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Protective efficacy of *Anopheles minimus* CYP6P7 and CYP6AA3 against cytotoxicity of pyrethroid insecticides in *Spodoptera frugiperda* (Sf9) insect cells

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Abstract. Cytochrome P450 monooxygenases (P450s) are enzymes known to metabolize a wide variety of compounds including insecticides. Their overexpression leading to enhanced insecticide detoxification could result in insecticide resistance in insects. The increased mRNA expression of two P450 genes, *CYP6P7* and *CYP6AA3*, has been previously observed in laboratory-selected deltamethrin-resistant *Anopheles minimus*, a major malaria vector in Southeast Asia, suggesting their role in detoxification of pyrethroids. In this study CYP6P7 and CYP6AA3 were expressed in insect *Spodoptera frugiperda* (Sf9) cells via baculovirus-directed expression system. Insecticide detoxification capabilities of Sf9 cells with and without expression of CYP6P7 or CYP6AA3 were evaluated using 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. The results revealed that CYP6P7- or CYP6AA3-expressing cells showed significantly higher cytoprotective capability than parental Sf9 cells against cytotoxicity of pyrethroids including permethrin, cypermethrin and deltamethrin. Such cytoprotective effect was not observed for bioallethrin (pyrethroid), chlorpyrifos (organophosphate) and propoxur (carbamate). Moreover, expression of CYP6AA3, but not CYP6P7, could protect cells against λ-cyhalothrin cytotoxicity. In MTT assays upon co-incubation with piperonyl butoxide (P450 inhibitor), cytoprotective ability of CYP6P7 and CYP6AA3 against deltamethrin was diminished, implying that pyrethroid detoxification was due to activities of P450 enzymes. Insecticide detoxification capabilities of CYP6P7 and CYP6AA3 observed from MTT assays were correlated to their pyrethroid metabolizing activities observed from *in vitro* reconstitution enzymatic assays. Thus MTT assays using cells expressing P450 enzymes of interest could be primarily used to determine detoxification activities of enzymes against cytotoxic insecticides.

INTRODUCTION

Control of mosquito vectors relying on applications with chemical insecticides is an important strategy in preventing transmission of vector-borne diseases, including malaria. Pyrethroid insecticides are currently used worldwide in agriculture and for vector control, however long-term extensive use of insecticides has been a major cause of development of insecticide resistance among mosquito populations (Coleman & Hemingway, 2007). Consequently, resistance can lead to reduced efficacy of vector control programme. Pyrethroid resistance in mosquito vectors of malaria has been reported in several countries in Africa and Southeast Asia, including Thailand (Charoenviriyaphap *et al.*, 1999; Bortel *et al.*, 2008; Munhenga *et al.*, 2008; Cuamba *et al.*, 2010; Matowo *et al.*, 2010).

Cytochrome P450 monooxygenases (P450s) comprise a superfamily of enzymes that catalyze metabolisms of endogenous and xenobiotic compounds, including

insecticides (Feyereisen, 1999). Examinations of various insects have suggested involvement of P450s in resistance to different insecticides, by virtue of enhanced expression of P450s and thus increased detoxification of insecticides. (Feyereisen, 1999). Overexpression of P450s has been observed in several pyrethroid resistant insects such as increased expression of *CYP6D1* has been found in pyrethroid resistant *Musca domestica* (Tomita *et al.*, 1995), *CYP6P3* and *CYP6M2* in multiple pyrethroid resistant *Anopheles gambiae* (Djouaka *et al.*, 2008).

Anopheles minimus is one of malaria vectors in Southeast Asia, including Thailand, Loas, Cambodia, and Vietnam. We previously determined that P450 enzymes might act as a primary route of deltamethrin detoxification during laboratory selection for deltamethrin resistance in *An. minimus* (Chareonviriyaphap *et al.*, 2003). In parallel during selection, increased mRNA expression of *CYP6P7* and *CYP6AA3* was observed (Rongnoparut *et al.*, 2003; Rodpradit *et al.*, 2005). Thus CYP6P7 and CYP6AA3 could be implicated to play a role in detoxification of deltamethrin in this mosquito.

