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An Expression of an Insect Membrane-bound Cytochrome P450 CYP6AA3 in the *Escherichia coli* in Relation to Insecticide Resistance in a Malarial Vector

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Abstract: This laboratory investigation was carried out at the Faculty of Sciences, Mahidol University, Thailand during October 2007 to May 2009. The objectives of this study include: the search for heterologous expression of the cytochrome P450 CYP6AA3 enzyme of the *Anopheles minimus* mosquitoes in relation to Malaria disease and to provide some information on molecular mechanism of insects' pyrethroid resistance. The polymerase chain reaction aided by the *Pfu* DNA polymerase and some specific generated primers were used to modify the CYP6AA3 gene. The PCR product was ligated with a predigested pET-3a at the *Nde*I and *Bam*HI restriction sites. The modified CYP6AA3 enzyme was expressed in the *Escherichia coli* BL21 (DE3) pLysS in order to achieve a high amount of soluble form of its expression. The results showed that the use of the isopropyl-beta-D-thiogalactopyranoside (IPTG) and incubation together with ferric chloride and δ-aminolevulinic acid did not increase any soluble form of the CYP6AA3 enzyme. A significant amount of soluble enzyme was produced upon the replacement of the 30 N-terminal residues with a short peptide where it gave LΔ30CYP6AA3 protein and after purification process was taken place, it yielded up to 10.64 mg 10 L^{-1} or approximately 1 mg L^{-1} of the homogenous LΔ30CYP6AA3. When this purified LΔ30CYP6AA3 protein was used in a metabolizing process with the cypermethrin, deltamethrin and permethrin substrates, it gave their apparent K_m values for cypermethrin and deltamethrin of 12.5 and 23.5 μM, respectively. The heterologous expression carried out with the use of the *E. coli* gave a high amount of soluble CYP6AA3 enzyme of the *An. minimus* mosquitoes hence the modified technique being used was successfully achieved.

Key words: *Anopheles minimus*, CYP6AA3, heterologous expression, malaria, pyrethroid metabolism, pyrethroid resistance

INTRODUCTION

It was advocated that approximately a half of the world population is now facing with a severe disease widely known as Malaria. It is a tropical disease caused by *Plasmodium* parasites. In general, this disease could transmit to human by *Anopheles* mosquitoes. The cases on the spreading out of Malaria disease have been reported annually and it was found that nearly 250 million cases with two mortalities minute^{-1} have been recorded. The infected populations were found mainly among children with an age below 5 years old (Malaria Key Facts, Roll Back Malaria Partnership, http://www.rollbackmalaria.org/keyfacts.html). For the past decades, the prevention of this disease was carried out through the control of mosquito vectors primarily through the use of pyrethroid insecticides. With a long term exposure to insecticides, the mosquitoes have built up its adaptability against the chemicals where they are able to possess a high resistance to the chemical compounds. There have been a number of published data concerning pyrethroid insecticide resistance in *Anopheles* mosquitoes, e.g., with *An. albimanus* (Brogdon and Barber, 1990), *An. gambiae* in Africa (Chandre *et al.*, 1999a, b) *An. culicifacies* in India (Singh *et al.*, 2002), *An. stephensi* (Enayati *et al.*, 2003) and an outstanding one in Thailand, viz. *An. minimus* species A. The deltamethrin resistance being built up by *An. minimus* mosquitoes have been detected through deltamethrin selection carried out in laboratory and this insect species was found to attain a significant increase in the level of cytochrome P450 CYP6AA3 transcript when compared with other susceptible strain (Rongnoparut *et al.*, 2003; Rodpradit *et al.*, 2005). Thus the increases in the cytochrome P450 CYP6AA3 found with *An. minimus* species of mosquitoes may be considered as the cause related to the elevated expression of the encoded enzyme, hence responsible for an increase in insecticide resistance of this particular mosquito species.

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It was found that cytochrome P450 mono-oxygenase (CYP) enzyme plays its significant role in the metabolism of many endogenous and exogenous compounds, e.g., in human, both CYP3A4 and CYP2D6 enzymes have involved in human drug metabolism (Yu *et al.*, 2002; Ekroos and Sjogren, 2006) and also steroids biosynthesis (Guengerich, 2005). In plants, the CYP enzymes could be catalyzed in the process of detoxification of both herbicides and insecticides, as well as the synthesis of isoflavonoids, phenylpropanoids, furanocoumarins, alkaloids and terpenes (Schuler, 1996a, b). These enzymes could also involve in microorganisms mediate lipid and fatty acid metabolisms (Narhi and Fulco, 1986). In some insects, these enzymes play their important role in the metabolism of steroid hormones, e.g., juvenile and molting hormones and also pheromones (Feyereisen, 1999). In addition, an expression of some insect CYPs is normally induced by the presence of toxins in foods or by a long term exposure to insecticides hence the insects perform its adaptability by detoxification activities (Berenbaum *et al.*, 1990; Frank and Fogleman, 1992). The detoxification mechanism was found to possess more of its activeness in some insects where it increases level of insecticide resistance (Taylor and Feyereisen, 1996). The case on the building up of its resistance against insecticides in many insect species may be considered to be one of the major problems in controlling many emerging diseases those transmitted to human through insect vectors. They include malaria, dengue and yellow fever diseases.

