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Cholinesterase-based biosensor using *Lates calcarifer* (Asian Seabass) brain for detection of heavy metals

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ABSTRACT

Heavy metals are sometimes needed for nutrient uptake but only in low concentrations and the nervous system is the most susceptible to be affected. Cholinesterase (ChE) inhibition has been used extensively as a biomarker for heavy metals detection. In this study, the potential of ChE from *Lates calcarifer* brain as an alternative biosensor for heavy metals is evaluated. There are many Malaysian rivers such as Sungai Juru and Sungai Merbok that are greatly polluted by industrial effluents. Chronic exposure to heavy metals may cause nervous system disorders. Thus, a fast and simple biomonitoring technique will help in alerting government agencies and the public to such a threat. In this study, ChE from the brain of *L. calcarifer* (Asian seabass) was purified through ammonium sulphate precipitation and ion exchange chromatography. Enzyme recovery was 5.31% with a specific activity of 5.472 U mg^{-1}. The Michaelis-Menten constant (K_m) value was 0.3075 mM and a V_{max} of 0.0304 µmol min^{-1} mg^{-1}. ChE from *L. calcarifer* brain showed higher affinity to acetylthiocholine iodide (ATC) compared to butyrylcholinesterase (BTC) and propionylcholinesterase iodide (PTC). Maximum activity of ChE was detected when the enzyme was assayed at the temperature of 20-30°C and incubated in Tris-HCl buffer pH 8.0. Silver (Ag), arsenic (As), cadmium (Cd), chromium (Cr) and mercury (Hg) showed more than 50% inhibition amongst the 10 types of heavy metals that have been tested for inhibition study. These results can be used to further develop an alternative way to detect heavy metals that are low cost and give faster results compared to existing biosensor kits.

Keywords: Cholinesterase, Biomarker, Biomonitoring, Heavy metals.

INTRODUCTION

Many water bodies have been polluted by the direct or indirect release of natural or artificial molecules that aimed to kill or mitigate any harmful organism. Non target living organisms were also affected by these compounds. They can cause poisoning in the human body through the inhibition of cholinesterase enzyme (ChE), which plays the role in human detoxifying process. The inhibition reaction is very important in human nervous system although they are far more lethal against insects and small animals. Water rehabilitation followed by monitoring is the best technique to ensure safer environment. Preliminary screening of contamination can be carried out using enzymatic biosensors before a high performance technology is needed for analytical quantification. ChE-based biomarker was considered as an effective method for the monitoring of environmental contamination. Fish are documented to be very sensitive towards toxicants, thus many studies used it as a biomarker along with ChE to indicate the existence of toxicants. This study highlights the capability of a ChE-based biosensor for detecting contamination caused by pesticides, which can later become one beneficial method for bioremediation.

Acetylcholinesterase (AChE) is a fast acting enzyme that is involved in hydrolysation of the neurotransmitter acetylcholine (ACh) that is located at the synaptic cleft. AChE is the target for inhibitors such as heavy metals due to its critical function in the nervous system. The majority of AChE can be found in the brain muscle (Massoulie, 2002). Due to its sensitivity, AChE has been manipulated to be used in biomonitoring and bioassay for those contaminants and has been recently used in various species of fish (Dembélé, 2000; Matozzo and Marin, 2005; Narbonne, 2005). AChE activity in invertebrates such as shellfish (Brown, 2004; Lehtonen, 2006) and crustaceans (Bocquene, 1997) has been proved to be useful in biomonitoring programmes. It has been discovered that AChE from *P. pangasius* is useful for detecting heavy metals and its sensitivity towards heavy metals such as copper,

silver and chromium (Tham, 2009). Thus, ChE is an effective biochemical indicator of toxic stress and serves as sensitive parameter for testing water quality for the presence of various toxicants affecting its activity (Bocquene and Cadiou, 1992; Narbonned, 1991; Kirby, 2000). In order to monitor the effects of contaminants, inhibitive ChE-based assay has been used for years on either human or wild life as a multiple detection marker of toxicant exposure.

