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Molecular Cloning, Purification, and Characterization of a Superoxide Dismutase from a Fast-Growing *Mycobacterium* sp. Strain JC1 DSM 3803

Ji-Sun Nam[1], Jee-Hyun Yoon[1], Hyun-Il Lee[1#], Si Wouk Kim[2], and Young-Tae Ro[1*]

[1]*Department of Biochemistry, Graduate School of Medicine, Konkuk University, Chungju 380-701, Republic of Korea*
[2]*Department of Environmental Engineering, Chosun University, Gwangju 501-759, Republic of Korea*
[#]*Present address: Department of Orthopedic Surgery, Samsung Medical Center, Seoul 135-710, Republic of Korea*

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A cytosolic superoxide dismutase (SOD) was purified and characterized from a fast-growing *Mycobacterium* sp. strain JC1 DSM 3803 grown on methanol. The native molecular weight of the purified SOD was estimated to be 48 kDa. SDS-PAGE revealed a subunit of 23 kDa, indicating that the enzyme is a homodimer. The enzyme activity was inhibited by H_2O_2 and azide. The purified SOD contained 1.12 and 0.56 g-atom of Mn and Fe per mol of enzyme, respectively, suggesting that it may be a Fe/Mn cambialistic SOD. The apo-SOD reconstitution study revealed that Mn salts were more specific than Fe salts in the SOD activity. The gene encoding the SOD was identified from the JC1 cosmid genomic library by PCR screening protocol. The cloned gene, *sodA*, had an open reading frame (ORF) of 624 nt, encoding a protein with a calculated molecular weight of 22,930 Da and pI of 5.33. The deduced SodA sequence exhibited 97.6% identity with that of *Mycobacterium fortuitum* Mn-SOD and clustered with other mycobacterial Mn-SODs. A webtool analysis on the basis of SOD sequence and structure homologies predicted the SOD as a tetrameric Mn-SOD, suggesting that the protein is a dimeric Mn-SOD having tetramer-specific sequence and structure characteristics.

***Keywords*:** Mn-SOD, *Mycobacterium*, PCR gene screening, sequence analysis, superoxide dismutase

Reactive oxygen species (ROS), such as superoxide radical anion ($^{\bullet}O_2^-$) and hydrogen peroxide (H_2O_2), are produced as by-products of adventitious electron transfers to oxygen during aerobic bacterial growth (Imlay, 2003). They can damage DNA, RNA, proteins, and lipids. In order to offset the harmful effects of the ROS, most organisms have developed protective mechanisms that utilize antioxidant enzymes, such as superoxide dismutases (SODs) and hydroperoxidases (i.e., catalases, peroxidases, and KatG), which scavenge $^{\bullet}O_2^-$ and H_2O_2, respectively (Fridovich, 1995).

SODs (EC 1.15.1.1) are metalloenzymes that catalyze the dismutation of the $^{\bullet}O_2^-$ to O_2 and H_2O_2, and they play a key role in cellular primary protection against oxidative stress conditions (Fridovich, 1995). There are several forms of SOD enzymes that are generally classified according to their metal cofactors and protein structures (Bannister *et al*., 1987). The Cu/Zn-SODs are predominantly found in eukaryotes and some bacteria including *Mycobacterium tuberculosis* (Dussurget *et al*., 2001), and they are dimers with two identical subunits containing one each of the Cu and Zn atoms. Most bacteria contain Fe- and/or Mn-SODs, and often they exist as subunits that link into dimmers or tetramers. The metal content of both enzymes can vary between one and two atoms per dimer, and they are mostly cytosolic enzymes, although a few are located on or secreted through the cytoplasmic membrane (Beaman *et al*., 1983). In addition, Ni-SODs have been discovered in several *Streptomyces* species (Youn *et al*., 1996).

Mycobacterium sp. strain JC1 DSM 3803 is a facultative chemolithotrophic carboxydobacterium and is capable of growing aerobically on both carbon monoxide (CO) and methanol as sole carbon and energy sources (Ro *et al*., 1997a, 2000). These distinct types of nutrition are also present in all mycobacterial strains tested, except for *M. tuberculosis* which is able to grow on CO but not on methanol, suggesting that CO and/or methanol utilization is an intrinsic ability of all known mycobacteria (Park *et al*., 2003). We previously reported that this bacterium possessed three types of catalases, including a catalase-peroxidase (KatG) (Shin *et al*., 1994). This KatG exhibited typical enzymatic characters of mycobacterial KatGs and the antibodies raised against it strongly cross-reacted with other mycobacterial KatGs (Ro *et al*., 2003). Recently, we cloned a DNA fragment containing two complete open reading frames (ORFs) encoding a putative ferric uptake regulator A (FurA) and a putative KatG protein (Lee *et al*., 2010). Genetic analysis of the cloned *furA* and *katG* genes revealed that their gene organization and sequence homology were highly homologous to those of other mycobacteria.

