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Molecular cloning, characterization, and the response of manganese superoxide dismutase from the Antarctic bivalve *Laternula elliptica* to PCB exposure

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ABSTRACT

Manganese superoxide dismutase (leMnSOD) cDNA was cloned from the Antarctic bivalve *Laternula elliptica*. The full-length cDNA of leMnSOD is 1238 bp in length and contains an open reading frame of 681 bp encoding 226 amino acid residues including a putative mitochondrial targeting peptide of 26 amino acids in the N-terminal region. The calculated molecular mass is 24.8 kDa with an estimated isoelectric point of 6.75. leMnSOD signatures from 185 to 192 (DVWEHAYY) and four conserved amino acids (H52, H11, D185, and H192) responsible for binding manganese were observed. Sequence comparison showed that leMnSOD had high levels of identity with MnSOD from *Haliotis discus discus*, *Mizuhopecten yessoensis*, and *Crassostrea gigas* (68%, 66%, and 59%, respectively). RT-PCR analysis revealed the presence of leMnSOD transcripts in all tissues examined. Quantitative real-time RT-PCR assay indicated that treatment with polychlorinated biphenyls (PCBs) significantly increased leMnSOD mRNA expression in an organ-, time-, and dose-dependent manner. The mRNA expression with exposure to PCBs at 0.1 and 10 ppb reached the highest level at 6 h and then recovered slightly from 6 to 48 h in the gill. In contrast, the expression of leMnSOD mRNA showed a different expression pattern related to PCB concentration in the digestive gland. The mRNA expression at 0.1 ppb PCBs increased up to 12 h and then decreased by 48 h, but increased immediately at 10 ppb PCBs. The leMnSOD was overproduced in *Escherichia coli* and purified. The recombinant leMnSOD showed maximum activity at pH 9.0, and it retained more than 50% of its original activity after incubation for 30 min at 40 °C.

1. Introduction

Polychlorinated biphenyls (PCBs), which are major persistent organic pollutants (POPs), have been suggested to be the result of atmosphere subsidence into the Antarctic environment and anthropogenic activities [1]. These organic pollutants can be found in aquatic and terrestrial environments and tend to bioaccumulate through the food chain, thus threatening ecosystems. Several studies have reported the relationships between POPs and immune defense capabilities, such as hemocyte number, ribosomal enzymes, and reactive oxygen metabolites [2,3]. Arkoosh et al. [4] reported that PCBs can disrupt immune function in fish, and Liu et al. [5] also found that PCBs had significant toxic effects on various immune parameters, including the number of hemocytes, the proportion of granulocytes, phagocytosis, lysosomal membrane stability, bacteriolytic activity, and reactive oxygen species (ROS) generation, in the scallop *Chlamys farreri*. In addition, susceptibility to infectious diseases, such as *Perkinsus marinus*, was significantly affected by organic chemicals in oysters, suggesting that exposure to organic chemicals could compromise immune function [6,7].

ROS are produced as by-products of normal cell metabolism. Although small amounts of ROS are a cellular requirement because they are involved in signaling pathways and in regulation of a variety of cellular activities and gene expression, excess ROS production increases oxidative damage in the cell, possibly by altering or inactivating proteins, lipid membranes, and DNA [8]. To protect their cellular components, organisms have several enzymatic and chemical antioxidant systems that remove these harmful oxygen species. The primary defense system against ROS is the superoxide dismutase family (EC 1.15.1.1) [9]. Superoxide dismutases (SODs) are ubiquitous metalloenzymes found not only in all oxygen-respiring organisms but also in some obligate anaerobes [10]. These enzymes, which catalyze the dismutation of superoxide radicals ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2) and oxygen (O_2), have

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been suggested to play an important role in protecting living cells from the cytotoxic effects of the ROS formed from superoxide anion radicals such as H_2O_2, hydroxyl radicals, and singlet oxygen [11]. SODs are classified into four groups depending on their redox-active metal cofactors: cytosolic Cu/ZnSOD (containing copper and zinc), MnSOD (containing manganese), FeSOD (containing iron), and NiSOD (containing nickel) [12–15]. Cu/ZnSOD is a dimeric protein with $M_r = 16$ kDa per subunit, and NiSOD is a tetrameric protein with subunits of 13 kDa. MnSOD in the mitochondria of eukaryotes, which is synthesized as a precursor protein in the cytoplasm and is then imported into the mitochondria after cleavage of its signal peptide, is a tetrameric protein with subunits of 22 kDa [16]. In contrast, the cytosolic MnSOD in the blue crab, *Callinectes sapidus*, is a dimeric protein [17]. MnSOD has been shown to play a major role in promoting cellular differentiation and tumorigenesis [18] and in protecting cells against hyperoxia-induced pulmonary toxicity [19].

