Earthworm cytochrome P450 determination and application as a biomarker for diagnosing PAH exposure

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We developed a new microsome purification method and used the Omura and Sato method to measure the total content of cytochrome (Cyt) P450 in earthworm (*Eisenia fetida*) microsomes. In method development, two different pretreatments, *i.e.* solubilization or manual separation were used to purify worm microsomes. Solubilization was more effective than manual separation and difference spectra showed a peak at 450 ± 1 nm in microsomes received solubilization pretreatment. We conducted a 48 h contact test by exposing worms on pyrene (Py) and benzo[*a*]pyrene (BaP) spiked filter paper. A dose-response relationship was established between total P450 content and the concentration of Py or BaP ranging from 10^{-6} mg mL^{-1} to 10^{-2} mg mL^{-1}. Results show that total Cyt P450 content in earthworms is a promising biomarker for diagnosing PAHs exposure at sublethal dose ranges.

Introduction

Cytochrome (Cyt) P450 exists widely in living organisms and plays a crucial role in the detoxification and inactivation of xenobiotics.[1–2] Cyt P450 comprises a large family of isoenzymes. Xenobiotic phase I biotransformation in organism is mainly mediated by the Cyt P450-dependent monooxygenase system.[3–4] Cyt P450 content represents total P450 proteins. Although most of Cyt P450 proteins do not show any response to xenobiotics, selective induction of some P450 isoenzymes may cause a significant elevation in total P450 level.[5] This has made it a potential biomarker for diagnosing early response of organisms to xenobiotics. Alteration of total P450 level is often observed in laboratory studies with various fish species exposed to trace PAHs.[6–11]

The most frequently measured parameters related to P450 are total P450 content, specific isoenzyme activity (*e.g.*, benzoxyresorufin-*O*-dealklase (BROD), ethoxyresorufin-*O*-deethylase (EROD), and ethoxycoumarin-*O*-dealkylase (ECOD)), and CYP1A protein level, which are often determined by means of spectrophotometric, fluorescence, or immunological methods. Research on using P450 as biomarker has mainly focused on aquatic species such as fishes or algae.[6–8,12–16] Few studies have been documented using P450 in earthworms to evaluate soil ecotoxicity.

Earthworms are considered to be one of the most important species in soil fauna. They are often used as model species in the ecotoxicological assessment because of their direct contact with soil.[17–19] There is a growing amount of evidence showing the existence of P450 in earthworms.[20–23] However, most studies were limited to exploring the usefulness of total P450 content and isoenzyme activities (*e.g.*, BROD, ECOD and EROD) in earthworms as a biomarker for assessing ecotoxic response of contaminants in soil, owing mainly to technical difficulties in obtaining purified microsomal fraction for measuring P450 content without interference.[21,24–26]

Polycyclic aromatic hydrocarbons (PAHs) are persistent and recalcitrant contaminants with soil being their largest sink. Some PAHs such as benzo[*a*]pyrene (BaP) are proven carcinogens.[3,27] In this study we developed a new method to purify earthworm microsomes and attempted to establish a dose-response relationship between total P450 content in purified worm microsomes and exposure concentrations of two PAHs, pyrene (Py) and benzo[*a*]-pyrene (BaP). Our aim was to evaluate the feasibility of using P450 as a biomarker to diagnose PAHs contamination in soil.

Experimental

Sample

Earthworms (*Eisenia fetida*) were purchased from a commercial breeder in Tianjin, China. Adult worms with well-developed clitella and body weight of 300–400 mg each were picked and conditioned in a pristine meadow brown soil before use. The soil (0–20 cm) was collected from the Ecological Experimental Station of Chinese Academy of Sciences in Shenyang, Liaoning Province, China. It had the following characteristics: pH 6.2, K-N 0.091%, total P 0.04%, total K 0.18%, organic matter content 1.65%, cation exchange capacity 12.3 cmol kg^{-1}, water holding capacity 32%, sand (>50 μm) 22%, silt (1–50 μm) 64%, and clay (<1 μm) 14%.

