Journal of
Biological Sciences

Bioactivation of Chlorpyrifos in the Riceland Prawn, *Macrobrachium lanchesteri*

W. Tongbai and P. Damrongphol

Department of Biology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand

Abstract: Metabolic activation of chlorpyrifos to chlorpyrifos-oxon via cytochrome-P450 (CYP450) in the riceland prawn *Macrobrachium lanchesteri* was examined. Direct effects of chlorpyrifos-oxon on Acetylcholinesterase (AChE) activity was studied *in vitro* by exposing *M. lanchesteri* supernatants to chlorpyrifos-oxon. And the role of CYP450 in the bioactivation was investigated *in vivo* by treating *M. lanchesteri* with piperonyl butoxide, a potent CYP450 inhibitor, prior to chlorpyrifos exposure. The study indicated that chlorpyrifos-oxon was a more potent AChE inhibitor than chlorpyrifos. However, chlorpyrifos concentrations which did not inhibit AChE activity *in vitro* exerted AChE inhibition *in vivo*. CYP450 played an important role in the bioactivation of chlorpyrifos since *M. lanchesteri* pretreated with 500 μg L^{-1} piperonyl butoxide for 24 h prior to 1.5 μg L^{-1} chlorpyrifos exposure for 96 h significantly attenuated the inhibition of AChE activity caused by chlorpyrifos. Nevertheless, pretreatment with piperonyl butoxide did not alter the effects of chlorpyrifos in reducing catalase activity, increasing lipid peroxidation as evidenced by increased thiobarbituric acid reactive substance levels and inducing histopathological changes such as swelling of the gills and degeneration of the hepatopancreatic cells. In conclusion, chlorpyrifos was bioactivated to chlorpyrifos-oxon in *M. lanchesteri* via CYP450. Chlorpyrifos-oxon was a strong AChE inhibitor but did not alter the effects of chlorpyrifos in inducing oxidative stress and histopathological changes.

Key words: Chlorpyrifos, cytochrome-P450, acetylcholinesterase, piperonyl butoxide, oxidative stress, prawn

INTRODUCTION

Organophosphate Pesticides (OPs) have been widely used to control agricultural pests but are frequently harmful to non-target aquatic organisms due to contamination of the aquatic environment by drainage from agricultural areas (Roche *et al.*, 2007; Joseph and Raj, 2011). Chlorpyrifos is one of the most widely used OPs; it is highly toxic to non-target organisms, particularly arthropods (Crane *et al.*, 2003; Demirel, 2007; Deligeorgidis *et al.*, 2008). The primary effect of chlorpyrifos is an irreversible inhibition of Acetylcholinesterase (AChE), an enzyme at the cholinergic synapses in the nervous systems of both vertebrates and invertebrates, resulting in an accumulation of neurotransmitter acetylcholine at the synapses (Taylor, 1990; Mohammad *et al.*, 2007). Such accumulation causes hyperactivity, paralysis and finally death of the organism (Fulton and Key, 2001).

Chlorpyrifos is a relatively weak AChE inhibitor but it undergoes metabolic activation within an organism to become a more potent AChE inhibitor, chlorpyrifos-oxon, via a group of enzymes called cytochrome-P450 (CYP450) isozymes (Ankley and Collyard, 1995; Mutch and Williams, 2006). Studies in mammalian system indicate that the rate of activation is dependent on the level of CYP450 (Smith *et al.*, 2009). The presence of piperonyl butoxide, a non-toxic and potent CYP450 inhibitor, reduced the toxic effects of chlorpyrifos on mortality, malformation and AChE activity in water flea *Ceriodaphnia dubia* and South African clawed frog *Xenopus laevis* (El-Merhibi *et al.*, 2004). AChE also plays a role in development of neurons and network formation (Paraoanu and Layer, 2008). Chlorpyrifos was found to interfere with both cholinergic and neural developmental effects of AChE. Exposure to chlorpyrifos-oxon using rat (Yang *et al.*, 2008) or zebrafish (Jacobson *et al.*, 2010) embryo model disrupted neural growth. However, information on chlorpyrifos bioactivation and the role of AChE in neural development in aquatic invertebrates especially freshwater crustaceans are limited.