A vast number of deleterious xenobiotics including heavy metals, pesticides, industrial chemicals, and pathogens, that bioaccumulate in marine organisms, may cause toxicity to fish, handlers and eventually humankind as the ultimate consumer (Hejkal, 1983; WHO/FAO, 1989; Almroth, 2008). The investigation of cholinesterase or AChE activity in fish tissues as early-warning biomarker was made recently for the assessment of pollution in ponds or lakes that receive sewage wastewater revealed site and tissue-specific variations in AChE responses. In this study, the brain sample of *L. calcarifer* was used as the sample as AChE is concentrated in the brain.

MATERIALS AND METHODS

Chemicals: Silver (II), arsenic (V), cadmium (II), chromium (VI), copper (II), mercury (II), plumbum (II), zinc (II), acetylthiocholine iodide (ATC), 5, 5-dithiobis (2-nitrobenzoic acid) (DTNB), butyrylthiocholine iodide, and propionylthiocholine iodide purchased from Sigma-Aldrich.

Extraction and purification: Fish were obtained from Pusat Sains Marin UPM in Telok Kemang, Port Dickson. The fish was killed in a box full of ice and then its head was dissected to extract the brain. The sample was then weighed followed by homogenisation process using Ultra-Turrax T25 Homogeniser in 0.1 M sodium phosphate buffer pH 7.5 containing 1 mM phenylmethylsulfonyl fluoride with buffer ratio of 1: 4 (brain: buffer). After centrifugation process at 10,000 xg for 30 minutes at 4°C, the supernatant of the sample was collected and stored at -20°C for purification process. Thawing process of the sample takes place after it was left at ambient temperature. The ion exchange column containing DEAE-Cellulose with the dimensions of 5 cm diameter and 80 cm height was poured with 15 mL of supernatant for the next purification process. 1.5 L of 25 mM sodium phosphate buffer pH 7.0, which is the washing buffer, was loaded into the column with the flow rate calibrated at 0.1 mL min^{1}. During the washing stage, elimination of the unbounded protein was done and the elution of *L. calcarifer* ChE that is bounded to the matrix was done by loading 25 mM sodium phosphate buffer pH 7.0 containing 1 M NaCl into the column. Enzyme activity and protein concentration determination was obtained by collecting 1 mL fractions and assaying to find the fraction that show the highest enzyme activity and then the sample was concentrated using Sartorius Vivaspin 20 at 5000 xg at 4°C for 10 minutes. The purified ChE sample was stored at −20°C. According to Laemmli (1970), Native polyacrylamide gel electrophoresis (Native-PAGE) was done to visualise the efficiency of the purified ChE. Coomasie Brilliant Blue G-250 was used to visualise the protein that has been separated. A broad range protein marker (Bio-Rad, UK) was used, which contains myosin, beta-galactosidase, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme and aprotinin with molecular weight (MW) of 198, 103, 58, 41, 27, 20, 15 and 6 kDa, respectively. The stained gel was visualised using a calibrated G-800 densitometer (Bio-Rad, UK). The division of protein marker migration value with the migration value of silver blue was used to calculate the retention factor (*rf*).

ChE activity and protein content determination: A slight modification of Ellman et al., (1961) method that used 96-well microplate was chosen to be the method for examining the enzyme activity of *L. calcarifer* at the wavelength of 405 nm. 200 μL of sodium phosphate buffer (0.1 M, pH 7.0), 20 μL of DTNB (0.1 mM) and 10 μL ChE were loaded into the microplate wells, mixed and incubated for 15 minutes. 20 μL of the substrates (5.0 mM ATC, BTC and PTC) was then added to the mixture and then 10 minutes of incubation time was added for the reaction to take place. ChE activity was stated as the amount of substrate (μM) broken down by ChE per minute (U) with the extinction coefficient of 13.6 mM^{-1} cm^{-1}, while the specific activity is expressed as μmole min^{-1} mg^{-1} of protein or U mg^{-1} of protein. Protein content determination was studied using the method created by Bradford. In this study, Bovine serum albumin (BSA) was used as a standard for the quantitative determination of the protein. All tests were carried out in triplicates and the assays were run in a dark environment.

Optimal substrate specificity: Different synthetic substrates, namely ATC, BTC, and PTC with concentrations of 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 mM were used for determination of the substrate specificity for *L. calcarifer* ChE in sodium phosphate buffer (0.1 M, pH 7.0). The substrate was added into the reaction mixture and the absorbance (405 nm) was recorded after 10 minutes. Michaelis-Menten curves were obtained using Graph Pad Prism Software version 5 and were used to determine the biomolecular constants (K_m) and the maximal velocities (V_{max}) of ChE activity.

