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Characterization of Copper/Zinc-Superoxide Dismutase from *Pagrus major* cDNA and Enzyme Stability

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A full-length cDNA of 794 bp encoding a putative copper/zinc-superoxide dismutase (Cu/Zn-SOD) from *Pagrus major* was cloned by the PCR 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 (53−91%) with the sequences of Cu/Zn-SOD from other species. Computer analysis of the residues required for coordinating copper (His-47, 49, 64, and 121) and zinc (His-64, 72, 81, and Asp-84), as well as the two cysteines (58 and 147) that form a single disulfide bond, were well conserved among all reported Cu/Zn-SOD sequences. To further characterize the *Pagrus major* Cu/Zn-SOD, the coding region was subcloned into an expression vector, pET-20b(+), and transformed into *Escherichia coli* BL21(DE3). 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 superflow. Dimer was the major form of the enzyme in equilibrium. The dimerization of the enzyme was inhibited under acidic pH (below 4.0 or higher than 10.0). The half-life was 8.6 min and the inactivation rate constant (k_d) was 9.69×10^{-2} min^{-1} at 70 °C. The enzyme activity was not significantly affected under 4% SDS or 0.5 M imidazole. The enzyme was resistant to proteolysis by both trypsin and chymotrypsin.

KEYWORDS: ***Pagrus major*****; expression;** ***Escherichia 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 eucaryotes in the cytosolic fraction and is very sensitive to cyanide and hydrogen peroxide. Mn-SOD is associated with mitochondria and is insensitive to cyanide and hydrogen peroxide. Fe-SOD is found in prokaryotes and is not sensitive to cyanide but is inhibited by hydrogen peroxide.

The role of SOD is to catalyze the dismutation of the superoxide ion (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 (*4*). Antioxidant enzymes have been proposed as bioindicators for environmental impact assessment (*5*, *6*), because both metals and certain organic xenobiotics generate oxidative stress (*7*). Increased levels of several detoxifying and antioxidative enzymes have been described in

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1   AGGCATGTTGGAGACCTGGGGAACGTGACTGCAGGAGCAGATAATGTTGCCAAGATAGACATCACGGACAAG
73  ATG GTG CAG AAA GCA GTG TGT GTG CTG AAA GGA GCC GGG GAG ACC ACC GGG GTC
            RpCu-1 (→)
1   M   V   Q   K   A   V   C   V   L   K   G   A   G   E   T   T   G   V
127 GTT CAT TTT GAG CAG GAG AGT GAG TCA GCC CCT GTG ACG CTC AAA GGA GAA ATC
19  V   H   F   E   Q   E   S   E   S   A   P   V   T   L   K   G   E   I
181 TCG GGA CTT ACT CCT GAT GAG CAT GGT TTC CAT GTC CAT GCA TTT GGA GAC AAT
37  S   G   L   T   P   D   E   H   G   F   H   V   H   A   F   G   D   N
235 ACA AAT GGG TGC ATC AGT GCA GGC CCT CAC TTC AAT CCC CAC AAT AAG AAT CAT
55  T   N   G   C   I   S   A   G   P   H   F   N   P   H   N   K   N   H
289 GCC GGT CCT ACT GAT GCA GAG AGG CAT GTT GGA GAC CTG GGG AAC GTG ACT GCT
                                        RpCu-3 (→)
73  A   G   P   T   D   A   E   R   H   V   G   D   L   G   N   V   T   A
343 GGA GCA GAT AAT GTT GCC AAG ATA GAC ATC ACG GAC AAG ATG CTC ACT CTC AAT
                                            RpCu-4 (←)
91  G   A   D   N   V   A   K   I   D   I   T   D   K   M   L   T   L   N
397 GGG CCC TTC TCC ATC ATT GGC AGA ACC ATG GTG ATC CAC GAG AAG GCA GAC GAC
109 G   P   F   S   I   I   G   R   T   M   V   I   H   E   K   A   D   D
451 CTG GGA AAA GGA GGC AAC GAG GAG AGT CTA AAG ACA GGC AAT GCT GGT GGA CGT
127 L   G   K   G   G   N   E   E   S   L   K   T   G   N   A   G   G   R
505 CTG GCC TGT GGA GTC ATT GGC ATC TGC CAA TAA AGATAACATGGAGCACTGAAAACGTCT
                    RpCu-2 (←)
145 L   A   C   G   V   I   G   I   C   Q   *
565 TTTCCCCTGAGCACTTAATAAGACCAACCTAGCTACTTGATGTGTCAGTTTGTCCTTTTCACTTCTCTGGCATTTTA
642 CTGACTAGTCAAGAGAGTAGATGAGCCATGTCTGACCCTGTCCGTTCTTCATGACAATTGTATGTATGGGTTTATAT
719 GTCTGCTGTTTAGTTTTGGTCCCAAAGAATTGGTAACGCACAATTAAAATAAACGGATGTATACAATTTGAAAAGCT
757 AACCAATAAATTGTAAGTTCATCTGAAAAAAAAAAAAA
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Figure 1. Nucleotide sequence of the *Pagrus major* 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. Underlines denote primers for PCR.

