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The use of *Lates calcarifer* as a biomarker for heavy metals detection

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Abstract Fish are ubiquitous organisms that have many features that designate their potential as a biomarker of heavy metals pollution. Thus, an investigation was done to detect the effect of heavy metals on cholinesterase (ChE) activity from *Lates calcarifer* organs which were gill and muscle. Ammonium sulphate precipitation was performed along with ion exchange chromatography to purify the enzyme. In the substrate specificity study, ChE from *L. calcarifer* gills was capable of breaking down acetylthiocholine iodide (ATC) at a faster rate compared to the other two synthetic substrates, which are butyrylthiocholine iodide (BTC) and propionylthiocholine iodide (PTC). In contrast, the muscle ChE has a higher affinity towards PTC. The maximum activity of ChE observed at the temperature ranging from 20 to 30 °C in Tris–HCl buffer pH 8. ChE from the two organs of *L. calcarifer* showed an inhibitive reaction towards heavy metals, but with different effects. ATC from gills showed 50 % inhibition by Cu, Hg and Pb, while PTC from muscle showed 50 % inhibition by Pb. The variation of inhibitory effect that was shown by ChE from *L. calcarifer* organs can be further studied in designing a biosensor kit that is sensitive towards heavy metal.

Keywords Biomarker · Biosensor · Cholinesterase · Heavy metals

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1 Introduction

Pollution of aquatic ecosystems can have serious consequences over time when it reaches a point at which it may be too late to take effective countermeasures. Development of a range of biological responses measured in number of different species has been done because of the increasing need to detect and assess the impact of pollutants, particularly at low, sub lethal concentrations, on environmental quality (Livingstone and Goldfarb 1998; Fent 2004). Changes in molecular, physiological or behavioural responses, prior to death or overt sickness indicate the response to stress by organisms. A growing interest in cholinesterase based biomarker in the last two decades has been observed as it provides an integrative measurement of the overall neurotoxic risk that arise in contaminated areas by several classes of pollutants (Lionetto et al. 2013; Shukor et al. 2013).

According to Carajaville et al. (2000), a sensitive tool for biological effect measurement in environmental quality assessment has been introduced with the use of biomarkers measured at the molecular level. Acetylcholine is the primary neurotransmitter in the sensory and

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464

Rend. Fis. Acc. Lincei (2016) 27:463–472

neuromuscular system of fish. The activity of this system is important for normal behaviour and muscular function (Payne et al. 1996). Cholinesterase (ChE) based biomarkers are well-developed markers of neurotoxicity that was being applied in invertebrates and fish for pollution monitoring studies (Kirby et al. 2000; Porte et al. 2002; Lionetto et al. 2004). In the muscle tissues of marine fish species, pseudocholinesterases such as butyrylcholinesterase (BChE) and propionylcholinesterase (PChE) (EC 3.1.1.8) are also present along with AChE. Sturm et al. (2000) stated that in some cases, pseudocholinesterases are more sensitive to anti-ChE chemicals. Thus, this study aimed to assess the fish muscle tissue cholinesterase inhibition by pesticides and heavy metals as there is abundance of cholinesterase in that organ.

2 Materials and method

2.1 Extraction and purification

The fish was obtained from Pusat Sains Marin UPM in Telok Kemang, Port Dickson. The gills of *L. calcarifer* were dissected and then weighed. Homogenisation of the sample was performed using Ultra-Turrax T25 Homogeniser in 0.1 M sodium phosphate buffer pH 7.5 containing 1 mM phenylmethylsulfonyl fluoride with the ratio of 1:4 (w/v). The sample was later centrifuged at $10,000 \times g$ for 30 min at 4 °C. Collected supernatant was stored at −20 °C for the purification process. The next step was done by leaving the sample to thaw at the ambient temperature followed by loading 15 mL of the supernatant into the ion exchange column containing DEAE-Cellulose with the dimensions of 5 cm diameter and 80 cm height. 1.5 L of 25 mM sodium phosphate buffer pH 7, which is the washing buffer, was filled into the column with the flow rate calibrated at 0.1 mL min^{-1}. The unbounded protein need to be eradicated. The ChE of *L. calcarifer* bounded to the matrix was eluted by loading 25 mM sodium phosphate buffer pH 7 containing 1 M NaCl into the column. 1 mL fractions were collected and assayed for enzyme activity and protein concentration determination. The fraction that shows the maximum activity was later concentrated using Sartorius Vivaspin 20 at $5000 \times g$ at 4 °C for 10 min. Purified ChE was stored at −20 °C (Hayat et al. 2015). Native polyacrylamide gel electrophoresis (Native-PAGE) was done to see the efficiency of the ChE purification as described by Laemmli (1970). Proteins that have been separated were stained using Coomasie Brilliant Blue G-250.

