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Cholinesterase activities as potential biomarkers: Characterization in two freshwater snails, *Potamopyrgus antipodarum* (Mollusca, Hydrobiidae, Smith 1889) and *Valvata piscinalis* (Mollusca, Valvatidae, Müller 1774)

Beatrice Gagnaire [a], Olivier Geffard [a], Benoit Xuereb [a], Christelle Margoum [b], Jeanne Garric [a,*]

[a] *Cemagref, Unité Biologie des Ecosystèmes Aquatiques, Laboratoire d'Ecotoxicologie, 3 bis quai Chauveau, CP 220, 69336 Lyon Cedex 09, France*
[b] *Cemagref, Unité Qualité des Eaux et Prévention des Pollutions, Laboratoire des Micropolluants Organiques, 3 bis quai Chauveau, CP 220, 69336 Lyon Cedex 09, France*

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Abstract

Anti-cholinesterase insecticides constitute a major portion of modern synthetic pesticides and the assessment of cholinesterase (ChE) inhibition is widely used as a specific biomarker for evaluating the exposure of non-target organisms to these pollutants. However, most studies on this biomarker were developed on vertebrates and among invertebrates, gastropod mollusks are rarely used. Gastropods are important members of aquatic habitats and therefore present a high ecological relevance for freshwater ecosystems. In this context, ChE activities were characterized in two freshwater gastropod mollusks, *Potamopyrgus antipodarum* and *Valvata piscinalis*, in order to ascertain their value as sentinel species. Firstly, characterization of ChE activities was performed using different substrates (acetylcholine iodide, butyrylcholine iodide and propionylcholine iodide) and specific inhibitors (eserine, *iso*-OMPA and BW284c51). Secondly, *in vivo* effect of a widely used organophosphate insecticide, chlorpyrifos, was tested on ChE activity in both species. Results suggested that *P. antipodarum* possesses two isoforms of cholinesterases, one isoform which properties are intermediate between an acetyl and a propionyl ChE, and one minor isoform which correspond to a butyryl ChE, while *V. piscinalis* seems to possess only one isoform which displays typical properties of an acetyl ChE. Chlorpyrifos induced no effect on *V. piscinalis* ChE. In contrast, *P. antipodarum* activity was significantly decreased by environmental realistic chlorpyrifos concentrations (2.86 and 14.2 nM) after seven days of contact. The present study suggests that *P. antipodarum* may be employed as a biological indicator for assessing pesticide contamination.

Keywords: Cholinesterase activity; Substrates; *Potamopyrgus antipodarum*; *Valvata piscinalis*; Chlorpyrifos; Biomarker

1. Introduction

The measurement of the exposure to pollution and of the biological effects of toxicants has become of major importance for the assessment of the quality of the environment (van der Oost et al., 2003). The use of biological markers at the molecular or cellular level have been proposed as sensitive 'early warning' tools for biological effect measurement (van der Oost et al., 2003). This approach has been widely used both *in vivo* and *in vitro* for the evaluation of xenobiotic effects on animals (Binelli et al., 2006).

Among anthropogenic contaminants, pesticides are widely detected in freshwater and estuarine ecosystems. These molecules are spread on terrestrial cultures and enter waterways from agricultural and urban run-off. Pesticides may have major ecological consequences (Ozretic and

* Corresponding author. Tel.: +33 4 72 20 89 05; fax: +33 4 78 47 78 75.
E-mail address: garric@lyon.cemagref.fr (J. Garric).

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Krajnovic-Ozretic, 1992). The organophosphates (OPs) and carbamates (Cs) are modern synthetic insecticides and are potent neurotoxic molecules (Ashauer et al., 2006). They exert acute toxicity by blocking the breakdown of acetylcholine by the enzyme acetylcholinesterase (AChE: E.C.3.1.1.7) in vertebrate and invertebrate organisms (Fulton and Key, 2001). Acetylcholine is the primary neurotransmitter in the sensory and neuromuscular systems in most species. The activity of this system is vital to muscular function and represents a prime target on which OPs and Cs can exert a detrimental effect (Sarkar et al., 2006).

Monitoring AChE activity in wildlife populations has been proposed as a general method for detecting environmental contamination from OPs and Cs, particularly since many of these chemicals have relatively short half-lives in the aquatic environment and are not water soluble. The World Health Organization (Paris) recognizes AChE biomonitoring as a preventive measure against OP overexposure in non-target species (Romani et al., 2005). Its use as a specific biomarker to assess the exposure of aquatic organisms to these compounds is widely applied in laboratory and field studies (Bocquené et al., 1997; Scaps et al., 1997; Galloway et al., 2002; Binelli et al., 2006).

