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Purification and Characterization of Black Porgy Muscle Cu/Zn Superoxide Dismutase

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Chi-Tsai Lin, Tung-Liang Lee, Kow-Jen Duan and Jong-Ching Su (2001) Purification and characterization of black porgy muscle Cu/Zn superoxide dismutase. *Zoological Studies* **40**(2): 84-90. Superoxide dismutase (SOD) has been proposed to be used as a bioindicator for environmental impact assessment. From a survey of SOD activity in black porgy, *Acanthopagrus schlegeli*, we found that Cu/Zn SOD was distributed rather evenly in 6 tissues, and in addition, only heart, liver, and testis had Mn SOD. We purified Cu/Zn SOD from muscle to homogeneity by a procedure that includes heating at 65 °C and fractionation on 2 chromatographic columns. The molecular mass of the native enzyme was 33 kDa and that of the subunit mass, deduced from a cDNA sequence, was 15.85 kDa. Thus the native enzyme appeared to be a homodimer. It had an N-terminal sequence of VLKAVCVLKGAGQTTGVV. The specific activity was 3318 u/mg. The enzyme had a broad optimum pH range of 5.8 to 11.2 and was resistant both to proteolysis by trypsin and chymotrypsin and to heat denaturation. The thermal inactivation rate constant of the enzyme at 80 °C was -0.0237 min^{-1} and the half life for inactivation was 27.8 min.

Key words: Black porgy, *Acanthopagrus schlegeli*, Cu/Zn superoxide dismutase, Thermal stability.

Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide ion (O_2^-) to hydrogen peroxide and oxygen molecule 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). Recently, 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, Spain, where the Tinto River brings Fe and Cu from pyrite mines, and organic xenobiotics such as industrial pollutants and pesticides are released. So, molluscs and fish caught in that zone show significant increases in SOD activity (Rodriguez-Ariza et al. 1991, Rodriguez-Ariza et al. 1992).

Based on such reasoning, the study of SODs and their application as biomarkers have become important areas in environmental impact assessment. SODs are metalloproteins and can be classified into 3 types, Cu/Zn, Mn, and Fe SODs, depending on the metal found in the active site (Brock and Walker 1980, Harris et al. 1980, Fridovich 1986). Cu/Zn SOD is predominantly associated with the cytosolic fraction of eukaryotes 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. Previously, we cloned and se-

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quenced the cDNA of Cu/Zn SOD from sweet potato (Lin et al. 1993) and analyzed its gene structure (Lin et al. 1995a). Subunit interaction enhanced enzyme activity, and its thermal stability has been established (Lin et al. 1995b). Furthermore, Arg-141 to Ser substitution in sweet potato by site-directed mutagenesis showed unusual thermal stability (Lin et al. 1996). We also cloned Mn SOD cDNA from sweet potato callus tissues (Lin et al. 1997) and Cu/Zn SOD cDNA from papaya fruit (Lin et al. 1998), and expressed the latter in *Escherichia coli*, and demonstrated dimer-monomer equilibrium and its equilibrium shift (Lin et al. 1999). From this experience with plant SODs, we noted that it would be of interest from a comparative biochemical standpoint to study SODs of fish and then use them to assess environmental pollution. So far, only a few reports on these SODs have appeared, and there should be much room left for exploring the physiological roles related to pollution played by SODs in aquatic animals.

Liver cell-free extracts of fish (*Mugil* sp.) from polluted environments showed new Cu/Zn SOD isozymes (Pedrajas et al. 1993) because of high levels of metals (Cu ions) and organic compounds (waste spills from chemical industries and from an intensively cultivated 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. Fish of this species are males for the first 2 y of life, but then some reverse sex to become females (Chang et al. 1994). It is a high-quality and economically valuable species in Taiwan. Here we report on the purification and characterization of Cu/Zn SOD from its muscle, with the enzyme source selected from the results of a preliminary tissue distribution survey. Based on these studies, it will be easy to monitor water quality by checking changes of fish SODs due to environmental pollution.

MATERIALS AND METHODS

Fish sample

A live 2.5-y-old black porgy, *Acanthopagrus schlegeli*, weighing 263 g, provided by Dr. Ching-Fong Chang (Dept. of Aquaculture, National Taiwan Ocean Univ.) was used. Freshly dissected tissues were frozen in liquid nitrogen and stored at −70 °C until use.

