ToxEHS

Proteomic Analysis of *Daphnia magna* Exposed to Glyphosate and Methidathion

Thai-Hoang Le[1,*], Ji-Young Ahn[2,*], Jihee Yoon[3], Simranjeet Singh Sekhon[2], Jiho Min[1,3] & Yang-Hoon Kim[2]

[1]Department of Bioprocess Engineering, Chonbuk National University, 664-14 Duckjin-dong, Jeonju 561-756, Korea
[2]Department of Microbiology, Chungbuk National University, 1 Chungdae-ro, Seowon-gu, Cheongju 362-763, Korea
[3]Division of Chemical Engineering, Chonbuk National University, 664-14 Duckjin-dong, Jeonju 561-756, Korea
*These authors contributed equally to this work.
Correspondence and requests for materials should be addressed to Y.-H. Kim (kyh@chungbuk.ac.kr), J. Min (jihomin@jbnu.ac.kr)

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Abstract

Proteomic analysis was performed to identify proteins involved in the stress responses of *Daphnia magna* to glyphosate and methidathion. Lethal concentration of chemicals causing 50% mortality (LC50) determined by acute toxicity assays were used to expose to daphnias from 24 h to 21 days prior to total protein extraction. The proteomic profile of tested organisms was analyzed by the two-dimensional electrophoresis method. The average intensity of each spots was then analyzed to determine the differently expressed proteins (DEPs). The DEPs was considered as down- or up-regulations only if their expression level in control samples are linearly higher than LC50 or lower than LC50, respectively. The result showed that there are 22 and 27 DEPs responding to the exposure of glyphosate and methidathion, respectively. The expression pattern of established DEPs is helpful to understand the molecular response of *D. magna* to glyphosate and methidathion, and discover potential candidates as novel biomarkers.

Keywords: Glyphosate, Methidathion, Two-dimensional electrophoresis, Protein expression, *Daphnia magna*

Introduction

Pesticides and chemicals, commonly used in agriculture, to control pests, pathogens, and weeds have seriously contaminated aquatic environment through spray drift, volatilization, drainage, and leaching[1,2]. Methidathion is a highly toxic insecticide used to control a wide spectrum of agricultural insect and mite pests. On the other hand, glyphosate, the active ingredient in many commercial weed-killing formulation (e.g., Roundup), is widely used in agricultural, silvicultural, and urban environments[3]. Increasing quantities of these pesticides have been detected in the environment, especially in aquatic systems which may have many ecotoxicological impacts on non-target aquatic organisms[4].

Protein is synthesized from mRNA, the product of gene expression in the transcription process. Protein expression based on the expression of the respective gene is difficult to predict because the relation between gene and protein is not necessarily one-to-one[5,6]. Thus, analyzing protein expression in organisms exposed to toxic chemicals is very helpful to study the toxicity of the chemicals. Notably, when a cell is in unfavorable environmental conditions, some proteins under stress are over expressed. The expression patterns of specific proteins are produced in response to a specific stress. Therefore, such proteins can be employed as indicators for certain chemicals in the environment[7].

In this study, the proteomic analysis of the water flea, *Daphnia magna*, has been performed to study the expression of an entire set of proteins in the organism in response to glyphosate and methidathion toxicity. The total protein samples extracted from *daphnia* were separated by the method of two-dimensional electrophoresis (2-DE) with pH range from 3 to 10 before and after exposure to the two chemicals. The protein spots were identified using the matrix assisted laser desorption time of flight mass spectrometry (MALDI-TOF) method and analyzed by Progenesis software to identify the differently expressed proteins (DEPs) Identification of these stress-induced-proteins is helpful to understand the stress mechanism and uncover the potential biomarkers.

