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Enantioselective Effects of Methamidophos on the Coelomocytes Lysosomal Membrane Stability of *Eisenia fetida*

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Abstract Many of organophosphorous insecticides are chiral compounds. In this study, the enantioselective effects of organophosphate insecticide methamidophos on the coelomocytes lysosomal membrane stability of earthworm *Eisenia fetida* were studied: (1) The enantiomers of methamidophos were absolutely separated by high-performance liquid chromatography with a commercial chiral column; (2) The neutral red retention assay was used to judge the lysosomal membrane stability. The results showed that with the concentration increasing, lysosomal membranes have been significantly destroyed by individual stereoisomers and racemate of methamidophos. The neutral red retention times were significantly descended from 76.88 to 29.78 min. Both (+)- and (−)-methamidophos showed more prone to destroy the integrity of the lysosomal membrane than the racemate. However, the different effect between stereoisomers is slight.

Keywords Methamidophos · Chiral · Earthworm · Neutral red

With the increasing application of chiral herbicides, more and more attention has been focused on the different biological activity between their enantiomers (Liu et al. 2005). Most chiral pesticides are produced and released into the environment as racemates, but the residue in the circumstance behaves as drastically different compounds in respect that enantioselective degradation may cause one enantiomer to be more persistent in the environment than the other (Ma et al. 2009). Therefore, the effects and the environmental fate of the enantiomers of chiral pollutants need to be investigated separately. Methamidophos is a highly efficient, broad-spectrum organophosphate insecticide, which is used in great quantities worldwide (Yu and Zhou 2005). It contains one chiral center at the phosphorus atom and thus has two stereoisomers. Several recent studies focused on the ecotoxicity of methamidophos to soil ecosystems due to its relatively short half-life, readily degradable and high mobility in soils. However, the potential adverse effects on ecosystem health and environmental safety can't be ignored, because the long-term and persistent input of methamidophos to agricultural soils made the amount of methamidophos residual in agricultural soils has even exceeded the self-purifying capacity of soil ecosystems (Liao et al. 2003; García-de la Parra et al. 2006). On the other hand, so far little attention has been given to enantioselectivity in chronic toxicities such as based on the molecular or cellular level.

The species *Eisenia fetida* is considered to be a standard species for standardization of acute and chronic ecotoxicological assays by the OECD and the EEC. Much work has been done to evaluate the acute toxicological effects on the earthworm. However, once death happened, the contaminant would make irreversiblely adverse effect to the ecosystem. Hence, the assessment of subacute effect of pollutant by using biomarkers (measured at the molecular or cellular level) is significantly important to ecotoxicology. The use of biological markers has been proposed as sensitive 'early warning' tool for biological effect measurement in environmental quality assessment (Cajaraville

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et al. 2000). Lysosomes is a target for the toxic action of xenobiotics, involved primarily in intracellular digestion (Moore et al. 1982). Neutral red staining is generally applied to evaluate cell toxicity. The purpose of this experiment was to evaluate enantioselectivity of methamidophos by the earthworm, and the biomarker of coelomocytes lysosomal membrane stability was chosen as an indicator.

Materials and Methods

Methamidophos [(RS)-O,S-dimethyl phosphoramidothioate, purity >99.0%] was purchased from Kefa New Technology Development Co.(Shenyang, China). Neutral Red (3-amino-6-dimethylamino-2-methyl-phenazine hydrochloride) was obtained from Amresco (Solon, OH, USA). Other solvents used in this study were HPLC grade.

Earthworms (*Eisenia fetida*) were purchased from a commercial feed lot in Tianjin, China, and cultured in our laboratory in moist sphagnum peat soil under darkness at room temperature. Before beginning the experiment, the worms were acclimatized for 2 weeks. Adult healthy worms with a well-developed clitellum and weight of 300–600 mg were selected for the bioassay.

