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Short communication

Identification of a myeloperoxidase-like ortholog from rock bream (*Oplegnathus fasciatus*), deciphering its transcriptional responses to induced pathogen stress

Don Anushka Sandaruwan Elvitigala [a, b], Ilson Whang [a, b], Bo-Hye Nam [c], Hae-Chul Park [d, **], Jehee Lee [a, b, *]

[a] Department of Marine Life Sciences, School of Marine Biomedical Sciences, Jeju National University, Jeju Self-Governing Province 690-756, Republic of Korea
[b] Fish Vaccine Development Center, Jeju National University, Jeju Special Self-Governing Province 690-756, Republic of Korea
[c] Biotechnology Research Division, National Fisheries Research and Development Institute, 408-1 Sirang-ri, Gijang-up, Gijang-gun, Busan 619-705, Republic of Korea
[d] Graduate School of Medicine, Korea University, Ansan, Gyeonggido 425-707, Republic of Korea

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ABSTRACT

Myeloperoxidases (MPOs) are heme-linked oxidative stress-generating enzymes found abundantly in azurophilic granules of polymorphonuclear neutrophils. Mature MPOs act as potent antimicrobial agents by producing hypohalous acids using hydrogen peroxide and halide ions as substrates. These acids can readily oxidize reactive groups of biomolecules on invading microbes. In this study, we identified and characterized a homolog of MPO from rock bream (*Oplegnathus fasciatus*), designated as RbMPO. We analyzed the *RbMPO* gene for its basal expression level in physiologically important tissues and for transcriptional changes under different pathogenic stress conditions. The complete coding sequence of RbMPO consisted of 2652 nucleotides encoding an 884 amino acid sequence with a predicted molecular mass of 99.7 kDa. Our *in silico* analysis confirmed the typical MPO domain arrangement in RbMPO, including the propeptide, large chain and heavy chain, along with the heme peroxidase signature. Intriguingly, a C1q domain was also identified in the C-terminal region of the derived amino acid sequence. Most of the known functionally important residues of MPOs are found to be well conserved in RbMPO, showing a close evolutionary relationship with other teleostan MPOs, particularly with that of mandarin fish. *RbMPO* exhibited a ubiquitous basal expression in physiologically relevant tissues, with particularly high expression levels in blood cells. Basal transcript levels of *RbMPO* in gill and spleen tissues were found to change upon different pathogen or pathogen-derived mitogen stimulation, with detectable inductive responses. Together, these data suggest the potential involvement of RbMPO in the innate immune response in rock bream.

1. Introduction

Reactive oxygen species (ROS) formed in cellular environments play a critical role in first line host defense mechanisms by efficiently eradicating invading microorganisms [1]. In this regard, ROS are frequently produced in the cells of the innate immune system, including neutrophils and macrophages, mostly through enzyme-catalyzed reactions [1]. Among the diverse enzymes involved in this catalytic process, myeloperoxidase (MPO) plays an indispensable role, catalyzing the formation of bactericidal hypochlorous acid (HOCl) using hydrogen peroxide (H_2O_2) produced by the cells in the presence of chloride ions [2,3].

Myeloperoxidases are heme-linked lysosomal glycoproteins that are found abundantly in azurophilic granules of polymorphonuclear neutrophils, with lower concentrations in monocytes and macrophages [4,5]. Myeloperoxidase is encoded by a single gene as a preproenzyme; subsequently, it is proteolytically

* Corresponding author. Marine Molecular Genetics Lab, Department of Marine Life Sciences, College of Ocean Science, Jeju National University, 66 Jejudaehakno, Ara-Dong, Jeju 690-756, Republic of Korea. Tel.: +82 64 754 3472; fax: +82 64 756 3493.
** Corresponding author. Tel.: +82 31 412 6712; fax: +82 31 412 6729.
E-mail address: jehee@jejunu.ac.kr (J. Lee).

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cleaved into a signal peptide, propeptide, heavy subunit, and light subunit to form the mature enzyme with a heterodimeric structure consisting of two heavy (55–63 kDa) and two light (10–15 kDa) subunits [6–8]. In this heterodimer, the heavy subunit is linked to a prosthetic heme group and four or five mannose oligosaccharide side chains [6,9].

Upon pathogen invasion, phagocytic leukocytes in host organisms are activated, resulting in the production of superoxide anions by phagocyte oxidases. These anions are then dismutated by superoxide dismutase enzymes (SODs) to yield hydrogen peroxide. Some of these hydrogen peroxide molecules can be utilized by mature MPOs to oxidize halide ions, especially Cl^- ions, to produce hypohalous acids such as HOCl [10]. These acids are highly reactive compounds that can readily react with most oxidizable groups in any substrate, including thiols, thiol esters, heme groups, and unsaturated fatty acids, particularly in invading microbial pathogens [11]. Apart from halide ions, the MPO-H_2O_2 system can also use pseudohalides such as thiocyanate ions, nitrite ions, and some other inorganic and organic molecules as substrates in its oxidation process [11]. On the other hand, oxidation of halides by this system can also yield variety of products in addition to hypohalous acids, including chloramines, hydroxyl radicals, singlet oxygen and ozone [11,12].

