

Characterization of Fe/Mn–Superoxide Dismutase from Diatom *Thalassiosira weissflogii*: Cloning, Expression, and Property

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A cDNA clone of 1114 bp encoding a putative Mn–superoxide dismutase (Mn–SOD) from diatom *Thalassiosira weissflogii* was cloned by the PCR technique. Nucleotide sequence analysis of this cDNA clone revealed that it was translated into 201 amino acid residues. When the sequence was compared with Mn–SODs from *Vibrio mimicus* and *Escherichia coli*, as well as two Fe–SODs from *E. coli* and *Photobacterium leiognathi*, this SOD showed higher homology to Mn–SOD. The amino acid residues required to coordinate the single manganese ion were conserved in all reported Mn–SOD sequences. This cDNA was introduced in an expression vector, pET-20b(+), and transformed into *E. coli* BL21(DE3)pLysS. The expressed SOD protein was then purified by a His-tag column. The recombinant enzyme was heated at 55 °C with a time-dependent assay; the time interval for 50% inactivation was 23 min, and its thermal inactivation rate constant K_d was $3.03 \times 10^{-2} \text{ min}^{-1}$. The enzyme was inactivated either in acidic pH (below 4.0) or in the presence of imidazole (above 1.6 M) and had only a moderate effect under SDS (above 4%), whereas it was not affected under an alkaline pH (above 9.0). The atomic absorption spectrometric assay showed that 0.6 atom of iron/manganese (3:1) was present in each subunit of SOD. Reconstitution study was suggested that diatom SOD was cambialistic (Fe/Mn)–SOD. The finding of this SOD cDNA could be used for a reference in comparing the differences among marine phytoplankton species and as a probe to detect the transcription level of this enzyme, which can be applied in cosmetics for skin protection or defending unesthetic effects caused by oxygen-containing free radicals.

KEYWORDS: Diatom; *Thalassiosira weissflogii*; expression; cambialistic-superoxide dismutase (Fe/Mn–SOD)

INTRODUCTION

Superoxide dismutases (SODs) form the first line of defense system in various organisms against reactive superoxide radicals and are vital to the survival of cells (1). SODs can be classified into four types, Cu/Zn–, Mn–, Fe–, and Ni–SOD, depending on the metal bound at the active site (2–5). Cu/Zn–SOD occurs primarily in the cytosol of eukaryotic cells and the chloroplasts of plants and is also found in the periplasm of gram-negative bacteria and in the intermembrane space of mitochondria. Mn–SOD is distributed among prokaryotic and eukaryotic organisms associated with mitochondria and is insensitive to cyanide and hydrogen peroxide. Fe–SOD is found in plants as well as in prokaryotes and is not sensitive to cyanide but is inhibited by hydrogen peroxide. Ni–SOD was purified from several aerobic

soil bacteria of the *Streptomyces* species that is distinct from the Mn–, Fe–, or Cu/Zn–SODs on the basis of amino acid sequence, immunological cross-reactivity, and spectroscopic properties (6). Fe–SOD and Mn–SOD appear to be closely related in structure but have no resemblance to Cu/Zn–SOD. An anaerobic bacteria synthesized either Fe– or Mn–SOD with identical protein moiety depending on the metal supplied (7). Fe-containing Mn–SOD in *E. coli* grown in tryptic soy yeast extracts medium suggested the possibility that Fe-substituted Mn–SOD could have some physiological function (8). Even if a Mn–SOD had been classified as having highly metal-specific enzyme activity, it may exhibit at least some activity with iron at acidic pH. The camphor SOD more resembled Mn–SODs in primary sequence, but its bound metal (0.5–1 atom of Fe per camphor SOD subunit) and the ultraviolet and visible spectra were similar to those of iron-containing enzymes (9). These represented a type of SODs that could accept either Fe or Mn as metal cofactors and was named “cambialistic” SOD. In this study, we report that a SOD cDNA was cloned from diatom

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and the active form of SOD was purified from *Escherichia coli*. Taking this primary sequence to compare with two Mn–SODs from *Vibrio mimicus* and *E. coli* as well as that of two Fe–SODs from *E. coli* and *Photobacterium leiognathi* (10–12), this SOD is more similar to Mn–SOD in sequence, but SOD purified from *E. coli* grown in LB medium containing iron indicated that there was 0.6 atom per SOD subunit; the ratio of Fe to Mn was 3-fold. Thus, this SOD could belong to be cambialistic-SOD and was first reported from unicellular eukaryotes.

