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Biochemical Characterization of a Cambialistic Superoxide Dismutase Isozyme from Diatom *Thallassiosira weissflogii*: Cloning, Expression, and Enzyme Stability

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A cDNA clone of 1081 bp encoding a second putative superoxide dismutase (SOD) from diatom *Thallassiosira weissflogii* was cloned by the polymerase chain reaction technique. The cDNA encodes a protein of 286 amino acid residues. Alignment of the truncated SOD sequence containing 217 amino acid residues with Mn−SODs from *Vibrio mimicus* and *Escherichia coli*, as well as two Fe−SODs from *E. coli* and *Photobacterium leiognathi*, this SOD showed greater homology to Mn−SOD. The residues required to coordinate the manganese ion were conserved in all reported Mn−SOD. The recombinant SOD has a half life of deactivation of 14.7 min at 65 °C. Its thermal inactivation rate constant K_d was 3.21×10^{-2} min^{-1}. The enzyme was stable in a broad pH range from 4 to 12. The presence of imidazole (up to 0.8 M) and sodium dodecylsulfate (up to 4%) had little effect on the enzyme's activity. The atomic absorption spectrometric assay showed the presence of 0.3 atom of iron/manganese (2:1) in each SOD subunit. Reconstituted activity suggested that diatom SOD was cambialistic Fe/Mn−SOD.

KEYWORDS: diatom; *Thallassiosira 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 cell survival (*1*). Reactive oxygen species (ROS) can be produced by irradiation of visible light in the presence of a photosensitizer, 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 $O_2^{\bullet -}$, which dismutates to oxygen and hydrogen peroxide, producing highly reactive HO• through the metal ion catalyzed Haber−Weiss reaction. Even under nonstress conditions, this ROS-generating mechanism can do 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 $O_2^{\bullet -}$ is a precursor of several other reactive species, control over the steady-state $O_2^{\bullet -}$ levels by SOD is critical.

SODs are classified based on the type of metal bound at the active site: Cu/Zn−SOD, Mn−SOD, Fe−SOD, and Ni−SOD (*2*−*5*). Cu/Zn−SOD has been found primarily in cytosol of eukaryotes, chloroplasts of plants, intermembrane space of mitochondria, and periplasm of gram negative bacteria. Mn−SOD is found in both prokaryotic and eukaryotic organisms associated with mitochondria. The enzyme is insensitive to cyanide and hydrogen peroxide. Fe−SOD has been found in both eukaryotes and prokaryotes. It is insensitive to cyanide but is inhibited by hydrogen peroxide. Ni−SOD has been isolated from several aerobic soil bacteria of the *Streptomyces* species. Ni−SOD is distinct from the Mn-, Fe-, or Cu/Zn−SODs on amino acid sequence, immunological crossreactivity, and spectroscopic properties (*6*). Fe−SOD and Mn−SOD appear to be closely related in structure. It has been reported that some bacterial species can use a common SOD for both Fe and Mn and utilize either of these two metals as the active metal cofactor, depending on the metal supplied (*7*). A SOD protein that is enzymatically active and can accommodate either Fe or Mn metal ligand is known as "cambialistic" Fe/Mn−SOD (*8*). The first eukaryotic cambialistic Fe/Mn−SOD was purified from mature seeds of camphor tree. The camphor SOD closely resembled Mn−SODs in primary sequence, but its bound metal (0.5−1 atom of Fe

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per camphor SOD subunit) and the ultraviolet and visible spectra were similar to those of iron-containing enzymes (*9*).

The antioxidant response to oxidative and environmental stress has not been investigated in diatom at the molecular level. These are a diverse group of unicellular eukaryotes containing bioluminescent, photosynthetic, heterotropic, 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 has been studied in the dinoflagellate (*10*), there is a paucity of data for marine diatom *T. weissflogii*. We are interested in diatom because of its importance to the marine phytoplankton community. Recently, we reported cloning, expression, and characterization of the first cambialistic Fe/Mn−SOD from diatom (*11*). In this study, we report cloning and expression of a second functional SOD cDNA from diatom. From amino acid sequence alignment of the present SOD to the published first Mn−SOD from diatom, two Mn−SODs from *V. mimicus* and *E. coli*, as well as that of two Fe−SODs from *E. coli* and *P. leiognathi* (*12*−*14*), this SOD is more closely related to Mn−SOD in sequence. Our results showed that the recombinant SOD purified from *E. coli* grown in LB medium contained 0.3 atom of iron/manganese per SOD subunit, and the ratio of Fe to Mn was 2:1. In addition, reconstitution of the present apoSOD with Fe, Fe and Mn, or Mn recovered 80, 100 or 90% of the activity, respectively. Thus, it was concluded that this diatom SOD is a cambialistic Fe/Mn−SOD. The cDNA may 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 unaesthetic effects caused by oxygen-containing free radicals (*15*−*17*).

