Article

CHARACTERIZATION OF MOSQUITO CYP6P7 AND CYP6AA3: DIFFERENCES IN SUBSTRATE PREFERENCE AND KINETIC PROPERTIES

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Cytochrome P450 monooxygenases are involved in insecticide resistance in insects. We previously observed an increase in CYP6P7 and CYP6AA3 mRNA expression in Anopheles minimus mosquitoes during the selection for deltamethrin resistance in the laboratory. CYP6AA3 has been shown to metabolize deltamethrin, while no information is known for CYP6P7. In this study, CYP6P7 was heterologously expressed in the Spodoptera frugiperda (Sf9) insect cells via baculovirus-mediated

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expression system. The expressed CYP6P7 protein was used for exploitation of its enzymatic activity against insecticides after reconstitution with the An. minimus NADPH-cytochrome P450 reductase enzyme in vitro. The ability of CYP6P7 to metabolize pyrethroids and insecticides in the organophosphate and carbamate groups was compared with CYP6AA3. The results revealed that both CYP6P7 and CYP6AA3 proteins could metabolize permethrin, cypermethrin, and deltamethrin pyrethroid insecticides, but showed the absence of activity against bioallethrin (pyrethroid), chlorpyrifos (organophosphate), and propoxur (carbamate). CYP6P7 had limited capacity in metabolizing λ-cyhalothrin (pyrethroid), while CYP6AA3 displayed activity toward λ-cyhalothrin. Kinetic properties suggested that CYP6AA3 had higher efficiency in metabolizing type I than type II pyrethroids, while catalytic efficiency of CYP6P7 toward both types was not significantly different. Their kinetic parameters in insecticide metabolism and preliminary inhibition studies by test compounds in the flavonoid, furanocoumarin, and methylenedioxyphenyl groups elucidated that CYP6P7 had different enzyme properties compared with CYP6AA3. © *2011 Wiley Periodicals, Inc.*

Keywords: cytochrome P450; pyrethroid; CYP6P7; CYP6AA3; kinetic study

INTRODUCTION

Cytochrome P450 monooxygenases (P450s or CYPs) constitute a superfamily of heme-containing monooxygenases that play roles in the metabolisms of endogenous and exogenous compounds, including insecticides (Feyereisen, 1999). In insects, increased expression level of P450s, leading to enhanced detoxification of insecticides, is suggested to play a role in insecticide resistance (Feyereisen, 1999; Scott, 2008). A link between insecticide resistance, high level of monooxygenase activity, increased P450 expression, and ability of P450s to metabolize insecticides has been noted in various insects. For instance, an increase in CYP6D1 mRNA and protein expression level has been observed in the Learn Pyrethroid Resistant (LPR) strain of *Musca domestica*, and CYP6D1 microsomal enzyme has been shown to metabolize pyrethroids at a higher level in LPR strain than the susceptible strain (Wheelock and Scott, 1992; Tomita et al., 1995; Zhang and Scott, 1996). Overexpression of CYP6BQ9 and CYP6P3 in association with pyrethroid resistance has been reported in deltamethrin-resistant strain of *Tribolium castaneum* and permethrin-resistant field *Anopheles gambiae* mosquitoes, respectively, and heterogously expressed enzymes of *CYP6BQ9* and *CYP6P3* demonstrate activities in pyrethroid metabolism (Müller et al., 2008; Zhu et al., 2010).

Anopheles minimus is one of the primary malaria vectors in Thailand. We previously selected a laboratory strain of *An. minimus* species A for deltamethrin resistance (Chareonviriyaphap et al., 2002). We observed elevated enzyme activities of mixed function oxidases in the resistant mosquitoes, suggesting that P450s could act as a primary route of insecticide detoxification (Chareonviriyaphap et al., 2003). Further studies demonstrated that, among the CYP6 P450 cDNA fragments obtained, *CYP6P7*, *CYP6P8*, and *CYP6AA3* genes were overexpressed in deltamethrin-resistant mosquitoes (Rongnoparut et al., 2003; Rodpradit et al., 2005). The increase in CYP6P7 and CYP6AA3 transcripts was correlated with increased resistance to deltamethrin in