Chlorpyrifos induces oxidative stress in an organism by over-production of Reactive Oxygen Species (ROS), which are highly reactive thus damaging biological molecules and subsequent increased lipid peroxidation (Livingstone, 2001). Chlorpyrifos also causes alterations in activity of antioxidant enzymes such as catalase (Kavitha and Rao, 2008) and causes histopathological alterations in intoxicant organisms (Ahmed *et al.*, 2010).

Macrobrachium lanchesteri De Man are small freshwater prawns commonly found in the vicinity of agricultural areas throughout Southeast Asia. They are subject to risks of exposure to agrochemicals including OPs and are good bioindicator for freshwater

ecotoxicological assessment (Shuhaimi-Othman *et al.*, 2006). The objectives of the present study were to investigate chlorpyrifos bioactivation in freshwater crustaceans using *M. lanchesteri* as a model. Direct exposure of *M. lanchesteri* supernatants to chlorpyrifos-oxon was carried out to study the effects of chlorpyrifos-oxon on AChE *in vitro*. And pretreatment of *M. lanchesteri* with piperonyl butoxide prior to chlorpyrifos exposure was performed to investigate the role of CYP450 *in vivo*. Furthermore, the effects of chlorpyrifos on catalase activity, lipid peroxidation and histopathology were also examined.

MATERIALS AND METHODS

Chemicals and supplies: Chlorpyrifos (99.5% purity) and chlorpyrifos-oxon (94.0% purity) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Piperonyl butoxide, Acetylthiocholine iodide (ATChI), 5,5-dithio-bis-2-nitrobenzoate (DTNB), 1-chloro-2,4-dinitrobenzene(CDNB) and 1,1,3,3-tetramethoxypropane were purchased from the Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Duration of study: The studies were carried out from June 2009 to December 2010.

Prawn samples: Adult *M. lanchesteri* (0.3-0.5 g in weight and 3.0-4.0 cm in length) were purchased from a local market in Bangkok, Thailand. They were acclimatized under laboratory conditions in an aerated glass aquarium (50x120x50 cm) containing about 200-300 L of water at 25-27°C, 12 h : 12 h light: dark cycle for about one wk. They were starved for 24 h prior to experimentation.

Preparation of supernatant: The whole bodies of *M. lanchesteri* instead of separate organs were used for the study due to their small size. The exoskeleton of each prawn was removed; and the whole body was cut into small pieces and homogenized in 5 mL ice-cold homogenization buffer (100 mM sodium phosphate buffer, pH 7.2). Crude homogenate was centrifuged twice at $10,000 \times g$, 4°C for 20 min each. Supernatant was collected for biochemical assay immediately or stored at -70°C until use.

Effects of chlorpyrifos-oxon on AChE activity *in vitro*: Stock solutions of chlorpyrifos-oxon and of chlorpyrifos were prepared in absolute ethanol. Five μL of various concentrations of chlorpyrifos-oxon or chlorpyrifos solution were incubated with 495 μL of pooled prawn supernatant to give final concentrations of chlorpyrifos-oxon or chlorpyrifos ranging from 10^{-9} to 10^{-4} M. Five μL of water or absolute ethanol were used as control and vehicle control. The mixture was incubated at 25°C for 30 min. AChE activity was determined using the method of Ellman *et al.* (1961). Briefly, 75 μL mixture were incubated with 875 μL reaction buffer (100 mM sodium phosphate buffer, pH 8.0 and 0.36 mM DTNB) at 25°C for 10 min. Then, 50 μL of substrate solution (10 mM ATChI in 100 mM sodium phosphate buffer, pH 8.0) were added. The absorbance was recorded every 30 sec for 3 min at 412 nm. AChE activity was expressed as nmol substrate hydrolyzed min^{-1} mg total protein^{-1}. Four replicate experiments were performed.