Pyrethroids are known to commonly target on sodium channels of nervous system. Other toxic effects of pyrethroids toward cells include causing DNA damage, inhibition of mitochondrial complex I, and induction of reactive oxygen species (ROS) accumulation (Gassner *et al.*, 1997; Villarini *et al.*, 1998; Naravaneni *et al.*, 2005; Patel *et al.*, 2007). Cytotoxic effects of organophosphate and carbamate insecticides have also been reported such as oxidative stress, alteration of mitochondria function (Swann *et al.*, 1996; Schmuck & Mihail, 2004; Maran *et al.*, 2010). Thus treatment of cells with cytotoxic insecticides in cytotoxicity assays can cause cell mortality unless cells possess ability to detoxify insecticides. This is supported by that *Spodoptera frugiperda* (Sf9) insect cells expressing CYP6AA3 have significantly higher cell viability upon treatment with deltamethrin than cells without CYP6AA3 expression in MTT cytotoxicity assay, suggesting that CYP6AA3 could detoxify deltamethrin in Sf9 cells (Boonsuepsakul *et al.*, 2008).

In this study, we further employed MTT cytotoxicity assays to investigate detoxification capability of Sf9 cells expressing CYP6P7 or CYP6AA3 against pyrethroid, organophosphate and carbamate insecticides that are commonly used in Thailand. Enzymatic assays of CYP6P7 and CYP6AA3 against insecticides were performed and compared to results obtained from MTT assays.

MATERIALS AND METHODS

Chemicals

The chemical compounds including deltamethrin, permethrin, cypermethrin, λ-cyhalothrin, bioallethrin, chlorpyrifos, propoxur, leupeptin, phenylmethylsulphonyl fluoride (PMSF), dimethyl sulfoxide (DMSO), and piperonyl butoxide (PBO) were purchased from Sigma-Aldrich (St. Louis, MO). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from USB (Cleveland, OH).

Cell culture and baculovirus-mediated insect cell expression of P450 proteins

The Sf9 insect cell line and SF-900 II SFM culture media were purchased from Invitrogen (Carlsbad, CA), and Sf9 cells were cultured in SF900 II serum-free media at 28°C. The recombinant baculovirus, either containing CYP6AA3 or CYP6P7, was produced following manufacturer's instruction as previously described (Kaewpa *et al.*, 2007). Briefly, CYP6P7 or CYP6AA3 cDNA isolated from deltamethrin resistant *An. minimus* (Rongnoparut *et al.*, 2003; Rodpradit *et al.*, 2005) was subcloned into the transfer vector pBacPAK8 (BD Biosciences, Palo Alto, CA), co-transfected with a linearized BacPAK6 viral DNA into Sf9 cells. The resulting recombinant virus, upon purification, was used in the expression of enzymes. For cytotoxicity assays, Sf9 cells were infected with CYP6P7 or CYP6AA3 recombinant virus at multiplicity of infection (moi) equal to 1. After 72 hours post-infection,

cells expressing CYP6P7 or CYP6AA3 were harvested and seeded onto 24-well plate for cytotoxicity assays. These conditions followed the methods described by Kaewpa *et al.* (2007) and Boonsuepsakul *et al.* (2008). Expression of P450 proteins was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Expression of P450s for *in vitro* enzymatic activity assay was performed as described by Boonsuepsakul *et al.* (2008). In brief, Sf9 cells were infected with either CYP6P7 or CYP6AA3-expressed virus at moi equal to 3. The infected cells were harvested at 70-80 hours post-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. The harvested cells were lysed and subjected to differential centrifugation, resulting in microsomal pellet which was solubilized in sodium phosphate buffer pH 7.2 containing 150 mM KCl and 1% (v/v) Triton X-100. The microsomes of Sf9 cells infected with either CYP6P7 or CYP6AA3 recombinant virus were used in enzymatic reconstitution assays as described in Kaewpa *et al.* (2007). The expressed proteins in microsomal fractions were observed by SDS-PAGE and total P450 content was measured by reduced-CO difference spectrum analysis according to Omura & Sato (1964).

