Vol.1, No.1, 20-27 (2012)
http://dx.doi.org/10.4236/jacen.2012.11004

Journal of Agricultural Chemistry and Environment

Biotransformation of endosulfan by the tiger worm, *Eisenia fetida*

Byeoung-Soo Park[1], Jae-Hong Yoo[2], Jeong-Han Kim[3], Jang-Eok Kim[4], Sung-Eun Lee[4*]

[1]Resarch Station, Nanotoxtech Inc., Ansan, South Korea
[2]Agricultural Microbiology Division, National Academy of Agricultural Science, RDA, Suwon, South Korea
[3]School of Agricultural Biotechnology, Seoul National University, Seoul, South Korea
[4]Division of Applied Biology and Chemistry, Kyungpook National University, Daegu, South Korea;
[*]Corresponding Author: selpest@knu.ac.kr

Received 6 October 2012; revised 9 November 2012; accepted 18 November 2012

ABSTRACT

This study assesses the role of the earthworm, *Eisenia fetida*, in the breakdown of endosulfan in a soil environment. Two strains of *E. fetida* were used in this study to assess the effect of salinity on toxicity and metabolism of endosulfan in these earthworms. One strain of *E. fetida* (R) was reared in high salinity soil (over 2.0 dS/m of electric conductivity) from Shiwha lake, Korea. A control strain (W) was reared in pig manure compost. Acute toxicity of endosulfan was lower in the R strain when endosulfan was injected. *In vitro* metabolic studies of endosulfan based on microsomal preparations showed that both strains produced two major metabolites, endosulfan sulfate and endosulfan diol. The production rate of endodulfan sulfate was not significantly different between the strains, while endosulfan diol production was significantly different. *In vivo* metabolism studies showed only one primary metabolite, endosulfan sulfate, was produced by both strains. HPLC-MS/MS analysis showed annetocin was the indicative protein newly expressed in the R strain in relation to salinity exposure. These findings suggest salinity may induce hydrolyzing enzymes to produce endosulfan diol from endosulfan.

Keywords: Endosulfan; Endosulfan Diol; Endosulfan Sulfate; *Eisenia fetida*; Salinity; LC-MS/MS; Annetocin

1. INTRODUCTION

Soil-dwelling invertebrates, especially earthworms, are often exposed to pesticides applied for the control of pests. As the broad-spectrum activities of a variety of pesticides are rarely limited to target pests, pesticides may be lethal to non-target avian, aquatic or terrestrial organisms. It is, therefore, necessary to develop an adequate evaluating method for determining impact of pesticides on non-target species. Several scientific approaches have demonstrated that earthworms, such as *Lumbricus terrestris* and *Eisenia fetida*, are excellent models for assessing effects of pesticides on soil-dwelling invertebrates [1-3]. Earthworms show high susceptibility to residual pesticides in soil. Standardized protocols have been proposed to determine pesticide toxicity [4,5]. Such screening tests generally determine effects of pesticides using filter paper or artificial soil containing quartz, kaolinite clay, sphagnum peat, and $CaCO_3$. On the other hand, earthworms are also used in acute toxicity, with plant seed germination, root elongation, and plant genotoxicity bioassyas, for the evaluation of post-emediation of a pollutant-contaminated soil [6].

Recently, many reports demonstrated the role of earthworms in soil ecosystems. Earthworms increase mineralization and humification of organic matter and transform biologically hazardous compounds to nontoxic metabolites. However, little information is available to understand metabolism of pesticides by earthworms, despite their importance in testing soil ecosystems. *L. terrestris* degraded isoproturon under laboratory conditions, after exposure to soils contaminated with different concentrations of the herbicide [3]. Interestingly, the herbicide did not show any lethal effect to the earthworms even at the highest concentration (1.4 g/kg soil) after 60 days of exposure. However, residues of the herbicide caused a significant reduction of in the growth rate and total soluble proteins. No metabolites of the herbicide were reported [3]. There are some reports on metabolism of industrial pollutants by earthworms. When earthworms were treated with antibiotics to minimize gut microflora, two xenobiotic compounds, 4-fluoroaniline and 4-fluorobiphenyl, were metabolized to *N*-*β*-glucoside conjugates,

at high doses, and to -glutamyl conjugates, at lower dose levels [7]. Earthworms also hydrolyze phthalic acid that is eventually degraded to CO_2 from procatechuic acid and *β*-carboxylmucoix acids with assistance of intestinal microflora of species of *Pseudomonas* [8].