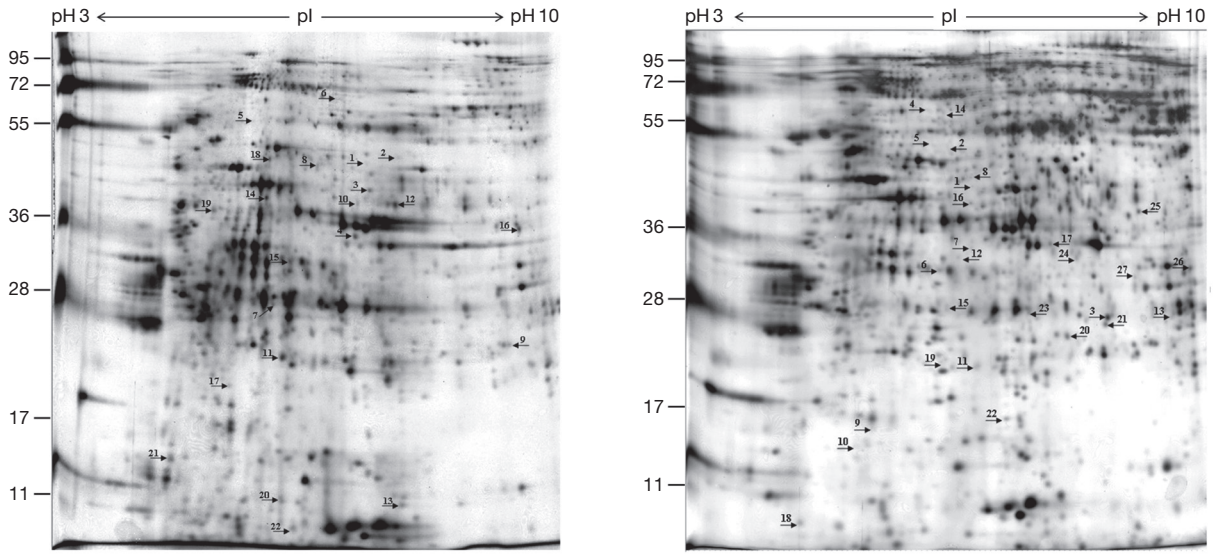

Figure 1. The 2-DE map showing the location of the DEPs in response to toxicity of glyphosate and methidathion. The proteins were separated with pH gradient ranging from 3 to 10 and the molecular weight (MW) are also indicated.

Results and Discussion

Proteomic Analysis of *D. magna* Exposed to Glyphosate and Methidathion

To obtain insight into proteome expression in *D. magna* in response to glyphosate and methidathion toxicity, adult *D. magna* (21 days old) were exposed to LC50 concentrations of glyphosate (234 ppm) and methidathion (0.044 ppm) over 24 hours. The total protein samples containing the proteomes of *D. magna* were separated by the 2-DE method with pH gradient ranging from 3 to 10 and by molecular weight (Figure 1). The protein spots were detected by silver staining and analyzed by the Progenesis software for screening DEPs. In comparison to the 2-DE image of control sample, DEPs were determined by increasing or decreasing intensities of protein spots in at least 2 fold change.

A total of 22 DEPs induced by the toxicity of glyphosate were found in this study. Among these, 10 proteins were up-regulated and 12 proteins were down-regulated by glyphosate exposure (Table 1). In case of methidathion, the organisms exposed to LC50 concentration were analyzed for proteomic expression (Figure 1b) and compared to the control. The results showed that 27 DEPs responded to methidathion toxicity. The 19 up-regulated proteins and 8 down-regulated proteins in *D. magna* responded in response to LC50 methidathion (Table 1).

Notably, the 2-DE map showed DEPs in two dimensions of pH 3-10 range and molecular weight, although many DEPs formed in response to glyphosate (Figure 1a) and methidathion (Figure 1b) toxicity. Only 2 overlapped proteins (DEP11 and DEP15 by glyphosate or DEP6 and DEP19 by methidathion) were found to change their expression by either glyphosate or methiathion (Figure 2). Interestingly, the DEP11 and DEP15 by glyphosate (or DEP19 and DEP6 by methidathion) were found to repressed by glyphosate toxicity, but induced by methidathion toxicity (Table 1). This implies that glyphosate and methidathion may have the different toxic modes while influencing *D. magna* as well as other non-target aquatic organisms. These two DEPs are thus the good candidates for discovery of novel biomarkers in glyphosate and methidathion toxicity. In future work, all DEPs should be analyzed by MALDI-TOF techniques in order to identify the specific proteins.

