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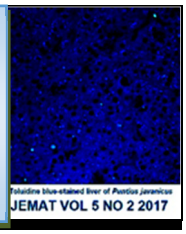

Assay for Heavy Metals Using an Inhibitive Assay Based on the Acetylcholinesterase from *Puntius schwanenfeldii*

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ABSTRACT

Acetylcholinesterase (AChE) is frequently used as an inhibitive assay for insecticides. Relatively little is known about AChE inhibition properties on heavy metals. This present study assesses the potential of the AChE from the brain of *Puntius schwanenfeldii*, which forms as a waste from the aquaculture industry, as an inhibitive assay for heavy metals. The results of the study revealed that Ag^{+}, Cu^{2+} and Hg^{2+} completely inhibited AChE activity during initial screening. Furthermore, when tested at various concentrations, the heavy metals demonstrated exponential decay type inhibition curves. The calculated IC_{50} for the heavy metals Ag^{+}, Cu^{2+} and Hg^{2+} were observed to be 0.2498, 0.2427 and 0.2255 mg/L, respectively. The present assay for copper was comparable in sensitivity to assay methods such as immobilized urease, 15 min Microtox™, 48 h *Daphnia magna*, 96 h Rainbow trout, papain and bromelain assays while the present assay for mercury was significantly more sensitive than immobilized urease, equivalent in sensitivity to the rest of the assays, but less sensitive than the papain and bromelain assays. The present assay for silver was more sensitive to all of the assays with the exception of the rainbow trout assay. In conclusion, the findings in this study indicate that the assay, which can be carried out in less than 30 min at ambient temperature can be a useful assay for monitoring both insecticides and heavy metals pollution.

INTRODUCTION

The increasing level of heavy metals in developing countries like Malaysia with an agricultural agenda posed a greater threat. Heavy metals cause severe health problems, which result in the loss of millions of ringgits to both healthcare and river monitoring. The presence of high concentrations of heavy metals in the body may perhaps adversely affect the physiological function due to bioaccumulation of heavy metals at vital organs and overproduction of reactive oxygen species [1,2,3]. Neurotoxic compounds such as copper, cadmium, mercury, and chromium are capable of deterring the enzymatic activities of acetylcholinesterase (AChE) [4]. AChe, most present in brain tissue, plays a significant part in signal termination at cholinergic synapses by speedy hydrolysis of the neurotransmitter acetylcholine. The Malaysian Department of environment (2013), documented that between 5 to 10 % river basins in Malaysia are polluted with both organics and inorganics contaminants. Thus, monitoring of these rivers is highly crucial. However, owing to the high cost the yearly monitoring of levels of heavy metals in these polluted rivers has reduced tremendously. Fortunately, the use of biomonitoring has greatly reduced the cost of monitoring river pollution. The USEPA has recognized microbial- and enzyme-based methods to biomonitor toxicants [5-8].

Microbial-based methods such as Microtox and Polytox are not amenable for field trial works as they require bulky incubators. An enzymes such as urease [9] and proteases [11,12] could be used, but they are time-consuming as each measurement takes more than one hour to complete. Basically, acetylcholinesterase is typically used for biomonitoring of insecticides. Nonetheless it might also be used for detecting heavy metals [10,11,12]. The fruit fly *Drosophila melanogaster*

and the electric eel (*Electrophorus electricus*) enzyme for pesticide bioassay and biosensor are relatively very expensive [13]. The use of fish cholinesterase such as in *Anabas testudineus* [3] *Electrophorus electricus* [4], *Periophtalmodon schlosseri* [5], *Lates calcarifer* [cholinesterase (ChE) from the kidney] [6] *Osteochilus hasselti* [7] and *Pangasius hypophthalmus* [8], for bioassay of a variety of toxicants including heavy metals have been reported. Fish can simply be exposed to and pick up heavy metals present in the aquatic environment [14-19].

Fish serves as a source of protein for human and as well as part of the food chain. They are considered as an indicator of water quality. Similarly, due to bioaccumulation and bio-magnification of heavy metals the health of fish reflects the harshness of water pollution [20]. Inhibitive determination of heavy metals via enzymatic assay is an evolving technology to report the infrequent amount of heavy metal contamination in water, soil and air. Enzymatic assays are usually prompt, cost-effective, able to detect bioavailable metal ions, and do not require highly trained personnel. Previous studies have demonstrated the used of enzymes such as glucose oxidase [21], invertase, peroxidase, glucose oxidase, urease [1,2] and proteases like bromelain, trypsin, and papain [11,12] for the inhibitive assay to detect trace amount of heavy metals.

