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Role of Cytochrome P450 in Mechanism of Pyrethroid Resistance*

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Cytochrome P450 monooxygenases are important metabolic enzymes involved in catalyzing a number of compounds and thus are essential for most creatures. One of the major roles of P450s in insects is detoxification or activation of pesticides including pyrethroid insecticides. In this paper, our recent attempts to elucidate the role of P450s in mechanisms of pyrethroid resistance in *Culex* mosquitoes, houseflies and fruit flies are summarized. Possible applications to insect pest management and to the development of novel insect regulators are also discussed.

Keywords: cytochrome P450 monooxygenases, pyrethroids, *Culex quinquefasciatus,* insecticide resistance, target site, microarray.

INTRODUCTION

Cytochrome P450 monooxygenases are vital metabolic enzymes involved in catalyzing a number of endogenous and exogenous lipid-soluble compounds. The major roles of P450s in insects are the synthesis and degradation of hormones including ecdysteroids and juvenoids, and detoxification or activation of chemicals such as plant toxins and insecticides. Insecticide resistance is a major problem in controlling agriculturally and medically important insect pests and one of the most important mechanisms of resistance to pyrethroids and to many other classes of insecticides is enhanced detoxification activity mediated by P450 monooxygenases. Herein, our recent efforts to elucidate the role of P450s in the mechanisms of pyrethroid resistance in three insect species are summarized.

PERMETHRIN RESISTANCE AND P450S IN *CULEX* MOSQUITOES

Culex mosquitoes are distributing worldwide and are important vectors of human diseases including filariasis and West Nile encephalitis. Using a pyrethorid-resistant strain of *C.* quinquefasciatus (JPal-per) originally collected from Saudi Arabia, we examined the relationship between P450s and the resistance. After selection for 12 consecutive generations, JPal-per developed extremely high levels of resistance (2430–4160 fold) to a group of pyrethroids (permethrin, phenothrin and etofenprox), which contain a 3-phenoxybenzyl moiety. Given that P450 inhibitors such as PBO (piperonyl butoxide) and PTPE (2-propynyl 2,3,6-trichlorophenyl ether) had synergistic effects on the toxicity of permethrin against 4th instar larvae, a contribution by P450 monooxygenases was suspected. We then quantified the total amount of P450 and b_5 by using a CO difference spectrum. The levels of P450 and b_5 were about 2.5 times higher in the JPal-per strain than in the susceptible strain. Accordingly, experiments were conducted *in vitro* using [^{14}C]-*trans*-permethrin as a substrate so as to prove the increased activity of P450s in JPal-per larvae. P450 monooxygenases in microsomes prepared from the guts and other body parts metabolized permethrin to 4′-HO-permethrin and other compounds, and most of the metabolic activity was dependent on NADPH. Since the activity of degrade permethrin was much greater for the P450 enzymes from strain JPal-per than from the susceptible strain and the activity was inhibited by PBO and PTPE, P450 monooxygenases were clearly shown to play a major role in the resistance to permethrin in JPal-per larvae.

In order to identify the P450 isoform(s) contributing to the resistance, multiple degenerate primers were designed based on conserved amino acid domains reported in insects including houseflies, fruit flies, anopheline mosquitoes and cotton bollworms. PCR products amplified from gut cDNA of 4th instar larvae were purified, cloned into the plasmid vector, and sequenced. Overall, 11 novel P450s were identified and they

* See Part II for the full Japanese article.

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were classifiable into subfamilies 4 and 6. We then screened two of these clones from a gut cDNA library and determined the full-length cDNA sequences, which were designated *CYP6E1* and *CYP6F1*. The deduced amino acid sequences of these P450s possess conserved domains of a membrane-anchoring signal, reductase binding sites, heme-binding sites, and substrate recognition sites for a typical P450. Moreover, real time quantitative PCR revealed that expression levels of these two P450 genes were several times higher in JPal-per than the susceptible strain suggesting the possible involvement of these P450s in pyrethroid resistance.

