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Copper/Zinc−Superoxide Dismutase from *Epinephelus malabaricus* cDNA and Enzyme Property

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A full-length cDNA of 803 base pairs encoding a putative copper/zinc−superoxide dismutase (Cu/Zn-SOD) from *Epinephelus malabaricus* was cloned by the polymerase chain reaction approach. Nucleotide sequence analysis of this cDNA clone revealed that it comprises a complete open reading frame coding for 154 amino acid residues. The deduced amino acid sequence showed high similarity (65−91%) with the sequences of the Cu/Zn-SOD from other species. Computer analysis of the residues required for coordinating copper (His-49, -64, and -121) and zinc (His-64, -72, and -81 and Asp-84), as well as the two cysteines (58 and 147) that form a single disulfide bond, was well-conserved among all reported Cu/Zn-SOD sequences. To further characterize the *E. malabaricus* Cu/Zn-SOD, the coding region was subcloned into an expression vector, pET-20b(+) and transformed into *Escherichia coli* BL21(DE3)pLysS. The expression of the Cu/Zn-SOD was confirmed by enzyme activity stained on a native gel and purified by Ni^{2+}−nitrilotriacetic acid Sepharose. The enzyme activity was inhibited under basic pH (higher than 10.0). The enzyme retained 65% activity after heating at 60 °C for 10 min. The inactivation rate constant (k_d) was 6.64×10^{-2} min^{-1} at 60 °C. The enzyme activity was only some decrease under 3% sodium dodecyl sulfate. The enzyme was resistant to proteolysis by trypsin and chymotrypsin. The finding of Cu/Zn-SOD cDNA could be used as a probe to detect the transcription level of this enzyme, which can be used as an early biomarker of environmental pollution. The property of this enzyme could provide a reference as compared to the oxidized forms or new isoforms, which could be induced under the experiments of pollution.

KEYWORDS: ***Epinephelus malabaricus*; copper/zinc-superoxide dismutase (Cu/Zn-SOD); expression; *Echerichia coli*; PCR; pET-20b(+)**

INTRODUCTION

Superoxide dismutases (SODs) are metalloproteins and can be classified into three types, Cu/Zn-, Mn-, and Fe-SOD, depending on the metal found in the active site (*1*−*3*). Cu/Zn-SOD is predominantly associated with eukaryotes in the cytosolic fraction and is very sensitive to cyanide and hydrogen peroxide. Mn-SOD is associated with mitochondria and insensitive to cyanide and hydrogen peroxide. Fe-SOD is found in prokaryotes and is not sensitive to cyanide but is inhibited by hydrogen peroxide.

Reactive oxygen species (ROS) such as superoxide ion (O_2^-) are normal byproducts of oxidative metabolism and have the potential to give rise to hydroxyl radicals (HO•). Some ROS may function as important signaling molecules that alter gene expression and modulate the activity of specific defense proteins (*4*); all ROS may be harmful and pose a threat to aerobic organisms. Oxidative damage to DNA, proteins, and lipids can lead to mutagenesis, carcinogenesis, and alterations in cell structure. The role of SOD is to catalyze the dismutation of the O_2^- to hydrogen peroxide and molecular oxygen during oxidative energy processes. The reaction diminishes the destructive oxidative processes in cells. The level of scavenging enzymes has been extensively used as an early warning indicator of marine pollution (*5*). Antioxidant enzymes have been proposed as bioindicators for environmental impact assessment (*6*, *7*), because both metals and certain organic xenobiotics generate oxidative stress (*8*). Increased levels of several detoxifying and antioxidative enzymes have been described in molluscs and fish from the Spanish South Atlantic littoral in response to environmental pollution. This is particularly important in the Huelva estuary of the country, where the Tinto river brings Fe and Cu from pyrite mines and organic xenobiotics, such as industrial pollutants and pesticides, are released. Thus, molluscs and fish caught in that zone showed significant increases in SOD activity (*9*, *10*).

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Liver cell-free extracts of fish (*Mugil* sp.) from polluted environments showed new Cu/Zn-SOD isozymes (*11*) due to high levels of metals (Cu ions) and organic compounds (waste spills from chemical industries and from intensive agricultural areas). The combined effect of Pb and Zn caused changes in the liver SOD−catalase detoxication system of carp (*12*).

On the basis of such reasoning, the study of SODs and their application as biomarkers has become an important area in environmental impact assessment. We have noted that it would be of interest from the comparative biochemical standpoint to study the SODs of the fish and then to assess environmental pollution. So far, only a few papers on this topic have appeared, and there should be much room left for exploring the physiological roles played by SODs related to pollution in aquatic animals. In our prestudy of pollution, we find zebrafish Cu/Zn-SOD amounts decrease in proportion to concentration (10, 100, 200, and 300 ppm) of paraquat but Cu/Zn-SOD amounts increase in proportion to Cu^{2+} concentration (1, 10, 100, and 500 ppm). Because zebrafish is not economically valuable, we chose *Epinephelus malabaricus* as our study sample.