Chemicals

All chemicals were of analytical grade. They were purchased from following sources: BaP from Fluka (Switzerland),

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albumin bovine (BSA) and Coomassie brilliant blue G-250 (CBG) from BBI (Toronto, Canada), sodium cholate from Aldrich (Deisenhofen, Germany), and Tris and DTT from Amresco (USA). Other chemicals were all obtained from commercial suppliers in Shenyang, China. Carbon monoxide with a purity of 99.99% was provided by the Gas Station of Shenyang, China.

Microsomes preparation

Worms were transferred onto moistened filter paper, and kept up to 12 h at 20 °C in darkness for defecation before microsome preparation. Then the worms were immobilized in ice-cold glycerol solution (20% glycerol in distilled water) for 1 h. Each worm was cut into 9–10 pieces in cold KCl solution (0.15 mol L^{-1}) for bleeding. After 10 min, the samples were collected and transferred onto a gauze and washed with large volumes of KCl (0.15 mol L^{-1}) until the outflow was colorless. The worm pieces were placed in HB buffer (250 mmol L^{-1} sucrose, 50 mmol L^{-1} Tris pH 7.5, 1 mmol L^{-1} DTT, 1 mmol L^{-1} EDTA) and homogenized in a tissue homogenizer at 8000 rpm for 30 s (Xinzhi, Ningbo, China). The homogenate was centrifuged at 15 000 *g* for 30 min. The supernatant was collected and centrifuged again at 150 000 *g* for 1.5 h in a hypervelocity centrifuge (Hitachi CP-80MX, Japan) to obtain microsome pellets in the bottom fraction. The above procedures were all carried out at 4 °C.

Pretreatment tests

Two pretreatments, *i.e.*, solubilization treatment (ST) or manual separations (MS) were adopted to remove interfering substances from worm microsomes. For comparison, microsome pellets were also directly suspended with no additional pretreatment (NT) in HB supplemented with 20% glycerol and used for P450 determination.

Solubilization treatment (ST)

Microsomal pellets were resuspended in HB buffer supplemented with 20% glycerol, and the microsomal suspension was gradually made up to 0.5% sodium cholate by adding cholate (20%) dropwise. After 30 min of stirring on ice, the suspension was centrifuged at 100 000 *g* for 45 min. The supernatant was collected carefully and used for P450 determination.

Manual separation (MS)

Microsome pellets obtained by differential centrifugation were carmine in color with a buffy coat. To make manual separation easier, the microsome pellets were transferred onto ice and stored in fridge until frozen. Then, the less dense buffy overlying coat was collected carefully and suspended in HB supplemented with 20% glycerol for determination of P450 content.

Determination of total Cyt P450

The total content of Cyt P450 in microsomal suspension was determined according to Omura and Sato[28] by means of the sodium dithionite reduced carbon monoxide difference spectrum using a UV-2550 dual beam spectrophotometer (Shimadzu, Japan). Total microsomal protein content was determined by the Bradford method using BSA as a reference protein.[29]

Contact test

Earthworm contact test was performed in accordance with the OECD guideline.[30] Filter paper was cut to fit the inside of test vials ($\varnothing$ 3.6 cm × 7.0 cm) to give an exposure area of 78 cm^2. In each vial, 1.0 ml of acetone (control) or the Py or BaP solution (in acetone) was added to the filter paper and was allowed to evaporate for 2 h at 20 °C in darkness. The filter paper was then rehydrated with 1 ml of water and one adult worm was placed in each vial. After 48 h incubation at 20 ± 1 °C, worms were taken out from the vials for total P450 determination with solubilization pretreatment in microsome preparation. There were six treatments including a solvent control and five concentrations logarithmically spanning from 10^{-6} to 10^{-2} mg mL^{-1} for each compound. Each tretment had eight replicates (worms). For each determination, two worms of the same treatment were used.

All experiments were performed in compliance with the relevant state and local laws and guidelines. The use of earthworms did not require approval from any institutional committees.

Statistical analysis

Parametric tests were preceded by tests for homogeneity of variances. All experimental results were subjected to analysis of variance (ANOVA) using SPSS v12.0 for Windows. The LSD test was used to determine differences among means.