Within the genus *Mycobacterium*, SOD has been identified as one of the major protein antigens (Zhang *et al*., 1991). Because of the highly conserved amino acid sequence within the SOD, the SOD gene has been used as a target in the screening of mycobacteria presence on the genus level (Zolg and Philippi-Schulz, 1994). Currently, numerous papers have reported the distribution, characterization, and biological significance of SODs in mycobacteria (Mayer and Falkinham, 1986; Dussurget *et al*., 2001). For example, *M. tuberculosis* is a major human pathogen that produces a tetrameric Fe-SOD (SodA) and a dimeric Cu/Zn-SOD (SodC) (Zhang *et al*., 1991;

* For correspondence. E-mail: ytaero@kku.ac.kr; Tel: +82-2-2030-7825; Fax: +82-2-2049-6192

Wu *et al*., 1998). The Fe-SOD is a major 23 kDa antigen and is secreted to medium during growth (Andersen *et al*., 1991). The Cu/Zn-SOD possesses a putative signal peptide and is located in the periphery of *M. tuberculosis* (Wu *et al*., 1998; Dussurget *et al*., 2001). *M. leprae* expressed a major 28 kDa antigen identified as SOD on the basis of the high degree of homology with known Mn-SOD sequences (Thangaraj *et al*., 1990). In addition, it is reported that the deduced amino acid sequence of SOD gene cloned from *M. fortuitum* was similar to those of SODs from several other origins and that this enzyme required Mn as a cofactor (Menéndez *et al*., 1995). Thus, many papers described the existence of *SodA* gene encoding either Fe- or Mn-SODs in mycobacteria. However, a few works describing the enzymatic nature of Mn-SOD found in the genus *Mycobacterium* have been published as well (Escuyer *et al*., 1996).

In our research, we study the physiological and biochemical properties of SOD from a fast-growing *Mycobacterium* sp. Strain JC1 DSM 3803, and subsequently report the cloning, characterization, and expression of the SOD gene in order to investigate the diversity of bacterial SODs. Biochemical properties of the SOD protein and SOD sequence analysis reveals that this protein is a dimeric Mn-SOD having tetramer-specific sequence and structure characteristics.

Materials and Methods

Bacterial strains and cultivation

Mycobacterium sp. JC1 and other mycobacteria used in this study were cultivated at 37°C in standard mineral base (SMB) medium supplemented with 1% (v/v) methanol (Ro *et al*., 2000). *Escherichia coli* strains for cloning and expressing SOD gene and a Western blot analysis were grown at 37°C in Luria-Bertani (LB) medium. Growth was measured with a spectrophotometer by determination of turbidity at 436 nm.

Preparation of cell-free extracts

Cells were collected by centrifugation and washed once with 50 mM potassium phosphate buffer (pH 7.0, standard buffer). The washed cells were resuspended in the B-PER Reagent (in Phosphate Buffer, Thermo, USA). Cells in the suspension were briefly disrupted by sonic treatment for efficient cell lysis and then centrifuged at 15,000×g for 15 min. The resulting supernatant was used as the cell-free extract.

Enzyme assays and protein determination

All assays were carried out at 25°C unless otherwise noted. SOD activity was detected by an indirect method based on the ability of SOD to inhibit the reduction of nitro blue tetrazolium (NBT) via scavenging superoxide anion radicals produced by the xanthine/xanthine oxidase (Sigma, USA) system (Beauchamp and Fridovich, 1971). One unit (U) of SOD activity was defined as the amount of enzyme that gave 50% inhibition of NBT reduction. Protein was determined by Bradford reagent (Sigma) using bovine serum albumin as a standard as described in the manufacturer's protocol.

Purification of JC1 SOD

Small-scale purification of SOD was performed with a Pharmacia FPLC system equipped with columns listed below (Amersham Pharmacia, USA) and all purification steps were carried out at room temperature except when noted otherwise. Crude cell-free extract prepared from a 2.5 g of cells was heat-treated at 60°C for 10 min and centrifuged at 15,000×g for 15 min at 4°C. The heat-treated extract was concentrated and separated into a Sephacryl S-300 HR column (HiPrep 16/60) with the standard buffer at a flow rate of 1 ml/min. SOD fractions were applied into a Mono-Q column (HR 5/5) equilibrated with 0.2 M KCl in a standard buffer. Elution was performed with a linear gradient of 0.2-0.7 M KCl in a standard buffer at a flow rate of 0.5 ml/min. SOD fractions were concentrated and desalted with Centricon YM10 filter units. Then, the ammonium sulfate (AS)-treated sample (final concentration, 1 M) was applied onto a 5 ml HiTrap Phenyl Sepharose HP column equilibrated with 1 M AS in the standard buffer and the column was washed with the same buffer at a flow rate of 1 ml/min. The active fractions collected in the washing step were combined, concentrated, and applied to a 5 ml HiTrap desalting column. Elution was done with pure water and the desalted fractions were used as the purified SOD fraction.

Electrophoresis and SOD activity staining

Polyacrylamide gel electrophoresis (PAGE) was performed in accordance with the procedure of Laemmli (1970) and proteins were stained with Coomassie brilliant blue R-250 (CBB). SOD activity was visualized on a non-denaturing polyacrylamide gel as previously described (Beauchamp and Fridovich, 1971). The SOD activity area appeared as a clear zone on a blue-violet background.

Characterization of JC1 SOD protein

Native molecular mass of the purified SOD was determined by gel filtration chromatography on a Sephacryl S-300 HR column (HiPrep 16/60) calibrated with a molecular weight standard marker kit (MW-GF-1000; Sigma). Molecular mass of the SOD subunit was determined by a SDS-denaturing PAGE (10% polyacrylamide gel) with prestained broad-range protein standards (Bio-Rad, USA).

