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- Note -

Expressional Analysis of Superoxide Dismutase in Olive Flounder (*Paralichthys olivaceus*) against Viral Hemorrhagic Septicemia Virus Infection

Young Mee Lee, Jung-Eun Kim, Jae Koo Noh, Hyun Chul Kim, Choul-Ji Park, Jong-Won Park, Kyung-Kil Kim and Jeong-Ho Lee*

Genetics and Breeding Research Center, NFRDI, Geoje 656-842, Korea

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Superoxide dismutase is a family of important antioxidant metalloenzymes and catalyzes the dismutation of toxic superoxide anions into dioxygen and hydrogen peroxide. A recent study identified the partial superoxide dismutase (SOD) gene in olive flounder (*Paralichthys olivaceus*). The same study reported that it strongly induced benzo[a]pyrene and that it was an indicator of aquatic oxidative stress responses. However, its transcriptional response against viral infection has not been investigated. In the present study, the spatial and temporal expression profiles were analyzed to investigate the function of Of-SOD in the antiviral response. The *Of-SOD* transcripts were ubiquitously detected at various levels in diverse tissues in a real-time PCR. The expression of *Of-SOD* was significantly higher in the muscles, liver, and brain but extremely low in the stomach and spleen. Following a VHSV challenge, the expression of *Of-SOD* increased within 3 h in the kidneys and decreased to the original level 2 days postchallenge. In muscle, liver, and brain, *Of-SOD* mRNA was similarly up-regulated at 3 - 6 h postchallenge and then decreased to the basal level. Although the expression pattern and induction time differed slightly depending on the tissue, the transcript of *Of-SOD* consistently increased in the acute infection response, but the expression was low in the chronic response. The expression of *Of-SOD* was induced after the VHSV infection, and *Of-SOD* was probably involved in the immune response against the viral challenge. These results suggest that SOD may play important roles in the immune defense system of *P. olivaceus* and perhaps contribute to the protective effects against oxidative stress in olive flounder.

Key words : Gene expression, olive flounder, *Paralichthys olivaceus*, superoxide dismutase (SOD), viral hemorrhagic septicemia virus (VHSV)

Introduction

The olive flounder (*Paralichthys olivaceus*) is an economically important fish species in Korea. However, various infectious diseases caused severe financial losses in the aquaculture of olive flounder. Most of the flounder diseases are caused by a bacterial in the low water temperature period until the mid-2000s, but the rapidly spreading parasites and virus disease all the year round thereafter. Mortality of olive flounder caused by the viral hemorrhagic septicemia virus disease (VHS) and scuticociliatosis that account for about 70% of the total and damage cost is 120 billion a year.

Viral haemorrhagic septicaemia (VHS) is one of the most important viral diseases of olive flounder aquaculture that causes fetal septicemia in all life stages of fresh water and marine fish. Viral haemorrhagic septicaemia virus (VHSV) is a member of the genus *Novirhabdovirus* from the family *Rhabdoviridae*. It was first isolated in 1963 from freshwater cultured rainbow trout in Denmark [8]. The virus remained predominantly a pathogen of European in fresh water species as well as a few marine species over the next 25 years [15, 20]. Thereafter, it was reported from various marine fishes in European countries and North America [12, 13] and also isolated from olive flounder in Japan and South Korea in 1999 and 2001, respectively. A high mortality rate was found to be 40 to 60% in cultured olive flounder from winter to spring in Korea. Regarding the problem, a better understanding of its immune system should help to reduce diseases.

