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Gene Expression of Cytochrome P450 1A in Hybrid Catfish (*Clarias gariepinus* × *Clarias macrocephalus*) Exposed to Chlorpyrifos or Carbaryl Insecticides

Chawanrat Somnuek [a], Chuta Boonphakdee[b], Voravit Cheevaporn [a], Keiichi Tanaka [c] and Khobkul Nongnutch [d]

[a]*Department of Environmental Science, Faculty of Science and Technology, Rambhai Barni Rajabhat University, Chanthaburi 22000, Thailand*
[b]*Department of Biology, Faculty of Science, Burapha University, Chonburi 20131, Thailand*
[c]*Faculty of Pharmacy, Osaka Ohtani University, Tondabayashi, Osaka 584-8540, Japan*
[d]*Faculty of Industrial Technology, Rajamangala University of Technology Isan Sakonnakhon campus, 47160, Thailand*

Abstract

Cytochrome P450 1A (CYP450 1A) induction is used widely as a biomarker when assessing exposure to contaminants in environmental systems including pesticides. The effect of chlorpyrifos and carbaryl on CYP450 1A induction was assessed both by measurements of the induction of the EROD activity and the CYP450 1A gene expression in hybrid catfish (*Clarius gariepinus x Clarius macrocepharus*). Fish were treated with several concentrations of chlorpyrifos (0.43, 4.3 or 43 μM), or carbaryl (1.19, 11.9 or 119 μM) for 24 or 48 h. The livers were then assayed for changes. Chlorpyrifos and carbaryl at all concentrations slightly increased the levels of EROD activity after 24 and 48 h and increased gene expression of CYP450 1A mRNA in dose-dependently. Exposure to chlorpyrifos and carbaryl at high concentrations resulted in significant elevation of CYP450 1A gene expression in comparison to control fish ($P<0.05$) at both time intervals. These results showed that the responses of CYP450 1A were more pronounced in gene expression analysis than in EROD assay. This suggests that the induction of CYP450 1A at the gene level was more sensitive than at the protein level. The induction of CYP450 1A following chlorpyrifos and carbaryl exposure indicates the possible use of CYP450 1A as a biomarker for detecting effects of these pesticides in hybrid catfish.

Keywords: cytochrome P450 1A; chlorpyrifos; carbaryl; hybrid catfish; EROD activity; real-time PCR

1. Introduction

Chlorpyrifos (*O,O*- diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothionate) and carbaryl (1-naphthol N-methylcarbamate) insecticides are commonly used in agricultural areas for pest control. Unfortunately, many of these chemicals have had unintended effects on aquatic organisms by aerial overspray or run-off (Somnuek *et al*., 2009).

Exposure to chemicals can be revealed with biomarkers. CYP450 1A induction is one of the best studied parameters as a biomarker for measuring the response of aquatic organisms when assessing exposure to contaminants in environmental systems (Flammarion *et al*., 1998; Cheevaporn and Beamish, 2007). The cytochromes P450 superfamily is one of the largest and functionally most diverse protein families. The CYP450 enzyme system has been detected in all organisms examined, from bacteria to mammals. These enzymes are associated with an extremely important metabolic system because of their involvement in regulating the titers of endogenous compounds such as hormones, fatty acids, and steroids. Additionally, this enzyme system plays a central role in the metabolism of xenobiotics such as drugs, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides (Stegeman and Hahn, 1994; Scott, 1999).

Hybrid catfish (*Clarias macrocephalus X Clarias gariepinus*) are mostly nocturnal scavengers which like taking oxygen on surface water. They live near the bottom in shallow waters which may be contaminated by insecticides; therefore they may be exposed to toxins at all levels of water. As a consequence, toxins can transfer to humans by the food chain. In fish, the liver plays an important role in several vital functions of basic metabolism and it is also a major organ of accumulation, biotransformation and excretion of contaminants, including degradation and bioactivation of insecticides. The ability of fish to biotransform xenobiotics can help predict their susceptibility to contaminants in the environment (Nabb *et al*., 2006).

