Purification, molecular cloning, and some properties of a manganese-containing superoxide dismutase from Japanese flounder (*Paralichthys olivaceus*)

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Abstract

Manganese-superoxide dismutase (Mn-SOD) from Japanese flounder (*Paralichthys olivaceus*) hepatopancreas has been purified with high purification (781-fold) and recovery (10.8%). The molecular mass of the purified enzyme was estimated to be 26 kDa by SDS-PAGE under reducing conditions. In activity staining by native-PAGE, the Japanese flounder Mn-SOD gave three active bands and exhibited KCN-insensitive activity. In addition, the electrophoretic mobility of this enzyme was observed to be faster than that of Japanese flounder Cu,Zn-SOD. On the other hand, the N-terminal amino acid sequence of this Mn-SOD was determined to be 16 amino acid residues, and the sequence showed high homology to other Mn-SODs but not Japanese flounder Cu,Zn-SOD. Analysis of nucleotide and deduced amino acid sequences revealed that the Mn-SOD cDNA consisted of a 64 bp 5'-non-coding region, a 675 bp open reading frame encoding 225 amino acids, and a 465 bp 3'-non-coding region. The first 27 amino acids containing a mitochondria-targeting signal were highly conserved among other Mn-SODs.

Keywords: Manganese-superoxide dismutase; Japanese flounder; Hepatopancreas; Purification; Amino acid sequence; cDNA sequence; Mitochondria-targeting signal

1. Introduction

Generally, reactive oxygen species (ROS) are produced in organisms as the natural products of oxidative metabolism (Fridovich, 1978). ROS, such as superoxide anion and hydrogen peroxide, can be toxic to cells and tissues to cause oxidative stress, including oxidation of proteins, DNA/RNA breaks, lipid peroxidation and cell death (Matés and Sánchez-Jiménez, 1999; Ikebuchi et al., 2006). However, living cells in organisms have the antioxidative defense system against ROS and to maintain redox homeostasis. The antioxidative defense system includes enzymatic and non-enzymatic components. Among the enzymatic system, superoxide dismutase (SOD; EC 1.15.1.1) is a family of important antioxidant metalloenzymes. It catalyzes the dismutation of toxic superoxide anion into dioxygen and hydrogen peroxide (McCord and Fridovich, 1969; Whittaker, 2000), which is subsequently reduced to water by glutathione peroxidase in the cytosol, or by catalase in the peroxisomes (Beyer et al., 1991; Whittaker, 2000; Hermes-Lima and Zenteno-Savın, 2002). Increasing evidence has shown that SOD is related to numerous physiological and pathological situations, such as aging, tumors, autoimmunity diseases and radioprotection (Oberley and Buettner, 1979; Warner, 1994; Lih-Brody et al., 1996; Zhang et al., 2008), so it has fine foreground of development and application.

It is well known that there are three major types of SODs characterized by their redox-active metals such as copper and zinc (Cu,Zn-SOD), manganese (Mn-SOD), and iron (Fe-SOD) at the catalytic sites (Fridovich, 1986). Cu,Zn-SOD is found predominantly in the cytosolic fraction of eukaryotes, and shows very sensitive

characteristics to cyanide and hydrogen peroxide (Weisiger and Fridovich, 1973). Mn-SOD is mostly present in the mitochondrial matrix of eukaryotes, and is also detectable in prokaryotes. It is inhibited by chloroform-alcohol but not by either cyanide or hydrogen peroxide (Kawaguchi et al., 1989). Fe-SOD is found in prokaryotes as well as in a few plants, and can be inhibited by hydrogen peroxide but not cyanide (Asada et al., 1980). Moreover, a kind of extracellular SOD (EC-SOD), distinct from cytosolic Cu,Zn-SOD, has also been found in eukaryotes (Marklund, 1982).

In our previous studies, Cu,Zn-SOD has been purified from Japanese flounder (*Paralichthys olivaceus*), and the purified enzyme has been analyzed with the N-terminal amino acid sequence and compared with other Cu,Zn-SODs (Osatomi et al., 2001). In contrast with Cu,Zn-SOD, little information on purification and biochemical characterization of Mn-SOD from aquatic animals, especially Japanese flounder, was known.

There was a report that in *Hemibarbus mylodon*, Mn-SOD expression in the liver and kidney were remarkably up-regulated by LPS injection, *Edwardsiella tarda* challenge, and heavy metal exposure (Cho et al., 2009). Moreover, Wang and colleagues (2010) recently found that the gene expression of Mn-SOD was significantly increased after immunostimulation in red swamp crayfish. Several reports also have evaluated differential SOD modulation in fish organs during exposure to xenobiotic compounds (Shi et al., 2005; Vega-López et al., 2007; An et al., 2008; Cho et al., 2009). Given that at least 90% of generated ROS originated from mitochondria in most vertebrate species (Boveris and Chance, 1973; Wilhelm Filho, 2007), we believe that Mn-SOD, a mitochondria protein, plays an important role in the host`s defense against oxidative stress in various species, including marine fishes. Therefore, in this study, we

attempt to purify Mn-SOD from Japanese flounder (*P. olivaceus*) hepatopancreas and investigate its biochemical characteristics including amino acid sequence and cDNA structure.

2. Materials and methods

2.1. Materials

Cultured Japanese flounders (*P. olivaceus*) with mean body weight of approximately 800 g were purchased from a local commercial supplier (Nagasaki, Japan). Fifteen fishes were decapitated, and their hepatopancreases were collected and immediately stored at −35°C until use.