To determine the stability of SOD protein on different pHs, purified SOD in triplicate was incubated for 90 min at various pHs ranging from 3.0 to 10.0, and the residual SOD activity was measured by routine enzyme assay. The following buffer systems were used at 50 mM concentration: pH 3.0 to 5.0 sodium citrate buffer, pH 6.0 to 8.0 sodium phosphate buffer, and pH 9.0 to 10.0 glycine buffer. To determine the resistance to heat inactivation, purified SOD in duplicate was incubated for 60 min at various temperatures and then analyzed for residual SOD activity at room temperature, and compared with the SOD activity of a control sample incubated at 25°C for the same period. For inhibitor study, purified SOD in duplicate was incubated for 90 min with 1 mM and 10 mM concentrations of inhibitors and SOD activity was then measured as described above.

Determination of metal contents

Metals present in the purified enzyme were analyzed using spectrophotometers (Korea Basic Science Institute, Korea). Cu, Mn, Ni, and Zn contents were measured with X5 inductively coupled plasma-mass spectrometer (ICP-MS, ThermoElemental, UK). The Fe content was measured with Optima 4300DV ICP-atomic emission spectrometer (ICP-AES, Perkin-Elmer, USA).

Preparation and reconstitution of the apoenzyme

Apoenzyme was prepared by a metal removal procedure (Kirby *et al*., 1980) with minor modifications. Briefly, purified SOD was dialyzed against 20 mM 8-hydroxyquinoline, 2.5 mM guanidium chloride, 5 mM Tris, and 0.1 mM EDTA at pH 3.8, at 4°C for 18 h. For the reconstitution, apoenzyme solution was dialyzed against 5 mM Tris

buffer (pH 7.8), containing 1 mM of either Fe, Mn, or Fe/Mn salts for 18 h. The purified protein was dialyzed against the reconstitution buffer containing both Fe and Mn salts and it was used as a native SOD control. Excess amounts of metal salts were removed by further dialysis against the same buffer. The resulting samples were then clarified by centrifugation and used for SOD activity staining.

DNA manipulation

Chromosomal DNA was isolated from the cells of *Mycobacterium* sp. JC1 according to a method previously reported (Goldberg and Ohman, 1984). Plasmid and cosmid DNAs from *E. coli* were isolated by using Exprep Plasmid SV kit as described in the manufacturer's instruction (GeneAll, Korea).

Polymerase chain reaction (PCR)

Two degenerated primers, Sod-DF (5′-GTGGCTGAATACACYYT G-3′) and Sod-DR (5′-YYAGCCGAAGATCAGRCC-3′), were synthesized to amplify the region covering the SOD gene of JC1, based on the consensus amino acid sequences VAEYTL and GFILG(Q/S/N) that are present at the N- and C-termini of other mycobacterial SODs, respectively. The PCR was performed in a 50 μl reaction mixture containing 100 ng JC1 genomic DNA, 50 pmol of each primer, and 1 unit of HotStar Taq plus DNA polymerase (QIAGEN, USA). The amplification conditions consisted of 35 cycles of 45 sec at 94°C, 45 sec at 55°C and 1 min at 72°C after primary activation for 2 min at 94°C, with subsequent 10 min extension at 72°C. The PCR products were directly cloned into pGEM-T-Easy cloning vector (Promega, USA) as the manufacturer's instruction. Positive clones were subjected to DNA sequencing performed by Cosmo (Korea). DNA sequences were analyzed by using ExPASy proteomic server (http://ca.expasy.org/) to find the SOD sequence homology.

SOD gene cloning

Two nested primers, Sod-NF (5′-CAGATCAACGAGCTTCACCA-3′) and Sod-NR (5′-TAGTCCGCCTTCACGTTCTT-3′), were synthesized to screen the previously constructed JC1 genomic cosmid library (Lee *et al*., 2010), based on the real JC1 partial SOD gene sequence identified above. The PCR screening was carried out in a 20 μl reaction mixture containing 1 μl of JC1 cosmid DNA, 50 pmol of each primer, and 1 unit of HotStar Taq plus DNA polymerase. The amplification conditions consisted of 35 cycles of 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C after primary activation for 2 min at 94°C, with subsequent 10 min extension at 72°C. A positive cosmid clone (#3-57) was selected and subjected to DNA sequencing directly with the nested primers.

Computer-assisted sequence analysis and phylogenetic analysis

DNA and protein sequence analyses were carried out using ExPASy proteomics server. Homologous genes and proteins were searched in Basic Local Alignment Search Tool (BLAST) either of National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/) or of network service of ExPASy (http://ca.expasy.org/tools/blast/). Phylogenetic analysis of SOD proteins were performed using the MEGA3 program after multiple alignments of data using the CLUSTAL W program.

Overproduction of SOD gene in *E. coli*

The entire *sodA* coding region of *Mycobacterium* sp. JC1 was amplified by PCR using genomic DNA as a template and the following primer pair based on the SOD gene sequence found in this study: 5′-CCATGG*CTGAATACACCCTGCCAG*-3′ (JCSOD-N) and 5′-AGATCT*GCCGAAGATGAGGCCGTT*-3′ (JCSOD-C), consisting of a *Nco*I and a *Bgl*II restriction sites (underlined) followed by the sequences (in italic) encoding the six N- and C-terminal amino acids of JC1 SOD, respectively. The PCR fragment was cloned into pGEM-T-Easy vector and *Nco*I/*Bgl*II-digested fragment was further cloned into pQE-60 expression vector (QIAGEN, USA) to create pQE60-JC1SodA which directed the expression of the recombinant C-terminal His-tag fusion protein with the authentic JC1 SOD sequence. The resulting pQE60-JC1SodA was introduced into *E. coli* XL1-Blue and induced for the recombinant SOD protein expression by 0.5 mM IPTG.