Effects of piperonyl butoxide: Chlorpyrifos and piperonyl butoxide were dissolved in acetone. Prawns were randomly divided into five groups each of 30 prawns. Group I was the control; group II was the vehicle control exposed to 0.05 % acetone; group III was the piperonyl butoxide treated control exposed to 500 μg L^{-1} piperonyl butoxide for 24 h; group IV was the experimental group pretreated with 500 μg L^{-1} piperonyl butoxide for 24 h prior to 1.5 μg L^{-1} chlorpyrifos exposure for 96 h and group V was the experimental group exposed to 1.5 μg L^{-1} chlorpyrifos for 96 h. At the end of the experiment, prawns were collected for biochemical and histological studies. Piperonyl butoxide and chlorpyrifos concentrations were selected from preliminary studies. Three replicate experiments were performed.

Biochemical assay: AChE activity was determined as described previously. Catalase activity was determined using the method of Aebi (1984). Lipid peroxidation level was determined by measuring Thiobarbituric Acid Reactive Substance (TBARS) level according to the method of Buege and Aust (1978). The protein content of prawn supernatant was determined following the method of Bradford (1976).

Histological study: The gills and the hepatopancreases were dissected out and fixed in Bouin's solution for 24 h before dehydrating through a graded series of ethanol solutions. They were cleared with xylene, embedded in paraffin, cut at 5 μm thickness and stained with hematoxylin-eosin. Histological alterations were observed using an Olympus BX51 microscope (Olympus Optical Co., Tokyo) and the images were captured with an Olympus DP50 digital camera.

Statistical analysis: Statistical significance of differences were set at p<0.05 calculated with one-way Analysis Of Variance (ANOVA) followed by Duncan's test using the SPSS statistical package program (SPSS, Chicago, IL.).

RESULTS

Effects of chlorpyrifos-oxon on AChE activity *in vitro*:
The presence of 10^{-9} M chlorpyrifos-oxon did not inhibit
M. lanchesteri AChE activity *in vitro*; however, a
concentration at 5×10^{-9} M significantly inhibited (p<0.05)
AChE activity and a concentration at 10^{-8} M or higher
almost completely inhibited AChE activity (Fig. 1).
However, chlorpyrifos at the same concentrations did not
have any effect on AChE activity; a high concentration of
chlorpyrifos at 10^{-4} M was required to significantly inhibit
(p<0.05) AChE activity (Fig. 1).

Effects of piperonyl butoxide on biochemical alteration:
There were no significant differences in *M. lanchesteri*
AChE activities among the control, the vehicle control
and the piperonyl butoxide treated control. Pretreatment
with 500 μg L^{-1} piperonyl butoxide for 24 h prior to
chlorpyrifos exposure caused significantly lower (p<0.05)
AChE activity than those of the controls but higher
(p<0.05) activity than those exposed to chlorpyrifos
without piperonyl butoxide pretreatment. Exposure to
1.5 μg L^{-1} chlorpyrifos for 96 h markedly inhibited AChE
activity (Fig. 2a). In contrast, piperonyl butoxide
pretreatment did not change the effect of chlorpyrifos on
catalase activity or TBARS level. Exposure to 1.5 μg L^{-1}
chlorpyrifos for 96 h with or without piperonyl butoxide

pretreatment reduced catalase activity (Fig. 2b) but
increased TBARS level (Fig. 2c). There were no significant
differences (p>0.05) in the activities of catalase
or in TBARS levels between these two groups. Again, no
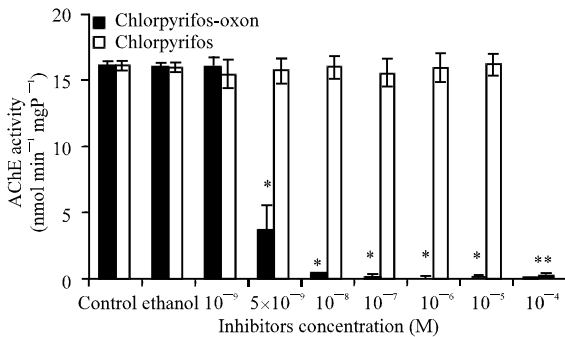significant differences (p>0.05) in the activities of catalase

