BULLETIN OF ENVIRONMENTAL SCIENCE AND MANAGEMENT
Website: http://journal.hibiscuspublisher.com

An inhibitive assay for insecticides using the acetylcholinesterase from *Osteochillus hasselti*

Sabullah, M.K.,[1,2]Ahmad, S.A.[1*],Ishak,I.,[1]Sulaiman, M.R.,[2] Shukor, M.Y.,[1] Syed, M.A.[1] and Shamaan, N.A.[3]
[1]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, University Putra Malaysia, UPM 43400 Serdang, Selangor, Malaysia
[2]School of Food Science and Nutrition, Universiti Malaysia Sabah, Jalan UMS, 88400, Kota Kinabalu, Sabah, Malaysia.
[3]Faculty of Medicine and Health Sciences, Universiti Sains Islam Malaysia, 13th Floor, Menara B, Persiaran MPAJ, Jalan Pandan Utama, Pandan Indah, 55100 Kuala Lumpur, Malaysia
Coresponding author; Siti Aqlima Ahmad, Phone no: +603-89478292, Fax no: +603-89430913, Email: aqlimaahmad@gmail.com

History
Received: 30 September 2013
Received in revised form: 25 November 2013
Accepted: 2 December 2013
Available online: 25 December 2013

KEYWORDS
Osteochillus hasselti
Acetylcholinesterase
Carbamates
Organophosphates

ABSTRACT

The aim of this study is to investigate the ability of acetylcholinesterase from fresh water carp, *Osteochillus hasselti*as an assay to detect insecticides.The IC_{50} values for the carbamates carbofuran, carbaryl, methomyl, bendiocarb were 0.0550, 0.0497, 0.0845, 0.0470µg/l, respectively, and the IC_{50} values for the oxonated organophosphates parathion, malathion, diazinon and chlorpyrifos were 0.0660, 0.0681, 0.0991 and 0.0632µg/l, respectively. The carbamates carbaryl, and the oxonated organophosphate diazinon showed lower IC_{50}s in *O. hasselti* compared to *E. electricus* while parathion and diazinon showed similar sensitivity to *E. electricus*. Carbofuran, methomyl, bendiocarb and malathion exhibited lower IC_{50} confidence interval in *E. electricus* than in *O. hasselti*. This suggests that in overall, AChE from *Osteochillus hasselti*is a suitable source of enzyme for the detection of insecticides.

INTRODUCTION

Many studies concerning the detection and toxicity analysis of organophosphates and carbamates using fish by measuring acetylcholinesterase activity have been carried out to develop biomarkers and not for invitro assays [1,2].The use of fish AChE as a source of enzyme for in vitro assay of insecticides is still not widely pursued. Currently, the commercial source of AChE comes from *Drosophila melanogaster* and the electric eel *Electophorus electricus*[3]. The sensitivity of marine organisms especially fishto toxicants such as detergents [4], pesticides [5], textile dyes [6] and heavy metals [7,8]are widely known. This reflects the sensitivity of fish to toxicants. The use of AChE from several fish species to detect organophosphates has been carried out. However, the IC_{50}s obtained are above 1 mg l^{-1}[9]and not adequate for in vitro assay. Recently, AChE from *C. batrachus*with better sensitivity towards insecticides has been reported [10].In this work we demonstrate that the AChE isolated from *Osteochillus hasselti*, commonly found in Malaysia and scattered all over South East Asia [11], showed comparable sensitivity to the AChE from *E. electricus* making it a suitable and cheaper source of AChE for in vitro assay of insecticides.

MATERIALS AND METHODOLOGY

Chemicals

Acephate, bendiocarb, carbaryl, carbofuran, methomyl, propoxur, trichlorfon, chlorpyrifos, diazinon, dimethoate, malathion, parathion, acetylthiocholine iodide (ATC), β-mercaptoethanol and procainamide hydrochloride were purchased from Sigma-Aldrich. 5'-dithio-bis (2-nitrobenzoic acid) (DTNB) were purchased from Fluka Chemie GmbH. Vivaspin4 was from Vivascience. All other chemicals used in this study were of analytical or special grade. Commercial AChE preparation from eel (*Electrophorus electricus*, Lot No. 044K7655, 349 units/mg solid) was purchased from Sigma (St. Louis, USA).

