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# Highly selective biomarkers for pesticides developed in *Eisenia fetida* using SELDI-TOF MS

*Doo-San Park*[a,1], *Hwang-Ju Jeon*[a,1], *Eun-Sil Park*[a], *In Kyung Bae*[a], *Yong-Eun Kim*[b], *Sung-Eun Lee*[a,*]

[a] *School of Applied Biosciences, Kyungpook National University, Daegu 702-701, Republic of Korea*
[b] *Division of Environmental Science and Ecological Engineering, Korea University, Seoul 136-713, Republic of Korea*

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ABSTRACT

The repeated use of pesticides, and their subsequent residues, has contributed to severe adverse effects on the environment, including risks to human health. Therefore, it is important to assess the quality of the environment to ensure it remains free from pesticide residues. The six pesticides tested in this study showed high mortality on *Eisenia fetida* with $LC_{50}$ values ranging from 7.7 to 37.9 g $L^{-1}$. The strongest lethal effect resulted from the organochlorine insecticide endosulfan ($LC_{50}$ = 7.7 g $L^{-1}$). Following exposure to the carbamate pesticides, acetylcholinesterase activity in *E. fetida* decreased dramatically in comparison to the control. Carboxylesterase activity was only lowered in *E. fetida* exposed to propoxur, when compared to the control. The remaining five pesticides had no significant effect on carboxylesterase activity in *E. fetida*. In order to discover pesticide-specific biomarkers with differentially expressed proteins after exposure to pesticides, protein patterns of pesticide-treated *E. fetida* were analyzed using SELDI-TOF MS with Q10 ProteinChips. Protein patterns were compared with their intensities at the same mass-to-charge ratios (*m*/*z*). All 42 peaks had intensities with associated *p*-values less than 0.089, and 40 of these peaks had associated *p*-values of 0.05. Using SELDI-TOF MS technology, selective biomarkers for the six pesticides tested were found in *E. fetida*; four proteins with 5425, 5697, 9523, and 9868 *m*/*z* were consistently observed in the earthworms following exposure to the carbamates.



## 1. Introduction

Pesticides are typically applied during crop cultivation to efficiently control a variety of agricultural pests and maintain the standards of agricultural products to a level suitable for human and livestock consumption. However, the repeated use of pesticides, and the residues that remain following application, has contributed to severe adverse effects on the environment, including risks to human health (Damalas and Eleftherohorinos, 2011). Therefore, it is important to assess the quality of the environment to ensure it remains healthy, with no pesticide residue. It is, thus, really important to develop accurate, precise detection methods to assess the present state of the environment and to validate the effects caused by residual pesticides (Kapka-Skrzypczak et al., 2011).

* Corresponding author. Tel.: +82 53 950 7768; fax: +82 53 953 7233.
E-mail address: selpest@knu.ac.kr (S.-E. Lee).
[1] These authors equally contributed to this paper as first authors.

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In this respect, biomarker discovery for environmental purposes plays an important role in building a safety zone for expectation of less pesticide contamination.

Biomarkers found in aquatic and terrestrial organisms following exposure to pesticides have been intensively studied in order to predict the presence of pesticides in the environment (Henson-Ramsey et al., 2011; Jamec et al., 2010). Recently, many studies have reported the use of terrestrial organisms for developing biomarkers in response to residual pesticides (Henson-Ramsey et al., 2011; Radwan and Mohamed, 2013; Stepić et al., 2013), and among these, earthworms were widely used to understand the impacts of pesticides in the environment. In two earthworm species, *Eisenia fetida* and *Lumbricus terrestris*, multiple esterases, including acetylcholinesterase (AChE), butyrylcholinesterase, and carboxylesterase (CE), have been assessed as biomarkers for malathion exposure (Henson-Ramsey et al., 2011). Several studies have also reported AChE, catalase (CAT), and glutathione-S-transferase (GST) as biochemical biomarkers in *Eisenia Andrei* for the insecticides, endosulfan, temephos, malathion, and pirimiphos-methyl (Stepić et al., 2013), and AChE, CAT, CE, and the efflux pump as biomarkers in *E. andrei* and *Octolasion lacteum* for dimethoate exposure (Velki and Hackenberger, 2012). However, these studies have demonstrated that environmental biomarkers are not specific to the individual burden experienced by the organism, resulting in misleading data interpretation.

