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A proteomic analysis of larval midguts of *Boettcherisca peregrina* in response to cadmium exposure

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Abstract

In this study, the changes of protein expression in larval midgut cells of *Boettcherisca peregrina* Robineau-Desvoidy (Diptera Sarcophagidae) after exposure to 800 μg cadmium (Cd) per g diet for 48 h were investigated by two-dimensional polyacrylamide gel electrophoresis (2DE) plus with mass spectrometric analysis. The results showed the expression of ten proteins of midgut cells increased in response to acute Cd stress. Of these, nine were successfully identified, and seven of them are relevant to cellular detoxification processes. Functions of these seven proteins include removal of Cd (P-glycoprotein, P-gp), stress response (Heat shock protein, Hsp), osmoregulation and metal ion transport (Cd-inducible lysosomal protein CDR). This study will provide valuable information for the cell defense mechanism against heavy metals.

Key words: *Boettcherisca peregrina*, midgut, cadmium, proteomics.

Introduction

Heavy metal pollution has become a global environmental problem and severely threatened the biological diversity and people's health. Studies from an environmental metal intoxication process will help us gain valuable information on the metabolism of essential metals and the cell defense machinery. More and more attention has been paid to the potential effects of heavy metal pollution on insects, one of the important groups of global biological diversity (Sun *et al.*, 2007). Selecting biomarkers from such insects, therefore, would be of utmost interest and feasible. The flesh fly, *Boettcherisca peregrina* Robineau-Desvoidy (Diptera Sarcophagidae) is confronted with heavy metals in the environment, predominantly by the uptake of food from polluted sites. Heavy metals are absorbed in special metal-specific tissues of the fly. After severe exposure, cadmium (Cd) is mainly absorbed in midgut epithelium cells (Aoki *et al.*, 1984). Epithelium cells in the midgut take up Cd both from the luminal side and the hemolymph. Cellular absorption of Cd may in the first place lead to the induction of some proteins, which bind Cd to prevent cellular toxicity. Since Cd itself is nondegradable, this form of detoxification could be classified as sequestration. In addition, the midgut epithelium cells are also able to sequester Cd by compartmentalization, i.e., storage in membrane-enclosed vesicles (granules) as part of the lysosomal fraction in the cell, where each type of Cd bounds irreversibly to a specific compound in the granule (Wu *et al.*, 2009). However, the mechanism of Cd transported to membrane-enclosed vesicles (granules) is still not clear.

In recent years, two dimensional (2D) gel proteomic strategies have been widely resorted to identifiy original biomarkers of toxicity, discover novel mechanistic pathways and provide new insights into complex mechanisms (Kanitz *et al.*, 1999; Kennedy, 2002). Surprisingly, to date, few proteomic studies have been reported on protein expression profiles in insects following Cd insult. Using the proteomic approach to identify insect biomarkers for Cd pollution can be particularly attractive for environmental risk assessment, since insect samples may be readily collected from polluted environments (Xu *et al.*, 2007).

In this study, several Cd-induced proteins were identified in *B. peregrina* midguts by using proteomic methods. The functions of the several Cd-induced proteins included removal of Cd, stress response, osmoregulation and metal ion transport. This study will provide valuable information for the cell defense mechanism against heavy metals.

Materials and methods

Insect culture and Cd treatment

The strain of flesh fly *B. peregrina* was a laboratory reared population at Institute of Insect Sciences, Zhejiang University, China. The larvae were fed on an artificial diet that was mainly consisted of wheat flour, water and porcine liver (w : w : w = 3 : 5 : 6) (Wu *et al.*, 2009). Cultures of the fleshfly were maintained in an environmental chamber at 25 ± 1 °C with a photoperiod of 14:10 (L:D). For the treatment, 2 d old larvae were fed with artificial diets homogenized with 800 μg Cd per g diet in 500 mL glass jars for the following 2 d. Untreated controls were placed in the same conditions for the same duration.