Enzyme assay in solution

SOD activity was measured using a RANSOD kit (RANDOX, Ardmore, UK). 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 initial 3-min time interval, measured as the absorbance at 505 nm, that 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 native PAGE

The enzyme separated on 10% native PAGE was assayed by the SOD activity staining method as described by Beauchamp and Fridovich (1971). Proteins were stained with coomassie brilliant blue. The area and intensity of activity and protein bands were measured by a computing densitometer (Molecular Dynamics, Sunnyvale, CA). KCN at 8 mM was added to the riboflavin solution for activity staining to distinguish between Cu/Zn and Mn SOD activities.

Protein concentration measurement

Protein concentration was measured by A_{280} or by a dye-binding method (Bio-Rad Protein Assay Kit, Hercules, CA) using crystalline bovine serum albumin as the standard.

SDS-PAGE

The purified enzyme was boiled in dye-buffer containing 2-mercaptoethanol and SDS for 5 min and was electrophoresed on 10% SDS-PAGE. Proteins were stained with coomassie brilliant blue.

Amino terminal sequencing

The amino terminal sequence of the purified enzyme was determined by the procedure of Matsudaira (1987). The active fraction from Sephacryl S-100 was subjected to 15% SDS-PAGE. The protein band in the gel was transferred to PVDF film and stained with coomassie brilliant blue R250.

The desired protein band was analyzed in a model 477A protein sequencer from Applied Biosystems (Foscity, CA).

Enzyme stability

The homogeneous enzyme sample recovered from a Sephacryl S-100 column was concentrated before use. An aliquot of 10 μl of enzyme solution containing 0.3 μg protein, after being treated as described in the following tests (1~5), was electrophoresed in 10% native PAGE and stained for activity.

(1) Thermal stability. The enzyme in the assay buffer was heated to 80 °C for 1, 2, 4, or 8 min.

(2) pH stability. Enzyme in 4 μl each of 3 buffer solutions, 0.2 M citrate, at pH 2.2, 3.0, or 5.4; 0.2 M Tris-maleate, at pH 6.6, 7.8, or 9.0; and 0.2 M 2-[N-cyclohexylamino]ethanesulfonic acid (CHES), at pH 9.0, 10.4, or 11.2, was incubated at 37°C for 1 h.

(3) SDS effect. The enzyme solution was supplemented with SDS to 1%, 2%, or 4% and incubated at 37 °C for 1 h.

(4) Imidazole effect. The enzyme solution was supplemented with imidazole at 0.1, 0.3, 0.5, or 1 M, and incubated at 37 °C for 1 h.

(5) Proteolytic susceptibility. The enzyme was incubated with 1/20 w/w of trypsin or chymotrypsin at pH 8.8 and 37 °C for up to 1, 2, or 3 h. For chymotrypsin digestion, $CaCl_2$ was added to 20 mM. Aliquots were removed from time to time and analyzed by native PAGE.

Preparation of crude enzyme extract for activity distribution

Two-tenths gram each of heart, liver, spleen, and testis was homogenized in 0.4 ml of 10 mM Tris-HCl, pH 6.8, containing 1% glycerol (buffer A) at 4 °C for 15 min. One-half gram of gill and muscle was ground to powder in liquid nitrogen, and 0.2 g each of the powders was stirred with 0.4 ml of buffer A at 4 °C for 15 min. All samples were centrifuged at 9000 ×g for 15 min. Ten microliters of the supernatant was diluted 1000 fold, and 200 μl of this was taken for protein measurement. Twenty-five-microliter samples from 10- to 25-fold diluted supernatant were used for determining SOD activity. Two-tenths milligram of proteins in the supernatant was pipetted out for 10% native PAGE.

Muscle enzyme purification

A muscle sample weighing 24 g was ground with liquid nitrogen to a powder in a ceramic mortar. The powder was extracted twice with a 24-ml aliquot of buffer A, and after being centrifuged, the supernant was pooled for further purification. All steps were carried out at 4 °C unless otherwise indicated.

(1) Heat treatment. Crude extract (50 ml) was heated to 65 °C for 10 min and centrifuged at 9000 ×g for 15 min. The supernatant (45 ml) was saved.