In eukaryotes, the innate immune system is a critical means of host defense against microbial infections. One of these protective defenses is the generation of microbicidal reactive oxygen species (ROS). ROS could be constantly

***Corresponding author**
Tel : +82-55-639-5811, Fax : +82-55-639-5809
E-mail : jhlee7124@korea.kr

produced in response to both external and internal stimuli in aerobic organisms and may play multiple functions in many biological processes. Low levels of ROS have been shown to be involved in many biochemical processes like intracellular signaling in the cell differentiation and cell progression or the arrest of growth, apoptosis, immunity and defense against microbial infection. Whereas the high levels of ROS and reactive oxygen intermediates (ROI) may lead to cell damage. Therefore, elimination or detoxification of residual ROS/ROI on time is critical for host to protect itself from damage. The antioxidant enzymatic system is recruited for protecting the host from the toxic effects by the activated oxygen species. These enzymes include superoxide dismutase (SOD), catalase (CAT), glutathione reductase, glutathione peroxidase, glutareodoxin and thioredoxin reductase [9].

Superoxide dismutases (SODs) play a crucial role in the defense against oxidative cell damage, through catalyzing the breakdown of superoxide anion to oxygen and hydrogen peroxide [2, 7]. Several forms of SOD have been described, that can be distinguished by their primary structure, cellular compartmentalization, primary function, and the metal required for activity. These are copper zinc SOD (CuZnSOD), manganese SOD (MnSOD), and iron SOD (FeSOD) [17]. CuZnSOD is an important type of SOD because of its physiological function and therapeutic potential [6]. This enzyme requires Cu^{2+} which has a role as oxidized and reduced alternation and Zn^{2+} helps to maintain enzyme stability. Loss of Cu^{2+} results in its complete inactivation and induces many diseases in human and animals [11, 18]. Also, SOD activity has been reported to be present in many aquatic and other species and plays an important role in immunity against diseases [14].

In the recent report, single nucleotide polymorphisms (SNPs) in SOD genes of mollusk, *Argopecten irradians* have been correlated with their resistance or susceptibility to *Vibrio anguillarum* [3], suggesting that polymorphisms in SOD gene could impair the anti-oxidative capacity and may influence the resistance to *V. anguillarum*. Recently, many studies have been conducted to CuZnSOD as this enzyme has multiple functions against pathogenic infection [4, 7]. CuZnSOD has been cloned from a few fish species, including black porgy, Antarctic teleost, red sea bream, grouper, zebra fish, cartilaginous shark species, rock bream, and olive flounder [19, 20]. However, the immunological functions of CuZnSOD in olive flounder remain poor characterized.

Olive flounder, *Paralichthys olivaceus* is an important fish species for aquaculture in Korea. However, various infectious diseases caused serious economic losses in the aquaculture of olive flounder. Regarding the problem, a better understanding of its immune response should help to reduce diseases in addition to immune-related genes. In this study, we demonstrated the spatial and temporal expression of *Of-SOD* from olive flounder in order to investigate the distribution and functional roles of Of-SOD against viral infection.

Materials and Methods

Fish maintenance and sample preparation

Olive flounders were obtained from Genetics and Breeding Research Center, National Fisheries Research and Development Institute (NFRDI; Geoje, Republic of Korea), and maintained in 10 tons flow through tank at 20±1°C under a natural photoperiod. The tissues including brain, eye, fin, gill, intestine, kidney, liver, muscle, spleen and stomach were dissected out from five healthy olive flounder (approximately 1 kg) and grounded in the Trizol Reagent (Invitrogen) and kept at -70°C until use.

RNA extraction and real-time PCR

Total RNAs were extracted from the frozen sample according to the manufacturer's instructions. The total RNA concentration was quantified by spectrophotometer and 1 μg of total RNA was used for reverse transcribed into cDNA using Transcriptor First Strand cDNA synthesis kit (Roche) using oligo$(dT)_{18}$ primer after digestion with recombinant DNase I (RNase free) (TaKaRa) to eliminate the genomic contamination. Synthesized cDNAs were stored at -20°C until use. Primers for *Of-SOD* were designed using highly conserved regions of olive flounder (GenBank accession no. ABS12626).