Endosulfan (1,2,5,6,7,7-hexachloro-5-norbornene-2,3-dimethanolcyclic sulfite) is an organochlorine insecticide mainly used to control *Helicoverpa* species in the upland soil in Korea and this insecticide is highly toxic to fish [9]. Several intensive studies on the degradation of endosulfan have been conducted showing the primary metabolites to normally be endosulfan sulfate and endosulfan diol (endodiol) [10-14]. Endodiol is much less toxic to fish and other organisms than the parent compound. However, endosulfan sulfate has a similar toxicity as endosulfan and has a much longer half-life in the soil compared to endosulfan. Therefore, production of endosulfan sulfate is by biological systems possess an ecological hazard in that it contributes to long persistence of endosulfan in soil.

Herein, we report toxicity levels of endosulfan on and its metabolism by the earthworm *E. fetida* and how these can be influenced by salinity in the soil.

2. MATERIALS AND METHODS

2.1. Earthworms

E. fetida was obtained from Rural Development Administration, Suwon, Korea. The earthworms were held in a medium consisting of pig manure compost (S strain) and high salinity soil (over 2.0 dS/m of electric conductivity) obtained from Shiwha lake, Korea, (R strain) fortified with cornmeal at 13˚C. Only sexually mature worms weighing 300 - 500 mg having well-developed citella were used.

2.2. Chemicals

α-Endosulfan, *β*-endosulfan, endosulfan sulfate, endodiol, endosulfan ether, and endosulfan hydroxyether were purchased from Chem Service Inc. (West Chester, PA). $NaNO_3$, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $CaCl_2$, ferric citrate, and ethylenediaminetetraacetate (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals used were of the highest grade commercially available.

2.3. Toxicity Tests

Endosulfan was dissolved in a reagent grade of acetone, except for the injection experiments the compound was dissolved in ethanol. The ethanolic solutions were then diluted to an appropriate concentration with distilled water (1:99) as previously reported. Prior to injection, worms were anesthetized by placement on ice for 15 min. Ethanolic solutions (1 µL) of endosulfan were into the hemocoel, directly behind the citellum, using a microapplicator [1]. Mortality of worms exposed for 48 h to each concentration of pesticide was recorded. These data were used to estimate median lethal concentration (LC_{50}) using Probit analysis [15].

Another assay used the paper-contact toxicity method [4]. Briefly, sides of flat-bottom glass vials 8 cm in length and 3 cm in diameter were lined, with no overlap, with Whatman filter paper No. 1. Test chemicals were dissolved in acetone, predetermined concentrations were loaded on filter paper with 1 mL of solution, and vial was rotated for uniform distribution of endosulfan. Treated paper was allowed to dry using a slow stream of compressed air. Controls were also run in parallel with the carrier solvent alone. After drying, 1 mL of deionized water was added to each vial. The acclimated worms (fasted 3 h, on moist filter paper) were washed, dried and randomly divided into groups of 20 each. Worms were exposed (one earthworm per vial) to different concentrations. Mortality of worms exposed for 14 days to each concentration of pesticide was recorded. These data were used to estimate median lethal concentration (LC_{50}) using Probit analysis [15].

2.4. Preparation of Earthworms Homogenates

Approximately, 10 g of earthworms was homogenized in 30 mL 100 mM phosphate buffer, pH 7.4, using a glass homogenizer, on ice. The resultant homogenates were filtered through four layers of cheesecloth, the filtrate centrifuged at 12,100 g at 4˚C for 20 min using an Eppendorf Centrifuge 5417R and the supernatants reserved as crude enzyme extracts.

Crude extracts of earthworms were transferred to 15 mL polycarbonate ultracentrifuge tubes and centrifuged at 100,000 g at 4˚C for 1 h, including acceleration time, in a Beckman L8-M ultracentrifuge using a Ti 50 rotor. The supernatant was reserved as the cytosolic fraction. The microsomal pellet was washed twice and fully resuspended to a volume of 2 mL in resuspension buffer, 200 mM phosphate buffer, pH 7.4, 1 mM EDTA.

2.5. Metabolism of Endosulfan Using Microsomal Preparations

Metabolism of endosulfan was studied using an incubation mixture (250 µL final volume) consisting of 92 mM sodium phosphate buffer (pH 7.4), 0.5 mM NADPH, and 1.0 mg/mL protein of the microsomal preparations. After a pre-incubation period of 10 min at 37˚C, endosulfan was added as substrate to the reaction mixture. After 1 h, incubation reactions were stopped by adding 1 mL phosphoric acid. For extraction of endosulfan and

metabolites, ethyl acetate (1 mL) was added to the reaction mixture twice and vortexed for 2 min. Then, the mixture was centrifuged at 12,100 g for 10 min at room temperature. The ethyl acetate layer (2 μL) was analyzed by ECD-GC.

