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Full Length Article

Purification and Anticholinesterase Sensitivity of Cholinesterase Extracted from Liver Tissue of *Puntius Javanicus*

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Abstract

The purification of a soluble cholinesterase (ChE) from *Puntius javanicus* liver using affinity chromatography was studied. Affinity matrix was synthesised through the cooling system of ligands procainamide to epoxy-activated Sephacryl 6B and purification process was performed using calibrated flow rate at 0.2 mL/min. Non-denaturing electrophoresis condition was employed and the single band native form of ChE was detected at 66.267 kDa after being stained with commasie brilliant blue. ChE detection was performed using gel filtration; ZORBAX column attached to the HPLC with the flow rate of 1 mL/min. Only a single peak was detected at the retention time of 3.720. From the assay evaluation, the final purified ChE procedure displayed the highest sensitivity of detecting the anticholinesterase namely mercury, copper, malaoxon and carbofuran compared to the impure ChE and the results were further discussed in detail to the potential application of ChE from *P. javanicus* as a biomarker for those toxicants. © 2015 Friends Science Publishers

Keywords: Purification; Cholinesterase; *Puntius Javanicus*; Anticholinesterase

Introduction

The liver is the primary target for the accumulation of metabolites especially for the regulation of protein metabolism, glycogen deposition, toxicant degradation and detoxification (Charlton, 1996; Berg *et al*., 2002; Ferrari *et al*., 2007; Nyakudya *et al*., 2014). Detoxifying enzymes such as glutathione S-transferase, glutathione peroxidase and cholinesterase play a major role in the degradation or biotransformation of toxic to non-toxic compounds (Holovská *et al*., 2005; Cho and Kong, 2007; Ramsey *et al*., 2010; Nicolet *et al*., 2003).

Cholinesterase is an enzyme in the group of serine hydrolases that falls broadly into two functionally and structurally identical, yet different enzymes and distributions known as acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE). Both enzymes can be distinguished based on their own substrate specificities, which are acetylcholine (ACh) for AChE and butyrylcholine (BuCh) for BuChE (Principato *et al*., 1989; Barbosa *et al*., 2001; Pohanka *et al*., 2011; Romani *et al*., 2011). BuChE is also known as pseudocholinesterase as its capability to hydrolyse both ACh and BuCh at different frequencies (Forget *et al*., 2002; Khan *et al*., 2008). It has been shown that BuChE works as a co-regulator for choligernic activity (Giacobini, 2003). Previous studies mentioned that BuChE detoxifies xenobiotic compounds before they reach to AChE at physiologically important target sites (Tougu, 2001; Çokugra, 2003; Nicolet *et al*., 2003; Gandahi *et al*., 2013). BuChE is predominantly synthesised in the liver tissue (Santarpia *et al*., 2013).

Utilisation of ChE as a biosensor tool is well known (Fulton and Key, 2001; Rao, 2006; Aker *et al*., 2008). However, previous studies had mentioned that different species exhibited various individual sensitivity results (Kuca *et al*., 2005; Gbaye *et al*., 2012; Santarpia *et al*., 2013) especially fish, which happens to be a preferred tool for the early detection of contaminant existence (Oliveira *et al*., 2007; Sabullah *et al*., 2014). However, more data are

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required to identify any alternative sources of biosensors that are rapid and sensitive to toxicant especially pesticides and heavy metals contamination.

To obtain a reliable result, purification is needed to increase the sensitivity of ChE to react with the toxicant efficiently. Thus, the objective in this study were: 1) to isolate ChE from the liver tissue of *P. javanicus* through several purification procedures, 2) to measure the molecular weight of purified ChE based on SDS PAGE and HPLC analysis, and 3) to compare the remaining activity of crude and purified ChE after treated with toxicant such insecticides and metal ion to prove the sentitive for detection purposes.

