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Earthworm activity increases pesticide-sensitive esterases in soil

Juan C. Sanchez-Hernandez [a,*], S. Martínez Morcillo [a], J. Notario del Pino [b], P. Ruiz [c]

[a] Ecotoxicology Lab, Fac. Environmental Science and Biochemistry, University of Castilla-La Mancha, Toledo, Spain
[b] Department of Soil Science and Geology, University of La Laguna, Canary Islands, Spain
[c] Microbiology Lab, Fac. Environmental Science and Biochemistry, University of Castilla-La Mancha, Toledo, Spain

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ABSTRACT

Carboxylesterases (CbEs) are serine hydrolases involved in the detoxification of anticholinesterase (organophosphorus and methylcarbamate) pesticides. Past studies have documented the occurrence of these esterases in soil, but little is known about their origin, function, and particularly, their reactivity against agrochemicals. In this study, it was compared the potential CbE activity in earthworm-treated and control (earthworm-free) soils by enzyme kinetics with multiple carboxylic esters and native polyacrylamide gel electrophoresis. After 12 weeks of inoculation, CbE activity was between two- and four-fold higher in the earthworm-treated soils (α-naphthyl acetate = 4.85 ± 1.58 μmol h^{-1} g^{-1} dry soil, α-naphthyl butyrate = 2.93 ± 1.60, α-naphthyl valerate = 2.64 ± 1.27, 4-nitrophenyl acetate = 1.41 ± 0.37, 4-nitrophenyl butyrate = 0.87 ± 0.15 and 4-nitrophenyl valerate = 0.89 ± 0.11; values are presented as mean ± standard deviation) than in controls. Although this enhanced esterase activity remained unchanged for 1 month following earthworm removal, it decreased under soil desiccation (31%–60%) or thermal denaturing (43%–82%). The potential sources for enhanced soil CbE activity were also examined through plate-count of microorganisms and zymographic techniques. The earthworm gut microenvironment was a significant source of soil CbE activity, and the casts were found to be the main contributors to the esterase activity analyzed. Soil CbE activity was strongly inhibited by organophosphorus (chlorpyrifos-oxon, paraoxon-ethyl and paraoxon-methyl) and, at less extent, by methylcarbamates (carbaryl and carbofuran). In vitro inhibition kinetics showed a biphasic curve that revealed at least two sensitive esterases and a resistant fraction; the latter varied widely depending on the enzyme substrate (7–68% of control activity). Likewise, spiking of earthworm-treated soils with 4 mg kg^{-1} (wet weight) of chlorpyrifos-oxon led to a significant inhibition of CbE activity 2 (40–72% inhibition) and 6 days (37–53%) after its application. Current results suggest that the soil-dwelling earthworm *Lumbricus terrestris* may be used as a promoter of soil enzyme activities with a direct benefit for pesticide bioremediation.

1. Introduction

Earthworms significantly impact many soil physicochemical and biological processes through their continuous burrowing and casting activities (Blouin et al., 2013). Changes in nutrient availability, soil respiration, microbial biomass and composition, and bacterial-to-fungal ratio are ecological endpoints commonly used to assess the impact of earthworm activity in soil. However, alterations that these annelids may cause in soil extracellular enzymes have been comparatively paid less attention (Kizilkaya et al., 2010). Most of the studies agree that earthworm casts (or excreta) display higher levels of hydrolytic activity than the bulk soil (Tao et al., 2009), although this difference is highly dependent on earthworm diet (Flegel and Schrader, 2000) and enzyme type (Kizilkaya and Hepsen, 2004; Dempsey et al., 2013).