Irradiation by visible light in the presence of a photosensitizer leads to the production of reactive oxygen species (ROS), which in plants and algae is linked to photosynthesis. Because of the elevated oxygen concentration and intense electron flux within chloroplasts, electrons inevitably react with oxygen, thereby generating $O_2^{\cdot-}$, which dismutates to oxygen and hydrogen peroxide, producing the highly reactive HO^{\cdot} through the metal-ion-catalyzed Haber–Weiss reaction. Even under nonstress conditions, this ROS-generating mechanism can harm and inactivate the photosystem II reaction center, resulting in photoinhibition. Thus, tolerance of photosynthetic organisms to oxidative challenge is enhanced by defense responses that prevent oxidative damage to chloroplasts. Because $O_2^{\cdot-}$ is a precursor of several other reactive species, control over the steady-state $O_2^{\cdot-}$ levels by SOD is critical.

In contrast to plants, the antioxidant response to oxidative and environmental stress has not been investigated in diatoms at the molecular level. These are a diverse group of unicellular eukaryotes containing bioluminescent, photosynthetic, heterotrophic, and symbiotic members having important ecological roles as primary producers and consumers in aquatic environments. Diatoms are responsible for red tides, with those that are toxic, having the potential for producing serious health and economic problems. Diatoms have unique genomic features, including large amounts of DNA packed in permanently condensed chromosomes and an absence of classical histones, which make their mechanisms of genetic regulation of great interest.

Although the regulation of Fe–SOD expression was studied in the dinoflagellate (13), there is a paucity of data for marine diatom *Thalassiosira weissflogii*. A diatom was chosen because of its importance to the marine phytoplankton community. Further reason was that the sequence and properties of SOD was not reported in marine diatom.

The cDNA could be used as a reference for comparison of differences among the marine phytoplankton species as well as a probe to detect the transcription level of this enzyme. This enzyme can be used for several beneficial applications in cosmetics for the protection of the skin or unesthetic effects caused by oxygen-containing free radicals (14–16).

MATERIALS AND METHODS

Culture Conditions. Cells of the diatom *T. weissflogii* were cultured at 25 °C on a 12:12-h light–dark cycle with cool white fluorescent light at an irradiance of $150 \mu E \cdot m^{-2} \cdot s^{-1}$. Typically, 115 mL medium (100 mL seawater containing 75 μg $NaNO_3$, 50 μg $NaH_2PO_4 \cdot H_2O$, 300 μg $Na_2SiO_3 \cdot 9 H_2O$, 980 ng $CuSO_4 \cdot 5 H_2O$, 220 ng $ZnSO_4 \cdot 7 H_2O$, 100 ng $CoCl_2 \cdot 6 H_2O$, 18 μg $MnCl_2 \cdot 4 H_2O$, 630 ng $Na_2MoO_4 \cdot 2 H_2O$, 315 μg $FeCl_3 \cdot 6 H_2O$, 436 μg Na_2EDTA , 200 ng biotin, 560 ng vitamin B_{12} , 10 μg thiamine-HCl) was inoculated with 15 mL of dense culture and used after 1 week of growth.

Total RNA Preparation and Single-Strand cDNA Synthesis. Fresh diatom (2.0 g) was put into liquid nitrogen and ground to powder in a ceramic mortar. The sample was dissolved in 20 mL of TRIzol reagent (GIBCO, Frederick, MD), incubated for 5 min at room temperature, and then 3 mL of chloroform was added. The mixture was shaken

vigorously for 15 s, incubated at room temperature for 2–3 min, then centrifuged at 12 000g for 15 min at 4 °C. The aqueous phase was transferred to a new tube, 7.5 mL of isopropyl alcohol was added, and the tube was incubated at 4 °C for 10 min and then centrifuged at 12 000g for 10 min at 4 °C. The total RNA (264 μg) was obtained. Then, we pipetted 3 μg total RNA for synthesizing single-strand cDNA using a kit (GeneRacer) from Invitrogen (Grand Island, NY).

