Soil Biology & Biochemistry 79 (2014) 1–4

Contents lists available at ScienceDirect

Soil Biology & Biochemistry

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

Short communication

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

Juan C. Sanchez-Hernandez [a], Manuel Aira [b, *], Jorge Domínguez [b]

[a] Laboratorio de Ecotoxicología, Facultad de Ciencias Ambientales y Bioquímica, Universidad de Castilla-La Mancha, 45071 Toledo, Spain
[b] Departamento de Ecoloxía e Bioloxía Animal, Universidade de Vigo, 36310 Vigo, Spain

ARTICLE INFO

Article history:
Received 4 April 2014
Received in revised form
13 August 2014
Accepted 14 August 2014
Available online 1 September 2014

Keywords:
Carboxylesterases
Soil detoxification
Earthworms
Chlorpyrifos
Enzymatic bioremediation

ABSTRACT

We provide evidences that indicate *Aporrectodea caliginosa* detoxifies extracelullarly 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.

There is a growing recognition that pesticide applications represent a serious threat to non-target organisms (Devine and Furlong 2007), groundwater and surface waters (Arias-Estévez et al. 2008), and soil components that provide beneficial agricultural services such as microorganisms and soil enzymes (Gianfreda and Rao 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; Rodriguez-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 Vejares 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 α-naphthol

* Corresponding author.
E-mail address: aira@uvigo.es (M. Aira).

http://dx.doi.org/10.1016/j.soilbio.2014.08.012
0038-0717/

(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-

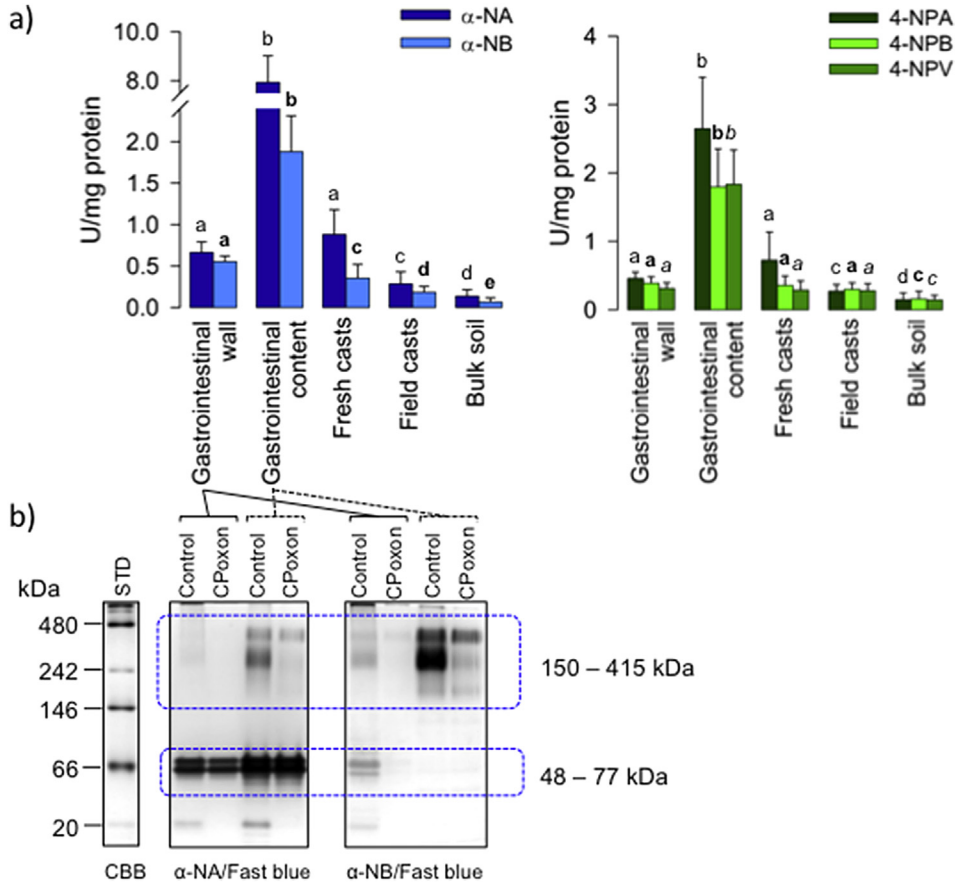

Fig. 1. Panel a) Mean (±standard deviation) carboxylesterase (CbE) activity in the gastrointestinal wall, gastrointestinal content, fresh casts, field casts and bulk soil. Different letters denote significant differences ($P < 0.05$) after a post-hoc LSD test (normal font for α-naphthyl acetate [α-NA] 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).

