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Development of an inhibitive assay using commercial *Electrophorus electricus* acetylcholinesterase for heavy metal detection

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Abstract

Near-real-time assay is an assay method that the whole process from sampling until results could be obtained in approximately less than one hour. The Ellman assay for acetyl cholinesterase (AChE) has near real-time potential due to its simplicity and fast assay time. The commercial acetylcholinesterase from Electrophorus electricus is well known for its uses in insecticides detection. A lesser known fact is AChE is also sensitive to heavy metals. A near real-time inhibitive assay for heavy metals using AChE from this source showed promising results. Several heavy metals such as copper, silver and mercury could be detected with IC_{50} values of 1.212, 0.1185 and 0.097 mg l^{-1}, respectively. The Limits of detection (LOD) for copper, silver and mercury were 0.01, 0.015 and 0.01 mg l^{-1}, respectively. TheLimits of quantitation (LOQ) for copper, silver and mercury were 0.196, 0.112 and 0.025 mg l^{-1}, respectively. The LOQ values for copper, silver and mercury were well below the maximum permissible limit for these metal ions as outlined by Malaysian Department of Environment. A polluted location demonstrated near real-time applicability of the assay with variation of temporal levels of heavy metals detected. The results show that AChE from *Electrophorus electricus* has the potential to be used as a near real-time biomonitoring tool for heavy metals.

Key words

AChE, *Electrophorus electricus*, Heavy metals, Near real-time assay

Introduction

The concentration of heavy metals in the aquatic environment has reached alarming level over the years. Hence, simple and field trial friendly methods have been developed to monitor the levels of heavy metals in the environment (Malik *et al.,* 2011; Aksu *et al.,* 2012). Inhibitive determination of heavy metal using enzymes is a new technology developed for this purpose. Inhibitive enzyme assays are usually rapid, able to detect bioavailable metal ions, do not require skilled technician and are amenable to field trial works (Jung *et al.,* 1995). Enzymes that have been used for inhibitive determination of heavy metal traces include peroxidase, xanthine oxidase, invertase, glucose oxidase and the proteases bromelain and papain (Shukor *et al.,* 2006; Shukor *et al.,* 2008). However, other sources of enzymes are needed since none of these assays, in their own, are sensitive to several heavy metals. The use of acetylcholinesterase as an inhibitive enzyme assay for insecticides are well known (Villatte *et al.,* 1998; Tham *et al.,* 2009) but very few research have been done on the potential of acetylcholinesterase as an assay for heavy metals. Previously, Frasco *et al.* (2007) reported that AchE from *Electrophorus electricus* is sensitive to mercury but use of this finding as an inhibitive assay for heavy metals was not pursued further. In this work, the acetylcholinesterase from *E. electricus* is demonstrated to be very sensitive not only to mercury but to copper and silver at the sub parts per million level and this sensitivity could be used for the detection of heavy metals. In addition, due to the rapidity of the assay, a near-real-time format for assaying heavy metals have been developed and was successfully used in monitoring the presence of heavy metals in waters from industrial site.

Materials and Methods

Heavy metal estimation through AChE inhibition: The heavy metals to be incorporated in the screening assay were dissolved in deionized water. The reaction mixtures contained 150 µl of 0.1 M potassium phosphate buffer pH 8.0, 20 µl of 5,5'-dithiobis-(2-nitrobenzoic acid) or DTNB (0.067 mM) followed by 50 µl of metal ions and subsequently 10 µl of commercially available *E. electricus* AChE. The mixture was incubated in dark for 30 min at room temperature. 20 µl of acetylthiocholine iodide or ATC (0.5 mM) was then added. The mixture was then left to stand for 10 min at room temperature before the AChE was assayed. The control was run through the same procedure except substituting samples with potassium phosphate buffer at pH 8.0. Activity of AChE was measured according to the method of Ellman *et al.* (1961) with modification for microassay using 96 well micro plate readers. The intensity of yellow color was measured at 405 nm using a spectrophotometer. The hydrolysis of ATC was calculated from the extinction coefficient of DTNB (13.6 $mM^{-1}cm^{-1}$). Activity of AChE was expressed as the production of one micromole of thiocholine per minute. All reagents were prepared in 0.1 M potassium phosphate buffer pH 8. The protein concentration of AChE was determined as described by Bradford (1976) using bovine serum albumin as a standard. The concentration of heavy metal that cause 50% of inhibition or IC_{50} was obtained using one phase exponential decay model available from Graphpad PRISM 4 from www.graphpad.com.

Near-real-time trial: One location was used to demonstrate the near-real-time applicability of the developed assay. Water samples from a heavy metals-contaminated site (Shukor *et al.,* 2006) at the Prai Industrial Park, Penang (05° 20.87'N; 100°24.692'E) were initially filtered with 0.45 µm syringe filter and then immediately assayed using the AChE-based inhibitive assay as described above using a portable spectrophotometer (Axiom, Germany). The assay was carried out every two hours for a period of 24 hrs. Heavy metals in the samples were determined using Atomic Emission Spectrometer on a Perkin Elmer Optima 3000 ICP-AES. All experiments were performed in triplicate.