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                        1                                          41
Pagrus major            M VQKAVCVLKG AGETTGVVHF EQESESAPVT LKGEISGLTP
black porgy             M .L........ .......... .........K .T...K....
swordfish                 .l......R. ......T.Y. ...GNANA.G KGIILK.
shark                      M........ T..V..T.L. ..-AADG..K ...S.T....
mouse                   . AM........ D.PVQ.TI.. ..KASGE..V .S.Q.T...E
human                   . AT........ D.PVQ.IIN. ..KESNG... VW.S.K...E
X.laevis                   V......A. S.DVK...R. ..QGDDGD.K VE.K.E...D
C.elegans               . SNR..A..R. E.TV..TIWI T.K..NDQAV IE...K....
fruit fly               . .V.....IN. DAK--.T.F. ....SGT..K VS..VC..AK
yeast                   . ..-..A.... DAGVS...K. ..A...E.T. VSY..A.NS.
tomato                  A TK...A.... NSNVE...TL S.DGDDG.T. VNVR.T..A.
sweet potato            . .-...A..SS SEGVS.TIF. S..GDG-.T. VT.NVK..K.
pea                     . .-...A..SN SN.VS.TIN. S..GNGK.T. VT.TLA..K.

              42     * *         o      *        *        *   *     91
Pagrus major   DE-HGFHVHAF GDNTNGCISA GPHFNPHNKN HAGPTDAERH VGDLGNVTAG
black porgy    G.-........ .......... ...L...G.. .G....E... ..........
swordfish      G.-......G. .......... ......AS.K ....K.ED.. .........D
shark          GK-........ .......... ...Y..FS.. .G..D.E... .......E.N
mouse          GQ-......QY ....Q..T.. .......S.K .G..A.E... ..........
human          GL-......E. ....A..T.. ......LSRK .G..K.E... .........D
X.laevis       GN-....I.V. .......L.. ......Q... .GS.K..D.. .........E
C.elegans      GL-......QY ..S....... ......FG.T .G..KSEI.. .......E..
fruit fly      GL-......E. .......M.S ......YG.E .GA.V.EN.. L.....IE.T
yeast          NAER...I.E. ..A....V.. ......FK.T .GA...EV.. ...M...KTD
tomato         GL-....L.EY ..T....M.T .A....NKLT .GA.G.EI.. A.....IV.N
sweet potato   GL-.......L ..T....M.T ......AG.E .GA.G.DN.. A.....I.V.
pea            GL-....I..L ..T......T ......NG.E .GA.E.ET.. A.....INV.