2.2 ChE activity and protein content determination

A slight modification of Ellman et al. (1961) method was used to determine the enzyme activity of *L. calcarifer* using 96-well microplate 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 and incubated for 15 min. 20 μL of the substrates (5.0 mM ATC, BTC and PTC) were then added to the mixture and incubated for 10 min. ChE activity was specified 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/mg of protein or U mg^{-1} of protein. Protein content determination was calculated as described by Bradford (1976). Bovine serum albumin (BSA) was used as standard. The assays were run in the dark and all of the tests were carried out in triplicates.

2.3 Optimal substrate specificity

The substrate specificity for *L. calcarifer* ChE was determined at the ambient temperature with three different synthetic substrates, which are ATC, BTC and PTC, with concentrations ranging from 0.1, 0.5, 1, 2, 5 and 10 mM of the substrates in sodium phosphate buffer (0.1 M, pH 7). The substrate was added into reaction mixture and the reading of absorbance at 405 nm was recorded after 10 min. Graph Pad Prism Software version 5 was used to plot Michaelis–Menten curves to determine the maximal velocity (V_{max}) of ChE activity and biomolecular constant (K_m) (Sabullah et al. 2015a).

2.4 Optimal pH and temperature profile

Lates calcarifer ChE was incubated 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) to determine the optimum pH for the enzyme. The reaction mixture was incubated in different temperatures ranging from 15 to 50 °C. The optimal temperature of the enzyme was then determined. Beyond this range of temperature, ChE was considered fully denatured.

2.5 The effect of metal ion

Ten types of heavy metals, namely silver (Ag), arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb) and zinc (Zn) were tested on *L. calcarifer* ChE to study the effect of these metal ions on the enzyme activity. These metals were selected because of their capability to give an adverse impact to the

environment. The reaction mixture contains 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^{-1}, 20 μL of DTNB (0.1 mM), and 10 μL of ChE. The reaction mixture was then incubated for 15 min followed by the addition of 20 μL of the substrate into the mixture. The mixture was left for another 10 min of incubation before the absorbance was recorded at the end of the incubation time at the wavelength of 405 nm. The sample was then incubated for 30 min with selected heavy metals. An enzyme assay was performed to determine IC$_{50}$ value for the sample.

3 Results and discussion

3.1 Sample extraction and purification

Lates calcarifer was chosen to be the source of the ChE because it is one of the most economically important species among our native Malaysian fishes. Most Asian sea bass are produced by commercial aquaculture in many Asian countries such as Malaysia, Taiwan, Thailand and Indonesia. It is also a relatively hardy species that can tolerate crowding and has wide physiological tolerances. There are other types of fish such as *Tilapia mossambica* (Al-Ghais 2013), *Osteochillus hasselti* (Sabullah et al. 2013) and *Puntius javanicus* (Sabullah et al. 2014) that were reported to be a sensitive biomarker with toxicants especially heavy metals. Thus, fish is considered as a sensitive biomarker tool and a highly sensitive enzyme such as ChE allows the detection of lower contamination levels of pollution (Sabullah et al. 2015b). In this study, the extraction of ChE from the gill and muscle of *L. calcarifer* was done using ammonium sulphate precipitation and ion exchange chromatography steps. Gills are normally full of blood that contains ΨChE, thus the gill was extensively washed before homogenization process to ensure that ΨChE activity did not alter the activity or properties of the enzyme studied. While performing the ion exchange chromatography, DEAE-Cellulose was chosen to be the matrix. Enzyme recovery of 7.79 % was recorded at the end of this study and the purified gill ChE displayed a specific activity of 4.254 *U* mg^{-1} with purification folds of 30 (Table 1). While in muscle, enzyme recovery of 8.32 % with 26 purification folds was obtained at the end of this study (Table 1). The enzyme recovery for both gill and muscle ChE were rather low with almost 90 % of the activity lost throughout the purification process. This purification approach is necessary as the purified enzyme is more sensitive toward inhibitors compared to the crude enzyme. The molecular weight for the purified ChE was determined after the graph of log$_{10}$ (kDa) that was plotted. Based from the results, the molecular weight obtained for gill was 136 kDa (Fig. 1a). The result was in contrast with

the study done by Bocquene et al. (1997), in which the molecular weight of gill ChE extracted from the common oyster (*Crassostrea gigas*) was in the range of 200–330 kDa when electrophoresis was performed in non-denaturing conditions. While for muscle ChE, the molecular weights obtained were 18 and 16 kDa, respectively (Fig. 1b). The two different molecular weights that were obtained for muscle were the same monomers, but with different aggregation. There is no recent study on the molecular weight of muscle ChE, thus no comparison can be made on the native form of muscle ChE. However, the variation of gill and muscle ChE activity has been proved by Tu et al. (2009) in which they show different activity and inhibition frequencies after exposed with serine, iso-OMPA, BW284c51 and also pesticides. The purification process does not affect the enzyme activity although it seems to modify the molecular structure. The enzyme activity only can be affected by temperature, pH and concentration of the enzyme and substrate. These results proved that purified ChE was electrophoretically homogenous. In the study, the specific activity of the enzyme increases throughout the purification process at the end of the experiment.