Many studies have summarized the role of MnSOD in protection against ROS along with their physiological functions [20–22]. Although SODs are known housekeeping genes, the expression of SODs are significantly modulated by stimuli, such as infection, heavy metals, organic chemicals and thermal stress [23,24] and its immunostimulation has been also reported by β-glucan and polysaccharide in crustacean species [25]. However, insufficient information is available regarding immunotoxic effects of organic chemicals on mollusks. Here, we describe the cDNA cloning and characterization of the MnSOD gene from the Antarctic bivalve *Laternula elliptica*, which shows a widespread geographic distribution in the Antarctic region and represents an important species in coastal water ecosystems. The biochemical properties of the purified recombinant leMnSOD expressed in *Escherichia coli* are also discussed. To gain insight into the immunotoxic effects of PCBs in *L. elliptica*, we also explored the induction of leMnSOD at the transcriptional level *in vivo*.

2. Materials and methods

2.1. Organisms and PCB exposure experiments

Specimens of *L. elliptica* (shell length ~80 mm) were hand-collected by scuba divers from depths of 20–30 m in Marian Cove, near King Sejong Station, on the northern Antarctic Peninsula (62°13′S, 58°47′W) in January 2006. *L. elliptica* specimens were exposed to PCBs under artificial conditions as described previously [26]. Briefly, samples were divided into three groups and acclimated under conditions equivalent to those in the field (temperature ~1.0 °C) with a constant air supply. After acclimation for 2 days, *L. elliptica* were transferred to 40-L temperature-controlled aquaria filled with filtered (~0.2 μm) natural seawater. Each aquarium contained 15 animals and the water was exchanged every 24 h. PCB exposed groups were treated with a final concentration of 0.1 and 10-ppb PCB mixture, Aroclor 1254, in 1 mL of methanol carrier, respectively, and the control group was treated with 1 mL of methanol without Aroclor 1254. Seawater was aerated and water temperature was maintained at 1.0 °C ± 0.1 °C throughout the experimental period. After 0, 6, 12, 24 and 48 h, three specimens were randomly selected and removed from each of the three aquaria (i.e. $n = 3$ per treatment) for total RNA isolation.

2.2. Cloning of MnSOD and phylogenetic analysis

Total RNA was isolated using the TRIzol procedure (Invitrogen, Frederick, MD) and precipitated in ethanol. The mRNA was isolated from total RNA using Oligotex mRNA spin columns (Qiagen, Valencia, CA, USA). The cDNA library was constructed using the ZAP-cDNA synthesis kit and ZAP-cDNA Gigapack III Gold Cloning Kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. In total, 2592 randomly collected clones were sequenced using an ABI Prism 3730 automated sequencer (Applied Biosystems, Foster City, CA, USA). The partial MnSOD cDNA sequences were obtained from this expressed sequence tag (EST) library (Park et al., 2008). To obtain the full-length cDNA of the MnSOD gene, the 3′ and 5′ ends were amplified using the Capfishing full-length cDNA kit (Seegene, Seoul, Korea) according to the manufacturer's instructions, with each gene-specific forward and reverse primers (for 5′-RACE, 5′-GAG GTG ACC GCC TCC ATT GAA TT-3′; for 3′-RACE, 5′- GGT CTC GTG CCA TTA TTT GGA ATT GA-3′). The RACE-PCR products, cloned into pCR2.1-TOPO, were sequenced from both the 5′ and 3′ ends using an ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (PerkinElmer, Waltham, MA) and an automated sequencer. Complete sequences were analyzed by searching for similarities in the National Center for Biotechnology Information (NCBI) databases using the BLASTX search program. Putative signal peptide region of leMnSOD coding sequence was identified by TargetP V1.1 World Wide Web Server www.cbs.dtu.dk/services/TargetP/ [27]. Phylogenetic trees were constructed using the amino acid sequences from various organisms, including vertebrates and invertebrates. The amino acid sequences were aligned using Clustal W [28], and molecular phylogenetic trees were constructed using the neighbor-joining method. Support for major nodes was evaluated by bootstrapping, with generation of 1000 bootstrap replicates of the whole data set. The following proteins were used in the alignment: human (*Homo sapiens*; CAA30687), tufted capuchin monkey (*Cebus apella*; BAC20359), rat (*Rattus norvegicus*; CAA39937), chicken (*Gallus gallus*; AAG46055), zebrafish (*Danio rerio*; AAP34300), tetraodon (*Tetraodon nigroviridis*; CAG03626), grouper (*Epinephelus coioides*; AAW29024), cobia (*Rachycentron canadum*; ABC71306), scallop (*Mizuhopecten yessoensis*; BAE78580), abalone (*Haliotis discus discus*; ABF67504), and oyster (*Crassostrea gigas*; ABZ90958). The 3-D structural models were constructed by submitting the deduced amino acid sequences of *L. elliptica* SOD cDNA to the SWISS-MODEL server [29] using the human MnSOD (Protein Data Bank code no. 1luvA) as the structure template due to the sequence similarity of 65.6% between human MnSOD and *L. elliptica* MnSOD. SWISS-Pdb viewer 4.01 was used to generate a three-dimensional (3-D) image.

