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Purification and Characterization of Manganese Superoxide Dismutase from *Staphylococcus sciuri*

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Abstract The intracellular superoxide dismutase (SOD) from *Staphylococcus sciuri* was isolated to homogeneity by continuous steps, including ammonium sulfate fractionation, DEAE-ion-exchange chromatography, gel filtration, and phenyl hydrophobic gel chromatography. Pure SOD had a specific activity of 4,625 U/mg and was purified 158-fold with a yield of 31% from a cell free extract. The molecular weight of the purified SOD was determined to be approximately 35.5 kDa by gel filtration and the enzyme was also shown to be composed of dimeric subunits on denaturing SDS-PAGE. The enzyme activity remained stable at pH 5 to 11 and also to heat treatment of up to 50°C at pH 7.8, with 80% relative activity. The enzyme was insensitive to cyanide, hydrogen peroxide, and azide, indicating that it is a manganese-containing SOD. The EPR spectrum showed the enzyme containing manganese as a cofactor.

Key words: Purification, superoxide dismutase, *Staphylococcus sciuri*

The univalent reduction of molecular oxygen occurs favorably by the Pauli's exclusion principle and relative sluggishness of spin inversion [23]. The intermediates of the univalent reduction, namely, the activated oxygen species, are the superoxide radical (O_2^-), hydroxy radical (HO^-), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$). Although these oxygens may serve as a cell defense system, for example, superoxides produced by leukocytes eliminate the opsonized particles in phagocytosis, most of the active oxygens overproduced are not only from epinephrine oxidation [1] and normal metabolism by flavoenzymes like xanthine oxidase [12] and glutathione reductase [17], but also from an abnormal state such as inflammation or medicinal poisoning, which can cause direct toxicities against the body. They also participate in the free radical reaction of unsaturated fatty acids in a cell membrane to induce the peroxidation of lipids [15], and cause structural damage in cells and disorders like inflammation or cancer [22] by reacting nonspecifically with cell components such as carbohydrates, protein, amino acids, peptide enzyme, or DNA [10]. Superoxide dismutase (SOD; superoxide oxidoreductase, EC 1.15.1.1.), which is a metalloenzyme catalyzing the dismutation of superoxide radicals (O_2^-) to yield H_2O_2 and O_2, seems to be present in all oxygen metabolizing organisms and is considered as the major enzymatic defense mechanism against O_2^- radicals [8]. Most SODs are intracellular enzymes which are classified as Cu,Zn-SOD, Mn-SOD, and Fe-SOD according to the metal ion in their active sites. However, there is an extracellular SOD (EC-SOD) in mammalian external fluids. Studies on SOD have been performed in various fields including the relations between oxygen radicals and inflammation and the physiological functions of SOD as an anti-inflammatory effect. In addition, the industrial applications of SOD are extending rapidly to the production of recombinant human SOD (rh-SOD) in yeast [21] and chinese hamster ovary cells [24], modified SODs such as liposome-SOD, polyethyleneglycol (PEG)-SOD [3], styrene maleimide (SM)-SOD, low MW components with SOD functions, and the improvement of food functions by the addition of SOD.

We have screened various food microorganisms to identify superoxide dismutases. Among them, it was found that *Staphylococcus sciuri* exhibited a higher SOD activity [5]. *S. sciuri,* which is non-pathogenic, coagulase and hemolysin negative, is currently distributed in processed

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meat products such as dry-cured ham [4] and fermented milk products [7]. As some species of *Staphylococcus* have a proteolytic activity [20], as well as the ability of nitrate reduction and bacteriocin production [27], the value of its application may be very important. Accordingly, in this study, SOD from *S. sciuri* with high intracellular activity was purified and its property was characterized.

MATERIALS AND METHODS

Materials

Horse heart cytochrome c (Type III), xanthine, xanthine oxidase (grade IV), nitroblue tetrazolium, and phenyl Sepharose were obtained from Sigma Co. Sephadex G-75 and protein MW standard markers were purchased from Pharmacia LKB Biotechnology (Sweden). DEAE Toyopearl 650C from Toyo Soda Co. (Japan) was used for ion-exchange chromatography and all other chemicals were of analytical grade.

