THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

MOLECULAR CLONING AND CHARACTERIZATION OF ANTIOXIDANT GENES FROM SCUTICOCILIATE, *Miamiensis avidus*

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Part II. Molecular cloning and characterization of glutathione peroxidase (GPx) gene from scuticociliate, *Miamiensis avidus*

요 약 문

한국 어류양식 산업에 있어서 스쿠티카충에 의한 피해는 매년 증가하고 있는 추세이다. 스쿠티카충은 섬모충의 일종으로 어류의 조직을 파고 먹음으로써 어류의 폐사를 가져온다. 현재 감염성 스쿠티카충 종류에는 *Mianiensis avidus*, *Philasterides dicentrarchi*, *Uronema nigricans*, *Tetrahymena* sp. 등이 알려져 있다. 이들이 어류 조직에 한번 감염되기 시작하면 조직을 파고 먹어 들어감으로써 혈관을 통해 아가미, 심장, 뇌, 장, 근육조직 등까지 전체적으로 퍼져나간다. 여러 스쿠티카충 중에서도 특히나 *Mianiensis avidus* 는 가장 강력한 감염성을 지녔다는 보고가 있다. 이 종은 어류조직에 감염되면 빠른 속도로 전체조직으로 퍼져나가 대량의 어류폐사를 일으킨다. 그러나 *Mianiensis avidu* 의 대한 연구는 subunit ribosomal RNA (SSU rRNA)에 의한 종 동정과 *in vitro* 상에서의 배양방법, 감염 시 숙주 어류에서 일어나는 면역반응 등에 대한 것만이 발표되었다.

생물에 있어서 활성산소(reactive oxygen species, ROSs)는 물질대사에 의해 발생된다. 이러한 활성산소는 생물 물질대사에 있어 신호전달이나 면역 등에 중요한 역할을 담당하지만 그 양이 과다하게 되면 세포나 조직에 피해를 주게 된다. 자외선, 약품, 독소, 항체, 대식세포의 면역작용에 의해 활성산소가 과다하게 생성되면, 이들 활성산소들은 단백질, 지질, 색산, 당의 생합성에 영향을 주며, DNA 의 변형을 가져오고 결국 세포의 사멸을 가져온다. 이러한 활성산소의 부작용을 막기 위하여 생물체는 여러 항산화 체계를 발달시켜왔다. 그 중에서 항산화 효소로 superoxide dismutase (SOD), glutathione peroxidase (GPX), catalases (CAT), peroxiredoxin (PRX)가 알려져 있다. SOD 는 산소 라디칼($O_2^{\cdot -}$)을 과산화수소(H_2O_2)와 산소(O_2)로 전환시키는 효소로 활성부위에 어떠한 금속을 갖는 가에 따라 분류된다. 현재, copper/zink containing SOD (CuZn-SOD), Manganese containing SOD (Mn-SOD), iron containing SOD (Fe-SOD), nickel containing SOD (Ni-SOD) 등이 보고되었다. GPX 는 SOD 에 의해 생성된 H_2O_2 나 유기과산화물을 물이나 알코올 형태로 분해하는 효소이다. GPX 의 활성부위에 seleno-cysteine (Se-Cys)을 갖는 GPX 가 포유류와 어류, 식물 등에서 주로 보고되었고 몇몇의 종에서 활성부위에 Se-Cys 대신 보통의 cysteine 를 갖는 nonselenium GPX (또는 seleno-indenpendent GPX)가 보고되고 있다.

많은 기생충들은 숙주의 면역체계를 방어하기 위해 여러 가지 전략을 가지고 있으며 특히 대식세포에서 분비되는 활성산소나 포식작용을 이겨내기

위한 항산화효소가 발달되어있다. 이미 말라리아충이나 장내 기생하는 nematode 등에서 이러한 항산화효소가 발표되었다.
Mianiensis avidus 가 스쿠티카충에서도 강력한 공격자로 알려져 있으나 이에 대한 생활사나 분자유전학적 연구가 미미하다. 이에 이 논문에서는 *Mianiensis avidus* 의 항산화 효소인 SOD 와 GPX 에 대해 분자유전학적으로 분석함으로써 앞으로 이 종에 대한 유전학적, 면역학적 기전 연구에 있어 도움을 주고자 한다. 또한 간접적으로 스쿠티카충이 숙주 면역작용과 여러 환경적 스트레스에 어떻게 살아남는지에 대한 이해를 높이고자 한다.

저자가 속해있는 실험실에서는 이미 *Mianiensis avidus* 에 대한 선행연구로써, 발현되는 유전자에 대해 GS-FLX 분석을 실시한 바 있다. 이를 통해 *Mianiensis avidus* 유전자들의 contig 와 singleton 들을 얻었고, GS-FLX blast 결과를 통해 SOD 와 GPX 로 추정되는 부분 서열을 확인하였다. 이 부분 서열을 토대로 full-length SOD 와 GPX 를 얻기 위해 RACE PCR 용 primer 를 제작하였고, 이를 이용하여 SOD 와 GPX 서열을 클로닝 하였다.

클로닝된 *Mianiensis avidus* SOD (maSOD)는 총길이 958bp 로 663bp 의 open reading frame (ORF)를 포함하고 있었으며 220 개의 아미노산을 암호화하고 있었다. 단백질서열 분석을 통해 분자량 약 25 kDa 과 isoelectric point (pI, 등전점) 6.65 를 확인하였다. NCBI BLAST 분석을 통해 maSOD 가 Fe-SOD 와 Mn-SOD 와 유사하다는 것을 확인하였고 이를 토대로 알려진 여러 Fe-SOD 와 Mn-SOD 과 비교 및 분석하였다. maSOD 에는 Fe 이나 Mn 과 결합하여 그 기능을 수행할 수 있게 해주는 4 개의 amino acid residue 를 포함하고 있었으며, Fe/Mn-SOD 의 보존된 2 개의 motif 와 1 개의 signature 서열이 있었다. Pairwise ClustalW 분석을 실시하여 maSOD 단백질 서열이 *Tetrahymena thermophlia*, *Tetrahymena pyriformis* 와 각각 유사성 81, 75 %로 가장 가까웠으며, 다른 Fe/Mn-SOD 들과는 45%에서 62%의 유사도를 보였다. maSOD 의 계통수와 비교분석을 살펴보면 maSOD 가 다른 기생충이나 병원체에서 알려진 Fe-SOD 보다 고등진핵생물의 Mn-SOD 와 유사함을 알 수 있다. 계통수에서 maSOD 의 위치는 Fe-SOD 가지가 아닌 Mn-SOD 가지에 속해있다. 실제로 Fe-SOD 와 Mn-SOD 는 매우 유사한 서열을 지니고 있으며, 고등생물에서 주로 Mn-SOD 가 보고되고 하등생물에서 Fe-SOD 가 보고되고 있다. maSOD 단백질의 특성을 알기 위해 재조합단백질을 이용한 항산화효과를 측정하였다. *Miamiensis avidus* 와 같은 섬모충의 유전자를 박테리아인 *E. coli* 에서 발현시키기 위해서는 별도의 과정이 필요하다.

섬모충과 *E. coli* 의 codon usage 에 차이가 있기 때문에 maSOD 의 발현서열 중에서 *E. coli* 에서 없는 codon 들을 *E. coli* 에 특이적인 codon 으로 바꿔준 다음, 이 변형된 서열을 기초로 만든 oligodeoxynucleotide 와 aseembly PCR 를 통해 변형 maSOD ORF 를 합성하였다. 'pMAL™ protein fusion and purification system'과 *E. coli* BL21 (DE3)를 이용하여 재조합 단백질을 발현시켜, maltose binding protein (MBP)를 포함한 재조합 퓨전 maSOD 를 얻어내었다. 그 다음, 이 재조합 단백질의 최적온도와 최적 pH, maSOD 의 금속특성을 확인하였다. 효소활성 실험에서 maSOD 는 0℃에서 가장 높은 활성을 보였고 0 ~ 15℃에서 높은 활성을 가졌으며 20℃ 이후부터 그 활성이 급격히 감소하였다. maSOD 의 최적 pH 는 7.5 이며 pH 7.0 에서 pH 10 까지 전반적으로 높은 활성을 보였다. Fe-SOD 와 Mn-SOD 가 유사함에 따라 이를 분류하기 위한 실험으로 특정억제제를 이용하여 금속특성을 확인 할 수 있다. 억제제인 H_2O_2 와 NaN_3 를 처리하였을 때 H_2O_2 를 처리한 maSOD 가 억제제를 처리하지 않은 대조구에 비해 활성이 급격히 억제됨을 알 수 있었다. 이를 통해 maSOD 가 Mn-SOD 와 Fe-SOD 의 중간형태를 지녔으나 그 활성을 보아 Fe-SOD 임을 확인할 수 있었다.

Mianiensis avidus GPX (maGPX)는 총길이 901bp 로 684 bp 의 ORF 를 포함하고 있으며, 227 개의 아미노산을 암호화하고 있었다. 분자량은 25 kDa 이고 pI 는 6.1 로 분석되었다. maGPX 아미노산 서열을 NCBI protein BLAST program 과 Motif scan, SMART program 으로 분석하였을 때, 활성부위에 Se-Cye 대신 보통의 cysteine 을 갖는 seleno-independent GPX 로 분류되었다. 또한 포유류의 NS-GPX 달리 1-cys peroxiredoxin (1-cys PRx)의 기능을 가진 것으로 나타났다. maGPX 는 알려진 seleno-independent GPX 및 1-cys PRx 와의 비교를 통해, 보존된 활성부위인 PVCTTE 를 포함하고 있는 것이 확인되었고, 1-cys PRx 의 기능 중 phospholipase A_2 활성을 나타내는 유사한 서열도 포함하고 있었다. Pairwise ClustalW 분석을 통해 maGPX 는 알려진 NS-GPX 와 1-cys PRx 들과 56 %에서 73 %의 큰 유사도를 보였으며 계통수에서는 Se-Cys 을 포함한 GPX 와는 다른 가지에 속해있었다. maGPX 의 단백질적 특성을 확인하기 위해 maSOD 와 같은 방법으로 재조합단백질을 얻어내었다. 단백질 활성실험의 기준을 세우기 위한 선행실험에서 재조합 maGPX 가 대조구와 비교했을 때 GPX 활성이 없는 것으로 나타났다. 이는 여러 연구에서 재조합 NS-GPX 나 1-cys PRx 가 in vitro 상에서 peroxidase 활성이 나타나지 않을 수도 있다는 것이 보고된 적이 있다. 활성에

관한 실험은 아직 수행 중에 있으며 추가적으로 GPX 활성과, phospholipase 활성 등을 확인하고자 한다.

maSOD 와 maGPX 유전자 동정을 통해 *Mianiensis avidus* 또한 항산화 효소로써 SOD 와 GPX 를 발현시킨다는 것을 알 수 있었다. 이 논문을 위해 *Mianiensis avidus* 에 산화적 스트레스를 주었을 때 실제로 이들 유전자가 특이적으로 발현되는지 확인하고자 했으나, 실험적으로 큰 유의성을 보이지 않았다. 이는 아직 이 종에 대한 in vivo 실험조건과 기준이 제대로 확립되어있지 않았고, 산화적 스트레스를 줄 수 있는 조건이 확인되지 않았기 때문인가 추측하고 있다. 앞으로 기생섬모충 중 가장 큰 영향력을 보이는 *Mianiensis avidus* 가 실제 숙주 면역체계에 대해서 어떠한 반응을 보이는지에 대한 여러 분자유전학적 및 면역학적, 생화학적 연구가 진행되어야 할 것이다. 이 논문은 그러한 연구의 기초단계로써 항산화 효소의 유전자를 동정했다는 의미를 가진다고 할 수 있다.

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Introduction

1. Scuticociliate, *Miamienesis avidus*

Scuticociliates are recognized as serious infectious parasite that causes important economic losses in marine aquaculture worldwide including Korea. They were reported in farming fishes such as olive flounder *Paralichthys olivaceus* (1993), turbot *Scophthalmus maimus* (1994), sea bass *Dicentrarchus labrax* (1995), southern Bluefin tuna *Thunnus maccoyii* (1997), seahorse *Hippocampus erectus* (1964) and silver pomfret *Pampus argenteus* (2007). Especially farming of olive flounder, *Paralichthys olivaceus* in Jeju Island, South Korea is taken heavy damage in production from 1990s in consequence of increasingly high mass mortalities by disease of ciliates, term scuticociliatosis. The ciliates occur in fish tissues and organs including gill, skin, heart, brain, muscles and intestine. Scuticocliatosis is highly histophagous and destroys infected tissues and organs. These are known mainly histophagous ciliates such as *Miamiensis avidus*, *Philasterides dicentrarchi*, *Uronema nigricans*, *Uromema* sp. and *Tetrahymena* sp. in cultivated marine fishes. However, it is not clear whether all the scuticociliates species cause mortalities and which species invade first and main.

Miamiensis avidus is strong invader of histophagous scuticociliates into the host fishes directly from seawater, causing high mortality. This ciliate was reported from Olive flounder, *Paralichthys olivaceus* by Jung et al. (2005) in South Korea and identified as *Philasterides dicentrarchi* through small subunit ribosomal RNA (SSU rRNA) gene sequencing of scucicoclilates 8 strains isolated from olive flounder by Jung et al. (2005). *M. avidus* is a drastic pathogen that can lead to primary infection and rapidly invade and proliferate in the skin and gills. In infected fish we can confirm the large

numbers at these tissues. Then they eat host cells and body tissues and spread to the other internal organs, quickly. According to Jung et al. (2007), invading *M. avidus* once to the host, they should spread at high speed via blood vessels or an oral route to other tissues and raise a systemic infection. After all *M. avidus* exhibit systemic invasion of brain, digestive tract, liver, kidneys and gonads.

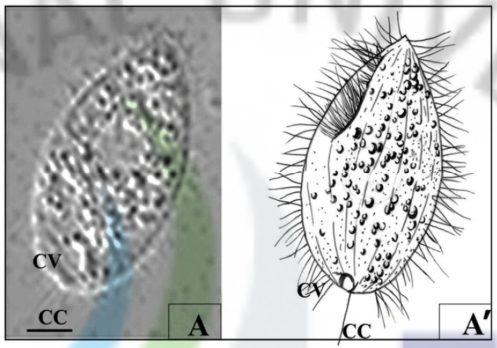


Figure 1. Picture and diagram of *Miamiensis avidus* isolated from olive flounder. CC: caudal cilium; CV: contractile vacuole. Scale bars = 5μm. The ciliate picture and diagram was adapted from Jung et al. (Jung et al., 2007).

