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Effects of Hypoxia and Sedimentary Naphthalene on the Activity of *N*-acetyl-*β*-Glucosaminidase in the Epidermis of the Brown Shrimp, *Penaeus aztecus*

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Abstract The brown shrimp, *Penaeus aztecus*, is subject to dual stresses of environmental hypoxia and contamination of polycyclic aromatic hydrocarbons (PAHs) in the northern Gulf of Mexico. The effects of hypoxia and sedimentary naphthalene, administered alone and in combination, on epidermal activity of *N*-acetyl-*β*-glucosaminidase (NAG), a biomarker for molt-interfering effects in *P. aztecus*, were investigated. It was found that hypoxia and sedimentary naphthalene, when given simultaneously, significantly inhibited epidermal NAG activity, suggesting that these two environmental stressors together can have adverse effects on molting of the brown shrimp. The results of this study also show that sedimentary naphthalene potentiates hypoxia effects on epidermal NAG activity.

Keywords *N*-acetyl-*β*-glucosaminidase · Hypoxia · Naphthalene · *Penaeus aztecus*

Because of coastal eutrophication as a result of the input of nutrients from the Mississippi and Atchafalaya Rivers, occurrence of hypoxic zone, where oxygen concentration in the bottom of water column lower than 2 mg/L, has become an annual event in the recent years in the northern Gulf of Mexico (NGM) (Walker and Rabalais 2006). Rabalais et al. (2002) found that hypoxia occurs not merely in the bottom water, but well up into 10% to over 80% (typically, 20%–50%) of the entire water column. The largest hypoxic zone covering an area of 22,000 km^2 was documented in 2002, and the 2007 hypoxic zone encompassed an area of 20,460 km^2, the third largest on record according to the US National Oceanic and Atmospheric Administration. The depletion of dissolved oxygen in the NGM certainly constitutes an environmental stressor to aerobic organisms inhabiting these waters. Besides the stress from environmental hypoxia, aerobic aquatic organisms in the NGM are also subject to pollution of polycyclic aromatic hydrocarbons (PAHs) from activities of gas and petroleum production. The continental shelf of the NGM has been heavily mined for gas and petroleum, and the operations of gas and petroleum production are always associated with contamination of petroleum hydrocarbons, particularly the PAHs. Because of their generally high hydrophobicity, PAHs tend to be associated with sediments. An earlier investigation by Wade et al. (1988) showed that the PAH content in the NGM sediments ranges from 0.005 to 36.7 ppm. In the Mobile Bay of the NGM, sedimentary PAH concentration ranges from 0 to 25.77 ppm according to a recent study by Peachey (2003). It is apparent that aerobic aquatic organisms living in the NGM are subject to two environmental stressors simultaneously, hypoxia and PAH contamination.

The brown shrimp, *Penaeus aztecus*, also known as *Farfantepenaeus aztecus*, is one of the commercially important crustaceans of the US Gulf coast. Its juvenile stages are spent in inshore waters, such as bays, lakes, estuaries and bayous. The adult *P. aztecus* emigrates to offshore waters for reproduction, with the peak emigration taking place during the period of May through August. *P. aztecus* of the NGM spawns all year around primarily in offshore waters, and the peak spawning occurs in two periods, September through November and April to May (Lassuy 1983). After larval development, shrimps migrate back to inshore waters to complete the life cycle. There is an apparent overlap between the life history of *P. aztecus* and

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the hypoxic zone in the NGM. *P. aztecus*, like other penaeid shrimps, spends a large amount of time on the bottom, an interface between water column and sediments, where oxygen concentration is most likely to be low and the concentration of PAHs is likely to be high. Shrimps may be acutely exposed to high doses of PAHs when the sediments are disturbed. Therefore, *P. aztecus* in the NGM is prone to exposure to hypoxia and PAH contamination. Only recently have the interactive effects of theses two environmental stressors in *P. aztecus* started to be investigated. Zou and Stueben (2006) found that the presence of naphthalene, a representative PAH, reduces the oxyregulating capacity of *P. aztecus*, making the brown shrimp more susceptible to hypoxia. Ye and Zou (2008) investigated whether hypoxia can promote bioaccumulation of naphthalene in *P. aztecus*. These investigators found that hypoxia does not increase bioaccumulation of naphthalene in the brown shrimp, presumably owing to increased naphthalene biotransformation in this shrimp. The objective of the present study was to investigate the effects of hypoxia and sedimentary naphthalene, administered alone and in combination, on the activity of *N*-acetyl-β-glucosaminidase (NAG) (also known as chitobiase), a chitinolytic enzyme found in the epidermis of *P. aztecus*. Epidermal NAG, an enzyme indispensable for degradation of exoskeletal chitin, has previously been shown to be a product of the gene regulated by the molting hormone in Crustacea (Zou and Fingerman 1999a, b). The activity of chitinolytic enzymes has been used as biomarker for the effects of environmental agents on crustacean molting (Zou and Fingerman 1999c, d; Zou and Bonvillain 2004). Therefore, the results of the present study can be used to assess the impacts of hypoxia and sedimentary naphthalene on molting of the brown shrimp.