The induction of 7-ethoxyresorufin O-deethylase (EROD) activity is a commonly used biomarker for exposure to CYP450 1A inducers. However, the gene expression of CYP450 1A is another interesting biomarker which is dose-responsive to environmental

concentrations of different xenobiotics (Fent, 2001). The most accurate method for transcript evaluation is reverse transcription-real time PCR (Dixon *et al*., 2002; Funkenstein *et al*., 2004). Therefore, the objective of this study was to investigate the effect of two broadly used insecticides on both induction of EROD activity and the molecular expression in hybrid catfish. Specifically, we investigated whether this gene expression could be used as a monitoring tool for detecting the effects of these insecticides in hybrid catfish.

2. Materials and Methods

2.1. Animal preparation

Juvenile hybrid catfish weighing 100 to 150 g (approximately 3 months old) were purchased from a local supplier and transported live to the laboratory in aerated tanks. They were acclimatized in glass aquaria containing 200 L with continually aerated water under natural photoperiod. During the acclimatization period, the catfish were fed daily with fish food pellets (Safe feed 7711, Charoen Pokphand Foods PCL, Thailand) weighing about 1% of the body weight, and were then fasting for 24 hours before the experiment. The ambient water temperature was 23±2°C, pH 7.4±0.5 and dissolved oxygen 5±1.5 mg/L. Fish were held for at least 15 days before exposure to the insecticides.

2.2. Insecticide exposure

Chlorpyrifos (95.0% purity) and carbaryl (85.0% purity) were purchased from Gharda Chemicals Limited, Maharashtra, India and Hunan Haili Chemical Industry Co. Ltd., Hunan, China, respectively. The insecticides LC_{50} values were determined and then no effect concentration (NOEC) was subsequently used in this study. The insecticide compounds were diluted in 0.02% acetone in water to obtain the desired concentration then added to the aquaria. In a continued flow system, juvenile hybrid catfish were exposed to a range of NOEC of chlorpyrifos (0.43, 4.3 or 43 µM) and carbaryl (1.19, 11.9 or 119 µM) for 24 and 48 h. In the control tank, only 0.02% acetone was added. Fish were not fed on the day proceeding an initiation of the experiment or during the length of the exposure. At the end of each treatment, surviving fish were sacrificed; the liver was rapidly removed and excised for enzyme determination and CYP450 1A gene expression analysis.

2.3. Enzymatic determinations

Liver from fish was weighed and homogenized in 5 volumes of homogenizing buffer (100 mM K_2HPO_4, 1 mM EDTA, 150 mM KCl and 1 mM dithiothreitol). The homogenates were centrifuged at 10,000 g for 5 minutes at 4 °C. The supernatant (S9) was used for an enzymatic assay performed at room temperature. The principle of the assay is based on the hydrolyzation of the substrate ethoxyresorufin to the fluorescent compound resorufin, a procedure carried out in a 96-well microplate (Stagg and Mcintosh, 1998). Excitation and emission wavelengths for measuring resorufin formation were, respectively, 530 and 590 nm. Resorufin formation was measured every 2 min for 10 min. EROD activities were measured as pmol/min/mg protein. Total protein analysis was conducted with 100 µl of tissue homogenate from each fish using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Life Sciences Group, CA, U.S.A.). Colorimetric analysis was performed following the standard procedure outlined in the protein assay instructions. Bovine serum albumin (BSA) was used to obtain a standard curve from which relative measurements of protein concentration in the sample were made.

2.4. Primer design

Initially, CYP450 1A gene was isolated from liver of hybrid catfish by reverse transcription-polymerase chain reaction (RT-PCR) using degenerate primers designed from conserved regions of CYP450 1A genes in closest species from GENBANK. PCR products from a pair of degenerate primers were cloned and sequenced for specific primer designing. The specific primers of the CYP450 1A gene were then synthesized and used in real-time PCR analysis. Housekeeping genes, 28S rRNA, designed in conserved region of fish and other organisms (Boonphakdee *et al*., 2008) were used to compare with the CYP450 1A gene. The primers used for this study are listed in Table 1.