E. malabaricus is a high quality and valuable species in Taiwan. Although primary structures of fish Cu/Zn-SOD are known by the protein sequence analysis method from swordfish and shark (*13*), no fish Cu/Zn-SOD cDNA sequence has been reported. Recently, we cloned full-length Cu/Zn-SOD cDNA clones from zebrafish (*14*), black porgy (*Acanthopagrus schlegeli*) (*15*), and *Pagrus major*. Although both cDNAs were introduced into expression vector, we did not obtain enough pure enzymes to study their properties. Here, we report the cDNA sequence and deduced amino acid sequence from *E. malabaricus* Cu/Zn-SOD cDNA; in comparison to zebrafish and black porgy, there are still differences. In addition, the coding region of Cu/Zn-SOD cDNA was introduced into an expression vector, pET-20b(+), and transformed into *Escherichia coli* BL21(DE3)pLysS. This Cu/Zn-SOD cDNA clone could overexpress Cu/Zn-SOD enzyme in *E. coli*. Enough pure enzyme could be obtained for further property analysis. Thus, the finding of Cu/Zn-SOD cDNA could be used as a probe to detect transcription levels of this enzyme, which can be used as an early biomarker of environmental pollution. The property of this enzyme could provide a reference as compared to the oxidized forms or new isoforms, which could be induced under the experiments of pollution.

MATERIALS AND METHODS

Fish Sample. A live 2 year old *E. malabaricus* weighing 400 g, provided by a local market, was used. Freshly dissected tissues were frozen in liquid nitrogen and stored at −70 °C until use.

Total RNA Preparation and Single Strand cDNA Synthesis. Gill (2 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) and 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, and then centrifuged at 12 000*g* 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 12 000*g* for 10 min at 4 °C. The total RNA (740 μg) was obtained. Single-strand cDNA was synthesized using a kit (GeneRacer) from Invitrogen (Grand Island, NY).

Subcloning, DNA Sequence Analysis, and Transformation. According to the cDNA sequence of the black porgy Cu/Zn-SOD (EMBL accession no. AJ00249), two primers (RpCu-1, 5′CCC ATG GTG CTT AAA GCC GTG TG3′; RpCu-2, 5′GGA ATT CTG GGT GAT GCC AAT CAG TCC3′) 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 0.45 kbp cDNA was amplified by the polymerase chain reaction (PCR) technique (25 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s). The 0.45 kbp cDNA was subcloned into pCR2.1 cloning vector (Invitrogen) using TOPO 10 as a host. The nucleotide sequence of the insert was determined in both directions by autosequencing (ABI PRISM 377-96 DNA Sequencer, Perkin-Elmer, Cypress, CA). On the basis of this cDNA sequence, a GrCu-1 primer (5′ AGG CAT GTT GGA GAC CTG GG 3′), which was the sense sequence for 3′-RACE, and a GrCu-2 primer (5′ TGA TTA TCT TGT CCG TGA TGT C 3′), which was the antisense sequence for 5′-RACE, were synthesized. To a 0.5 mL microtube containing 0.1 μg of the single strand cDNA from *E. malabaricus* as the template was added 8 pmol of GeneRacer 3′ primer (from GeneRacer kit) and 10 pmol of GrCu-1 primer. Eight picomoles of GeneRacer 5′ primer (from GeneRacer kit) and 10 pmol of GrCu-2 primer were added to another 0.5 mL microtube containing 0.1 μg of single strand cDNA from *E. malabaricus*. One 0.55 kbp cDNA (3′-RACE: 3′-cDNA end) and one 0.4 kbp cDNA (5′-RACE: 5′-cDNA end) were amplified by the PCR technique (25 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 30 s). Both 0.55 kbp and 0.4 kbp cDNA fragments were subcloned into pCR2.1 cloning vector using TOPO 10 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 3′-RACE and 5′-RACE covered the full-length Cu/Zn-SOD cDNA (803 bp). On the basis of 5′-RACE and 3′-RACE sequences, using 0.1 μg of single strand cDNA as a template, one full-length cDNA of Cu/Zn-SOD was created by the PCR technique (EMBL accession number AY035854). The coding region of this full-length cDNA could encode for 154 amino acid residues. Using the program of the University of Wisconsin Genetics Computer Group, this amino acid sequence was compared with other organism species.