Subcloning and cDNA Sequence Analysis. In accordance with the amino acid sequences of SOD from *Nostoc sp.* PCC 7120 (EMBL accession no. AF173990) and *Nostoc commune* (EMBL accession no. AF177945), two primers (5'TTC CAC CAC GAT AAG CAC CAC3' and 5'GAG GTA GTA AGC GTG TTC CCA3') were synthesized. Using 0.1 μg of the single-strand cDNA as a template, 10 pmol of each of the two primers was added. One 0.4-kb DNA fragment was amplified by polymerase chain reaction (PCR) techniques (25 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s). The 0.4-kb DNA fragment was subcloned into pCR2.1 cloning vector (Invitrogen, Grand Island, NY) using TOPO10 as a host. The nucleotide sequence of the insert was determined in both directions according to autosequencing (ABI PRISM 377-96 DNA sequencer, Perkin-Elmer, CA). On the basis of this DNA sequence, a DiFe-14 primer (5'ATA ACC GCC ACC ATT ATT ACG3') and a DiFe-18 primer (5'GAG GTT TGC TCA AGT GCG AAC CAG3') were synthesized. To a 0.5-mL microtube containing 0.1 μg of the single-strand cDNA as template was added 10 pmol GeneRacer 5' primer and 10 pmol DiFe-14 primer. Ten picomoles GeneRacer 3' primer and 10 pmol DiFe-18 primer were added to another 0.5 mL microtube containing 0.1 μg of the single-strand cDNA. One 0.5-kb DNA (5'-RACE; 5'-DNA end) and one 0.5-kb DNA (3'-RACE; 3'-DNA end) were amplified by the PCR technique (25 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s). Both 0.5-kb DNA fragments were subcloned into pCR2.1 cloning vector using TOPO10 as a host. The nucleotide sequences of these inserts were determined in both directions according to the dideoxy technique using autosequencing. Sequence analysis revealed that 5'-RACE and 3'-RACE covered an open reading frame of SOD cDNA (1.1 kb, EMBL accession no. AF478456).

Recombinant DNA Preparation for Transformation. According to the 1.1-kb cDNA sequence, a 5' upstream primer (5'GGATCCG ATG GCT TTT GAA TTA CCA GAT C 3') and a 3' downstream primer (5'CTCGAG TTT TCC AGC GGC ATA TCT TCT TG 3') were synthesized. Using 0.1 μg of the single-strand cDNA as a template, 10 pmol of each 5' upstream and 3' downstream primer was added and a 0.6-kb fragment was amplified by PCR (25 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s), ligated with pCR2.1, and then transformed into an *E. coli* TOPO10 host. A positive clone was selected by hybridization with ^{32}P -labeled SOD cDNA as the probe, and the plasmid DNA was prepared. A suitable amount of the plasmid DNA was digested with *Bam* HI and *Xho* I and then run on a 0.8% agarose gel. A 0.6-kb insert DNA containing *Bam* HI and *Xho* I sites was recovered and subcloned into pET-20(+) expression vector (pretreated with *Bam* HI and *Xho* I) from Novagen (Madison, WI). The recombinant DNA was then transformed into *E. coli* BL21(DE)pLysS. The recombinant DNA was expressed in *E. coli* BL21(DE)pLysS and its protein was identified by activity staining.

Culture and Enzyme Purification. The transformed *E. coli* was grown at 32 °C in 250 mL of Luria Bertani medium containing 50 $\mu g/mL$ ampicillin and 12.5 $\mu g/mL$ chloramphenicol until A_{600} reached 0.9. Isopropyl β -D-Thiogalactopyranoside (IPTG) was added to a concentration of 1 mM, and 25 μL of 0.1 M Fe^{2+} was added. The culture was incubated at 32 °C for 7 h at 150 rpm, and then the bacterial cells were harvested by centrifugation at 6000g for 5 min. Cells were suspended in 2 mL of PBS buffer containing 1% glycerol and 1 g glass beads, and then they were vortexed for 5 min and centrifuged at 10 000g for 5 min. The extraction procedure was repeated two times, and the supernatants were pooled together. The final crude enzyme (6 mL) was loaded on a Ni–nitrilotriacetic acid Sepharose superflow (Qiagen) column (with a bed volume of 2 mL), and then the column was washed with 12 mL PBS buffer containing 5 mM imidazole. The enzyme was eluted with 6 mL PBS buffer containing 100 mM imidazole (at a flow rate of 0.4 mL/min and 1.5 mL/fraction at room temperature). The purified enzyme (3 mL) that was dialyzed against 200 mL PBS buffer containing

1% glycerol at 4 °C for 4 h twice was used for analysis or stored at -20 °C for further analysis.