MPO was identified as a strong microbicidal agent in neutrophils and monocytes, using the substrates H_2O_2 and halides to form an effective and potent defense against invading pathogens through facilitating ROS formation [13,14]. HOCl is the major product of the MPO-H_2O_2 system, which not only involves in direct destruction of pathogens, but also plays a role in the modulation of immune responses and inflammatory reactions [15]. For instance, HOCl was found to activate nuclear factor-κB and tyrosine phosphorylation in T and B cells, triggering calcium-mediated signaling and tumor necrosis factor α (TNFα) production [16,17].

Although MPOs from higher vertebrate lineages, especially mammals, have been extensively studied, those from lower vertebrates, such as teleosts, have been studied in very few species, including the catfish (*Ictalurus punctatus*) [18] and turbot (*Scophthalmus maximus*) [19]. These studies show that teleostean MPOs can demonstrate detectable *in-vitro* peroxidase activity and are expressed abundantly in tissues important for host immunity in fish, such as kidney tissues.

Rock bream (*Oplegnathus fasciatus*) is an in-demand fish delicacy cultivated through commercial mariculture farming in East and South-East Asian countries like Korea and Japan. However, as reported in recent productions, this aqua crop has begun to decline significantly due to bacterial and viral infections, necessitating the establishment of an effective disease management strategy. Studying host defense mechanisms at the molecular level will give insight into one potential way to manipulate its natural immunity to combat pathogenic threats. Here, we identified and characterized a homolog of MPO from rock bream, showing its basal expression pattern in physiologically important tissues and its transcriptional modulation in gill and spleen tissues under pathogen stress.

2. Materials and methods

2.1. Sequence identification and in-silico characterization

A homolog of myeloperoxidase was identified from our cDNA sequence database of formerly constructed rock bream transcriptome library [20] by using the NCBI Basic Local Alignment Tool (BLAST) and designated as RbMPO. The identified *RbMPO* sequence was then used to demarcate the putative coding region and derive its corresponding amino acid sequence using the DNAssist 2.2 software package. Sequence comparisons of the predicted protein sequence through pairwise sequence alignment and multiple sequence alignment strategies were carried out by the EMBOSS needle (http://www.ebi.ac.uk/Tools/psa) and ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2) programs, respectively. The evolutionary relationship of RbMPO with its vertebrate counterparts was analyzed with the Neighbor-joining method with 1000 bootstrap support using the Molecular Evolutionary Genetics Analysis (MEGA) software, version 5 [21]. Characteristic domain signatures of the RbMPO were predicted by the ExPASy-prosite server (http://prosite.expasy.org), and some of the physicochemical properties were identified using the ExPASy ProtParam tool (http://web.expasy.org/protparam). In addition, potential glycosylation sites of the protein were identified using the NetNGlyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc).

2.2. Animal husbandry and tissue extraction

Healthy Rock bream with an average body weight of 50 g were obtained from Jeju Special Self-Governing Province Ocean and Fisheries Research Institute (Jeju, Republic of Korea). Animals were acclimatized for 1 week prior to experiments in a controlled environment (salinity 34 ± 1 psu, pH 7.6 ± 0.5, 22–24 °C) in 400 L tanks and fed with commercially available fish feed. After acclimatization, these animals were killed for tissue collection. Whole blood (1 mL/fish) was collected from the caudal fin using a sterilized syringe coated with 0.2% heparin sodium salt (USB, USA) as the anticoagulant, and samples were immediately centrifuged at 3000 × *g* for 10 min at 4 °C to separate the blood cells from the plasma. The collected cells were snap-frozen in liquid nitrogen. Meanwhile, the sampled fish was killed and the gill, liver, skin, spleen, head kidney, kidney, heart, muscle, and brain were excised. These tissues were immediately snap-frozen in liquid nitrogen and stored at −80 °C until use for total RNA extraction.

