

# The Assessment of Toxicity on Organic Sludge Using Acetylcholinesterase, Cytochrome P<sub>450</sub>, and Hsp70 Extracted from Earthworm (*Eisenia fetida*)

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The toxicity of organic sludge such as municipal sewage sludge (MSS), industrial sewage sludge (ISS), alcohol fermentation processing sludge (AFPS) and leather processing sludge (LPS) were evaluated with three environmental biomarkers as acetylcholinesterase, cytochrome P<sub>450</sub>, and heat shock protein 70 extracted from earthworm (*Eisenia fetida*). Their toxicities were compared with those of pig manure compost (PMC). MSS, ISS, LPS, and AFPS did not significantly affect the acetylcholinesterase activity, whereas only the elutriate of PMC slightly was increased the activity. MSS, AFPS, and PMC tended to slightly inhibit the cytochrome P<sub>450</sub> activity, but ISS and LPS showed significantly the inhibitory effect on cytochrome P<sub>450</sub>. The hsp70 expression began to increase after treatments and showed high induction at 6 hour, followed by zero level at around 12 hour. The quantity of the hsp70 expressed by elutriate treatments of PMC, AFPS, MSS, ISS, and LPS was 1.9, 3.0, 3.3, 4.4, and 4.7 fold higher than that of distilled water. These results indicate that in toxicity tests of five organic waste materials, four kinds of sludge materials appeared more toxic than PMC. Results of AChE, P<sub>450</sub>, and hsp70 of earthworm might be useful for expecting or assessing an effect by exposure of organic wastes to earthworms in soil.

**Key words:** Earthworm, Organic waste, Heavy metals, Contaminants. Acetylcholinesterase, Cytochrome P<sub>450</sub>, Heat shock protein 70

## Introduction

The annual production of sewage sludge amounts up to two and half million tonnes in 2005 in Korea (Anonymous, 2006). Disposal of organic sludge from wastewater treatment plants is of considerable environmental concern. In many countries, current rules for controlling the use of organic wastes on agricultural land have been criticized because they apparently do not take into consideration of the potential adverse effects of inorganic heavy metals and organic compounds produced in organic waste-treated soils on soil organisms (McGrath, 1994).

Recently, the use of biological markers or biomarkers measured at the molecular or cellular level have been proposed as sensitive 'early warning' tools for biological effect measurement in environmental quality assessment

(McCarthy and Shugart, 1990). One of the most important features of molecular/cellular biomarkers is that they have the potential to anticipate changes at higher levels of biological organization, i.e. population, community or ecosystem. Thus these 'early warning' biomarkers can be used in a predictive way, allowing the initiation of bioremediation strategies before irreversible environmental damage of ecological consequences occurs.

Objectives of this study were to assess toxicity for four organic sludges and pig manure using three environmental biomarkers such as acetylcholinesterase, cytochrome P<sub>450</sub>, and heat shock protein 70 extracted from the earthworm.

## Materials and Methods

**Chemical properties of four sludges and pig manure compost** Four waste materials used in this study were as follows: municipal sewage sludge (MSS) collected from

municipal waste treatment plants in 1994 on Gwacheon city, Gyeonggi Province, Korea; industrial sewage sludge (ISS) and alcohol fermentation sludge (AFPS) from Ansan industrial complex, Gyeonggi Province, in 1994; leather processing sludge (LPS) from leather treatment plant on Cheongju, Chungnam Province, Korea, in 1994. Pig manure compost (PMC) was purchased from Anjung Nong-hyup located in Anjung, Gyeonggi Province, in 1994. These materials were kept in deep-freezer to be applied annually from 1994 to 2001.

Chemical properties of test waste materials and pig manure were described by Na (2004). MSS had characteristics that all components of heavy metal were low level in comparison with legally allowed limit values (Cu 300, Zn 900, Cr 300, Cd 5, Pb 150, Ni 50 mg kg<sup>-1</sup>) for potentially toxic elements, although the content of Zn was relatively high as 797 mg kg<sup>-1</sup>. ISS was characterized by the excess level in four components that the contents of Cu, Zn, Cr, and Ni appeared 19.3, 3.1, 3.7, and 22.8 times higher than limit values respectively. LPS was marked by the excess level in two heavy metals that the contents of Cr and Cd were 34.3 and 1.5 times higher than limited values respectively. AFPS contained 56 mg kg<sup>-1</sup> of Ni and this value was 1.5 times higher than the limited value. PM had characteristics that one component, Cu was 1.9 times higher in comparison with limited level and the other components were low levels except for Zn. In terms of total PAHs concentrations, AFPS was the highest (1,221.7) followed by MSS (928.4), LPS (369.4), ISS (206.6), and PMC (168.7 g kg<sup>-1</sup>).