MATERIALS AND METHODS

Culture Conditions. Cells of the diatom *T. weissflogii* were cultured as described previously (*11*). Briefly, cells were grown at 25 °C on a 12:12-h light−dark cycle with cool white fluorescent light at an irradiance of 150 $\mu E \cdot m^{-2} \cdot S^{-1}$.

Total RNA Preparation and Single-Strand cDNA Synthesis. Fresh diatom (wet weight 2.0 g) was frozen in liquid nitrogen and ground to powder in a ceramic mortar. Total RNA was prepared using TRIzol reagent (GIBCO, Frederick, MD) as described before (*11*). The total RNA (264 μg) was obtained. Some of the total RNA (3 μg) was used for single-strand cDNA synthesis using a GeneRacer kit from Invitrogen (Grand Island, NY).

Cloning of a Second Putative SOD Gene and cDNA Sequence Analysis. We have recently cloned for the first time an Fe/Mn−SOD cDNA from diatom *T. weissflogii* using RACE polymerase chain reaction (PCR) techniques (*11*). In that paper, we amplified a 0.4-kb fragment using single-strand cDNA of diatom as a template and two primers (5′TTC CAC CAC GAT AAG CAC CAC3′ and 5′GAG GTA GTA AGC GTG TTC CCA3′) based on the conserved sequences of SOD from *Nostoc sp.* PCC 7120 (EMBL accession no. AF173990) and *Nostoc commune* (EMBL accession no. AF177945). The 0.4-kb fragment was subcloned and sequenced. In the present study, we used the same primers and single-strand cDNA of diatom as a template for the amplification. A different 0.4-kb fragment was obtained as determined by subcloning and sequencing (except for the sequence of the primers used in PCR). On the basis of this DNA sequence, two primers near both ends, a DiFe-3 primer (5′AAT CAA GAC AAT CCT TTG ATG3′) and a DiFe-4 primer (5′GTG TCC TCC TCC GTT GTT GCG3′), were synthesized. The primers allowed sequence extension from both ends of the 0.4-kb fragment using GeneRacer primers. Two PCRs were carried out each using 0.1 μg of the single-strand diatom cDNA as the template. The primer pairs in each reaction were GeneRacer 5′ primer and DiFe-4 primer and GeneRacer 3′ primer and DiFe-3 primer. A 0.5-kb DNA (5′-RACE; 5′-DNA end) and a 0.5-kb DNA (3′-RACE; 3′-DNA end) were amplified by the PCR techniques. Both DNA fragments were subcloned into a pCR2.1 cloning vector using TOPO10 as a host. The nucleotide sequences of these inserts were determined in both strands.

Truncated Recombinant DNA Preparation. The cDNA region homologous to other Mn or Fe−SODs covers amino acid residues 71 to 286, were amplified using two gene-specific primers. The 5′ upstream primer contains ATG and *Eco* RI recognition site (5′GAATTCG ATG GCC TAC GCC GTC CCC GAC CTT A3′), and the 3′ downstream primer contains the *Not* I recognition site (5′ GCGGCCGC TCC ACG GAC AGG TAC TCC AGA 3′). By use of 0.1 μg of the single-strand cDNA as a template and 10 pmol of each 5′ upstream and 3′ downstream primers, a 0.6-kb fragment was amplified by PCR. The fragment was ligated into pCR2.1 and transformed into *E. coli* TOPO10. A positive clone was selected by hybridization with ^{32}P-labeled diatom SOD cDNA. Plasmid DNA was isolated from the clone and double digested with *Eco* RI and Not I. The digestion products were separated on a 0.8% agarose gel. The 0.6-kb insert DNA was gel purified and subcloned the into *Eco* RI and *Not* I sites of a pET-20(+) expression vector (Novagen, Madison, WI). The recombinant SOD protein was overexpressed in *E. coli* BL21(DE)pLysS, and the functional protein was identified by activity staining as described below.

Overexpression and Purification of the Truncated Recombinant SOD. The conditions for overexpression of the diatom SOD gene are the same as described before (*11*). The transformed *E. coli* BL21(DE)-pLysS was grown at 32 °C in 250 mL of Luria Bertani medium containing 50 μg/mL ampicillin and 12.5 μg/mL chloramphenicol until A_{600} reached 0.9. Protein expression was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The culture was incubated for an additional 8 h at 150 rpm, and then the bacterial cells were harvested by centrifugation. Cells were suspended in 2 mL of phosphate-buffered saline (PBS) containing 1% glycerol and 1 g of glass beads. The content was vortexed for 5 min and centrifuged at 10 000 g for 5 min. The extraction procedure was repeated three times, and the supernatants were combined. The final crude extract (6 mL) was loaded on a Ni−nitrilotriacetic acid Sepharose superflow (Qiagen) column (with 2 mL of bed volume). The column was washed with 12 mL of PBS containing 5 mM imidazole. The enzyme was eluted with 6 mL of PBS containing 100 mM imidazole (at a flow rate of 0.4 mL/min and 1.5 mL/fraction at room temperate). The purified enzyme (3 mL) was dialyzed against 200 mL of PBS containing 1% glycerol at 4 °C for 4 h. Fresh PBS containing 1% glycerol was changed once during dialysis. The dialyzed sample was either used directly for analysis or stored at −20 °C until use.