              92                              *                   138
Pagrus major   ADNVAKIDIT DKMLTLNGPF SIIGRTMVIH EKADDLGKGG N---EESLKT
black porgy    .......... ......T..L .......... ..T....... .---......
swordfish      .NG....... ..-IS.T..Y .......... .......R.. .---......
shark          GNG..EFE.K .RQ.H.S.ER ......L.V. ..E....... D---....R.
mouse          K.G..NVS.E .RVIS.S.EH ........V. ..Q....... .---...T..
human          K.G..DVS.E .SVIS.S.DH C.....L.V. .......... .---...T..
X.laevis       G-G..QFKF. .PQIS.K.ER ......A.V. ..Q....... D---D.....
C.elegans      ..G....KL. .TLV..Y..N TVV..S..V. AGQ....E.V GDKA...K..
fruit fly      G.CPT.VN.. .SKI..F.AD ......V.V. AD.....Q.. H---.L.KS.
yeast          ENG...GSFK .SLIK.I..T .VV..SV... AGQ......- --DT......
tomato         ..G..EVTLV .NQIP.T..N .VV..AL.V. .LE....... H---.L..T.
sweet potato   E.GT.SFT.. ..QIP.T.AN .V...AV.V. GDP....... H---.L.KS.
pea            D.GTVSFT.. .NHIP.T.TN .....AV.V. ADP....... H---.L.KT.

             139       o     154
Pagrus major   GNAGGRLACG VIGICQ
black porgy    .......... ....T.
swordfish      ....S. ... TE
shark          ....S..... ....AKD
mouse          ....S..... ....A.
human          ....S..... ....A.
X.laevis       .......... ...FTP
C.elegans      ....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. *Pagrus major*: this study (EMBL no. AF329278); black porgy (AJ00249); shark and swordfish (*12*); 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 *Pagrus major*. A dot refers to identities with *Pagrus major*. A dash denotes deletion. Residues coordinating copper and zinc are indicated with asterisks. The two cysteines that form a disulfide bridge are circled.

molluscs and fish from the Spanish South Atlantic littoral in response to environmental pollution. This is particularly important in the Huelva estuary of Spain , 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 have shown significant increases in SOD activity (*8*, *9*).

Liver-cell-free extracts of fish (*Mugil sp.*) from polluted environments showed new Cu/Zn-SOD isozymes (*10*) due to high levels of metals (Cu ions) and organic compounds (waste spills from chemical industries and from the intensive agricultural area). The combined effect of Pb and Zn caused changes in the liver SOD-catalase detoxication system of carp (*11*).

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 the environmental pollution. So far only a few reports 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.

Pagrus major is a high quality and economically valuable species in Taiwan. Although primary structures of fish Cu/Zn-SOD are known by protein sequence analysis method from swordfish and shark (*12*), no fish Cu/Zn-SOD cDNA sequence has been reported. Recently, we have cloned full-length Cu/Zn-SOD cDNA clones from zebrafish (*13*) and black porgy (*Acanthopagrus schlegeli*) (*14*). Although both cDNAs were introduced into an expression vector, we did not obtain enough

Table 1. Comparison (% identity, % similarity) of Amino Acid Sequences for Cu/Zn-SOD of *Pagrus major* and Other Organisms

English name	EMBL no.	Genus species	% identity	% similarity
Pagrus major	AF329278	*Pagrus major*		
black porgy	AJ00249	*Acanthopagrus schlegeli*	91	91
zebrafish	Y12236	*Danio rerio*	79	84
swordfish[a]		*Xiphias gladius*	77	84
shark[a]		*Prionace glauca*	72	82
mouse	X06683	*Mus musculus*	69	78
human	X02317	*Homo sapiens*	60	75
X. laevis	X16585	*Xenopus laevis*	68	78
C.elegans	L20135	*Caenorhabditis elegans*	58	70
fruit fly	Y00367	*Drosophila melanogaster*	59	70
yeast	J03279	*Saccharomyces cerevisiae*	60	70
sweet potato	X73139	*Ipomoea batatas*	56	66
pea	M63003	*Pisum sativum*	55	67
tomato	X14041	*Solanum lycopersicum*	53	67

[a] Ref. 12 (Calabrese et al., 1989).

pure enzymes to study their properties. Here we report the cDNA sequence and deduced amino acid sequence from a *Pagrus major* Cu/Zn-SOD cDNA clone. 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). This Cu/Zn-SOD cDNA clone can overexpress Cu/Zn-SOD enzyme in *E. coli.* Enough pure enzyme could be obtained for biochemical analysis.

