Gene expression of acetylcholinesterase in hybrid catfish (Clarias gariepinus X Clarias macrocephalus) exposed to chlorpyrifos and carbaryl

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Abstract: After serving as a neurotransmitter, acetylcholine is hydrolyzed by acetylcholinesterase (AChE). Inhibition of AChE is considered to be a specific biomarker for exposure to organophosphate and carbamate insecticides. In this study, the AChE gene was isolated from hybrid catfish brain by RT-PCR methods using degenerate primers. The amplified fragment of 966 nucleotides generated by PCR was cloned, and sequence analysis showed 82% nucleotide identity with AChE of the Electrophorus electricus. Specific primers of the hybrid catfish brain by RT-PCR methods using degenerate primers. The amplified fragment of 966 nucleotides generated by PCR was then synthesized and used in the examination of AChE gene expression in brain tissue of hybrid catfish exposed to sublethal concentrations of chlorpyrifos (0.43, 4.3 and 43 μM) and carbaryl (1.19, 11.9 and 119 μM) for 24 hr. Real-time PCR was used to compare with the amplified 28S rRNA gene. AChE gene expression was significantly elevated 12.4 times in catfish exposed to 43 μM chlorpyrifos in comparison to the control group (p<0.05). Carbaryl did not produce any significant change. These results indicate that AChE is more sensitive to administration of chlorpyrifos than carbaryl. The induction of the AChE gene indicates the possibility of using this gene as biomarker for detecting effects of organophosphate insecticides in hybrid catfish.

Key words: Acetylcholinesterase, Organophosphate, Carbamate, Chlorpyrifos, Carbaryl, Hybrid catfish, Real-time PCR

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Introduction

Organophosphate (OP) and carbamate (CB) insecticides are widely used in agriculture. The primary molecular mechanisms of action of the OP and CB are based on the inhibition of the enzyme acetylcholinesterase (AChE) (Triak et al., 2003), which hydrolyses the neurotransmitter acetylcholine in cholinergic synapses of the central and peripheral nervous system, both in vertebrates and invertebrates (Agrahari et al., 2006). Inhibition of AChE leads to the accumulation of acetylcholine in the nerve terminals, resulting in excessive stimulation of postsynaptic cholinergic receptors (Pope, 1999) and the continuous stimulation of the post-synaptic membrane, a process that may lead to death. OP and CB can distribute to aquatic environments through surface runoff, sprays and soil leachate. Therefore, both invertebrates and vertebrates might be exposed to these insecticides, especially fishes which consume aquatic organisms. Several studies have used cholinesterase activity as a tool to diagnose the exposure of fish to OP and CB insecticides or to assess their neurotoxic effects (Boone and Chambers, 1997; Bocquene et al., 1990; Gruber and Munn, 1998; Varo et al., 2003, Tahara et al., 2005; Johal et al., 2007).

Hybrid catfish (Clarias macrocephalus X Clarias gariepinus) are a favorite food of people. Catfish are mostly nocturnal scavengers which like to take 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. Most studies monitoring of the AChE activities in fish have been based on measuring the enzyme levels using method of Ellman et al. (1961), and this has been suggested as a diagnostic biomarker, with decreased activities indicating water contamination by OP and CB insecticides (Coppage and Braidech, 1976; Zinkl et al., 1987; Roriguez-Fuentes and Gold-Bouchot, 2000; Ferrari et al., 2004). In addition, changes at the gene level can be used as indicators of chemical contamination, such as metallothionein gene expression reflecting metal toxicity (Lam et al., 1998; Chan et al., 2006) and expression of the cytochrome P450 gene in organisms exposed to pesticides (Ledirac et al., 1997; Alimi et al., 2002), or polycyclic aromatic hydrocarbons (PAHs) (Barouki and Morel, 2001). AChE gene expression is obviously the basis for the cholinergic neuronal signaling, both in the central and the peripheral nervous system (Damodaran et al., 2003). Even moderate changes in neuronal excitability may lead to overt modulations in brain gene expression (Kaufert al., 1999), yet gene expression analysis of AChE has only rarely been performed in fish exposed to OP and CB insecticides.

Therefore, the aims of this study were to clone, sequence the AChE gene, and to design a specific primer of the AChE gene. Specifically, we investigated the usefulness of this molecular
technique as a monitoring tool for detecting the effects of OP and CB insecticide in hybrid catfish.

Materials and Methods

Animal preparation: Hybrid catfish weighing an average 100 to 150 g (approximately 2-3 months old) were purchased from a local supplier and transported live to the laboratory in aerated tanks. They were acclimatized in glass aquaria (200 l) with continuously aerated tap water under natural photoperiod for at least 15 days. 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 fasted for 24 hr before the experiment. Fish were sacrificed, the brain was rapidly removed, weighed, and dissected for RNA extraction and sequencing.