2. Antioxidant enzymes

In aerobic organisms, ROS (Reactive oxygen species) such as the superoxide anion radical ($O_2\cdot^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($HO\cdot$) are generated by metabolic reaction. In particular several organisms need to protect against ROS that biotransformation process from exposure to radiation, redox-cycling drugs, toxins, antibiotics or stimulated host phagocytes. ROS cause damage to all biological

macromolecules by oxidation of proteins, lipids and nucleic acid, depolymerization of polysaccharides, DNA modifications and strand breaks, etc. As a result this is lead to the modification of membranes, receptors, cytoskeleton proteins, enzymes inactivation and damage to the genome, seriously. Therefore, organisms have developed enzymatic and non-enzymatic antioxidant systems for protection against these ROS and repair systems of ROS-derived damage. The well-known antioxidant enzyme families in eukaryotic organisms include superoxide dismutases (SODs), glutathione peroxidases (GPXs), and catalases (CATs) and peroxiredoxins (PRXs), etc.

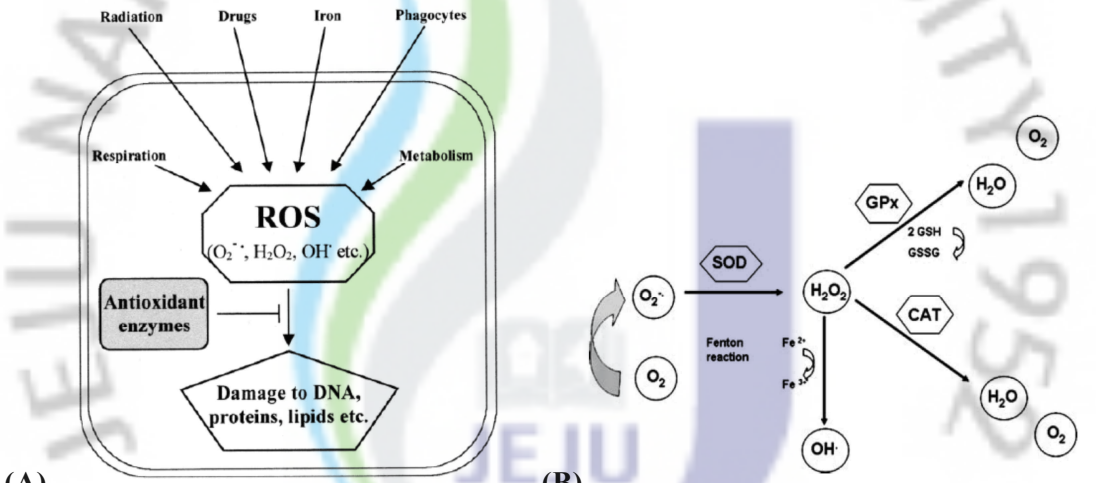


Figure 2. Reactive Oxygen species (ROSs) and antioxidant systems. (A) Schematic and simplified presentation of the processes leading to ROS and the defense against them. (B) Enzymatic activity of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidas (GPX). (A) and (B) was adapted from Henkle-Dührsen et al (Henkle-Dührsen et al, 2001) and Chiumiento et al (Chiumiento et al, 2009), respectively.

SOD is metalloenzyme which catalyze the dismutation of superoxide radicals ($O_2\cdot^{-}$) to H_2O_2 and oxygen (O_2). SOD family is classified four groups depending on their metal cofactors; (i) cytosolic CuZn-SOD containing copper and zinc, (ii) Mn-SOD containing manganese, (iii) Fe-SOD containing iron, (iv) Ni-SOD containing nickel.

GPX is the common name for a family of multiple isozymes that catalyze the reduction of H_2O_2 or organic hydroperoxides to water or alcohols using reduced glutathione (GSH), H_2O_2 +2 GSH → GSSH + $2H_2O$. In mammals, there are known GPXs groups which contain reactive selenocysteine (Se-Cys) residue in its active site. These Se-Cys containing GPXs (selenoGPXs) are regenerated to their reduced form by reduced GSH (Dayer et al., 2008). However seleno-independent GPXs, also named non-selenium GPX (NS-GPX), are isolated from yeast, plant, cyanobacteria and some animals. The NS-GPX has cysteine residue at active site in place of the Se-Cys.

3. Host and parasite immune interaction

Many parasites have the best strategies to avoid host immune system such as DNA repair system, substrate scavenging, and an antioxidant enzyme system. These strategies involve restriction of reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (NOIs) (Sitjà-Bobadilla, A., 2008). Parasites can inhibit the phagosomes and lysosomes from macrophage cells using secreting enzymes. The antioxidant enzyme of parasite is a well-known defense and protection system against host attack of oxidative killing by immune cells. Especially SOD and GPX are effective to inactivate two important ROIs: superoxide anion and hydrogen peroxide. From scuticociliates parasitizing fish, it have been reported that this ciliates have scavenging

activity against intracellularly produced ROIs and protection skill from host-phagocyte-mediated oxidative damage as to destroy phagocytes (Leiro et al., 2004; kwon et al., 2002).

4. Propose

In the recently worldwide aquaculture, diseases from pathogenic organisms include parasites has increased with serious risks to fish and shellfish production. Accordingly research of fish immune systems has developed steadily and also knowledge of the immune response against fish parasites. However study about scuticociliatic parasite such as *M. avidus* has not improved thoroughly. Identification of ribosomal RNA, pathogenicity and response against host immune system of *M. avidus* were just reported by Jung et al (2005, 2007), Song et al (2009) and J. Leiro et al (2004, 2008). For more understanding *M. avidus* and interaction with host immune, we need to perform the molecular genetics and immunologic research.

In this study as basically molecular genetics step, we cloned superoxide dismutase gene (maSOD) and glutathione peroxidase gene (maGPX) from *M. avidus*. These genes were known as antioxidant to protect from ROSs and host attack, example phagocytosis. Through this analysis of *M. avidus* antioxidant genes, we will be more understanding how can survive *M. avidus* from the marine or aquaculture environment and host immune system.

Part I. Molecular cloning and characterization of superoxide dismutase (SOD) gene in scuticociliate, *Miamiensis avidus*

Abstract

Scuticociliates are regarded as serious pathogens in marine aquaculture worldwide, causing mass mortalities in fish and crustaceans such as olive flounder, turbot, sea bass, American lobster and seahorse. The scavenging activity against intracellularly produced reactive oxygen intermediates (ROIs) of the host has been reported for these organisms. For inhibition of ROIs: superoxide anion ($O2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-), scuticociliates have enzymes, such as superoxide dismutase (SOD), glutathione peroxidase, catalase and peroxiredoxin. The SOD is one of the antioxidant enzymes involved in cellular defense against oxidative stress and catalyzes the conversion of superoxide ($O_2^{\cdot -}$) into the H_2O_2. To improve our understanding that scuticociliatida how can survive from the host immune system, we have characterized the SOD gene of one scuticociliate strain, *Miamiensis avidus*. The full-length cDNA of *M. avidus* SOD was cloned using degenerate primers by the method of 3' and 5' rapid amplification of cDNA ends PCR (RACE PCR). The full length sequence was analyzed using BLAST and standard analytical programs. The nucleotide sequence consisted of 958bp with a 5' UTR of 165bp, and 3' UTR of 130bp with a poly-A tail. The *M. avidu*s SOD open reading frame encoded 663bp, which corresponded to 220 amino acids. Predicted molecular mass and theoretical isoelectric point (pI) were 25 kDa and 6.65, respectively. The protein sequence was compared for sequence similarity with known homologous genes such as superoxide dismutase from *Tetrahymena thermophila* (81%), iron superoxide dismutase from *Tetrahymena*

pyriformis (75%), *Plasmodium vivax* (46%) and *Escherichia coli* (47%), that were available in GenBank. We confirmed biochemical characterization of SOD by enzyme assay and metal identification of recombinant *M. avidus* SOD protein. From 0 to 15 ℃, the recombinant protein had 100 to 90 % relative activity. The optimal pH was 7.5 and it was stable at pH 7.0 to 10.0 with more than 80% relative activity. The recombinant enzyme was sensitive by H_2O_2 but not by NaN_3.

Materials and Methods

1. Selecting superoxide dismutase gene and design primers

Our laboratory have constructed *M. avidus* cDNA sequence database by the genome sequence FLX^{TM} (GS-FLX) genome sequencing technique. After GS-FLX analysis, we found a partial sequence of superoxide dismutase and designed primers for full-length sequence analysis by 5'- and 3'-rapid amplification of cDNA ends (RACE) Polymerase Chain Reaction (PCR) (Table 1).

Table 1. Primers designed for RACE PCR of superoxide dismutase

Primer name	Primer sequence	Tm(℃)
maSOD F1 primer (3'-end)	5'- ATAGAAGACGCTACCGCCTAAGGA -3'	59.0
maSOD F2 primer (3'-end)	5'- TGCCGCCATTTAAGGAAGTGGATG -3'	59.8
maSOD R1 primer (5'-end)	5'- CGTCAGCCCAGTTGACAATTTACC -3'	58.3
maSOD R2 primer (5'-end)	5'- ACTTCCTTAAATGGCGGCAGCTCT -3'	60.9

2. PCR amplification of maSOD gene

2.1. Miamienesis avidus

M. avidus (YS2) was isolated from olive flounder by Jung et al. (2005). For YS2 culture, the Chinook salmon embryo-214 (CHES-214) cells (CRL-1681™, ATCC) were cultured in MEM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 IU penicillin (Gibco, USA) and 100 μg/ml streptomycin (Gibco, USA) at 20℃. After 3 to 7 days, YS2 was grown in the CHSE-214 cells medium at 15℃. Growth was continued for around 1 week to stop of cell division. The YS2 were harvested by centrifugation at 3000 rpm, 30 minutes, 4℃. The cells were immediately snap-frozen in liquid nitrogen and stored at -70℃.

2.2. RNA extraciton and cDNA synthesis

The total RNA was extracted from pooled cells using Tri Reagent™ (Sigma, USA) according to the manufacturer's protocol. The RNA concentration was measured at 260nm in a UV-spectrometer (Bio Rad, USA). Originally purified RNA was diluted up to a concentration of 1 μg / μl and for RACE PCR 4 μg RNA was used to synthesize cDNA using a SMART™ RACE cDNA Amplification protocol.

2.3. RACE PCR and Cloning into easy T-vector

The procedures for the generation of the 5' and 3' regions for partial maSOD sequence were carried out consulting the commercial protocol for the rapid amplification of 5' and 3' cDNA ends (SMART™ RACE cDNA Amplification kit, Clontech Laboratories,

Inc., USA) using gene specific primers. RACE PCR primers were diluted to 10 pmole per 1μl with reaction water.

First 3'-RACE PCR was carried out with 1 μl of synthesized cDNA, 1 μl of 2 μM Universal Primer A Mix (UPM) (Clontech Laboratories, Inc., USA), 1 μl of maSOD F1 primer, 1 μl of 10mM dNTP mix (Clontech Laboratories, Inc., USA), 5 μl of 10X *Ex Taq* Buffer (Mg^{2+} plus) (TaKaRa, Japan), 1 μl of TaKaRa *Ex Taq*^{TM} DNA polymerase (5U/ μl) (TaKaRa, Japan) and 40 μl of reaction water. PCR conditions for first step were: 1 cycle at 94℃ for 2 min; 25 cycles at 94℃ for 30 sec, 68℃ for 30 sec, 72℃ for 3 min; 1 cycle at 72℃ for 5 min. The 1st 3'-RACE PCR product was diluted to 50 times with reaction water. For second PCR of nested reaction, 5 μl of the diluted 1st 3'-RACE PCR product was mixed with 1 μl of 10 μM Nested Universal Primer A (NUP) (Clontech Laboratories, Inc., USA), 1 μl of maSOD F2 primer, 1 μl of 2 μM Universal Primer A Mix (UPM) (Clontech Laboratories, Inc., USA), 1 μl of maSOD F1 primer or maSOD R1 primer, 1 μl of 10mM dNTP mix (Clontech Laboratories, Inc., USA), 5 μl of 10X *Ex Taq* Buffer (Mg^{2+} plus) (TaKaRa, Japan), 1 μl of TaKaRa *Ex Taq*^{TM} DNA polymerase (5U/ μl) (TaKaRa, Japan) and 36 μl of reaction water. Second PCR conditions were same with the 1st 3'-RACE PCR.

For first 5'-RACE PCR, 1 μl of synthesized cDNA was mixed with 1 μl of 2 μM UPM, 1 μl of maSOD R1 primer, 1 μl of 10mM dNTP mix, 5 μl of 10X Advantage 2 PCR Buffer (Clontech Laboratories, Inc., USA), 1 μl of 50X Advantage 2 Polymerase Mix (Clontech Laboratories, Inc., USA) and 40 μl of reaction water. PCR conditions for first step were: 1 cycle at 94℃ for 1 min; 25 cycles at 94℃ for 30 sec, 68℃ for 30 sec, 72℃

for 2 min; 1 cycle at 72℃ for 1 min. Second RACE PCR was carried out on the 1st 5'-RACE PCR conditions with 1 μl of the 1st 5'-RACE PCR product, 1 μl of NUP, 1 μl of maSOD R2 primer, 1 μl of 10mM dNTP mix, 5 μl of 10X Advantage 2 PCR Buffer, 1 μl of 50X Advantage 2 Polymerase Mix and 40 μl of reaction water.

The generated cDNA fragments were inserted into pGEM-T easy vector (Promega, USA) and determined by a sequencing reaction using the termination kit, Big Dye, and an ABI 3700 sequencer (Macrogen Co., Korea). After sequencing analysis, we got the full-length maSOD cDNA sequence.

2.4. Sequence analysis

The NCBI BLAST program was used to search for nucleotide and protein sequence similar to maSOD. The SOD family sequence domains were determined by means of a motif scan program (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and SMART sequence analysis progrom (http://smart.embl-heidelberg.de/smart). Pair-wise and multiple sequence alignments of the maSOD protein with known SOD proteins were analyzed, using ClustalW version 2.0. The amino acid sequences were aligned using ClustalW software and phylogenetic tree was constructed using Neighbor-Joining method from the MEGA 4.0 software. The bootstrap values were replicated for 1000 times to obtain the confidence value for the analysis.

3. Cloning and expression of maSOD

3.1 Synthesis maSOD gene for expression

3.1.1 Analysis codon usage for over-expression by E. coli BL21 DE3

Expression of ciliate gene in bacteria, E. coli, is limited by codon usage (Lin et al., 2002). We analyzed maSOD codon usage and changed maSOD codons, which are rare codons in bacteria, to E. coli codons for protein expression in E. coli BL21 DE3. Glutamine TAA, normally stop codon in bacteria, to CAG and Glycine GGA to GGT, Arginine AGA to CGT, Proline CCC to CCG, Isoleucine ATA to ATC, we changed for designed assembly PCR oligodeoxynucleotides.

