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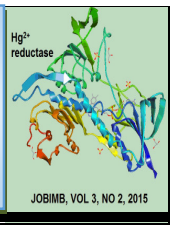

Assessment of Inhibitive Assay for Insecticides Using Acetylcholinesterase from *Puntius schwanenfeldii*

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ABSTRACT

In this study, the substrate specificity and the inhibition kinetics of various types of insecticides to the acetylcholinesterase (AChE) from a local fish; *Puntius schwanenfeldii* were investigated**.** The substrate specificity determination was done using three thiocholine substrates, which were ATC, PTC and BTC**.** The results showed that he partially purified cholinesterase from *Puntius schwanenfeldii* that preferred ATC is a true AChE. The K_m and V_{max} values of AChE for these substrates were 16.61 mmol and 286.5 U/mg for ATC, 19.92 mmol and 245.3 U/mg for PTC, and 48.64 mmol and 219.6 U/mg for BTC, respectively. The IC_{50} values for the carbamates bendiocarb, carbaryl, propoxur, carbofuran and methomyl were 0.838, 7.045, 29.441, 1.411 and 8.335 mg/L, respectively, which were comparable to the IC_{50} values for carbamates from several AChE from fish.

INTRODUCTION

Organophosphorous (OP) and carbamate (CB) insecticides are widely used today especially in the agricultural field. The increase in world food demand encourages farmers to use these pesticides extensively in order to minimize the losses of crops to pests. Unfortunately, only about 10% of the applied pesticides reach the target organisms while the rest of the pesticides disperses in the environment and causes toxicity to non-target organisms. OPs and CBs are responsible for many major accidents such as the Sandoz accident in Switzerland in 1986 [1].

The primer toxicity of OP and CB is through the inhibiting the neurotransmitter-regulating enzyme; acetylcholinesterase (AChE) (Gordon et al., 2006). Both types of insecticide have similar behaviors of action with the exception that the inhibition caused by CBs is generally irreversible [2] while OP does not [3]. These compounds will bind at the enzyme's active site and cause the accumulation of the neurotransmitter acetylcholine on the synaptic spaces and postsynaptic membranes, which can be lethal [4,5].

The inhibition of AChE by the pesticides provides an excellent method as an *in vitro* assay and biomarker for the detection and monitoring of pesticides in the environment as well as other products including agricultural [4,6–9]. The common chromatographic methods for the detection of the presence and concentrations of OPs and CBs are not suitable for a rapid, immediate and a large-scale sample analysis. Chromatographic techniques are expensive, time-consuming, involve complicated procedures, and do not yield the information about the toxicity of the compounds to organisms.

Recently, more attention has been given to evaluate the potential of AChE from fish as bioindicator or *in vitro* assay to monitor OP and CB pollutions [2,3,10–16]. Some of the species that have been used for this purpose are *Cynoscion striatus, Piaractus mesopotamicus* [17], *Sparus aurata* [18], *Oreochromis niloticus* [19], *Carassius auratus* [20], *Aphanius iberus* [21], *Clarias batrachus* [5,22], *Osteochilus hasselti* [9], *Pangasius* sp. [14], *Periophthalmodon schlosseri* [23] *Channa micropeltes* [15] and *Puntius javanicus* [3]. In this study, the sensitivity of AChE from the local fish *Puntius Schwanenfeldii* towards OP and CB insecticides is evaluated for the first time. The aim of this study is to assess the potential of AChE from

this fish as an *in vitro* bioassay for monitoring insecticides pollution.

MATERIALS AND METHODS

Chemicals and specimens

Specimens of the fish *P. Schwanenfeldii* were purchased from Keng Hin Aquarium, Serdang, Selangor Darul Ehsan. Bendiocarb, carbaryl, carbofuran, methomyl, acephate, chlorpyrifos, diazinon, dimethoate, malathion, parathion, acetylthiocholine iodide (ATC), β-mercaptoethanol and procainamide hydrochloride were obtained from Sigma-Aldrich. 5, 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) was purchased from Fluka Chemie GmbH. All other chemicals used in this study were of analytical grade.

Preparation of brain AChE extracts from *Puntius Schwanenfeldii*

The fish were killed by decapitation and the whole brain was dissected out immediately. The extraction procedure was carried out using 0.1 M sodium phosphate buffer pH 8.0. The brains of *P. Schwanenfeldii* were homogenized in Toto 1:5(W/V) for 1 minute on ice using Ultra-Turrax T25 homogenizer fitted with a Teflon pestle. The crude extract was then centrifuged at 15 000×g for 1 hour minutes at 4 ºC using Sorval® Ultra Pro 80-TH-641 to remove debris. The pellet was discarded and the supernatant was used in the further study [5].