Materials and Methods

Extraction and Purification

Specimen Preparation: Live *Puntius javanicus* was brought from Aquaculture Development Centre, Bukit Tinggi, Pahang, Malaysia to laboratory and acclimatised for 15 days with fully aerated and chlorine-free tap water. Water was changed two times per week to ensure water cleanness. Prior the experiment, the fish were not fed for 24 h to minimise the dietary effects. The fish were killed by immersing it in a box of ice for approximately 30 minutes. The liver was dissected and weighed. Mortar and pestle were first used to crush the liver to facilitate the homogenization process. The extraction process was performed using Ultra Turax homogeniser with 0.1 M sodium phosphate buffer, pH 7.5, containing crushed liver at the ratio of 1:4 (w (sample)/v (buffer)). The crude extract was then ultracentrifuged at 100,000 × g for 1 h at 4°C. The supernatant was collected and stored at -25°C.

Procainamide-sephacrayl 6B synthetization: Procainamide-based affinity matrix was synthesised based on the method of Ralston *et al*. (1983) with slight modification from Son *et al*. (2002). Sephacryl 6B (50 mL settled gel) was washed with 10 volumes of deionised water using a sintered glass tunnel as recommended by the manufacturer, the Sigma Chemical Company (Poole, UK), sucked dry to a wet cake, then suspended in a volume of 0.6 M NaOH containing 50mM sodium borohydrate (Sigma, St. Louis, USA) and stirred. A volume of 1,4-butanediol diglycidyl ether (Sigma, St. Louis, USA) was slowly added with constant stirring and left overnight at room temperature. Epoxy activated sephacryl waswashed with 10 volumes of deionised water and finally washed using one volume of acetone to completely remove the bioxirane group. The activated gel slurry was washed with distilled water and further washed using 10 volumes of 0.1 M sodium acetate buffer (pH 4.5) 0.012 M sodium borate buffer (pH 10) and 10 volumes of deionised water. The gel slurry was then transferred onto a coupling buffer of 12 mM borate buffer (pH 11.0) containing 0.2 M of procainamide (Sigma, St. Louis, USA) and the pH of the mixture was adjusted by the addition of 1.0 M NaOH, which was then incubated for 96 h at room temperature on an orbital shaker. A volume of 1.0 ethanolamine (pH 9.0) was used to block all of the remaining active groups on the amine-reactive supports of the gel. The mixture was shaken for 6 h at room temperature. Finally, the gel was washed with 10 volumes of 1.0 M NaCl followed by 10 L of deionised water. The gel was then immersed in 30% of ethanol and stored at 4°C.

Purification of ChE using affinity procainamide-based chromatography: The affinity matrix was packed in a column(0.9 cm diameter) and allowed to sink to obtain a bed height of 5 cm. The matrix was first washed with 10 column volumes of washing buffer; 20 mM sodium phosphate buffer, pH 7.5, to clean and calibrate the flow rate at 0.2 mL/min. The supernatant was directly loaded onto the column. After all of the supernatant has been dissolved in the matrix, 3 batch volumes of washing buffer were loaded, followed by the collection of 1 mL fractions using microcentrifuge tubes and kept on ice. Then, the elution buffer; 20 mM sodium phosphate buffer containing 100 mM sodium chloride, pH 7.5, was applied onto the matrix. The elution phase was continued using a series of elution buffers with NaCl concentration sranging from 0.2 M to 1.0 M. ChE activity (Ellman *et al*., 1961) and protein content determination (Bradford, 1976) was carried out for all the fractions collected. Fractions that displayed high ChE activity were then pooled. The purified samples were concentrated and desalted using VivaSpin® tubes at 5000 × g for 10 min at 4°C. The dialysed purified ChE was stored at -25°C until subsequently used.

Purity and Molecular Weight Determination

Gel electrophoresis: Native-PAGE was carried by following the method of Laemmli (1970) which included the preparation 4% stacking polyacrylamide gels and 10% resolving polyacrylamide gels followed by polymerisation using the addition of N, N, N', N'-tetramethylethylenediamine (TEMED) and 10% of ammonium persulfate (APS). The crude extract, supernatant and concentrated purified *P. javanicus* ChE were run through the gel. Each protein sample was dissolved in sample buffer containing 0.2 M Tris buffer (pH 6.8), 10% (w/v) SDS, 20% (w/v) glycerol, 0.05% (w/v) bromphenol blue at the ratio of 5:1 (v/v). Broad range of molecular weight (MW) was chosen, containing myosin, β-galactosidase, BSA, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, lysozyme and aprotinin with MW of 202.403, 114.802, 73.058, 47.891, 34.111, 27.046, 17.014 and 6.026 kDa, respectively (Bio-Rad, UK). The prepared samples and protein markers were loaded in to each well and electrophoresis was performed in a Mini Protean 3 system (Bio-Rad, UK) at a constant current of 16 mA until the dye front reached the resolving gel (10% acrylamide) and then at 25 mA until the bromophenol dye reached the bottom of the gel. At the end of the process, the