2.3. Quantification of leMnSOD transcript

The transcriptional responses of the leMnSOD gene to PCB exposure were examined in the digestive gland and gill of *L. elliptica* by quantitative real-time RT-PCR. leMnSOD mRNA and β-actin mRNA (GenBank accession number EF198331), used as an internal PCR control, were amplified with the appropriate gene-specific primers: SODF (5′-GGA AGT GTC TCA GCC CCA CT-3′) and SODR (5′-CCT GAA GTG GGT CCT GGT TT-3′), and actF (5′-CAA CAG AGA AAA GAT GAC AC -3′) and actR (5′-CTG CTG TCT GCA TTT CCT GT-3′). PCR amplification was performed in 25-μl reaction mixtures containing cDNA generated from 2 μg of the original RNA template, 0.2 μM of the gene-specific primers used in RT-PCR, and 12.5 μL of Brilliant II SYBR Green QPCR mix (Stratagene, La Jolla, CA). The amplified signals were monitored continuously using the Mx3000P QPCR System (Stratagene), with the following amplification protocol: an initial 15 s of denaturation and enzyme activation at 95 °C, followed by 45 cycles at 95 °C for 5 s, 50 °C for 15 s, and 72 °C for 15 s. The β-actin gene of *L. elliptica* was used as a reference to normalize the expression levels between samples. The leMnSOD transcript expression of treated samples were compared to control samples collected at the same culture times. All experiments were repeated at least three times in each specimen. The relative fold-change in

gene expression was determined by the $2^{-\Delta\Delta Ct}$ method [30]. All data are presented as means ± standard deviation and were subjected to one-way analysis of variance (ANOVA), followed by Duncan's multiple range test, using SPSS version 12.0 (SPSS Inc., Chicago, IL).

2.4. Expression and purification of recombinant leMnSOD

The complete coding region of the leMnSOD cDNA was amplified by PCR with leMnSODF (5′-CAT ATG TTG TCA ACC GCT ATC TCA GC-3′, including an NdeI site) and leMnSODR (5′-CTC GAG TTA GTT GTT CAA TCT TGC AT-3′, including an XhoI site). The PCR products were cloned into the pCR2.1-TOPO cloning vector (Invitrogen), and leMnSOD was subsequently cloned into the pET28a expression vector (Novagen, Madison, WI), which facilitates $(His)_6$ affinity tag introduction, utilizing the engineered restriction sites. The identity of the insert was verified by sequencing. The recombinant enzyme was overexpressed in *E. coli* BL21(DE3)pLysE cells. The transformants were cultivated in 250 mL of Luria–Bertani (LB) medium at 37 °C in the presence of 50 μg mL^{-1} ampicillin until the absorbance at 600 nm (A_{600}) reached approximately 0.6. At this point, the temperature was changed to 25 °C, and the expression of the His_6-tagged proteins was induced by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG), followed by a further 15 h of culture with shaking. To increase the proportion of active recombinant leMnSOD recovered, 0.2 mM $MnCl_2$ was added to the bacterial cultures at the time of IPTG induction. The cells were collected by centrifugation at 6000 × *g* for 15 min at 4 °C. The cell pellet was resuspended in 20 mL of ice-cold phosphate-buffered saline (PBS) and lysed by sonication on ice. The cell debris was removed by centrifugation at 12 000 × *g* for 20 min, and the supernatant was loaded onto a column of Ni-NTA resin (Amersham Biosciences, Piscataway, NJ). The column was washed with 50 mM PBS (pH 8.0) containing 20 mM imidazole and then eluted with 50 mM PBS (pH 8.0) containing 500 mM imidazole. The purity of eluted samples was analyzed by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [31], and stained with Coomassie brilliant blue R-250. Protein concentrations were determined by the method of Bradford using bovine serum albumin (BSA) as a standard [32]. Proteins separated by SDS-PAGE were transferred onto polyvinylidene difluoride membranes (Immobilon PVDF membrane; Millipore, Billerica, MA) for Western blotting analysis according to the method of Towbin et al. [33]. The blotted PVDF membranes were incubated in 5% BSA in PBS-T buffer [200 mM Tris (pH 7.0), 138 mM, 2.7 mM KCl, 0.05% Tween 20] at room temperature for 2 h. The membranes were then incubated with anti-His G-HRP antibody (1:5000; Invitrogen) at room temperature for 3 h, rinsed, and washed three times with 0.1% PBS-T for 60 min at room temperature. Detection was accomplished with an ECL Plus Western Blotting kit (Amersham Biosciences).

2.5. Enzyme activity assay

SOD activity was measured using a spectrophotometric method based on the ability of SOD to inhibit the reduction of cytochrome *c* by O_2^- produced by the xanthine oxidase/xanthine reaction [34]. Briefly, 0.1 mM xanthine was dissolved in 50 mM $NaCO_3$ buffer. A dilute stock solution was added to a 10 μM solution of cytochrome *c*, 50 μM xanthine, and 0.1 mM ethylenediaminetetraacetic acid (EDTA) to produce a change in absorbance at 550 nm (A_{550}) of 0.025 min^{-1}. One unit of SOD was defined as the amount of sample needed to achieve 50% inhibition of the rate of cytochrome *c* reduction.