Real-time PCR was performed in a reaction mixture of 20 μl containing 1 μl of total transcribed cDNA, *Of-SOD* specific primer and 10 μl of Fast SYBR Green PCR Master Mix (Applied Biosystems, UK). The standard cycling conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. The real-time PCR reactions was monitored with melting curve analysis using 7,500 software (version 2.0.5) and the β-actin gene was employed as the internal standard. Amplification efficiency was determined

by a serial dilutions. All experiments were repeated in triplicate. Each reactions displayed an efficiency 93.3% (R^2= 0.997) and 95% (R^2=0.999), *Of-SOD* and β-actin, respectively.

Gene expressions of *Of-SOD* against VHSV infection

The pathogenicity of the virus obtained from from the National Fisheries Research and Development Institute (NFRDI, CHSE cell line) was examined using olive flounder. Experimental fish were controlled at 15°C using re-circulation system, which without flowing and feeding. Five fish were injected intraperitoneally with 100 μl $10^{4.8}$ $TCID_{50}$ virus/fish per each treatment time. After challenge, fish were transferred to new 3 ton tank and 5% of the water was exchanged everyday. Samples were collected at 0, 1, 3, 6, 9, 12, 18, 24 hr and 2, 3, 4, 5, 6, 7, 10, 15, 20 days post-injection to collect above the described tissues and total RNA was extracted from the tissues and cDNA was synthesized as described.

Statistical analysis

Gene expression levels of *Of-SOD* were normalized to those of an internal control gene. Expressions were taken as the average value of 5 fish and were expressed as the fold change relative to the value of the control group. The significance of differences between groups was assessed with anova.

Results

Spatial expression profile of *Of-SOD*

To investigate the tissue distribution profile of *Of-SOD* transcripts, total RNA from the tissues of brain, eye, fin, gill, intestine, kidney, liver, spleen and stomach was excised from healthy olive flounder. The real-time PCR analysis was performed using olive flounder β-actin as an invariant control and relative mRNA expression-fold was derived by comparing the transcript level in each tissue with that of stomach. As a result, *SOD* transcripts were detected in all the examined tissues, but the relative levels of basal expression were variable. Of the ten tissues examined, the highest level of expression was observed in the muscle, followed by liver, brain. The remaining tissues, including eye, fin, gill, intestine, kidney showed similar expression level. The stomach and spleen showed the lowest level of *SOD* transcription (Fig. 1).

Temporal expression profile of *Of-SOD* during VHSV challenge.

To investigate the function of Of-SOD in the viral-infection response, the olive flounder fish were challenged with VHSV and the kidneys dissected out in a variety of infection time of the fish owing to its importance in the immune system, and the expression of *Of-SOD* was assayed by real-time PCR. The expression of *Of-SOD* was increased a 2.7-fold at 3 hr post-challenge with VHSV, then decreased level was maintained basal status to 24 hr. Thereafter, the transcript of *Of-SOD* was complete bottom level at 2 days post-challenge, decreased transcript was increased again to basal level showing a lower bell-shaped curve (Fig. 2). In contrast, transcript level of *Of-SOD* was not changed when without VHSV challenge.

Fig. 3 shows the expression changes of *Of-SOD* in the highly expressed tissues. In the muscle, the expression of *Of-SOD* was increased at 1 hr post-challenge, and then returned to the original value at 9-24 h and entirely declined 2 days post-challenge. The transcript of *Of-SOD* was risen again and maintained certain basal level. Whereas the transcript of *Of-SOD* was obviously increased and decreased during acute infection and completely reduced and maintained after 2 days post-challenge in the liver. The ex-