Subsequently, the treated and control larvae were removed from the diets and washed well with distilled water. The washed larvae were kept in contact with water for one day to let the diet in the larvae purge from the gut. Then, they were dissected on ice with the aid of a stereomicroscope. After being rinsed with deionized water once and 10 mM Tris buffer three times, the midguts were collected and stored at −80 °C prior to use.

Sample preparation

The stored midguts were homogenized in liquid-nitrogen. The resulted powder was then suspended in prechilled TCA/acetone (1 : 9, w/v) and sit at −20 °C overnight. Subsequently, samples were centrifuged for 30 min at 35,000 g and 4 °C. The precipitate was again re-suspended in prechilled acetone containing 0.2% DTT and let sit at −20 °C for one hour before a 30 min centrifugation at 35,000 g and 4 °C. The precipitate was placed on ice in a dust-free cabinet to air dry. Rehydration buffer (8 M urea, 4% CHAPS, 50 mM DTT, and 0.2% Bio-Lyte) was added for direct lysis. The solution was centrifuged for 30 min at 40,000 g and 4 °C to remove cell debris. The clear supernatant was collected as protein samples and stored in aliquots at −80 °C until analysis. Protein concentrations were measured by a Bradford assay.

Two-dimensional gel electrophoresis (2-DE) analysis

The 2-DE analysis was performed using pH 4-7 17 cm ReadyStrip IPG strips following protocols provided by the manufacturer (Bio-Rad, USA). Briefly, 1 mg protein from each sample was added to the rehydration buffer (8 M urea, 2% CHAPS, 10 mM DTT, 0.2% Bio-Lyte) to a final volume of 350 μL. An aliquot of 350 μL was then transferred into the focusing tray. ReadyStrip IPG strip was drawn gel side down through the solution that contained sample and buffer. After mineral oil was applied over each ReadyStrip IPG strip to prevent precipitation of the urea, the isoelectric focusing (IEF) was initiated in a PROTEAN IEF cell (Bio-Rad, USA). Rehydration and focusing were performed at 17 °C and zero voltage for 12 h, followed by 6,000 V power for a total of 40,000 Vh and a current limit of 50 μA. After IEF, the strips were removed from the focusing tray and equilibrated with gentle shaking for 15 min in 5 mL SDS equilibration buffer (375 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, 20% glycerol, 2% DTT). A second 15 min equilibration was followed in 5 mL volume of the same buffer with 2.5% iodoacetamide substituted for the DTT. At the end of equilibration, the ReadyStrip IPG strips were embedded on top of a 15% homogeneous SDS-PAGE gel and overlaid with 0.5% agarose in Laemmli buffer for electrophoresis. The second dimensional electrophoresis was carried out using a PROTEAN II xi system (Bio-Rad, USA) and at 15 mA/gel for 10 h.

After gel electrophoresis, gels were rinsed three times in deionized water. They were stained with Coomassie R-250 solution consisting of 45% v/v methanol, 45% v/v glacial acetic acid, and 0.1% w/v Coomassie R-250, and destained with 10% v/v methanol and 10% v/v glacial acetic acid successively until the background was cleared. Stained gels were imaged using a GS-800™ calibrated Densitometer (Bio-Rad, USA). After imaging, the PDQuest™ 2-D (Bio-Rad, USA) image analysis software was utilized to find proteins differentially expressed after 48 h Cd treatment. Spots whose expression level increased greater than 2 fold were considered significant for playing roles in cellular defense against Cd and selected for MS analysis.

After PDQuest™ 2-D image analysis software relatively quantified every protein spots according to the photometric value of protein spots on the gel image, we inputed the data and finished the histogram plot in Excel. The 2-DE analysis experiment repeated three times.