3. Results

3.1. cDNA cloning and sequencing of the MnSOD cDNA of L. elliptica

The complete cDNA sequence of the MnSOD of *L. elliptica* was obtained by 3′ and 5′ RACE-PCR based on the expressed sequence tag (EST) sequences. The nucleotide and deduced amino acid sequences of the MnSOD gene is shown in Fig. 1S in the Supplementary materials. The full length of leMnSOD comprised 1238 bp (GenBank accession no. GQ202272) and contained an open reading frame of 681 bp (including a stop codon) encoding a product of 226 amino acid residues. The nucleotide sequences from 1 to 61 and 743 to 1238 included the 5′ untranslated region (UTR) and 3′ UTR containing a consensus polyadenylation site (ATTAAA) at position 1198, respectively. The predicted molecular mass of the product was 24.8 kDa and the estimated isoelectric point was 6.75. Fig. 1A shows an alignment of leMnSOD with selected mollusk MnSOD sequences using CLUSTALX. On multiple sequence alignment, the deduced sequence of *L. elliptica* MnSOD was shown to have conserved potential metal-binding ligands for manganese (H52, H101, D185, and H189) [16,35] and consensus features of MnSOD (DVWEHAYY) from residues 185–192. leMnSOD contained eight α-helices and five β-sheets, and shows the greatest similarity to MnSOD from the abalone *H. discus discus* (68% identity and 83% similarity). A molecular 3-D model of leMnSOD was constructed by homology-based modeling using the SWISS-MODEL server. Human MnSOD was used as a template structure for constructing the 3-D model. Seven α-helices and five β-sheets were observed in the leMnSOD monomer (Fig. 2B). The N-terminus of the protein was composed mainly of α-helices and the middle domain consisted of three antiparallel β-sheets consisting of an ααβααββββααβα structural motif. A range of 12 vertebrate and invertebrate MnSOD amino acid sequences was analyzed to build a phylogenetic tree. All mollusk MnSOD clustered together as a subgroup, and leMnSOD first clustered with abalone *H. discus discus*, then with the scallop *M. yessoensis* and the oyster *C. gigas* (Fig. 3).

3.2. Expression of the leMnSOD transcript

leMnSOD expression in five tissues of *L. elliptica* was investigated at the transcriptional level by RT-PCR. β-Actin was used as an internal control gene. Specific primers (SODF and SODR) for leMnSOD were used to amplify a 210-bp fragment with cDNA from the digestive gland, gill, gonad, mantle, and intestine tissues using β-actin as an internal control. On RT-PCR, the leMnSOD transcript was detected in all of these tissue samples (see the Fig. 2S in the Supplementary materials).

To confirm the response of the leMnSOD transcript, mRNA levels of leMnSOD from the gill and digestive gland were investigated after exposure to PCB mixture (Aroclor 1254) using quantitative real-time RT-PCR. Fig. 3 shows the expression profiles of leMnSOD in the digestive gland (A) and gill (B) on exposure to 0.1 ppb and 10 ppb PCBs.

In the control group (treated with 1 mL of methanol without Aroclor 1254), leMnSOD mRNA levels were not changed during exposure period (see the Fig. 3S in the Supplementary materials). The levels of leMnSOD mRNA in the gill increased up to 6 h of exposure to PCBs at 0.1 ppb, reaching 4.1-fold compared to time 0. In the case of the digestive gland, the leMnSOD mRNA level increased gradually from 6 to 12 h, and reached the highest level of 6.7-fold at 12 h compared to time 0. However, the levels of leMnSOD increased up to 6 h in response to exposure to 10 ppb PCBs in both organs (10.5-fold in the digestive gland and 14.5-fold in the gill) compared to time 0, and then decreased gradually from 6 to 48 h. The highest levels of induction of leMnSOD mRNA in the