Bacterial Strain and Culture Condition

Staphylococcus sciuri was used for SOD production. The cultivation was carried out in a 250-ml Erlenmeyer flask containing 100 ml of the medium, which consisted of 7% soluble starch, 1% yeast extract, and 2% NaCl, at 45°C with 300 rpm for 9 h. The initial pH of the medium was adjusted to 7.0.

Assay of SOD Activity

The assay system for SOD activity was based on the ability to inhibit the reduction of cytochrome c by xanthine/xanthine oxidase. After adding SOD sample to the reaction mixture containing 30 mM sodium phosphate, pH 7.8, 0.1 mM EDTA, 3.8 nM cytochrome c, and 38 nM xanthine, the reaction was initiated by adding 30 µl of xanthine oxidase diluted with 2.3 M ammonium sulfate, and the reduction change of cytochrome c became 0.02 absorbance per min at 550 nm. The total volume of the reaction mixture was adjusted to 1 ml and one unit of SOD activity was defined as the amount of enzyme required to inhibit the reduction of cytochrome c by 50%.

Enzyme Purification

Cells (60 g of wet weight) were harvested, suspended in 300 ml of 50 mM phosphate buffer, pH 7.8, and disrupted with a sonicator. The lysate was centrifuged at 12,000 rpm for 15 min at 4°C and a crude enzyme solution was obtained from the supernatant. Ammonium sulfate was added to produce 60% saturation in the above solution and centrifuged. Then, ammonium sulfate was added to adjust to 70% and 90% saturation levels, respectively. The recovered supernatant was applied to DEAE-Toyopearl 650C column (2.5×40 cm) equilibrated with 50 mM phosphate buffer, pH 7.8, and eluted with a linear gradient of NaCl (0-500 mM) in the same buffer. Fractions containing the enzyme were then reapplied to a DEAE-Toyopearl 650C column (1.9×33 cm) equilibrated with 5 mM phosphate buffer containing 50 mM NaCl and eluted with a linear gradient of NaCl (50-300 mM) in the same buffer. These fractions were pooled and subjected to Sephadex G-75 column (2.6×100 cm) chromatography. The enzymatically active portions of the fractions were pooled and dialyzed against 30% ammonium sulfate and 50 mM phosphate buffer, pH 7.8. For further purification, hydrophobic gel chromatography was carried out with a phenyl Sepharose CL-4B column (2.5×10 cm) equilibrated with 50 mM phosphate buffer, pH 7.8, containing 30% ammonium sulfate, and fractions were eluted with a linear gradient of ammonium sulfate (0-30%) in the same buffer. The enzyme fractions obtained were reapplied to the Sephadex G-75 column (2.6×100 cm) and eluted with 50 mM phosphate buffer, pH 7.8, at an 8 ml/h flow rate.

Determination of Molecular Weight and Electrophoresis

The molecular weight of the purified enzyme was determined by Sephadex G-75 column chromatography using standard marker proteins, such as bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (25 kDa). Electrophoresis for the active staining of the SOD was carried out using 7.5% polyacrylamide disc gel. Proteins were stained with 1% Coomassie Brilliant blue R-250 and the SOD activity was detected using the NBT (nitroblue tetrazolium) reduction method as described by Maral *et al.* [16]. Denaturing PAGE was performed as described by Laemmli [13].

Characterizations of SOD

The pH stability of the enzyme was investigated by measuring the remaining activity after 12 h incubation at 4°C, using potassium acetate buffer (for pH 3-6), potassium phosphate buffer (for pH 6-8), and Tris-HCl (for pH 8-9). The thermal stability was examined after incubation at 20 to 70°C for 20 min in 50 mM phosphate buffer, pH 7.8. Effects of specific inhibitors on SOD activity were observed under 0.1-5 mM concentrations of KCN, H_2O_2, and NaN_3, respectively. Electron paramagnetic resonance (EPR) spectra of native enzyme and the supernatant, obtained by boiling the solution containing 3.7 mg purified SOD per ml of 0.2 N HCl at 100°C for 2 min, were recorded at 77 K. The microwave power and the frequency were 10 mW and 9.446 GHz, respectively. Field modulation was 5 G (gauss) and receive gain was 1×10^5.