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mosquitoes during selection, while there was no such correlation observed for CYP6P8 (Rodpradit et al., 2005). The results suggested that both CYP6P7 and CYP6AA3 might play a role in deltamethrin resistance. However, the fold of increase in CYP6P7 and CYP6AA3 transcripts was different (Rodpradit et al., 2005). Thus, the knowledge of catalytic activities and differences between CYP6P7 and CYP6AA3 in insecticide metabolism may provide a better understanding of insecticide detoxification mechanisms in this mosquito. We previously cloned and expressed the functional CYP6AA3 protein via baculovirus-mediated insect cell expression system and CYP6AA3 showed ability to metabolize deltamethrin in vitro (Kaewpa et al., 2007; Boonsuepsakul et al., 2008). In this study, we expressed CYP6P7 in Sf9 insect cells via baculovirus-mediated expression and investigated CYP6P7 enzymatic activity in insecticide metabolism after reconstituting it with *An. minimus* NADPH-cytochrome P450 reductase (CPR) in a NADPH-regenerating system in vitro. The ability of CYP6P7 in metabolizing pyrethroids and insecticides in the organophosphate and carbamate groups was compared with CYP6AA3. The results revealed that CYP6P7 and CYP6AA3 were capable of metabolizing both types I and II pyrethroids with different substrate preference, but no detectable activity against test organophosphate and carbamate compounds was observed. Kinetic properties and results of preliminary inhibition studies indicated that CYP6P7 was different from CYP6AA3.

MATERIALS AND METHODS

Materials

The chemical compounds, including deltamethrin, permethrin, cypermethrin, λ-cyhalothrin, bioallethrin, chlorpyrifos, propoxur, benzyloxyresorufin, ethoxyresorufin, methoxyresorufin, penthoxyresorufin, 1, 2-didodecanoyl-rac-glycero-3-phosphocholine (DLPC), glucose-6-phosphate, leupeptin, nicotinamide adenosine diphosphate reduced form (NADPH), nicotinamide adenosine diphosphate ($NADP^+$), phenylmethylsulphonyl fluoride (PMSF), dimethyl sulfoxide, α-naphthoflavone, β-naphthoflavone, 5-methoxypsolaren (bergapten), 8-methoxypsoralen (xanthotoxin), piperonyl butoxide (PBO), piperine, and glucose-6-phosphate dehydrogenase (G6PDH) enzyme, were purchased from Sigma-Aldrich (St. Louis, MO). The organic solvents, including acetonitrile (ACN) and ethyl acetate, were of high-performance liquid chromatography (HPLC) grade, obtained from Fisher Scientific (Fair Lawn, NJ). The Sf9 insect cell line and SF-900 II SFM culture media were from Invitrogen (Carlsbad, CA).

Cell Culture and Baculovirus-Mediated Expression of P450s

Expression of CYP6AA3 protein via baculovius-mediated expression in Sf9 cells was prepared following the protocol of Kaewpa et al. (2007). Production of recombinant baculovirus for the expression of CYP6P7 was carried out as previously described (Kaewpa et al., 2007). Briefly, CYP6P7 cDNA isolated from deltamethrin-resistant *An. minimus* (Rodpradit et al., 2005) was subcloned into the transfer vector pBacPAK8 (BD Biosciences, Palo Alto, CA), and co-transfected with linearized BacPAK6 viral DNA into Sf9 cells cultured in SF900 II SFM media at 28°C. For the expression of CYP6P7 protein, Sf9 cells were infected with the CYP6P7 expressed virus (2.5×10^8 plaque-forming units/ml) at multiplicities of infection of 3 (Kaewpa et al., 2007). The infected

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cells were harvested at 70–80 hr after infection and resuspended in sodium phosphate buffer pH 7.2 containing 1 mM EDTA, 0.5 mM PMSF, 5 μg/ml leupeptin, 0.1 mM DTT, and 20% glycerol, and were subjected to microsome preparation using differential centrifugation as described (Boonsuepsakul et al., 2008). The CYP6AA3 or CYP6P7 expressed protein in membrane fraction was observed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and P450 content was measured by reduced-CO difference spectrum analysis according to Omura and Sato (1964).

In Vitro Reconstitution of CYP6P7 and CYP6AA3 Enzymatic Activities Against Insecticides

The in vitro reconstitution assays in the presence of insecticide substrate were performed as previously described with some modifications (Kaewpa et al., 2007). In brief, membrane fraction containing 10 pmol of either CYP6P7 or CYP6AA3 was reconstituted with purified *An. minimus* CPR in the ratio of 3:1 in enzymatic assays (Kaewpa et al., 2007). Insecticides used in the assays included type I pyrethroid (bioallethrin and permethrin), type II pyrethroid (cypermethrin, deltamethrin, and λ-cyhalothrin), carbamate (propoxur), and organophosphate (chlorpyrifos) compounds. The reconstituted P450-reductase system was assayed at 30°C in the presence of DLPC, NADPH-regenerating system (0.6 mM $NADP^+$, 6 mM glucose-6-phosphate, 2 mM $MgCl_2$, 1 U of G6PDH), and 80 μM of insecticide in the total volume of 250 μl. Reaction was initiated by the addition of NADPH-regenerating system. Bioallethrin (100 μM) was used as an internal standard and was added after the reaction was terminated with 30 μl of 2 N HCl at different time points. When testing enzyme activity with bioallethrin as a substrate, deltamethrin was used as an internal standard. The remaining substrate was extracted with 750 μl ethyl acetate, and the extract was dried under a stream of N_2 gas, dissolved with ACN before subjected to HPLC analysis using C18-reverse phase column (Nova-Pak® C18 4 μM 3.9 × 150 mm; Waters, Milford, MA). HPLC gradient was initiated at 50% ACN in water (v/v) for 5 min, a linear gradient from 50 to 100% ACN over 3 min, 100% ACN was held for 8 min, followed by a linear gradient returning to 50% ACN over 3 min, and final equilibration with 50% ACN for 6 min. Unmetabolized insecticide and internal standard peaks were monitored by UV detection at 220 nm. The substrate peak area in each reaction was compared with that of time zero. The enzyme activity was determined as substrate disappearance/min/pmol P450. Each of reconstitution experiments was performed in three independent repetitions and internal standard was used for normalization among reactions. Control reactions were performed including reactions without NADPH, in the presence of PBO (P450 inhibitor), and using parental Sf9 membrane.

