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A manganese superoxide dismutase (MnSOD) from red lip mullet, *Liza haematocheila*: Evaluation of molecular structure, immune response, and antioxidant function

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ABSTRACT

Manganese superoxide dismutase (MnSOD) is a nuclear-encoded antioxidant metalloenzyme. The main function of this enzyme is to dismutase the toxic superoxide anion (O_2^-) into less toxic hydrogen peroxide (H_2O_2) and oxygen (O_2). Structural analysis of mullet MnSOD (*MuMnSOD*) was performed using different bioinformatics tools. Pairwise alignment revealed that the protein sequence matched to that derived from *Larimichthys crocea* with a 95.2% sequence identity. Phylogenetic tree analysis showed that the MuMnSOD was included in the category of teleosts. Multiple sequence alignment showed that a SOD Fe-N domain, SOD Fe-C domain, and Mn/Fe SOD signature were highly conserved among the other examined MnSOD orthologs. Quantitative real-time PCR showed that the highest *MuMnSOD* mRNA expression level was in blood cells. The highest expression level of *MuMnSOD* was observed in response to treatment with both *Lactococcus garvieae* and lipopolysaccharide (LPS) at 6 h post treatment in the head kidney and blood. Potential ROS-scavenging ability of the purified recombinant protein (rMuMnSOD) was examined by the xanthine oxidase assay (XOD assay). The optimum temperature and pH for XOD activity were found to be 25 °C and pH 7, respectively. Relative XOD activity was significantly increased with the dose of rMuMnSOD, revealing its dose dependency. Activity of rMuMnSOD was inhibited by potassium cyanide (KCN) and N-N′-diethyl-dithiocarbamate (DDC). Moreover, expression of *MuMnSOD* resulted in considerable growth retardation of both gram-positive and gram-negative bacteria. Results of the current study suggest that MuMnSOD acts as an antioxidant enzyme and participates in the immune response in mullet.

1. Introduction

Red lip mullet (*Liza haematocheila*), a member of the family *Mugilidae,* is an economically important and high-yielding aqua crop. Red lip mullet inhabits coastal areas of Japan, China, and Korea. It shows high reproductive ability and a high rate of growth since it is well adapted to the environment [1]. However, increase in intensive aqua farming combined with poor handling caused higher prevalence of diseases and injuries in the cultured fish. Furthermore, hazardous environmental conditions, specifically the presence of pollutants including heavy metals and toxicants, may cause oxidative stress in the fish, which leads to high susceptibility towards pathogenic infection [2,3].

Oxidative stress promotes the elevation of levels of harmful metabolites, including reactive oxygen species (ROS) and reactive oxygen intermediates (ROI), in fish [4,5]. These harmful metabolites are superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl radicals ($OH^·$) [6]. Phagocytosis increases oxygen consumption, which leads to respiratory burst but also generates ROS. Therefore, these ROS play an important role in cell defense mechanism by killing invading microorganisms and harmful agents [5,7]. However, excess ROS can have an adverse effect on the internal organs of fish and interfere with the survival of fish through cellular injuries, which may ultimately lead to death [8].

The antioxidant system can be divided into enzymatic and non-enzymatic components. In the enzymatic antioxidant system, superoxide dismutases (SODs) are the most important metalloenzymes; they are involved in dismutation of toxic superoxide anions (O_2^-) into hydrogen

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peroxide (H_2O_2) and oxygen (O_2) [9,10]. Further decomposition of H_2O_2 into a non-toxic component, water, is catalyzed by catalase (CAT), glutathione peroxidase (GSH-PX), glutathione S-transferase (GST), peroxiredoxin, and glutathione reductase (GSR) [11]. Thus, these antioxidant enzymes are essential for the regulation of ROS by converting them to harmless compounds [12].

Superoxide dismutases (SODs) can be classified into 4 groups based on the metal residue that binds to the active site: manganese superoxide dismutase (MnSOD), copper/zinc superoxide dismutase (Cu/ZnSOD), iron superoxide dismutase (FeSOD) and nickel superoxide dismutase (NiSOD) [10,13]. Cu/Zn-SODs are usually homodimeric and are mainly present in the eukaryotic and prokaryotic organisms [9,14]. These SODs are present in the cytoplasm and chloroplasts of plant cells, as well as in mitochondria and in gram-negative bacterial cells [10,13]. FeSODs are predominant in prokaryotes, algae, and plant chloroplasts [9,15]. Prior investigations have shown that MnSOD and FeSOD have evolved from a common ancestor and are similar in structure [16]. NiSOD has been recently isolated from the aerobic *Streptomyces* present in soil [17].

MnSOD helps to protect the cells from the adverse effects of excess ROS in aerobic organisms. It is mainly present in the mitochondria of eukaryotes and in prokaryotic cells. It has the capacity to convert the superoxide anion into H_2O_2 and O_2 [16]. There are two types of MnSOD: mitochondrial and cytosolic. The mitochondrial form is homotetrameric and is predominantly found in eukaryotic cells while the cytosolic form is homodimeric and is found in prokaryotic cells [18]. It has been shown that expression of MnSOD could be upregulated by lipopolysaccharide (LPS) [19], ROS, vascular endothelial growth factor (VEGF) [20], and cytokines [21]. After injection of *V. harveyi* into the *Penaeus monodon*, clear changes in MnSOD expression were observed at different time points [22]. Therefore, MnSOD acts as an immune responsive anti-oxidative protein in the host defense system.

In this study, MnSOD was recognized from the constructed red lip mullet cDNA data base and characterized its structural and functional properties. The main objectives of this study were to investigate the potent antioxidant function and antibacterial activity of the mullet MnSOD through functional assays and to compare the gene expression of healthy tissues and those stimulated with various pathogen-associated molecular patterns (PAMPs).

2. Material and methods

2.1. Development of mullet cDNA database

The transcriptome database of mullet cDNA sequences was developed using *de novo* assembly. Mullet fish purchased from Sangdeok fish farm in Hadong, Korea were used for RNA extraction and their average body weight and length were 100 g and 24 cm, respectively. The fish were acclimatized in the laboratory aquarium tanks at 20 °C for a week prior to the experiment. In brief, total RNA content was extracted from kidney, spleen, head kidney, gill, skin, liver, intestine, stomach, heart, eye, brain, and blood tissues of 5 mullet fish. The extracted RNA was then sent to Insilicogen, Korea. Sequencing was run on a Pacbio platform at Insilicogen, Korea.

2.2. In silico analysis

The multiple sequence alignment was performed using Clustal omega online software (https://www.ebi.ac.uk/Tools/msa/clustalo/). Construction of the phylogenetic tree was done by the Neighbor-Joining method using Molecular Evolutionary Genetics Analysis (MEGA) version 5. The domain framework of MuMnSOD was analyzed using ExPASy PROSITE (http://prosite.expasy.org/) and SMART tool (http://smart.embl-heidelberg.de/). The location of the MuMnSOD in the cell was predicted using the MultiLoc online tool (http://abi.inf.uni-tuebingen.de/Services/MultiLoc/). Identity and sequence similarities were predicted using pairwise sequence alignment with EMBOSS Needle tool (https://www.ebi.ac.uk/Tools/psa/emboss_needle/). *N*-linked glycosylation sites of protein were identified using the NetNGlyc web tool (http://www.cbs.dtu.dk/services/NetNGlyc/). Molecular weight and isoelectric point of MuMnSOD were identified using the ProtParam online tool (https://web.expasy.org/protparam/).

2.3. Rearing of experimental mullet fish and tissue collection

Red lip mullets were purchased from Sangdeok fishery center in Hadong, Korea. Laboratory aquarium tanks were used to acclimatize the fish at 20 °C for a week prior to experiment. Five fish with an average body weight of 100 g were selected for the analysis of tissue distribution. The guidelines for the health and welfare monitoring of the fish were followed through out the experiment [23]. Each fish was anesthetized using 40 mg/L of Tricaine mesylate-MS222. Sterile syringes coated with heparin sodium salt (USB, USA) were used to collect the blood. Then, the peripheral blood cells were separated by immediate centrifugation at 3000 × *g* for 10 min at 4 °C. Other tissues such as head kidney, spleen, liver, gill, intestine, kidney, brain, muscle, skin, heart, and stomach were sampled and immediately snap-frozen in liquid nitrogen and stored at −80 °C.

2.4. Immune challenge for mullet fish

Healthy mullet fish with an average body weight of 100 g were used for the immune challenge experiment. Four groups of fish were prepared for the immune challenge experiment; each group contained five fish. Then, 1.25 μg/g of lipopolysaccharide (LPS), 1.5 μg/g of polyinosinic:polycytidylic acid (poly I:C), and 1×10^3 CFU/μL of *Lactococcus garvieae* were prepared in phosphate-buffered saline (PBS) as immune stimulants. 100 μL of PBS was injected intraperitoneally into the fish serving as a control group. The peripheral blood cells and head kidney were collected from five individuals at 0, 6, 24, 48, 72 h post-injection as described in section 2.3.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted (n = 5 for tissue distribution and immune challenge) using RNAiso plus (TaKaRa, Japan) and cleaned up with the RNeasy spin column (Qiagen, Germany). Additionally, RNA quality was inspected using 1.5% agarose gel electrophoresis. The concentration of RNA was then measured at 260 nm in μDrop Plate (Thermo Scientific). First-strand cDNA was synthesized with PrimeScript™ II 1st strand cDNA Synthesis Kit (TaKaRa, Japan) in a 20 μL reaction mixture containing 2.5 μg of RNA. The synthesized cDNA was diluted 40-fold in nuclease-free water and stored at −80 °C.

