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RESEARCH ARTICLE

Biomarker discovery and proteomic evaluation of cadmium toxicity on a collembolan species, *Paronychiurus kimi* (Lee)

Jino Son[1], Sung-Eun Lee[2]*, Byeoung-Soo Park[2], Jinho Jung[1], Hyung Soon Park[3], Joo Young Bang[3], Gum-Yong Kang[3] and Kijong Cho[1]*

[1] Division of Environmental Science and Ecological Engineering, College of Life Sciences and Biotechnology, Korea University, Seoul, South Korea
[2] Research Station, Nanotoxtech Inc., Gyunggi Technopark Technology Development Center, Ansan, South Korea
[3] ProBiond Co., Ltd., Information Center for Admissions, Konkuk University, Seoul, South Korea

The goal of this study was to identify promising new biomarkers of cadmium by identifying differentially expressed proteins in *Paronychiurus kimi* after exposure to cadmium. Through proteomic analysis of *P. kimi* using 1-D PAGE and nano-LC-MS/MS, 36 downregulated proteins and 40 upregulated proteins were found. Some of the downregulated and upregulated proteins were verified by LC-MS/MS analysis after 2-D PAGE. Downregulated proteins in response to cadmium exposure were involved in glycolysis and energy metabolism, chaperones, transcription, reproduction, and neuron growth. In contrast, proteins involved in glycolysis and energy production, neurogenesis, defense systems response to bacteria, and protein biosynthesis were upregulated in cadmium-treated collembolans. Cubulin may be a potential biomarker for the detection of cadmium in *P. kimi* since this biomarker was able to low levels (3.5 mg/kg) of cadmium. The 14-3-3 ζ was also found to be a potential biomarker for the detection of medium levels (14 mg/kg) of cadmium. Collembolans may be an alternative tool to humans because many collembolans proteins show a high homology to human proteins.

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1 Introduction

Healthy soil is crucial for both biota and not-biota to sustain the function of natural ecosystems. However, soil pollution with metals has become a widespread environmental problem throughout the world and over the past decades soil has been polluted with a variety of metals originating from both natural sources and humans. In particular, cadmium is a naturally occurring, non-essential metal pollutant of the environment that originates from a variety of anthropogenic sources such as agricultural, mining and industrial activities [1]. Soil pollution by cadmium is of considerable concern due to its high toxicity and high solubility in water. It has also been reported that cadmium affects soil organisms such as earthworms [2] and Collembola [3–5]. In this respect, cadmium has been regarded as an extremely significant and important pollutant.

Over the past decades, chemical analysis of environmental samples has been an essential component in assessing the status of pollution of a specific medium in

Correspondence: Professor Kijong Cho, Division of Environmental Science and Ecological Engineering, College of Life Sciences and Biotechnology, Korea University, Seoul 136-701, South Korea
E-mail: kjcho@korea.ac.kr
Fax: +82-2-3290-3060

Abbreviations: CI, confidence interval; **HIF-1,** hypoxia-inducible factor-1; **LC_{50},** lethal concentration; **NR,** net response; **VCP,** valosin-containing protein

*These authors contributed equally to the work.

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environmental monitoring. However, while these methods are the most simplistic way to assess the levels of pollution in environmental samples, they provide little information about the biological risk posed by pollutants [6, 7]. In addition, there are technical limitations and high costs involved in quantifying all pollutants found in polluted soils.

Recently, monitoring biological effects has become a common tool used to complement chemical analysis in environmental monitoring. Moreover, the use of biomarkers has been gaining popularity in environmental monitoring over the past two decades, serving as early warning tools of exposure (monitoring the environmental pollution) and in understanding the effects of exposure (assessing the biological response that can be directly linked to exposure) [8]. Although biomarkers can be used to detect chemicals of potential concern (COPCs) and their potential effects on organisms in the early stages, no single biomarker, be it an indicator of exposure or its effects, will be sufficient to fully describe pollutant-induced responses. In this respect, the use of biomarkers in environmental monitoring is moving toward a panel of multiple biomarkers rather than a single biomarker [9]. Because of its potential ability as an early warning tool, multiple biomarker-based approaches capable of understanding the impact of pollutants on organisms should be incorporated into environmental monitoring. Recent advances in ecotoxicoproteomic technologies, such as MS, have improved the ability to discover and validate protein biomarkers, specifically indicators that reflect both chemical exposure and the subsequent biological effect [10]. In this respect, ecotoxicoproteomics, which is a high-throughput technology, may be a promising tool for identifying multiple biomarkers. Moreover, ecotoxicoproteomic approaches have been successfully applied to aquatic organisms such as midge, fish and molluscs in the past few years [9, 11–13]. However, it has been stressed that the number of presently available biomarkers for soil organisms are too limited to allow realistic environmental monitoring of polluted soils [14].

Collembola, which are small arthropods, are abundant in soil and are often the most sensitive species to toxic compounds [15, 16]. These properties make them to be important indicator organisms for evaluating potential impacts of chemicals on soil animals [17]. Recently, *Paronychiurus kimi* (Lee) native to Korean soils has been selected as an alternative Collembola species to *Folsomia candida* for toxicity testing [4, 5, 18, 19]. This collembolan species was chosen as a test species in this study.

The primary goal of this study was to discover multiple biomarkers of *P. kimi* after short-term exposure (7 days) to sublethal levels of cadmium using ecotoxicoproteomic analysis. The sublethal levels of cadmium were determined in simultaneous 28-day Collembola reproduction test [17]. On the basis of EC_{50} values, differential protein expression of *P. kimi* after short-term exposure to sublethal levels of cadmium was investigated using one-dimensional (1-D)- and two-dimensional (2-D)-polyacrylamide gel electrophoresis (PAGE) with nano-LC/MS/MS.

2 Materials and methods

2.1 Test species

A population of *P. kimi* collected from paddy soils in Korea in 1996 [20] was maintained in the laboratory using a culture method to the one developed for *F. candida* [21–23]. Adult animals cultured by this procedure were used for this study.

2.2 Toxicity test

Artificial soil was prepared by mixing 70% quartz sand, 20% kaolin clay and 10% finely ground Sphagnum peat (<0.05 mm) as previously described [19]. The soil pH (1:5 soil–water ratios) was adjusted to 6.0 ± 0.5 through the addition of $CaCO_3$ (≥99% pure, Sigma-Aldrich, St. Louis, MO, USA). Cadmium ($CdCl_2 \cdot 2\ ^1/_2H_2O$, Sigma-Aldrich, purity >98%) was added to the artificial soils as aqueous solutions of chloride dissolved in the correct amount of deionized water to achieve a soil moisture content equal to 50% of the water-holding capacity.

In the reproduction test, the following nominal test concentrations of cadmium were prepared; 0, 12.5, 25, 50, 100 and 200 (in mg cadmium/kg dry weight of soil) with six replicates per concentration. This experiment was carried out followed by the method previously reported [22, 23]. From the concentration-response relationships, the 28-day effective concentration (EC_{50} for reproduction; i.e. EC_{50} which reduce a 50% offspring production compared to the control) values were determined using a logistic model described previously [24]. The calculated 28-day EC_{50} value for cadmium was used in subsequent tests.

