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Cadmium exposure on tissue-specific cadmium accumulation and alteration of hemoglobin expression in the 4th-instar larvae of *Propsilocerus akamusi* (Tokunaga) under laboratory conditions

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ABSTRACT

The expression of hemoglobin (Hb) genes has considerable potential as a biomarker for environmental monitoring in *Chironomus*. However, no sequence information is available regarding Hb genes in *Propsilocerus akamusi* (Tokunaga), thus the change in Hb mRNA gene expression caused by environmental pollutants remains unknown. In this study, we cloned two Hb gene fragments (PaHbV and PaHbVII) from *P. akamusi*, analyzed the expression patterns of the PaHbV and PaHbVII transcripts in different tissues using Real-Time quantitative PCR (RT-qPCR), and also measured the Cd levels in different tissues exposed to a sublethal concentration. The results showed significantly increased Cd concentrations and tissue-specific Cd distribution patterns in all of the tissues tested, including the hemolymph, during all time courses. A model describing the roles of specific tissues in Cd uptake and accumulation dynamics was also determined. The Malpighian tubules, gut, and epidermis were the primary sites of Cd accumulation, whereas the hemolymph was the temporary target organ of Cd accumulation, with the Cd being transferred to other internal tissues via the hemolymph. The relative mRNA expression profiles of PaHbV and PaHbVII indicated that their expression levels differed across the different tissues, indicating a tissue-specific response. Our results suggested a reverse effect between Hb expression and Cd accumulation during long-term Cd exposure in comparison with previous studies. The expressions of Hb genes in *P. akamusi* could be developed as biomarkers for assessing the general health conditions of freshwater ecosystems.

1. Introduction

Cadmium (Cd) is a highly toxic heavy metal that is widely distributed in the environment (Kikuchi et al., 2007; Kabata-Pendias, 2010; Prakash et al., 2011). This trace element is of particular concern due to its toxic properties associated with physiological disturbances, such that exposure via bioaccumulation in the food chain often results in deleterious effects on organisms, including health dysfunction and cancer (Obata and Umebayashi, 1997; Thevenod, 2009). It has been reported that Cd accumulates in river sediments through various industrial and anthropogenic activities, and the concentration is much higher in the sediment than in the water samples (Korte, 1983; Jung et al., 2005; Prakash et al., 2011).

Chironomidae larvae constitute a substantial part of the sediment-dwelling fauna in all freshwater systems and are important prey species for fish and aquatic birds. They have been widely used as test organisms in aquatic ecotoxicological studies due to their important role in the aquatic food chain and association with benthic sediments (Bouché et al., 2000; OECD, 2001; Nair et al., 2011). Some studies have demonstrated that chironomids are physiologically tolerant to a range of adverse environmental conditions, including exposure to industrial byproducts such as metals (Choi et al., 2000; Haas et al., 2005; Hassell et al., 2006; Nowak et al., 2007; Vogt et al., 2007; Béchard et al., 2008). Among Chironomidae, *Propsilocerus akamusi* (previously *Tokunagayusurika akamusi*) is a common species of midge found in Siberia, Japan, and China, and its larvae are morphologically and ecologically quite unique. Previously, we reported that *P. akamusi* has an extraordinary capacity to resist Cd, as indicated by the extreme LC50 in our laboratory experiment (Zheng et al., 2008, 2011).

Heavy metal resistance is a complex process involving a variety of underlying biochemical, behavioral, and physiological changes (Hall, 2002; Naimo, 1995; Krantzberg and Stokes, 1990; Hall et al., 1979;

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Posthuma et al., 1992). Some reports have suggested that underlying changes in metal accumulation may contribute towards metal resistance (Weis, 2002; Gale et al., 2003; Xie and Klerks, 2004).

In addition, heavy metals have also been found to induce genotoxic effects on chironomids. Studying the single gene mRNA expressions involved in biological processes using Real-Time PCR can facilitate the diagnosis of environmental contamination, which can be a useful biomarker of stress in animals (Bustin, 2002; Michailova et al., 2001; Warchałowska-Sliwa et al., 2005; Nair et al., 2011). Hemoglobin (Hb), though widely distributed throughout the animal kingdom, is restricted to only a few representatives of large invertebrate taxonomic groups, including Insecta and Crustacea. The function of invertebrate Hb is essentially the same as that of vertebrates in that it carries oxygen from the environment to the respiring tissues (Ha and Choi, 2008). *Chironomus* Hb exists as monomers or dimers and exhibits a high O_2 affinity and a high degree of polymorphism (Das and Handique, 1996; Osmulski and Leyko, 1986; Fukuda et al., 1993; Kao et al., 1994; Gruhl et al., 1997; Hankeln et al., 1997). The expression of Hb genes has considerable potential as a biomarker for environmental monitoring in *Chironomus* (Lee et al., 2006; Ha and Choi, 2008). Some studies have reported the isolated hemoglobin components from the 4th-instar larvae of *T. akamusi* by gel filtration using Sephadex G-50 and DEAE-cellulose chromatography, as well as the crystal structures of components V and VII from *P. akamusi* (Fukuda et al., 1993; Kuwada et al., 2007). However, to our knowledge, no sequence information is available regarding Hb genes in *P. akamusi,* thus the changes in Hb mRNA gene expression as a result of environmental pollutants remains unknown.