MATERIALS AND METHODS

Fish Sample. A live $2^1/_2$-year-old *Pagrus major* weighing 450 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 (2.2 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), 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 to 3 min, then centrifuged at 12000*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, then centrifuged at 12000*g* for 10 min at 4 °C. The total RNA (516 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′; and RpCu-2, 5′GGA ATT CTG GGT GAT GCC AAT CAG TCC3′) were synthesized. Using 0.05 μg of the single-strand cDNA as a template, 10 pmol of each of the two primers was added. One 0.47-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 0.47-kbp cDNA was subcloned into a 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 RpCu-3 primer (5′ AGR CAT GTT GGA GAC CTG GG 3′), which was the sense sequence for 3′-RACE, and a RpCu-4 primer (5′ TGA KYA TCT TGT CYG TGA TGT C 3′), which was the antisense sequence for 5′-RACE, were synthesized. To a 0.5-mL microtube containing 0.05 μg of the single-strand cDNA from *Pagrus major* as template was added 8 pmol of GeneRacer 3′primer (from GeneRacer kit) and 10 pmol of RpCu-3 primer. GeneRacer 5′primer (8 pmol, from GeneRacer kit) and 10 pmol of RpCu-4 primer were added to another 0.5-mL microtube containing 0.05 μg of single-strand cDNA from *Pagrus major*. One 0.5-kbp cDNA (3′-RACE: 3′-cDNA end) and one 0.3-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 0.5-kbp and 0.3-kbp cDNA fragments were subcloned into the 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 cover the full-length Cu/Zn-SOD cDNA (794 bp). On the basis of 5′-RACE and 3′-RACE sequences, using 0.05 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 AF329278). 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 those of other organism species.

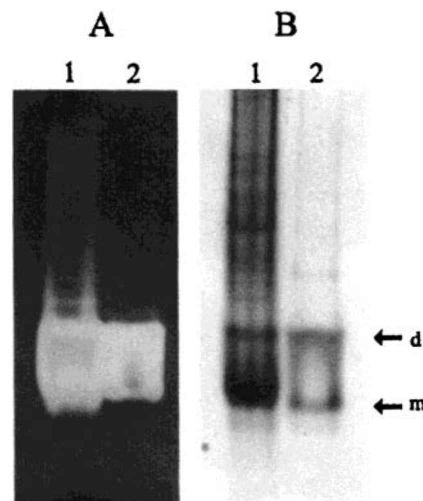

Figure 3. Total soluble protein profile of IPTG-induced *E. coli* BL21-(DE3) containing the pET-20b(+)–Cu/Zn-SOD clone and one step purification: 15 μL of induced crude extract (lane 1) (2.56 μg/μL), and purified enzyme after dialysis (lane 2) (0.1 μg/μL) were subjected to a 15% native-PAGE followed by activity staining (A panel), and Coomassie blue staining (B panel), respectively ("d" denotes dimer; "m" denotes monomer).

According to the 794 bp cDNA sequence, a 5′ upstream primer (5′ GAA TTC GAT GGT GCA GAA AGC AGT GTG TG 3′) and a 3′ downstream primer (5′ CTC GAG TTG GCA GAT GCC AAT GAC TCC 3′) were synthesized. Using 0.05 μg of *Pagrus major* single-strand cDNA as a template, 10 pmol of each 5′ upstream and 3′ downstream primer was added, 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 (*15*) with ^{32}P-labeled Cu/Zn-SOD cDNA as probe, and plasmid (recombinant DNA) was prepared. Appropriate plasmid DNA was digested with *Eco* RI and *Xho* I 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) as a host. A transformed clone was selected by hybridization with ^{32}P-labeled Cu/Zn-SOD cDNA as probe.