An. minimus CYP6AA3 was found to express in the baculovirus expression system where it possessed a deltamethrin-metabolizing activity (Boonsuepsakul *et al.*, 2008). Although the enzyme is known to have its expression in terms of a functional form, its relatively low amount being detected in the microsomal fraction may be insufficient for thoroughly studies of the enzyme including the determination on its structure. Thus it is a common problem for any membrane-bound CYP enzymes studies. Therefore, some efforts have involved in the process towards any heterologous expression of the CYP enzymes as to overcome its limitation. It was reported that microsomal CYP enzymes have been successfully produced with its heterologous form in bacterial system in rabbit liver CYP2B5 when it exhibited a high level of expression in the *Escherichia coli* JM-109. It also showed a catalytic activity towards the 4-nitroanisole and androstenedione (Lehnerer *et al.*, 1995). For the enzymes of the CYP2B1, CYP2B4, CYP2B6, CYP2B11, as well as the CYP2C5 when they are required for use in laboratory, it requires some modifications in order to increase both its amount and solubility and enable to achieve its high expression when modified and expressed in the *E. coli*

bacteria (Cosme and Johnson, 2000; Scott *et al.*, 2001). With a recent study, it was found that *An. gambiae* CYP6Z2 was functionally expressed in the *E. coli* using bacterial OmpA signal peptide with a co-expression of the *An. gambiae* P450 reductase where it inhibited by the cypermethrin or permethrin yet unable to metabolize pyrethroid insecticides (McLaughlin *et al.*, 2008). *An. gambiae* CYP6P3 could perform heterologous expression in the *E. coli* upon the replacement of the natural N-terminal sequence with a short peptide MALLLAVF. It was shown to degrade both deltamethrin and permethrin (Muller *et al.*, 2008). These two insect CYP enzymes, however, are normally expressed as membrane-bound forms, which may pose a solubility problem for their structural determinations (McLaughlin *et al.*, 2008; Muller *et al.*, 2008). The objective of this investigation was to try to produce a high amount of soluble form of the *An. minimus* CYP6AA3 with the use of the *E. coli* system through a modification process of the CYP6AA3 enzyme, which may allow an expression of its function and the soluble form where the produced amount is adequate for further crystallization and structural studies. It seems more likely that up to present the data dealing with the *An. minimus* mosquitoes on this particular respect has not been advocated. The results derived from this investigation may be of tangible value for further enzymes studies and to understand more clearly on some mechanisms in building up insecticide resistance in insect vectors so that the controlling on the spreading out of Malaria disease to man, particularly with the *An. minimus* mosquitoes could be carried out more effectively.

MATERIALS AND METHODS

The establishment of plasmid expression: A full-length of the CYP6AA3 enzyme in the pGEM-T Easy vector being established and described by Rongnoparut *et al.* (2003) was amplified with the use of the Polymerase Chain Reaction (PCR) method using Pfu DNA polymerase (Agilent Technologies, CA, USA) for sub-cloning into pET-3a vector (Merck KGaA, Darmstadt, Germany). The *Nde*I and *Bam*HI recognition sites were incorporated to both upstream and downstream of the CYP6AA3 through the forwarding and reversing primers, respectively (Table 1). The codons of the six histidine residues with a linker peptide DGTS were added to the 3'-end of the CYP6AA3 gene to facilitate purification process of the attained protein. The addition of a linker was carried out aiming to extend the His_6-tag from the core of the CYP enzyme structure. The obtained PCR product was ligated with a predigested pET-3a at the *Nde*I and *Bam*HI restriction sites and then the product was again used for

Table 1: Different primers were used for the establishment of the modified *CYP6AA3* genes

Established enzymes	Oligonucleotide primers
Full-length CYP6AA3	5'-CTCTCTCC*ATATG*GGGTTACGTTAATGTGGTG-3'
Δ22CYP6AA3	5'-CTCTCTCC*ATATG*GCGAAGCAACACTATCGTTACTGG-3'
Δ23CYP6AA3	5'-CTCTCTCC*ATATG*GCGCAACACTATCGTTACTGGGCC-3'
Δ24CYP6AA3	5'-CTCTCTCC*ATATG*GGCGCACTATCGTTACTGGGCCAACC-3'
Δ25CYP6AA3	5'-CTCTCTCC*ATATG*GCGTATCGTTACTGGGCCAACCGG-3'
Δ26CYP6AA3	5'-CTCTCTCC*ATATG*GCGCGTTACTGGGCCAACCGGAACATTCC-3'
LΔ22CYP6AA3	5'-CTCTCTCC*ATATG*GCGAAAAAAACCTCTTCTAAGCAACA CTATCGTTACTGGGCCAACCGGAACATTCC-3'
LΔ30CYP6AA3	5'-CTCTCTCC*ATATG*GCGAAAAAAACCTCTTCTAACCGGAA CATTCCGCAGCTGGAGGCATCATTCCCGG-3'
Reverse primer[a]	5'-GT*GGGATCC*ACGGCTTAGTGATGATGATGATGATGAGAGGTA CCATCCAACTTGGTTGCATTCAACCAGATGCCTCCTTCGACC-3'

The underlines are nucleotides corresponded to parts of the native *CYP6AA3* sequences, whilst the *Nde*I (in the forwarding primers) and *Bam*HI (in a reverse primer) sites are indicated in italics, [a]: This reverse primer was used in all established or constructed enzymes

the transformation of the *E. coli* XL1-Blue (Agilent Technologies, CA, USA). The resistant clones carrying the pET-3a/full-length CYP6AA3 plasmid were selected with 100 μg mL^{-1} ampicillin on LB agar plates and they were cultured for plasmid extraction and then sequenced its verification with the use of the DNA sequencing. The verified plasmid was again used in the transformation of the *E. coli* BL21 (DE3) pLysS (Merck KGaA, Darmstadt, Germany) for the determinations of the subsequent established proteins.