2.6. *In Vivo* Study of Endosulfan Metabolism by Earthworms in a Vial Coated with Endosulfan and Artificial Soil

Application of endosulfan (10 μL) was accomplished using a microapplicator (100 μL Hamilton Syringe fitted with a 27-gauge needle) into the bottom of assay vials. The solvent acetone was evaporated until dryness. Controls were only treated with acetone. Each vial contained one ml of deionized water and an earthworm. After 7 days, the earthworms were homogenized in 10 mL of acetone using a glass homogenizer immersed in ice. The homogenates were vortexed for 2 min and then centrifuged at 12,000 g for 10 min at 4˚C. Extracts were concentrated and redissolved in ethyl acetate to a final volume of 2 mL. Vials containing endosulfan residues and earthworm feces were extracted with 5 ml of acetone twice. The extracts were then evaporated and redissolved with ethyl acetate (2 mL). Aliquots were analyzed using ECD-GC.

A method was used to study metabolism of endosulfan by *E. fetida* in artificial soil [16], with the following changes: 1 mL of each concentration of endosulfan dissolved in acetone was mixed with the diets of earthworms (5 g/day) and dried for 1 h. The dried diet containing endosulfan was added to 200 g of soils consisting of quartz, kaolinite clay, sphagnum peat (7:3:1, w/w) and $CaCO_3$. Twenty worms were exposed to the diet per jar. Three replicates of each concentration were performed. Upper layer (5 cm from the surface) of the soils were extracted with 200 mL acetone three times by mechanical shaking for 3 h. Acetone solutions were evaporated and 2 mL of ethyl acetate were added. Aliquots (2 μL) were used to ECD-GC analysis.

2.7. ECD-GC Analyses

Remaining endosulfan and its metabolites were extracted as described above and the organic layer was collected and evaporated. Two milliliters of ethyl acetate was added to the dried sample, and a 2 μL volume of each extract was subjected to ECD-GC analysis (Agilent 4890D) with an electron capture detector. The column was an HP-1 (30 m × 0.25 mm i.d., film thickness 0.25 μm, Hewlett-Packard, CA, USA), and the oven temperature profile; 100 to 280˚C at 10˚C/min with a 3 min hold at 100˚C and 5 min hold at 280˚C. Nitrogen was used as the carrier gas at 1.0 mL/min. The temperature of injection port and detection block was 260 and 320˚C, respectively. The split ratio was 1/100. The retention time of endosulfan ether, endosulfan lactone, endodiol, α-endosulfan, β-isomer and endosulfan sulfate was 15.7, 17.6, 18.4, 19.0, 19.7, and 20.5 min, respectively.

2.8. Peptide Identification of Oxytocin-Vasopressin Neuropeptide by HPLC-ESI-MS/MS

All procedures were followed by the methods previously reported [17,18].

2.8.1. Sample Preparation

Each earthworm sample (0.6 ng protein/L) was reduced by DTT (dithiothreitol, Sigma) and alkylated with iodoacetic acid (1 M in NaOH solution, Sigma), then the reduced samples were treated with trypsin (1:50 w/w) at room temperature for 12 h.

2.8.2. Two-Dimensional HPLC and Mass Spectrometry Measurements

HPLC was performed on a Surveyor LC system (Thermo-Finnigan, San Jose, CA). Flow rate was maintained at 150 μL/min before splitting and at 1.5 μL/min after the flow split. The eluting gradient was started at 2% AcCN dissolved in 0.1% formic acid for 3 min, ramped to 60% AcCN in 180 min, and finally ramped to 80% AcCN for another 20 min. An aliquot (10 μL) of the sample solution (in a 100 μL sample loop) was injected by an autosampler onto a C-18 capillary column (Biobasic C-18, Thermo Keystone-Hypersil, 180 μm × 10 cm), which was connected to an ion source chamber (orthogonal) with a sheath gas flow at 3 units for MS analysis. The 2D separations were performed on a ProteomeX system, having an auto sampler, two HPLC pumps, a 10-port column-switching valve, and a Deca-XP^{Plus} iontrap mass spectrometer with a micro-electrospray interface. The 10-port valve allowed loading of a subsequent ion-exchange fraction onto the second C-18 column. For capillary separations, a flow rate of 2 μL/min was used. In the first step, a strong cation exchanger (BioBasic SCX, 0.32 mm × 10 cm, Thermo Keystone/Hypersil, Allentown, PA) was used, and then a reversed-phase (BioBasic C18, 300 Å, 5 μm silica, 180 μm × 10 cm, Thermo Keystone/Hypersil, Allentown, PA) capillary column was used for the second dimension.