Materials and Methods

Brown shrimps, 3.1–8.3 g in wet weight, were purchased from a bait shop in Chauvin, Louisiana. Upon arrival in the laboratory, shrimps were released into aquaria containing artificial seawater made from Instant Ocean synthetic sea salt (Aquarium Systems, Mentor, OH) at a salinity of 14 ppt. This salinity was similar to that of the local bay where the shrimps were caught. Shrimps were reared at 19–21°C and under the light regime of 14 h light versus 10 h dark and fed shrimp tail meat once 3–4 days. Shrimps were allowed to acclimate to laboratory conditions for at least 4 days before use in an experiment.

Since NAG activity in the epidermis varies during the molting cycle (Zou and Fingerman 1999a), only intermolt shrimps, selected according to the method of Robertson et al. (1987), were used in the experiment. Four groups of 18 intermolt shrimps each were, respectively, exposed to normoxia with clean artificial sediments, hypoxia with clean sediments, normoxia with sedimentary naphthalene, and hypoxia with sedimentary naphthalene. The hypoxia system used in the present study was similar to that of Seidman and Lawrence (1985). It consisted of degassing, exposure and reoxygenation tanks. The dissolved oxygen in the 38 L exposure tank varied between 1.0 and 2.6 mg/L over the duration of the exposure. The exposure tank contained 5 kg artificial sediments, which was made by mixing humic substances (pulverized, decomposed peat) (Carolina Biological, Burlington, NC), ASP400 clay, ASP900 clay (both hydrous aluminosilicates) (Engelhard Co., Iselin, NJ), and sand (40–100 mesh) (Acros Organics, Geel, Belgium) in a weight ratio of 1:6:6:7. Normoxia was created through continuous aeration. Naphthalene (purity > 99%, Sigma, St. Louis, MO) was first dissolved in 50 mL pure ethanol and then spiked in artificial sediments at a concentration of 40 mg/kg. Clean sediments ware spiked with 50 mL pure ethanol. After chemical spiking, the exposure tank was filled with artificial seawater (14 ppt) at a temperature of 19–20°C, and shrimps introduced. Shrimps were not fed during the experiment, but there was cannibalism behavior among the shrimps exposed to normoxia with clean sediments, and two deaths of that group were attributed to cannibalism. Following a 24-h exposure survivors of each treatment were snap-frozen with liquid nitrogen and stored at −80°C until enzymatic analysis. The mortalities ranged from 11% to 44%.

To assay NAG activity, epidermal tissue from carapace region was homogenized on ice in 0.15 M pH 5.5 citrate-phosphate buffer containing 0.04% v/v proteinase inhibitor cocktail (Sigma, St. Louis, MO). After centrifugation at 10,000*g* for 3 min, 20 µL of supernatant was incubated with 100 µL of 2 mM 4-nitrophenyl *N*-acetyl-β-D-glucosaminide (Sigma, St. Louis, MO), a specific substrate for NAG, at 25°C for 15 min. The reaction was stopped by addition of 0.9 mL 0.5 M NaOH. The liberated nitrophenol was quantified at 405 nm with the Beckman DU730 Life Science UV/VIS Spectrophotometer. Protein concentrations in supernatant were determined using the Bradford method. Enzymatic activity was expressed as nmol nitrophenol liberated $(\mu g \text{ protein})^{-1}$ $(15 \text{ min})^{-1}$.

One way analysis of variance (ANOVA) and Tukey's test (SPSS 16.0) were used to test the significance of difference between NAG activities of shrimps subjected to various treatments. A probability value of less than 0.05 was deemed as significant.

Results and Discussion

No significant difference in epidermal NAG activity was found between shrimps exposed to normoxia with clean

sediments and those treated with normoxia with sedimentary naphthalene ($p = 0.977$, Fig. 1), suggesting that under normoxic condition naphthalene administered in sediments at a concentration of 40 mg/kg had no effect on epidermal NAG activity in *P. aztecus*. Hypoxia alone was found to have no significant effect on epidermal NAG activity since there was no statistical difference between NAG activities in the epidermis of *P. aztecus* exposed to normoxia with clean sediments and hypoxia with clean sediments (Fig. 1). However, when hypoxia and sedimentary naphthalene were given together, there was a 47% decrease in epidermal NAG activity relative to that for the shrimps exposed to normoxia with clean sediments ($p = 0.02$, Fig. 1), suggesting that these two environmental stressors, when administered simultaneously, inhibit NAG activity in the epidermis of *P. aztecus*.

