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Effects of Selenium on the Antioxidant Enzymes Response of *Neocaridina heteropoda* Exposed to Ambient Nitrite

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Abstract The effects of dietary Selenium (Se) supplementation on muscle superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) activities and haemolymph superoxide anions (O_2^-) of *Neocaridina heteropoda* exposed to ambient nitrite were investigated. The results showed supplementation of Se in diet could enhance the resistance of shrimp to low concentration ambient nitrite. The results demonstrated that Se might have a potentially useful role as an effective antioxidant and resistance to aqueous nitrite in shrimp and the effect of the organic Se was better than that of the inorganic Se.

Keywords Selenium · Nitrite · Antioxidant enzyme · *Neocaridina heteropoda*

Nitrite is the most common pollutant in aquaculture systems. Nitrite might accumulate in aquatic systems as a result of inadequate nitrification to nitrate by *Nitrobacter* sp. (Mevel and Chamroux 1981). The toxicity of nitrite to crustaceans has been studied by several investigators (Lewis and Morris 1986; Chen and Lee 1997; Cheng and Chen 1999). In single exposure, ammonia and nitrite concentrations were related to the mortality rate (Alcaraz et al. 1997). Nitrite in water caused a depressed immune response in *Litopenaeus vannamei* and increased susceptibility to *Vibrio alginolyticus* infection along with an increase of superoxide anion production, possibly to cytotoxic levels for the host (Tseng and Chen 2004). *Penaeus japonicus,* following 24 h exposure to nitrite as low as 0.076 mmol L^{-1}, increased its ammonia-N excretion by a factor of 1.9, its urea-N excretion by 200, and its organic-N excretion by 37, as compared to those in the control solution (Cheng and Chen 2001).

Various stresses have been associated with enhanced free radical generation causing oxidative damage (Siers 1985). Free radicals, including reactive oxygen species (ROS), such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), and singlet oxygen ($^1O_2$), are produced during normal aerobic metabolism. Under normal physiological conditions, continuous production of ROS induces oxidative stress and can result in damage to cell membranes, inactivation of enzymes, and damage to genetic material and other vital cell components. Radical damage can be significant because it can proceed as a chain reaction (Chien et al. 2003). The effective control and rapid elimination of ROS is essential to the proper functioning and survival of organisms. This is performed by antioxidant defense systems, which combat in vivo oxidation, maintain health and prevent oxidation-induced lesions (Jacob 1995).

The health of aquatic organisms is linked to overproduction of reactive oxygen species and antioxidants protect cell membranes against the production of free radicals. Thus, normal metabolism depends upon the ratio of free

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radical production and the activity of lipid peroxidation protection factors (Mourente et al. 2002). These antioxidant defense systems include both enzymatic and non-enzymatic components. The enzymes include radical scavenging enzymes such as catalase (CAT) and superoxide dismutase (SOD) acting on H_2O_2 and O_2^-, respectively, and glutathione peroxidase (GPX), which scavenges H_2O_2 and lipid hydroperoxides (Winston and Digiulio 1991; Halliwell and Gutteridge 1996). Small molecules that act as antioxidants include water-soluble compounds as well as lipid-soluble molecules (Packer 1991; Sies et al. 1992).

Selenium (Se) is an essential trace element for organisms, as determined from its biochemical role as a part of the active site in selenoproteins. The influence of dietary Se upon the activity of GPX has been well documented (Bügel et al. 2001). The Se requirement of animals presents an appropriate dose response which plays an essential role in the nutritional condition. This is called the "Se Weinberg dosage- effect principle".

So far, several studies on Se requirements have been reported (Hilton et al. 1980; Gatlin and Wilson 1984). However, there is little information on the relationship between Se and environment stress. The purpose of this research is to determine the effects of dietary Se on the antioxidant response of *N. heteropoda* exposed to ambient nitrite; and to evaluate the effects of different sources of Se on the activities of antioxidant enzymes. This study may provide some useful information for improving local aquaculture by increasing the nitrite tolerance of *N. heteropoda*, a commercially important small species found in freshwater throughout China.