The modification of the CYP6AA3 enzyme: All of the N-terminal modifications were made through the PCR reaction using the forwarding and reversing primers as stated earlier. The resultants of the PCR products were ligated through the predigested pET-3a vector at *Nde*I and *Bam*HI sites and then relatively used to transform the *E. coli* XL1-blue as described earlier in order to achieve the established products. The Plasmids carrying the modified CYP6AA3 were extracted and subjected to the DNA sequence verification prior to its expression when carried out in the *E. coli* BL21 (DE3) pLysS.

The expressions of both the full-length and the truncated CYP6AA3 enzymes: The *E. coli* BL21 (DE3) pLysS harboring the desired plasmids for the various CYP6AA3 resultants were incubated overnight at 37°C in the LB medium containing 100 μg mL^{-1} ampicillin and 35 μg mL^{-1} chloramphenicol. An amount of 1% of each overnight culture was transferred to a 250 mL modified Terrific broth (10 g tryptone, 12 g yeast extract, 2 g peptone, 4 mL glycerol, 2.31 g KH$_2$PO$_4$, 12.7 g K$_2$HPO$_4$ for 1 L solution) and then supplemented with 1 mM MgCl$_2$, 2.5 mM (NH$_4$)$_2$SO$_4$ and 50 μM FeCl .6H₂O together with the appropriate antibiotics. Cells were cultured at 37°C through the use of a shaking incubator at a rate of 250 rpm until the OD$_{600}$ reached a range from 0.7-1.0. Each expression of individual CYP6AA3 was induced with the added amount of 0.5 mM IPTG together with 0.5 mM

δ-aminolevulinic acid in order to facilitate the haem synthesis. Each of the modified CYP6AA3 protein was exposed to a temperature of 15°C for 24 h with a shaking speed of 180 rpm prior to the harvest of cells through the use of a centrifuge at 8,600 g for 10 min at 4°C. The attained cell pellets were re-suspended in a 50 mM NaH$_2$PO$_4$, pH 7.4 buffer containing 0.2 M NaCl, 20% glycerol, 1% CHAPS, 1% sodium cholate and 0.25 mM PMSF and then sonicated at a temperature below 10°C. Cell debris (P1 fraction) was removed with the use of a centrifuge at 39,000 g for 30 min at 4°C and the attained supernatant was subjected to the ultracentrifugation at 100,000 g for 1 h. This was carried out in order to separate the cell pellets (P2 fraction) those containing large molecular units where they were aggregated from the soluble materials. The partition of each enzyme of the pellets and the cytosolic fractions was compared with the use of the SDS-PAGE. To determine whether the established CYP6AA3 enzymes had expressed into a soluble form or not, the supernatant fraction was then subjected to a pre-equilibrated, a small-scaled nickel affinity column, then briefly washed with a buffer containing 30 mM imidazole and again eluted with a buffer containing 150 mM imidazole for the SDS-PAGE analysis.

The purification of the LΔ30CYP6AA3 enzyme: The cytosolic fraction containing the LΔ30CYP6AA3 enzyme was applied to Ni-NTA column (Qiagen, GmbH, Hilden, Germany), pre-equilibrated with 10 mM imidazole, 0.2 M NaCl and 20% glycerol in 50 mM NaH$_2$PO$_4$, pH 7.4. The column was washed with the use of the ten-column volumes of the equilibration buffer followed by the fifteen-column volumes of a washing buffer (50 mM imidazole, 0.5% Triton X-100, 10 mM β-mercaptoethanol, 0.5 M NaCl and 20% glycerol in 50 mM NaH$_2$PO$_4$, pH 7.4). The Triton X-100 was then removed from the column by washing with the ten-column volumes of the washing buffer without the Triton X-100 prior to the elution of the LΔ30CYP6AA3 with 150 mM imidazole, 0.2 M NaCl and

20% glycerol in 50 mM NaH_2PO_4, pH 7.4. The fractions containing the LΔ30CYP6AA3 were pooled and dialyzed twice against a hundred-sample volume of 50 mM NaCl and 20% glycerol in 50 mM NaH_2PO_4, pH 7.0 before applying to the SP Sepharose column (GE Healthcare Life Sciences, Buckinghamshire, UK) and then pre-equilibrated with the same buffer. The LΔ30CYP6AA3 was eluted with a linear gradient, 100-500 mM NaCl. The fractions containing a single band of the LΔ30CYP6AA3 were pooled and dialyzed against 50 mM NaH_2PO_4, pH 7.4 containing 20% glycerol. Eventually, the enzyme solutions were attained and then all of the aliquots were kept at-80°C until use.

