Short communication

Extracellular pesticide detoxification in the gastrointestinal tract of the earthworm *Aporrectodea caliginosa*

Juan C. Sanchez-Hernandez, Manuel Aira, Jorge Domínguez

*Laboratorio de Ecotoxicología, Facultad de Ciencias Ambientales y Bioquímica, Universidad de Castilla-La Mancha, 45071 Toledo, Spain*  
*Departamento de Ecología e Bioloxía Animal, Universidade de Vigo, 36310 Vigo, Spain*

**Abstract**

We provide evidences that indicate *Aporrectodea caliginosa* detoxifies extracellularly organophosphorus (OP) pesticides through its digestive system. A high carboxylesterase activity (a group of esterases able to bind OP pesticides) was found in the luminal microenvironment of its gastrointestinal tract compared to casts (faeces) or bulk soil. In vitro inhibition kinetics and microcosm trials showed that this luminal CbE activity was highly sensitive to inhibition by chlorpyrifos-oxon. Our data suggest that CbE activity was an efficient bioscavenger for OP pesticides during gastrointestinal transit of soil, which increases our understanding of how earthworms are able to tolerate OP-contaminated soils.

**Keywords:** Carboxylesterases  
Soil detoxification  
Earthworms  
Chlorpyrifos  
Enzymatic bioremediation

There is a growing recognition that pesticide applications represent a serious threat to non-target organisms (Devine and Furlong 2007), and soil components that provide beneficial agricultural services such as microorganisms and soil enzymes (Gianfreda and Varone 2008). These environmental risks have driven the search for in situ biological methods for degrading pesticides (Singh et al. 2009). In the last decade, earthworms have been used for soil restoration and bioremediation (Butt 2008; Boyer and Wratten 2010; Rodríguez-Campos et al. 2014), and some studies suggest that symbiotic relationships between microbes and endogeic earthworms contribute to pesticide breakdown. For example, proliferation of Alphaproteobacteria in burrow walls and casts of *Aporrectodea caliginosa* significantly degraded the herbicide 2-methyl-4-chlorophenoxyacetic acid (Liu et al. 2011). Other authors have described the occurrence of pesticide-detoxifying esterases in the digestive tract of *Lumbricus terrestris* (Sanchez-Hernandez et al. 2009).

Based on these studies, we hypothesize that the digestive tract of *A. caliginosa* (Savigny) is able to detoxify OP pesticides through the existence of luminal carboxylesterases (CbEs, EC 3.1.1.1), which act as an enzymatic barrier against pesticide uptake, and also would provide a potential mechanism for pesticide dissipation in soil. Thus, the aims were: 1) to compare the hydrolytic activity of this esterase in the gastrointestinal tract (luminal content and wall), field casts, freshly egested casts and bulk soil; and 2) to examine the sensitivity of gastrointestinal CbE activity to chlorpyrifos as a model OP pesticide. Because earthworm CbE activity is represented by multiple isozymes with a high variation in substrate preference and pesticide sensitivity (González Vejarás et al., 2010), the enzyme kinetics were performed using multiple ester substrates and supported by zymographic analysis.