Preparation of SOD antiserum and immunoblot analysis

Because the recombinant SOD protein was expressed as an insoluble form, it was purified from the inclusion body using B-PER reagent (Thermo, USA) according to the manufacturer's protocol. The purified protein was then subjected to 10% SDS-PAGE. After electrophoresis, the protein was cut out from the gel and used as an immunogen. Antiserum production in rabbits was conducted in a custom antibody service company (AbFrontier, Korea). A Western blot analysis was performed as described previously (Ro *et al*., 1997b). Briefly, protein sample was resolved by SDS-PAGE (10% acrylamide) and electroblotted to a polyvinylidene difluoride (PVDF) membrane with Semi-Dry Transfer Cell unit (Bio-Rad). The blot was incubated in a 1/5,000 dilution of the JC1 recombinant SOD antiserum produced in this study as a primary antibody and in a 1/10,000 dilution of alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Promega) as a secondary antibody. The alkaline phosphatase color development reaction was performed with a Fast BCIP/NBT kit recommended by the manufacturer (Sigma).

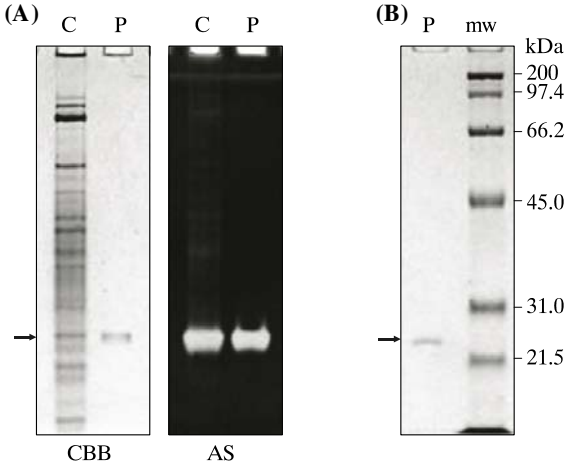

Fig. 1. The analysis of SOD protein on polyacrylamide gels. (A) Non-denaturing PAGE of SOD protein. The cell-free extract (40 μg, lane C) and purified SOD (2 μg, lane P) from *Mycobacterium* sp. JC1 were subjected to PAGE on a 7.5% acrylamide gel and stained by Coomassie Brilliant Blue R-250 (CBB) or SOD activity (AS). (B) Denaturing PAGE of purified SOD. SDS-PAGE (10% acrylamide, 0.1% SDS) was performed with 2 μg of purified SOD (lane P) and molecular mass marker proteins (lane mw; Prestained SDS-PAGE standards, Broad range, Bio-Rad) and stained with CBB. Arrow indicates the SOD protein.

Table 1. Purification of a superoxide dismutase (SOD) from *Mycobacterium* sp. JC1

Purification step	Total protein (mg)	Total activity (U)[a]	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	123.46	2707.3	21.9	100.0	1.0
Heat-treated extract	25.75	1489.7	57.8	55.0	2.6
Sephacryl S-300	2.49	1211.0	97.0	44.7	4.4
Mono-Q	1.39	1065.5	768.5	39.4	35.0
Phenyl Sepharose & HiTrap desalting	0.17	432.9	2523.7	16.0	115.1

[a] One unit (U) was defined as the amount of enzyme that gave 50% inhibition of NBT reduction

Nucleotide sequence accession number

The nucleotide sequence reported in this study has been deposited to the GenBank under accession number GQ454924.

Results

Detection and purification of SOD

A single band with SOD activity was detected from the cell-free extract of *Mycobacterium* sp. JC1 (Fig. 1A). As summarized in Table 1, the SOD was purified 115-fold in five steps to homogeneity, with a yield of 16% and the specific activity of 2,523 U/mg of protein. Non-denaturing PAGE of the purified protein showed only a single band, illustrating the purity of the enzyme. When judged by SOD activity staining (AS), it exhibited SOD activity, showing that the purified enzyme is active (Fig. 1A).

