Molecular Cloning, Characterization, and Expression of a cDNA Coding Copper/Zinc Superoxide Dismutase from Black Porgy

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A full-length complementary DNA (cDNA) clone encoding a putative copper/zinc superoxide dismutase (Cu/Zn-SOD) was amplified by a Polymerase Chain Reaction (PCR) based technique from cDNA synthesized from black porgy, *Acanthopagrus schlegeli*, mRNA. Nucleotide sequence analysis of this cDNA clone revealed that it comprised a complete open reading frame coding for 154 amino acid residues. The deduced amino acid sequence showed slightly higher identity (72.8−78.1%) with shark and swordfish Cu/Zn-SOD than with Cu/Zn-SOD from mammalian (68.1−70.7%) and plant (55.5−56.5%) sources. The residues required for coordinating copper and zinc are conserved as they are among all reported Cu/Zn-SOD sequences. The deduced amino acid sequence lacks mitochondria targeting sequence, which suggests that the black porgy cDNA clone encodes a cytosolic Cu/Zn-SOD. The coding region of Cu/Zn-SOD from black porgy was introduced into an expression vector, pET-20b(+), and transformed into *Escherichia coli* AD494(DE3)pLysS. A predominant achromatic zone was detected by activity staining of native PAGE. This indicates that the Cu/Zn-SOD cDNA clone can express active Cu/Zn-SOD enzyme in *E. coli*.

Keywords: *Acanthopagrus schlegeli; expression; Escherichia coli; PCR; pET-20b(+)*

INTRODUCTION

The role of superoxide dismutase (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 (Buhler and Williams, 1988). Antioxidant enzymes have been proposed as bioindicators for environmental impact assessment (Livingstone, 1991; Winston and Giulio, 1991) due to the fact that both metals and certain organic xenobiotics generate oxidative stress (Sies, 1986). 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 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 showed significant increases in SOD activity (Rodriguez-Ariza et al., 1991, 1992).

On the basis of such reasoning, the study of SODs and their application as biomarkers has become an important area in environmental impact assessment. SODs are metalloproteins and can be classified into three types, Cu/Zn-, Mn-, and Fe-SODs, depending on the metal found in the active site (Brock and Walker, 1980; Fridovich, 1986; Harris et al., 1980). 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. Previously, we had worked on the Cu/Zn-SOD from sweet potato by cloning cDNA (Lin et al., 1993) and genomic DNA (Lin et al., 1995a), expressing it in *Escherichia coli*, demonstrating subunit interaction (Lin et al., 1995b), and mutating Arg-141 to Ser (Lin et al., 1996) to enhance the enzyme activity and thermal stability. We also cloned an Mn-SOD cDNA from sweet potato callus tissues (Lin et al., 1997) and a Cu/Zn-SOD cDNA from papaya fruit (Lin et al., 1998), expressed them in *E. coli*, and demonstrated a dimer−monomer equilibrium and its equilibrium shift (Lin et al., 1999). From these experiences on the plant SODs, 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 reports on them have appeared, and there should be much room left for exploring the physiological roles related to pollution played by the SODs in the aquatic animals.

Liver cell-free extracts of fish (*Mugil* sp.) from polluted environments showed new Cu/Zn-SOD isozymes (Pedrajas et al., 1993) due to high levels of metals (Cu ions) and organic compounds (waste spills from chemical industries and from intensive agricultural area). The combined effect of Pb and Zn caused changes in the liver SOD−catalase detoxication system of carp (Dimitrova et al., 1994).

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Black porgy, *Acanthopagrus schlegeli*, a marine protandrous hermaphrodite, is widely distributed in many areas of Asia (Chang and Yueh, 1990). It has an annual reproductive cycle with a multiple spawning pattern occurring in late winter and spring. They are males for the first two years of life but then some reverse sex to females (Chang et al., 1994). It 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 (Calabrese et al., 1989), no fish Cu/Zn-SOD cDNA sequence has been reported. Recently, we have cloned full-length Cu/Zn-SOD cDNA clones from zebrafish and black porgy, *A. schlegeli*. Here we report the cDNA sequence and deduced amino acid sequence from a black porgy Cu/Zn-SOD cDNA clone. In addition, the coding region of Cu/Zn-SOD cDNA from black porgy was introduced into an expression vector, pET-20b(+), and transformed into *E. coli* AD494(DE3)pLysS. This Cu/Zn-SOD cDNA clone can express the Cu/Zn-SOD enzyme in *E. coli*.