3.2 Kinetic study

ATC, BTC, and PTC were used at various concentrations to study the specificity of the ChE towards the substrate. Figure 2a, b shows a Michaelis–Menten kinetics curves that was obeyed and this result explained the hydrolysation of the substrate by ChE. An increase in substrate concentration will increase the activity of the enzyme until the maximum points reached. Above 5 mM substrate concentration, the enzyme, however, displayed a plateau state. A comparison was made among the three substrates and it was concluded that ATC showed the lowest K_m value, which is 0.2345 mM and the highest V_{max} value of 0.0354 μmol min^{-1} mg^{-1} as shown in Table 2 for gill ChE. On the other hand, in muscle tissue, the purified ChE showed a preference for the substrate PTC, as seen by the maximum hydrolysis rates and greater catalytic efficiencies of ChE with this substrate as displayed in Table 2. Furthermore, when the purified sample was incubated with PTC, there was a maximum ChE activity of 8.431 μmol min^{-1} mg^{-1} protein at 5 mM of PTC. Furthermore, the highest catalytic efficiency (V_{max}/K_m) was shown by ATC and PTC. Figure 3a, b also proved that purified ChE from the gill and muscle of *L. calcarifer* had a strong affinity towards substrate ATC and PTC, respectively, as they showed the highest V_{max} and lowest K_m value. These results were in agreement with the study reported by Srivastava et al. (2013) as the highest ratio of V_{max}/K_m was obtained in this study explaining that the purified ChE hydrolysed ATC and PTC with a maximum efficiency.

466

Rend. Fis. Acc. Lincei (2016) 27:463–472

Table 1 ChE from *L. calcarifer* gill and muscle purification table

Procedure	Total protein (mg)		Total ChE activity (U)		Specific activity (U/mg)		Purification fold		Yield (%)	
	G	M	G	M	G	M	G	M	G	M
Crude	103.20	47.60	14.73	15.51	0.14	0.33	1	1	100	100
Ammonium sulphate precipitation	3.20	2.36	2.78	6.38	0.87	2.70	6	8	18.89	41.13
DEAE-cellulose	0.27	0.15	1.15	1.29	4.25	8.43	30	26	7.79	8.32

The specific activity from each step of purification is displayed in ($U\ mg^{-1}$)

G gill, *M* muscle

Fig. 1 The molecular weight of the purified ChE from *L. calcarifer* by interpolation of the retention factor (*rf*) of protein markers. **a** Gill; **b** muscle. Overlapping of purified ChE with other protein was indicated by the *triangle*