Avoidance test with *P. kimi* was carried out according to ISO [25] with some modifications. Additional artificial soils spiked with aqueous solutions of cadmium chloride at concentrations of 6.25, 12.5, 25 and 50 mg/kg were prepared. Cadmium non-spiked artificial soil was used as a control soil. Cylindrical plastic Petri dishes (10 cm diameter × 4 cm height) were used as test container. Each plastic Petri dish was divided into two equal sections with plastic wall, and 30 g wet of spiked soil was placed in one of the Petri dishes, while the opposite side was filled with the same amount of control soil. After the soil addition, the plastic wall gently removed and thirty collembolans, 42–44 days old, were placed on the contact line between the two sections. Three replicates per each combination were tested. Additionally, control–control test (with cadmium non-spiked soil at the both sides of Petri dish) was conducted to verify the random distribution of collembolans. The Petri dishes were kept in continuous darkness at $20 \pm 1^{\circ}C$ for 48 h. At the end of the test, the removable wall was inserted

in the center and the number of collembolan in each side was counted, separately.

2.3 Statistical analysis for the toxicity test

All statistical analyses were carried out using the statistical software SAS [26]. The median lethal concentration (LC_{50}) and 95% confidence intervals (CI) were calculated by plotting a graph of percent mortality (probit value) against log concentrations using the probit analysis. The EC_{50} was estimated by fitting the data to a previously described logistic model (Eq. 1) [24].

$$y = \frac{c}{[1+\exp(b(x-a))]} \quad (1)$$

where y is the number of progeny, x is the natural logarithm of the test concentrations, a is the natural logarithm of EC_{50}, b is the slope parameter and c is the number of progeny per adult collembolan of the control [23].

The behavioral toxicity of cadmium was expressed as avoidance net response (NR) and calculated as follows:

$$\mathrm{NR} = ((C-T)/N) \times 100 \quad (2)$$

where C is the collembolans observed in the control soil; T the collembolans observed in test soil and N the total number of collembolans per replicate.

A positive NR indicates avoidance and a negative NR indicates an attraction to the chemical tested in a given concentration. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was conducted to determine the effect of cadmium on avoidance NR. A χ^2 test was conducted to test the random distribution of collembolans in the control–control combination. The concentration causing 50% avoidance response (EC_{50} for avoidance response) was calculated using Probit regression, after verifying that 50% of the collembolans are in each side of Petri dish in the control–control combination (random distribution).

2.4 Preparation of Collembola samples for profiling proteins

Based on the EC_{50} value determined in the reproduction test, artificially contaminated soils were prepared in the same way as described in Section 2.2. The test cadmium concentrations were as follows: $EC_{50}/16$, $EC_{50}/8$, $EC_{50}/4$, $EC_{50}/2$ and EC_{50} with three replicates per concentration. A total of 50 synchronized (42–44 days old) *P. kimi* per concentration were introduced into a polystyrene vessel containing 90 g wet weight of contaminated soil. The test was conducted under the same conditions as those used in the reproduction test, except for the exposure duration (7 days). No food was added at the beginning of the test. After 7 days of exposure, the surviving Collembolans were collected and flash-frozen in liquid nitrogen and then stored at −70°C for further proteomic analysis. The extraction procedure of proteins were followed by the method previously reported [12]. The mortality rates were less than 5% at all cadmium exposure levels.

2.5 1-D SDS-PAGE

Protein samples (each 20 μg) were mixed with SDS-PAGE sample buffer and heated at 100°C for 5 min. The denatured proteins were separated on 10–20% gradient SDS-PAGE mini gels (9 × 10 cm, PAGEr Gold Precast Gel, Cambrex Bioscience, Rockland, ME, USA) followed by Coomassie Blue dye (G-250) staining. The samples were separated by SDS-PAGE and then the stained gel bands were sliced and chopped with a knife in a dimension of 1 mm × 1 mm. The sliced gel pieces were destained with 50% ACN in 50 mM NH_4HCO_3 and vortexed until the Coomassie dye (G-250) was completely removed. The gel pieces were then dehydrated in 100% ACN and vacuum-dried for 10 min using a SpeedVac. For the reduction, gel pieces were added to a 10 mM DTT, 50 mM NH_4HCO_3 buffer solution for 45 min at 56°C, followed by alkylation of cysteines in 55 mM iodoacetamide, 50 mM NH_4HCO_3 for 30 min in dark. Finally, the proteins in each gel piece were digested by 12.5 ng/μL sequencing grade modified trypsin (Promega, Madison, WI, USA) in 50 mM NH_4HCO_3 buffer (pH 7.8) at 37°C overnight. After digestion, the tryptic peptides were harvested twice by 5% formic acid in 50% ACN solution at room temperature for 20 min. The supernatants were collected and dried using a SpeedVac. Peptides that were re-suspended in 0.1% formic acid in water were purified and concentrated using C18 ZipTips (Millipore, MA, USA) before MS analysis.

2.6 Nano-electrospray LC-MS/MS and analysis of peptide sequences

LC-MS/MS analysis was conducted by the method previously reported [27]. The obtained LC-ESI-MS/MS fragment spectra were searched in the BioWorksBrowser™ (version Rev. 3.3.1 SP1, Thermo Fisher Scientific, CA, USA) with the SEQUEST search engines against National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) non-redundant human, *Drosophila*, mosquito and springtail database. The search conditions included trypsin for enzyme specificity, a permissible level for two missed cleavages, peptide tolerance; ±2 amu, a mass error of ±1 amu on fragment ions and fixed modifications of carbamidomethylation of cysteine (+57 Da) and oxidation of methionine (+16 Da) residues.

In order to compare the protein expression profile of each sample, a label-free protein quantification method, which counts the number of spectra used to identify each protein, was employed.

2.7 2-D PAGE, Coomassie staining and analysis of gel images of 2-D PAGE

2-D PAGE, Coomassie staining and analysis of gel images for 2-D PAGE were performed according to the method previously reported [12, 28].

3 Results

3.1 Toxicity of cadmium to survival, reproduction and behavior of *P. kimi*

In the reproduction test, adult survival was significantly affected by cadmium concentration at concentrations of 100 mg/kg and higher according to the Tukey's test ($p<0.05$) after 28 days of exposure to cadmium. The predicted 28-day LC_{50} value of cadmium was 128.9 (112.0–149.3) mg/kg (Table 1). Reproduction also decreased in a concentration-dependent manner after 28 days of exposure to cadmium ($p<0.05$) (Fig. 1A). The EC_{50} value with corresponding 95% CI for reproduction was 28.3 (23.7–33.8) mg/kg (Table 1). The 28-day EC_{50} value for reproduction of cadmium was used as a basis for sample preparation in the subsequent proteomic study.

