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The evaluation of *Periophtalmodon schlosseri* as a source of acetylcholinesterase for the detection of insecticides

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Abstract

The aim of this study is to investigate the ability of acetylcholinesterase from mudskipper, *Periophtalmodon schlosseri*, as an assay to detect various pesticides, especially carbamate and organophosphate, and selected metal ions. The assay is sensitive to several carbamates with IC50of 0.0993, 0.0945, 0.0532 and 0.0488 mg/L for carbaryl, methomyl, propoxur and bendiocarb, respectively. Carbofuran highly inhibited the activity (0.0313) with the limit of detection (LOD) of 0.0176 and the limit of quantitation (LOQ) of 0.0526. The organophosphate group is less inhibiting compared to carbamate group.

INTRODUCTION

Pesticides are used in agriculture to control the activity of the pests by an external contact on the surface reaction or by internal contact which is by consuming[1]. The crisis of this compound is improper applications which contaminate rivers and other water source endangered the aquatic organism, birds and human[2, 3]. The impact of pesticides toxicology are major public health concerns globally [3].

Acetylcholinesterase (AChE) is a functional protein in the brain that is widely used as a traditionally analysis as to biomonitor the environmental pollution [4].The alteration of the AChE activity in the tissues of fish and other is caused by inhibition of organophosphorous and carbamates exposure determine of this enzyme have potent as biomarker for such compounds [5-8].Organophosphorous and carbamates insecticides are able to interact with the cholinergic system by binding at the activie site of AchE [9]. Heavy metals also affected to the inhibition of cholinesterase activity such chromium, arsenic, mercury and others [10, 11]. The persistence of heavy metals may accumulate in the ecosystem food chain and cannot be detoxified through degradation [12].

Inhibition of AChE is not compulsory lethal, but the purpose of a highly sensitive AChE source from fish as a viewpoint species can detect the lowest detection level of contaminant. In this work, AChE was obtained source from the brain of mudskipper, Periopthalmodon schlosseri and will be purified by using procainamide-base affinity chromatography and level of inhibition will be determined. This study was carried out to add on data for further comparative studies on the local sea fish in Malaysia.

MATERIALS AND METHODOLOGY

Chemicals

Carbofuran, methomyl, carbaryl, parathion, malathion, diazinon, bendiocarb, chlorpyrifos, acephate, dimethote and trichlorfon were purchased from Dr. Ehrenstorfer (Augsburg, Germany). Bromine, acetylthiocholine iodide (ATC), propionylthiocholine chloride (PTC), β-mercaptoethanol and procainamide hydrochloride were purchased from Sigma-Aldrich. 5‟-dithio-bis (2-nitrobenzoic acid) (DTNB) and butyrylthiocholine iodide (BTC) were purchased from Fluka Chemie GmbH. Biorad Protein Assay were purchased from from Bio-Rad Laboratories Inc. Vivaspin4 was sourced from Vivascience. All other chemicals used in this study were of analytical or special grade.

Specimens

AChE from mudskippers, Periophtalmodon Schlorsseri was obtained from Pulau Melaka, Malacca, Malaysia (N 2o10‟47.82 E 102o15‟19.62”). Only healthy and disease-free fishes were used for the experiment. The specimens were brought alive to the laboratory and were frozen immediately. They were decapitated and their brains were dissected out immediately and weight.

Preparation of Affinity Chromatography Columns; Epoxy (Bisoxirane) Activation

Affinity procainamide chromatography was prepared according to the modified method of Ralston et al. (1983) [13]. Briefly, Sephacryl S-1000 (100ml settled gel) (Sigma, St. Louis, USA) was washed with 1 L of deionized water in a sintered glass tunnel, sucked dry to a wet cake, and then transferred to a 500 ml beaker. The gel was suspended in 75 ml of 0.6 M NaOH containing 150 mg sodium borohyride (Sigma, St. Louis, USA) and stirred. About 75 ml of 1,4-butanediol diglycidyl ether (Sigma, St. Louis, USA) was slowly added with constant stirring. The reaction mixture was stirred at room temperature overnight. The activated gel was thoroughly washed with water to remove excess reagent. The washing was continued until there was no longer evidence of an oily film on the surface of the gel representing the remaining epoxy compound. Acetone was used to aid in the complete removal of bisoxirane groups. The gel was resuspended in water for ligand coupling.

Ligand Coupling of Procainamide–Sephacryl S-1000 gel

The epoxy-activated Sephacryl S-1000 was washed with deionized water on a sintered glass filter. The gel slurry was transferred onto a coupling solution of 12 mM of borate buffer (pH 11.0) containing 0.2 M of procainamide (Sigma, St. Louis, USA). The pH of the gel slurry was then adjusted to 12 by the addition of 1.0 M NaOH. The mixture was incubated at 25°C for 96 hours on a shaking incubator. The gel was washed in sequence with 10 volumes each of 0.1 M sodium acetate (pH 4.5), 12 mM sodium borate (pH 10) and deionized water. The excess active groups on the gel were blocked by suspending the gel in 100 ml of 1.0 M ethanolamine (pH 9.0) which acted to block all remaining active groups on the amine-reactive supports. The mixture was stirred at room temperature for 6 hours. Finally, the gel was washed thoroughly with 1 L of 1.0 M NaCl followed by 5 L of deionized water.