MATERIALS AND METHODS

Fish Sample. A live two and half-year-old black porgy, *A. schlegeli*, weighing 263 g, provided by Dr. Ching-Fong Chang (Department of Aquaculture, National Taiwan Ocean University), was used. Freshly dissected tissues were frozen in liquid nitrogen and stored at −70 °C until use.

mRNA Preparation and cDNA Synthesis. Muscle (1.7 g) was put into liquid nitrogen and ground to powder in a ceramic mortar. The sample was dissolved in 15 mL of TRIzol reagent (GIBco BRL) and incubated for 5 min at room temperature; 3 mL of chloroform was then added. The mixture was shaken vigorously for 15 s, incubated at room temperature for 2−3 min, and 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 mixture was incubated at 4 °C for 10 min and then centrifuged at 12000*g* for 10 min at 4 °C. The total mRNA pellet was obtained. The poly(A)+ RNA was isolated according to oligo-(dT) affinity chromatography. Double-strand blunted cDNA was synthesized using a kit (cDNA synthesis module RNP 1256) from Amersham (Little Chalfont, Buckinghamshire, U.K.).

Subcloning and DNA Sequence Analysis. One microgram of blunted cDNA was ligated with 30 pmol of Marathon cDNA adaptor (Clontech, Palo Alto, CA) at 4 °C for 16 h. According to the amino acid sequence (DEDRHVGD, DDLGRGGN) of the swordfish (Calabrese et al., 1989) Cu/Zn-SOD, two degenerate primers were synthesized. Using 0.05 μg of the ligated cDNA as template, 10 pmol of each of the two degenerate primers was added. One 0.15 kbp cDNA was amplified by a Polymerase Chain Reaction (PCR) technique (25 cycles of 94 °C for 30 s, 46 °C for 1 min, 68 °C for 1.5 min). The 0.15 kbp cDNA was subcloned into pGEM-T cloning vector (Promega, Madison, WI) using JM109 as a host. The nucleotide sequence of the insert was determined in both directions according to the dideoxy technique using a Taq Track sequencing system kit (Promega). On the basis of this cDNA sequence, a BpCu-3 primer (5′ CCT CCT CTT CCC AGG TCA TC 3′) and a BpCu-4 primer (5′ GAC CTG GGC AAC GTG ACT GC 3′) were synthesized. To a 0.5 mL microtube containing 0.05 μg of the ligated cDNA as template was added 10 pmol of Clontech adaptor primer and 10 pmol of BpCu-3 primer. Ten picomoles of Clontech adaptor primer and 10 pmol of BpCu-4 primer were added to another 0.5 mL microtube containing 0.05 μg of ligated cDNA. One 0.3 kbp cDNA (5′-RACE: 5′-cDNA end) and one 0.4 kbp cDNA (3′-RACE: 3′-cDNA end) were amplified by the PCR technique (25 cycles of 94 °C for 30 s, 46 °C for 1 min, 72 °C for 2 min). Both 0.3 kbp and 0.4 kbp cDNA fragments were subcloned into pGEM-T using JM109 as a host. The nucleotide sequences of these inserts was determined in both directions according to the dideoxy technique using the Taq Track sequencing system kit. Sequence analysis revealed that 5′-RACE and 3′-RACE cover the full-length Cu/Zn-SOD cDNA (0.8 kbp). Using the 5′-RACE and 3′-RACE as template, one fused full-length cDNA of Cu/Zn-SOD was created by the PCR technique (EMBL accession no. is AJ00249). 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 species.