Extracellular enzyme activities are critical in the decomposition of soil organic matter and nutrient cycling (Arnosti et al., 2014). Accordingly, most research efforts have been addressed to understand the functional role of hydrolytic enzymes involved in the biogeochemical cycles of C (e.g., β-glucosidase, cellulase), N (e.g., urease, proteases), P (e.g., phosphomonoesterases, phosphodiesterases), and S (e.g., arylsulfatase). Over the past two decades, other extracellular enzyme activities, particularly oxidases, have gained widespread relevance to bio-remediate contaminated soils (Bollag, 1992; Gianfreda and Rao, 2008; Burns et al., 2013). Thus, laccases, lignin peroxidases, and Mn-dependent peroxidases have shown to be excellent catalysts to degrade polycyclic aromatic hydrocarbons,

* Corresponding author. Tel.: +34 925268800; fax: +34 925268840.
E-mail address: juancarlos.sanchez@uclm.es (J.C. Sanchez-Hernandez).

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polychlorinated biphenyls, azo dyes, or trinitrotoluene (Gianfreda and Rao, 2004). However, the role of enzymes other than oxidases with potential capability to remove soil pollutants has been scarcely investigated.

The hypothesis of this study was as follows: earthworm activity increases the hydrolytic activity of soil carboxylesterases (CbEs, EC 3.1.1.1), an important group of pesticide-detoxifying esterases. This hypothesis was supported by former studies that documented the occurrence of CbE activity in soil (Satyanarayana and Getzin, 1973; Cacco and Maggioni, 1976), as well as by the stimulation of several soil enzyme activities attributed to earthworm activity. Carboxylesterases are α/β-serine hydrolases with an important function in the detoxification of organophosphorus and methylcarbamate pesticides (Sogorb and Vilanova, 2002; Wheelock et al., 2008). These pesticides constitute an important group of widely used agrochemicals, despite their regulatory and market restrictions (Casida and Durkin, 2013). In spite of their short half-lives in the environment, they are not exempted from causing deleterious environmental side effects. Many of these pesticides are toxic to microbial communities, being also capable to inhibit the hydrolytic and oxidative activities of some soil biochemical processes (Sannino and Gianfreda, 2001; Gianfreda and Rao, 2008). In this study, it is expected to provide some insight on earthworm-induced soil CbE activity of ecotoxicological concern. Within the framework of this hypothesis, the aims were: 1) to determine whether earthworm activity increases soil CbE activity, 2) to explore the main routes of enhanced soil CbE activity during earthworm activity, and 3) to determine whether this esterase activity is sensitive to inhibition by anticholinesterase pesticides.

2. Materials and methods

2.1. Chemicals

Reagents for CbE assays, that is, α-naphthyl acetate (α-NA), α-naphthyl butyrate (α-NB), α-naphthyl valerate (α-NV), 4-nitrophenyl acetate (4-NPA), 4-nitrophenyl butyrate (4-NPB), 4-nitrophenyl valerate (4-NPV), α-naphthol, 4-nitrophenol, Fast Red ITR and Fast Blue RR salts, were purchased from Sigma–Aldrich (Madrid, Spain). The organophosphorus pesticides (>98% purity) chlorpyrifos-oxon (*O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphate), paraoxon methyl (*O,O*-dimethyl *O*-(4-nitrophenyl) phosphate), paraoxon ethyl (*O,O*-diethyl *O*-(4-nitrophenyl) phosphate), and the methylcarbamate insecticides (>98% purity) carbofuran (2,2-dimethyl-2,3-dihydro-1-benzofuranyl-7-methylcarbamate) and carbaryl (1-naphthyl methylcarbamate) were obtained from Dr. Ehrenstorfer (Augsburg, Germany). Substrates for CbE activity were prepared daily at a concentration of 20 mM in ethanol. Pesticide solutions (10^{-4} M) used in inhibition kinetic assays were initially made in dimethyl sulfoxide and kept at 4–5 °C; dilutions from these stock solutions were prepared in distilled water when required.

