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Acetylcholine Receptor-based Biosensor Derived from Asian Swamp Eel, *Monopterus Albus* for Heavy Metals Biomonitoring

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ABSTRACT

Cholinesterase-based biosensor well known as a sensitive method to detect the existence of harmful dissolved compounds in any type of water source, especially the river. This alternative biosensor can be used to determine the level of pollution of the water in a short period of time as well as to evaluate the low cost and simple service. The aim of this study was to exceed the effectiveness of acetylcholinesterase source extracted from the brain tissue of Asian swamp eel; *Monopterus albus* as a potential environmental biosensor. Purified acetylcholinesterase exposed to a different type of metal ions and mercury showed the highest percentage of inhibition at 62.9% followed by chromium at 59.22% while silver, arsenic, cadmium, cobalt, copper, nickel, zinc and lead at not more than 50% (approximately 37-50%). Metal ions such as mercury, zinc, chromium and copper showed exponential decay type inhibition curves with calculated half maximal inhibitory concentration; IC_{50} in the ascending sensitivity order 0.005, 0.595, 0.687 and 1.329 mgL^{-1}, respectively. Field trial works exhibited that the acetylcholinesterase was applicable in sensing heavy metals pollution from the river which closed to the industrial and agricultural sites at near real-time and verified using ICP-OES. This study proves

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the potential use of acetylcholinesterase sourced from *M. albus* as a biomonitoring tool to assess the contamination level of the river.

Keywords: Acetylcholinesterase, biosensor, heavy metal, IC_{50}, *Monopterus albus*

INTRODUCTION

The main enzyme found in the neuromuscular junction and cholinergic nervous system, brain cholinesterase, specifically acetylcholinesterase (AChE), is triggered to suppress synaptic transmission. The function of AChE is to hydrolyse of acetate and choline neurotransmitter acetylcholine (ACh) (Colovic et al., 2013). Anticholinesterase can block the activity of AChE and causes synaptic aggregation of Ach, including insecticides and toxic substances, (Sussman et al., 1991). Throughout aquatic organisms such as fish, ubiquitous cholinesterase (ChE) was also found. Fish can thus be a high potential biomarker for controlling pollution in terms of analysing their developmental and behavioural modifications as well as the evaluation of ChE activity for heavy metal treatments.

Biomarkers are considered as one of the methods most capable of ecotoxicological implementations, able to provide early recognition of toxic water disclosure and the main indication of potential effects at advanced levels of biological organisation, such as workforce and environmental effects (Quintaneiro et al., 2016). Previous studies showed that several freshwater systems in Malaysia, including the Klang river, the Langat Basin river and also the Mamut river in eastern Malaysia, have become a concern about their heavy metal toxicity levels, such as copper (Cu), cadmium (Cd), zinc (Zn), and lead (Pb) (Alam et al., 2015 & Naji et al., 2014).

In this research, AChE was commonly used for insecticide biomonitoring and is also useful for heavy metal detection (Bocquene et al., 1990; Frasco et al., 2008; Olson & Christensen, 1980). The AChE assay's rate and versatility make it desirable to use a portable spectrophotometer in the field. We realized in this work that *Monopterus albus,* AChE was sensitive to pollutants such as mercury, copper, silver and chromium. Therefore, we used this enzyme to trap heavy metals from a variety of aquatic bodies in the several river samples in Malaysia.

MATERIALS AND METHODS

Chemicals

Significantly, MERCK had imported the products for the processing of heavy metals from atomic absorption spectrometry standard solutions like; chromium (Cr^{6+}), nickel (Ni^{2+}), zinc (Zn^{2+}), silver (Ag^{2+}), arsenic (As^{5+}), cadmium (Cd^{2+}), cobalt (Co^{2+}), copper (Cu^{2+}), mercury (Hg^{2+}) and lead (Pb^{2+}) while acetylthiocholine iodide (ATC) and 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich. These solutions were freshly prepared, before being used.

