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Deciphering of the Dual oxidase (Nox family) gene from kuruma shrimp, *Marsupenaeus japonicus*: Full-length cDNA cloning and characterization

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ABSTRACT

In many physiological processes, including the innate immune system, free radicals such as nitric oxide (NO) and reactive oxygen species (ROS) play significant roles. In humans, 2 homologs of Dual oxidases (Duox) generate hydrogen peroxide (H_2O_2), which is a type of ROS. Here, we report the identification and characterization of a Duox from kuruma shrimp, *Marsupenaeus japonicus*. The full-length cDNA sequence of the *M. japonicus* Dual oxidase (*Mj*Duox) gene contains 4695 bp and was generated using reverse transcriptase-polymerase chain reaction (RT-PCR) and random amplification of cDNA ends (RACE). The open reading frame of *Mj*Duox encodes a protein of 1498 amino acids with an estimated mass of 173 kDa. In a homology analysis using amino acid sequences, *Mj*Duox exhibited 69.3% sequence homology with the Duox of the red flour beetle, *Tribolium castaneum*. A transcriptional analysis revealed that the *Mj*Duox mRNA is highly expressed in the gills of healthy kuruma shrimp. In the gills, *Mj*Duox expression reached its peak 60 h after injection with WSSV and decreased to its normal level at 72 h. In gene knockdown experiments of free radical-generating enzymes, the survival rates decreased during the early stages of a white spot syndrome virus (WSSV) infection following the knockdown of the NADPH oxidase (*Mj*Nox) or *Mj*Duox genes. In the present study, the identification, cloning and gene knockdown of the kuruma shrimp *Mj*Duox are reported. Duoxes have been identified in vertebrates and some insects; however, few reports have investigated Duoxes in crustaceans. This study is the first to identify and clone a Dual oxidase from a crustacean species.

1. Introduction

In many physiological processes, free radicals such as nitric oxide (NO) and reactive oxygen species (ROS) play significant roles [1]. ROS, including superoxide (O_2^-) and the superoxide-derived hydrogen peroxide (H_2O_2), can function independently or in conjunction with other free-radical intermediates and are predominantly associated with antibacterial and antiviral host defense mechanisms [2,3]. Examples of enzymes that generate ROS in humans are the members of the Nox family, which consists of 5 homologs of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox) and 2 homologs of Dual oxidase (Duox). ROS generated from these enzymes include O_2^-, which is generated by the 5 Noxes (Nox1, Nox2, Nox3, Nox4 and Nox5), and H_2O_2, which is generated by the 2 Duoxes (Duox1 and Duox2) [4].

Because Duoxes were first described in the thyroid gland, they were originally called thyroid oxidases [5,6]. These proteins with homologous sequences were later identified in the roundworm *Caenorhabditis elegans*, and they were renamed "Dual oxidase" based on the structural features of the proteins: a peroxidase-like domain on the N-terminal side and an NADPH oxidase domain on the C-terminal side [7]. Although Duoxes have a peroxidase-like domain, they have no peroxidase activity because they lack the histidines that are essential for peroxidase activity [7–9]. In humans, Duox1 and Duox2 have high expression levels in the thyroid gland. They are also expressed in the epithelial cells of the gastrointestinal tract and the respiratory tract [10,11]. Inactivating mutations in the Duox2 gene are associated with a loss of thyroid hormone synthesis and cause

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congenital hypothyroidism [12]. H_2O_2 derived from the Duoxes serves as a primary immune activator and a signaling molecule with multifaceted roles, such as thyroxin synthesis, matrix cross-linking and host defense in human cells [4,13]. Regarding the regulation of the Duoxes, the expression of Duox1 mRNA is induced by interleukin-4 (IL-4) and interleukin-13 (IL-13). In contrast, the expression of Duox2 mRNA is induced by interferon-gamma (IFN-γ) [14]. Additionally, Ca^{2+} is known as another regulatory factor of Duox1 and Duox2. Duoxes are activated by Ca^{2+} via a pair of EF-hands that are known Ca^{2+} binding motifs [9].