3.3 Optimal pH and temperature

Acetate, phosphate, and Tris–HCl buffer were used with a different range of pH to determine the optimal pH of ChE. The activity of ChE was at maximum in between pH 7 and 8 of potassium phosphate 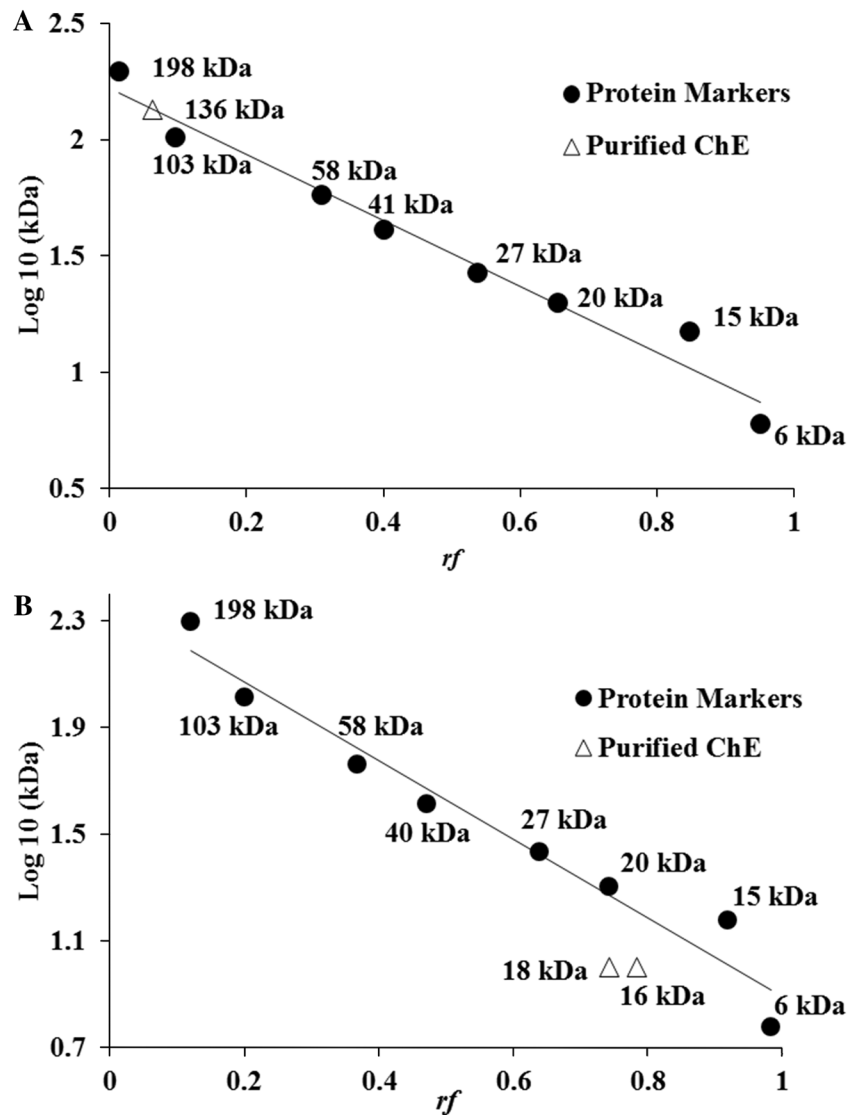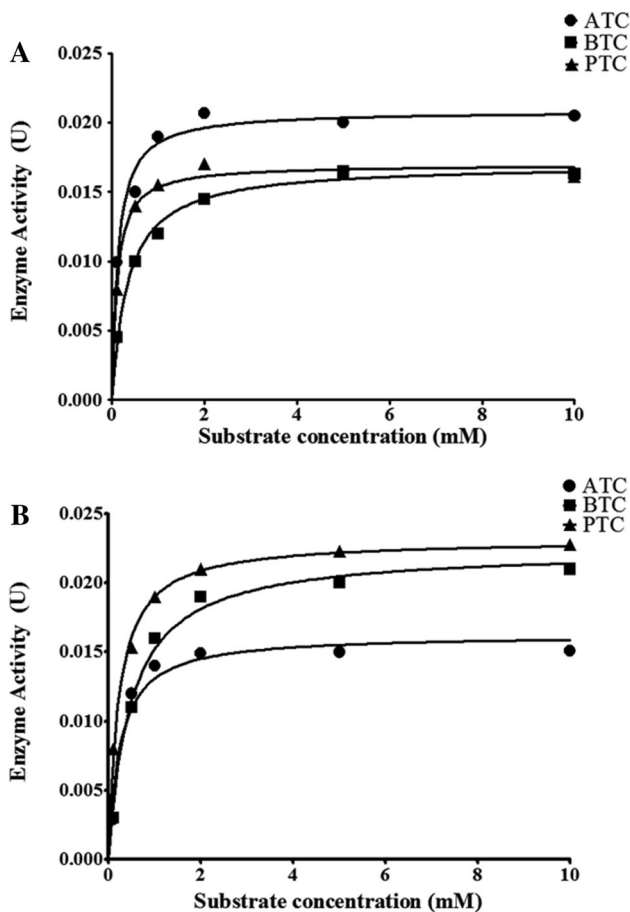and Tris–HCl buffer as shown in Fig. 3a, b. Tris–HCl buffer (0.1 M, pH 8) has been determined to be the best buffer as the ChE activity was the highest and showed the highest value. Tris–HCl buffer gave an optimum condition for the purified ChE as the highest mean point of data that was showed when the results from two types of buffer were compared. According

Fig. 2 Incubation of purified ChE from *L. calcarifer* **a** gill; **b** muscle in different synthetic substrates; ATC, BTC and PTC with different concentrations

to (Masson et al. 1996), changes in pH caused protonation of imidazole group of histidine that exist at the catalytic triad thus affect the formation of enzyme-substrate complex.

Figure 4 shows different temperatures displayed different effects on ChE activity. The optimum ChE activity was determined to be at 25 °C. At lower temperature, minimum activity of ChE was observed. As the temperature increases, the activity rose and reached maximum value where the given velocity was reached and a bell shaped curve was later demonstrated. However, at higher temperatures the ChE activity decreased abruptly. The maximum activity of

L. calcarifer ChE was achieved at the range of 20–30 °C. Gaudy et al. (2000) reported that the protein will undergo denaturation process when at higher temperature resulting loss of protein stability. Hence, this theory explained the sudden decrease in enzyme activity at temperature above 30 °C shown in Fig. 4.