According to the 803 bp (base pair) cDNA sequence, a 5′ upstream primer (5′ GAA TTC GAT GGT TTT GAA AGC TGT GTG TG 3′) and a 3′ downstream primer (5′ CTC GAG CTG GGA GAT GCC AAT GAC ACC 3′) were synthesized. Using 0.1 μg of *E. malabaricus* single strand cDNA as a template, 10 pmol of each 5′ upstream and 3′ downstream primer was added, and a 0.45 kbp fragment was amplified by PCR and ligated with pCR2.1 and then transformed into *E. coli* TOPO 10 host. A positive clone was selected by hybridization (*16*) with ^{32}P-labeled Cu/Zn-SOD cDNA as the probe and plasmid (recombinant DNA) were prepared. Appropriate plasmid DNA was digested with *Eco* RI and *Xho* I and then electrophoresed on an 0.8% agarose gel. An 0.45 kbp insert DNA containing *Eco* RI and *Xho* I sites was recovered and ligated with pET-20b(+) (pretreated with *Eco* RI and *Xho* I) from Novagen (Madison, WI) and then transformed into BL21(DE3)pLysS as a host. A transformed clone was selected by hybridization with ^{32}P-labeled Cu/Zn-SOD cDNA as the probe.

Culture and Enzyme Purification. The transformed *E. coli* were grown at 37 °C in 250 mL of Luria Bertani medium containing 50 μg/mL ampicillin until A_{600} reached 1.0. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM. The culture was incubated at 31 °C for 4 h at 150 rpm, and then, the bacterial cells were harvested by centrifugation at 10 000*g* for 5 min. The cells were suspended in 4 mL of 10 mM PBS buffer (pH 8.0) containing 0.1% glycerol and 1 g of glass beads and then vortexed for 5 min and centrifuged at 10 000*g* for 5 min. The extraction procedure was repeated two times, and the supernatants were pooled together. The final crude enzyme (12 mL) was loaded on a Ni−-nitrilotriacetic acid Sepharose superflow (Qiagen) column (bed volume = 4 mL), and then, the column was washed with 20 mL of 20 mM imidazole. The enzyme was eluted with phosphate-buffered saline (PBS) containing 250 mM imidazole (flow rate: 0.4 mL/min, 2 mL/fraction, at room temperature). The purified enzyme (4 mL) that was dialyzed against 600 mL of PBS containing 0.1% glycerol at 4 °C for 4 h immediately was used for analysis or stored at −20 °C for further analysis.

Protein Concentration Measurement. Protein concentration was determined by the 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

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1                CATGGACTGAAGGAGTAGAAAAGAAACTTAGGCGTCTACTGGAACTGCTACGAAG
56  ATG GTT TTG AAA GCT GTG TGT GTG CTG AAA GGA GCT GGA GAG ACC AGC GGG
1    M   V   L   K   A   V   C   V   L   K   G   A   G   E   T   S   G
109 ACC GTG TAT TTT GAG CAG GAG ACT GAT TCA GCC CCT GTG AAG CTG ACA GGA
18   T   V   Y   F   E   Q   E   T   D   S   A   P   V   K   L   T   G
158 GAA ATC AAA GGC CTC ACT CCT GGT GAG CAT GGT TTC CAA GTC CAT GCT TTT
35   E   I   K   G   L   T   P   G   E   H   G   F   Q   V   H   A   F
209 GGA GAC AAT ACA AAC GGG TGC ATC AGT GCA GGG CCT CAC TTC AAC CCC CAC
52   G   D   N   T   N   G   C   I   S   A   G   P   H   F   N   P   H
260 AAC AAG CAT CAT GCC GGT CCT ACT GAT GCG GAG AGG CAT GTT GGA GAC CTG
69   N   K   H   H   A   G   P   T   D   A   E   R   H   V   G   D   L
311 GGA AAT GTG ACT GCA GGA GGC GAT AAT GTT GCC AAG ATA GAC ATC ACG GAC
86   G   N   V   T   A   G   G   D   N   V   A   K   I   D   I   T   D
362 AAG ATA ATC ACC CTC AAT GGC CCA TAC TCC ATC ATT GGC AGA ACC ATG GTG
103  K   I   I   T   L   N   G   P   Y   S   I   I   G   R   T   M   V
413 ATC CAT GAG AAG GCT GAT GAC CTG GGA ACA GGG GGC AAT GAA GAG AGT CTA
120  I   H   E   K   A   D   D   L   G   T   G   G   N   E   E   S   L
464 AAG ACA GGC AAC GCT GGT GGA CGT CTA GCC TGT GGT GTC ATT GGC ATC TCC
137  K   T   G   N   A   G   G   R   L   A   C   G   V   I   G   I   S
515 CAG TAAATGATCAACACAATGCACTGGAAACAATTTTTCCCCCACAGCTCTTACTAAGACCAACAC
154  Q   *
581 AGCTACTTAACGTGACAGTTTGTCCTGTTCAGTACACTTTTTATTTACAGAGTAGATGAGCCATGCTT
649 TACCCTGTCAGTTCCTCATGACAATTGTTTGTTTGGGTTTATATGTCTGCTGAGTAGTTTTGGGTCCC
717 AAAGAACTGATAACGCACAAGTGATAATCAGATGTATACAATTTACAAAGCTGATGAATAAATTGTCC
785 ATCTGGAAAAAAAAAAAAA
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Figure 1. Nucleotide sequence of the *E. malabaricus* Cu/Zn-SOD cDNA and its deduced amino acid sequence. Numbers to the left refer to nucleotide and deduced amino acid residues. The asterisk denotes the translation stop signal.