Preparation of Brain AChE Extractions

M. albus were brought from supplier at Selangor, Malaysia. The fish were delivered to the laboratory alive and picked for the experiment (according to apparent health conditions). The fish were starved a day before the experiment to reduce the potential dietary factors on the fish's organic metabolite status. The fish was slaughtered in an ice-filled box, called as freeze-killed followed by the dissection of the fish brain. The brain samples extracted from the eel were then homogenized using an Ultra-Turrax T25 Homogenizer with a buffer proportion of 1:4(brain(w): Buffer(v) of a 0.1 M sodium phosphate buffer pH 7.5 comprising 1mM of phenylmethylsulfonyl fluoride. The sample supernatant was collected and stored at-20°C for the purification method process after a centrifugation cycle at 10,000 x *g* at 4°C for 10 minutes. The thawing process of the sample took place after it was left at ambient temperature.

Preparation of Affinity Purified AChE

The AChE was purified using procainamide chromatography (Sabullah et al., 2014) with a slight-modification using a different matrix which was procainamide-Sepharose CL-6B. Chromatography of the procainamide affinity was used to partially purify the AChE after precipitation from the selected fraction. The matrix was packed to a bed height of 4 cm in a column syringe, 1 mL of the supernatant was loaded into the column of affinity containing procainamide-sepharose CL-6B and washed with 6 ml of buffer containing 20 mM of pH 7.0 sodium phosphate buffer into the column with gravity flow rate. This stage is essential for removing the unbounded protein from the column to the matrix. For eluting buffer, 20 mM sodium phosphate buffer pH 7.0 containing 1M NaCl was then loaded to elute the ChE from *M. albus,* which was bounded to the affinity matrix. A 1 mL of fractions was obtained and examined for the determination of enzyme activity and protein concentration. The maximum enzyme activity fraction was taken and deposited at -25°C.

ChE Activity and Protein Content Determination

The activity of *M. albus* ChE was determined using a slight modification of Ellman et al. (1961) method and read at the wavelength of 405 nm using 96-well microplate. A 200 µL sodium phosphate buffer (0.1 M, pH 7.0), 20 µL DTNB (0.1 mM) and 10 µL ChE sample were inserted into the wells of the microplate and incubated for 15 minutes, and initial reading was obtained. Subsequently, 20 µL of a substrate (5.0 mM ATC) was applied to the mixture and incubated for 10 min. ChE activity was determined as the amount of substrate (µM) broken down by ChE per minute (U) with an extinction coefficient of 13.6 mM^{-1} cm^{-1} while the specific activity was expressed as µmole/min/mg of protein or U mg-1 of protein. The determination of the protein content has been evaluated, as stated by Bradford, 1976. The standard quantitative value of the protein was Bovine serum albumin (BSA). The

assay were conducted in the dark area, and all the triplicates were done. For the non-linear regression analysis software available on the Internet (www.graphpad.com), IC_{50} of heavy metals was calculated using a one-phase exponential decline model on Graphpad PRISM 5.

Field Trials

During good and hot weather, sampling was done but stopped at the rainy season to prevent dilution of the sample. Water samples (Table 1) were immediately checked using the inhibitive assay, as mentioned above, with the spectrophotometer for the present of