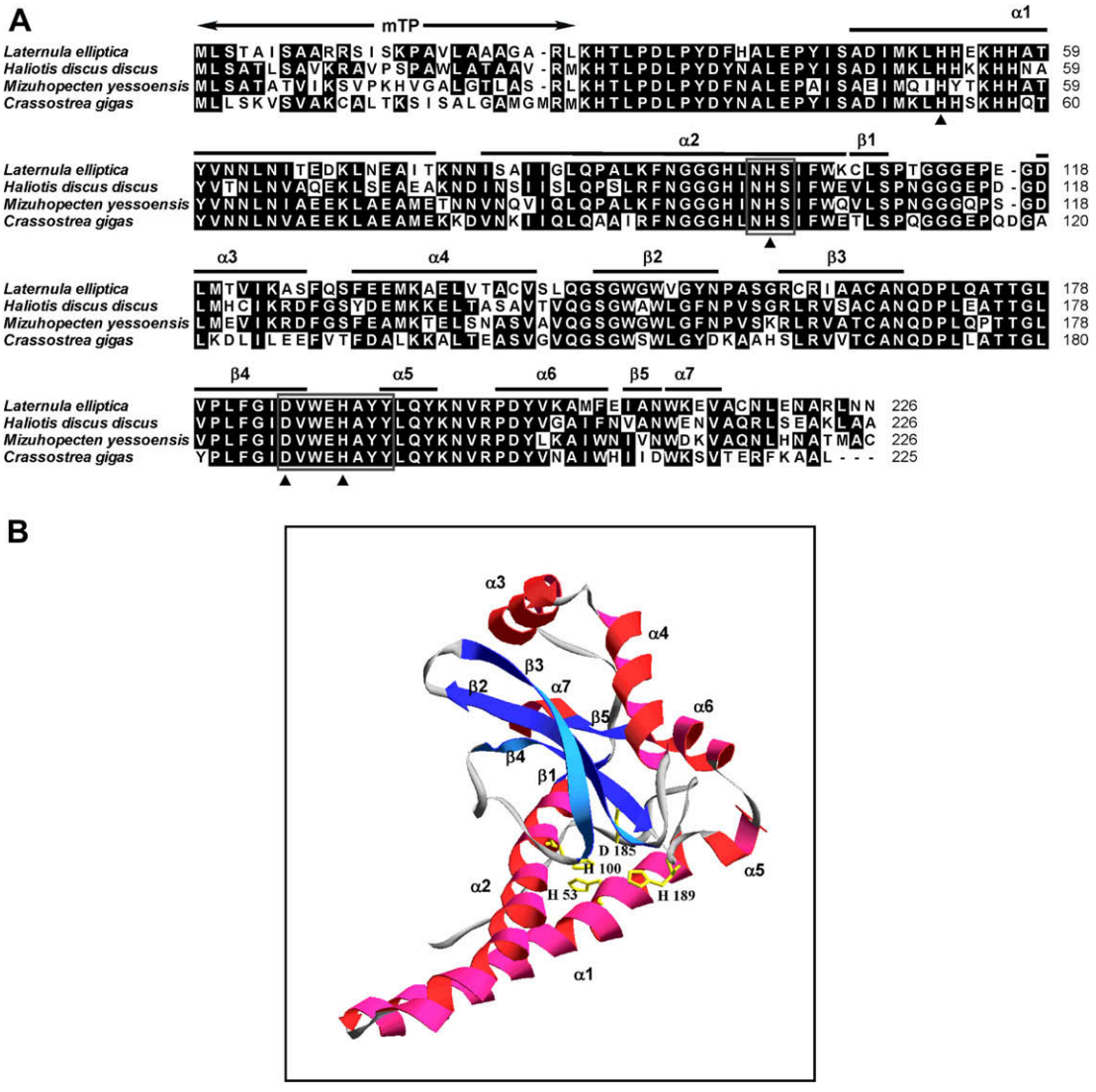

Fig. 1. Alignment of leMnSOD deduced amino acid sequence of *L. elliptica* with other known mollusk MnSODs (A). Amino acids identical in all sequences are highlighted in dark gray. The MnSOD signature (DVWEHAYY) and putative N-glycosylation site (NXS) are boxed. Four residues involved in the metal center are indicated (▲). The arrow denotes residues involved in mTP, and bold lines indicate residues involved in α-helices and β-sheets. Predicted 3-D structure of the leMnSOD monomer (B). The figure was produced by homology-based modeling using the SWISS-MODEL server. The structure of human MnSOD (PBD ID 1luvA) was chosen as a template. Red ribbons represent α-helices and blue arrows indicate β-sheets.

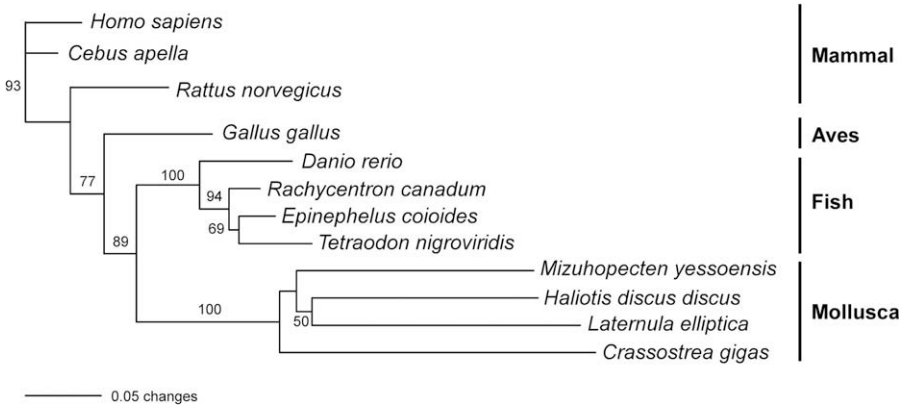

Fig. 2. Phylogenetic analysis of the leMnSOD proteins compared to those of other species. Numbers at each branch indicate the percentage of the times a node was supported in 1000 bootstrap pseudoreplicates by the neighbor-joining method.