Results and discussion

Comparison between ST and MS pretreatments

Unlike fish, earthworms as an annelid among soil fauna, has no liver, and the whole body was used for microsome preparation. Previous studies[21,23] and our own preliminary investigations both indicate that determination of Cyt P450 by the Omura and Sato method[28] was impossible without additional separation of giant hemoglobin from microsomes. As shown in Fig. 1, the CO-difference spectra of microsomes prepared with ST or MS pretreatment as well as that prepared without pretreatment (NT) have similar shape. One peak occured at 420 nm and the other around 450 nm. However, the second peak of the NT spectra (Fig. 1a) shifted from 450 nm to about 454–456 nm, indicating the existence of interfering substances that overlaid with P450 proteins. After manual separation, the second spectrum peak moved forward to 451–453 nm (Fig. 1c). The spectra of the microsome received solubilization treatment (ST) peaked at 450 ± 1 nm (Fig. 1b). These results suggest that interfering substances were partially removed by MS and that a higher purity was reached by ST.

Milligan *et al.*[22] also reported that manual separation was effective for partial purification of microsomes. They found that the less dense part of the microsome, *i.e.* the buffy coat overlying fraction showed a higher purity of P450. However, manual treatment has some disadvantages. For instance, there

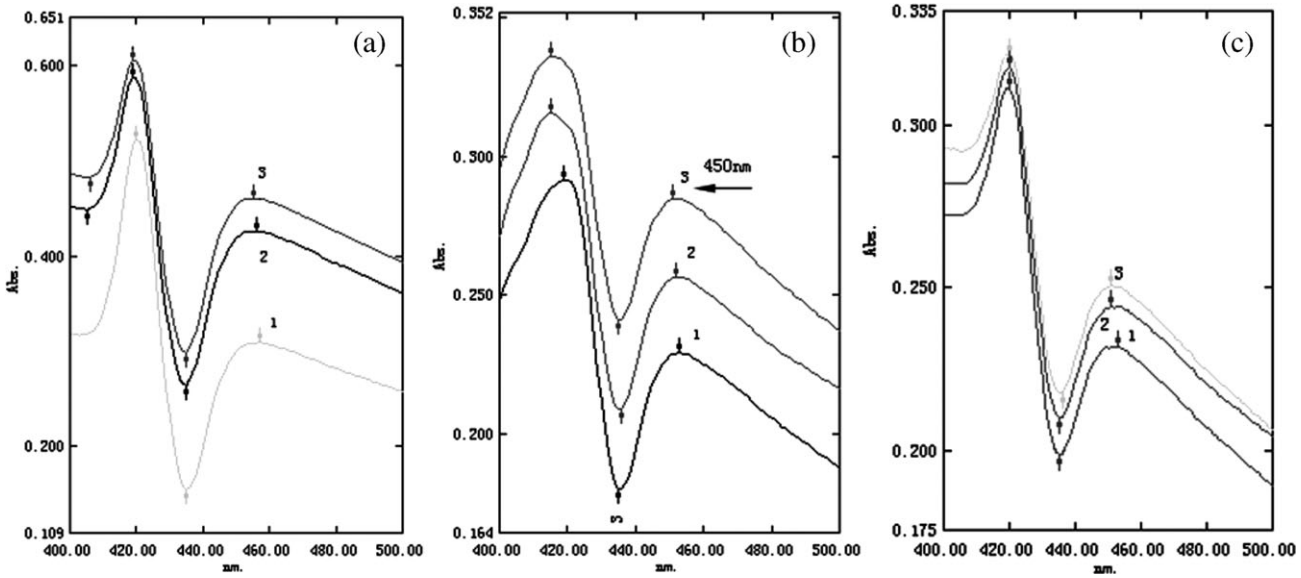

Fig. 1 Carbon monoxide (CO)-difference spectra in microsomes with and without pretreatment: (a) no pretreatment (NT); (b) solubilization treatment (ST); and (c) manual separation (MS).

are some uncertainties in manually separating the upper layer from the dense fraction of sedimentation, which may cause inconsistence in the precision and reproducibility for this method. Solubilization pretreatment was more effective than manual separation, probably because configuration of membrane-bound proteins (Cyt P450) was broken up by solubilization.