2.6. Expression analysis for MuMnSOD

Quantitative real-time PCR (qPCR) was performed to analyze the expression profile of the *MuMnSOD* with specifically designed primers (Table 1). A 10 μL reaction volume including 3 μL diluted cDNA template, 5 μL 2 × TaKaRa Ex Taq™ SYBR premix, 0.4 μL each of the forward and reverse primers (10 pmoL/μl), and 1.2 μL of H_2O was used for the qPCR in a Thermal Cycler Dice™ TP950 (TaKaRa, Japan). The qPCR cycle profile consisted of one cycle of 95 °C for 10 s, followed by 45 cycles of 95 °C for 5 s, 58 °C for 10 s and 72 °C for 20 s, and a final single cycle of 95 °C for 15 s, 60 °C for 30 s and 95 °C for 15 s. The experiment was performed in triplicate to enhance the credibility. The relative mRNA expression level for each sample was calculated using the Livak method [24]. Mullet *Elongation Factor 1 alpha* (*EF1α*) (MH017208) was used as the internal control gene. Temporal expression of post injected samples was compared to the PBS-injected control and 0 h un-injected control at each time point to determine the relative fold changes after the immune challenge experiment.

Table 1
Description of primers.

Primer Name	Primer sequence (5′-3′)
MuMnSOD forward (*Eco*R I)	gagagagaattcATGAACATGCTGTGCAGAGTTGGTC
MuMnSOD reverse (*Hin*d III)	gagagaaagcttCTACTTTTTGGCAATCTGAAGACGGTCG
MuMnSOD forward (qPCR)	TGTCTGCTGCTACCGTTGCA
MuMnSOD reverse (qPCR)	GGGCCGCACATTCTTGTACTGAA
MuEF1α forward (qPCR)	CCCTGGTCAGATCAGTGCTGGTTAT
MuEF1α reverse (qPCR)	AGCGTCGCCAGACTTTAGGGATTT

2.7. Construction of the recombinant plasmid (pMAL-c5X)

The coding sequence (CDS) of the cDNA fragment was amplified by using gene-specific cloning primers (Table 1). The size of the amplicon was 684 bp and it was isolated from a 1% agarose gel by using a gel purification kit (BIONEER, Korea). EcoRI and HindIII were used as restriction enzymes to digest the amplicon and *pMAL*-c5X plasmid containing the ampicillin marker. Digested products were ligated using Mighty Mix DNA Ligation Kit (TaKaRa, Japan). Then, the recombinant plasmids were transformed into *Escherichia coli* competent cells (DH5α). Transformed DH5α cells were grown on an LB ampicillin plate at 37 °C overnight. Successive clones were recognized and the sequence was verified by sequencing (Macrogen, Korea). The clone confirmed by sequencing was transformed into *E. coli* ER2523 competent cells for expression of the relevant protein.

2.8. Overexpression and purification of recombinant MuMnSOD

Recombinant MuMnSOD (rMuMnSOD) protein was overexpressed as a fusion protein with a maltose binding protein tag (MnSOD-MBP) in *E. coli* ER2523 cells. Transformed *E. coli* ER2523 cells were grown in LB rich ampicillin medium (1 L) which contained 100 mg mL^{-1} ampicillin and 0.2% glucose. The culture was incubated at 37 °C and 200 rpm in a shaking incubator. On the following day, the seed culture was inoculated into LB rich ampicillin medium and incubated at 37 °C and 200 rpm until the OD_{600} reached 0.5. Isopropyl-b-thiogalactopyranoside (IPTG; 0.25 mM) was added into the culture medium to induce the expression of the protein at 20 °C and 200 rpm for 10 h. Next, the induced cell culture was pre-chilled for 30 min on ice and harvested at 3500 × *g* for 30 min at 4 °C. Harvested cells were re-suspended in the column buffer, which contained 20 mM Tris-HCl, 200 mM NaCl, pH 7.4, and stored at −20 °C overnight.

On the following day, the suspension of the cells was thawed and 1 mg mL^{-1} of lysozyme was added. Afterwards, the lysed cell sample was sonicated and the cell lysate was centrifuged at 20,000 × *g* at 4 °C for 30 min. The supernatant was separated and purified on a column with amylose resin (New England Biolabs, USA). The purified rMuMnSOD was eluted using elution buffer composed of column buffer and 10 mM maltose. Simultaneously, overexpression and purification of the recombinant MBP (rMBP) was completed. Concentrations of purified rMuMnSOD and rMBP were measured using Bradford technique [25]. Efficacy of protein purification was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% SDS-PAGE) under reducing conditions.

2.9. Functional assays of rMuMnSOD

2.9.1. Conventional xanthine/xanthine oxidase (XOD) assay

Antioxidant activity of rMuMnSOD was evaluated by the XOD assay. The reaction mixture for the XOD assay containing 160 μL of 0.1 M phosphate buffer (pH 7), 0.75 mM of nitroblue tetrazolium (NBT), 0.15% of bovine serum albumin (BSA), 3 mM of xanthine and rMuMnSOD was mixed and incubated at 25 °C for 10 min. Then, 6 mU of xanthine oxidase was introduced to the mixture and incubated at 25 °C for 20 min. Finally, OD_{560} values were measured in each sample using a microplate reader (Multiskan GO, Thermo Scientific, USA). Additionally, positive (rMBP) and negative (without any protein) controls were used in this experiment.

2.9.2. Determination of biochemical properties of rMuMnSOD

Biochemical properties such as optimum temperature and pH, effect of inhibitors, and protein concentration were analyzed by the XOD assay described in section 2.9.1. XOD assay was performed at 9 different pH buffers, such as citrate (pH 3, 4, 5), phosphate (pH 5, 7, 8) and glycine-NaOH (pH 9, 10, 11). Optimum temperature was determined by using 9 different temperatures (5, 15, 25, 35, 45, 55, 65, 75, and 85 °C), and 8 different enzyme concentrations (10, 20, 30, 40, 50, 60, 70 and 80 μg) were used to determine the effect of rMuMnSOD concentration on superoxide scavenging ability. Then, 6.25 mM of potassium cyanide (KCN), sodium azide (NaN_3), N-N′-diethyl-dithiocarbamate (DDC) and ethylene diamine tetraacetic acid (EDTA) were used as inhibitors to determine the effect of inhibitors on the rMuMnSOD superoxide scavenging activity. Each inhibitor was incubated with the recombinant protein at 25 °C and pH 7 for 15 min prior to commencement of the XOD assay. Residual activity of the enzyme was determined in this experiment.

2.9.3. Antibacterial activity of rMuMnSOD

Antibacterial activity of rMuMnSOD was evaluated using *E. coli* as a gram-negative bacterium and *L. garvieae* and *Micrococcus luteus* as gram-positive bacteria [26,27]. Specific media for each bacterium were used for culturing of bacteria and for the assay. Briefly, LB medium was used for *E. coli*, while BHIS (1.5% sodium) medium was used for *L. garviae* and BHI medium was used for *M. luteus*. All the bacteria were cultured until their mid-logarithmic phase. Then, bacterial cells were harvested by centrifugation at 5000 × *g* for 20 min at 4 °C. Harvested cell pellets were washed with PBS. Each bacterial pellet was re-suspended in fresh culture media and diluted to a final concentration of 2×10^7 cells/mL. Bacterial cells were then seeded into a 96-well plate, and incubated with rMuMnSOD (100 μg/mL and 200 μg/mL) at their optimum temperatures (*E. coli*: 37 °C, *L. garvieae*: 30 °C, and *M. luteus*: 30 °C) for 10 h. Finally, absorbance values of each sample were measured using a microplate reader (Biotek Instruments, Korea Ltd.) at 600 nm in 1 h interval for 10 h.

2.10. Statistical analysis

All the experimental data were presented as mean ± standard deviation (mean ± SD) of a triplicate measurement. Mean comparisons of the results obtained from qPCR and other experiments were performed by the unpaired, two-tailed *t*-test using the GraphPad statistical software (GraphPad Software, Inc., USA). Mean values of the antibacterial assay and XOD assay for the effect of inhibitors were statistically analyzed by One-way analysis of variance (ANOVA) following the Duncan's Multiple Range Post-Hoc test using the SPSS 16 software.

3. Results and discussion

3.1. Structural characterization of the MuMnSOD sequence

The full-length MuMnSOD cDNA sequence was deposited in GenBank under the accession no of MH018137. Coding sequence of MuMnSOD was recognized by homology screening from the mullet cDNA database. The 5′ untranslated region (UTR) of the MuMnSOD contained 230 bp; the CDS contained 684 bp which encoded 227 amino acids; and the 3′UTR contained 437 bp. The predicted molecular weight of the encoded protein of the MuMnSOD was 25.39 kDa and its

isoelectric point was 8.37. The instability index of the relevant protein was 34.99. This index suggested that MuMnSOD protein is a stable protein [52]. Signal peptide could not be identified in the sequence. Therefore, MuMnSOD might be identified as an intracellular protein. Results obtained from the MultiLoc tool suggest that MuMnSOD was present in the mitochondria. The mitochondrial matrix is important as the site that generates energy for cells. Thus, high amounts of ROS may be released in the electron transport system [28]. MnSOD is the principal antioxidant enzyme present in the mitochondrial matrix of eukaryotic organisms. Cytosolic forms of MnSOD isoforms have been found throughout the cellular compartments in various organisms displaying their antioxidant capacity. However, mitochondrial MnSOD could play a distinct function in protecting cellular components against ROS in eukaryotic organisms [28,29].

