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Response of antioxidase in viscera of *Pagrosuma Major* larvae to water soluble fraction of hydrocarbons in No.0 diesel oil

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Abstract: *Pagrosomus major* larvae were exposed to the water-soluble fraction of hydrocarbon in No.0 diesel oil (corresponding to No.2 fuel oil) at concentrations of 0, 0.17, 1.22 and 8.82 mg/L for up to 15 days. Larvae were sampled on days 9 and 15 of the experiment. Supernatants of viscera tissue extractions were assayed for biochemical response in terms of oxidative stress-superoxide dismutase(SOD), activity of selenium-dependant glutathione peroxidase(Se-GPx) and catalase(Ca), and the concentration of reduced glutathione(GSH). On day 9 of exposure, statistically significant dose-related increases in Se-GPx and SOD activity, and GSH concentration were observed in all cases except for Se-GPx activity under the highest dosage of hydrocarbon. However, on day 15 of exposure, a similar dose-related response was only observed for Se-GPx activity. GSH concentration decreased and SOD activity showed no statistical difference as compared to controls. However, a significant decrease in compared to day 9 Se-GPx activity and GSH concentration, in contrast to increase SOD activity at day 15 as indicates an accelerated accumulation of H_2O_2 and potential oxidative damage under long term exposure of larvae to hydrocarbons. No statistical changes were observed in Ca activity throughout the experiment, possibly owing to the high efficiency of Se-GPx.

A recovery experiment was performed on indicating that the response of antioxidants measured tending to return to their control levels. These results prove the function of the antioxidant defense system of the larvae to the water-soluble fraction of hydrocarbons in No.0 diesel oil.

Keywords: antioxidant system; water-soluble fraction of hydrocarbon; *Pagrosomus major*; larvae

Introduction

Aquatic farms larger than 14 km^2 are located in the northern part of West Harbor, Xiamen Municipality of China. These farms are under great environmental stress owing to rapid economic development. In recent years, deteriorating fresh water fishery, concomitant with increasing mortality and/or occurrence of diseases related to worsening water quality has been extersively observed. Studies concerning the acute and chronic toxicity of pesticides and heavy metals to fish have been performed(Dai, 1996; 1997). However, no laboratories have examined hydrocarbon pollutants even through a study of organic pollutants in the sediment of West Harbor, Xiamen found that concentrations of aliphatic and aromatic hydrocarbons ranged from 3.1 to 32.9 and 2.9 to 61 μg/g(d.w.), respectively(Hong, 1995). These concentrations were that higher than those found in other coastal regions with pollutants inputs were primarily caused by oil pollution(Hong, 1995). The content of petroleum hydrocarbon in oyster collected from four stations in Xiamen coastal regions(Ferry Harbor, Xinlin Bay, Tong'an Bay and Huangcuo Beach) were 380.68, 112.34, 27.31, 20.37 μg/g wet, respectively(Chen, 2001). The results indicated that the higher rate of accumulation of hydrocarbons in the oyster from the located regions is consistent with higher diesel oil pollution. It has been shown that No.0 diesel(corresponding to No.2 fuel oil), one of the fuels most often consumed in China, is the main source of petroleum hydrocarbon pollution in West Harbor environment. Laboratory experiments have indicated that hydrocarbons in diesel are more toxic and more similar to those found in aquatic environments than other petroleum hydrocarbons(Jia, 1990; 1998). However few studies have been aimed toward understanding the toxic mechanisms of diesel hydrocarbons in aquatic animals.

A possible toxic mechanism of pollutants is to increase the levels of reactive oxygen radicals such as hydrogen peroxide, resulting in oxidative damage to DNA and other molecules (Livingstone, 1990; 1991;

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Winston, 1991; Ericson, 2000; Akcha, 2000). It has been reported that P4501A proteins are primarily responsible for hydrocarbon metabolism in fish, but with respect to toxic mechanisms of hydrocarbons in aquatic species, biochemical reactions associated with cytochrome P450 system are also important processes (Stegeman, 1992; Goksoyr, 1992). Livingstone *et al*. (Livingstone, 1992) have reported that these reactions include both detoxification and toxic injury from reactive metabolites cytochrome P450 can alter the balance between oxidative and conjugate metabolism and, accordingly, it acts as an important source of oxygen-radicals induced by pollution(Stegeman, 1992).