Fig. 1: Effects of chlorpyrifos-oxon on *M. lanchesteri*
AChE activity *in vitro*. Five μL of chlorpyrifos-
oxon or chlorpyrifos solution were incubated with
495 μL prawn supernatant at 25°C for 30 min; the
AChE activity was determined by adding 75 μL of
mixture into 875 μL reaction buffer (100 mM sodium
phosphate buffer, pH 8.0 and 0.36 mM DTNB) at
25°C for 10 min; then, 50 μL of substrate solution
(10 mM ATChI in 100 mM sodium phosphate
buffer, pH 8.0) The absorbance was recorded
every 30 sec for 3 min at 412 nm. Each bar
represents mean±SE from four replicate
experiments. *Indicates significantly different from
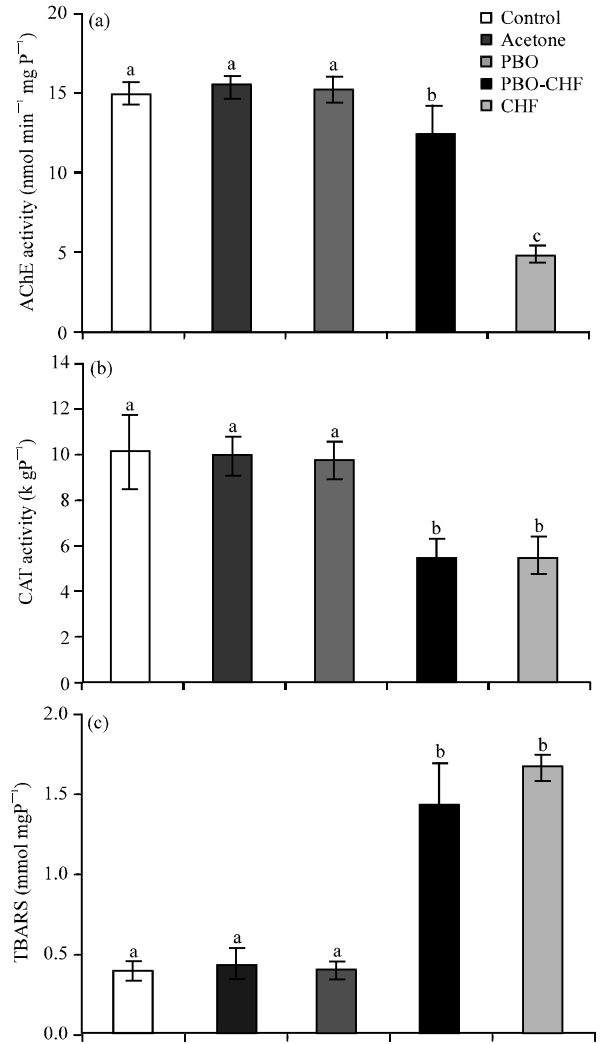the control

Fig. 2(a-c): Effects of piperonyl butoxide on
M. lanchesteri (a) AChE activity, (b) catalase
activity and (c) TBARS level. Each experiment
comprised five different groups: the control,
the vehicle control, the 500 μg L^{-1} piperonyl
butoxide treated control, the group pretreated
with 500 μg L^{-1} piperonyl butoxide for 24 h
prior to 1.5 μg L^{-1} chlorpyrifos exposure for
96 h and the group exposed to 1.5 μg L^{-1}
chlorpyrifos for 96 h. Each bar represents
mean±SE from three replicate experiments.
Different letters indicate significant differences
(p<0.05) between groups. PBO = Piperonyl
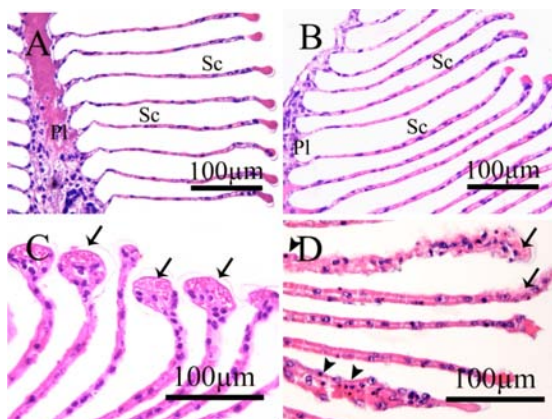butoxide; CHF = Chlorpyrifos