MuMnSOD might be a glycoprotein owing to the presence of two *N*-glycosylation sites (^{68}NVTE^{71} and ^{102}NHTI^{105}). Two main domains can be detected in the protein sequence of MuMnSOD, including an iron/manganese superoxide dismutase C-terminal domain (SOD_Fe_C domain) and an iron/manganese superoxide dismutase N-terminal domain (SOD_Fe_N domain). Four distinct binding sites for Mn^{2+} were predicted (H^{55}, H^{99}, $D^{188,}$ and H^{192}), which may be important for its catalytic function [30] (Fig. 1). ExPASy PROSITE web tool revealed that the manganese and iron superoxide dismutase signature (Mn/Fe SOD) can be recognized in the protein sequence of the MuMnSOD. SMART web tool identified the HAMP domain, which is mainly found in chemotaxis proteins and in histidine kinases as a sensor of bacteria [31] and of eukaryotic cells. The HAMP domain may associate with numerous domains including the histidine kinase, the bacterial chemotaxis sensory transducer domain, the protein phosphatase 2C-like domain, and the guanylate cyclase domain. The HAMP domain facilitates the transmission of conformational changes from the periplasmic ligand binding domain to the cytoplasmic signal kinase domain and the methyl acceptor domain. Therefore, it regulates the methylation and phosphorylation functions of homodimeric receptors [31–33]. Moreover, conformational changes of the HAMP domain caused a decrease of the kinase enzyme activity due to the binding of MnSOD [34–36]; this adversely affects phosphorylation [37]. Therefore, conformational changes of the HAMP domain presented in the MuMnSOD might be involved in reducing bacterial growth by decreasing the kinase activity of bacteria.

3.2. Assessment of evolutionary relationship and homology of MuMnSOD

Construction of a phylogenetic tree was completed with the well-known orthologs of MnSOD by the Neighbor-Joining method using their sequences. Phylogenetic tree analysis showed that MuMnSOD was included in the category of teleosts and was separated from other counterparts (Fig. 2). Interestingly, vertebrates and the invertebrates were split into two main groups and all the counterparts were cladded within their taxonomic groups.

Conservation of MuMnSOD within the evolutionary process was determined using ClustalW pairwise alignment. According to the results of ClustalW pairwise alignment, highest identity and similarity were shown by the *Larimichthys crocea* MnSOD (95.2% identity and 98.8% similarity) followed by *Totoaba macdonaldi* (95.2% identity and 97.8% similarity) and *Epinephelus coioides* (95.2% identity and 97.4% similarity)(Table 2). Moreover, MuMnSOD shared significant sequence identity and similarity with avian (~78% identity and ~86% similarity) and mammalian (> 70% identity and > 80% similarity) MnSODs. Results of the sequence alignment revealed that the SOD_Fe_N domain, the SOD_Fe_C domain, *N*-glycosylation sites and the Mn/Fe SOD signature were highly conserved among the other orthologs inspected in the present study (Fig. 2). Interestingly, it is stated in the literature that MnSOD sequences are well conserved through the evolution, especially the domains and the active sites [38]. Collectively, structural conservation observed through the homology analysis suggested that the antioxidant function of MuMnSOD may also be conserved in its orthologs.

3.3. Transcriptional examination of MuMnSOD mRNA level

3.3.1. Modulation of gene expression level of MuMnSOD in different tissues

Different tissues were collected from healthy fish and cDNA was synthesized after extraction of RNA to investigate the relative transcription content of *MuMnSOD*. The mRNA expression levels of *MuMnSOD* were observed in all twelve tissues, with different transcription levels in each tissue. The highest mRNA expression level was observed in the blood, followed by the liver, kidney, muscle, and heart (Fig. 3). Since blood is the main tissue which transports oxygen into host cells [39], generation of ROS in the blood may be greater than in other organs. Therefore, higher expression of *MuMnSOD* in blood may be important for the antioxidant defense mechanism of mullet in order to combat the hazardous ROS. Similar results have been published in a previous study supporting the current results [30]. However, previous studies showed that *MnSOD* expression was highest in the ovaries in *Hippocampus abdominalis* [38], gills in *Mytilus coruscus* [40] and heart in *Hemibarbus mylodon* [41]. Moreover, *MuMnSOD* expression level was high in the heart, liver, and kidney, which are metabolically active organs. It might be due to the presence of high mitochondrial content in these organs/tissues, and MnSOD may act as a fundamental ROS scavenger throughout the aerobic metabolism in the eukaryotic system [42]. Therefore, these results suggest that *MnSOD* expression variation may be species-specific.

3.3.2. Temporal transcriptional expression profile of MuMnSOD

Immune response of *MuMnSOD* was examined by its expression profile after treatment with different pathogenic stimulants at different time points. Head kidney and blood cells were selected as immune-related tissues to investigate the immune modulated response of *MuMnSOD* against immune stimulants. Head kidney is the fundamental immune structure responsible for the process of phagocytosis [43], formation of immunoglobulin (IgM), antigen processing function, and memory of immune activities *via* melanomacrophagic centers [44]. In the phagocytosis process, production of ROS results in respiratory burst [6]. Therefore, antioxidants may be used to maintain the redox homeostasis. Moreover, the blood is an important medium for innate immune functions in fish body [38]. Modulated expressional profile of *MuMnSOD* was observed in both head kidney and blood tissues upon infection of different PAMPs (Fig. 4).

Poly I:C injected mullet fish indicated significant upregulations of *MuMnSOD* at each time point. The highest upregulation of *MuMnSOD* was seen at 6 h post injection, and then the expression level drastically declined and elevated again 72 h post injection in the head kidney. Reduction of the expression level of *MuMnSOD* might be due to mRNA turnover [45]. In the blood cells, significant upregulation of *MuMnSOD* mRNA transcript was observed at 24 h post injection with poly I:C. Moreover, *L. garvieae* showed similar results in both tissues. However, expression pattern against LPS was somewhat different from that for the other two stimulants. The highest *MuMnSOD* mRNA expression level was observed 72 h and 6 h post injection with LPS in the head kidney and blood, respectively. In addition, *MuMnSOD* expression was highly enhanced at 6 h post injection of LPS in the blood cells. However, the modulation patterns of *MuMnSOD* against PAMPs in the head kidney and blood cells were different. Therefore, it could be suggested that mRNA expression of *MuMnSOD* might be tissue-specific in the presence of different immune stimulants used in the challenge experiments.

Results of the previous studies revealed that MnSOD is an ordinary stress response agent that could be modulated by PAMPs, heavy metals and adverse environmental factors [19,40]. Antioxidant balance of the mammalian cells has been changed due to virus-generated ROS [46]. In rock bream, *MnSOD* expression was elevated during the challenge of poly I:C [30]. Moreover, *MnSOD* of seahorse manifested an