ethylenediaminetetraacetic acid (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 the rate of INT reduction at 25 °C over the first 3 min time interval, 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 Cu/Zn-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 illuminating in a solution containing 0.45% tetramethylenediamine 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 section of the gel was stained with Coomassie blue. The area and intensity of activity and protein bands were measured by a computing densitometer (Molecular Dynamics Co., CA).

Enzyme Characterization. Each enzyme sample was 5.7 μg/30 μL for 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 polyacrylamide gel (PAGE) to determine the changes of activity and protein.

(1) Thermal Stability. The enzyme samples were heated at 60 °C for 5, 10, 15, or 20 min.

(2) pH Stability. The enzyme sample was amended with a half volume of buffer in different pH values: 0.2 M citrate buffer (pH 2.3, 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 or 11.0). Each sample was incubated at 37 °C for 1 h.

(3) Sodium Dodecyl Sulfate (SDS) Effect. SDS was added into the enzyme sample to the levels of 1.0, 2.0, 3.0, or 4.0% and then incubated at 37 °C for 1 h. Both SDS and imidazole are protein denaturing reagents.

(4) Imidazole Effect. Imidazole was added into the enzyme to the levels of 0.1, 0.5, 1.0, or 1.6 M and then incubated at 37 °C for 1 h.

Table 1. Comparison (% Identity, % Similarity) of Amino Acid Sequences for CuZn-SOD of Malabar Rockcod and Other Organisms

organism	EMBL no.	genus species	identity (%)	similarity (%)
malabar rockcod		*E. malabaricus*		
black porgy	AJ00249	*A. schlegeli*	91	91
swordfish[a]		*Xiphias gladius*	80	86
zebrafish.	Y12236	*Danio rerio*	78	85
shark[a]		*Prionace glauca*	69	80
mouse	X06683	*Mus musculus*	69	80
X. laevis	X16585	*X. laevis*	67	77
human	X02317	*Homo sapiens*	62	77
fruit fly	Y00367	*Drosophila melanogaster*	61	74
C. elegans	NM063030	*C. elegans*	58	71
yeast	J03279	*Saccharomyces cerevisiae*	56	68
sweet potato	X73139	*Ipomoea batatas*	56	66
pea	M63003	*Pisum sativum*	55	65
tomato	X14041	*Solanum lycopersicum*	53	65

[a] Ref *13*.

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                          1                                                 41
M. rockcod                M VLKAVCVLKG AGETSGTVYF EQETDSAPVK LTGEIKGLTP
Black porgy               . .......... ....T.V.H. ...SE..... ..........
Swordfish                   .l......R. ....T..... ...GNANA.G KGIIL..
Shark                       M..... T..VT...L. ..-AADG... .K.S.T....
Mouse                     . AM..... D.PVQ..IH. ..KASGE..V .S.Q.T...E
Human                     . AT..... D.PVQ.IIN. ..KESNG..T VW.S.....E
X.laevis                    V......A. S.DVK.V.R. ..QG.DGD.. VE.K.E...D
C.elegan                  . SNR..A..R. E.TVT..IWI T.KSENDQAV IE.......
Fruit fly                 . .V.....IN. DAK--...F. ...SSGT... VS..VC..AK
Yeast                     . .Q-..A.... DAGV..V.K. ..ASE.E.TT VSY..A.NS.
Tomato                    A TK...A.... NSNVE.V.TL S.DG.DG.TT VNVR.T..A.
Sweet potato              . .-...A..SS SEGV...IF. S..G.G-.TT V..NV...K.
Pea                       . .-...A..SN SN.V...IN. S..GNGK.TT V..TLA..K.