Cell treatment and MTT cytotoxicity assays

Cytotoxicity effect of insecticides evaluated by MTT assays was performed as previously described (Boonsuepsakul *et al.*, 2008). Insecticides used in this study included bioallethrin, cypermethrin, chlorpyrifos, deltamethrin, permethrin, propoxur and λ-cyhalothrin. We previously used cells infected with baculovirus containing BacPak6 viral DNA as control in parallel with uninfected parental Sf9 cells, and both control cells showed same response to deltamethrin (Boonsuepsakul *et al.*, 2008). In this study we thus used only uninfected Sf9 cells as control in MTT assays, and cells treated with DMSO solvent alone was a negative control. CYP6P7- or CYP6AA3-expressing cells and control Sf9 cells were seeded at 2×10^5 cells per well in 24-well culture plates. Cells were allowed to attach to wells for 3 hours, insecticides ranging from 0.1 to 500 µM final concentrations were subsequently added into each well and cells were left exposing to each insecticide for 72 hr. When insecticides were used at higher concentration than 500 µM, they were precipitated in culture media, particularly for pyrethroids. Thus concentrations of pyrethroids over 500 µM were not evaluated in MTT assays. Following insecticide treatment, culture media were removed and each well was washed twice with Luckhoff's buffer (132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 20 mM HEPES). Cell viability was determined using MTT assay. This was accomplished by addition of 200 µl MTT solution (5 mg/ml in Luckhoff's buffer) and further subjected to 4-hour incubation at 28°C. Subsequently, MTT solution was removed and DMSO was added to dissolve the purple formazan precipitate formed by activity of mitochondrial enzymes in live cells (Mosmann, 1983). The absorbance of formazan product was measured at 540 nm using Multiskan EX microtiter plate reader (Thermo Labsystems, Finland). Cell viability upon incubation with each insecticide was expressed as percentage of viable cells relative to cells treated with DMSO solvent alone which was assigned as 100% viability. The value of 50% lethal concentration (LC_{50}) was evaluated from the plot of percentage of cell viability against different concentrations of each insecticide. Higher LC_{50} value represents higher ability to survive toxicity of insecticides.

In enzyme inhibition study, cells were co-incubated with 100 µM deltamethrin and PBO (known P450 inhibitor) at concentrations of 0.1, 1 and 10 µM (in each case the final concentration of DMSO in media was 1%). This range of PBO concentrations was less than LD_{50} value (25 µM) of PBO against Sf9 cells pre-determined by MTT assays (data not shown). Inhibition of P450s by PBO was measured as cell survival against deltamethrin cytotoxicity in the presence of PBO, after normalization with that of cells treated with PBO only. Each MTT assay measuring cytotoxic effect of insecticides and

measuring inhibitory effect by PBO was done in triplicate.

In vitro insecticide metabolism by CYP6P7 and CYP6AA3

The *in vitro* reconstitution assays in the presence of insecticide substrate were performed as previously described (Kaewpa *et al.*, 2007). Briefly reconstitution reactions were carried out at 30°C in 0.1 M sodium phosphate buffer pH 7.2 in the presence of 10 pmol of either CYP6P7 or CYP6AA3 and was reconstituted with purified *An. minimus* NADPH-dependent cytochrome P450 reductase (P450 redox partner) in the ratio of 3:1, 80 µM of test insecticide, and NADPH-regenerating system. Internal standard (bioallethrin) and remaining of insecticide at each reaction time were analyzed by HPLC analysis and peaks were monitored by UV detection at 220 nm. The substrate peak area at each time point was calculated as percentage of remaining residual substrate compared to that of time zero which was assigned to be 100%. When bioallethrin was a substrate, deltamethrin was used as internal standard. To determine metabolisms mediated by CYP6P7 and CYP6AA3, amount of each insecticide consumed at each incubation time was calculated as percentage of depletion of each insecticide compared to the initial amount at time zero. Each of reconstitution experiments was performed in triplicate and internal standard was used for normalization. Control reactions were performed by incubating reactions without NADPH-regenerating system, and reaction using microsome of uninfected Sf9 cells.