Earthworms (*n* = 10), field casts (*n* = 10) and surrounding bulk soil (*n* = 10, sieved at <5 mm) were collected in a grassland ecosystem (University of Vigo, Galicia, NW Spain). Freshly egested casts (*n* = 24 samples) were obtained after putting earthworms on moistened filter paper in Petri dishes (24 h, dark and 10 °C). Carboxylesterase activity was measured according to Thompson (1999), and using supernatants (9000 g, 10 min, 4 °C) obtained after homogenization of the gastrointestinal wall (1:10, w/v) in cold 10 mM Tris–HCl (pH 7.4), supplemented with 1 mM EDTA and 0.1% Triton X-100, or by mixing vigorously (1:2.5, w/v) the gastrointestinal content, soil or casts with the same buffer for one minute. The pharynx-to-gizzard segment of the digestive tract was chosen in this study because previous work with *L. terrestris* has documented a maximum CbE activity in the luminal microenvironment of that digestive region (Sanchez-Hernandez et al., 2009). Enzyme activity was calculated using calibration curves made with 2-naphthol.
5–50 μM) or 4-nitrophenolate (5–100 μM), and expressed as U/mg of total protein (1 U = 1 μmol min⁻¹ mg⁻¹ of protein). Native polyacrylamide gel electrophoresis were performed according to González Vejares et al. (2010). Sensitivity of CbE activity to chlorpyrifos was evaluated by in vitro and microcosm experiments (full methodological details are provided in the Supplementary Information). Because chlorpyrifos needs to be metabolized to its highly toxic metabolite chlorpyrifos-oxon to gain affinity for the active site of CbE (Wheelock et al., 2008), microcosm experiments were performed with soils treated separately with chlorpyrifos (10 mg kg⁻¹ dry soil, chemical analysis of pesticide residues described in Supplementary Table S1) and chlorpyrifos-oxon (7.3 mg kg⁻¹).

The highest CbE activity was found in the gastrointestinal content (Tukey post-hoc test, $P < 0.05$), and the short-chain esters α-naphthyl-acetate (α-NA) and 4-nitrophenyl-acetate (4-NPA) provided the highest hydrolytic rates compared to longer chain length esters (e.g., α-naphthyl butyrate [α-NB]) (Fig. 1a). Zymographic analysis corroborated the enzyme kinetic results, with a higher staining intensity for protein bands of the gastrointestinal content (Fig. 1b). However, the use of two naphthyl esters resulted in a different zymographic profile. The substrate α-NB evidenced a group of bands with apparent native molecular masses between 150 and 415 kDa, which were strongly stained in the samples of gastrointestinal content. However, α-NA gave a higher staining for CbE bands distributed in the range of 44–60 kDa (Fig. 1b). These findings contrast with results reported for small mammals, for instance, in which CbE activity was up to 3-fold higher in the mucosa of the small intestine than in its luminal compartment (Hänninen et al., 1987). Soil-eating behavior of endogeic earthworms and their symbiotic relationships with gut microbes may account for this difference. Digestive enzymes in endogeic earthworms mainly originate from symbionts (Lavelle et al. 1995). Accordingly, maximum activity of glycolytic enzymes occurs in the foregut (the region of the digestive tract located after the gizzard) and gradually decreases towards the distal gut (Supplementary Fig. S2). In our study, localization of maximum CbE activity occurs in the crop-gizzard region, which leaves open the possibility of the major source of esterase activity being the gastrointestinal wall rather than symbionts.

In vitro inhibition kinetics showed that gastrointestinal CbE activity was highly sensitive to chlorpyrifos-oxon, although it was substrate-dependent as evidenced in the zymograms (Fig. 1b, Supplementary Fig. S3). Incubation of some samples in the presence of 1.2 × 10⁻⁶ M chlorpyrifos-oxon resulted in a decrease of staining intensity in two groups of CbE bands corresponding to molecular masses of 150–415 kDa and 48–77 kDa. Although in-gel staining using α-NB showed a higher number of CbE isozymes within the range of 17–130 kDa, there was a high inter-