The characterization activity of the LΔ30CYP6AA3: With the metabolism of pyrethroids where it catalyzed by the purified LΔ30CYP6AA3, this was determined in a reconstituted system with the use of the cytochrome P450 reductase (Kaewpa *et al.*, 2007). The reaction was assayed in a total volume of 250 μL which contained a ratio of 1:3 of cytochrome P450 reductase: LΔ30CYP6AA3 in the 0.2 M sodium phosphate buffer, pH 7.4 containing 20 μg mL^{-1} dilauroylphosphatidylcholine, 50 μM pyrethroid (deltamethrin, permethrin, or cypermethrin) and 0.5 U glucose-6-phosphate dehydrogenase. The reaction mixture was pre-incubated at 30°C for 15 min before the adding up of the 6 mM glucose-6-phosphate, 0.6 mM $NADP^+$ and 2 mM $MgCl_2$. This was aiming to initiate the reaction and regenerate the NADPH for a prolonged incubation. After 15, 30, or 45 min of incubation, the reaction was stopped by the addition of 30 μL of 2 M HCl. The bioallethrin was then added with 100 μM as an internal standard followed by an extraction with the use of 750 μL ethyl acetate. Each reaction tube was mixed for 2 min prior to centrifugation at 2,000 g for 10 min. The clear upper layer of the ethyl acetate extracted sample containing un-metabolized pyrethroid and bioallethrin in each tube was transferred to new tubes and dried under nitrogen stream. Acetonitrile (50 μL) was used to dissolve the dried pyrethroids and then an amount of 20 μL of the attained clarified solution was injected into the Nova-Pak C18-reverse phase column (3.9×150 mm, 4 μm, Waters, MA, USA) for HPLC analysis (Agilent Technology, series 1100). The column was pre-equilibrated with 50% acetonitrile at a flow rate of 1 mL min^{-1} for 30 min prior to the injection of the sample. Both bioallethrin and un-metabolized pyrethroid were eluted using 2 mL 50-80% acetonitrile gradient, followed by 1 mL of 80-100% gradient and 8 mL of 100% acetonitrile and then a UV absorbance monitored at 220 nm. Peak area of each pyrethroid was normalized with that of the bioallethrin and

the attained activity was calculated from the amount of un-metabolized substrate. Standard curves of the pyrethroids were determined within a range between 20-100 μM for deltamethrin, 6-37 μM for cypermethrin and 7.5-37.5 μM for permethrin. Enzymatic activity was determined as molar disappearances of the deltamethrin, cypermethrin, or permethrin minute^{-1}. Kinetic parameters of the LΔ30CYP6AA3 were determined using assay conditions described earlier except that of the concentrations of either deltamethrin or cypermethrin, which was varied from 2.5 to 100 μM. The initial rates of the pyrethroid disappearance were fitted to the Michaelis-Menten non-linear equation with the use of the KaleidaGraph computer programme (Synergy Software, PA, USA).

RESULTS

Heterologous expressions of both the full-length and the truncated CYP6AA3 enzymes: An induction with the use of the IPTG at 37°C produced a full-length of the CYP6AA3 enzymes in the *E. coli* with a theoretical molecular weight of 58 kDa found in the pellet fraction. When the induction was subjected to a temperature of 15°C, it yielded a high CYP6AA3 expression per cell when determined using the SDS-PAGE with the normalized based samples on cell density. Nevertheless, a large amount of the expressed enzyme was still observed in the pellet fraction. When supplemented with the δ-aminolevulinic acid to the culture, it was found that the medium did not affect the enzyme expression level. The putative trans-membrane region at the N-terminus of the CYP6AA3 was then modified based on its alignment of amino acid sequences. The modification was carried out with those of the P450s whose structures have been successfully determined (Fig. 1). In this context, a series of the CYP6AA3 was established with the deletion of the N-terminal residues where they were eventually generated its residual effect (Table 2). In addition, either alanine or a short positively charged peptide (AKKTSS) was added to the N-termini of these established proteins after the initiation of the methionine. This was carried out aiming to promote a high expression of the enzymes in the *E. coli*. Except for the establishment of the LΔ30CYP6AA3, all of the modified CYP6AA3s exhibited a higher expression level than the native full-length clone, but were largely localized in the membrane fraction with a relatively low amount of soluble enzymes. The LΔ30CYP6AA3 enzyme, whose 30 native amino acids at the N-terminus were replaced with the MAKKTSS-peptide, it displayed significant reduction of the expressed protein in the

Table 2: The schematic representation of the established products with its positive and negative expressions

	Established products			Soluble expression[a]
	TM			
CYP6AA3	1-22	23-505	DGTSHHHHHH	+
Δ22CYP6AA3	MA	23-505	DGTSHHHHHH	–
Δ23CYP6AA3	MA	24-505	DGTSHHHHHH	–
Δ24CYP6AA3	MA	25-505	DGTSHHHHHH	–
Δ25CYP6AA3	MA	26-505	DGTSHHHHHH	–
Δ26CYP6AA3	MA	27-505	DGTSHHHHHH	–
LΔ22CYP6AA3	MAKKTSS	23-505	DGTSHHHHHH	–
LΔ30CYP6AA3	MAKKTSS	31-505	DGTSHHHHHH	+++

[a]: This was detected with the use of a small-scaled nickel affinity column, TM letters refer to the putative trans-membrane region

