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Inhibition of cholinesterase activity by azinphos-methyl in two freshwater invertebrates: *Biomphalaria glabrata* and *Lumbriculus variegatus*

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Abstract

In this study, some biochemical features and the extent of inhibition induced by the organophosphorous pesticide azinphos-methyl on the cholinesterase (ChE) activity present in whole soft tissue of two freshwater invertebrate species, the gastropod *Biomphalaria glabrata* and the oligochaete *Lumbriculus variegatus* were investigated. Both invertebrate organisms presented marked differences in ChE activity, type of enzymes and subcellular location. Acetylthiocholine was the substrate preferred by *B. glabrata* ChE. The enzyme activity was located preferentially in the supernatant of 11,000 × *g* centrifugation and was inhibited by increasing concentrations of substrate but not by *iso*-OMPA. Results showed that there were progressive inhibitions of the enzyme activity, with values 21%, 59%, 72%, 76%, and 82% lower than the control at levels of 1, 10, 50, 100 and 1000 μM of eserine, respectively. In contrast, *L. variegatus* ChE activity was distributed both in the supernatant and pellet fractions, with values approximately 6 and 20 times higher than *B. glabrata*, respectively. Studies with butyrylthiocholine and *iso*-OMPA suggested that about 72% of the activity corresponded to butyrylcholinesterase. A strong enzyme inhibition (88–94%) was found at low eserine concentrations (1–10 μM). ChE activity from *L. variegatus* and *B. glabrata* was inhibited by in vivo exposure to azinphos-methyl suggesting that both species can form the oxon derivative of this pesticide. However, both invertebrate species showed a very different susceptibility to the insecticide. The NOEC and EIC_{50} values were 500 and 1000 times lower for *L. variegatus* than for *B. glabrata*, reflecting that the oligochaetes were much more sensitive organisms. A different pattern was also observed for the recovery of the enzymatic activity when the organisms were transferred to clean water. The recuperation process was faster for the oligochaetes than for the gastropods. Mortality was not observed in either of the experimental conditions assayed, not even at concentrations that induced 90% of ChE inhibition. The differences in substrate specificity, sensitivity to inhibitors, and subcellular location between the ChEs of *B. glabrata* and *L. variegatus* could be the main factors contributing to the differential susceptibility to azinphos-methyl ChE inhibition found in the present study.

Keywords: Cholinesterase; Azinphos-methyl; *Biomphalaria glabrata*; *Lumbriculus variegatus*

Abbreviations: ChE, cholinesterase; AChE, acetylcholinesterase; AsCh, acetylthiocholine; BChE, butyrylcholinesterase; BsCh, butyrylthiocholine; DTNB, 5,5′-dithio-2-bis-nitrobenzoate; *iso*-OMPA, tetraisopropyl pyrophosphoramide

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1. Introduction

For decades, the activity of the enzyme acetylcholinesterase (AChE, EC 3.1.1.7) has been used as a sensitive biomarker of exposure to organophosphorous and carbamate pesticides (Timbrell, 2000; Walker et al., 2001). Its inhibition is linked with the pesticide mechanism of toxic action, irreversible or reversible binding to the esteratic site of the enzyme and potentiation of cholinergic effects in the nervous system.

As AChE is readily inhibited by organophosphorous compounds it is considered a Type B-esterase (Peakall, 1992). This group also includes the butyrylcholinesterase (BChE: acylcholine acyl hydrolase, EC 3.1.1.8), neurotoxic esterases, and carboxilesterases (Peakall, 1992). AChE is regarded as a true-cholinesterase, since it hydrolyzes preferentially acetylcholine and apart from the nervous system, it is also present in erythrocytes (Huggett et al., 1992). BChE, regarded as a pseudocholinesterase, hydrolyzes preferentially butyrylcholine and it is found in various tissues, e.g. in liver, intestine, serum, heart, and lung (Huggett et al., 1992; Li et al., 2000). In addition, both cholinesterases exhibit different sensitivity towards inhibitors. AChE presents inhibition by an excess of substrate, it is inhibited by eserine but insensitive to *iso*-OMPA, while BChE is specifically inhibited by *iso*-OMPA (Hyne and Maher, 2003).

The classification, characteristics and tissue localization given before are generally valid for cholinesterases (ChEs) derived from vertebrates, in particular from mammalian species, but at least some of them cannot be directly applied to non-mammalian species. In the last case, the ChEs may exhibit a wide variety of substrate specificities. For instances, in the lake trout *Salvelinus namaycush* predominates AChE, in the crab *Uca pugmax* it is BChE, and in ducks it is a ChE propionylcholine specific (Huggett et al., 1992).

On the other hand, ChEs from different sources may present a large variety of molecular forms, different solubility and mode of membrane anchorage (Massoulié et al., 1993).

Although aquatic invertebrates have been widely used as bioindicator organisms in many monitoring programs, the use of AChE inhibition as a biomarker in these species has been largely neglected (Hyne and Maher, 2003). Inhibition of ChE activity by organophosphorous and carbamate pesticides has been measured in a few aquatic invertebrate species, such as bivalve mollusks (Bocquené et al., 1997; Basack et al., 1998; Mora et al., 1999; Doran et al., 2001; Cooper and Bidwell, 2006), and crustaceans (Day and Scott, 1990; Lundebye et al., 1997; Sturm and Hansen, 1999; Varó et al., 2002). Since the properties of ChE may differ from species to species, it is important to characterize the type of enzyme(s) present in the species to be studied before its use as biomarker. Moreover, more than one ChE may be present and these ChEs may show different sensitivities to anticholinesterase agents. In this sense, Bocquené et al. (1997) described the presence of two ChE in the common oyster, one insensitive to organophosphates and carbamates, anchored to membrane and that hydrolyses AsCh but not BsCh, and the other, hydrophilic and highly sensitive to organophosphates and carbamates that hydrolyses AsCh and to a much lesser extent BsCh. Mora et al. (1999) also described a ChE that was poorly inhibited by organophosphates in marine mussels and in a freshwater bivalve. In addition, Basack et al. (1998) described two ChE activities in *Corbicula fluminea*, one present in the pellet of 10,000 × *g* centrifugation sharing all the features of vertebrate AChE, and another localized in the supernatants of 10,000 × *g* relatively insensitive to eserine.

In this work two main aspects were investigated. Firstly, a preliminary biochemical characterization of the ChE enzymes present in two freshwater invertebrate species, the gastropod *Biomphalaria glabrata* and the oligochaete *Lumbriculus variegatus*, was undertaken. Secondly, a comparative study of the extent of ChE inhibition induced by azinphos-methyl, a widely used pesticide in the Northern Patagonia agricultural areas, was performed.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide (AsCh), butyrylthiocholine iodide (BsCh), 5,5′-dithio-2-bis-nitrobenzoate (DTNB), physostigmine hemisulfat salt (eserine), and tetraisopropyl pyrophosphoramide (*iso*-OMPA) were obtained from Sigma (St. Louis, MO). Azinphos-methyl (98.3% pure) was a gift from Bayer, S.A., Argentina. All other chemicals used were of analytical reagent grade.

2.2. Organisms selected

B. glabrata snails were originally obtained from a laboratory culture established at the Invertebrate Laboratory, Department of Biology, Faculty of Exact and Natural Sciences, University of Buenos Aires. The organisms were then cultured in our laboratory under standard conditions in aerated glass aquariums (17–20 L), at a temperature of 22 ± 2 °C, and under a 14–10 h artificial light–dark photoperiod regime. For the cultures animals were fed lettuce leaves ad libitum (Fried et al., 1992). For all the experiments, adult snails of similar size