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Tissue distribution, isozyme abundance and sensitivity to chlorpyrifos-oxon of carboxylesterases in the earthworm *Lumbricus terrestris*

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The measurement of carboxylesterase inhibition in earthworm is a sensitive and complementary biomarker of pesticide exposure.

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ABSTRACT

A laboratory-based study was conducted to determine the basal carboxylesterase (CbE) activity in different tissues of the earthworm *Lumbricus terrestris*, and its sensitivity to the organophosphate (OP) pesticide chlorpyrifos-oxon (CPx). Carboxylesterase activity was found in the pharynx, crop, gizzard, anterior intestine, wall muscle and reproductive tissues of *L. terrestris*, and multiple tissue-specific isozymes were observed by native gel electrophoresis. Esterase activity and sensitivity to CPx inhibition varied on a tissue- and substrate-specific basis, suggesting isoforms-specific selectivity to OP-mediated inhibition. Three practical issues are recommended for the use of earthworm CbE activity as a biomarker of pesticide exposure: (i) CbE should be measured using several routine substrates, (ii) it should be determined in selected tissues instead of whole organism homogenate, and (iii) earthworm CbE activity should be used in conjuncture with other common biomarkers (e.g., ChE) within a multibiomarker approach to assess field exposure of OPs, and potentially other agrochemicals.

1. Introduction

Carboxylesterases (CbEs; EC 3.1.1.1) are hydrolases that cleave carboxyl esters to yield the corresponding alcohol and carboxylic acid (Sogorb and Vilanova, 2002; Wheelock et al., 2005a,b). These enzymes participate in the detoxification of pyrethroid (PYD), carbamate (CB) and some organophosphorus (OP) insecticides. Inhibition of CbE activity by OP and CB pesticides has been used as a biomarker of pesticide exposure in many aquatic and terrestrial organisms (Wheelock et al., 2008). Laboratory and field studies have shown that CbE activity can be more sensitive than cholinesterase (ChE) activity to OP-mediated inhibition (Wogram et al., 2001; Fourcy et al., 2002; Galloway et al., 2002), which is the most common biomarker of OP and CB exposure. Multiple ChEs with varying sensitivity to pesticide inhibition are frequently found in the same tissue, potentially leading to contraindicative data if total activity is used (Stenersen, 1980; Bocquené et al., 1997; Aamodt et al., 2007). It is therefore recommended that the tissue distribution and biochemical activity of ChE are characterized before use as a biomarker of pesticide exposure. It has been suggested that similar concerns exist for CbE activity because multiple isozymes are present in the same tissue (Satoh and Hosokawa, 1998). In earthworms, ChE activity has been enzymologically characterized using specific ChE inhibitors and substrates (Stenersen, 1980; Caselli et al., 2006; Rault et al., 2007); however, less attention has been placed on the characterization of CbE activity and its potential biomarker applications in these terrestrial invertebrates.

The importance of developing new biomarkers in earthworms has been previously stressed as well as the need to validate those biomarkers, such as CbEs, that are commonly used in other species (Scott-Fordsmand and Weeks, 2000; van Gestel and Weeks, 2004; Sanchez-Hernandez, 2006; Rodriguez-Castellanos and Sanchez-Hernandez, 2007). To the best of our knowledge, only three studies have investigated CbE activity in the earthworms *Eisenia fetida* (Øien and Stenersen, 1984; Oneto et al., 2005) and *Lumbricus terrestris* (Haites et al., 1972). Similar to mammals, multiple CbE isoforms have been identified in several tissues of *L. terrestris* (Haites et al., 1972). Øien and Stenersen (1984) found four and three esterase isozymes in *E. unicolor* and *E. fetida*, respectively. These esterases were identified as CbEs because they were fully inhibited by paraoxon and evidenced high activity towards α- and β-naphthyl acetate. Earthworm CbE activity appears to be equally sensitive as ChE activity to OP inhibition (Oneto et al., 2005), which would justify its use in a multibiomarker approach for assessing field exposure to OPs. Despite these studies, earthworm CbE activity has not been as extensively investigated as ChE activity. Several practical and toxicological aspects still need to be explored to determine the appropriate applications of earthworm CbE activity in assessing field exposure to anti-ChE pesticides, including the

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impact of confounding environmental and biological variables on basal CbE activity, isozyme sensitivity to OP inhibition (as well as other xenobiotics), and assay format for enzyme activity measurements.

The purpose of this study was to increase our knowledge of CbE activity in the earthworm *L. terrestris* and evaluate its use as a complementary biomarker of anti-ChE pesticide exposure. Towards this end, we examined the tissue distribution of total CbE activity using three model substrates (α-naphthyl acetate, 4-nitrophenyl valerate and 4-nitrophenyl acetate), the variations of enzyme activity levels with sexual maturation (presence or absence of clitellum), the long-term stability of CbE activity, the tissue-specific differences in CbE isozyme abundance, and the sensitivity of CbEs to chlorpyrifos-oxon (CPx) inhibition. These data expand our knowledge of CbE tissue distribution and activity as well as provide useful information on the suitability of using earthworm CbE activity as a biomarker of exposure to agrochemicals.

2. Materials and methods

2.1. Reagents

The chemicals for CbE activity assays, α-naphthyl acetate (α-NA), 4-nitrophenyl acetate (4-NPA), 4-nitrophenyl valerate (4-NPV) and 4-nitrophenol, were purchased from Sigma–Aldrich (Madrid, Spain). The OP pesticide (>98% purity) chlorpyrifos-oxon (*O*,*O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphate) was obtained from Dr. Ehrenstorfer (Augsburg, Germany), whereas its metabolite 3,5,6-trichloro-2-pyridinol (TCP) was supplied from ChemService (West Chester, PA, USA). Acetylthiocholine iodide (AcSCh), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) were supplied by Scharlab (Barcelona, Spain). Porcine liver esterase was purchased from Sigma–Aldrich (catalog no. E-E3019-20KU, 27 U mg^{-1} solid, lot no. 026K7029). All other chemicals were obtained from commercially available sources and used as provided.