2.2. Chemicals

Q-Sepharose Fast Flow and Sephacryl S-200 were purchased from Pharmacia LKB Biotechnology (Uppsala, Sweden). Nitroblue tetrazolium and riboflavin were products of Sigma Chemical Company (St. Louis, MO, USA). Xanthine oxidase was obtained from Boehringer Mannheim (Mannheim, Germany). Sulfanilic acid and *N*-1-Naphthylethylene-diamine dihydrochloride were purchased from Wako Pure Chemicals Inc. (Osaka, Japan). Xanthine monosodium salt and hydroxylamine-*O*-sulfolic acid were from Nakarai Chemicals Ltd. (Kyoto, Japan). All other chemicals used were reagent grade.

2.3. SOD activity assays

SOD activity was measured by two types of assay methods. The spectrophotometric method was based on the inhibition of xanthine/xanthine oxidase-induced production of

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azo pigment in 5 mM phosphate buffer (pH 7.8) at 37°C through the nitrite method (Oyanagui, 1984). One unit of SOD activity was defined as the amount of protein required to inhibit the rate of production of azo pigment by 50% under the assay conditions as described above. On the other hand, visualization of the SOD activity on native-PAGE gels was performed by the nitroblue tetrazolium (NBT) illumination method (Beauchamp and Fridovich, 1971). The gel was soaked in an NBT solution. After 30 min, the NBT solution was poured off and replaced by a riboflavin solution for 20 min. Subsequently, the gel was illuminated until it became uniformly blue. Staining for SOD activity was also carried out in the presence or absence of 5 mM KCN.

2.4. Purification of Japanese flounder Mn-SOD

All purification procedures were carried out at 0‑4°C according to the modified method of Crapo et al. (1978), and the SOD activity at each step was measured by the nitrite method. Pooled Japanese flounder hepatopancreases (150 g) were homogenized with a Polytron homogenizer under cooling in equal volumes of 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA and 0.1 mM PMSF. The homogenate was centrifuged at 13,200 × g for 30 min. The resultant precipitate was resuspended in two volumes of the same buffer and then centrifuged again. The two supernatants were combined and treated under stirring at 65°C for 5 min. The crude extract were prechilled and removed by centrifugation at 13,200 × g for 20 min, and the resulting supernatant was fractionated with ammonium sulfate from 60% to 80% saturation. The precipitate was collected by centrifugation and dissolved in a minimum volume of 5 mM potassium phosphate buffer (pH 7.8) containing 0.01 mM EDTA, and the solution

was dialyzed over 24 h against the same buffer. The dialysate was applied to a column of Q-Sepharose Fast Flow (1.4 × 26 cm) equilibrated with 5 mM potassium phosphate buffer (pH 7.8) containing 0.01 mM EDTA and was eluted with a convex gradient of NaCl (0‑0.5 M) in the same buffer. The active fractions were pooled and dialyzed against 50 mM potassium phosphate buffer (pH 7.8) containing 0.1 mM EDTA and 0.1 M NaCl buffer. The enzyme solution was loaded onto a Sephacryl S-200 column (1.4 × 100 cm) equilibrated with the same buffer, followed by a Bio-Sil SEC-125 column (7.8 × 300 mm) equilibrated with Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl. The active fractions were pooled and stored at -35°C.

2.5. *Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis of SOD*

SDS-PAGE analysis of SOD was performed according to the method reported by Laemmli (1970) with slight modifications. The SOD samples were mixed with equal volume of SDS-PAGE sample buffer (final 62.5 mM Tris-HCl; pH 6.8, 10% glycerol and 2% SDS). After heating at 100°C for 5 min, proteins from each sample were subjected to SDS-PAGE (12.5% gel). After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250.

Western blot analysis for Mn-SOD was performed as reported previously (Cao et al., 2000). In brief, the SOD samples were separated by SDS-PAGE (12.5%), and electrotransferred to a nitrocellulose membrane. Non-specific protein sites were blocked with 3% (w/v) gelatin in Tris-HCl buffered saline (TBS: 20 mM Tris-HCl; pH 7.5, containing 0.5 M NaCl) at room temperature for 1 h. Subsequently, blotted proteins

were incubated with rabbit anti-human Mn-SOD antibody (StressGen Biotechnologies, Canada) at 4°C overnight. The membranes were washed twice in TBS containing 0.1% Tween-20 (TBST), followed by incubation with HRP-conjugated goat anti-rabbit IgG antibody (Wako, Osaka, Japan) at room temperature for 1 h. After washing for three times by TBST, immunodetection was carried out using a Konica Immunostaining HRP-1,000 substrate (Konica Inc., Tokyo, Japan) for horseradish peroxidase.

2.6. Determination of the N-terminal amino acid sequence

The purified Mn-SOD was subjected to electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, MA, USA). After staining with Coomassie brilliant blue R-250, the 26 kDa band was cut out and submitted to amino acid sequence analysis using Edman degradation with a Procise Model 492 protein sequencer (Applied Biosystems Division of Perkin-Elmer, CA, USA).

2.7. RNA isolation, cDNA synthesis and RACE

Total RNA was extracted from fresh Japanese flounder hepatopancreas (120 mg) with an ISOGEN Kit (Nippon Gene, Toyama, Japan). The RNA was first denatured and then reverse-transcribed with SuperScript RT (Gibco BRL, Paisley, UK) using the oligo (dT)-adaptor primer (5'-GGCCACGCGTCGACTAGTAC(T)^{17}-3') for first-strand cDNA synthesis.