                        42     * *         o       *        *        *   *     91
M. rockcod                GE-HGFQVHAF GDNTNGCISA GPHFNPHNKH HAGPTDAERH VGDLGNVTAG
Black porgy               ..-...H.... .......... ...L...G.N .G....E... ..........
Swordfish                 ..-...H..G. .......... ......AS.K ....K.ED.. .........D
Shark                     .K-...H.... .......... ...Y..FS.N .G..D.E... .......E.N
Mouse                     .Q-...H..QY ....Q..T.. .......S.K .G..A.E... ..........
Human                     .L-...H..E. ....A..T.. ......LSRK .G..K.E... .........D
X.laevis                  .N-...HI.V. .......L.. ......Q..N .GS.K..D.. .........E
C.elegan                  .L-...H..QY ..S..... ......FG.T .G..KSEI.. .......E..
Fruit fly                 .L-...H..E. .......M.S ......YG.E .GA.V.EN.. L.....IE.T
Yeast                     NAER..HI.E. ..A....V.. ......FK.T .GA...EV.. ...M...KTD
Tomato                    .L-...HL.EY ..T....M.T .A....NKLT .GA.G.EI.. A.....IV.N
Sweet potato              .L-...H...L ..T....M.T ......AG.E .GA.G.DN.. A.....I.V.
Pea                       .L-...HI..L ..T......T ......NG.E .GA.E.ET.. A.....INV.

                       92                                 *                   138
M. rockcod                GDNVAKIDIT DKIITLNGPY SIIGRTMVIH EKADDLGTGG N---EESLKT
Black porgy               A......... ..ML..T..L .......... ..T....K.. .---......
Swordfish                 ANG....... ..-IS.T... .......... .......R.. .---......
Shark                     .NG..EFE.K .RQLH.S.ER ......L.V. ..E....K.. D---....R.
Mouse                     K.G..NVS.E .RV.S.S.EH ........V. ..Q....K.. .---...T..
Human                     K.G..DVS.E .SV.S.S.DH C.....L.V. .......K.. .---...T..
X.laevis                  .-G..QFKF. .PQ.S.K.ER ......A.V. ..Q....K.. D---D.....
C.elegan                  A.G....KL. .TLV..Y..N TVV..S..V. AGQ....E.V GDKA...K..
Fruit fly                 ..CPT.VN.. .SK...F.AD ......V.V. AD.....Q.. H---.L.KS.
Yeast                     ENG...GSFK .SL.K.I..T .VV..SV... AGQ....K.- --DT......
Tomato                    A.G..EVTLV .NQ.P.T..N .VV..AL.V. .LE....K.. H---.L..T.
Sweet potato              E.GT.SFT.. ..Q.P.T.AN .V...AV.V. GDP....K.. H---.L.KS.
Pea                       D.GTVSFT.. .NH.P.T.TN .....AV.V. ADP....K.. H---.L.KT.

                      139       o     154
M. rockcod                GNAGGRLACG VIGISQ
Black porgy               .......... ....T.
Swordfish                 ....S. ... TE
Shark                     ....S..... ....AKD
Mouse                     ....S..... ....A.
Human                     ....S..... ....A.
X.laevis                  .......... ...FTP
C.elegan                  ....A.A... ..ALAAPQ
Fruit fly                 ....A.IG.. ....AKV
Yeast                     ....P.P... ...LTN
Tomato                    .......... .V.LTPI
Sweet potato              ......V... I..LQG
Pea                       ......V... I..LQG
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Figure 2. Optimal alignment of Cu/Zn-SOD among several species. *E. malabaricus*: this study (EMBL no. AY035854); black porgy (AJ00249); swordfish and shark (*13*); mouse (X06683); human (X02317); *X. laevis* (X16585); *C. elegans* (L20135); fruit fly (Y00367); yeast (J03279); tomato (X14041); sweet potato (X73139); and pea (M63003). Numbers refer to amino acid residues of *E. malabaricus*. A dot refers to identities with *E. malabaricus*. A dash denotes deletion. Residues coordinating copper and zinc are indicated with asterisks. The two cysteines that form a disulfide bridge are circled.

Proteolytic Susceptibility. The enzyme was incubated with 1/20 in weight of trypsin or chymotrypsin at pH 8.8 and 37 °C for up to 1, 2, or 3 h. In the chymotrypsin digestion, $CaCl_2$ was added to 20 mM. Aliquots were removed from time to time and analyzed.

RESULTS AND DISCUSSION

Cloning and Characterization of a cDNA Coding for *E. malabaricus* Cu/Zn-SOD. Figure 1 shows the nucleotide and deduced amino acid sequence of one *E. malabaricus* Cu/Zn-SOD clone. Sequence analysis found that the cDNA was full-length, comprising a complete open reading frame coding for 154 amino acid residues. The DNA sequence translation start site (GAAGATGG) matches the consensus sequence (AACAATGG) reported (*18*).