Interestingly, diagnosing human diseases using biomarkers is much more specific than environmental detection of pesticides because highly specific and novel biomarkers are used (Bickers and Aukim-Hastie, 2009). Recently, surface-enhanced laser desorption/ionization-time-of-flight (SELDI-TOF) mass spectrometry (MS) has contributed dramatically to the identification of more accurate, precise biomarkers specific for human cancers (Silsirivanit et al., 2014). Thus, it is worthwhile in applying SELDI-TOF MS to aid in the discovery of novel, specific biomarkers for use in environmental situations. SELDI-TOF MS has been used to identify biomarkers that are specific for endosulfan exposure in Japanese rice fish (*Oryzias latipes*) (Lee et al., 2013).

In this study, sensitive, specific biomarkers were identified in *E. fetida* against six pesticides using SELDI-TOF MS. Additionally, biochemical biomarkers, such as AChE and CE were also studied in comparison with the pesticide exposure.

## 2. Materials and methods

### 2.1. Chemicals

Captan, carbaryl, carbofuran, and α-endosulfan were purchased from Riedel-de Haën (Seelze, Germany). Chlorpyrifos was purchased from Chem Service Inc. (West Chester, PA). Propoxur, acetylthiocholine iodide (ATChI), and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals used were of the highest grade commercially available.

### 2.2. Earthworms

*E. fetida* were obtained from Sekyung Farm Inc. (Gimhae, Korea). The earthworms were maintained in a medium consisting of pig manure compost fortified with steamed sweet potatoes at 25 °C. Only sexually mature worms weighing 100–200 mg having well-developed clitellum were used.

### 2.3. Topical treatments of pesticides on earthworms

Each pesticide was dissolved in reagent grade dimethyl sulfoxide (DMSO). The DMSO solutions were then diluted to an appropriate concentration and 1 μL of pesticide-containing DMSO solution was applied into the clitellum using a microapplicator. Mortality of worms exposed for 48 h to each concentration of pesticide was recorded and the data were used to estimate median lethal concentrations ($LD_{50}$) using Probit analysis.

### 2.4. Biochemical biomarkers and preparation of earthworm homogenates

Approximately 10 g of earthworms was homogenized in 30 mL of 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 was centrifuged at 12,100 × *g* at 4 °C for 20 min using an Eppendorf Centrifuge 5417R, and the supernatants were reserved as crude enzyme extracts. The activities of the enzymes were measured with absorbance difference using a 96 well system (Thermo Multiskan GO, Thermo Fisher Scientific Inc., Vantaa, Finland).

For the determination of acetylcholinesterase (AChE) activity of earthworms, crude enzyme extracts were incubated with an appropriate volume of phosphate buffer (100 mM, pH 7.4) and the reaction was initiated by the addition of 100 μL of the substrate (acetylthiocholine iodide, ATChI) and DTNB [5′,5′-dithio(2-nitrobenzoic acid)] at 25 °C in the microtiter plate.

Carboxylesterase assay, using *p*-nitrophenylacetate (pNPA) in ethanol as a substrate, was conducted. The reaction mixture (total volume of 300 μL) consisted of 290 μL of 10 mM Tris–HCl buffer (pH 7.5), 5 μL of crude enzyme, and 5 μL of 150 mM pNPA. The reaction was initiated by the addition of pNPA. After incubation at 25 °C for 4 min, absorbance was determined at 405 nm.

### 2.5. SELDI-TOF MS analysis

#### 2.5.1. Sample collection and protein extraction

The procedure was modified from a previously reported method (Lee et al., 2013). Pesticide-exposed whole *E. fetida* were collected and homogenized using a mortar and pestle in ice-cold phosphate buffer (0.1 M, pH 7.2) and then centrifuged for 20 min at 10,000 × *g*. The supernatant fractions were then loaded into each well of a ProteinChip® (Q10 array chips). The ProteinChip array incorporates with quaternary ammonium groups (positively charged) of peptides or proteins, acting as a strong anion exchanger. Before addition of samples, each ProteinChip well was activated by adding 150 μL of 0.1 M Tris–HCl buffer (pH 8.0) containing 0.1% Triton-X 100, and then incubated for 5 min with gentle shaking at 250 rpm. After the ProteinChip-activation buffer was removed, 50 μL of prepared sample was added to each well and incubated for 30 min with shaking at 250 rpm. The samples were removed from the wells of the ProteinChip and each well was washed 3 times with