Comparative Biochemistry and Physiology, Part C 150 (2009) 503–511

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

Carboxylesterase activity in earthworm gut contents: Potential (eco)toxicological implications

Juan C. Sanchez-Hernandez [a,*], Christophe Mazzia [b], Yvan Capowiez [c], Magali Rault [b]

[a] Laboratory of Ecotoxicology, Faculty of Environmental Science, University of Castilla-La Mancha, Avda. Carlos III s/n, 45071, Toledo, Spain
[b] Universite d'Avignon et des Pays de Vaucluse, UMR 406 Abeilles et Environnement, Site AGROPARC, F-84914, Avignon Cedex 09, France
[c] INRA, Unite PSH, Site AGROPARC, F-84914 Avignon Cedex 09, France

ARTICLE INFO

Article history:
Received 12 June 2009
Received in revised form 21 July 2009
Accepted 22 July 2009
Available online 3 August 2009

Keywords:
Gut secretion
Lumbricus terrestris
Luminal carboxylesterases
Organophosphorus pesticides
Pesticide-detoxifying esterases

ABSTRACT

Carboxylesterases (CbEs) are key enzymes in pesticide detoxification. These esterases are involved in the biochemical mechanism for pesticide resistance in some pest species, and further they are considered an efficient protective mechanism against acute toxicity by organophosphate (OP) pesticides in mammals. To gain knowledge on the role of CbEs in pesticide toxicity and natural tolerance in earthworms, we performed an enzyme kinetic analysis to investigate whether these annelids are able to secrete them into their gut lumen. We determined levels of CbE activity and isozyme abundance in the gut wall and ingested soil collected from different portions of the gastrointestinal tract of *Lumbricus terrestris*. Moreover, modulation of enzyme activity by selected substrates (α-naphthyl acetate [α-NA], 4-nitrophenyl valerate [4-NPV] and 4-nitrophenyl acetate [4-NPA]) and OP pesticides was examined to compare the response between tissue and soil CbEs. We found a high CbE activity in the ingested soil extracts from the crop/gizzard (α-NA-CbE = 8.43 ± 2.76 U mg^{-1} protein and 4-NPA-CbE = 5.98 ± 2.11 U mg^{-1} protein) compared to the gut wall. Three lines of evidences suggest that the gut epithelium is the main source of this luminal CbE activity. First, the effect of substrate concentrations on CbE activity from both the ingested soil extracts and gut tissues resulted in similar apparent K_m and V_{max} values. Second, native PAGE gels revealed that some of the CbE isozymes in the gut tissue were also present in the soil extracts. Third, tissue and soil CbEs showed the same sensitivity to inhibition by OPs. The concentrations of insecticide causing 50% of esterase inhibition (IC_{50}) was comparable between tissue (IC_{50}s range = 4.01–9.67 nM dichlorvos and 8480–6880 nM paraoxon) and soil (IC_{50}s range = 6.01–11.5 nM dichlorvos and 8400–7260 nM paraoxon). Our results suggest a set of (eco)toxicological implications and environmental applications derived from the ability of earthworms to secrete these pesticide-detoxifying enzymes.

1. Introduction

Earthworms occupy a notable place in terrestrial ecotoxicology. They are common standard organisms in soil toxicity testing to assess either harmful effects of new registered chemicals or toxicity of historically contaminated soils (van Straalen and van Gestel, 1998). Moreover, these organisms are demonstrated to be excellent bioindicators in the field monitoring of soil pollution by metals (Spurgeon and Hopkin, 1999), although some earthworm species are able to tolerate highly metal-contaminated soils (Spurgeon and Hopkin, 2000). The induction of metal-sequestrating proteins is among the more plausible causes for earthworm survival in heavily metal-polluted soils (Vijver et al., 2004). Likewise, pesticide-detoxifying enzymes such as carboxylesterases (CbE, EC 3.1.1.1) can make the pesticide ineffective against individuals that naturally have high levels of these enzymes (Stenersen, 2004). These esterases detoxify organophosphate (OP) and carbamate (CB) pesticides and synthetic pyrethroids (SPs) by two main ways; hydrolysis of the ester bond (CBs, SPs and those OPs containing this type of bond such as malathion) and binding of the pesticide (OPs) to the active site of CbE (Maxwell 1992; Chambers et al., 1994; Wheelock et al., 2005; Crow et al., 2007). Inhibition of CbE activity by OPs involves nucleophilic attack of the catalytic serine residue in the enzyme active site on the electronic deficient oxidized phosphorous to form a stable enzyme–inhibitor complex (Satoh and Hosokawa, 1998). This inhibition is considered a stoichiometric mechanism for decreasing the OP concentration at the target site, i.e., acetylcholinesterase (Maxwell, 1992; Chanda et al., 1997).