In Vitro Reconstitution of CYP6P7 and CYP6AA3 Enzymatic Activities With Fluorescent Substrates

The P450-mediated *O*-dealkylation reaction of each of resorufin derivatives (10 μM final concentration) was performed in 50 mM Tris–HCl buffer pH 7.5, in a total volume of 500 μl. Membrane fractions containing either CYP6P7 or CYP6AA3 (~25 pmol each) were used and enzymatic activities were assayed after reconstituted with purified CPR in the ratio of 3:1. Fluorescent substrates dissolved in dimethyl sulfoxide (1% final concentration) were benzyloxyresorufin, ethoxyresorufin, methoxyresorufin, and penthoxyresorufin. Each reaction was initiated by the addition of NADPH to a final concentration of 1 mM. Resorufin product was measured at

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λ_{ex} = 530 and λ_{em} = 590 nm using RF-5301 PC spectrofluorophotometer (Shimadzu, Kyoto, Japan). The amount of resorufin product was calculated referring to the resorufin standard curve. Rate of resorufin formation was expressed as pmol resorufin/min/pmol P450.

Steady-State Kinetic Parameter Analysis

To obtain the steady-state kinetic parameters (K_m and V_{max}), reaction rates were measured under linear conditions using different substrate concentrations ranging from 0 to 320 μM for permethrin, 0–640 μM for deltamethrin, λ-cyhalothrin, and cypermethrin, and 0–16 μM for benzyloxyresorufin. The kinetic parameters K_m and V_{max} were determined from the plot of substrate concentrations vs. initial velocity (V_0) by fitting experimental data to the Michaelis–Menten equation, using nonlinear regression analysis of GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). The catalytic efficiency, defined as V_{max}/K_m, was calculated from the estimated K_m and V_{max} values.

Fluorescence-Based CYP6P7 and CYP6AA3 Inhibition Assay

In this study, we used benzyloxyresorufin as a substrate for preliminary studies of CYP6P7 and CYP6AA3 inhibition assays. Reaction conditions were performed as described for fluorescence reconstitution assays. Inhibitors tested were α-naphthoflavone, β-naphthoflavone, xanthotoxin, bergapten, PBO, and piperine. Assays were carried out by incubating benzyloxyresorufin substrate at the concentration approximately K_m value (0.5 μM for CYP6P7 and 2 μM for CYP6AA3) in the presence of different concentrations of individual test inhibitors dissolved in dimethyl sulfoxide (1% final concentration). Inhibitory effect of each compound at each concentration was calculated as percent relative inhibition compared with the vehicle control reaction.

Time- and NADPH-dependent inhibition activity of test compounds against benzyloxyresorufin-*O*-debenzylation (BROD) activities of CYP6P7 and CYP6AA3 was preliminarily investigated as a primary indication of mechanism-based inhibition. Assays were performed by pre-incubating enzyme with various concentrations of test compounds in the presence or absence of NADPH for 30 min before the addition of benzyloxyresorufin. The IC_{50} values were determined and compared between reactions pre-incubating with and without NADPH. An indication of mechanism-based inhibition is the IC_{50} shift to lower range in the assay pre-incubating with NADPH (Fowler and Zhang, 2008). Type of inhibition and apparent inhibition constant K_i were determined if the test inhibitor gave reversible inhibition pattern. Various concentrations of inhibitors and substrates were used for the generation of Lineweaver–Burk plots using GraphPad Prism 5. Type of inhibition was determined graphically from Lineweaver–Burk plots. The K_i values were obtained via secondary plots of the slopes from Lineweaver–Burk plots.