For pesticide exposure in gene expression analysis, chlorpyrifos (95.0% purity) and carbaryl (85.0% purity) were purchased from local distributors. The compounds were diluted in 0.02% acetone in water to obtain the desired concentration and then added to the aquaria. In a static flow system, the sublethal concentrations tested were 0.43, 4.3 and 43 µM of chlorpyrifos and 1.19, 11.9 and 119 µM of carbaryl for 24 hr. In the control tank, only 0.02% acetone was added. Treated fish were sacrificed, the brain was rapidly removed for RNA extraction followed by reverse transcription and fold induction of gene expression between AChE and 28S rRNA genes, and were then analyzed by real-time PCR.

Total RNA isolation: Total RNA was extracted from the brain of hybrid catfish using RNeasy Mini Kit (QIAGEN GmbH, Germany) (Miller and Yolken, 2003), according to the manufacturer’s instructions. RNA was analyzed in 1% agarose gel, containing ethidiumbromide and visualized with UV light. The 1 Kb DNA ladder plus and 100 bp DNA ladder plus (Fermentas, USA) was used as molecular marker. RNA was quantified by spectrophotometry and all samples were adjusted to 500 ng µl⁻¹.

Reverse transcription-polymerase chain reaction (RT-PCR): Complementary DNA (cDNA) was synthesized by using First Strand cDNA Synthesis kit for RT-PCR (AMV) (Roche Diagnostics, GmbH, Germany), according to the manufacturer’s instructions. PCR amplification used degenerate primers. Primers of AChE gene were designed in conserved region of fishes and other organisms from GENBANK using CODEHOP program. Primer sequences were; FL1: 5'- GAA CCC CAA CAG GGA GAT G A/T C/G A/C/T/G GA A/G GA C/T TG-3' and FR1: 5'- CAC GGG GCA GAT CAC GT G/T A/G T A/G A/G T C A/G/T/C CC-3'.

For the PCR reaction, 4 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 MgCl₂, 5 U of Taq DNA polymerase (Fermentas, USA). Twenty µM of each pair of the 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 45 sec, annealing at 57°C for 30 sec, and extension at 72°C for 1 min, followed by a final extension period of 5 min. PCR products were analyzed by electrophoresis on 1% agarose gels stained with GelStar Nucleic Acid Gel Stain (Cambrex Bio Science Rockland, Inc.).

Fig. 1: (A) Gel electrophoresis of RNA extraction from hybrid catfish’s brain (M1 = 1 Kb DNA ladder plus, lane 1 = Bands of 28S rRNA and 18S rRNA) (B) RT-PCR product size 966 bp of AChE gene amplified from FL1 and FR1 primers (M2 = 100 bp DNA ladder plus, lane 2 = Band of RT-PCR product of 966 bp)
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Fig. 2: Alignment of the AChE hybrid catfish nucleotide sequence with *Danio rerio* acetylcholinesterase (AChE), mRNA (GenBank Accession No. NM_131846), *Electrophorus electricus* acetylcholinesterase catalytic subunit precursor gene, complete cds (GenBank Accession No. gb|AF030422.1| AF030422) and *Carassius auratus* acetylcholinesterase mRNA, partial cds (GenBank Accession No. AY847745)
Cloning and sequencing: PCR products were isolated from gel by QiAquick Gel Extraction Kit (QiAGEN) and ligated into pCR 2.1-TOPO vectors (Invitrogen™ Life Technologies, Carlsbad, USA) according to the manufacturer’s instructions, and transformed into high efficiency E. coli competent cells (Invitrogen). Positive clones (white colonies) which have insert size of AChE gene were confirmed by PCR with M13 primer pairs. The plasmids containing insert of the correct size were grown in 1.5 ml of LB broth with appropriate antibiotic and shaken at 37°C for 18 hr. Plasmids were purified using a QiAprep Spin Miniprep Kit (QiAGEN) and completely sequenced on both strands analyzed by First Base Laboratories Sdn Bhd, Malaysia.

Gene expression of AChE in hybrid catfish: The sequences of the AChE gene designed by the Primer3 program for use as specific primers (Rozen and Skaltsky, 2000) were Int. L (5’ - GGT GGT GGT CTC CAT GAA CT) and Int. R (5’ - CAT CAA CCA CAG GCA CAA AC). Housekeeping genes, 28S rRNA, were designed in conserved region of fishes and other organisms from GENBANK to compare with the AChE gene. The sequences of 28S rRNA were 28S-L (5’- CGA AGC CAG AGG AAA ATC TG) and 28S-R (5’- CCG GGC TTC TTA CCC ATT TA).

