Comparative Biochemistry and Physiology, Part C 160 (2014) 42–53

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

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

Zonation in the digestive tract of *Eisenia fetida*: Implications in biomarker measurements for toxicity assessment

A. Irizar, U. Izagirre, O. Diaz de Cerio, I. Marigómez, M. Soto *

Cell Biology & Environmental Toxicology Research Group, Research Centre for Experimental Marine Biology & Biotechnology (PIE) & Zoology & Animal Cell Biology Department (Faculty of Science & Technology), University of the Basque Country, P.O. Box 644, E-48080 Bilbo, Basque Country, Spain

ARTICLE INFO

Article history:
Received 1 October 2013
Received in revised form 22 November 2013
Accepted 23 November 2013
Available online 1 December 2013

Keywords:
Zonation
Digestive tract
Lysosomal markers
Molecular biomarkers
Toxicity
Eisenia fetida

ABSTRACT

Eisenia fetida is a model species for soil health assessment and different biomarkers that detect either the presence of bioavailable contaminants or their biological effect have been developed. These parameters are performed in a target tissue or whole earthworm, without considering the marked zonation in histological organisation, enzyme activities and gene expression pattern existing along the body. Thus, the present work was aimed at (a) characterising the morphofunctional heterogeneity along the digestive tract of *E. fetida* in tissue morphology and turnover, lysosomal enzyme markers (β-glucuronidase, β-GUS; hexosaminidase, HEX), lipofuscin contents (LPF) and metallothionein (MT) and catalase (CAT) gene expression; and (b) determining whether the responsiveness to Cd exposure varies among tissues and along the digestive tract. HEX and β-GUS exhibited a heterogeneous distribution pattern along and across the digestive tract and Cd exposure caused a marked decrease of HEX and an increase of β-GUS activity. Likewise, the significant decrease of cell turnover and the induction of MT transcription were was zone-dependent. Therefore, in was concluded that the consideration of the zonation when applying biomarker for toxicity assessment would reduce the intrinsic variability that results from overlooking the marked morphofunctional heterogeneity that exists in annelids along their body axis.

1. Introduction

Eisenia fetida is widely used in soil toxicity assessment due to its sensitivity to a wide range of pollutants and because it is easy to handle and cultivate in laboratory conditions. Overall, earthworms have a great impact on decomposition activity, nutrient mineralization and primary production and can perceive a diverse range of chemical stresses and respond through certain reactions accordingly (Lukkari et al., 2005). Among the biological responses to soil pollutants, biomarkers are those measurable functional responses that are elicited at the subindividual level of biological organisation within a relatively short time (Ricketts et al., 2004). Thus, they are claimed to be used as early warning tools in environmental monitoring and risk assessment. A variety of biomarkers is currently available in earthworms, and particularly in *E. fetida*, suitable to be used for soil toxicity assessment and health status screening (Scott-Fordsmand and Weeks, 2000; Di Marzio et al., 2005; Brulle et al., 2006; Asensio et al., 2007; Plytycz et al., 2009; Chen et al., 2011; Asensio et al., 2013). It has been proposed that a suite of biomarkers rather than a selective choice in *E. fetida* could serve as a sensitive tool for use in soil contamination surveys (Saint-Denis et al., 1999; Yang et al., 2012b; Asensio et al., 2013). Likewise, during the last decade, the analysis of gene expression profiles has contributed to understand the mechanisms of toxic action of chemical pollutants as well as to identify toxicity profiles in earthworms (Brulle et al., 2011; Mo et al., 2012; Asensio et al., 2013).

Most of these investigations deal with the analysis of a target tissue (muscle, chloragogenous tissue, tegument) or the whole earthworm, without considering that, in annelids, there exists a marked zonation along the body, with regions with different histological organisations, enzyme activities and responsiveness against pollutants (Morgan and Morgan, 1990; Ferreira-Cravo et al., 2009; Fernández et al., 2012; Gao et al., 2013). Basically, the digestive tract is morphologically and functionally divided in different regions (foregut, midgut and hindgut). Evidences of such morphofunctional zonation are available since very early reports. Needham (1962) found that the wet weight per unit length of the gut and body wall is smoothly graded along the body of earthworms. This gradient varies in detail with the tissue and with dietary regime. Also, the bacterial communities and their metabolic activities are heterogeneously distributed along the gut (Jolly et al., 1993; Lattaud et al., 1997; Garvín et al., 2000; Mendez et al., 2003). As regards zonation in the distribution of enzyme activities along the body, arginase activity in *Lumbricus* sp. and in *Eisenia* sp. is maximal in the anterior hindgut and declines progressively towards the crop–gizzard region, being inversely related to the amount of chloragogen tissue (Needham, 1962). Carboxylesterase activity is the highest in gizzard/crop and the lowest in pharynx (Sánchez-Hernández and Wheelock, 2009; Sánchez-Hernández et al., 2009). Heterogeneous antioxidant enzyme

* Corresponding author.
E-mail address: manu.soto@ehu.es (M. Soto).

