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Changes of exoskeleton surface roughness and expression of crucial participation genes for chitin formation and digestion in the mud crab (*Macrophthalmus japonicus*) following the antifouling biocide irgarol

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ABSTRACT

Irgarol is a common antifoulant present in coastal sediment. The mud crab *Macrophthalmus japonicus* is one of the most abundant of the macrobenthos in the costal environment, and its exoskeleton has a protective function against various environmental threats. We evaluated the effects of irgarol toxicity on the exoskeleton of *M. japonicus*, which is the outer layer facing the environment. We analyzed transcriptional expression of exoskeleton, molting, and proteolysis-related genes in the gill and hepatopancreas of these exposed *M. japonicus*. In addition, changes in survival and exoskeleton surface characteristics were investigated. In the hepatopancreas, mRNA expression of chitinase 1 (*Mj-chi1*), chitinase 4 (*Mj-chi4*), and chitinase 5 (*Mj-chi5*) increased in *M. japonicus* exposed to all concentrations of irgarol. *Mj-chi1* and *Mj-chi4* expressions from 1 to 10 μg L^{-1} were dose- and time-dependent. Ecdysteroid receptor (*Mj-EcR*), trypsin (*Mj-Tryp*), and serine proteinase (*Mj-SP*) in the hepatopancreas were upregulated in response to different exposure levels of irgarol at day 1, 4, or 7. In contrast, gill *Mj-chi5*, *Mj-Tryp*, and *Mj-SP* exhibited late upregulated responses to 10 μg L^{-1} irgarol compared to the control at day 7. *Mj-chi1* showed early upregulation upon exposure to 10 μg L^{-1} irgarol and *Mj-chi4* showed no changes in transcription in the gill. Gill *Mj-EcR* presented generally downregulated expression patterns. In addition, decreased survival and change of exoskeleton surface roughness were observed in *M. japonicus* exposed to the three concentrations of irgarol. These results suggest that exposure to irgarol induces changes in the exoskeleton, molting, and proteolysis metabolism of *M. japonicus*.

1. Introduction

Emerging booster biocide contamination has focused attention on marine ecosystem health. Irgarol has been widely used as a booster biocide in combination with copper in antifouling paints (Bao et al., 2013). It is considered an environmental contaminant because of its relatively high stability and persistence in the marine environment (Konstantinou and Albanis, 2004). In marine ecosystems, irgarol levels up to 254 ng L^{-1} in water and up to 9 ng g^{-1} dry weight in sediment have been recorded in southern California marinas (Sapozhnikova et al., 2013). In estuaries, reported irgarol concentrations have reached 0.68 μg L^{-1}, and in sediments, they can be up to 300 times that of the concentration in the overlying waters (Voulvoulis et al., 2000). Irgarol was detected at concentrations of 1.7 μg L^{-1} in marinas and ports (Readman et al., 1993), and 1.4 μg L^{-1} in mooring areas of a UK coast (Thomas et al., 2001). Maximum concentration of measured irgarol was 2021 ng L^{-1} in coastal waters of peninsular Malaysia (Ali et al., 2013). Irgarol was also detected at concentrations up to 67.64 ng L^{-1} along Korean coasts (Lee et al., 2010a). Irgarol may cause problems for top coastal predators because of bioaccumulation up the food chain. It induced apoptosis and increased levels of intracellular reactive oxygen species in HepG2 cell (Wang et al., 2013). Irgarol poses potential risks to the health of marine species even at low levels of exposure because it has strong adsorption characteristics and degrades slowly, having a half-life of 100 days (Okamura et al., 2000).

Macrophthalmus japonicus is one of the most abundant macrobenthic species of southwestern costal mudflats in Korea (Kitaura et al., 2002; Park et al., 2014). It contributes to the purification functions in the benthos of the tidal flats region because it burrows into organic matter in the intertidal zone (Herman et al., 1999). *Macrophthalmus japonicus* is an ecologically important species in Korea, and it can tolerate a wide range of natural and

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anthropogenic stressors (Lee et al., 2010b; Nikapitiya et al., 2014). Thus, mud crabs inhabiting the sediment, such as *M. japonicus*, are a suitable indicator species for monitoring the effects of antifouling irgarol on the marine benthic community. The organic matrix of the crab exoskeleton has a complex structure composed mainly of chitin microfibrils embedded in a protein matrix (Kuballa et al., 2007). The molting process, by which crabs shed their exoskeleton, involves the partial breakdown of the old exoskeleton and the synthesis of a new cuticle (Taylor et al., 2007). Toxic stress in *M. japonicus* can be measured by changes in transcriptional and physiological processes of the exoskeleton components.