RESULTS

Characterization of CYP6P7 and CYP6AA3 Activities in Insecticide Metabolism

The *An. minimus* CYP6P7 protein was successfully expressed in Sf9 insect cells via baculovirus-directed expression system. Total P450 content calculated from the characteristic spectrum was approximately 200–300 pmol per milligram membrane protein, and membrane preparation of the functional CYP6P7 was used for the

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reconstitution of enzymatic assays as previously described (Boonsuepsakul et al., 2008). In this study, we examined CYP6P7 enzymatic activity against insecticides commonly used in mosquito vector control programs and agricultural practices in Thailand (Chareonviriyaphap et al., 1999; Thapinta and Hudak, 2000; Vector Borne Disease Annual Report, 2002–2003). These included type I and type II pyrethroids, (bioallethrin, permethrin, λ-cyhalothrin, cypermethrin, and deltamethrin), organophosphate (chlorpyrifos), and carbamate (propoxur) insecticides. As shown in Figure 1, CYP6P7 could metabolize both type I and type II pyrethroids, namely permethrin, cypermethrin, and deltamethrin, while there was no detectable enzyme activity against bioallethrin and λ-cyhalothrin using methods described in this study. In parallel, there was an absence of CYP6P7 activity toward chlorpyrifos and propoxur. The absence of CYP6P7 activity in the reactions without NADPH and in the presence of PBO (P450 inhibitor) indicated that the detected enzyme activities in insecticide metabolism were due to the cytochrome P450 CYP6P7 protein (unreported data). When tested these insecticide compounds with CYP6AA3, the results demonstrated that CYP6AA3 could metabolize all test pyrethroids except bioallethrin. Similar to CYP6P7, there was absence of CYP6AA3 activity in chlorpyrifos and propoxur degradation. The cypermethrin consumption rate of CYP6P7 was higher than CYP6AA3, whereas its permethrin consumption rate was lower than CYP6AA3 (Fig. 1).

Kinetic Analysis of CYP6P7 and CYP6AA3 Enzymes

The steady-state kinetics of CYP6P7 in metabolizing pyrethroids were investigated at various substrate concentrations and compared with CYP6AA3. The apparent K_m, V_{max} and catalytic efficiency (V_{max}/K_m) were determined from the best nonlinear fit of data to the Michaelis–Menten equation ($r^2 > 0.9$). As shown in Table 1, the catalytic efficiency (V_{max}/K_m) values of CYP6P7 for permethrin, cypermethrin, and deltamethrin were not significantly different. On the other hand, CYP6AA3 could metabolize type I pyrethroid permethrin with significantly higher efficiency than all type II pyrethroids. The better catalytic efficiency of CYP6AA3 toward permethrin was contributed by both its lower K_m and higher V_{max} values than toward type II pyrethroids.

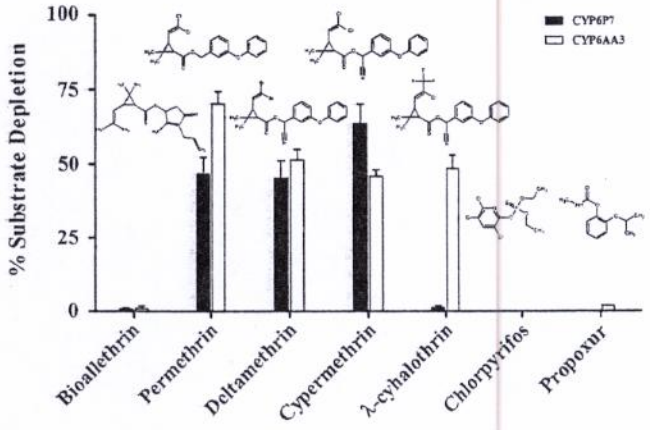

Figure 1. The CYP6P7- and CYP6AA3-mediated insecticide metabolism. The reconstitution assays were performed using 80 μM final concentration of each insecticide substrate. Insecticide metabolisms were measured and expressed as percent substrate depletion over 30 min of incubation. Values are mean ± SD of three replicated experiments.

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Table 1. Kinetic Constants for CYP6P7- and CYP6AA3-Mediated Pyrethroid Metabolism

	CYP6P7[a]			*CYP6AA3*[a]		
Compounds	K_m (μM)	V_{max}[b]	V_{max}/K_m	K_m (μM)	V_{max}[b]	V_{max}/K_m
Type I pyrethroids						
Permethrin	69.7±10.5	65.7±1.6	0.96±0.13	41.0±8.5	124.2±1.2	3.03±0.34[c]
Type II pyrethroids						
Cypermethrin	97.3±6.4	83.3±7.6	0.86±0.05	70.0±7.1	40.0±7.1	0.57±0.04
Deltamethrin	73.3±2.9	55.3±5.7	0.75±0.05	80.2±2.0	60.2±3.6	0.75±0.03
λ-Cyhalothrin	N.D.[d]	N.D.[d]	N.D.[d]	78.3±7.0	60.7±1.1	0.78±0.06

[a]Values are mean±SD of three replicated experiments.
[b]V_{max} was measured as pmol substrate disappearance/min/pmol P450.
[c]Significant differences between V_{max}/K_m values of CYP6AA3 in pyrethroid metabolisms, as determined by one-way ANOVA Tukey's multiple comparison test ($P<0.05$). All V_{max}/K_m values of CYP6P7 in metabolizing pyrethroids were not significantly different.
[d]N.D., not determined.