Recombinant DNA Preparation and Transformation. According to the 0.8 kbp cDNA sequence, a 5′ upstream primer (5′ CCC ATG GTG CTT AAA GCC GTG TG 3′) and a 3′ downstream primer (5′ GGA ATT CTG GGT GAT GCC AAT GAC TCC A 3′) were synthesized. Using 0.1 μg of black porgy blunted cDNA as 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 pGEM-T and then transformed into *E. coli* JM109 host. A positive clone was selected by hybridization with ^{32}P-labeled Cu/Zn-SOD cDNA (created by 5′-RACE and 3′-RACE) as probe, and plasmid DNA was prepared. Appropiate plasmid DNA was digested with *Nco*I and *Eco*RI and then electrophoresed on an 0.8% agarose gel. A 0.45 kbp insert DNA containing *Nco*I and *Eco*RI sites was recovered and ligated with pET-20b(+) (pretreated with *Nco*I and *Eco*RI) from Novagen (Madison, WI) and then transformed into AD494(DE3)pLysS as a host. A transformed clone was selected by hybridization with ^{32}P-labeled Cu/Zn-SOD cDNA as probe.

Culture and Enzyme Extraction. The transformed *E. coli* were grown at 37 °C in 3.0 mL of Luria Bertani medium containing 50 μg/mL ampicillin, 30 μg/mL kanamycin, and 34 μg/mL chloramphenicol until A_{600} reached 0.9. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to a concentration of 1 mM. The culture was incubated at 37 °C for 3 h at 120 rpm, and then the bacterial cells were harvested by centrifugation at 6000*g* for 5 min. The cells were suspended in 0.2 mL of 10 mM Tris buffer (pH 8.0) containing 0.1% glycerol and 0.01 g of glass beads and then vortexed for 5 min and centrifuged at 13000*g* for 5 min. The enzyme extract contained active Cu/Zn-SOD.

Enzyme Assay by Activity Staining on Native PAGE. Two 10 μg samples of the enzyme extract were electrophoresed on 10% 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 [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% tetramethylenediamine and 10 μg/mL riboflavin. During illumination, the gel became uniformly blue except at positions containing SOD, which showed an achromatic zone, the insolubility of the blue reduction product of NBT by superoxide anion.] as described previously (Beauchamp and Fridovich, 1971), and the other was stained with Coomassie Blue.

RESULTS AND DISCUSSION

Figure 1 shows the nucleotide and deduced amino acid sequence of one black porgy 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 (Lütcke et al., 1987).

Table 1 shows higher identity with the amino acid sequence of the Cu/Zn-SOD from two other aquatic species (swordfish and shark, 78.1−72.8%) than with the Cu/Zn-SOD sequences from other organisms including mammalian (mouse, 70.7%; human, 68.1%), *X. laevis* (66.0%), and sweet potato (56.5%).

Figure 2 shows that seven residues coordinating copper (His-47, -49, -64, and -121) and zinc (His-64, -72,

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                                         TCTAGCGGCCCCCGGGCAGGTAGCAAAGAAG
32  ATG GTG CTT AAA GCC GTG TGT GTG CTG AAA GGA GCC GGG GAG ACC ACC GGG
 1   M   V   L   K   A   V   C   V   L   K   G   A   G   E   T   T   G

93  GTC GTT CAT TTT GAG CAG GAG AGT GAG TCA GCA CCT GTG AAG CTC ACA GGA
18   V   V   H   F   E   Q   E   S   E   S   A   P   V   K   L   T   G

134 GAA ATC AAA GGA CTT ACT CCC GGT GAG CAC GGC TTC CAT GTC CAT GCA TTT
35   E   I   K   G   L   T   P   G   E   H   G   F   H   V   H   A   F

185 GGA GAC AAT ACA AAT GGG TGC ATC AGT GCA GGC CCT CAC TTA AAT CCC CAC
52   G   D   N   T   N   G   C   I   S   A   G   P   H   L   N   P   H

236 GGT AAG AAT CAT GGC GGT CCT ACT GAT GAA GAG AGG CAT GTT GGA GAC CTG
69   G   K   N   H   G   G   P   T   D   E   E   R   H   V   G   D   L

287 GGC AAC GTG ACT GCA GGA GCA GAT AAT GTT GCC AAG ATA GAC ATC ACG GAC
86   G   N   V   T   A   G   A   D   N   V   A   K   I   D   I   T   D

338 AAG ATG CTC ACT CTC ACT GGG CCG TTG TCC ATA ATT GGC AGA ACC ATG GTG
103  K   M   L   T   L   T   G   P   L   S   I   I   G   R   T   M   V

389 ATC CAC GAG AAG ACA GAC GAC CTG GGA AAA GGA GGC AAC GAG GAG AGT CTA
120  I   H   E   K   T   D   D   L   G   K   G   G   N   E   E   S   L

