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

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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, metoxyrl, 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 *Electrophorus electricus* [3]. The sensitivity of marine organisms especially fish to 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$_{50}$ 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 4°20'7.98" E 102°23'41.1" 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 000g for 10 minutes at 4 ºC to remove debris and the resulting homogenate was then subjected to ultracentrifugation at 100,000xg 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 °C. The dialyzed partially purified AChE was stored at -20 °C 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 in a 96 well microplate assay format using according to Ellman 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'-dithiobis-2-nitrobenzoate (DTNB) to produce a yellow color. AChE activity is expressed as the amount of acetylthiocholine 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 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.

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

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% confidence interval was performed [14].

RESULTS AND DISCUSSION

**IC\(_{50}\) values of insecticides**

Bendiocarb, carbaryl, carbofuran, methomyl, acephate, chlorpyrifos, diazinon, dimethoate, 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. Schehenker 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.](image)

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.

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REFERENCES