In the behavior toxicity test, no significant difference in the NR was observed in the control–control combination ($p>0.05$), verifying that the random distribution of collembolans in the control–control combination. The mean NR ranged from 33.8 (6.25 mg/kg) to 67.3 (50 mg/kg), showing that avoidance response significantly increased with increasing cadmium concentration ($p<0.05$) (Fig. 1B). The EC_{50} value with corresponding 95% CI for avoidance response was 17.1 (10.4–27.6) mg/kg.

Table 1. LC_{50} and EC_{50} values with the corresponding 95% CI of adult survival and reproduction of *P. kimi* after 28 days of exposure in artificial soil

Metals	28 days LC_{50} (mg/kg)	28 days EC_{50} (mg/kg)
Cadmium	128.9 (112.0–149.3)	28.3 (23.7–33.8)

The 95% CI are given in parentheses.

3.2 Analysis of proteins in response to cadmium exposure in *P. kimi*

The protein levels in *P. kimi* between exposed to cadmium and control were compared. Proteins displaying altered levels were quantified and identified from the 1-D gels and analyzed by LC-MS/MS as previously reported [12]. Some of the differentially expressed proteins identified by 1-D PAGE and LC-MS/MS were confirmed through further isolation by 2-D PAGE using PD-Quest software with LC-MS/MS analysis. A typical 1-D gel is presented in Fig. 2 and the three lanes designated as the control, 3.5 and 14.0 mg/kg were cut into 14 pieces for further analysis. 2-D PAGE with numbers are shown in Figs. 3 and 4 indicating altered and excised protein spots after analysis by the PD Quest program. By comparing the intensity of the protein spots (Fig. 2) between *P. kimi* exposed to a cadmium concentration of 3.5 mg/kg or 14 mg/kg and the control, the spot intensities of 36 proteins were found to be lower after cadmium exposure (Table 2) and 40 proteins appeared to be over-expressed after exposure to cadmium (Table 3).

3.3 Identification and classification of proteins in relation to cadmium toxicity

Through the use of proteomic techniques, differentially expressed proteins in the cadmium-exposed *P. kimi* in relation to the control group were identified. According to the UniProtKB/Swiss-Prot classification of proteins, the downregulated proteins were involved in glycolysis, energy production, protein biosynthesis and degradation, protein folding, signal transduction, chaperones and so on. Thus, these results clearly demonstrated that cadmium affected several aspects of cell function (Fig. 5).

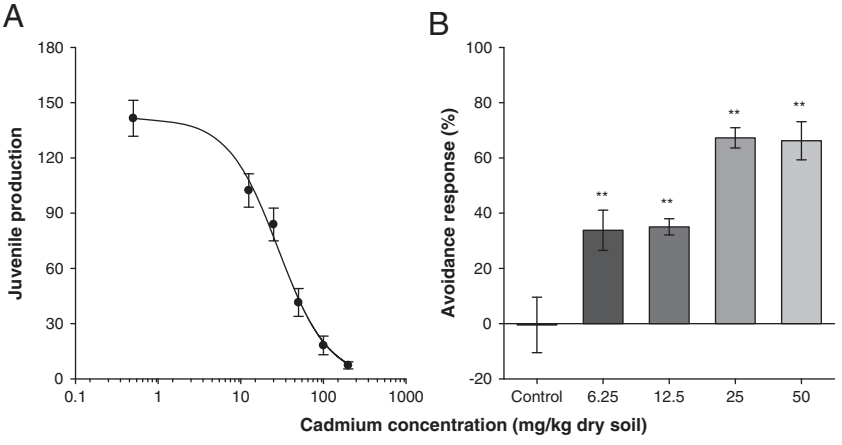

Figure 1. Juvenile reproduction (A; mean ± SE) and avoidance response (B; mean ± SE) of *P. kimi* after 2 and 28 days exposure to cadmium in OECD artificial soil. ** indicates significantly different ($p<0.05$) compared with the control.

Proteins in *P. kimi* that were quantitatively upregulated in response to 3.5 mg/kg of cadmium were involved in the defense, transcription, glycolysis, development, TCA cycle, binding protein, protein biosynthesis, detoxifying system and signal transduction according to the UniProtKB/Swiss-Prot classification of proteins.

Proteins in *P. kimi* that were over-expressed in response to 14.0 mg/kg of cadmium were involved in neurogenesis, ATP biosynthesis, defense, binding protein, translation, protein biosynthesis, detoxifying system and TCA cycle according to the UniProtKB/Swiss-Prot classification of proteins. Among them, only 14-3-3 ζ was simultaneously over-expressed in *P. kimi* after exposure to both 3.5 and 14.0 mg/kg of cadmium.

Using 2-D PAGE analysis, five over-expressed proteins were found in *P. kimi* in response to 14.0 mg/kg of cadmium, while 26 proteins in *P. kimi* were quantitatively downregulated in response to 14.0 mg/kg of cadmium.

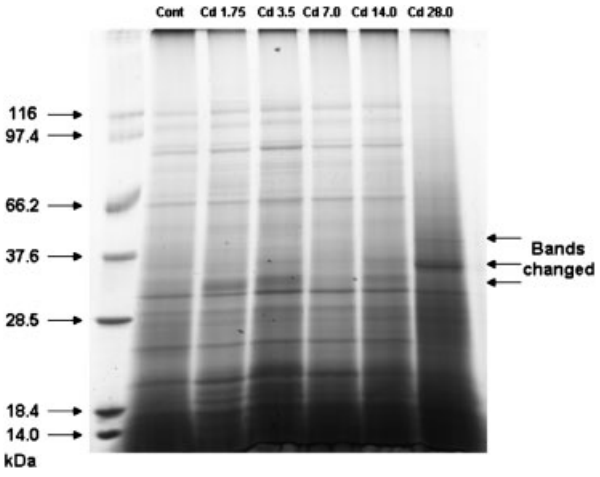

Figure 2. 1-D SDS-PAGE images of *P. kimi* proteins after exposure to a series of cadmium concentrations. Fifty *P. kimi* samples were analyzed at each treatment. Cont indicates control and a series of concentration at 1.75, 3.5, 7.0, 14.0 and 28.0 mg/kg of cadmium were applied to artificial soil.

Among them, four proteins identified using 1-D PAGE were reconfirmed using 2-D PAGE analysis and they were phenylalanyl-tRNA synthetase, lysine-specific histone demethylase, crumbs, and CDKN1A interacting zinc finger protein.

4 Discussion

Exposure to heavy metals has been detected by biomarker expression and metallothioneins in *Orchesella cincta*. In addition, biomarkers of heavy metal exposure have also been identified in *D. melanogaster*. However, metallothioneins were not included in this study because they have been widely studied in response to cadmium toxicity in collembolans and their molecular weights were too low to be efficiently separated by gel-electrophoresis. In this study, differentially expressed proteins over the size of 10 kDa after exposure to cadmium in a collembolan species, *P. kimi*, were identified using a proteomic technique with 1D- or 2-D PAGE in conjunction with nano LC-MS/MS analysis.