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                  10        20        30        40        50        60        70
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CYP6AA3        ----MGYVNVVFYLVLPALGLLYYYVKQHYRYWANRNIPQLEASFPVGNMKGVGSKLHFNDVLGEAYDKG 66
LΔ30CYP6AA3    --------------------------MAKKTSSNRNIPQLEASFPVGNMKGVGSKLHFNDVLGEAYDKG 43
CYP3A4         MALIPDLAMETWLLLAVSLVLLYLYGTHSHGLFKKLGIPGPTPLPFLGNILSYHKG-FCMFDMECHKKYG 69
CYP3A4 1TQN    -------------------MALYGTHSHGLFKKLGIPGPTPLPFLGNILSYHKG-FCMFDMECHKKYG 48
CYP2C5         --------MDPVVVLVLGLCCLLLLSIWKQNSGRGKLPPGPTPFPIIGNILQIDARDISKSLTKFSECYG 62
CYP2C5 1DT6    ------------------------MAKKTSSKGKLPPGPTPFPIIGNILQIDARDISKSLTKFSECYG 44
CYP2A6         ----MLASGMLLVALLVCLTVMVLMSVWQQRKSKGKLPPGPTPLPFIGNYLQLNTEQMYNSLMKISERYG 66
CYP2A6 1Z10    ------------------------MAKKTSSKGKLPPGPTPLPFIGNYLQLNTEQMYNSLMKISERYG 44
CYP2D6         ----MGLEALVPLAVIVAIFLLLVDLMHRRQRWAARYPPGPLPLPGLGNLLHVDFQNTPYCFDQLRRRFG 66
CYP2D6 2F9Q    ------------------------MAKKTSSKGKLPPGPLPLPGLGNLLHVDFQNTPYCFDQLRRRFG 44
CYP102A1 1ZO9  ---------------------------GAMTIKEMPQPKTFGELKNLPLLNTDKPVQALMKIADELG 40
CYP101A1 1AKD  --------MTTETIQSNANLAPLPPHVPEHLVFDFDMYNPSNLSAGVQEAWAVLQESNVPDLVWTRCNGG 62
CYP121 1N4G    -------------MTATVLLEVPFSARGDRIPDAVAELRTREPIRKVRTITGAEAWLVSSYALCTQVLED 57
CYP154A1 1ODO  -----------MATQQPALVLDPTGADHHTEHRTLREGGPATWVDVLGVQAWSVSDPVLLKQLLTSSDVS 59
CYP119 1F4U    ------------------------MYDWFSEMRKKDPVYYDGNIWQVFSYRYTKEVLNNFSKFS 40
CYP175A1 1N97  ------------------------MKRLSLREAWPYLKDLQ----QDPLAVLLEWGRAHP 32
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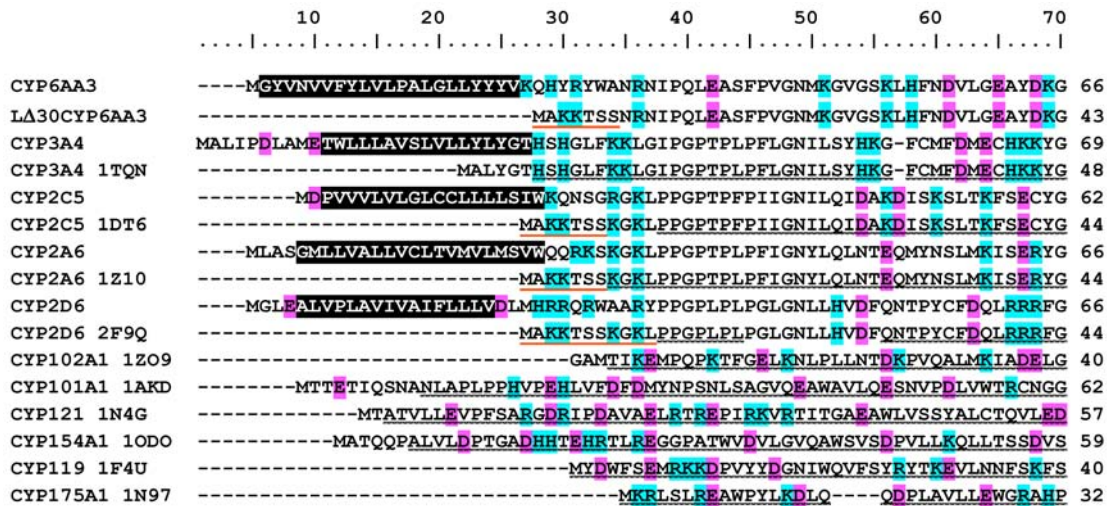
Fig. 1: The alignment of the native N-terminal (1–66) and the modified sequences of the CYP6AA3 with those of the CYPs with its available structures (Yano *et al.*, 2000, 2003, 2004, 2005; Rowland *et al.*, 2006; Hegde *et al.*, 2007; Schlichting *et al.*, 1997; Leys *et al.*, 2003; Podust *et al.*, 2004). The putative trans-membrane regions are highlighted with the white letters on black shades and the modified sequences are indicated with underlined in red. Structures of the CYP102A1, CYP101A1, CYP121, CYP154A1, CYP119 and CYP175A1 were determined without modified sequence. The double squiggled being underlined indicated the regions presented in the corresponding structures. Cyan and magenta shadings represent residues with positive and negative charges, respectively. Sequence alignment was made with the use of the ClustalX computer programme (Thompson *et al.*, 1997)