Preparation of Brain AChE Extracts

Osteochillus hasselti (water-breathing and omnivore) was obtained from Malaysia National Park, Kuala Atok, Pahang Malaysia at coordinate N 4º20'7.98" E 102º23'41.1"and brought alive to the laboratory. Only healthy and disease-free fishes were used for the experiment. They were decapitated and the brains were dissected out immediately and weighed. Homogenization of the brain was carried out using an Ultra-Turrax T25 homogenizer fitted with a Teflon pestle. Briefly, one gram of brain was homogenized in 20% (w/v) of 0.1 M sodium phosphate buffer pH 8.0. The crude extract was subjected to centrifugation at 15 000×g for 10 minutes at 4 ºC to remove debris and the resulting homogenate was then subjected to ultracentrifugation at 100,000×g in a Sorval® Ultra Pro 80-TH-641 ultracentrifuge for one hr at 4 ºC. The pellet was discarded and the supernatant was used in the purification procedures.

Isolation and Partial Purification of Cholinesterase

Affinity chromatography was performed using procainamide, a ligand specific for the choline-binding site [12]. 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 each Eppendorf tube 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 1 ml fractions into each 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 Viva Spin tubes at 2500 rpm at 4 ℃. The dialyzed partially purified AChE was stored at -20 ℃ until subsequent use.

Activation of organophosphate

OPs were subjected to activation according to the modified method of Villate et al. (1998) [3]. The organophosphates (25 µl) were incubated with 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.

Activity and substrate specificity

AChE activity was measured ina 96 well microplate assay format using according toEllman et al. (1961) with modification [13]. Acetylthiocholine iodide (ATC) was used as a synthetic substrate for AChE. Acetylthiocholine iodide 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. AChE activity is expressed as the amount of acethylthiocholine iodide (µmol) which is broken down by AChE per minute. The specific activity is given as µmole ATC hydrolyzed/min/mg of protein or U/mg of protein and was calculated on the basis of an extinction coefficient of 13.6 $mM^{-1}.cm^{-1}$[13].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. The IC_{50} value was statistically analyzed using Graphpad PRISM 4

Table 1. Comparisons of the sensitivity of *O. hasselti*AChE with AChEs from *E. electricus*to various insecticides.

Insecticides	*O. hasselti* IC_{50} (Confidence Interval) □ g/l	*E. electricus* IC_{50} (Confidence Interval)□ □ g/l
Carbofuran	0.0550 (0.0515-0.0670)	0.0060 (0.0063-0.0065)
Carbaryl	0.0497 (0.0414-0.0620)	0.1330 (0.1220-0.1450)
Methomyl	0.0845 (0.0747-0.0973)	0.0260 (0.0240-0.0280)
Bendiocarb	0.0470 (0.0409-0.0553)	0.0150 (0.0150-0.0160)
Parathion-oxon	0.0660 (0.0580-0.0766)	0.0680 (0.0660-0.0690)
Malathion-oxon	0.0681 (0.0592-0.0802)	0.0140 (0.0130-0.0140)
Diazinon-oxon	0.0991 (0.0906- 0.1094)	0.1770 (0.1690-0.1860)
Chlorpyrifos-oxon	0.0632 (0.0570-0.0709)	0.0600 (0.0550-0.0650)

Note: All values have a Correlation coefficient of 0.99. non-linear regression analysis and the model used was radioactive decay [10].

Statistical Analysis

Values are means ± SE. All data were analyzed using Graphpad Prism version 3.0 and Graphpad InStat version 3.05. Comparison between groups was performed using a Student's t-test or a one-way analysis of variance (ANOVA) with post hoc analysis by Tukey's test with the 95% confidential interval was performed [14].