PYRETHROID RESISTANCE AND HOUSEFLY P450S

The housefly is an important insect not only as a nuisance pest but also as a transmitter of bacteria including enterohemorrhagic *Escherichia coli* O157. The LPR strain of housefly was originally collected from a dairy farm in New York State. After selection, this strain developed immensely high levels of resistance to pyrethroid insecticides. Scott *et al*. previously demonstrated that the major mechanism of the resistance was detoxification mediated by P450 monooxygenases. The P450 involved in the resistance (CYP6D1) was identified and it was revealed that the level of *CYP6D1* mRNA was about 10 times higher in LPR than the susceptible strain, due to increased gene transcription. In order to identify the regulatory elements of *CYP6D1* gene expression and the gene sequences which they bind, we analyzed the 5′ flanking region of *CYP6D1* from 5 pyrethroid-susceptible strains. The most notable result was that there was a 15-bp fragment in the sequence of LPR close to the transcription initiation site and the insert was found only in the resistant LPR strain. Eventually we identified a nuclear protein, termed *Gfi*-1, which was a predicted gene repressor interacting with the 5′ flanking sequence of *CYP6D1* in pyrethroid-susceptible strains. In order to further clarify the mechanism of *CYP6D1* expression, we screened a housefly genomic library using a DNA fragment of the *CYP6D1* 5′ flanking sequence as a probe. A clone of approximately 14 kbp, which contains a part of *CYP6D1*, was chosen and the whole sequence was determined. One remarkable feature of the sequence was that there was another P450 about 4 kbp upstream of the *CYP6D1* gene. The newly identified P450, termed *CYP6D3*, had a similar genetic structure to *CYP6D1* (5 exons and 4 introns of similar length) and the deduced amino acid sequence was 78% identical to CYP6D1 protein. Thus, it was suggested that one of these genes might have been the result of a duplication event. The expression of *CYP6D3* mRNA was also increased in LPR compared to the susceptible CS strain, suggesting the involvement of a common mechanism(s) in the regulation of the gene expression. On the other hand, despite their nucleotide similarity, there are some differences in the expression pattern of these genes; for instance, *CYP6D1* is expressed only in adults while *CYP6D3* is expressed in larvae as well as adults. Further comparison of these two genes and identification of their respective promoter regions will help to identify the factor(s) involved in their regulation and elucidate the mechanism of insecticide resistance mediated by P450s.

EXHAUSTIVE ANALYSIS OF P450S USING MICROARRAY TECHNIQUES

Microarray analysis is ideal for comparing the expression of a number of genes simultaneously among multiple samples. This system is especially suitable for the analysis of genes of a superfamily like P450s. In order to explore new target sites for novel insecticides, which are safer for mammals and highly specific to the pest insects, and to better understand the mechanisms of insecticide resistance, we utilized this promising method to investigate P450s of fruit flies. We designed gene-specific primers for all P450s of *Drosophila melanogaster* based upon the information obtained from the genome project. The cDNAs of 86 P450s were cloned and sequenced, then insert DNAs were amplified, purified and spotted onto nylon membranes. We first compared the transcriptional levels of P450s between male and female flies. Of the 86 P450s, several isoforms were expressed significantly higher levels in either gender. After an independent verification of the transcriptional levels, *Cyp312a1* expression was confirmed to be more than 80 times higher in male than female flies. This unique phenomenon was universally conserved among three strains of *Drosophila* originally collected from the USA, the UK and Japan. Furthermore, the expression of *Cyp312a1* was mostly observed in the abdomen, and its transcriptional level gradually increased from the pupal stage and peaked in the 5-day-old adult. Given that *Cyp312a1* is a male-specific P450 and its expression was observed only in the abdomen, Cyp312a1 protein is suspected to be involved in catalyzing endogenous active substance(s) such as hormones or pheromones. The discovery of this unique characteristic was achieved with a microarray analysis of P450s. Similarly, the exhaustive analysis of P450s with microarrays should provide an enormous amount of information regarding the function of each isoform and mechanisms of transcriptional regulation, leading to elucidation of the mechanism of insecticide resistance and discovery of new targets for insecticides.