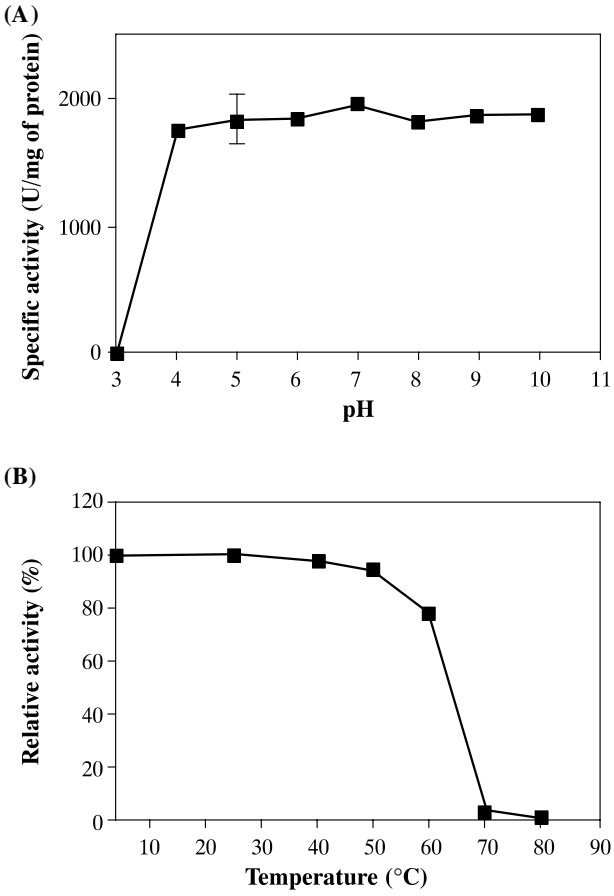

Fig. 2. Stability to pH and temperature on the SOD protein. (A) pH stability of the purified protein was determined in different pHs, as described in 'Materials and Methods' section. (B) Thermal stability was determined after incubation of the purified protein for 60 min at the indicated temperatures. The initial activity at 4°C was set as a rate of 100% and it corresponded to 1,877 U/mg of protein.

Molecular properties of SOD

The molecular weight of the native enzyme was estimated to be 48,000 by gel filtration on a Sephacryl S-300 HR column (HiPrep 16/60) with a molecular weight standard marker kit. Denaturing PAGE of the purified protein showed a single band of approximately 23 kDa (Fig. 1B), indicating that the JC1 SOD is a homodimer.

The UV-visible absorption spectra of the purified protein showed a typical protein peak at 280 nm with a shoulder at 294 nm, but did not show eminent peaks at the visible light range (data not shown).

Stability to pH and temperature

After 90 min incubation at pH 3.0, the purified enzyme completely lost the SOD activity. In contrast it remained active over pH ranges of 4.0 to 10.0, suggesting that the SOD is stable at broad ranges of pH (Fig. 2A). The purified protein was stable after 1 h of incubation at 60°C, which means the SOD is relatively thermostable. However the activity rapidly declined when the protein was incubated at 70°C above for 1 h (Fig. 2B).

Inhibitor study

SOD activity in the purified enzyme was assayed with various types of inhibitors. After the purified SOD was incubated for 90 min in the presence of 10 mM H_2O_2, 85% of its activity

Table 2. Effect of inhibitors on *Mycobacterium* sp. JC1 SOD activity

Inhibitor	Concentration (mM)	Inhibition (%)
KCN	1	3.4
	10	5.8
NaN_3	1	10.6
	10	12.0
H_2O_2	1	36.8
	10	84.6
EDTA	1	0.0
	10	0.0
Cuprizone	0.1	0.0

Table 3. Metal contents of *Mycobacterium* sp. JC1 SOD

Metals	Metal contents[a]		Culture medium (M)
	g/mg of enzyme	g-atom metal/mol of enzyme	
Cu	0.15	0.05	0.40
Fe	1.35	0.56	8.96
Mn	2.67	1.12	2.96
Ni	0.0	0.0	0.0
Zn	0.23	0.08	1.74

[a] The mean values of two different analysis are given.

was inhibited. On comparison, when the SOD was incubated with KCN or NaN_3 at the same concentration, only 6% or 12% of the SOD activity was inhibited, respectively. Both EDTA as a metal chelator and cuprizone as a copper chelator did not inhibit the SOD activity at the concentration of 10 mM and 0.1 mM, respectively (Table 2).

Metal at the active site

As shown in Table 3, atomic absorption analysis for metals revealed that the purified SOD was found to contain 1.12 g-atom of Mn and 0.56 g-atom of Fe per mol enzyme. Trace amounts of Cu and Zn were detected in the purified protein fraction, but Ni was absent (Table 3).

The specificity of metal ion at the active site in the SOD was determined by metal reconstitution study with the apo-SOD. After dialysis in buffer containing Mn and Fe/Mn ions, 19.1% and 19.8% of the SOD activity were recovered, respectively. However, 4.3% of the SOD activity was restored after dialysis in buffer containing Fe ions (Table 4). This indicated that Mn salts were more specific than Fe salts to *Mycobacterium* sp. JC1 SOD, although they were not able to completely restore the enzyme activity. Since the purified SOD (native SOD) dialyzed against both Fe and Mn salts exhibited much lower activity than the purified protein itself, this may be due to the permanent denaturation of the native enzyme during the reconstitution experiment and possibly also links to the failure of the apo-SOD in restoring the metals.

Table 4. Specific activities of native, apo, and metal-reconstituted SODs[a]

Enzymes	Specific activity[b]	Relative activity[c]
Native SOD[d]	278	100
Apo-SOD	<1	<0.4
Fe-reconstituted SOD	12	4.3
Mn-reconstituted SOD	53	19.1
Fe/Mn-reconstituted SOD	55	19.8

[a] Values are means of three individual tests.
[b] U/mg of protein. One U of activity was defined as the amount enzyme required to give a 50% decrease in the rate of reduction of NBT.
[c] Native SOD activity was set as 100%.
[d] The purified protein was dialyzed against the reconstitution buffer containing both Fe and Mn salts to use as a control.