3.4 Metal ion inhibition study

In aquatic species, ChE activity has been widely studied and employed as a biomarker for chemical exposure detection in natural ecosystem (Dellali et al. 2001; Roméo et al. 2003; Lavado et al. 2006). AChE is mainly considered to be a biomarker for the detection of exposure to organophosphate (OP) and carbamate (CB) pesticides, other than that, heavy metals, other pesticides, polycyclic aromatic hydrocarbons, detergents, and components of complex mixtures of contaminants are also able to inhibit ChE activity as reported in recent years, (Lionetto et al. 2004; Jebali et al. 2006; Vioque-Fernandez et al. 2007; Kopecka-Pilarczyk 2009). Thus, in this study, we aim to study the inhibitory effects of heavy metals on ChE activity. Silver (Ag), arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni) and zinc (Zn) with the concentration of 10 ppm were selected and incubated with gill samples from *L. calcarifer*. Only Cu, Hg and Pb showed 50 % inhibition when the samples were treated with ATC as the substrate (Fig. 5a). On the other hand, when BTC was used, all of heavy metals selected showed inhibition. However, the effect of heavy metals on ChE activity differs when PTC was used as substrate. Based on the results, Cu and Pb showed an inhibition towards the purified ChE activity. Among all of the selected heavy metals, only three heavy metals showed >50 % inhibition which were Cu, Hg and Pb. The in vitro studies of muscle ChE as shown in Fig. 5b displayed that muscle inhibited almost all of selected heavy metals when ATC and BTC were used as the substrates. On the other hand, only Pb showed inhibition of >50 % after the ChE muscle was incubated with PTC as the activity was lowered to 8.113 %. When gill and muscle ChE was incubated in specific substrate, they were inhibited by Cu, Hg and Pb. Pb^{2+} is an important in vitro inhibitor of ChE activity in which they stated that the maximum decrease observed was up to 65 % in earthworms, 49 % in

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

	ATC		BTC		PTC	
	G	M	G	M	G	M
V_{max} (µmol/min/mg)	0.0354	0.0164	0.0288	0.0230	0.0251	0.0241
K_m (mM)	0.2345	0.2641	0.2844	0.4926	0.2600	0.2391
V_{max}/K_m	0.1508	0.0622	0.1012	0.0466	0.0965	0.1009