![Fig. 1. Panel a) Mean (±standard deviation) carboxylesterase (CbE) activity in the gastrointestinal wall, gastrointestinal content, fresh casts, field casts and bulk soil. Different letters denote significant differences ($P < 0.05$) after a post-hoc LSD test (normal font for α-naphthyl acetate [α-NA] and 4-nitrophenyl acetate [4-NPA], bold font for α-naphthyl butyrate [α-NB] and 4-nitrophenyl butyrate [4-NPB], and italic font for 4-nitrophenyl valerate [4-NPV]). Panel b) Native polyacrylamide gel electrophoresis of gastrointestinal wall (19 μg protein) and content (6.6 μg protein) samples previously incubated in vitro (30 min, 22 °C) with 1.2 × 10⁻⁶ M chlorpyrifos-oxon (CPoxon). Reference samples were incubated with the same volume of distilled water (Control). Post-electrophoresis visualization was performed with the substrates α-NA and α-NB. The native proteins standard (Invitrogen NativeMark™ protein standard) was composed of soybean trypsin inhibitor (20 kDa), bovine serum albumin (66 kDa), lactate dehydrogenase (146 kDa), β-phycoerythrin (242 kDa) and apoferritin (480 kDa), and stained with coomassie brilliant blue (CBB).](image-url)
individual variation in isozyme abundance, which hindered the effect of chlorpyrifos-oxon (Supplementary Fig. S1). Microcosm experiments revealed the potential role of luminal CbE activity as an extracellular bioscavenger for OPs (Fig. 2). Chlorpyrifos-oxon-spiked soils caused a significant ($P < 0.03$, Mann–Whitney test) inhibition of both tissue (Fig. 2a) and luminal (Fig. 2b) CbE activities after four days of exposure. Similar results were achieved with chlorpyrifos exposure (Fig. 2c and d), but inhibition of luminal CbE activity was lower (22–52% compared with controls) than that obtained with chlorpyrifos-oxon exposure (63–75%). Depression of CbE activity was not detected with $\alpha$-NA and 4-NPA (Supplementary Table S2), which was in agreement with in vitro outcomes. These findings suggest that the gastrointestinal lumen of A. caliginosa is able to detoxify extracellularly OP pesticides in the way illustrated in Fig. 2. Most OPs need a bioactivation step that consists of an oxidative desulfuration to yield the oxygen metabolite (i.e., chlorpyrifos-oxon), which displays a higher affinity for the active site of CbEs than the parent compound (Wheelock et al. 2008). In our study, exposure to chlorpyrifos caused greater percentages of CbE inhibition in the gastrointestinal wall than in the lumen (Fig. 2c and d), which would indicate a higher bioactivation rate of chlorpyrifos in the digestive epithelium. Moreover, the occurrence of cytochrome P450-dependent monooxygenases in the digestive system of lumbricid earthworms supports this assumption (Berghout et al., 1991), because this enzyme system is involved in OP bioactivation (Hodgson, 2010). Likewise, the higher sensitivity of CbE activity for chlorpyrifos-oxon inhibition than chlorpyrifos, would lead to a lesser gastrointestinal uptake for the former. This explains the lower percentages of enzyme inhibition in the gastrointestinal wall of earthworms exposed to soils contaminated with chlorpyrifos-oxon (Fig. 2a), compared with those of the luminal CbEs (Fig. 2b). Because the earthworm gastrointestinal gut is anoxic (Wust et al., 2009), the role of symbionts in chlorpyrifos bioactivation may be considered as negligible, and this reaction likely occurs by biotic and abiotic transformations in soil (Racke, 1993).

Fig. 2. Proposed conceptual model that illustrates how Aporrectodea caliginosa may detoxify the organophosphorus (OP) pesticide chlorpyrifos through its gastrointestinal carboxylesterase (CbE) activity. Luminal CbE activity represents a source of binding proteins for chlorpyrifos, which reduces its gastrointestinal uptake. This stoichiometric mechanism of extracellular detoxification is more effective with ‘oxon’ metabolites of phosphorothionate-type OPs (e.g., chlorpyrifos-oxon). Comparison of percentages of CbE inhibition (indicated above bars) between the gastrointestinal wall and lumen compartments of earthworms exposed to 7.3 mg kg$^{-1}$ chlorpyrifos-oxon (Fig. 2a) and the control (Fig. 2b) and 10 mg kg$^{-1}$ chlorpyrifos (graphs c and d) supports this assumption. Bars display the mean and standard deviation (n = 10), and decrease of CbE activity was statistically significant for all exposed groups compared with the corresponding controls ($P < 0.03$, Mann–Whitney test). Substrate abbreviations are as in Fig. 1.

Acknowledgments

This work was supported by the Ministerio de Ciencia e Innovación (CTM 2011-25788/TECNCO). We are grateful to an anonymous reviewer for their valuable comments.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.soilbio.2014.08.012.

References