4.1 Proteins involved in survival and reproduction under cadmium stress

The 28-day LC_{50} value of cadmium was comparable to that of our previous studies; 113.9 (102.6–126.6) and 90.1 (28.3–139) mg/kg [4, 5]. However, the 28-day EC_{50} value of cadmium in the present study was only comparable to the value of 28.0 (21.8–36.0) reported in our previous study [4]. When cadmium toxicity to *P. kimi* was compared with those of other Collembola species, *P. kimi* appeared to be more sensitive to cadmium than other Collembola species. The 28-day LC_{50} value for cadmium determined in this study for *P. kimi* (128.0 mg/kg) was lower than that previously determined for *F. candida* (617 mg/kg) [29]. In addition, this cadmium 28-day EC_{50} value (28.3 mg/kg) was lower than that reported for *F. candida* (112 mg/kg) [30]. These results clearly showed that *P. kimi* holds promise for use as an

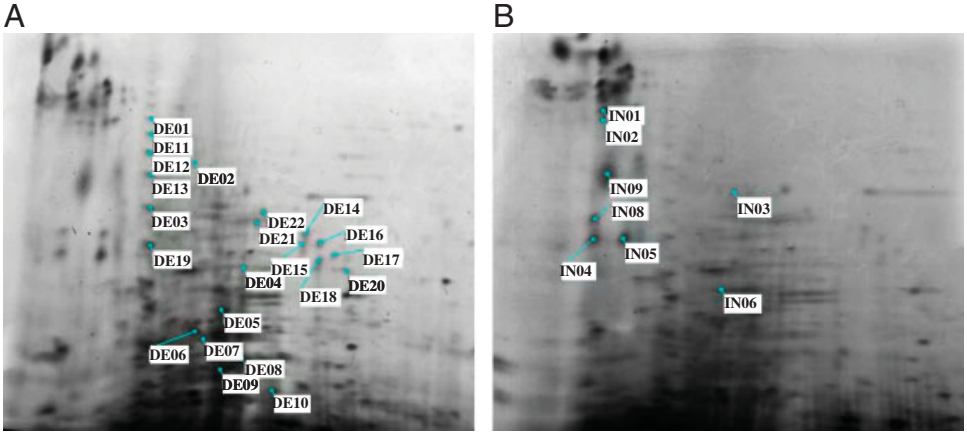

Figure 3. Image analysis after 2-D PAGE (p*I* ranges of 4–10) of *P. kimi* proteins after exposure to 14.0 mg/kg of cadmium using PD-QUEST program. (A), ''DE'' indicates the proteins that were downregulated in the *P. kimi* after cadmium exposure: (B), ''IN'' indicates the proteins that were upregulated in the *P. kimi* after cadmium exposure.

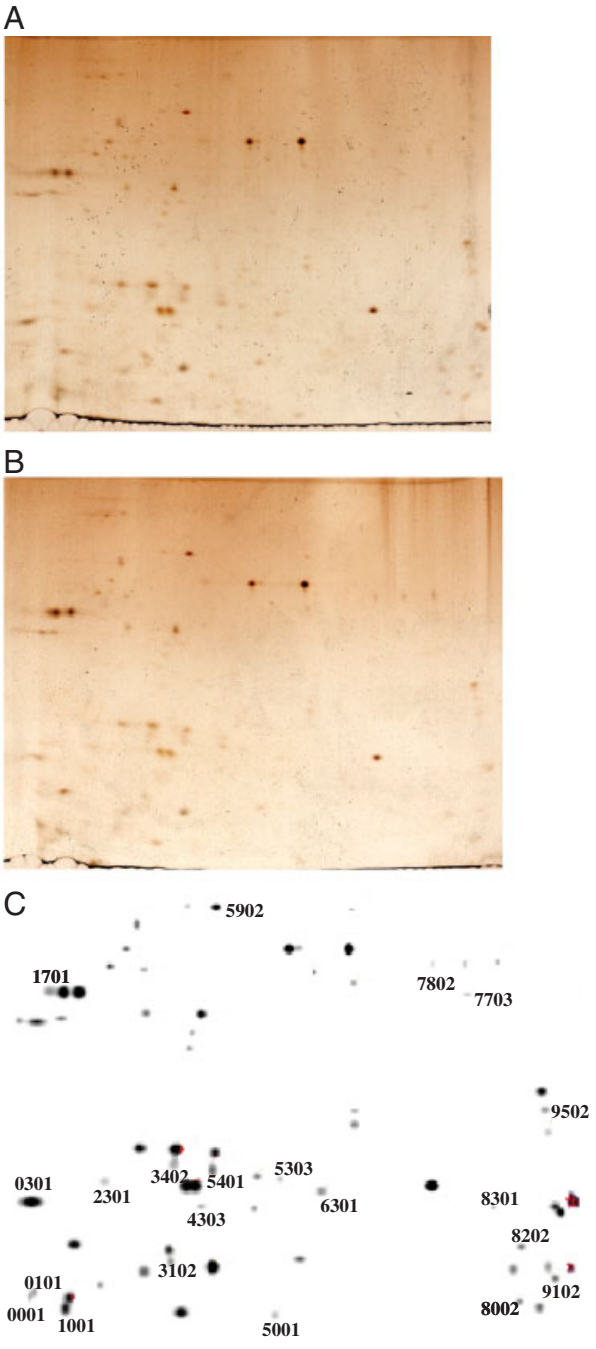

Figure 4. 2-D PAGE images (p*I* ranges of 4–7) of *P. kimi* proteins after exposure to 14.0 mg/kg of cadmium. (A) control, (B) cadmium exposure at a concentration of 14.0 mg/kg, (C) Image analysis of the 2-D PAGE gels using PD-QUEST program.

ecologically relevant bioindicator, since this species was highly sensitive to a wide range of toxicants and has an acceptable rate of reproduction in artificial soil.

P. kimi populations in vessel decreased in a cadmium-dependant manner (Fig. 1A). In relation to these results, reproduction systems in the tested Collembolans seemed to be affected by cadmium toxicity according to the proteomic analysis. LC-MS/MS analysis after 1-D PAGE showed that *P. kimi* over-expressed the clathrin heavy chain, fibrous sheath CABYR-binding protein and kismet (Table 3). However, these responses were only determined at the concentration of 3.5 mg/kg of cadmium. On the other hand, *P. kimi* downregulated Ypsilon schachtel, short stop and PIWI proteins after exposure to cadmium. Downregulation of Ypsilon schachtel was only determined at the concentration of 3.5 mg/kg of cadmium, while downregulation of PIWI protein was determined only at the concentration of 14.0 mg/kg of cadmium. Downregulation of short stop protein was found with both concentrations. Therefore, differential response in relation to the cadmium toxicity on reproduction of *P. kimi* is dependent on the concentration of the heavy metal in the environment.