2.2. Earthworms and tissue dissection

Adult earthworms (*L. terrestris*) were obtained from a local commercial supplier (Armeria20, Toledo, Spain), who imported them from a commercial vermiculture supplier (Vivastic, Elsenheim, France). Earthworms were delivered by post (~4 °C, transportation temperature) to the local commercial supplier and kept there at 10 °C until they were transported to the laboratory (not more than 1 day). In the laboratory, earthworms were kept under continuous dark, at 12 °C and in natural non-contaminated soils (pH = 7.58 ± 0.05, organic matter = 2.03 ± 0.32% and conductivity = 218 ± 41.2 μs cm^{-1}; mean and standard deviation of three soil subsamples) for a month. When experiment was initiated, the worms were left on wet filter paper in Petri dishes for 48 h to void the gut contents and the weight was then recorded. The earthworms were divided into two groups composed of 20 individuals each; one group comprised of well-developed clitellated (fresh weight = 3.77 ± 0.53 g, mean and standard deviation) and the other was composed of non-clitellated individuals (3.11 ± 0.79 g). Earthworms were placed in Petri dishes and kept at 4–5 °C for 15 min prior to dissection to relax the muscles, and there were killed by the dissection process. This was performed on the dorsal side from the clitellum towards the mouth, with the following tissues and organs dissected: seminal vesicles, seminal receptacles, pharynx, crop, gizzard, anterior intestine and wall muscle (Fig. 1). The anterior intestine was invested with chlorogogen tissue. Intestinal tissues were rinsed with deionized water to eliminate any soil particles and all tissues were weighed and kept on ice.

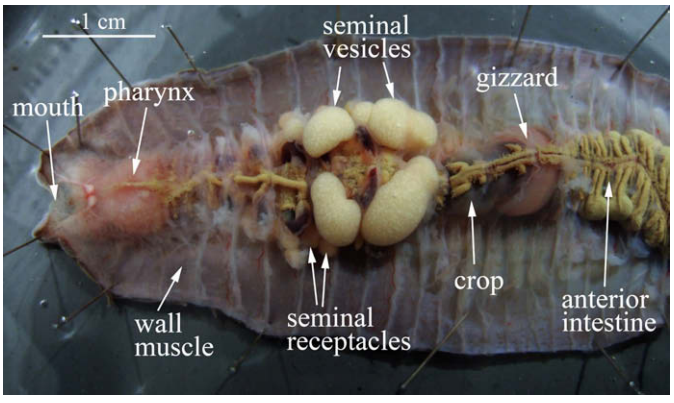

Fig. 1. Dissection of *Lumbricus terrestris* showing the tissues and organs used for carboxylesterase analysis.

Weighed samples were added (1:5 w/v ratio for seminal receptacles and 1:10 w/v ratio for the other tissues) to ice-cold 25 mM Tris–HCl buffer (pH = 8.0) containing 0.1% Triton X-100. Samples were homogenized using either a glass–Teflon Potter–Elvehjem homogenizer or a conical hand-held homogenizer connected to a bench-top overhead stirrer (seminal receptacles). The homogenate was then centrifuged at 9000×*g* at 4 °C for 10 min to obtain the post-mitochondrial fraction. Mean (±standard deviation) total proteins of clitellated earthworms ($n = 20$ individuals) were 4.16 ± 1.00 (pharynx), 4.59 ± 1.15 (crop), 4.60 ± 1.13 (gizzard), 5.32 ± 1.71 (anterior intestine), 8.45 ± 1.63 (wall muscle), 4.76 ± 1.00 (seminal vesicles) and 1.29 ± 1.08 mg ml^{-1} (seminal receptacles), whereas the mean total proteins in the extracts of non-clitellated earthworms ($n = 20$) were 4.60 ± 1.52 (pharynx), 3.93 ± 1.66 (crop), 3.62 ± 1.01 (gizzard), 4.25 ± 1.48 (anterior intestine), 6.93 ± 3.49 (wall muscle), 3.69 ± 1.41 (seminal vesicles) and 1.02 ± 0.74 mg ml^{-1} (seminal receptacles). The supernatant S9-fraction was stored at −80 °C until use. The homogenates of the dissected tissues from a minimum of 20 clitellated earthworms were pooled and used for the in vitro inhibition assays, to determine the effect of enzyme concentration and stability, to measure the basal levels of pesticide-metabolising enzymes and in the electrophoretic study.

2.3. Carboxylesterase activity assays

Carboxylesterase activity was assayed spectrophotometrically (Jenway 6400 Spectrophotometer, Barloword Scientific, Essex, UK) using the substrates α-NA, 4-NPA and 4-NPV, and specific enzyme activity was expressed as nmol min^{-1} mg^{-1} of total protein. Total protein was quantified by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

Carboxylesterase activity using α-NA was determined according to Gomori (1953), as adapted by Bunyan and Jennings (1968). The reaction medium (final volume [F.V.] = 1950 μl) contained 25 mM Tris–HCl, 1 mM $CaCl_2$ (pH = 7.6) and the sample. The reaction was initiated by addition of 50 μl α-NA (1.04 mg ml^{-1} in acetone) after a preincubation period of 5 min at 25 °C. The formation of naphthol was stopped after 10 min of incubation by the addition of 500 μl 2.5% SDS and subsequently 500 μl 0.1% Fast Red ITR in 2.5% Triton X-100 in water. The solutions were allowed to stand for 30 min at room temperature in the dark, and the absorbance of the naphthol–Fast Red ITR complex was read at 530 nm (molar extinction coefficient of 33.225 × 10^3 M^{-1} cm^{-1}). Hydrolysis of 4-NPV by CbE was determined as described by Carr and Chambers (1991). Samples were preincubated in 50 mM Tris–HCl (pH = 7.5) for 5 min at 25 °C (F.V. = 1980 μl), and the reaction was initiated by the addition of 20 μl 4-NPV (5 × 10^{-4} M, final concentration). After 15 min, the reaction was stopped by the addition of a solution 2% (w/v) SDS and 2% (w/v) Tris base. The 4-nitrophenol liberated was read at 405 nm and quantified by a calibration curve (5–100 μM). Carboxylesterase activity assay using 4-NPA followed the method by Chanda et al. (1997). The incubation mixture contained 20 mM Tris–HCl (pH = 8.0), 1 mM EDTA and the sample (F.V. = 1980 μl). The reaction was initiated by the addition of 20 μl 4-NPA (5 × 10^{-4} M, final concentration), and the formation of 4-nitrophenolate was monitored for 1 min at 405 nm and quantified using the 4-nitrophenol standard curve. Blanks (reaction mixture free of sample) were periodically checked for non-enzymatic hydrolysis of 4-NPA.

2.4. Effect of enzyme concentration and stability on enzyme activity

Direct proportionality between sample volume (enzyme concentration) and initial velocity of the reaction was tested for all tissues. Increasing volumes of the supernatant S9-fractions were separately assayed using the three substrates under the corresponding conditions for measuring CbE activities. Kinetics were carried out in duplicate at 25 °C. This experiment enabled the optimization of the homogenate sample volume for subsequent CbE activity determinations.

The stability of CbE activity at 25 °C was also tested over 24 h to evaluate eventual loss of enzyme activity during the 30-min period of inhibition experiments in the presence of CPx. Tissue homogenate was incubated in an agitation bath at 25 °C for 24 h, and periodically (0, 0.5, 1, 2, 3, 6 and 24 h) an aliquot was assayed for CbE activity using the three selected substrates.