All aerobic organisms have developed an antioxidant defense system which plays an important role in controlling oxygen-radicals generated during detoxication of pollutants. Indeed, some major components of the very effective defense system can be induced by stress from the pollutants themselves. Increased activity of antioxidant enzymes has been shown in a number of fish species samples obtained from both polluted sites and exposure experiments (Livingstone, 1992; Mather-Mihaich, 1986; 1991a; Markovics, 1987; Iqbal, 2000). These studies also indicate that pollution-mediated oxidants may be responsible for animal fitness(Di, 1989; Winston, 1991). Epidemic studies show that the increase in rates of idiopathic lesions and neoplasia among some populations of aquatic animals inhibiting a polluted environment is related to oxidative stress(Malins, 1988; Van, 2000). In contrast to the considerable work on the cytochrome P450 system, relatively little has been done regarding the antioxidant defense system and hydrocarbons in environment(Thomas, 1984; Wofford, 1988).

Early life stages of fish are very sensitive to environmental changes(Cameron, 1992) that has been shown in pollutant exposure experiments(Buhl, 1991; Goksoyr, 1992; Peters, 1992; Schoor, 1991) and studies of site samples (Peters, 1994). In this study, the biochemical response of the antioxidant defense system under oxidative stress was examined in larvae of *Pagrosomus major* exposed to the water-soluble fraction of hydrocarbons in No.0 diesel oil. *Pagrosomus major* was selected for this study because it is one of the most economically important cultured fish species in the Xiamen coastal waters and never has been studied in this regard.

1 Materials and methods

1.1 Reagents

Analytical and biochemical reagents were obtained from Chemical Reagent Company, Shanghai.

1.2 Preparation of oil stock solution

Oil stock solution was prepared according to James *et al*. (James, 1996). Oil was mixed with sea water (1∶10, V/V) blended with an agitator at low speed for 18 hours. The mixture was then set aside for 5 hours, sea water in lower layer siphoned off, then the mixture stored stock solution in the darkness at 4℃. The concentration of oil in stock solutions was measured by fluorospectrometry according to the method of the IOC(IOC, 1984) with some minor modifications, i.e. commercial product of No.0 diesel oil was used as standard instrumental parameters were set as $E_x = 310$ nm, $E_m = 365$ nm according to the spectrum of No.0 diesel oil. Concentration of 1 mg/L oil means 1 milligram No.0 diesel oil per liter. Table 1 lists the oil dosage in this experiment.

Table 1 Oil dosage in experiment ($n = 6$)

Groups	Dosage, mg/L
Control	0
Low concentration	0.17 ± 0.02
Middle concentration	1.22 ± 0.06
High concentration	8.82 ± 0.05

1.3 Experimental species and exposure conditions

Pagrosomus major larvae were bought from a local aquatic farm for use in the experiment. Each larvae weighed 1.80 ± 0.18g. Before the exposure experiment, larvae were acclimated in a cultivation container 30 cm in diameter and 80 cm in depth for 5 days. Each container twelve larvae in five liters of either clear sea-water or oil-dosed sea water. The containers were well-aerated and the larvae fed freshly mashed fish

flesh two hours before replacing the water. This process was repeated every other day. The water temperature was maintained at 24 ± 2℃. Duplicate experiments were performed for each exposure. No mortality was observed during the experiment.

1.4 Sample preparation

On days 9 and 15 of the exposure experiment, 3—6 tails of live larvae were taken from each experimental container and killed. The viscera tissues were quickly dissected, weighed and homogenized at 4℃ with double-distilled water. Supernatants were separated by centrifuging at 5000 r/min for 10 minutes and stored at −20℃ until biochemical assay.

1.5 Biochemical analysis

The activity of superoxide dismutase (SOD) was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) using the method of Beauchamp and Fridovich (Beauchamp, 1971). The activity of catalase was determined utilizing the method of Rong Cuiqin (Rong, 1989) with minor modification. Glutathione peroxidase was assayed by spectrophotometry according to the method of Xia Yiming (Xia, 1987). GSH concentration was determined using a method based on Xia Yiming (Xia, 1991).