3.1.2 Design assembly PCR oligodeoxynucleotides

Using Aseembly PCR Oligo Maker on the web at http://publish.yorku.ca/~pjohnson/AssemblyPCRoligomaker.html by Rydzanicz et al (2005), we designed fourteen assembly PCR oligodeoxynucleotides and two flanking primers, shown Table 2. Flanking forward primer and reverse primer was made to include the digestion enzyme site for cloning into pMal-c2x vector for over-expression, BamH I (ggatcc) and Pst I (ctgcag), respectively. Oligodeoxynucleotides ranging from 61 to 80 were synthesized by Integrated DNA Technologies (IDT, USA).

1 **Table 2. Oligodeoxynucleotides designed for superoxide dismutase assembly PCR**

Name	Oligodeoxynucleotide sequence
SOD assembly-1	5'-ATGAAATCTCTTACTAAATTAATTCGTTATCGTTTTTCTTCTTATACTCCTGCCACTTTAC-3'
SOD assembly-2	5'-GCTGATAAAACCGGTTCTAATTCACCATATTCAAAATCCAACTTAGGTAAAGTGGCAGGAGTATAAGAAGAAA-3'
SOD assembly-3	5'-GTGAATTAGAACCGGTTTTATCAGCTAATTTATTATCTTTCCACCACGGTAAACACCATCAGACTTATGTC-3'
SOD assembly-4	5'-GCGGTAGCGTCTTCGATCTGTTTGTAAATTGCATTTAAGTTGTTGACATAAGTCTGATGGTGTTTACC-3'
SOD assembly-5	5'-ATCGAAGACGCTACCGCCCAGGGTGACGCCAAAAAAGTTGCTTCATTACAACCGGCCTTACGTTTC-3'
SOD assembly-6	5'-GGGCTAAGTTTTTCCAGTAAATAGAGTGGTTCAAGTGACCACCTAAATTGAAACGTAAGGCCGGTTGTAA-3'
SOD assembly-7	5'-CTCTATTTACTGGAAAAACTTAGCCCCTAAAGGTAAAGGTGGTGGTGTTTTACCTGAAGCTTCTTCTCCTCTT-3'
SOD assembly-8	5'-TTGCATAAAACTTTCATAACCACCATACTGCTGTTGAATAGCCTGAGAAAGAGGAGAAGAAGCTTCAGGTAAA-3'
SOD assembly-9	5'-GTATGGTGGTTATGAAAGTTTTATGCAAGAATTCAACAAACGTGCTGCCGCCATTCAGGGTAGTGGTTGGG-3'
SOD assembly-10	5'-TAGCTAATTCAAATAAACGCAAATTTTTATTGACAGTGTCATAGGCTAACCAACCCCAACCACTACCCTGAAT-3'
SOD assembly-11	5'-GTCAATAAAAATTTGCGTTTATTTGAATTAGCTAATCAGGAAATCCCTGATTTTTCTGATTGCATCCCTTTATTAACTAT-3'
SOD assembly-12	5'-TTAACGTTCTGGTAATCAAGATAGAAAGCATGTTCCCACATATCAATAGTTAATAAAGGGATGCAATCAGAAAAATC-3'
SOD assembly-13	5'-CTTTCTATCTTGATTACCAGAACGTTAAAGTTAAATATTTAAGCGATATCTGGCAGATTGTCAACTGGGCT-3'
SOD assembly-14	5'-TCAATTCTGTTTAATAGCATCAAGATAACGTGCTTCAACGTCAGCCCAGTTGACAATCTGCC-3'
Flanking-Forward	5'-gagagaggatcc (BamH I) ATGAAATCTCTTACTAAATTAATTCGTTATCGTT-3'
Flanking-Reverse	5'-tctctcctgcag (Pst I) TCAATTCTGTTTAATAGCATCAAGATAACG-3'

3.1.3 Assembly PCR

Assembly PCR oligodeoxynuclotieds were diluted to 10 pmole per 1 μl and flanking primers to 20 pmole per 1 μl with reaction water. First assembly PCR was carried out with 2 μl of each oligo, 4 μl of 2.5mM dNTPs, 5 μl of 10X Herculase reaction buffer (Stratagene, USA), 0.5 μl of Herculase hotstart DNA polymerase (500U/100μl) and 12.5 μl of reaction water. PCR conditions for first step were: 1 cycle at 94℃ for 7 min; 10 cycles at 94℃ for 1.5 min, 54℃ for 2 min, 72℃ for 3 min; 1 cycle at 72℃ for 5 min. For second PCR, 1 μl of the first PCR product was mixed with 2 μl of flanking forward primer, 2 μl for flanking reverse primer, 4 μl of 2.5mM dNTPs, 5 μl of 10X Herculase reaction buffer (Stratagene, USA), 0.5 μl of Herculase hotstart DNA polymerase (500U/100μl) and 35.5 μl of reaction water. Second PCR conditions were: 1 cycle at 94℃ for 5min; 30 cycles at 94℃ for 30 sec, 54℃ for 1 min, 72℃ for 1.5 min; 1 cycle at 72℃ for 5min. The last PCR for amplification of assembly PCR product was performed with 1 μl of diluted 10 times 2^{nd} PCR product, 4 μl of flanking forward primer, 4 μl for flanking reverse primer, 8 μl of 2.5 mM dNTPs, 10 μl of 10X LA PCR^{TM} buffer II (Mg^{2+} free) (TaKaRa, Japan), 10 μl of 25mM $MgCl_2$ (TaKaRa, Japan), 1 μl of *TaKaRa LA taq* ^{TM} polymerase (5 unit/μl), 62 μl of reaction water. Third PCR steps were: 1 cycle at 94℃ for 5 min; 10 cycles at 94℃ for 30 sec, 54℃ for 1 min, 72℃ for 1.5 min; 1 cycle at 72℃ for 5 min. The PCR products were analyzed by 1% agarose gel electrophoresis.

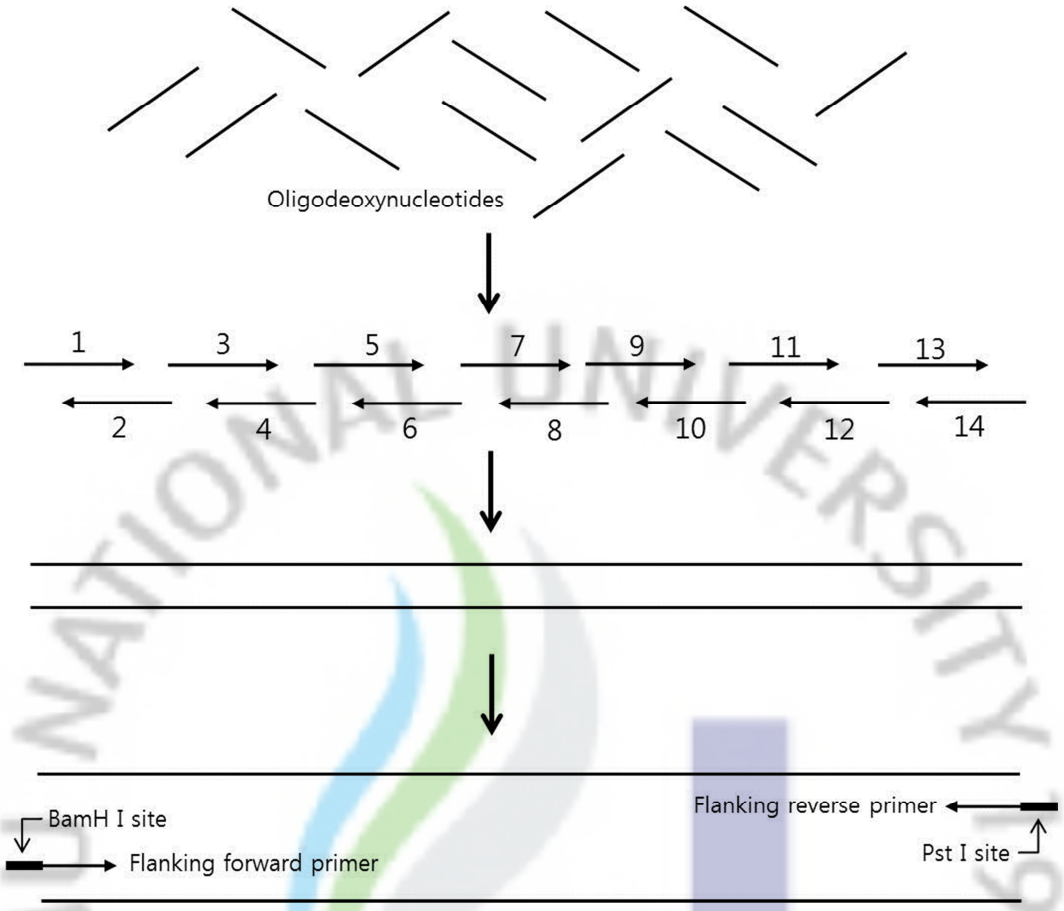


Figure 3. The assembly PCR steps for amplification of recombinant maSOD gene

3.2 Cloning of maSOD ORF into the pMAL-c2x expression vector

The aseembly PCR product was purified using the Accuprep^{TM} gel purification kit (Bioneer Co., Korea) and both insert and pMAL-c2x (NEG, USA) were digested with the respective restriction enzymes. The ligated product was transformed into competent cells of *E. coli* BL21 (DE3).

3.3 Over-expression and purification of recombinant maSOD

The recombinant maSOD was over-expressed in *E. coli* BL21 (DE3) cells in the presence of isopropyl-β-thiogalactopyranoside (IPTG). Briefly, a 5 ml of *E. coli* BL21 (DE3) starter culture was inoculated into 500 ml Luria broth with 500 μl ampicillin (100 mg/ml) and 1 g glucose (0.2 % final concentration). The culture was incubated at 25℃ while shaken at 180 rpm until the cell count reached 0.5 at 600nm and induced by IPTG at 0.5mM final concentration. After 6hours of induction, the cells were cooled on ice for 30 min, and harvested by centrifugation at 3500 rpm for 30 min at 4 ℃, after which the supernatant was removed. The recombinant fusion protein, which was fused with maltose binding protein (MBP), was purified by means of the pMAL^{TM} protein fusion and purification system.

3.4 SDS-PAGE

The purified protein was collected in 500 μl aliquots, and respective samples were run on 12% SDS-PAGE with a protein molecular weight marker (low) (TaKaRa, Japan). Gels were stained using 0.05 % coomassie Blus R-250, followed by a standard de-staining procedure. All the activity tests mentioned in this study were conducted using this purified recombinant maSOD fused with MBP. The concentration of the purified

protein was determined via the Bradford method, using bovine serum albumin (BSA) as the standard.

4. Enzyme assay of recombinant maSOD

4.1 Superoxide dismutase activity assay

The maSOD activity was determined using the xanthine oxidase method described by Ekanayake et al (2006). The reaction mixtures contained 480 µl of 50mM sodium carbonate buffer (pH10.5), 20 µl of 3mM Xanthine (sigma, USA), 20 µl of 3mM EDTA, 20 µl of 0.15% BSA (Sigma, USA), 20 µl of 0.75mM NBT (sigma, USA) and 20 µl of maSOD enzyme sample. After incubation at 25℃ for 10 min, the reaction was started by adding 6 mU xanthine oxidase (sigma, USA) and maintaining at 25℃ for 20 min. The reaction was finished by adding 20 µl fo 6mU CuCl. At 560nm, the absorbance was checked in a UV-spectrometer (Bio Rad, USA). One unit of enzyme activity was considered as the amount of the enzyme required to reduce the reaction by 50%. The protein concentration was determined by the Bradford assay using BSA as the standard.

4.2 Optimal temperature

The optimal temperature was carried out at 0, 5, 10, 15, 20, 25, 30, 35 and 40℃ at standard pH and incubation time.

4.3 Optimal pH

The optimal pH of maSOD was tested at pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10, and 10.5 by standard assay method. 50mM phosphate buffers were used for pH 6.0 to pH 8.5 and for pH 9.0 to pH 10.5, 50mM carbonate buffers were used.

4.4 Identification metal cofactor of maSOD

To identify the metal cofactor of maSOD, recombinant protein was mixed with 10mM sodium azide (NaN_3) or 10mM hydrogen peroxide (H_2O_2) and incubated at 20℃ for 30 min. After incubation, the enzyme activity was assayed and residual enzyme activity was compared to the control without any inhibitor. Normally, Fe-SOD activity is inhibited by H_2O_2, but Mn-SOD is insensitive to H_2O_2 (Barra et al., 1990; Mehlotra, R. K, 1996).

Results

1. Cloning and sequence analysis of maSOD

The full-length maSOD gene was completely cloned by RACE PCR with specific primers designed based on the *M. avidus* SOD partial sequence from GS-FLX result. The nucleotide sequence and the deduced amino acids sequence are shown Figure 4. The full-length maSOD gene is 958bp including an open reading frame (ORF) of 663bp, encoding 220 amino acid residues. The molecular weight and isoelectric point (pI) are 25 kDa and 6.65, respectively.

The maSOD amino acid sequence was confirmed to have two putative conserved Fe-SOD domains by NCBI BLAST and SMART program. At position 17 to 98 and position 105 to 213 were each shown as Fe-SOD N-terminal domain and Fe-SOD C-terminal domain. The four amino acid residues known to be putatively required for binding to Fe or Mn, His-42, His-90, Asp-179 and His-183 were conserved in the maSOD sequence (Figure 4). The ^{82}F-N-L-G-G-H-L-N-H^{90}-S-I-Y-W^{94} motif was shown as characteristic motif that distinguishes Fe-SOD and Mn-SOD. According to Jackson and Cooper (1998), Fe-SOD and Mn-SOD have (F-N-N-A-A-Q)-X-(W-N)-H-X-(F-Y)-W motif and (Base-F-N-G-G)-G-(H)-X-(N)-H-X-(L-F)-W, respectively. As shown figure 5 the (F-N-N-A-A-Q)-X-(W-N)-H-X-(F-Y)-W motif is conserved on the others Fe-SOD, but the maSOD amino acid sequence has a middle type motif of these conserved motifs. Also the G-S-G-W-X-W-L consensus sequence was identified at position 141 to 147 amino acids. The ^{179}D-M-W-E-H^{183}-A-F-Y^{186} was analyzed a putative Fe or Mn SOD signature.

The results of pairwise ClustalW analysis using the maSOD deduced amino acid sequence with selected known Fe or Mn SOD proteins are shown in Table 3. The maSOD exhibited the high level of similarity percentage with *Tetrahymena thermophlia* SOD (81.5%) and Tetrahymena pyriformis Fe-SOD (75.9 %), *Dictyostelium discoideum* AX4 SOD (62.2 %), *Hydro vulgaris* Mn-SOD (62 %), *Spirometra erinaceieuropaei* Mn-SOD (61.8 %), *Caenorhabditis elegans* Mn-SOD (60.2 %). Generally similarity percentages with others Fe or Mn SOD were shown ranging 45 to 59 %. Interestingly the maSOD was similar both others Fe-SOD and Mn-SOD. Multiple sequence alignment by ClustalW of maSOD and others SOD protein are shown in figure 5. These results show that maSOD has higher conserved sequence or motif with others SOD. After all the analysis, we concluded that type of maSOD is Fe-SOD. To improve this result we performed enzyme assay of identification metal cofactor.

