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

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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 especially important 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 antioxidant 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 Mn ions and organic compounds (waste spills from chemical industries and from intensive agricultural area). The high levels of metals and certain organic xenobiotics (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 and then to assess environmental pollution. So far, only the role of SODs 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 especially important 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 antioxidant 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 were shown to have significantly increased 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, and demonstrated 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 detoxification system of carp (Dimitrova et al., 1994).
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 12000g 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 12000g 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 blunt 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 blunt 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 (DEDRHVG DDLRGRGN) 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 subcloned into pGEM-T cloning vector (Promega, Madison, WI) using M109 as a host. The nucleotide sequence of the insert 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 AJ 00249). 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 CTG AAA GCC GTG TG 3′) and a 3′ downstream primer (5′ GAA ATT C TG GTG GAT GCC AAT GAC TCC A 3′) were synthesized. Using 0.1 μg of black porgy blunt 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 J M109 host. A positive clone was selected by hybridization with 32P-labeled Cu/Zn-SOD cDNA (created by 5′-RACE and 3′-RACE) as probe, and plasmid DNA was prepared. Appropriate plasmid DNA was digested with NcoI and EcoRI and then electrophoresed on an 0.8% agarose gel. A 0.45 kbp insert DNA containing NcoI and EcoRI sites was recovered and ligated with pET-20b(+) (pretreated with NcoI and EcoRI) from Novagen (Madison, WI) and then transformed into AD494(DE3)pLysS as a host. A transformed clone was selected by hybridization with 32P-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 A600 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 6000g 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 13000g 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 (AAACAATGG) 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,
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 another cysteine between Val-6 and Val-8 not found in Caenorhabditis 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.

**CONCLUSION**

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

LITERATURE CITED


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