Protein concentrations in the supernatants were analyzed according to the method of Lowery Rosebrough (Lowery, 1951).

All enzymatic activity assays were carried out at 25℃。

1.6 Data processing

Data were processed to give the mean value ± standard deviation ($n = 3$—6). The results were tested using single factor analysis of variance (ANOVA) and the significance level between data sets was examined by one-tailed t-test. Results were considered significant when $p < 0.05$.

2 Results

The results are summarized in Table 2. It was shown that water-soluble hydrocarbon in No.0 diesel oil caused significant changes for all biochemical indicators except catalase.

Table 2 Results of water-soluble hydrocarbon exposure on activities of SOD, Se-GPx and Ca, and GSH concentrations in *Pagrosomus major* larvae viscera tissue

	SOD, unit/mg	Se-GPx, nmol/(min·ml)	Ca, mol/(min·g)	GSH, nmol/ml
Days 9				
I	105.51 ± 6.25	13.55 ± 1.83	17.16 ± 0.97	27.82 ± 5.14
II	175.03^{a} ± 0.56	24.94^{a} ± 2.82	21.55 ± 3.69	97.42^{a} ± 2.87
III	336.30^{a} ± 0.33	31.29^{a} ± 4.07	26.71 ± 7.97	69.99^{a} ± 7.91
IV	429.15^{a} ± 0.94	8.62^{a} ± 1.65	27.38 ± 2.53	69.58^{a} ± 3.41
Days 15				
I	799.36^{b} ± 57.22	2.96^{b} ± 0.91	16.63 ± 0.05	56.52 ± 1.40
II	834.62^{b} ± 47.03	$9.97^{a,b}$ ± 4.23	16.39 ± 1.62	52.12^{b} ± 3.24
III	617.30 ± 54.16	$15.45^{a,b}$ ± 0.71	22.11 ± 2.81	$20.12^{a,b}$ ± 0.78
IV	1182.86 ± 70.93	2.73^{b} ± 0.97	14.96^{b} ± 0.23	$16.35^{a,b}$ ± 0.85
(IV)	(235.63 ± 20.15)	(15.35 ± 6.77)	(21.79 ± 0.87)	(56.20 ± 7.50)

Notes: SOD. superoxide dismutase; Se-GPX. selenium-pendant glutathione peroxidase; Ca. catalase; GSH. reduced glutathione; Figures are in mean value + standard deviation, $n = 3$—6; a. significantly different from the corresponding control sample, $p < 0.05$; b. significantly different among samples with different exposure time, $p < 0.05$

2.1 Variation of enzyme activity in the 9 days exposure experiment

During the exposure experiment of 9 days, all enzyme activity showed clear parabolic variation. The correlation coefficients between enzyme activity and hydrocarbon concentration were $r = 0.9950$, 0.9323, and 0.9298 for SOD, Se-GPx, and Ca, respectively. The activity of SOD and Ca increased 1.66, 3.19,

4.07 and 1.26, 1.56, 1.60 times, respectively; over controls for samples in groups II, III and IV. The activity of Se-GPx increased 1.84 and 2.3 times over controls for groups II and III at low dosages. However, only 64% of controls showed inhibition of activity for group at maximum dosage. The great increase in SOD activity accompanied by the minor increase in Ca activity and significant decrease of Se-GPx activity clearly indicates an imbalance between production and elimination of oxygen-radicals at maximum dosage.

While changes of SOD and Se-GPx activity was statistically significant as compared with controls, variation of Ca activity was small and insignificant.

2.2 Variation of enzyme activity in exposure experiment of 15 days

Prolong exposure of larvae to water-soluble hydrocarbons for 15 days resulted in further adverse changes to the antioxidant system as compared to 9 days of exposure. SOD activity at 15 days increased 7.58, 4.77, 1.84, and 2.76 times greater than it had for the 9 days exposure for groups I, II, III and IV respectively. However, neither a dose-related connection nor a significant statistical difference was observed in compared exposed and control samples. In contrast to the reaction of SOD, suppression of Se-GPx activity was observed. Se-GPx activity was 22%, 40%, 49%, and 32% lower than that from short-time (9 days) experiment groups I, II, III and IV, respectively. The parabolic dose-response seemed more consistent with Se-GPx activities than SOD. The variation of Ca activity also showing a parabolic dose-response pattern, was similar to that of SOD in the 9-day exposure experiment and to that of Se-GPx in the 15 – day exposure.