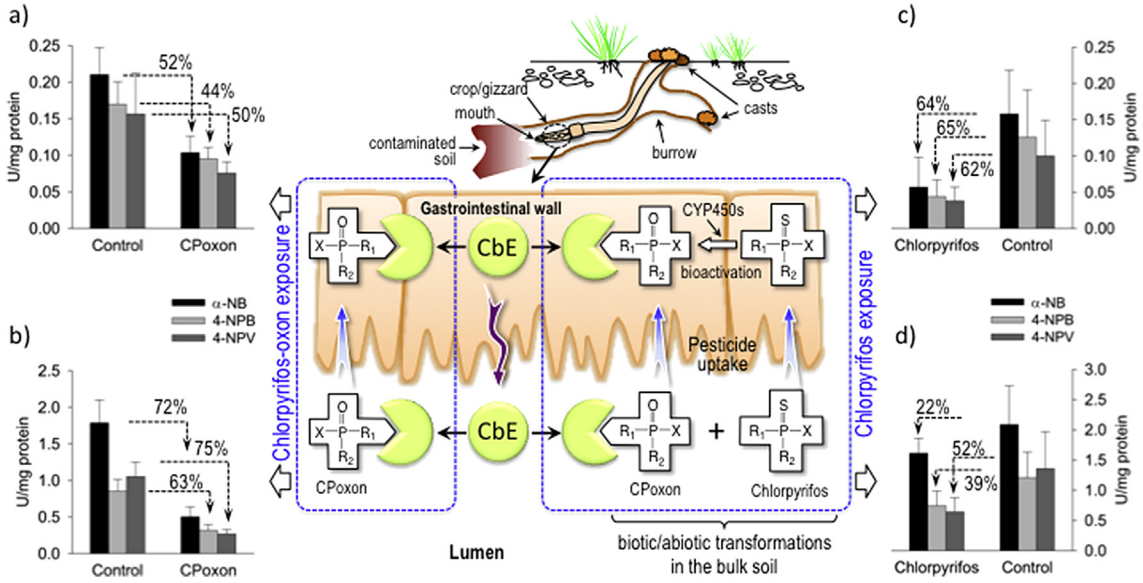

Fig. 2. Proposed conceptual model that illustrates how *Aporrectodea caliginosa* may detoxify the organophosphorus (OP) pesticide chlorpyrifos through its gastrointestinal carboxylesterase (CbE) activity. Luminal CbE activity represents a source of binding proteins for chlorpyrifos, which reduces its gastrointestinal uptake. This stoichiometric mechanism of extracellular detoxification is more effective with 'oxon' metabolites of phosphorothioate-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 (CPoxon, graphs a and b) 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.

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 α-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 extracelullarly 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).

Acknowledgments

This work was supported by the Ministerio de Ciencia e Innovación (CTM 2011-25788/TECNO). 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

Arias-Estévez, M., López-Periago, E., Martínez-Carballo, E., Simal-Gándara, J., Mejuto, J.-C., García-Río, L., 2008. The mobility and degradation of pesticides in soils and the pollution of groundwater resources. Agriculture, Ecosystems and Environment 23, 247–260.

Berghout, A.G., Wenzel, E., Büld, J., Netter, K.J., 1991. Isolation, partial purification, and characterization of the cytochrome P-450-dependent monooxygenase system from the midgut of the earthworm *Lumbricus terrestris*. Comparative Biochemistry and Physiology 100C, 389–396.

Boyer, S., Wratten, S.D., 2010. The potential of earthworms to restore ecosystem services after opencast mining – a review. Basic Applied Ecology 11, 196–203.

Butt, K.R., 2008. Earthworms in soil restoration: lessons learned from United Kingdom case studies of land reclamation. Restoration Ecology 16, 637–641.

Devine, G.J., Furlong, M.J., 2007. Insecticide use: contexts and ecological consequences. Agriculture and Human Values 24, 281–306.

Gianfreda, L., Rao, M.A., 2008. Interactions between xenobiotics and microbial and enzymatic soil activity. Critical Reviews of Environmental Science and Technology 38, 269–310.

González Vejares, S., Sabat, P., Sanchez-Hernandez, J.C., 2010. Tissue-specific inhibition and recovery of esterase activities in *Lumbricus terrestris* experimentally exposed to chlorpyrifos. Comparative Biochemistry and Physiology 151C, 351–359.

Hänninen, O., Lindström-Seppä, P., Pelkonen, K., 1987. Role of gut in xenobiotic metabolism. Archives of Toxicology 60, 34–36.

Hodgson, E., 2010. Metabolism of pesticides. In: Krieger, R. (Ed.), Hayes' Handbook of Pesticide Toxicology. Elsevier Inc., London, pp. 893–921.

Lavelle, P., Lattaud, C., Trigo, D., Barois, I., 1995. Mutualism and biodiversity in soils. Plant and Soil 170, 23–33.

Liu, Y.-J., Zaprasis, A., Liu, S.-J., Drake, H.L., Horn, M.A., 2011. The earthworm *Aporrectodea caliginosa* stimulates abundance and activity of phenoxyalkanoic acid herbicide degraders. ISME Journal 5, 473–485.

Racke, K.D., 1993. Environmental fate of chlorpyrifos. Reviews of Environmental Contamination and Toxicology 131, 1–154.

Rodriguez-Campos, J., Dendooven, L., Alvarez-Bernal, D., Contreras-Ramos, S.M., 2014. Potential of earthworms to accelerate removal of organic contaminants from soil: a review. Applied Soil Ecology 79, 10–25.

Sanchez-Hernandez, J.C., Mazzia, C., Capowiez, Y., Rault, M., 2009. Carboxylesterase activity in earthworm gut contents: potential (eco)toxicological implications. Comparative Biochemistry and Physiology 150C, 503–511.

Singh, A., Kuhad, R.C., Ward, O.P., 2009. Biological remediation of soil: an overview of global market and available technologies. In: Singh, A., Kuhad, R.C., Ward, O.P. (Eds.), Advances in Applied Bioremediation. Springer-Verlag, Berlin, pp. 1–19.

Thompson, H.M., 1999. Esterases as markers of exposure to organophosphates and carbamates. Ecotoxicology 8, 369–384.

Wheelock, C.E., Phillips, B.M., Anderson, B.S., Miller, J.L., Miller, M.J., Hammock, B.D., 2008. Applications of carboxylesterase activity in environmental monitoring and toxicity identification evaluations (TIEs). Reviews of Environmental Contamination and Toxicology 195, 117–178.

Wust, P.K., Horn, M.A., Drake, H.L., 2009. In situ hydrogen and nitrous oxide as indicators of concomitant fermentation and denitrification in the alimentary canal of the earthworm *Lumbricus terrestris*. Applied and Environmental Microbiology 75, 1852–1859.