

# Characterisation of cholinesterase from kidney tissue of Asian seabass (*Lates calcarifer*) and its inhibition in presence of metal ions

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## Abstract

**Aim :** The cholinesterase (ChE) based inhibition studies from fish were investigated and presented here emerged to be one of the great potential biomarkers for heavy metals monitoring.

**Methodology :** In this study, the capability of ChE extracted from the kidney of *Lates calcarifer* was assessed for of metal. ChE was purified through ammonium sulphate precipitation and ion exchange chromatography.

**Results :** The purified enzyme gave 12 fold purification with the recovery of 12.17% with specific activity of 2.889 U mg<sup>-1</sup>. The Michaelis-Menten constant ( $K_m$ ) and  $V_{max}$  value obtained was 0.1426 mM and 0.0217  $\mu\text{mol min}^{-1}\text{mg}^{-1}$ , respectively. The enzyme has the ability to hydrolyse acetylthiocholine iodide (ATC) at a faster rate compared to other two synthetic substrates, propionylthiocholine iodide (PTC) and butyrylthiocholine iodide (BTC). ChE gave highest activity at 20-30°C in Tris-HCl buffer pH 8.0. The results showed that cholinesterase from *L. calcarifer* kidney was very sensitive to sensitive to copper and lead after being tested argentum, arsenic, cadmium, chromium, copper, cobalt, mercury, nickel, lead and zinc.

**Interpretation :** The effect of heavy metals studied on the activity of ChE differed from each other. The result of the study can be used as a tool for further developing a biomarker for the detection of heavy metals in aquatic ecosystems. In addition, the information can also be used for designing a kit, that would give a rapid and accurate result.

### Cholinesterase as a biomaker

### Extraction of cholinesterase from kidney of *Lates calcarifer*



### Purification of cholinesterase from kidney of *Lates calcarifer*

- ammonium sulphate precipitation
- ion exchange chromatography

### Optimization of cholinesterase

- Substrates
- pH
- Temperature

### Effect of 10 metal ions

- silver (II) • arsenic (V) • cadmium (II) • chromium (VI) • cobalt (II) • copper (II) • mercury (II) • nickel (II) • lead (II) • zinc (II)

– Half maximal inhibitory concentration ( $IC_{50}$ )

### Development of biomarker for the detection of heavy metals in aquatic ecosystems

## Introduction

Cholinesterases (ChE) are serine hydrolases, which can be divided into two molecular types, namely acetylcholinesterase (AChE) and pseudocholinesterase (PChE) based on their response towards selective inhibitor and substrate preferences (Villatte and Bachmann, 2002). In biological systems, some heavy metals are present in the form of metal ions and are required only in trace amount for biochemical reactions (Cohen *et al.*, 2000; Ma *et al.*, 2009). Bioaccumulation that occurs mainly in vital organs due to high concentration of heavy metals will give an adverse effect to the physiological functions (Singh *et al.*, 2011).

Formation of enzyme-substrate complex is facilitated by metal ions. However, they have a tendency to react with terminal -OH and -SH functional groups as they bind with the allosteric sites that caused the conformational changes making the substrate failed to bind at specific site of the enzyme (Glusker *et al.*, 1999). Metal ions have the capability to inhibit ChE activity as they are considered as neurotoxic compounds (Elumalai *et al.*, 2002). Metal ions such as copper, cadmium, mercury and chromium are strong inhibitors of ChE (Tilton *et al.*, 2011; Hayat *et al.*, 2015).

Fish are sensitive to toxicants as they are exposed directly to these pollutants through skin by absorption, breathing and oral intake (Mathur and Singh, 2006). The most studied biomarkers are ChE based, which use two types of ChE, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) (Ahmad *et al.*, 2016a; Hayat *et al.*, 2016). Further more, aquatic organisms exhibit heavy metals intoxication prior to their death and hence, are used as model organisms (Cokugras, 2003; Adedeji, 2011).

BChE are crucial for different parts of immune system and considered as one of the core detoxifying enzymes (Archana *et al.*, 2011). BChE protects AChE from being inhibited by nerve agent such as pesticides (Soreq and Zakut, 1993). Biomarkers are the best tools in determining the effect of pesticides and heavy metals. However, they show irreversible effects on ChE (Sabullah *et al.*, 2015a; Ahmad *et al.*, 2016b).