2.5. Native gel electrophoresis

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was performed on a Bio-Rad Tetra Cell Electrophoresis Unit (Bio-Rad, USA). Supernatant S9-fractions (10 μl) were loaded on 4% stacking and 12.5% resolving 0.75 mm polyacrylamide gel (25 mM Tris, 192 mM glycine as running buffer), and electrophoresed at a constant voltage of 30 V for 30 min and then 150 V until bromophenol blue tracking dye reached the bottom of the gel. Carboxylesterase bands were visualized by incubation (30 min at room temperature) of the gels with a staining solution containing 100 mM Na-phosphate buffer (pH = 7.4), 0.5 mg ml^{-1} α-NA and 0.025 g of Fast Blue RR salt, which was prepared and filtered immediately before use. Stained gels were scanned using the Kodak Digital Science 1D Image Analysis Software, version 2.0.3 (Rochester, NY, USA). The sensitivity of CbE isozymes to CPx inhibition was assessed by incubation of the gel, post-electrophoresis, with 5 × 10^{-7} M of CPx and subsequent staining for CbE activity after washing with 100 mM Na-phosphate buffer.

2.6. In vitro inhibition of carboxylesterase activity

Aliquots of each tissue extract were incubated in the corresponding reaction medium for each CbE activity in the absence of substrate and spiked with CPx. The OP concentration ranged from 10^{-15} to 10^{-5} M depending on the tissue. Stock solutions (100 mM) of CPx were initially dissolved in dimethyl sulfoxide, and the final concentration of the solvent in the reaction medium was kept below 0.1%, which had no effect on CbE activity. After 30 min of inhibition at 25 °C, the enzymatic reaction was initiated by addition of the substrate (α-NA, 4-NPA or 4-NPA). All inhibition kinetics were carried out in at least duplicate. To obtain comparable IC50 values among tissues, the incubation conditions (temperature, time, pH and substrate concentration) were kept equal for all assays. However, we examined the effects of extrinsic factors on CbE sensitivity to CPx inhibition other than tissue-specific differences. Thus, the effect of the amount of tissue used in the incubation medium on the inhibition curve profile was investigated.

2.7. Glutathione S-transferase, cholinesterase and phosphotriesterases activities

Levels of glutathione S-transferase (GST, EC 2.5.1.18), phosphotriesterase (PTE, EC 3.1.8.1) and ChE (EC 3.1.1) activity were also determined as potential confounding factors in the inhibition CbE curves. Glutathione S-transferase activity was determined by the method described by Habig et al. (1974). The enzyme kinetics were read at 340 nm in a reaction mixture containing 100 mM Na-phosphate buffer (pH = 6.5), 2 mM CDNB, 5 mM GSH and the sample (10 μl). Enzyme activity was expressed as nmol min^{-1} mg^{-1} of total protein using a molar extinction coefficient of 9.6×10^3 M^{-1} cm^{-1}. Cholinesterase (EC 3.1.1) activity was determined in a reaction medium that contained 25 mM Tris–HCl (pH = 7.6) supplemented with 1 mM $CaCl_2$, 0.3 mM DTNB, 2 mM AcSCh and 10 μl of earthworm homogenate (Ellman et al., 1961). The variations in optical density were recorded at 412 nm for 1 min, and ChE activity was expressed as nmol of AcSCh hydrolyzed min^{-1} mg^{-1} of total protein using a molar extinction coefficient of 14.15×10^3 M^{-1} cm^{-1}. All kinetics were carried out in triplicate at 25 °C and blanks (reaction mixture free of sample) were periodically checked for non-enzymatic hydrolysis of substrates. Phosphotriesterase activity was evaluated by the hydrolysis of CPx to yield TCP and diethylphosphate. The continuous kinetic assay was performed according to methods described by Mortensen et al. (1996). The reaction medium contained 100 mM Tris–HCl (pH = 8.5), 2 mM $CaCl_2$ and 20 μl of earthworm extract. After 15 min of incubation, the reaction was initiated by addition of CPx (1 mM, final concentration) and the appearance of TCP was monitored for 30 min at 5-min intervals using a Spectronic Genesis-5 spectrophotometer (Spectronic Instruments, Rochester, NY, USA) set at 310 nm. Non-enzymatic hydrolysis of CPx was checked with blanks without the sample. As earthworm PTEs are strongly inhibited by EGTA (Lee et al., 2001), hydrolysis of CPx by esterases other than PTEs was also checked by substitution of $CaCl_2$ by 1 mM EGTA in the reaction mixture, and no substrate hydrolysis was observed under these conditions. Phosphotriesterase activity was quantified using a TCP standard curve (10–100 nmol ml^{-1}).

2.8. Statistical analysis

Significant differences in the CbE activity among tissues were tested using the Kruskal–Wallis ANOVA test, whereas differences in CbE activity between clitellated and non-clitellated earthworms were tested by the Mann–Whitney *U* test. The percent of CbE inhibition was plotted against the logarithmic transformed molar concentration of CPx, and the concentration of pesticide causing a 50% reduction in enzyme velocity (IC50) was calculated for each tissue and CbE activity. The inhibition curves were fitted using a non-linear method based on the algorithm of Marquardt–Levenberg (SigmaPlot Program, version 9.01, Systat Software, Point Richmond, CA, USA) and the best fits were found with the logistic model $y = y_0 + a/(1 + (x/x_0)^b)$ and the third order logarithmic model $y = y_0 + a \ln x + b (\ln x)^2 + c (\ln x)^3$. A level of probability less than 0.01 was taken as statistically significant.

3. Results

3.1. Tissue distribution of CbEs

The effect of sample volume (enzyme/protein concentration) was first evaluated for each tissue. The reaction rate of CbE activity was directly proportional to a sample volume range from 5 to 80 μl, irrespective of the substrate or tissue (data not shown), with linear correlation coefficients of 0.993–0.999. Selected volumes for subsequent CbE determinations ranged from 20 μl for seminal receptacles to 40 μl for intestinal tract tissues in a 1-ml final volume of mixture reaction.