RESULTS AND DISCUSSION

Purification of Superoxide Dismutase

Superoxide dismutase from *S. sciuri* was purified by ammonium sulfate precipitation and five successive

Table 1. Purification of intracellular SOD from *Staphylococcus sciuri.*

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude cell extract	330.0	3,300	96,820	29	1	100
$(NH_4)_2SO_4$ 70-90%	10.8	191	51,365	269	9	53
1st DEAE-Toyopearl	12.6	81	45,680	563	19	47
2nd DEAE-Toyopearl	9.4	35	35,900	1,017	35	37
1st Sephadex G-75	12.4	14	33,260	2,446	84	34
Phenyl Sepharose	14.9	8	30,884	3,813	130	32
2nd Sephadex G-75	33.9	6	29,832	4,625	158	31

chromatographies, including DEAE-ion-exchange, Sephadex G-75, and phenyl Sepharose CL-4B. The purification profile is summarized in Table 1. The purified SOD obtained from the final step showed 29,800 units of total activity with a 31% yield, 4,625 U/mg of specific activity, and a 158-fold purification. The most effective purification was accomplished through ammonium sulfate precipitation with 9-fold purification, since most proteins except the enzyme were precipitated and removed at the concentration of 70% ammonium sulfate. Most superoxide dismutases precipitated by a high concentration of ammonium sulfate were found in Mn-SOD from *Gluconobacter cerinus* (60-100% ammonium sulfate) and Fe-SOD from *Methanobacterium bryantii* (90-100% ammonium sulfate). The specific activity of the SOD from *S. sciuri* was lower than the SOD (5,500 U/mg) from *Streptococcus mutans*, but higher than the Mn-SOD (340 U/mg) from *Halobacterium cultirubrum*. The final recovery yield of the SOD from *S. sciuri* was lower than that from *H. cultirubrum* (79%), but similar to that from *Streptococcus mutans* (34%).

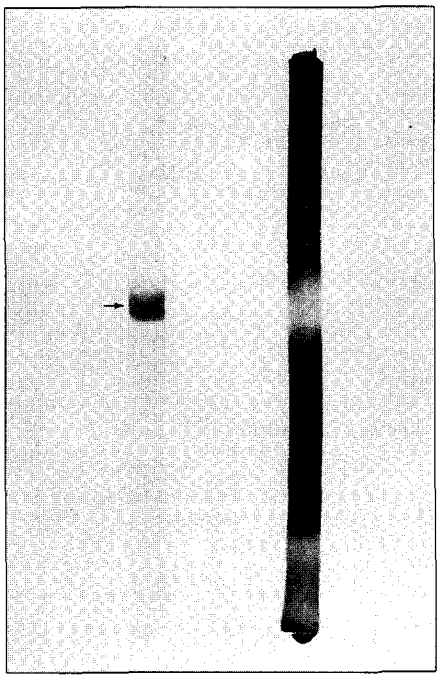

Fig. 1. Polyacrylamide disc gel electrophoresis.
The purified enzyme was fractionated in disc gel at pH 8.6. The protein was migrated from cathode to anode. The arrow indicates the SOD protein stained with Coomassie Brilliant blue R-250 (left). The achromatic zone shows the SOD activity (right).

Characteristics of SOD from *S. sciuri*

To confirm the homogeneity of the purified SOD from *S. sciuri*, PAGE was carried out. Protein was visualized by staining with 1% Coomassie Brilliant blue R-250 and the SOD activity was measured as an achromatic zone according to its ability to prevent the reduction of nitroblue tetrazolium by photochemically generated superoxide radicals. As can be seen in Fig. 1, the SOD from *S. sciuri* was fractionated as a single band, whereas the SOD activity was localized at a zone corresponding to the stained protein band in disc gel electrophoresis, indicating that the position of SOD activity coincided with the protein band.