Fig. 3(a-d): Light micrographs of *M. lanchesteri* gill. (A) The control showing primary lamella (Pl) and stacks of secondary lamellae (Sc). (B) The 500 µg L^{-1} piperonyl butoxide treated control showing similar morphology as the control. (C) The group pretreated with 500 µg L^{-1} piperonyl butoxide for 24 h prior to 1.5 µg L^{-1} chlorpyrifos exposure for 96 h showing enlargement at the distal end of secondary lamellae (arrows). (D) The group exposed to 1.5 µg L^{-1} chlorpyrifos for 96 h showing swollen gills, thickening of the secondary lamellae (arrows) and increased hematocyte infiltration (arrow head)

Fig. 4(a-d): Light micrographs of *M. lanchesteri* hepatopancreas. (A) Cross-section of hepatopancreatic tubules of the control showing star-like structure of the lumen and large, relatively round hepatopancreatic cells (B) The 500 µg L^{-1} piperonyl butoxide treated control showing similar morphology as the control. (C) The group pretreated with 500 µg L^{-1} piperonyl butoxide for 24 h prior to 1.5 µg L^{-1} chlorpyrifos exposure for 96 h showing accumulation of hematocytes (He) in the hemocoel, degeneration and detachment of hepatopancreatic cells (arrow head) from the tubules. (D) The group exposed to 1.5 µg L^{-1} chlorpyrifos for 96 h showing degeneration of hepatopancreatic cells in the tubules (Tu)

or the levels of TBARS among the control, the vehicle control and the piperonyl butoxide treated control were observed (Fig. 2b, c).

Effects of piperonyl butoxide on histological alteration:
All controls did not show any histopathological changes of the tissues. The gills of the control and the piperonyl butoxide treated control consisted of a primary lamella and plate-like secondary lamellae (Fig. 3a, b). Exposure to 1.5 µg L^{-1} chlorpyrifos for 96 h with or without pretreatment with 500 µg L^{-1} piperonyl butoxide caused similar histopathological alterations which included edema of the gills, enlargement of the distal ends of the secondary lamellae forming swollen club-shaped ends (Fig. 3c), increased hematocyte infiltration and lifting off of the epithelium from the secondary lamellae (Fig. 3c, d). The hepatopancreas of the control and the piperonyl butoxide treated control contained branched, tubular structures. Cross-section of the tubules showed star shaped lumens; four different types of hepatopancreatic cells: embryonic cells, fibrillar cells, blister-like cells and resorptive cells, were observed
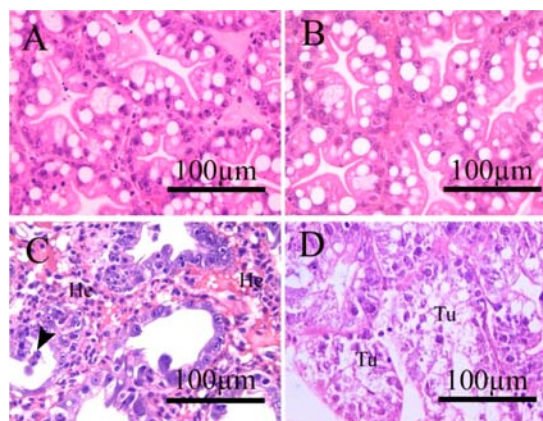
(Fig. 4a, b). Exposure to 1.5 µg L^{-1} chlorpyrifos for 96 h with or without pretreatment with 500 µg L^{-1} piperonyl butoxide for 24 h induced degeneration of the tubules, hematocyte infiltration into the intertubular hemocoels and the lumens became relatively flattened loosing the star-shaped (Fig. 4c, d).

DISCUSSION

In vitro study in the present investigation indicated that chlorpyrifos-oxon was a more potent AChE inhibitor than chlorpyrifos. The concentrations which significantly inhibited AChE activity by chlorpyrifos-oxon did not alter AChE activity by chlorpyrifos. OPs are weak AChE inhibitors because the sulfur moiety of OPs has a lower electronegativity than the oxygen moiety. In an organism, OPs undergo bioactivation into OP-oxon by replacing sulfur with oxygen, which readily binds to a serine hydroxyl group in the active site of AChE leading to the inhibition of AChE activity (Fukuto, 1990). The present