There are several different opinions in explaining the first peak occurred at 418–420 nm in the spectra of all samples. First, it was considered a Cyt P450 degradation product (*e.g.* Cyt P420)[31,32] or a degradation product from other hemoprotein.[33] This was based on the fact that some denaturing factors existed in the microsome preparation, which may result in the generation of degradation product. Several agents such as chelators (*e.g.*, EDTA), antioxidants, proteolysis inhibitors, and P450 protective agent (glycerol) have been used in attempt to prevent the production of P420 proteins, but none of them worked effectively.[26,34,35] Second, some investigators believed that the peak at 420 nm was earthworm hemoglobin. However, Rocha-e-Silva *et al.*[1] disagreed to this opinion and proved by spectroscopic analysis that the peak was distinguishable from those of carboxy-hemoglobin. Third, the substance was considered to be a new hemoprotein with some specific function, which was tightly bound to microsomal membrane.[1] This new hemoprotein consistently appeared in microsomes of different animals,[31–33,36,37] that possibly contributes to the adaptation of animals to unfavorable environment.

Optimization of CO aeration

As Omura and Sato[28] indicated, carbon monoxide (CO) plays an important role in P450 determination. Deficiency in CO supply would result in incomplete reduction. Tests based on ST pre-treatment were carried out to optimize CO supply for P450 determination. Results (Fig. 2) indicate that insufficient CO supply resulted in a significant shift of the target peak far from 450 nm, when CO was flushed at a flow rate of 10 cm^3 min^{-1} for 0.5 min (curve 1). As the aeration duration of CO increased from 1 min to 2 and 3 min, the peak moved closer to 450 nm. Further increase to 4 min resulted in a peak at 450 nm. More than 4 min of CO aeration did not change the

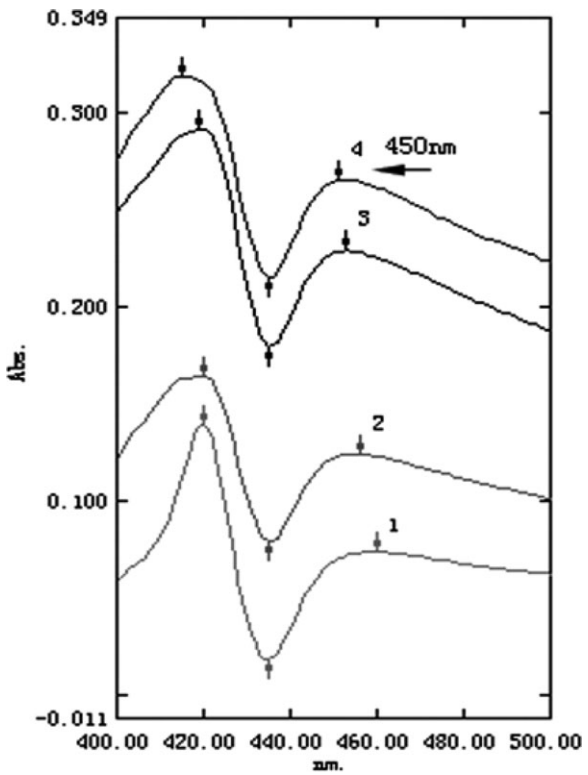

Fig. 2 Carbon monoxide (CO)-difference spectra in microsomes prepared with different length of CO aeration: (1) 1 min; (2) 2 min; (3) 3 min; and (4) 4 min.