2.8.3. Bioinformatics

Sequences of uninterpreted CID spectra were identified by correlation with peptide sequences present in the nonredundant protein sequence database (OWL Version 30.3) using the SEQUEST algorithm (Version C1) incorporated into ThermoFinnigan Bioworks (Version 3.1). The SEQUEST search results were initially assessed by

examination of the Xcorr (cross correlation) and ΔCn (delta normalized correlation) scores. Bioworks is a new version of TurboSequest in which the three matching factors (Sp, Xcorr, and ΔCn) are used to construct a unified ranking score.

2.9. Protein Determination

Protein was determined using 50 μL of sample solution and 2.5 mL of diluted (1 to 5) Bio-Rad Coomasie Blue Concentrate [19].

2.10. Statistical Analysis

For all assays conducted, the means of three replicates were compared and tested for significant differences to controls using Scheffe's test at the P = 0.05 level [20].

3. RESULTS AND DISCUSSION

LC_{50} and LC_{95} values for earthworms (*E. fetida*) exposed to endosulfan for 14 days, filter paper assay, and 2 days, injection assay, shown in **Table 1**. While no mortality was observed in control groups of the two strains, mortality in the endosulfan-treated groups increased with concentration of insecticide. Interestingly, the LC_{95} value for the salinity soil strain (R) was higher, and therefore less susceptible to endosulfan, in comparison to the normal soil strain (**Table 1**). Recently, toxicity of endosulfan was assessed for *L. terrestris* using the soil incorporation assay. The LC_{50} values of these tests were 12.29, 5.82, and 3.36 ppm for 2, 7, and 14 day exposure [21]. With our results, endosulfan toxicity to earthworms was quite apparent. Exposure to endosulfan resulted in reduction of total protein content and rate of growth [21]. Differences in LC_{95} values between the two strains examined suggested there may be, endosulfan metabolizing enzyme involved in its detoxification via production of non-toxic metabolites. Generally, salinity has no effect on uptake and elimination in aquatic species [22]. However, microsomal biotransformation by organisms present in a high salinity environment may prolong the pesticide toxicity by production of oxidative metabolites from the parent pesticide. For example, *in vitro* metabolic studies in a Japanese medaka, *Oryzia latipes*, showed 6-fold and 9-fold higher aldicarb sulfoxide production in a male and female, respectively, after exposure to a variety of salt concentrations. This enhanced production was not related to cytochrome P450-dependent monooxygenase [22,23]. Salinity-induced enhancement of aldicarb toxicity to *O. latipes* might be partly attributed to up-regulation of flavin-containing monooxygenases to produce sulfoxide, a potent inhibitor of acetylcholinesterase [23], while total cytochrome P450 does not appear to be altered by salinity [24]. Therefore, it was of interest to assess *in vitro* and *in vivo* endosulfan degradation in the two strains of *E. fetida*.

Another possible explanation for difference in endosulfan toxicity between the two strains is a lower penetration rate of endosulfan through dermis. Earthworms take up several organic compounds through their skin as well as from their foods [25]. When *E. fetida* was exposed to carbofuran and oxamyl in aqueous solution, carbofuran, which is more lipophilic and less water-soluble than oxamyl, was much more toxic [26]. The rate of absorption of carbofuran by the earthworm was 13-fold greater than for oxamyl [27]. Rates of uptake of pesticides from aqueous solutions by earthworms increased with increasing lipophilicity (log Kow or Log P) of the compounds [28].

Salinity may contribute to alterations in membrane lipids in the skin. Modifications in membrane lipid composition play a major role in adaptation of diverse organisms to specific environment and physiological circumstance [29]. Restructuring in acyl chain and molecular species in phospholipids are usual adaptations to environmental insult, being implicated in membrane adjustments to temperature, pressure, water activity, pH and salinity. In contrast, other adaptations [e.g. modulation of anionic phospholipids (salinity adaptation), trehalose content (dehydration) and PC/PE ratio (temperature acclimation)] appear to be more context specific [29]. Therefore, enhanced detoxifying enzymes and lower penetration of endosulfan via modifications in membrane lipid composition protect the earthworm strain living in a high salinity soil from endosulfan toxicity.

There are two important metabolic pathways for endosulfan degradation in biological systems, oxidation or hydrolysis. Endosulfan sulfate and endodiol are the representative products of oxidation and hydrolysis, respectively. Endodiol is a much less toxic metabolite to organisms, while endosulfan sulfate has a similar toxicity compared to the parent compound endosulfan. Endosulfan sulfate has a long persistence in the soil compared to

Table 1. Toxicity of endosulfan to two strains of the tiger earthworm, *Eisenia fetida*, via injection into the hemocoel: W, control earthworm grown in pig manure compost; R, a strain of *E. fetida* grown in the high salinity-soil from Shihwa lake, Korea.