Culture and Enzyme Purification. The transformed *E. coli* were grown at 32 °C in 400 mL of Luria Bertani medium containing 50 μg/mL ampicillin until A_{600} reached 0.9. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM. The culture was incubated at 32 °C for 8 h at 150 rpm, and then the bacterial cells were harvested by centrifugation at 6000*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, then vortexed for 5 min and centrifuged at 10000*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) colum (bed volume = 4 mL), and then the column was washed with 20 mL of 20 mM imidazole. The enzyme was eluted with PBS

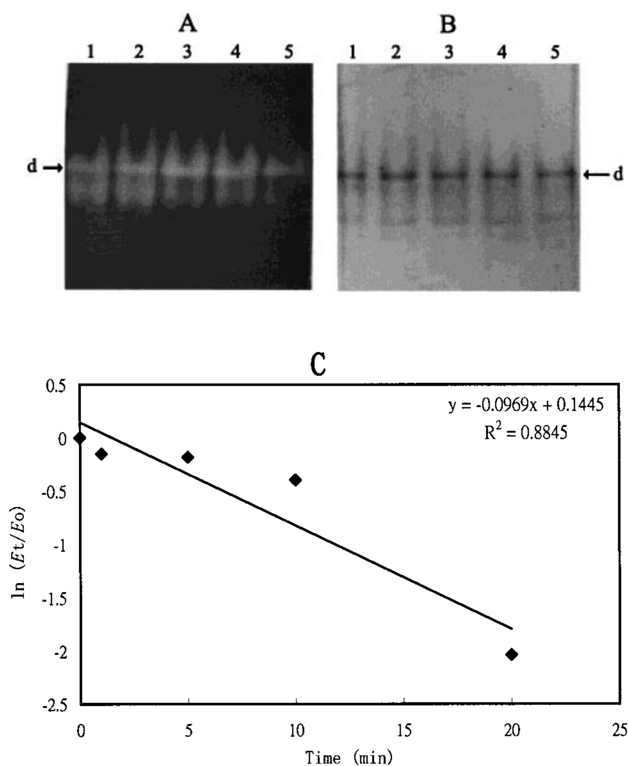

Figure 4. Effect of temperature on enzyme stability. The enzyme samples were heated at 70 °C for various times then performed on a 15% native-PAGE: (A) activity staining (1 μg/lane); (B) Coomassie blue staining (1 μg/lane), lanes 1–5 (control, 1, 5, 10, and 20 min, respectively); (C) plot of thermal inactivation kinetics. The PAGE data were quantitated by a densitometer for calculation. The areas of activity measured by the densitometer were 1431.3 ± 43.5 (control), 1231.7 ± 35.6 (1 min), 1194 ± 31.2 (5 min), 962.3 ± 38.7 (10 min), and 186.7 ± 5.6 (20 min) ("d" denotes dimer). Triplicate experiments were done.

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 was immediately used for analysis or stored at −20 °C for further analysis.

Protein 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, UK). The assay solution (1 mL) 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 so 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, 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 was assayed for Cu/Zn-SOD activity staining. This gel was soaked in 0.6 mg/mL nitro blue tetrazolium (NBT) solution for 15 min in the dark with gentle shaking, followed by immersion (with illumination) into 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 zone of the insolubility of the blue reduction product of NBT by superoxide anion. This procedure was described previously (*16*). 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, Sunnyvale, CA).

Enzyme Characterization. Each enzyme sample was used at a concentration of 2 μg/20 μL for the following tests. After the treatments as described below, each sample was divided into 2 parts, and each part of the sample was electrophoresed onto a 15% native-polyacrylamide gel to determine the changes of activity and protein.

(1) Thermal Stability. The enzyme samples were heated at 70 °C for 1, 5, 10, or 20 min.

(2) pH Stability. Each enzyme sample was amended with half-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 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) SDS Effect. The enzyme sample was added with SDS to 1.0, 2.0, 3.0, or 4.0% and incubated at 37 °C for 1 h. Both SDS and imidazole are protein-denaturing reagents.

(4) Imidazole Effect. The enzyme was added with imidazole to 0.1, 0.5. or 1.0 M and incubated at 37 °C for 1 h.

(5) Proteolytic Susceptibility. The enzyme was incubated with one-twentieth 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 *Pagrus major* Cu/Zn-SOD. Figure 1 shows the nucleotide and deduced amino acid sequence of one *Pagrus major* 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 (CAAGATGG) matches the consensus sequence (AACAATGG) previously reported (*17*).

Table 1 shows higher identity with the amino acid sequence of the Cu/Zn-SOD from four other aquatic species (91−72%) than with the Cu/Zn-SOD sequences from other organisms including mammalian (mouse, 69%; human, 60%), *X. laevis* (68%), and sweet potato (56%).