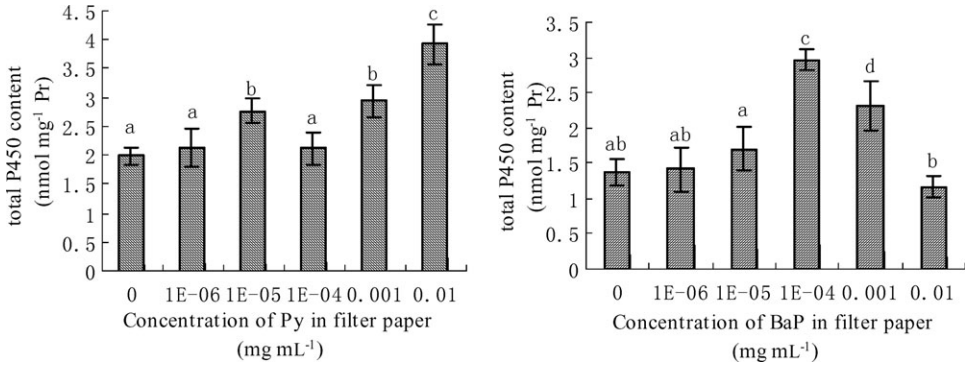

Fig. 3 Effect of 48 h exposure to pyrene (Py) or benzo[*a*]pyrene (BaP) on total cytochrome P450 content (mean ± SD, $n = 4$). Letters over the columns represent significant difference at $p < 0.05$ among treatments (ANOVA).

peak any further and the peak stayed at 450 nm (not shifted), suggesting that 4 min aeration was sufficient. Therefore, the optimum CO supply was 4 min at a flow rate of 10 cm^3 min^{-1}.

Dose–response relationship between Cyt P450 and Py or BaP

As shown in Fig. 3a, a dose-dependent response in total worm P450 was observed after 48 h exposure to Py at doses ranging from 10^{-2} to 10^{-6} mg mL^{-1}. P450 content in worms exposed to 10^{-5}, 10^{-3} or 10^{-2} mg mL^{-1} Py was significantly higher than that in the control. However, exposure to 10^{-4} mg mL^{-1} Py did not cause any significant change in worm P450 level (LSD test, $p > 0.05$). Worm P450 responded in a different manner to BaP exposure. The two lowest concentrations of BaP (10^{-6} and 10^{-5} mg mL^{-1}) as well as the highest one (10^{-2} mg mL^{-1}) showed no effect on total P450 content. But, at the two median concentrations, *i.e.* 10^{-4} and 10^{-3} mg mL^{-1} BaP, a significant increase in P450 content was observed.

The elevation of P450 contents in Py or BaP treated worms demonstrated that biotransformation of PAHs in worms might have activated some specific P450 isoenzymes that mediate catalyzing reactions, which ultimately enhanced the level of total P450 proteins. However, with the exposure concentration of BaP increasing, P450 content was reduced in worms exposed to 10^{-3} and 10^{-2} mg mL^{-1} BaP. The reason might be that BaP could act as an inducer for some specific P450 isoenzymes, but an inhibitor for others. This would cause a considerable alteration in isoenzyme levels, whereas the amount of total P450 was not affected.

Cyt P450 as a potential biomarker

The filter paper contact test demonstrated that worm P450 was affected by stress of Py and BaP exposure, indicating the usefulness of cyt P450 as a potential biomarker in soil matrix. However, the P450 family has lots of isoenzymes. For a certain chemical stressor, each individual isoenzyme may respond differently, such as induction, inhibition and inactivation. As a whole, the total content of P450 may not reflect the actual exposure level. It was thought that specific P450 isoenzymes (*e.g.* EROD, ECOD, AHH, and PROD) might be better indicators than total P450.[21,24–26] However, reported results suggest that they are general as good as total P450 in terms of selectivity, sensivity and effectiveness, probably owing to the diversity and complexity of P450 isoenzymes. For example, Brown *et al.*[26] tried to use EROD activity as a biomarker for diagnosing the ecotoxicity of Py exposure, but found that EROD was not detected in earthworm *L. rubellus*. ECOD, PROD and BROD activities were not induced by BaP in either *A. caliginosa* or *L. rubellus* or *E. fetida*. EROD was reduced and PROD was unchanged in *E. crypticus* after short-term exposure to BaP.[21,25] However, Saint-Denis *et al.*[24] showed MROD activity was particularly sensitive as significant changes were observed at the lowest concentration of BaP (50 μg kg^{-1} soil). Therefore, the effectiveness of P450 isoenzymes as biomarker depends on the choice of the appropriate isoenzyme responding to the specific stressor.

Without prior knowledge about which enzyme or enzymes would react to a specific stressor, it is common to measure multiple isoenzymes in combination with total P450 content. This approach can generate valuable information that may help to understand metabolic mechanisms of the chemical.

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