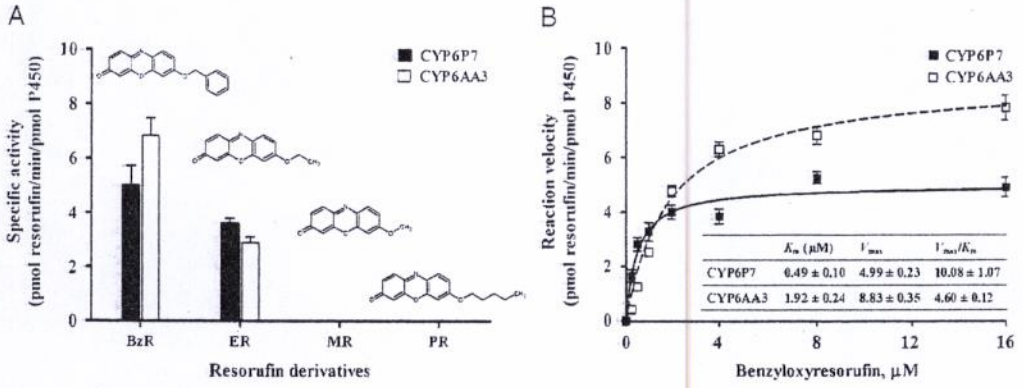

Figure 2. Specific activities of CYP6P7 and CYP6AA3 toward resorufin derivatives (**A**) and Michaelis–Menten plot of CYP6P7- and CYP6AA3-BROD activities (**B**). The resorufin derivatives are benzyloxyresorufin (BzR), ethoxyresorufin (ER), methoxyresorufin (MR), and penthoxyresorufin (PR). Data are mean±SD of triplicates.

Metabolism of the Fluorescent Substrates

To characterize for potential fluorogenic substrate probe and for use in inhibition studies of CYP6P7 and CYP6AA3, four resorufin fluorogenic substrates containing different alkyl groups including benzyloxyresorufin, ethoxyresorufin, methoxyresorufin, and penthoxyresorufin were screened. Both CYP6P7 and CYP6AA3 could metabolize benzyloxyresorufin and ethoxyresorufin, with higher specific activities toward benzyloxyresorufin than ethoxyresorufin, while there was absence of activities against methoxyresorufin and penthoxyresorufin (Fig. 2A). Thus, benzyloxyresorufin was used as a substrate in inhibition studies. From kinetic studies, CYP6P7 could metabolize benzyloxyresorufin with approximately twofold higher efficiency than CYP6AA3, contributed by low binding constant to benzyloxyresorufin (Fig. 2B).

Analysis of CYP6P7 and CYP6AA3 Enzyme Inhibition

To further characterize CYP6P7 and CYP6AA3 enzymes, we used known P450 inhibitors with different structures to compare their inhibition profile against these two

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Table 2. IC_{50} Values for Inhibition of CYP6P7- or CYP6AA3-Mediated BROD Activities

	IC_{50} (μM)[a]			
	CYP6P7, pre-incubation		*CYP6AA3, pre-incubation*	
Inhibitor	*(−) NADPH*	*(+) NADPH*	*(−) NADPH*	*(+) NADPH*
Flavonoid				
α-Naphthoflavone	2.90 ± 0.27	3.03 ± 0.45	0.37 ± 0.06	0.38 ± 0.06
β-Naphthoflavone	17.25 ± 3.67	33.35 ± 9.90	19.22 ± 3.13[b]	34.44 ± 5.95[b]
Furanocoumarin				
Bergapten	52.76 ± 6.77[b]	114.00 ± 11.81[b]	93.77 ± 10.87[b]	170.30 ± 16.88[b]
Xanthotoxin	33.77 ± 3.54[b]	78.93 ± 10.04[b]	51.04 ± 2.15	52.17 ± 2.86
MDP compound				
PBO	31.77 ± 3.21[b]	16.22 ± 1.81[b]	9.91 ± 0.81[b]	4.04 ± 0.31[b]
Piperine	52.86 ± 6.92[b]	3.48 ± 0.36[b]	15.26 ± 1.21[b]	4.86 ± 0.79[b]

[a]Values are mean ± SD of triplicate experiments.
[b]Significant differences between (−) NADPH and (+) NADPH, $P < 0.05$, Student's *t*-test.