The exoskeleton of a crab is a natural composite consisting of highly mineralized chitin-protein fibers arranged in a twisted plywood or Bouligand pattern (a stack of layers that have completed a 180° rotation). There is a high density of pore canal tubules in the direction normal to the surface (Chen et al., 2008). The exoskeleton consists of the epicuticle, exocuticle, and endocuticle. The outermost region is the epicuticle, a thin, waxy layer, which is the primary waterproofing barrier. Crustaceans changes from its natural condition in response to environment factors, such as changes in water pH and temperature, and the presence of chemical toxicants and pathogens (Biggers and Laufer, 2004; Walker et al., 2005; Robohm et al., 2005; Glenn and Pugh, 2006). Chitin is one of the major components of the exoskeleton of crustaceans and provides mechanical rigidity, protection to extracellular structures, protection against the environment and microorganisms, and resistance to desiccation (Mali et al., 2004; Chen et al., 2008). Chitinases are hydrolytic enzymes that breakdown glycosidic bonds in chitin (Nikapitiya et al., 2014). They are generally found in organisms that need to either reshape their own chitin or dissolve and digest the chitin of fungi or animals (Sámi et al., 2001). Ecdysteroids, molting hormones of arthropods, are secreted by crustacean Y-organs that are homologous to the prothoracic glands of insects (Techa and Chung, 2015). In arthropods, ecdysteroids regulate molting, limb regeneration, and reproduction through activation of the ecdysone receptor (EcR) (Gong et al., 2015). EcR also regulates gene expression associated with molting and metamorphosis in insects (Szamborska-Gbur et al., 2014). The proteolysis process is an essential protein digestion function that enables growth and can occur during cellular homeostasis or can be induced by external stress stimuli, such as heat, or biological or chemical disturbance (Pham et al., 2014). Serine proteinases (SPs) are crucial proteolytic enzymes responsible for digestion and other processes, including signal transduction and immune responses (Song et al., 2013; Sun et al., 2015). The enzymes regulate the proteolytic cascade in multiple biological processes (Liu et al., 2015). Trypsin, in the serine protease family, is a proteolytic enzyme with important receptors related to allergic responses (Larsen et al., 2013). Exoskeleton development and molting are important for external protection following toxic stress in crustaceans. However, there is no information regarding the molecular response of genes related to the molting and proteolysis process or effects of antifouling chemicals on the crustacean exoskeleton.

The crustacean hepatopancreas has digestive, synthetic, storage, immunity, and detoxifying functions (Gibson and Baker, 1979; Horst et al., 2007). Gill tissue has direct contact with the environment, and thus has a high tolerance capability for many contaminants (Nikapitiya et al., 2014). The goal of our study was to elucidate the effects of irgarol on *M. japonicas* developmental process following chitin formation and digestion. To accomplish, we investigated changes in survival, exoskeleton surface structure, and transcriptional expressions of six genes in the mud crab *M. japonicus* after irgarol exposure. Differential expressions of exoskeleton (chitinase 1; *Mj-chi1*, chitinase 4; *Mj-chi4*, and chitinase 5; *Mj-chi5*), molting (ecdysteroid receptor; *Mj-EcR*), and proteolysis-related genes (trypsin; *Mj-Tryp*, and serine proteinase; *Mj-SP*) were analyzed in gill and hepatopancreas from *M. japonicus* exposed to irgarol for 1, 4, and 7 days. Our data will be useful in the prediction of the potential risk of irgarol toxicity on the aquatic environment.

2. Materials and methods

2.1. Experimental M. japonicus maintenance

Crabs (weight = 7.0 ± 3.0 g; width = 3.5 ± 0.5 cm; height = 3.0 ± 0.5 cm; mean ± SD (n = 220)) caught in Suncheon Bay were collected from local aquatic markets of Haeryongmyeon in Korea. They were transported to the Laboratory of Marine Technology, Chonnam National University. The crabs were acclimatized to laboratory conditions for 24 h in tanks containing natural seawater (SW) (45.7 × 35.6 × 30.5 cm) with continuous aeration at 17 ± 0.5 °C and were fed a small amount (~180 mg) of Tetramin (Tetra-Werke, Melle, Germany) every day.

2.2. Irgarol treatment of M. japonicus and tissue extraction

Irgarol (2-(tert-butylamino)-4-(cyclo-propylamino)-6-(methylthio)-s-triazine) was purchased from Sigma Aldrich (St. Louis, MO, USA). Because of its low water solubility, a stock solution was prepared by dissolving 10 mg of irgarol in 1 mL of 99% acetone at room temperature. Test solutions of 1, 10, and 30 μg L^{-1} were made by diluting the stock solution with seawater. In the irgarol exposure study, crabs were separated into five groups (corresponding to the 1, 10, and 30 μg L^{-1} irgarol solutions, Seawater and seawater more solvent controls; n = 200), which were then subsequently distributed in two groups: one for survival and the other for gene expression and surface roughness analyses. Of the two subgroups (from each of the five groups), one set (n = 15) was used to determine survival rate after 7 days. The second set (n = 25 per replicate) was used for tissue sampling for RNA extraction at different time intervals (day 1, 4, and 7) after exposure to the three irgarol concentrations, after which 10 shells were used for surface roughness analyses. Experimental measurements were repeated three times. The irgarol concentrations were 1 μg L^{-1}, 10 μg L^{-1}, and 30 μg L^{-1}, and seawater was used as control.

Mortality was recorded daily until 7 days post irgarol exposure and the cumulative survival rate was determined. Gill and hepatopancreas tissue were extracted at each time point from three animals in each irgarol exposure concentration, as well as from control crabs. Sampled tissues were frozen in liquid nitrogen and were stored at −80 °C until RNA isolation. Throughout the experiment, water temperature, salinity, dissolved oxygen, and irgarol concentration were measured daily for all the treatments and the control.

2.3. RNA extraction and cDNA synthesis

Total RNA was extracted as previously described (Nikapitiya et al., 2014) from *M. japonicus* gill and hepatopancreas tissues from each irgarol treatment and control group (~35 mg $crab^{-1}$) using the TRIzol® reagent (Life Technologies, USA) according to the manufacture's protocol. Extracted RNA was treated by Recombinant DNase I (RNase free) (Takara, Japan) to remove genomic DNA contamination. RNA quantity was measured using a NanoDrop-1000 (Thermo Fisher Scientific, USA) and the concentration was equalized using nuclease-free water. RNA integrity was checked by 1.0% agarose gel electrophoresis, and the samples were stored at −80 °C. The total RNA from gill and hepatopancreas tissues was used as a template to synthesize cDNA using PrimeScript™ 1st strand cDNA synthesis kit (Takara, Japan) according to the manufacture's protocol as described by Nikapitiya et al. (2015).