Statistical analysis: Values are means ± SE for at least three replicates. Data were analyzed using Graphpad Prism version 3.0. A Student's t-test or a one-way analysis of variance (ANOVA) with post hoc analysis by Tukey's test was used for comparison between groups (Miller and Miller, 2000). $P < 0.05$ was considered statistically significant.

Results and Discussion

Effect of heavy metals to AChE activity: ANOVA analysis showed that out of the 6 metals tested at the final concentration of 5 mg l^{-1}, only copper, silver and mercury showed more than 50% significant inhibition ($p<0.05$) (Fig. 1). When tested at various concentrations, copper, silver and mercury exhibited exponential decay type inhibition curves (Fig. 2-4). The calculated IC_{50} using the GraphPad software (GraphPad Software, Inc., San Diego, CA) using the one phase exponential decay model for copper, silver and mercury were 1.212, 0.1185 and 0.097 mg l^{-1}, respectively. The limit of detection (LOD) is the lowest concentration which can be detected with confidence (99% confidence interval) and is usually assigned as three times the standard deviation of the blank for the y-intercept while the limit of quantitation (LOQ) is assigned as the concentration that can be determined with acceptable precision (usually RSD < 10 to 25%) and accuracy (usually 80-120% recovery) and is usually assigned as ten times the standard deviation of the blank for the y-intercept (Miller and Miller, 2000).The LOD for copper, silver and mercury were 0.01, 0.015 and 0.01 mg l^{-1}, while the LOQ for copper, silver and mercury were 0.196, 0.112 and 0.025 mg l^{-1}, respectively. The LOQ values for copper, silver and mercury were well below the maximum permissible limit for these metal ions as outlined by Malaysian Department of Environment (DOE, 2007).

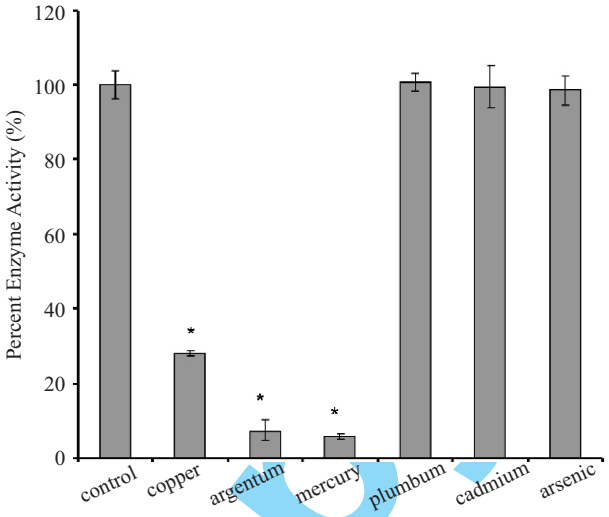

Fig. 1 : Effect of various heavy metals on the activity of AChE from E. electricus. Data were expressed as % AChE activity. Values significantly different from those obtained from control are marked by stars ($p<0.05$). Data is mean standard error (n=3)

The comparative LC_{50}, EC_{50} and IC_{50} data for the metals; presented as 95% confidence intervals (where available) for different toxicity tests is shown in Table 1. The results of this study were also compared with fish (rainbow trout), daphnids *(Daphnia magna)*, immobilized urease, *R. meliloti*, papain, bromelain and Microtox™ toxicity data in the same table. The present assay for silver was significantly more sensitive ($p<0.05$) to the papain and *Daphnia magna* assays, and significantly less sensitive $p<0.05$) to the rainbow trout assay. The present assay for

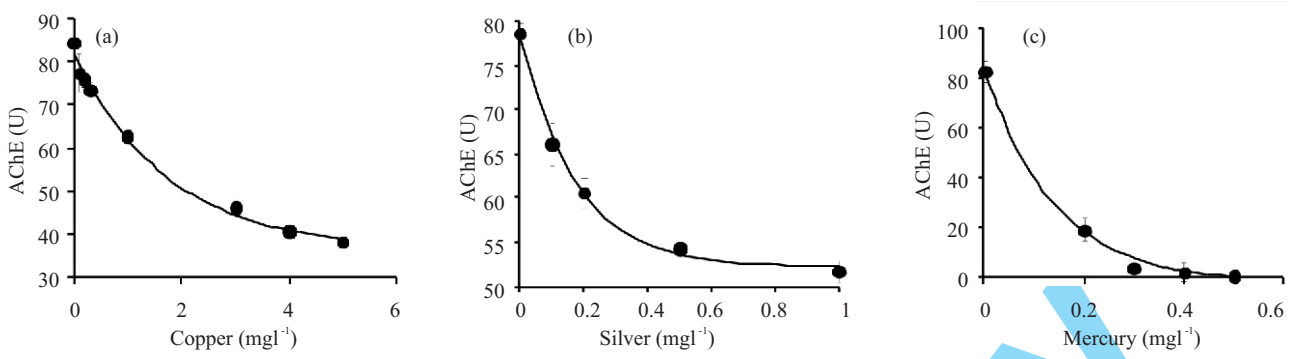

Fig. 2 : Effect of (a) copper, (b) silver and (c) mercury on the AChE activity of *E. electricus*. Values are mean of three replicates ± SE