study indicated that chlorpyrifos was first bioactivated into chlorpyrifos-oxon before eliciting AChE inhibition in *M. lanchesteri* since a chlorpyrifos concentration of 1.5 µg L⁻¹ (equivalent to 4.28 nM) did not inhibit *M. lanchesteri* AChE activity *in vitro* but exerted AChE inhibition in *M. lanchesteri in vivo*. Moreover, *M. lanchesteri* pretreated with a CYP450 inhibitor, piperonyl butoxide, reduced the degree of AChE inhibition caused by chlorpyrifos, suggesting an involvement of CYP450 in the bioactivation of chlorpyrifos into a more potent AChE inhibitor *in vivo*. Such findings are consistent with previous studies. Piperonyl butoxide has been reported to decrease the degree of AChE inhibition caused by malathion in Nile tilapia *Oreochromis niloticus* (Pathiratne and George, 1998), diazinon in Medaka *Oryzias latipes* (Hamm *et al.*, 2001), chlorpyrifos in water flea *C. dubia* and South African clawed frog *X. laevis* (El-Merhibi *et al.*, 2004) and to be responsible for insecticide resistance in cotton ballworm *Helicoverpa armigera* (Ramasubramanian and Regupathy, 2004). Furthermore, CYP450 isozymes, CYP2B6 and CYP3A4, have been shown to be major enzymes mediating bioactivation of chlorpyrifos into chlorpyrifos-oxon in the human liver (Mutch and Williams, 2006).

Pretreatment with piperonyl butoxide attenuated the inhibition of *M. lanchesteri* AChE activity by chlorpyrifos but did not alter the effects of chlorpyrifos on catalase activity, lipid peroxidation level and histopathological conditions. Chlorpyrifos-oxon did not seem to mediate these reactivities. Chlorpyrifos itself directly induced oxidative stress in *M. lanchesteri*. This is consistent with previous studies in the amphipods (Steevens and Benson, 1999) and the land snail *Helix aspersa* (Salama *et al.*, 2005). Oxidative stress occurs by over-generation of ROS and/or disruption of antioxidant enzymes. Increased lipid peroxidation production as evidenced by an increased TBARS level is induced by oxidative decomposition of polyunsaturated fatty acids damaged by ROS (Bagchi *et al.*, 1995). Catalase, on the other hand, is a ROS scavenging enzyme decomposing H_2O_2 to H_2O and O_2 (Escobar *et al.*, 1996). The reduction of catalase activity in the present study caused an accumulation of H_2O_2 which in turn enhanced oxidative stress.

Chlorpyrifos induced histopathological alterations in the gills and the hepatopancreas of *M. lanchesteri*. Edema of the gills and swelling at the distal ends of the secondary lamellae are defense mechanisms in response to toxicants in aquatic animals to increase the diffusion distance between external and internal environments. The

marked increase in hematocyte infiltration into the intertubular hemocoels of the hepatopancreas seemed to be an inflammatory response stimulated by damaged tissues. That more striking tissue damage was observed in the hepatopancreas than in the gills may be due to the fact that the hepatopancreas is a high lipid containing organ, thus enhancing accumulation of lipophilic pesticides including chlorpyrifos. Similar histopathological alterations have previously been reported in the giant freshwater prawn *M. rosenbergii* exposed to trichlorfon (Chang *et al.*, 2006), the estuarine crab *Chasmagnathus granulatus* exposed to methyl parathion (Bianchini and Monserrat, 2007) and even in freshwater fish *Glossogobius giuris* obtained from polluted environment (Venkataraman *et al.*, 2007). Histopathological alterations may be the result of oxidative stress. ROS are highly reactive and readily attack cellular molecules leading to loss of membrane permeability and integrity, inactivation of protein, lipid and DNA and eventually dysfunction of cellular homeostasis and cell death (Livingstone, 2001; Ahmed *et al.*, 2010).

CONCLUSION

In conclusion, chlorpyrifos is bioactivated in *M. lanchesteri* into chlorpyrifos-oxon, which is more potent than chlorpyrifos in inhibiting AChE activity. Exposure to chlorpyrifos in the present study reduced catalase activity, increased lipid peroxidation production and induced histopathological alterations in *M. lanchesteri*.

ACKNOWLEDGMENTS

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