**Test elutriates of four sludges and pig manure compost** The volumes of water and wet waste to be added into a jar to hold 800 ml of distilled water plus 200 g of dry waste in elutriate jar were calculated as follows:

$$\begin{aligned} &\text{Water added to elutriate jar (ml)} \\ &= (800 \text{ ml}) - (\text{water content} \times 200 \text{ g dry sample}) \\ &\text{Wet waste weight (g)} \\ &= (200 \text{ g dry sample}) + (\text{water content} \times 200 \text{ g dry sample}) \end{aligned}$$

The calculated amount of wet sample was placed into a elutriate jar and then inputted the amount of calculated deionized water. The content of the jar was mixed end-over-end in darkness at 20°C ± 2 for 48 hr. The suspension was centrifuged at 10,000 g for 10 min at 4°C.

**Test organism** The earthworms, *Eisenia fetida* were maintained in an artificial soil after catching them from

the heap of compost made from rice straw. They were kept in a darkened incubator at 21 ± 1°C. The adult earthworms with well-developed clitellum were used in this assay.

**Acetylcholinesterase activity** The effect of elutriates on acetylcholinesterase activity was determined by the method of Ellman *et al.* (1961). A homogenate of 10 earthworms (3 g) was made in 20 ml of 0.1 M phosphate buffer (pH 7.5) using a glass homogenizer. This procedure was repeated three times. The homogenates were centrifuged at 10,000 g for 20 min at 4°C and the supernatant was used as crude extract. The reaction mixture was consisted of 0.1 ml of samples (each elutriate, 250 ppm of chlorpyrifos-oxon), 0.58 ml of 0.1 M phosphate buffer (pH 7.5), 0.1 ml of 0.01 M DTNB, 20 µl of 0.075 M ATChI, and 0.2 ml of homogenate in a total volume of 1.0 ml. 250 ppm of chlorpyrifos-oxon was used as positive control because chlorpyrifos was reported as inhibitor of AChE and its predicted environmental concentration (PEC) was 250 ppm in soil. Optical density at 412 nm was read on a spectrophotometer (Pharmacia Biotech, Ultrospec® 2000, Sweden) for 200 s. since the reaction mixture was started.

**Cytochrome P450 activity** For preparation, earthworms were kept on moist paper towels at room temperature up to four days to completely purge the gut of the earthworm. Contents (soil, organic matter, etc) in gut was completely removed for this time. After being inactive in a refrigerator at 8°C, earthworms were immersed afterwards in ice-cold glycerol solution (20% glycerol in distilled water) for about 1.5 hr. After removal of the head and tail, the animals were cut into five to six pieces. All subsequent operations were carried out at 4°C. The pieces were transferred into homogenization buffer HB1 [250 mM sucrose, 50 mM Tris (pH 7.5), 1 mM DTT (dithiothreitol), 1 mM EDTA (ethylenediaminetetra - acetic acid)]. After about 30 min, they were collected and homogenized in 10 volumes of HB1 in a glass homogenizer. After filtering the crude homogenate through gauze, a second homogenization was performed. The crude homogenate was centrifuged at 10,000 g for 20 min. Microsomal fractions were used immediately or after storing at -70°C. The effect of the elutriate on cytochrome P<sub>450</sub> activity of the earthworm microsomal fraction was determined with the inhibition level of

desulfuration activity. Desulfuration activity was determined with chlorpyrifos to chlorpyrifos-oxon as a substrate. Chlorpyrifos oxon produced was determined using GLC and an electron capture detector (ECD). The adequate volume of chlorpyrifos oxon solution was analyzed using GLC (3 m × 2.0 mm custom made unsilanised borosilicate glass column containing SE-30 (5%): DC-200 (5%) (3:1) on Gaschrom Q 80/100 mesh; column temperature 210°C with an ECD. A standard curve was prepared in the range of 0 to 5 pmol for chlorpyrifos oxon. All measurements were carried out in triplicate.