Carboxylesterase activity was found in all tissues analyzed, although marked variations in activity were observed on a tissue- and substrate-specific basis (Fig. 2). Significant differences in CbE activity using α-NA were detected among the tissues of both clitellated ($H_{6,151} = 95.32$, $P < 0.0001$, Kruskal–Wallis ANOVA test) and non-clitellated earthworms ($H_{6,169} = 95.73$, $P < 0.0001$). The highest CbE activity using α-NA was found in the gizzard (Fig. 2A). Similarly, CbE activity towards 4-NPV was significantly different among the tissues of clitellated ($H_{6,155} = 105.13$, $P < 0.0001$) and non-clitellated ($H_{6,182} = 128.46$, $P < 0.0001$) earthworms. Contrary

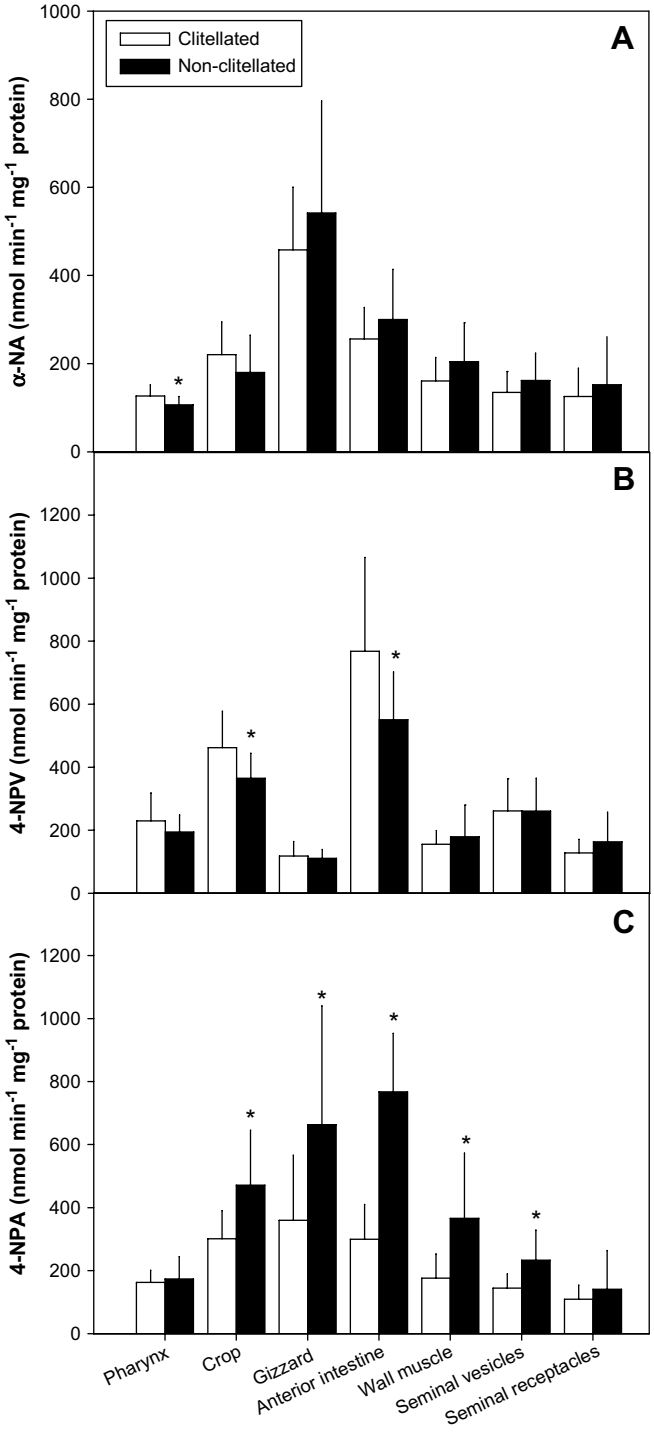

Fig. 2. Basal levels (mean ± standard deviation) of carboxylesterase (CbE) activity in different tissues of clitellated ($n = 20$) and non-clitellated ($n = 20$) adults of *Lumbricus terrestris*. Activity was measured with (A) α-naphthyl acetate (α-NA), (B) 4-nitrophenyl valerate (4-NPV) and (C) 4-nitrophenyl acetate (4-NPA). Asterisks indicate significant differences in CbE activity between clitellated and non-clitellated earthworms (Mann–Withney *U* non-parametric test, $P < 0.01$).

to CbE activity using α-NA, the gizzard showed the lowest esterase activity towards 4-NPV, whereas the highest CbE activity using this substrate was found in the anterior intestine (Fig. 2B). When 4-NPA was employed to assay CbE activity, statistically significant differences were also observed among the tissues of both clitellated ($H_{6,184} = 94.38$, $P < 0.0001$) and non-clitellated ($H_{6,172} = 119.41$, $P < 0.0001$) individuals. The highest CbE activity using 4-NPA was observed in gizzard and anterior intestine (Fig. 2C). In addition, CbE activity for 4-NPA was significantly higher in the crop, gizzard, anterior intestine, wall muscle and seminal vesicles of non-clitellated earthworms compared to those of clitellated individuals (Mann–Whitney U test, $P < 0.01$). The sexual maturation had less impact on CbE activity using α-NA or 4-NPV (Fig. 2A and B).

Significant linear correlations between CbE activity with α-NA and that using 4-NPA were found in clitellated ($y = 71.14 + 0.71x$, $r = 0.88$, $P < 0.01$) and non-clitellated ($y = 106.8 + 1.25x$, $r = 0.76$, $P < 0.05$) earthworms.

3.2. Tissue-specific sensitivity of CbEs to chlorpyrifos-oxon

Although the parameters pH, temperature, time of incubation and substrate concentration were kept constant in the incubation medium during acquisition of the inhibition kinetics, the impact of other extrinsic factors and long incubation periods on CbE sensitivity to CPx inhibition were evaluated. Three variables were examined; loss of enzyme activity during extended incubation intervals, concentration of earthworm homogenate in the reaction mixture, and the impact of pesticide-metabolising (and sequestration) enzymes. Carboxylesterase activities were stable for 1 h of incubation at 25 °C for all tissues, although a significant loss of CbE activity to 4-NPV (20–67% decrease) was observed for all tissue except anterior intestine when the incubation period was prolonged up to 24 h (Fig. 3). The amount of tissue homogenate used in the inhibition kinetics affected the observed IC50 (Fig. 4). The concentration of CPx needed to inhibit 50% of CbE activity decreased by 2–5 orders of magnitude when samples were diluted 1:500 in the reaction medium, although this effect was not as pronounced for gizzard and seminal receptacles. In addition, a high tissue-specific variability in IC50 values (from 10^{-6} to 10^{-11} M) was evidenced when dilution of homogenate in the reaction mixture was 1:500 (Table 1). Moreover, ChE, PTE and GST activities were measured in all earthworm tissues and they were highly variable among tissues (Table 1). No relationship was observed between the activities of these detoxifying enzymes and the CbE IC50s to CPx.