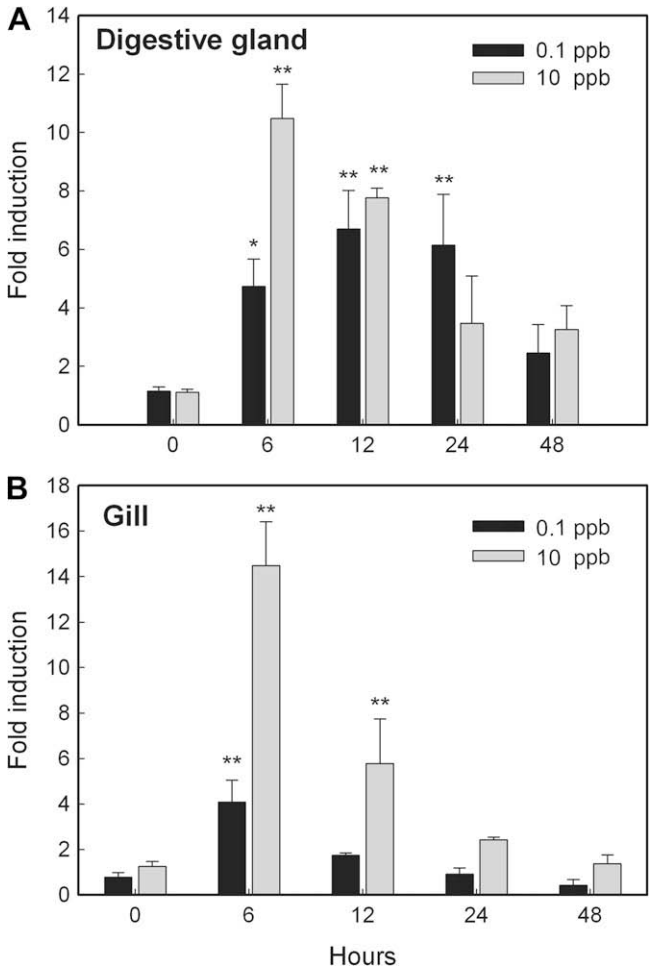

Fig. 3. Expression levels of leMnSOD mRNAs at different time points after exposure to the PCB mixture Aroclor 1254 in the digestive gland (A) and gill (B). Transcript levels for all samples were assessed by quantitative real-time RT-PCR with SYBR Green; leMnSOD expression was measured relative to β-actin expression. Values are presented as means ± SD. $^{*}P < 0.05$ and $^{**}P < 0.01$.

digestive gland and the gill were 1.6-fold and 3.5-fold higher, respectively, in samples exposed to PCBs at 10 ppb compared to 0.1 ppb.

3.3. Characterization of the recombinant leMnSOD

The overexpression of recombinant leMnSOD was carried out with *E. coli* BL21(DE3)pLysE and the expression vector pET28a. Induction of the expression construct, pET28a/leMnSOD, resulted in high yields of the recombinant protein. The leMnSOD purified by Ni-NTA resin column with the His_6 tag yielded a single band of ~24 kDa on SDS-PAGE and Western blotting analysis (Fig. 4). A total of 2.12 mg of leMnSOD was purified from 250 mL of LB medium, which had a specific activity of 20.1 U mg^{-1} protein when no metal ions were added to the bacterial culture. In contrast, the specific activity increased to 1853 U mg^{-1} protein in the presence of Mn^{2+} ions. The recombinant leMnSOD showed the greatest specific activity at pH 9.0 and retained more than 50% of its original activity at pH 8.0–10.5 (Fig. 5A). leMnSOD was incubated at pH 9.0 at various temperatures for 30 min and residual activity was measured. The results indicated that leMnSOD retained more than 50% of its original activity after incubation for 30 min at 40 °C (Fig. 5B).

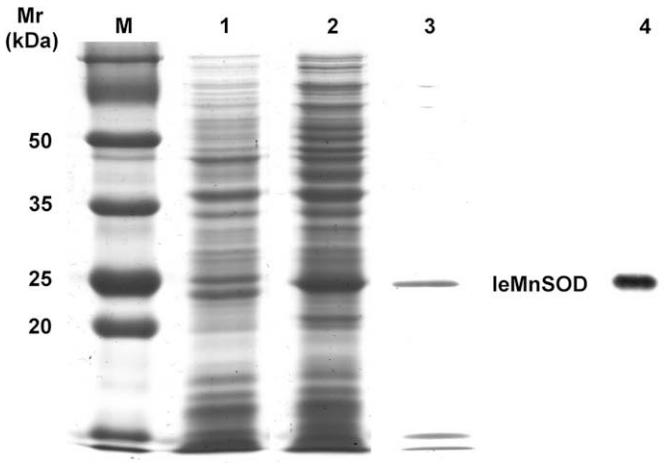

Fig. 4. SDS-PAGE and Western blotting analysis of expression and purification of the recombinant leMnSOD. M, molecular mass markers with sizes shown on the left in kDa: lanes 1–3 leMnSOD (lane 1, control before induction; lane 2, total soluble extract; lane 3, purified recombinant leMnSOD; lane 4, Western blotting with anti-His antibody).