Materials and Methods

In this research, Na_2SeO_3 was generously provided by Zhonglian Chemical Industry Reagent Plant, Chaoyang district, Beijing, China. $NaNO_2$ was bought from Baoding Chemistry Reagent Plant in Hebei province, China, and Se-enriched yeast was provided by Jamieson, Canada.

The composition of the experimental diet is given in Table 1. According to our research (Wang et al. 2009), the optimum dietary Se for *N. heteropoda* was about 0.45 μg g^{-1}. The selenium concentrations of the three diets were determined by atomic fluorescence photometry (Dagnall et al. 1969) to be 0.005 μg g^{-1} (un-supplemented control), 0.455 μg g^{-1} (inorganic Se diet), 0.455 μg g^{-1} (organic Se diet). Na_2SeO_3 was used as an inorganic Se source and Se-enriched yeast (added the selenium element in culturing process of yeasts, the yeast absorbs the selenium and transforms as the biological selenium) as an organic Se source. All ingredients were thoroughly mixed with soybean oil, and water was added to produce pellets of ~2 mm in diameter. After drying, the diets were stored at −30°C in sealed plastic bags during the experiments.

Common shrimp, *N. heteropoda* were collected from Baiyangdian Lake, Hebei Province, China in October 2008, and acclimated in a holding tank for 1 week. Shrimp were selected for the experiments. They had a mean weight of 0.30 ± 0.11 g and were reared in nine freshwater recirculation systems. Each tank of 50 × 50 × 20 cm^3 was aerated with an air pump attached to an aeration stone. Shrimp of similar sizes were randomly distributed into these nine tanks. Among these tanks, each diet was randomly assigned to three aquariums. During the experiment, water was replaced daily at 10%, and water temperature was kept at 20 ± 1°C, pH at 8.0 ± 0.2. The Se concentration in the water of aquariums was 0.01 mg L^{-1} (Dagnall et al. 1969). The three diets contained inorganic and organic Se and the control. The shrimp were fed twice daily in the morning and evening at a daily rate about 5% of their body weight. Feces and excess feed were removed in the morning before feeding. Shrimp were weighed at the beginning and end of the experiment. The intermolt shrimp, defined by Peebles (1977), from the diet trial were starved 2 days, and then they were used for a short-term nitrite toxicity test (Wang et al. 2006).

Static bioassay tests to assess nitrite toxicity of *N. heteropoda* were conducted. Shrimp were removed from the holding tank and individually transferred to a 10 L glass tank containing 5 L of test solution. The different solutions were prepared by adding nitrite to freshwater until the desired concentration was attained. The concentrations of nitrite were 0, 10, 30, 50, 70 and 90 mg L^{-1}. Twenty

Table 1 Composition of the basal diet (/kg)

Ingredient	Bean cake (g)	Soybean oil (ml)	Wheat starch (g)	Soybean meal (g)	Corn starch (g)	Vitamin premix[a] (g)	Mineral premix[b] (g)
Basal diet	412	31	227	237	52	21	20

[a] Supplied (per kg diet): vitamin A 300,000 IU; riboflavin 480 mg; pyridoxine 360 mg; cyanocobalamin 1.2 mg; thiamin 20.0 mg; menadione 20 mg; folic acid 170 mg; biotin 10 mg; a-tocopherol 3,000 IU; myo-inositol 8,000 mg; calcium pantothenate 800 mg; nicotinic acid 200 mg; choline chloride 8,000 mg; vitamin D 40,000 IU

[b] Supplied (per kg diet): $ZnSO_4 \cdot 7H_2O$, 0.817 g; $CaCO_3$, 3.28 g; NaH_2PO_4, 2.96 g; KH_2PO_4, 6.752 g; $CaCl_2$, 1.3328 g; $MgSO_4 \cdot 7H_2O$, 1.6 g; KCl, 0.448 g; $AlCl_3 \cdot 6H_2O$, 0.0192 g; $MnSO_4 \cdot (4–6)H_2O$, 0.229 g; $CuCl_2$, 0.52 g; $FeSO_4 \cdot 7H_2O$, 1.8 g; $CoCl_2$, 0.0282 g; KI, 0.036 g

shrimp were put in each aerated glass tank. Levels of nitrite were determined every morning using the spectrophotometer method described by Solorzan (1969) before replacing with fresh solutions.

