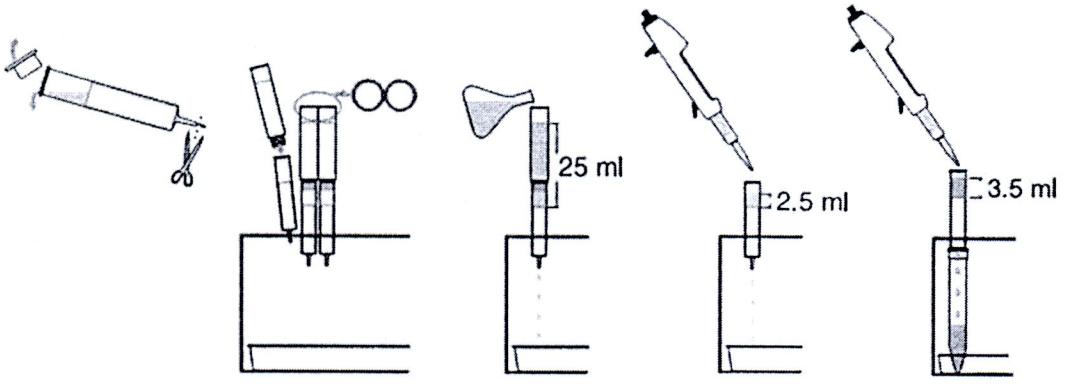

Figure 1 A schematic diagram of the PD-10 desalting column (Amersham Biosciences).

Table 1 Susceptibility of PMD-R larvae to permethrin after 24-h exposure

Permethrin (ppb)	**Experiment I**		Mortality (%)	**Experiment II**		Mortality (%)	**Experiment III**		Mortality (%)	**Experiment IV**		Mortality (%)
	Death	Survive		Death	Survive		Death	Survive		Death	Survive	
5	1	24	4	1	24	4	1	24	4	2	23	8
10	5	20	20	6	19	24	6	19	24	8	17	32
50	20	5	80	18	7	72	18	7	72	18	7	72
100	21	4	84	22	3	88	21	4	84	22	3	88
500	25	0	100	25	0	100	25	0	100	25	0	100

Table 2 Susceptibility of PMD-R larvae to permethrin in the presence of PBO after 24-h exposure

Permethrin (ppb)	**Experiment I**		Mortality (%)	**Experiment II**		Mortality (%)	**Experiment III**		Mortality (%)	**Experiment IV**		Mortality (%)
	Death	Survive		Death	Survive		Death	Survive		Death	Survive	
0.5	1	24	4	4	21	16	0	25	0	2	23	8
1	2	23	8	3	22	12	3	22	12	4	21	16
5	7	18	28	9	16	36	10	5	40	8	17	32
7.5	10	15	40	12	13	48	13	12	52	11	14	44
10	16	9	64	18	7	72	16	9	64	15	10	60
50	19	6	76	21	4	84	20	5	80	19	6	76
100	24	1	96	23	2	92	22	3	88	22	3	88

Table 3 Susceptibility of PMD-R larvae to permethrin in the presence of BNPP after 24-h exposure

Permethrin (ppb)	**Experiment I**		Mortality (%)	**Experiment II**		Mortality (%)	**Experiment III**		Mortality (%)	**Experiment IV**		Mortality (%)
	Death	Survive		Death	Survive		Death	Survive		Death	Survive	
1	0	25	0	0	25	0	0	25	0	0	25	0
5	3	22	12	1	24	4	1	24	4	0	25	0
7.5	3	22	12	3	22	12	4	21	16	3	22	12
10	11	14	44	7	18	28	13	12	52	12	13	48
50	21	4	84	23	2	92	25	0	100	23	2	92
100	25	0	100	25	0	100	25	0	100	25	0	100

Table 4 Susceptibility of PMD larvae to permethrin after 24-h exposure

Permethrin (ppb)	**Experiment I**		Mortality (%)	**Experiment II**		Mortality (%)	**Experiment III**		Mortality (%)	**Experiment IV**		Mortality (%)
	Death	Survive		Death	Survive		Death	Survive		Death	Survive	
0.1	1	24	4	0	25	0	0	25	0	0	25	0
0.3	1	24	4	3	22	12	4	21	16	3	22	12
0.5	6	19	24	8	17	32	7	18	28	7	18	28
0.75	8	17	32	8	17	32	9	16	36	7	18	28
1	15	10	60	11	14	44	13	12	52	13	12	52
5	25	0	100	25	0	100	25	0	100	25	0	100