2.3 Variation of GSH concentration during exposure experiment

For the 9 days exposure, a dose-related increase in GSH concentrations were observed as 2.04, 1.46 and 1.46 times greater than for groups II, III and IV in controls respectively. Conversely, the 15 – day exposure experiment showed a dose-related decrease GSH concentration. The concentration of GSH were 92%, 35% and 29% of that the controls for groups II, III and IV respectively. It is interesting that GSH concentrations were similar for groups III and IV in both exposure experiments. These results may indicate that there is a type of saturation regulation mechanism of GSH when there is pollution stress.

2.4 Results of recovery test

To further examine the behavior of the antioxidant system in *Pagrosomus major* larvae exposed to water-soluble hydrocarbons, six larvae from group were transferred to clear sea-water after exposure to hydrocarbons for 9 days. The results reveal that activity of all enzymes tends to resume to various degrees after six days of recovery. The activities of Se-GPx and Ca, respectively, returned to 1.1 and 1.3 times of previous levels for control samples. Activity of SOD declined to only 2.2 times of that of the control sample after recovery, much lower than the 11.2 times increase during the exposure experiment. These results suggest that the generation of oxygen-radicals decreased and that the function of the antioxidant system recovered to some extent after exposure to certain types of pollution with means of eliminating oxygen-radical production.

3 Discussion

Biological systems are continuously producing oxygen-radicals in a number of processes involving both endogenous and xenobiotic compounds (Diguiseppi, 1984). Potential sources of pollution-stimulated oxygen-radical production include redox reactions involving transition metals (Anst, 1985), free organic radicals (Winsterbourne, 1987), O_2^-. production by cytochrome P450 reductase and other microsomal reductases and O_2^-. and/or H_2O_2 production by cytochrome P450 (Ortiz, 1986; Premereur, 1986) with relation to oxygen stress of the aquatic environment. Laboratory studies have been done on the effects of the herbicide paraquat (Gabryelak, 1985; Vig, 1989), metal ions (Radi, 1988) and mercaptan-containing

compounds(Mather-Mihaich, 1986). Results indicated that there is a relationship between antioxidant response and xenobiotic exposure. The present time it is also known that the response is variable and its quantification difficult. Obviously, further study is urgently needed.

Most investigations of the biochemical response of fish to hydrocarbons have focused on the function of bio-transformation enzymes in the cytochrome P450 system(Livingstone, 1987; 1999; Goksoyr, 1992) rather than the oxidant-mediated response. However, Roberts *et al*.(Roberts, 1987) observed that the activity of SOD in spot samples(*Leiostomus xanthurus*) from PAH-polluted areas was usually higher than that in reference samples. In addition, the increase of SOD activity was associated with an increase of arylhydrocarbon hydroxylase (AHH). Thomas and Wofford (Thomas, 1984) also reported that the concentration of GSH in liver of stripped mullet(*Mugil cephalis*) increased after exposure to cadmium and fuel oil. Di Giulio (Di, 1989) reported similar results when examining catfish exposed to PAH-contaminated sediment. All these results indicate a relationship between antioxidant defense response and PAH-exposure. In this study, water soluble hydrocarbon from No.0 diesel oil were used to test the possible mechanism of toxicity in *Pagrosumus major* larvae. The variation of antioxidant response shown in this study indicates that this type of hydrocarbon can stimulate the generation of oxygen-radicals in larvae. Although the antioxidant defense system in the larvae was reactive to the exposure stress and may be effective in controlling the pollution-induced oxygen-radicals, it can be damaged under greater oxidative stress(i.e., high dose or low dose with a longer time of exposure).