Enzyme Activity and Protein Content Determination

The activity of AChE was determined using the Ellman's method [24] with some modification using a 96 well microplate assay. The reaction mixtures contained 50 µl of 0.1 mM ATC, 20 µl of 0.067 M DTNB, 120 µl of 0.1 M sodium phosphate buffer pH 8, and 10 µl of enzyme. The produce was conducted in dark condition at room temperature. The activity of the enzyme was measured using microplate reader at 405nM. The extinction coefficient of the product (thionitrobenzoate ion) is 13.67 $mM^{-1}cm^{-1}$ and the activity was expressed in Unit (1 U=1 µmol of product produced per minutes) per gram of total protein. The protein content was determined quantitatively using the Bradford's method with Bovine Serum Albumin as a standard [25].

Substrate Specificity

The substrate specificity of both AChEs was compared using four selected substrates (ATC, BTC, and PTC). The Michaelis constant (K_m) and V_{max} was determined for the three substrates at concentrations ranging from 0.5 to 2.5 mmol.

Insecticides Screening and IC_{50} Assays

The insecticides to be incorporated in the screening assay were dissolved in the suitable solvents and diluted to desirable concentration using distilled water. The reaction mixtures contained 120 µl of 0.1 M potassium phosphate buffer pH 8.0, 20 µl DTNB (0.067 mM) followed by 50 µl of the insecticides and subsequently 10 µl of enzymes. The mixtures of enzyme preparation were incubated in the dark for 30 minutes at room temperature. Finally, 50 µl of ATC (0.5 mM) was then added. The final concentration of insecticides in the mixtures was 1 mg/ml. Again, the mixture was left to stand for 10 minutes at room temperature before the absorbance was read at 405 nm.

The IC_{50} studies were conducted by using insecticides at different concentrations. The control was run through the same procedure except substituting samples with potassium phosphate buffer pH 8.0.

Statistical Analysis

Values are means ± SE of at least two replicates. IC_{50} values were calculated through nonlinear regression using the one-phase exponential decay model available from Graphpad Prism version 5.0. Comparison between groups was performed using a Student's t-test or a one-way analysis of variance with post hoc analysis by Tukey's test. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Substrate specificity and substrate inhibition

The substrate specificity of the fish brain AChE was investigated using three thiocholine iodide substrates; ATC, PTC and BTC. **Fig.** 1 shows that the enzyme preferred ATC compared to other substrates. The K_m and V_{max} values of these three substrates are shown in **Table** 1. The order of the enzyme's affinity towards the substrates was ATC > PTC > BTC. The large number of hydrocarbon group in the substrate's structure will result in a weaker interaction between the carbonyl group of the substrate and the hydroxyl group of serine at the AChE esterification site, and resulting in a less enzyme's affinity towards the substrate [20]. Our result was in accordance with the result since the order of hydrocarbon group size for these three substrates is BTC > PTC > ATC. This result also indicates that the enzyme is a true AChE. One of the properties that distinguishes AChE from other cholinesterase group member is its preference to hydrolyze acetylcholine and its derivatives than other substrates including BTC and PTC. The acetylcholine substrate ATC is also reported being the preferred substrate of AChE from *Carassius auratus* [20] and many other fish species [3,5,9,23].

Fig. 1. Initial rates of AChE from *Puntius Schwanenfeldii*of on three substrates. The error bars represent mean ± standard deviation of three replicates.

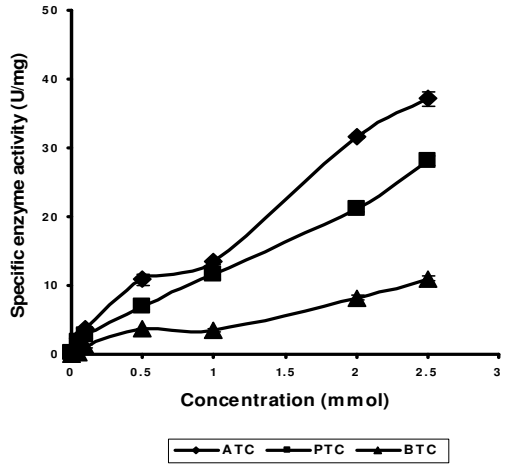

Table 1. K_m and V_{max} values of different substrates hydrolyzed by *Puntius Schwanenfeldii* acetylcholinesterase.