Mortality	W (ppm, 95% confidence limits)	R (ppm, 95% confidence limits)	Ratio (R/W)
LC_{50}	2.54 (1.62 - 3.68)	3.55 (2.41 - 5.38)	1.4
LC_{95}	18.9 (10.1 - 80.2)	66.8 (27.9 - 406.4)	3.6

endosulfan [30]. Hence, with regard to the concept of environmental safety, detoxification of endosulfan by would be the preferred pathway.

In vitro metabolic studies showed the two major metabolites of endosulfan as endodiol and endosulfan sulfate produced by the different strains of *E. fetida* (**Table 2**). Interestingly, endodiol production was significantly different in that the wild strain produced less of the non-toxic metabolite, endodiol, than the high salinity strain. However, endosulfan sulfate formation was not significantly different (**Table 2**). On the other hand, *in vivo* metabolic studies showed only endosulfan sulfate was produced by both strains levels not statistically different. In contrast, many species of soil microbes produce two principle metabolites, endodiol and endosulfan sulfate (**Table 3**).

Several microorganisms including *Phanerochaete chrysosporium* [11,13], *Mucor thermohyalospora* MTCC 1384 [12], *Trichoderma harzianum* [11], and *Anabaena* sp. PCC 7120 produce endodiol and endosulfan sulfate [14]. Martens demonstrated that in flooded soil containing several fungi species degradation of endosulfan lead to production of endodiol as the primary metabolite followed by endosulfan sulfate [31]. Guerin determined endodiol was formed when endosulfan was incubated with bacteria under anaerobic conditions [32]. Recently, Lee et al. reported endosulfan was metabolized to endosulfan sulfate, endodiol, endosulfan ether, endosulfan hydroxyether, and endosulfan lactone in an aqueous nutrient medium inoculated with *Anabaena* sp. 7120 [14]. Sutherland *et al*. found a tentative metabolite endosulfan monoaldehyde, as a hydrolysis metabolite and endosulfan sulfate in a strongly buffered culture medium (pH 6.6), to minimize chemical hydrolysis [33].

The present study was also undertaken to determine if there was induction of certain protein in earthworms in relation to high soil salinity. After proteomics studies, we found an important neuropeptide, annetocin, in the earthworm grown in high salinity soil (**Figure 1**).

Neuropeptides in the annelid nervous central systems has been well documented. These peptides are considered to have hormonal-like activity [34]. Angiotensins, oxytoxin/vasopressin peptide family, and opoids are groups of neuroendocrine signaling molecules of annelids localized in ganglionic regions [34]. Angiotensins may play an important role in enhancing the increase in body weight and in osmoregulation [35]. The oxytoxin/vasopressin family commonly present in animal species shares at least five of nine residues and a disulfide-linked ring structure, which puts severe constraints on conformational flexibility [36].

An oxytocin-vasopressin-related peptide, Cys-Phe-Val-Arg-Asn-Cys-Pro-Thr-Gly-NH_2, was isolated from the lumbricid earthworm, *E. fetida* and termed annectocin [37]. Annetocin potentiated not only spontaneous contractions of the gut but also pulsatory contractions and bladder-shaking movement of nephridia. Annetocin may be involved in osmoregulation through nephridial function. Annetocin is an earthworm oxytocin-related peptide that induces egg-laying-like behaviors in *E. fetida* [38]. Annetocin expression in R-strain is directly related to the evidence that R-strain was exposed to higher salinity in comparison to the W-strain. It was not related to the enhanced metabolism or other aspects in the R-strain.

In conclusion, salinity may induce some osmoregulating peptide hormones in earthworms. However, it does not seem to be related to susceptibility to endosulfan toxicity. By direct injection of endosulfan, an earthworm strain from high salinity soil was less susceptible than a strain from normal soil. *In vitro* metabolic studies show-

Table 2. Microsomal metabolism of endosulfan by two different strains of the earthworm, *Eisenia fetida*: W, control earthworm grown in pig manure compost; R, a strain of *E. fetida* grown in the high salinity-soil from Shihwa lake, Korea.

Strain	Endodiol formation (nmoles/min/mg protein)	Endosulfan sulfate formation (pmoles/min/mg protein)
W	0.35 ± 0.029 *a*	8.93 ± 1.40 *a*
R	0.50 ± 0.075 *b*	13.1 ±2.80 *a*

The means in the column followed by the different italic letters are significantly different from the control ($P < 0.05$) using Sheffe's test when $n = 9$.