2.3. Immune stimulation

In order to analyze the temporal modulation of *RbMPO* basal expression under pathogenic stress, *Edwardsiella tarda*, *Streptococcus iniae*, lipopolysaccharides (LPS) and polyinosinic:polycytidylic acid (Poly I:C) were used as immune stimulants in a time course immune challenge experiment. The two bacterial pathogens were obtained from the Department of Aqualife Medicine, Chonnam National University, Korea. The bacteria were incubated at 25 °C for 12 h using brain heart infusion (BHI) broth (Eiken Chemical Co. Japan) supplemented with 1% sodium chloride. The cells were collected by centrifugation and resuspended in sterile phosphate buffered saline (PBS) and subsequently diluted to the desired concentration (5×10^3 CFU/μL for *E. tarda* and 1×10^4 CFU/μL for *S. iniae*) by examining the colony count in serially diluted culture in PBS at desired optical density value (0.8) at 600 nm, to inject fish. LPS (*E. coli* 055:B5) and Poly I:C were purchased from the Sigma company, USA and resuspended in sterilized PBS at a concentration of 1.25 μg/μL and 150 μg/μL, respectively for administration. Each individual fish was

Table 1
Primers used in the study.

Name	Purpose	Sequence (5′ → 3′)
RbMPO-qF	qPCR for *RbMPO*	AGGAGTACAAGGAGAGCCGTGAATAC
RbMPO-qR	qPCR for *RbMPO*	GACCCAAGATCCAGCGCCAATTTA
Rb-βF	qPCR for rock bream β-actin gene	TCATCACCATCGGCAATGAGAGGT
Rb-βR	qPCR for rock bream β-actin gene	TGATGCTGTTGTAGGTGGTCTCGT