Table 1 shows a higher identity with the amino acid sequence of the Cu/Zn-SOD from four other aquatic species (69−91%) than with the Cu/Zn-SOD sequences from other organisms including mammalian (mouse, 69%; human, 62%), *Xenopus laevis* (67%), and sweet potato (56%).

Figure 2 shows that seven residues coordinating copper (His-49, -64, and -112) and zinc (His-64, -72, and -81 and Asp-84), as well as the two cysteines (58 and 147) that form a disulfide bridge, are conserved as they are all reported Cu/Zn-SOD sequences (*2*). There is an another cysteine between Val-6 and Val-8 not found in *Caenorhabditis elegans* and all known plant species in which Cu/Zn-SOD was thermostable. This Cys-7 may compete to form a disulfide bridge with Cys-58 or Cys-147

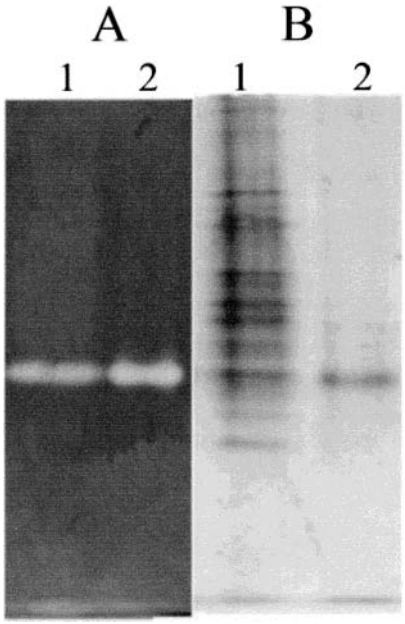

Figure 3. Total soluble protein profile of IPTG induced *E. coli* BL21-(DE3) pLysS containing the pET-20b(+)–Cu/Zn-SOD clone and one step purification. Fifteen microliters of induced crude extract (lane 1) (2.54 μg/μL) and purified enzyme after dialysis (lane 2) (0.35 μg/μL) was subjected to a 15% native PAGE followed by activity staining (A) and Coomassie blue staining (B), respectively.

during thermal denaturation, consequently leading to irreversible denaturation. Further studies via point mutation of zebrafish Cu/Zn-SOD at position 7 from Cys to Ala demonstrated that the mutant Cu/Zn-SOD possessed more thermostability than wild type (data not shown).

Transformation and Expression of *E. malabaricus* Cu/Zn-SOD. One goal of this study was to clone and express the *E. malabaricus* Cu/Zn-SOD coding sequence in *E. coli*. Using single strand cDNA as the template and two specific primers corresponding to the translation initiation and termination sequences, respectively, the 0.45 kb DNA fragment coding for the *E. malabaricus* Cu/Zn-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 induced with IPTG, and their total cellular proteins were analyzed by a 15% native PAGE with activity and protein staining (**Figure 3**, lane 1).

Purification of *E. malabaricus* Cu/Zn-SOD. The *E. malabaricus* Cu/Zn-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 Sephrose (Qiagen) according to the instruction manual. The yield was 0.38 mg from 0.25 L of culture. The specific activity was 3883 units/mg. The purified enzyme showed one active enzymatic form (**Figure 3**, lane 2) on a 15% native PAGE.