Table 1
Sampling location was conducted at nine rivers from six different state

Location	Classification	SAMPLE Code	SAMPLING COORDINATE	Remark
Penang	Class IV	SJP1	N 5°14'45.81", E 100°28'20.3"	Dark in colour and smelly
		SJP2	N 5°14'22.23", E 100°28'30.2"	
		SJP3	N 5°14'28.13", E 100°28'57.4"	
Melaka	Class III	SMM1	N 2°13'32.55", E 102°15'16.5"	Yellowish brown colour and smelly
		SMM2	N 2°13'37.17", E 102°15'39.7"	
		SMM3	N 2°13'48.27", E 102°15'33.2"	
Selangor	Class IV	SKS1	N 3°00'57.87", E 101°42'36.0"	Yellowish brown colour and smelly
		SKS2	N 3°01'31.52", E 101°42'31.3"	
		SKS3	N 3°01'58.69", E 101°42'08.4"	
Pahang	Class II	SBP1	N 3°31'17.22", E 101°54'37.1"	Yellow color but sometime clear
		SBP2	N 3°31'16.75", E 101°54'27.9"	
		SBP3	N 3°31'34.68", E 101°54'13.8"	
Terangganu	Class II	STT1	N 5°21'05.66", E 103°03'56.5"	Yellow-green color
		STT2	N 5°18'21.41", E 103°05'23.1"	
		STT3	N 5°18'53.47", E 103°05'37.4"	
Sabah	Class II	SIS1	N 5°58'58.16", E 116°07'6.84"	Yellowish color
		SIS2	N 6°00'14.43", E 116°07'6.02"	
		SIS3	N 6°00'43.52", E 116°6'51.53"	
	Class III	SSS1	N 5°58'08.72", E 116°04'13.9"	Yellowish brown colour and smelly
		SSS2	N 5°57'59.59", E 116°04'14.3"	
		SSS3	N 5°57'54.04", E 116°04'10.7"	
	Class II	STS1	N 6°05'25.84", E 116°12'12.7"	Yellowish color with strong smell
		STS2	N 6°04'55.97", E 116°12'34.3"	
		STS3	N 6°05'07.56", E 116°12'21.8"	
	Not Recorded	SKT1	N 5°41'05.82", E 116°22'45.8"	Clean and clear. Considered as Class I river.
		SKT2	N 5°40'49.95", E 116°22'35.5"	
		SKT3	N 5°40'46.78", E 116°22'29.1"	

Note. River classification is based on Water Quality Index reported by DOE, 2018
Sample code abbreviation: SJP = Sungai Jawi, Penang; SMM = Sungai Melaka, Melaka; SKS = Sungai Kuyuh, Selangor; SIS = Sungai Inanam, Sabah; SSS = Sungai Sembulan, Sabah; STE = Sungai Telipok, Sabah; STA = Sungai Keinop-Tambunan, Sabah.

bioavailable heavy metals. Atomic emission spectrometry (ICP-OES, Optima 3700DV, Perkin-Elmer, USA) had been used to measure the determination of heavy metal content in our samples. Distilled water had been used as a study control. Using 90 mm Whatman filter paper, 50 mL of each sample was initially filtered, followed by ultrafiltration using 0.45-micron nylon syringe filters. The sample was treated with a few drops of 70% nitric acid analytical grade to prevent the metal ion from reacting and sticking to the sample tube wall. ICP-OES has been tested using a standard multi-element solution containing; Cr^{6+}, Ni^{2+}, Zn^{2+}, Ag^{2+}, As^{5+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+} and Pb^{2+} (excluded in this study) and optimized standardization according to the recommendation of the manufacturer. All the experiments were carried out in triplicate.

Data and Statistical Analysis

The percent of inhibition level was calculated according to the formula:

$$\%\text{Inhibition} = \frac{\text{Activity of control} - \text{Activity of sample} \times 100}{\text{Activity of control}}$$

The values shown are means ± standard deviation. Graphpad Prism version 5.0 was used to analyse all data. A comparison between groups was obtained by one-way analysis of variance (ANOVA) with post hoc analysis by Tukey's test or by using a student's t-test. $P<0.005$ was regarded to be statistically significant.

RESULTS AND DISCUSSIONS

Sample Extraction and Purification

Table 2 shows that ChE was purified with a yield of 38.73% at 20.53-fold. The yield decreased due to the loss of enzyme activity caused by such an external temperature factor that was higher than the optimal enzyme temperature (Robinson, 2015). The total protein amount decreased while the activity of the enzyme increased throughout this phase of purification. The purification is required to reduce any inference from other proteins

Table 2
Purification table for purification of ChE from M. albus

Procedure	Total protein (µg)	Total ChE activity (U)	Specific activity (U/µg)	Purification folds	Yield (%)
Crude homogenate	0.53	47.68	89.96	1	100
Procainamide-Sepharose CL-6B	0.01	18.47	1847	20.53	38.73

Note. The specific activity from each step of purification is displayed in (U/µg), which means µmole/min/mg of protein.

to ensure that the desired purified protein can achieve maximum performance (Forget et al., 2002; Gao & Zhu 2001; Talesa et al., 2001). The purification process does not affect the activity of the enzyme because it appears the molecular structure and the activity of the enzyme can only be affected by the enzyme and substratum temperature, pH and concentration (Bisswanger, 2014). In this study, the enzyme's specific activity increased at the end of the experiment throughout the purification process.