Two degenerate primers were designed based on the N-terminal amino acid sequence from the purified Japanese flounder hepatopancreas Mn-SOD. The degenerate

oligonucleotide primer sequences were: 5'-A(G/A)(C/T)T(A/G/C/T)CA(C/T)CA(C/T)(A/T)(C/G)(A/G/C/T)AA(G/A)CA(C/T)CA-3' for the sense primer and 5'-C(A/G)TT(C/T)TT(A/G)TA(C/T)TG(A/G/C/T)A(A/G)(A/G)TA(A/G)TA-3' for the antisense primer. PCR was performed with 1 cycle of 10 min at 95°C, 45 cycles of 1 min at 94°C, 1 min at 45°C, 2 min at 72°C, and 1 cycle of 7 min at 72°C. The PCR products were purified from an agarose gel and cloned into a pGEM-T Easy vector (Promega, Madison, WI, USA). Plasmids were prepared for double-stranded DNA sequencing using an alkali-SDS method.

The first-stranded cDNA for 5'-RACE was synthesized using a specific primer (5'-GGCCACGCGTCGACTAGTAC-3') and added poly(A) into the 3'-terminus by terminal deoxynucleotidyl transferase (Gibco BRL). The PCR reaction used both the sense (5'-GAGGTTTGTCCAGAAGATGGTGTG-3') and the antisense primers (5'-GGCCACGCGTCGACTAGTAC(T)^{16}-3'). PCR was performed with 1 cycle of 10 min at 95°C, 45 cycles of 1 min at 94°C, 1 min at 45°C, 2 min at 72°C, and 1 cycle of 7 min at 72°C. 3'-RACE was amplified using both the sense (5'-AAGGAGAGCGGAAGACTTCGCATC-3') and the antisense primers (5'-GGCCACGCGTCGACTAGTAC-3'). Parameters used for PCR amplification were the same as mentioned above. Both the 5'-RACE (400 bp) and the 3'-RACE (700 bp) products were cloned into pGEM-T Easy vector, and sequences of each of five independent clones were verified.

2.8. DNA sequencing and analysis

DNA sequencing was performed by the dideoxy chain termination method using

BigDye™ Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, CA, USA) with a Model 377 DNA sequencer (Applied Biosystems Division of Perkin-Elmer, CA, USA). Sequence analysis and comparisons were performed with DNASIS-MAC Ver 3.6 (Hitachi Software, Kanagawa, Japan), and the BLAST program on the DDBJ/GenBank databases. The GenBank accession number for the sequence of Japanese flounder Mn-SOD cDNA is AB604157.

2.9. Determination of protein concentration

Protein concentration was determined by the Lowry method (Lowry et al., 1951) or a bicinchoninic acid kit using bovine serum albumin as a standard.

3. Results

3.1. Purification of Mn-SOD

Mn-SOD was purified from Japanese flounder hepatopancreas and the purification results are summarized in Table 1. Approximately 1.3 mg of the purified enzyme was obtained from approximately 150 g of hepatopancreas, and the specific activity was increased by approximately 781-fold with a 10.8% recovery from the crude extract. The homogeneity of the final preparation was tested by 12.5% SDS-PAGE. As shown in Fig. 1, the purified enzyme gave a main band with molecular mass of 26 kDa on SDS-PAGE under reducing conditions.

3.2. Western blot analysis of purified Mn-SOD

The purified Mn-SOD was subjected to a Western blot analysis using an anti-human Mn-SOD antibody, only one band with a molecular mass of 26 kDa was observed in the presence of 2-mercaptoethanol (2-ME; Fig. 2, lane 2), suggesting that the 26 kDa protein represented purified Mn-SOD. In the absence of 2-ME, the purified Mn-SOD gave a main band with a molecular mass of 26 kDa and a minor band with a molecular mass of 44 kDa (Fig. 2, lane 3), which seems to represent the monomer and homodimer, respectively. In addition, the ammonium sulfate fraction was observed to have a slight band with 26 kDa (Fig. 2, lane 4). On the contrary, the purified Cu,Zn-SOD from Japanese flounder hepatopancreas was undetectable using the same anti-human Mn-SOD antibody (Fig. 2, lane 5).

3.3. Molecular properties of purified Mn-SOD

The polyacrylamide gel electrophoresis (native-PAGE) was also employed to evaluate SOD activities. In the native-PAGE, six active bands (arrows a−f) were observed when the ammonium sulfate fraction was tested (Fig. 3, lane 1). However, the three upper bands (arrows a−c) disappeared in the presence of 5 mM KCN, a specific inhibitor of Cu,Zn-SODs (Fig. 3, lane 2). These results suggested that the three lower active bands represented Mn-SOD, whereas the three upper ones were Cu,Zn-SOD. This result was consistent with our previous study in which purified Japanese flounder Cu,Zn-SOD showed three bands in native-PAGE (Osatomi et al., 2001). In addition, when the purified Mn-SOD was applied into the native-PAGE, only the three lower active bands were observed, and the location was consistent with that of Mn-SOD bands in lanes 1 and 2 (Fig. 3, lane 3), which suggested that Cu,Zn-SOD was completely abolished from purified Mn-SOD samples.

3.4. N-terminal amino acid sequence

The N-terminal amino acid sequence of the Mn-SOD (26 kDa protein band in SDS-PAGE) was determined to be 16 amino acid residues. The sequence was compared with the reported Mn-SOD cDNA clones from other sources and Japanese flounder Cu,Zn-SOD (Fig. 4). The sequence of Japanese flounder Mn-SOD showed high homology with these Mn-SODs but not Japanese flounder Cu,Zn-SOD.

3.5. Cloning and sequence comparison of Mn-SOD cDNA

The nucleotide and deduced amino acid sequences of Japanese flounder Mn-SOD cDNA (1,204 nucleotides) were shown in Fig. 5. The sequence consisted of a 64 bp 5'-non-coding region, a 675 bp open reading frame encoding 225 amino acids, and a 465 bp 3'-non-coding region. The 3'-non-coding region contained a putative poly(A) signal, AATAAA (Fig. 5). The processing site between pre-region (27 amino acids) and mature form (198 amino acids) was confirmed by N-terminal amino acid sequence of the purified Japanese flounder Mn-SOD.