In the 9 day-exposure experiment, a significant parabolic variation of dose-activity was noted for both SOD and Se-GPx. The parabolic change of dose-response is one of the most commonly observed patterns. The inflexion of the parabola is critical, which merits special attention since enzyme activity will increase along with increase of dosage up to this level, then decrease with further increase of dosage. Accordingly, the organism being studied is considered to be able to eliminate the possible adverse effects of contamination resulting from polluting at levels below the inflexion, while higher dosages cause toxicity due to the inability of the organism to adapt. From this study, the significant increase of SOD and Se-GPx activity and GSH concentration in groups II andIII can be regarded as the adaptive response of the larvae to oxygen-radical production caused by hydrocarbon contamination. The extremely great increase of SOD activity, concomitant with a slight increase of both Ca activity and GSH concentration, and the significant decrease of Se-GPx activity in group IV clearly indicates that the ability of the antioxidant system of the larvae to combat the greater oxidative stress (especially the higher production of H_2O_2) has been weakened.

The higher level of SOD activity shown at 15 day exposure times indicates that the larvae adapted to the increased dosage and longer exposure to hydrocarbons. It also indicates higher production of H_2O_2. However, the activity of both Se-GPx and Ca, which are the main eliminators of H_2O_2, declined instead of increasing. This response pattern of SOD and Se-GPx and Ca suggests that there is an accumulation of H_2O_2 in larvae tissues. Although H_2O_2 is not a real free radical, it is very reactive and can play a key role in hydroxyl radical production such as with Haber-Weiss reaction(Stegeman, 1992). Thus oxidative stress and/or oxidative damage are more likely to be triggered under long term exposure to pollution. The negative effects caused by long-term contamination should be given special attention because fish in natural waters may be continuously exposed to sublethal pollutants.

The phenomenon that Se-GPx activity significantly increases with increased hydrocarbon dosage, together with the insignificant change in Ca activity, is similar to that observed for carp exposed to copper. The latter is explained as being due to the high activity of Se-GPx(Radi, 1988). This is contrary to the case in which channel catfish where exposed craft mill effluent where in there was a significant dose-related increase of Ca activity and insignificant change is GPx activity. These different results may be caused by

different characteristics of the pollutants and species.

Low molecular weight scavengers have been detected in several aquatic species and their concentrations increase as a function of oxidative stress in some cases. Increased fluxes of oxygen-radicals may reasonably alter GSH status and/or the metabolism of the species in several ways. Previous studies, where striped mullet (*Mugil cephalis*) were exposed to cadmium or fuel oil(Thomas, 1982; 1984) and channel catfish exposed to sediment(Di, 1989) have shown that complex variations in GSH concentration can be induced under different contamination stress patterns(i.e. changes in exposure time to pollutant dosage). The GSH concentration often quickly increases a maximum level, then may be maintained at the peak or decreased according to exposure time and/or dosage.

The response of antioxidant defense systems to environmental change was further revealed during our recovery experiment, the results of which indicate that both antioxidant enzyme activity and free radical scavengers tended to return to control levels after six days. Compared to the response of gastropods (*Littorina littorea*) under anoxic stress and the results of aerobic recovery experiments, similar reactions and recovery of abilities of antioxidant defense systems were obtained(Pannunzio, 1998). These results clearly suggested the antioxidant defense system in marine animals to be an adaptable system consisting of components that are reactive to environmental change.

3 Conclusion

The results are indicative of the relationships between response of larvae antioxidant defense system and water-soluble hydrocarbons stress. The dose-response and variation of the activity of the enzymes at different exposure time indicate that oxygen-radical generation can be exacerbated by environmental pollution, especially at high contaminant level and/or long time of exposure. What is difficult to gauge, however, is the threshold at which oxygen-radical generation could cause serious oxidative damage and pose a threat to the fitness of aquatic animals. However, non-contaminant influences cannot be overlooked as indicated by the variations of Se-GPx and SOD activity in control groups and as concluded from *L. Limanda* from the North Sea (Livingstone, 1992). The response of antioxidant enzymes to pollution exposure was transient either because of the intervention of small molecular radical scavenger or possible existence of other mechanism such as "neutralizing" or packaging-away of the potential reactive oxidant chemical species. Further studies should address these problems and focus on the demonstration of oxygen-radical generation in vivo.

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