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Identification and characterization of myeloperoxidase in orange-spotted grouper (*Epinephelus coioides*)

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ABSTRACT

Cryptocaryon irritans is an important protozoan ciliate, which has led to heavy economic losses in marine aquaculture. Previous studies have indicated that *C. irritans* infection could induce the migration of neutrophils to infection sites. Myeloperoxidase (MPO) mainly exists in the cytoplasmic granules of the neutrophil and performs its function by a unique enzymatic capacity to produce hypohalous acid and other toxic oxidants. To determine the involvement of MPO and neutrophils against *C. irritans* infection in the host, we amplified MPO cDNA (EcMPO) from orange-spotted grouper (*Epinephelus coioides*). The open reading frame (ORF) of EcMPO encodes a putative polypeptide of 770 amino acids and has typical structural characteristics of mammalian MPO, including a signal peptide, a propeptide, a light chain, a heavy chain, and a peroxidase domain. Bioinformatics analysis has demonstrated that the most important functional sites in mammalian MPO were also conserved in grouper and other piscine MPO, implying the functional conservation of this protein during evolution. A rabbit *anti*-MPO recombinant protein polyclonal antibody was produced, which could recognize the native MPO protein. The expression of EcMPO was higher in the lympho-hematopoietic organs, such as head kidney, trunk kidney, spleen, but lower in muscle, heart, and brain. After infection with *C. irritans*, the EcMPO transcript was significantly up-regulated at specific time points in the infection sites (skin and gill) and systemic immune organs (head kidney and spleen); The number of EcMPO positive cells first increased and then decreased in the gill, but was still higher than the control after 7 days. These results demonstrated that EcMPO and its positive cells may be involved in *anti-C. irritans* infection in the grouper, which is attributed to the innate immune mechanisms of the host against parasite infection.

1. Introduction

Cryptocaryon irritans, a ciliated obligate parasite, infects almost all marine bony fish reared in tropical and subtropical regions, and causes heavy economic losses in aquaculture. The life cycle of *C. irritans* contains four stages, namely living trophont, off-host protomont, reproductive tomont, and infective theront [1]. Trophont is the only parasitic stage that mainly resides under the epidermis of the skin and gills, where it impairs the physiological function of these organs and induces host immune responses. Luo et al. and Yambot et al. have reported that a specific antibody titer was significantly increased in grouper after exposure to *C. irritans*, and played a crucial role in host defense against re-infection of this parasite [2,3]. However, Bai et al. have indicated that after intraperitoneal injection with hydrocortisone, although the antibody titer was not significantly changed, the immune protection was largely reduced, implying that except for specific antibodies, the cellular immune response may also be implicated in *anti-C. irritans* infection (unpublished data). Our studies have also demonstrated that at 3 days post-*C. irritans* infection, large numbers of leucocytes, including neutrophil-like cells, eosinophilic granule cells, lymphocytes, and monocytes are infiltrated into the gills, and the expression of IL-8, an important chemokine that mainly recruits neutrophils into the local inflammatory or injury sites, was significantly up-regulated in the infection sites [4]. These results suggest that the neutrophils may participate in grouper defense against *C. irritans* infection.

Neutrophils are important innate immune components, which constitute the first line of defense in the host immune system against invading pathogen. Neutrophils are typically the first cells recruited to the inflammatory site, where they attract additional neutrophils, macrophages, and T cells by releasing immune factors [5]. Upon activation,

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neutrophils become powerful killers, which can release toxic intracellular granules, produce reactive oxygen species (ROS), and secrete neutrophil extracellular traps [6]. Myeloperoxidase (MPO), a heme peroxidase, exists primarily in the cytoplasmic granules of neutrophil and contributes to the antimicrobial function by producing ROS, such as hypohalous acid (HOCl, HOBr, and HOI) and other toxic oxidants produced from H_2O_2 and halides, and these oxidants can oxidize organic molecules and kill microbes [7,8]. Moreover, MPO and these toxic agents can be released to the outside of the cell and damage a variety of microorganisms that are too large to be ingested, such as protozoa, amoebae, and helminths [9]. On the other hand, there is considerable evidence that the inappropriate activity of MPO results in host tissue damage, and this damage has been implicated in the pathogenesis of diverse inflammatory diseases, including atherosclerosis, demyelinating disorders of the central nervous system, and certain tumors [10]. Nevertheless, the complex and widespread biological activities of MPO, which are accomplished in humans, are still poorly understood in teleosts. In fact, MPOs have only been identified in a few fish species [11–15].

In this study, to assess whether MPO and its positive cells are involved in host defenses against *C. irritans* infection, an MPO cDNA sequence (EcMPO) was identified in grouper, *Epinephelus coioides* and *anti*-EcMPO serum was prepared using recombinant proteins. The expression pattern of EcMPO was analyzed in healthy and grouper infected by *C. irritans*. Lastly, the dynamic profile of EcMPO positive cells was determined in the gills after *C. irritans* infection.