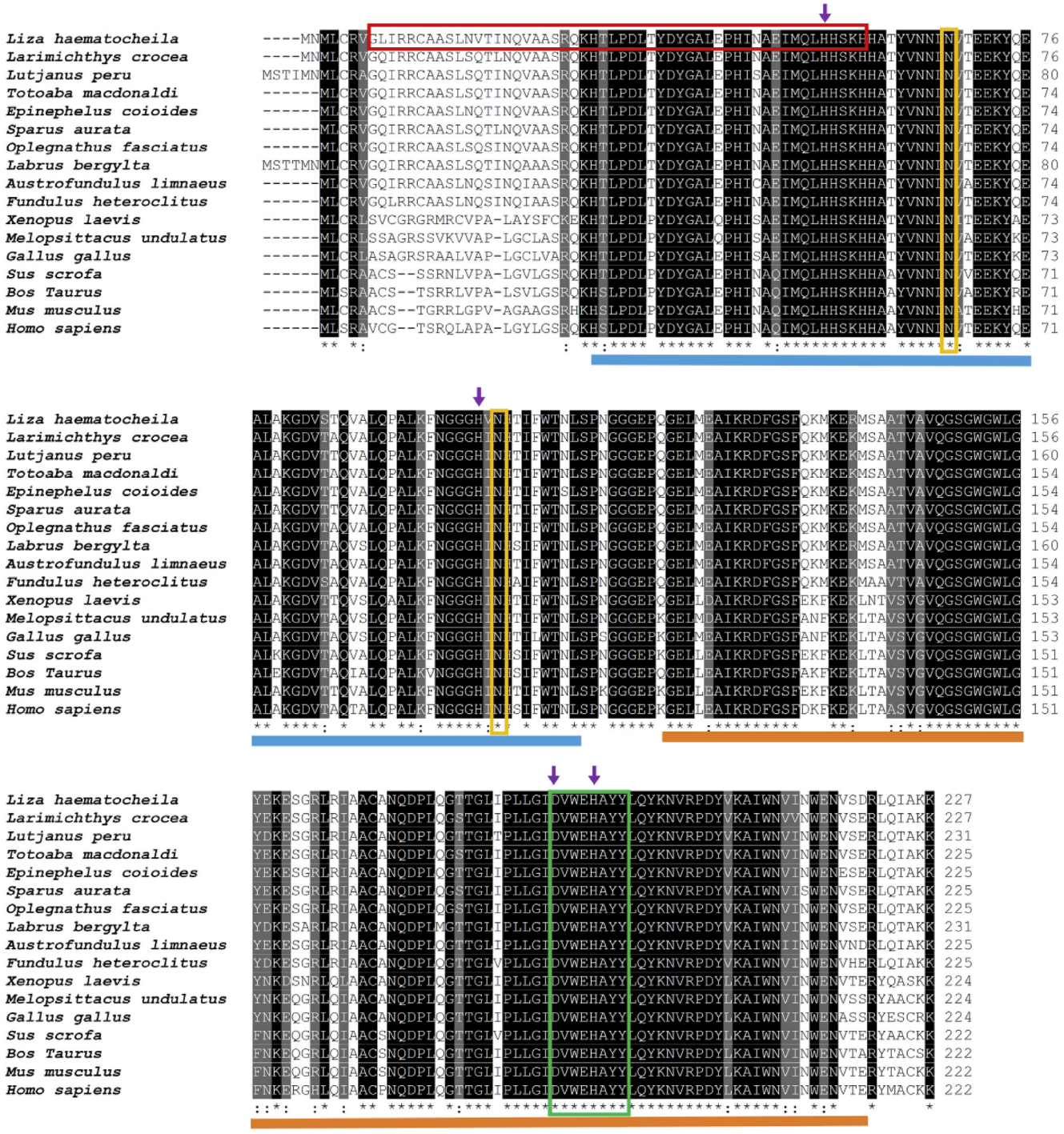

Fig. 1. Multiple sequence alignment of MuMnSOD. Identical residues are marked in black and partially conserved residues are marked in grey. The SOD_Fe_N domain is marked by a blue line whereas the SOD_Fe_C domain is indicated by an orange line; the HAMP domain present in the mullet protein sequence is indicated by a red box. Manganese and iron superoxide dismutase signature and *N*-glycosylation sites are indicated by yellow and green boxes, respectively. Four distinct Mn^{2+} binding sites are shown by four arrows. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

upregulation at late phase of the poly I:C challenge [38]. According to these findings, the present data suggest that MnSOD might play an important role in protection against viral infections in mullet.

L. garvieae is a gram-positive bacterium able to survive a wide range of environmental factors and a major causative agent of streptococcosis and lactococcosis in fish species [47,48]. Moreover, it has been reported that cultured grey mullet in Taiwan was highly affected by *L. garvieae* and it caused high mortality [49]. Previous findings showed that the *MuMnSOD* transcriptional level was significantly induced by *Streptococcus iniae*, a gram-positive bacterium in seahorse [38]. However, the expression of *MnSOD* mRNA decreased during *L. garvieae* challenge at 3 h post infection in freshwater prawn [50]. Collectively, results of these studies showed that modulation of *MnSOD* expression level might differ from species to species; thus, *MnSOD* expression might be species-specific. However, results obtained in the current study suggest that *MuMnSOD* may protect the fish cells against the bacteria-generated ROS.

LPS is a harmful endotoxin present in gram-negative bacteria [51].

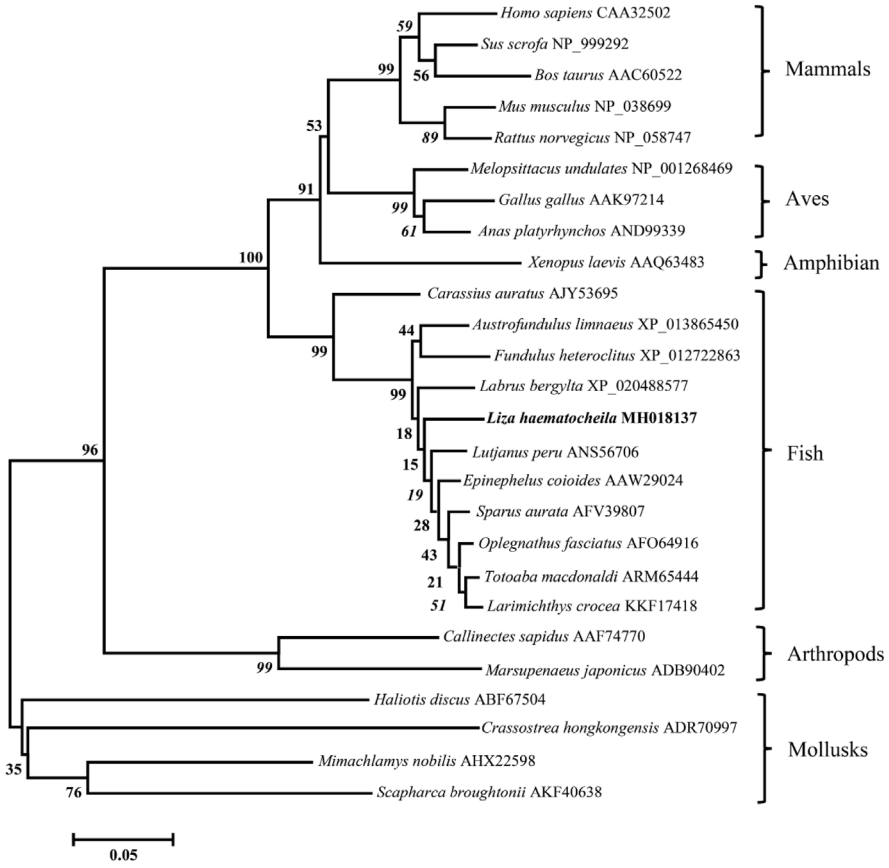

Fig. 2. Phylogenetic tree of MuMnSOD with its known MnSOD members. The tree was built using known amino acid sequences of SODs from different species, and the numerical value of each node displays the percentage of bootstrapping after 5000 replications. GenBank accession numbers are displayed adjacent to each organism.

Table 2
Determination of the homology of mullet MnSOD.

Organisms	Taxonomy	Identity%	Similarity %	Gaps %	AA
Larimichthys crocea	Teleostei	95.2	98.8	0.0	227
Totoaba macdonaldi	Teleostei	95.2	97.8	0.9	225
Epinephelus coioides	Teleostei	95.2	97.4	0.9	225
Sparus aurata	Teleostei	94.3	97.8	0.9	225
Oplegnathus fasciatus	Teleostei	94.3	97.4	0.9	225
Austrofundulus limnaeus	Teleostei	93.8	96.9	0.9	225
Lutjanus peru	Teleostei	93.1	96.1	1.7	231
Fundulus heteroclitus	Teleostei	92.5	96.5	0.9	225
Labrus bergylta	Teleostei	92.2	95.2	1.7	231
Gallus gallus	Aves	78.4	86.8	1.3	224
Sus scrofa	Mammalia	78.1	86.8	3.1	222
Melopsittacus undulatus	Aves	78.0	86.3	1.3	224
Homo sapiens	Mammalia	77.3	85.2	3.9	222
Mus musculus	Mammalia	76.8	85.5	3.1	222
Xenopus laevis	Amphibia	75.3	86.6	4.8	224
Bos taurus	Mammalia	74.3	83.0	4.8	222

Both endotoxin stress and oxidative stress result in the elevated expression levels of *MnSOD* during inflammation process in mammalian cells [52]. Studies of rock bream *MnSOD* [30], bay scallop *Cu/ZnSOD* [53], and spotted barbell *MnSOD* [41] revealed that significantly elevated SOD expression upon LPS bacterial stimulant. Previous studies reported that the LPS-stimulated secretion of TNF-α and expression of SOD proceed through ERK-1 activation process during inflammation [54], and it has been suggested that MnSOD might be a cytokine-regulated gene [55]. Altogether, modulation of the expression profile suggests that *MuMnSOD* might be involved in the defense mechanism against excess ROS levels.

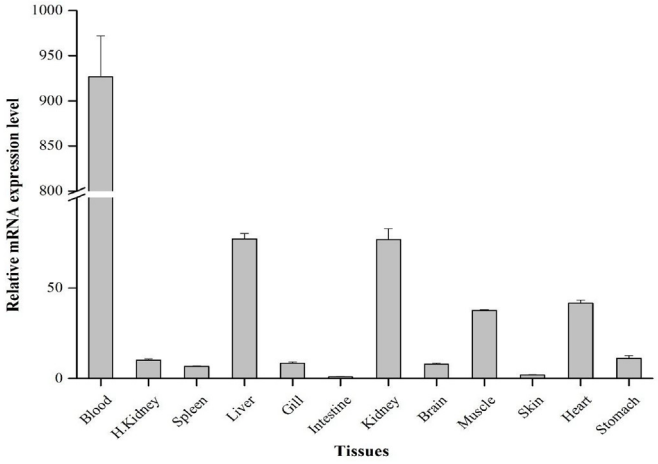

Fig. 3. Relative mRNA expression level of *MuMnSOD* in different tissues. The tissues were collected from healthy mullet fish, and expression levels in each tissue were analyzed using real-time qPCR. All data are presented as mean values (n = 3) and error bars show standard deviations (± SD). (H. kidney-Head kidney).