Duoxes are also present in invertebrates, but only a few reports of this observation are available. Insects such as *Drosophila* have a homolog of the human Duoxes [15]. It has been reported that *Drosophila* that includes a specific RNAi sequence exhibits an increased mortality rate [15,16]. In *Anopheles gambiae* (African malaria mosquito), a peroxidase/Duox system protects the microbiota by preventing the activation of epithelial immunity [17]. Thus, Duoxes play an important role in the biological defense system of insects.

In crustaceans, the role of ROS in innate immunity is to exclude microbes. When a pathogen enters the hemolymph, ROS, such as O_2^-, the hydroxyl radical (OH^-), H_2O_2 and singlet oxygen ($^1O_2$), are produced and play important roles in the anti-microbial activity of the Pacific white shrimp *Litopenaeus vannamei* [18,19]. In this report, the transcript levels of superoxide dismutase (SOD) increased transiently 1 h following infection with white spot syndrome virus (WSSV) and decreased 12 h post-infection. Hemocyte SOD induction has been proposed to be part of an early ROS detoxification response. The steady decrease in SOD expression as the viral infection progressed would be expected to generate higher local levels of ROS and may be an important mechanism to limit viral replication [19]. Regarding bacterial stimulation, in the hemocytes of Pacific white shrimp (*L. vannamei*), the production of O_2^- is dependent on the concentration of bacteria (*Escherichia coli*) [18]. It has previously been reported that in the hemocytes of the giant freshwater prawn *Macrobrachium rosenbergii*, O_2^- generation clearly reveals the involvement of Nox and phenoloxidase pathways [20]. In the hemocytes of the kuruma shrimp *Marsupenaeus japonicus*, early gene up-regulation of Nox (*Mj*Nox) was confirmed after *Vibrio penaeicida* or poly (I:C) stimulations [21]. Kuruma shrimp also generate nitric oxide (NO) after stimulation by LPS [22], and the gene expression of nitric oxide synthase (*Mj*NOS) increases after *V. penaeicida* injection in the gills [23].

These reports suggest that free radicals, including ROS and NO, and the enzymes involved in their generation are important in the immunity of shrimp. However, the existence of Duox, which is capable of generating H_2O_2, is unclear in shrimp. For these reasons, we identified the full-length cDNA encoding the Duox gene from the kuruma shrimp, denoted as *Mj*Duox. Additionally, we investigated gene expression and survival rates after WSSV infection and gene knockdown of *Mj*NOS, *Mj*Nox and *Mj*Duox to better understand the role of free radical-generating enzymes in biological defense mechanisms. In the present study, the identification, cloning and gene knockdown of the kuruma shrimp *Mj*Duox gene are reported for the first time, and these data include some new information about enzymes that generate free radicals in crustaceans.

2. Materials and methods

2.1. Animals

Adult kuruma shrimp, *M. japonicus* (average weight: 15 g), were obtained from a shrimp farm in Miyazaki, Japan. They were fed once a day with a commercial diet at 1% of their body weight and acclimatized in aerated seawater at 22 °C.

2.2. Designing degenerate primers

A partial Duox cDNA was initially obtained using RT-PCR with degenerate primers that were designed based on the conserved regions of the fruit fly (*Drosophila melanogaster*) and red flour beetle (*Tribolium castaneum*) Duox genes in the NCBI (GenBank accession numbers: NM_134871 and XM_965755) using a ClustalW alignment with the ClustalW program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The degenerate primers dg-Duox-F and dg-Duox-R (Table 1) were designed to anneal to highly conserved DNA sequences.