Statistical analysis

Data were statistically analyzed by GraphPad Prism version 5 (GraphPad Software Inc., San Diego, CA) using ANOVA with Tukey's Multiple comparison test. Results with $p < 0.05$ were considered to be significantly different.

RESULTS

In this study, cytotoxicity of insecticides was examined with Sf9 insect cells, with and without expression of CYP6P7 or CYP6AA3, using MTT cytotoxicity assays. CYP6P7 and CYP6AA3 were expressed via baculovirus-directed expression system. The results revealed higher cell viability against cytotoxic effects of permethrin, cypermethrin and deltamethrin in CYP6P7- or CYP6AA3-expressing cells than parental Sf9 cells for all given insecticide concentrations (Figure 1B-D). This is demonstrated by 5- to 13-fold higher LC_{50} values observed in CYP6P7- and CYP6AA3-expressing cells than control Sf9 parental cells. It could be noted that response to λ-cyhalothrin cytotoxicity was different between cells expressing CYP6P7 and CYP6AA3, as demonstrated by 3.7-fold higher LC_{50} value observed in CYP6AA3-expressing cells than in CYP6P7-expressing cells and parental Sf9 cells (Figure 1E). Upon treatment with bioallethrin, chlorpyrifos, and propoxur, percentage of cell viability of CYP6P7- and CYP6AA3-expressing cells and LC_{50} values were similar to control Sf9 cells (Figures 1A, F, and G).

In order to determine whether increased cell survival against pyrethroid cytotoxicity found in cells expressing CYP6P7 or CYP6AA3 was due to activity of P450s, we assayed deltamethrin cytotoxicity in the presence of different concentrations of PBO, P450 inhibitor. Decrease of cell viability against deltamethrin cytotoxicity was observed when cells expressing CYP6P7 and CYP6AA3 were treated with increasing concentrations of PBO (Figure 2). Since PBO also possessed cytotoxic effect against Sf9 cells (see Materials and Methods section), thus percent cell viability of P450-expressing cells shown in Figure 2 was obtained after normalization with that of cells treated with PBO only. Percentage of cell survival against deltamethrin of CYP6P7- or CYP6AA3-expressing cells in the presence of 10 µM PBO was significantly decreased, compared to those without PBO ($p<0.05$). In Sf9 control cells, after normalization, percentage of cells surviving deltamethrin cytotoxicity remained similar throughout treatment with different PBO concentrations (Figure 2).

Enzymatic activities of CYP6P7 and CYP6AA3 against pyrethroid, organo-phosphate, and carbamate insecticides were