G gill, *M* muscle

468

Rend. Fis. Acc. Lincei (2016) 27:463–472

Fig. 3 pH profile of *L. calcarifer* **a** gill; **b** muscle on the purified ChE activity
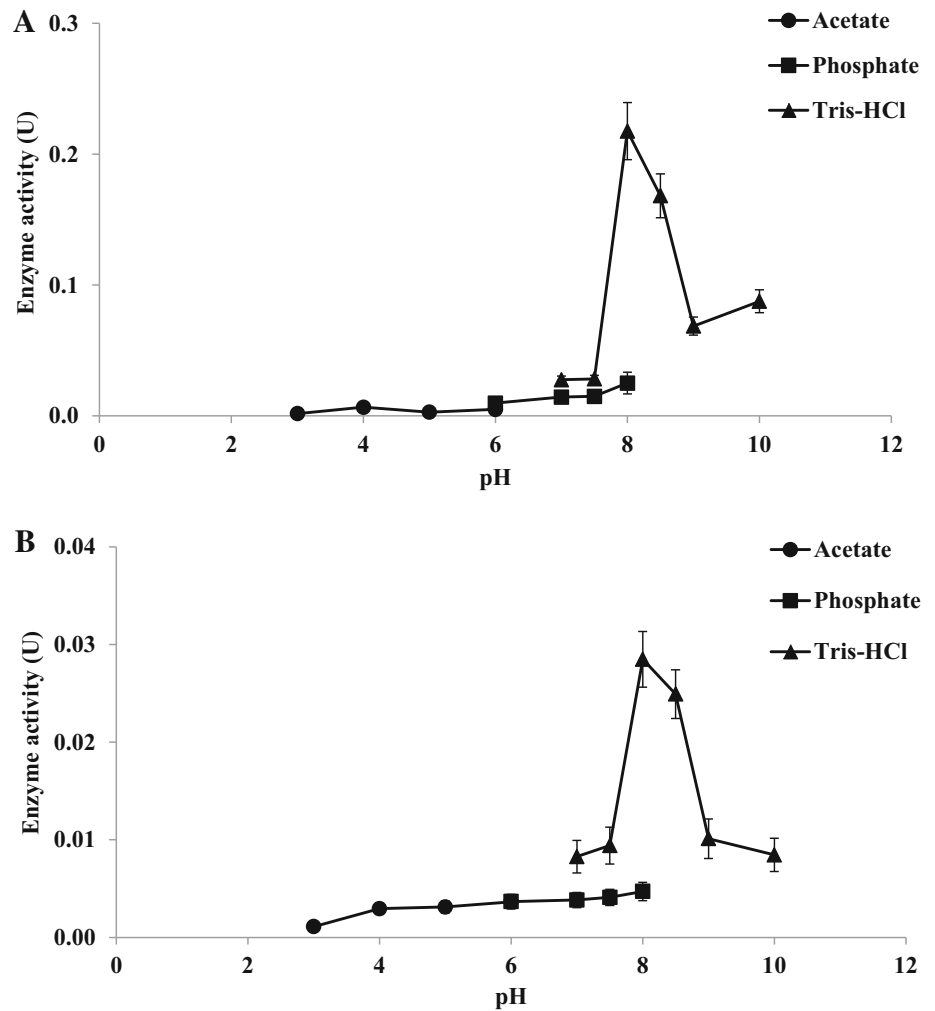
Fig. 4 Temperature profile of *L. calcarifer* gill and muscle on the activity of purified ChE
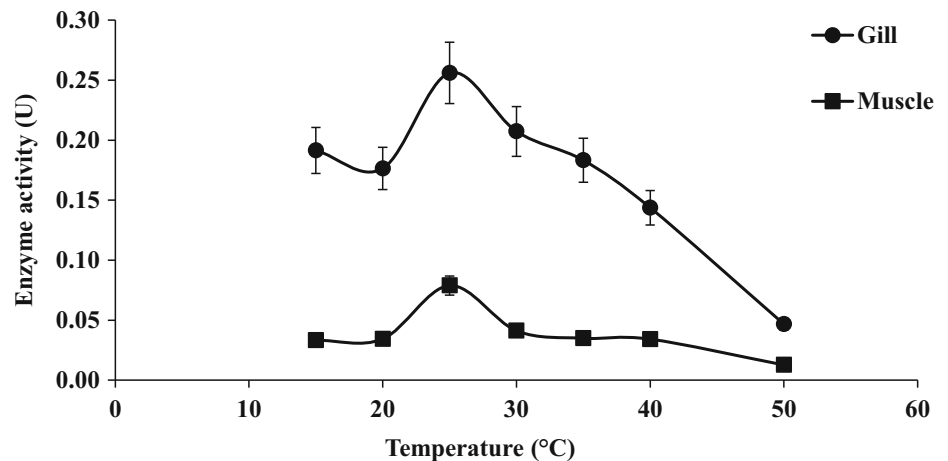

clams, and 35 % in fish for the dose of 2.5 g Pb L^{-1} (Labrot et al. 1996).

Three selected heavy metals were then incubated again for 30 min with ChE for IC$_{50}$ value determination. The IC$_{50}$ value of gill ChE for Cu was at 1 ppm and the enzyme fully inhibited at higher concentrations (Fig. 6a). IC$_{50}$ value for Hg was determined to be at 0.5 ppm as shown in Fig. 6b. On the other hand, Fig. 6c shows the IC$_{50}$ value to