Table 3. *In vivo* metabolism of endosulfan to endosulfan sulfate (ES) by two different strains of the earthworm, *Eisenia fetida*: W, control earthworm grown in pig manure compost; R, a strain of *E. fetida* grown in the high salinity-soil from Shihwa lake, Korea. The studies were undertaken in two different assay systems as earthworms in endosulfan containing vials without food or soil and earthworms in endosulfan containing diet on artificial soils.

Strain	ES production in vial application (ppm, 7 days incubation)	ES production in artificial soils (ppm, 14 days incubation)
W	0.27 ± 0.063 *a*	0.19 ± 0.02 *a*
R	0.24 ± 0.079 *a*	0.13 ± 0.04 *a*

The means in the column followed by the different italic letters are significantly different from the control ($P < 0.05$) using Sheffe's test when $n = 9$.

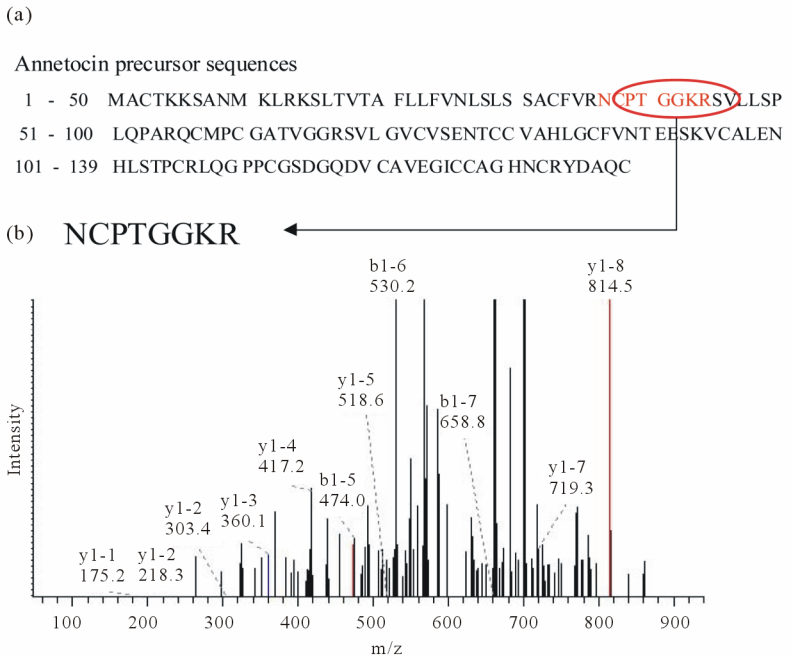

Figure 1. Annetocin precursor sequence (a) and its mass spectrum (b) using LC MS/MS.

ed a higher rate of endodiol formation in the high salinity soil strain compared to a wild strain. Earthworms can metabolize injected endosulfan into two principal metabolites, endodiol and endosulfan sulfate. But only endosulfan sulfate is produced when earthworms are exposed to endosulfan from the soil, *in vivo*. Our results show that earthworms may contribute to prolonged endosulfan toxicity in soil systems through production of endosulfan sulfate.

4. ACKNOWLEDGEMENTS

This work was carried out with the support of "Cooperative Research Program for Agriculture & Technology Development (Project No. PJ008544)" Rural Development Administration, Republic of Korea.

REFERENCES

[1] Fisher, S.W. (1984) A comparison of standardized methods for measuring the biological activity of pesticides to the earthworm, *Lubricus terrestris*. *Ecotoxicology and Environmental Safety*, **8**, 564-571. doi:10.1016/0147-6513(84)90016-2

[2] Addison, J.A. and Holmes, S.B. (1995) Comparison of forest soil microcosm and acute toxicity studies for determining effects of fenitrothion on earthworms. *Ecotoxicology and Environmental Safety*, **30**, 127-133. doi:10.1006/eesa.1995.1016

[3] Mosleh, Y.Y., Paris-Palacios, S., Couderchet, M. and Vernet, G. (2003) Effects of the herbicide isoproturon on survival, growth rate, and protein content of mature earthworms (*Lumbricus terrestris* L.) and its fate in the soil. *Applied Soil Ecology*, **23**, 69-77. doi:10.1016/S0929-1393(02)00161-0

[4] OECD (1984) Earthworms acute toxicity tests. In: *OECD Guidelines for Testing of Chemicals* (*TEST* 207), Organization for economic co-operation and development (OECD), Paris.