From the analysis of biological functions it is evident that the clathrin heavy chain is involved in sperm individualization, fibrous sheath CABYR-binding protein is involved in spermatogenesis and kismet is involved in border follicle cell migration. They were all over-expressed against cadmium exposure. On the other hand, Ypsilon schachtel, short stop and PIWI proteins play an important role in oogenesis, oocyte fate determination and cell fate determination, respectively. These proteins were downregulated after cadmium exposure. Therefore, the variance in the reproduction rates of *P. kimi* may be resulted from sexually different responses to cadmium toxicity. However, *P. kimi* are too small to collect them by sexual difference for the toxicity tests and for the proteomic studies.

However, it was not easy to interpret precisely for the functions of the differentially expressed proteins in relation to the reproduction of *P. kimi*. Further studies on the molecular level will be needed to validate possible mechanisms of identified proteins in relation to the reproduction toxicity of cadmium.

4.2 Proteins involved in behavioral changes under cadmium stress

No comparable data on other collembolan species exist for avoidance response of *P. kimi* in the literature. When the EC_{50} value of cadmium for avoidance response was compared to that value of *Enchytraeus albidus* (Enchytraeids), the EC_{50} value of *P. kimi* was about 20 times lower than that of *E. albidus* (362 mg/kg) [31]. It is likely that *P. kimi* species is more sensitive to *E. albidus* to the cadmium presence (Fig. 1B).

In relation to the results of avoidance test, neurotoxicity of cadmium was observed in collembolans, which was also previously observed in zebrafish [32]. Cadmium-induced neurotoxicity might be caused by impaired neurogenesis, resulting in markedly reduced neuronal differentiation and axonogenesis, leading to neuronal cell death.

Wnt genes are, in most cases, expressed in specific domains during central nervous system (CNS), limb and tail

Table 2. Identification of proteins that were downregulated in response to cadmium toxicity in the collembolan species, *P. kimi*

No.	Protein name	Species match	Accession no.	Fold change concentration (mg/kg)		Gene ontology (general annotation) (biological or molecular function)
				3.5	14.0	
1	Heat shock protein cognate 2	*Drosophila melanogaster*	17737941	0.41	–	Response to stress
2	Purine-rich binding protein-α, isoform D	*D. melanogaster*	24638642	0.48	–	Binding protein
3	CG7998	*D. melanogaster*	24647881	0.46	0.52	Glycolysis, malate dehydrogenase
4	Ypsilon schachtel	*D. melanogaster*	24663131	0.48	–	Oogenesis
5	Heat shock protein cognate 1	*D. melanogaster*	24663992	0.46	0.35	Response to stress
6	CG5847	*D. melanogaster*	24667472	0.45	0.40	Unknown
7	Calcium ATPase at 60A	*D. melanogaster*	24762457	0.28	0.52	ATP biosynthetic process
8	Myosin heavy chain, isoform H	*D. melanogaster*	28574239	0.17	0.15	Myosin
9	Elongation factor 1 α 100E	*D. melanogaster*	45553816	0.40	–	Protein biosynthesis
10	Short stop	*D. melanogaster*	221330235	0.47	0.42	Oocyte fate determination, sensory organ development
11	Arginine kinase, isoform F	*D. melanogaster*	221331031	0.30	0.43	ATP binding
12	Glyceraldehyde 3-phosphate dehydrogenase	*D. melanogaster*	18110149	–	0.45	Glycolysis
13	Hyrax	*D. melanogaster*	21355767	0.35	0.32	Wnt receptor signaling pathway, compound eye morphorgenesis
14	CG9391	*D. melanogaster*	21357329	–	0.25	Inositol (1 or 4)-monophosphatase activity
15	Hexokinase	*D. melanogaster*	24640843	–	0.37	Glycolysis
16	CG7461	*D. melanogaster*	24655582	–	0.34	Oxidation reduction, acyl-CoA dehydrogenase activity
17	CG6129	*D. melanogaster*	28571853	–	0.40	Unknown
18	Nipped-A, isoform D	*D. melanogaster*	161076331	–	0.41	Binding, phosphotransferase
19	Glucosamine phosphate isomerase	*Tomocerus* sp. ''Tom2''	262304399	0.40	0.57	Glucosamine catabolic pathway, deaminase, fructose 6-phosphate production
20	Heat shock protein	*F. candida*	134269646	–	0.46	Response to stress
21	Heat shock protein 70 cognate	*F. candida*	134269648	–	0.46	Response to stress
22	NADH dehydrogenase	*Sminthurus viridis*	171473614	–	0.41	Respiratory chain
23	*S*-Adenosylmethionine synthetase	*Aedes aegypti*	157106365	0.47	0.32	One carbon metabolism, SAM biosynthetic process
24	Initiation factor 5a	*A. aegypti*	157138466	0.37	–	Protein biosynthesis
25	Hairy	*Culex quinquefasciatus*	170037964	0.23	–	Transcription
26	Apyrase	*C. quinquefasciatus*	170041898	0.37	0.36	Nucleotide catabolic process
27	PIWI	*C. quinquefasciatus*	170050287	–	0.50	Cell fate determination, female or male germ-line stem cell division
28	Virilizer	*C. quinquefasciatus*	170057859	–	0.50	Predicted
29	Chymotrypsin	*C. quinquefasciatus*	170058224	–	0.47	Digestion, proteolysis
30	Regulating synaptic membrane exocytosis protein	*C. quinquefasciatus*	170063125	–	0.50	Calcium ion-dependent exocytosis, synaptic vesicle exocytosis, visual perception
31	Dihydrolipoyl dehydrogenase	*Homo sapiens*	91199540	0.49	0.35	Reactive oxygen species (ROS) generator
32	Centromere-associated protein E	*Homo sapiens*	71061468	0.36	–	Cell division, microtubule-based movement
33	Glutathione reductase, mitochondrial precursor	*Homo sapiens*	50301238	–	0.30	Cell redox homeostasis
34	E3 SUMO-protein ligase RanBP2	*Homo sapiens*	150418007	0.20	0.42	Protein folding, intracellular transport

Table 2. Continued

No.	Protein name	Species match	Accession no.	Fold change concentration (mg/kg)		Gene ontology (general annotation) (biological or molecular function)
				3.5	14.0	
35	CDKN1A interacting zinc finger protein 1	*Homo sapiens*	196115147	0.38	–	Nucleic acid binding, zinc ion binding
36	Crumbs homolog 1 precursor	*Homo sapiens*	41327708	–	0.47	Cell–cell signaling, response to stimuli, visual perception

Table shows results using LC-MS/MS and their homology alignments.

development. The hyrax protein activates Wnt/Wg target gene transcription by direct association with β-catenin/armadillo [33]. In the present study, a downregulation of the hyrax protein was observed in response to cadmium exposure, indicating that the CNS was damaged in the presence of cadmium. *P. kimi* lowered the Wnt signaling pathway to the levels of 3.5 and 14.0 mg/kg cadmium after 28-day exposure. However, some proteins that were related to cell development were over-expressed in cadmium-treated collembolans. The sciribble protein, which is involved in neurogenesis, was over-expressed as was 14-3-3 ζ, which is required for Raf-dependent cell proliferation and photoreceptor differentiation during eye development. Heart development was also damaged by cadmium treatment [34]. In relation to heart development, *P. kimi* over-expressed laminin A chain, which is involved in heart development and locomotory behavior, after cadmium treatment.