3.4. Bacterial overexpression and rMuMnSOD purification

MuMnSOD was expressed in ER2523 E. *coli* cells (NEB Express) and purified. Samples acquired at different stages of purification process were subjected to SDS-PAGE analysis, and a specific band corresponding to the target protein was observed in the cell lysate. Purified recombinant protein fused with the MBP showed the molecular weight of approximately 68 kDa (Fig. 5). Therefore, we have shown that rMuMnSOD was successfully expressed and purified in the *E. coli* system.

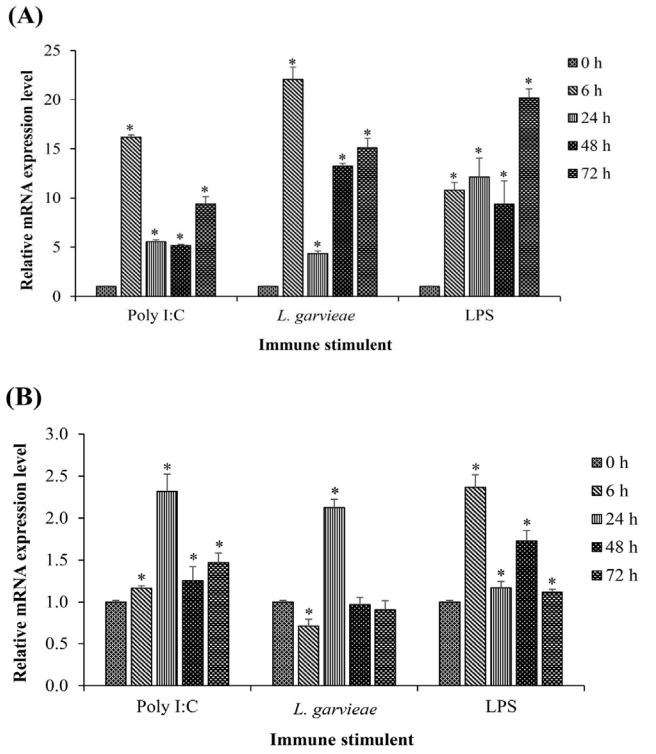

Fig. 4. Transcriptional expression levels of *MuMnSOD* in head kidney (A) and blood (B) after immune challenge with different pathogenic stimulants such as polyinosinic: polycytidylic acid (Poly I:C:), *Lactococcus garvieae (L. garvieae)* and lipopolysaccharide (LPS). Real-time qPCR was used to examine the relative expression levels at each time point. All data are indicated as mean values (n = 3) and error bars show standard deviations (± SD).

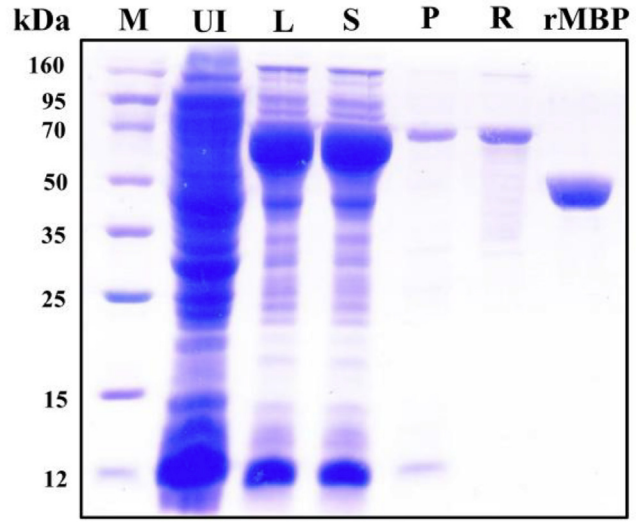

Fig. 5. Recombinant protein expression of *MuMnSOD* in *E. coli* ER2523. (M) protein marker; (UI) total cellular extracts from *E. coli* ER2523; (L) total cellular extract after protein induction at 20 °C for 10 h (S) soluble fraction after lysis of the cells by sonication. (P) insoluble fraction after centrifugation of the sonicated cellular extracts. (R) purified recombinant *MuMnSOD*. (rMBP) recombinant maltose binding protein.

3.5. Determination of the antioxidant activity of rMuMnSOD

Superoxide molecules generated during mitochondrial metabolism are effectively decomposed by the dismutation process catalyzed by the MnSOD enzyme [56]. Therefore, rMuMnSOD protein was used to perform the antioxidant assay and evaluate the antioxidant activity of MuMnSOD. XOD assay was conducted to determine the superoxide scavenging potential of MuMnSOD in the presence of a chromogenic indicator. Uric acid and hydrogen peroxide are generated in the reaction of xanthine and xanthine oxidase, and superoxide radicals are produced as a byproduct. NBT was converted into NBT-diformasan by the superoxide radicals. Therefore, the color of the solution was changed; this was monitored at a 560 nm wavelength. MnSOD converts superoxide to less harmful H_2O_2 and this conversion lowers the NBT-diformasan levels, which could be quantified as a relative activity of SOD [30,38].

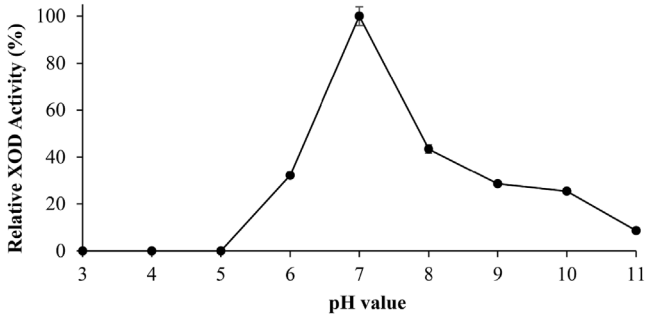

Fig. 6. Xanthine oxidation (XOD) assay for the identification of optimum pH for the recombinant MuMnSOD activity.

3.5.1. Determination of antioxidant ability of rMuMnSOD at different pH values

Relative SOD activity of rMuMnSOD at different pH values is shown in Fig. 6. Highest relative activity of rMuMnSOD was identified at pH 7. Hence, pH 7 was selected for XOD assays to decipher other properties of MuMnSOD. Present data showed that the antioxidant activity of rMuMnSOD was inhibited at more acidic pH values. pH of the medium highly affected the metal-ligand interaction. At acidic pH, imidazole rings of the manganese ion ligand His residues might be protonated. Therefore, metal-ligand complex might be more stable at higher pH levels [55]. Previous reports showed that SOD activity of rock bream, sea horse, and manila clam SODs were high at alkaline pH values [30,38,57]. However, purified ark shell MnSOD activity was high at acidic pH values [27].

3.5.2. Determination of antioxidant activity of rMuMnSOD at different temperatures

Xanthine oxidase assay was performed in the temperature range of 5–85 °C. The optimum temperature for the rMuMnSOD function was identified to be 25 °C (Fig. 7). The existence of MnSOD activity at a wide range of temperatures (5–55 °C) showed that rMuMnSOD might be stable under thermal stress conditions. Previous findings revealed that

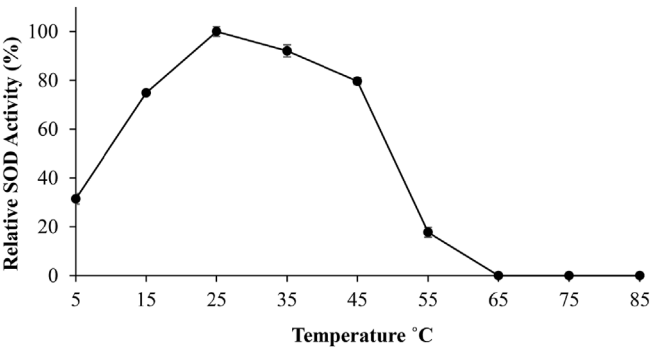

Fig. 7. Xanthine oxidation (XOD) assay for the identification of optimum temperature for the recombinant MuMnSOD activity. The tested temperatures were 5, 15, 25, 35, 45, 55, 65, 75, and 85 °C.

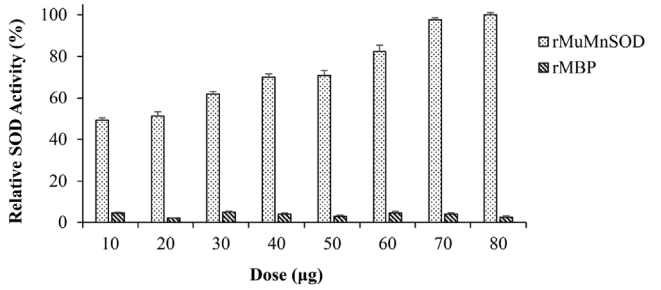

Fig. 8. Xanthine oxidation (XOD) assay for the identification of the effect of recombinant MuMnSOD dose (µg) at optimum pH and temperature conditions.

the optimum temperature for SOD activity was 25 °C. Therefore, these findings support the current findings related to the optimum temperature [38,58]. In ark shell, 50 °C was the optimum temperature for its MnSOD activity [27], while the rock bream MnSOD activity was highest at 20 °C [30]. Therefore, these studies suggest that MnSODs displayed species-specific differences in their antioxidant activities at different temperatures.