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

SOD Activity Assay. The SOD activity was measured spectrophotometrically using a RANSOD kit (RANDOX, Ardmore, UK) according to the instruction manual. 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-nitrophenol)-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, measured as the absorbance at 505 nm fell within the percentage of inhibition that could be transformed into units of SOD by referring to a standard curve.

SOD Assay by Activity Staining on a Native Gel. Duplicate samples containing the SOD enzyme were electrophoresed on a 15% native gel for 2.5 h at 100 V. The duplicate lanes were sliced into two parts. One part was stained for SOD activity as previously described (*18*). The gel was soaked in 0.6 mg/mL nitro blue tetrazolium (NBT) solution for 15 min in the dark with gentle shaking, followed

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5'   1 TTT GTG GCC TTT TGG ATC GTA GCC ATT ATC CAC TTT CCA CTT CTG AAG CTC TTC
     1  F   V   A   F   W   I   V   A   I   I   H   F   P   L   L   K   L   F

    55 CGT CCT ATT TGC CGT CCT AAT ATC GTT AAT ACT ATT ATT TCT CCC ACA ACA ATC
    19  R   P   I   C   R   P   N   I   V   N   T   I   I   S   P   T   T   I

   109 AAA ATC GAC ACA AAA TTA GAG ACC GGA TCC AAC ACC CGT CGT GAA ATT TTG CAG
    37  K   I   D   T   K   L   E   T   G   S   N   T   R   R   E   I   L   Q

   163 AAA GGA GCC GCC CTC GCC ACT GTC GCA TCC GTC CCC GCT GCT GCC AAT GCC TAC
    55  K   G   A   A   L   A   T   V   A   S   V   P   A   A   A   N   A   Y

   217 GCC GTC CCC GAC CTT AAA TAC CCC TTC GAA GCA CTG GAA CCA TAC ATT GAC GCA
    73  A   V   P   D   L   K   Y   P   F   E   A   L   E   P   Y   I   D   A

   271 CCC ACC ATG AAA ATC CAC CAC GAT AAA CAC CAC GCC ACC TAC GTC GCC AAT ATC
    91  P   T   M   K   I   H   H   D   K   H   H   A   T   Y   V   A   N   I

   325 AAC AAG GCC ACT GAA GGC AAG CCT GAT GTC GAT ATT CTC GAG CTC CAA CTC AAC
   109  N   K   A   T   E   G   K   P   D   V   D   I   L   E   L   Q   L   N

   379 GCC CTC GAG GCA GGA CCC GCC GTC CGC AAC AAC GGA GGA GGA CAC TAC AAC CAT
   127  A   L   E   A   G   P   A   V   R   N   N   G   G   G   H   Y   N   H

   433 GCC TTC TTC TGG GAT GAA ATG GCT CCT CCC GAG GAG GCA AAG AAA ACT AAG CCC
   145  A   F   F   W   D   E   M   A   P   P   E   E   A   K   K   T   K   P

   487 AGT GCC GAG CTG GAG GCC ATG ATC AAC AAA TCT TTT GGA TCC ATA GAT GAG ATG
   163  S   A   E   L   E   A   M   I   N   K   S   F   G   S   I   D   E   M

   541 AAG TCC GCA TTT GAG GCC CGA GCC GCC CCC GGT GCG CTC TTT GGA TCT GGA TGG
   181  K   S   A   F   E   A   R   A   A   P   G   A   L   F   G   S   G   W

   595 GTG TGG ATC TGT GTC AAT GCC GCA GGG AAT GAG TTG AAG CTC GTT GGA ACT CCT
   199  V   W   I   C   V   N   A   A   G   N   E   L   K   L   V   G   T   P

   649 AAT CAA GAC AAT CCT TTG ATG AAG GGC GTG GCT GAC GAG GTC ATG TTC CCT ATC
   217  N   Q   D   N   P   L   M   K   G   V   A   D   E   V   M   F   P   I

   703 CTT GGC TTG GAT GTT TGG GAA CAT GCT TAC TAC CTC AAG TAT CAA AAC CGC CGA
   235  L   G   L   D   V   W   E   H   A   Y   Y   L   K   Y   Q   N   R   R

   757 CCG GAA TAT GTT TCT AAC TGG TGG AAC GTT GTT AAC TGG GAT AAG GTT TCG GAG
   253  P   E   Y   V   S   N   W   W   N   V   V   N   W   D   K   V   S   E