Metal Ion Inhibition Study

Figure 1 shows that Cr and Hg showed the highest inhibition, decreasing ChE activity to less than 50% and showing a sense of value ($P<0.005$) between them. ChE also inhibited *in vitro* by silver (Ag^{2+}), arsenic (As^{5+}), cadmium (Cd^{2+}), chromium (Cr^{6+}), cobalt (Co^{2+}), copper (Cu^{2+}), mercury (Hg^{2+}), nickel (Ni^{2+}), zinc (Zn^{2+}) and lead (Pb^{2+}) by lowering activity to 52.88%, 52.22%, 57.35%, 40.78%, 56.54%, 51.73%, 37.10%, 62.59%, 50.47% and 57.79% respectively when ATC was used as the substrate at the concentration of 10 ppm. Other than that, Hg and Cu were rated as the highest rank of heavy metal toxicity, and this assertion (Alam & Maughan, 1992; Apartin & Ranco, 2001; Bellas et al., 2001; Martin et al., 1981; Ramakritinan et al., 2012; Strubelt et al., 1996) has been confirmed by other previous studies. Besides, the high inhibition of Hg on ChE activity was in line with studies using fishes such as *Puntius javanicus, Anabas testudineus* and *Clarias gariepinus* (Ahmad et al., 2016; Padrillah et al., 2017; Sabullah et al., 2014). Various heavy metals normally

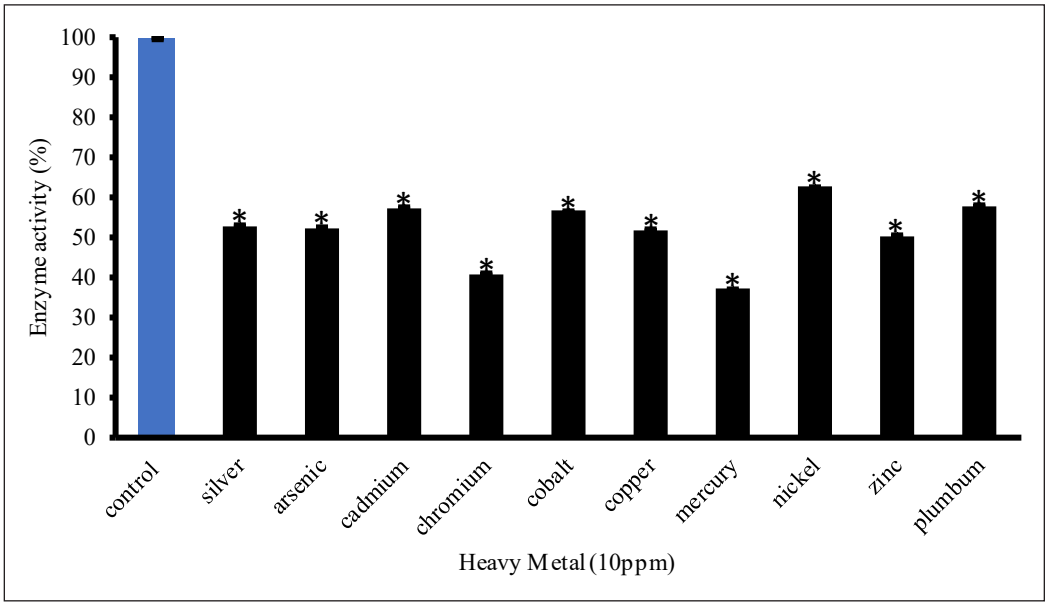

Figure 1. Effect of different types of heavy metals on the enzymatic activity of purified ChE from *M. albus* brain after inhibition at 10ppm. Error bars represent mean ± standard error (n=3). Statistical significance of different from control: *P <0.005.