The amino acid sequence of Japanese flounder Mn-SOD showed high homology compared with other Mn-SODs (human 84.8%, rat 82.8%, tobacco 57.9%, rice 58.9% and *Caenorhabditis elegans* 68.6%). The three residues coordinating manganese (His-26, His-30, and His-31) were conserved among Mn-SODs (Fig. 6).

As shown in Fig. 7, the pre-regions of Mn-SOD from Japanese flounder contained 27 amino acids, which were subsequently compared with the pre-regions of other Mn-SODs including signal peptides. Although they were not highly homologous, they consisted of some common basic amino acids, in which several hydrophobic amino acids were inserted.

4. Discussion

In fishes, the main physiological source of reactive oxygen species (ROS) is mitochondria (Wilhelm Filho, 2007). The oxygen-induced cell injury in mitochondria includes the increased production of superoxide anion, mitochondrial injury, and decreased ATP production, which result in cell dysfunction and death (Crapo et al., 1980; Hyslop et al., 1988; Schoonen et al., 1990). Mn-SOD, a mitochondrial protein, has the ability to catalyse the toxic superoxide anion into molecular oxygen and hydrogen peroxide (McCord and Fridovich, 1969), then hydrogen peroxide will be removed by catalase and glutathione peroxidase (Beyer et al., 1991; Whittaker, 2000). Therefore, Mn-SOD in mitochondria is able to protect cells against the damage caused by ROS. Considering the significance of Mn-SOD, in this study, we focus attention on the evaluation of purification to homogeneity and some properties of Mn-SOD from Japanese flounder (*P. olivaceus*), which is one of the most economically important marine species in Northeast Asia (Castaño-Sánchez1 et al., in press).

Thus far there were some reports that Mn-SOD from human, *Ganoderma microsporum* (a fungus) or *Plectonema boryanum* (a blue-green alga) did not bind to an anion exchange column (Asada et al., 1975; Crapo et al., 1978; Pan et al., 1997). However, in our present study, the Japanese flounder Mn-SOD was adsorbed by the Q-Sepharose Fast Flow column. Such contradictory phenomena has suggested that the Japanese flounder Mn-SOD may carry much more negative charges compared with the Mn-SODs from other species. Although the reason is not yet known, it may be associated with the special properties of Japanese flounder. Hence, it will become one of our next important issues in the future studies.

In the Western blot analysis, Japanese flounder Mn-SOD was detectable by an anti-human Mn-SOD antibody, suggesting that the reactivity of anti-human Mn-SOD polyclonal antibody was cross-reactive with the purified Japanese flounder Mn-SOD. Moreover, the subunit molecular mass of the purified Mn-SOD was shown to be 26 kDa. Since another slight band of 44 kDa was observed under non-reducing conditions, we have speculated that this 44 kDa protein may be a homodimer of Mn-SOD (Fig. 2). Furthermore, the protein with an apparent molecular mass of 70 kDa was also separated by the gel filtration (data not shown), which indicated that the Mn-SOD may be composed of four subunits. The molecular mass of the tested Mn-SOD (70 kDa) is less than that expected for a tetrameric enzyme with 26 kDa subunits. Similar discrepancies between subunit molecular mass and the molecular mass of native Mn-SOD have been previously reported (Meier et al., 1994; Brouwer et al., 1997). In addition, all of these findings were consistent with previous reports that Mn-SOD was usually a homodimer or homotetramer (Abe and Okazaki, 1987; Streller et al., 1994; Brouwer et al., 1997; Blackman et al., 2005).

In the analysis of SOD activity by native-PAGE, Mn-SOD of Japanese flounder was shown as three fast moving bands, while Cu,Zn-SOD occurred as three slow moving bands. Since the abovementioned excessive negative charges carried by Japanese flounder Mn-SOD, it was possible that much more negative charges are carried by Japanese flounder Mn-SOD than Cu,Zn-SOD, which might cause the faster electrophoretic mobility of Mn-SOD (Fig. 3). Similar results were also observed in Mn-SOD from Japanese flounder skin (Nakano et al., 1993). However, it seems that the electrophoretogram of Japanese flounder SOD was different from other species, because in human, *Escherichia coli*, white-rot fungus, tube worm and clam, the electrophoretic

mobility of Mn-SOD was slower than that of Cu,Zn-SOD (Blum and Fridovich, 1984; Ohkuma et al., 1987; Belinky et al., 2002). Thus, the property of electrophoretic separation pattern of SOD seems to be dependent on the species, and the property of Japanese flounder SOD appears unique, although the underlying reason is still unknown.

Moreover, it was notable that the Japanese flounder Mn-SOD gave three active bands in the SOD activity staining. Although there were reports that some plants and bacteria had two or four Mn-SOD encoding genes (Miao et al., 1993; Zhu and Scandalios, 1993; Brouwer et al., 1997; Blackman et al., 2005), it was very interesting whether three active bands of Japanese flounder Mn-SOD in this study represented the expressed products from multiple gene compositions or not. We sequenced the independent clone containing the entire Japanese flounder Mn-SOD gene using its genomic DNA. It was judged that Japanese flounder Mn-SOD was a single-copy gene consisting of five exons interrupted by four introns (data not shown). Also, it was confirmed that the obtained sequence (five exons) was the same as the abovementioned cDNA sequence of Japanese flounder Mn-SOD (Fig. 5). These results were consistent with previous reports in which Mn-SODs from human and bovine were also single-copy genes (Wan et al., 1994; Meyrick and Magnuson, 1994). Therefore, we speculate that the purified Japanese flounder Mn-SOD may consist of three distinctly charge isomers of identical molecular size, as is the case of Japanese flounder Cu,Zn-SOD (Osatomi et al., 2001).