   811 AAC TTC GCT TAC GTT GTT GAG AAG AAG TCT GGA GTA CCT GTC CGT GGA TAA
   271  N   F   A   Y   V   V   E   K   K   S   G   V   P   V   R   G   *

   862 CTTTGGATATTCTTGGTTCGGCCTTCATTGAGCAATCGCATCAATTGTTGCACG
   916 AATGTCTACTGCAGCTGCATCCAACAGACTATTTAAACAAAACAAAGGCTCAAA
   970 CAGAATTAGGGCCCACATTGGTGCGCCAAATCTTTGGTCTTGATCCGTGTAGAA
  1024 GGCTATCTATCATACTAAACATAATACAATCTTCTTATGTATAAAAAAAAAAAAAAAA 1081-3'
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Figure 1. Nucleotide sequence of a diatom SOD cDNA and the deduced amino acid sequence (accession no. AF450119). Numbers to the left refer to nucleotide and amino acid residues. The asterisk denotes the stop signal.

by an immersion with illumination in a solution containing 0.45% tetramethylenediamine and 10 μg/mL riboflavin. During illumination, the gel became uniformly blue except at the position of SOD, which showed achromatic 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. The enzyme sample was tested for stability under various conditions as described before (*11*) and below. Aliquots of the SOD sample underwent the following treatment:

(1) Thermal Stability. Enzyme sample was heated to 65 °C for 2, 4, 8, or 16 min.

(2) pH Stability. Enzyme sample was adjusted to desired pH by adding a half volume of buffer with different pHs: 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, a denaturing reagent, was added to the enzyme sample to the levels of 1, 2, or 4% and incubated at 37 °C for 1 h.

(4) Imidazole Effect. During protein purification, the SOD enzyme was eluted with imidazole. Therefore, the effect of imidazole on activity/stability of the SOD was examined. 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 one-twentieth its weight of trypsin or chymotrypsin at pH 8.0, 37 °C, for up to 1, 2, or 3 h. In the chymotrypsin digestion, $CaCl_2$ was added to 20 mM. Aliquots were removed at various time intervals for analysis.

Figure 2. Alignment of the amino acid sequences of diatom SOD (Fe/Mn diatom 2; this study, accession no. AF450119); Fe/Mn diatom 1, first diatom Fe/Mn–SOD (accession no. AF478456); Mn *V. mimicus*, *V. mimicus* Mn–SOD (accession no. AAL26843); Mn *E. coli*, *E. coli* Mn–SOD (accession no. P00448); Fe *E. coli*, *E. coli* Fe–SOD (accession no. P09157); Fe *P. leiognathi*, *P. leiognathi* Fe–SOD (accession no. A26707). Identical amino acids in all sequences are shaded black; conservative replacements are shaded gray. A dash denotes gap.

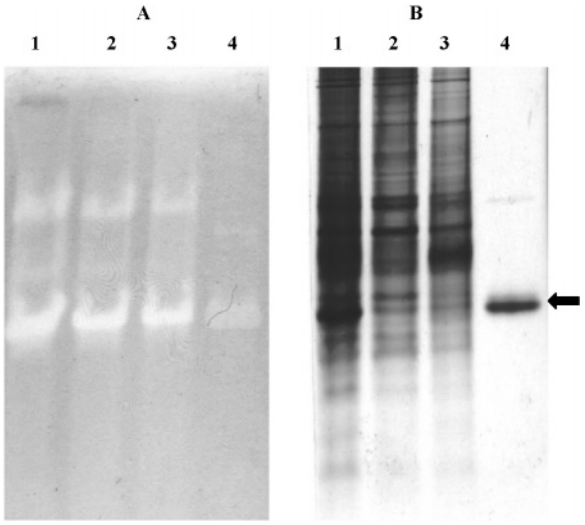

Figure 3. Purification of the recombinant diatom SOD. Crude extract (6 mL) was obtained from 200 mL of culture. Ten μL of each sample was performed on a 15% native PAGE followed by (**A**) activity stain. (**B**) Coomassie blue stain. Lane 1, crude extract; lane 2, flowthrough; lane 3, wash; lane 4, eluted fraction containing Fe/ Mn–SOD. An arrow denotes purified diatom SOD.

After each treatment, duplicate sets of sample were electrophoresed onto a 15% native gel to determine the changes in activity and protein levels.

Determination of Fe and Mn Compositions in the 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 from diluting 1000 mg L^{-1} stock solution (J. T. Baker) with PBS.