Fig. 5 shows the concentration–response curves of CbE activity using a dilution factor of the homogenate of 1:500 in the incubation mixture. The responses of the intestinal tract and wall muscle CbE activity to increasing CPx concentrations were fitted ($r > 0.97$, $P < 0.0001$) to the sigmoid model $y = y_0 + a/(1 + (x/x_0)^b)$, whereas concentration–response curves for reproductive tissues gave an improved fit with the logarithmic equation $y = y_0 + a \ln x + b (\ln x)^2 + c (\ln x)^3$ ($r > 0.98$, $P \leq 0.003$). The sensitivity of CbE activity to CPx was highly dependent on the tissue and substrates used for the activity assay. Concentrations of CPx that resulted in 50% inhibition of CbE activity varied from 1070 (gizzard) to 0.02 nM (seminal vesicles) for α-NA, from 69.1 (seminal receptacles) to 0.015 nM (anterior intestine) for 4-NPV, and from 6160 (crop) to 0.014 nM (seminal vesicles) for 4-NPA (Table 1). In general, CbE activity using 4-NPV was more sensitive to CPx inhibition than CbE activity using α-NA or 4-NPA. Gizzard CbE activity measured with 4-NPV was resistant (33% of control activity remaining) to CPx at concentrations as high as 3.7×10^{-5} M (Fig. 5B). Similarly, the CbE activity from all tissues showed a resistant fraction (19–47% of control activity remaining) to the OP when 4-NPA was used as the substrate (Fig. 5C). There was a marked difference in CbE sensitivity

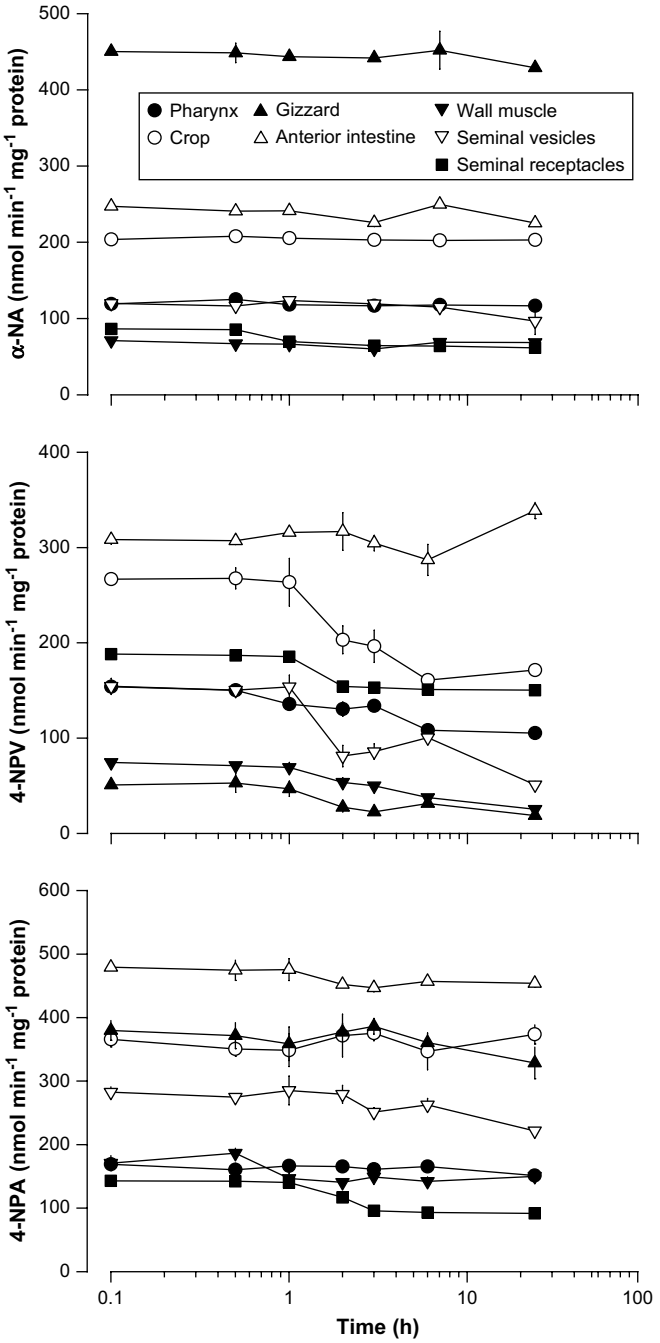

Fig. 3. Variations of carboxylesterase activity towards α-naphthyl acetate (α-NA), 4-nitrophenyl valerate (4-NPV) and 4-nitrophenyl acetate (4-NPA) for 24 h incubation at 25 °C. Data are depicted as the mean (±standard deviation) of three independent determinations.

to CPx between male and female reproductive organs, being CbE activity in the seminal vesicles more sensitive to CPx inhibition than that of seminal receptacles (Table 1).

3.3. Native PAGE

Native PAGE was used to detect CbE isozyme abundance, with esterases visualized by activity staining (Fig. 6). A maximum of 12 protein bands were distinguished (designated as ES1–ES12), but the number of stained bands and the intensity of the staining (corresponding to the hydrolytic activity towards α-NA) varied with

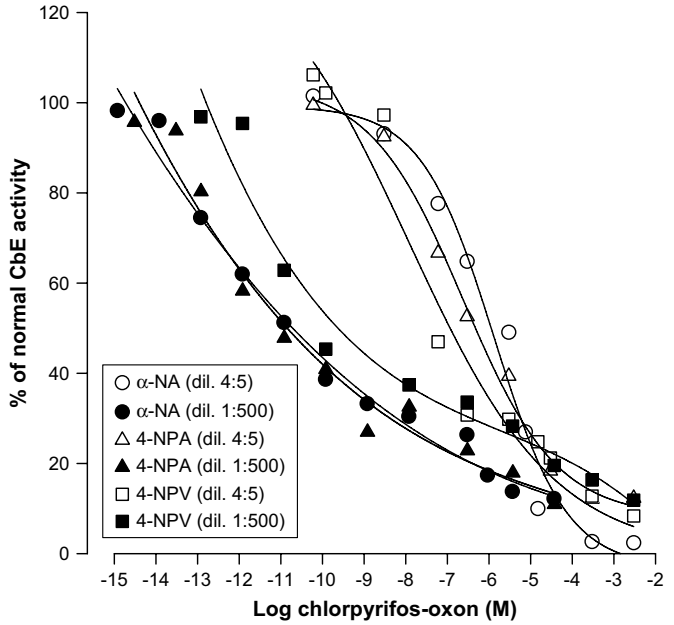

Fig. 4. An example of tissue dilution effect on carboxylesterase (CbE) inhibition curves of seminal vesicles of *Lumbricus terrestris*, using α-naphthyl acetate (α-NA), 4-nitrophenyl valerate (4-NPV) and 4-nitrophenyl acetate (4-NPA) as the substrate. Each point represents the average of two independent assays. White symbols are CbE inhibition curves when dilution factor of earthworm homogenate was 4:5, whereas black symbols are CbE inhibition curves when dilution factor was 1:500.

tissue. Two groups of CbE isozymes (ES5–ES7 and ES9–ES11) were present in all earthworm tissues. Elevated hydrolytic activity was observed for CbE activity in the crop, gizzard, anterior intestine, wall muscle and seminal receptacles. However, the CbE fractions corresponding to the bands ES9–ES11 were more pronounced in the pharynx and the reproductive tissues. The intensity of staining for all CbE bands, with the exception of ES5–ES8, strongly decreased when the gel was incubated in the presence of 5×10^{-7} M CPx for 30 min.