440 AAG ACG GGC AAC GCT GGT GGA CGT CTG GCC TGT GGA GTC ATT GGC ATC ACC
137  K   T   G   N   A   G   G   R   L   A   C   G   V   I   G   I   T

491 CAA TAAATCTGACATGGAGCACTGAAAATATCTTTTCCCCGGAGCACTTAAGACCAACCTAGCTA

154  Q   *

    CTTGATTTGTCAGTTTGTCCTTTTCACTACTCTGGCATTTTACTGACTAGTCGAGAGTAGATGAGC
    CATGTCTAACCTTGTCCGTTCCTCATGACAATTGTATGTATGGGTTTATGTCTGCCGTTTAGTTTT
    GGTCCCAAAGAATTGGTAACGCACAAGTAGAATAAACGGATGTATACAATTTGAAAAGCTAACCAA
    TAAATTGTACATTCAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 1. Nucleotide sequence of a black porgy Cu/Zn-SOD cDNA and the deduced amino acid sequence. Numbers to the left refer to nucleotide and its deduced amino acid residues. Consensus sequence of the translation start site is underlined. The asterisk denotes the stop signal.

Table 1. Comparison (Percent Identity, Percent Similarity) of Amino Acid Sequences for Cu/Zn-SOD of Black Porgy and Other Organisms

English name	EMBL no.	genus species	% identity	% similarity
black porgy	AJ00249	*Acanthopagrus schlegeli*		
swordfish[a]		*Xiphias gladius*	78.1	82.1
shark[a]		*Prionace glauca*	72.8	77.5
mouse	X06683	*Mus musculus*	70.8	76.6
human	X02317	*Homo sapiens*	68.2	72.7
X. laevis	X16585	*Xenopus laevis*	66.0	72.0
C. elegans	L20135	*Caenorhabditis elegans*	62.1	69.3
fruit fly	Y00367	*Drosophila melanogaster*	61.8	67.8
yeast	J03279	*Saccharomyces cerevisiae*	59.5	64.7
sweet potato	X73139	*Ipomoea batatas*	56.6	63.2
pea	M63003	*Pisum sativum*	56.6	61.8
tomato	X14041	*Solanum lycopersicum*	55.6	64.1

[a] Calabrese et al. (1989).

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 (Fridovich, 1986). There is an another cysteine between Val-6 and Val-8 not found in *C. elegans* 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.

As shown in Figure 3, an achromatic zone (lane 2, ←) denotes the activity of recombinant Cu/Zn-SOD from crude enzyme extract compared with control. This indicates that this recombinant Cu/Zn-SOD cDNA can express active Cu/Zn-SOD enzyme in *E. coli* AD494(DE3)pLysS.

CONCLUSION

A full-length cDNA encoding a putative Cu/Zn-SOD from black porgy was amplified by a PCR technique.

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                     1                                             41
Black porgy          M VLKAVCVLKG AGETTGVVHF EQESESAPVK LTGEIKGLTP
swordfish              ........R. ......T.Y. ...GNANA.G KGIIL.....
shark                   M........ T..V..T.L. ..-AADG..T .K.S.T....
mouse                . AM........ D.PVQ.TI.. ..KASGE..V .S.Q.T...E
human                . AT........ D.PVQ.IIN. ..KESNG... VW.S.....E
X.laevis                V......A. S.DVK...R. ..QGDDGD.T VE.K.E...D
C.elegan             . SNR..A..R. E.TV..TIWI T.K..NDQAV IE........
fruit fly            . .V.....IN. DAK--.T.F. ....SGT..K VS..VC..AK
yeast                . .Q-..A.... DAGVS...K. ..A...E.TT VSY..A.NS.
tomato               A TK...A.... NSNVE...TL S.DGDDG.TT VNVR.T..A.
sweet potato         . .-...A..SS SEGVS.TIF. S..GDG-.TT V..NVS..K.
pea                  . .-...A..SN SN.VS.TIN. S..GNGK.TT V..TLA..K.