[5] Zhou, S.P., Duan, C.Q., Fu, H., Chen, Y.H., Wang, X.H. and Yu, Z.F. (2007) Toxicity assessment for chlorpyrifos-contaminated soil with three different earthworm test methods. *Journal of Environmental Sciences*, **19**, 854-858. doi:10.1016/S1001-0742(07)60142-9

[6] Chang, L.W., Meier, J.R. and Smith, M.K. (1997) Application of plant and earthworm bioassays to evaluate remediation of a lead-contaminated soil. *Archives of Environmental Contamination and Toxicology*, **32**, 166-171. doi:10.1007/s002449900170

[7] Bundy, J.G., Lenz, E.M., Osborn, D., Weeks, J.M., Lindon, J.C. and Nicholson, J.K. (2002) Metabolism of 4-fluoroaniline and 4-fluorobiphenyl in the earthworm *Eisenia veneta* characterized by high-resolution NMR spectroscopy with directly coupled HPLC-NMR and HPLC-MS. *Xenobiotica*, **32**, 479-490. doi:10.1080/00498250210124156

[8] Albro, P.W., Corbett, J.T. and Schroeder, J.L. (1993) The metabolism of di(2-ethylhexyl)phthalate in the earthworm *Lubricus terrestris*. *Comparative Biochemistry and Physiology C*, **104**, 335-344.

[9] Gupta, P.K. and Gupta, R.C. (1979) Pharmacology, toxicology and degradation of endosulfan: A review. *Toxicology*, **13**, 115-130.

[10] Katayama, A. and Matsumura, F. (1993) Degradation of

organochlorine pesticides, particularly endosulfan, by *Trichoderma harzianum*. *Environmental Toxicology and Chemistry*, **12**, 1059-1065.

[11] Kullman, S.W. and Matsumura, F. (1996) Metabolic pathways utilized by *Phanerochaete chrysosporium* for degradation of the cyclodiene pesticide endosulfan. *Applied and Environmental Microbiology*, **62**, 593-600.

[12] Shetty, P.K., Mitra, J., Murthy, N.B.K., Namitha, K.K., Savita, K.N. and Raghu, K. (2000) Biodegradation of cyclodiene insecticide endosulfan by *Mucor thermo-hyalospora* MTCC 1384. *Current Science*, **79**, 1381-1383.

[13] Kim, Y.K., Kim, S.H. and Choi, S.C. (2001) Kinetics of endosulfan degradation by *Phanerochaete chrysosporium*. *Biotechnology Letters*, **23**, 163-166. doi:10.1023/A:1010308332581

[14] Lee, S.E., Kim, J.S., Kennedy, I.R., Park, J.W., Kwon, G.S., Koh, S.C. and Kim, J.E. (2003) Biotransformation of an organochlorine insecticide, endosulfan, by *Anabaena* species. *Journal of Agricultural Food and Chemistry*, **51**, 1336-1340. doi:10.1021/jf0257289

[15] Finney, D.J. (1971) Probit analysis. 3rd Edition, Cambridge University, London.

[16] Karnak, R.E. and Hamelink, J.L. (1982) A standardized method for determining the acute toxicity of chemicals to earthworms. *Ecotoxicology and Environmental Safety*, **6**, 216-222. doi:10.1016/0147-6513(82)90012-4

[17] Son, J., Lee, S.E., Park, B.S., Jung, J., Park, H.S., Bang, J.Y., Kang, G.Y. and Cho, K. (2011) Biomarker discovery and proteomic evaluation of cadmium toxicity on a collembolan species, *Paronychiurus kimi* (Lee). *Proteomics*, **11**, 2294-2307. doi:10.1002/pmic.200900690

[18] Lee, S.E., Yoo, D.H., Son, J. and Cho, K. (2006) Proteomic evaluation of cadmium toxicity on the midge *Chironomus riparius* Meigen larvae. *Proteomics*, **6**, 945-957. doi:10.1002/pmic.200401349

[19] Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*, **72**, 248-254. doi:10.1016/0003-2697(76)90527-3

[20] SAS (1995) SAS user's guide: Statistics. SAS Institute: Cary.

[21] Mosleh, Y.Y., Paris-Palacios, S., Couderchet, M. and Vernet, G. (2003) Acute and sublethal effects of two insecticides on earthworms (*Lumbricus terrestris* L.) under laboratory conditions. *Environmental Toxicology*, **18**, 1-8. doi:10.1002/tox.10095

[22] El-Alfy, A.T., Bernache, E. and Schlenk, D. (2002) Gender differences in the effect of salinity on aldicarb uptake, elimination, and *in vitro* metabolism in Japanese medaka, *Oryzias latipes*. *Aquatic Toxicology*, **61**, 225-232. doi:10.1016/S0166-445X(02)00059-0

[23] El-Alfy, A.T. and Schlenk, D. (1998) Potential mechanisms of the enhancement of aldicarb toxicity to Japanese medaka, *Oryzias latipes*, at high salinity. *Toxicology and Applied Pharmacology*, **152**, 175-183. doi:10.1006/taap.1998.8479

[24] Stegeman, J.J. and Hahn, M. (1994) Biochemistry and molecular biology of monooxygenase: Current perspectives on forms, functions and regulation of cytochrome P450 in aquatic species. In: Malin, D and Ostrander, G. Eds., *Aquatic Toxicology*: *Molecular*, *Biochemical*, *and Cellular Perspetives*, Boca Raton, 87-204.