The stereoconfiguration of methamidophos is given in Fig. 1. Stereoisomeric separation conditions were similar to the methods reported in a previous study (Lin et al. 2006). In brief, a Jasco LC-2000 series HPLC system (Jasco, Tokyo, Japan) with a chiralcel OD column (250 nm × 4.6 mm) was used. A volume of 20 μL was injected for chiral analysis with the mobile phase of *n*-hexane/isopropyl alcohol solution (70:30). The flow rate of the mobile phase was 0.5 mL min^{-1}. The detection wavelength of CD was set at 230 nm. The resolved enantiomers were individually manually collected at the HPLC outlet, evaporated to dryness and redissolved in acetone. Concentrations of the enantiomers were determined on an Agilent 6890N gas chromatograph (GC) equipped with a nitrogen-phosphorus detector (NPD).

Methamidophos exposure was done in filter paper according to OECD test guideline (1984). The earthworms were transferred to flat-bottomed glass vial approximately 8 cm in length and 3 cm in diameter. Filter paper was cut to fit the inside of the test vials. 2 mL of certain concentration test chemical was pipetted into the vial which was dissolved in ultrapure water (18.3 MΩ-cm resistivity) produced by Milli-Q system (Millipore, MA, USA). Earthworms were exposed to five concentrations (0.0016, 0.0032, 0.0159, 0.0318 and 0.1592 μg cm^{-2} equivalent to 0.05, 0.1, 0.5, 1, 5 mg L^{-1}). These concentrations were based on the preliminary range-finding test, and ultrapure water was used as control. One adult worm was added per vial. Prior to exposure, the earthworms were allowed to depurate their gut contents on damp filter paper for 1 day, then washing and drying for use, and five replicates were used for one concentration treatment. Then each vial was sealed with a plastic film with a small ventilation hole, and kept in the darkness at test temperature of 20 ± 2°C for 24 h.

The effect of methamidophos exposure on lysosomal membrane stability was measured using the neutral red retention assay (NRRA) as described by Weeks and Svendsen (1996). A volume of 20 μL of coelomic fluid containing coelomocytes was collected by inserting a hypodermic needle directly into the coelomic cavity posterior to the clitellum of the worm, and mixed with an equal volume of earthworm physiological Ringer solution. A stock solution of neutral red was prepared by dissolving 20 mg of neutral red in 1 mL of dimethyl sulfoxide (DMSO), and a working solution of 80 μg mL^{-1} was prepared by diluting 10 μL of the stock solution with 2.5 mL physiological Ringer solution. Then the coelomic fluid (20 μL) was placed on clean microscope slides and the cells were allowed to adhere to slides for 30 s before application of the neutral red working solution (20 μL) and a coverslip sealed with. Slides with the coelomic fluid were continuously observed at 2-min intervals under a Microscope (Leica Dmirb, equipped with Leica DFC300FX camera, 400× magnification), and the number of stained and unstained cells were counted. Observation was stopped when the cells with fully stained cytosols were over 50% of the total number of cells counted. This time was recorded as the neutral-red retention time. Coelomic fluid from each individual worm was collected and processed separately for each assay.

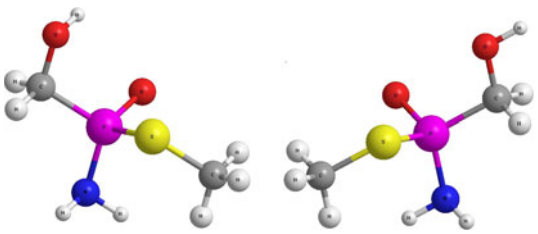

Fig. 1 Enantiomers of methamidophos

Results and Discussion

To examine the enantioselective effects of methamidophos on lysosomal membrane stability, earthworms were treated with different concentrations of individual enantiomer or racemate of methamidophos for 24 h. The lysosomes of healthy earthworm can retain neutral red. Conversely, when the lysosomes membrane was injured, the neutral red