Table 5 Susceptibility of PMD larvae to permethrin in the presence of PBO after 24-h exposure

Permethrin (ppb)	**Experiment I**		Mortality (%)	**Experiment II**		Mortality (%)	**Experiment III**		Mortality (%)	**Experiment IV**		Mortality (%)
	Death	Survive		Death	Survive		Death	Survive		Death	Survive	
0.1	0	25	0	0	25	0	1	24	4	1	24	4
0.3	10	15	40	7	18	28	7	18	28	6	19	24
0.5	14	11	56	12	13	48	12	13	48	10	15	40
0.75	18	7	72	20	5	80	25	5	80	16	9	64
1	25	0	100	21	4	84	24	1	96	22	3	88
5	25	0	100	25	0	100	25	0	100	25	0	100

Table 6 Susceptibility of PMD larvae to permethrin in the presence of BNPP after 24-h exposure

Permethrin (ppb)	**Experiment I**		Mortality (%)	**Experiment II**		Mortality (%)	**Experiment III**		Mortality (%)	**Experiment IV**		Mortality (%)
	Death	Survive		Death	Survive		Death	Survive		Death	Survive	
0.1	0	25	0	0	25	0	0	25	0	0	25	0
0.3	0	25	0	0	25	0	0	25	0	0	25	0
0.5	0	25	0	0	25	0	1	24	4	2	23	8
1	7	18	28	4	21	16	9	16	36	8	17	32
3	24	1	96	24	1	96	25	0	100	23	2	92
5	25	0	100	25	0	100	25	0	100	25	0	100

Table 7 Equivalent units of P450s levels in crude, cytosol and microsomal fraction of PMD strain

Strains	OD (nm)	Calculated P450	Protein conc. (mg/20µl)	Equivalent unit of P450/mg protein	Mean±SD
Crude PMD	0.491	0.088	0.154	0.568	0.562±0.02
	0.500	0.089	0.154	0.574	
	0.458	0.084	0.154	0.543	
Cytosol PMD	0.581	0.098	0.131	0.751	0.718±0.03
	0.525	0.092	0.131	0.701	
	0.525	0.092	0.131	0.701	
Microsomal PMD	0.199	0.038	0.025	1.523	1.493±0.03
	0.190	0.038	0.025	1.493	
	0.181	0.037	0.025	1.463	

Table 8 Equivalent units of P450s levels in crude, cytosol and microsomal fraction of PMD-R strain

Strains	OD (nm)	Calculated P450	Protein conc. (mg/20µl)	Equivalent unit of P450/mg protein	Mean±SD
Crude PMD-R	0.491	0.088	0.175	0.501	0.526±0.03
	0.575	0.097	0.175	0.557	
	0.517	0.091	0.175	0.519	
Cytosol PMD-R	0.554	0.095	0.149	0.636	0.639±0.01
	0.556	0.095	0.149	0.637	
	0.567	0.096	0.149	0.646	
Microsomal PMD-R	0.331	0.049	0.034	1.432	1.466±0.04
	0.362	0.052	0.034	1.507	
	0.343	0.050	0.034	1.461	

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ORIGINAL PAPER

Enzymes-based resistant mechanism in pyrethroid resistant and susceptible *Aedes aegypti* strains from northern Thailand