Cloning of the SOD gene

Attempt to identify the N-terminal amino acid sequence from the purified SOD was unsuccessful possibly due to the N-terminal block. Therefore, two degenerated primers, Sod-DF and -DR, were synthesized based on the amino acid sequences conserved in several mycobacterial Mn-SODs, and they were used to amplify the SOD gene. Using the primers, a 0.6 kb DNA fragment was amplified by PCR with JC1 genomic DNA as a template (data not shown). The fragment was sequenced after cloning into pGEM-T-Easy vector and its sequence was found to be highly homologous to those of the *M. fortuitum* and *M. smegmatis sodA* genes.

For cloning the entire JC1 SOD gene, we performed PCR

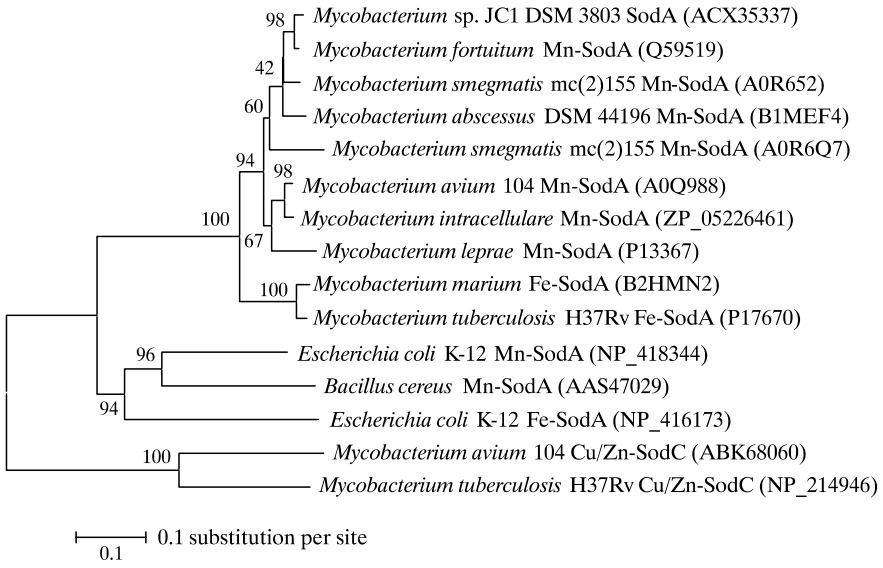

Fig. 3. Phylogenetic tree of the deduced protein sequences of *Mycobacterium* sp. JC1 SOD protein. Amino acid sequence alignments were performed using CLUSTAL W. The tree was generated by neighbor-joining (Poisson correction model) using the MEGA 3 program. The gaps in the alignment were completely deleted. The GenBank accession nos. for the sequences were given in parentheses. Bootstrap values were calculated from 100 replicates.

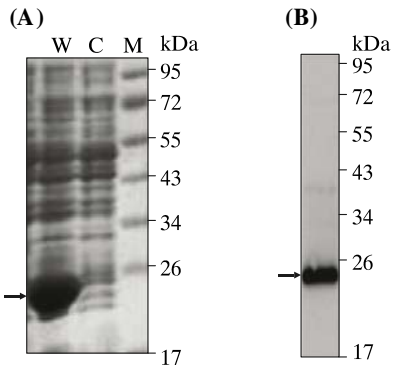

Fig. 4. Overproduction of the recombinant SOD protein in *E. coli* and the Western blot analysis. (A) The recombinant SOD protein was induced by addition with 0.5 mM IPTG. The whole cell extract (W) and soluble cell-free extract (C) from the IPTG-induced cells were subjected to SDS-denaturing PAGE on a 10% acrylamide and the gel was stained with CBB. Arrow indicated an overproduced recombinant protein. M: Prosi prestained protein marker (GenDEPOT, USA). (B) The Western blot analysis. The cell-free extract (10 μg) from *Mycobacterium* sp. JC1 grown on methanol was resolved by SDS-PAGE (10% acrylamide) and subjected to a Western blot analysis with antiserum raised against the recombinant SOD protein. Arrow indicates the SOD protein.

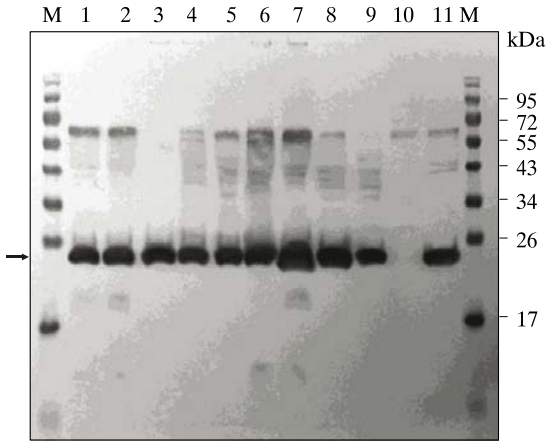

Fig. 5. Immunoblotting of SOD in mycobacteria. Cell-free extracts (10 μg each) prepared from mycobacteria and *E. coli* grown on methanol and LB, respectively, was resolved by SDS-PAGE (10% acrylamide) and subjected to a Western blot analysis with antiserum raised against the recombinant SOD protein. Arrow indicates the SOD protein. Lanes: 1 and 11, *Mycobacterium* sp. JC1; 2, *M. parafortuitum* (ATCC 19686); 3, *M. flavescens* (ATCC 14474); 4, *M. gastri* (ATCC 15754); 5, *M. neoaurum* (ATCC 25795); 6, *M. phlei* (ATCC 11758); 7, *M. vaccae* (ATCC 15483); 8, *M. smegmatis* mc^2 (ATCC 700084); 9, *M. peregrinum* (ATCC 14467); 10, *E. coli* XL-1 blue; M, Prosi prestained protein marker (GenDEPOT, USA).