P450s. The compounds included flavonoids (α- and β-naphthoflavone), furanocoumarins (bergapten and xanthotoxin), methylenedioxyphenyl, or MDP-containing compounds (PBO and piperine). To initially examine whether test compounds could inhibit both P450s in a mechanism-based inhibition pattern, the experiments were carried out by pre-incubating each test compound with the enzyme mixture in the presence or absence of NADPH for 30 min before the addition of benzyloxyresorufin substrate. Both PBO and piperine showed time and NADPH-dependent inhibition activities against both enzymes, indicating a typical mechanism-based inhibition characteristic (Table 2). CYP6P7 was less efficiently inhibited by PBO than CYP6AA3.

Among all test inhibitors, α-naphthoflavone had the most potent inhibitory activity against both enzymes, and it inhibited CYP6P7 with IC_{50} and K_i values higher than CYP6AA3 (see Table 2, Fig. 3). The IC_{50} values of α-naphthoflavone against both enzymes were similar under pre-incubations with and without NADPH. The CYP6P7 activity was inhibited by bergapten and xanthotoxin more efficiently when pre-incubated without NADPH than in the presence of NADPH. This was observed by the significantly higher IC_{50} value shift on pre-incubations of bergapten and xanthotoxin with NADPH. For CYP6AA3, the significantly higher IC_{50} value shift was observed for β-naphthoflavone and bergapten, but not for xanthotoxin.

Differences in higher IC_{50} value shift of xanthotoxin observed between CYP6P7 and CYP6AA3 prompted us to further investigate type of enzyme inhibition by xanthotoxin compared with bergapten and, in addition, to α-naphthoflavone which is the most potent inhibitor. Study of type of inhibition was performed and Lineweaver–Burk plots are shown in Figure 3. The K_i values shown in Figure 3 were comparable to IC_{50} values in Table 2. The results elucidated that α-naphthoflavone could uncompetitively inhibit both enzymes, while bergapten inactivated both enzymes in a mixed-type inhibition pattern. The difference in type of inhibition was noted for xanthotoxin as it uncompetitively inhibited CYP6AA3 enzyme, while inhibition against CYP6P7 was mixed type.

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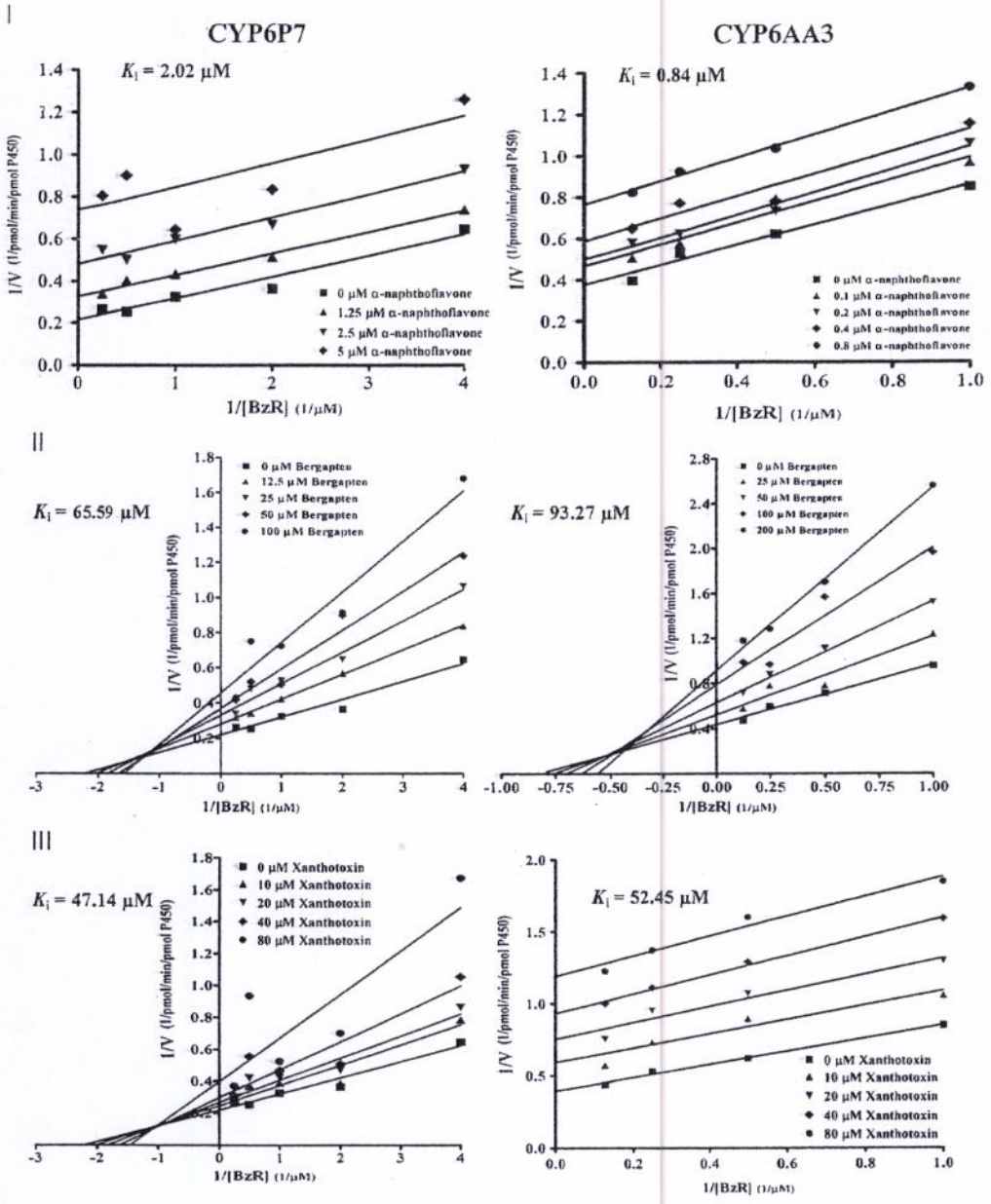

