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Immune responses of prophenoloxidase and cytosolic manganese superoxide dismutase in the freshwater crayfish *Cherax quadricarinatus* against a virus and bacterium

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ABSTRACT

Prophenoloxidase (proPO) and cytosolic manganese superoxide dismutase (cytMnSOD) play crucial roles in crustacean innate immunity. In the present study, both of the above genes were cloned from hemocytes of the red claw crayfish *Cherax quadricarinatus*. A phylogenetic analysis of the amino acid sequences showed that *C. quadricarinatus* proPO and cytMnSOD were more closely related to the proPO and cytMnSOD of other crayfish than to those of penaeids, crabs, lobsters, or freshwater prawns. A tissue distribution analysis revealed that proPO was primarily expressed in hemocytes, gills, and the heart, while cytMnSOD was detected in all tissues examined. All of the crayfish artificially infected with white spot syndrome virus (WSSV) died within 4 days. According to a non-lethal dose, there was no mortality in crayfish when infected deliberately with *Aeromonas hydrophila*. Total hemocyte counts (THCs) had significantly decreased in crayfish at 48 and 72 h after infection with WSSV compared to the control group. In contrast, THCs of crayfish after *A. hydrophila* challenge had recovered by 48 and 72 h from a lower level at 24 h. There were similar responses in enzyme activities toward WSSV and *A. hydrophila* infection. Phenoloxidase (PO) and superoxide dismutase (SOD) activities per hemocyte significantly increased from 48 to 72 h compared to the control group. After WSSV challenge, expressions of proPO and cytMnSOD transcripts in hemocytes significantly decreased at 12 h, then had respectively recovered and increased at 24 h. At 48–72 h, transcript levels were finally downregulated. No significant differences in the expression profiles of proPO and cytMnSOD were observed between the *A. hydrophila*-infected and control groups, besides the significant upregulation at 24 h post-infection. These results implicate proPO and cytMnSOD in the immune response, and they presented similar expression patterns, although different defense mechanisms may exist for crayfish induced by WSSV and *A. hydrophila*.

1. Introduction

Freshwater crayfish including *Cherax quadricarinatus*, *Procambarus clarkii*, and *Pacifastacus leniusculus* are extensively involved in research of crustacean immunity and disease resistance. Some species are used for aquaculture purposes and have suffered outbreaks of infectious diseases. The Australian red claw crayfish, *C. quadricarinatus*, is currently the only commercially farmed crayfish in Taiwan. The worldwide shrimp culture industry is seriously affected by viral pathogens, particularly white spot syndrome virus (WSSV), which have led to significant economic losses. WSSV (genus *Whispovirus*, family Nimaviridae) is a large, enveloped, ellipsoid, double-stranded DNA virus (Chou et al., 1995; Nakano et al., 1994; Wang et al., 1995) that also attacks crabs, lobsters, and many other crustaceans with a broad host range (Lo et al., 1996b; Maeda et al., 1998). In addition to experimental infection (Shi et al., 2000; Edgerton, 2004), there have been many cases of natural infection of red claw crayfish by WSSV on crayfish farms (Longshaw, 2011; Wang et al., 2012). In cultured penaeid shrimp, WSSV can cause a cumulative mortality of up to 100% within 3–7 days of infection. The main targets for WSSV replication include many tissues of ectodermal and mesodermal origin. In moribund shrimp, most tissues and organs are heavily infected with the virus, and they exhibit severe multifocal necrotic areas. The observed

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clinical signs of WSSV infection in shrimp are white spots in the exoskeleton and epidermis. Infected animals are lethargic, reduce their food consumption, and display a delayed (or completely absent) clotting reaction due to lysis of infected hemocytes (Leu et al., 2010). However, the body surface of infected *C. quadricarinatus* showed no obvious white spots (Wang et al., 2012). *Aeromonas hydrophila*, ubiquitous in freshwater environments, is a Gram-negative bacterium and sometimes isolated from apparently healthy crayfish but is considered to have the potential to cause diseases under culture conditions (Longshaw, 2011). It was suggested that in freshwater prawns *Aeromonas* spp. may function as opportunistic pathogens and only cause disease in prawns that are stressed by unfavorable environmental conditions (Sung et al., 2000). Jiravanichpaisal et al. (2009) isolated a highly virulent strain of *A. hydrophila* from apparently healthy *P. leniusculus*. Following culture of the bacteria in broth, the crude extracellular toxins obtained from culture supernatant of *A. hydrophila* were able to kill crayfish by injection by destroying the hemocytes or other types of cells by remarkable cytoplasmic vacuolization. At 6 h after an injection with *A. hydrophila*, moribund crayfish exhibited lethargic symptoms with the tail curved down to the abdomen and had necrotic lesions in various tissues. It is firmly believed that crustaceans rely solely on their innate immune system which has a critical role in recognizing pathogen infections and mounting subsequent responses. Consequently, investigating the mechanisms of immune defense against pathogenic invaders of *C. quadricarinatus* might be beneficial to the management of crayfish farming practice.