1532-0456/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.cbpc.2013.11.006

distribution and activities have also been reported in annelids (da Rosa et al., 2005; Ferreira-Cravo et al., 2009). The gene expression pattern also varies considerably in the three earthworm regions, as well as its responsiveness to pollutant exposure. For instance, the midgut is more sensitive to the veterinary drug Albendazole than the foregut and the hindgut for the expression of target genes such as l-rRNA and HSP90 (Gao et al., 2013). It has been therefore recommended that the responses to pollutants of target genes must be focused to different regions of the digestive tract in order to provide reliable information for soil toxicity assessment (Gao et al., 2013).

Thus, the present work was aimed at (a) characterising the morphofunctional heterogeneity along the digestive tract of *E. fetida* in tissue morphology and turnover, lysosomal enzyme markers (β-glucuronidase, β-GUS; Hexosaminidase, HEX), lipofuscin contents (LPF) and metallothionein (MT) and catalase (CAT; EC 1.11.1.6) gene expression; and (b) determining whether the responsiveness to Cd exposure varies among tissues and along the digestive tract from the midgut to the hindgut.

Cadmium was selected as a model pollutant because it is globally relevant as soil pollutant and highly toxic (Alloway, 1990). Cadmium concentration in non-polluted agricultural soils in the USA ranges from 0.005 to 2.4 mg/kg (mean value: 0.27 mg/kg; median value: 0.20 mg/kg; Holmgren et al., 1993); whilst in contaminated soils in China it ranges from 1.09 to 27.9 mg/kg (Wang, 1997). Moreover, Cd is accumulated in earthworm tissues, although at long exposure times a certain metabolisation has been reported (Asensio et al., 2007; Sandrini et al., 2008; Yang et al., 2012a).

Metallothionein induction is a renowned biomarker of exposure to metals, as demonstrated in a wide range of aquatic and terrestrial organisms including earthworms (Cajaraville et al., 2000; Brulle et al., 2006; Zorita et al., 2007; Asensio et al., 2007). Metallothionein expression is induced in earthworms on exposure to Cd, even at a concentration as low as 0.6 mg Cd/kg soil dry wt (Brulle et al., 2006; Asensio et al., 2007; Bernard et al., 2010; Mo et al., 2012). Thus, MT induction in *E. fetida* has been proposed as a potential effective biomarker of exposure to Cd in soils (Brulle et al., 2006; Mo et al., 2012). Interestingly, MTs have been isolated and fully characterised in *E. fetida* (Gruber et al., 2000). Metallothioneins show dose- and time-dependent increases in protein and the number of transcripts coding MT when *E. fetida* is exposed to metals, especially Cd (Gruber et al., 2000; Brulle et al., 2006; Asensio et al., 2007; Demuynck et al., 2007). Cd-*mt* expression has been studied in earthworms exposed to spiked soils (Galay-Burgos et al., 2005; Brulle et al., 2006, 2007) and earthworms exposed to polluted field soils (Spurgeon et al., 2005; Svendsen et al., 2007; Bernard et al., 2010; Brulle et al., 2011; Asensio et al., 2013).

