Acetylcholinesterase Characterization in the Terrestrial Isopod *Porcellionides pruinosus*

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(Received 25 January 2010; accepted 21 February 2010)

**Abstract**—In the last decades biomarkers have been widely used for the assessment of effects and/or exposure to environmental contaminants, but to our knowledge few data has been disseminated for isopods. Here, the cholinesterase of the isopod *Porcellionides pruinosus* was characterized using three substrates (acetylthiocholine iodide, propionylthiocholine iodide, and S-butyrylthiocholine iodide) and three ChE inhibitors (eserine hemisulfate, BW284C51, and iso-OMPA). The results showed that this organism has only one cholinesterase form, the acetylcholinesterase with a mean basal level of $113.6 \pm 4.7$ U/mg protein. The present study highlights the relevance of ChE characterization before its use as a biomarker in ecotoxicology and biomonitoring studies.

Keywords: biomarkers, acetylcholinesterase, acetylcholinesterase characterization, kinetic curves

**INTRODUCTION**

Biomarkers can be described as any biological response to an environmental stressor below the individual level, measured as biochemical, molecular, genetic, immunologic, physiologic signals or even organism products (e.g., urine, faeces, hair, feathers, etc.) or events in biological systems (van Gestel and van Brummelen, 1996). These events indicate a shift from the normal status or fitness and most of the times can not be detected by phenotypic or life trait changes, but will be possibly linked to them in a near future. The definition of biomarkers also comes associated to two predominant features: (1) their sensitivity and quick response may act as early alarms to toxicant impacts on organisms, before ecological disturbances can be observed, (2) they may also provide a more accurate relationship between toxicant exposure and biological response before causing irreversible effects (Morgan et al., 1999).

The knowledge of biomarkers basal levels can build a threshold from where changes can be indicative of the health status of organisms and how stressors may act upon exposure.

In ecotoxicological studies or biomonitorization procedures, enzymatic
biomarkers are usually used to screen specific chemical groups. Among these, acetylcholinesterase is usually related to carbamate and organophosphate exposures which have a direct mode of action inducing an overstimulation of the central nervous system and causing neurotoxic effects (Barata et al., 2004).

In terrestrial ecosystems edaphic organisms are often exposed to xenobiotics that may jeopardize all decomposition and fragmentation processes, causing a decrease in soil quality and soil services (MEA, 2005). As macrodecomposers, isopods play an important role in decomposition processes by the fragmentation of litter material and in the re-cycling of nutrients (Zimmer, 2002; Zimmer et al., 2003; Loureiro et al., 2006). Terrestrial isopods and particularly the species *Porcellionides pruinosus* have been described as good sensors for soil contamination or changes in their habitat (Takeda, 1980; Vink et al., 1995; Jansch et al., 2005; Loureiro et al., 2005, 2009; etc.).

The main goal of this study was divided into two parts: i) to determine the best homogenization methodology for isopod test-species: the use of homogenizer vs. sonicator; ii) to characterize the cholinesterase in this isopod species. This information and methodology will be crucial as a foundation for future studies where the effects of contaminants or other stressors will be assessed in the terrestrial isopod *Porcellionides pruinosus*.

**MATERIALS AND METHODS**

*Test organism and culture procedure*

The organisms used in this study belong to the species *Porcellionides pruinosus* (Brandt, 1833), and were previously collected from a horse manure pill and maintained for several generations in laboratory cultures. In culture, isopods were fed *ad libidum* with alder leaves (*Alnus glutinosa*) and maintained at 25 ± 2°C, with a 16:8 h (light:dark) photoperiod. Twice a week cultures were water sprayed and food provided. Only adult animals (15–25 mg wet weight) were used in the experiments and no distinction between sexes was made, although pregnant females were excluded.

*Experimental procedure*

To optimize the methodology for the enzymatic measurements two procedures were applied to our sampling animals. Ten organisms were processed using a homogenizer (*Ystral GmbH D-7801*, Dottingen, Germany) and the other ten using a sonicator (*Kika Labortechnik, V200Scontrol*, Germany). Total protein and AChE activity were measured for each homogenization method.

Test organisms were collected from culture boxes, weighted and cautiously observed: animals with abnormalities, moulting and pregnant females were discarded.