Melanization through the prophenoloxidase (proPO)-activating system and phagocytosis respectively represent the humoral and cellular responses of the crustacean innate immunity (Jiravanichpaisal et al., 2006). Upon recognition of non-self molecules, such as lipopolysaccharide, β 1,3-glucan, and peptidoglycan, the proPO cascade is activated to release proPO which is synthesized in hemocytes and stored in granules, and this leads to the limited proteolysis of proPO to the active PO. Then PO catalyzes the oxidation of phenolic substances to quinines, which are further polymerized non-enzymatically to melanin (Cerenius et al., 2008). The activated innate immune system also engages in phagocytosis to eliminate microbes or foreign particles. Intracellular microbe killing, after phagocytosis, is mediated by lysosomal enzymes and oxygen radicals. The accumulation of reactive oxygen species (ROS) and reactive oxygen intermediates (ROI) may lead to irreversible cell damage and eventually to cell death. The antioxidant enzyme, superoxide dismutase (SOD), converts superoxide anions into water and hydrogen peroxide, which is then transformed into water and oxygen by catalase and glutathione peroxidase, resulting in post-phagocytosis self-protection (Lin et al., 2010). SOD requires a metal cofactor for catalysis, and eukaryotic animal cells commonly possess two types of SODs: a cytosolic copper and zinc (CuZn)SOD and a mitochondrial manganese (mtMn)SOD. However, crustaceans that use copper-based hemocyanin for oxygen transport were shown to have a cytosolic (cyt)MnSOD which was proposed to replace the function of CuZnSOD in other systems (Gomez-Anduro et al., 2006).

During melanization and phagocytosis, highly reactive and toxic intermediates are produced. Thus, it is obvious that transcriptional regulation of proPO and cytMnSOD coordinated with enzyme activity are tightly regulated to eliminate invading microorganisms and minimize potential toxic side effects. In this study, proPO and cytMnSOD mRNAs were first cloned from hemocytes of *C. quadricarinatus* and compared to other known sequences to conduct a phylogenetic analysis. Gene expression profiles of the two genes in different tissues were further compared. Lastly, the gene expression of proPO and cytMnSOD and total enzymatic activity of PO and SOD were investigated when *C. quadricarinatus* were artificially injected with WSSV and *A. hydrophila*.

Table 1
PCR primers used in this study.

Name	Sequence (5′ → 3′)
P1: proPO degenerate-F	GCNATGGCNGCNWSNTGYAAC
P2: proPO degenerate-R	NARNCCNGCNCKRCANGGRCACAT
P3: proPO 5′RACE-R	TCTGCCGGAGTTATTAGAAGTGA
P4: proPO 3′RACE-F	CCTCTGGTCAGAGATGGATAAGTT
P5: proPO RT PCR-F	TGAAAAAGTGGGTCGAGGAC
P6: proPO RT PCR-R	AACACTGAGTCTTTCCATGTCGT
P7: proPO QPCR-F	GCCAAGGATCTTTGTGATGTCTT
P8: proPO QPCR-R	CGGCCGGCCAGTTCTAT
P9: cytMnSOD degenerate-F	CCAGCTATMAAGTTCAAYGGWGG
P10: cytMnSOD degenerate-R	TCHGCACGBAGGTTCTTGTACTG
P11: cytMnSOD 5′RACE-R	AATCAGACCGTGAGTGATCTGG
P12: cytMnSOD 3′RACE-F	TCTCAACCACACAATCTTCTGGA
P13: cytMnSOD RT PCR-F	TCTCAACCACACAATCTTCTGGA
P14: cytMnSOD RT PCR-R	TAAATGCTCATTCTTGGGTTGG
P15: cytMnSOD QPCR-F	AGGTCGAGCAAGCAGGTGTAG
P16: cytMnSOD QPCR-R	GTGGGAATAAACTGCAGCAATCT
P17: β-actin RT PCR-F	GAYGAYATGGAGAAGATCTGG
P18: β-actin RT PCR-R	CCRGGGTACATGGTGGTRCC
P19: β-actin QPCR-F	TCTGGCTCCTGCTACCATCAA
P20: β-actin QPCR-R	CACCGATCCAGACGGAGTACTT

2. Materials and methods

2.1. Animal preparation

Freshwater crayfish, *C. quadricarinatus*, were purchased from Pingtung, Taiwan and maintained in aerated tap water at 26–30 °C. Crayfish were kept in 60 cm × 45 cm × 45 cm aquaria with a circulating biofilter system for acclimation and quarantine prior to the experiments. During the acclimation and experimental periods, the crayfish were fed once daily with commercial feed. Then each group of crayfish was confirmed to be WSSV-free by a nested polymerase chain reaction (PCR) using primers designed by Lo et al. (1996a). Only animals determined to be healthy were used for the experiments.

2.2. RNA isolation and cloning of proPO and cytMnSOD complementary (c)DNAs

Hemolymph was withdrawn from the ventral sinus of mature crayfish (30–40 g) and immediately diluted 1:1 with an anticoagulant solution (0.14 M sodium chloride, 0.1 M glucose, 0.03 M trisodium citrate, 0.026 M citratic acid, and 0.01 M EDTA; pH 4.6) (Lee et al., 2000). Diluted hemolymph was centrifuged at 800 × *g* and 4 °C for 20 min to collect hemocytes, and then total RNA was extracted using the REzol™ C&T reagent (Protech, Taipei, Taiwan). For the reverse-transcription (RT)-PCR, first-strand cDNA was synthesized using 1 μg of total RNA pre-digested with DNase I (Promega, WI, USA), the oligo (dT) primer, and SuperScript™ III reverse transcriptase (Invitrogen, CA, USA) at 50 °C for 1 h. Four degenerate primers (P1, P2, P9, and P10; Table 1) were designed from conserved sequences of *P. leniusculus* (X83494), *P. clarkii* (EF595973, EU254488), *Litopenaeus vannamei* (EU284136, DQ298208), *Penaeus monodon* (AF099741, FJ467929), and *Necora puber* (FM242563). First-strand cDNA for 5′ rapid amplification of cDNA ends (RACE) and 3′ RACE was synthesized from total RNA using a FirstChoice RLM-RACE Kit (Ambion, TX, USA) according to the manufacturer's protocol. To obtain full-length cDNA sequences for proPO and cytMnSOD, four gene-specific primers (P3, P4, P11, and P12; Table 1) were designed based on nucleotide sequences of cDNA fragments amplified by the RT-PCR and used in the RACE PCRs.