2.2. Earthworms and soils

Adult and clitellated earthworms (*Lumbricus terrestris*) were purchased from a local supplier (Poisson Fenag, Madrid, Spain) and kept in an acclimatized chamber (15 °C and darkness). Two agricultural soils were employed in this study. Soil A was collected from an abandoned agricultural land located at Montes de Toledo (Toledo, Spain), and used in a first experiment (experiment-1) aimed to examine the effect of earthworms on soil CbE activity. Particle sizes of this soil were distributed as follows: 10.7% clay, 10.8% silt, 54.5% coarse sand and 23.7% fine sand (data from three subsamples dried at 105 °C for 48 h). Table 1 summarizes the total organic carbon (TOC), pH and electrical conductivity (EC) values of this soil. Soil B was collected from a rural area close to Polán town (Toledo, Spain), and was used for examining the sensitivity of CbE activity to chlorpyrifos by soil spiking trials (experiment-2). The physicochemical properties of this soil (<2 mm) were as follows: pH = 7.8 ± 0.05, EC = 116.5 ± 9 μS cm^{-1}, TOC = 1.6 ± 0.06% and maximum water holding capacity (WHC) = 0.31 ± 0.01 g H_2O g^{-1} dry soil, particle size distribution = 7.95% clay, 11.6% silt, 62.4% coarse sand and 17.9% fine sand (n = 3 subsamples dried at 105 °C for 48 h).

2.3. Experiment-1. Earthworm-induced changes in soil carboxylesterase activity

Samples of soil A (1 kg wet weight, wetted at 50% of maximum WHC which was 0.30 ± 0.03 g H_2O g^{-1} dry soil, n = 3) were added to five plastic containers (14.5 × 14 × 12 cm) and left at 15 °C for 48 h for equilibration before earthworm inoculation. Prior to inoculation, earthworms were placed in Petri dishes over wet filter paper (at 15 °C for 48 h) for voiding their guts, and subsequently weighted (3.92 ± 0.78 g wet weight, n = 20). Four individuals were released in each container, whereas five earthworm-free containers with equivalent amounts of soil A were set as controls. All the containers were kept at 15 °C and in darkness for 12 weeks. Water losses were measured by periodically weighting the containers, and moisture was corrected by adding distilled water when needed. Unsterile cattle manure (approx. 10 g fresh weight per box) was applied to the soil surface every two weeks.

Periodically (5, 17, 53 and 89 d), earthworms were removed from each replicate and placed in Petri dishes (at 15 °C in darkness for 48 h) to collect casts and determine possible changes in earthworm weight. A soil sample (15 g) was also collected from each container. Casts and soil samples were frozen at −80 °C, a recommended temperature to prevent the loss of enzyme activities after a long storage time (Wallenius et al., 2010). Toward the end of this experiment, earthworms were killed by freezing at −80 °C and kept at this temperature until tissue collection (no later than one month). Samples (145 g wet weight/replicate) from the earthworm-treated soils were air-dried (22–23 °C) for one month, whereas another set of samples (145 g wet weight/replicate) was kept at 15 °C and constant moisture for one month. These two soil batches were used to assess the aging of soil CbE activity after the earthworm removal.

2.4. Experiment-2. Sensitivity of soil CbEs to anticholinesterase insecticides

Soil B was inoculated with *L. terrestris* (4 individuals/kg wet weight) as what was done for the experiment-1. After 2 months of earthworm activity, these soils were used for testing the interaction between CbE activity and selected OP insecticides. Treatments consisted of chlorpyrifos-, chlorpyrifos-oxon-spiked and control soils. Each replicate (n = 9/treatment) was made by placing 100 g of soil (wet weight) into a Petri dish (∅ 110 mm). Pesticides were dissolved in acetone and applied (4 ml) onto the soil surface to yield nominal concentrations of 5 mg kg^{-1} wet wt (chlorpyrifos) and 4 mg kg^{-1} (chlorpyrifos-oxon); control soils received 4 ml of acetone. The Petri dishes were placed in a fume hood for solvent evaporation (≈45 min), and two 2.5-g subsamples were immediately taken from each replicate to determine the pesticide concentrations (Supplementary methodology and Fig. S1). After 2 and 6 d of pesticide spiking, soil samples (4 g) were taken from each treatment for CbE determination.