Protein digestion and mass spectrometric analysis

According to the intensity of Coomassie-stain in 2-D gels, 10 differential proteins spots (not found in Control or increased at least twofold in the amount when treated with Cd) were resolved to be analyzed by mass spectrometry, and were excised from different gels and transferred into Eppendorf tubes. Each spot was washed twice in milli-Q water, destained by washing with a 1 : 1 solution of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate, and equilibrated in 200 mM ammonium bicarbonate for 20 min to pH 8.0. The gels were then repeatedly washed twice in milli-Q water, dehydrated by addition of acetonitrile, and dried in a SpeedVac (Thermo Savant, Holbrook, NY, USA). Subsequently, gel pieces were rehydrated in 20 μL of TPCKTrypsin (Sigma) solution (20 μg/mL in 40 mM NH_4HCO_3 and 9% ACN) and incubated at 37 °C for 10 h. The mixture was then added to 50 μL extraction solution containing 50% ACN and 5% TFA to extract the trypsin-digested peptides. Later on, the extracted mixtures were concentrated to 4-5 μL in a lyophilizer. This protocol often gave a good signal-to-noise ratio on MALDI-TOF spectra. If not, peptides were treated with ZipTips (Millipore, USA) prior to being applied onto the sample plate. The peptide mixture (1 μL) was mixed with an equal volume of 10 mg/mL α-Cyano-4-hydroxycinnamic acid (Sigma, USA) saturated with 80% ACN in 0.05% TFA and analyzed with a Voyager-DE STR MALDI-TOF MS (Applied Biosystems, USA). The instrument was set to reflector mode with 100 ns delay extraction time, positive polarity, 65% grid voltage and 20,000 V accelerating voltage. The external calibration was carried out using Peptide Mass Standard Kit (Perspective Biosystems, USA). The matrix and the autolytic peaks of trypsin were used as internal standards for mass calibration.

Database searching

To interpret the mass spectra, the monoisotopic peptide mass fingerprints (PMF) of the tryptic peptides from MALD-TOF MS data were used for protein identification by searching the NCBI Nr database using Mascot software. Database search was performed using the following parameters: unrestricted, trypsin digest (allowed up to 1 missed cleavage), cysteines modified by carbamidomethylation, and mass tolerance of 100 ppm using internal calibration.

Results

Identification of differentially expressed proteins following Cd treatment

A representative example of the midgut proteins separated on a 2-DE gel was shown in figure 1. Approximately 70 protein spots were detected on the gels. Majority of these spots had pI values ranging from 4.2 to 7.0 and molecular weights from 7 to 72 kDa. After the

matching analysis, the expression levels of ten protein spots were found to be significantly increased following Cd-treatment (figure 1).

Protein identification by MS

Protein spots with significant changes were cut out and subjected to identification. Nine spots were successfully identified by MALDI-TOF mass based on PMF (table 1). Seven of these 9 proteins were relevant to cellular detoxification processes. They were assigned to the functions related to the removal of Cd (P-glycoprotein, P-gp), stress function (Heat shock proteins, Hsp), osmoregulation and transportation of metal ions (Cd-inducible lysosomal protein CDR).