As can be seen in Fig. 2A, the molecular weight of the native enzyme from *S. sciuri* was measured to be approximately 35.5 kDa using gel filtration on a column of Sephadex G-75 with standard protein markers. Molecular weights of various SODs were diverse according to their origin. It has been previously reported that the molecular weight of Mn-SOD from human liver is 23 kDa [2], Cu,Zn-SOD of bovine erythrocyte is 33 kDa, Mn-SOD from *Streptococcus mutans* is 40 kDa, Mn-SOD from *E. coli* is 45.8 kDa, Mn-SOD from bovine erythrocyte is 89 kDa, and human EC-SOD is 135 kDa, respectively. When the purified SOD (5 μg) was fractionated on denaturing polyacrylamide gel, as shown in Fig. 2B, the molecular weight of the SOD was estimated to be about 17.5 kDa by comparison with standard marker proteins, indicating that the native enzyme was consisted of two equal sized subunits, that is, a homodimeric subunit.

The optimum pH range of this enzyme was shown to be 5 to 11 with 90% of the remaining activity. This result is similar to the pH stability of Mn-SOD (pH 6-11) from *Gluconobacter cerinus* [25], but is more stable than that from *Pseudomonas polycolor* (pH 9-11) [14]. The SOD from *S. sciuri* was shown to maintain 80% activity after

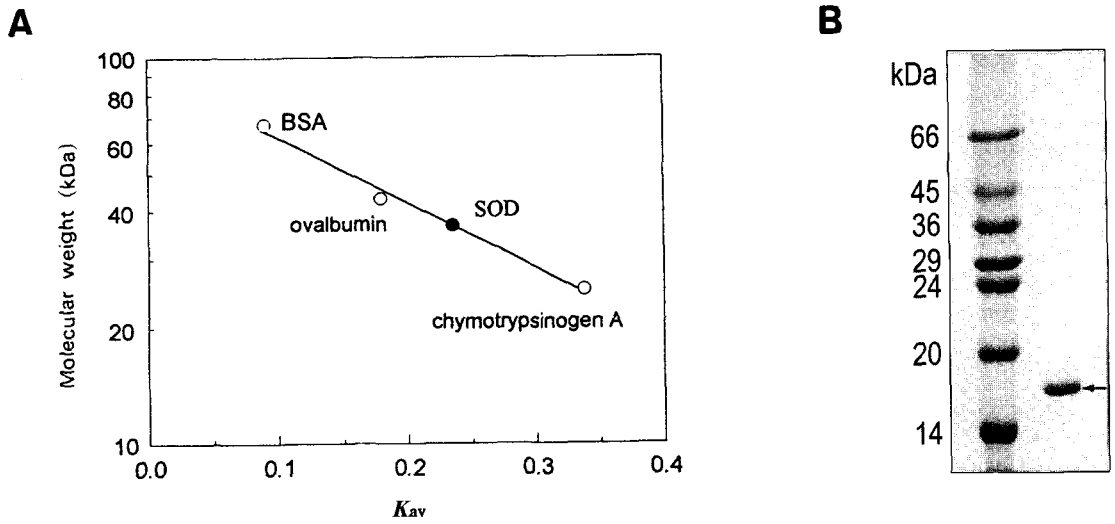

Fig. 2. Determination of the molecular weight of superoxide dismutase.
A. Gel filtration on Sephadex G-75: The molecular weight of the native enzyme was estimated by Sephadex G-75 gel filtration, using a column (2.6×100 cm) with 50 mM phosphate buffer (pH 7.8). The standard proteins are bovine serum albumin (67 kDa), ovalbumin (43 kDa), and chymotrypsinogen A (23 kDa). B. SDS-PAGE: The purified SOD (5 μg) was fractionated on 12% polyacrylamide gel. The molecular weight was estimated by comparison with standard marker proteins such as bovine albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-p-dehydrogenase (36 kDa), bovine carbonic anhydrase (29 kDa), bovine pancreas trypsinogen (24 kDa), soybean trypsin inhibitor (20 kDa), and bovine milk α-lactalbumin (14 kDa). The arrow indicates the position of the SOD.

heating at 50°C and the activity remained above 60% at 60°C; however, 80% of the activity was lost at 70°C. The thermal stability of SOD from *S. sciuri* was higher than Fe-SOD from *Pseudomonas polycolor* which exhibited 60% of remaining activity with heat treatment at 50°C for 20 min, whereas it was lower than Mn-SOD from *Gluconobacter cerinus* exhibiting 60% of remaining activity at 70°C for 40 min.