RESULTS AND DISCUSSION

IC_{50} values of insecticides

Bendiocarb, carbaryl, carbofuran, methomyl, acephate, chlorpyrifos, diazinon, dimethote, malathion, parathion, trichlorfon caused 97, 92, 97, 93, 9, 75, 71, 9, 72, 74 and 11 % inhibition of AChE activity, respectively (Figure 1). ANOVA analysis showed that all of the inhibition seen was significant compared to control ($p<0.05$) in the absence of insecticide. However, insecticides that caused less than 20% inhibition were excluded for IC_{50} determination. The IC_{50}s for the various insecticides chosen for further studies are shown in Table 1 in comparison with *E. electricus* AChE.Scehenker and Gentleman (2001)[15] demonstrated that non-overlap of confidence interval usually signifies significant difference at the $p<0.05$ level while overlapped interval does not necessary means difference or no significant differences at the $p<0.05$ level. Overlapped confidence interval provides a general view that more data and experimentation are needed to assess non-significance. Thus, the carbamates; carbaryl, and the oxonated OPs; diazinon showed significantly lower IC_{50}s in *O. hasselti* compared to *E. electricus* while parathion and diazinon showed overlapped IC_{50}s. Carbofuran, methomyl, bendiocarb and malathion exhibited significantly lower IC_{50} confidence interval in *E. electricus* than in *O. hasselti* (Table 1). This suggests that in overall, AChE from *O. hasselti* is sensitive to insecticides compared to the AChEs from *E. electricus*.

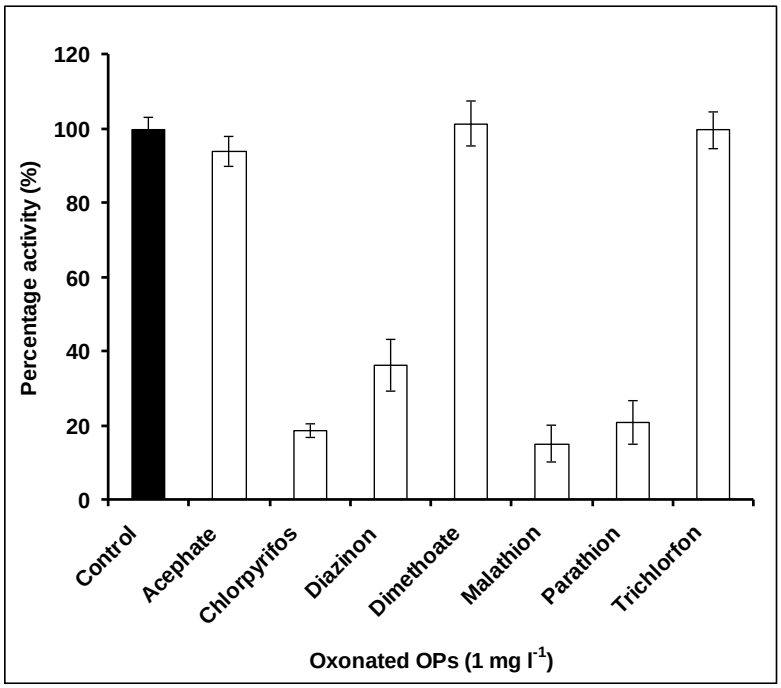

Figure 1: Effect of carbamates and various oxonated OP on the enzymatic activity of the partially purified AChE from *Osteochilus hasselti.* Data represents mean± SEM, n=3.

CONCLUSIONS

Screening of the effects of xenobiotics towards the partially purified AChE activity showed that nine out of twelve insecticides tested showed significant inhibition. Since most of the insecticides tested gave lower IC_{50} than the commercial AChE from *E. electricus*, a more sensitive inhibitive assay for insecticide can be developed. For further studies, more insecticides will be screened and field study works will be carried out.

ACKNOWLEDGMENT

Department of Wildlife and National Park (PERHILITAN), Cheras, Selangor, Malaysia

REFERENCES

[1] De La Torre FR, Ferrari L, Salibian A. Freshwater pollution biomarker: response of brain acetylcholinesterase activity in two fish species.CompBiochemPhysiolC Toxicol Pharmaco.2002; 131:271–280.

