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Short communication

Differential protein expression and localization of CYP450 enzymes in three species of earthworm; is this a reflection of environmental adaptation?

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HIGHLIGHTS

- CYP1A2, CYP2E1 and CYP3A4 proteins were detected in 3 species of earthworm.
- CYP450 protein levels varied by species and usually increased with age.
- CYP protein levels is higher in anterior parts of 2 species and posterior parts in a third.
- CYP450 proteins were often concentrated in the body wall and intestine.
- We propose CYP450 protein expression reflects the ecological niche of earthworm species.

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ABSTRACT

Cytochrome P450 (CYP450) is a hemoprotein superfamily, among which CYP1, CYP2 and CYP3 play a major role in the metabolism of vast array of xenobiotics and endobiotics. This paper reports on three CYP enzyme variants (CYP1A2, CYP2E1 and CYP3A4) in three species of earthworm (*Eisenia fetida, Metaphire guillelmi* and *Amynthas carnosus)*. The relative expression levels and localization of the three associated proteins were investigated at three life-cycle points (juvenile, sub-adult and adult), through comparison of anterior and posterior body tissue and between specific organs (body wall, intestine and reproductive tissues) using western blot analysis. This study confirmed the presence of CYP3A4, CYP1A2 and CYP2E1 in all three species of earthworm tested. The levels of expression varied with earthworm species, age, and body location. These differences in occurrence of the three CYP enzymes appeared to reflect the ecological niche (the spatial and temporal location and functional relationship of each individual or population in populations or communities), and the likelihood of contact with soil contaminants of the respective species. These results may help to explain why earthworms are capable of adapting to very different and extensively polluted soil environments and provide important data for subsequent ecotoxicology and ecological adaptability studies.

1. Introduction

In terrestrial ecosystems, earthworms play an important role in the formation and the development of soil (Lee, 1985). They can successfully adapt to various types of polluted environments, and ameliorate the contamination, thus there must be a robust detoxification capacity in their tissues and organs, to resist the harmful effects of the many xenobiotics encountered in the environments they inhabit. However, there have been very few studies to date investigating differences in protein expression in the detoxification systems of earthworms and whether there is variability linked to the habitat occupied. For example, the different burrowing habits of epigeic (shallow-burrowing) or endogeic (deep-burrowing) species may result in differences in pattern of contaminant exposure.

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Similarly, little is known about the behavioural ecology of many earthworm species, so that if juveniles and adults of a species have differing habitats or behaviours then, logically, differences in protein expression could be expected.

CYP450 enzymes have an important role in microsomal oxidase function across a broad range of taxa (Pakharukova et al., 2012). They are involved in metabolism and detoxification of both internally generated compounds and xenobiotics as the phase I metabolizing enzymes and play an important role in maintaining internal homeostasis (Anzenbacher, 2001). In some cases, however, the metabolites of CYP action on xenobiotics such as benzo(*a*)pyrene are also toxic or carcinogenic (Minacapelli et al., 2013). In the CYP super family, CYP1A2, CYP2E1 and CYP3A4 have significant roles in drug metabolism in humans and other mammals (Snyder, 2000; Huang and Yang, 2001). They are the three most studied sub-enzymes in the P450 super family, accounting for approximately 13%, 7% and 50% respectively, of the total CYP450 protein content in human liver and are found in similar abundance in many other mammals (Schumacher and Jose, 2012). Because earthworms are able to adapt to a wide range of polluted soil environments, live in direct contact with, and ingest the various pollutants, it can be hypothesized that their body tissues require a high detoxification capacity compared with many other organisms and that these three CYP enzymes would be involved in such a detoxification processes.

The expression of CYP1A2, CYP2E1 and CYP3A4 proteins has been reported for many animal species (Czerwinski et al., 2015). However, very few reports are available on CYP enzymes in earthworms. Song et al. (2015). reported using CYP3A4 enzymatic activity as the biomarker for exposure to pyrethyroids, but without quantification of CYP protein expression. Saint-Denis et al. (1999) found that methoxyresorufin-*O*-deethylase (CYP1A2) in the earthworm *E. fetida* exposed to benzo(*a*)pyrene was induced at low concentrations (0.05 and 1 mg kg^{-1} soil) but inhibited at high concentrations (100 and 1000 mg kg^{-1} soil). At the time of writing we are unaware of any studies reporting specifically on the expression of 2E1 in earthworms. Current knowledge of CYP450 enzymatic activity in earthworms is largely derived indirectly from detoxification studies with specific compounds likely to accumulate chemical residues in the environment (Song et al., 2015; Berghout et al., 1991; Achazi et al., 1997; Li et al., 2007). Given the increasing human impact on the environment, the importance of CYP450 enzymes for detoxification and the role of earthworms in maintaining soil health, it is important to have more specific information about CYP450 protein expression and regulation in earthworms.