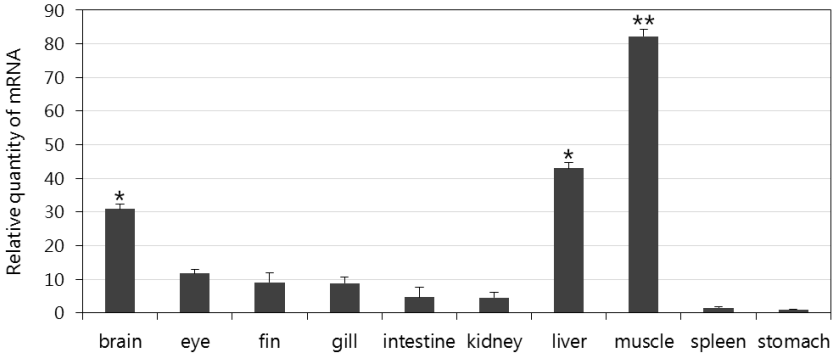

Fig. 1. Distribution of the *Of-SOD* gene transcript in different tissues by real-time PCR. Analyzed tissue is: brain, eye, fin, gill, intestine, kidney, liver, muscle, spleen and stomach. This experiment was performed for three times and the data demonstrated means of triplicates. Error bars in dicate SD. Statistical significance is indicated with an asterisk (*: p<0.05; **: p<0.01).

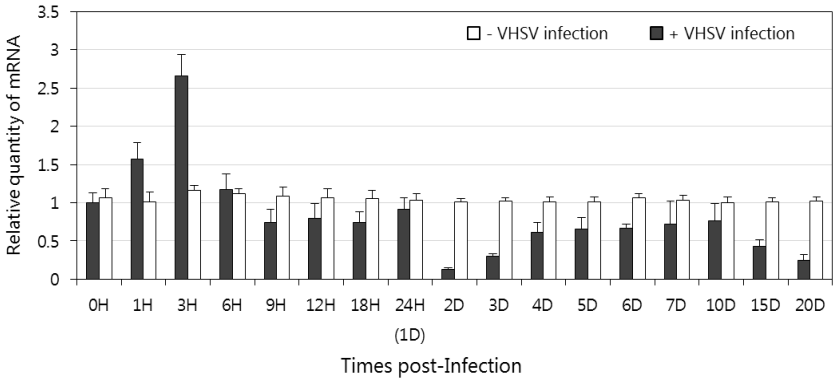

Fig. 2. Temporal expression profiles of *Of-SOD* without (white box) versus with VHSV challenge (gray box) in the kidney. The mRNA expression level is calculated relative to β-actin expression. Each symbol and vertical bar represents SD. Significant difference from control is indicated with an asterisk (*: $p<0.05$; **: $p<0.01$).