4. Discussion

4.1. Tissue distribution of earthworm CbEs

Tissue-specific variations in CbE activity have been observed extensively in aquatic invertebrates and vertebrates (Wheelock et al., 2008). Current results in the earthworm *L. terrestris* also observed tissue-dependent CbE activity, and showed that the enzyme activity is highly dependent on the substrate used in the kinetic assay. The majority of the ecotoxicological studies published to date have preferentially used the substrates 4-NPA or α-NA for measurement of CbE activity. These substrates are convenient for employment in esterase activity assays due to their commercial availability and ubiquitous use in the literature. However, the relevancy of these substrates to either biological or toxicological mechanisms has not been established. A recommended strategy is therefore the use of a battery of substrates to establish CbE activity profiles (Wheelock et al., 2005a,b, 2008). In *L. terrestris*, inhibition kinetic curves of CbE activity and native PAGE patterns have shown the presence of multiple CbE isozymes with a clear tissue-specific hydrolysis efficiency for the three substrates used in this study, and a tissue-specific CbE sensitivity to CPx inhibition. Furthermore, some inhibition curves like that observed for seminal receptacle CbE activity using α-NA (Fig. 5A) showed a biphasic pattern, suggesting that multiple CbE isoforms with different sensitivities to CPx are involved in the response to OP inhibition. A biphasic response has been also previously documented in rat brain CbE activity in response to CPx (Chanda et al., 1997). The significant correlations found between CbE activity using α-NA and that with 4-NPA suggest that the same CbE isoforms participate in the hydrolysis of these acetyl esters.

High levels and isozyme abundance of CbEs can contribute to pesticide tolerance because this esterase hydrolyses CBs and PYDs (Jokanovic, 2001; Sogorb and Vilanova, 2002), and it is able to bind to the OP decreasing therefore the effective concentration of pesticide (Maxwell and Brecht, 2001). Carboxylesterase activity was abundant in the intestinal tract of *L. terrestris*. Moreover, a higher number of CbE isozymes were distinguished by native electrophoresis in pharynx, crop and anterior intestine compared to the other tissues. However, Haites et al. (1972) found that the reproductive tissue and wall muscle of *L. terrestris* showed a higher number of CbE isoforms. Probably, electrophoresis conditions could account for this discrepancy. In the study by Haites et al. (1972), a 7.5% polyacrylamide gel was used, however, a higher percent of acrylamide results in a high resolution of CbE bands. It could be speculated that earthworm exposure to pesticides through its alimentary surfaces would be lead to a less degree of toxicity than exposure to pesticides by dermal uptake, because the high CbE activity levels and isozyme abundance present in the intestinal tissues would contribute to a more effective OP detoxification.

Sexual maturation of *L. terrestris* seems to be a confounding factor to consider when CbE activity is used as a biomarker of pesticide exposure. Significant differences in CbE activity were found between clitellated and non-clitellated earthworms, particularly towards 4-NPV and 4-NPA in tissues such as intestinal tract or seminal vesicles. Previous studies have also reported that CbE

Table 1
Basal glutathione S-transferase (GST), cholinesterase (ChE) and phosphotriesterase (PTE) activities[a] in different tissues of *Lumbricus terrestris*, and concentrations of chlorpyrifos-oxon causing 50% carboxylesterase (CbE) inhibition

Tissue	GST	ChE	PTE	Median inhibition concentration (IC50) of chlorpyrifos-oxon (nM)		
				CbE (α-NA)[b]	CbE (4-NPV)	CbE (4-NPA)
Pharynx	377 ± 45.6	359 ± 37.2	1.67 ± 0.18	0.602	0.016	4460
Crop	757 ± 70.1	40.9 ± 3.0	2.97 ± 0.46	3.16	0.575	6160
Gizzard	68.0 ± 3.4	4.71 ± 0.5	2.37 ± 0.21	1070	63.1	1070
Anterior intestine	545 ± 25.8	25.1 ± 2.0	6.78 ± 0.94	13.1	0.015	151
Wall muscle	323 ± 29.3	391 ± 8.9	6.94 ± 1.06	67.6	0.025	3800
Seminal vesicles	295 ± 80.9	12.8 ± 0.5	0.67 ± 0.02	0.02	0.234	0.014
Seminal receptacles	139 ± 10.0	38.5 ± 2.1	1.24 ± 0.29	524	69.1	3230
Porcine liver esterase	N.D.	N.D.	N.D.	0.229	0.038	0.049

N.D. = not determined.
[a] Mean (±standard deviation) activity of three independent assays and expressed as nmol min^{-1} mg^{-1} of total protein.
[b] α-NA = α-naphthyl acetate, 4-NPV = 4-nitrophenyl valerate, 4-NPA = 4-nitrophenyl acetate.