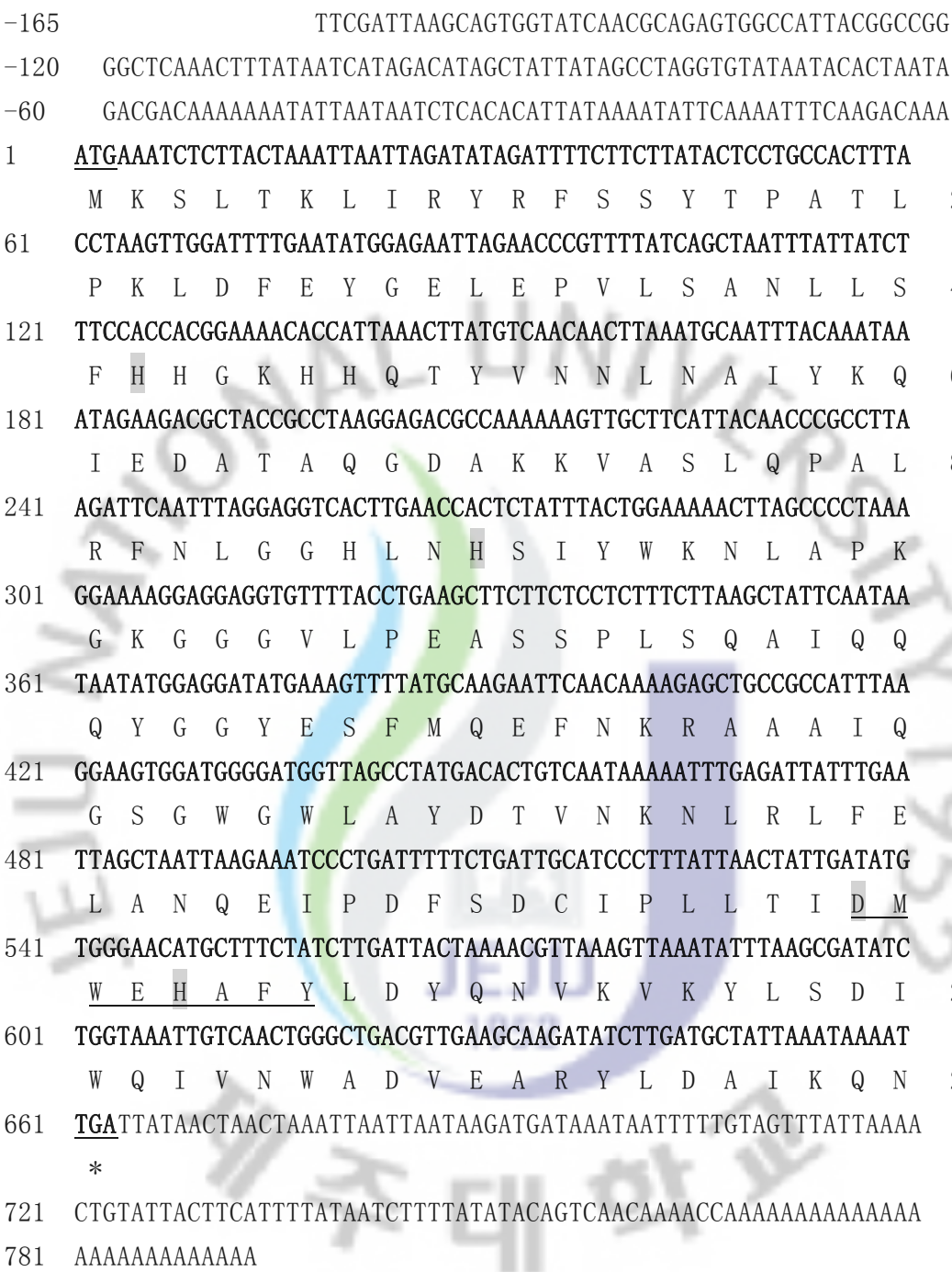
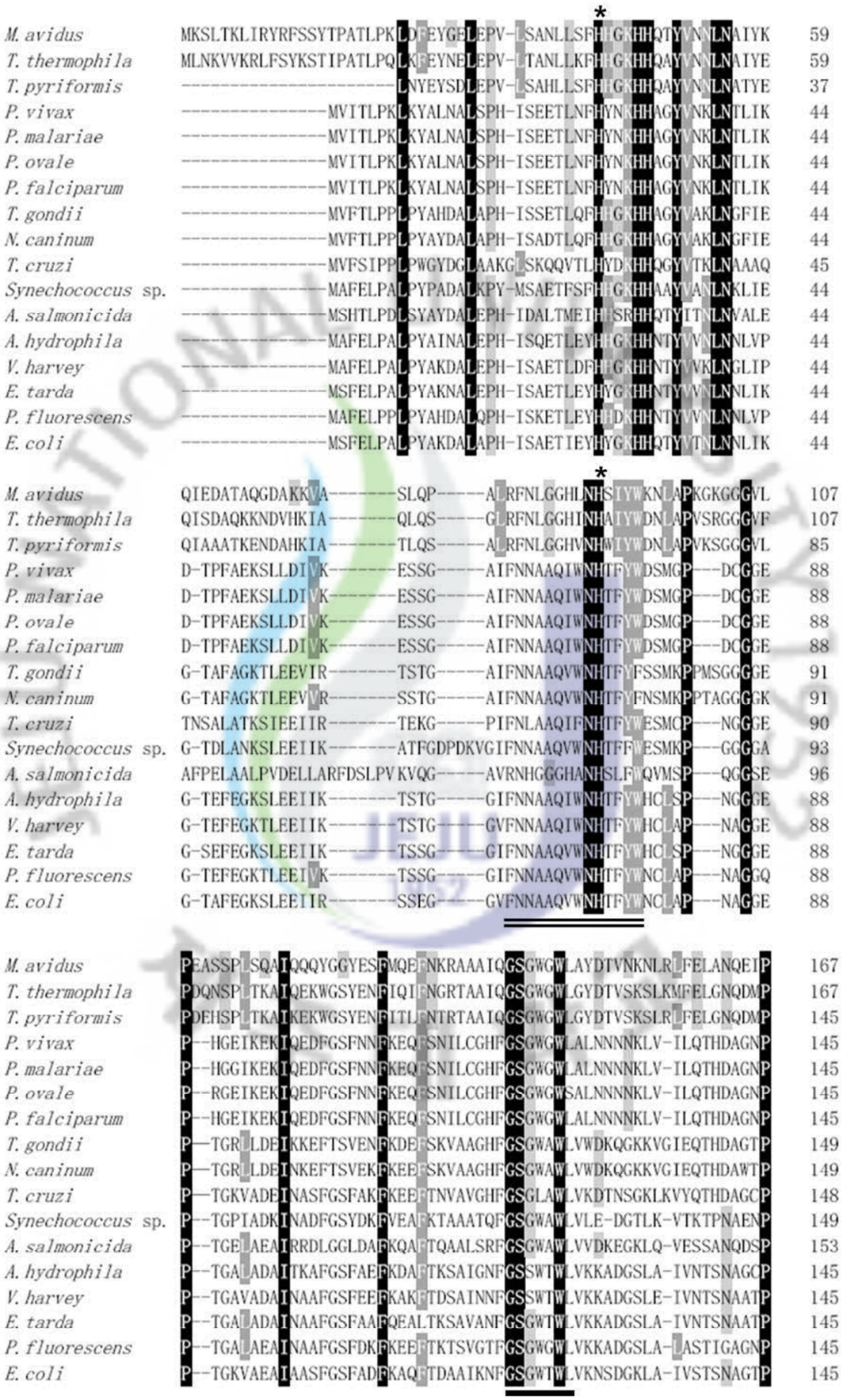


Figure 4. Nucleotide sequence and the deduced amino acid sequence of maSOD. The coding sequence (from 1 to 663) is in bold letters. Amino acid sequence corresponding to mature protein consists of 220 amino acids from start codon ATG to stop codon TGA (*). The shade box is marked as the amino acid residues for binding to Fe. The putative Fe-SOD signature is indicated by *underline*.



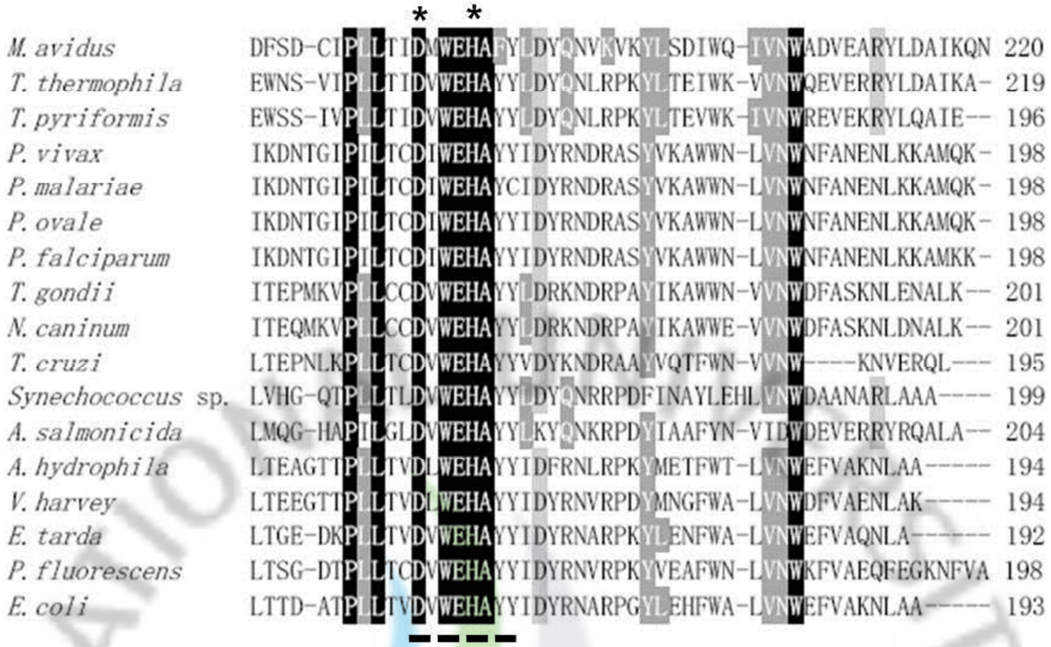


Figure 5. Multiple sequence alignment of predicted maSOD amino acid sequence with known Fe-SODs. Residues shaded are conserved across all species aligned sequences. The four amino acid residues responsible for binding to Fe are indicated by *asterisks* (*). The *bold double line* on the sequence is the (F-N-N-A-A-Q)-X-(W-N)-H-(F-Y)-W motif of the critical characteristics that distinguish Fe-SOD and Mn-SOD (Jackson and Cooper, 1998). The G-S-G-W-X-W-L motif is marked as *bold single line* on the sequence. The *bold dotted line* is the putative Fe-SOD signature. Accession numbers for the amino acid sequence of SODs are the same as in Table 3.

Type of SOD	Species	Gene Bank accession No.	Protein length (amino acid)	Similarity percentage (%)
SOD	*Tetrahymena thermophila*	XP_001010506	219	81.5
Fe-SOD	*Tetrahymena pyriformis*	P19666	196	75.9
Fe-SOD	*Plasmodium vivax*	AF139529	198	46.2
Fe-SOD	*Plasmodium malariae*	AF139528	198	45.8
Fe-SOD	*Plasmodium ovale*	AF139527	198	45.8
Fe-SOD	*Plasmodium falciparum*	Z49819	198	46.2
Fe-SOD	*Toxoplasma gondii*	XP_002364798	201	45.2
Fe-SOD	*Neospora caninum*	AAL62028	201	47.2
Fe-SOD	*Trypanosoma cruzi*	U39401	195	45.7
Fe-SOD	*Synechococcus sp.* JA-2-3B	YP_478710	199	47.4
SOD-A	*Aeromonas salmonicida* subsp. A449	AAP85516	204	53.1
Fe-SOD	*Aeromonas hydrophila*	AAL26891	194	45.6
Fe-SOD	*Vibrio harveyi* iDA3	ZP_06176456	194	44.6
SOD	*Edwardsiella tarda* ATCC23685	ZP_06714789	192	46.7
Fe-SOD	*Pseudomonas fluorescens* Pf-5	AAY94056	198	45
Fe-SOD	*Escherichia coli* strain K-12	AC_000091	193	47.1
Mn-SOD	*Spirometra erinaceieuropaei*	AAT79388	222	61.8
Mn-SOD	*Paragonimus westermani*	AAT79387	222	59.4
Mn-SOD	*Hydra vulgaris*	ABC25024	219	62
Mn-SOD	*Danio rerio*	AY195857	224	58.9
Mn-SOD	*Homo sapiens*	X14322	222	58.3
Mn-SOD	*Caenorhabditis elegans*	D85499	221	60.2
SOD	*Dictyostelium discoideum* AX4	XP_645815	226	62.2

Table 3. Analysis of the deduced amino acid similarity percentage of maSOD with parasitic protozoa, bacteria and other Fe or Mn SOD by EMBOSS Pairwise Alignment Algorithms (http://www.ebi.ac.uk/Tools/emboss/align/).

2. Phylogenetic analysis of maSOD

To determine the evolutionary relationship of maSOD with other known SODs, a phylogenetic tree was composed as figure 6 using the Neighbor-joining method in MEGA 4.0. Phylogenetic results showed that maSOD was closely related with *Tetrahymena* sp. which is ciliate protozoa like *M. avidus*. These ciliate protozoa SODs are placed in Mn-SOD large cluster but identified as Fe-SOD. Different cluster was observed within bacteria and parasites Fe-SOD. As before result with multiple sequence alignment and pairwise by ClustalW, it was shown that maSOD has motif and active site both Mn-SOD and Fe-SOD.