Table 1 : Comparison of the assay to MicrotoxTM, Daphnia magna, fish, papain and bromelain assays

	Ic_{50} (mg l^{-1})							
Metals	**Immobilized urease**	**15-min. Microtox™[a]**	**48 hr *Daphnia magna*[a]**	**96 hr Rainbow trout[a]**	**Papain[b]**	**Bromelain[c]**	**Mo-reducing enzyme**	**Present study (95% CI*)**
Ag	ND	ND	1.930a	0.05	0.33-0.49	NI	NI	0.074-0.29
Cu	0.41±0.14	0.076-3.8	0.020-0.093	0.25	0.004 (LOQ‡)	0.163-0.305	0.099±0.013	0.64-1.690
Hg	0.330.021	0.029-0.05	0.005-0.21	0.033-0.21	0.24-0.62	0.130 -0.160	NI	0.084-0.115

[a] Jung *et al.,* 1995; [b] Shukor *et al.*, 2006; [c] Shukor *et al.*, 2008; [d] Shukor *et al.*, 2009; ND No Data; NI No inhibition; ‡ Limit of Quantitation; *Confidence Interval

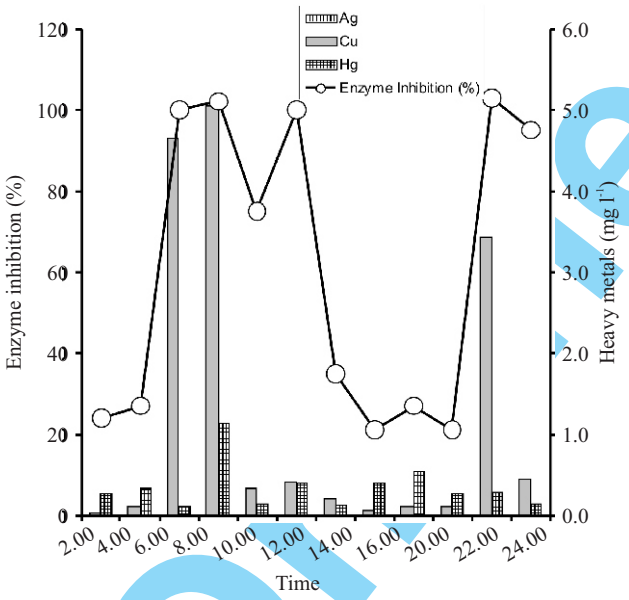

Fig. 3 : Near real-time biomonitoring of heavy metals using AChE from *E. electricus* validated using ICP-OES. Data is mean standard error (n=3)

copper was significantly less sensitive ($p<0.05$) than all of the other assays. The *E. electricus* AChE assay for mercury was significantly more sensitive ($p<0.05$) to papain, bromelain and immobilized assays, and was significantly less sensitive ($p<0.05$) to the microtox assay. Repeated measurements of the assay suggested that they were reproducible with Coefficient of Variation (CV) of the replicated data ranging from 4.3 to 5.5%.

It is well known that fish are sensitive to toxicants and the use of fish for the bioassay or bioindicator of heavy metals (Tham *et al.,* 2009) have been reported. Thus, the inhibition by heavy metals added a new dimension to the use of AchE as an inhibitive assay. The elevated level of mercury and copper were detected in near real-time and validated using ICP-OES with good correlation between biological and instrumental results (Fig. 3). Since metal-related industries can be found in abundance in this area (Shukor et al., 2006), it is suspected that these types of industries are responsible for the elevated levels of heavy metals. Temporal variation in heavy metals level seen in this work highlights problem in heavy metals monitoring in running water bodies. This variation is probably caused by clandestine release of wastes containing heavy metal pollution into rivers to evade detection by enforcement agencies (Birch *et al*., 2001). Globally, application of real-time or near real-time monitoring of heavy metals is almost nonexistent since instrumental and biological-based assays require bulky instrument or take too long (Lopez-Roldan *et al*., 2012). Near-real-time biomonitoring is an exciting recent trend as realtime or near-realtime biomonitoring of heavy metals would allow forensic approach towards apprehending environmental criminals (Girotti *et al*., 2008). Currently, samples need to be transported to the laboratory and hence such monitoring systems can be defined as batch system monitoring (Shukor *et al*., 2006). Heavy metals could be loss via evaporation especially in the case of mercury or be irreversibly attached to sampling container walls (Shukor *et al*., 2008). A field trial assessment of the assay reveal near real-time applicability

in temporal monitoring capability. This assay would allow a temporal analysis of heavy metals pollution to be carried out. In the future temporal capability of inhibitive assays can be extended to spatial variation of pollution either in sedimentary.

Acknowledgments

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