3.5.3. Determination of antioxidant ability of rMuMnSOD at different dosages

Relative superoxide scavenging ability of rMuMnSOD was evaluated using a range of dosages (10–80 µg) of rMuMnSOD protein (Fig. 8). Approximately, 50% of relative SOD activity was observed even with the lowest dosage of the recombinant protein. Relative SOD activity gradually increased with the increasing dose of the recombinant protein. However, relative SOD activity of recombinant MBP was not significantly changed with the change in dose. It showed that rMBP was not an active protein. Earlier reports also revealed that considerable SOD activity was detected even at the lower dosages of recombinant MnSOD protein and relative SOD activity increased with increasing dosage of the recombinant protein up to its maximum SOD activity [38,58]. The results of previous studies and the current study revealed that low levels of the MnSOD protein might be adequate for the antioxidant function in mullet.

3.5.4. Determination of the effect of inhibitory factors on rMuMnSOD activity

Impact of inhibitory factors on the rMuMnSOD activity was determined using potassium cyanide (KCN), sodium azide (NaN_3), N-N′-diethyl-dithiocarbamate (DDC), and ethylene diamine tetraacetic acid (EDTA). SOD activity was strongly inhibited by KCN and DDC. However, EDTA and NaN_3 did not significantly affect SOD activity (Fig. 9). Similarly, previous findings also showed that EDTA [58,59] and NaN_3 [60] did not significantly affect SOD activity while KCN and

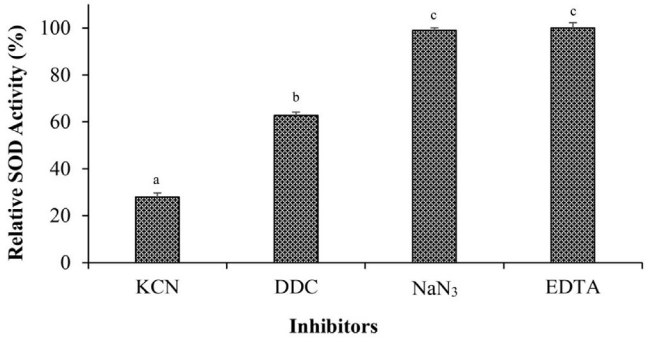

Fig. 9. Xanthine oxidation (XOD) assay for the identification of the effect of different inhibitors on recombinant MuMnSOD at optimum pH and temperature conditions. The letters on the error bars indicate significant differences ($P < 0.05$) between each treatment.

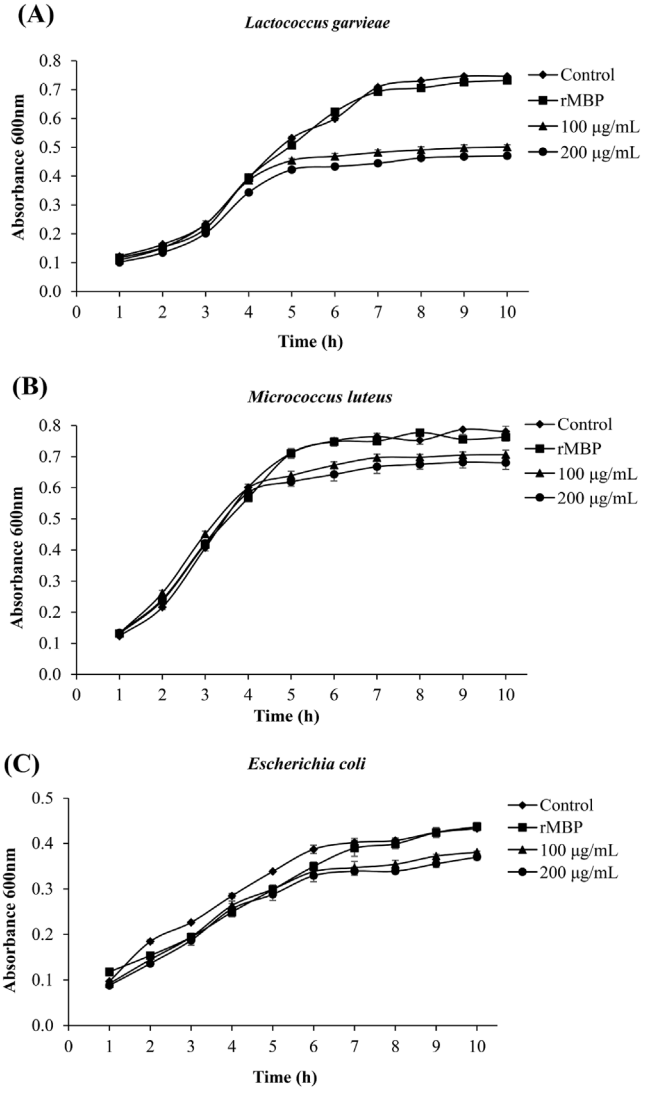

Fig. 10. The growth rate of *Lactococcus garvieae* (A), *Micrococcus luteus* (B), and *E. coli* (C) after incubating with recombinant MuMnSOD protein. All data are indicated as mean value (n = 3). Significant variations are observed from 5 h incubation with *L. garvieae* and *M. luteus* and 2 h incubation with *E. coli*, compared to the respective control samples ($P < 0.05$).

DDC may adversely affect SOD function [38,58,61,62]. Therefore, these findings suggested that the relative MuMnSOD activity could be disrupted by some inhibitors (KCN and DDC) and is specifically resistant to others (EDTA and NaN_3).

3.6. Evaluation of antibacterial activity of rMuMnSOD

Antibacterial activity possessed by rMuMnSOD was evaluated by observing the growth rates of selected bacteria in the presence of rMuMnSOD (Fig. 10). Remarkably, growth retardation was observed both in gram-positive bacteria *M. luteus* and *L. garvieae* and in the gram-negative bacterium *E. coli*, but significantly affect the *L. garvieae* growth. Fascinatingly, highest temporal expression of *MuMnSOD* was indicated at 6 h post-injection with *L. garvieae* and antibacterial activity against *L. garvieae* was commenced at 5 h after treatment of rMuMnSOD. Therefore, results of both transcriptional and antibacterial assays indicated that the MuMnSOD play a crucial role to combat *L. garvieae* after its infection. Moreover, present results suggest that rMuMnSOD possesses an antibacterial activity against both gram-positive and gram-negative bacteria and it might be due to the indirect

activity of HAMP domain of the protein sequence. Similarly, earlier reports indicated that ark shell MnSOD also showed antibacterial activity against both gram-positive and gram-negative bacteria [27]. Examination of the *Crassostrea gigas* recombinant SOD revealed its LPS binding ability [63]. Moreover, *C. gigas* SOD may have opsonic function acting *via* identification of bacterial cells and prevention of bacterial growth through binding to the integrins of hemocytes. It was reported that the amino acid sequence of the oyster recombinant SOD includes a LPS-binding motif identified in the endotoxin CD14 receptor, which showed an affinity to *E. coli* and to the LPS stimulant [63]. Nevertheless, studies of the antibacterial mechanism of SOD have been limited. Thus, the conserved structural sites present in the MuMnSOD sequence might be important for its antibacterial activity in the mullet defense system.

4. Conclusion

In conclusion, a mullet cDNA library was used for cloning and characterization of the structural features of MuMnSOD. *In silico* analysis revealed that MuMnSOD was present in the mitochondria and was highly conserved compared to other MnSOD counterparts. In addition, gene expression of *MuMnSOD* was examined through the transcriptional profiling using different immune stimulants including poly I:C, LPS, and *L. garvieae,* which are involved in the upregulation of *MuMnSOD* expression at different time points. The antioxidant function was evaluated by xanthine oxidase (XOD) assay and the results highlighted the antioxidant activity of the rMuMnSOD protein. Moreover, the present study explored the potential antibacterial activity of rMuMnSOD. Overall, it can be suggested that MuMnSOD acts as an antioxidant enzyme and as an immune-related substance that might be important to protect mullet fish cultures against pathogenic diseases.