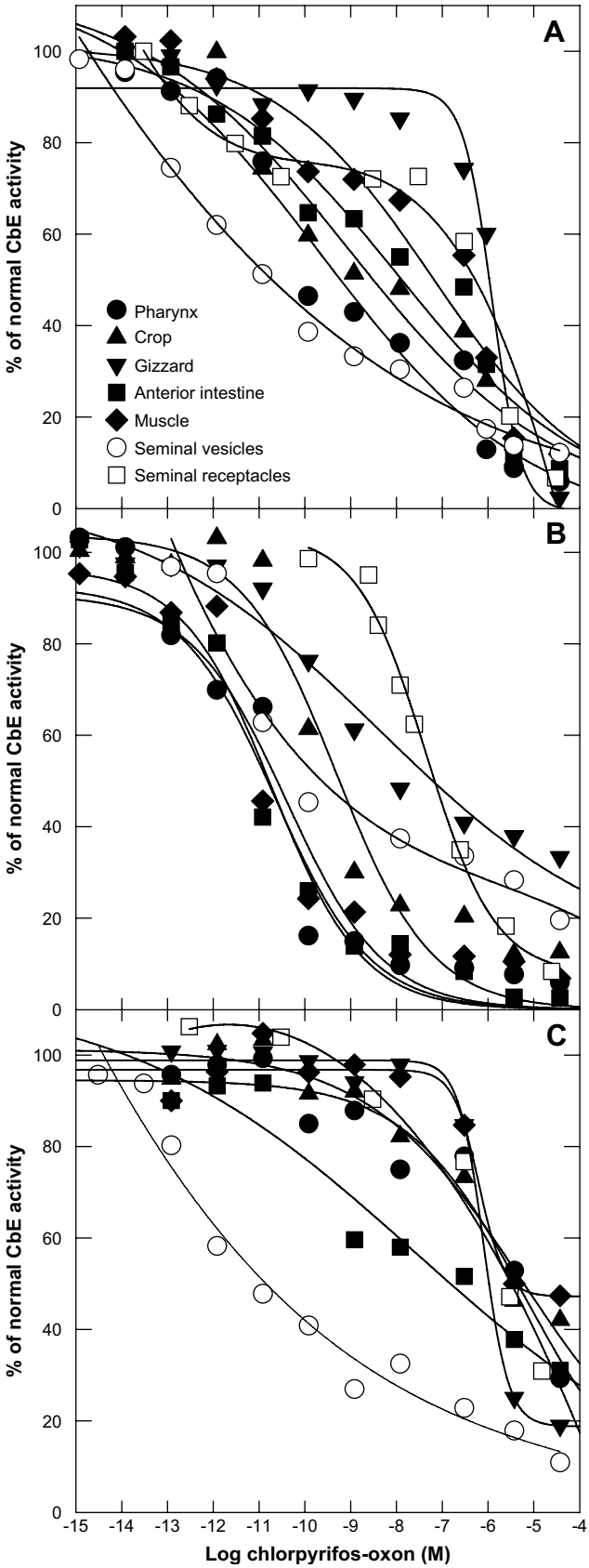

Fig. 5. In vitro inhibition of carboxylesterase (CbE) activity towards α-naphthyl acetate (A), 4-nitrophenyl valerate (B) and 4-nitrophenyl acetate (C) by increasing concentrations of chlorpyrifos-oxon in different tissues of *Lumbricus terrestris*. Each point represents the average of two independent assays.

activity varies substantially during organism development. For example, Barron et al. (1999) found that CbE activity of rainbow trout was up to 12-fold higher in the juvenile and adult than in the embryo. In rats, a significant variation in CbE activity using 4-NPA was observed in the plasma, liver and lung of different age groups (Karanth and Pope, 2000).

4.2. Sensitivity of CbEs to chlorpyrifos-oxon

Earthworm CbE activity was very sensitive to CPx inhibition, with IC50s varying in the μM to nM range on a tissue and substrate selective basis. We also determined CbE IC50 values for a commercial porcine liver esterase as a comparison, which were in the nM range (Table 1). Wheelock et al. (2005a,b) reported a mean CbE IC50 value of 1.55 nM for porcine esterase activity using 4-NPA, which was higher than that measured in this study (0.049 nM). There are a number of potential causes for the observed differences in activity. This commercial esterase preparation has been used extensively in the literature and has become an ex post facto standard, due to its commercial availability. However, it has been shown that the activity and isozyme abundance of this preparation can vary from lot to lot, making direct inter-study comparisons often inappropriate (Wheelock et al., 2006). In addition, differences in laboratory assay methods could account for this discrepancy. These results further demonstrate that caution should be exercised when directly comparing IC50 values between different laboratories and it is often more appropriate to only compare rank order potency in the absence of the dissociation constant for the enzyme-inhibitor complex (K_i).

In a comparative context, earthworm CbE activity using 4-NPA was as sensitive to in vitro inhibition by CPx as CbE activity in a number of organisms. For example, CbE activity of rat brain, plasma and liver had IC50 values ranging from 0.75 to 100 nM (Chanda et al., 1997). Similarly, CPx IC50s varied between 0.28 (neonatal rats) and 0.77 nM (aged rats) for liver CbE activity, and between 0.20 (aged rats) and 1.44 nM (adult rats) for lung CbE activity (Karanth and Pope, 2000). Chlorpyrifos-oxon IC50 values for liver CbE activity in Chinook salmon (*Oncorhynchus tshawytscha*) and CbE activity in whole body homogenates of medaka (*Oryzias latipes*) and splittail (*Pogonichthys macrolepidotus*) were 0.14, 6.12 and 8.42 nM, respectively (Wheelock et al., 2005a,b). Using 4-NPV as substrate, liver CbE IC50 of mosquitofish (*Gambusia affinis*) for CPx was 1.34 nM (Boone and Chambers, 1997), whereas *L. terrestris* CbE IC50 values varied from 69.1 (seminal receptacles) to 0.015 nM (anterior intestine) when 4-NPV was used. Accordingly, CbE sensitivity to CPx in earthworms is well within the range observed in a number of different vertebrate species.

Traditionally, in vitro sensitivity of AChE activity to anti-ChE pesticides has been a powerful predictor tool for acute toxicity. Many studies have reported good correlations between the IC50s of AChE activity and the LD50 (or LC50) values as a measurement of in vivo acute toxicity. However, others have questioned the use of the in vitro sensitivity of AChE activity to make predictions regarding acute toxicity because extrinsic factors related to incubation conditions (e.g., tissue dilution) can mask the true sensitivity of esterases to pesticides (Mortensen et al., 1998). In line with this assumption, we found that the amount of tissue used for inhibition kinetics determines the IC50 outcome. For the majority of earthworm tissues, the IC50 increased with homogenate concentration in the incubation mixture, varying between 10^{-7} and 10^{-5} M (Fig. 4). However, tissue-specific differences in IC50s were more marked when inhibition kinetics were obtained by using a homogenate dilution of 1:500 in the incubation medium (Table 1). This shows that extrinsic factors other than the sensitivity of CbE activity to CPx could be involved in the inhibition curve outcomes. Similar results were previously reported by Mortensen et al. (1998),

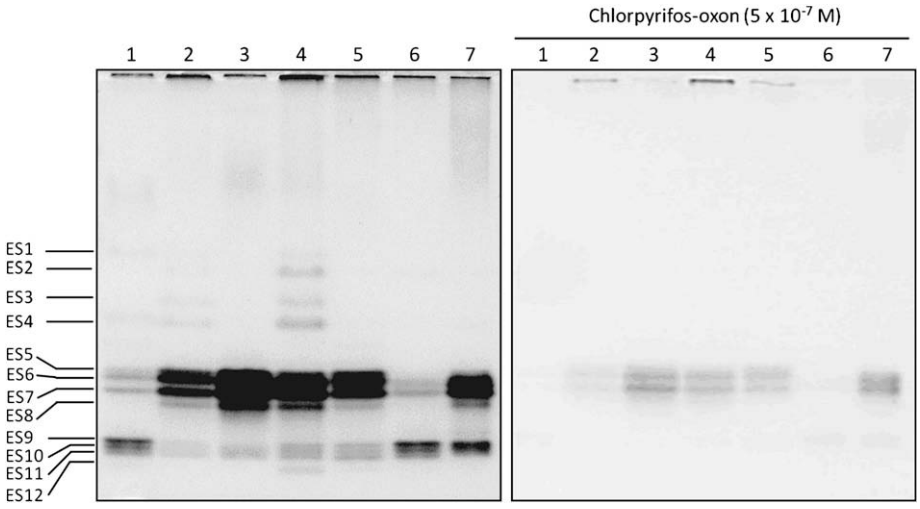

Fig. 6. Non-denaturing polyacrilamide gel electrophoresis of crude extracts of diverse tissues of *Lumbricus terrestris* followed by staining for esterase activity using α-naphthyl acetate. Lane 1: pharynx (14.7 μg protein), lane 2: crop (12.5 μg protein), lane 3: gizzard (10.9 μg protein), lane 4: anterior intestine (16.3 μg protein), lane 5: wall muscle (21.6 μg protein), lane 6: seminal vesicles (14.0 μg protein), and lane 7: seminal receptacle (21.3 μg protein). Right gel was incubated with 5×10^{-7} M chlorpyrifos-oxon for 30 min at room temperature prior to visualization.