In the present study we analyzed Cd accumulation in the Malpighian tubules, entire gut, epidermis, and hemolymph of 4th-instar larvae *of P. akamusi* exposed to a sublethal concentration of cadmium chloride. Furthermore, two cDNA clone-encoding parts of Hb genes were identified and the mRNA expression profile of Hb under sublethal Cd exposure was also studied in these four tissues (including the hemolymph). This in vitro data helps us to account for the role of specific tissues in Cd uptake and accumulation dynamics, as well as to assess whether the expression patterns of Hb genes could be used as potential biomarkers for environmental monitoring in *P. akamusi.*

2. Materials and methods

2.1. Biological samples and Cd^{2+} preparation

Propsilocerus akamusi samples were collected from the Chaobai River (Baodi district, Tianjin City, China; latitude 40.068; longitude 116.821) in April 2014 and transported live to the laboratory. Upon arrival, the samples were transferred into a clean plastic plate and kept at 5 °C prior to experimentation. We measured the head capsule widths to determine the larval instars. The 4th-instar larvae weighed 0.017 ± 0.004 g and their length was 0.95 ± 0.11 cm. Prior to exposure in the control and experimental tanks, the larvae were reared on an artificial diet of fish flake food (90105942, Xiamen Mincheng Imp & Exp Co., Ltd, Xiamen, Fujian, China) in glass chambers containing 2 L of dechlorinated tap water and 1–2 cm of acid-washed sand for 48 h, with aeration under a 16–8 h light-dark photoperiod and 20 ± 1 °C temperature conditions.

Cadmium chloride (purity, 99%) was purchased from Tianjin Reagent Factory (Tianjin, China). The nominal concentrations of Cd^{2+} were reported as $CdCl_2{\cdot}2.5H_2O$. The test medium was dechlorinated tap water with a water hardness of 260 mg/L as $CaCO_3$; the pH was 7.6; dissolved oxygen (DO) was 5.2 mg/L; biochemical oxygen demand (BOD) was 0.55 mg/L; chemical oxygen demand (COD) was 7.8 mg/L; Cd^{2+} and other trace metals were not detectable.

2.2. Cd exposure and determination of Cd content

The acute toxicity bioassays were performed for 96 h using 4th-instar *P. akamusi* larvae consisting of five groups of 20 individuals in 100 mL glass aquaria, comprising four experimental groups and one control. Experimental groups were exposed for 96 h to four concentrations of Cd^{2+} including 10.0, 20.0, 40.0, and 50.0 mmol/L. The control group was simultaneously exposed to clean water alone. The experiments were performed in static systems and the insects were not fed during the period of exposure. The tests were conducted in triplicate. Those individuals that exhibited no body movement when gently prodded with a spatula were considered dead. The 96h-LC50 of Cd^{2+} on inhibition of the growth of *P. akamusi* was calculated according to the lethal rates of the samples. The results showed that the 96h-LC50 of Cd^{2+} on *P. akamusi* was 24.0 mmol/L. Therefore, 10% 96h-LC50 (2.4 mmol/L) was subsequently used as the sublethal Cd^{2+} dose for the remainder of the study.

The larvae were transferred to 250 mL glass beakers containing 200 mL of aerated dechlorinated Hamilton tap water (80 chironomids per beaker) and exposed to a sublethal concentration of cadmium chloride for 48, 72, and 96 h. All the exposure and control treatments were done in triplicate in each exposure performed in beakers. An initial 70 larvae were sampled on the day the experiment was started (day 0), treated with the controls (CK), then 70 larvae from each treatment (each replicate) were sampled at 48, 72, and 96 h for Cd tissue concentration analysis. No feeding was done during exposures.

After each exposure period, the larvae were transferred to fresh water for 5 min to remove non-specifically bound Cd and then blotted dry using filter paper (Gillis and Wood, 2008). The epidermis, entire gut, and the attached Malpighian tubules were dissected out of the 4th-instar larvae under a binocular microscope in physiological saline. The tubules were then dissected from the entire gut. Hemolymph droplets were collected and then transferred by pipette to microcentrifuge tubes. Before transferred to the microcentrifuge tubes, the epidermis, gut, and Malpighian tubules were washed with fresh water to remove the hemolymph.

For Cd analysis, the digestion was completed using aqua regia solution (McGrath and Cunliffe, 1985; HCl: HNO3, 3:1, v/v) followed by microwave-assisted digestion at 185 °C for 20 min (Multiwave 3000, Anton Paar, Australia). After cooling, the residues were transferred to 50 mL flasks and diluted with deionized water. Prior to analysis, the samples were filtered through a 0.45 μm membrane filter (Chahid et al., 2014). The Cd contents were eventually measured by flame atomic absorption spectrometry (FAAS, AA240FS, Varian, Palo Alto, CA). Procedural blanks containing no biological materials were also analyzed with each experimental run.

2.3. Identification and expression analysis of Hb genes

2.3.1. Two P. akamusi hemoglobins (PaHb) cDNA fragments

Two amino acid sequences of component V (L-type Hb) and component VII (N-type Hb) from *P. akamusi* were obtained from a published study (Kuwada et al., 2007) and were designated as PaHbV and PaHbVII. These two amino acid sequences of the PaHb were aligned with that of other chironomid species using ClustalW (Thompson et al., 1994) and the conservative region was used to design the degenerate primers. Two pairs of degenerate primers for these two genes were used to amplify the cDNAs by reverse transcription PCR (RT-PCR) using cDNA templates prepared from the entire insect body (Table 1). The RT-PCR products were run on a 1.5% agarose gel, purified using a Gel Extraction Kit (Omega, Doraville, CA, USA), subcloned into the pEASY-T3 cloning vector (TransGen Biotech Co., Ltd. Beijing, China), and then sequenced by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China).

The similarities and characteristics of the two PaHb fragments were compared with those of known amino acid sequences from *P. akamusi*. The nucleic acid features were analyzed using DNAman (Version 4.0, Lynnon Corp., Quebec, Canada) and BioEdit (version 7.0.0, Ibis Therapeutics, a division of Isis Pharmaceuticals, Inc., Carlsbad, USA) as well as the online BLAST program provided by NCBI (Madden, 2002).