The survival of tested shrimp in each container was determined at 24-, 48-, 72- and 96-h. The median lethal concentration (LC_{50}) was analyzed by probit analysis (Zhou and Zhang 1989).

The starved shrimp fed the basal and selenium (inorganic and organic) diet were transferred to a 9 L plastic tank containing 5 L of the test solution. There were six treatments (0 control, 2.2, 7.0, 12.0, 17.0 and 21.7 mg L^{-1} nitrite) (with three repetitions per group). After 24 h of nitrite stress, shrimp were immediately frozen in liquid nitrogen after collection and preserved at −84°C until analysis.

After 24 h, shrimp were randomly taken for two samples from each treatment (with three repetitions) and the tissues were provided for the assay of antioxidant enzyme activities.

The protein content of the homogenate was measured by the method of Bradford (1976) with spectrophotometer using bovine serum albumin as standard. Absorbance of samples was detected at 595 nm.

The haemolymph was drawn from the shrimp heart using a 250-Al glass syringe and placed in plastic tubes on ice. The haemolymph of five shrimp from each exposure was pooled for the following measurements of reactive oxygen intermediate production in haemocytes of the shrimp, according to a procedure already described by Munoz et al. (2000). Before measuring, the haemolymph was homogenated in 30 volumes of 50 mmol L^{-1} phosphate buffer (pH 7.2) and the homogenates were centrifuged at 9960×*g*. The resultant supernatant was used for the O_2^- assays. Fifty microliter of culture medium (MHBSS buffer with 6 mmol L^{-1} $Ca^{2+}$and 13 mmol L^{-1} Mg^{2+}) were placed in wells of a 96-well microtiter-plate and incubated in humid conditions and room temperature. After 30 min, the supernatant was eliminated and replaced with 50 μL medium. Then, 50 μL of 0.3% NBT in the appropriate medium was immediately distributed to each well. Following 2 h incubation, the supernatants were removed and the haemocytes were fixed by the addition of 200 μL absolute methanol and washed twice with 70% methanol, then dried. The formazan precipitates were solubilized in 120 μL, 2 mmol L^{-1} KOH and 140 μL dimethyl sulfoxide (DMSO; Sigma). After homogenization of the contents in the wells, the absorbance was read at 620 nm with a spectrophotometer.

The muscles were taken from the shrimp, immediately freeze-dried in liquid nitrogen and homogenised in nine volumes of 20 mmol L^{-1} phosphate buffer pH 7.4, 1 mmol L^{-1} EDTA and 0.1% triton X-100 and the homogenates were centrifuged at 600×*g*, to remove debris, and the resultant supernatants was used directly for enzyme assays.

Total SOD activity in solution was determined by using the method of Marklund and Marklund (1974) based on the autoxidation of pyrogallol.

GPX activity was measured by following the rate of NADPH oxidation at 340 nm and by the coupled reaction with glutathione reductase (Bell et al. 1985).

CAT activity was measured by ultraviolet spectrophotometer (Beers and Sizer 1952). A 10 μL sample was added to 3.0 mL of H_2O_2 phosphate buffer pH 7.0 (0.16 mL of 30% H_2O_2 to 100 mL of 67 mmol L^{-1} phosphate buffer) and the variation of H_2O_2 absorbance in 60 s was measured with a UV-2100 spectrophotometer at 250 nm. One unit of enzyme activity is defined as the amount of enzyme which decreases the concentration of H_2O_2 by 50% in 100 s at 25°C.