Although hypoxia alone had no statistically significant effects on enzymatic activity, exposure to hypoxia alone did result in a 31% decrease ($p = 0.14$, Fig. 1) in reference to the shrimps under normoxia with clean sediments. Interestingly, in the presence of sedimentary naphthalene, hypoxia caused a significant reduction in epidermal NAG activity in the shrimps subjected to hypoxia with sedimentary naphthalene compared with that for the shrimps exposed to normoxia with sedimentary naphthalene ($p = 0.04$, Fig. 1). This result clearly shows that sedimentary naphthalene can potentiate hypoxia's inhibitory effect on epidermal NAG activity in *P. aztecus*.

Molting in crustaceans is regulated by a multi-hormonal system, but is under immediate control of the steroid

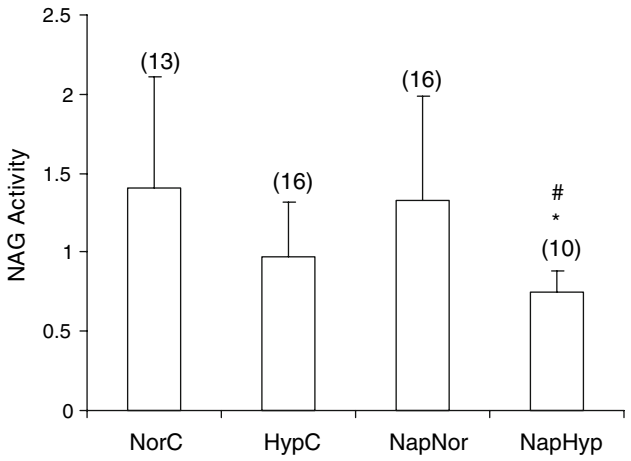

Fig. 1 Effects of hypoxia and sedimentary naphthalene on activity of *N*-acetyl-*β*-glucosaminidase (NAG) in the epidermis of *Penaeus aztecus*. Enzymatic activity was expressed as nmol nitrol phenol liberated (μg protein)$^{-1}$ (15 min)$^{-1}$. *NorC* Normxia + Clean sediments; *HypC* Hypoxia + Clean Sediments; *NapNor* Naphthalene in sediments + Normoxia; *NapHyp* Naphthalene in sediments + Hypoxia. *Error bars* represent standard deviation. Sample size is shown in *brackets*. *$p = 0.02$ relative to NorC, #$p = 0.04$ relative to NapNor

hormones called ecdysteroids (Chang et al. 1993). In decapods, ecdysteroids are produced in the Y-organs whose activity is controlled by the molt-inhibiting hormone (MIH) from the X-organ-sinus gland complexes. In epidermal cells, ecdysteroids regulate gene activities at the transcriptional level through interaction with the ecdysteroid receptor (EcR), which then heterodimerizes with crustacean retinoid X receptor (RXR) (Durica and Hopkins 1996; Chung et al. 1998). This EcR/crustacean RXR dimer binds to the DNA response elements of the genes regulated by the molting hormones. Among the products of the genes regulated by the molting hormones are enzymes responsible for degradation of the old exoskeleton, such as the chitonlytic enzyme NAG. Therefore, NAG production in the epidermis represents the terminal event in the endocrine cascades for molting control in decapod crustaceans, and epidermal NAG action can be used as a biomarker for adverse effects on crustacean molting (Zou 2005). The significant inhibition of epidermal NAG action after simultaneous exposure to hypoxia and sedimentary naphthalene suggests that these two environmental stressors, when administered in combination, can render inhibitory effects on brown shrimp molting in view of the fact that NAG activity is essential for exoskeleton degradation and that this chitinolytic enzyme is a marker for actions of the molting hormone.

Molting is a major physiological event in the life of a crustacean. The periodic shedding of the confining exoskeleton must be a hugely energy-consuming process. It is not unexpected that when ambient oxygen is limited the animal would slow down the molting process by downregulating enzymes involved in molting for the sake of energy conservation. This could account for the insignificant decline in epidermal NAG activity observed in the shrimps exposed to hypoxia alone (Fig. 1). Ye and Zou (2008) showed in a recent study that naphthalene can readily accumulate in tissues of *P. aztecus* and induce two naphthalene-metabolizing enzymes, ethoxyresorufin O-deethylase (EROD) and glutathione *S*-transferase (GST). It is well known that EROD catalyzes monooxygenation of naphthalene, which is then conjugated with glutathione through a phase II reaction catalyzed by the GST. Both monooxygenation and glutathione conjugation reactions are energy-consuming, which calls for additional oxygen. This demand for additional oxygen could aggravate the hypoxic stress, leading to further downregulation of molting-related activities, such as the production of NAG in the epidermis. This could explain why when naphthalene was present hypoxia produced a significant inhibitory effect on epidermal NAG in *P. aztecus*.

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