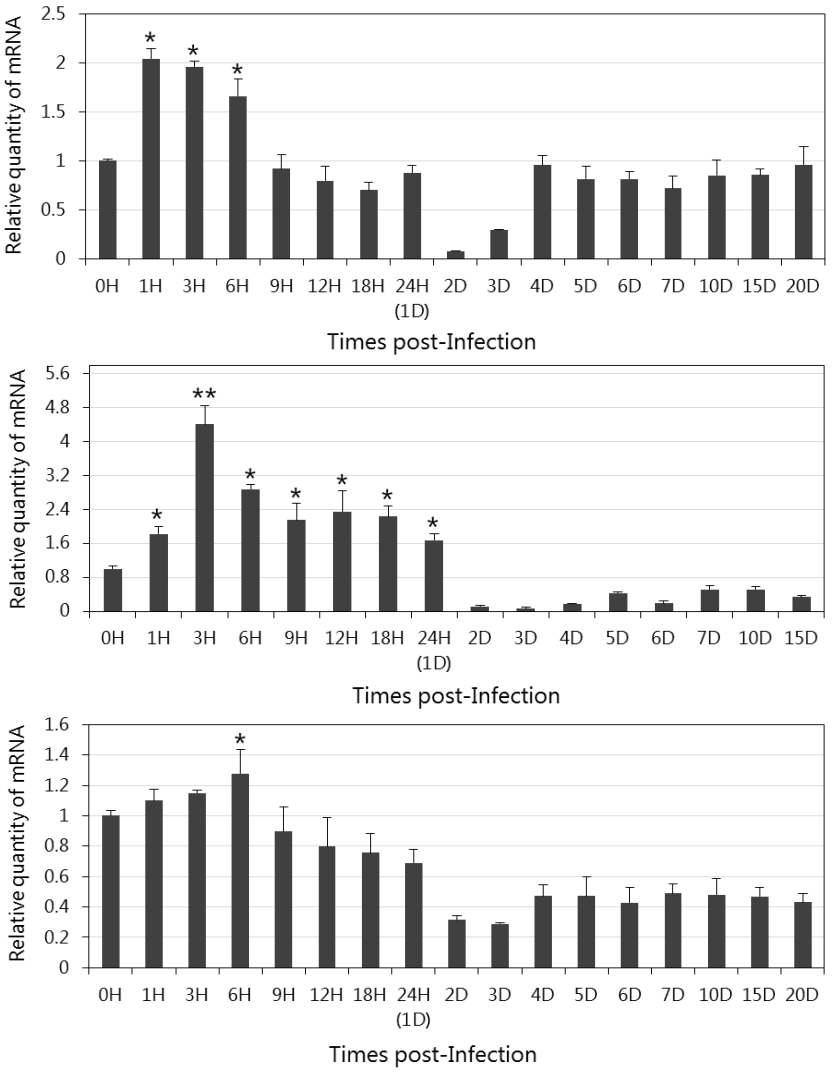

Fig. 3. Relative gene expression of *Of-SOD* transcript in the (A) muscle, (B) liver and (C) brain of olive flounder infected VHSV measured by real-time PCR. *Of-SOD* expression was quantified in relation to internal control of olive flounder β-actin gene expression. Results are expressed as SD and significantly different from 0 h are indicated with an asterisk (*: $p<0.05$; **: $p<0.01$).

pression pattern in the brain was matched with the induced profile at the kidney against VHSV infection though the expression is weak. The transcript of *Of-SOD* was dominantly increased in acute infection period in the tested all organ and rapidly declined at 2 day post-challenge, but the expression showed difference depending on the organ in the chronic treatment. The variable expression pattern of *Of-SOD* was speculated to be related with tissue-dependent mitochondrial content and oxidative load. Which points toward a potential role for Of-SOD in activating the antioxidant system in this species and this perhaps to protect the organs from the production of oxy radicals upon acute

infection.

Discussion

Reactive oxygen species (ROS) are reported to be released by oxidative stress generated in aquatic organisms in response to invading microorganisms (pathogens) such as viruses, bacteria, and fungi [4, 16]. Aquatic animals have well developed defense systems against ROS during infection, by both limiting the formation of ROS and by instituting their removal. Superoxide itself can kill microbes, but does not diffuse across membranes efficiently and is rapidly dismutated to hydrogen peroxide (H_2O_2) by Cu/Zn-superoxide dismutase (SOD). H_2O_2 causes direct oxidative damage to many pathogens such as oxidation of proteins, lipid-peroxidation, DNA/RNA breaks, base oxidation and deamination, and oxidation of methionine residues, can be directly tracked in microbes exposed to respiratory burst.

In the recent report, An *et al* [1] was reported olive flounder SOD for the first time that partial SOD cDNA consists of 326 bp and encodes a protein of 109 amino acids and belong to CuZnSOD. The expression level of *Of-SOD* mRNA was increased with benzo[a]pyrene (BaP) exposure and they suggested that SOD play an important role in the detoxification of ROS caused by BaP exposure, and thus may be indicators of oxidative stress responses. However, the immunological functions of SOD in fish remain poorly characterized.