Carboxylesterases have been described in earthworms (Haites et al., 1972; Øien and Stenersen, 1984). The intestinal tract of *Lumbricus terrestris* presents a wide variety of enzyme; among them, CbE activity is predominant in the peripherical chloragog tissue (Prento, 1987). Recently, Sanchez-Hernandez and Wheelock (2009) reported up to twelve CbE isozymes in the gastrointestinal tract, body wall muscle and reproductive tissues of *L. terrestris*, being particularly abundant in the crop, gizzard and foregut. The abundance of CbEs in the gastrointestinal

* Corresponding author. Tel.: +34 925 268800; fax: +34 925 268840.
E-mail address: juancarlos.sanchez@uclm.es (J.C. Sanchez-Hernandez).

1532-0456/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpc.2009.07.009

tract of earthworms leads to hypothesize if these enzymes represent an enzymatic barrier for reducing pesticide uptake from contaminated ingested soil, or even to speculate if these esterases can be secreted into the gut lumen by earthworms themselves as this occurs with other invertebrates such as spiders (Mommsen, 1978), lepidopterans (Turunen and Chippendale, 1977), mosquitoes (Geering and Freyvogel, 1974) or cockroaches (Cook et al., 1969).

The aims of this study were therefore to determine the presence of CbEs in the earthworm gut content and to investigate whether gut wall is a source of luminal CbE activity. We measured the levels of enzyme activity and isozyme abundance in tissue homogenates from the pharynx, crop/gizzard, foregut and midgut sections of the alimentary canal of *L. terrestris*. Soil samples collected from these gut regions were also used for CbE determination. In addition, the effect of selected substrates and inhibitors (OP insecticides) on enzyme activity was compared between tissue and soil CbE activities. These in vitro experiments provided solid evidences for a gut secretion of these pesticide-metabolizing enzymes that suggest some toxicological implications and environmental applications of concern in earthworm ecotoxicology.

2. Materials and methods

2.1. Reagents

The chemicals for CbE activity assays, α-naphthyl acetate (α-NA), 4-nitrophenyl acetate (4-NPA), 4-nitrophenyl valerate (4-NPV), 4-nitrophenol, Fast Red ITR salt, Fast Blue RR salt and 4-methylumbelliferyl acetate (4-MUBA) as well as lipase type II from porcine pancreas (100–400 U mg^{-1} protein) and lipase from *Candida cylindracea* (20.7 U mg^{-1} protein) were purchased from Sigma-Aldrich (Madrid, Spain). The OP pesticides (>98% purity) dichlorvos (2,2-dichlorovinyl dimethyl phosphate) and paraoxon (*O,O*-diethyl *O*-(4-nitrophenyl) phosphate) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). We used dichlorvos and paraoxon (the active oxon metabolite of parathion) as model pesticides because they are potent esterase inhibitors (Chambers et al., 1994).

2.2. Experimental setup

Adults clitellate *L. terrestris* (3.4–5.0 g fresh mass) were obtained from a commercial supplier (Decathlon, France) and kept individually in boxes containing 100 g of field soil (collected in uncontaminated area) during 1 week at 12–15 °C and darkness. Earthworms were anesthetized by cooling (individuals were placed in petri dishes and kept on ice) to collect gut tissues and ingested soil. Dissection was initiated in the mid-dorsal line by a longitudinal incision. Pharynx, crop/gizzard, foregut (the gut segment between the gizzard and the clitellum) and midgut (the gut segment after the clitellum towards the anus) were removed and open separately to collect carefully the ingested soil avoiding to scrape the epithelium. Soil present in the last portion of the intestine (hindgut) corresponded to fresh cast.

Gut tissues, ingested soil, control soil (bulk soil from the box) and fresh cast were immediately transferred to 1.5-mL microcentrifuge tubes and weighed. Tissue samples were homogenized in 10% (w/v) buffer containing 10 mM Tris–HCl (pH 7.3), 10 mM NaCl supplemented with a cocktail of protease inhibitors (aprotinin, leupeptin and pepstatin = 5 μg mL^{-1}; antipain = 1 μg mL^{-1}; trypsin inhibitor = 1 mg mL^{-1}) using a conical hand-held homogenizer connected to a bench-top overhead stirrer (IKA RW14 Basic). Fresh soil samples were suspended in 10% (w/v) homogenization buffer and homogenized by rapid mechanical stirring for 1 min. Tissue homogenates and soil suspensions were centrifuged at 3000 g for 10 min at 4 °C to obtain the tissue post-mitochondrial supernatants (PMS) and the soil extracts, which were stored at −20 °C until analysis (within two months) with 10% glycerol as an enzyme stabilizing agent.