insoluble P2 fraction when compared with the expression profile of the full-length counterpart (Fig. 2). Furthermore, some considerable amounts of the soluble LΔ30CYP6AA3 were observed in the cytosolic fraction after an enrichment with the nickel affinity column. When the LΔ30CYP6AA3 was compared with the full-length enzyme, it gave much higher solubility efficacy than the full-length enzyme.
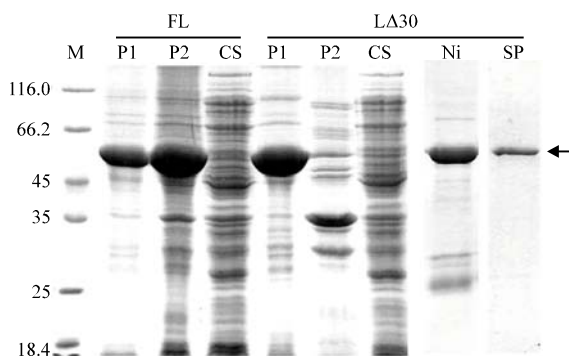
Fig. 2: The SDS-PAGE, displaying an expression and purification of the full-length CYP6AA3 and LΔ30CYP6AA3. The cells were expressed in the full-length CYP6AA3 (FL) and LΔ30CYP6AA3 (LΔ30) enzymes. They were normalized on cell density prior to cell lysis. The pellet P1 derived from centrifugation, pellet P2 obtained from an ultra-centrifugation and Cytosolic Fractions (CS). They were analyzed on the SDS-PAGE. Significant amount of the soluble LΔ30CYP6AA3 was recovered from the Ni-NTA-affinity column (Ni) with some impurities. The impurities were removed out using the SP Sepharose chromatography (SP). An arrow indicated the expected bands of the CYP6AA3 enzymes

Purification of the LΔ30CYP6AA3 enzyme: The purification of the LΔ30CYP6AA3 was carried out using the Ni-NTA column, it yielded as a partially purified LΔ30CYP6AA3 enzyme with approximately 80-90% homogeneity (Fig. 2). The LΔ30CYP6AA3 pool was further purified using the SP Sepharose column, to which the LΔ30CYP6AA3 could efficiently bind at a pH 7.0 with the presence of 50 mM NaCl and 20% glycerol. An elution with the 100-500 mM NaCl gradient resulting in the LΔ30CYP6AA3 fractions, it gave over 95% of homogeneity where it showed itself as a single protein band on the SDS-PAGE. The total yield of the purified LΔ30CYP6AA3 enzyme was up to 10.64 mg 10 L^{-1} of the cell culture or approximately 1 mg L^{-1}.

Metabolism of pyrethroids by LΔ30CYP6AA3: To determine whether the heterologous expression of the LΔ30CYP6AA3 enzyme was enzymatically active, its ability to metabolize pyrethroids was characterized using the described HPLC elution protocol, the retention times of the bioallethrin, deltamethrin, cypermethrin and permethrin were 8.8, 9.6, 9.5 and 9.7 min, respectively. With the analysis of the remaining pyrethroids after
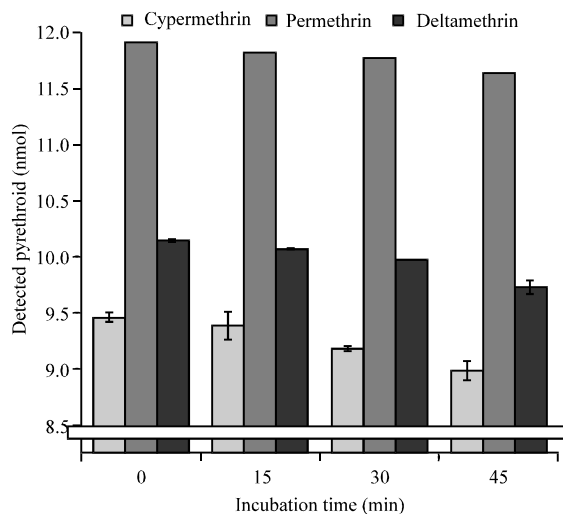

Fig. 3: Metabolism of pyrethroids against incubation time on the purified LΔ30CYP6AA3, the remaining pyrethroids from the assay reactions were extracted at various incubated periods (0, 15, 30 and 45 min) when subjected to the HPLC analysis. The amounts of the detected normalized pyrethroids with the use of an internal standard bioallethrin compared with those of a zero minute. Vertical lines represent standard errors (±)

45 min of incubation, it gave 2.4-5.1% of pyrethroid consumption (Fig. 3). The LΔ30CYP6AA3 had metabolized in the substrates of deltamethrin, cypermethrin and permethrin in a pyrethroid-concentration at a dependent manner. When plotting initial velocities against pyrethroid concentrations for deltamethrin and cypermethrin, it was well fitted to the Michaelis-Menten kinetics. The metabolism of the pyrethroids by the purified LΔ30CYP6AA3 enzyme had expressed and possessed its function at a considerable manner. Although its K_m value towards deltamethrin was nearly twice that of the cypermethrin, its catalytic efficiency for deltamethrin was three times higher than the cypermethrin (Fig. 4a,b).