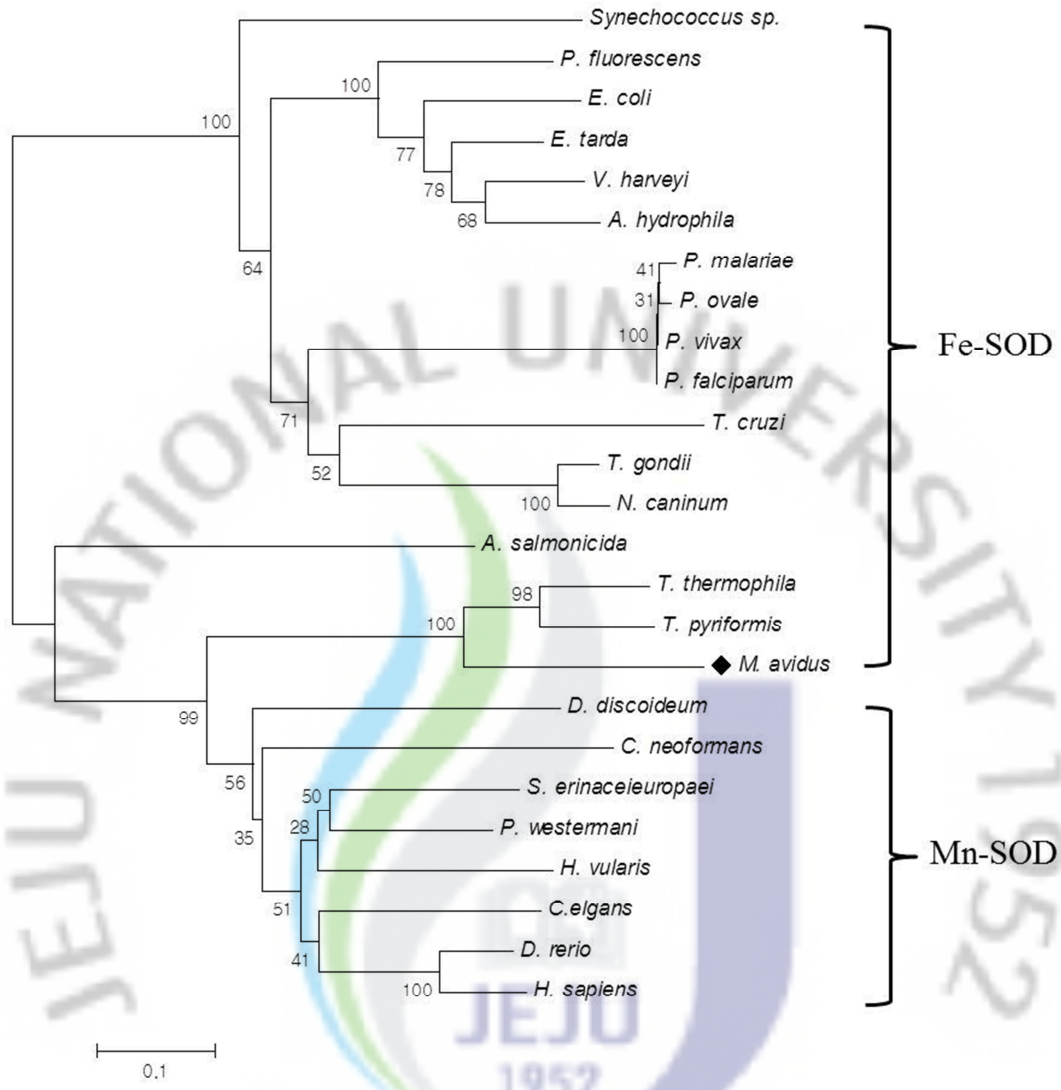


Figure 6. Phylogenetic analysis of msSOD protein (◆) comparing with bacteria, parasitic protozoa and other species Fe or Mn SOD proteins by the Neighbor-Joining method with the PAM matrix model (MEGA 4.0). The phylogenetic tree is based on an alignment corresponding to the full length of the SOD amino acid sequences. The numbers of the branches are bootstrap confidence values through 1000 replications. The GeneBank accession codes for the sequence designations as follows. Cianobacteria (*Synechococcus* sp. JA-2-3B) Fe-SOD, YP_478710; *Pseudomonas fluorescens* Fe-SOD, AAY94056; *Escherichia coli* strain K-12 Fe-SOD, AC_000091; *Edwardsiella tarda*

ATCC23685 SOD, ZP_06714789; *Vibrio harveyi* iDA3 Fe-SOD, ZP_06176456; *Aeromonas hydrophila* Fe-SOD, AAL26891; *Plasmodium malariae* Fe-SOD, AF139528; *Plasmodium ovale* Fe-SOD, AF139527; *Plasmodium vivax* Fe-SOD, AF139529; *Plasmodium falciparum* Fe-SOD, Z49819; *Trypanosoma cruzi* Fe-SOD, U39401; *Toxoplasma gondii* Fe-SOD, XP_002364798; *Neospora caninum* Fe-SOD, AAL62028; *Aeromonas salmonicida* subsp. A449 SOD-A, AAP85516; *Tetrahymena thermophile* SOD, XP_001010506; *Tetrahymena pyriformis* Fe-SOD, P19666; *Dictyostelium discoideum* AX4 SOD, XP_645815; *Cyptococcus neoformans* Mn-SOD, AAW56834; *Spirometra erinaceieuropaei* Mn-SOD, AAT79388; *Paragonimus westermani* Mn-SOD, AAT79387; *Hydra vulgaris* Mn-SOD, ABC25024; *Caenorhabditis elegans* Mn-SOD, D85499; *Danio rerio* Mn-SOD, AY195857; *Homo sapiens* Mn-SOD, X14322.

3. Overexpression of recombinant maSOD protein

3.1 Change codon usage of maSOD gene for protein expression by *E. coli*

The SOD gene of ciliate *M. avidus* has limited codons for protein expression using *E. coli*. The TAA codon is standard stop codon in wide species include *E. coli* but in ciliate like scuticociliate *M. avidus*, this codon specially encode glutamine (Glu, E). We analyzed that maSOD gene has eleven TAA codons and changed to CAG codon, which encode codon of glutamine in *E. coli*. Also we modified rare codons as follows. Twelve GGA codons to GGT for glycine (Gly, G); six AGA codons to CGT for arginine (Arg, R); two CCC codons to CCG for proline (Pro, P); one ATA codon to ATC for Isoleucine (Ile, I). Base on the gene of changed codon, we designed oligodeoxynucleotides for assembly PCR to get modified ORF of maSOD. After assembly PCR, we successfully cloned modified maSOD ORF with restriction enzyme sites on the 5' and 3' ends, BamH I (ggatcc) and Pst I (ctgcag), for expression by pMAL™ protein fusion system and *E. coli* BL21 (DE3). We checked forty-nine gaps between original maSOD ORF and modified msSOD ORF but they encoded match protein amino acid.

3.2 Purification of recombinant maSOD protein

For expression the recombinant maSOD protein, the modified msSOD gene was cloned into pMAL-c2x expression vector, which encodes a maltose binding protein (MBP) tag at the N-terminal of fusion protein under the strong tac promoter (P_{tac}) and *malE* translation initiation signal. The recombinant maSOD was over-expressed in *E. coli* BL21 (DE3) cells by IPTG induction. The 12% SDS-PAGE results of recombinant maSOD is shown figure 7. The approximate 67.5 kDa strong band was confirmed in the

induced cells (lane 3) but not in the un-induced cells (land 2). The purified recombinant maSOD was shown as a specific band of approximate 67.5 kDa (land 4). This result was in agreement with our predicted molecular weight of 25 kDa, because the MBP has 42.5 kDa molecular mass.

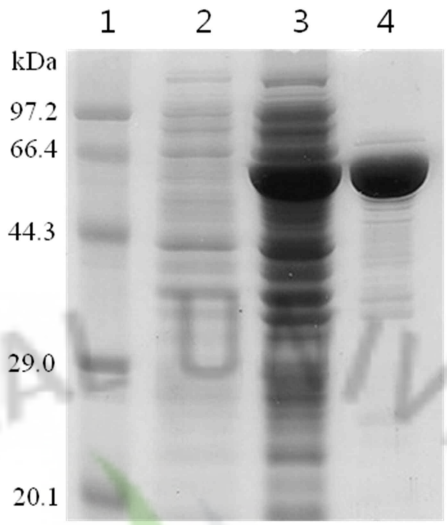


Figure 7. SDS-PAGE analysis of recombinant maSOD protein expressed in *E. coli* BL21 (DE3) cells following purification in 12% denaturing polyacrylamide gel. Lane 1: protein molecular weight marker (Low) (TaKaRa, Japan); lane 2: total cellular extract before induction with IPTG; lane 3: total cellular extract after cells were induced with 0.5mM IPTG and grown at 25 ℃ for 6 hours; lane 4: purified recombinant maSOD fusion protein.

4. Recombinant maSOD enzyme assay

4.1 Optimal temperature

The optimal temperature of purified recombinant maSOD was determined at different temperature ranging from 0 to 40 ℃ at intervals of 5 ℃ as shown figure 8 (a). From 0 ℃ to 15 ℃, recombinant maSOD protein had 100 to 90 % relative activity. When the temperature was increased from 20 ℃, the relative activity was started to decrease. At the temperature of 30 ℃, relative activity was remained 50% and at the 40 ℃ almost was no activity.

4.2 Optimal pH

As shown figure 8 (b), the optimal pH was 7.5 in phosphate buffer and it was stable at pH 7.0 to 8.5 in phosphate buffer and pH 9.0 to 10.0 in sodium carbonate buffer with more than 80% relative activity.

4.3 co-metal identification assay of maSOD

Sensitivity of maSOD to inhibitors is shown figure 8 (c). The maSOD enzyme activity was inhibited by 10mM H_2O_2 but not by 10mM NaN_3. Control means the activity assayed in the absence of any inhibitor.

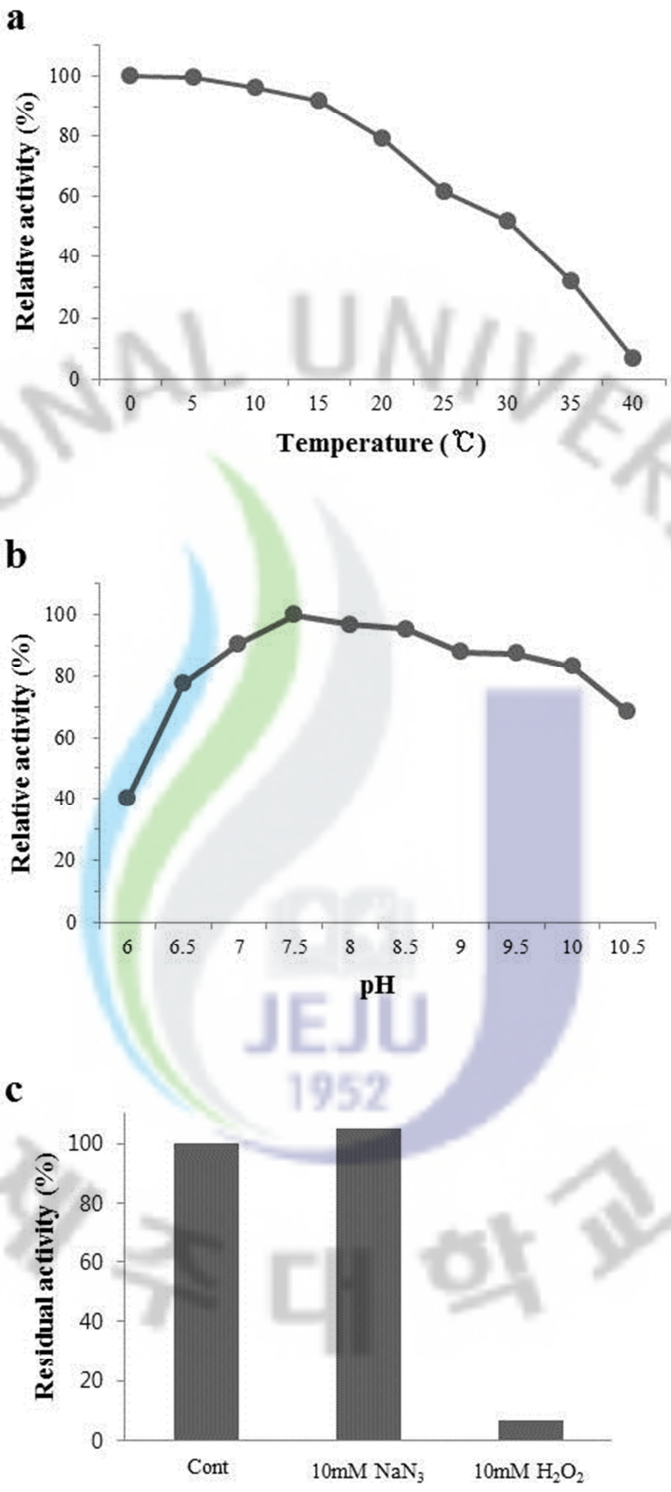


Figure 8. Biochemical properties of maSOD. (a) Optimal temperature: The effect of temperature on the enzyme activity was determined under standard assay condition at

temperature ranging 0 to 40 ℃. (b) Optimal pH: Enzyme activity was assayed in various pH buffers ranging from pH 6.0 to 10.5. Maximal activity was shown as 100%. (c) Inhibition assay for identification metal co-factor and sensitivity of maSOD to inhibitors: maSOD was incubated with 10mM NaN_3 or H_2O_2 at 20℃ for 30 min, and the enzyme activity was determined and compared to the control without any inhibitor.

Discussion

SOD is reported in many organisms from bacteria to high vertebrate as antioxidant to protect damage from ROSs, drugs, environment stress, infection or host immune system. Specially, analysis of antioxidant such as SOD is important and basic step for understanding parasite and host interaction. However study for strong invader includes scuticociliate in aquaculture is not enough.

In this study, we identified SOD gene from *M. avidus* and also analyzed characterization of the sequence and recombinant protein. The putative partial sequence of SOD was isolated from *M. avidus* GS-FLX blast analysis. Based on this sequence, we designed primers of RACE PCR and cloned the full-length maSOD gene, completely. The analysis of the maSOD amino acid sequence was shown that maSOD has conserved residues, His-42, His-90, Asp-179 and His-183 which are putatively required for binding Fe or Mn to SOD activity. These residues were confirmed in Fe SOD from wide species includes parasitic orgarnisms (Barra et al., 1990; Baert et al., 1999; Ödberg-Ferragut et al., 2000; Cho et al., 2004; Wilkinson et al., 2006; Kang et al,. 2008). Amino acid sequence of Fe-SODs and Ma-SODs is similar and difficult to distinguish by sequence analysis. But some differences between these sequences were identified by structural analysis (Jakson and Coopre, 1998). Fe-SOD has (F-N-N-A-A-Q)-X-(W-N)-H-X-(F-Y)-W motif, while in Mn-SOD the motif is (Base-F-N-G-G)-G-(H)-X-(N)-H-X-(L-F)-W and this motif is at least 50% conserved in known Fe-SODs and Mn-SODs. In our maSOD amino acid sequence we verified the putative motif before mention but also checked interesting difference. These residues at position 82 to 94 (^{82}F-N-L-G-G-H-L-N-H^{90}-S-I-Y-W^{94}) in maSOD was shown to conserve both Mn

and Fe-SOD motifs. Normally Mn and Fe SODs are found in wide life such as eubacteria, archaebacteria, organelles of eukaryotes, chloroplasts of plants and mitochondria. Especially Mn-SODs are found in higher living things and Fe-SODs in lower living things (van Camp et al., 1990; Almana et al., 1991). Since *M. avidus* might have metabolism between prokaryote and eukaryote, maSOD seems to have middle type motif of Mn and Fe-SOD. But Barra et al. (1990) discussed about this motif that the residues for distinguishing properties of the metal binding specificity, called discriminating residue rules, may hold for prokaryotic Fe-SODs and eukaryotic Mn-SODs, but not for eukaryotic Fe-SODs. Also G-S-G-W-X-W-L consensus sequence was confirmed in maSOD, ^{141}G-S-G-W-G-W-L^{147}. This consensus sequence was fully conserved in the Fe-SODs (as shown figure 5) and Mn-SODs (Jackson and Cooper, 1998). The maSOD exhibited the highest level of similarity with *T. termophila* (XP_001010506, 81.5 %) and *T. pyriformis* (P19666, 75.9 %) in the pairwise alignment but showed analogous similarity range 45 to 61 % both Mn and Fe-SODs. And also we confirmed that the maSOD was belonged to Mn-SOD cluster in phylogenetic tree (figure 6). These results indicated the maSOD gene has characteristics of Mn and Fe-SODs as mentioned above.