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Abstract Previous studies have shown that permethrin resistance in our selected PMD-R strain of *Aedes aegypti* from Chiang Mai, Thailand, was associated with a homozygous mutation in the knockdown resistance (*kdr*) gene and other mechanisms. In this study, we investigated the metabolic mechanism of resistance of this strain compared to the PMD strain which is susceptible to permethrin. The permethrin susceptibility of larvae was determined by a dose–response bioassay. Two synergists, namely piperonyl butoxide (PBO) and bis(4-nitrophenyl)-phosphate (BNPP), were also added to determine if the resistance is conferred by oxidase or esterase enzymes, respectively. The LC_{50} value for PMD-R (25.42 ppb) was ~25-fold higher than for PMD (1.02 ppb). The LC_{50} was reduced 3.03-fold in PMD-R and 2.27-fold in PMD when the oxidase inhibitor (PBO) was added, but little or no reduction was observed in the presence of BNPP, indicating that oxidative enzymes play an important role in resistance. However, the LC_{50} previously observed in the heterozygous mutation form was reduced ~eightfold, indicating that metabolic resistance is inferior to *kdr*. The levels of cytochrome P450 (P450) extracted from fourth instar larvae were similar in both strains and were about 2.3-fold greater in microsomal fractions than in crude supernatant and cytosol fractions. Microsome oxidase activities were determined by incubation with each of three substrates, i.e., permethrin, phenoxybenzyl alcohol (PBOH), and phenoxybenzaldehyde (PBCHO), in the presence or absence of nicotinamide adenine dinucleotide phosphate (NADPH), nicotinamide adenine dinucleotide (NAD^+), PBO, and BNPP. It is known that hydrolysis of permethrin produces PBOH which is further oxidized to PBCHO by alcohol dehydrogenase (ADH) and then to phenoxybenzoic acid (PBCOOH) by aldehyde dehydrogenase (ALDH). When incubated with permethrin, a small amount of PBCOOH was detected in both strains (about 1.1–1.2 nmol/min/mg protein), regardless of the addition of NADPH. The addition of PBO resulted in about 70% and 50% reduction of PBCOOH in PMD and PMD-R, respectively. The addition of BNPP reduced PBCOOH about 50% and 35% in PMD and PMD-R, respectively. Using PBOH as substrate increased PBCOOH ~16-fold and ~40-fold in PMD and PMD-R, respectively. Using PBCHO as substrate increased PBCOOH ~26-fold and ~50-fold in PMD and PMD-R, respectively. The addition of NADPH, and particularly NAD^+, increased the level of PBCOOH. Together, the results have indicated the presence of a metabolic metabolism involving P450, ADHs, and ALDHs in both PMD and PMD-R strains, with greater enzyme activity in the latter.

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Introduction

The mosquito *Aedes aegypti* is the major vector of dengue and dengue hemorrhagic fever, which are serious public health problems in Thailand and elsewhere. Due to the lack of vaccine and specific treatment, control of transmission is mainly based on management of breeding places and

insecticide applications, by fogging and ultra-low-volume sprays and larvicides. Dichlorodiphenyltrichloroethane (DDT), organophosphates (e.g., malathion, fenitrothion, and temephos), and carbamate (e.g., propoxur) have been heavily used for mosquito control over 40 years before being replaced (except temephos) by pyrethroids in the early 1990s (Chareonviriyaphap et al. 1999). Like many other countries, the adverse effect of the heavy and long-term use of insecticides in Thailand is resistance of *A. aegypti*. In Thailand, resistance to DDT has been reported in *A. aegypti* since the mid 1960s (Neely 1966). At present, it is known to be resistant to several insecticides, particularly pyrethroids (i.e., permethrin and deltamethrin), organophosphate compounds (i.e., temephos and fenitrothion), and carbamate compounds (i.e., propoxur) (Somboon et al. 2003; Paeporn et al. 2004; Ponlawat et al. 2005; Jirakanjanakit et al. 2007a, b; Pethuan et al. 2007). This problem has severely hampered the control of vectors using insecticides.

There are two broad classes of resistance mechanism that play an important role in mosquito resistance to insecticides: target site insensitivity and metabolic enzyme-based resistance (Hemingway and Ranson 2000). Target site insensitivity to pyrethroids and DDT in mosquitoes and other insects is associated with single or multiple mutations, commonly referred to as knockdown resistance (kdr). These mutations modify the voltage-gated sodium channel protein, making it less susceptible to the binding of pyrethroids and DDT (Soderlund and Knipple 2003). Metabolic enzyme-based resistance is principally associated with three enzyme groups: cytochrome P450 monooxygenases (P450s), esterases, and glutathione-S-transferases, depending on the insect species/strain and the insecticide (Hemingway and Ranson 2000).