In this study we investigated three earthworm species commonly found in the Shanghai area; the epigeic *Amynthas carnosus*, the endogeic *Metaphire guillelmi* and the widely studied composting earthworm *Eisenia fetida*. The expression of CYP1A2, CYP2E1 and CYP3A4 proteins was investigated at different developmental stages and in different body locations (anterior and posterior body regions as well as relative quantification between earthworm body wall, reproductive, and intestinal tissues), in each of the 3 species studied.

2. Materials and methods

To obtain earthworm specimens, we identified 5 sites in Shanghai which were previously farmed (Soil physical and chemical properties: pH:7.5, EC 129.5 μS cm^{-1}, organic matter: 32.1 g kg^{-1}, total P: 0.958 g kg^{-1}, total N: 1.85 g kg^{-1}, total K: 2.53 g kg^{-1}), but where farming ceased more than five years ago, and chose the two most frequently encountered species: *A. carnosus* and *M. guillelmi* as the test species. *E. fetida*, is the most widely used species for commercial breeding and ecotoxicity testing and so this species was also selected for inclusion in this study and purchased from a local commercial supplier. The specimens collected included adults, sub-adults and juveniles. The worms were depurated by placing on filter paper soaked with isotonic salt solution at 22 ± 1 °C for 2 d prior to the experiments (Wang et al., 2010). All subsequent operations were conducted at temperatures below 4 °C.

Depurated adult, sub-adult and juvenile earthworms were each used to make microsomal fractions to verify the expression of CYP1A2, CYP2E1 and CYP3A4 at different developmental stages of the earthworm. Between 1 and 1.5 g earthworm tissue (2–5 individual earthworms) were used for each treatment. In a second investigation, adult earthworms were cut transversely just behind the clitellum into anterior and posterior parts to identify expression of CYP1A2, CYP2E1 and CYP3A4 in the respective body regions. In this way, two samples of anterior and posterior were obtained and homogenized to prepare microsomal fractions. Each treatment had three replications.

The body wall, intestine, seminal receptacle and seminal vesicle in the adult worms were also isolated and homogenized to make microsomal fractions to investigate the relative expression of CYP1A2, CYP2E1 and CYP3A4 in these different organs. In order to get sufficient mass, about 20 individual adult worms were dissected. There were three replicates for each treatment. The clitellum of *E. fetida* (0.497 g ± 0.046) is located at body segments xxv–xxxiii, and the gizzard, seminal receptacles and seminal vesicles are located posterior to the clitellum. The clitella of *A. carnosus* (1.563 g ± 0.035) and *M. guillelmi* (1.604 g ± 0.022) are located at segments xiv–xvi, and the gizzard, seminal receptacles, seminal vesicles, testes, prostate and appendix are located posterior to the clitellum (Chen, 1959). There are two pairs of seminal receptacles in *E. fetida* located at segments ix–x or x-xi and four pairs of seminal vesicles located at ix–xii. There are three or four pairs of receptacles in *A. carnosus* located at segments v/vi–viii/ix, three pairs of seminal receptacles in *M. guillelmi* located at segments vi/vii–viii/ix, and three or four pairs of seminal vesicles in *A. carnosus* and *M. guillelmi* located at segments v/vi–viii/ix (Chen, 1956, 1959).

Microsomal fractions of earthworm tissue were prepared as per a previous study with modifications (Zhang et al., 2006). Briefly, the earthworm tissue samples transferred into cold 0.15 mol l^{-1} KCl solution and were cut into shreds. The remaining solid tissue homogenized for 30 min at 8000 $r.min^{-1}$. The supernatant centrifuged at 14,700 $r.min^{-1}$ for 30 min and then the supernatant ultracentrifuged at 46,500 $r.min^{-1}$ for 90 min. After dissolving precipitation, the mixed liquor was centrifugal at 38,000 $r.min^{-1}$ for 45 min at 4 °C. Finally, the supernatant was collected and stored at −80 °C. The protein levels of CYP1A2, CYP2E1 and CYP3A4 in the microsomes were measured by Western blot. Rabbit polyclonal antibody anti-human, rat, mouse CYP 1A2, CYP2E1 and CYP3A4; mouse monoclonal anti-β-actin antibody; horseradish peroxidase labelled goat anti-rabbit IgG, goat anti-mouse IgG were used. The relative fluorescence intensity (RFI) of the three CYPs compared to β-actin were used to assess relative protein expression level. Statistical analyses were performed with SPSS Software Version 17.0 (SPSS Inc, Chicago, USA) and the data were expressed as the mean ± SD.

3. Results

3.1. Expression of CYP1A2, CYP2E1 and CYP3A4 at different developmental stages

Western blots showed that all three CYPs were present in all three developmental stages of the three earthworm species investigated, with expression generally highest in adults and