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Low-molecular-weight-chitosan ameliorates cadmium-induced toxicity in the freshwater crab, *Sinopotamon yangtsekiense*

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ABSTRACT

Cadmium (Cd) has been shown to induce oxidative stress. Low-molecular-weight-chitosan (LMWC) has been demonstrated to exhibit potent antioxidant effects. We investigated the regulation role in Cd^{2+}-induced oxidative damage in the hepatopancreas of the freshwater crab *Sinopotamon yangtsekiense* and the protective effect of LMWC. The results showed that Cd^{2+} significantly increased the hepatopancreatic metallothionein (MT) mRNA levels and protein kinase C (PKC) activity while decreasing the activities of Na^+,K^+-ATPase and Ca^{2+}-ATPase in crabs relative to the control group. Co-treatment with LMWC suppressed the levels of MT and PKC but raised the activities of Na^+,K+-ATPase and Ca^{2+}-ATPase in hepatopancreatic tissues compared with the crabs exposed to Cd^{2+} alone. We postulate that LMWC may exert its protective effect through regulating the expressions of MT, PKC, Na^+,K^+-ATPase and Ca^{2+}-ATPase, thereby enhancing antioxidant defense. These observations suggest that LMWC may be beneficial because of its ability to alleviate the Cd^{2+}-induced damages to the crabs.

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1. Introduction

Chitosan (deacetylated chitin) is derived from chitin, the component of cell walls of fungi and the shells of arthropods (Shahidi and Abuzaytoun, 2005). It has been demonstrated to exhibit a wide array of biological effects, including anti-oxidative, anti-bacterial and anti-fungal effects (No et al., 2002; Yen et al., 2007; Seyfarth et al., 2008; Dutta et al., 2009; Zhao et al., 2010). Generally, the common chitosan with a relatively high molecular weight is insoluble in water. However, lower-molecular-weight-chitosan (LMWC) with an average molecular weight of 10 kDa, obtained by persulfate-induced depolymerization of chitosan, is soluble in water, which may make it easy to be absorbed by the digestive tract. Some researchers had identified that LMWC has many outstanding health benefits such as immunity regulation, anti-tumor, liver protection, blood lipids lowering, anti-diabetic, antioxidant and anti-obesity (Maeda and Kimura, 2004; Baek et al., 2007; Yin et al., 2009), and especially Tomida et al. (2009) reported that LMWC has more potent antioxidant effects than high molecular weight chitosan. Hence the biological properties of LMWC and its applications in some aspects involving diet, medicine and feed additives have attracted considerable attention.

Cadmium (Cd), a toxic metal, is released into the air, water and soil environment mainly from industrial processes (Satarug et al., 2003). The importance of Cd as the ecological environmental and health problem has received great attention over the past 50 years (Jarup and Akesson, 2009; Nordberg, 2009). In aquatic ecosystem Cd may first bioaccumulate in one species (Honda et al., 2008; Wang and Wang, 2009; Chora et al., 2009), and then gets biomagnified along food chains, and ultimately poses risks to the ecosystem and human health (Shore and Douben, 1994; Satarug et al., 2003). For example, Zhu et al. (2005) found that Cd pollution was one of the main factors that resulted in the decrease in the quantity and the individual specification of population of freshwater crab, *Sinopotamon chekiangense Tai et Song*, on the Shuanqing Stream of Tianmu Mountain in Zhejiang China. Kobayashi et al. (2009) showed that drinking and/or cooking with Jinzu River (Japan) water and eating Cd-polluted rice influenced the occurrence of Itai–itai disease, which refers to a syndrome that principally consists of a painful skeletal condition resulting from weak and deformed bones induced by chronic Cd poisoning.

Previous study has identified that Cd poisoning is mainly embodied in oxidative damages to body such as the liver and kidney of rats through the excessive accumulation of reactive oxygen species (ROS) and the decrease of the activities of cellular anti-oxidative enzymes including superoxide dismutase (SOD),

Abbreviations: Cd, cadmium; CaM, calmodulin; CAT, catalase; GSH-Px, glutathione peroxidase; LPO, lipid peroxidation; LMWC, lower-molecular-weight-chitosan; MDA, malondialdehyde; MT, metallothionein; ANOVA, one-way analysis of variance; PKC, protein kinase C; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase

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catalase (CAT) and glutathione peroxidase (GSH-Px) (Nigam et al., 1999; Jurczuk et al., 2004). Also, metallothionein (MT), protein kinase C (PKC), Na^+,K^+-ATPase and Ca^{2+}-ATPase have been shown to play an important role in regulating cellular functions and are related to the oxidative damage process (Mense et al., 1997; Golod and Savina, 1997; O'Brien and Salacinski, 1998; Kumari et al., 1998; Gopalakrishna and Jaken, 2000; Asagba et al., 2004). MT, a low-molecular-weight, cystein-rich and metal-binding protein, is mainly implicated in metal ion detoxification (Kumari et al., 1998). MT gene expression is predominantly regulated by metals at the level of transcription, while the induction of MT has been proposed to be an important adaptive mechanism in decreasing Cd toxicity such as $CdCl_2$-induced oxidative damage, lethality and hepatoxicity (Goering et al., 1995; Liu et al., 1995). PKC plays a prominent role in the cellular transcriptional regulation of cell growth and differentiation (Reyland and Bradford, 2010). Cd may stimulate the nuclear targeting of PKC and activate transcription factors by phosphorylation, which in turn stimulates the expression of proliferation genes. Na^+,K^+-ATPase, also known as Na^+/K^+-pump, maintains Na^+ and K^+ gradients across cell membranes. This enzyme is found in almost all animal cells. It is responsive to multiple metabolic factors and hormones, since it contributes to the establishment of an electrochemical gradient that is necessary to drive a variety of secondary active transport processes across the membrane. Besides Na^+,K^+-ATPase, the Ca^{2+}-ATPase is an important regulator of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]i$) in most animal cells. Since Ca^{2+} outward transport by the Ca^{2+}-ATPase is activated by small increments in $[Ca^{2+}]i$, the activity of this transport system modulates the $[Ca^{2+}]i$ changes induced by various agonists. Cd had been shown to disrupt the functioning of the Na^+,K^+-ATPase and Ca^{2+}-ATPase in the process of cell membrane injury. The activation of PKC is associated with the transcriptional regulation of MT and the activities of Na^+,K^+-ATPase or Ca^{2+}-ATPase (Yu et al., 1997; Kosk-Kosicka and Zylińska, 1997), and such interaction is related to oxidative damages induced by Cd^{2+}.

Accordingly, we hypothesized in the present study that Cd would induce oxidative damage, which is related to the expression of MT and the activities of PKC, Na^+,K^+-ATPase and Ca^{2+}-ATPase, while antioxidants would prevent the damaging effect via regulating the expressions of the biological parameters above. To test this hypothesis, we evaluated the effects of Cd on MT, PKC, Na^+,K^+-ATPase and Ca^{2+}-ATPase as well as the preventive effects of LMWC against Cd^{2+}-induced cell injury using *Sinopotamon yangtsekiense* as the model crustacean.

S. yangtsekiense, a freshwater crab in the northern China, is an important link in the aquatic food web. The field and laboratory studies showed that Cd^{2+} impaired the growth and survival of freshwater crabs and increased the mortality in Cd^{2+} exposed crabs (Wang et al., 2003; Zhu et al., 2005; Mi et al., 2008). The hepatopancreas is a digestive organ with diverse functions, including secreting enzyme, storing nutrients, metabolism and detoxification. Ma et al. (2008) reported the bioaccumulation of Cd^{2+} in the hepatopancreas of *S. yangtsekiense* followed the concentration–response relationship, and the Cd^{2+} induced severe oxidative damages to the hepatopancreas. Moreover, Li et al. (2010) reported that Cd^{2+} significantly inhibited the activities of total antioxidant capacity (T-AOC), SOD, CAT, GSH-Px and peroxidase (POD), while it markedly increased malondialdehyde (MDA) levels in the hepatopancreas *S. yangtsekiense* exposed to Cd^{2+} (58 mg/L for 96 h). It was implied that crab hepatopancreas was the site of multiple oxidative reactions and may, therefore, be a site of intense free radical generation. This result is in agreement with the notion that hepatopancreas is the main metabolic center for ROS production in crustaceans (Pinho et al., 2003). So it is necessary to investigate in depth the toxicological roles of Cd^{2+} in oxidative damage in the hepatopancreas of *S. yangtsekiense* and the protective effects of the antioxidants to the animals.

To our knowledge, there have been no studies on LMWC's roles in ameliorating the Cd^{2+}-induced oxidative stress through regulating the expressions of MT, PKC, Na^+,K^+-ATPase and Ca^{2+}-ATPase in the hepatopancreas of *S. yangtsekiense*. This work would provide an important experimental evidence for elucidating the mechanisms of Cd^{2+}-induced oxidative damages and the anti-oxidative mechanisms of LMWC.