screenings of *Mycobacterium* sp. JC1 genomic cosmid library, instead of Southern hybridizations, with two nested primers, Sod-NF and -NR that are synthesized based on the DNA sequences of the PCR product above. Through the screening of only 94 cosmid DNAs among a total of 720 cosmid clones, we found that a cosmid DNA from #3-57 clone contained the JC1 SOD gene (data not shown). The cosmid DNA was directly sequenced with the nested primers and 900 bp nucleotide sequences containing the entire JC1 SOD ORF was deposited to the GenBank under accession number GQ454924.

The SOD gene and phylogenetic analysis

The identified SOD ORF consisted of 624 nucleotides and could encode for a protein of 207 amino acid residues. The calculated molecular mass and pI of the protein were 22,930 Da and 5.33, respectively. A putative ribosome-binding site (GGAAGGAA) localized at 5 to 12 bp upstream of the GTG initiation codon (data not shown). BLAST similarity search showed that the SOD sequence had overall identities exceeding 79% with other mycobacterial SodAs including *M. tuberculosis* H37Rv and had the highest identity of over 97% to *M. fortuitum* Mn-SOD (GenBank accession no. Q59519).

Phylogenetic analysis using the MEGA3 program revealed that the JC1 SOD clustered with other mycobacterial Mn-SodAs, but not with the Cu/Zn-SodCs from other mycobacteria (Fig. 3).

Overproduction of the SOD in *E. coli* and Western blot analysis

A pQE-60 plasmid containing the entire JC1 SOD gene was introduced into *E. coli*, and the recombinant protein was induced by the addition of IPTG. Judged by a SDS-PAGE, an overproduced protein with 23 kDa size was seen only in whole cell extracts, but not in soluble cell-free extract (Fig. 4A, lane W and C, respectively). This indicated that the recombinant SOD was expressed as an insoluble form. Changes of culture temperatures (22, 30, and 37°C) and IPTG concentrations (0.1, 0.5, and 1 mM) did not enhance the solubility of the recombinant protein (data not shown).

To generate polyclonal antibody against the recombinant SOD protein, the purified insoluble proteins were retrieved from polyacrylamide gel and used in polyclonal antibody generation in rabbits. The Western blot analysis revealed that the antiserum raised against the insoluble protein clearly recognized a 23 kDa protein in *Mycobacterium* sp. JC1 cell-free extract (Fig. 4B), showing the specificity of the produced SOD antiserum. Thus, immunoblotting of a gel after SDS-denaturing PAGE of cell-free extracts proved that a 23 kDa protein from all mycobacteria including *Mycobacterium* sp. JC1, except *E. coli*, cross-reacted with the antiserum raised against the JC1 SOD protein (Fig. 5). Consequently it is illustrated that the tested mycobacteria have a SOD protein similar in subunit size and antigenicity to those of the *Mycobacterium* sp. JC1 SOD protein.

Discussion

All bacteria, including obligate anaerobes, generally produce either Fe-SOD, Mn-SOD, or both. The SODs exist as subunits (molecular masses 19 to 23 kDa) that are linked into a dimer or a tetramer. Fe-SOD predominates in anaerobic organisms whereas Mn-SOD is more commonly found among aerobic organisms. Furthermore they are cytoplasmically located, although a unique SOD from *Nocardia asteroids* is associated to the outer cell wall and the SOD is not excreted to the

medium (Beaman *et al*., 1983).

A single band with SOD activity was detected from the cell-free extract of *Mycobacterium* sp. JC1. The SOD protein was not found in the concentrated medium after growth (data not shown), indicating that the SOD is a cytoplasmic protein. To characterize the nature of the SOD protein, we purified the protein 115-fold with homogeneity in five steps as outlined in Table 1. The SOD protein was a homodimer of an approximately 23 kDa subunit, similar to those of other bacterial Fe- or Mn-SODs described above. The purified enzyme was relatively stable at 60℃ and the activity dropped to 45% after 60 min at 65℃, suggesting that the SOD is thermostable. The purified SOD also showed the stability in a broad pH range (4.0-10.0), similar to an extracellular Fe-SOD from *M. bovis* BCG (Kang *et al*., 1998).

In general, Mn-SOD was inhibited only by azide, whereas Fe-SOD is sensitive to H_2O_2 and cyanide (Misra and Fridovich, 1978; Beyer and Fridovich, 1987). Inhibitor study of the purified SOD showed that the protein was sensitive to H_2O_2 and azide. This inhibition pattern is similar to that of Fe-SOD. Moreover, metal analysis in the purified protein revealed that it contained 1.12 g-atom of Mn and 0.56 g-atom of Fe per mol of enzyme, suggesting that the JC1 SOD may be a new member of the so called cambialistic SOD that contains Fe and/or Mn (Whittaker, 2003). As Fe- and Mn-SODs are highly homologous and exhibit structural similarity (Stallings *et al*., 1984), it is assumed that they originate from a common ancestry. However, it is often quite difficult to distinguish the metal specificity on the basis of their protein structures (Wintjens *et al*., 2004). Despite this similarity, they can be distinguished by the presence of some key discriminating amino acids in and close to the active site (Parker and Blake, 1988) and also by their different sensitivities to H_2O_2 inhibition (Beyer and Fridovich, 1987). However, these criteria are sometimes misleading, and the identity of the metal cofactor still needs to be confirmed by direct analysis of the purified holoproteins. Indeed, direct metal reconstitution study of the purified SOD revealed that Mn ions were more specific than Fe ions in *Mycobacterium* sp. JC1 SOD, indicating that the SOD may be a Mn-SOD. To clarify the nature of metal ions at the active site in the SOD further experiments would be required.