Optimal pH and temperature: Determination of the optimum pH for the enzyme was done by incubating the ChE of *L. calcarifer* with an overlapping buffer system consisting of 0.1 M acetate buffer (pH 3-5), 0.1 M sodium phosphate buffer (pH 6-8), and 0.1 M tris-HCl buffer (pH 7-10). The optimal temperature of the enzyme was

determined after incubating the ChE sample at different range of temperatures from 15 to 50°C. ChE was observed to change its structure beyond this range of temperature.

The effects of metal ions: The study on the effect of metal ions towards the enzyme activity was done by incubating 10 types metal ions, which are silver (II), arsenic (V), cadmium (II), chromium (VI), cobalt (II), copper (II), mercury (II), nickel (II), plumbum (II), and zinc (II) with *L. calcarifer* ChE. These metals were selected because of their toxicity and threat to the environment. The reaction mixture contained 150 μL of sodium phosphate buffer (0.1 M, pH 7.5), 50 μL of the metal ion with the final concentration of 10 mg/L, 20 μL of DTNB (0.1 mM), and 10 μL of the ChE. The reaction mixture was incubated for 15 minutes followed by the addition of 20 μL of the substrate into the mixture. The mixture was left for another 10 minutes of incubation before the reading of the absorbance at the wavelength of 405 nm.

RESULTS AND DISCUSSION

Sample extraction and purification: ChE from *L. calcarifer* brain was extracted and purified through ammonium sulfate precipitation and ion exchange chromatography that used DEAE-Cellulose as the matrix. 5.31% of enzyme recovery was recorded at the end of this study. The specific activity with a value of 5.472 U mg^{-1} was obtained with purification folds of 45 (Table 1). The molecular weight of the purified ChE was determined to be 41 kDa after the $\log_{10}$ (kDa) graph was plotted and interpolated as shown in Figure 1. A main protein band for ChE sample of *L. calcarifer* brain is displayed in Figure 2 after DEAE-Cellulose ion exchange chromatography was performed at lane 2.

Previous studies have reported the use of ion exchange chromatography using DEAE-Cellulose as the matrix purifies ChE from various samples (Ding, 2011; Lockridge and La Du, 1986). The efficiency of the purification processes was evaluated after Native-PAGE was carried out. The obvious differences between purification stages were displayed by removing large amounts of foreign proteins until a main band was obtained after being purified using DEAE-Cellulose. Thus, this result proved that the matrix was capable to purify ChE from the brain tissue of *L. calcarifer*. Based from recent studies, it has been reported that ChE exists in native form as two molecular forms in *Pacu serum* (Salles, 2006), three molecular forms in rat (Andres, 1990) and four ChE bands were displayed by *curimbata serum* (Salles, 2006). However, unlike the studies mentioned, the purified ChE from *L. calcarifer* brain obtained in this study only showed one major protein band on nondenaturing PAGE gel after it was stained with silver blue, suggesting a major molecular form of ChE.

Berg et al., (2002) mentioned that to accurately study the biochemical functions, molecular weight determination, identification and structure of the targeted protein or individual analysis, protein purification is required. Food processing and drug development study also used purified protein. In this study, elimination of foreign proteins that interfere with enzyme action by purification process enhanced the sensitivity of ChE towards toxicants. When the toxicants does not bind with or inhibit the ChE or they bind to other proteins through various interactions, it will caused an interference in the enzyme reaction. Inhibition study on the characteristics of toxicants that inhibit ChE activity were done by previous studies where the purified ChE from various samples were widely used (Hsiao, 2004; Yang, 2013).

Table.1.Table of purification for ChE from *L. calcarifer* brain. The specific activity from each step of purification is displayed in (U mg^{-1}).