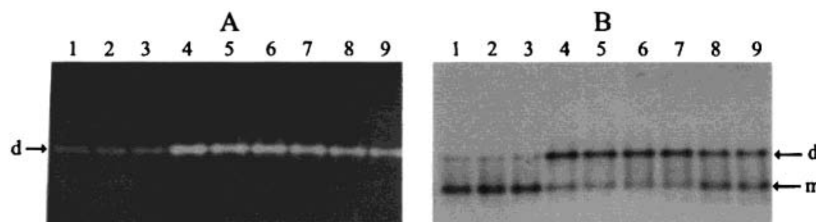

Figure 5. Effect of pH on enzyme stability. The enzyme samples were incubated in buffer with different pH values at 37 °C for 1 h, and then performed in a 15% native-PAGE: (A) activity staining (0.7 μg/lane); (B) Coomassie blue staining (1.3 μg/lane), lanes 1–9 (pH 2.3, 3.0, 4.0, 5.0, 7.0, 8.0, 9.0, 10.0, and 11.0, respectively). The areas of activity measured by a densitometer were 113.8 ± 1.3 (pH 2.3), 98.5 ± 3.2 (pH 3), 113.5 ± 0.6 (pH 4), 562.2 ± 27.4 (pH 5), 554.8 ± 33.2 (pH 7), 578.8 ± 34.9 (pH 8), 472.3 ± 58.3 (pH 9), 463.2 ± 31.9 (pH 10), and 448.2 ± 32.6 (pH 11) ("d" denotes dimer; "m" denotes monomer). Triplicate experiments were done.

Figure 2 shows that seven residues coordinating copper (His-47, 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 disulfide bridge, are conserved as they are all reported Cu/Zn-SOD sequences (*2*). There is another cysteine between Val-6 and Val-8 not found in *C. elegan* and all known plant species. This Cys-7 may compete to form a disulfide bridge with Cys-58 or Cys-147. Further studies are currently underway to obtain more insight into the structure−function relationship in this enzyme.

Transformation and Expression of *Pagrus major* Cu/Zn-SOD. One goal of this study was to clone and express the *Pagrus major* 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 mature *Pagrus major* 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 staining or protein staining (**Figure 3**, lane 1).

Purification of *Pagrus major* Cu/Zn-SOD. The *Pagrus major* Cu/Zn-SOD was fused in the pET-20b(+)−6His-tag vector and expressed in *E. coli* BL21(DE3). 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 0.4 mg from 0.4 L of culture. The specific activity was 4880 units/mg. The purified enzyme showed two active enzymatic forms (dimer and monomer, **Figure 3**, lane 2) on a 15% native-PAGE.

Characterization of the Purified *Pagrus major* Cu/Zn−SOD. The enzyme inactivation kinetics at 70 °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 70 °C was 9.69×10^{-2} min^{-1}, and the half-life for inactivation was 8.6 min (**Figure 4**A−C). This showed the enzyme was more thermally stable than that from teleost fish skin that, when heated at 70 °C, was completely inactivated (*18*).

As shown in **Figure 5** (lanes 4−7), *Pagrus major* SOD was very stable in a broad pH range from pH 5−9, although the total activity decreased to 20−17% at pH 4.0 (lane 3) − pH 2.2 (lane 1). The decrease of the enzyme activity at acidic pH was due to the dissociation of dimer into monomer (**Figure 5B**, lanes 1−3). Quantitation of proteins by a densitometer revealed that the acidic and alkaline (10−11) pH conditions favored the monomer formation. This suggests that the charge interaction could be important for subunit association (*19*).

The enzyme activity showed no decrease in either 4% SDS or 0.5 M imidazole (data not shown). 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 damages generated by metals or organic pollutants (*4, 6*). The molluscs and fish caught from a metal-contaminated area showed significantly increased SOD activity (*8*, *9*). After exposure to cadmium (*20*), copper (*21*), and chromium (*22*), the SOD activities of crabs were increased. Thus, the cloned cDNA for Cu/Zn-SOD would be useful as a molecular probe in developing the diagnostic means to assess the environmental impact of marine pollutants.

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