In addition, in this study, the amino acid sequence of Japanese flounder Mn-SOD, except for the pre-regions, showed high homology with that of human, rat, tobacco, rice and *C. elegans* Mn-SODs (Fig. 6). Also, we have compared the homology

of Mn-SODs of Japanese flounder with four other kinds of fishes, including *Epinepelus coioides* (AAW29024), *Danio rerio* (NP_956270), *H. mylodon* (ACR23311) and *Anguilla anguilla* (ABF50548), which shared 91%, 83%, 83% and 81% identity, respectively (data not shown). Additionally, it was found that three histidine residues coordinated with the active center of manganese ion were conserved in all tested Mn-SODs of different species. Similar results were also observed in Mn-SODs of *Litopenaeus vannamei*, *Haliotis discus discus* and *H. mylodon* (Ekanayake et al. 2006; Gómez-Anduro et al., 2006; Cho et al., 2009). Therefore, these findings suggest that Mn-SOD is highly conserved in the biosphere, especially in vertebrates.

On the other hand, in this study, we compared the pre-regions of Japanese flounder Mn-SODs with other species. In general, Mn-SOD is synthesized in the cytosol, and after some structural modifications, transported into the mitochondrion, an organelle of extreme oxidative load (Ambrosone et al., 1999). In the process of mitochondrial import, the mitochondria-targeting signal of Mn-SOD plays important roles (Ryan and Jensen, 1995). Inefficient targeting of Mn-SOD caused mitochondria without their full defense system against superoxide radicals, leading to protein oxidation as well as mitochondrial DNA mutations (Rosenblum et al., 1996; Ambrosone et al., 1999). Thus, it is becoming increasingly clear that the mitochondria-targeting signal plays a crucial role in terms of Mn-SOD controlling dioxygen toxicity in the mitochondria (Fridovich, 1995; Richter et al., 1995). According to some reports, the mitochondria-targeting signal is characterized by the existence of some common basic amino acids and the insertion of several hydrophobic amino acids into these basic amino acids (Roise and Schatz, 1988; Wispe et al., 1989). In the present study, we found that the amino acid sequences of the pre-region of Japanese flounder Mn-SOD included the

above mentioned common basic amino acids and insertions (Fig. 7). Therefore, we speculate that the pre-region of Japanese flounder Mn-SOD include the mitochondria-targeting signal.

In conclusion, for the first time, we have described the purification and some characteristics of Mn-SOD from Japanese flounder hepatopancreas. Also, the nucleotide sequence of a cDNA encoded Mn-SOD has been determined. Since Mn-SOD plays an important immunomodulatory role in bacteria-infected organisms, this study provides valuable and reliable information to research the oxidative stress-induced regulation of fish Mn-SOD expression during bacterial invasion, which makes it possible to treat the bacterial fish disease and develop the vaccine.

Acknowledgements

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Figure legends

Fig. 1. SDS-PAGE analysis of the purified enzyme. Electrophoresis was carried out using 12.5% polyacrylamide gel. Lane 1, molecular weight markers; lane 2, the purified enzyme. The 26 kDa band (Mn-SOD) is indicated by an arrow.

Fig. 2. Western blot analysis of Mn-SOD from Japanese flounder hepatopancreas. Electrophoresis was carried out using 12.5% polyacrylamide gel. Lane 1, molecular weight markers; lane 2, the purified enzyme (+2-ME); lane 3, the purified enzyme (−2-ME); lane 4, ammonium sulfate fraction (+2-ME); lane 5, purified Cu,Zn-SOD from Japanese flounder hepatopancreas (+2-ME). The bands of Mn-SOD are indicated by arrows.

Fig. 3. SOD activity in Japanese flounder by native-PAGE analysis. Electrophoresis was carried out using 7.5% gel in Tris-glycine buffer (pH 8.0) and the gel was stained by NBT method. In some preparations, 5 mM KCN was added to inactivate Cu,Zn-SOD activity. The active bands are indicated by arrows a−f, respectively. Lane 1, ammonium sulfate fraction, without KCN; lane 2, ammonium sulfate fraction, with KCN; lane 3, final preparation, without KCN.

Fig. 4. Alignment of N-terminal amino acid sequences of the Mn-SOD, Cu,Zn-SOD from Japanese flounder hepatopancreas and other Mn-SODs. Amino acid residues identical to Japanese flounder Mn-SOD are boxed. The other listed sources are human (*Homo sapiens*; Barra et al, 1984), rat (*Rattus norvegicus*; Ho YS, 1987), maize (*Zea mays* L.; Zhu and Scandalios, 1993), pine (*Pinus sylvestris* L.; Streller et al., 1994), *E.*

coli (Steinman, 1978) and yeast (*Saccharomyces cerevisiae*; Ditlow et al., 1982).

Fig. 5. The nucleotide and deduced amino acid sequences of Mn-SOD gene from Japanese flounder hepatopancreas. The N-terminal amino acid sequence of the purified enzyme is underlined with a single line. Polyadenylation sequence of AATAAA is enclosed in a box.

Fig. 6. Multiple sequence alignment of Japanese flounder Mn-SOD and amino acid sequence of other Mn-SODs. Sequences from Japanese flounder (*P. olivaceus*, GenBank accession number AB604157), human (*H. sapiens*, P04179), rat (*R. norvegicus*, NP_058747), tobacco (*Nicotiana tabacum*, BAC75399), rice (*Oryza sativa*, AAA57131) and *C. elegans* (BAA02363) are compared. Identical residues with Japanese flounder Mn-SOD are marked by asterisks. Potential metal ligands for manganese are shown in boxes.