Protein of the Purified Enzyme Concentration Measurement. Protein concentration was determined by a Bio-Rad Protein Assay Kit (Richmond, CA) using bovine serum albumin as a reference standard.

Enzyme Assay in Solution. The SOD activity was measured by using a RANSOD kit (RANDOX, Ardmore, U.K.). One milliliter of the assay solution contained 40 mM CAPS at pH 10.2, 0.94 mM EDTA, 0.05 mM xanthine, 0.025 mM INT [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride], and 0.01 unit of xanthine oxidase. The amount of SOD added was adjusted to obtain a rate of INT reduction at 25 °C over the first 3-min time interval, was measured as the absorbance at 505 nm, and fell within the percentage of inhibition that could be transformed into units of SOD by referring to a standard curve according to the instruction manual.

Enzyme Assay by Activity Staining on a Native Gel. Samples of the enzyme were electrophoresed on a 15% native gel for 2.5 h at 100 V. The slab acrylamide gel was then cut into parts: one part was assayed, as previously described (17), for SOD activity staining. The gel was soaked in 0.6 mg/mL nitro blue tetrazolium (NBT) solution for 15 min in the dark with gentle shaking, followed by an immersion with illumination in a solution containing 0.45% tetramethylethylenediamine and 10 µg/mL riboflavin. During illumination, the gel became uniformly blue except at positions containing SOD, which showed acromatic zones revealing insolubility of the blue reduction product of NBT by superoxide anion. The other part was stained with Coomassie blue. The area and intensity of activity and protein bands were measured by a computing densitometer (Molecular Dynamics, CA).

Enzyme Characterization. Each enzyme sample underwent the following tests. After treatments as shown below, each sample was divided into two parts, and then each part of the sample was electrophoresed onto a 15% native gel to determine the changes of activity and protein levels.

(1) **Thermal Stability.** Enzyme samples were heated to 55 °C for 2, 4, 8, or 16 min.

(2) **pH Stability.** Enzyme sample was amended with half a volume of buffer in different pH values: 0.2 M citrate buffer (pH 2.2, 3.0, 4.0, or 5.0), 0.2 M Tris-HCl buffer (pH 7.0, 8.0, or 9.0), or 0.2 M glycine-NaOH buffer (pH 10.0, 11.0, or 12.0). Each sample was incubated at 37 °C for 1 h.

(3) **SDS Effect.** SDS was added to the enzyme sample to the levels of 1, 2, or 4% and incubated at 37 °C for 1 h. Both SDS and imidazole are protein denaturing reagents.

(4) **Imidazole Effect.** Imidazole was added to the enzyme sample to the levels of 0.2, 0.4, 0.8, or 1.6 M and incubated at 37 °C for 1 h.

(5) **Proteolytic Susceptibility.** The enzyme was incubated with 1/20 its weight of trypsin or chymotrypsin at pH 8.0 and 37 °C for up to 1, 2, or 3 h. In the chymotrypsin digestion, CaCl₂ was added until it reached 20 mM. Aliquots were removed from time to time and analyzed.

Determination of Fe and Mn in Diatom SOD. Composition of Fe and Mn in the purified SOD was measured by a Hitachi Z-8200 graphite furnace atomic absorption spectrometer (GFAAS). Calibration standards were prepared by diluting 1000 mg L⁻¹ stock solution (J. T. Baker) with PBS buffer.

Metal Replacement and Reconstituted Activity. The diatom SOD was dialyzed for 24 h with 50 mM acetate buffer (pH 5.5) containing 1 mM *o*-phenanthroline and 10 mM sodium ascorbate and subsequently was reconstituted by dialysis with 50 mM acetate buffer containing 1 mM ferrous ammonium sulfate, 0.5 mM ferrous ammonium sulfate/0.5 mM manganese chloride, or 1 mM manganese chloride followed by extensive dialysis with 50 mM potassium phosphate buffer (pH 7.8) (7, 18).

RESULTS AND DISCUSSION

Cloning and Characterization of a cDNA Coding for Mn-SOD. Figure 1 shows the nucleotide sequence, and the deduced amino acid sequence, of the diatom Mn-SOD clone. Sequence analysis found that the DNA comprised a complete open reading frame coding for 201 amino acid residues.