[2] PeebuaP, Kruatrachue M, Pokethitiyook P,Singhakaew S. Histopathological alterations of Nile tilapia, *Oreochromis niloticus* in acute and subchronic alachlor exposure. JEnvironBiol.2008; 29(3):325-331.
[3] Villatte F, Marcel V, Estrada-Mondac S, Fournier D. Engineering sensitive acetylcholinesterase for detection of organophosphate and carbamate insecticides. Biosens Bioelectron. 1998; 13:157–64.
[4] Kumar M, Trivedi SP, Misra A,Sharma S. Histopathological changes in testis of the freshwater fish, Heteropneustes fossilis (Bloch) exposed to linear alkyl benzene sulphonate (LAS). JEnvironBiol. 2007; 28:679-684.
[5] Arufe MI, Arellano JM, Albendin G,Sarasquete C. Toxicity of parathion on embryo and yolk-sac larvae of gilthead seabream (Sparus aurta l.): Effects on survival, cholinesterase and carboxylesterase activity. EnvironToxicol. 2010; 25:601-7.
[6] SoniP, Sharma S,Sharma S, Kumar S,Sharma KP. A comparative study on the toxic effects of textile dye wastewaters (untreated and treated) on mortality and RBC of a freshwater fish *Gambusia affinis* (Baird and Gerard). JEnvironBiol. 2006; 27(4):623-628.
[7] Singh D, Nath K, Trivedi SP, SharmaYK. Impact of copper on haematological profile of freshwater fish, *Channa punctatus*.J Environ Biol. 2008; 29:253-2570.
[8] Srivastava R and Srivastava N. Changes in nutritive value of fish, Channa punctatus after chronic exposure to zinc. JEnvironBiol. 2008; 29:299-302.
[9] Zhu XS, Meng FP, Zhu L, He DH, Yang Y.Selection study on the sensitivity of marine fishes brain acetylcholinesterase to organophosphorus pesticides. Environ Sci. 2006; 27(3):567-571.
[10] Tham LG, Perumal N, Syed MA, Shamaan NA, Shukor MY: Assessment of *Clarias batrachus* as a source of acetylcholinesterase (AChE) for the detection of insecticides. J Environ Biol. 2009; 30(1):135-8.
[11] Kottelat M, Whitten AJ, Kartikasari SN,Wirjoatmodjo S. Freshwater fishes of Western Indonesia and Sulawesi. Periplus Editions Ltd., Hong Kong. Xxxviii+221 pp., 84 pls 1993.
[12] Hoz DDL, Doctor BP,Robert JSR,Rush S,Wolfe AD. A simplified procedure for the purification of large quantities of fetal bovine acetylcholinesterase.Life Sci.1986; 39:195-199.
[13] Ellman GL, Courtney DK, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity.Biochem Pharmacol. 1961; 7:88–95.
[14] Miller JN and Miller JC. Statistic and chemometrics for Analytical Chemistry, (4th EDn.), Prentic Hall, Harlow, England. (2000)
[15] Scehenker N and Gentleman JF. On judging the significance of differences by examining the overlap between confidence intervals. The American Statistician.2001; 55:182-186.
[16] Mahttiessen P, Sheahan D, Harrison R, Kirby M, Rycroft R, Turnbull A, Volkner C,William R. Use of a *Grammarus pulex* bioassay to measure the effects of transient carbofuran runoff from farmland. Ecotoxicol Environ Saf.1995; 30:111-119.
[17] Waite DT, Grover R, Wescott ND, Sommerstad H,Karr L. Pesticides in ground water, surface water and spring runoff in a small Saskatchewan watershed. EnvironToxicolChem. 1992; 11:741-748.
[18] Farahani GHN, Sahid I, Zakaria Z, Kuntom A, Omar D. Study on the Downward Movement of Carbofuran in Two Malaysian Soils.BullEnviron ContamToxicol.2008; 81:294-298.
[19] Bastos VLFC, Bastos JC, Lima SS, Foria MVC.Brain acetylcholinesterase as an in vitro detector of organophosphorus and carbamate insecticides in water.Water res. 1991; 25(7):835-840.
[20] Keizer J, D'Agostino G, Nagel R, Volpe T, Gnemi P, Vittozzi L. Enzymological differences of AChE and diazinon hepatic metabolism: correlation of in vitro data with the selective toxicity of diazinon to fish species. Sci Total Environ.1995; 171(1-3):213-20.
[21] Metcalf RL."Insect Control" Ullman's Encyclopedia of Industrial Chemistry. Wiley-VCH Verlag GmbH & Co. KGaA: New York. 2002.
[22] Mutch E and Williams FM. Diazinon, chlorpyrifos and parathion are metabolised by multiple cytochromes P450 in human liver. Toxicology.2006; 224(1-2):22-32.
[23] Barber D, Correll L, Ehrich M. Comparative effectiveness of organophosphorus protoxicant activating systems in neuroblastoma cells and brain homogenates. J Toxicol Environ Health A. 1999; 57(1):63-74.
[24] Hajjar NP and Hodgson E. Sulfoxidation of thioether-containing pesticides by the flavin-adenine dinucleotide-dependent monooxygenase of pig liver microsomes.Biochem Pharmacol. 1982; 31(5):745-52.