[25] Jager, T., Fleuren, R.H., Hogendoorn, E.A. and De Korte, G. (2003) Elucidating the routes of exposure for organic chemicals in the earthworm, *Eisenia andrei* (Oligochaeta). *Environmental Science and Technology*, **37**, 3399-3404. doi:10.1021/es0340578

[26] Stenerson, J. (1979) Action of pesticides on earthworms. Part I. The toxicity of cholinesterase inhibiting insecticdes to earthworms as evaluated by laboratory tests. *Pesticide Science*, **10**, 66-74. doi:10.1002/ps.2780100109

[27] Stenerson, J. and Øien, N. (1980) Action of pesticides on earthworms. Part IV. Uptake and elimination of oxamyl compared with carbofuran. *Pesticide Science*, **11**, 396-400. doi:10.1002/ps.2780110405

[28] Lord, K.A., Briggs, G.G., Neale, M.C. and Manlove, R. (1980) Uptake of pesticides from water and soil by earthworms. *Pesticide Science*, **11**, 401-408. doi:10.1002/ps.2780110406

[29] Hazel, J.R. and Williams, E.E. (1990) The role of alterations in membrane lipid composition in enabling physiological adaptation of organisms to their physical environment. *Progress in Lipid Research*, **29**, 167-227. doi:10.1016/0163-7827(90)90002-3

[30] Guerin, T.F. and Kennedy, I.R. (1992) Distribution and dissipation of endosulfan and related cyclodienes in sterile aqueous systems: implications for studies on biodegradation. *Journal of Agricultural Food and Chemistry*, **40**, 2315-2323. doi:10.1021/jf00023a052

[31] Martens, R. (1977) Degradation of [8,9-^{14}C]endosulfan by soil microorganisms. *Applied and Environmental Microbiology*, **31**, 853-858.

[32] Guerin, T.F. (1999) The anaerobic degradation of endosulfan by indigenous microorganisms from low-oxygen soils and sediments. *Environmental Pollution*, **106**, 13-21. doi:10.1016/S0269-7491(99)00067-6

[33] Sutherland, T.D., Horne, I., Lacey, M.J., Harcourt, R.L., Russell, R.J. and Oakeshott, J.G. (2000) Enrichment of an endosulfan degrading mixed bacterial culture. *Applied and Environmental Microbiology*, **66**, 2822-2828. doi:10.1128/AEM.66.7.2822-2828.2000

[34] Lefebvre C. and Salzet M. (2003) Annelid neuroimmune system. *Current Pharmaceutical Design*, **8**, 99-110.

[35] Fewou, J. and Dhainaut-Courtois, N. (1995) Research on polychaete annelid osmoregulatory peptide(s) by immunocytochemical and physiological approaches. Computer reconstruction of the brain and evidence for a role of angiotensin-like molecules in Nereis (Hediste) diversicolor of muller. *Biology of the Cell*, **85**, 21-33.

[36] Satake, H., Takuwa, K., Minakata, H. and Matsushima, O. (1999) Evidence for conservation of the vasopressin/oxytocin superfamily in Annelida. *Journal of Biological Chemistry*, **274**, 5605-5611. doi:10.1074/jbc.274.9.5605

[37] Fujino, Y., Nagahama, T., Oumi, T., Ukena, K., Morishita, F., Furukawa, Y., Matsushima, O., Ando, M., Takahama,

H., Satake, H., Minakata, H. and Nomoto, K. (1999) Possible functions of oxytocin/vasopressin-superfamily peptides in annelids with special reference to reproduction and osmoregulation. *Journal of Experimental Zoology*, **284**, 401-406. doi:10.1002/(SICI)1097-010X(19990901)284:4<401::AID-JEZ6>3.0.CO;2-U

[38] Oumi, T., Ukena, K., Matsushima, O., Ikeda, T., Fujita, T., Minakata, H. and Nomoto, K. (1994) Annetocin: An oxytocin-related peptide isolated from the earthworm, *Eisenia foetida*. *Biochemical Biophysiscal Research Communication*, **198**, 393-399. doi:10.1006/bbrc.1994.1055