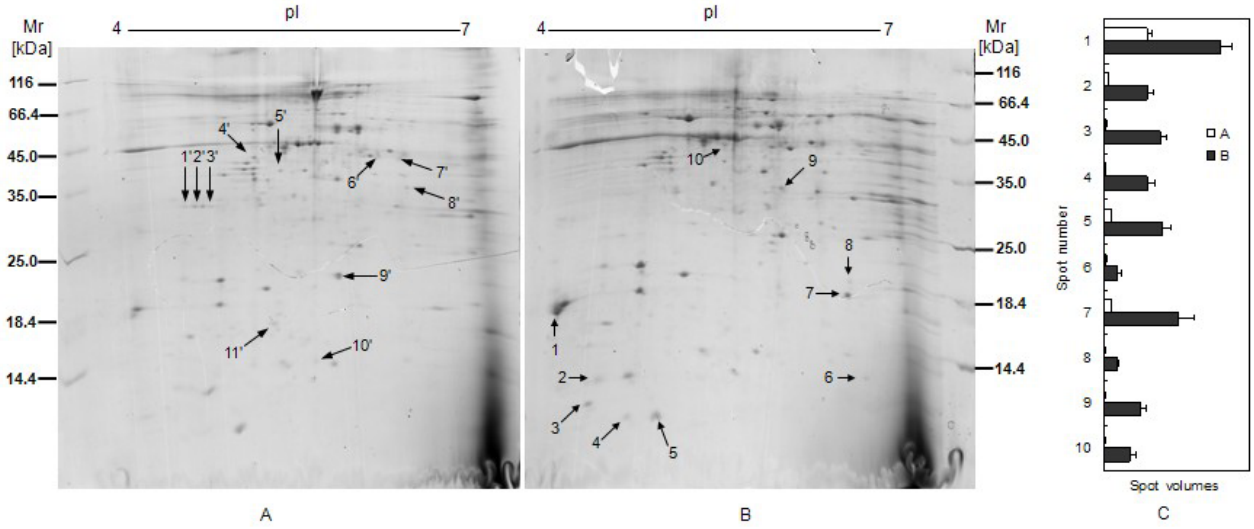

Figure 1. Two-dimensional gel electrophoresis analysis of the midgut proteins in *B. peregrina* larvae in response to cadmium. (A) Control. (B) Treatment with cadmium. (C) The relative quantifications of the 10 differential proteins (not found in Control or increased at least twofold in the amount when treated with cadmium) according to the intensity of Coomassie-stain in gels. Digits represent the differential spots between the two treatments. Digits 1'–11' were eleven differential spots that existed in untreated controls, but were undetected when treated with cadmium. Digits 1–10 were ten spots that were either absent or significantly decreased in control samples, but were present or increased at least twofold in the amount when treated with cadmium.

Table 1. The midgut proteins of *B. peregrina* larvae with altered expression levels following treatment with cadmium. [a] The protein spot numbers are indicated in figure 1; [b] Mowse score and threshold value are derived from Mascot algorithm, it is a probability indicator of the quality of the result match in the mascot software; [c] Matched = proportion of protein covered; [d] Theoretical molecular mass and pI of the protein; [e] Referred to protein entry code of the NCBI; [f] Functions are from the NCBInr database.

Spot no.[a]	Identified protein	MOWSE Score[b]	Matched (%)[c]	Protein MW (Da)/pI[d]	NCBInr accession number[e]	Function[f]
1					Unidentified	
2	MHC class II DR beta chain	44	61	9295/ 5.29	7108495	Protective immunization effects
3	odorant binding protein	43	28	12258/ 4.75	6634105	Perceiving exoteric odorant
4	DR beta-chain antigen binding domain	43	40	10229/5.36	1835672	Binding exogenous antigen
5	P-glycoprotein	50	30	9693/5.28	53748561	Transport, ion transport
6	CG11015-PA	59	36	13579/9.0	20129279	Catalytic oxidation, transferred electron
7	small heat shock protein	46	21	20125/6.06	1362582	Stress response, protein folding, transport, repair
8	cadmium-inducible lysosomal protein CDR-5	72	35	31914/ 6.83	52352460	Osmoregulation, maintaining salt balance
9	heat shock protein 40	47	20	39000/8.47	20086429	Posttranslational modification, protein turnover, chaperones
10	RE12057p	106	21	37816 / 5.36	21064361	Cytoskeleton, filament formation

Discussion

To gain insight into the Cd detoxification mechanism in *B. peregrina*, this study analyzed proteomic responses of *B. peregrina* to this toxic metal. Several proteins were found to be induced by Cd, including P-glycoproteins (P-gp), heat shock protein (Hsp), Cd responsive proteins (CDR) and some proteins with antioxidant properties.