Results in this paper were expressed as mean ± SD (n = 6) and analyzed using the SPSS version 11.5. Differences between mean values were analyzed by one-way analysis of variance followed when pertinent by a Tukey multiple comparison test. Differences in enzyme activities and biochemical variables were analyzed by two-way ANOVA, using three diet groups (control, inorganic Se, organic Se) as one independent variable, and six nitrite levels (0, 2.2, 7.0, 12.0, 17.0, 21.7 mg L^{-1}) as the second variable. The results were considered significant at $p < 0.05$.

Results and Discussion

After 30 days, all shrimp survived. Mean growth rates of body length (control diet, inorganic and organic selenium diet) were (17.08 ± 2.82)%, (32.02 ± 2.16)% and (35.48 ± 3.73)%, respectively. Mean growth rates of body weight were (15.18 ± 1.20)%, (28.91 ± 2.36)%, and (30.30 ± 2.72)% respectively. It was obvious that Se supplementation caused a significant ($p < 0.05$) increase in the mean growth rates of body length and weight. The shrimp in the organic Se-supplemented group had higher mean growth rates of both body length and weight than those in the control and the inorganic Se-supplemented groups (Table 2).

Table 2 The mean growth rates of body length and weight of *N. heteropoda*

Dietary groups	The mean growth rates of body length (%)	The mean growth rates of body weight (%)
Control	17.08 ± 2.82	15.18 ± 1.20
Inorganic Se	32.02 ± 2.16*	28.91 ± 2.36*
Organic Se	35.48 ± 3.73*	30.38 ± 2.72*

* Significantly different from the corresponding values in control group ($p < 0.05$)

Table 3 The acute toxicity test of nitrite to *N. heteropoda*

Nitrite concentration (mg L^{-1})	Mortality (%) n = 20			
	24 h	48 h	72 h	96 h
60	70.0 ± 2.5	85.0 ± 5.0	100.0 ± 0.0	100.0 ± 0.0
50	47.5 ± 2.5	80.0 ± 0.0	90.0 ± 0.0	100.0 ± 0.0
40	30.0 ± 0.0	57.5 ± 2.5	70.0 ± 0.0	100.0 ± 0.0
30	0.0 ± 0.0	30.0 ± 2.5	67.5 ± 2.5	70.0 ± 5.0
20	0.0 ± 0.0	10.0 ± 0.0	45.0 ± 2.5	47.5 ± 2.5
10	0.0 ± 0.0	0.0 ± 0.0	20.0 ± 0.0	20.0 ± 2.5
Lc50	50.4	38.8	25.1	21.7

The 24-, 48-, 72- and 96-h LC_{50} values of sodium nitrite at pH 8.0 were 50.4, 38.8, 25.1 and 21.7 mg L^{-1}, respectively for *N. heteropoda.* Twenty shrimp were used in each treatment (Table 3).

The O_2^- value (Fig. 1) of the shrimp in the groups of inorganic and organic Se diet was lower ($p < 0.05$) than that of the shrimp in the control group exposed to the same concentration of nitrite. In nitrite level (12.0 mg L^{-1}), the O_2^- value of the shrimp in the control group was significantly higher compared to that of the inorganic and organic Se supplemented groups ($p < 0.05$). TheO_2^- values of the shrimp in the inorganic and organic Se supplemented groups were decreased by 11.5% and 13.1%, respectively.

Total SOD (Fig. 2), GPX (Fig. 3) and CAT (Fig. 4) activities of shrimp in the groups supplemented with inorganic and organic Se diet were higher ($p < 0.05$) than that of the shrimp in the control group exposed to the same concentration of nitrite. At 12.0 mg L^{-1} nitrite concentration the activities of SOD (Fig. 2) in the inorganic and organic Se supplemented groups were increased by 25.4% and 45.4%, compared with the shrimp fed on the control diet. The GPX (Fig. 3) activities were enhanced by 20.8% and 54.2%. The CAT (Fig. 4) activities were increased by 10% and 36%.

After 30 days, both Se supplementations resulted in a significant increase ($p < 0.05$) in the specific growth rate of shrimp compared with the control group (Table 2). Additionally, the organic Se resulted in a significant growth rate increase over the inorganic ($p < 0.05$). Compared with shrimp under production environments, the growth rate in our study is lower. The reason should be as follows: Firstly, the space is limited under the laboratory environments. Secondly, the nutrition (organic detritus, phytoplankton, zooplankton, and so on) is inferior to the production environments.