Cadmium inhibits the release of acetylcholine, probably by interfering with calcium metabolism [35]. In our study, the regulating synaptic membrane exocytosis (RIMS1) protein was downregulated in response to cadmium treatment. Downregulation of RIMS1 seems to be related to the inhibition of acetylcholine secretion because it is involved in the regulation of vesicle exocytosis during short-term plasticity with RAB3A [36] and MUNC13 [37]. On the other hand, the clathrin heavy chain, which is involved in neurotransmitter secretion in collembolans, was upregulated 2.54 times in response to cadmium treatment. Two proteins involved in calcium ion metabolism, neurocalcin and cubulin, were over-expressed in the cadmium-exposed collembolans. Therefore, abnormality in neurotransmission in relation to cadmium toxicity was recovered by over-expression of these three proteins, at least, in collembolans.

Cadmium induces VCP-mediated aggresome formation [38]. Aggresome is a type of cellular organelle in which ubiquitinated and misfolded proteins accumulate and valosin participates in transporting ubiquitinated proteins to aggresomes. The aggresomes induced by cadmium are one of parameters associated with neurodegenerative disorders [39]. Therefore, induction of VCP by cadmium treatment in collembolans enhances formation of aggresomes, leading to neurodegenerative disorders.

4.3 Defense systems over-expressed in relation to cadmium toxicity

Cadmium is a heavy metal ion and its biological function is still unknown, however, it has been determined to be one of the most serious environmental pollutants. It has been shown to be a mutagen and its mutagenic effects are due to the generation of ROS, inhibition of several types of DNA repair proteins, depletion of glutathione and alteration of apoptosis [40].

As expected, the upregulation of proteins such as CG9674, known as glutamate synthase, and glutathione *S*-transferase, was related to scavenge ROS. These findings were similar to the results obtained in a previous study [12], which examined the effects of cadmium on *Chironomus riparius* larvae. In the tested collembolans, glutathione production systems were activated to suppress cadmium toxicity. Glutathione biosynthesis mechanisms and glutathione *S*-transferase were over-expressed in relation to cadmium exposure in *P. kimi*, but we could not explain why the glutathione reductase was downregulated.

Dihydrolipoyl dehydrogenase, which is an ROS-producing enzyme, was downregulated in response to cadmium exposure (Table 2). Therefore, the generation of ROS seems to be controlled by the upregulation of scavenging systems and downregulation of ROS generators in collembolans. Additionally, actin 57B, which is a detoxifying enzyme that possesses glucuronyltransferase activity, was over-expressed in the *P. kimi* after exposure to cadmium (Table 3). However, the role of this protein in cadmium detoxification is still not known.

Cadmium is also responsible for inactivating the DNA repair machinery, which is profoundly important because the repair systems are needed to constantly fix DNA damage associated with normal cell functions [41]. In regard to the mismatch repair systems, centrosomes may play an important role as DNA damage regulators. Inactivation of the centrosomal pathway, which is triggered by impaired DNA integrity, results from centrosome abnormalities that are linked to genomic instability and is considered a possible cause of cancer [42]. A previous study showed that injection of DNA-damaging agents into *D. melanogaster* embryos

Table 3. Identification of proteins that were over-expressed in response to cadmium toxicity in the collembolan species, *P. kimi*

No.	Protein name	Species match	Accession no.	Fold change concentration (mg/kg)		Gene ontology (general annotation) (biological or molecular function)
				3.5	14.0	
1	Actin 57B	*D. melanogaster*	17647135	2.47	–	Glucuronosyltransferase acitivity
2	Transcription factor IIF	*D. melanogaster*	17737791	2.11	–	Transcription initiation from RNA polymerase II promotor
3	CG13872	*D. melanogaster*	20130165	2.11	–	Unknown
4	CG18476	*D. melanogaster*	24646039	2.11	–	Nucleus, nucleic acid binding
5	CG10748	*D. melanogaster*	24663595	2.11	–	Glycolysis, malate dehydrogenase
6	Neurocalcin	*D. melanogaster*	28574621	2.91	–	Calcium ion binding (inhibits the phosphorylation of rhodopsin in a calcium-dependent manner)
7	14-3-3 ζ, Isoform 1	*D. melanogaster*	62471697	2.01	2.30	Neurogenesis (required in Raf-dependent cell proliferation and photoreceptor differentiation during eye development)
8	Scribble, isoform G	*D. melanogaster*	116008106	2.41	–	Neurogenesis, sensory transduction
9	Clathrin heavy chain, isoform F	*D. melanogaster*	161077850	2.54	–	Sperm individualization, neurotransmitter secretion
10	Kismet, isoform C	*D. melanogaster*	221330583	2.54	–	Border follicle cell migration (the directed movement of a border cell through the nurse cells to reach the oocyte), antimicrobial humoral response
11	ATP synthase-β	*D. melanogaster*	24638766	–	2.31	ATP synthesis
12	Knockdown, isoform A	*D. melanogaster*	24640126	–	2.22	TCA cycle, behavior, citrate synthase
13	Histone H2B	*D. melanogaster*	78707160	–	2.04	Defense response to bacterium
14	Histone H2A	*D. melanogaster*	78707162	–	3.97	Defense response to bacterium
15	Histone H3	*D. melanogaster*	78707164	–	3.47	Defense response to bacterium
16	CG14696	*D. melanogaster*	24645817	–	2.27	Protein binding
17	CG6059	*D. melanogaster*	24650536	–	2.73	Unknown
18	Ribosomal protein S15Ab	*D. melanogaster*	24652557	–	3.47	Translation
19	Ribosomal protein L23A	*D. melanogaster*	24655502	–	4.61	Translation
20	Bellwether	*D. melanogaster*	24658560	–	2.98	ATP synthesis, ATP synthase-α
21	Glycyl-tRNA synthetase	*D. melanogaster*	24664462	–	3.47	Protein biosynthesis
22	CG9674	*D. melanogaster*	28574881	–	4.67	Glutamate biosynthesis pathway, glutamate synthase
23	CG9468	*D. melanogaster*	24582929	–	2.27	Mannose metabolic process, α-mannosidase
24	CG32703	*D. melanogaster*	24640802	–	3.12	Phosphorylation, MAP kinase
25	CG31619	*D. melanogaster*	116007356	–	3.57	Metallopeptidase activity, proteolysis
26	CG15086	*D. melanogaster*	221330399	–	3.79	Unknown
27	Rest corepressor	*A. aegypti*	157114706	2.40	–	DNA binding
28	Laminin A chain	*A. aegypti*	157119736	2.20	–	Heart development, locomotion involved in locomotory behavior
29	Phenylalanyl-tRNA synthetase β chain	*C. quinquefasciatus*	170072129	–	2.33	Protein biosynthesis
30	Cubulin	*A. aegypti*	157127870	7.31	–	Calcium ion binding
31	Ubiquitin specific protease	*C. quinquefasciatus*	170060428	2.41	–	Protein desumoylation, SUMO-specific protease activity
32	Numb-associated kinase	*C. quinquefasciatus*	170035699	2.00	–	ATP binding
33	Serine protease inhibitor, serpin	*C. quinquefasciatus*	170041372	2.05	–	Apoptosis, response to cytokine stimulus
34	Conserved hypothetical protein	*C. quinquefasciatus*	170049245	3.67	–	Unknown
35	Microtubule-associated protein futsch	*C. quinquefasciatus*	170055227	3.05	–	Axonogenesis, negative regulation of neuron apoptosis