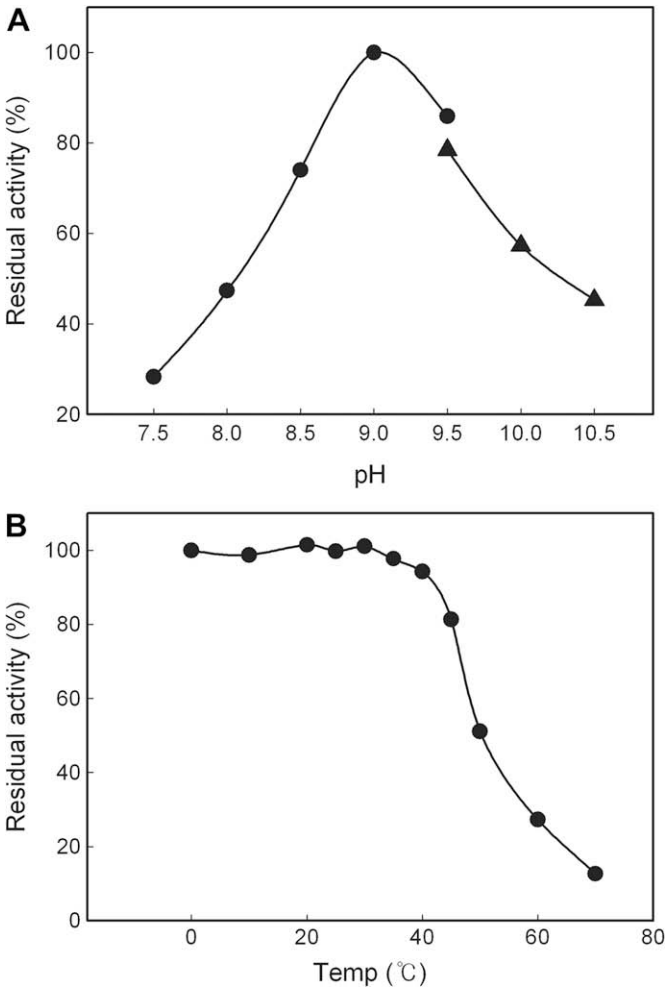

Fig. 5. Effects of pH (A) on purified recombinant leMnSOD activity. Activity was measured at 25 °C and various pH. The buffer used was 0.1 M Tris–HCl buffer (pH 7.5–9.5) (●) and 0.1 M Borax–NaOH buffer (pH 9.5–10.5) (▲). Thermostability of recombinant leMnSOD (B). The leMnSOD was incubated at various temperatures for 30 min, and its residual activity was measured at 25 °C and pH 9.0.

4. Discussion

SOD, which catalyzes the rapid two-step dismutation of the toxic superoxide anion to molecular oxygen and H_2O_2, has been implicated in protection against the damaging effects of oxidant stress in living organisms. Although MnSOD has been studied in various organisms, including prokaryotes and eukaryotes [21,22,24], only limited information is available regarding MnSOD from mollusks [36,37]. In the present study, we determined the full-length cDNA of manganese superoxide dismutase (denoted as leMnSOD) from the Antarctic bivalve *L. elliptica*. Sequence comparison of leMnSOD showed it was included in a monophyletic clade with other molluskan species in the phylogenetic analysis, and that it was most closely related to that of the abalone *H. discus discus*, with a relatively weak monophyletic relationship to the bivalves *M. yessoensis* and *C. gigas*.

Multiple alignment of the deduced amino acid sequence of leMnSOD with those of other organisms indicated retention of four highly conserved residues (H52, H101, D185, and H189), which are required for the coordination of manganese [16]. The MnSOD signature (DVWEHAYY) was completely conserved and a putative N-glycosylation site (Asn-X-Ser) was found from residue 99–101 as shown in other orthologs. MnSOD is generally localized in the mitochondrial matrix of eukaryotes and prokaryotes; it is synthesized as a precursor protein in the cytoplasm and then imported into the mitochondria after cleavage of its mitochondrial targeting peptide (mTP) [10]. However, two cytosolic types of MnSOD have been reported [17,38], which differ in their subunit structures: mitochondrial MnSODs are homotetramers, while cytosolic MnSODs are homodimers lacking the N-terminal signal peptide and are retained in the cytosol [17]. The putative mTP region of 26 amino acids was identified within the leMnSOD coding sequence by TargetP V1.1 [27]. Therefore, the leMnSOD cloned in this study belongs to the mitochondrial MnSODs. The overall sequence identity with *H. discus discus* MnSOD was 68%, but the mTP region (residues 1–26 of leMnSOD) showed a low level of identity (<46%), although the sequence of the mature region showed about 70% identity.