Partial Purification of AChE using Affinity Procainamide Chromatography

The matrix was packed in the column and allowed to settle to obtain a bed height of 3 cm. Flow rate was maintained at 0.2 ml min-1. The matrix was first washed with 5 batch volumes of washing buffer (20 mM sodium phosphate buffer, pH 7.5) to clean and equilibrate the column. The crude extract was then loaded onto the affinity matrix. At least 3 batch volumes of washing buffer were then applied directly to the matrix. Fractions of 1 ml were then collected in Eppendorf tubes and kept on ice. Washing was continued until all non-absorbed proteins were washed out. At least 3 batch volumes of elution buffer (20 mM sodium phosphate buffer containing 1.0 M sodium chloride, pH 7.5) were then applied directly to the matrix. Collection of 1ml fractions into Eppendorf tubes continued until the elution process was completed. Enzyme activity and protein content determination was carried out for all the fractions collected. Fractions exhibiting high AChE activity collected during the elution process were then pooled. The partially purified sample was concentrated and dialyzed with 3 batch volumes of washing buffer using VivaSpin® tubes at 2500 rpm at 4°C. The dialyzed partially purified AChE was stored at -20°C until subsequent use.

Determination of protein content

Bradford"s method (1976) was used for quantitative determination of proteins with bovine serum albumin (BSA) as a standard [14].

Activity and Effect of Substrates

AChE activity was measured using the method developed by Ellman et al. (1961) with modification for a 96 well microplate assay [15]. This method employs acetylthiocholine iodide (ATC) as a synthetic substrate for AChE. ATC is broken down to thiocholine and acetate by AChE and thiocholine is reacted with 5, 5'-dithio-bis-2-nitrobenzoate (DTNB) to produce a yellow color. The quantity of yellow color which develops over time is a measured of the activity of AChE and can be measure using a microplate reader. AChE activity is expressed as the amount of butirylthiocholine iodide (µmol) which is broken down by AChE per minute. The specific activity is given as µmole hydrolyzed/min/mg of protein or U mg-1 of protein and was calculated on the basis of an extinction coefficient of 13.6 mM-1.cm-1. Experiment was carried out in triplicates. The Km(app) was determined by analysis of Graphpad PRISM 4 for non-linear regression analysis software available from www.graphpad.com. OPs were subjected to activation according to the modified method of Villate et al. (1998) [16]. The pesticide (25 µl) was incubated in 5 µl of 0.01 M pure bromine solution at room temperature for 20 minutes. The activation process was stopped with 20 µl of 5% ethanol, which acted as a reducing agent. Preliminary experiments showed that bromine and ethanol at the given concentration in this bioassay system did not inhibit AChE activities. The IC50 was determined using at least five different concentrations of carbamate and OPs. The assay mixture in a well contained 150 µl of potassium phosphate buffer (0.1 M, pH 8.0), DTNB (20 µl, 0.067 mM), carbamate (50 µl) and enzymes (10 µl). The mixture was incubated in the dark for 10 minutes at room temperature. Acetylthiocholine iodide (20 µl, 0.5 mM) was then added. Again, the mixture was left to stand but for 10 minutes at room temperature before the absorbance was read at 405 nm [17].

Standard analysis

To compare the value between other group, statically analyze by a student,s t-test or a one-way analysis of variance with post hoc analysis by Tukey"' test with the 95% confidential interval was performed [18].

RESULTS AND DISCUSSION

Purification processes

Purification stage using affinity chromatography exhibited higher specific activity compared to the other stage after the sample was homogenized and centrifuged. AChE was successfully partial purified with the purification fold approximately 5.9 fold and yield 31.62% (Table 1).

Insecticides inhibition studies

The inhibition studies showed that carbamate group mostly inhibited the acetylcholinesterase activity while bendiocarp,cabaryl, carbofuran and propoxur inhibited more than 80% after incubated in 1 ppm (Figure 1).