Cholinesterase activity in the kidney of aquatic organisms is currently not well characterised and documented. Hence, in the present study importance of ChE activity in the Asian seabass kidney and its effect when exposed to metal ions.

## Materials and Methods

**Extraction, purification and enzyme activity of cholinesterase:** *L. calcarifer* measuring 25 cm in length and weighing approximately 1.0 kg were procured from Pusat Sains Marin UPM in Telok Kemang, Port Dickson. The kidney of *L. calcarifer* was dissected, weighed and homogenised. The supernatant of the sample was collected after centrifugation at

10,000 xg for 30 min at 4 °C and stored at -20 °C for purification. The crude extract derived from above was gradually added with ammonium sulfate powder to obtain 0–30, 30–40, 40–50, 50–60, 60–70 and 70–80% saturation levels. A 15 ml of supernatant was loaded into the ion exchange column containing DEAE-Cellulose for purification process. ChE of *L. calcarifer* which was bounded to the matrix was eluted by loading 25 mM sodium phosphate buffer (pH 7.0) containing 1 M NaCl into the column.

Enzyme activity and protein concentration determination was then experimented. Native polyacrylamide gel electrophoresis (Native-PAGE) was carried out following the method of Laemmli (1970). The separated protein was visualised using Coomassie Brilliant Blue G-250 (silver blue). Protein marker with a broad range of molecular weight (MW) was used that contained myosin, beta-galactosidase, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme and aprotinin with molecular weight of 198, 103, 58, 41, 27, 20, 15 and 6 kDa, respectively (Bio-Rad, UK). The stained gel was then visualised using calibrated G-800 densitometer (Bio-Rad, UK). Retention factor was calculated by dividing migration of protein marker with migration of silver blue.

A slight modification of Ellman *et al.* (1961) method using 96-well microplate was chosen to examine the enzyme activity of *L. calcarifer*, at wavelength of 405 nm. Protein content was determined using the method developed by Bradford (1976). Bovine serum albumin was used as standard for quantitative determination of protein. All the tests were carried out in triplicates and the assays were run in dark.

**Optimal assay determination :** Three different synthetic substrates, namely ATC, BTC and PTC at 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 mM were concentrations used for determining the substrate specificity for ChE extracted from the kidney of *L. calcarifer* in sodium phosphate buffer (0.1 M, pH 7.0).

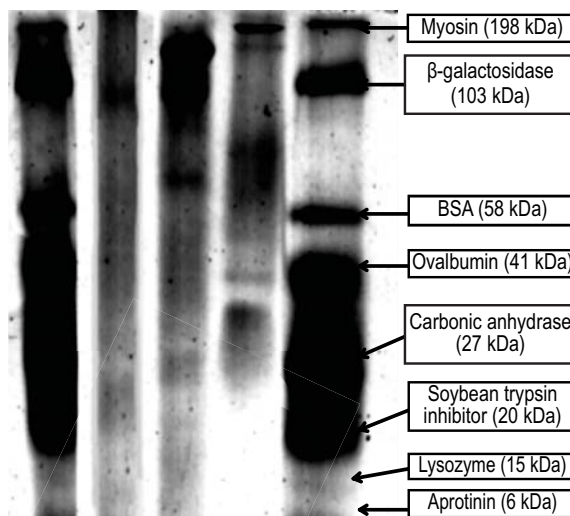
Determination of optimum pH for enzyme was done by incubating *L. calcarifer* ChE with an overlapping buffer system of 0.1 M acetate buffer (pH 3 to 5), 0.1 M sodium phosphate buffer (pH 6 to 8) and 0.1 M tris-HCl buffer (pH 7 to 10). The optimal temperature of the enzyme was determined after incubating ChE sample at different temperatures ranging from 15 to 50°C. Beyond this temperature range, ChE was observed to be denatured.

**Effect of metal ion :** The enzyme activity was experimented by incubating ten types of metal ions *viz.*, silver (II), arsenic (V), cadmium (II), chromium (VI), cobalt (II), copper (II), mercury (II), nickel (II), lead (II) and zinc (II) with *L. calcarifer* ChE. Half maximal inhibitory concentration (IC<sub>50</sub>) was determined by incubating the ChE sample with inhibitors of different concentrations for 30 min.