(2) DE-52 chromatography. The supernatant from the heat treatment was loaded onto a DE-52 (Whatman, Kent, UK) column (2.6 cm × 20 cm) which was equilibrated with buffer A. The column was washed with 50 ml of buffer A and eluted with

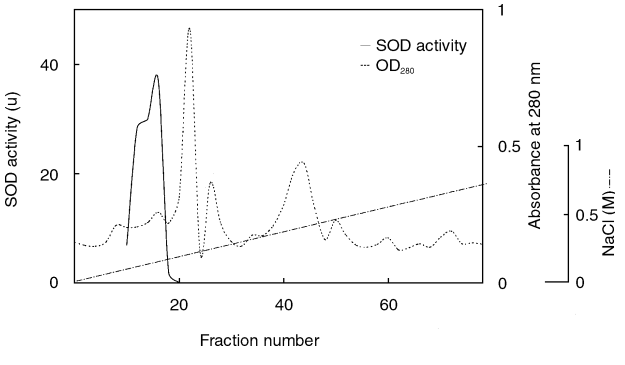

Fig. 1. Elution profile of Cu/Zn SOD from a DE-52 column. After heat treatment, the sample was applied to a DE-52 column (2.6 cm × 20 cm) equilibrated with 10 mM Tris-HCl and 1% glycerol, pH 6.8, and eluted with a linear gradient of 0 to 0.7 N NaCl in the same buffer (total volume, 400 ml). The flow rate was 1.0 ml/min and 4.3 ml/fraction.

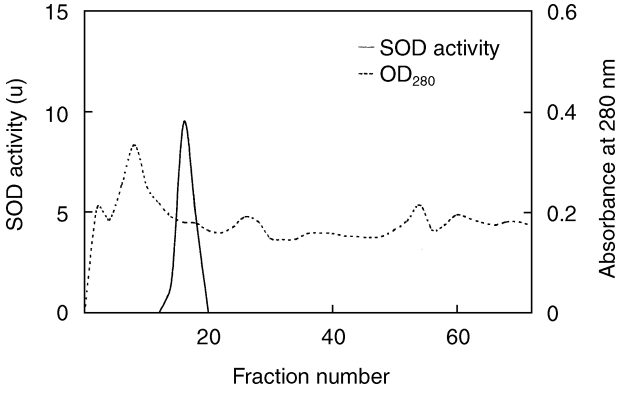

Fig. 2. Chromatographic profile on a Sephacryl S-100 column (2.6 cm × 90 cm). A sample containing 18 mg protein eluted from the DE-52 column was used, with elution buffer, 10 mM Tris-HCl, 1% glycerol, and 0.2 N NaCl, pH 6.8. The elution rate was 0.5 ml/min and 4.5 ml /fraction.

Table 1. Purification of black porgy Cu/Zn SOD from 24 g of muscle tissue

Fraction	Volume (ml)	Total activity (u)	Total protein (mg)	Specific activity u/mg	Multiples of purification	Yield (%)
Crude extract	50	531	562	0.94	1	100
65 °C	45	635	165	3.85	4	120
DE-52	38	801	24.4	32.8	35	151
Sephacryl S-100	60	365	0.11	3,318	3,529	69

a linear gradient of 0 to 0.7 M NaCl in buffer A at a flow rate of 1.0 ml/min. Fractions of 4.3 ml were collected.

(3) Sephacryl S-100 chromatography. The active fractions (38 ml) from DE-52 were pooled and dialyzed against buffer A. The sample was concentrated to 5.3 ml in a Centricon 10 (Amicon, Beverly, MA) with a cutoff range of 10 kDa. A 2.6-ml aliquot of the concentrate was put on a Sephacryl S-100 column (2.6 cm × 90 cm) and eluted with buffer A containing 0.2 M sodium chloride at a flow rate of 0.5 ml/min. Fractions of 4.5 ml were collected. The active fractions were pooled (60 ml) and concentrated in a Centricon 10.

(4) Superdex 75 chromatography. Six-tenths milliliter of each active fraction from Sephacryl S-100 was concentrated to 0.15 ml in a Centricon 10. Each of the concentrates was loaded onto a Superdex 75 column (1.0 cm × 30 cm) in an FPLC (Pharmacia) system. The column was eluted with PBS buffer (1 mM phosphate, 2.7 mM KCl, 137 mM NaCl) at a flow rate of 0.5 ml/min. Fractions of 0.2 ml were collected.