Earthworm digestive tract consists of gut epithelium, chloragogenous tissue and muscle. The chloragogenous tissue, composed by chloragocytes, is located between the gut epithelium and the coelom and constitutes a main site for metal accumulation and elimination (Cancio et al., 1995; Morgan et al., 2002; Giovanetti et al., 2010). Metals can be also accumulated in the gut epithelium (Morgan et al., 2002), which experiences remarkable morphological alterations as a result of pollutant insult (Amaral et al., 2006; Lourenço et al., 2011). Therefore, histopathological alterations in gut epithelium and chloragogenous tissue in earthworms are valuable markers of soil pollutant toxicity (Amaral et al., 2006; Giovanetti et al., 2010; Kiliç, 2011; Lourenço et al., 2011). Exposure to soil pollutants results in the loss of structural integrity and necrosis of the chloragogenous tissue and in the atrophy of the gut epithelium accompanied by fusion of the villi, enhanced mucus secretion and disorganisation of the associated circular and longitudinal muscles (Amaral et al., 2006; Kiliç, 2011; Lourenço et al., 2011). Moreover, pollutants may cause DNA damage (Di Marzio et al., 2005; Fourie et al., 2007; Sforzini et al., 2011) and/or alteration in the cell cycle (Bjerrengaard, 2007), which would result in impairment of tissue renewal. Alterations in cell renewal in the digestive epithelium of molluscs exposed to Cd have been determined by means of Bromodeoxiuridine (BrdU) immunohistochemistry (Zaldibar et al., 2007). This technique has been applied in a previous study to the reproductive tissue of *E. fetida* (Espinoza-Navarro and Bustos-Obregon, 2005).

Lysosomal enlargement and membrane destabilisation constitute general early responses to stressors in different organisms (Marigómez and Baybay-Villacorta, 2003; Izagirre and Marigómez, 2009). These lysosomal responses are usually quantified after histochemical demonstration of lysosomal marker enzymes such as β-GUS, Hex and acid phosphatase (AcP; Izagirre and Marigómez, 2009). In *E. fetida*, studies on lysosomal biomarkers in the digestive tract are almost lacking albeit there exists evidence of changes in AcP activity in chloragocytes after exposure to metals (Cancio et al., 1995). About 10% of the gut AcP resides in chloragocytes, both in lysosomes and a small proportion of the chloragosomes (Prentø, 1987; Cancio et al., 1995). β-GUS and esterase activities have also been demonstrated in chloragosomes (Varute and More, 1973).

Changes in antioxidant enzyme activities in response to pollutants in soils are extensively reported (Feret et al., 2003; Li, 2003; Brulle et al., 2006; Chen et al., 2011). For instance, in *E. fetida* Cd exposure results in excess production of reactive oxygen species (ROS) and then cells protect themselves against ROS damage by the action of antioxidant enzymes such as superoxide dismutase and CAT (Ribera et al., 2001; Yang et al., 2012a,b). Catalase is ubiquitous in archaea, prokaryotes, and eukaryotes (Xiong et al., 2013). In earthworms, CAT activity is partitioned between the chloragocyte's cytosol (60–70%) and the gut epithelium peroxisomes (30–40%) (Prentø, 1987). In the absence of environmental insult, one main function of the chloragocyte CAT is scavenging for H_2O_2 arising from the interaction between blood heme–protein and oxygen (Prentø, 1987) but on exposure to pollutants CAT activity is enhanced to protect against augmented ROS levels (Xiong et al., 2013).

2. Material and methods

2.1. Experimental soils

Two different soils were used: (a) a well known agricultural soil obtained from NEIKER, Basque Centre for Agricultural Research; and (b) OECD soil (OECD 1984, test N 207; sand 70%, kaolin clay 20%, sphagnum, peat 10%). The physico-chemical characteristics of NEIKER soil are given in Table 1. Cadmium was added as $CdCl_2$ dissolved in $_dH_2O$ to both soils in order to obtain a 40% WHC (determined as specified in ISO 11274) and concentrations of 5 and 25 mg Cd/kg soil wet wt in the OECD soil and 100 mg Cd/kg soil dry wt in NEIKER soil. The soils were stabilised for ~45 days, sealed and maintained at 4 °C.

2.2. Earthworms

E. fetida earthworms used for the experiments were obtained from the stock population provided by a commercial dealer (Mancha Verde, Ciudad Real, Spain) and maintained in the laboratory under controlled

Table 1
Physico-chemical characterisation of the agricultural soil.

Agricultural soil			
pH	6.9	K	454 mg/L
Electric conductivity	2520 mS/cm	Ca	4460 mg/L
Organic matter	14.35%	Mg	586 mg/L
N	0.64%	Cr	29.9 mg/kg
C/N	13	Cd	1.05 mg/kg
Structure	Lime and clay	Cu	48.3 mg/kg
Nitrates	95.3 mg/L	Ni	10.9 mg/kg
Phospates	>120 mg/L	Zn	176 mg/kg
Na	38 mg/L	Pb	46.5 mg/kg