Procedure	Total Protein (mg)	Total ChE activity (U)	Specific activity (Umg^{-1})	Purification fold	Yield (%)
Crude	93.00	11.43	0.123	1	100
Ammonium sulphate precipitation	4.14	2.599	0.628	5	22.74
DEAE-Cellulose	0.11	0.607	5.472	45	5.31

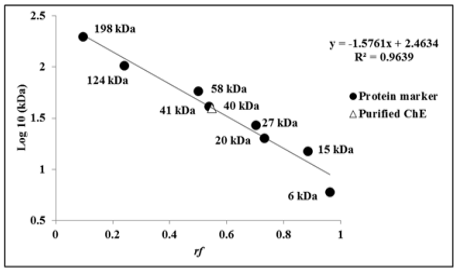

Figure.1. The determination of the molecular weight of the purified ChE from *L. calcarifer* brain by interpolating the retention factor (*rf*) of protein markers. Overlapping

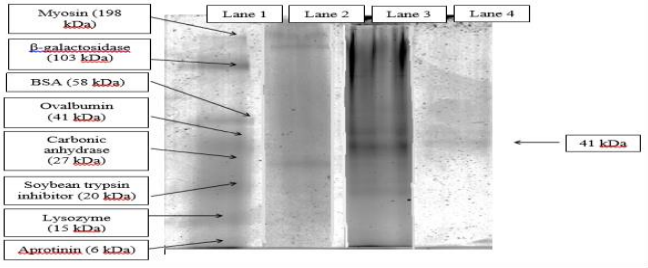

Figure.2. Diagram of Native-PAGE for ChE from brain of *L. calcarifer*. Lane 1 is broad range protein marker. Lane 2 is crude extract of *L. calcarifer* brain while Lane 3 show

of purified ChE with other protein was indicated by the triangle.

the pellet precipitated by ammonium sulphate of 50-60% saturation and Lane 4 is sample fraction from DEAE-Cellulose ion exchange chromatography.

Kinetic study: An obeyed Michaelis-Menten kinetics is shown in Figure 3 that displays the hydrolysation of the three different synthetic substrates, namely ATC, BTC, and PTC, at various concentrations by the ChE. According to the results shown in all three reactions, the increase in hydrolytic activity was affected by the increase in substrate concentrations. Contrariwise, at above 5mM substrate concentration, the enzyme demonstrated a plateau state. Table 2 shows the catalytic efficiency (V_{max}/K_m) of the enzyme when incubated with ATC as the substrate that showed the highest value. Thus, it can be concluded that the enzyme affinity towards ATC was greater compared to the other two substrates and was selected as the ideal substrate for this assay.

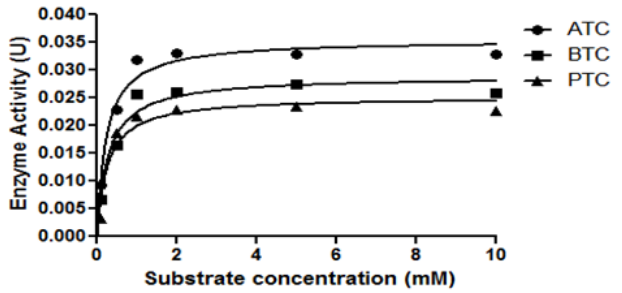

Figure.3. Incubation of purified ChE from *L. calcarifer* brain in three synthetic substrates with different concentrations.

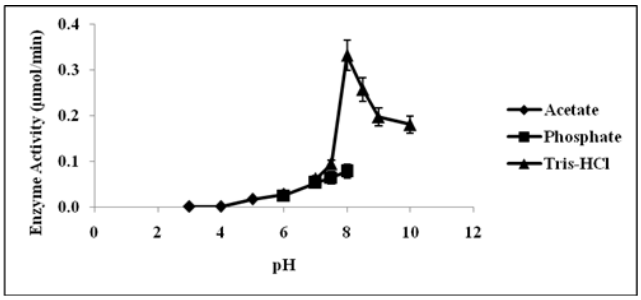

Figure.4. pH profile of *L. calcarifer* brain on the purified ChE activity with mean point of triplicate assay and Y error bars.

Table.2. Comparison table for maximum velocity (V_{max}) and biomolecular constant (K_m) for ATC, BTC, and PTC of ChE from *L. calcarifer* brain.