Heavy metals particularly mercury, silver, and copper are known to have abnormalities effects on fish thereby affecting their feed intake and decreased in swimming activity [22-26]. In addition, heavy metals lead to altered physiology in fish and hepatonuclear damage [27,24]. Toxic heavy metals have the ability to disturb the regular functioning of the animal central nervous system (CNS) [24,28]. Fish has been used in previous studies as a biomarker tool via application of che enzyme for the discovery of heavy metal exposure [4,29]. The promptness and easiness of the AChE assay make it attractive to be used in the field. Similarly, AChE from *Puntius schwanenfeldii* is observed to be sensitive to the heavy metals such as mercury, copper, silver and chromium. These enzymes were therefore used to detect heavy metals from several aquatic bodies in the juru river basins

MATERIALS AND METHODS

Chemicals

Heavy metals such as (i) silver, (ii) copper, and (iii) mercury were prepared from atomic absorption spectrometry standard solutions from Merck. Working solutions at the concentrations of 10, 5, 2.5, 1.0 and 0.5 mg/L were prepared by diluting them in deionized water, and all of them were stored in acid-washed polypropylene containers. These solutions were prepared fresh daily. Acetylthiocholine iodide (ATC), β-mercaptoethanol and 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) were purchased from sigma-Aldrich. Absorption spectrometry standard solutions from Merck (Merck, Darmstadt, Germany).

Preparation of Affinity Purified AChE

Puntius schwanenfeldii with an average weight of 750 g and about 25 cm in length were sourced from a local fish aquaculture farmer in Dengkil, Selangor in 2012. the whole brain was dissected out, and 10 g of brain was homogenized (Ultra-Turrax T25 homogenizer) in 20% (w/v) of 100 mm sodium phosphate buffer at pH 8.0. Unbroken tissues were removed by centrifugation at 15 000 × g for 10 min at 4 ºC. This is followed by ultracentrifugation of the supernatant at 100,000 × g (Sorval) for 1 h at 4 ºC. The supernatant was subjected to affinity purification.

A procainamide affinity chromatography was used to partially purify the AChE [30]. The matrix, packed in a glass column (1.6 cm x 20 cm) (Amersham) to a bed height of 10 cm matrix washed with 400 ml of buffer a (20 mm sodium phosphate buffer, pH 8.0). This procedure was to clean and equilibrate the column. About 10 mg of crude extract was loaded onto the affinity matrix and then washed with 500 ml of buffer a with a flow rate of 1 ml/min. A linear gradient of 1 m NaCl in the buffer a was used to elute AChE using a total volume of 100 ml. Fractions of 1 ml were then collected and assayed for activity and protein. Fractions exhibiting high AChE activity were then pooled and dialyzed in 2:l of the buffer at 4 ºC overnight. The dialyzed fraction was then concentrated (Viva Spin) and stored at -20 °C until subsequent use [19].

Determination of AChE activity

Ache activity was calculated on the basis of an extinction coefficient of 13.6 $mm^{-1}.cm^{-1}$ using the method developed by Ellman *et al*. [31]. One unit of activity is defined as one µ mole atc hydrolyzed/min. The reaction mixture was composed of 150 µl of potassium phosphate buffer (0.1 M, pH 8.0), DTNB (20 µl, 0.067 mm), carbamate (50 µl) and enzyme (10 µl). The mixture was incubated in the dark for 10 min at room temperature. Then, 20 µl of acetylthiocholine iodide (0.5 mm stock) was then added. Again, the mixture was left to stand but for 10 min at room temperature before the absorbance was read at 405 nm. The experiment was conducted in triplicates. IC_{50} of heavy metals was determined using a one-phase exponential decay model on GraphPad prism 4 for non-linear regression analysis software available from www.graphpad.com.

Data and statistical analysis

The percent inhibition was computed according to the following formula:

$$\% \text{ inhibition} = \frac{\text{test activity of control - test activity of sample x 100}}{\text{Test activity of control}}$$

Values are means ± se. All data were analyzed using GraphPad prism version 3.0. Comparison between groups was performed using a student's t-test or one-way analysis of variance (ANOVA) with post hoc analysis by Tukey's test. $P < 0.05$ was considered statistically significant.

RESULTS

Fig. 1 showed that Ag^{2+}, Cu^{2+} and Hg^{2+} inhibited almost 100% of AChE activity. When tested at various concentrations, the heavy metals exhibited exponential decay type inhibition curves (**Figs. 2 to 4**). The IC_{50} for the heavy metals Ag^{2+}, Cu^{2+} and Hg^{2+} including their 95% confidence interval were then calculated (**Table 1**).