We have previously studied two strains of *A. aegypti*, PMD and PMD-R, originating from Chiang Mai Province, Thailand, formerly called R^dS^p and R^dR^p, respectively (Prapanthadara et al. 2002). The PMD strain is resistant to DDT whereas PMD-R is resistant to both DDT and permethrin. Permethrin resistance in the PMD-R strain is highly associated with a homozygous mutation in codon F1552 of the *kdr* gene of *A. aegypti* (equivalent to F1534 in the house fly Vssc1 sequence) resulting in the replacement of phenylalanine by cysteine in segment six domain III of the voltage-gated sodium channel protein (Yanola et al. 2010). This mutation is common throughout Thailand (□0.8 allele frequency, Yanola et al. 2010; 2011) and has also been found associated with pyrethroid resistance in Vietnam and the British West Indies (Kawada et al. 2009; Harris et al. 2010). In addition, a genetic study involving crossing and backcrossing of the PMD and PMD-R strains indicated that a number of unlinked genes contribute to permethrin resistance (Yanola et al. 2010). Our previous biochemical characterization revealed that in both strains, there was □tenfold increase in DDTase activity and a fourfold increase in P450 activity compared to the Rockefeller (susceptible) strain, whereas the esterase and glutathione-S-transferase (GST) activities only slightly increased (Prapanthadara et al. 2002). DDT resistance activity in both strains is therefore considered mainly due to the increased DDTase. In addition, the kdr gene in PMD-R may be involved in DDT resistance because DDT and pyrethroid insecticides share a similar target site. However, the role of P450s in the detoxification of insecticides is not clear because the strains exhibit different susceptibility to permethrin, but have similar levels of P450s.

The P450s are known to be involved in the metabolism of xenobiotics including insecticides and have a role in endogenous metabolism (Scott 1999). In mammals, in vivo and in vitro studies (e.g., Guaghan et al. 1977; Choi et al. 2002; Hodgson 2003; Nakamura et al. 2007) have indicated that permethrin is rapidly hydrolysed by carboxylesterase and the phenoxybenzyl alcohol (PBOH) that is formed is further oxidized to phenoxybenzaldehyde (PBCHO) and phenoxybenzoic acid (PBCOOH). There have been indications that permethrin metabolites (PBOH and PBCHO) can be more cytotoxic than the parent permethrin (Stratton and Corke 1982). The oxidative enzymes that oxidize PBOH and PBCHO may be different depending on the organism. Choi et al. (2002) and Hodgson (2003) reported that alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) in human liver cytosolic fractions catalyze the oxidations to PBOH and PBCHO, respectively. They suggested that P450s play no role in the metabolism of permethrin and its hydrolysis products in mammals. However, Nakamura et al. (2007) showed that P450s in rat liver microsomes do play some role in oxidizing PBOH and PBCHO.

In mosquitoes including *A. aegypti*, several studies (e.g., Yaicharoen et al. 2005; Penilla et al. 2007; Pethuan et al. 2007; Awolola et al. 2009; Jagadeshwaran and Vijayan 2009) suggested that an increased level of P450s was often associated with pyrethroid resistance. In addition, significant elevation of esterases, glucose-6-phosphate dehydrogenase (G6PD), and GST was reported in a pyrethroid resistant A. aegypti strain (Jagadeshwaran and Vijayan 2009). However, little is known of how P450s and other oxidative enzymes are involved in the metabolic pathway of permethrin. In this study, we investigated the synergistic effect of piperonyl butoxide (PBO), a monooxygenase inhibitor and bis(4-nitrophenyl)-phosphate (BNPP), an esterase inhibitor, in a larval bioassay. We also demonstrated the enzymatic involvement of ADH, ALDH and, particularly, P450s in permethrin metabolism pathway by in vitro assays using enzymes extracted from the PMD and PMD-R A. aegypti strains. An understanding of the resistant mechanisms could help to develop and/or provide some novel management of vector control strategies.

Materials and methods

Mosquito strains

Two laboratory strains of *A. aegypti*, PMD and PMD-R, were compared for their ability to metabolize permethrin. Both originated from Ban Pang Mai Daeng, Mae Tang District, Chiang Mai Province, Thailand (Prapanthadara et al. 2002). The PMD-R strain is resistant to both DDT and permethrin while PMD is susceptible to permethrin but resistant to DDT, based on the WHO susceptibility test with impregnated papers (4% DDT and 0.25% permethrin). The adult mosquitoes were maintained under regular insecticide pressure (0.75% permethrin) using standard WHO kits (WHO 1975). The eggs were harvested and stored at room temperature.

Chemicals

The following chemicals were used: technical grade permethrin (99.6%) (Supelco, USA); PBO (90%), BNPP (99%); PBCHO (98%) and pyrene (99%) from Aldrich (USA); PBOH (≥99%), PBCOOH (≥98%) from Fluka (USA), tetramethylbenzidine (TMBZ); nicotinamide adenine dinucleotide phosphate (NADPH) (96%), nicotinamide adenine dinucleotide (NAD^+) (≥98%), and G6PD from Sigma (USA).