*Cholinesterase characterization*

Cholinesterase characterization was performed by the determination of
substrate preferences and selective inhibitor effects. A pool of twelve heads from culture organisms were homogenized using a sonicator in 6 ml of K-Phosphate buffer (0.1 M, pH 7.2) and centrifuged (1700 g, 3 min, 4°C) for cholinesterase activity determination, which was performed with six replicates, according to the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996).

In independent experiments, acetylthiocholine iodide (AcSCh), S-butyrylthiocholine iodide (BuSCh), and propionylthiocholine iodide (PrSCh) within a dose range (0.005 to 20.48 mM) were used as substrates. Eserine hemisulfate was used as selective inhibitor of the activity of all the ChE, tetraisopropyl pyrophosphoramide (iso-OMPA) as selective inhibitor of pseudocholinesterase (PChE) and 1,5-bis(4-allyldimethyl-ammonimphenyl) pentan-3-one dibromide (BW284C51) as selective inhibitor of AChE. In the selective inhibitor experiments, all enzymatic activities were determined using AcSCh as substrate after an incubation period of 30 min at 25 ± 1°C. For each inhibitor, 5 µl of a stock solution was incubated with 495 µl of homogenate sample extract. Inhibitor concentrations ranged from 6.25 to 200 mM (eserine and BW284C51) and from 0.25 to 8.0 mM (iso-OMPA). Ultrapure water was added to controls and an additional control with ethanol was used in the experiments with iso-OMPA.

**Acetylcholinesterase**

One isopod head per sample was homogenized using a homogenizer or sonicator in 500 µl of potassium phosphate buffer (0.1 M, pH 7.2), and the supernatants obtained after centrifugation of the homogenates (4°C, 1700 g, 3 min) were removed and stored at –80°C until enzymatic analysis.

In a 96 well microplate 250 µl of the reaction solution was added to 50 µl of the sample and the absorbance was read at 414 nm, after 10, 15 and 20 min. The reaction solution had 1 ml of 5,50-dithiobis-2-nitrobenzoic acid (DTNB) 10 mM solution, 1,280 ml of 0.075 M acetylthiocholine iodide solution and 28,920 ml of 0.1M phosphate buffer. The enzymatic activity is expressed as unit (U) per mg of protein. A U corresponds to one nmol of substrate hydrolyzed per minute, using a molar extinction coefficient of 1.36 × 10⁻³ M⁻¹ cm⁻¹.

**Protein quantification**

The protein concentration was determined according to the Bradford method (Bradford, 1976), adapted from BioRad’s Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as standard.

**Chemical compounds**

All chemicals used in these experiments were obtained from Sigma-Aldrich Europe, except the Bradford reagent, which was purchased from Bio-Rad (Germany).
Statistics

For the cholinesterase characterization, values for in vitro inhibition concentration (IC$_{50}$) were calculated using a nonlinear four parameter logistic curve for eserine hemisulfate and a nonlinear 2 parameters exponential decay curve for BW284C51 (SPSS, 1999). An analysis of variance (ANOVA) was performed to compare differences between inhibitor’s concentrations after data

Fig. 1. ChE activity measured in Porcellionides pruinosus as a function of acetylthiocholine iodide (AcSCh), propionylthiocholine iodide (PrSCh) and S-butyrylthiocholine iodide (BuSCh) concentrations. Values are means of 6 isopods’ heads with 4 enzymatic determinations per isopod and the corresponding standard error bars.

Fig. 2. Apparent $K_m$ value for acetylthiocholine iodide (ASCh) substrate presented in a Lineweaver and Burk graph.
transformation using natural logarithm (ln). Dunnett’s comparison test was carried out to discriminate statistical different treatments (SPSS, 1999).

The comparison between the two types of sampling processing (homogenize vs. sonicator) was made using the Students t-test (SPSS, 1999).
RESULTS

Homogenization methodology

The two homogenization procedures were compared and significant differences were found for the amount of protein extracted ($t_{18} = 5.959; p < 0.001$), and AChE activity ($t_{18} = 7.872; p < 0.001$). These biomarker activity, measured by the sonicator procedure, showed higher activities than when using an homogenizer. Therefore, all samples were processed with a sonicator.