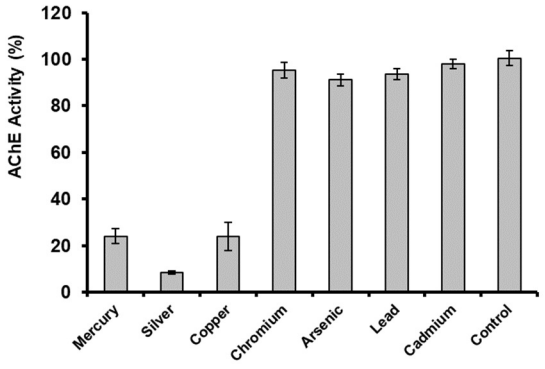

Fig. 1. The effect of metal ions on the activity of the partially purified AChE from *P. schwanenfeldii* (data represent means ± SEM, n = 3).

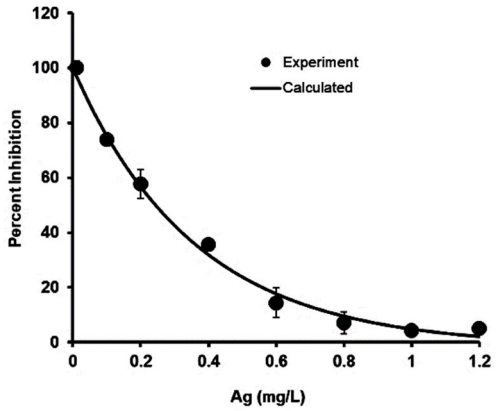

Fig. 2. Modelling the effect of silver on the activity of the partially purified AChE from *P. schwanenfeldii* using a one-phase exponential decay model (data represent means ± SEM, n = 3).

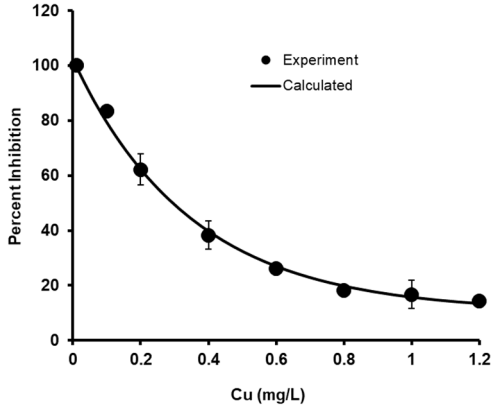

Fig. 3. Modelling the effect of copper on the activity of the partially purified AChE from *P. schwanenfeldii* using a one-phase exponential decay model (data represent means ± SEM, n = 3).

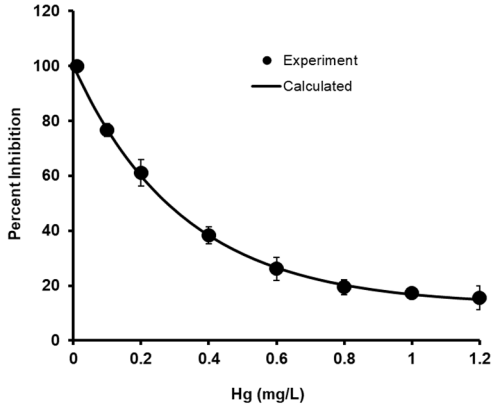

Fig. 4. Modelling the effect of mercury on the activity of the partially purified AChE from *P. schwanenfeldii* using a one-phase exponential decay model (data represent means ± SEM, n = 3).

Table 1. IC_{50} values for heavy metals that inhibit the fish cholinesterases enzyme activity.

Metal	R^2	IC_{50} (mg/l)	95% (CI)
Ag^{+}	0.99	0.2498	0.2177 to 0.2929
Cu^{2+}	0.99	0.2427	0.2110 to 0.2858
Hg^{2+}	0.99	0.2255	0.2002 to 0.2583

Table 2. Comparison of this assay to immobilized urease, Microtox™, *Daphnia magna*, fish bioassays (Rainbow trout), papain and bromelain assay.