AChE cannot be inhibited by malathion and chlorpyrifos even though they exhibited significant activation which was a small difference compared to 3% of the control. The organophosphate test, AChE did not show extreme inhibition or can consider there were no inhibitions. Generally, organophosphate group poor inhibit acetylcholinestrase as proven by previous investigators [17, 19]because these compounds need biotransformation of nontoxic OP to be active metabolites [20]. Further study to detect half life

(IC50) of carbamate group will be done in this work (Table 2). Result showed the IC50 values with the 95% confidence interval are 0.1124 (0.1025 to 0.1245), 0.0567 (0.0504 to 0.0648), 0.0633 (0.0537 to 0.0773), 0.0450 (0.0399 to 0.0517) and 0.0892 (0.0761 to 0.1077)for carbaryl, methomyl, bendiocarp, carbofuran and 0.01531, 0.03490, 0.03576, 0.03384 and 0.03247mg l-1, respectively. Among the variuos pesticides commonly applied in agriculture, carbafuran is more presistent than other carbamate. Carbofuran has been detected in ground, surface, and rain water due to its widespread use [21, 22].Carbofuran is widely used for the foliar feeding insects and control of soil dwelling. The use is broadly applied in paddy field, sugar cane, vegetables and fruits in propoxur, respectively. The LOD for carbaryl, methomyl, bendiocarp, carbofuran and propoxur, were 0.00824, 0.02722, 0.02544, 0.02997 and 0.01430mg l-1, respectively. The LOQ for carbaryl, methomyl, bendiocarp, carbofuran and propoxur, were

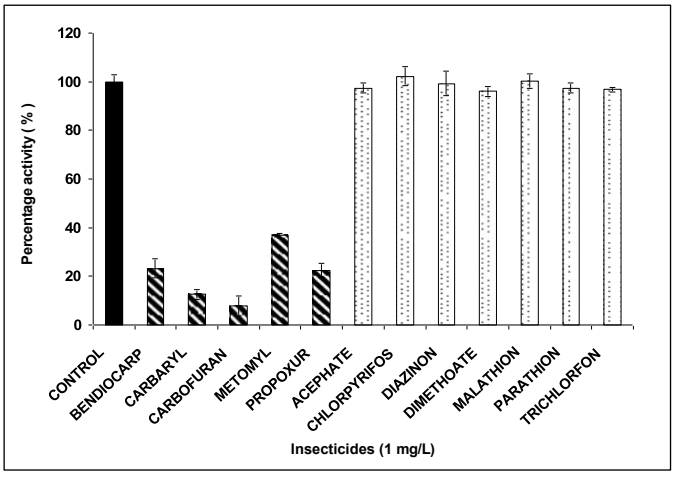
Malaysia making their detection important [23]. It is a broad spectrum of systemic acaride and insecticide which is typical examples exhibitin fairly high tovicity and more extensively [24, 25]. AcHE inhibition is a sensitive indicator elative to clinical effects of exposure to carbofuran in both animals and humans [24, 26].

Table 1: The purification table of various procedure of AChE from *Periophthalmodon schlosseri*. (U) is equal to nmole/min/mg.

Procedure	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor (fold)	Yield (%)
Homogenate	1229.76	5.6	219.74	1.0	100.00
Crude supernatant (Centrifugal 30,000g, 4ºC, 1 hour)	829.03	1.4	594.67	2.7	67.41
Affinity Chromatography (Procainamide Sepcharyl S-1000)	389.70	0.3	1292.07	5.9	31.62

Table 2: Half life (IC_{50}) of partial purified AChE activity expose with various carbamate

Carbamate Compound	IC_{50}(95% Confidence Interval) mg/L (ppm)	Limit of Detection (LOD) mg/L (ppm)	Limit of Quantitation (LOQ) mg/L (ppm)
Carbaryl	0.1124 (0.1025 to 0.1245)	0.00824	0.01531
Methomyl	0.0567 (0.0504 to 0.0648)	0.02722	0.03490
Bendiocarp	0.0633 (0.0537 to 0.0773)	0.02544	0.03576
Carbofuran	0.0450 (0.0399 to 0.0517)	0.02997	0.03384
Propoxur	0.0892 (0.0761 to 0.1077)	0.01430	0.03247



Carbamate Organophosphate

Figure 1: The inhibition of the activity of partially purifieds AChE from Periophthalmodon schlosseri by insecticides with the mean point of triplicate assay and Y error bars which incidate ± Standard deviation of the mean.

Each of these insecticides is toxic to fish when exposed in vivo and the mechanism of inhibition is probably through inhibiting cholinesterases function [27]. For example, carbaryl is toxic to Gobiocypris rarus (Chinese Rare Minnow) [26], Oncorhynchus mykiss (rainbow trout) [28] andCirrhina mrigala (Cyprinidae) [29]; Tetrodotoxin is toxic to Lagocephalus sceleratus (Pufferfish, Fugu) [30] and Mytus Vittatus („Baung“) [31]; and propoxur is toxic Carassius auratus [32]and Mytus Vittatus („Baung“) [31].
The organophosphates are not toxic in their own and need activation by oxonation. This is accomplished using bromine water in vitro and enzymes in vivo. Parathion is coverted to O,O-

diethyl O-(4-nitrophenyl) phosphorothioate in microsome made it potent as actylcholinesterase inhibitor [33, 34].

CONCLUSION

This work can be concluded that the acetylcholinesterase source from Periophthalmodon schlosseriis the best bioindicator for various carbamate xenobiotic insecticides such as carbaryl, metahomyl, bendiocarp, carbofuran and propoxur. For further studies, this enzyme will be tested with water polluted sample and compare with other AChE sources.

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