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Molecular characterization of a novel nitric oxide synthase gene from *Portunus trituberculatus* and the roles of NO/O_2^-- generating and antioxidant systems in host immune responses to *Hematodinium*

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ABSTRACT

Increasing evidences have established that the nitric oxide synthase (NOS) and NADPH oxidase (NOX) play important roles in host defense system by catalyzing the production of nitrogen oxide (NO) and superoxide anions (O_2^-), respectively. While, there are limited studies to explore the roles of NOS/NOX enzymes in crustacean immunity, and no studies as yet were attempted to elucidate their functions in host immune responses to parasites. In the present study, we cloned a full-length cDNA of *NOS* and two partial cDNA fragments of *NOX* and *GPx* from the economic valuable crab *Portunus trituberculatus*. The full-length cDNA of *NOS* was 4002 bp in length that encoded 1203 amino acids containing motifs of the NOS protein and conserved domains. The phylogenetic analysis showed that the NOS protein sequence was clustered together with those of crustacean species in the phylogenetic tree. All of the three novel genes showed high mRNA transcripts in the immune-related tissues (e.g. hemocytes, hepatopancreas) of *P. trituberculatus*. Striking fluctuation in the transcripts of the critical NO/O_2^-- generating/scavenging related genes (*NOS*, *NOX*, *CuZnSOD*, *CAT*, *GPx*) as well as in the enzymatic activities of NOS, NOX, SOD, CAT and GPx were observed in the hemocytes and hepatopancreas of *P. trituberculatus* post challenged with the parasitic dinoflagellate *Hematodinium*, indicating that the NO/O_2^-- generating and the antioxidant systems played vital roles in the crustacean innate immunity against the parasitic intrusion. The results indicated a novel respect of the host-parasite interaction between the crab host and the parasitic dinoflagellate *Hematodinium*.

1. Introduction

The free radical molecules, such as the nitric oxide (NO), superoxide anions (O_2^-), and their derivatives reactive nitrogen/oxygen species (RNS/ROS), were involved in versatile physiological processes including the cell proliferation, apoptosis, signal transduction and immune defense [1–4]. Among those important reactive free radicals, nitric oxide (NO) was produced by nitric oxide synthase (NOS) catalyzing the conversion of L-arginine to L-citrulline with the concomitant oxidation of the nicotinamide adenine dinucleotide phosphate (NADPH), while the O_2^- molecules were generated from the NADPH oxidase (NOX) [5,6]. In mammals, both NOS and NOX played important roles in host immune system [5–7]. Three isoforms of NOS, including neuronal NOS (nNOS, or NOS1), inducible NOS (iNOS) and endothelial NOS (eNOS, or NOS3) had been reported [5]. Whereas, seven members of the NOX family, with different patterns of tissue-specific distribution as well as distinct patterns of function in host defense, had been identified [6,7]. In invertebrates, the production of NO together with O_2^- and H_2O_2 was closely involved in host defense reactions against both microbial pathogens and eukaryotic parasites in the *Drosophila* [8]. Arumugam et al. [9] reported that NO and O_2^- played important roles in the defense system of *Mytilus galloprovincialis*, showing that both the NOX and NOS pathways were involved in phagocytosis of *Saccharomyces cerevisiae*. Meanwhile, increasing number of studies had also showed that NOS and NOX were closely associated with invertebrate innate immunity against various types of pathogens, including bacteria [10], viruses [11] and parasites [12].

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Table 1
Primers designed for fragments cloning, 5′- and 3′-RACE and qRT-PCR.

Primer	Sequence (5′ to 3′)	Remark
PtNOS -F1	GGTACATGGTGTCNGAGATCG	*PtNOS* fragment cloning
PtNOS -R1	TACATCATCGTCCAGGCAGAA	
PtNOS -F2	GAGACTTTCTGCCTGGACGAT	*PtNOS* fragment cloning
PtNOS -R2	GGCTCACCTTCTCTGGGATT	
PtNOX –F1	GCCATCACCTACGAGTCCSC	*PtNOX* fragment cloning
PtNOX –R2	CGGGATGTTGACGAACACGT	
PtGPX –F1	GCTDATCCAGAACACGGCG	*PtGPX* fragment cloning
PtGPX –R1	CTTCGTTKGGAATTTCTTGC	
PtNOS -5gsp1	TGTCCAGCCCCATCCTCTTCC	5′RACE
PtNOS -5gsp2	TGGCTGTCACACAAGTCCCGAG	
PtNOS -3gsp1	GAGACCCCACTGTGCCCATCAT	3′RACE
PtNOS -3gsp2	CATACCCCACAAAAAAATCCCAGA	
PtNOS-QRT-F	CATACCCCACAAAAAAATCCC	qRT-PCR
PtNOS-QRT-R	ATCCATCTTTCGTGTTCGGC	
PtNOX-QRT-F	TTGAGGTGTTTTACTGGACGCA	qRT-PCR
PtNOX-QRT-R	TAACGCTACGGTGGATTCGC	
PtGPX-QRT-F	GTCCTGGTAACAACTTTGAGCC	qRT-PCR
PtGPX-QRT-R	ATGATACACTTGGGGTCTGCC	
PtCAT-QRT-F	ATTTGAGGGACCCAAGGAGA	qRT-PCR
PtCAT-QRT-R	GTTCACCAATCGTTGCCGT	
PtCuZnSOD-QRT-F	GCGGTAGTGAACTTTGTGCC	qRT-PCR
PtCuZnSOD-QRT-R	GAATGTTGCCAAGGTCTCCA	
UPM	Long-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT Short-CTAATACGACTCACTATAGGGC	5′ and 3′RACE
NUP	AAGCAGTGGTATCAACGCAGAGT	5′ and 3′RACE