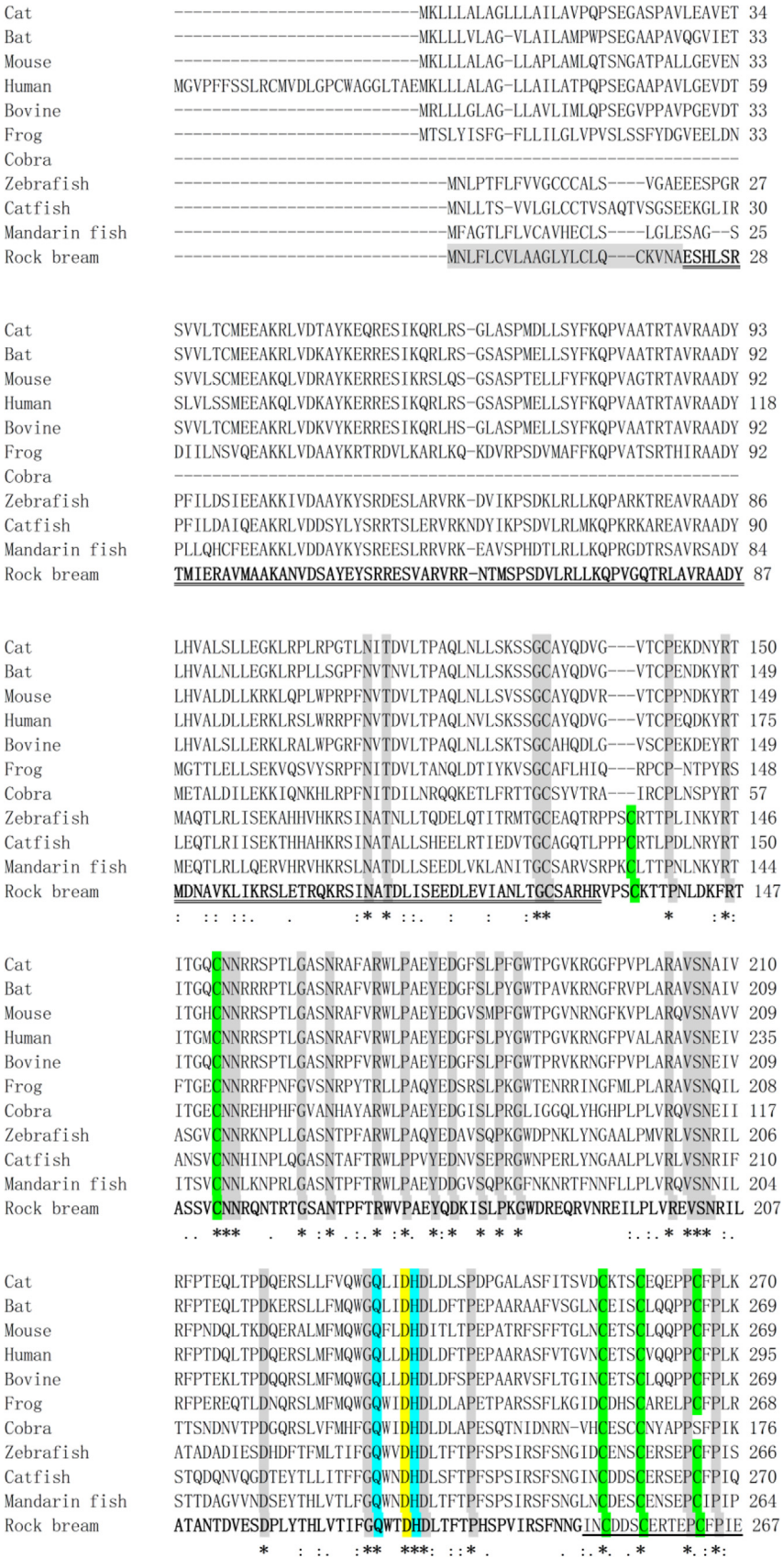

Fig. 1. Multiple sequence alignment of vertebrate MPO orthologs, including rock bream MPO (RbMPO). The signal peptide sequence of RbMPO is shaded in gray, and the corresponding propeptide, heavy chain, and light chain are denoted by underlined bold letters, bold letters, and underlined letters, respectively. The C-terminal C1q domain of RbMPO is indicated by underlined italicized letters. Conserved active site catalytic residues, heme linkage residues, and conserved cysteine residues are shaded in blue, yellow, and green, respectively. The putative Ca^{2+} binding residues conserved among the orthologs are highlighted in pink. All completely conserved residues among the sequences are shaded in gray and marked by (*), whereas semi-conserved residues are indicated by (.) or (:). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

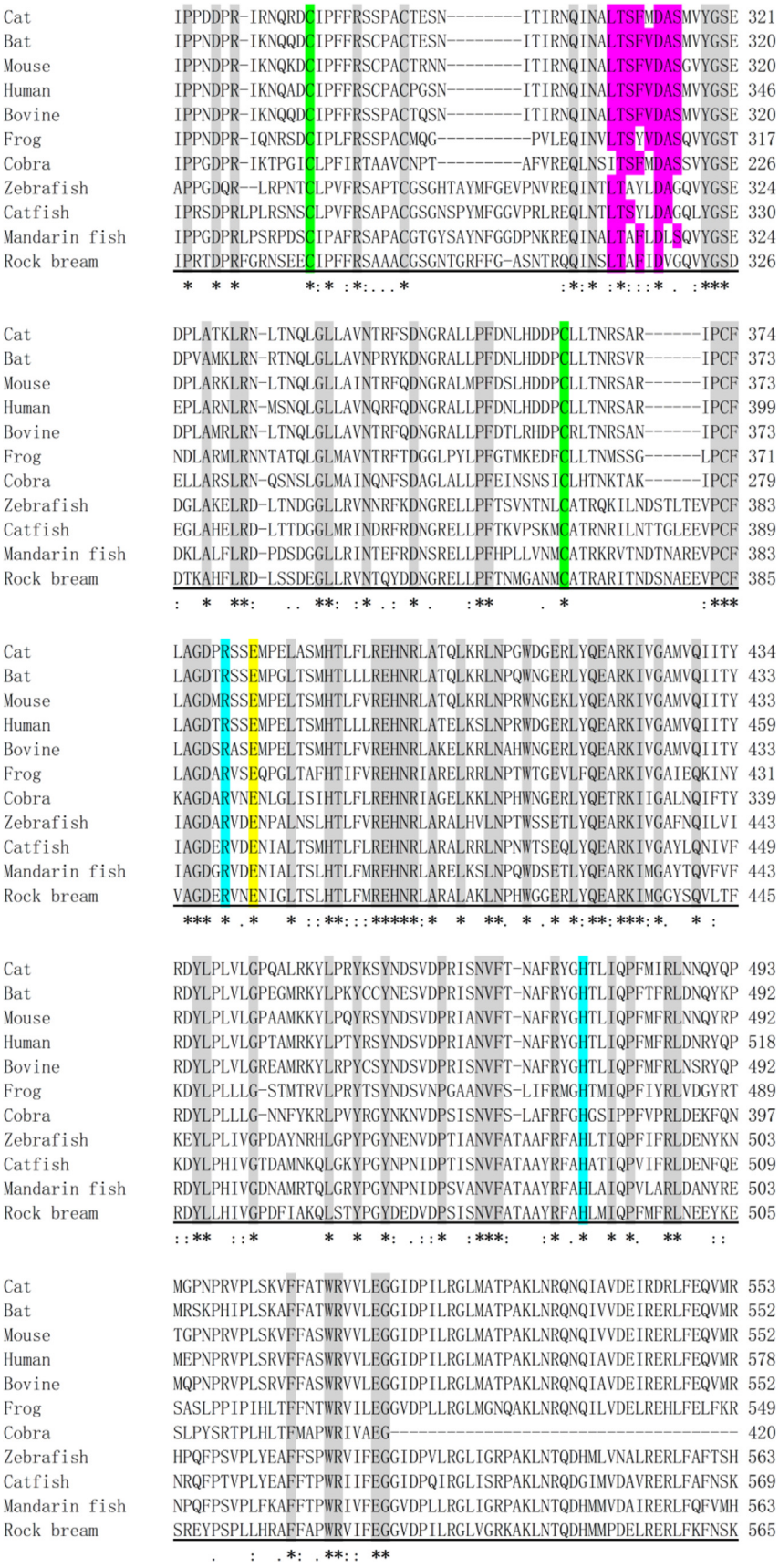

Fig. 1. (*continued*).