A 23 kDa protein predominates in mycobacteria as a major cytosolic antigen and it is identified as a mycobacterial SOD of the Fe or Mn type (Zhang *et al*., 1991; Escuyer *et al*., 1996). The Western blot analysis after denaturing PAGE of the cell-free extracts prepared from nine mycobacteria including *Mycobacterium* sp. JC1 and an *E. coli* strain revealed that all mycobacteria tested, except *E. coli*, contained an approximately 23 kDa protein cross-reacting with the antiserum raised against the recombinant JC1 SOD, suggesting that mycobacteria may have a common cytosolic SOD similar in subunit size (23 kDa) and antigenicity.

To precisely define the nature of the JC1 SOD, the gene encoding the SOD was cloned and the sequence was analyzed. Surprisingly, the deduced amino acid sequence from the cloned SOD gene was almost identical with *M. fortuitum* (97.6%) Mn-SOD (Menéndez *et al*., 1995). The deduced molecular mass of the SOD protein was 22,930 Da, similar to that of the purified protein calculated by SDS-PAGE (23 kDa). A gel filtration study estimated the molecular mass of the native JC1 SOD protein to be approximately 48 kDa, indicating that JC1 SOD is a dimeric protein of 23-kDa subunits. Phylogenetic analysis of the JC1 SOD revealed that it closely clustered with other mycobacterial Mn-SODs.

On the basis of 261 aligned SOD sequences and 12 superimposed x-ray structures, several conserved residues characteristic of either Mn-/Fe-specific and/or dimer-/tetramer-specific sequences and structures have been reported (Wintjens *et al*., 2004). Also, a webtool (http://babylone.ulb.ac.be/SODa/) is available to predict whether a target sequence corresponds to a Fe/Mn SOD. It can also estimate the metal ion specificity (Fe, Mn, or cambialistic) and the oligomerization mode (dimer or tetramer) of the target (Kwasigroch *et al*., 2008). When the JC1 SOD sequence was submitted in the webtool, the SOD was predicted to a Mn tetramer SOD and its fingerprint score was 66.54%. The six highly conserved residues (His^{30}, His^{31}, Tyr^{34}, Trp^{77}, Trp^{122}, and Trp^{158}) in the immediate environment of the metal cofactor in Mn-/Fe-SODs are well conserved in the SOD. Residues Thr^{22}, Asn^{65}, Phe^{118}, and Pro^{144} are systematically encountered in dimmers but never in tetramers, whereas Phe^{65} residue is observed among tetramers. In the JC1 SOD sequence, the dimmer-specific residues (Thr^{22}, Asn^{65}, Phe^{118}, and Pro^{144}) were interestingly substituted to Ile^{22}, Phe^{65}, Gln^{118}, and Asn^{144}, respectively, which were conserved in tetramers. Also, other tetramer-specific residues (Ile^{18}, Asn^{79}, Leu^{164}, Gln^{165}, and Tyr^{166}) were well conserved in the JC1 SOD, suggesting that the SOD can be a tetramer. Of the four Mn-specific residues (Met^{23}, Gly^{68}, Gln^{141}, and Asp^{142}) in both dimmers and tetramers, only Gly^{68} and Gln^{141} residues were found in the SOD, but the Met^{23} and Asp^{142} residues were substituted to Asn^{23} and Gln^{142}, respectively, which were found in Fe tetramer SODs. The results suggest that the *Mycobacterium* sp. JC1 SOD presents an atypical behavior and it is a dimeric Mn-SOD having tetramer-specific sequence and structure characteristics.

The SOD analysis represents that SOD dimmers, whether binding Fe or Mn, have common sequence and structure characteristics that differentiate them from tetramers. From this point of view, the ensemble of residues that ensures the metal specificity and/or oligomeric state of SOD enzymes can be used to define Fe-/Mn- and dimer/tetramer-specific fingerprints. If a given sequence shows some deviations from the typical fingerprints, it can be thought to adapt to alternative oligomeric states or to have cambialistic tendencies (Wintjens *et al*., 2004). Indeed, the analysis of a Mn-SOD from an extremely thermophilic *Thermus thermophilius* predicted that it had all the sequence and structural features of typical dimmers. However the biochemical evidence revealed an apparent contradiction to the above assumption since the SOD exhibited a tetrameric behavior in solution (Whittaker and Whittaker, 1999), reflecting the possibility that the oligomeric state of the SOD may depend on the experimental conditions such as temperature, pH, protein concentration, or ionic strength (Whittaker, 2003; Wintjens *et al*., 2004). Therefore, it is also possible that *Mycobacterium* sp. JC1 SOD can adopt to alternative oligomeric states, either dimer or tetramer, subject to the experimental conditions. More detailed experiments on the effect of different conditions could provide better understandings about the oligomeric states of the JC1 SOD.

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