P-gp, an ATP-dependent transporter, plays a physiologic role in the transport and detoxification of Cd (Ambudkar *et al.*, 2006; Broeks *et al.*, 1996; Callaghan and Denny, 2002; Einicker-Lamas *et al.*, 2003; Thevenod *et al.*, 2000). This study showed increased P-gp expression in larval midgut cells of *B. peregrina* after exposure to 800 μg Cd per g diet. This is similar to the report of Huynh-Delerme *et al.* (2005). Cd was extruded from the apical membrane of the cells probably due to the P-gp expression level (Endo *et al.*, 2002; Kimura *et al.*, 2005).

Hsp was found to be induced in midgut cells of *B. peregrina* larvae exposure to Cd. Similar to other reports where the level of Hsps increased under heavy metal stress (Barque *et al.*, 1996; Bournias-Vardiabasis *et al.*, 1990; Braeckman *et al.*, 1999; Courgeon *et al.*, 1984; Warchalowska-Sliwa *et al.*, 2005). The expression Hsp may serve an emergency function and rescue sensitive proteins from being damaged by Cd exposure, because Hsps function involved in 'housekeeping' in the cell, including prevention of aggregation of damaged proteins, transportation, folding and unfolding, assembly and disassembly of multi-structured units, and degradation of misfolded or aggregated proteins (Gething and Sambrook, 1992; Jolly and Morimoto, 2000; Lindquist, 1986; Parsell and Lindquist 1994).

The CDR-1 protein was predicted to be an integral membrane protein that constituted two transmembrane spanning domains, and it might play roles in osmoregulation to maintain salt balance in *Caenorhabditis elegans* (Maupas) (Dong *et al.*, 2005). Lysosomal membrane proteins may also contribute to the mechanism of Cd tolerance. They might pump Cd ion and other ions into lysosome cells, where the transition metals were reserved. Expression of the Cd-inducible lysosomal protein, CDR-5, increased in midgut cells of *B. peregrina* larvae exposure to Cd, suggesting that CDR-5 might contribute to Cd tolerance or Cd detoxification.

Data in this study suggested that Cd could induced RE12057p (a type of actin) in the cell of *B. peregrina* larvae. Some studies also have demonstrated that Cd interferes with the dynamics of actin filaments in various types of cells (DalleDonne *et al.*, 1997; Mills and Ferm, 1989; Prozialeck and Niewenhuis, 1991; Wang *et al.*, 1996; Wang and Templeton, 1996). It is likely that Cd could either affect the intracellular Ca^{2+} concentration or compete with Ca^{2+} for protein binding sites (Hinkle *et al.*, 1987; Swandulla and Armstrong, 1989; Tvermoes *et al.*, 2011), therefore exerting an effect on actin filaments whose assembly and/or disassembly are regulated by a number of calcium-dependent factors (Furukawa *et al.*, 2003; Oertner and Matus, 2005).

Major histocompatibility complex (MHC) class II DR beta-chain and DR beta-chain antigen binding domain are involved in the interaction about antigen processing and presentation of peptide or polysaccharide antigen via MHC class II and immune response in *Bos taurus* L. and *Ovis aries* L. (Takeshima *et al.*, 2011). Hitherto, MHC class II DR beta-chain and DR beta-chain antigen binding domain had not been reported to be important in detoxification after Cd exposure. However, MHC Class II proteins can bind Zn^{2+} and Ni^{2+} (Loh and Fraser, 2003). Maybe MHC Class II protein can bind Cd ion, and MHC Class II proteins (MHC class II DR beta-chain and DR beta-chain antigen binding domain) increase precisely for the detoxification of Cd.

In conclusion, when *B. peregrina* larvae were exposed to Cd, many proteins were found to be changed by Cd, including P-gp, Hsp, CDR and proteins with antioxidant properties. The proteins might contribute to Cd detoxification. But detoxification functions of the proteins must be revealed by further functional studies *in vivo* and *in vitro*. The identification of Cd induced proteins in midguts of *B. peregrina* larvae will set a basis for monitoring heavy metal pollutions in the environment and the Cd tolerance mechanism studies in insects.

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