offered different inhibition or vulnerability to purified ChE activity (Gbaye et al., 2012; Kuca et al., 2005; Santarpia et al., 2013). Past investigations studies had found heavy metal had blocked the use of substrates either specifically binding to the active enzyme site or binding to the allosteric site, leading in the conformation change and the failure of substrates to shape the complex of compound substrates (Ahmad et al., 2016; Basirun et al., 2018; Giedroc et al., 2007; Glusker et al., 1999; Mathonet et al., 2006; Padrillah et al., 2017).

Selected metal ion; Hg^{2+}, Zn^{2+}, Cr^{6+} and Cu^{2+}, showed exponential decay type inhibition curves with calculated half maximal inhibitory concentration; IC_{50} in the ascending sensitivity order of 0.005, 0.595, 0.687 and 1.329 mgL^{-1}, respectively in Table 3. The lower IC_{50} value will give a higher potency of the antagonist, and the lower the concentration of toxicant is required to inhibit the maximum biological response.

Table 3
The IC_{50} value using GraphPad Prism 5 with a type analysis of nonlinear regression by equation of one phase exponential decay

Metal ions	IC_{50} (95% confidence interval) mg L^{-1}	R^2
Cr^{6+}	0.687 (0.4440 to 0.9031)	0.9604
Cu^{2+}	1.329 (1.1460 to 1.5830)	0.9801
Hg^{2+}	0.005 (0.0020 to 0.0120)	0.9916
Zn^{2+}	0.595 (0.3387 to 0.9444)	0.9254

Field Trial

The purpose of this project was to test the ability of *M. albus* AChE to detect anti-AChE in various river samples (Figure 2) identified by DOE (2018) as polluted (Class IV and V), slightly polluted (Class II and III) and WQI-based clean river (Class I). *Ex-situ* testing was performed, and Table 4 shows the percentage of the remaining activity of purified AChE after exposure to tap water and river samples, while the red dotted line shows a percentage below 80%; or inhibition more than 20%, considered as contaminated as stated by Sabullah et al. (2015) which this idea supported by Ahmad et al., 2016 and Hayat et al., 2016. The result shows that 13 samples were considered unpolluted, where SEM 1 and SA 3 were not statistically significantly different ($p > 0.05$) with control. In contrast, the others showed less than 20% of AChE inhibition (about 10% to 20% of inhibition) such as SEM 3 and SA 1. Some samples showed slight contamination as the percentage of SA 4, SA 2 and SEM 4 to the unpolluted range was less than 3% to 10%. Shukor et al. (2013) reported that any sample inhibiting AChE activity by more than 50% was was classified as toxic and harmful to aquatic habitat. In this analysis, no toxic sample was detected. Meanwhile, according to Sabullah et al. (2015), the cholinesterase inhibition of more than 20% the sample was considered polluted. In this analysis, the polluted sample could be SEM 2 at 24.17% corresponding to the number of variables and the highest concentration of metal

Table 4
Quantification of metal ions concentration in river samples from Peninsular area and Sabah area using ICP-OES

Sample	Inhibition (%)	Metal ion concentration (ppb)								
		Ag	As	Cd	Cr	Cu	Hg	Pb	Ni	Zn
Tap Water	0	*nd	*nd	*nd	*nd	*nd	*nd	*nd	*nd	0.01 ± 0.001
SEM 1	0	0.632 ± 0.043	*nd	*nd	0.287 ± 0.075	*nd	*nd	*nd	*nd	*nd
SEM 2	24.17± 2.22	0.679 ± 0.021	*nd	*nd	1.317 ± 0.027	16.69 ± 1.190	*nd	42.125 ± 2.999	25.540 ± 0.978	579.81± 25.479
SEM 3	16.67± 3.14	0.704± 0.068	1.14± 0.02	*nd	0.077 ± 0.001	0.283± 0.007	*nd	*nd	*nd	3.760± 0.184
SEM 4	8.33 ± 1.55	0.349± 0.090	*nd	*nd	2.759 ± 0.006	4.970± 0.356	*nd	*nd	*nd	4.575± 0.677
SA 1	20.70± 2.47	0.821± 0.064	*nd	*nd	*nd	8.055 ± 0.003	*nd	0.191± 0.004	*nd	15.01± 3.511
SA 2	4.12 ± 0.112	0.459± 0.079	*nd	*nd	*nd	*nd	*nd	*nd	*nd	5.383± 0.649
SA 3	0	0.393± 0.025	*nd	*nd	*nd	*nd	*nd	0.034± 0.001	*nd	4.176± 0.615
SA 4	2.43± 0.287	0.935± 0.180	*nd	*nd	*nd	*nd	*nd	*nd	*nd	*nd