RESULTS

SOD activity distribution

From the results of the PAGE and solution assays, we found that SOD activity (u/mg fresh tissue) is evenly distributed in different tissues (gill 0.66, heart 0.47, liver 0.93, spleen 0.47, testis 0.82, and muscle 0.6), and only 2 types, Mn and Cu/Zn SODs, were present. Cu/Zn SOD was present in all tissues, but only the heart, liver, and testis contained Mn SOD (data not shown). From these data, we decided to use muscle as the source of Cu/Zn SOD.

Purification of muscle Cu/Zn SOD

The chromatographic elution profiles are shown in figures 1 and 2, and the purification is summarized in table 1. Apparently, obtaining a pure enzyme of very high specific activity (3318 u/mg) at a high yield (69%) was possible due to the unusually high

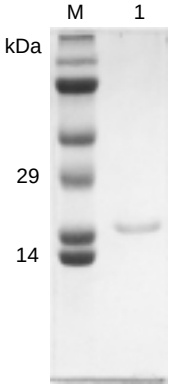

Fig. 3. Ten percent SDS-PAGE analysis of purified black porgy Cu/Zn SOD. A sample of 0.7 μg of purified enzyme was boiled for 5 min in the presence of loading dye containing 2% SDS. M, molecular mass markers; lane 1, pure enzyme from Sephacryl S-100.

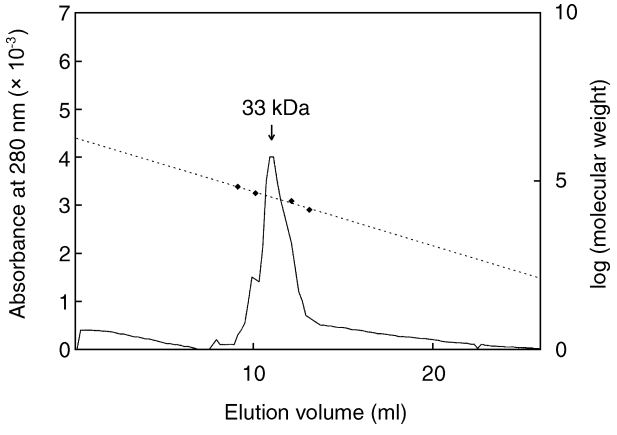

Fig. 4. Estimation of molecular mass by gel filtration. A 0.15-ml sample (1.2 mg/ml) was injected into a Superdex 75 (HR 10/30) column (Pharmacia) and eluted with PBS at 0.5 ml/min. The column was calibrated with albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease A (13.7 kDa).

thermal stability of the enzyme.

The enzyme (0.7 μg) from Sephacryl S-100 subjected to 10% SDS-PAGE (Fig. 3) showed a single protein band whose molecular mass was estimated to be 17 kDa. This indicates that the enzyme was purified to homogeneity. Thus subsequent experiments were carried out by using the active column (Sephacryl S-100) eluate which was concentrated in a Centricon 10 before use.

The molecular mass of the enzyme was estimated to be 33 kDa by molecular sieving through a Superdex 75 column (Fig. 4). Recently, we have cloned and sequenced a Cu/Zn SOD cDNA from black porgy (EMBL accession no. AJ00249). According to the deduced amino acid sequence, the molecular mass of the polypeptide was calculated to be 15.85 kDa. Together with the result of figure 3, we thus suggest that the enzyme has a homodimeric structure.

The amino terminal sequence of the purified enzyme is VLKAVCVLKGAGQTTGVV. This sequence is closely homologous with that of shark Cu/Zn SOD, which is VMKAVCVLKGTGEVTGTV (Calabrese et al. 1989), and fully matches that deduced from the cDNA sequence. These data further indicate that the purified enzyme is Cu/Zn SOD.

Characterization of the enzyme

The enzyme has a broad optimal pH range of 5.8 to 11.2 as shown in figure 5. The enzyme retained 24.2% and 11.3% of maximal activity (pH 9.0, lane 8) at pH 3.0 (lane 2) and pH 2.2 (lane 1), respectively. The decrease of enzyme activity at acidic pH values is probably due to dissociation of the more active dimer into the less active monomer as we also observed in the sweet potato enzyme (Lin et al. 1995b).

The enzyme inactivation kinetics at 80 °C were fitted to the 1st -order rate equation, ln(Et/Eo) = *k*d • t, where Eo and Et represent the original activity and residual activity that remains after being heated for time t, respectively (Fig. 6). The thermal inactivation rate constant, *k*d, was –0.0237 min^{-1}, and the half life for inactivation was 27.8 min. These results indicate that the enzyme is much more heat stable than the reportedly heat-stable plaice skin SOD (Nakano et al. 1995).