**Hsp70 induction** To determine content of stress protein (hsp 70), earthworms were kept in plastic petri dishes (90 mm diameter). The dishes were bedded with wetted filter paper after immersion of an earthworm into 20 ml solution of each elutriate and chlorpyrifos for 5 min and were sampled after 0, 3, 6, and 12 hr. For total RNA extraction, tissue samples that earthworms were cut into two or three pieces after removal of the head and tail were homogenized in TRI Reagent (100 mg tissue / 1 ml, Sigma) using a glass-Teflon. The total RNA amount was determined by spectrophotometer and then stored at -85°C until it was used.

The synthesis of the first strand of cDNA with RNA pellet was performed by adding 1 l of oligo (dT) 12-18 and 0.4 µl of 25 mM deoxynucleotide triphosphate (equimolar solution of dATP, dCTP, dGTP, and dTTP at neutral pH) to 1 g of total RNA using semi-quantitative reverse transcription and polymerase chain reaction (RT-PCR). This method was described by Ducas et al. (1993). PCR was performed with specific primer as follows:

Hsp 70 (forward; 5'-CGCTGGCTCGACTCCAACCAG-3' and reverse; 5'-ATGGTTGGTCCGCCAGAT-3'),

β-actin (forward; 5'-TATACTCCGCGCATGGTGTC-3' and reverse; 5'-CGTAGCACAGCTTCTCCTTG-3').

The β-actin of earthworm was used as an internal control to normalize sample for variations in the amount of initial RNA. To quantitate PCR products, each sample was electrophoresed in 8% polyacrylamide gel and stained with ethidium bromide. Band intensity was measured with an OptiQuant (v2.0) program (Packard bioscience). The value of hsp70 induction was calculated as the ratio of the amount of PCR product of hsp70 to that of control treated with distilled water.

**Protein assay** Protein was determined by the method of Bradford (1976) using 50 l of sample solution and 2.5 ml of diluted (1 to 5) coomassie blue concentrate.

**Statistical analysis** Percentage mortality was determined and transformed to arcsine square root values for analysis variance (ANOVA). Treatment means were compared and separated by Scheffe test at  $P = 0.05$  (SAS Institute, 1990). Means ± SE of untransformed data are reported.

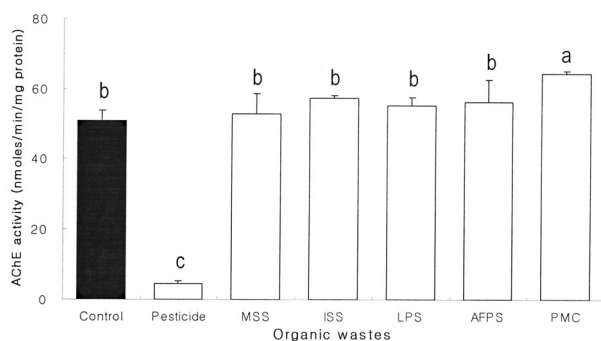
## Results and Discussion

Exposure to a contaminant may cause some physiological disruptions or morphological abnormalities of earthworms. Pižl and Sterzynska (1991) reported that rates of infection of earthworms by pathogens such as monocystid gregarines was significantly greater in field plots adjacent to roads than in plots in centers of parks, and they concluded that pollution stress including heavy metal may be increased the susceptibility of earthworms to infections by these pathogens. Additionally, earthworms in soil intensively treated with herbicides were easily infected by the pathogens (Pi 1, 1985), and a variety of types of morphological abnormalities including rupture of the cuticle, body constrictions and swellings, and fragmentation of posterior pars, or inhibition of AChE in them were caused by treatments of insecticides such as chlorpyrifos (Venkateswara Rao *et al.*, 2003).

Organic sludge and soil contaminants such as PAHs are generally ubiquitous ones that can be found at very high concentrations in sludge and contaminated soils (Walsh *et al.*, 1997; Na, 2004). PAHs are products of combustion processes of carbonaceous fuels and they are composed of two or more fused aromatic (benzene) rings. In this study, effects of organic wastes on AChE activity, cytochrome P<sub>450</sub> activity, and hsp70 induction of *E. fetida* were elucidated. PAHs including benzo(a)pyrene are known to be metabolized either by cytochrome P450-dependent monooxygenases or by the generation of free radicals (Saint-Denis *et al.* 1999).