Dose–response bioassay test for mosquito larvae

The dose–response bioassay was conducted according to the WHO standard method (WHO 1981). Stock and serial concentrations (0.1–500 ppb) of permethrin were prepared in ethanol and stored at 4°C. Batches of 25 early fourth instar larvae were placed in a 400-ml beaker containing 249 ml of distilled water and 1 ml of permethrin solution. There were four replicates per concentration. In the control experiments, 0.4% ethanol was included in 250 ml of water. In parallel with this, an extra set of bioassays with the addition of either PBO or BNPP (0.3 mg/ml each) was performed to determine if the resistance is conferred by oxidase or esterase enzymes, respectively (Paul et al. 2006). Larval mortality was recorded after 24 h exposure. Data were analyzed by standard probit analysis (Finney 1971).

Preparation of enzyme fractions

The fourth 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 conditions (Prapanthadara et al. 2002). 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, 1 mM EDTA, 100 mM phenylmethylsulfonyl fluoride, and 1 mM DTT]. The homogenates were centrifuged at 12,000 × g, 4°C for 20 min. The supernatants were further separated into cytosol and microsomes by centrifugation at 100,000 × g, 4°C, for 60 min in an ultracentrifuge (Optima™ -100XP ultracentrifuge, Beckman Coulter). The microsomes were washed with 10 ml homogenizing buffer by centrifugation at 100,000 × g, 4°C for 60 min. The microsomal pellets were resuspended with 1 ml of homogenizing buffer before being used.

Determination of cytochrome P450 (P450) activities

The procedure to determine P450 activity followed Penilla et al. (2007) with minor modifications. Twenty µl of microsomes were incubated with 80 µl of 0.0625 M potassium phosphate buffer pH 7.2 and 200 µl of 6.3 mM 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, prepared fresh daily) in a 96-well microtitre plate. Twenty-five µl of 3% H_2O_2 were added, and allowed to stand for 2 h at room temperature. Two controls per plate were prepared each with 20 µl of homogenizing buffer instead of the microsome suspension. The plate was read at 650 nm in a microtitreplate reader (Spectra MR, DYNEX Technologies) and the values were compared with known concentrations of cytochrome C from horse heart type VI (Sigma) and reported as equivalent units of P450/mg protein.

Protein concentrations of enzyme preparations were determined with a commercial Bio-Rad protein reagent (Life Science Research). In each microtitreplate well, 300 µl of Bio-Rad protein reagent (diluted 1:4 with distilled water) was added with 10 µl of enzyme solution. The absorbance was measured at 595 nm in the microtitreplate reader. Protein values were calculated from a standard curve of known concentrations of bovine serum albumin (0–0.5 mg/ml).

In vitro metabolism of permethrin, PBOH, and PBCHO

The method for the study of permethrin metabolism followed Choi et al. (2002) and Nakamura et al. (2007) with minor modifications. The 1 ml of individual reactions contained 0.8 mg of microsomal fraction in 50 mM sodium phosphate buffer pH 7.4, and 0.1 mM of permethrin was added as the substrate. To evaluate the role of P450 in detoxifying permethrin, the reaction was performed in the presence of 25 µl of NADPH regenerating system solution A [26 mM $NADP^+$ and 66 mM of glucose-6-phosphate in 50 mM phosphate buffer pH 7.4] and 10 µl of NADPH

Table 1 Susceptibility of PMD and PMD-R larvae to permethrin in the presence of PBO or BNPP after 24-h exposure

Strains	Permethrin		Permethrin + BNPP			Permethrin + PBO		
	LC_{50} (95% CI)	Slope (SE)	LC_{50} (95% CI)	Slope (SE)	SR	LC_{50} (95% CI)	Slope (SE)	SR
PMD	1.02 (0.87–1.29)	2.26 (0.28)	1.30 (1.18–1.45)	4.73 (0.42)	0.78	0.45 (0.41-0.50)	3.32 (0.28)	2.27
PMD-R	25.42 (21.38–30.29)	1.98 (0.16)	19.58 (16.26–23.51)	1.83 (0.15)	1.30	8.39 (6.85–10.31)	1.22 (0.08)	3.03

Values are parts per billion

SE standard error, SR synergist ratio (LC_{50} observed in the absence of synergist/LC_{50} observed in the presence of synergist), CI confidence interval, BNPP bis(4-nitrophenyl)-phosphate, PBO piperonyl butoxide

regenerating system solution B [40 U/ml of G6PD in 50 mM phosphate buffer pH 7.4], as an electron donor for P450. Additionally, the inhibition activity of PBO on P450, or BNPP on esterase enzymes, was investigated using further reactions to which of PBO 0.1 mM or 0.1 mM BNPP had been added. All reaction mixtures were incubated at 30°C for 2 h. After incubation, pyrene was added as an internal control and then the mixture was extracted three times with 1.5-ml chloroform. The chloroform extract was evaporated to dryness by N_2, the residue reconstituted with 200 µl of acetonitrile, then 10 µl of the suspension injected for analysis by high-performance liquid chromatography (HPLC).