The optimal temperature and pH of recombinant maSOD protein were range of 0℃ to 20 ℃ and 7.5, respectively. Most proteins of parasite show high sensitivity and activity at the host life condition. Growth temperature of *M. avidus* is 10℃ to 20℃ and the marine hosts live in around 15℃ to 20℃. Consequently we concluded why maSOD activity has at low temperature, similar plankton SOD (Perelman et al., 2006). From the co-metal identification assay, maSOD was inhibited by H_2O_2 but not NaN_3. This result means maSOD is Fe containing SOD not Mn. But we can't explain why

maSOD use Fe ions for activity despite maSOD gene is similar with Mn-SOD. For explanation of maSOD characterization, we need to discuss more and research about *M. avidus*.

To improve our understanding that scuticociliatida how can survive from the host immune system, we will check the maSOD mRNA expression patterns at various oxidative stress such as heavy metal, H_2O_2, organic peroxide and host phagocytosis.

Part II. Molecular cloning and characterization of glutathione peroxidase (GPX) gene from scuticociliate, *Miamiensis avidus*

Abstract

Scuticociliates are recognized as serious infectious parasite that causes important economic losses in marine aquaculture worldwide including Korea. The scavenging activity against intracellularly produced reactive oxygen intermediates (ROIs) of the host has been reported for these organisms. For inhibition of ROIs: superoxide anion ($O2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-), scuticociliates have enzymes, such as superoxide dismutase, glutathione peroxidase (GPX), catalase and peroxiredoxin. GPX is the common name for a family of multiple isozymes that catalyze the reduction of H_2O_2 or organic hydroperoxides to water or alcohols using reduced glutathione (GSH), H_2O_2 +2 GSH → GSSH + $2H_2O$. In this study as basically molecular genetics step for understanding that scuticociliatida how can survive from the host immune system, we cloned glutathione peroxidase gene from scuticociliate *Miamiensis avidus*. The full-length cDNA of *M. avidus* GPX was cloned using degenerate primers by the method of 3' and 5' rapid amplification of cDNA ends PCR (RACE PCR). The full length sequence was analyzed using BLAST and standard analytical programs. The nucleotide sequence consisted of 901bp with a 5' UTR of 86bp, and 3' UTR of 131bp. The *M. avidu*s GPX open reading frame encoded 684bp, which corresponded to 227 amino acid residues. Predicted molecular mass and theoretical isoelectric point (pI) were 25 kDa and 6.1, respectively. Resulted the NCBI BLASTp and pairwise alignment, *M. avidu*s GPX was shown the highest similarity with GPX from *Hymeniacidon perlevis* (ABB91779), 73 % and also with PRx 6 from Danio

rerio (NP_957099), 71%. There through we denoted maGPX as seleno-independent GPX containing 1-cys PRx functional domains.

Materials and Methods

1. Selecting glutathione peroxidase gene and design primers

After GS-FLX analysis, we selected a partial sequence of glutathione peroxidase and designed primers for full-length sequence analysis by 5'- and 3'-rapid amplification of cDNA ends (RACE) Polymerase Chain Reaction (PCR) (Table 1).

Table 4. Primers designed for RACE PCR of glutathione peroxidase

Primer name	Primer sequence	Tm(℃)
maGPX R1 primer (5'-end)	5'- CAGGGAAGTTGGCATCAGCTTCTT -3'	59.6
maGPX R2 primer (5'-end)	5'- TGGTGGGATCAGCGATGATAGGAA -3'	59.7

2. PCR amplification of maGPX gene

2.1. *Miamienesis avidus*

M. avidus (YS2) was isolated from olive flounder by Jung et al. (2005). For YS2 culture, the CHES-214 cells (CRL-1681™, ATCC) were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS), 100 IU penicillin (Gibco, USA) and 100 μg /ml streptomycin (Gibco, USA) at 20℃. After 3 to 7 days, YS2 was grown in

the CHSE-214 cells medium at 15℃. Growth was continued for around 1 week to stop of cell division. The YS2 were harvested by centrifugation at 3000 rpm, 30 minutes, 4℃. The cells were immediately snap-frozen in liquid nitrogen and stored at -70℃.

2.2. RNA extraciton and cDNA synthesis

The total RNA was extracted from pooled cells using Tri Reagent™ (Sigma, USA) according to the manufacturer's protocol. The RNA concentration was measured at 260nm in a UV-spectrometer (Bio Rad, USA). Originally purified RNA was diluted up to a concentration of 1 µg / µl and for RACE PCR 4 µg RNA was used to synthesize cDNA using a SMART™ RACE cDNA Amplification protocol.

2.3. RACE PCR and Cloning into easy T-vector

The procedures for the generation of the 5' and 3' regions for partial maSOD sequence were carried out consulting the commercial protocol for the rapid amplification of 5' and 3' cDNA ends (SMART™ RACE cDNA Amplification kit, Clontech Laboratories, Inc., USA) using gene specific primers. RACE PCR primers were diluted to 10 pmole per 1 µl with reaction water.

For first 5'-RACE PCR, 1 µl of synthesized cDNA was mixed with 1 µl of 2 µM Universal Primer A Mix (UPM) (Clontech Laboratories, Inc., USA), 1 µl of maGPX R1 primer, 1 µl of 10mM dNTP mix, 5 µl of 10X Advantage 2 PCR Buffer (Clontech Laboratories, Inc., USA), 1 µl of 50X Advantage 2 Polymerase Mix (Clontech Laboratories, Inc., USA) and 40 µl of reaction water. PCR conditions for first step were: 1 cycle at 94℃ for 1 min; 25 cycles at 94℃ for 30 sec, 68℃ for 30 sec, 72℃ for 2

min; 1 cycle at 72℃ for 1 min. Second RACE PCR was carried out on the 1st 5'-RACE PCR conditions with 1 μl of the 1st 5'-RACE PCR product, 1 μl of Nested Universal Primer A (NUP) (Clontech Laboratories, Inc., USA), 1 μl of maGPX R2 primer, 1 μl of 10mM dNTP mix, 5 μl of 10X Advantage 2 PCR Buffer, 1 μl of 50X Advantage 2 Polymerase Mix and 40 μl of reaction water.

The generated cDNA fragment was inserted into pGEM-T easy vector (Promega, USA) and determined by a sequencing reaction using the termination kit, Big Dye, and an ABI 3700 sequencer (Macrogen Co., Korea). After sequencing analysis, we got the full-length maGPX cDNA sequence.

2.4. Sequence analysis

The NCBI BLAST program was used to search for nucleotide and protein sequence similar to maGPX. The GPX or 1-cys PRx family sequence domains were determined by means of a motif scan program (http://myhits.isb-sib.ch/cgi-bin/motif_scan) and SMART sequence analysis progrom (http://smart.embl-heidelberg.de/smart). Pair-wise and multiple sequence alignments of the maGPX protein with known GPX and 1-cys PRx proteins were analyzed, using ClustalW version 2.0. The amino acid sequences were aligned using ClustalW software and phylogenetic tree was constructed using Neighbor-Joining method from the MEGA 4.0 software. The bootstrap values were replicated for 1000 times to obtain the confidence value for the analysis.

3. Cloning and expression of maGPX

3.1 Synthesis maGPX gene for expression

3.1.1 Analysis codon usage for over-expression by *E. coli* BL21 DE3

Expression of ciliate gene in bacteria, *E. coli,* is limited by codon usage (Lin et al., 2002). We analyzed maGPx codon usage and changed maGPx codons, which are rare codons in bacteria, to *E. coli* codons for protein expression in *E. coli* BL21 DE3. Glutamine TAA, normally stop codon in bacteria, to CAG or CAA and Glycine GGA to GGT or GGC, Arginine AGA to CGT or CGC, Proline CCC to CCG, we changed for designed assembly PCR oligodeoxynucleotides.

3.1.2 Design assembly PCR oligodeoxynucleotides

Using Aseembly PCR Oligo Maker on the web at http://publish.yorku.ca/~pjohnson/AssemblyPCRoligomaker.html by Rydzanicz et al (2005), we designed fourteen assembly PCR oligodeoxynucleotides and two flanking primers, shown Table 2. Flanking forward primer and reverse primer was made to include the digestion enzyme site for cloning into pMal-c2x vector for over-expression, BamH I (ggatcc) and Pst I (ctgcag), respectively. Oligodeoxynucleotides were synthesized by Integrated DNA Technologies (IDT, USA).

1 **Table 5. Oligodeoxynucleotides designed for glutathione peroxidase assembly PCR**

Name	Oligodeoxynucleotide sequence
GPx assembly-1	5'- ATGCAAACCCAGCAGCGTAACACTACTTTGCTCCTCGGTGATGAAGTTCCGAATTTTGCT -3'
GPx assembly-2	5'- GAATCTTTGATGTAATCGTGGAATTGAATATCGCCAGCGGAAGTTTTGGCAGCAAAATTCGGAACTTCATCACC -3'
GPx assembly-3	5'- TCAATTCCACGATTACATCAAAGATTCTTGGGCCATTCTTTTCTCTCACCCTGCTGACTACACTCCTGTTTG -3'
GPx assembly-4	5'- GTTTGGCGAATTCTGAAGTCAATTTTGCAACACGACCCAATTCGGTGGTACAAACAGGAGTGTAGTCAGCA -3'
GPx assembly-5	5'- AAATTGACTTCAGAATTCGCCAAACGCAATGTTAAAGTTTTAGCCCTTTCTTGTGATAGTGTCGAAGACCAC -3'
GPx assembly-6	5'- TGACTTCACAGCTGCTGTACTGGTTAACGTCACTAATCCAGCCATTGTGGTCTTCGACACTATCACAAG -3'
GPx assembly-7	5'- GTACAGCAGCTGTGAAGTCAAGTTTCCTATCATCGCTGATCCGACCCGTGATATCTCTCGTCAGTGGGG -3'
GPx assembly-8	5'- TAAGCGGCATACCTTTCTGGTCGGTAGGGCCTTCAGTTTGGATCATACCCCACTGACGAGAGATATCAC -3'
GPx assembly-9	5'- CCAGAAAGGTATGCCGCTTACTGTTCGCAGTGTTTTCATTATCGGCCCTGATCGTAAATTGAAATTATCCA -3'
GPx assembly-10	5'- GCGCAAAATTTCGTCAAAGTTACGACCTGTAGATGCCGGATAAGTGATGGATAATTTCAATTTACGATCAGGG -3'
GPx assembly-11	5'- CGTAACTTTGACGAAATTTTGCGCGTTGTCGACTCTTTACAATTGACAGCTGGCCGTAAACTGGCTACTC -3'
GPx assembly-12	5'- CTTTAGTAACAGAAGGTAAGATGATACAATCCTGACCATGTTTCCAGTCTACAGGAGTAGCCAGTTTACGGCC -3'
GPx assembly-13	5'- GATTGTATCATCTTACCTTCTGTTACTAAAGAAGAAGCTGATGCCAACTTCCCTGGCTACACTACAGCTGATTT -3'
GPx assembly-14	5'- TCATTTTTTGTCAGGGTTGAAAGAACGTAAGTATTGTTTTTTAGAAGGTAAATCAGCTGTAGTGTAGCCAG -3'
Flanking-Forward	5'- gagagaggatcc(BamH I) ATGCAAACCCAGCAGCG -3'
Flanking-Reverse	5'- tctctcctgcag(Pst I) TCATTTTTTGTCAGGGTTGAAAGAAC -3'

3.1.3 Assembly PCR

Assembly PCR oligodeoxynuclotieds were diluted to 20 pmole per 1 μl and flanking primers to 20 pmole per 1 μl with reaction water. First assembly PCR was carried out with 1 μl of each oligo, 4 μl of 2.5mM dNTPs, 5 μl of 10X Herculase reaction buffer (Stratagene, USA), 0.5 μl of Herculase hotstart DNA polymerase (500U/100μl) and 26.5 μl of reaction water. PCR conditions for first step were: 1 cycle at 94℃ for 7 min; 8 cycles at 94℃ for 1.5 min, 54℃ for 2 min, 72℃ for 3 min; 1 cycle at 72℃ for 5 min. For second PCR, 1 μl of the first PCR product was mixed with 2 μl of flanking forward primer, 2 μl for flanking reverse primer, 4 μl of 2.5mM dNTPs, 5 μl of 10X Herculase reaction buffer (Stratagene, USA), 0.5 μl of Herculase hotstart DNA polymerase (500U/100μl) and 35.5 μl of reaction water. Second PCR condition were: 1 cycle at 94℃ for 5min; 25 cycles at 94℃ for 30 sec, 54℃ for 2 min, 72℃ for 1.5 min; 1 cycle at 72℃ for 5min. The last PCR for amplification of assembly PCR product was performed with 1 μl of 2^{nd} PCR product, 2 μl of flanking forward primer, 2 μl for flanking reverse primer, 16 μl of 2.5 mM dNTPs, 10 μl of 10X LA PCR™ buffer II (Mg^{2+} free) (TaKaRa, Japan), 10 μl of 25mM $MgCl_2$ (TaKaRa, Japan), 1 μl of *TaKaRa LA taq* ™ polymerase (5 unit/μl), 53 μl of reaction water. Third PCR step were: 1cycle at 94℃ for 5 min; 10 cycles at 94℃ for 30 sec, 54℃ for 2min, 72℃ for 1min; 1cycle at 72℃ for 5min. The PCR products were analyzed by 1% agarose gel electrophoresis.

3.2 Cloning of maSOD ORF into the pMAL-c2x expression vector

The aseembly PCR product was purified using the Accuprep™ gel purification kit (Bioneer Co., Korea) and both insert and pMAL-c2x (NEG, USA) were digested with

the respective restriction enzymes. The ligated product was transformed into competent cells of *E. coli* BL21 (DE3).