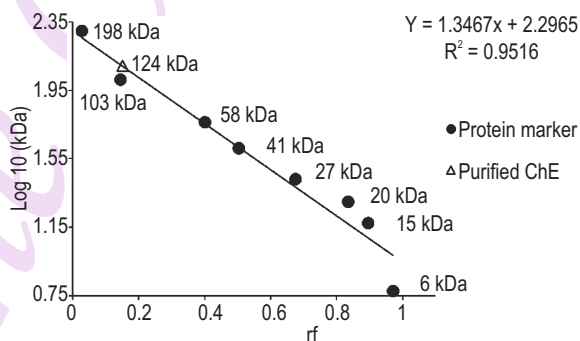
## Results and Discussion

ChE extracted from the kidney of *L. calcarifer* was purified by using three-step procedures such as sample extraction, ammonium sulfate precipitation and ion exchange chromatography by using DEAE-Cellulose column. At the end of the study, nearly 12.17% enzyme recovery was achieved. The purified ChE showed the specific activity of 2.889 U mg<sup>-1</sup> of protein (Table 1). The presence of ChE in fish kidney has already been reported by Sol'e *et al.* (2012). The presence of ChE in other organs of fish such as liver (Sabullah *et al.*, 2014), muscle (Rodríguez-Fuentes *et al.*, 2008), plasma and brain tissues (Tham *et al.*, 2009; Sabullah *et al.*, 2015b) have been well documented. The crude extract was eliminated by a large portion of proteins of low molecular mass ammonium sulfate precipitation with 50-60% saturation (Fig. 1). A single protein band for ChE from DEAE-Cellulose ion exchange chromatography at lane 2 as indicated, the purified ChE was determined to have a molecular weight of 124 kDa after molecular weight of standard proteins was interpolated as shown in Fig. 2.

An obeyed Michaelis-Menten kinetics was observed in hydrolysis of three different substrates, namely acetylthiocholine iodide (ATC), butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC) at varying concentrations by ChE (Fig. 3). Increasing substrate concentration influenced the increase in hydrolytic activity as shown in all the three reactions. Conversely, the enzyme exhibited a plateau state at above 1 mM substrate concentration. ATC recorded the lowest  $K_m$  values compared to PTC and BTC (Table 2). This result shows that the affinity of the enzyme towards ATC substrate was greater when BTC and PTC was used as substrate. In the study also, ATC displayed highest ratio of catalytic efficiency ( $V_{max}/K_m$ ), thus was selected as an ideal substrate for this assay. An increase in  $V_{max}/K_m$  value indicates the intensity of enzyme affinity. The results of the substrates study show that the value of  $V_{max}/K_m$ , when tested with ATC, was maximum as compared to PTC and BTC (Fig. 3). Thus, it can be concluded that ChE from *L. calcarifer* kidney had a



**Fig. 1** : Diagram of Native-PAGE for ChE extracted from *L. calcarifer* kidney. Lane 1 and 5 is broad range protein marker. Lane 2 is sample fraction from DEAE-Cellulose ion exchange chromatography. Lane 3 show the pellet precipitated by ammonium sulphate of 50-60% saturation and Lane 4 is crude extract of *L. calcarifer* kidney



**Fig. 2** : Retention factor ( $rf$ ) of interpolation of protein markers to determine the molecular weight of purified ChE from *L. calcarifer* kidney. The triangles indicated overlapping of purified ChE with other protein

**Table 1** : Total and specific activity of ChE extracted from the kidney of *L. calcarifer*

Procedure	Total protein (mg)	Total ChE activity (U)	Specific activity (U mg <sup>-1</sup> )	Purification fold	Yield (%)
Crude	39.555	9.615	0.243	1	100
Ammonium sulphate precipitation	0.725	1.633	2.254	9	16.98
DEAE-Cellulose	0.405	1.17	2.889	12	12.17

**Table 2** : Comparison of the maximum velocity ( $V_{max}$ ) and biomolecular constant ( $K_m$ ) for ATC, BTC and PTC of *L. calcarifer* kidney ChE

	ATC	BTC	PTC
$V_{max}$ ( $\mu\text{mol min}^{-1} \text{mg}^{-1}$ )	0.0217	0.0178	0.0178
$K_m$ (mM)	0.1426	0.3853	0.1214
$V_{max}/K_m$	0.1525	0.0461	0.1469

higher affinity towards ATC. Nevertheless, the enzyme inhibition was noticed in the presence of excess substrates. Based on these results, implication can be made that the purified ChE was a type of acetyl cholinesterase (AChE).