Table 1. List of primers used in this study

Name	Sequence (5'- 3')
Degenerate primer:	
CYP-L	TCA RYR AYG GMA AGA GYY TG
CYP-R	TTG GMG TTC TCR TCY AGY TT
Specific primer:	
sp-CL	CGA GGG TGA GAG TTC TGA GT
sp-CR	CAG CTT CCT GTC CTC ACA GT
28Soce_L99	CGA AGC CAG AGG AAA ATC TG
28Soce_R334	CCG GGC TTC TTA CCC ATT TA

2.5. RNA preparation and reverse transcription-PCR (RT-PCR)

Total RNA was extracted from liver tissue (~30 mg; *n =5*) from each individual fish using RNeasy Mini Kit (QIAGEN GmbH, Germany) (Miller and Yolken, 2003), according to the manufacturer's instructions. Following determination of RNA concentrations by measuring the absorbance at 260 nm, the relative purity was evaluated by computing the ratio of A_{260} to A_{280} where a ratio of 1.8-2.0 was considered highly pure. Five hundred nanograms per microlitre of RNA was used to generate first strand cDNA using First Strand cDNA Synthesis kit for RT-PCR (AMV) (Roche Diagnostics, GmbH, Germany), according to the manufacturer's instructions, which was stored at −20°C until proceeding.

For the PCR reaction, 2 µl of cDNA from each synthesis were added to 7 µl of "2X PCR master mix" containing 10X PCR buffer, 10 mM dNTP, 25 mM $MgCl_2$, 5 U of *Taq* DNA polymerase (Fermentas, U.S.A.). Twenty µM of each pair of the degenerate primers was added, and the final volume was adjusted to 14 µl with nuclease free water. The mixtures were denatured at 94°C for 3 min. Thirty five cycles of PCR were carried out, with denaturation at 94°C for 1 min, annealing at 60°C for 45 sec, and extension at 72°C for 1 min, followed by a final extension period of 3 min. PCR products were analyzed by electrophoresis on 1% agarose gels stained with GelStar Nucleic Acid Gel Stain (Cambrex Bio Science Rockland, Inc.). An expect size of CYP450 1A DNA band was isolated from gel by QIAquick Gel Extraction Kit (QIAGEN) for cloning and sequencing. The obtained sequences were further used for CYP450 1A specific primers designed.

2.6. Gene expression analysis

After specific primers were designed and tested, total RNA from liver of each treatment was isolated and cDNA synthesized. The cDNA was amplified by real-time PCR and compared with 28S rRNA gene. Fold inductions of CYP450 1A gene expression with the 28S rRNA gene were measured, 1 µl of cDNA was used in a total volume of 10 µl, using LightCycler FastStart DNA Master SYBR Green I (Roche) following the instructions from the supplier. Real-time PCR reaction was conducted for 10s at 95 ºC, 8s at 60 ºC, and 20s at 72 ºC for 45 cycles. The CYP450 1A and 28S rRNA gene ratios were established for each treatment using LightCycler Software 4.05.

2.7. Statistical analysis

All experiments were repeated at least five times. The results are reported as means ± S.E. Statistical differences between treatments and time of exposure were assessed by SPSS software package. Values of $P<0.05$ were considered to be significant.

3. Results

3.1. Effect of chlorpyrifos and carbaryl on EROD activity

Exposure of chlorpyrifos and carbaryl at all concentrations to hybrid catfish resulted in increased EROD activities, which, however, did not reach statistical significance. The levels of EROD activity showed the dose-response slightly increased after 24 and 48 h. The highest induction of EROD activity was found at 43 µM and 119 µM of chlorpyrifos and carbaryl, respectively (Fig. 1).