3.3 Over-expression and purification of recombinant maGPX

The recombinant maGPX was over-expressed in *E. coli* BL21 (DE3) cells in the presence of isopropyl-β-thiogalactopyranoside (IPTG). Briefly, a 5ml of *E. coli* BL21 (DE3) starter culture was inoculated into 500ml Luria broth with 500 μl ampicillin (100 mg/ml) and 1g glucose (0.2 % final concentration). The culture was incubated at 25℃ while shaken at 180 rpm until the cell count reached 0.5 at 600nm and induced by IPTG at 0.5mM final concentration. After 6hours of induction, the cells were cooled on ice for 30 min, and harvested by centrifugation at 3500 rpm for 30 min at 4 ℃, after which the supernatant was removed. The recombinant fusion protein, which was fused with maltose binding protein (MBP), was purified by means of the pMAL^{TM} protein fusion and purification system.

3.4 SDS-PAGE

The purified protein was collected in 500 μl aliquots, and respective samples were run on 12% SDS-PAGE with a protein molecular weight marker (low) (TaKaRa, Japan). Gels were stained using 0.05 % Coomassie Blus R-250, followed by a standard de-staining procedure. All the activity tests mentioned in this study were conducted using this purified recombinant maGPX fused with MBP. The concentration of the purified protein was determined via the Bradford method, using bovine serum albumin (BSA) as the standard.

Results

1. Sequence analysis of the full-length maGPX

The maGPX was cloned completely by RACE PCR with designed primers. The nucleotide sequence and amino acid sequence are presented in figure 9. The full-length of maGPX gene was 901bp contain of 684bp ORF encoding 227 amino acid residues. The moleculare mass and isoelectric point (pI) are each 25 kDa and 6.1.

By NCBI protein BLAST program (BLASTp), we identified that maGPX was one of glutathione peroxidase containing cysteine (C^{52}) in conserved active site replaced by selenocystein compare with classical seleno-dependent GPX. And also maGPX amino acid sequence was analyzed to have putative domains like 1-cys peroxiredoxin (peroxiredoxin 6) at position 12 to 148 of alkylhydroperoxide reductase-thiol specific antioxidant (AhpC-TAS) and 158 to 219 of 1-cys peroxiredoxin C-terminal domain using Motif Scan and SMART program. The ^{50}P-V-C-T-T-E^{55} was conserved in maGPX as a putative consensus region of catalytic center for the peroxidase activity. The Cys-52 residue was shown as conserved position with His-44 and Arg-134 residues for peroxidase activity of 1-cys PRx. This results was indicated that maGPX is non-selenuim glutathione peroxidase consist of 1-cys PRx active site and motif.

The pairwise ClustalW analysis using the maGPX deduced amino acid sequence with chosen known GPX or PRx proteins are shown in Table 6. The maGPX exhibited the high level of similarity percentage range 74 to 56 with others.

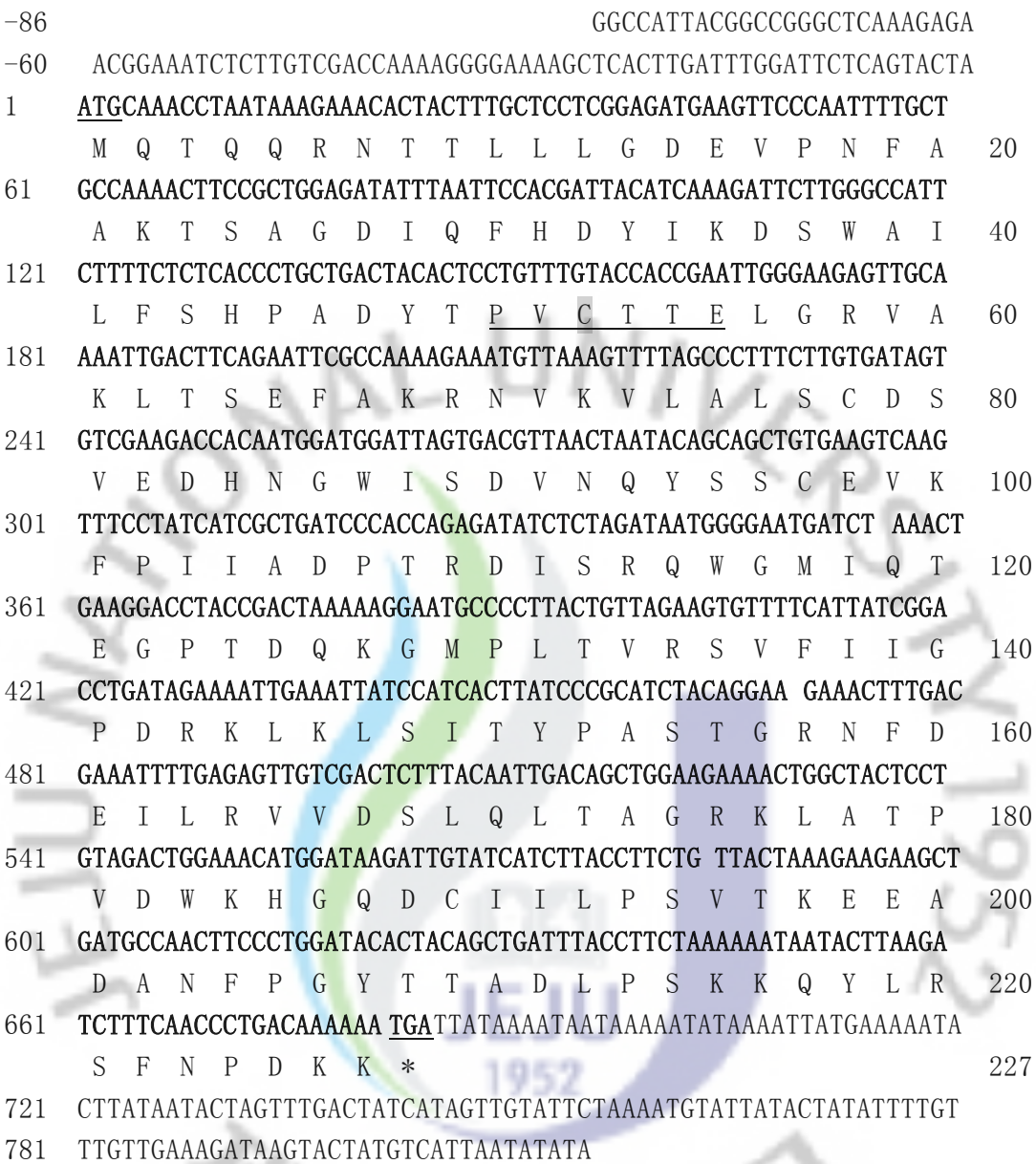


Figure 9. The complete nucleotide and deduced amino acid sequence of maGPX. The coding sequence (from 1 to 684) is in bold letters. Amino acid sequence corresponding to mature protein consists of 227 amino acids from start codon ATG to stop codon TGA (*). The *shade box* is marked as the conserved active cysteine residue. The catalytic centers residues of peroxidase activity (PVCTTE) are indicated by *underline*.

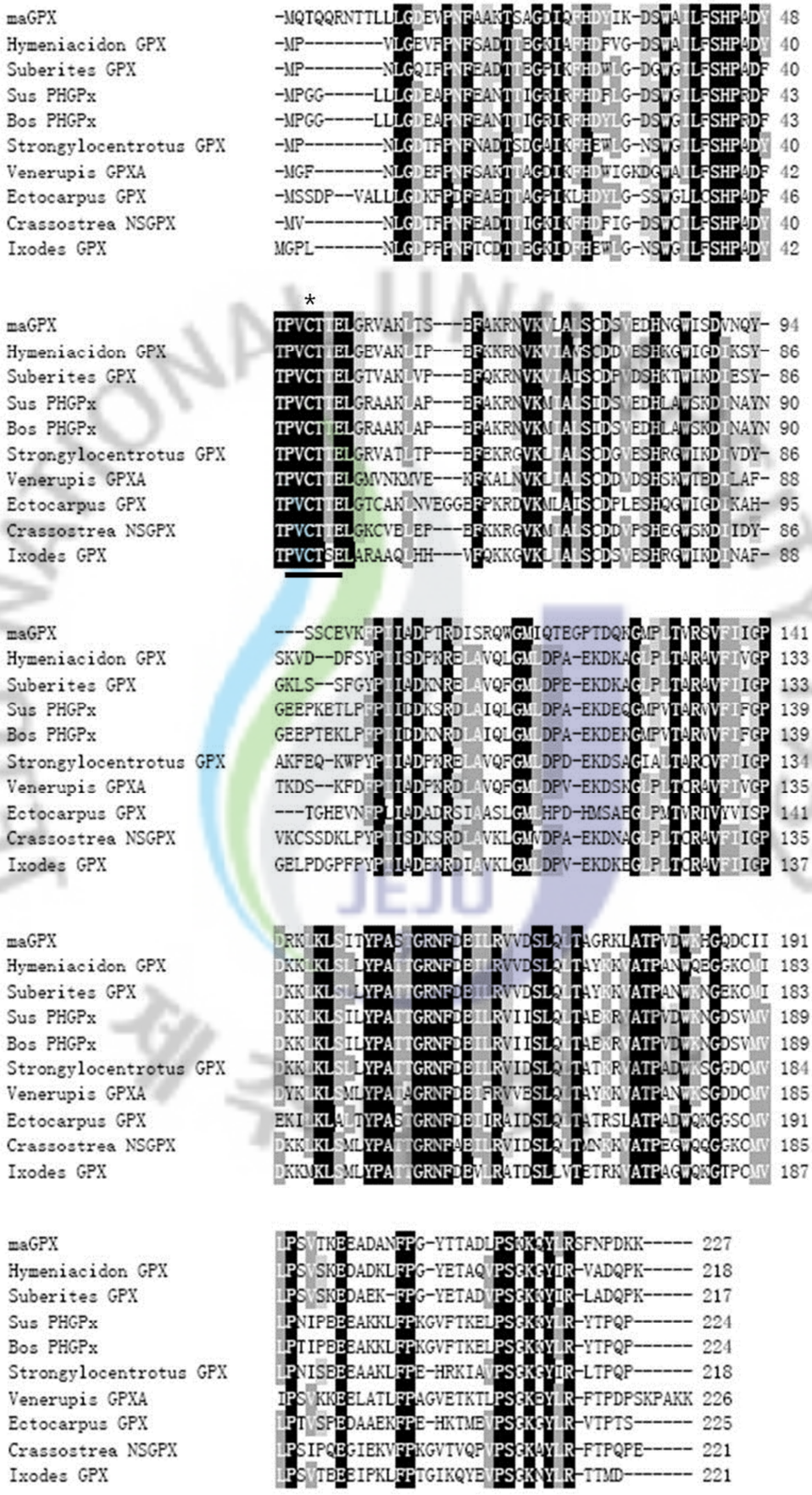


Figure 10. Multiple sequence alignments of predicted maGPX amino acid sequence with others known glutathione peroxidases. Identical residues in all sequences are highlighted in *black*. Conserved substitutions are shaded by *gray* color and semi-conserved substitutions are highlighted in *light gray* color. The conserved active cysteine is indicated by asterisk (*). The *bold single line* is catalytic centers residues of peroxidase activity (PVCTTE). Accession numbers of the amino acid sequences of GPXs are shown in Table 6.

Type of GPX or PRx	Species	Gene Bank accession No.	Protein length (amino acid)	Similarity percentage (%)
GPX	*Hymeniacidon perlevis*	ABB91779	218	73.7
GPX	*Suberites domuncula*	CAC38779	217	72.8
PHGPx (non-selenium)	*Sus scrofa*	CAB65456	224	71.6
PHGPx (non-selenium)	*Bos taurus*	CAB64802	224	71.1
GPX	*Strongylocentrotus intermedius*	ADK11694	218	70.7
GPX A	*Venerupis philippinarum*	ACU83219	226	69.6
GPX	*Ectocarpus siliculosus*	CBN79130	225	69.1
NS-GPX	*Crassostrea gigas*	CAK22382	221	67.5
GPX	*Ixodes ricinus*	ACI49692	221	65.7
PRx 6	*Danio rerio*	NP_957099	222	71.1
PRx 6	*Homo sapiens*	NP_004896	224	70
1-cys PRx	*Mus musculus*	AAC63376	224	69.7
PRx 6	*Xenopus laevis*	NP_001082669	224	69.1
PRx 6	*Haliotis discus discus*	ABO26614	218	69.0
1-cys PRx	*cyanobacterium UCYN-A*	YP_003421401	211	66.8
1-cys PRx	*Marinobacter aquaeolei VT8*	ABM18497	212	64.9
1-cys PRx	*Fagopyrum esculentum*	AAF12782	219	64.6
1-cys PRx	*Plasmodium falciparum 3D7*	XP_001349492	220	56.7

Table 6. Anlaysis of the deduced amino acid sequence similarity percentage of maGPX with known glutathione peroxidases (GPX), peroxiredoxin 6 (PRx 6) or 1-cys peroxiredoxin (1-cys PRx) by EMBOSS Pairwise Alignment Algorithms (http://www.ebi.ac.uk/Tools/emboss/align/).

2. Phylogenetic analysis of maGPx

To establish the evolutionary position of maGPX, we analyzed and constructed a phylogenetic tree with known GPX and PRx by the Neighbor-Joining method using MEGA 4.0 program as figure 11. Phylogenetic results showed that maGPX belonged to a large cluster of seleno-independent GPX and PRx. Different cluster was observed within plant, human, rat, zebrafish, worm and bacteria GPX groups. Interestingly, seleno-independent GPX and 1-cys RPx, PRx 6 were in closed position of the tree. This result was matched as multiple alignment and pairwise results and shown that maGPX is seleno-independent GPX which has active motif and domain of 1-cys PRx. According Herbette et al (2007), most nonselenium GPXs (NS-GPXs) includes the mammalian GPX4 and other various organisms GPXs, belong to the phospholipid hydroperoixde GPX (PHGPX) group. However in our result maGPX was not similar with human, rat, zebrafish, plant GPX4 or PHGPX except cattle and wild boar PHGPX. Also compare with known NS-GPXs such as *H. sapiens* GPX5, *E. coli* GPX, *P. falciparum* GPX, maGPX had low relationship in the tree.