Fig. 7. Pre-regions of Mn-SOD from Japanese flounder hepatopancreas and other Mn-SODs. The basic amino acid residues are shown in boxes and the hydrophobic amino acids are shown by gray.

Table 1. Purification result of Mn-SOD from Japanese flounder hepatopancreas*.

	Total protein (mg)	Total activity (Units)	Specific activity (Units/mg)	Purification (fold)	Yield (%)
Crude extract	9,350	6,000	0.64	1	100
Heat treatment	1,927	5,056	2.62	4.09	84.3
Ammonium sulfate fraction	466.1	3,147	6.75	10.5	52.4
Q-Sepharose F.F.	22.2	1,937	87.2	136	32.3
Sephacryl S-200	3.53	1,260	356.9	558	21.0
Bio-Sil SEC-125	1.3	650	500	781	10.8

*SOD activity at each step was measured as described in Materials and methods.
One unit of SOD activity was defined as the amount of protein required to inhibit the rate of production of azo pigment by 50% under the assay conditions.

Fig. 1.
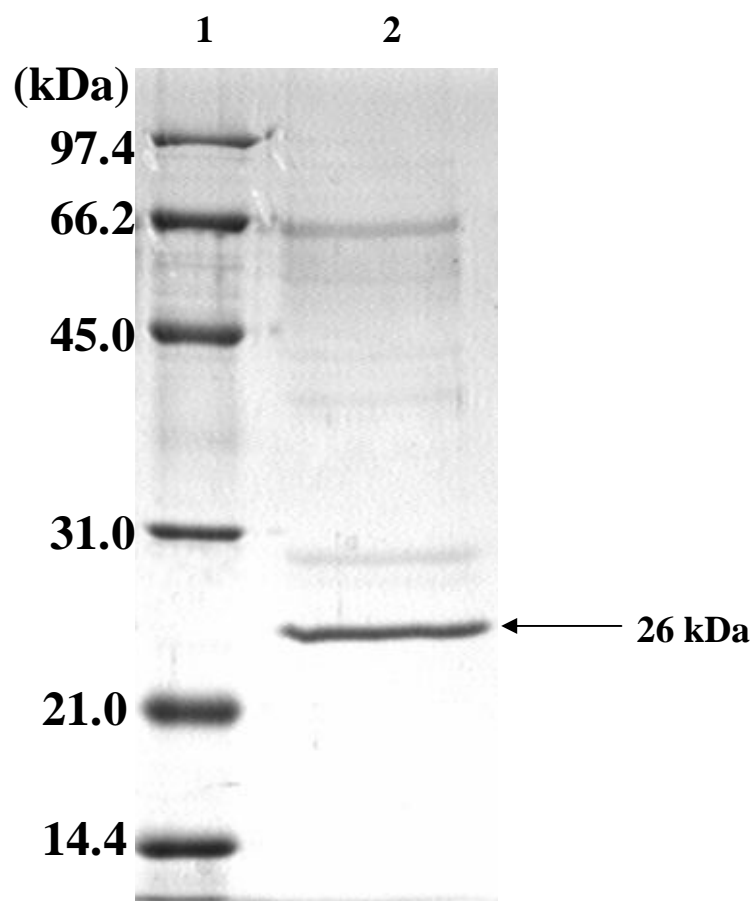
Fig. 2.
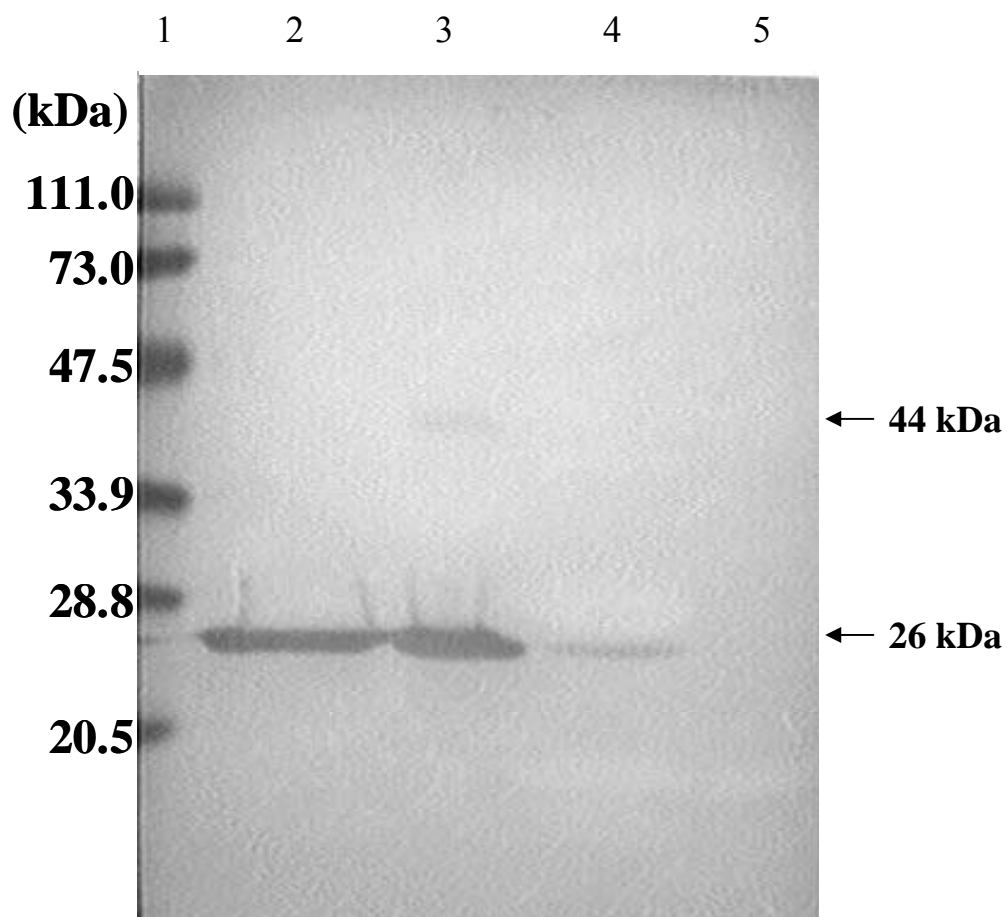
Fig. 3.
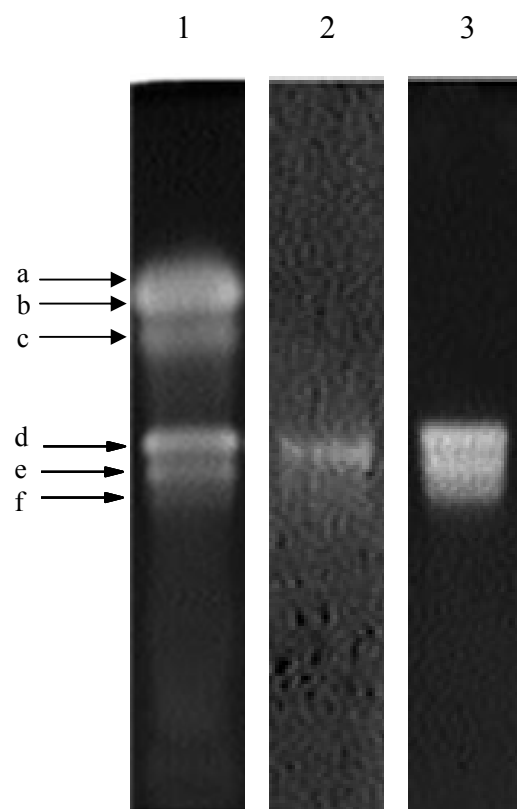
Fig. 4.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Mn-SOD	*P. olivaceus*	K	H	T	L	P	D	L	T	Y	D	Y	G	A	L	E	P
	H. sapiens	K	H	S	L	P	D	L	P	Y	D	Y	G	A	L	E	P
	R. norvegicus	K	H	S	L	P	D	L	P	Y	D	Y	G	A	L	E	P
	Z. mays L.	T	V	A	L	P	D	L	S	Y	D	F	G	A	L	E	P
	P. sylvestris L.	S	F	S	L	P	E	L	P	Y	E	Y	S	A	L	E	P
	E.coli	S	Y	T	L	P	S	L	P	Y	A	Y	D	A	L	E	P
	S. cerevisiae	K	V	T	L	P	D	L	K	W	D	F	G	A	L	E	P
Cu,Zn-SOD	*P. olivaceus*	A	L	K	A	V	C	V	L	K	G	A	G	E	T	S	G