Fig. 5 Effect of different types of heavy metals on the enzymatic activity of purified ChE from *L. calcarifer* **a** gill; **b** muscle when incubated with three different synthetic substrates (ATC, BTC and PTC)
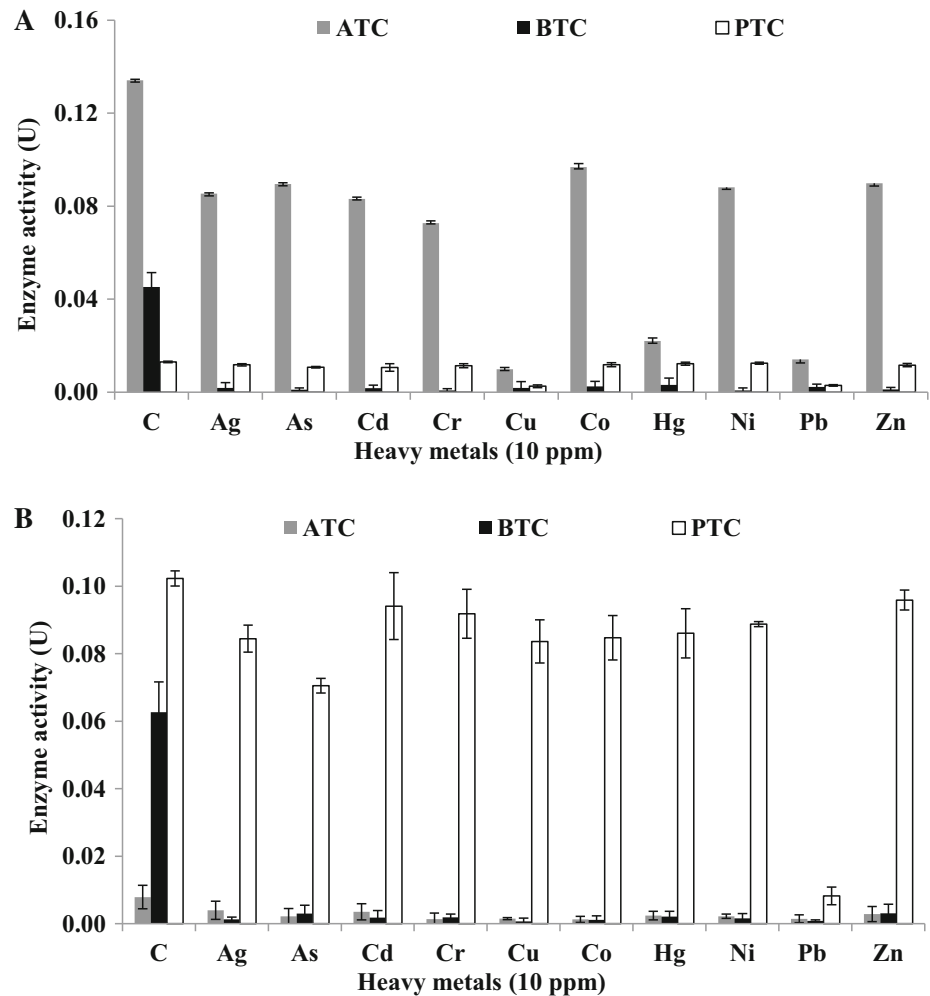

be at 1 ppm when gill ChE was incubated with Pb. Contrariwise, Fig. 7 demonstrated the half maximal inhibitory concentration (IC_{50}) of muscle ChE by Pb. The enzyme activity was inhibited >50 % at concentration of 0.1 ppm. The activity was further inhibited by Pb as the concentration was increased.

Different species showed different sensitivity results when tested with toxicants as reported in the recent studies (Kuca et al. 2005; Gbaye et al. 2012; Santarpia et al. 2013). Fish was chosen as a favoured tool for the early detection of toxicants as they were sensitive towards toxicants (Oliveira et al. 2007; Tham et al. 2009). Additional studies are needed to identify any alternative source of biosensors that are sensitive towards toxicant especially heavy metals and give rapid results. The level of heavy metals pollution is rising due to the abundance of metal-related industries in Malaysia (Shukor et al. 2006). In Malaysia, the use of real-time monitoring of heavy metals was lacking because of conventional chemical assays require numerous large instruments and a longer time period is needed to obtain the results (Lopez-Roldan et al. 2012). An exciting trend has
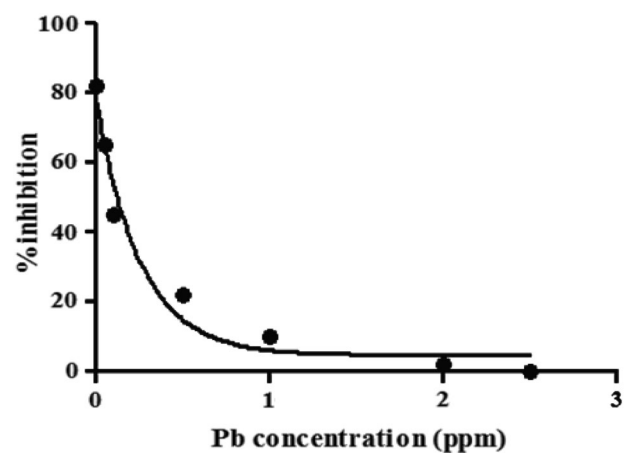
emerged recently, as reported by Girotti et al. (2008) which is near-real-time biomonitoring of heavy metals. The presence of amino acid residues promoted the toxicant to bind at either the active or allosteric sites of ChE. After the structure of the enzymes was altered by the interactions of heavy metals with negatively charged amino acids, the enzyme will be inhibited. Another reason for the inhibition to occur was the cleavage of the enzyme disulfide bonds (Masson et al. 1996; Najimi et al. 1997; Abdelhamid et al. 2007; Frasco et al. 2007).