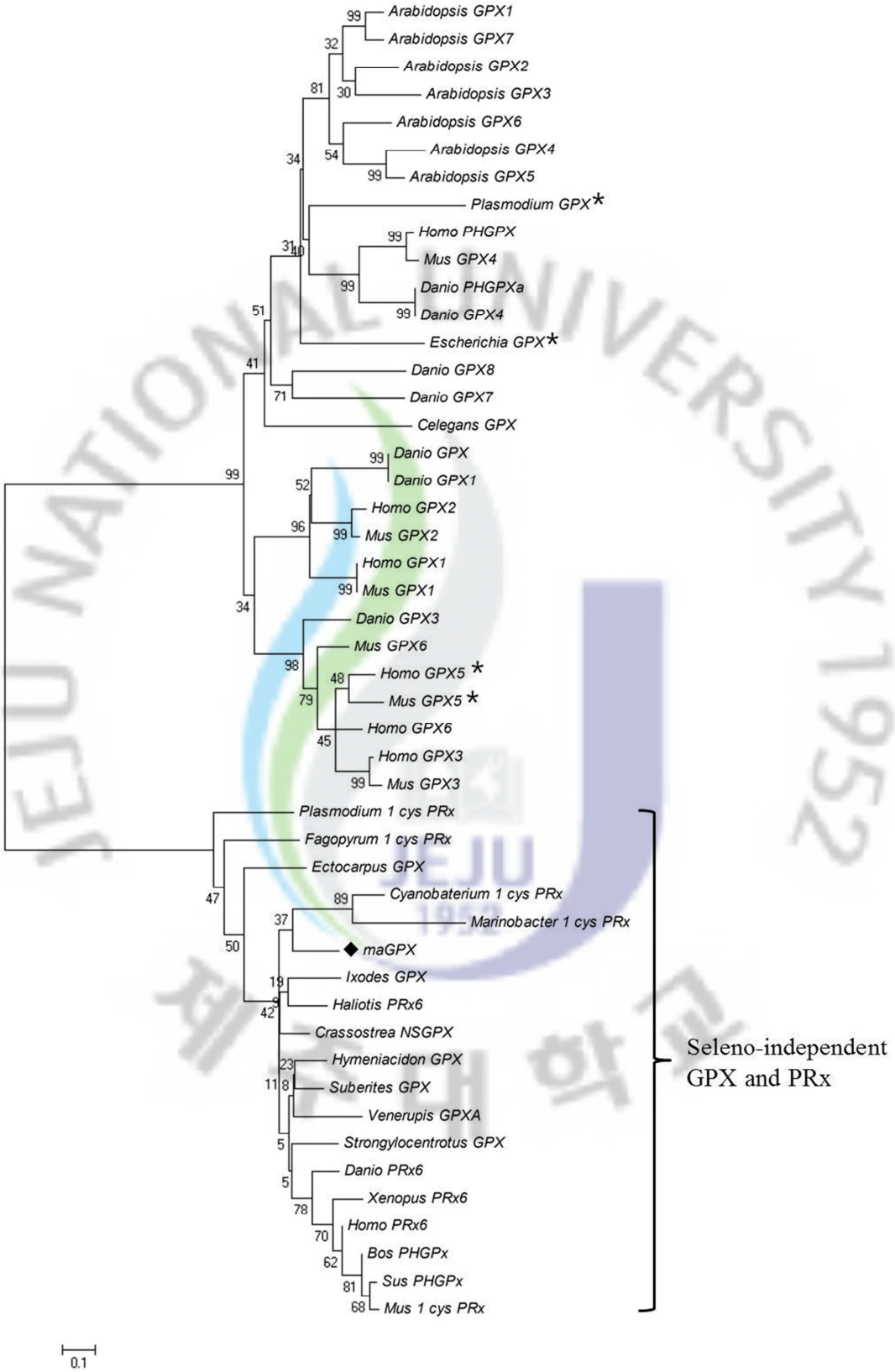


Figure 11. Phylogenetic analysis of maGPX protein (◆) comparing with other sepieces proteins or nucleotides of GPX and PRx 6, 1-cys PRx by Neighbor-Joining method (MEGA 4.0). The phylogenetic tree is based on an alignment corresponding to the full length of the GPX or PRx amino acid sequences. The numbers of the branches are bootstrap confidence values through 1000 replications. NS-GPX in the large cluster of Seleno GPX groups is indicated by *ariskstars* (*). The GeneBank accession code for the sequence designations as follows. *Arabidopsis thaliana* GPX1 (NP_180080), GPX2 (NP_180715), GPX3 (NP_181863), GPX4 (NP_566128), GPX5 (NP_191867), GPX6 (NP_192897), GPX7 (NP_194915); *Homo sapiens* GPX1 (P07203), GPX2 (P18283), GPX3 (P22352), PHGPX (CAA50793), GPX5 (NM_001509), GPX6 (NM_182701), PRx6 (NP_004896); *Mus musculus* GPX1 (P07203), GPX2 (BC054848), GPX3 (U13705), GPX4 (O70325), GPX5 (P21765), GPX6 (NP_663426), 1-cys PRx (AAC63376); *Danio rerio* PHGPXa (AY216590), GPX1 (AAO86703), GPX3 (NP_001131027), GPX4 (NP_001007283), GPX7 (NP_001018337), GPX8 (NP_956516), PRx6 (NP_957099); *Hymeniacidon perlevis* GPX (ABB91779); *Bos Taurus* PHGPx (non-selenium) (CAB64802); *Sus scrofa* PHGPx (non-selenium) (CAB65456); *Crassostrea gigas* NSGPX (CAK22382); Suberites domuncula GPX (CAC38779); *Strongylocentrotus intermedisu* GPX (ADK11694); *Venerupis philippinarum* GPXA (ACU83219); *Ectocarpus siliculosus* GPX (CBN79130); *Ixodes ricinus* GPX (ACI49692); *Plasmodium falciparum* GPX (CAA92396); *Plasmodium falciparum* 3D7 1-cys PRx (XP_001349492); *Escherichia coli* str. K-12 substr. MG1655 GPX (NP_416225); *Caenorhabditis elegans* GPX (Q95003); *Cyanobacterium* UCYN-A 1-cys RRx (YP_003421401); *Fagopyrum esculentum* 1-cys PRx (AAF12782); *Marinobacter aquaeolei* VT8 1-cys PRx (ABM18497); *Xenopus laevis* PRx6 (NP_001082669); *Haliotis discus discus* PRx6 (ABO26614).

3. Overexpression of recombinant maGPx protein

3.1 Change codon usage of maGPx gene for protein expression by *E. coli*

The GPX gene from ciliate *M. avidus* has restrictive codons using protein expression system by *E. coli*. The TAA codon is normally recognized as stop signal in wide organism include *E. coli* but this codon unusually encode glutamine (Glu, E) in ciliate such as *M. avidus*. We confirmed the *M. avidus* codon usage and analyzed that maGPX has six TAA codons and also thirty rare codons which are limited codon usage in *E. coli*. For protein expression, we modified these rare codons as follow. Six and three TAA codons to CAG or CAA (Gln, E); eight and three AGA codons to CGA or CGC (Arg, R); six and six CCA codons to GGT or GGC (Gly, G); four CCC codons to CCG (Pro, P). Based on this modified gene we designed assembly PCR oligodeoxynucleotides to get the converted maGPX ORF sequence with restriction enzyme sited on the 5' (BamH I, ggatcc) and 3' (Pst I, ctgcag) ends for pMAL^{TM} protein fusion system and *E. coli* BL21 (DE3). We checked fifty-two gaps between original maGPX and converted maGPX ORF but their protein sequence are completely matched.

3.2 Purification of recombinant maGPx protein

We cloned the converted maGPX ORF into pMAL-c2x expression vector which encodes a maltose binding protein (MBP) tag at the N-terminal of fusion protein. The recombinant maGPX protein was over-expressed in E. coli BL21 (DE3) cells by IPTG induction. The 12% SDS-PAGE results of recombinant maGPX protein is shown figure 12. We verified the strong band of the approximate 67.5 kDa size in the induced cells (lane 2) compare the un-induced cells (land 1). The purified recombinant maGPX

protein was exhibited as 67.5 kDa size for strong band (land 4). This result was in agreement with our predicted molecular weight of 25 kDa, since the MBP has a 42.5 kDa molecular mass.

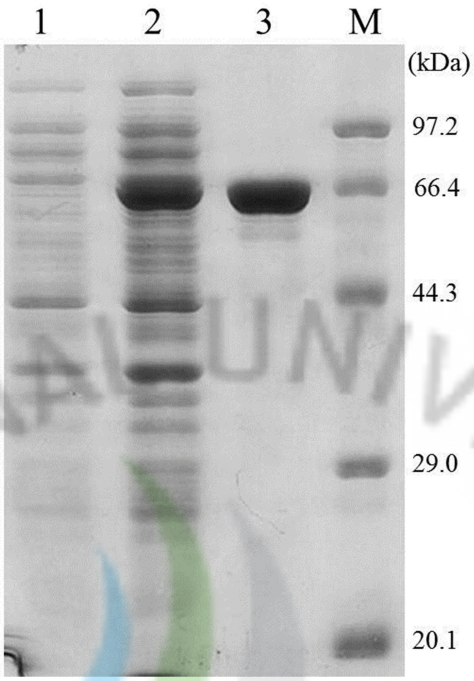


Figure 12. SDS-PACE analysis of recombinant maGPx protein expressed in *E. coli* BL21 (DE3) cells following purification in 12% denaturing polyacrylamide gel. Lane 1: total cellular extract before induction with IPTG; lane 2: total cellular extract after cells were induced with 0.5mM IPTG and grown at 25 ℃ for 6 hours; lane 3: purified recombinant protein; M: protein molecular weight marker (Low) (TaKaRa, Japan)

Discussion

In the present study, the full-length maGPX sequence was cloned and analyzed gene characterization. We confirmed the putative GPX contig sequence from GS-FLX blast results and designed primers for 5'end RACE PCR. We identified maGPX of 227 amino acid residues, which contain domains of alkly hydroperoxide reductase (AhpC) and thiol specific antioxidant (TAS), 1-cys peroxiredoxin C-termianl. Resulted the NCBI BLASTp and pairwise alignment, maGPX was shown the highest similarity with GPX from *Hymeniacidon perlevis* (ABB91779), 73.7 % and also with PRx 6 from Danio rerio (NP_957099), 71.1%. There through we denoted maGPX as seleno-independent GPX containing 1-cys PRx functional domains. The similar studies were reported from some species, for example *Crassostrea gigas* (David et al., 2007), *Bos taurus* (Leyens et al., 2003) and described PRx 6 as non-selenium glutathione peroxidase (Munz et al., 1997; Fisher et al., 1999; Manevich and Fisher, 2005). The maGPX amino acid shows a consensus sequence PVCTTE in the N-terminal which is a catalytic centre for the peroxidase activity in 1-cys PRx (Wood et al., 2003; Manevich and Fisher, 2005). This consensus region was also confirmed in type of non-selenium GPX (as shown figure 10) and 1-cys PRx (or PRx 6) (Data not shown). The seleno-independent GPX has cysteine in the active site replaced reactive selenocysteine (Se-Cys) residue encoded TGA codon, compared with classical selenium dependent GPXs, named seleno GPX or Se-GPX (Herbette et al., 2007; Dayer et al., 2008). In the seleno GPX 3' untranslated region of mRNA, there is a selenosysteine insertion sequence (SECIS) element (Arthur, 2000). On the other hand, in the maGPX sequence there is cysteine replaced Se-Cys and no SECIS in 3' untranslated region of maGPX mRNA. In case of green agar *Chlamydomonas reinhardtii* (Dayer et al., 2008), aquatic organisms are limited to

support selenium because aqua environment condition is lower oxygen levels and the selection pressure against Se-Cys.

In recently research, a new phospholipid hydroperoxide GPX (PHGPX or GPX4) was identified in mammals, which comprises cysteine instead of Se-Cys in the conserved catalytic region and named nonselenocysteine PHGPX (NPGPX). And also nonselenium GPXs (NS-GPX) were reported in higher plants (Cirqui et al., 1992; Holland et al., 1993; Roeckel-Drevet et al., 1998) and yeast (Inoue et al., 1999), nematode (Cookson et al., 1992), protozoan (Wilkinson et al., 2000), some animals. According to Herbette et al (2007), most NS-GPXs belong to the PHGPX family and distinct from other mammalian GPX groups. In our study, maGPX shows not to close with mentioned NS-GPXs by Herbette et al (2007) such as H. sapiens PHGPX (CAA50793), D. rerio PHGPXa(AY216590), M. musculus GPX4(O70325), P. falciparum GPX (CAA92396) and E. coli GPX (NP_416225). As shown figure 11, maGPX belongs to large cluster of seleno-independent GPX and PRX but mentioned NS-GPXs by herbette et al (2007) is formed another group in the different large cluster. Interestingly, the phylogenetic tree result is shown that maGPX is one of seleno-independent GPXs and also 1-cys RPx in agreement with our NCBI BLASTp and pairwise alignment.

1-cys PRx (same as PRx 6) is known one family of peroxiredoxins, which contains only the one N-terminal conserved cysteine residue compared with 2-cys PRx and atypical 2-cys PRx. This antioxidant is a bifunctional enzyme with glutathione peroxidase and phospholipase A_2 Activities (Fisher et al., 1999; Chen et al., 2000). In this enzyme coding sequence, two motifs are conserved such as PVCTTE residues for peroxidase

activity and GDSWG (GXSXG) lipase motif of the catalytic centre for phospholipase A2 activity. We confirmed the PACTTE consensus region in the N-terminal but not GDSWG motif in the maGPX amino acids. This point is different with other NS-GPXs (as shown figure 10) and 1-cys PRx or PRx 6 (data not shown). But the putative motif was verified at position ^{35}K-D-S-W-A^{39} and maGPX has lysine (K) and alanine (A) in place of glycine (G). We assumed that maGPX protein has function with peroxidase and phospholipase A_2 activities.

For enzyme assay, we modified maGPX ORF sequence and expressed the recombinant fusion maGPX protein using pMAL™ protein fusion and purification system and *E. coli* BL21 (DE3). In primary enzyme assay for GPX activity, the recombinant maGPX was not shown significant activity with control (data not shown). Similar studies were reported about this result that recombinant GPX or 1-cys PRx lost activity or showed low sensibility *in vitro* assay (Kang et al., 1998; Peshenko et al., 1998, 2001). It is reason (1) recombinant protein maybe be stable oxidized homodimer after interaction with peroxides, (2) 1-cys PRx acts a form of oligomer for activity but recombinant protein lose function and a structure of oligomer.

Future research should need to improve our understanding of the maGPX characterization and *M. avidus* follow as various enzyme assay and structure study, expression analysis in the oxidative stress.

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감사의 글

대학교 4년, 석사 2년. 그 시간 동안 여러모로 지도해주시고 이끌어주시고 항상 열정을 심어주신 이제희 교수님께 감사드립니다. 제주대학교 해양생명과학과에서 여러 흥미롭고 값진 학문을 가르쳐주신 이기완 교수님, 송춘복 교수님, 최광식 교수님, 허문수 교수님, 여인규 교수님, 전유진 교수님, 김기영 교수님, 정준범 교수님, 이영돈 교수님께 감사드립니다. 스쿠티카충 연구와 여러 실험을 지도해주신 전남대 정성주 교수님께도 감사인사를 드립니다. 스쿠버다이빙으로 바다의 아름다움을 알려주신 관광대 김재영 교수님과 김세민 교수님께 감사인사 드립니다.

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