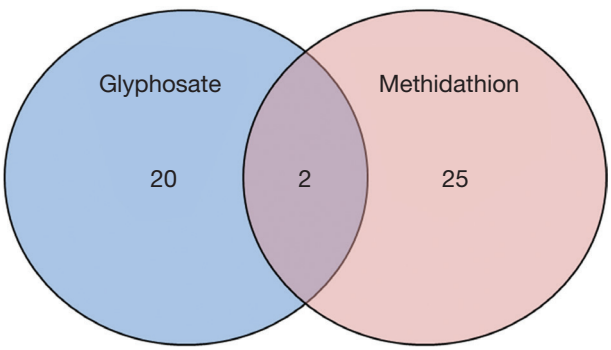

Figure 2. Venn diagram represents the number of DEPs shared by treatment of glyphosate and methidathion.

Table 1. Lists of the differently expressed proteins in *D. magna* in response to toxicity of glyphosate and methidathion at the LC50 concentration.

Spot number	Fold change		
	Control	Glyphosate	Methidathion
1	1	7.44	24.761
2	1	3.38	14.505
3	1	2.97	14.11
4	1	2.75	11.142
5	1	2.35	9.549
6	1	2.15	8.702
7	1	1.98	8.544
8	1	1.80	8.318
9	1	1.50	7.765
10	1	1.38	7.407
11	1	−5.91	6.028
12	1	−4.72	5.429
13	1	−3.63	4.723
14	1	−3.13	4.407
15	1	−3.11	4.384
16	1	−2.38	4.267
17	1	−1.98	4.195
18	1	−1.96	4.164
19	1	−1.87	4.092
20	1	−1.65	−11.202
21	1	−1.53	−4.527
22	1	−1.15	−3.979
23	1	ND	−3.648
24	1	ND	−3.525
25	1	ND	−3.482
26	1	ND	−3.456
27	1	ND	−3.384

ND, not detected.

Materials and Methods

Culture Conditions of *Daphnia magna*

D. magna was provided by the Korea Institute of Toxicology (Daejeon, Korea) for this study. The organisms were cultured and handled according to the US Environmental Protection Agency (USEPA, 2002) manual. The culturing and maintenance of *D. magna* was performed at 20 ± 1°C in 2 L glass beakers containing 1.5 L of hard reconstituted water (HRW) prepared by adding 0.12 g/L $MgSO_4$, 0.192 g/L $NaHCO_3$, 0.008 g/L KCl and 0.12 g/L $CaCO_3$ into deionized water distilled using a Minipore Milli-Q apparatus. This HRW was controlled at a pH of 8.2 ± 0.2 and aerated for at least 24 h prior to use. The medium for the *D. magna* culture was renewed with fresh HRW three times per week and fed with algae (chlorella) and YTC (a mixture of yeast, cerophyll, and trout chow) purchased from Aquatic Biosystem Inc. (Colorado, USA). The number of *D. magna* was adjusted to about 30 to 50 organisms per 2 L culture vessel. A photoperiod of 16 h light : 8 h darkness was applied[8].

Analysis of Protein Expression

LC50 concentration of *D. magna* was defined as the concentration when the percentage of dead testing *D. magna* was 50%. This sub-lethal concentration, 234 mg/L for glyphosate and 0.044 mg/L for methidathion, was determined by the 24 h acute toxicity test reported in a previous study[9]. The exposure test was performed according to the USEPA manual 2002. The 20 days old *D. magna* collected from less than 30 days old mother *D. magna* was used in this experiment. Before exposure to the chemicals, the organisms were starved for 24 h under the culturing conditions as described above. After the exposure tests were conducted using ten organisms per 300 mL test solution in 500 mL chambers with three replicates. Test conditions were controlled at 20 ± 1°C for 24 h with a photoperiod of 16 h light : 8 h darkness[9].

Protein Isolation and Concentration Determination

Total protein for testing *D. magna* was isolated after 24 h exposure to glyphosate and methidathion. Each group of ten animals were transferred into 1.5 mL tube and washed three times with DPBS buffer (Dulbecco's Phosphate Buffer Saline, Sigma Aldrich Co., USA). 200 μL mixture of lysis buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA pH 8.0, 150 mM NaCl, 1 mM EGTA pH 8.5, 1% v/v Triton X-100), PIC (Protein Inhibotor Cocktails, Roche, US), and PMSF (Phenylmethanesulfonyl Fluoride, Roche, USA) with ratio 100 : 1 : 1 was added into each tube before sonication, which was set at 50% amplification, 7 sec on/10 sec off, repeat 4 times. Samples were kept on ice for 5 min and then centrifuged for 15 min at 12,000 rpm and soluble protein concentration was measured in the supernatant using the Bradford assay. Protein samples were stored at −70°C until use.