Metal Replacement and Reconstituted Activity. ApoSOD was prepared from the purified his-tagged diatom SOD by dialysis for 24 h against 50 mM acetate buffer (pH 5.5) containing 1 mM *o*-phenan-

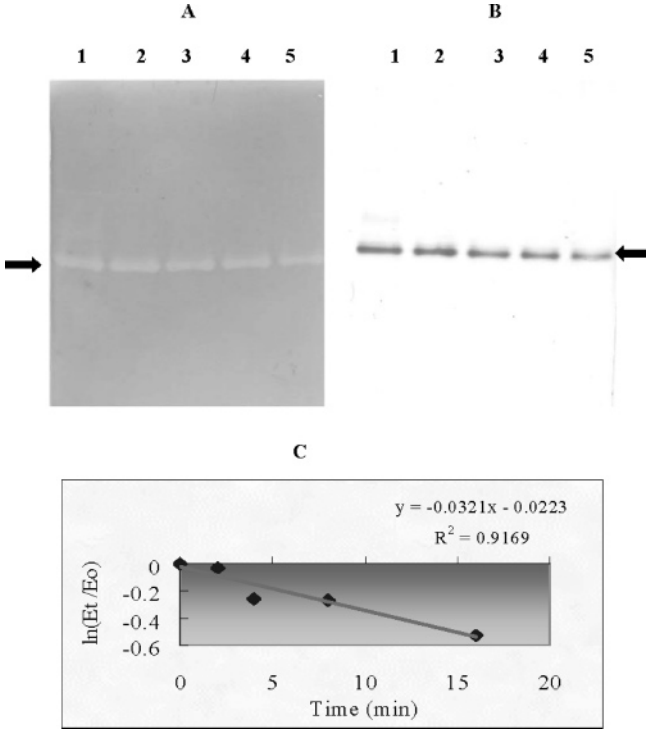

Figure 4. Effect of temperature on the purified diatom SOD. The enzyme samples heated at 65 °C for various times were analyzed by 15% native PAGE. (**A**) Staining for activity (6 μg/lane). (**B**) Staining for protein (2 μg/lane). Lanes 1–5 (control, 2, 4, 8, and 16 min). (**C**) Plot of thermal inactivation kinetics. The effect of temperature was determined by activity stain. The PAGE data were quantitated by a densitometer. E_o and E_t are original activity and residual activity after being heated for different times. The areas of activity measured by a densitometer were 184 ± 26 (control), 179 ± 30 (2 min), 143 ± 41 (4 min), 141 ± 34 (8 min), and 109 ± 11 (16 min).

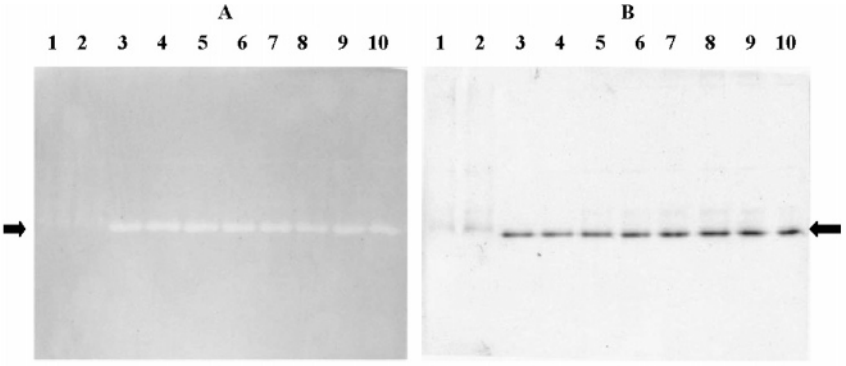

Figure 5. Effect of pH on stability of the purified diatom SOD. The enzyme samples were incubated in buffers with different pH value at 37 °C for 1 h and then analyzed by 15% native PAGE followed by activity staining (panel **A**, 6 μg/lane), Coomassie blue staining (panel **B**, 2 μg/lane). Lanes 1–10 (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 61 ± 4 (pH 2.3), 75 ± 11 (pH 3.0), 185 ± 5 (pH 4.0), 214 ± 32 (pH 5), 313 ± 28 (pH 7), 309 ± 24 (pH 8), 347 ± 40 (pH 9), 404 ± 27 (pH 10), 388 ± 34 (pH 11), and 326 ± 19 (pH 12). An arrow denotes both activity and protein.