	LC_{50} or IC_{50} (mg/L)						
Metals	Immobilized urease[a]	15 min. Microtox™ [a, b]	48 h *Daphnia magna*[a]	96 h Rainbow trout[a c]	Papain[b]	Bromelain[d]	This Assay
Cu	0.41±0.14	0.076-3.8	0.020-0.093	0.25	0.004 (LOQ)	0.163-0.305	0.2110 - 0.2858
Hg	0.33±0.021	0.029-0.05	0.0052-0.21	0.033-	0.24-0.62	0.13-0.16	0.2002 - 0.2583
Ag	n.d.	n.d.	1.930	0.210.05	0.33-0.49	n.d.	0.2177 - 0.2929

[a] [3]
[b] [4]
[c] [5]
[d] [6]
n.d. Not detected

The comparative LC_{50} (lethal concentration that causes 50% toxicity), LD_{50} (lethal dose that causes 50% toxicity), EC_{50} (effective concentration that causes 50% response) and IC_{50} (concentration that causes 50% inhibition) data for the metals; presented as 95% confidence intervals (CI) (where available) for different toxicity tests based on fish cholinesterases (**Table 2**) and other assays was then made. In general, the IC_{50} value or EC_{50} or LC_{50} values are usually used to benchmark bioassays [6-12]. Non-overlap of confidence interval usually signifies a significant difference at the $p<0.05$ level while overlapped interval does not necessary means difference or no significant differences at the $p<0.05$ level [7]. An overlapped confidence interval provides a general view that more data and experimentation are needed to assess non-significance.

Based on this, the present assay for copper was comparable sensitivity to all the assays as the confidence interval contained all of the values of this assay. Based on other inhibitive assays, the present assay for mercury was significantly more sensitive ($p<0.05$) than immobilized urease, equivalent in sensitivity to the rest of the assays, but less sensitive than the papain and bromelain assays. The present assay for silver was more sensitive to all of the assays ($p<0.05$) with the exception of the rainbow trout assay (**Table 2**).

DISCUSSIONS

The results in this study revealed that fish AChE could be used for the *in vitro* detection of heavy metals. Fish and aquatic organisms due to their aquatic environment are sensitive to toxicant such as heavy metals. For instance, the heavy metals chromium, copper, cadmium and mercury has been shown to be very toxic to *Gambusia affinis* [14], *Mytilus galloprovincialis* [15] and *Pomatoschistus microps* [16], respectively.

Metal ions inhibit enzymes because they could form ligands with amino and carbonyl groups, tryptophan (ring nitrogen), cysteine (thiol), methionine (thioether), serine, threonine, tyrosine (hydroxyl groups), asparagine and glutamine groups of the protein [11]. In addition, the metal ion mercury could disrupt cysteine bridges leading to protein denaturation [12]. The enzymatic activity of AChE is also inhibited by binding affinity of metal towards the amino acid side chain.

Histidine is most vulnerable to zinc and copper binding towards the side chain [13–15]. Thus, the imidazole group of histidine enhanced the strongest cation-π attraction that may perhaps interact with nitrogenous cations of substrates or free metal ions [16,17]. Conversely, Sarkarati et al. [24] revealed that the inhibition of ChE by metal ions is triggered by the attraction of the negative charge of amino acid side chains containing carboxyl groups such as glutamate and aspartate

which are present at the catalytic triad of ChE, leading to structural change of the active site [19,20]. Other amino acids such as cysteine, methionine, phenylalanine, threonine, asparagine, glutamine, tyrosine and tryptophan also contribute in the interaction with metal cations, either at the active site or at the allosteric site of the protein [21].

Studies have revealed that copper, cadmium and zinc demonstrate non-competitive inhibition actions to ChE activity, whereas mercury acts as an irreversible inhibitor [18]. The inhibition of ChE activity by metal ion is therefore associated with (a) obstruction of enzymatic active site, (b) modification of ChE structure, and (c) amino acid sequence variety which tend to be affected differently by the metals and other toxicants, thus preventing the formation of enzyme-substrate complex or protein denaturation, either reversible or irreversible [22,23].

Sabullah et al. [30] prove the ability of metal ion to inhibit the activity of *P. javanicus ChE* activity. Frasco et al. [18] and Opazo et al. [20], showed that copper is strong inhibitor of ChE. Recent studies also reported that nerve agent carbamate and organophosphate can inhibit the activity of ChE via carbamylation and phosphorylation at the activity side and through blocking of substrate binding [26–28]. In this present study the ability of mea tal ion to inhibit ChE activity extracted from *Puntius schwanenfeldii* has been verified.

Conclusively, the study showed that AChE from *Puntius schwanenfeldii* is sensitive to heavy metals with LOD values that can be used for biomonitoring works. Owing to the inadequate information vis-à-vis the use of AChE as a biomonitoring assay for heavy metals, this study develops new data and information that will be valuable for imminent biomonitoring studies using enzymes.

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