Previous studies have focused on the effects of nitrite on growth, molting and toxicity to shrimp (Chen and Chin 1988; Chen and Lee 1997; Cheng and Chen 1999). In this research, the increased activities of SOD and CAT at low nitrite concentrations could be ascribed to the generation of

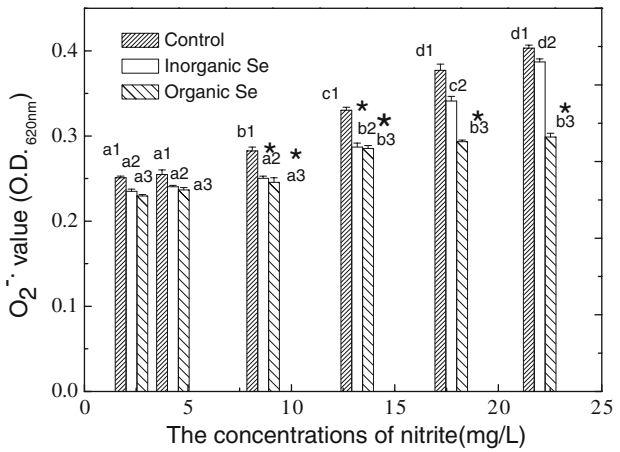

Fig. 1 The O_2^- value (OD 620 nm) of *N. heteropoda* fed two Se-supplemented diets and control diet under different concentrations of nitrite. Values (mean ± SD) in the same dietary group with *different letters* are significantly different ($p < 0.05$) among different concentrations of nitrite. *, significantly different from the corresponding values in control group ($p < 0.05$)

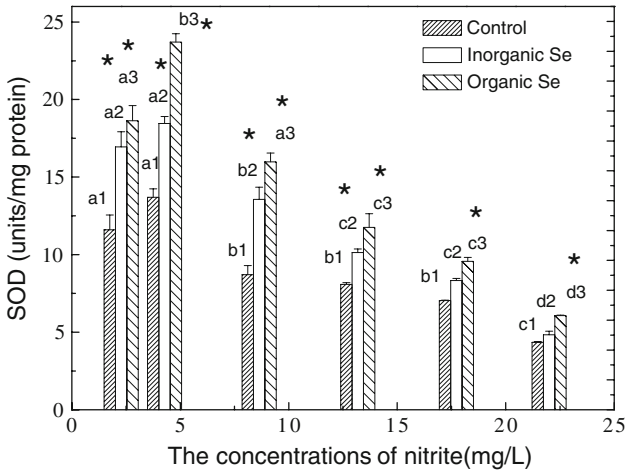

Fig. 2 The SOD activities (SOD units/mg protein) of *N. heteropoda* fed two Se-supplemented diets and control diet under different concentrations of nitrite. Values (mean ± SD) in the same dietary group with *different letters* are significantly different ($p < 0.05$) among different concentrations of nitrite. *, significantly different from the corresponding values in control group ($p < 0.05$)

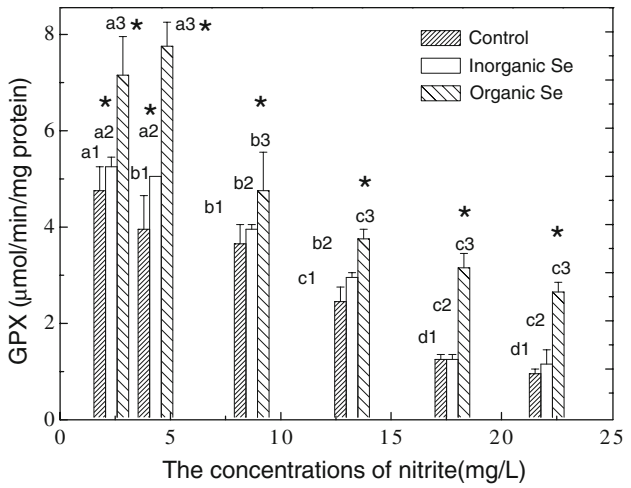

Fig. 3 The GPX activities (μmol/min/mg protein) of *N. heteropoda* fed two Se-supplemented diets and control diet under different concentrations of nitrite. Values (mean ± SD) in the same dietary group with *different letters* are significantly different ($p < 0.05$) among different concentrations of nitrite. *, significantly different from the corresponding values in control group ($p < 0.05$)