The optimum pH for ChE activity was determined after its purification. The effects of different range of pH towards ChE activity have been also investigated. The optimum pH for ChE from *L. calcarifer* kidney fell between pH 7.0 and 8.0 (Fig. 4) of potassium phosphate and Tris-HCl buffer ( $p > 0.05$ ). 0.1 M Tris-HCl buffer, pH 8.0 was selected as an optimum assay condition because it showed highest mean point of data when these two types of buffer were compared. Interaction of substrate with ChE at low pH was disrupted by high concentration of protons due to existence of protonated imidazole group of histidine at catalytic triad of the enzyme (Masson *et al.*, 2002). The mechanism of ChE may be affected due to alteration in histidine conformation (Masson, 2012). Whereas at high pH, binding of enzyme and substrate was also affected due to change of substrate charge.

The effects of different temperatures on ChE activity is depicted in Fig. 5. The optimum ChE activity was observed between 20 and 30°C ( $p > 0.05$ ). At lower temperature, the activity of ChE was minimum, but as the temperature increased the activity reached maximum until reaching the given velocity thus, exhibiting a bell shaped curve. After that, the ChE activity decreased abruptly at higher temperatures. The optimum temperature for *L. calcarifer* ChE matches with the ambient temperature. Although, kinetic energy was limited at low temperature, ChE activity was retarded but not denatured thus, PTC could not be hydrolysed completely. However, the activity significantly increased as temperature reached maximum point. In the present study, maximum activity of *L. calcarifer* ChE was achieved at 20–30°C, but the activity briskly decreased beyond this temperature. This is in agreement with the theory; protein will undergo denaturation because they lose their stability at high temperature (Gaudy *et al.*, 2000).

Ten selected metal ions with the concentration of 10 mg l<sup>-1</sup> were incubated with *L. calcarifer* kidney sample under combined optimal assay parameters. The *in vitro* studies showed that ChE was inhibited by silver, chromium, copper, cobalt, nickel and zinc by lowering the activity to 36.55, 48.10, 9.10, 40.18, 35.01 and 31.95%, respectively when incubated with PTC (Fig. 6). Meanwhile, all these metals showed more than 50% of inhibition when incubated with BTC. Cu and Pb displayed inhibition when incubated with ATC where the activities decreased by 60 and 40.4%. Frasco *et al.* (2007) and Wang *et al.* (2004) reported that copper is a strong ChE inhibitor. Based on the results, it can be concluded that the toxicity of tested metals at 10 ppm varied according to the types of substrate used. All these metal ions showed significant inhibition towards ChE activity, but differed in inhibition percentage ( $p < 0.005$ ). Earlier studies have reported that carbamate and organophosphate which also known as nerve

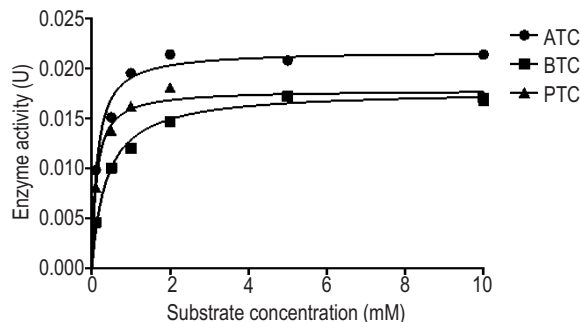


Fig. 3 : Purified ChE incubated in three synthetic substrates with different concentrations