34

Fig. 5.

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   1                                                             GGAT      4
   5 CTGTAGTTTGTGAGCGTCCCTCCTGTCTGTGCTGTCTGTGCTGTGGACACTGACACCATC     64

  65 ATGCTCTGCAGAGTTGCTCAGATACGCAGGTGTGCAGCCAGTCTCAGCCAAACCATAAGC    124
   1  M  L  C  R  V  A  Q  I  R  R  C  A  A  S  L  S  Q  T  I  S     20

 125 CAGGCAACCGCATCGAGGCACAAGCACACGCTCCCAGACCTGACCTACGACTATGGTGCC    184
  21  Q  A  T  A  S  R  H  K  H  T  L  P  D  L  T  Y  D  Y  G  A     40

 185 CTGGAGCCCCACATCAGTGCAGAGATCATGCAGCTGCACCACAGCAAGCACCACGCCACA    244
  41  L  E  P  H  I  S  A  E  I  M  Q  L  H  H  S  K  H  H  A  T     60

 245 TATGTGAACAACCTCAACGTCACAGAGGAGAAGTATCAAGAGGCACTGGCAAAGGGTGAC    304
  61  Y  V  N  N  L  N  V  T  E  E  K  Y  Q  E  A  L  A  K  G  D     80

 305 GTGACGGCACAAGTTGCCCTCCAGCCTGCTCTGAAATTTAATGGAGGAGGCCACATTAAT    364
  81  V  T  A  Q  V  A  L  Q  P  A  L  K  F  N  G  G  G  H  I  N    100

 365 CACACCATCTTCTGGACAAACCTCTCTCCAAATGGTGGAGATGGGCCACAGGGGGAACTA    424
 101  H  T  I  F  W  T  N  L  S  P  N  G  G  D  G  P  Q  G  E  L    120

 425 ATGGAAGCCATCAATCGGGACTTTGGCTCCCTCCAGAAGTTGAAGGAAAAGATGTCTGCT    484
 121  M  E  A  I  N  R  D  F  G  S  L  Q  K  L  K  E  K  M  S  A    140

 485 GCTACCGTTGCAGTGCAGGGCTCAGGGTGGGGCTGGCTGGGCTACGACAAGGAGAGCGGA    544
 141  A  T  V  A  V  Q  G  S  G  W  G  W  L  G  Y  D  K  E  S  G    160

 545 AGACTTCGCATCGCTGCTTGTGCAAACCAGGATCCTCTGCAGGGGACGACAGGTCTCATC    604
 161  R  L  R  I  A  A  C  A  N  Q  D  P  L  Q  G  T  T  G  L  I    180