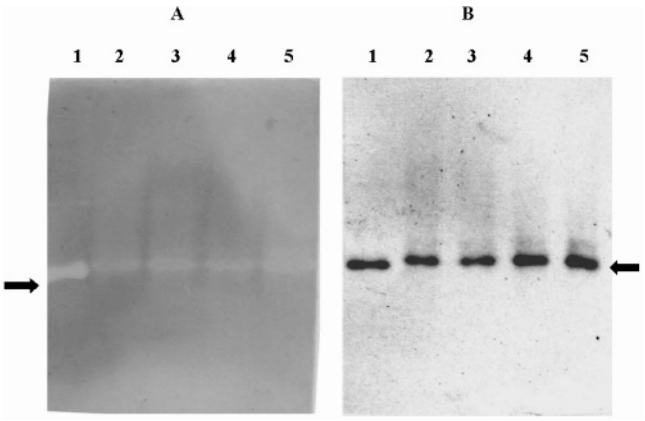

Figure 6. Effect of SDS on the purified diatom SOD. The enzyme samples were incubated with SDS of different concentrations at 37 °C for 1 h and then subjected to 15% native PAGE. (**A**) Staining for activity (6 μg/lane). (**B**) Staining for protein (2 μg/lane). Lanes 1–5 (control, 1, 2, 3, or 4%). The enzyme activity after SDS treatment, measured by a densitometer, were 275 ± 32 (control), 234 ± 22 (1%), 214 ± 19 (2%), 202 ± 27 (3%), 197 ± 17 (4%). An arrow denotes both the activity and the protein.

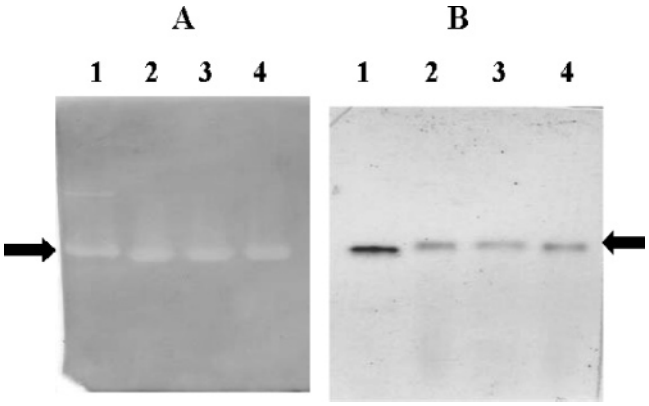

Figure 7. Effect of trypsin on the purified diatom SOD. The enzyme samples were incubated with trypsin (1/20 SOD) at 37 °C for different times and then subjected to 15% native PAGE. (**A**) Staining for activity (6 μg/lane). (**B**) Staining for protein (2 μg/lane). Lanes 1–4 (control, 1, 2, or 3 h). The enzyme activity after treatment with trypsin measured by a densitometer were 127 ± 13 (control), 122 ± 12 (1 h), 104 ± 20 (2 h), and 99 ± 6 (3 h). An arrow denotes both activity and protein.

throline and 10 mM sodium ascorbate (*19*). The holoenzyme was reconstituted with Fe and/or Mn by dialysis against 50 mM acetate buffer containing 1 mM ferrous ammonium sulfate or 0.5 mM ferrous ammonium sulfate/0.5 mM manganese chloride or 1 mM manganese chloride followed by extensive dialysis against 50 mM potassium phosphate buffer (pH 7.8) (*7, 19*).

RESULTS AND DISCUSSION

Cloning and Characterization of a Diatom cDNA Encoding Fe/Mn SOD. Sequence analysis revealed that combination of the 5′-RACE fragment, the 0.4-kb PCR fragment, and the 3′-RACE fragment covered an open reading frame of SOD cDNA which encodes a protein of 286 amino acid residues lacking ATG (Met) at the 5′ end. **Figure 1** shows the nucleotide and the deduced amino acid sequences of the diatom SOD clone (1081 bp, EMBL accession no. AF450119). The encoded protein contains a region (positions 71−286) homologous to Mn−SOD and Fe−SOD from several sources. The sequence appears to extend longer at the 5′ end than the previously reported Fe/Mn−SOD cDNA from diatom (*11*). The clone contains an additional 70 amino acid residues at the N-terminus that do not present in any of the published full-length SOD. The overlapping region of the two diatom SODs shared 48% identity at the protein level and 44% identity at the DNA level. **Figure 2** shows the amino acid sequence alignment of the present diatom SOD, Fe/Mn diatom 2 (amino acid positions 71 to 286, **Figure 1**), with Mn−SOD and Fe−SOD from several sources. Pairwise sequence alignment of the present diatom SOD to each of the previously published Fe/Mn−SOD from diatom (Fe/Mn diatom 1, accession no. AF478456), *V. mimicus* (accession no. AAL26843), and *E. coli* (accession no. P00448) showed approximately 44−48% identity. Pairwise alignment of the present diatom SOD to each of the Fe−SODs from *E. coli* (accession no. P09157) and *P. leiognathi* (accession no. A26707) showed approximately 35% identity. These results suggested that the present diatom SOD is more closely related to Mn−SOD than to Fe−SOD.