In this study, we analyzed the expression of *Of-SOD* spatial and temporal expression to investigate a role of Of-SOD against the invasion of pathogens. The results indicated that *Of-SOD* transcripts were constitutively expressed in all examined tissues, but the relative levels of expression were variable. Predominant expression of *Of-SOD* was detected in muscle, liver and brain, while relatively low in spleen and stomach . The expression pattern of teleost SODs in Hemibarbus mylodon [5] and silver carp [21] were somewhat different from that of *Of-SOD*, suggesting species-specific expression of SOD. However, all these teleost and human [10] SOD transcripts were considerably abundant in metabolically active tissues with high energy requirement such as heart, brain, liver and muscle. This could be related to the oxygen required configuration of those tissues. The differences in the tissue-distribution of *Of-SOD* expression may be attributed to the balance of various biological processes acting on the production of ROS in different environments.

During the VHSV infection, transcript level of the *Of-SOD* was commonly increased post-challenge at acute response in the all tested tissue, and then expression was rapidly decreased to the ground level at 2 day post-challenge. Reduced mRNA has been restored to basal level in the chronic response in the kidney, muscle and brain, except liver. The expression of *Of-SOD* generally increased in the acute response but decreased in the chronic infection in the liver, whereas restored from chronic response in other tissues. Liver is an important and critical component in the defense against pathogen infection and contains numerous innate and adaptive immune cells that specialize in detection and capture of pathogens. Also, liver is metabolically active tissues with high energy requirements and has relative abundant mitochondrial content and oxidative burden and these result suggest that *Of-SOD* exhibited opposite function in acute and chronic responses in the liver.

Antioxidant enzymes such as SOD, CAT and GPx provide the first line of cellular defense against toxic free radicals which cause oxidative stress. The SOD expression levels in many species can be significantly increased by exposure to pathogen, suggesting that they form part of an immune response to pathogen invasion, which could determine their suitability to be used as effective biomarkers of aquatic infection.

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초록 : Viral hemorrhagic septicemia virus (VHSV) 감염에 대한 넙치 superoxide dismutase (*Of-SOD*)의 발현분석

이영미 · 김정은 · 노재구 · 김현철 · 박철지 · 박종원 · 김경길 · 이정호*
(국립수산과학원 육종연구센터)

활성산소종(ROS)은 환경 스트레스 및 병원체의 침입에 의한 산화 대사의 자연적인 산물로써 생물에서 생산된다. 산화적 스트레스에 의해 생성되는 superoxide 음이온 및 과산화수소와 같은 ROS는 세포와 조직에 독성을 나타낼 수 있고, 이 과정에 관여하는 superoxide dismutase (SOD)는 중요한 metalloenzyme이다. 최근 연구는 올리브 넙치(*Paralichthys olivaceus*)에서 SOD의 부분 유전자가 benzo[a]pyrene에 의해 강하게 발현이 유도되고 산화 스트레스 반응의 지표라고 확인하였지만, 바이러스성 감염에 대한 전사적 반응에 대해서는 조사되지 않았다. 본 연구에서는 항바이러스 반응에서 넙치 SOD의 기능을 알아보기 위해 공간 및 시간적 발현 프로파일을 분석하였다. 넙치 SOD 전사체는 정도의 차이는 있지만 다양한 기관에서 보편적으로 발현되었으며, 근육, 간, 뇌에서는 높게 발현되었고, 위와 비장에서는 상대적으로 낮게 발현되었다. VHSV 감염 후 넙치 콩팥에서 SOD 발현은 3시간 이내에 증가하였으며 점차적으로 감소하여 감염 2일째 원래 수준으로 돌아갔다. 검사 조직에 따라 발현이 유도되는 시간의 차이는 있지만 근육, 간, 뇌에서도 콩팥과 유사한 발현양상을 보였으며, 공통적으로 급성적 면역반응에서는 발현이 증가하지만 만성적 면역반응에서는 감소하였다. 이상의 결과들을 종합해 볼 때, 넙치 SOD는 넙치(*P. olivaceus*)의 면역 방어 시스템에 중요한 역할을 하고 넙치의 산화 스트레스에 대한 보호 효과에 기여할 것으로 기대할 수 있다.