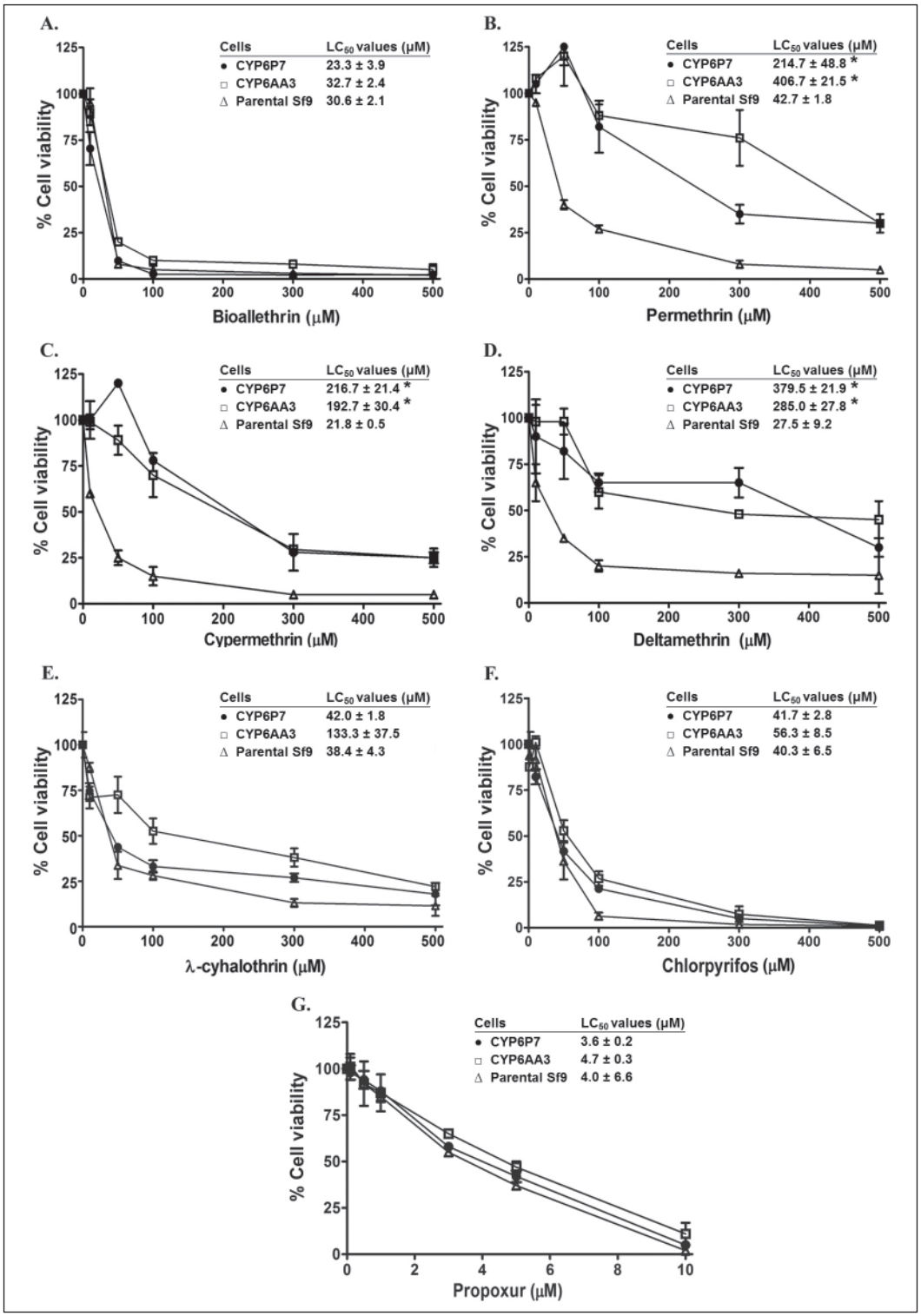

Figure 1. Viability of CYP6P7-expressing Sf9 cells (●), CYP6AA3-expressing Sf9 cells (□) and parental Sf9 cells (△) upon incubation with various insecticides. Percentage of cell viability was measured using MTT cytotoxicity assays as described in Materials and Methods. Data are means ± S.D. of three replicated experiments. Asterisk (*) represents the value significantly different from control cells ($p < 0.05$, one-way ANOVA, Tukey's Multiple Comparison Test)