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1                                     TGAATGTACACTTTACGTAACCGCTGGAACCATG
34 CGTGTATGTGTGCGGGAGCTCAACTACTGGCCAGCTGGGGCGATTGGTTTATGGAGCAAGTGATGCCAACGAGG
109 TTATTTCACGGCGGAAATAAATCTTTTGCATCCGAGAACAGAAATGAGTCAAGGATTGTAAGAGAAGAGAGTGA
184 AGCTTTGATGAAAAGTTTTTTTTCAAGCTCCGATAGTTTTTAAATTCGATCAAAATCAAAATTAATTTCAACT
259 ATG GCT TTT GAA TTA CCA GAT CTT CCT TAC GCA TTC CAT GCT TTT GAA CTT AAT ATT
  I M A F E L P D L P Y A F A D A L E P N I
316 GAC GCC AGA ACG ATG GAA ATC CAT CAT GAT AAG CAT CAC GCT GGC TAT ACT AGT AAA
  20 D A R T M E I H H D K H H A G Y T S K
373 TTA AAC CGA GCA GTG GCA GCA ACA GAC CTT GAA GGC AAG AGC ATC GAG GCA CTT TTG
  39 L N G A V A G T D L E G W K S I E V A L L
430 GCT GAC AAC TCC GAT AAC CGT GCC ATT CGT AAT AAT GGT GGC GGT TAT TAC AAC CAC
  58 A D N S D N G A I R N N G G G Y Y N H
487 AAC CTC TTT TGG ACC GTA ATG TCT CCT GAT GGT GGA GGT GAG CCG ACC GGA GAG CTG
  77 N L F W T V M S P D G G G G E H A Y E L
544 GCT GAT GCA ATC AAT GAA GCC TGT GGC TCA TTT GAT GAA TTC AAA ACG GCT TTT TCA
  96 A D A I N E A C G S F D E F K T A F S
601 AAT GCA CGG GCA ACC AGG TTT GGT TCT GGT TGG GCA TGG CTT TGT GTA CAG GCA GGA
  115 N A G A T R F G S G W A W L C V Q Y E L
658 GGA AAA GTA GAG GTT TGC TCA AGT GCG AAC CAG GAC AAT CCT TTA ATG CCT GGT ATT
  134 G K V E V C S S A N Q D N P L M P G I
715 GGT TGT GGA GGC ACG CCT ATC CTT GGG TTA GAT GTG TGG GAA CAC GCT TAC TAC CTT
  153 G C G G T P I L G L D V W E H A Y E L
772 AAC TAT CAA AAC AGA CGT CCT GAT TAC ATC AAT GCA TTC TTT AAT GTG ATA AAT TGG
  172 N Y Q N R R P D Y I N A F G F N V I N W
829 GAT GAA GTG GCA AGA AGA TAT GCC GCT GCA AAA TAG AGCTTGATTTTCAAACTAAGAAAATGA
  191 D E V A R R Y A A G K *
892 GGGCTCTTGGACCCCTTTTTTATGGCTGTCTATACGCTCTCTTACTTGTATGCTACCTGGGGCTATACGC
  967 CCAGGATTTGGAGCATCTTTTGTGAAATAGACATTTGAAGCAAGAGTGAACCAATCAAAATTTGACTTGGCTG
  1042 AGTGAATGAAAATGTTAAATAAATACTGCTGCTAAAAGTTTATTTAACTATGAAAAAATAAAAAAAAAA 1114

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Figure 1. Nucleotide sequence of a Mn-SOD cDNA and the deduced amino acid sequence. Numbers to the left refer to nucleotide and amino acid residues. The asterisk denotes the stop signal.