2.3. Carboxylesterase assays

Carboxylesterase activity was assayed spectrophotometrically using the substrates α-NA, 4-NPA and 4-NPV, and protocols were adapted to a microplate reader (Synergy HT, Bio-Tek). Carboxylesterase activity using α-NA was determined according to Gomori (1953), as adapted by Bunyan et al. (1968). The reaction medium (200 μL, final volume) contained 25 mM Tris–HCl (pH 7.6), 1 mM $CaCl_2$ and 2 mM α-NA, and it was incubated for 10 min at 25 °C with the sample. The enzymatic formation of α-naphthol was stopped by the addition of 50 μL of 2.5% SDS in 0.1% Fast Red ITR/2.5% Triton X-100. Solutions were allowed to stand for 30 min at 22–23 ºC and dark. The absorbance of the naphthol–Fast Red ITR complex was read at 530 nm, and the concentration of α-naphthol was determined using a α-naphthol standard curve that was measured under the same conditions. We used a reaction buffer supplemented with $CaCl_2$ because concomitant determination of Ca^{+2}-dependent A-esterase activity (data not shown), however Ca^{+2} had no effect on hydrolysis rate of α-NA.

Hydrolysis of 4-NPV and 4-NPA was determined as described by Carr and Chambers (1991) and Chanda et al. (1997), respectively. The reaction mixture (250 μL, final volume including enzyme sample) contained 1 mM 4-NPV and 50 mM Tris–HCl (pH 7.5) for assaying CbE activity towards 4-NPV, or 5 mM 4-NPA, 20 mM Tris–HCl (pH 8.0) and 1 mM EDTA when 4-NPA was used as substrate. In both methods, the formation of 4-nitrophenol was monitored for 5 min at 405 nm, 25 °C and quantified using a 4-nitrophenol standard curve. Blanks (reaction mixture free of enzyme sample) were periodically checked for nonenzymatic hydrolysis of 4-NPV and 4-NPA, and no significant activity was observed. Proteins were quantified according to the Lowry method as modified by Markwell et al. (1978), and using bovine serum albumin as the standard protein. All enzyme assays were run in triplicate. Specific CbE activities were expressed as units per milligram of total proteins (U mg^{-1} protein). One unit of enzyme activity is defined as one micromole of substrate hydrolyzed per minute under the experimental conditions described above.

2.4. Effect of substrate concentration on CbE activity

We examined the effect of the concentration of the substrates α-NA (range of tested concentrations = 0.012–2.5 mM, final concentration), 4-NPA (0.031–2.5 mM) and 4-NPV (0.012–5 mM) on tissue and soil CbE activities. The kinetic parameters K_m and V_{max} associated to substrate-mediated modulation of CbE activity were estimated using the Michaelis–Menten equation, i.e., $V_0 = V_{max} \times [S]/(K_m + [S])$. Concentrations of substrate higher than 5 mM were not assayed because of substrate precipitation in the reaction medium.

2.5. Kinetic inhibition analysis

We studied the sensitivity of tissue and soil CbE activities to dichlorvos and paraoxon. The molar concentration of inhibitor causing a 50% reduction of initial enzyme activity (IC_{50}) was calculated by plotting the logarithm of inhibitor concentration versus the percentage of remaining CbE activity. Dose–response curves were fitted to the four-parameter logistic equation $y = min + (max - min)/1 + (x/IC_{50})^{-HillSlope}$, where y is the percentage of residual CbE activity compared to controls after a 30-min incubation with the inhibitor, *min* is the y response to the highest concentration of inhibitor, *max* is y response to the lowest concentration of inhibitor, x is the logarithmic of inhibitor molar concentration and *Hillslope* describe the steepness of the dose–response curve (Motulsky and Christopoulos, 2003). Esterase activity was assayed using α-NA solely because of the higher enzyme activity previously observed in both PMS and soil extracts. Aliquots of tissue or soil samples were incubated in the presence of serial concentrations of dichlorvos ($7 \times 10^{-11} - 2.7 \times 10^{-7}$ M) or paraoxon ($7 \times 10^{-12} - 2.7 \times 10^{-4}$ M) for 30 min at 22–23 °C. Stock