2. Materials and methods

2.1. Fish and parasites

100 healthy orange-spotted grouper (weight 60.3 ± 7.3 g) were purchased from the Marine Fisheries Development Center of Guangdong Province, China, and were maintained at 25 ± 1 °C for two weeks in a recirculating seawater system before the challenge. *C. irritans*, used in this study, were maintained by a serial passage on pompano *Trachinotus ovatus* in our laboratory based on the method of Dan et al. [16].

2.2. C. irritans challenge and sampling

For infection, groupers were randomly divided into two groups (30 fish per group), namely the untreated control group and infected group. Fish in the infection group were challenged by immersing to live *C. irritans* at a dose of 25000 theronts per fish as previously described [17]. At day 4 post infection, fish were transferred into a new tank to avoid auto-reinfection. Fish in the control group were treated the same as the infection group with the exception of parasite infection. At 6 and 12 h, 1, 2, 3, 5, and 7 days post challenge, samples of the gill, skin, spleen, and head kidney were isolated from both groups for expression profile analysis. Three fish were sampled from each group at each time point. At the same time, tissues of blood, thymus, head kidney, gill, skin, muscle, liver, spleen, trunk kidney, heart, brain, midgut, and stomach were taken from three healthy groupers for tissue distribution analysis. All samples were immediately frozen in liquid nitrogen and then stored at −80 °C until RNA isolation. In addition, gills were sampled at the same time point and then fixed in Bouin's solution for immunohistochemistry.

2.3. RNA extraction and cDNA synthesis

Extraction of the total RNA from all samples was performed with a Magen HiPure Universal RNA Kit (Magen, China) according to the manufacturer's protocol. After removing DNA contamination with DNase I (Thermo Fisher Scientific, USA), first strand cDNA was then synthesized from total RNA with a ReverTra Ace-α-Kit (Toyobo, Japan) following the manufacturer's protocol.

Table 1
Primers used in this study.

Primer	Sequence (5′ to 3′)
MPOF	GATGCTTTTCTCTGTCCTTCTTCTG
MPOR	TGATGTCATGTGCTGGTGGTCC
MPOEPF	GCGGATCCTGCCGCACCACACCAAA
MPOEPR	CCGCTCGAGTTACAGAGCATCC
MPORTF	GACGACGGCATTTCCCTACC
MPORTR	TGACCACGCCTGCATCTGTT
β-actinF	TGCTGTCCCTGTATGCCTCT
β-actinR	CCTTGATGTCACGCACGAT

2.4. Cloning of EcMPO cDNA

We have previously obtained 80 G transcriptome data from the skin, gill, spleen and head kidney of grouper (unpublished data). A 3293 bp EST sequence containing the complete ORF of MPO has been identified from this data via the blast program. Primers MPOF/R (Table 1) were designed according to the identified EST sequences to amplify the ORF of EcMPO. The amplification products were ligated into a pEASY®-Blunt Cloning Vector (TransGen, China), transformed into competent *Escherichia coli* DH5α cells and sequenced (Invitrogen, USA).

2.5. Sequence analysis and phylogeny analysis

The potential open reading frame was identified using the ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The amino acid sequence was analyzed with DNASTAR LaserGene 7.1 software. Protein structure analysis was performed using the SMART program (http://smart.embl-heidelberg.de/index2.cgi) and ExPASy-prosite server (http://prosite.expasy.org). The protein *N*-glycosylation sites were predicted with NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/). Multiple sequence alignment was performed by ClustalX 2.1. A phylogenic tree was constructed using MEGA 5.04 program.

2.6. Expression of recombinant EcMPO and preparation of antiserum

The partial EcMPO sequence (residues 403–1662) was inserted into a pET-32a vector. In brief, primers of MPO EPF/EPR (Table 1) were designed to amplify the partial sequence of EcMPO. The PCR products and pET-32a vector were digested with restriction enzyme BamH I and Xho I (TaKaRa, Japan) and then combined with a DNA Ligation Kit (TaKaRa, Japan). The recombinant plasmid was transformed into *E. coli* BL21(DE3) and sequenced. The recombinant EcMPO protein (rEcMPO) was expressed according to a conventional method [18] with some modifications. In brief, the positive clone was incubated in 500 ml LB medium (containing 100 μg/ml ampicillin) at 37 °C with shaking at 180 rpm. When the culture medium reached 0.6–0.8 at OD600, isopropyl β-D-1-Thiogalactopyranoside (IPTG) was added to the medium to a final concentration of 1 mM, and then the cells were incubated at 37 °C for 4 h. Recombinant protein with a 6 × His tag was purified with nickel-nitrilotriacetic acid-agarose (Ni-NTA; Thermo, USA) on an affinity chromatography column according to the manufacturer's instructions. Purified protein was dialyzed extensively against gradient urea solution and PBS buffer, and concentrated with Amicon Ultra Centrifugal Filter (Millipore, USA). SDS-PAGE under reducing conditions was used to assess the expression and purification of the recombinant protein. The concentration of the recombinant protein was determined with a BCA Protein Assay Kit (CWBIO, China). The polyclonal antibody against the recombinant protein was produced by immunizing rabbits as described previously [19]. The specificity of the antiserum was detected by western blotting.