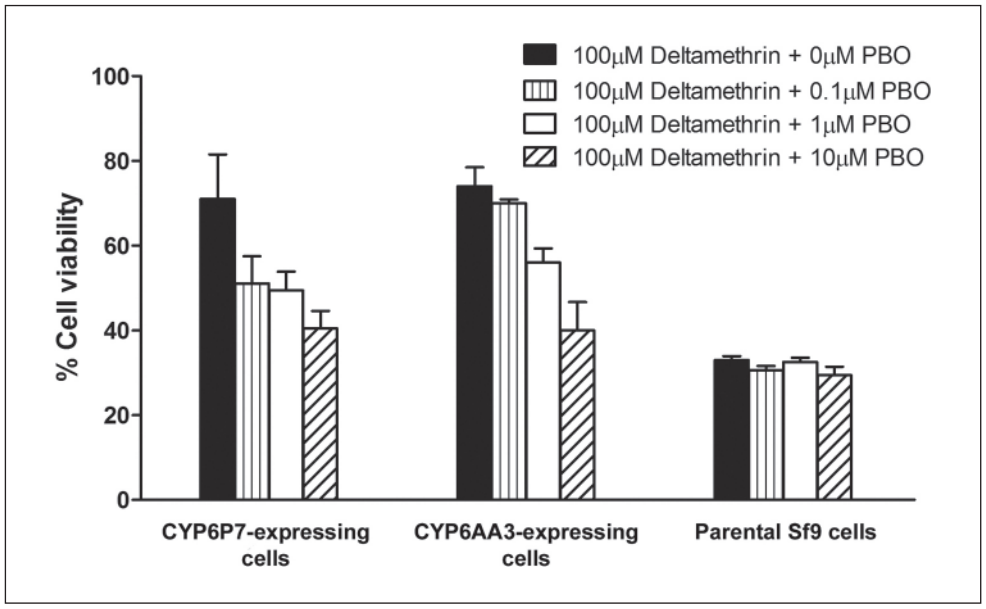

Figure 2. Effect of deltamethrin on viability of cells expressing CYP6P7, CYP6AA3, and parental Sf9 cells in the presence of PBO. Cells were co-incubated with deltamethrin and PBO, and percentage of cell viability after normalization with that treated with PBO only was obtained as described in Materials and Methods. Data are means ± S.D. of three replicated experiments

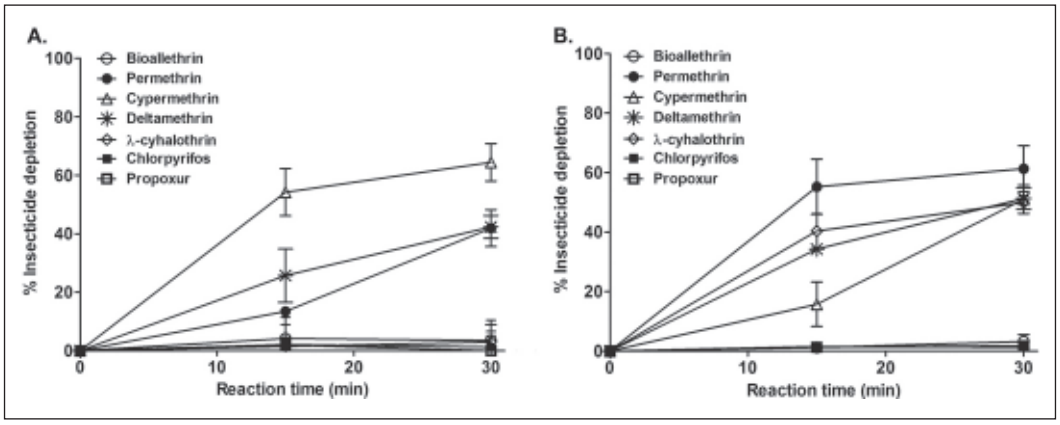

Figure 3. Plot of the time course of insecticide depletion mediated by CYP6P7 (**A**) and CYP6AA3 (**B**) enzymes using in vitro reconstitution assays, performed as described in Kaewpa *et al.* (2007). Degradation of insecticide mediated by CYP6P7 and CYP6AA3 was calculated as percentage of insecticide consumed in the reactions at each incubation time compared to time zero, after normalization with internal standard. Data are means ± S.D. of triplicate experiments

investigated using CYP6P7- or CYP6AA3 reconstituted with their redox partner enzyme, the NADPH-dependent cytochrome P450 reductase. As shown in Figure 3 CYP6P7 and CYP6AA3 showed enzymatic activities toward permethrin, cypermethrin and deltamethrin, but no detectable activities were observed with bioallethrin, chlorpyrifos, and propoxur. Moreover, in contrast to CYP6AA3, CYP6P7 had undetectable enzyme activity in metabolizing λ-cyhalothrin. These results were thus in agreement with the ability of CYP6P7 and CYP6AA3 in protecting cells against cytotoxic effects of test insecticides.