Figure 3. Lineweaver–Burk plots for the inhibition of CYP6P7- or CYP6AA3-BROD activities by α-naphthoflavone (I) bergapten (II), and xanthotoxin (III). Data are means of triplicate experiments.

DISCUSSION

In this study, the role of CYP6P7 and CYP6AA3 in the metabolisms of type I and type II pyrethroids, organophosphate, and carbamate was investigated. The reconstitution enzymatic assays measuring substrate depletion revealed that both CYP6P7 and CYP6AA3 could metabolize pyrethroids but were incapable of degrading test organophosphate and carbamate compounds, suggesting that both enzymes could

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play a role in detoxification of pyrethroids in mosquitoes in vivo. The ability of both enzymes in metabolizing permethrin, cypermethrin, and deltamethrin may partly rely on the presence of phenoxybenzyl group in the alcohol moiety of substrate (see Fig. 1). This is accentuated by that CYP6P7 and CYP6AA3 lacked enzymatic activities toward bioallethrin and more structurally distant chlorpyrifos and propoxur insecticides, determined by methods used in this study. A clear difference existed between CYP6P7 and CYP6AA3 activities as demonstrated by the absence of CYP6P7 activity in degradation of λ-cyhalothrin, while CYP6AA3 could metabolize λ-cyhalothrin with similar kinetic values as for deltamethrin. The presence of CF_3- at the dihalovinyl groups in the acid moiety of λ-cyhalothrin (see Fig. 1) could be responsible for the absence of CYP6P7 enzyme activity.

CYP6P7 could metabolize pyrethroids with values of catalytic efficiency not significantly different between the two pyrethroid types. In contrast, CYP6AA3 was more active toward type I than type II pyrethroids, similar to human CYP2C19 and rat CYP2C6, in having higher rate of eliminating permethrin than λ-cyhalothrin and cypermethrin (Scollon et al., 2009). Moreover, CYP6P7 and CYP6AA3 metabolized deltamethrin with similar efficiency (an equal value of 0.75), but were 5- to 10-fold less efficient than human CYP2C8 and CYP2C19 as well as rat CYP2C6 and CYP2C11 (values range from 4.2 to 6.9, Godin et al., 2007).

Both CYP6P7 and CYP6AA3 enzymes possess K_m values either higher or in the same range as other insect P450s, but they have better capacity in metabolizing pyrethroids than other insect P450s, attributed by their higher rate of pyrethroid degradation. For instance, *A. gambiae* CYP6P3 has higher affinity for deltamethrin ($K_m = 5.9\,\mu M$) but its low V_{max} value ($1.8\,min^{-1}$; Müller et al., 2008) makes CYP6P3 approximately two to threefolds less efficient than *An. minimus* enzymes. In *Helicoverpa zea*, CYP6B8, co-expressed with house fly CPR in baculovirus expression system, has K_m values in the same range as CYP6P7 and CYP6AA3, but its efficiency in degrading cypermethrin is approximately three to sixfold lower than those reported herein (Li et al., 2004). Higher P450 turnover rate could be due to efficient catalytic site of P450s or due to high electron transfer rate through CPR redox partner. For example, CYP6AB3v2 can metabolize imperatorin substrate at a rate of about threefold faster than the CYP6AB3v1 variant form, due to higher NADPH consumption rate (Mao et al., 2007).