The free radical molecules (NO/O_2^-) as well as their derivatives (RNS/ROS) served as a “two-side sword” to the host, it could not only benefit the antimicrobial, antiviral and antiparasitic potency [2,5,13], but also result in cellular damages (e.g. DNA damage, lipid peroxidation and protein oxidations) due to their high cytotoxicity [14,15]. Therefore, the production of free radical molecules and their derivatives must be controlled in a delicate way and maintained at a homeostasis status. The antioxidant system was generally employed as a potent arsenal to alleviate the damage from reactive free radicals [16]. The first line of antioxidant defense was composed of antioxidant molecules involving glutathione (GSH), vitamin C and E, carotenoids, which was carried out in a non-enzymatic way [17]. Another line of antioxidant defense consisted of the key antioxidant radical-scavenging enzymes, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), that operated in an enzymatic way [18]. The three important antioxidant enzymes could eliminate the excessive ROS (e.g. O_2^-, H_2O_2, organic hydroperoxides) and collaboratively contribute to the homeostasis of redox status in aerobic host organisms [19–21], and those enzymes were also closely involved in the crustacean immune responses [22–25].

The swimming crab *Portunus trituberculatus* is one of the most important commercial species distributed extensively in East Asia. It supports a large proportion of crab aquaculture in China, with the total yield exceeding 100, 000 tons in 2013 [26]. However, the sustainability of the crab aquaculture industry has been constantly threatened by the outbreaks of diseases caused by various pathogens (e.g. bacteria, viruses, fungi, parasites) [27,28]. Since 2007, the parasitic dinoflagellate in the genus of *Hematodinium*, a serious epidemic pathogen affecting the wild populations of commercially valuable crustaceans around the world [29–31], had started causing epidemic diseases in cultured *P. trituberculatus* and resulted in severe economic losses to local farmers [32,33]. Our recent findings showed that the proPO system, serine proteinase cascade reactions as well as the transcripts of the important pathogen-recognition receptor Toll were influenced remarkably in the host immune responses to the parasite [34,35], highlighting the preliminary response of *P. trituberculatus* against the parasitic invasion. Thus, to further understand the host-parasite interaction and explore the roles of NO/O_2^-- generating or scavenging related genes/enzymes during the immune responses in crustaceans, we isolated three novel genes (*NOS*, *NOX* and *GPx*) from *P. trituberculatus* and characterized the full-length cDNA of the novel *NOS* gene. Then, we further investigated the transcriptional regulation of the five important NO/O_2^-- generating or scavenging related genes (*NOS*, *NOX*, *CuZnSOD*, *CAT*, *GPx*), as well as the post-translational mechanisms of regulating the five corresponding enzymatic activities (NOS, NOX, SOD, CAT, GPx) in the hemocytes and hepatopancreas of *P. trituberculatus* after challenged with *Hematodinium* parasites.

2. Materials and methods

2.1. Experimental animals

The swimming crabs *P. trituberculatus* (140 ± 10 g) were collected from a local aquaculture farm (Jiaonan, Shandong Province, China). The crabs deployed in laboratory challenge experiments were screened to be free of *Hematodinium* infection (referred as healthy crabs in following paragraphs), using both the microscopic assay and the molecular assay as described in Stentiford & Shields [31] and Small et al. [36]. These crabs were acclimatized in an aerated recycling seawater system (30 ppt, 23 ± 0.5 °C) for at least one week before being used in laboratory trials. Additional crabs with heavy *Hematodinium* infection were collected simultaneously from a separate pond and held temporarily in a separate tank until being used as donors for the challenge experiment. During the experimental period, crabs were fed clam meat once a day at night and food residues were then removed in the next morning. Waters were changed periodically to ensure the water quality being within an acceptable limit (ammonia: 0–0.3 ppm, nitrite: 0–0.6 ppm, pH: 7.4–8.2).

2.2. cDNAs cloning of the NOS, NOX and GPx genes

To isolate the *NOS*, *NOX* and *GPx* genes from *P. trituberculatus*, hemolymph were withdrawn individually from five healthy crabs