3.2. Gene expression analysis

RT-PCR products of CYP450 1A gene amplified from RNA using degenerate primers was detected in agarose gel and showed a band at the size of 553 bp

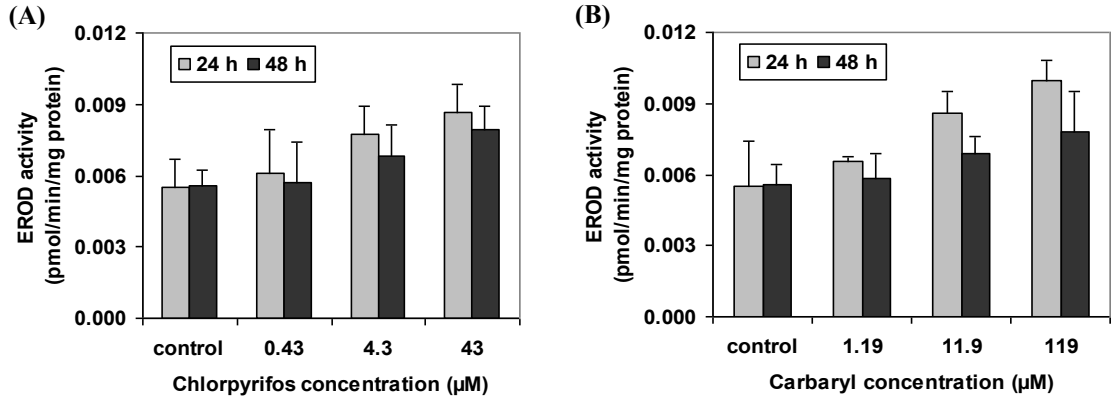

Figure 1. EROD activity of hybrid catfish exposed to chlorpyrifos (A) and carbaryl (B) at different concentrations and time exposure (*n=5*)

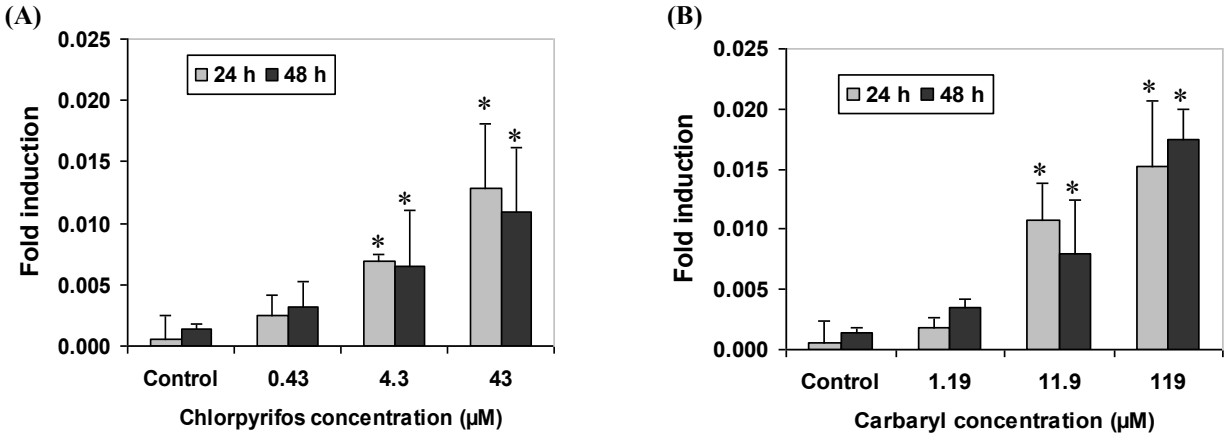

Figure 2. Fold induction of CYP450 1A gene expression of hybrid catfish exposed to chlorpyrifos (A) and carbaryl (B) at different concentrations and time exposure (* = significantly different from control, $P<0.05$). The highest induction was found in fish exposed to 43 μM of chlorpyrifos and 119 μM of carbaryl when compared to the control group

(data not shown). The fragment of PCR product was extracted from gel for cloning and sequencing. Specific primers for real-time quantitative analysis were then designed, and nucleotide sequences of the obtained CYP450 1A amplified products were 439 bp long.

Quantitative real-time PCR analysis of mRNA levels of CYP450 1A from hybrid catfish agreed with the trends obtained for EROD values. However, chlorpyrifos and carbaryl were shown to significantly induce CYP450 1A expression at higher concentrations. These insecticides were found to activate and up-regulate CYP450 1A at the transcriptional level which was demonstrated by dose-dependent increase in CYP450 1A mRNA levels after 24 and 48 h exposure (Fig. 2). The highest induction was observed in 43 μM of chlorpyrifos and 119 μM of carbaryl.