	ATC	BTC	PTC
V_{max} (µmol/min/mg)	0.0304	0.0199	0.0157
K_m (mM)	0.3075	0.2351	0.1979
V_{max}/K_m	0.0988	0.0845	0.0794

Optimal pH and temperature: After the ChE was purified, the optimal pH was determined. The effects of various pH values towards ChE activity were also investigated. The optimal pH for ChE from *L. calcarifer* brain was shown in Figure 4, where the maximum ChE activity falls between pH 7.0 and 8.0 of potassium phosphate and Tris-HCl buffer. Comparing these two types of buffer, 0.1M Tris-HCl buffer, pH 8.0 was selected as the best buffer although there is no significant difference of ChE activity (p>0.05) between the two groups of data. Figure 5 displayed the effects of different range of temperature on ChE activity. The optimum ChE activity was in between the temperature of 20–30°C and no significant difference (p>0.05) was shown in their mean points. At lower temperatures, the activity of ChE was the lowest but as the temperature increased, the activity reaches a maximum until and later demonstrated a bell shaped curve. However, the ChE activity decreased sharply at higher temperatures.

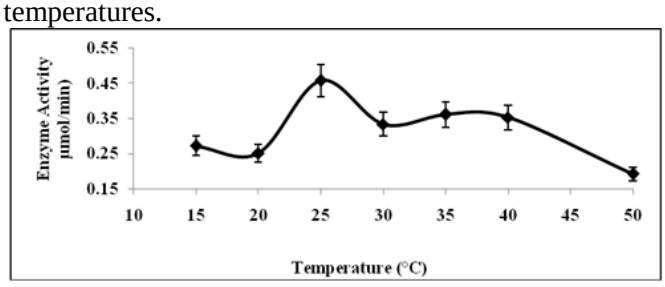

Figure.5. Temperature profile of *L. calcarifer* brain on the activity of purified ChE with mean point of triplicate assay and Y error bars.

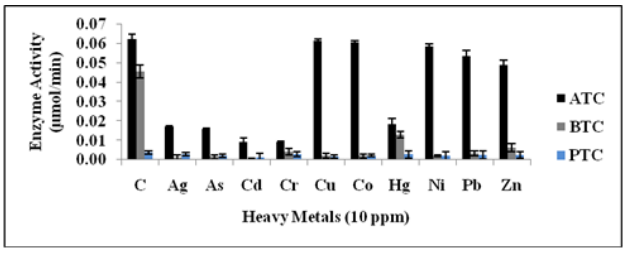

Figure.6. Effect of different types of heavy metals on the enzymatic activity of purified ChE from *L. calcarifer* brain when incubated with three different synthetic substrates (ATC, BTC and PTC)

Metal ion inhibition study: Ten selected metal ions with the concentration of 10 mg/L were incubated with *L. calcarifer* ChE from brain sample with joined optimal assay parameters. The in vitro studies of ChE inhibition was shown in Figure 6. Preliminary results recorded that the brain ChE was inhibited by Ag, As, Cd, Cr and Hg by

lowering the activity to 27.72, 25.48, 14.90, 14.74 and 20.67% respectively, when ATC was used as the substrate. Meanwhile, when the purified ChE was incubated with BTC, the enzyme activity was inhibited by all of the heavy metals as they showed more than 50% inhibition than the untreated control. However, when PTC was used, only Cd showed slight inhibition towards the activity of the purified ChE.

The binding of toxicants either at the active or allosteric site of ChE were determined by amino acid residue. Interaction of heavy metals such as mercury and copper with the negatively charged amino acids such as aspartate or imidazole group of histidine, or cleavage of disulfide bond are capable of inhibiting ChE because of changes in its structure (Masson et al., 1996; Najimi et al., 1997). Results shown in Figure 6 indicated that different heavy metals gave different inhibition on the activity of purified ChE. Furthermore, it was proved that most of heavy metals showed the capability to inhibit *in vitro* or *in vivo* ChE activity (Galloway and Cowling, 2004; Banni, 2005), especially in fish (Monserrat, 2002; Sturm, 2000).

CONCLUSION

In this study, the optimum assay parameters, namely pH and temperature for purified ChE from *L. calcarifer* brain were successfully determined and it can be concluded that ATC was preferred as the specific synthetic substrate. The sensitivity of ChE inhibition by selected heavy metals was determined and the results suggested a promising biosensor kit for the detection of heavy metals pollution in marine environment. Furtherwork is recommended to investigate the capability of the purifed ChE to detect other contaminants such as detergents, dyes, pesticides, and drugs.

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