Cholinesterase characterization

To investigate the substrate preferences of ChE in the head tissues of *Porcellionides pruinosus*, three substrates were assayed: AcSCh, PrSCh, and BuSCh (Fig. 1). Although the maximum activity of protein was obtained with AcSCh at 10.24 mM (201.94 ± 5.38 SE U/mg), in the stable zone of the graph, we have considered the value of 2.56 mM (99.55 ± 3.24 SE U/mg protein) at the end of the exponential phase of the enzymatic activity as the concentration of AcSCh to be used in future studies. Lower ChE activities were observed when using PrSCh and BuSCh as substrates (e.g., 70.69 ± 3.16 SE and 2.80 ± 0.87 SE for PrSCh and BuSCh, respectively, at 20.48 mM). Therefore, ChE activity measurements hereafter were performed using AsSCh as substrate at the concentration of 2.56 mM.

The apparent $K_m$ value for the AcSCh substrate calculated by the Lineweaver and Burk method was 356 μM (Fig. 2).
Eserine hemisulfate significantly inhibited ChE activity \((p < 0.001)\) (Fig. 3), and similar results were obtained with the selective inhibitor of AChE, BW284C51 \((p < 0.001)\), although data did not show a normal distribution (Fig. 4). Inhibition by eserine hemisulfate and BW284C51 was almost complete (>99%) at the highest concentrations tested. The effect of the selective inhibitor of BChE iso-OMPA did not affect \(P.\ pruinosus\) ChE activity \((p > 0.005)\) at concentrations up to 8 mM (Fig. 5). IC\(_{50}\) values for eserine hemisulfate and BW284C51 are 0.12 ± 3.22 SE U/mg protein and 0.26 ± 0.06 SE U/mg protein, respectively; IC\(_{50}\) values for iso-OMPA could not be determined since no significant inhibition was found up to the maximum concentration.

**DISCUSSION**

Sample’s preparation showed to be a very important step in the measurement of biomarkers activity. When applying two different methodologies for the homogenization procedure (homogenizer and sonicator), there were significant differences in the analyses. The amount of protein extracted and AChE activity was higher when using the sonicator.

The objective of this study, the characterization of the ChE activity in \(P.\ pruinosus\), included a first step to distinguish ChE from nonspecific esterases. This procedure is important because tissues may contain several nonspecific esterases, which contribute to the measured activity and may show different sensitivities towards anticholinesterase agents (Garcia \textit{et al.}, 2000). Nonspecific

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**Table 1. Examples of Michaelis-Menton constant \((K_m)\) for the AcSCh substrate in species used as test-organisms in ecotoxicological approaches. Values for this study on \(Porcellionides pruinosus\) are expressed as mean value of 6 replicates. Values for other species were reported in previous works.

<table>
<thead>
<tr>
<th>Species</th>
<th>(K_m) (µM)</th>
</tr>
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<tbody>
<tr>
<td>(Porcellionides pruinosus)</td>
<td>356</td>
</tr>
<tr>
<td>(Spodoptera frugiperda) (Yu, 2006)</td>
<td>33.5</td>
</tr>
<tr>
<td>(Mytilus galloprovincialis) (Mora \textit{et al.}, 1999)</td>
<td>34</td>
</tr>
<tr>
<td>(Nucella lapillus) (Cunha \textit{et al.}, 2007)</td>
<td>91</td>
</tr>
<tr>
<td>(Octopus vulgaris) (Talesa \textit{et al.}, 1995)</td>
<td>70</td>
</tr>
<tr>
<td>(Crassostrea gigas) (Bocquene \textit{et al.}, 1997)</td>
<td>30</td>
</tr>
<tr>
<td>(Pecten jacobaeus) gills (Stefano \textit{et al.}, 2008)</td>
<td>275</td>
</tr>
<tr>
<td>(Pecten jacobaeus) adductor muscle (Stefano \textit{et al.}, 2008)</td>
<td>234</td>
</tr>
<tr>
<td>(Monodonta lineate) (Cunha \textit{et al.}, 2007)</td>
<td>157</td>
</tr>
<tr>
<td>(Cathorops spixii) (Tortelli \textit{et al.}, 2006)</td>
<td>196</td>
</tr>
<tr>
<td>(Cnesterodon decemmaculatus) (de la Torre \textit{et al.}, 2002)</td>
<td>170</td>
</tr>
<tr>
<td>(Cyprinus carpio) (de la Torre \textit{et al.}, 2002)</td>
<td>230</td>
</tr>
<tr>
<td>(Haemulon plumieri) (Leticia and Gerardo, 2008)</td>
<td>310</td>
</tr>
<tr>
<td>(Microtubus flavissima) (Tortelli \textit{et al.}, 2006)</td>
<td>179</td>
</tr>
<tr>
<td>(Odonethes bonaerensis) (Monserrat \textit{et al.}, 2001)</td>
<td>40</td>
</tr>
<tr>
<td>(Odonethes argentinensis) (Monserrat and Bianchini, 2001)</td>
<td>50</td>
</tr>
<tr>
<td>(Oreochromis niloticus) (Rodriguez-Fuentes and Gold-Bouchot, 2002)</td>
<td>102</td>
</tr>
</tbody>
</table>
esterases contribution was estimated using the compound eserine hemisulfate, which is considered a specific inhibitor of ChE at low concentrations, in the $10^{-6}$–$10^{-5}$ M range (Eto, 1974). In the present study the measured enzymatic activity was almost fully inhibited by eserine hemisulfate at the lowest concentration tested, 6.25 $\mu$M, (98.49%), which indicates the predominant presence of ChE and not of other esterases.