The ability of CYP6P7 and CYP6AA3 in metabolizing pyrethroids lends support to their role in deltamethrin detoxification in *An. minimus* (Rodpradit et al., 2005). Consistently, CYP6P8 protein, expressed via baculovirus-mediated expression system, did not show detectable activity against pyrethroids with the methods used (unreported data). Thus, sequence similarity may not contribute to functions of P450 enzymes, since CYP6P7 has higher amino acid identity to CYP6P8 (61% identity) than to CYP6AA3 (40% identity) but the metabolic capacity toward insecticides of CYP6P7 is more related to CYP6AA3 than CYP6P8. An example is shown in *H. zea* that CYP6B8 and CYP321A1 proteins share only 32% primary sequence identity, but both can metabolize cypermethrin (Li et al., 2004; Sasabe et al., 2004; Rupasinghe et al., 2007). It could imply that three-dimensional structures of active sites of insect P450s may play a role in their substrate selectivity rather than sequence similarity among enzymes.

The fluorescence alkyloxyresorufins have been reported as substrates for several P450s (Scott et al., 2000; Mclaughlin et al., 2008). In this study, *An. minimus* CYP6P7 and CYP6AA3 could metabolize benzyloxyresorufin that contains planar aromatic benzene ring substituted group more efficiently than ethoxyresorufin that contains

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substituted hydrocarbon chain. CYP6P7 has higher affinity to benzyloxyresorufin than CYP6AA3, emphasizing differences in metabolic capability between these two enzymes. Using benzyloxyresorufin as substrate, we characterized the inhibition of CYP6P7- and CYP6AA3-BROD activities using known P450 inhibitors of different structures. The MDP-containing compounds (PBO and piperine) have been shown inhibiting human, rat, and insect P450s via mechanism-based inhibition (Scott et al., 2000; Bhardwaj et al., 2002; Correia and Ortiz de Montellano, 2005; Subehan et al., 2006). In this study, PBO and piperine also displayed mechanism-based inhibition on CYP6P7 and CYP6AA3. However, CYP6P7 was three to fourfold less susceptible to mechanism-based inhibition by PBO than CYP6AA3. Similarly, α-naphthoflavone was eight times less potent against CYP6P7 than CYP6AA3. In other insect P450s, α-naphthoflavone exhibited strong inhibitory effect against *An. gambiae* CYP6Z2-BROD activity with the IC_{50} value of 0.007 μM (Mclaughlin et al., 2008) and against housefly CYP6D1- methoxyresorufin-*O*-demethylation activities (MROD) activity with the IC_{50} value of 0.28 μM (Scott et al., 2000), a value approximately similar to CYP6AA3. In β-naphthoflavone, the position of the naphthyl group could account for approximately five to sevenfold lower inhibitory effect against CYP6P7 and CYP6AA3 than α-naphthoflavone.

It appears that both bergapten and xanthotoxin exhibited low inhibitory effect against both P450s. Moreover, pre-incubation with bergapten and xanthotoxin in the presence of NADPH decreased their inhibition effect on CYP6P7 activity, but only bergapten decreased the inhibition effect on CYP6AA3. Similar reduction of inhibition was observed for phenacetin and diclofenac, for example, against CYP1A2 and CYP2C9 activities, respectively (Yamamoto et al., 2002). Since phenacetin and diclofenac are also substrates of CYP1A2 and CYP2C9, respectively, thus it explains the reduced inhibitory effect of these compounds against P450 activities (Yamamoto et al., 2002). Further study of CYP6P7 and CYP6AA3 activities with bergapten in the absence of benzyloxyresorufin substrate to determine whether it could behave as substrate could help explaining the results of this study.

We further analyzed the type of inhibition and compared between CYP6P7 and CYP6AA3. It appears that xanthotoxin uncompetitively inhibited CYP6AA3, implicating that xanthotoxin could reversibly bind the CYP6AA3–benzyloxyresorufin complex. In contrast, xanthotoxin inhibited CYP6P7 in a mixed-type inhibition pattern, suggesting that it could competitively interact with free CYP6P7 at or near benzyloxyresorufin binding site and noncompetitively bind to the allosteric site (Segal, 1975; Correia and Ortiz de Montellano, 2005). Such inhibition results emphasize differences of CYP6P7 and CYP6AA3 properties.

The results obtained in this study address that CYP6P7 and CYP6AA3 may partly share structural properties that influence overlapping but different substrate specificity and catalytic efficiency. Moreover inhibition studies revealed that both CYP6P7 and CYP6AA3 were differently inhibited by test inhibitors. Taken together, it could imply that different properties of CYP6P7 and CYP6AA3 may be advantageous to *An. minimus* by their working together in detoxifying insecticides. Nevertheless, the results of this study do not exclude the possibility that other P450s in this mosquito could play role in insecticide detoxification. However, how CYP6P7 and CYP6AA3 have a redundancy of metabolizing overlapping set of pyrethroids and how they contribute to actual detoxification in *An. minimus* in vivo are not known. Finally, the results obtained from this study could contribute to better control of this mosquito vector.

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