DISCUSSION

For this laboratory investigation, heterologous expression of the *An. minimus* CYP6AA3 enzyme was carried out with the use of a bacteria viz. *Escherichia coli* (*E. coli*) in order to achieve a high level of expression of the enzyme in a soluble form to be used for further characterization process. With the initial study, an expression of the native full-length of the CYP6AA3 enzyme was determined in order to preserve the gene
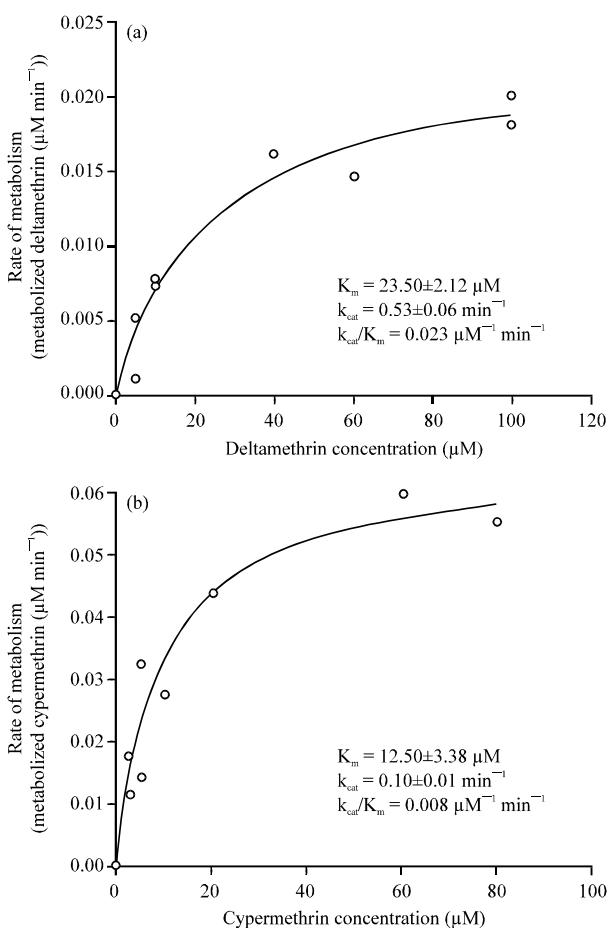
Fig. 4 (a,b): The Michaelis-Menten figures revealing rates of metabolism of the pyrethroid through the use of the LΔ30CYP6AA3 enzyme against different concentrations of both (a) deltamethrin and (b) cypermethrin, respectively

structure and encoded its native protein structure. However, it was found that the expression of the CYP6AA3 enzyme was only possible in the form of insoluble protein. To overcome the formation of the inclusion bodies and to increase its expression efficacy for a high level of solubility of the enzyme, some strategies were applied including the optimization of the IPTG concentration and incubation temperature for the induction of protein expression, as well as the supplementation of the culture media with the use of ferric chloride and δ-aminolevulinic acid, a precursor of the haem biosynthesis. It was found that none of them had aided in any increase in yield of a soluble expression form of the CYP6AA3 enzyme. This may be attributable to the N-terminal trans-membrane region of the enzyme still remained in all of the established protein. Thus the enzyme remains in its insoluble form.

Further exploration was carried out with the use of the *E. coli* C41 (DE3) as an alternative expression host, a co-expression of the CYP6AA3 enzyme with molecular chaperones, as well as, the expression of the CYP6AA3 as a fusion-protein with the thioredoxin. The *E. coli* C41 (DE3) is a bacterial strain derived from the BL21 (DE3) with unknown mutation, which prevents the death of cells due to possible toxicity of the expressed protein and thus allows for a high yielding of the targeted protein expression (Miroux and Walker, 1996). An expression of the recombinant membrane-bound rabbit CYP2E1, this strain gave a yield in a range from 9-14 folds where it gave higher expression than those being obtained in other *E. coli* hosts (Cheng *et al.*, 2004). The expression of the native CYP6AA3 enzyme in the *E. coli* C41 (DE3) was having a range of 2-3 folds higher yield than the expression of the attained result from the *E. coli* BL21 (DE3) pLysS. Nevertheless, it was found that the enzyme was still localized in the pellet fraction. This indicated that the changing of the expression host did not increase the solubility performance of the CYP6AA3 enzyme.

Heterologous expressions of several proteins have been facilitated by the co-expression of chaperone proteins, e.g., allergen of Japanese cedar pollen Cryj2; mouse endostatin, human oxygen-regulated protein ORP150 and human lysozyme have been co-expressed with the GroEL and GroES, with and without trigger factor, which prevented aggregation and assisted in the proper folding of the targeted proteins (Nishihara *et al.*, 1998, 2000). An attempt was upheld in collaboration with the use of the co-expression of the full-length of the CYP6AA3 enzyme with the C-terminal His$_6$-tag along with these chaperones in the *E. coli* C41 (DE3) system. Unfortunately, the CYP6AA3 was relatively expressed at a low level and was tightly bound to the GroEL component, within this case they were not able to separate through the use of conventional liquid chromatography. It may be possible that the expressed CYP6AA3 enzyme was not folded or had exposed its hydrophobic region that could possibly have been trapped inside the GroEL complex by a hydrophobic interaction.