Substrate	K_m (mmol)	V_{max} (U/mg)
ATC	16.61	286.5
PTC	19.92	245.3
BTC	48.64	219.6

Insecticides inhibition studies

In the screening study, the inhibition of twelve different types of OP and CB were tested on the AChE from this fish. The result in **Fig.** 2 shows that the CB insecticides inhibited AChE activity significantly higher ($p < 0.05$) than the OP insecticides. Among five of the CB insecticides, bendiocarb was the strongest inhibitor. All of the OP insecticides in this study were not inhibitory to the enzyme. We assumed that the fish brain's AChE was not sensitive to all of the OP pesticides tested in this study probably because the OPs pesticides were not activated through oxonation prior to assay. Based on the IC_{50} values, the brain fish AChE was most sensitivite towards bendiocarb followed by carbofuran, carbaryl, methomyl, and propoxur in descending order (**Table** 2). In comparison, *Carassius auratus* AChE was found to be sensitive towards carbofuran, propoxur, and methomyl with the IC_{50} values of 13.034, 0.230 and 0.933 mg/L, respectively [20]. Abou-donia et. al. 1966 reported the IC_{50} value of carbaryl for *Cymatogaster aggregate* AChE at 0.785 mg/L, which is more sensitive that the AChE in this study.

The AChE from *Channa micropeltes* is sensitive to carbofuran, carbaryl, methomyl, propoxur and bendiocarb and the oxonated OPs such as parathion-oxon, malathion-oxon, diazinon-oxon and chlorpyrifos-oxon with IC_{50} values of 0.0081, 0.07922, 0.0192, 0.0679, 0.0379, 0.0316, 0.0242, 0.0599 and 0.0522 mg/L while the AChE from *E. electricus* was sensitive to the same pesticides with IC_{50} values of 0.006, 0.133, 0.026, 0.060, 0.015, 0.068, 0.014, 0.177 and 0.060 mg/L [15].

Periophthalmodon schlosseri has also been reported to be sensitive to similar carbamates such as carbaryl, methomyl, bendiocarb, carbofuran and propoxur with IC_{50} values of 0.1124, 0.0567, 0.0633, 0.0450 and 0.0892 mg/L respectively [23]. Oxonation of the OPs utilized in this work should be carried out in future studies for comparison to data in other works. This result indicates that *P. schwenenfeldii* brain AChE can be potentially be used as an *in vitro* bioassay for detecting insecticides.

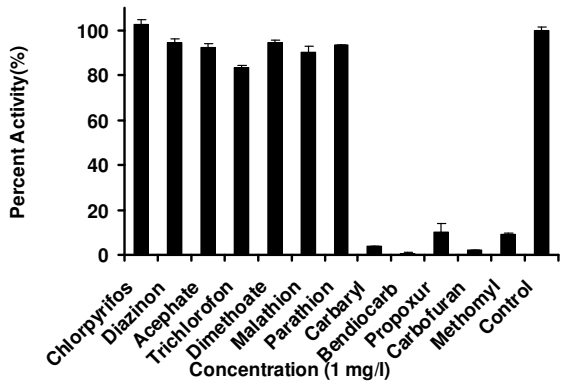

Fig. 2. The effect of various insecticides to the activity AChE from *Puntius Schwanenfeldii*. The error bars represent mean ± standard deviation of three replicates.

Table 2. Sensitivities of brain AChE (mg/L) in Puntius schwanenfeldii to five carbamate insecticides.

Pesticide	IC_{50} (mg/L)
bendiocarb	0.838
carbaryl	7.045
propoxur	29.441
carbofuran	1.411
methomyl	8.335

Among the CBs, carbofuran is a broad spectrum systemic insecticide which is commonly used throughout the world. Carbofuran has been detected in ground, surface, and rain water due to its widespread use [22]. The use of carbofuran is limited to oil palm plantation but is broadly applied in paddy field, vegetables and fruits in Malaysia making their detection an important exercise [26].

CONCLUSION

This study was meant to evaluate the potential of a local fish *Puntius schwanenfeldii* brain AChE as an *in vitro* assay for monitoring pesticides pollution and contamination. The enzyme shows good sensitivity to carbamates insecticide with sensitivity comparable to several i*n vitro* assay using fish AChE. *Puntius schwanenfeldii* is readily available throughout the year and can be harvested for its AChE year-round. Nevertheless, oxonation of the OPs utilized in this work is needed to compare the sensitivity of AChE to OPs from this fish to other available fish where oxonation of OPs resulted in a sensitive detection of OPs.

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