2.3. RNA extraction and cDNA preparation

The total RNA was extracted from the lymphoid organs of three kuruma shrimp using RNAiso Plus (TaKaRa, Japan) in accordance with the manufacturer's instructions and quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). The purity of the RNA samples treated with RNase-free DNase I

Table 1
PCR primers used for kuruma shrimp *Mj*Duox analysis.

Primers	Sequence (5′–3′)
Primers for degenerate PCR	
dg-Duox-F	GGTCCNTGGACSTGGAA
dg-Duox-R	AGCCACCTCRAAYTTRTACC
Primers for RACE PCR	
5′-RACE	
5′-DuoxR1	ACGGTCGGCTTTTGGTCGGAGGAG
5′-DuoxR2	TGAGCAGAAGCAGGGAGTAGCAGAAGGA
5′-DuoxR3	AGCAGGGAGTAGCAGAAGGAGAGGGAAG
5′-DuoxR4	TCGCTGACGGTGTTTGTTTTCG
5′-DuoxR5	GCCACTCCTTGACCATCATCTTGT
5′-DuoxR6	CACCACCAGCGTGTCCAAGTGAG
5′-DuoxR7	GCTTGTCGTCGGTCGTGCCCTTG
5′-DuoxR8	GGAGGACACCACCTGACCGAAGAAG
3′-RACE	
3′-DuoxF1	ACCGAAGGTCCGCCTGGATG
3′-DuoxF2	CCGCCTGGATGGACCTTTCG
Primers for cloning of partial sequence	
c-DuoxR1	CATCCAGGCGGACCTTC
c-DuoxR2	GAGGAGTTCGGGGTGGAC
c-DuoxF1	CTGGACGGCAACTTCGTG
c-DuoxR3	GTCATCAGCGTCCCACCA
Primers for qRT-PCR analysis	
*Mj*NOS-F	GCCCTGTCTCGTGAACCTAC
*Mj*NOS-R	TTTTCATCCCTCATCTGTAGCA
*Mj*Nox-F	ACGATGAAGCCCGAAAGG
*Mj*Nox-R	CCCGTGGAGGATTGTGAG
*Mj*Duox-F	TGGCTACACCAGAACCACAA
*Mj*Duox-R	ACCAGCGTGTCCAAGTGAG
*Mj*EF1α-F	GTCTTCCCCTTCAGGACGTA
*Mj*EF1α-R	GAACTTGCAGGCAATGTGAG
Primers for gene knockdown	
*Mj*NOS-i-F	GGCTTTTGGCATCATCGTC
*Mj*NOS-i-R	CTTCGTGGTATCTGTTTTCATCC
*Mj*NOS-dsF	GGATCCTAATACGACTCACTATAGGGGCTTTTGGCATCATCGTC
*Mj*NOS-dsR	GGATCCTAATACGACTCACTATAGGCTTCGTGGTATCTGTTTTCATCC
*Mj*Nox-F	ACGATGAAGCCCGAAAGG
*Mj*Nox-i-R	GCCGAAGAGATGGTGAAGG
*Mj*Nox-dsF	GGATCCTAATACGACTCACTATAGGACGATGAAGCCCGAAAGG
*Mj*Nox-dsR	GGATCCTAATACGACTCACTATAGGGCCGAAGAGATGGTGAAGG
*Mj*Duox-i-F	GCGTTCAGACCAACAAGGAG
*Mj*Duox-i-R	TCTACCAGAGAGCGGAGCA
*Mj*Duox-dsF	GGATCCTAATACGACTCACTATAGGGCGTTCAGACCAACAAGGAG
*Mj*Duox-dsR	GGATCCTAATACGACTCACTATAGGTCTACCAGAGAGCGGAGCA
GFP-F	ATGGTGAGCAAGGGCGAGGA
GFP-R	TTACTTGTACAGCTCGTCCA
GFP-i-F	GGATCCTAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGA
GFP-i-R	GGATCCTAATACGACTCACTATAGGTTACTTGTACAGCTCGTCCA