In order to determine the role of ADH and ALDH in the metabolic pathway, PBOH and PBCHO were used as substrates instead of permethrin. These assays followed the method for permethrin metabolism as mentioned above except that 0.25 mM of PBOH or 0.5 mM of PBCHO was used as the substrate. The reaction was performed in the presence of 2.5 mM NAD^+, instead of the NADPH regenerating system, as the electron donor for these enzymes. The reaction mixtures were incubated at 30°C for 2 h. The next steps of the experiment are the same as those described above when using permethrin as the substrate.

HPLC method

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 a Novapak® C18 mobile phase column (reverse phase C18, 4 µm, 150× 3.9 mm) and detected using a Shimadzu photodiode array (PDA) detector. For the assay of P450 activity, two solvents (solvent A: distilled water adjusted to pH 3.5 with 1 N acetic acid and solvent B: acetonitrile) were used for gradient elution (flow rate: 1 ml/min). The chromatographic analysis was conducted at 35°C, operating at a flow rate of 1 ml/min and at a wavelength of 230 nm.

Results

Dose–response bioassay test of mosquito larvae

The results of the larval susceptibility test of the PMD and PMD-R strains to permethrin with and without synergists (BNPP and PBO) are presented in Table 1 and Fig. 1. Without a synergist, the LC_{50} value for PMD-R (25.42 ppb) was ~25-fold higher than for PMD (1.02 ppb). The addition of esterase inhibitor (BNPP) appeared to have no effect on the LC_{50} of the PMD, but had a small effect on the PMD-R. By contrast, when the oxidase inhibitor (PBO) was added, the LC_{50} values were reduced 3.03-fold in PMD-R and 2.27-fold in PMD compared to the original levels.

P450s activity

The equivalent unit of total P450s, as determined by indirect assay with tetramethyl benzidine (Penilla et al. 2007), was significantly higher for the microsomal fractions than the cytosolic fractions and crude supernatant ($p<0.05$) (Fig. 2). Since there was no significant difference between

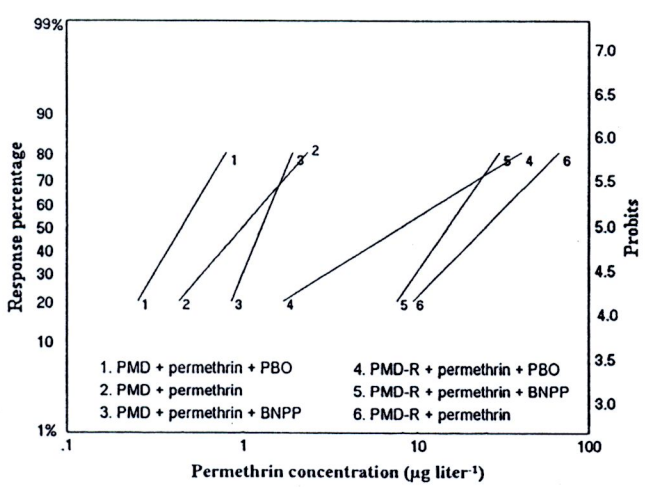

Fig. 1 The log dose-probit mortality lines of the PMD and PMD-R strains after exposure to permethrin, with and without synergists (PBO and BNPP)

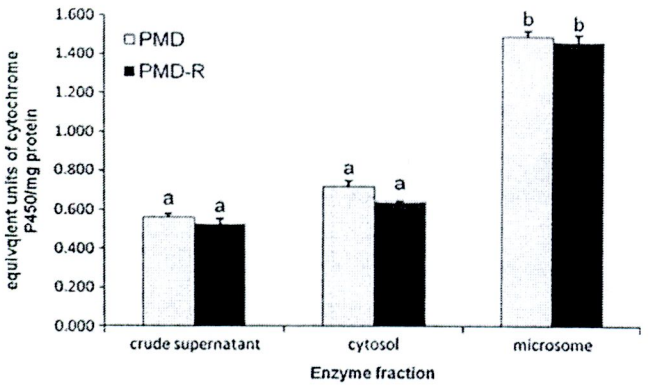

Fig. 2 The equivalent units of total P450/mg protein in the PMD and PMD-R strains. The different letters (a and b) indicate a significant difference ($p<0.05$) among the enzyme fractions

the PMD and PMD-R strains within each fraction ($p>0.05$), the microsomal fractions were used for further study.