	1	27	60
Mn Diatom	MAFELPDLPYAFDALLEPNIDARTMEIHDDKHAGYTSKLNAGVAGTDLGKSI EALLADN		
Mn V. mimicus	MPHLFPDLPYAYDAIEPYIDTKTMEVHYSKHRTYDKFLSAIKGTHEDR----	PLSE	
Mn E. coli	MSYTLPSLPHYAYDALLEPHFDKQTMIEHHTKHQYVNNANALESLEPELANPVEELITK		
Fe E. coli	-SPELPAIPYAKDALAPHSI SAETIEYHYGKHQYVYVNLNLIKGTAFEGK----	SLSE	
Fe P. leiognathi	-AFELPALPFAMNALLEPHISQETLEHYHGHNTYVVKLNGLVBTLEAK-----	SLSE	
		* * *	*
	61	76	114
Mn Diatom	SDNG-----AIRNNGGGYVNNLFWTMSPDGGGEPTGELADIANEACGSDFEFKTAFS		
Mn V. mimicus	IFAHYSALPAAVRNHGGGYNNHYVYVCTKPSAGGEPQGLAAEIERQFSGFAQFKAFS		
Mn E. coli	LDLQPADKKTVLRNAGGHANHSFPWGLKK--GTTLQGLKAAIERDFGSDNFKAEFE		
Fe E. coli	IIRSS---EGGVFNAAQVWNTHTFYVWCLAPNAGGEPTGKVAEIAASFGSFAFKAQFT		
Fe P. leiognathi	IIKTS---TCGVFNAAQVWNTHTFYVWCLAPNAGGEPTGVEAAAIEKAFGSAEFAKAKFT		
	* * * * *	* * *	*
	115		163 167 172
Mn Diatom	NAAATRFSGSWAWLQVAGGKVEVSSANQDNPLMPG--IGCGTPIGLDVEHAYYLN		
Mn V. mimicus	QAAVNTFGSGYVWLIVQ--QGQLSITSTSNQDNPLMDVV--AVRGEPIALDVEHAYYIR		
Mn E. coli	KAASRFSGSWAWLVLKGD-KLAVSTANQDSPLMGEAISGASGFPIMGLDVEHAYYLK		
Fe E. coli	DAAIKNFGSGWVWLVKNSDGKLAIVSTSNAGTPLTDD-----ATPLLTVDVEHAYYID		
Fe P. leiognathi	DSAINFGSSWVWLVKNSGLAIVNTSNAGCPIITEG-----VTPLLTVLVEHAYYID		
		* * *	*
	173	201	
Mn Diatom	YQNRDPDYINAFVNIWDEVARRYAAGK----		
Mn V. mimicus	YQNRPEYIDAWVNVNWEAVSENYAIALTQAA		
Mn E. coli	FQNRDPDYIKEFVNVNWEDEAARFAAKK----		
Fe E. coli	YRNARPGYLEHFVWLVNWEFVAKNLAA-----		
Fe P. leiognathi	YRNLRPSYMDGFVWLVNWEFVSKNLAA-----		
	*	* * *	*

Figure 2. Optimal alignment of diatom Mn-SOD cDNA and other organisms. Mn Diatom (this study); Mn V. mimicus, *V. mimicus* Mn-SOD DNA (accession no. AAL26843); Mn E. coli, *E. coli* Mn-SOD DNA (accession no. P00448); Fe E. coli, *E. coli* Fe-SOD DNA (accession no. P00448); Fe P. leiognathi, *P. leiognathi* Fe-SOD DNA. A dash denotes deletion. Residues designated with a star pinpoint as potential discriminators between the iron and manganese SOD.

Figure 2 shows that four residues (H-27, H-76, D-163, and H-167) which are required to coordinate the metal are conserved in all reported Fe/Mn-SODs (*V. mimicus*, *E. coli*, *P. leiognathi*). Residues H-27 through Y-35 which contained four histidines, as well as the alpha helix (residues P-158 through Y-173 which contain H-167, a patch of four aromatic residues), are conserved and presumably form an ion binding region. Stallings et al. (19)

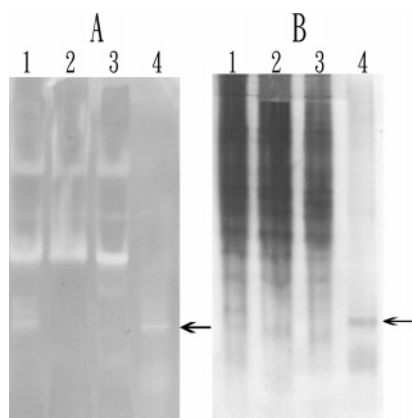


Figure 3. Purification of diatom Fe/Mn-SOD. Six milliliters of crude extract was obtained from 250 mL culture. Ten microliters of each sample was performed on a 15% native PAGE followed by (A) activity stain, (B) Coomassie blue stain. Lanes 1~4: 1, crude extract; 2, pass-through; 3, wash; 4, fraction containing Fe/Mn-SOD. An arrow denotes purified diatom SOD.

suggested that the observed helical conformation is required to juxtapose the three residues (H-27, H-31, and Y-35), and the arrangement is crucial for catalysis on the basis of the structure of Mn-SOD from *Thermus thermophilus* at 2.4 Å resolution. The primary structure of this SOD in comparison with two known Mn-SODs and two Fe-SODs showed the major differences of residues 71(Gly), 72(Gly), 79(Phe), 144(Gln), and 145(Asp). It can be concluded that this diatom SOD belongs to Mn-SOD in sequence.