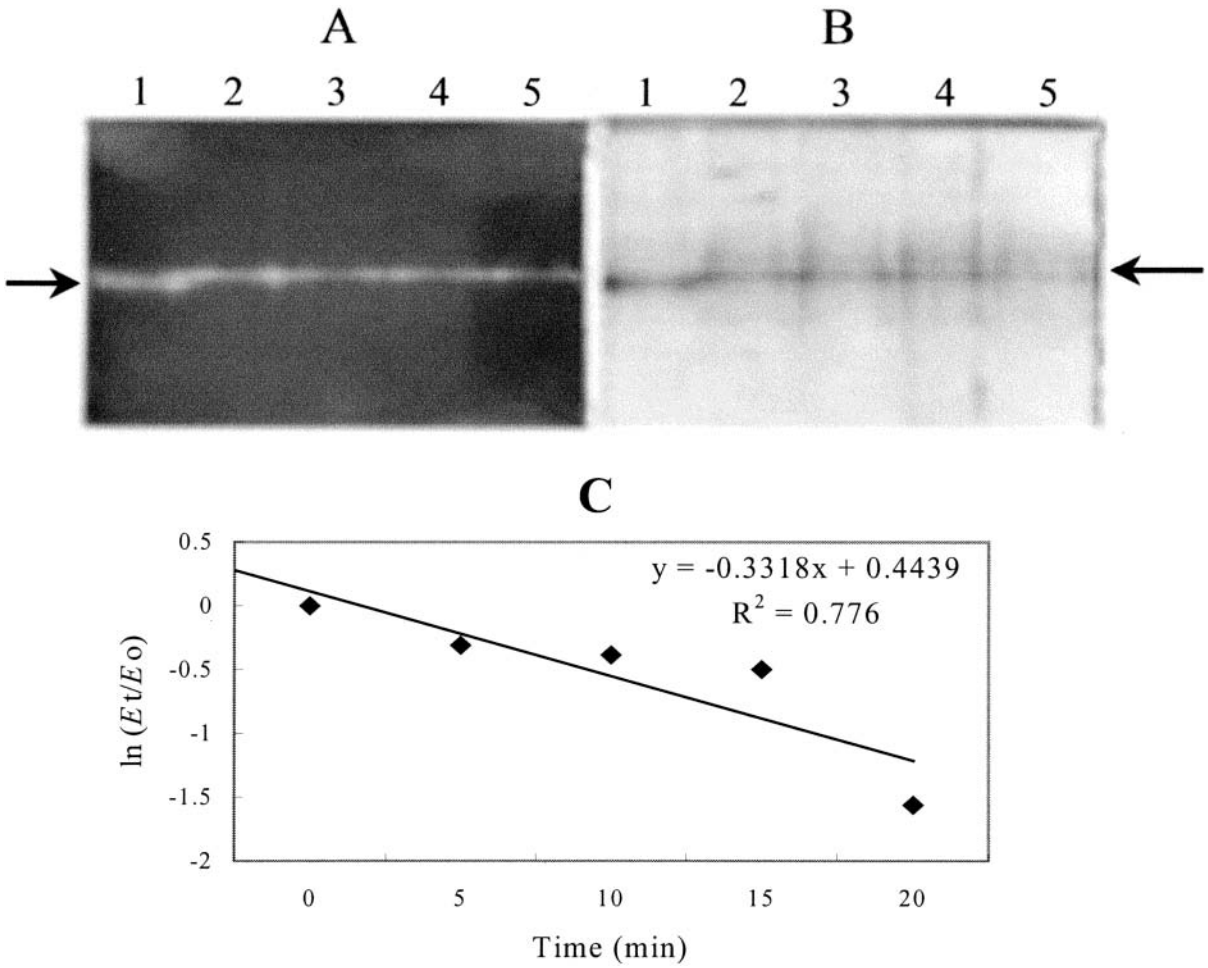

Figure 4. Effect of temperature on enzyme stability. The enzyme samples were heated at 60 °C for various times and then performed on a 15% native PAGE. (A) Activity staining (1.9 μg/lane); (B) Coomassie blue staining (3.8 μg/lane), lanes 1–5 (control, 5, 10, 15, or 20 min); and (C) plot of thermal inactivation kinetics. The PAGE data were quantitated by a densitometer for calculation. The areas of activity measured by a densitometer were 454.7 ± 31.8 (control), 333.9 ± 22.0 (5 min), 309.0 ± 17.1 (10 min), 276.2 ± 10.8 (15 min), or 95.1 ± 10.0 (20 min). Triplicate experiments were done.