resolving gel was stained using commasie brilliant blue. The stained gel was then visualised using calibrated G-800 densitometer (Bio-Rad, UK). Retention factor was calculated based on the migration of protein per the migration of bromophenol blue.

HPLC Detection: Purified ChE and broad range molecular weight standard marker (myosin, 202.403 kDa; β-galactosidase, 114.802 kDa; BSA, 73.058 kDa; ovalbumin, 47.891 kDa; carbonic anhydrase, 34.111 kDa; soybean trypsin inhibitor, 27.046 kDa; lysozyme, 17.014 kDa; aprotinin, 6.026 kDa) were injected into the ZORBAX GF-250 gel filtration column that was attached to the HPLC and the eluted protein was then monitored using an HPLC UV detector at a fixed wavelength of 280 nm. Degassed mobile phase containing 0.2 M sodium phosphate buffer and 0.2 M KCl with added 1 mM NaN_3 as a bacteriosatic agent was used and the flow rate was maintained at 0.5 mL/min. Each protein molecular weight was plotted versus the retention time (RT) on a semilog scale and the purified ChE molecular weight was estimated through interpolation of this standard curve.

Sensitivity Improvement Study

P. javanicus ChE; the supernatant and purified ChE were tested by exposure to 10 mg/L concentration of mercury, copper, malaoxon and carbofuran using 1.0 mM butyrylthiocholine iodide as the specific substrate of the test. The absorbance was read from 0 to 10 min at the wavelength of 405 nm. Inhibition were calculated based on remaining percentage activity compared to untreated (control) ChE.

Statistical Analysis

The data reported in the Fig. 4 and Table 1 were averages of triplicate observations (n=3). The data were subjected to determined their diferrences at the level of $p<0.05$ using Student T-Test and One-Way Analysis of Variance (ANOVA) based on Tukey post-hoc analyses. All of statistical analyses in this study were automatically calculated using GraphPad Prism version 5.0 (GraphPad Software, California, USA [http://www.graphpad.com/]).

Results

Purification and Molecular Mass Determination

The purification profile of *P. javanicus* ChE and protein concentrations are displayed in Fig. 1. Two peaks of ChE were obtained with increasing salt concentration, where the first peak was eluted at 200 mM (corresponding to fractions 14th to 16th) and the second peak was at 500 mM (corresponding to fractions 31st to 34th). The highest peak was pooled for subsequent use. ChE was purified to about 100.813 purification folds with 17.593% total recovery compared to supernatant with the purification fold of 2

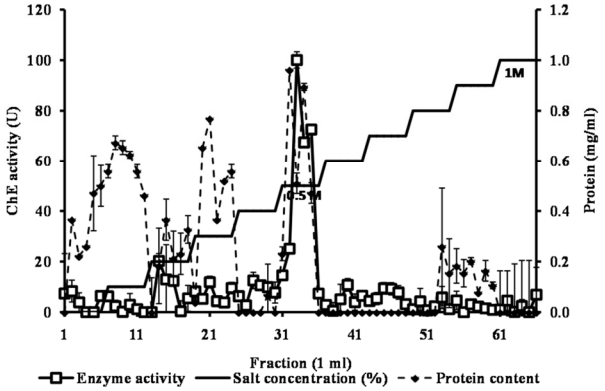

Fig. 1: The purification profile of BChE from liver tissue of *P. javanicus*

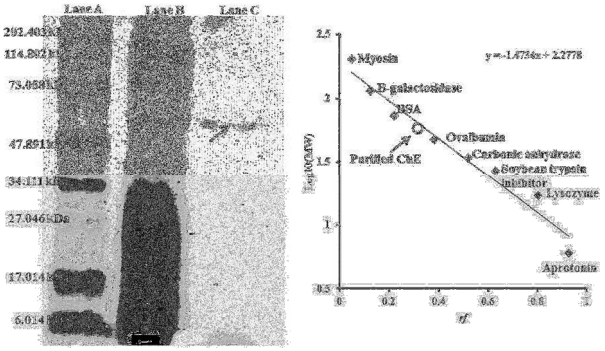

Fig. 2: Non-denaturing polyacrylamide gel electrophoresis (Native-PAGE) of purified ChE from *P. javanicus* in a 10% polyacrylamide gel.