In vertebrates two isoforms occur, acetylcholinesterase (AChE) which preferentially hydrolyses acetyl esters such as acetylcholine, and butyrylcholinesterases (BChE) which preferentially acts on butyrylcholine. The main function of AChE is the rapid hydrolysis of the neurotransmitter, whereas BChE has no known specific natural substrate, although it is able to hydrolyse acetylcholine (Fulton and Key, 2001; Valbonesi et al., 2003). Another isoform, propionylcholinesterase (PChE), has been characterized (Mora et al., 1999). Since the properties of ChE may differ between species, it is important to characterize the type of enzyme present in the species studied before its use as a biomarker (Kristoff et al., 2006).

Whilst ChEs have been extensively studied in vertebrates and insects, few data are available in molluscs (Mora et al., 1999). Molluscs, in particular bivalves, are often used as sentinel organisms: their world-wide distribution, their sedentary mode of life and their filter-feeding behaviour susceptible to induce pollutant bioaccumulation make them ideal species for the assessment of environmental pollution (Rittschof and McClellan-Green, 2005). Prosobranch snails including *Potamopyrgus antipodarum* (Hydrobiidae) and *Valvata piscinalis* (Valvatidae) are important members of aquatic habitats and possess a high ecological relevance for freshwater ecosystems (Mouthon and Charvet, 1999). They have proved to be sensitive test organisms in several studies (Oetken et al., 2005) and *P. antipodarum* has been recommended for toxicity tests by the Invertebrate testing group of OECD (Duft et al., 2007). Using these animals might facilitate the linking of laboratory data to field studies and field experiments could be undertaken on autochthonous or caged animals.

The aim of this study was to characterize and to investigate the relevance of ChE activities as early warning tools of neurotoxic stress in two freshwater mudsnails. Activities in *P. antipodarum* and *V. piscinalis* were firstly characterized *in vitro* by using different substrates acetylthiocholine (ASCh), propionylthiocholine (PSCh) and butyrylthiocholine (BSCh) and specific inhibitors (eserine for ChE, BW284c51 for AChE, *iso*-OMPA for BChE). Secondly, *in vivo* effects of a model insecticide, chlorpyrifos, on ChE activities were then studied in order to assess the value of *P. antipodarum* and *V. piscinalis* as sentinel species of freshwater insecticide contamination.

2. Material and methods

2.1. Chemicals

Acetylthiocholine iodide (ASCh), butyrylthiocholine iodide (BSCh), propionylthiocholine iodide (PSCh), 5,5-dithio-bis-2-nitrobenzoate (DTNB), eserine, BW284c51 (1,5-bis(4-allydimethylammoniumphenyl)-pentan-3-one dibromide), *iso*-OMPA (tetra-(monoisopropyl)pyrophosphor-tetra-mide) and chlorpyrifos were obtained from Sigma-Aldrich (Villefranche, France).

2.2. Organisms

P. antipodarum and *V. piscinalis* were obtained from the laboratory culture established in the laboratory (CEMAGREF, Lyon, France). Animals were reared under standard conditions in aerated glass aquariums (17–20 l), at a temperature of 22 ± 1 °C, and under a 16–8 h artificial light-dark photoperiod regime. For the cultures, animals were fed using Tetramin®. For all experiments, adult snails of similar size (4 mm) were used.

2.3. Cholinesterase activity

The whole animals with shell were weighed and homogenized with an Ultra-Turrax T25 basic® at 24 000 rpm for 40 s in 1:10 (W:V) for *V. piscinalis* and 1:20 for *P. antipodarum* 0.1 M phosphate buffer, pH 7.8, plus 0,1% Triton X-100. Homogenates were centrifuged at 9000×*g* for 15 min at 4 °C. Supernatants were used as the enzyme source.

The enzyme activity was measured following the Ellman method (1961). In a typical assay, 330 µl of 0.1 M phosphate buffer pH 7.8, 20 µl of 7.6 mM the chromogenic agent DTNB and 20 µl of sample were successively added in a 96 wells microtitre plate. Measurement of enzyme activity was initiated by the addition of 10 µl of freshly prepared acetylthiocholine iodide solution in distilled water. Absorption of the 2-nitro-5-thiobenzoate anion, formed from the reaction, was then recorded at 405 nm every 60 s for 9 min at room temperature using a TECAN® *Safire*® spectrofluorimeter. Spontaneous substrate hydrolysis was assessed using a blank without sample. Kinetic was calculated in the linear range. Each sample was analyzed in triplicates. Total protein was determined according to the