Fig. 1. (*continued*).

injected with 100 μL of corresponding stimulant. The immune challenge experiments were carried out as previously described [22], and at least 3 animals were killed for tissue collection from each challenge group at each time point. Thereafter, gill tissues and spleen tissues were collected as described in section 2.2.

2.4. Total RNA extraction and cDNA synthesis

Total RNA was extracted by Tri Reagent™ (Sigma-USA) from blood, gill, liver, spleen, head kidney, kidney, brain, skin, muscle, intestine, and brain tissues from healthy rock breams and from gill and spleen tissues collected at various time points from immune-challenged animals following the vendor's protocol. Subsequently, cDNA were synthesized from each set of RNA (2.5 μg) as previously described [20].

2.5. Quantification of mRNA expression using quantitative real-time PCR

Quantitative real-time PCR (qPCR) was used to analyze the basal mRNA expression levels of *RbMPO* in the aforementioned tissues of healthy fish and its expression changes in gill and spleen tissues after immune challenge. qPCR was performed using a Dice™ Real-Time System thermal cycler (TP800; TaKaRa, Japan) in a 15 μL reaction volume containing the following: 4 μL of diluted cDNA from each tissue, 7.5 μL of 2 × TaKaRa ExTaq™ SYBR premix, 0.6 μL of each primer (RbMPO-qF and RbMPO-qR; Table 1), and 2.3 μL of ddH_2O, as per the essential MIQE guidelines [23]. The qPCR conditions were as follows: 95 °C for 10 s; 35 cycles of 95 °C for 5 s, 58 °C for 10 s, and 72 °C for 20 s; and a final cycle of 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s. The threshold was set automatically by the Dice™ Real Time System software (version 2.00). *RbMPO* expression was determined using the Livak ($2^{-\Delta\Delta CT}$) method [24]. Rock bream β-actin (GenBank ID: FJ975145) was used as the internal control gene (Primer sequences in Table 1). The data are presented as the mean ± standard deviation (SD) of the relative mRNA expression in experiments performed in triplicate. To determine statistical significance ($P < 0.05$) between the experimental and uninjected control groups, two-tailed unpaired Student's *t*-tests were performed. For immune challenge experiments, the expression levels of *RbMPO* mRNA relative to that of the rock bream β-actin gene (internal control) were calculated. Values were further normalized to the corresponding PBS-injected controls at each time point to eliminate any plausible interference of injection medium on mRNA expression. The relative expression level in the uninjected control at the 0 h time point was used as the baseline reference.

3. Results and discussion

The *O. fasciatus* MPO homolog consisting of a 2652 bp putative coding region was identified from our rock bream cDNA database using NCBI-BLAST and designated as RbMPO. The complete cDNA sequence of RbMPO was comprised of 2947 nucleotides, and the identified coding region was found to encode an 884 amino acid sequence with a predicted molecular mass of 99.7 kDa and theoretical isoelectric point of 6.84. Sequence data were deposited in the NCBI-GenBank database under the accession number KP245917. The amino acid sequence of RbMPO contained the typical characteristics of MPOs, including the proximal signal peptide sequence (residues 1–22), propeptide (residues 23–132), light chain (residues 133–250), heavy chain (251–884), heme peroxidase superfamily signature (133–731), and three N-glycosylation sites (residues 108, 123 and 375), along with a C1q domain profile as a unique feature (Fig. 1). Presence of a signal peptide hints at the secretory nature of RbMPO. The predicted glycosylation sites may be involved in the modulation of its peroxidase activity, plausibly via maintaining its correct structural conformation, as reported from previously published studies on MPOs and some other peroxidases [19,25,26]. Intriguingly, the presence of a C-terminal C1q domain suggests that RbMPO belongs to the C1q domain

Table 2
Percentage sequence similarity and identity values of RbMPO with its orthologs.