 605 CCTCTTCTTGGTATTGACGTATGGGAGCACGCCTATTACCTTCAGTACAAAAATGTGCGG    664
 181  P  L  L  G  I  D  V  W  E  H  A  Y  Y  L  Q  Y  K  N  V  R    200

 665 CCGGACTATGTTAAGGCGATCTGGAACGTAATCAACTGGGAGAATGTGACTGAGCGTCTT    724
 201  P  D  Y  V  K  A  I  W  N  V  I  N  W  E  N  V  T  E  R  L    220

 725 CAGATCGCCAAAAAGTAGAATCTGACCCTCGCAAATCCCTGAAAACCTCTCTCATCTTGA    784
 221  Q  I  A  K  K  *                                             225

 785 CCTGGGCCTGGTTCAAATAGTCGCTGCAAATAATTCTTAGTTTTACCATGCGTGAAGTAT    844
 845 CAGTGGTGGTGTTCTTCGAATTTTAGATTGTCAATTGCATTAAAGTTGATTACCTTTGAC    904
 905 TTGGAAATACTTTCTATATTGAAAACATACAAATTAAAAATCATTTGCAAGTGCTTTTTA    964
 965 AATCTCAGGTCTGTCCTCAAACACCAGACTCTTCAAAGCGCACAAAAATGGCTGCCTTTC   1024
1025 ACGTCAAGGCTGGCTTTTGTTACCCAAATAGTAGTTGACGCAATACCAGAGAGCATATTC   1084
1085 AGTGAGCTGTGTCATTGTCGGAATTACTCACACAAAAATCAGGCAATGTTATTGCAGTCC   1144
1145 ATGTTTTTGATGTAGAATAATAAAATTATATGATTTCAGTTGTAAAAAAAAAAAAAAAAA   1204
```

35

Fig. 6.

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P.olivaceus      1 MLCRVAQIRRCAAS--LSQTISQATA-SRHKHTLPDLTYDYGALEP---H      50
H.sapiens        1 ..S.AV----.GT.RQ.PPVLG-YLG-..Q..S....P...........---.    50
R.norvegicus     1 ....A.-----.S.GRR.GPAA.-TAG-.....S....P...........---.    50
N.tabacum        1 .AL.TLVS..TL.TG----LGFRQQLRGLQTFS....P........AIS-       50
O.sativa         1 .AL.TLAS.KTL.AAA.-PLAAA.A.RGVTTVA....P........AIS-       50
C.elegans        1 ..QST.R----T..KLVQPVAGVLA--V.S.......PF..AD...VIS.       50
                   *                              ****  **  ***


P.olivaceus     51 ISAEIMQLHHSKHHATYVNNLNVTEEKYQEALAKGDVTAQVALQPALKFN      100
H.sapiens       51 .N.Q.........A.........................I.........      100
R.norvegicus    51 .N.Q.....................H.........T.............      100
N.tabacum       51 --GD.....QN..Q...T.Y.KAL.QLHD.IS...APTVAK.HS.I...     100
O.sativa        51 --GE..R...Q........A.Y.KAL.QLDA.V....AP.IVH..S.I...   100
C.elegans       51 E----..Q...Q.........QI...LH..VS..NLKEAI.........    100
                        ** ***  **  ** * *    *    *  **       *  * ***


P.olivaceus    101 GGGHINHTIFWTNLS--PNGGDGPQGELMEAIN-RDFGSLQKLKEKMSAA      150
H.sapiens      101 .......S.........--....GE.K...L...K-L....FD.F...LT..   150
R.norvegicus   101 .......S.........--.K..GE.K...L...K-.....FE.F...LT.V   150
N.tabacum      101 .......S...K..APVRE..GE.PKGSLGWAIDTN....EA.VQ..N.E    150
O.sativa       101 ....V..S...N..KPISE..GD.PHAKLGWAIDE....FEA.VK....E    150
C.elegans      101 .......S...T..AKD---.GE.SK...DT.K-......DN.QKRL.DI    150
                   **** ** *** **         *  *           ***


P.olivaceus    151 TVAVQGSGWGWLGYDKESGRLRIAACANQDPLQGTTG-LIPLLGIDVWEH      200
H.sapiens      151 S.G...........FN..R.H.Q....P.........-...........    200
R.norvegicus   151 S.G...........FN..Q...Q....S.H.......-...........    200
N.tabacum      151 GA.L.....V..V...LK..V.ETT......VSKGAN.V..........    200
O.sativa       151 GA.L.....V..AL...AKK.SVETT......VTKGAN.V..........   200
C.elegans      151 .I............C.KDKI.K..T.......E.MVP-.F---........   200
                       ***** **    *    *         ***      *   *******


P.olivaceus    201 AYYLQYKNVRPDYVKAIWNVINWENVTERLQIAKK      250
H.sapiens      201 ...........L...............YMAC..      250
R.norvegicus   201 ...........L...............SQ.YIVC..   250
N.tabacum      201 ...........L.N..K.M..KYAN.VYEKECP    250
O.sativa       201 ...........LSN..K.M..KYAG.VYEN.TA    250
C.elegans      201 ............H...KIA..K.IS..FAN.RQ    250
                   *************    **    **
```

Fig. 7.

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                                  10          20          30
P.olivaceus    MLCR-VAQI-  RRCAASLSQT  ISQATA-SRH
H.sapiens      MLSRAVCGTS  RQLPPVL-G-  ----YLGSRQ
R.norvegicus   MLCRAACSAG  RR-LGPAA--  -S--TAGSRH
N.tabacum      MALRTLVS--  RRTLATGLGF  RQQL----RG
O.sativa       MALRTLAS--  RKTLAAAALP  LAAAAAA-RG
C.elegans      MLQSTA----  R-TASKLVQP  VAGVLA-VRS
```