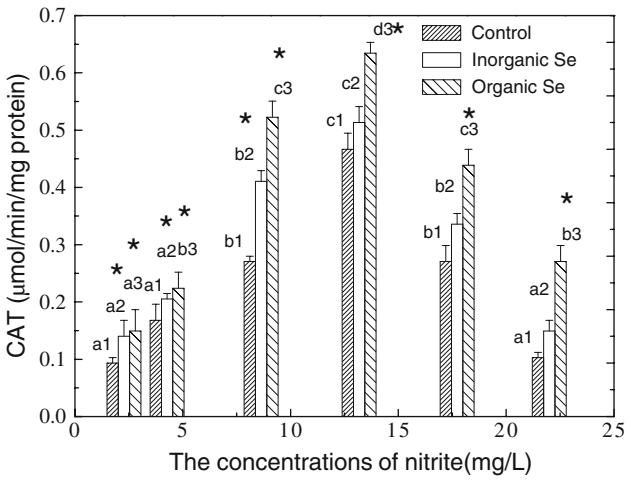

Fig. 4 The CAT activities (μmol/min/mg protein) of *N. heteropoda* fed two Se-supplemented diets and control diet under different concentrations of nitrite. Values (mean ± SD) in the same dietary group with *different letters* are significantly different ($p < 0.05$) among different concentrations of nitrite. *, significantly different from the corresponding values in control group ($p < 0.05$)

O_2^- and H_2O_2. The activity of SOD decreased with an increased concentration of nitrite by the process "phenomenon of suppression" (Buttke and Sandstorm 1994). Sequentially, the activities of GPX and CAT also declined.

There is a positive correlation between Se deficiency and O_2^- formation (Bell et al. 1986). In our study, it was clear that the value of O_2^- was influenced by Se concentration. The increasing O_2^- value of the shrimp exposed to different concentrations of nitrite in the control group might be ascribed to the deficiency of selenium, and the decreasing O_2^- value of the shrimp fed the Se supplemented diet revealed that Se contributed to reducing the stress caused by nitrite.

There have been several studies on the effects of dietary Se on the antioxidant enzyme activities (Bell et al. 1985). In our study, dietary Se resulted in an increase in antioxidant potential through enhancing the activities of SOD, GPX and CAT. Our results suggest that the resistance to nitrite stress of the shrimp fed diets supplemented with Se is greater when compared with the shrimp fed the control diet. These changes of antioxidant enzymes indicate that an adequate protection against oxidative stress can be achieved by a moderate dose of Se.

In our study, the organic Se has a more obvious effect in defending against oxidative stress than the inorganic Se. The method of inorganic Se absorption by animals is through passive diffusion; while the mode of organic Se assimilation is active transport. Therefore, absorption efficiency of organic Se is higher than that of inorganic Se. Bell and Cowey (1989) reported that Atlantic salmon had higher absorption efficiency when organic Se was supplemented. These results were also confirmed by a similar study on Atlantic salmon (Lorentzen et al. 1994).

In conclusion, nitrite in the aquatic environment potentially induces stress in *N. heteropoda*. Se diet-supplementation was found effective in relieving the stress and enhancing the growth ability against difficult conditions through increasing the activities of antioxidative enzymes and decreasing of O_2^- value. Therefore, it is necessary to supply moderate Se in the diet under stressful conditions. Organic Se plays a more significant role in defending against oxidative stress caused by nitrite than inorganic Se. Further research is needed to investigate the molecular mechanism of Se effects on the antioxidant enzymes response of *N. heteropoda*.

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