Transformation and Expression of Diatom Mn-SOD. One goal of this study was to clone and express the diatom *T. weissflogii* Mn-SOD coding sequence in *E. coli*. Using cDNA as the template and two specific primers corresponding to the translation initiation and termination sequences, the 0.6-kb DNA fragment coding for the diatom *T. weissflogii* Mn-SOD was amplified by PCR and successfully subcloned into the expression vector, pET-20b(+). Positive clones were verified by DNA sequence analysis. The transformants were incubated in LB containing 10 μM iron and induced with IPTG, and their total cellular proteins were analyzed by a 15% native PAGE with activity staining or protein staining.

Purification of Diatom SOD. The diatom SOD was fused in the pET-20b(+)-6His-tag vector and expressed in *E. coli* BL21(DE3)pLysS. The enzyme containing His-tag in the C-terminus was purified by affinity chromatography with nickel chelating Sepharose (Qiagen) according to the instruction manual. The yield was 0.25 mg from 0.25 L of culture. The specific activity was 2780 units/mg. The purified enzyme showed active enzymatic form (Figure 3, lane 4) on a 15% native PAGE.

Metal Composition of Diatom SOD and Reconstituted Activity. GFAAS assay indicates that 0.6 atom of iron/manganese (3:1) is present per subunit of SOD. Dialysis of the SOD with *o*-phenanthroline and sodium ascorbate for 24 h and subsequent dialysis with ferrous ammonium sulfate, ferrous ammonium sulfate/manganese chloride (1:1), or manganese chloride reconstituted activity (recoveries of 37, 43, or 64%, respectively), suggesting that diatom SOD was cambialistic (Fe/Mn)-SOD.

Characterization of the Purified Diatom SOD. The enzyme inactivation kinetics at 55 °C fit the first-order inactivation rate equation $\ln(E_t/E_0) = -k_d t$, where E_0 and E_t represent the original activity and the residual activity that remained after

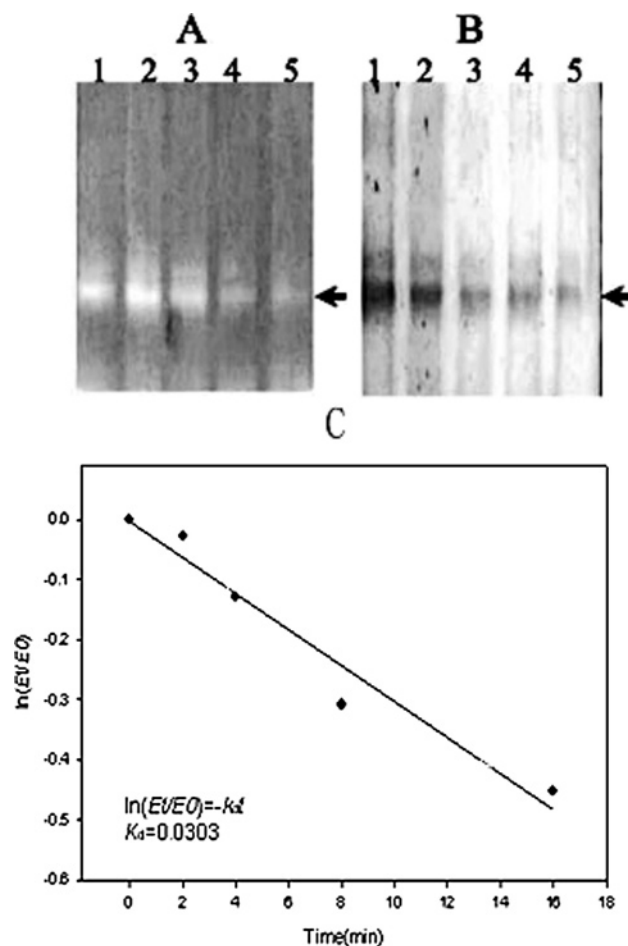


Figure 4. Effect of temperature on the purified Fe/Mn-SOD. The enzyme samples heated at 55 °C for various times were analyzed by 15% native PAGE. (A) Staining for activity (4 μg/lane), (B) staining for protein (4 μg/lane). Lanes 1~5 (control, 2, 4, 8, 16 min). (C) Plot of thermal inactivation kinetics. The effect of temperature was determined by activity stain. The PAGE data were quantitated by a densitometer for calculation. E_0 and E_t are original activity and residual activity after being heated for different times. The areas of activity measured by a densitometer were 324 ± 23 (control), 315 ± 28 (2 min), 285 ± 39 (4 min), 238 ± 26 (8 min), and 206 ± 11 (16 min). An arrow denotes both activity and protein.