Species	GenBank accession number	Amino acids	Identity (%)	Similarity (%)
1. *Danio rerio* (zebrafish)	AAH68379	839	56.9	71.8
2. *Siniperca chuatsi* (Mandarin fish)	ABC72122	763	51.8	62.9
3. *Ictalurus punctatus* (Channel catfish)	NP001187253	771	47.1	59.8
4. *Bos taurus* (Cattle)	NP001106769	719	38.9	52.1
5. *Felis catus* (Cat)	ACA61193	720	38.5	51.1
6. *Pteropus alecto* (Bat)	ELK01715	719	38.4	51.6
7. *Mus musculus* (Mouse)	AAH53912	718	38.3	52.6
8. *Homo sapiens* (Human)	AAA59863	745	37.6	50.6
9. *Xenopus laevis* (Frog)	NP001081108	725	37.1	51.8
10.*Ophiophagus hannah* (Cobra)	ETE71501	564	30.2	41.3

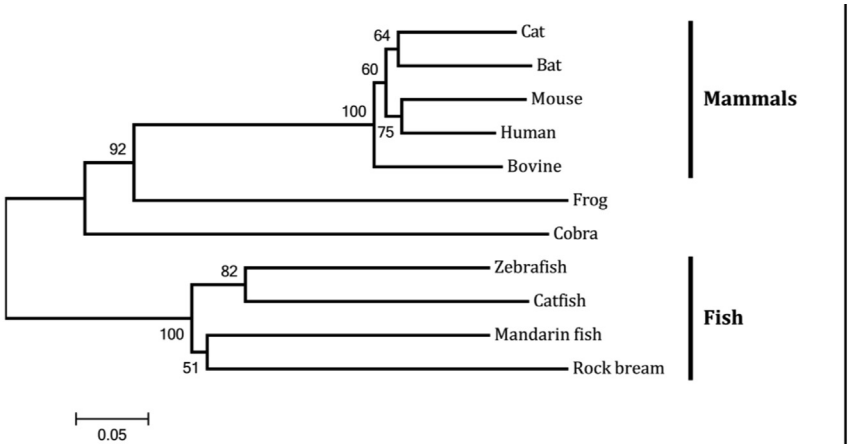

Fig. 2. The phylogenetic relationship of RbMPO with its orthologs. The evolutionary relationship of the different MPOs was analyzed based on ClustalW alignments of the respective protein sequences by using the neighbor-joining method. Corresponding bootstrap values are indicated on the nodes of the tree. NCBI GenBank accession numbers of the MPOs of the different species used in this construction were listed in Table 2.

containing (C1qDC) protein family. This *in silico*-derived evidence further supports the potential participation of RbMPO in host immune responses through several mechanisms, including pathogen recognition [27], activation of the complement system [28], or mediating cell migration [29] by binding self or non-self ligands [30] in addition to its main role in peroxidation. Our sequence analysis further hints that RbMPO is likely synthesized as a preproprotein and is subsequently proteolytically processed to form a mature protein consisting of heavy and light chain dimer pairs similar to human MPO [7,8].

Our pairwise sequence alignment revealed that RbMPO shares relatively low sequence identity but moderate sequence similarity with its homologs. The highest identity (56.9%) and similarity (71.8%) was with zebrafish (*Danio rerio*) MPO (Table 2). However, as

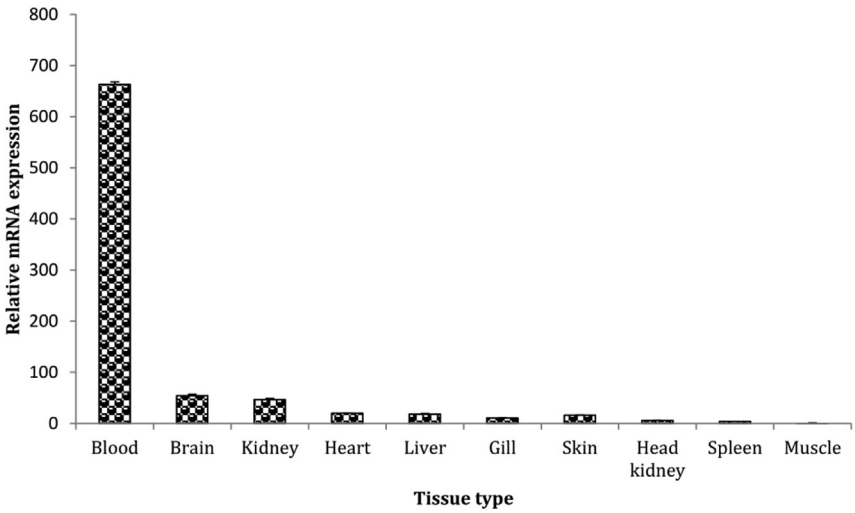

Fig. 3. Tissue-specific distribution of *RbMPO* mRNA expression in rock bream measured using qPCR. Fold-change expression is shown relative to the mRNA expression level in muscle tissue. Error bars represent SD (n = 3).

expected, relatively higher sequence compatibility was noted with teleostean MPO counterparts. The low percent identity of RbMPO with its homologs may reflect its merely distinct properties compared to the homologs. As per the multiple sequence alignment, most of the functionally important residues, including the active site catalytic residues, heme linkage residues, some residues in the putative Ca^{2+} binding motif [26], and cysteine residues potentially involved in intra- or interchain disulfide bond formation, were well conserved in RbMPO. However, most of the residues in the Ca^{2+} binding motif of non-mammalian MPOs, particularly those of teleost species were found to be different from mammalians, suggesting the plausible alteration of their Ca^{2+} affinity from mammalian counterparts.