               42     * *        o       *        *        *   *    91
Black porgy     GE-HGFHVHAF GDNTNGCISA GPHLNPHGKN HGGPTDEERH VGDLGNVTAG
swordfish       ..-......G. .......... ...F..AS.K .A..K..D.. .........D
shark           .K-........ .......... ...Y..FS.. ....D..... ........E.N
mouse           .Q-......QY ....Q..T.. ...F...S.K ....A..... ..........
human           .L-......E. ....A..T.. ...F..LSRK ....K..... .........D
X.laevis        .N-....I.VF .......L.. ...F..QN.. ..S.K.AD.. .........E
C.elegan        .L-......QY ..S....... ...F..F..T ....KS.I.. .......E..
fruit fly       .L-......E. .......M.S ...F..Y..E ..A.V..N.. L.....IE.T
yeast           NAER...I.E. ..A....V.. ...F..FK.T ..A....V.. ...M...KTD
tomato          .L-....L.EY ..T....M.T .A.F..NKLT ..A.G..I.. A.....IV.N
sweet potato    .L-.......L ..T....M.T ...F..A..E ..A.G.DN.. A.....I.V.
pea             .L-....I..L ..T......T ...F..N..E ..A.E..T.. A.....INV.

               92                               *                  138
Black porgy     ADNVAKIDIT DKMLTLTGPL SIIGRTMVIH EKTDDLGKGG N   EESLKT
swordfish       .NG....... ..-IS.T..Y .......... ..A....R.. .---......
shark           GNG..EFE.K .RQ.H...ER ......L.V. ..E....... D---....R.
mouse           K.G..NVS.E .RVIS...EH .........V. ..Q....... .---...T..
human           K.G..DVS.E .SVIS...DH C.....L.V. ..A....... .---...T..
X.laevis        G-G..QFKF. .PQIS.K.ER ......A.V. ..Q....... D---D.....
C.elegan        ..G....KL. .TLV..Y..N TVV..S..V. AGQ....E.V GDKA...K..
fruit fly       G.CPT.VN.. .SKI..F.AD ......V.V. ADA....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
Black porgy     GNAGGRLACG VIGITQ
swordfish       ....S..... ...TE
shark           ....S..... ....AKD
mouse           ....S..... ....A.
human           ....S..... ....A.
X.laevis        .......... ...FCP
C.elegan        ....A.A... ..ALAAPQ
fruit fly       ....A.IG.. ....AKV
yeast           ....P.P... ...L.N
tomato          .......... .V.L.PI
sweet potato    ......V... I..LQG
pea             ......V... I..LQG
```

Figure 2. Optimal alignment of Cu/Zn-SOD among several species. Black porgy: this study (EMBL AJ00249); shark and swordfish (Calabrese et al., 1989); mouse (X06683); human (X02317); *X. laevis* (X16585); *C. elegans* (L20135); fruit fly (Y00367); yeast (J03279); tomato (X14041); sweet potato (X73139); pea (M63003). Numbers refer to amino acid residues of black porgy. A dot indicates identity with black porgy; a dash denotes deletion. Residues coordinating copper and zinc are indicated with asterisks. The two cysteines that form a disulfide bridge are circled.

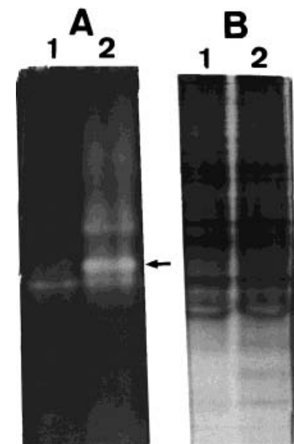

Figure 3. Activity staining and Coomassie Blue staining of the crude extract of *E. coli* harboring recombinant black porgy Cu/Zn-SOD cDNA: (A) staining for activity; (B) staining for protein by Coomassie Blue; (lanes 1) AD494(DE3)pLysS carrying pET-20b(+) as control; (lanes 2) AD494(DE3)pLysS carrying recombinant Cu/Zn-SOD cDNA. An arrow denotes Cu/Zn-SOD activity (panel A).

This clone comprises a complete open reading frame coding for 154 amino acid residues. The coding region was introduced into an expression vector, pET-20b(+),

and transformed into *E. coli* AD494(DE3)pLysS. The expression of the recombinant Cu/Zn-SOD cDNA clone was confirmed by enzyme activity staining on native acrylamide gel.

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