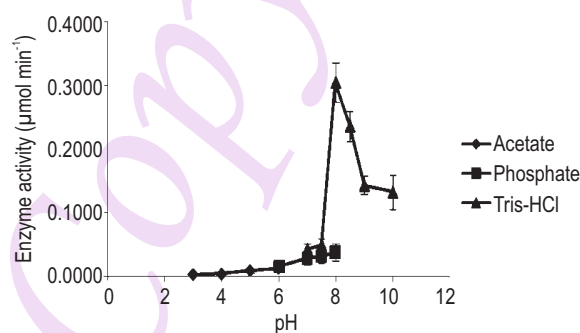


Fig. 4 : pH profile of *L. calcarifer* kidney on the activity of purified ChE

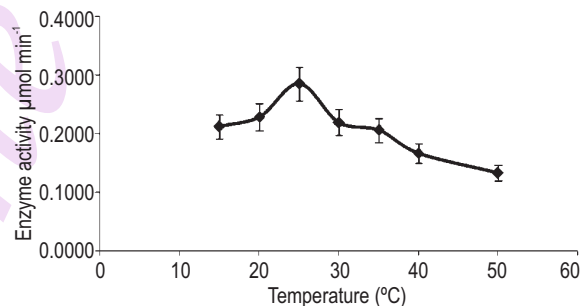


Fig. 5 : Temperature profile of *L. calcarifer* kidney on the activity of purified ChE

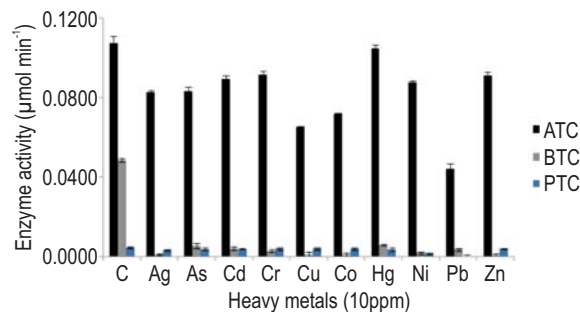


Fig. 6 : Effect of various heavy metals on the enzymatic activity of purified ChE from *L. calcarifer* kidney tested with three different synthetic substrates (ATC, BTC, PTC)

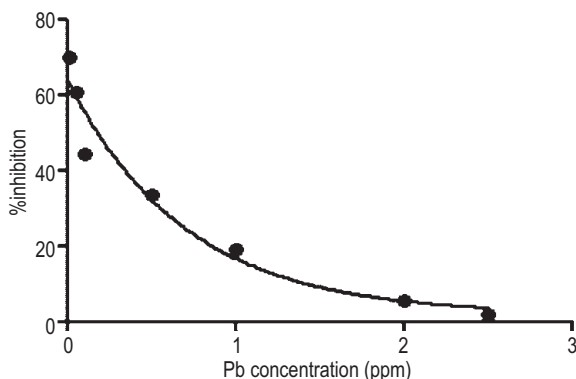


Fig. 7 : Effect of Pb concentrations on kidney ChE activity

agents can inhibit ChE activity through the process of carbamylation and phosphorylation at the active site and by blocking the binding of substrate (Kwong, 2002; Weinbroum, 2004; Rosenberry *et al.*, 2005). Unlike pesticides, metal ions inhibition is caused by the affinity binding towards the amino acid side chain. Proteins that have histidine residues are most susceptible to metal binding such as copper and zinc (Abdelhamid *et al.*, 2007; Rajesh *et al.*, 2009). In the present study capability of metal ion to inhibit ChE activity extracted from the kidney of *L. calcarifer* has been verified. The intensive study has been done to know the mechanism of heavy metals inactivation of ChE by mercury (Frasco *et al.*, 2007). They mentioned that inactivation of mercury is due to action of sulfhydryl reacting agent. The other heavy metals mechanism of inhibition still remains unknown, but in some studies it can be ventured to be caused by conservation of catalytic triad Ser-His-Glu in both AChE and BChE (Podoly *et al.*, 2009).

Screening of heavy metals showed that kidney ChE of *L. calcarifer* was sensitive towards Pb as it inhibited the activity by more than 50%. The purified kidney ChE was very sensitive to Pb, which acted as an inhibitor for the enzyme.  $IC_{50}$  study for Pb was obtained for further studies is shown in Fig. 7. Enzyme activity of kidney ChE was inhibited >50% at 0.1 ppm concentration. Heavy metals pollution is a great health threat and the emerging development of an assay for heavy metals is anticipated to increase the efficiency of toxic xenobiotics biomonitoring (Sani *et al.*, 2010; Kavita *et al.*, 2011). Agrahari *et al.* (2006) in his study used fish, *Clarias punctatus* as a biomarker for monitoring the presence of insecticide. Thus, there is a possibility that ChE from *L. calcarifer* kidney can be used as an alternative method for detection of not only heavy metals, but other contaminants such as detergents, dyes and pesticides.

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