Journal of Microbiology and Biotechnology
Vol. 3, No. 3, 188-193, 1993

Purification and Characterization of Superoxide Dismutase from *Pseudomonas polycolor*

LEE SANG-OK, IL-CHUN SEO, SOOK-HYUN CHUNG[1] AND TAE-HO LEE*

Department of Microbiology, College of Natural Sciences,
Pusan National University, Pusan 609-735, Korea
[1]Department of Food Engineering, Dongseo University, Pusan 616-010, Korea

Superoxide dismutase (SOD) was purified from *Pseudomonas polycolor* to an electrophoretically homogeneous state and partially characterized. SOD was purified by ammonium sulfate fractionation, column chromatography on DEAE-Sephadex A-50, phenyl-Toyopearl 650 M, and gel filtration on Sephadex G-100. The molecular weight and subunit molecular weight of the purified enzyme were estimated to be 40,000 and 20,000, respectively. The purified enzyme remained stable at pH 9.0~11.0, 25°C for 40 hr, but rapidly became inactive below 9.0. SOD was stable up to 45°C at pH 9.0 with about 80% relative activity, but rapidly became inactive at temperature above that. The enzyme was insensitive to cyanide and fluoride, and sensitive to hydrogen peroxide and azide. The results suggest that the enzyme be an iron-containing SOD.

The superoxide anion (O_2^-) is the first reactive species formed by the univalent reduction of oxygen, and O_2^- can lead to the formation of more reactive oxidants, hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) (18). These active oxygen species threaten living cells by oxidizing their nucleic acids, proteins and membrane lipids. Superoxide dismutase (SOD, EC 1.15.1.1) is the first line of defense enzyme against them by scavenging the superoxide free radical through its disproportionation: $O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$ (15, 18).

The family of SOD includes three metallo-forms, Cu, Zn-SOD, Mn-SOD, and Fe-SOD (4, 15, 18). There are three kinds of Cu, Zn-SODs with different distributions, the cytosol of most eukaryotic cells, plasma from several mammalian species (EC-SOD)(12, 13), and some bacteria (bacteriocuprein) (14, 21, 22). Two kinds of Mn-SODs are observed, one characteristic in mitochondrial matrix of eukaryotes and the other characteristic in the cytoplasm of bacteria (4, 8, 15, 25, 27). Fe-SOD shows wider distribution than other forms and is present in bacteria, algae, protozoa, and plants (4, 18). Mn- and Fe-SOD are closely related proteins since the two isozymes display extensive amino acid sequence homology and similar tertiary structure and are unlike the Cu, Zn-SOD, suggesting a common ancestral protein for the two isozymes (17). However, some differences between Mn- and Fe-SOD are observed(19). In general, aerobes and facultative anaerobes have Mn-SOD and/or Fe-SOD, but anaerobes have Fe-SOD with some exceptions (Bacteroides spp) (5, 20). Furthermore, whereas Mn-SOD is known to be induced by the increase of intracellular production of superoxide radical, Fe-SOD is not inducible but constitutive (7, 17).

Although the differences or similarities between Mn- and Fe-SODs have been demonstrated by many investigators in several points of view (19), little is known about the differences in the physiological functions of the two isozymes, and necessity of Fe-SOD being present in anaerobic cells which need not defend against oxygen toxicity (23).

Thus, to extend understanding of the functional differences between the two isozymes and of what function Fe-SOD has, studies were undertaken on the SOD isozymes from some bacteria previously (9, 11). In the present paper, *Pseudomonas polycolor* considered to produce relatively a large amount of Fe-SOD was selected and investigated. This paper deals with the purification and the characterization of some properties.

*Corresponding author
Key words: Superoxide dismutase (SOD), Fe-SOD

MATERIALS AND METHODS

Strain and Cultivation

Several stocked strains in our laboratory were grown in test tubes (22×200 mm) containing 8 ml of nutrient broth under aeration (120 strokes/min) at 30℃ for 24 hr. The crude enzyme solution, prepared from each bacteria, was used to measure SOD activity.

In order to investigate how the carbon and nitrogen sources, inorganic salts, initial pH, aeration and methyl viologen affect on SOD production, the selected strain, *Pseudomonas polycolor* IFO 3918, was grown at 30℃ for 24 hr in a 500 ml shake flask containing 100 ml of each medium composition with the addition of 2 ml of seed culture.

Preparation of Cell-free Extract

The cultured cells were harvested by centrifugation, washed twice with 0.85% NaCl solution, resuspended in 0.01 M sodium phosphate buffer (pH 7.0) and then disrupted by sonic oscillation for 3 min with 1-min pause for cooling on ice. The cell debris was removed by centrifugation at 13,000×*g* for 20 min and then the resulting cell-free extract was used as the crude enzyme solution to assay SOD activity.

SOD Assay

SOD activity was conveniently assayed in terms of its ability to inhibit the autoxidation of epinephrine to adrenochrome, which has an absorption maximum at 480 nm (16). The enzyme assay was done at 20℃ by mixing 0.3 ml of an adequately diluted enzyme solution, 3.0 ml of sodium carbonate buffer (pH 10.2) containing EDTA, and 0.3 ml of an epinephrine stock solution (pH 2.0), the final concentrations being 0.3 mM epinephrine, 0.1 mM EDTA and 0.05 M carbonate buffer. The reaction was done in a cuvette for 3 min with a time-scanning recording of the absorbance at 480 nm. For more precise data, the xanthine oxidase/cytochrom c method was used (18). One unit of SOD was defined as the amount that causes a 50% inhibition of blank autoxidation in the epinephrine method and a 50% inhibition in the rate of cytochrome c reduction in the xathine oxidase/cytochrome c method. Spectrophotometric assays were done with a Shimadzu UV-240 spectrophotometer.