Jonz and Zaccone (2009) reported that fish gill is a sophisticated organ that performs many processes including respiration and receives extensive innervations from nervous system in the form of sensory and motor pathways. Fish gills also illustrated a quick recovery and it has been determined as one of the organ that is sensitive to pesticide and toxicant effects according to the study done by McHenery et al. (1997). They also observed that fast recovery of the ChE activity from the gills of *Mytilus edulis* occurred when ChE was exposed to serial concentrations of dichlorvos (10, 100 and 1000 μg/L) and then replaced in

470

Rend. Fis. Acc. Lincei (2016) 27:463–472
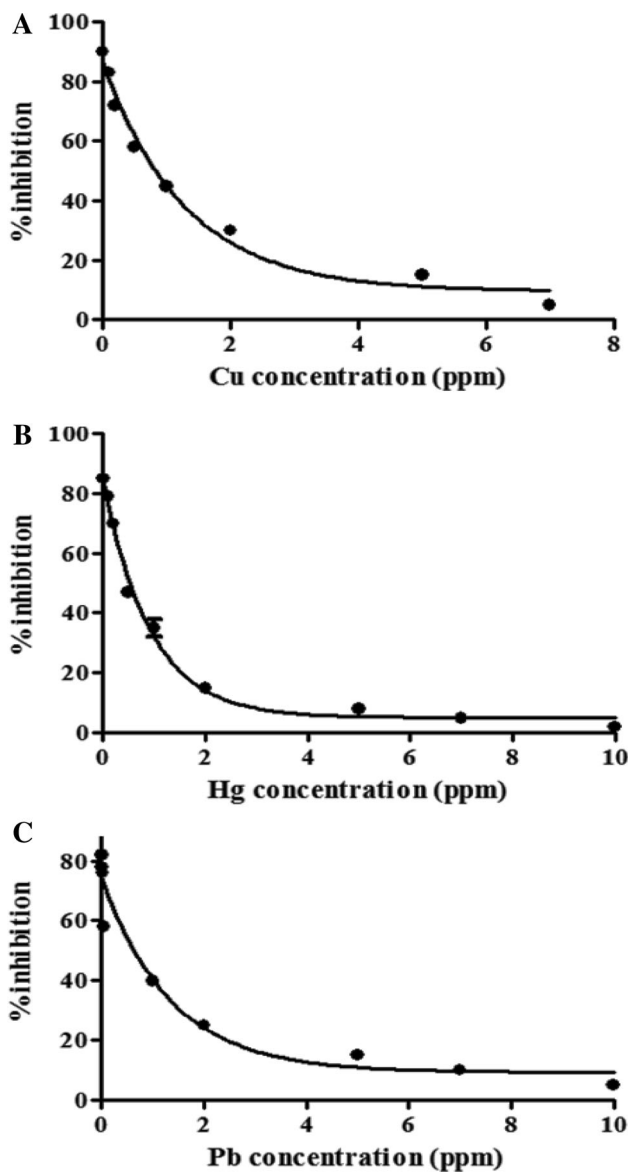
Fig. 6 Effect of **a** Cu; **b** Hg; **c** Pb concentrations on ChE activity from *L. calcarifer* gill

Fig. 7 Effect of Pb concentrations on ChE activity from muscle sample of *L. calcarifer*. The inhibitor with a different concentration was mixed with ChE sample and the activity was determined after the mixture was incubated for 30 min

calcarifer was inhibited in vitro, in agreement with study done by Bocquene et al. (1990). Hence, this study suggests that *L. calcarifer* ChE could be a possible new source of ChE to replace the existing commercial ChE.

4 Conclusions

The optimal assay parameters for purified ChE from *L. calcarifer* namely pH and temperature were successfully determined. In this study, ATC and PTC were chosen as the preferred specific synthetic substrate for *L. calcarifer* gill and muscle, respectively. A promising biosensor kit for heavy metals detection was suggested to reduce the pollution in marine environment after studying the sensitivity of ChE inhibition by selected heavy metals. Investigation on the capability of purified ChE to detect other contaminants such as detergents, dyes, pesticides and drugs is suggested for further study on this field.

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clean media for 7 days. Kopecka-Pilarczyk (2009) reported that as for the sensibility of various tissues, gills seem to be the best tissue as a biomarker of pesticides and heavy metals exposure because AChE from gills proved to be the most susceptible to inhibition by the pollutants tested. Purified ChE from *L. calcarifer* gill and muscle have the ability to show inhibition of activity by selected heavy metals with different levels of inhibition, which later can be expanded and developed into an alternative method for biosensing based from these results. Heavy metals such as copper, mercury and lead showed significant inhibition towards gill ChE activity as they reduced to half of its activity. These results proved that at some concentrations of heavy metals, the activity of ChE extracted from *L.*

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472

Rend. Fis. Acc. Lincei (2016) 27:463–472

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