Two-dimensional Electrophoresis

Before 2-DE, all protein samples (50 μg for each sample) were purified using 2-D cleanup kit (GE Healthcare Bioscience, USA) with an accompanied protocol to remove the contaminants and to concentrate protein samples. Then, protein pellets were redissolved into a rehydration buffer containing 350 μL of ET buffer (6 M urea, 2 M thiourea, 0.5% v/v tritonX-100, 1% bromophenol blue), 35 μL of 1 M DTT (dithiothreitol, Duchefa Biochemie Co., Netherland), and 1.75 μL of IPG buffer

(Immobilized pH gradient, Bio-Rad Co., USA). Next, the protein solution was loaded on an Immobiline Dry strip 18 cm, pH 3-10 (GE Healthcare Bio-Sciences Co., Sweden). A 2.5 mL mineral oil (Bio-Rad Co., USA) was added to cover onto the strip. The rehydration conditions were maintained at 50 mV for 12 h at 20°C using a Protein IEF Cell (Bio-rad Co., USA).

After rehydration step, paper wicks were inserted between the IPG strip and each strip holder electrode just before IEF to adsorb excess water. The IEF step was carried out at 20°C at 500 V (2 h), 1000 V (0.5 h), 2000 V (0.5 h), 4000 V (0.5 h), 8000 V (70,000 Vh), 500 V (0.25 h). The strips were stored at −70°C until running SDS-PAGE. Before performing SDS-PAGE, each IEF strip was equilibrated for 15 min by 4 mL of solution A (2% SDS, 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 0.5% bromophenol blue, and 80 mg DTT). Then, the strip was continuously soaked for 15 min in solution B (2% SDS, 50 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 0.5% bromophenol blue, and 100 mg Iodoacetamide (Sigma, USA). In SDS-PAGE, the IEF strip was loaded onto the 12.5% SDS-PAGE gel (16.8 mL 30% bis/acrylamide (Biorad), 10 mL 1.5 M Tris HCl pH 8.8, 13 mL distilled water, 800 μL 10% SDS (Sodium Dodecyl Sulfate) (Sigma Co., USA), 800 μL 10% APS (Ammonium Persulfate) (Sigma Co., USA), 80 μL TEMED (Tetramethylethylenediamine) (Sigma Co., USA). Then, approximately 3 mL of sealing gel solution (0.5% agarose, 0.2% bromophenol blue) was added onto the surface of the IEF strip. The SDS-PAGE separation was performed at 200 V, 400 mA for 6 h at room temperature. The separation gels were then fixed with fixing buffer (50% methanol, 12% acetic acid, 38% distilled water, and 0.00053% formaldehyde) for at least 2 h.

Silver Staining

After fixing, the gels were washed 2 times with 50% ethanol for 20 min. Then the gels were shaked in the sensitizing solution (0.2 g/L $Na_2S_2O_3$) for 75 sec. Next, the gels were washed 3 times with distilled water by shaking at 100 rpm for 20 sec prior to its reaction with 2 g/L $AgNO_3$ solution during 30 min. After that, the stained gels were washed 2 times with DW before developing in a solution (60 g/L $Na_2S_2O_3$, 20 mL 0.2 g/L $Na_2S_2O_3$, 0.00053% formaldehyde). It normally takes about 5-9 min to display all protein spots on the gel. The developing process was stopped by transferring the gels into the stopping solution (50% methanol, 12% acetic acid, and 38% distilled water) and shaking at 100 rpm for at least 2 h.

Analysis of Spots

The staining 2-DE gel was washed 2 times with distilled water at 100 rpm for 15 min each before being scanned by the TIFF format, 300 dpi. The protein spots in the gel image were detected by Progenesis software (Nonlinear Dynamic Co., UK). To determine the differently expressed spots, the protein spots from the control *D. magna* samples were used as standard in comparison with the protein spots from the protein samples of *D. magna* exposed to LC50 concentration of methidathion and glyphosate. Three replicate gels for control and each exposure concentration of glyphosate and methidathion were employed to make an average gel before doing analysis to find the differentially expressed protein spots.

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