In vitro metabolism of permethrin, PBOH, and PBCHO by microsomal enzymes

The in vitro metabolism of permethrin, PBOH, and PBCHO incubated with the microsomes of the fourth instar larvae of the PMD and PMD-R strains is shown in Table 2. The data presented in this paper are based on the principle of oxidoreduction catalysed by the in vitro activity of P450s, which is dependent on the presence of NADPH as a cofactor. When permethrin was used as the substrate (reactions 1–2), the metabolic product, PBCOOH, was detected equally in both strains, irrespective of the presence of an exogenous NADPH regenerating system. This indicates that the endogeneous NADPH within the microsome preparation is sufficient for the reaction assay. The intermediate products, PBOH and PBCHO, were also detectable, but were not detected in the absence of the microsomes or in the absence of substrates (data not shown). Addition of either BNPP or PBO resulted in a significant reduction of PBCOOH formation in both strains. The inhibition effect was significantly lower ($p<0.05$) in PMD-R which produced more metabolite. The difference in inhibition to enzyme activity suggests the qualitative difference of the enzymes. When PBOH was used as the substrate (reactions 3–4), much more of the PBCOOH was detected than when using permethrin, with a higher quantity in the PMD-R than the PMD samples. The addition of an NADPH-regenerating system significantly increased PBCOOH in PMD, but only slightly increased it in PMD-R. The highest amount of PBCOOH was produced in the presence of NAD^+. A similar tendency was observed when PBCHO was the substrate (reactions 5–6). The product was significantly increased in the presence of NADPH but a much greater quantity was detected when NAD^+ was added. Overall, the PMD-R microsomes produced more PBCOOH in all cases.

Discussion

Permethrin resistance in PMD-R has recently been demonstrated to be associated mainly with a homozygous mutation in the voltage-gated sodium channel gene (Yanola et al. 2010). They showed in the larval bioassay that the resistance level of the heterozygous forms (determined by the permethrin LC_{50}) was reduced ~eightfold compared to the homozygous form (25.64 ppb), but that this reduced level was still ~threefold higher than that of the PMD strain (1.03 ppb). The present results show that the LC_{50} of PMD-R was reduced only ~threefold (from 25.42 to 8.39 ppb) by PBO (Table 1 and Fig. 1). This therefore implies that the resistance ability of PMD-R is due mainly to the kdr gene (about three quarters) and the rest due to oxidase-based

Table 2 Amount of PBCOOH formation after incubation of the PMD and PMD-R microsomal fractions (Mic) with the substrates, permethrin, PBOH, and PBCHO in the presence or absence of inhibitors and co-enzymes (NADPH, NAD^+, PBO, and BNPP)

Reactions	PBCOOH formation (nmole/min/mg protein)				
	No treatment	NADPH	NAD^+	PBO + NADPH	BNPP
1. Mic (PMD) + permethrin	1.13±0.00	1.18±0.11	–	0.31±0.01	0.58±0.01
2. Mic (PMD-R) + permethrin	1.18±0.07	1.17±0.03	–	0.61±0.04	0.77±0.04
3. Mic (PMD) + PBOH	18.06±1.76	26.79±1.41	49.06±0.42	–	–
4. Mic (PMD-R) + PBOH	46.17±0.21	49.44±2.06	69.18±0.61	–	–
5. Mic (PMD) + PBCHO	29.73±0.27	51.30±1.67	157.92±5.43	–	–
6. Mic (PMD-R) + PBCHO	60.03±4.38	82.17±2.26	198.32±2.51	–	–

Values are represented as means ± SD (n=3)

NADPH nicotinamide adenine dinucleotide phosphate, NAD^+ nicotinamide adenine dinucleotide, PBO piperonyl butoxide, BNPP bis(4-nitrophenyl)-phosphate, PBOH phenoxybenzyl alcohol, PBCHO phenoxybenzaldehyde

resistance. These results agree with our previous (unpublished) observations that the addition of PBO in the larval bioassay of the heterozygous larvae reduced the LC_{50} close to the susceptible PMD strain, commented in Yanola et al. (2010). The LC_{50} of PMD was reduced 2.27-fold in the presence of PBO which was similar to PBO's synergistic effect on PMD-R (3.03-fold) (Table 1). The level of total P450s was similar in both strains (Fig. 2), agreeing with a previous study showing that both strains had an activity of P450s fourfold higher than the susceptible Rockefeller strain (Prapanthadara et al. 2002). These results reflect the in vivo involvement of P450s in detoxification of permethrin in both strains, agreeing with the result in the in vitro assay (Table 2, reactions 1–2).