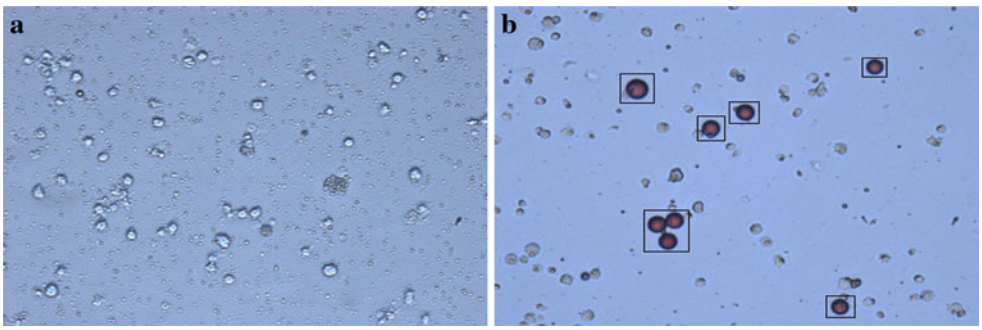

Fig. 2 Coelomocyte of earthworm **a** the coelomocyte of health earthworm wasn't dyed by neutral red **b** the lysosomal membrane was completely injured and dyed by neutral red

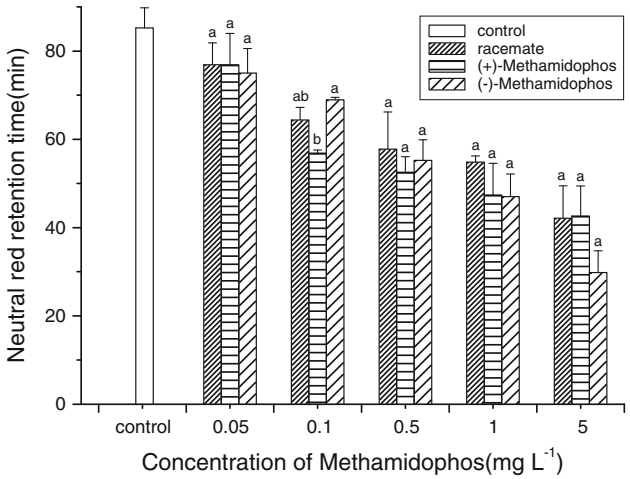

Fig. 3 Neutral red retention times (min) of earthworm after exposure to enantiomers and racemate of methamidophos with different concentration. *Different letters* above *adjacent bars* indicate a significant difference ($p < 0.05$, n = 5) between individual enantiomer and racemate, while the *same letter* indicates no significant difference

would release and the coelomocyte became red (Fig. 2). The dose–response relationships of neutral red retention time induced by the methamidophos enantiomers are shown in Fig. 3. The neutral red retention time of coelomocytes from earthworms in the control was 85.26 min in average. This result is relatively close to Weeks's research of 90 min. It illuminates that the earthworms we chose for our study is healthy enough to be researched. Lysosomal membranes have been significantly destroyed by individual stereoisomers and racemate of methamidophos. It is concluded that the methamidophos could damage the immune system of the earthworm, and the neutral red retention times were significantly descended from 76.88 to 29.78 min with the concentration increased. Both (+)-methamidophos and (−)-methamidophos showed more prone to destroy the integrity of the lysosomal membrane than racemate of methamidophos.

During our study, the earthworm didn't appear dead and show any morphological changes. These results demonstrate that, although the earthworm is exposed is exposed to low concentrations of methamidophos, the changes in the lysosomal membrane stability are significant. Responses of the lysosomal system are generally thought to provide a first answer to pollutant exposure, since injurious lysosomal reactions frequently precede cell and tissue pathology (Moore et al. 2006). In addition, many other researchers also have demonstrated that the NRRA responds far more rapidly than other physiological responses, e.g. growth, cocoon production, cocoon viability (Booth and O'Halloran 2001; Svendsen and Weeks 1995). Therefore, associated with our methamidophos exposure, NRRT is a reliable, sensitive, dose–effect relationship biomarker and could be an early warning of toxicant impacts on population of earthworm in agreement with previous research (Lowe et al. 1992). To the best of our knowledge, this is the first study to determine the enantioselectivity of methamidophos on lysosomal membrane stability in earthworm coelomocytes. The results of the study slightly showed the different effect between stereoisomer of methamidophos. Nevertheless the enantiomer of methamidophos inhibition toward acetylcholinesterases assay showed that (−)-methamidophos was about 8.0–12.4 times more potent to the enzymes than its (+)-form (Lin et al. 2006). This maybe attribute to the histochemical lysosomal stability technique, it unfortunately has several technical limitations at the present moment. The quantification of staining may be initially subjective. There is still a tendency to think about techniques in cell biology as not fully quantitative. Also the mechanism of enantioselectivity in the lysosomal membrane is not clear, requiring further research. On the other side, we can realize that it is easy to use one biomarker to judge the variation in single toxicant to organisms. It is fundamental to develop appropriate mathematical instruments to objectively and correctly evaluate the effects of pollution on the health of organisms by integrating the results obtained with different biomarkers (Cajaraville et al. 2000).