2. Material and methods

2.1. Chemicals and apparatus

LMWC ($MW \leq 5000$ Da) was purchased from Golden-Shell Biochemical Co.,Ltd (Zhejiang, China). TRIzol reagent, oligo (dT) (18) primer, and dNTPs were obtained from Biomed Biochemistry (Beijing, China). Avian Myeloblastosis Virus Reverse Transcriptase (AMV RT), RNasin, and Taq DNA polymerase were purchased from Takara (Liaoning, China). PepTag Assay for non-radioactive detection of PKC system was acquired from Promega Co. (USA), and the detection kits of Na^+,K^+-ATPase and Ca^{2+}-ATPase activities were obtained from the Nanjing Jiancheng Biochemistry(Jiangsu, China).

2.2. Animals and treatments

Freshwater crabs, *S. yangtsekiense*, were purchased from a local dealer in the Taiyuan Dong-an aquatic product wholesale market in Shanxi province of China. Only healthy adult crabs with a homogeneous weight (20.0 ± 0.5 g) were collected. Prior to experiment, crabs were acclimated for 2 weeks in glass aquaria (45 cm $\times$ 30 cm $\times$ 30 cm) filled with dechlorinated, carbon-filtered city tap water (pH 7.5, dissolved oxygen 8.0–8.3 mg/L). A regime of 12 h light/12 h dark was applied and the temperature was kept at 20 ± 2 °C. Aquaria were shielded using a black plastic to reduce disturbance. Crabs were fed a commercial feed three times a week.

After acclimatization, animals were randomly numbered and divided into 7 equal groups of 4 animals each including normal control group and chemicals-exposure groups with Cd^{2+} or Cd^{2+} plus LMWC. The treatment protocol was: Control, Group A (29 mg/L of Cd^{2+}), Group B (58 mg/L of Cd^{2+}), Group C (87 mg/L of Cd^{2+}), Group D (58 mg/L of $Cd^{2+}$and 20 mg/L of LMWC), Group E (58 mg/L of $Cd^{2+}$and 40 mg/L of LMWC) and Group F (58 mg/L of $Cd^{2+}$and 80 mg/L of LMWC). The concentrations of Cd^{2+} were chosen according to three sublethal concentrations of Cd^{2+}, 29, 58 and 87 mg/L, corresponding to 12.5%, 25% and 37.5% of the Cd^{2+} 96 h LC_{50} (Yan et al., 2007). The concentrations of LMWC in this study were used as 20, 40 and 80 mg/L for 96 h. Crabs were not fed during the experimental period. All other conditions were kept the same as those used for acclimation.

2.3. Sample preparation

After the 96 h exposure, crabs were put to lethargy by subjecting them on ice for about 15 min. The tissues of hepatopancreas were immediately excised, washed with ice-cold saline (0.9% NaCl, w/v) and weighted. A piece of the hepatopancreas was frozen in liquid nitrogen for RNA and PKC extraction, and the rest samples were homogenized at 10% (w/v) in 0.1 mM phosphate buffered saline (PBS, pH 7.4) for determination of the activities of Na^+,K^+-ATPase and Ca^{2+}-ATPase at 4 °C, using a Potter-Elvehjem type motor-driven homogenizer. The homogenates were centrifuged at 1468*g* for 15 min at 4 °C, and the supernatants were collected and stored at -80 °C in polypropylene tubes until assay.

2.4. Measurement of Na^+,K^+-ATPase and Ca^{2+}-ATPase activities

Activities of Na^+,K^+-ATPase and Ca^{2+}-ATPase were determined spectrophotometrically using corresponding kits from the Nanjing Jiancheng Biochemistry according to the manufacturer's protocol.

2.5. Real-time quantitative RT-PCR

Total RNA was isolated from less than 100 mg of tissue using TRIzol Reagent according to the manufacturer's instructions. RNA quality was insured by 1% gel electrophoresis (28S/18S RNA) and spectrophotometric analysis (OD260/280). The OD260/OD280 ratio was in the range of 1.8–2.0. Total RNA was quantified by determination of optical density at 260 nm. First-strand cDNA was synthesized following a protocol of AMV RT with a slight modification. In reverse transcription reaction system, 1.0 μg total RNA, 2.0 μL AMV RT Buffer, 0.5 μL oligo(dT) (18), 0.5 μL AMV Reverse Transcriptase and 4.5 μL diethylpyrocarbonate (DEPC)-treated water were contained in a total 10 μL reaction volume. The mixture was incubated at 37 °C for 15 min, and heated to 85 °C for 5 s to inactivate the reverse transcriptase. The cDNA product was stored at -80 °C until use.

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