Phylogenetic reconstruction generated using different vertebrate MPOs clearly exhibited a close and independent clustering pattern of teleost MPOs in which RbMPO clustered separately with the genetically similar mandarin fish, reflecting a relatively closer evolutionary relationship to each other than to zebrafish or catfish (Fig. 2). Interestingly, this clustering pattern within teleostan clade reflects a discrepancy with the outcomes obtained in our pairwise sequence alignment study, where RbMPO shared higher percent similarity and identity with the counterpart of zebrafish than that of mandarin fish (Table 2). As expected, MPOs from mammalian species also clustered together in a separate clade, in which bovine MPO showed a deviation from other orthologs, suggesting its evolutionary distant relationship to other mammalian MPOs. Amphibian MPO (frog) shares a common ancestral origin with mammals, whereas reptilian MPO (cobra) appeared to diverge before the evolution of the mammalian and amphibian counterparts from a common ancestor. Altogether, these results provide evidence for the homology of RbMPO with its teleostean counterparts, further suggesting its common vertebrate ancestral origin.

As detected by qPCR analysis, expression of *RbMPO* was observed in all tissues examined, albeit in different magnitudes, with the highest level of mRNA expression in blood (Fig. 3). This universal expression was different from what is observed in catfish [18], in which MPO expression is restricted to several tissues, such as spleen and kidney. On the other hand, the high level of *RbMPO* expression in blood, which contains leukocytes such as neutrophils, is consistent with human MPO, which is expressed in azurophils in neutrophils [31]. Moreover, the high level of RbMPO in blood is not surprising, since MPOs are generally found abundantly in azurophilic granules