Acknowledgments

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References

[1] W. Wang, Culture and Enhancement of Fishes, China Agriculture Press, Beijing, 2000.
[2] Z. Qi, X.H. Zhang, N. Boon, P. Bossier, Probiotics in aquaculture of China - current state, problems and prospect, Aquaculture 290 (2009) 15–21, https://doi.org/10.1016/j.aquaculture.2009.02.012.
[3] A. Valavanidis, T. Vlahogianni, M. Dassenakis, M. Scoullos, Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants, Ecotoxicol. Environ. Saf. 64 (2006) 178–189, https://doi.org/10.1016/j.ecoenv.2005.03.013.
[4] Charles A.J. Janeway, R. Medzhitov, Innate immune recognition, Annu. Rev. Immunol. 20 (2002) 197–216, https://doi.org/10.1146/annurev.immunol.20.083001.084359.
[5] M.W. Johansson, P. Keyser, K. Sritunyalucksana, K. Söderhäll, Crustacean Haemocytes and Haematopoiesis, Aquaculture vol. 191, (2000) 45–52, https://doi.org/10.1016/S0044-8486(00)00418-X.
[6] B.M. Babior, R.S. Kipnes, J.T. Curnutte, Biological defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent, J. Clin. Invest. 52 (1973) 741–744, https://doi.org/10.1172/JCI107236.
[7] B.P. Yu, Cellular defenses against damage from reactive oxygen species, Physiol. Rev. 74 (1994) 139–162, https://doi.org/10.1152/physrev.1994.74.1.139.
[8] R.A. Cairns, I.S. Harris, T.W. Mak, Regulation of cancer cell metabolism, Nat. Rev. Canc. 11 (2011) 85–95, https://doi.org/10.1038/nrc2981.
[9] A. Anju, J. Jeswin, P.C. Thomas, K.K. Vijayan, Molecular cloning, characterization and expression analysis of F-type lectin from pearl oyster *Pinctada fucata*, Fish Shellfish Immunol. 35 (2013) 170–174, https://doi.org/10.1016/j.fsi.2013.03.359.
[10] J.K. Huang, L. Wen, H. Ma, Z.X. Huang, C.T. Lin, Biochemical characterization of a cambialistic superoxide dismutase isozyme from diatom *Thallassiosira weissflogii*: cloning, expression, and enzyme stability, J. Agric. Food Chem. 53 (2005) 6319–6325, https://doi.org/10.1021/jf0507011.
[11] M.M. Helmy, Potential hepato-protective effect of a-tocopherol or simvastatin in aged rats, Pharmacol. Reports, 2012, pp. 698–705, , https://doi.org/10.1016/S1734-1140(12)70864-2.
[12] D. Marques, A.I. Esteves, M. Almeida, J. Xavier, M. Humanes, Superoxide Dismutase in the Marine Sponge *Cliona celata*, (2008), https://doi.org/10.1007/s00227-007-0853-3.
[13] V.C. Culotta, M. Yang, T.V. O'Halloran, Activation of superoxide dismutases: putting the metal to the pedal, Biochim. Biophys. Acta Mol. Cell Res. 1763 (2006) 747–758, https://doi.org/10.1016/j.bbamcr.2006.05.003.
[14] A. Bafana, S. Dutt, S. Kumar, P.S. Ahuja, Superoxide dismutase: an industrial perspective, Crit. Rev. Biotechnol. 31 (2011) 65–76, https://doi.org/10.3109/07388551.2010.490937.
[15] F. Wang, Q. Wu, Z. Zhang, S. Chen, R. Zhou, Cloning, expression, and characterization of iron superoxide dismutase in *Sonneratia alba*, a highly salt tolerant mangrove tree, Protein J. 32 (2013) 259–265, https://doi.org/10.1007/s10930-013-9482-5.
[16] F. Wolfe-Simon, D. Grzebyk, O. Schofield, P.G. Falkowski, The role and evolution of superoxide dismutases in algae, J. Phycol. 41 (2005) 453–465, https://doi.org/10.1111/j.1529-8817.2005.00086.x.
[17] M. Lü, R. Cai, S. Wang, Z. Liu, Y. Jiao, Y. Fang, X. Zhang, Purification and characterization of iron-cofactored superoxide dismutase from *Enteromorpha linza*, Chin. J. Oceanol. Limnol. 31 (2013) 1190–1195, https://doi.org/10.1007/s00343-013-3049-3.
[18] I.A. Abreu, D.E. Cabelli, Superoxide dismutases-a review of the metal-associated mechanistic variations, Biochim. Biophys. Acta Protein Proteonomics 1804 (2010) 263–274, https://doi.org/10.1016/j.bbapap.2009.11.005.
[19] G.A. Visners, C.W. Dougall, J.M. Wilson, I.A. Burr, S.H. Nick, Regulation of Manganese Superoxide Dismutase by lipopolysaccharide, interleukin-1, and tumor necrosis factor-Role in the acute inflammatory response, J. Biol. Chem. 265 (1990) 2856–2864.
[20] M.R. Abid, I.G. Schoots, K.C. Spokes, S.Q. Wu, C. Mawhinney, W.C. Aird, Vascular endothelial growth factor-mediated induction of manganese superoxide dismutase occurs through redox-dependent regulation of forkhead and IκB/NF-κB, J. Biol. Chem. 279 (2004) 44030–44038, https://doi.org/10.1074/jbc.M408285200.
[21] P.L. Jones, D. Ping, J.M. Boss, Tumor necrosis factor alpha and interleukin-1beta regulate the murine manganese superoxide dismutase gene through a complex intronic enhancer involving C/EBP-beta and NF-kappaB, Cell Biol. 17 (1997) 6970–6981.
[22] S. Nayak, S.K. Singh, N. Ramaiah, R.A. Sreepada, Identification of upregulated immune-related genes in *Vibrio harveyi* challenged Penaeus monodon postlarvae, Fish Shellfish Immunol. 29 (2010) 544–549, https://doi.org/10.1016/j.fsi.2010.05.010.
[23] R. Johansen, J.R. Needham, D.J. Colquhoun, T.T. Poppe, A.J. Smith, Guidelines for health and welfare monitoring of fish used in research, Lab. Anim. 40 (2006) 323–340, https://doi.org/10.1258/002367706778476451.
[24] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method, Methods 25 (2001) 402–408, https://doi.org/10.1006/meth.2001.1262.
[25] M.M. Bradford, A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding, Anal. Biochem. 72 (1976) 248–254, https://doi.org/10.1016/0003-2697(76)90527-3.
[26] B. Wu, Z. Liu, L. Zhou, G. Ji, A. Yang, Molecular cloning, expression, purification and characterization of vitellogenin in scallop *Patinopecten yessoensis* with special emphasis on its antibacterial activity, Dev. Comp. Immunol. 49 (2015) 249–258, https://doi.org/10.1016/j.dci.2014.12.004.
[27] L. Zheng, B. Wu, Z. Liu, J. Tian, T. Yu, L. Zhou, X. Sun, A. Yang, A manganese superoxide dismutase (MnSOD) from ark shell, *Scapharca broughtonii*: molecular characterization, expression and immune activity analysis, Fish Shellfish Immunol. 45 (2015) 656–665, https://doi.org/10.1016/j.fsi.2015.05.003.
[28] Q. Zhang, F. Li, B. Wang, J. Zhang, Y. Liu, Q. Zhou, J. Xiang, The mitochondrial manganese superoxide dismutase gene in Chinese shrimp *Fenneropenaeus chinensis*: cloning, distribution and expression, Dev. Comp. Immunol. 31 (2007) 429–440, https://doi.org/10.1016/j.dci.2006.08.005.
[29] G.A. Gomez-Anduro, R.R. Sotelo-Mundo, A. Muhlia-Almazan, G. Yepiz-Plascencia, Tissue-specific expression and molecular modeling of cytosolic manganese superoxide dismutases from the white shrimp *Litopenaeus vannamei*, Dev. Comp. Immunol. 31 (2007) 783–789 https://doi.org/10.1016/j.dci.2006.11.007.
[30] N. Umasuthan, K. Saranya Revathy, S.D.N.K. Bathige, B.S. Lim, M.A. Park, I. Whang, J. Lee, A manganese superoxide dismutase with potent antioxidant activity identified from *Oplegnathus fasciatus*: genomic structure and transcriptional characterization, Fish Shellfish Immunol. 34 (2013) 23–37, https://doi.org/10.1016/j.fsi.2012.09.011.
[31] L. Aravind, C.P. Ponting, The cytoplasmic helical linker domain of receptor histidine kinase and methyl-accepting proteins is common to many prokaryotic signalling proteins, FEMS Microbiol. Lett. 176 (1999) 111–116, https://doi.org/10.1016/S0378-1097(99)00197-4.
[32] M. Hulko, F. Berndt, M. Gruber, J.U. Linder, V. Truffault, A. Schultz, J. Martin, J.E. Schultz, A.N. Lupas, M. Coles, The HAMP domain structure implies helix rotation in transmembrane signaling, Cell 126 (2006) 929–940, https://doi.org/10.1016/j.cell.2006.06.058.
[33] S. Dunin-Horkawicz, A.N. Lupas, Comprehensive analysis of HAMP domains: implications for transmembrane signal transduction, J. Mol. Biol. 397 (2010) 1156–1174, https://doi.org/10.1016/j.jmb.2010.02.031.
[34] C.M. Khursigara, X. Wu, P. Zhang, J. Lefman, S. Subramaniam, Role of HAMP domains in chemotaxis signaling by bacterial chemoreceptors, Proc. Natl. Acad. Sci. Unit. States Am. 105 (2008) 16555 LP-16560 http://www.pnas.org/content/105/43/16555.abstract.
[35] G.L. Hazelbauer, J.J. Falke, J.S. Parkinson, Bacterial chemoreceptors: high-