Results from this study suggest that enantiomers of methamidophos could damage the immune system of the earthworm by inducing the lysosomal membrane. The neutral red retention time is a sufficiently sensitive, quick assessment biomarker for monitoring the pollution of methamidophos, and could be used as early warning indicators of an adverse impact of pesticides on earthworm populations. Both (+)- and (−)-methamidophos are higher toxicity to the racemate of methamidophos. The chiral compounds are involved in biochemical interactions, and receptors generally distinguish between their enantiomer. Monitoring of the racemate concentration will give an inadequate or misleading basis for assessing the environmental risk of chiral pesticides (Liu et al. 2005). Particular biomarkers of earthworm for estimating the enantioselectivity of chiral chemical needs further studies.

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References

Booth LH, O'Halloran K (2001) A Comparison of biomarker responses in the earthworm *aporrectodea caliginosa* to the organophosphorus insecticides diazinon and chlorpyrifos. Environ Toxicol Chem 20:2494–2502

Cajaraville MP, Bebianno MJ, Blasco J, Porte C, Sarasquete C, Viarengo A (2000) The use of biomarkers to assess the impact of pollution in coastal environments of the Iberian Peninsula: a practical approach. Sci Total Environ 247:295–311

García-de la Parra LM, Bautista-Covarrubias JC, Rivera-de la Rosa N, Betancourt-Lozano M, Guilhermino L (2006) Effects of methamidophos on acetylcholinesterase activity, behavior, and feeding rate of the white shrimp (*Litopenaeus vannamei*). Ecotoxicol Environ Saf 65:372–380

Liao M, Xie XM, Subhani A (2003) Combined effect of nutrient and pest management on soil ecological quality in hybrid rice double-cropping system. Pedosphere 13:129–138

Lin KD, Zhou SS, Xu C, Liu WP (2006) Enantiomeric resolution and biotoxicity of methamidophos. J Agric Food Chem 54:8134–8138

Liu WP, Gan JY, Schlenk D, Jury WA (2005) Enantioselectivity in environmental safety of current chiral insecticides. Proc Natl Acad Sci 102:701–706

Lowe DM, Moore MN, Evans BM (1992) Contaminant impact on interactions of molecular probes with lysosomes in living hepatocytes from dab *Limanda limanda*. Mar Ecol Prog Ser 91:135–140

Ma Y, Xu C, Wen YZ, Liu WP (2009) Enantioselective separation and degradation of the herbicide dichlorprop methyl in sediment. Chirality 21:480–483

Moore MN, Pipe RK, Farrar SV (1982) Lysosomal and microsomal responses to environmental factors in Littorina littorea from Sullom Voe. Mar Pollut Bull 13:340–345

Moore MN, Icarus Allen J, McVeigh A (2006) Environmental prognostics: an integrated model supporting lysosomal stress responses as predictive biomarkers of animal health status. Mar Environ Res 61:278–304

Svendsen C, Weeks JM (1995) The use of a lysosome assay for the rapid assessment of cellular stress from copper to the freshwater snail *Viviparus contectus* (Millet). Mar Pollut Bull 31:139–142

The Organisation for Economic Co-operation and Development (OECD) (1984) Earthworm acute toxicity tests. OECD Guideline for testing of chemicals, No. 207. OECD, Paris

Weeks JM, Svendsen C (1996) Neutral red retention by lysosomes from earthworm (*Lumbricus Rubellus*) colemocytes: a simple biomarker of exposure to soil copper. Environ Toxicol Chem 15:1801–1805

Yu Y, Zhou QX (2005) Adsorption characteristics of pesticides methamidophos and glyphosate by two soils. Chemosphere 58:811–816