Inspired by a high level of expression of the two proteins with double trans-membrane regions that derived from the cystic fibrosis trans-membrane conductance regulator (Therien *et al.*, 2002), therefore, the fusion protein of the thioredoxin and the native full-length of the CYP6AA3 enzyme was established. The subsequent expression in the *E. coli* Origami (DE3) revealed a high expression of the thioredoxin-CYP6AA3 fusion protein as inclusion bodies with a small amount of detectable soluble enzyme. The result indicated that the thioredoxin fusion partner did not assist in a proper folding of the CYP6AA3 enzyme.

Apparently the results revealed that the presence of the N-terminal trans-membrane region of the CYP6AA3 enzyme could probably have been the cause of its membrane-associated behaviour. Thus the removal of such trans-membrane regions in the other P450 enzymes had led to their successful heterologous expression in the *E. coli* system (Cosme and Johnson, 2000; Scott *et al.*, 2001). The truncations of the 22-26 N-terminal residues of the CYP6AA3 enzyme failed to improve its soluble expression, whereas the deletion of a 30 amino-acid together with the use of a buffer containing the 3-[(3-Cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) and sodium cholate (detergent buffer) were able to increase some certain amounts of the soluble LΔ30CYP6AA3 enzyme in the cytosolic fraction. The replacement of the 30 N-terminal residues with a short MAKKTSS-peptide was mainly based on the primary sequences of the various soluble P450s as well as those modified human, i.e., the CYP2A6, CYP2C5 and CYP2D6, whose structures have been determined by Cosme and Johnson (2000), Yano *et al.* (2005) and Rowland *et al.* (2006). This indicated that the solubility of the CYP6AA3 enzyme expressed in *E. coli* is mostly depended on the proper length of the N-terminal sequence. Even though the entire trans-membrane region (residues 1-22) and the four additional residues were deleted, the resulted truncated CYP6AA3 enzymes may not be able to fold properly hence it causes aggregation in the insoluble fractions. An addition of the KKTSS-peptide linker to the Δ22CYP6AA3 enzyme was probably not sufficient for the proper folding. The use of a 30-amino-acid truncation with the addition of the MAKKTSS-peptide could have been effectively changed the integral membrane protein of the CYP6AA3 enzyme into a weak membrane-associated to the LΔ30CYP6AA3 enzyme, which could be solubilized by both the CHAPS and Cholate. It may be of interest to note that the *An. gambiae* CYP6P3 could also be a heterologous one when expressed in the *E. coli* with the replacement of the native N-terminal amino acids with a short peptide MALLLAVF (Muller *et al.*, 2008). This emphasizes the importance of the N-terminal sequences of the P450 enzymes. In addition, the presence of the alanine after the starting of the methionine residues in the added peptide may promote the soluble expression of the LΔ30CYP6AA3 enzyme in the *E. coli*. This finding is similar to the results reported by John *et al.* (1994) for the CYP2B1 and by Von Wachenfeldt *et al.* (1997) for the CYP2C3.

Metabolism of the pyrethroids with the use of the purified LΔ30CYP6AA3 enzyme signified that the soluble expression of the expressed LΔ30CYP6AA3 was a functional enzyme. Although its K_m value towards the deltamethrin was nearly twice that of the cypermethrin, its catalytic rate for the deltamethrin was five times higher, resulting in a high catalytic efficiency for the deltamethrin, which is three times higher than that of the cypermethrin. The results indicated that deltamethrin could be a better substrate for the LΔ30CYP6AA3 enzyme than that of the cypermethrin. The K_m values attained in this work are at the same range as those deltamethrin metabolisms of those available commercial rat enzymes viz. CYP2C6 and CYP2C11, whose K_m values are within a range from 21.6±9.4 μM to 31.9±25.7 μM, respectively as well as those commercial human enzymes, i.e., CYP2C8 and CYP2C19, whose K_m values are within a range from 10.2±9.5 μM to 9.0±5.6 μM, respectively (Godin *et al.*, 2007). With the previous study on the deltamethrin metabolism with the use of the native CYP6AA3 enzyme expressed in the baculovirus system, the result showed that its apparent K_m value was 80±2.0 μM (Boonsuepsakul *et al.*, 2008) which is over three folds greater than the value attained in this investigation. The differences may be due to the reason that the native CYP6AA3 enzyme attained from the baculovirus system, which is localized in the microsomal fraction, may have much lower effective concentration than that of the purified soluble LΔ30CYP6AA3 enzyme, thus accounted for the discrepancy in the K_m values.

For this current study, the *An. minimus* LΔ30CYP6AA3 enzyme was established in terms of heterologous expression where it was expressed in the *E. coli* system, which was considered as a soluble and functional protein. Up to this report, no known three dimensional structures of any insect P450 had been reported, possibly due to many reasons such as the difficulty in obtaining sufficient amount of the expressed enzymes. This study provided a starting material that may allow scientists to be able to obtain structures of the insect CYPAA3 enzyme, which could be a key to understand more of the molecular mechanism of any insect's pyrethroid resistance. Furthermore, this work may contribute some insight facts for the building up of some strategies that able to produce a high expression of the soluble P450 enzymes of other insects, as well as, for those having some similar membrane-associated proteins.

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