AChE is responsible for hydrolysing acetylcholine into choline and acetic acid. Its inhibition is linked directly with the mechanism of toxic action of organophosphorus and carbamate insecticides, namely irreversible or reversible binding to the catalytic site of the enzyme, and potentiation of cholinergic effects. The quantification of this enzyme was applied to laboratory and field studies

with both vertebrates and invertebrates to assess exposure to organophosphorus and carbamate insecticides (Mineau, 1991). Anticholinesterase insecticides and a few other contaminants including mercury are good inhibitors (Stansley, 1993; Escartin and Porte, 1996). In this study, MSS, ISS, LPS, and AFPS did not produce significant inhibitory effect on AChE activity. However, PMC produced significant boot-up effect, although it was a slight (Fig. 1). These results indicate that MSS, ISS, LPS, and AFPS might not contain substances to inhibit the AChE, and PMC includes a constituent to enhance its activity. However, the exact mode of action for the boot-up effect by PMC remains unknown.

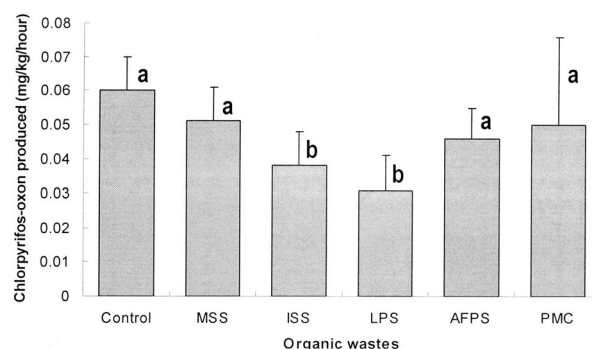


**Fig. 1. Effect of elutriates on acetylcholinesterase activity.**

Bars with the different letters were significantly different beyond the level of  $p < 0.05$ .

The cytochrome P<sub>450</sub> monooxygenase system comprises a family of structurally and functionally related heme proteins. In this family, multiple subfamilies are recognised to be active in the oxidative metabolism of diverse substrates, including drugs and environmental chemicals, as well as endogenous compounds such as steroids, neurohormones, fatty acids, and prostaglandins (Nelson *et al.*, 1996). The cytochrome P4501A (CYP1A) subfamily has attracted particular attention due to its key role in the biotransformation of many foreign compounds including dioxins, furans, PCBs, and PAHs (Nebert, 1989; Parkinson, 1995). Exposure of organisms to these compounds leads to an induction of CYP1A via the cytosolic Ah (arylhydrocarbon) receptor (Hankinson, 1995). The induced CYP1A protein catalytically converts the lipophilic xenobiotics to more water soluble compounds, and the step is the first for excretion and detoxification of toxic materials. In this study, MSS, AFPS, and PMC did not produce a significant inhibitory effect on the P<sub>450</sub> activity. However, ISS and LPS showed a potent inhibitory activity (Fig.2). ISS and LPS caused

potent mortality by inhibiting the P450 activity of earthworm. These data might explain the result of high toxicity obtained from the microcosm soil test in which ISS and LPS-treated soils caused the high mortalities of earthworms (Na *et al.*, 2007).

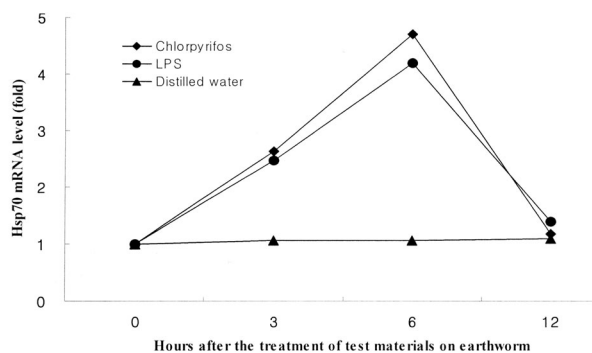


**Fig. 2. Effect of elutriates on the cytochrome P450 desulfuration activity.**

Bars with the different letters were significantly different beyond the level of  $p < 0.05$ .