performance signaling in networked arrays, Trends Biochem. Sci. 33 (2008) 9–19 https://doi.org/10.1016/j.tibs.2007.09.014.
[36] J.J. Falke, G.L. Hazelbauer, Transmembrane signaling in bacterial chemoreceptors, Trends Biochem. Sci. 26 (2001) 257–265 https://doi.org/10.1016/S0968-0004(00)01770-9.
[37] G. Manning, D.B. Whyte, R. Martinez, T. Hunter, S. Sudarsanam, The protein kinase complement of the human Genome, Science (80-.) 298 (2002) 1912 LP-1934 http://science.sciencemag.org/content/298/5600/1912.abstract.
[38] N.C.N. Perera, G.I. Godahewa, S. Lee, M.J. Kim, J.Y. Hwang, M.G. Kwon, S.D. Hwang, J. Lee, Manganese-superoxide dismutase (MnSOD), a role player in seahorse (*Hippocampus abdominalis*) antioxidant defense system and adaptive immune system, Fish Shellfish Immunol. 68 (2017) 435–442, https://doi.org/10.1016/j.fsi.2017.07.049.
[39] I. Tirado, J. Mateo, J.M. Soria, A. Oliver, E. Martínez-Sánchez, C. Vallvé, M. Borrell, T. Urrutia, J. Fontcuberta, The ABO Blood Group Genotype and Factor VIII Levels as Independent Risk Factors for Venous Thromboembolism, National Center for Biotechnology Information (US), Bethesda (MD), 2005, https://doi.org/10.1160/TH04-04-0251.
[40] J. Wu, M. Bao, D. Ge, L. Huo, Z. Lv, C. Chi, Z. Liao, H. Liu, The expression of superoxide dismutase in *Mytilus coruscus* under various stressors, Fish Shellfish Immunol. 70 (2017) 361–371, https://doi.org/10.1016/j.fsi.2017.08.018.
[41] Y.S. Cho, S.Y. Lee, I.C. Bang, D.S. Kim, Y.K. Nam, Genomic organization and mRNA expression of manganese superoxide dismutase (Mn-SOD) from *Hemibarbus mylodon* (Teleostei, Cypriniformes), Fish Shellfish Immunol. 27 (2009) 571–576, https://doi.org/10.1016/j.fsi.2009.07.003.
[42] G. Benard, B. Faustin, E. Passerieux, A. Galinier, C. Rocher, N. Bellance, J.-P. Delage, L. Casteilla, T. Letellier, R. Rossignol, Physiological diversity of mitochondrial oxidative phosphorylation, AJP Cell Physiol 291 (2006), https://doi.org/10.1152/ajpcell.00195.2006 C1172–C1182.
[43] J. Galindo-Villegas, H. Hosokawa, Immunostimulants: towards temporary prevention of diseases in marine fish, adv. En nutr. Acuicola VII memorias del VII simp, Int. Nutr. Acu'\icola. (2004) 16–19 http://www.uanl.mx/utilerias/nutricion_acuicola/VII/archivos/16JorgeGalindo.pdf.
[44] N.S. Trede, D.M. Langenau, D. Traver, A.T. Look, L.I. Zon, The use of zebrafish to understand immunity, Immunity 20 (2004) 367–379, https://doi.org/10.1016/S1074-7613(04)00084-6.
[45] R. Parker, H. Song, The enzymes and control of eukaryotic mRNA turnover, Nat. Struct. Mol. Biol. 11 (2004) 121 https://doi.org/10.1038/nsmb724.
[46] K.B. Schwarz, Oxidative stress during viral infection: a review, Free Radic. Biol. Med. 21 (1996) 641–649, https://doi.org/10.1016/0891-5849(96)00131-1.
[47] A.C. Barnes, C. Guyot, B.G. Hansen, K. Mackenzie, M.T. Horne, A.E. Ellis, Resistance to serum killing may contribute to differences in the abilities of capsulate and non-capsulated isolates of *Lactococcus garvieae* to cause disease in rainbow trout (Oncorhynchus mykiss L.), Fish Shellfish Immunol 12 (2002) 155–168, https://doi.org/10.1006/fsim.2001.0361.
[48] O. Byadgi, Y.C. Chen, A.C. Barnes, M.A. Tsai, P.C. Wang, S.C. Chen, Transcriptome analysis of grey mullet (Mugil cephalus) after challenge with *Lactococcus garvieae*, Fish Shellfish Immunol 58 (2016) 593–603, https://doi.org/10.1016/j.fsi.2016.10.006.
[49] S.C. Chen, L.L. Liaw, H.Y. Su, S.C. Ko, C.Y. Wu, H.C. Chaung, Y.H. Tsai, K.L. Yang, Y.C. Chen, T.H. Chen, G.R. Lin, S.Y. Cheng, Y.D. Lin, J.L. Lee, C.C. Lai, Y.J. Weng, S.Y. Chu, *Lactococcus garvieae*, a cause of disease in grey mullet, Mugil cephalus L., in Taiwan, J. Fish Dis. 25 (2002) 727–732, https://doi.org/10.1046/j.1365-2761.2002.00415.x.
[50] W. Cheng, Y.H. Tung, T.T. Chiou, J.C. Chen, Cloning and characterisation of mitochondrial manganese superoxide dismutase (mtMnSOD) from the giant freshwater prawn *Macrobrachium rosenbergii*, Fish Shellfish Immunol 21 (2006) 453–466, https://doi.org/10.1016/j.fsi.2006.02.005.
[51] C. Galanos, M.A. Freudenberg, Bacterial endotoxins: biological properties and mechanisms of action, Mediators Inflamm 2 (1993), https://doi.org/10.1155/S0962935193000687 S11–S16.
[52] R. Abe, T. Shimosegawa, S. Moriizumi, Y. Kikuchi, K. Kimura, A. Satoh, M. Koizumi, T. Toyota, Lipopolysaccharide induces manganese superoxide dismutase in the rat pancreas: its role in caerulein pancreatitis, Biochem Biophys Res Commun 217 (1995) 1216–1222, https://doi.org/10.1006/bbrc.1995.2898.
[53] Y. Bao, L. Li, F. Xu, G. Zhang, Intracellular copper/zinc superoxide dismutase from bay scallop *Argopecten irradians*: its gene structure, mRNA expression and recombinant protein, Fish Shellfish Immunol 27 (2009) 210–220, https://doi.org/10.1016/j.fsi.2009.04.005.
[54] M. Marikovsky, V. Ziv, N. Nevo, C. Harris-Cerruti, O. Mahler, Cu/Zn superoxide dismutase plays important role in immune response, J. Immunol. 170 (2003) 2993–3001, https://doi.org/10.4049/jimmunol.170.6.2993.
[55] A. Dolashki, R. Abrashev, S. Stevanovic, L. Stefanova, S.A. Ali, L. Velkova, R. Hristova, M. Angelova, W. Voelter, B. Devreese, J. Van Beeumen, P. Dolashka-Angelova, Biochemical properties of Cu/Zn-superoxide dismutase from fungal strain *Aspergillus Niger* 26, Spectrochim. Acta - Part A Mol. Biomol. Spectrosc. 71 (2008) 975–983, https://doi.org/10.1016/j.saa.2008.02.023.
[56] A. Phaniendra, D.B. Jestadi, L. Periyasamy, Free radicals: properties, sources, targets, and their implication in various diseases, Indian J. Clin. Biochem. 30 (2015) 11–26, https://doi.org/10.1007/s12291-014-0446-0.
[57] N. Umasuthan, S.D.N.K. Bathige, K.S. Revathy, Y. Lee, I. Whang, C.Y. Choi, H.C. Park, J. Lee, A manganese superoxide dismutase (MnSOD) from *Ruditapes philippinarum*: comparative structural- and expressional-analysis with copper/zinc superoxide dismutase (Cu/ZnSOD) and biochemical analysis of its antioxidant activities, Fish Shellfish Immunol 33 (2012) 753–765, https://doi.org/10.1016/j.fsi.2012.06.024.
[58] N.C.N. Perera, G.I. Godahewa, J. Lee, Copper-zinc-superoxide dismutase (CuZnSOD), an antioxidant gene from seahorse (*Hippocampus abdominalis*); molecular cloning, sequence characterization, antioxidant activity and potential peroxidation function of its recombinant protein, Fish Shellfish Immunol 57 (2016) 386–399, https://doi.org/10.1016/j.fsi.2016.08.052.
[59] S. Marklund, G. Marklund, Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, Eur. J. Biochem. 47 (1974) 469–474, https://doi.org/10.1111/j.1432-1033.1974.tb03714.x.
[60] K.C. Bhuyan, D.K. Bhuyan, Superoxide dismutase of the eye relative functions of superoxide dismutase and catalase in protecting the ocular lens from oxidative damage, BBA - Gen. Subj. 542 (1978) 28–38, https://doi.org/10.1016/0304-4165(78)90229-5.
[61] H. Saggu, J. Cooksey, D. Dexter, F.R. Wells, A. Lees, P. Jenner, C.D. Marsden, A selective increase in particulate superoxide dismutase activity in parkinsonian substantia nigra, J. Neurochem. 53 (1989) 692–697, https://doi.org/10.1111/j.1471-4159.1989.tb11759.x.
[62] B. Maitre, L. Jornot, A.F. Junod, Effects of inhibition of catalase and superoxide dismutase activity on antioxidant enzyme mRNA levels, Am. J. Physiol. Cell. Mol. Physiol. 265 (1993) L636–L643.
[63] M. Gonzalez, B. Romestand, J. Fievet, A. Huvet, M.C. Lebart, Y. Gueguen, E. Bachère, Evidence in oyster of a plasma extracellular superoxide dismutase which binds LPS, Biochem. Biophys. Res. Commun. 338 (2005) 1089–1097, https://doi.org/10.1016/j.bbrc.2005.10.075.