The highest ChE activity in *P. pruinosus* was obtained with AcSCh, showing a distinct preference over the other substrates. Furthermore, there was an almost complete inhibition when BW284C51 was used, while no significant inhibition was observed with iso-OMPA. Thus, it seems that only one ChE form is present in this species, with typical characteristics of an AChE. Cholinesterase forms in terrestrial invertebrate species have been less studied. To our knowledge there are no other studies where this characterization have been carried out for isopod species, therefore no comparisons could be made within isopoda. Considering other crustaceans, these results are in agreement with those obtained for several marine and freshwater species, since, in general, only AChE is present, such as in decapods (e.g., Key and Fulton, 2002; Quintaneiro *et al*., 2006), amphipods (Xuereb *et al*., 2007) and copepods (Forget and Bocquene, 1999). However, some crustacean species have shown different results, displaying a ChE form with atypical characteristics, as is the case of the cladoceran *Daphnia magna* (Diamantino *et al*., 2003).

The $K_m$ value obtained for AcSCh was higher than the ones published for other invertebrates (Table 1) such as the fall armyworm *Spodoptera frugiperda*, with 33.5 $\mu$M (Yu, 2006), the mollusc bivalve *Mytilus galloprovincialis*, 34 $\mu$M (Mora *et al*., 1999), *Nucella lapillus*, 90.83 $\mu$M (Cunha *et al*., 2007), *Octopus vulgaris*, 70 $\mu$M (Talesa *et al*., 1995) or *Crassostrea gigas*, 30 $\mu$M (Bocquene *et al*., 1997), but similar to the earthworm *Eisenia andrei*, 160 $\mu$M (Gambi *et al*., 2007), the mollusc bivalve *Pecten jacobaeus*, 274.8 $\mu$M in gills and 233.9 $\mu$M in the adductor muscle (Stefano *et al*., 2008) and *Monodonta lineate* 157.04 $\mu$M (Cunha *et al*., 2007). Values obtained were also very similar to others found for several fish species (Monserrat and Bianchini, 2001; Monserrat *et al*., 2001; de la Torre *et al*., 2002; Rodriguez-Fuentes and Gold-Bouchot, 2002; Tortelli *et al*., 2006; Leticia and Gerardo, 2008).

Results reported here will be used as a basis for future studies on the evaluation of biomarkers in the species *Porcellionides pruinosus* upon xenobiotic exposures in the laboratory, but also as a possible biomonitorization tool for *in situ* testing.

We also believe that these results and the approach carried out can be used as a control tool to evaluate isopods status in laboratory cultures.

Acknowledgments—The study was supported by the Portuguese project Agromix-Mixture Toxicity evaluation in agricultural soils (PTDC/AGR-AAM/68676/2006), financed by Fundação para a Ciência e a Tecnologia. The authors would like to thank the laboratorial support given by Dr. Abel Ferreira.
REFERENCES


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