The 3-dimensional structure of Mn−SOD from *Thermus thermophilus* HB8 has been revealed at 2.4-Å resolution (*20*). The Mn ion coordination site and some near-neighbor nonpolar residues have been identified. As expected, the four residues (H-27, H-82, D-177, and H-181) that are required to coordinate the metal are totally conserved in all reported Mn or Fe−SODs. Residues H-27 through Y-35 contained four histidines, as well as the alpha helix (residues P-172 through Y-187 which contain H-181, a patch of four aromatic residues) are conserved and presumably form an ion binding region. On the basis of the structure of *Thermus thermophilus* Mn−SOD, Stallings et al.

(*20*) 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.

Expression and Purification of the Recombinant Diatom SOD. The 0.6-kb DNA fragment covered amino acids coding region from positions 71 to 286 was amplified from diatom cDNA by PCR and subcloned into the expression vector, pET-20b(+) as described in the Materials and Methods. Positive clones were verified by DNA sequence analysis. Overexpression of the target protein was induced with IPTG, and their total cellular proteins were analyzed by a 15% native PAGE with activity staining or protein staining (**Figure 3**, **lane 1**). The diatom SOD was expressed as a 6His-tag fusion protein. The enzyme was purified by affinity chromatography with nickel-chelating Sepharose. The yield was 1.14 mg from 200 mL of culture. The recombinant SOD is enzymatically functional with specific activity of 2150 units/mg. the activity was also detected on a native gel. As can be seen in **Figure 3**, the purified SOD (lane 4) seems to be less active than that of the crude extract, flowthrough, and wash (lanes 1−3). This may be due to the presence of 100 mM imidazole in the purified SOD.

Metal Composition of Diatom SOD and Reconstituted Activity. GFAAS assay indicates that 0.3 atom of iron/manganese (2:1) is present per subunit of the SOD. This is different from the diatom SOD reported previously (*11*), which showed 0.6 atom of iron/manganese (3:1). Reconstitution of the present apoSOD with Fe, Fe and Mn, or Mn recovered 80, 100, or 90% of the activity, respectively, suggesting that the diatom SOD was cambialistic (Fe/Mn)−SOD. The present SOD appears to reconstitute more efficiently than the previously reported SOD (*11*), suggesting a higher affinity of this SOD toward Fe or Mn.

Characterization of the Purified Diatom Mn/Fe−SOD. The enzyme-inactivation kinetics at 65 °C fit the first-order inactivation rate equation $\ln(E_t/E_o) = -K_d t$, where E_o and E_t represent the original activity and the residual activity after heating for time t, respectively. The thermal inactivation rate constant (K_d) values calculated for the enzyme at 65 °C was 3.21×10^{-2} min^{-1}, and the half-life of inactivation was 14.7 min (**parts A−C of Figure 4**).

As shown in **Figure 5 (lanes 3−10)**, the SOD was stable in a broad pH range from pH 4 to 12. It appears to be more stable and more resistant to changes in pH environments than the previously reported diatom SOD (*11*).

The enzyme activity had little effect by SDS up to 4% (**parts A and B of Figure 6**) or imidazole up to 0.8 M (data not shown). The enzyme was somewhat resistant to digestion by trypsin (**parts A and B of Figure 7**) and chymotrypsin (data not shown) even at a high enzyme/substrate (w/w) ratio of 1/20.

The importance of SOD in antioxidant defense in nearly all cells exposed to oxygen has been well established. Although many SODs are known, the SOD in diatoms has not been extensively studied. Diatoms are unicellular photosynthetic eukaryotes that are thought to contribute as much as 25% of global primary productivity. They are thought to be the most important group of eukaryotic phytoplankton. Diatoms were used as food for the larvae of echinoderms, mollusks, and shrimp. They have also been an indicator of water quality and pollution, as they are able to uptake and bind both organic and inorganic pollutants. Despite their ecological importance in the world's oceans, very little information is available at the molecular level about the novel aspects of their biology. Scientists have been wondering for centuries what the molecular secrets are behind diatoms' success. One possibility is that they have extraordinary capacities for finding adaptive solutions to different environments. We believe that the ability of diatoms to defend themselves from ROS plays an important role in their survival. Therefore, cloning and characterization of diatom antioxidant enzyme SOD is an important step toward understanding how diatoms defend against oxidative and environment stress.

Conclusion. Very recently, we cloned and characterized the first cambialistic Fe/Mn−SOD (*11*). In this study, we reported cloning, expression, and characterization of an isozyme of diatom Fe/Mn−SOD. This enzyme appears to be more stable than the previously published SOD (*11*) under a broad range of pH, higher temperature, and in the presence of proteases. These properties are beneficial for potential applications described in the Introduction. Our results clearly demonstrated that diatoms have at least two SOD isozymes to regulate oxidative stress.

LITERATURE CITED

(1) Halliwell, B.; Gutteridge, J. M. Role of free radicals and catalytic metal ions in human disease: an overview. *Methods Enzymol.* **1990**, *186*, 1−85.