Lane A, broad range molecular weight proteins. Lane B, Supernatant. Lane C, protein was recovered from procainamide-sephacrayl 6B affinity column. Each protein from the gel were detected by commasie brilliant blue staining. Molecular weight of purified ChE from *P. javanicus* estimated from broad range molecular weight calibration plot was 66.267kDa as indicated by an arrow

(Table 1). The non-reducing-PAGE analysis showed a single protein band for ChE sample that was resolved in 10% polyacrylamide gel at lane C after being stained by commasie blue (Fig. 2). The molecular mass of the native form of *P. javanicus* ChE was estimated by interpolating the molecular weight of the standard proteins and displayed a relatively distinctive 66.267 kDa protein of the purified ChE. HPLC analysis showed the single peak that was detected at the RT of 3.72 min, while the molecular mass of ChE was 69.715 kDa. Statistical analysis using Student's t-test showed no significant difference for both molecular weight with triplicate data ($p>0.05$).

Inhibition Study

ChE from *P. javanicus* was tested by incubating in three different toxicants namely copper, malaoxon and carbofuran with the final concentration of 10 mg/L. Based on ANOVA, the results showed that both sample were significantly

Table 1: Purification table of ChE from the liver tissue of *P. Javanicus*

Procedure	Total Activity (U)	Total Protein (mg)	Specific Activity (Umg^{-1})	Purification factor	Recovery (%)
Crude extract	1429.412**	107.796	13.273	1	100
Supernatant	1137.794**	42.779	26.597	2.004	79.599
Purified (Procainamide-Sephacrayl 6B)	251.471	0.1879	1338.67	100.813	17.593

**marked as statistically no significant differences

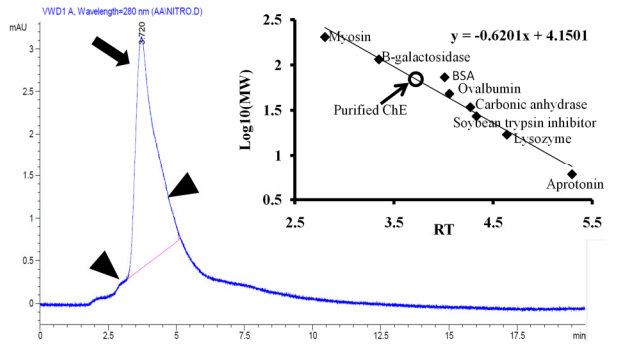

Fig. 3: Profile of purified ChE from *P. javanicus*on Zorbax GF-250 column attached to HPLC

ChE was detected at the RT of 3.720 min. The broad range molecular weight marker was injected in the same column and the logarithm data was ploted versus RT. Molecular weight of purified ChE from *P. javanicus* estimated from broad range molecular weight calibration plot was 69.715 kDa as indicated by an arrow. Triangles show other protein overlapped with ChE

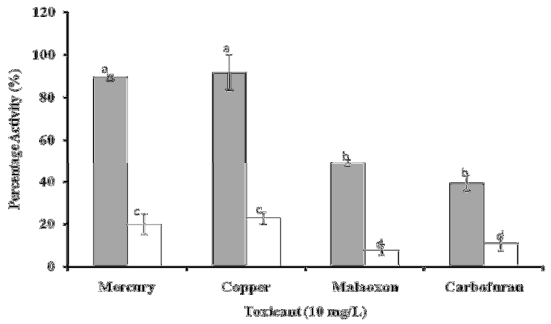

Fig. 4: Comparison the ChE activity incubated with different toxicants at the concentration of 10 mg/L

The error bar stand for standard deviation of mean (n=3) which all the coeficient value lower than 20%. Similar letter show no significant different between each mean point (p>0.05)

inhibited by these toxicants ($p<0.05$) (Fig. 3). However, the purified sample displayed the highest inhibition, where the activity was reduced to 19.97, 22.82, 7.93 and 11.14% after being treated by mercury, copper, malaoxon and carbofuran, respectively. In comparison, impure ChE the activity was only reduced to 50%. This study proved that the purified ChE is more sensitive compared to the impure ChE.