*Note. *nd = not detected*

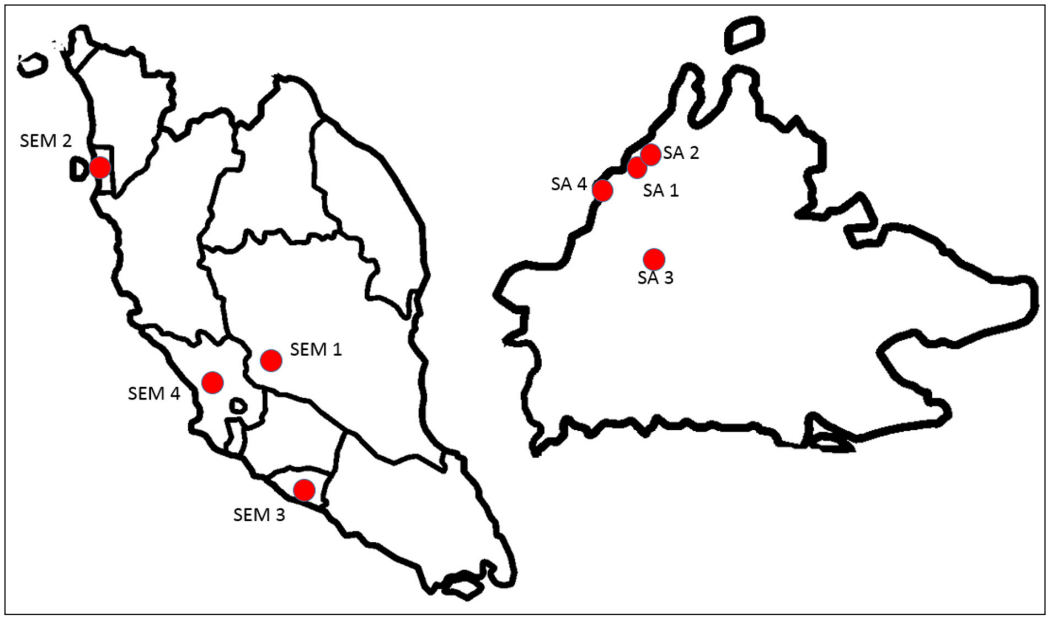

Figure 2. The map of sampling locations from six different states; SEM 1,2,3 and 4 denoted River Bentong, River Jawi, Penang, River Melaka and River Kuyuh river while SA 1,2,3 and 4 marked as River Telipok, River Sembulan, River Keinop and River Tuaran

ions, especially zinc at 579.8 ppb and lead 42.125 ppb. The results obtained in this study show the potential applicability of the biomonitoring assay. Biomonitoring allows only a positive sample, indicating inhibition of enzyme activity to be sent for instrumental testing. The cost of control will, therefore, be reduced dramatically.

CONCLUSION

In this study, purified *M. albus* brain ChE demonstrates activity inhibition after exposure to heavy metals that can later be extended and evolved into an alternative biosensing approach based on these findings. Furthermore this study shows significant inhibition of heavy metals, particularly mercury and chromium. This study, therefore, indicates that *M. albus* may be a potential new source of ChE to replace the existing commercial ChE. This work may add new data and information that are useful for future biomonitoring studies using this enzyme.

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