The effect of esterase inhibitor, BNPP, was not distinct in the larval bioassay, but was observed in the in vitro assay in which a significant reduction of PBCOOH was detected (Table 2, reactions 1–2). These results suggest a role for esterase enzymes in the hydrolysis of permethrin in both strains. In mosquitoes, elevated esterase enzymes are the primary mechanism for organophosphorus insecticide resistance as well as a secondary mechanism for carbamate resistance. Additionally, elevated levels of esterases also confer pyrethroid resistance in some insect species, including *A. aegypti*, (Hemingway and Karunaratne 1998; Flores et al. 2005; Jagadeshwaran and Vijayan 2009). Studies in several areas in Thailand found that elevated esterases in A. aegypti were associated mainly with organophosphorus insecticide resistance (Pethuan et al. 2007). The PMD and PMD-R strains probably had only a slightly increased esterase activity (Prapanthadara et al. 2002) because they originated from an area where resistance to organophosphorus insecticides was not observed (Somboon et al. 2003). No resistance to organophosphorus insecticides (temephos, fenthion, and malathion) was also observed in India (Tikar et al. 2008). However, one should be aware that in vitro experiments with model substrates do not necessarily reflect the in vivo situations of insect metabolizing insecticide molecules.

Interestingly, PMD-R produced more PBCOOH than PMD in most reactions, particularly when PBOH and PBCHO were the substrates (Table 2). This was observed in both NADPH and NAD^+ generating systems. These results have suggested a difference in the oxidative enzyme system between the two strains. NADPH is known to be cofactor of P450s which can also oxidize alcohol and aldehyde in insects and mammals (Asai et al. 1996; Guo et al. 2010). Although both strains had an equivalent level of P450 activity, it does not necessarily mean they have identical P450 genes because resistance can occur due to detoxification by only a few P450s (Scott 1999; Nakamura et al. 2007). Of the 160 P450 active genes found in *A. aegypti*, Strode et al. (2008) reported that 23 genes were overexpressed in the PMD-R strain relative to a susceptible strain; however, only a few P450s genes may be involved in pyrethroid resistance. The P450 genes in the PMD strain are still not known and require further study.

Replacement of the NADPH generating system with NAD^+ resulted in a considerably higher production of PBCOOH, suggesting that, besides P450s, there were other oxidative enzymes playing a role in oxidizing the substrates. The enzymes that are known to metabolize PBOH and PBCHO are ADHs and ALDHs, respectively, and both groups require NAD^+ as a cofactor (Devlin 2006; Gibson and Skett 1994). These enzymes are involved in detoxifying alcohol and aldehyde in mammals and insects (Guo et al. 2010), but it has not yet been shown that they are involved in permethrin metabolite oxidation in insects. Our present study demonstrates for the first time in insects that oxidation of PBHO and PBCHO in *A. aegypti* can also be mediated by ADHs and ALDHs, respectively, as previously reported in mammals (Choi et al. 2002; Hodgson 2003).

It has been demonstrated that PBO can also inhibit the activity of ADHs in insects (Guo et al. 2010) and this might occur in our larval bioassay (Table 1). Our ongoing study revealed that the ALDH genes (AAEL014080 and AAEL009948 in VectorBase) were overexpressed in the PMD-R larvae compared with the PMD larvae (Lumjuan N., unpublished data). This may partially explain the difference of PBCOOH production between the two strains. Our in vitro results suggest that overexpression of ADH genes might also occur in both strains studied (Table 2).

In conclusion, the permethrin resistance mechanism in the *A. aegypti* PMD-R strain is conferred mainly by the kdr gene and partially by oxidative enzymes involving P450s, ADHs, and ALDHs. It is likely that both types of resistance mechanism are present in many Thai populations of *A. aegypti*, occurring either singly or in combination. In this situation, the reduction of vector populations using pyrethroid will be difficult. It is important that care is taken in the appropriate choice of insecticide, using those that are less affected by *kdr* and oxidase-based resistant mechanisms were necessary.

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