Discussion

ChE from *P. javanicus* was purified through their basic biospecific interaction with an immobilized procainamide ligand. Previous researches have reported the use of procainamide-based affinity chromatography to purify ChE from various samples (Forget *et al*., 2002; Li and Han, 2002; Salles *et al*., 2006; Tham *et al*., 2009; Ralph *et al*., 2011). This affinity matrix was selected due to the isolation of targeted molecule from the abudance of foreign protein which can be purified using high recovery and fold in single step through a specific binding of ChE bound to the matrix and selectively removed under elution phase by the increase of ionic strength using high salt concentration.

Native-PAGE was carried out to evaluate the efficiency of the purification processes. The results displayed the obvious differences between purification stages with large amounts of foreign proteins being removed until a single band was left after being purified using Procainamide-Sephacrayl 6B. This proved the purification ability of the matrix to isolate ChE from the liver tissue of *P. javanicus*. ChE exists mostly in various native forms such as two molecular forms in *Corbicula fluminea* (Mora *et al*., 1999) and *Pacu serum* (Salles *et al*., 2006), three molecular forms in rat (Andres *et al*., 1990) and cotton aphid (Li and Han, 2002) while curimbata serum showed four ChE bands (Salles *et al*., 2006). In contrast, results with the purified ChE from *P. javanicus* obtained from non-denaturing PAGE showed only one major protein band after staining with commasie brilliant blue, suggesting a major molecular form of ChE in our purified sample. However, HPLC analysis showed several small peaks with undetected RT and perhaps, the detected single peak (marked with arrow) was being over lapped with other proteins (marked with triangles) (Fig 3).

Protein purification is required to accurately study the biochemical functions, molecular weight determination, identification and structure of the targeted protein or individual analysis (Berg *et al*., 2002). The purified protein canthen be used for other purposes such as food processing and drug development. In this study, purification was done carried out to increase the sensitivity of ChE towards toxicants by eleminating foreign proteins that interfere with enzyme action. This interference means that the toxicants are not binding or inhibiting the ChE, but instead bind to other protein through various interactions. Previous studies also reported purified ChE from various samples to study the inhibition characteristics of toxicant (Li and Han, 2002; Hsiao *et al*., 2004; Yang *et al*., 2013).

Amino acid residue is the main factor for the binding of toxicant either at the active or allosteric site of ChE. Heavy metals such as mercury and copper are capable of

inhibiting ChE by interacting with the negatively charged amino acids such as aspartate or imidazole group of histidine, or cleavage of disulfide bond that leads structural changes (Masson *et al*., 1996; Najimi *et al*., 1997; Abdelhamid *et al*., 2007; Frasco *et al*., 2008). Mercury and copper both caused low inhibition of unpurified ChE due to their capability to bind with other thiol and amino groups in protein (Fig. 4) as mentioned by Letelier *et al*. (2005), but, malaoxon and carbofuran showed higher inhibition rates with slight difference compared to the tested pure ChE. This result indicated that malthion and carbofuran are selective inhibitors with greater affinity to bind with the active site of ChE through the process of phosphorylation and carbamoylation that block the metabolisation of substrate (Boublik *et al*., 2002; Stojan *et al*., 2008).

Conclusion

ChE from *P. Javanicus* was successfully purified using Procainamide-Sephacrayl 6B, which was proven by the appearance of single major band on the nondenaturing electrophorotic gel. Purified ChE was also proved to be more sensitive toward toxicants, namely mercury, copper, malaoxon and carbofuran compared to the impure ChE. This means that, the pure ChE can be selected for future studies such as for biomarker development and further characterisation.

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