(2) Brock, C. J.; Walker, J. E. Superoxide dismutase from *Bacillus stearothermophilus*. Complete amino acid sequence of a manganese enzyme. *Biochemistry* **1980**, *19*, 2873−2882.

(3) Fridovich, I. Superoxide anion radical ($O_2^{\bullet -}$), superoxide dismutases, and related matters. *J. Biol. Chem.* **1997**, *272*, 18515−18517.

(4) Harris, J. I.; Auffret, A. D.; Northrop, F. D.; Walker, J. E. Structural comparisons of superoxide dismutases. *Eur. J. Biochem.* **1980**, *106*, 297−303.

(5) Youn, H. D.; Kim, E. J.; Roe, J. H.; Hah, Y. C.; Kang, S. O. A novel nickel-containing superoxide dismutase from *Streptomyces* spp. *Biochem. J.* **1996**, *318*, 889−896.

(6) Youn, H. D.; Youn, H.; Lee, J. W.; Yim, Y. I.; Lee, J. K.; Hah, Y. C.; Kang, S. O. Unique isozymes of superoxide dismutase in *Streptomyces griseus*. *Arch. Biochem. Biophys.* **1996**, *334*, 341−348.

(7) Meier, B.; Barra, D.; Bossa, F.; Calabrese, L.; Rotilio, G. Synthesis of either Fe- or Mn-superoxide dismutase with an apparently identical protein moiety by an anaerobic bacterium dependent on the metal supplied. *J. Biol. Chem.* **1982**, *257*, 13977−13980.

(8) Beyer, W. F., Jr.; Fridovich, I. In vivo competition between iron and manganese for occupancy of the active site region of the manganese-superoxide dismutase of *E. coli*. *J. Biol. Chem.* **1991**, *266*, 303−308.

(9) Chen, H. Y.; Hu, R. G.; Wang, B. Z.; Chen, W. F.; Liu, W. Y.; Schroder, W.; Frank, P.; Ulbrich, N. Structural studies of an eukaryotic combialistic superoxide dismutase purified from the mature seeds of camphor tree. *Arch. Biochem. Biophys.* **2002**, *404*, 218−226.

(10) Okamoto, O. K.; Robertson, D. L.; Fagan, T. F.; Hastings, J. W.; Colepiclo, P. Different regulation mechanisms modulate the expression of a dinoflagellate iron-superoxide dismutase. *J. Biol. Chem.* **2001**, *276*, 19989−19993.

(11) Ken, C. F.; Hsiung, T. M.; Huang, Z. X.; Juang, R. H.; Lin, C. T. Characterization of Fe/Mn-superoxide dismutase from diatom *Thallassiosira weissflogii*: cloning, expression, and property. *J. Agric. Food Chem.* **2005**, *53*, 1470−1474.

(12) Isobe, T.; Fang, Y. H.; Muno, D.; Okuyama, T.; Ohmori, D.; Yamakura, F. Difference between amino acid residues in the metal−ligand environments of iron- and manganese-superoxide dismutases. *Biochem. Int.* **1988**, *16*, 495−501.

(13) Parker, M. W.; Blake, C. C. F. Iron- and manganese-containing superoxide dismutase can be distinguished by analysis of their primary structures. *FEBS Lett.* **1988**, *229*, 495−501.

(14) Yamakura, F.; Kobayashi, K.; Ue, H.; Konno, M. The pH-dependent changes of the enzymatic activity and spectroscopic

properties of iron-substituted manganese superoxide dismutase. *Eur. J. Biochem.* **1995**, *227*, 700−706.
(15) Wilder, M. S.; Mass, A. Composition for preventing or alleviating skin irritation by formulations containing superoxide dismutase. U.S. Patent 4,957,740, 1990; pp 1−12.
(16) N'Guyen, Q. L.; Colin, C. Cosmetic composition containing a superoxide dismutase and a porphyrin. U.S. Patent 5,650,137, 1997; pp 1−6.
(17) Colin, C.; N'Guyen, Q. L. Cosmetic composition containing, in combination, a superoxide-dismutase and a melanin pigment. U.S. Patent 5,925,363, 1999; pp 1−9.
(18) Beauchamp, C.; Fridovich, I. Improved assays and an assay applicable to acrylamide gel. *Anal. Biochem.* **1971**, *44*, 276−287.
(19) Martin, M. E.; Byers, B. R.; Olson, M. O.; Salin, M. L.; Arceneaux, J. E.; Tolbert, C. A Streptococcus mutans superoxide dismutase that is active with either manganese or iron as a cofactor. *J. Biol. Chem.* **1986**, *261*, 9361−9367.
(20) Stallings, W. C.; Pattridge, K. A.; Strong, R. K.; Ludwig, M. L. The structure of manganese superoxide dismutase from *Thermus thermophilus* HB8 at 2.4-Å resolution. *J. Biol. Chem.* **1985**, *260*, 16424−16432.

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