Table 3. Continued

No.	Protein name	Species match	Accession no.	Fold change concentration (mg/kg)		Gene ontology (general annotation) (biological or molecular function)
				3.5	14.0	
36	Lysine-specific histone demethylase	*A. aegypti*	157129233	–	2.59	Demethylation, transcription
37	Paternally expressed 3, isoform1	*Homo sapiens*	226053169	5.64	–	Apoptosis
38	Exosome component 10	*Homo sapiens*	50301240	3.84	–	RNA processing
39	Fibrous sheath CABYR-binding protein	*Homo sapiens*	209977046	2.93	–	Cell projection
40	Valosin-containing protein (VCP)	*Homo sapiens*	6005942	–	2.37	Protein ubiquitination, protein transport

Table shows results using LC-MS/MS and their homology alignments.

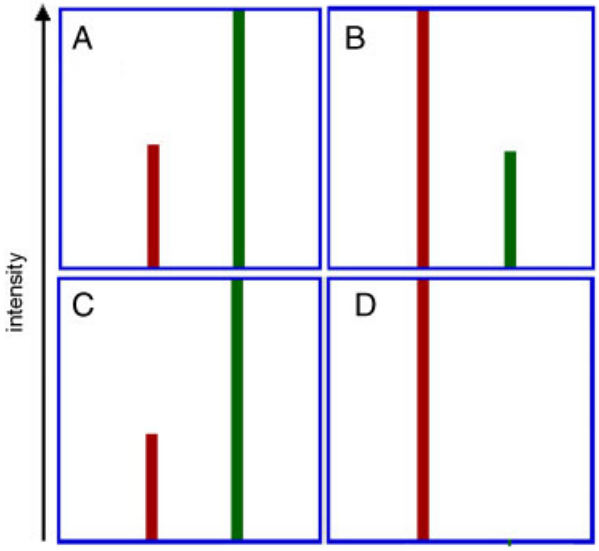

Figure 5. Four proteins commonly determined in 1-D and 2-D PAGE analyses and their intensities of differentially expressed in *P. kimi* after exposure to 14.0 mg/kg of cadmium. (A), phenylalanyl-tRNA synthetase α chain; (B), crumbs; (C), Lysine-specific histone demethylase; (D), CDKN1A interacting zinc finger protein 1 isoform 1.

triggered a message to the centrosomes that lead to their inactivation and the subsequent failure of the damaged chromosomes to segregate [43]. Cadmium treatment caused the downregulation of microtubule-associated protein and centromere-associated protein, resulting in abnormal centrosomes in cadmium-exposed collembolans. Thus, cadmium exposure may prevent collembolans from controlling DNA-damaged cells.

4.4 Various proteins affected by cadmium toxicity in *P. kimi*

Cadmium induces caspase-mediated cell death via suppression by Bcl-2 [44]. Apoptosis is a process of active cell death after activation of caspase proteins. In relation to cadmium toxicity, caspase-3 is activated and involved in apoptosis process in Rat-1 fibroblasts [44]. In the present work, several proteins in relation to apoptosis were over-expressed in cadmium-treated collembolans. Caspase-1 isoform γ precursor was downregulated in response to cadmium treatment in *P. kimi* (Table 4). The protein regulates positively I-κB or NF-κB cascade in animals and the significance of caspase-1 in prostate cancer and neurons has recently been documented [45, 46]. Therefore, down-regulation of caspase-1 in *P. kimi* against cadmium toxicity seems to be not enough to activate I-κB or NF-κB cascade at the level of 14mg/kg of cadmium concentration. In regard to caspase protein expression, a protein designated as paternally expressed 3 is believed to suppress tumor generation in ovarian cells [47]. A serine protease inhibitor,

Table 4. Identification of proteins differentially expressed in response to cadmium toxicity in the collembolan species, *P. kimi*

No.	No. on 2-D PAGE	Protein name	Species match	Accession no.	Score (expression status)	Gene Ontology (general annotation) (biological or molecular function)
1	0301	Dermcidin preproprotein	*Homo sapiens*	16751921	20.15 (down)	Defense response to bacterium
2	1701	Phenylalanyl-tRNA synthetase α chain	*C. quinquefasciatus*	170044713	10.16 (up)	tRNA processing
3	3402	CG6232	*D. melanogaster*	45550179	10.15 (down)	Proteinaneous extracellular matrix, metalloendopeptidase activity
4	5401	Crumbs homolog 1 precursor	*Homo sapiens*	41327708	10.15 (down)	Cell–cell signaling, response to stimuli, visual perception
5	6301	Protease m1 zinc metalloprotease	*A. aegypti*	157133549	20.15 (down)	Proteolysis
6	7802	Lysine-specific histone demethylase	*A. aegypti*	157129233	10.16 (up)	Histone demethylation, negative regulation of transcription factor activity
7	9102	PRKR-interacting protein 1	*Homo sapiens*	55741661	10.19 (down)	Negative regulation of protein kinase activity, protein kinase inhibitor activity
8	9502	Conserved hypothetical protein	*C. quinquefasciatus*	170052379	20.16 (down)	Predicted kinase
9	0001	Wnt inhibitory factor 1	*C. quinquefasciatus*	170053924	10.19 (down)	Wnt receptor signaling pathway, signal transduction
10	0101	Trypsin-3 isoform 1 preproprotein	*Homo sapiens*	62122917	10.25 (down)	Proteolysis
11	1001	Hypothetical protein LOC57653	*Homo sapiens*	55741661	20.20 (down)	Unknown
12	2301	Dermcidin preproprotein	*Homo sapiens*	16751921	20.16 (down)	Defense response to bacterium
13	3102	Caspase-1 isoform γ precursor	*Homo sapiens*	15431330	10.15 (down)	Apoptosis, positive regulation of I-kappaB kinase/NF-kappaB cascade
14	4303	Protease m1 zinc metalloprotease	*C. quinquefasciatus*	157133549	20.14 (down)	Proteolysis
15	5001	AGAP003775-PA	*Anopheles gambiae* str.	158288453	10.21 (down)	Predicted
16	5303	Dermcidin preproprotein	*Homo sapiens*	16751921	20.17 (down)	Defense response to bacterium
17	5902	AGAP004731-PA	*A. gambiae* str.	158297984	10.18 (down)	Phospholipid metabolic process, Phospholipase A2 activity
18	7703	CDKN1A interacting zinc finger protein 1 isoform 1	*Homo sapiens*	196115147	10.17 (down)	Nucleic acid binding, zinc ion binding
19	8002	AGAP011938-PA	*A. gambiae* str.	158300735	20.13 (down)	Predicted
20	8202	AGAP011938-PA	*A. gambiae* str.	158300735	20.15 (down)	Predicted
21	8301	Small optic lobes, isoform B	*D. melanogaster*	45549036	10.15 (down)	Proteolysis, zinc ion binding, calcium-dependent cyctein-type endopeptidase activity