heating for time t , respectively. The thermal inactivation rate constant (k_d) values calculated for the enzyme at 55 °C was $3.03 \times 10^{-2} \text{ min}^{-1}$, and the half-life for inactivation was 23 min (Figure 4A–C).

As shown in Figure 5 (lanes 4–10), SOD was stable in a broad pH range from pH 5 to 12.

The enzyme activity showed decrease in either SDS or imidazole (data not shown). The enzyme was resistant to digestion by trypsin (data not shown) and chymotrypsin even at a high enzyme/substrate (w/w) ratio of 1/20 (Figure 6 A–B).

CONCLUSION

In summary, neither the Mn-SOD cDNA sequence of diatom nor the properties of recombinant Fe/Mn(3:1)-SOD were reported; the results from the experiments illustrate that the cloned cDNA from this microbe not only overexpressed Fe/Mn-SOD in prokaryotes but also remained stable under a broad range of pH, higher temperature, and proteases. These properties are beneficial for applications described in the Introduction.

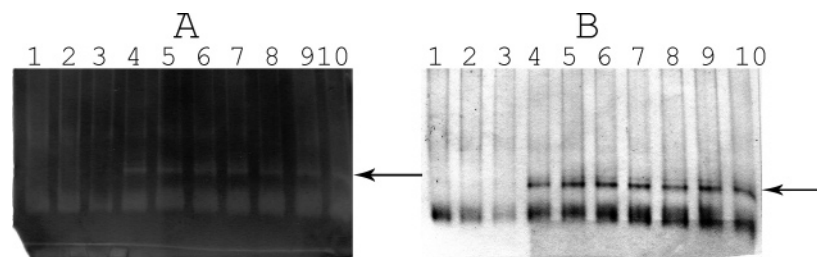


Figure 5. Effect of pH on enzyme stability. The enzyme samples were incubated in buffers with different pH values at 37 °C for 1 h and then analyzed by 15% native PAGE followed by activity staining (A panel, 4 μg/lane) and Coomassie blue staining (B panel, 4 μg/lane). Lanes 1~10 (pH 2.3, 3.0, 4.0, 5.0, 7.0, 8.0, 9.0, 10.0, 11.0, or 12.0). The total areas of activity measured by a densitometer were 16 ± 3 (pH 2.3), 32 ± 14 (pH 3.0), 40 ± 7 (pH 4.0), 194 ± 25 (pH 5), 236 ± 25 (pH 7), 253 ± 33 (pH 8), 245 ± 31 (pH 9), 250 ± 28 (pH 10), 251 ± 17 (pH 11), and 238 ± 32 (pH 12). An arrow denotes both activity and protein.

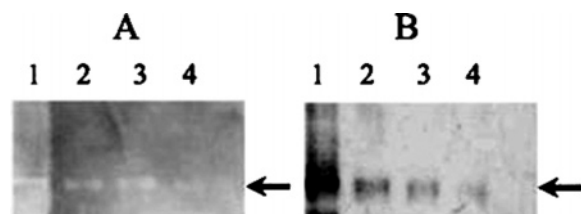


Figure 6. Effect of chymotrypsin (A and B). The enzyme samples were incubated with chymotrypsin ($1/20$ SOD) at 37 °C for different times and then subjected to 15% native PAGE. (A) Staining for activity (4 μg/lane). (B) Staining for protein (4 μg/lane). Lanes 1~4 (control, 1, 2, or 3 h). The enzyme activities after treatment with chymotrypsin measured by a densitometer were 312 ± 16 (control), 257 ± 21 (1 h), 250 ± 21 (2 h), and 160 ± 29 (3 h). An arrow denotes both activity and protein.

NOTE ADDED AFTER ASAP PUBLICATION

The original posting of February 9, 2005, contained an incorrect version of Figure 4. The correct version is shown in the posting as of February 14, 2005.

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