who demonstrated that the dilution of rat plasma significantly affected the ChE IC50 curves to CPx, but did not have any effect on the activity of a commercial pure mouse AChE preparation. In addition to the sample dilution effect, three extrinsic factors could also be involved in the observed in vitro earthworm CbE sensitivity to CPx and therefore contribute to the high tissue-specific variation of CbE IC50s found in this study. First, the presence of PTEs able to hydrolyse the oxon form of OPs (Vilanova and Sogorb, 1999), second, the existence of GST activity in the crude homogenate that can detoxify the CPx (Jokanovic, 2001), and third, the presence of ChEs that binds to the OP irreversibly, thereby reducing the effective concentration of CPx in the reaction medium. We measured these enzyme activities in the earthworm tissues to evaluate the impact of these potential sources on CbE IC50 variation (Table 1). Basal levels of these enzyme activities were in general agreement with literature values. Thus, ChE activity measured in wall muscle of *L. terrestris* was similar to that determined for this earthworm species by Rault et al. (2007) (209 ± 0.57 nmol min^{-1} mg^{-1} protein). Glutathione S-transferase activity in this study was also in the same range of normal variation as that measured for other earthworm species such as *E. andrei* (381–529 nmol min^{-1} mg^{-1} protein, Stokken and Stenersen, 1993; Ribera et al., 2001), *E. fetida* (227 nmol min^{-1} mg^{-1} protein; Oneto et al., 2005) or *Aporrectodea caliginosa* (49–101 nmol min^{-1} mg^{-1} protein; Booth and O'Halloran, 2001). With regard to earthworm PTE activity, current data are in line with those previously reported by Lee et al. (2001), who found a 90% of total PTE activity (using paraoxon as substrate) in the gut tissue of *E. andrei*. Tissue-related differences in CbE IC50s were not a function of pesticide metabolism or sequestration, because no significant relationships were observed between IC50 values and GST, ChE or PTE activities. We therefore suggest that the high tissue-specific variations of CbE IC50s were due solely to variations in the sensitivity of CbE activity, and those extrinsic factors such as OP-metabolising enzymes had a minimum contribution. The lack of CbE sensitivity to CPx when high concentrations of homogenate were used in the incubation mixture might be due to inactivation of the OP by binding to lipids and/or unspecific proteins present in the homogenate.

The sensitivity of CbE activity to in vitro inhibition by OPs could be a useful determinant of the safeguarding capacity of the tissue against the toxic impact of OPs. Although the *L. terrestris* gizzard was one of the selected tissues evidencing the highest CbE activity towards α-NA and 4-NPA, the sensitivity of CbE to CPx inhibition was low. Conversely, the seminal vesicles showed relatively low CbE activity levels compared to the intestinal tract; however, the sensitivity of CbE to CPx was very high as evidenced by the IC50s when α-NA or 4-NPA was used as the substrate (IC50 = 0.020 and 0.014 nM). This high sensitivity of seminal vesicle CbE activity could have serious toxicological implications considering that this esterase can be involved in sperm differentiation and maturation as reviewed by Mikhailov and Torrado (1999). These authors suggest that CbE activity levels in the male reproductive tract play an important role as a protective mechanism against toxicity from anti-ChE and PYD pesticides. On the other hand, some studies have given solid evidences for a role of CbE activity in reproductive toxicology. For example, the thiocarbamate herbicide molinate caused testicular toxicity in rats by inhibition of CbE activity (designated as hydrolase A) located in the Leidig cells and likely involved in the synthesis of testosterone (Jewell and Miller, 1998). In earthworms, the CB insecticide benomyl caused severe damage at the morphological and developmental level of spermatid and spermatozoa (Sorour and Larink, 2001). Current in vitro results encourage future research to test the hypothesis that the high sensitivity of seminal vesicle CbE activity to CPx is directly related to adverse effects on sperm number and morphology. If so, earthworm CbE activity measured in this tissue would be an attractive biochemical biomarker of reproductive toxicity with significant detrimental effects at the population level. Moreover, levels of PTE activity found in the seminal vesicles of *L. terrestris* are relatively low and the oxon form of OPs would persist for a long time in this tissue, increasing therefore the risk for CbE inhibition and reproductive toxicity.

5. Conclusions

Three practical issues should be taken into account before using earthworm CbE inhibition to monitor for exposure to OP pesticides. First, the selection of appropriate substrates for CbE assay is recommended because of the presence of multiple isoforms and lack of substrate specificity of CbE activity in *L. terrestris*. Our results have demonstrated a high variation in the substrate hydrolysis efficiency by CbE activity depending upon tissue and the CbE isozyme abundance. Second, CbE activity should be measured in selected tissues and not in whole organism homogenates. We have

found a clear tissue-specific distribution of CbE isozymes in *L. terrestris* with a marked difference not only in enzyme activity levels, but also in sensitivity to CPx inhibition. Third, the measurement of CbE inhibition should be used jointly with other biomarkers of pesticide exposure such as ChE activity. The fact that earthworm CbE activity shows a certain degree of resistance to CPx inhibition, depending on tissue and substrate, makes advisable the use of complementary biomarkers of pesticide exposure such as ChE activity. Furthermore, a number of studies have demonstrated that CbE activity is as useful for monitoring OP inhibition as ChE activity (Wogram et al., 2001; Galloway et al., 2002; Wheelock et al., 2008). The inclusion of CbE activity measurements in a multibiomarker scheme is justified not only because of its high sensitivity to OP pesticides, but also because comparisons of activity levels, i.e., hydrolysis efficiency and sequestration capacity, can be useful to examine the tolerance of organisms to agrochemicals. Accordingly, CbE activity could serve not only as a biomarker of OP exposure, but also as a biomarker of susceptibility to OP-induced toxicity. These data suggest that further studies should continue to validate the use of CbE activity in earthworms as biomarkers of agrochemical exposure.

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