DISCUSSION

The results of this study showed that expression of CYP6P7 and CYP6AA3 could help protecting insect Sf9 cells from cytotoxicity of several pyrethroids. Presence of PBO diminished CYP6P7- and CYP6AA3-mediated cytoprotection of Sf9 cells against deltamethrin cytotoxicity, suggesting that cytoprotection against pyrethroids was due to CYP6P7 and CYP6AA3 enzymes. Moreover, results from MTT cytotoxicity assays corresponded to those of *in vitro* reconstitution enzymatic assays. In this study we detected cytotoxic effect of PBO against Sf9 cells, PBO toxicity has also been reported, as it causes tumors and adverse effects in laboratory rodents (Takahashi *et al*., 1997; Okamiya *et al*., 1998).

As shown in this study, Sf9 parental cells were susceptible to cytotoxicity of test insecticides with LC_{50} values less than 50 µM. Sensitivity of insect Sf9 cells to cytotoxic effects of compounds such as fungal metabolites (Fornelli *et al*., 2004), pyridalyl insecticide (Saito *et al*., 2005) have been reported. Thus susceptibility of Sf9 parental cells to insecticide cytotoxicity observed in the present study is advantageous to determining capability of P450 enzymes in detoxifying insecticides. Capability of P450 in detoxifying cytotoxic xenochemicals were previously reported using insect cells expressing P450 enzyme via baculovirus-directed expression (Grant *et al*., 1996). Moreover Sf21 cells expressing human CYP1A1 were used to investigate toxicity of naphthalene (Greene *et al*., 2000).

The results of this study revealed that CYP6P7 and CYP6AA3 could function in metabolizing of permethrin, cypermethrin, and deltamethrin, however role in metabolisms of bioallethrin, chlorpyrifos and propoxur was not observed. Thus abilities of CYP6P7 and CYP6AA3 in detoxifying pyrethroids support their role in conferring deltamethrin resistance in *An. minimus* mosquito (Rodpradit *et al*., 2005). A difference was noted in that while CYP6AA3 showed metabolic activity and cytoprotective activity against λ-cyhalothrin, these capabilities were not observed for CYP6P7. The results thus indicated differences in properties of these two enzymes.

A characteristic of P450s that has been noted is their variation in substrate specificity such as a single P450 could metabolize more than one substrate (Scott, 1999). *In vitro* metabolisms of insecticides by insect P450s have been reported such as ability of CYP6P3 of *An. gambiae* in metabolizing more than one pyrethroid substrates including deltamethrin and permethrin (Mûller *et al*., 2008), and CYP6D1 of *M. domestica* in metabolisms of deltamethrin and cypermethrin (Wheelock & Scott, 1992; Zhang & Scott, 1996). In this study, results of both in situ detoxification assays and *in vitro* enzymatic assays demonstrated variation of CYP6P7 and CYP6AA3 activities toward different pyrethroids and with different specificities, suggesting their abilities to metabolize and detoxify several pyrethroid insecticides in *An. minimus* mosquito. Moreover, ability of CYP6P7 and CYP6AA3 to detoxify pyrethroids observed in this study, although without co-expression of *An. minimus* cytochrome P450 reductase, indicated that the NADPH-dependent cytochrome P450 reductase enzyme of Sf9 cells could help supporting activity of these mosquito P450s.

In conclusion, the results obtained in this study elucidate the ability of CYP6P7 and CYP6AA3 in detoxification of several pyrethroids and emphasize the possible role of CYP6P7 and CYP6AA3 enzymes in conferring pyrethroid resistance in *An. minimus* mosquito. A correlation of results from MTT cytotoxicity assays and *in vitro* enzymatic activity assays emphasizes usefulness of employing P450-expressing Sf9 cells to pre-screen detoxification capability of P450s against cytotoxic insecticides using MTT cytotoxicity assays.

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