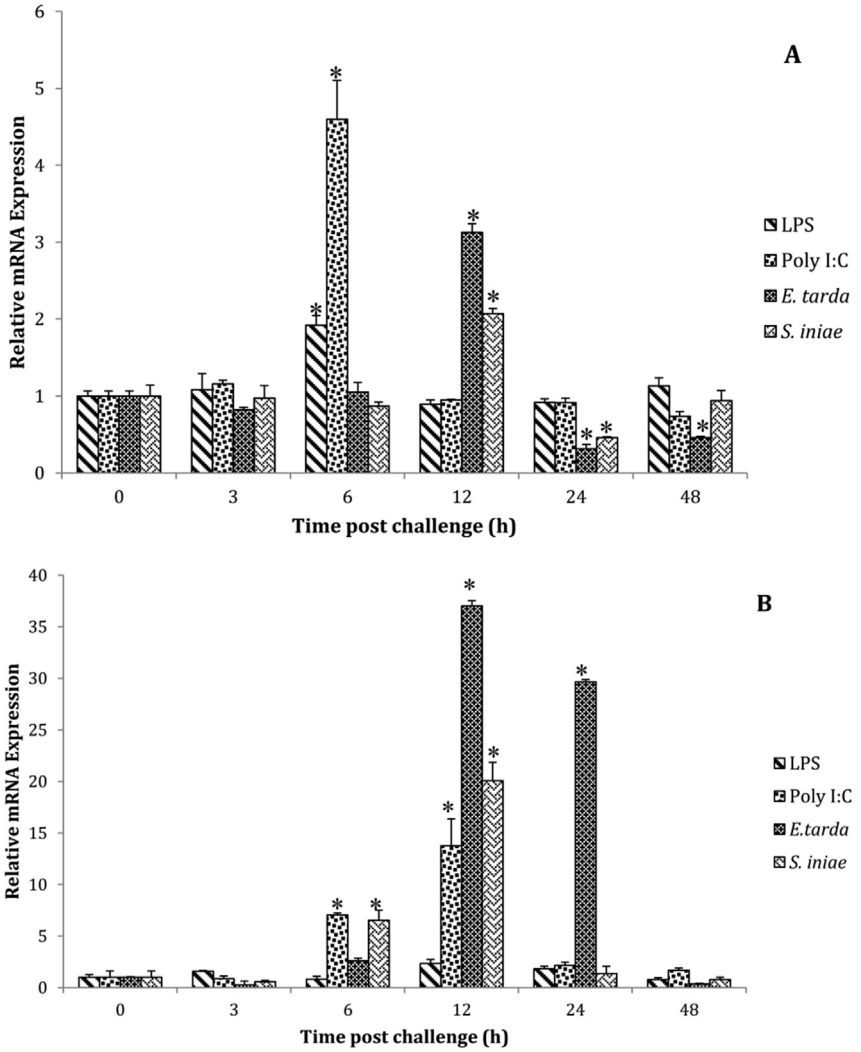

Fig. 4. Temporal modulation of *RbMPO* transcription in (A) gill and (B) spleen tissues upon immune stimulation with lipopolysaccharides (LPS) and polyinosinic:polycytidylic acid (poly I:C), *E. tarda* and S. *iniae* as measured using qPCR. The fold difference in mRNA expression is represented as relative to that of corresponding PBS-injected controls. The relative fold-change in expression at 0 h post-injection was used as the baseline. Error bars represent SD (n = 3); $^{*}P < 0.05$.

of polymorphonuclear neutrophils and secreted into extracellular fluid during an inflammatory process.

We analyzed the transcriptional changes in *RbMPO* expression upon mitogen stimulation with different pathogens and pathogen-derived molecules in rock bream gill and spleen tissues. The gills are in direct contact with the outer environment, reflecting a higher susceptibility to pathogen infection. On the other hand, spleen is considered as one of the largest lymphoid organs in teleosts, which harbors cellular component of innate immunity, including the cells involve in macrophage phagocytosis of antigens [32]. According to our qPCR assay, invasion of gill tissues by the live bacterial pathogens, *S. iniae* (Gram positive) and *E. tarda* (Gram negative), elicited a significant ($p < 0.05$) inductive transcriptional response at 12 h post injection (p.i.) with a significant ($p < 0.05$) down-regulation at 24 h p.i. (Fig. 4A). Thereafter, the transcript level of *RbMPO* returned to its basal expression level with respect to *E. tarda* treatment, while *S. iniae* treatment further maintains diminished expression levels of *RbMPO*. These down-regulations may reflect some immune evasion mechanism orchestrated by bacterial pathogens at the early-late phase of its infection. Similarly, *E.tarda* could markedly up-regulate the basal *RbMPO* transcription at 12 h p.i. along with 24 h p.i. in spleen, whereas *S.iniae* invasion could significantly elevate its transcription at 6 h and 12 h p.i. with noticeable fold differences (Fig. 4B). Treatment with the potent Gram negative bacterial endotoxin LPS also resulted in significant ($p < 0.05$) upregulation of *RbMPO* mRNA expression at early phase after injection (6 h p.i.) in gill tissue (Fig. 4A), but no significant transcriptional modulation could be observed in spleen tissues (Fig. 4B). These modulations of the basal transcript level of *RbMPO* upon treatment with live bacteria in both tissues or bacterial mitogens in gills suggests that bacterial infections may trigger the host immune defense mechanisms mediated by RbMPO, probably after sensing pathogen-associated molecules likes LPS in gills whereas under different mechanism in spleen. Interestingly, upon the treatment with viral stimulant poly I:C, which mimics the viral double stranded RNA, a prominent transcriptional inductive response at 6 h p.i. was observed, showing a 4.5-fold increase in expression over basal levels of *RbMPO* (Fig. 4A). Moreover, after the treatment of same stimulant, basal expression of RbMPO was significantly elevated at both 6 h and 12 h p.i., in spleen (Fig. 4B). These observations suggest the plausible involvement of MPOs in antiviral immune responses in fish, which deserves further investigation. Consistent with this hypothesis, virucidal effects of human MPO against HIV were reported previously [33]. Moreover, MPO was found to be highly expressed in neutrophils in the lungs of pigs infected by swine influenza virus [34]. The profound overall inductive transcriptional response trigged by live pathogens in spleen tissues relative to its basal *MPO* expression, compared to that in gill tissues may reflects the presence of significantly higher number of innate immune cells such as macrophages in spleen tissues than in gills. On the other hand, RbMPO expression might have markedly elevate to meet the required quantity of MPO in spleen cells to eradicate pathogens, since its basal expression in spleen is lesser than that in gills (Fig. 3.)

In conclusion, we have molecularly characterized RbMPO, analyzing its tissue distribution at the mRNA level and its transcriptional modulation under pathogen stress. We observed that *RbMPO* is expressed ubiquitously in physiologically important tissues, with extremely high expression in blood cells. Basal mRNA expression in gill and spleen tissues was upregulated upon live bacterial pathogen invasion and stimulation with pathogen derived mitogens. Together, these results suggest the potential mediation of RbMPO in host immune responses. However, further investigations, especially those focusing on RbMPO function, are needed to elucidate its exact role in rock bream.

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