Inhibition effects of specific inhibitors on the SOD were observed to confirm the type of metal ion present in the active site of the SOD. The SOD activity was inhibited by NaN_3, but not inhibited by H_2O_2 at all (Table 2). It has been reported that Cu,Zn-SOD was inhibited completely by 1 mM KCN [6] and the activity of Fe-SOD was decreased to 50% by 4 mM NaN_3 [9], while Mn-SOD was inactivated by only azide [19]. Therefore, the purified SOD from *S. sciuri* is most likely to be Mn-SOD because 10% of the SOD activity was inhibited by 5 mM sodium azide. Also, our previous findings indicated the SOD of *S. sciuri* to be Mn-SOD by experiments using metal and metal chelators [5].

In order to identify the metal ion present in the active site of the enzyme, electron paramagnetic resonance (EPR) spectroscopy was carried out for the native enzyme and the clear supernatant obtained after discarding the protein. When manganese is present, superoxide dismutase may be expected to display a specific EPR spectrum. As shown in Fig. 3, there was no distinctive metal ion spectrum identifiable in the native enzyme (upper tracing), whereas signals corresponding to Mn^{+2} appeared in the clear supernatant after discarding the protein (lower tracing) due to the denaturation of the enzyme liberating the

Table 2. Effects of specific inhibitors on the SOD activity.

Inhibitor	Relative activity (%)		
	0.1 mM	1 mM	5 mM
KCN	100	100	96
H_2O_2	100	100	99.2
NaN_3	100	98.5	91.3

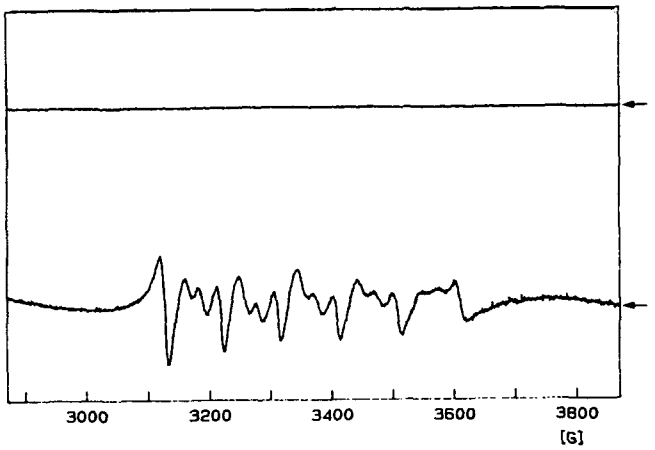

Fig. 3. Electron paramagnetic resonance for detecting manganese in SOD from *S. sciuri*.
The purified SOD was solubilized in 0.2 N HCl (3.7 mg/ml) and heated to 100°C for 2 min. After cooling, the extract was centrifuged and the clear supernatant obtained was used for the EPR spectroscopy at 77 K. The upper spectrum indicates the spectrum of the native enzyme and the lower one is that of manganese which showed up after discarding the protein by heat treatment.

manganese; the signal agreed with the EPR pattern of $MnCl_2$ [11] which showed 6 major peaks for manganese in the range of 3,000 to 3,600 gauss [26] confirming the Mn-SOD from *Streptococcus mutans*. Although there was a slight difference in the spectrum shape by minor peaks, the data suggests that the superoxide dismutase from *S. sciuri* contains manganese as a prosthetic group.

In conclusion, the SOD from *S. sciuri* was purified and characterized. In view of the results obtained using the inhibition effect and EPR spectrum, the SOD from *S. sciuri* was confirmed to be Mn-SOD. In further studies, experiments to investigate the isoelectric point, kinetic characteristics, and amino acids composition of the Mn-SOD will be carried out.

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