DISCUSSION

The enzyme activity showed no decrease in either 4% SDS or 1 M imidazole (data not shown), possibly due to a strong hydrophobic interaction at the subunit interface. The native enzyme was completely resistant to digestion by trypsin and chymotrypsin even at a high enzyme/substrate (w/w) ratio of 1/20 (data not shown).

Our study has shown that black porgy Cu/Zn SOD is unique in that it is stable in a broad pH range and at an elevated temperature of as high as 80 °C, and resistant to detergent (SDS) and proteolytic enzyme treatments; furthermore a homogeneous preparation can be obtained with only 3 steps of purification. These properties will be useful in comparison with SODs of fish raised in polluted environments. Furthermore, it might have potential

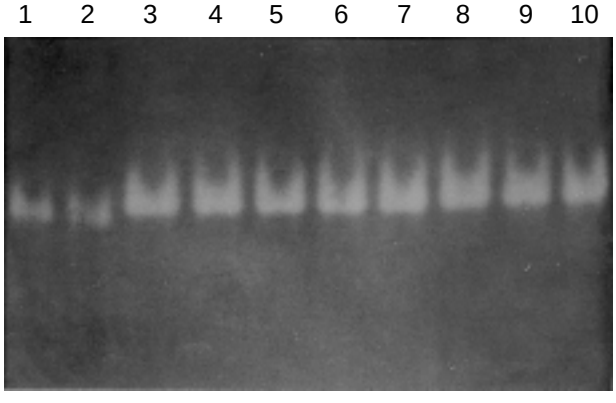

Fig. 5. Effect of pH on enzyme stability. Samples (0.3 μg each) were incubated in buffer solutions of different pH values at 37 °C for 1 h, and run on 10% native PAGE. Lanes 1-3, in citrate at pH 2.2, 3.0, or 5.8; lanes 4-7, in TM at pH 5.4, 6.6, 7.8, or 9.0; lanes 8-10, in CHES at pH 9.0, 10.4, or 11.2, respectively.

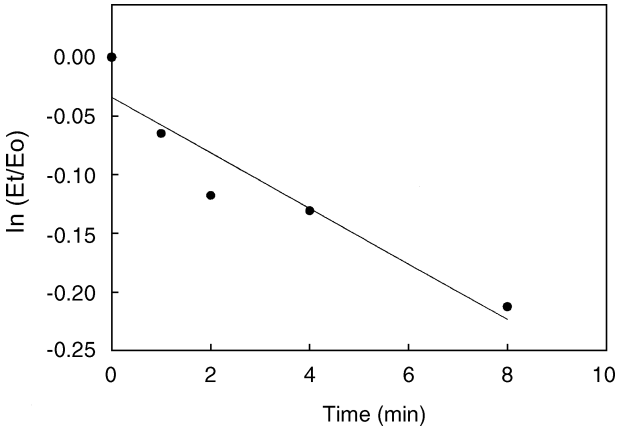

Fig. 6. Plot of thermal inactivation kinetics. The PAGE data were quantitated with a densitometer for calculation. Eo and Et are the original activity and residual activity after being heated for time t, respectively. The areas of activity were 651 (Eo), 610 (E_1), 578 (E_2), 571(E_4), and 526 (E_8).

applications in medicine, such as the protection of skin against inflammatory reactions and for periodontal use (Wilder and Mass 1987, Nakano 1989).

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黑鯛銅鋅型超氧歧化酶之純化及其性質之研究

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生物體內超氧歧化酶可當作對環境污染程度之生物指標，顯見其重要性。以黑鯛為材料，經測定其銅鋅型超氧歧化酶存在六種組織中，但心臟、肝臟及雄性生殖器同時亦含猛型超氧歧化酶。以其肌肉為材料，其粗抽取液經加熱及兩種管柱層析，可得到均質之純酵素。測得原態分子為 33 kDa，次單元體為 15.85 kDa，N端之胺酸為 VLKAVCVLKGAGQTTGVV，比活性每毫克含 3318 單位，此酶對 pH、 蛋白質水解酶及 80 °C 加熱處理均甚穩定。

關鍵詞：黑鯛，銅鋅型超氧歧化酶，熱穩定性。

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