4. Discussion

The detoxifying system protects aquatic species from endogenous and exogenous compounds. Induction of CYP450 1A is extensively used as an indicator of exposure and response to organic pollutants in teleost fish and other vertebrates (Stegeman and Hahn, 1994). The induction of CYP450 1A is known to be regulated by the cytosolic aryl hydrocarbon receptor (AhR) complex. The receptor-inducer complex apparently binds to a translocating factor, which allows the complex to enter the nucleus. Once inside the nucleus, the complex attaches to specific sites on DNA, distorting the DNA chain and resulting in transcription of mRNA that codes for CYP450 1A. The mRNA subsequently translates into new CYP450 1A protein (Hahn *et al*., 1992)

Organophosphate (OP) and carbamate (CB) insecticides, including chlorpyrifos and carbaryl, are primarily recognized by their anticholinesterase action. However, they are also metabolized by CYP450. Chlorpyrifos is activated to chlorpyrifos-oxon through a CYP450-catalyzed desulfuration reaction (Fukuto, 1990), and Matsumusa (1975) demonstrated that carbaryl can be hydrolyzed by oxidized CYP450 to form both hydrolysis and hydroxylation products, respectively.

In this study, chlorpyrifos and carbaryl were found to increase CYP450 1A both on protein and gene levels. The induction was characterized by a dose-dependent increase of EROD activity, which is correlated well with CYP450 1A mRNA levels in liver. Induction of CYP450 1A caused by exposure to organic compounds, by which chemicals stimulate the rate of gene transcription, results in increased levels of messenger RNA. The CYP450 protein is then synthesized and modified to give the catalytically active enzyme (Goksoyr and Forlin, 1992). The highest induction was found in fish exposed to 43 μM of chlorpyrifos and 119 μM of carbaryl both in EROD activity and CYP450 1A gene expression when compared to the control group. This result may be due to a rapid induction of CYP450 1A which can be detected within 24 h after exposure.

With chlorpyrifos, the effects on CYP450 occur similarly to that described by Eamkamon et al. (2005). They found in shrimps (*Peneaus monodon*), that exposure to a high concentration of chlorpyrifos (1000 μg/L) resulted in significant elevation of CYP450 gene expression in comparison to control. Induction of CYP450 1A in fish exposed to carbaryl has been studied by Ledirac *et al*. (1997), Denison *et al*. (1998), Delescluse *et al*. (2001), and Ferrari *et al*. (2007).

There are several hypotheses explained the inductions of CPY450 1A. For example, Danison *et al*. (1998) demonstrated that carbaryl can competitively bind to the AhR and that can activate AhR-dependent gene expression in cultured cells. They proposed that the compound mentioned above may in fact be

very weak ligands of AhR, but the demonstration of their ability to competitively bind to the receptor has been difficult, due to the extremely high AhR-binding affinity of strong inducers. Ledirac *et al*. (1997) stated that CYP450 1A is induced without binding to the AhR, but may act through another signaling pathway.

In conclusion, organophosphate and carbamate insecticides are primarily recognized by their anticholinesterase action. However, they have been reported to affect detoxifying responses in various species, including fish (Delescluse *et al*., 2001). In this study, we demonstrated that these insecticides have genotoxic effects in hybrid catfish. An early increase of CYP450 1A mRNA levels may be an adaptative response to detoxify itself from insecticides. The responses observed in liver showed similar trends in both measurements, EROD activity and CYP450 1A gene expression, in hybrid catfish exposed to chlorpyrifos and to carbaryl. However, the CYP450 1A gene was found prominently expressed in fish exposed to these insecticides. Therefore, our studies suggest that CYP450 1A gene expression could be used as an effective tool for biomonitoring of environmental contamination by chlorpyrifos and carbaryl insecticides.

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Correspondence to
Dr.Chawanrat Somnuek
Department of Environmental Science,
Faculty of Science and Technology,
Rambhai Barni Rajabhat University,
Chanthaburi 22000,
Thailand
Email: schwanrat@hotmail.com