Table shows results using 2-D PAGE (pH 4–7) and LC-MS/MS analysis, and their homology alignments.

serpin, which is involved in the inflammatory response, was also over-expressed in *P. kimi* after exposure to cadmium.

In this study, glycolysis and energy production were damaged by cadmium treatment due to the downregulation of hexokinase, glyceraldehyde 3-phosphate dehydrogenase, and NADH dehydrogenase in relation to cadmium treatment against *P. kimi* (Table 2). For energy production in the cadmium-treated collembolans, ATPase synthase α, ATPase synthase β and citrate synthase were over-expressed to compensate energy production pathway (Table 3).

Hypoxia-inducible factor-1 (HIF-1) α subunit was downregulated in response to cadmium in collembolans (Table 5). A similar result was previously reported [48]. In this previous study, expression of the HIF-1 α subunit was found to be downregulated in response to cadmium exposure to human cell lines [48]. However, a contradictory

Table 5. Identification of proteins that were differentially regulated in response to cadmium toxicity (14.0 mg/kg) in the collembolan species, *P. kimi*

No.	Protein name	No. of matched peptides	MASCOT score (value $P = 0.05$)	Accession no.	Species	Cellular function
DE2	Hypothetical protein SO0804	2	55 (54)	gil24372393	*Shewanella oneidensis* MR-1	Hypothetical protein
DE3	Trypsin	–	–	–	–	–
DE4	Trypsin	–	–	–	–	–
DE5	3-phosphoinositide-dependent protein kinase-1 (ISS)	2	64 (54)	gil116056580	*Ostreococcus tauri*	Kinase activity (cellular response to insulin stimulus)
DE8	Tubulin	1	89 (62)	gil121544009	*Maconellicoccus hirsutus*	GTP binding (protein polymerization)
DE9	Dermcidin preproprotein	1	67 (53)	gil16751921	*Homo sapiens*	Defense response to bacteria or fungus
DE10	Nucleoside diphosphate kinase A	2	87 (53)	gil12700713	*Cavia porcellus*	Kinase activity (nucleotide metabolism)
DE19	HIF-1 α subunit	2	96 (53)	gil3790535	*Homo sapiens*	Transcription regulation (oxygen homeostasis)
IN3	Hypothetical protein TTHERM_00933210	1	63 (54)	gil118377396	*Tetrahymena thermophila* SB210	Hypothetical protein
IN4	Trypsin	–	–	–	–	–
IN5	Keratin	–	–	–	–	–
IN6	Myosin	7	363 (62)	gil25469362	*D. melanogaster*	Structural protein
IN8	Glutathione transferase	2	77 (54)	gil84402	*Schistosoma japonicum*	Transferase (detoxification)
IN9	Lactate dehydrogenase	1	67 (53)	gil15616499	*Bacillus halodurans* C-125	Anaerobic glycolysis
IN3	Hypothetical protein TTHERM_00933210	1	63 (54)	gil118377396	*T. thermophila* SB210	Hypothetical protein

Table shows results using 2-D PAGE (pH 4–10) and LC-MS/MS and their homology alignments. Numbers absent in the table indicate ''no homology'' after proteomic analysis. Numbers are paired with numbers in the Figs. 3 and 4.

result was also reported. In this study, a gene encoding HIF-1 was induced in the collembolans after exposure to cadmium [49]. Based on these conflicting results, it is difficult to fully understand the effect of cadmium on the HIF-1 expression. However, it is certain that hypoxia stresses occur in collembolans due to cadmium treatment, which ultimately affects normal biological processes.

Upregulation of coagulation, hemostasis and wound healing have been thought to be involved in protecting from invading microorganisms in cadmium-exposed collembolans [50]. Nota et al. [49] found increased levels of penicillin and cephalosporin biosynthesis pathway in response to cadmium exposure in collembolans.

In regard to the induction of innate immune systems and antibiotic proteins after cadmium treatment, histone H2A, histone H2B and histone H3 were over-expressed after collembolans were exposed to cadmium for 28 days (Table 3). Histone proteins are alkaline proteins that undergo posttranslational modifications which alter their interaction with DNA and other nuclear proteins, leading to stabilized nuclear chromatin. Interestingly, histones are secreted by epithelial cells and released during degranulation of leukocytes [51]. Also, histones have potent antibacterial activities [52, 53], and they have been found in terrestrial and aquatic organisms. In this study, upregulation of histones contributed to stabilization of nuclear chromatin under cadmium toxic conditions and protection of collembolans from bacterial attack during cadmium exposure.

4.5 Concluding remarks

In this study, *P. kimi* exposed to cadmium chloride were subjected to proteomic analysis. In this analysis, 36 downregulated proteins and 40 upregulated proteins were identified by LC-MS/MS analysis after 1-D PAGE. Some of the downregulated and upregulated proteins were also verified by LC-MS/MS analysis after 2-D PAGE. According to the UniProtKB/Swiss-Prot protein classification, most of the downregulated proteins were involved in glycolysis and energy metabolism, chaperones, transcription, reproduction

and neuron growth. However, many proteins involved in glycolysis and energy production, neurogenesis, defense systems in response to bacteria, and protein biosynthesis were induced in cadmium-treated collembolans. These proteins play an important role in cell adaptation to cadmium toxicity by sequestering and removing ROD generated from cadmium. Based on the combined findings of this study, we suggest that cubulin may be used as a biomarker of collembolans to detect low levels (3.5 mg/kg) of cadmium in soil environments. In addition to this suggestion, 14-3-3 ζ may also be a potential biomarker for the detection of medium levels (14 mg/kg) of cadmium in soil environments. Interestingly, many of the proteins found in collembolans have a high homology to human proteins. Therefore, collembolans may be sued to detect changes in protein expression in relation to stress conditions without the use of human cell lines or animal species during the initial monitoring step. Further studies on changes in DNA, mRNA and metabolomic levels in *P. kimi* will be necessary to understand how these changes are related to human responses to cadmium exposure on the molecular level.

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