Fold inductions of AChE gene expression with the 28S rRNA gene were measured by real-time PCR 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 5s at 95°C, 5s at 60°C, and 15s at 72°C for 55 cycles. The AChE and 28S rRNA gene ratios were established for each treatment using LightCycler Software 4.05.

Results are expressed as means ± SE. ANOVA were used to test for differences between each treatment.

Results and Discussion

Cloning and sequencing the AChE gene in hybrid catfish: Intact RNA displaying the expected 2:1 ratio between 28S and 18S ribosomal RNA bands are shown in Fig. 1A. RT-PCR products of the AChE gene amplified from brain of hybrid catfish were detected as a band in 1% agarose gel at the approximate size of 1,000 bp. The fragment of AChE was extracted from the gel and cloned before sequencing. Nucleotide sequences of AChE cDNA were obtained 966 bp long (Fig. 1B). A search for homology of the nucleotide sequence with other AChEs in the GenBank/EMBL Data Bank (January 10th, 2008) revealed that the hybrid catfish AChE nucleotide sequences were highly similar to those of other teleost sequences. The AChE sequence from hybrid catfish was 82% identical with Electrophorus electricus AChE catalytic subunit precursor gene, complete codons (GenBank Accession No. AF030422.1), 77% identical with Danio rerio acetylcholinesterase (AChE), mRNA (GenBank Accession No. NM_131846) and Carassius auratus acetylcholinesterase mRNA, partial cds (GenBank Accession No. AY847745) (Fig. 2).

Gene expression of AChE in hybrid catfish: After comparing the AChE gene with the 28S rRNA gene in catfish exposed to different levels of chlorpyrifos and carbaryl, the results indicated that there was no alteration in 28S rRNA levels at any concentrations of the insecticides. The results also revealed that the level of fold induction of the AChE gene increased with increasing concentrations of chlorpyrifos and was significantly increased 12.4 times in catfish exposed to 43 µM chlorpyrifos when compared to the control group (Fig. 3). In the carbaryl group, the fold induction increased with higher concentrations of carbaryl, but the increase did not reach statistical significance at any concentration (p>0.05) (Fig. 4). AChE gene expression is an important part of the cholinergic neuronal systems for the maintenance of central nervous system homeostasis (Damodaran et al., 2003). Even moderate changes in neuronal excitability may lead to overt modulations in brain gene expression (Kaufer et al., 1999).

In our previous study, we showed that sublethal concentrations of chlorpyrifos and carbaryl can rapidly inhibit AChE activity within 24 hr in hybrid catfish, and that there is an increased inhibition of AChE with increasing concentrations of the insecticides.
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(Somnuek et al., 2007). Inhibition of AChE enzyme levels can be correlated to induction levels of AChE mRNA in the midbrain of Sprague-Dawley rats (Khan et al., 2000). In order to verify whether or not the AChE gene could be modulated when catfish were exposed to the insecticides we therefore investigated gene expression of AChE. The results showed that chlorpyrifos and carbaryl can alter the gene expression of AChE in hybrid catfish brain. However, only the highest concentration of chlorpyrifos (43 μM) resulted in a significant elevation of the gene expression level (p<0.05) in comparison to the control group. This up-regulation may be ascribed to the feedback response to transcription to depressed cholinergic neurotransmission, leading to elevated levels of brain acetylcholine following OP and CB exposure. An autologous feedback response could regulate transcriptional elevation from the AChE gene through insecticide complexes acting on signaling intracellular pathways, as suggested by Kaufer et al. (1999). They found in mouse brain, that diisopropylfluorophosphonate (DEF) (an OP insecticide) and pyridostigmine (a CB insecticide) increased levels of AChE mRNA over controls within 30 minutes; using sagittal hippocampal brain slices of mice. Similar results have been obtained by in Sprague-Dawley rats within 1 hr after exposure to sarin by Damodaran et al. (2003) who found differences in induction of AChE mRNA levels between different regions of the brain.

These results show that inhibition of AChE can be correlated to the AChE gene expression. The sublethal concentrations of the insecticides used in the present study reduced AChE activity. However, it appears that the concentrations of carbaryl were not high enough to produce any significant change in AChE gene in hybrid catfish. Therefore, we conclude that AChE gene expression is more sensitive to chlorpyrifos than to carbaryl, as reflected by the up-regulation of AChE gene expression within 24 hr.

The early biological changes caused by xenobiotics may not be manifest as pathological findings, but rather as inductions in the expression of the genes. In the case of the AChE gene, we observed such inductions in the hybrid catfish following the exposure to chlorpyrifos, indicating a potential biomarker for the early detection of organophosphate contamination. Results from previous (Somnuek et al., 2007) and this study indicated that gene expression technique is quite sensitive, rapid and reliable than AChE enzyme activities and should be applied as a screening tool for detection of the insecticides contamination in fish.

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References