Many organisms are able to synthesize proteins which offer some protection from cellular damages (Hartl, 1996). These proteins were first described in cells from *Drosophila melanogaster* Meigen during exposures to high temperature (Ritossa, 1962), and so the term "heat shock protein" (hsp) was coined. Since then, a range of environmental stresses have been known to induce heat shock proteins, and the term "Stress Protein" has subsequently been used to describe these proteins. The environmental stresses which can induce these proteins include trace metal exposure (Williams *et al.*, 1996), organic pollutants (Sanders, 1990), changes in temperature (Currie and Tufts, 1997), hypoxia and anoxia (Myrnel *et al.*, 1994), and ultraviolet radiation (Nepple and Bachofen, 1997). In the present study, the hsp70 expression began to increase after treatments and showed high induction at 6 hr, followed by zero level at around 12 hr (Fig.3). The quantity of the hsp70 expressed by treatments of PMC, AFPS, MSS, ISS, and LPS was 1.9, 3, 3.3, 4.4, and 4.7 fold higher than that with distilled water (Fig. 4). These results show that the stress of earthworm gave the most weak by PMC and was much more strong by ISS and LPS. These data might explain the result of toxicity levels obtained from the microcosm soil test that PMC-treated soil did not affect survival rate of the earthworm, whereas MSS and AFPS-treated soil began to the death after 8 weeks and ISS and LPS-treated soils caused the death of earthworms after 4 weeks (Na *et*





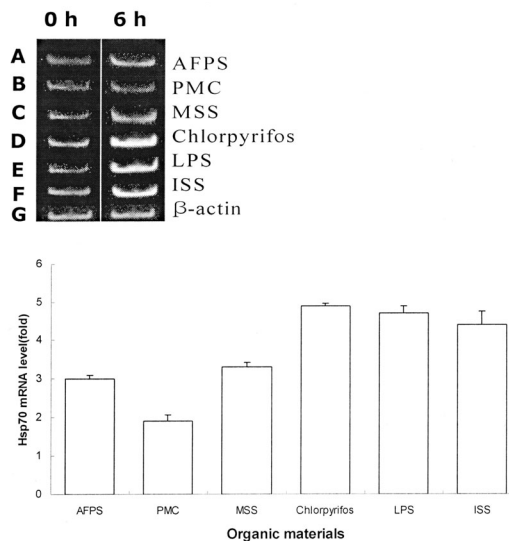
**Fig. 3.** Effect of chlorpyrifos and LPS on hsp70 mRNA accumulation.

al., 2007).

Results of AChE, P<sub>450</sub>, and hsp70 of earthworm and the microcosm soil test (Na *et al.*, 2007) about the same organic sludges might be useful for expecting or assessing an effect by exposure of organic wastes to earthworms in soil.

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**Fig. 4.** Expressed electrophoresis (upper) and quantitation (lower) of hsp70 mRNA after 6 hours exposure of earthworm on chlorpyrifos, four organic sludges, and pig manure compost.

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## 지렁이에서 추출한 Acetylcholinesterase, Cytochrome P<sub>450</sub>, and Heat shock protein 70을 이용한 유기성슬러지 독성 평가

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4 종류의 폐기물(생활하수오니, 공단하수오니, 피혁오니, 주정오니)과 대조구로서 돈분퇴비가 지렁이에게 미치는 독성을 평가하기 위하여 대표적인 유해성 평가 biomarker 3종류 (acetylcholinesterase, cytochrome P<sub>450</sub>, heat shock protein 70)를 사용하였다. 유기성 폐기물에 대한 acetylcholinesterase의 활성은 돈분퇴비의 경우 활성이 약간 촉진된 반면 생활하수오니, 공단하수오니, 피혁오니, 주정오니는 영향을 미치지 않았다. Cytochrome P<sub>450</sub>의 활성은 공단하수오니와 피혁오니는 활성을 억제하였고 생활하수오니, 주정오니, 돈분퇴비는 영향을 미치지 않았다. 또한 Hsp70의 발현량은 증류수보다 돈분퇴비는 1.9배, 주정오니는 3.0배, 생활하수오니는 3.3배, 공단오니는 4.4배, 피혁오니는 4.7배 순으로 지렁이 (*Eisenia fetida*)에게 스트레스를 많이 주었다. 이상의 결과로부터, 4 종류의 폐기물(생활하수오니, 공단하수오니, 피혁오니, 주정오니)은 돈분퇴비보다 독성이 강한 것으로 판단하였다. 또한 AChE, Cytochrome P<sub>450</sub>과 Hsp70은 추후 유기성 폐기물의 유해성을 모니터링하기에 적합한 biomarker로서 가치가 있다고 생각한다.