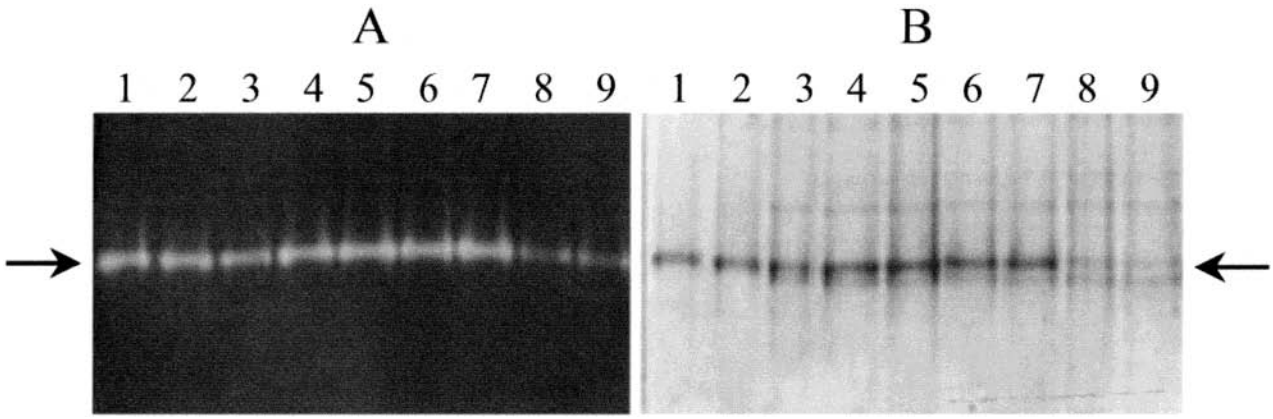

Figure 5. Effect of pH on enzyme stability. The enzyme samples were incubated in buffer with different pH values at 37 °C for 1 h and then performed in a 15% native PAGE. (A) Activity staining (1.9 μg/lane); (B) Coomassie blue staining (3.8 μg/lane), lanes 1–9 (pH 2.3, 3.0, 4.0, 5.0, 7.0, 8.0, 9.0, 10.0, or 11.0). The areas of activity measured by a densitometer were 905.7 ± 57.0 (pH 2.3), 895.5 ± 28.7 (pH 3), 793.9 ± 13.1 (pH 4), 993.9 ± 8.6 (pH 5), 1162 ± 23.6 (pH 7), 1070 ± 27.4 (pH 8), 988.9 ± 31.8 (pH 9), 508.0 ± 19.8 (pH 10), or 491.1 ± 8.0 (pH 11). Triplicate experiments were done.

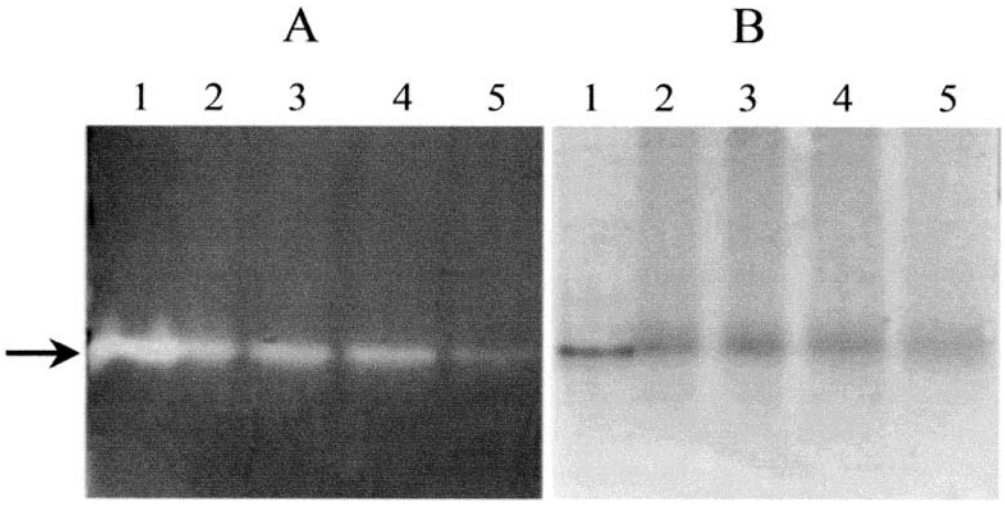

Figure 6. Effect of SDS on enzyme stability. The enzyme samples were incubated in various SDS concentrations at 37 °C for 1 h and then applied to a 15% native PAGE determined by SOD activity staining (A, 1.9 μg/lane) and Commassie blue staining (B, 3.8 μg/lane). Lanes 1–5 (control, 1, 2, 3, or 4% SDS, respectively). The arrow areas of activity measured by a densitometer were 966.0 ± 15.8 (control), 662.2 ± 156.6 (1%), 484.7 ± 1.1 (2%), 463.7 ± 41.4 (3%), and 126.8 ± 6.1 (4%). Triplicate experiments were done.

Characterization of the Purified *E. malabaricus* Cu/Zn-SOD. The enzyme inactivation kinetics at 60 °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 that remained after heating for time t, respectively. The thermal inactivation rate constant k_d value calculated for the enzyme at 60 °C was 6.64×10^{-2} min^{-1}, and the half-life for inactivation was 10.4 min (**Figure 4A−C**).

As shown in **Figure 5** (lanes1−7), *E. malabaricus* SOD was stable in a broad pH range from pH 2.3−9, although the total activity decreased to 56−54% at a high of pH 10.0 (lane 8) to pH 11 (lane 9).

The enzyme activity showed only a 52% decrease in a high concentration of SDS up to 3% (**Figure 6**, lane 4). The enzyme was resistant to digestion by trypsin and chymotrypsin even at a high enzyme/substrate (w/w) ratio of 1/20 (data not shown).

In aquatic organisms, SODs play an important role in defending damage from early biomarkers generated by metals or organic pollutants. Thus, the cDNA would be useful as a molecular probe as well as the enzyme as a reference in developing the diagnostic means to assess the environmental impact.

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