Many environmental contaminants that may accumulate in living organisms, such as PCBs, promote cellular defense systems with alterations of phase I and phase II metabolic enzyme activities. The production of ROS as by-products during phase I metabolism during the biotransformation of organochlorine contaminants is a common pathway of toxicity [39,40]. Previously, the gene encoding the phase II metabolic enzyme glutathione S-transferase was shown to be upregulated by PCB exposure [26]. In the present study, induction by PCB exposure of the major phase I metabolic enzyme, SOD, a mediator of ROS, was investigated. SOD scavenges the intermediates of oxygen reduction, which deprotonate superoxide anions (O_2^-) to H_2O_2. We compared SOD mRNA expression as a mechanism of protection against oxidative stress caused by PCB exposure in the gill and digestive gland using quantitative real-time RT-PCR analysis. Experimental exposure of *L. elliptica* to PCBs significantly altered MnSOD mRNA expression. The alterations were toward upregulation almost instantly, although large differences were observed depending on PCB concentration and tissue examined. In the digestive gland, leMnSOD mRNA was upregulated up to 12 h at the lower PCB concentration of 0.1 ppb, but the highest level of expression was observed at 6 h at the higher PCB concentration of 10 ppb. Subsequently, the level of leMnSOD mRNA decreased gradually to 48 h. In the gill, leMnSOD expression showed an increase in both tissues at 6 h, and the level of expression was 3.5-fold higher at the higher PCB concentration than at the lower concentration; this was likely because the gill is the most sensitive organ to external stimuli as it is directly exposed to the external environment [41]. Liu et al. [5] reported that Aroclor 1254 had a significant toxic effect on various immune parameters in the scallop *C. farreri*, including ROS production. Superoxide anion production on Aroclor 1254 treatment showed a significant increase on the first day and then became stable at an elevated level after long-term exposure. Therefore, we concluded that high levels of ROS generation due to PCB exposure at 10 ppb induced strong leMnSOD mRNA expression. In addition, the decreases in leMnSOD mRNA expression after a specific period of exposure may be caused by reduced metabolic capacity of the organism; this is consistent with the results of several previous studies indicating antioxidant enzyme mRNA expression is time- and dose-dependent as related to metabolic capacity [42,43].

Although MnSOD has been cloned, purified, and characterized from various organisms [10,17,24,44], few studies have reported on the cDNA structure, amino acid sequence, and characterization of those from marine mollusks [23,36,45]. The molecular weight of cloned leMnSOD was calculated to be 25 kDa based on the deduced amino acid sequence, which was almost the same as those of MnSODs from other organisms [23,46,47]. The MnSODs were stable over a wide pH range [48,49], although the optimum pH showed slight differences among species. The optimum pH of leMnSOD was 9.0, which was more alkaline than those of other marine mollusks [36,37,48]. Ken et al. [49] reported that acidic pH favors monomer formation. Therefore, the decrease in enzyme activity at acidic pH was due to dissociation to the monomeric forms caused by changes in equilibrium to subunit dissociation. The thermostability data indicated that leMnSOD retained more than 50% of its original activity below 40 °C. Yu et al. [50] reported that *Chlamydia pneumoniae* MnSOD showed higher thermostability due to the hydrophobic interactions formed by side chains of six conserved Leu residues of the N-terminal α-helices. The leMnSOD showed four conserved Leu residues (residues 64, 71, 86, and 90), which may explain the reduced thermostability of leMnSOD [48]. We compared the 3-D structure of leMnSOD with those of other organisms via homology-based modeling. The overall 3-D structure of leMnSOD monomer showed a high degree of similarity with other known MnSODs, but significant differences from scallop MnSOD models were found in that leMnSOD lacks two small α-helices corresponding to α1 and α6 of scallop MnSOD. These two α-helices are also absent in *Aspergillus* MnSOD [36]. Thus, leMnSOD was more similar to *Aspergillus* MnSOD in terms of α-helices. No previous reports have described relevant structural information on marine mollusk SOD. Thus, further studies of structural features should be conducted to clarify the structure/activity relationships and to evaluate the evolutionary relationships among organisms.

In conclusion, a leMnSOD gene was cloned from the Antarctic bivalve *L. elliptica*, and the recombinant leMnSOD expressed in *E. coli* was characterized. The deduced amino acid sequence showed a high degree of sequence identity to the MnSOD from the abalone *H. discus discus*. The main function of SOD is the elimination of superoxide radicals generated during normal physiological processes, as well as those due to external stimuli. The results reported here provide a basis for studying PCB detoxification processes in marine bivalves, and suggest that leMnSOD plays a role as an antioxidant protein in response to extracellular stimuli such as organotoxicants. This information on leMnSOD will be useful in studies of MnSODs in other marine mollusks. In the future, it will be important to extend our knowledge concerning the roles of other types of SOD (*i.e.*, Cu/ZnSOD, FeSOD, and NiSOD) to elucidate the evolutionary relationships and functions of the SOD systems in these organisms.

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Appendix. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi 10.1016/j.fsi.2009.07.008.

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