Homogeneity of the Purified Enzyme

Two identical 7.5% polyacrylamide disc gels were prepared, one for protein detection and the other for the detection of the enzyme, with the modification that both the resolving and the concentration gels were photopolymerized with riboflavin. Discontinuous gel electrophoresis was done on the gels at 2 mA per gel at 4℃ by the method of Davis (1964) (3). Protein bands were visualized after staining gels with 1% Amido Black 10B. Zones of SOD activity were visualized by the NBT method in which the gels were negatively stained by competition with nitroblue tetrazolium for a photochemical flux of O_2^- by riboflavin (2).

Measurement of Molecular Weight

The molecular weight of the purified enzyme was measured by gel filtration on Sephadex G-100 by the method of Andrews *et al.* (1). The subunit molecular weight of the enzyme was estimated by disc gel electrophoresis on a 10% polyacrylamide gel in the presence of sodium dodecyl sulfate with 2-mercaptoethanol, as described by Weber and Osborn (26). The standard proteins used for calibration were lysozyme (M.W. 14,000), α-chymotrypsinogen A (M.W. 25,700), ovalbumin (M.W. 45,000), and bovine serum albumin (M.W. 66,000).

Chemicals

Epinephrine, DEAE-Sephadex A-50, Sephadex G-100, methyl viologen, Ampholines, EDTA, and standard proteins were obtained from Sigma Chemical Co. Nitrobluetetrazolium was purchased from Fluka. Phenyl-Toyopearl 650 M was obtained from Tosoh in Japan. All other chemicals were of analytical reagent grade.

RESULTS

Culture Conditions for SOD Production from *Peudomonas polycolor*

In order to derermine the culture media and conditions for efficient enzyme production, effects of carbon nitrogen, inorganic source, initial pH, cultivation temperature and time, and aeration were investigated. The addition of methyl viologen, a compound that is known to increase intracellular production of superoxide radicals and to be an inducer of Mn-SOD, could not enhance the synthesis of *P. polycolor* SOD (data not shown) (7). Proper culture conditions for SOD production is summarized in Table 1.

Table 1. The optimum culture condition for the SOD production

Medium	Glycerin	3.0%
	Polypeptone	1.0%
	Meat extract	0.5%
	KCl	0.2%
	Initial pH	9.0
Other condition	Temperature	30℃
	Culture time	15 hr
	Agitation	120 Rev. stroke (reciprocal)

100 ml of medium per 500 ml shake flask

Purification of SOD

A seed culture was grown in a 500 m*l* shake flask containing 100 m*l* of nutrient broth with continuous reciprocal shaking for a day. It was then inoculated, at a concentration of 2% (v/v), into 4 *l* of the medium shown in Table 1 by using a 10 *l* fermentor (B. Braun Melsurgen Co.). The culture was continuously aerated at 10 *l* per min and agitated at 300 rpm and 30℃ for 15 hr. Cells obtained from broth were suspended in a 0.01 M sodium phosphate buffer (pH 7.0), disrupted by sonication, and then centrifuged. Solid ammonium sulfate was gradually added to the enzyme solution to 40% saturation and left for 4 hr at 4℃. The precipitate formed was removed by centrifugation and discarded. Solid ammonium sulfate was again added to the supernatant to 80% saturation, then left overnight at 4℃. The resulting precipitate was collected, dissolved in a small amount of 0.01 M sodium phosphate buffer (pH 7.0) and then thoroughly dialyzed against the same buffer at 4℃ with several changes. The precipitate formed during the dialysis was removed by centrifugation. Solid sodium chloride was added to the supernatant to a final concentration of 0.1 M, and then the mixture was applied to a column of DEAE-Sephadex A-50 (28×240 mm) previously equilibrated with sodium phosphate buffer containing 0.1 M NaCl. After the column had been washed with the same buffer thoroughly, elution was performed with 300 m*l* of the buffer containing 0.2 M NaCl, at which point a linear gradient of NaCl (0.2 M-0.5 M) in the buffer was applied. The active fractions were pooled, dialyzed against sodium phosphate buffer and the precipitate during dialysis was removed by centrifugation. Solid sodium chrolide was added to the enzyme solution to a final concentration of 0.1 M and secondary DEAE-Sephadex ion exchange chromatography was performed according to the first (column size: 20×160 mm). Fractions with SOD activity were pooled and solid ammonium sulfate was added to the enzyme solution to 30% saturation. The enzyme was put on a column of phenyl-Toyopearl 650 M (29×80 mm) previously equilibrated with 0.01 M sodium phosphate buffer containing 30% ammonium sulfate. After the column had been washed with the same buffer containing 30% ammonium sulfate, linear gradient of ammonium sulfate (30%-0%) in this buffer was applied in a total volume of 400 m*l*. The enzyme showing SOD activity was eluted as a single peak but three electrophoretically different bands were detected. Therefore, the active portion was pooled, dialyzed against the 0.01 M sodium phosphate buffer and concentrated by ultrafiltration through a CX-10 membrane. The concentrated enzyme solution was put on a Sephadex G-100 column (25×100 mm) previously equilibrated with sodium phosphate buffer containing 0.1 M NaCl. The enzyme, which was yellow in color, was adsorbed at the upper part of the column, suggesting that *P. polycolor* SOD be Fe-SOD containing iron as a cofactor. On elution of the column with the same buffer, the enzyme showing SOD activity was eluted as a single peak, with a constant ratio of protein to SOD activity in each fraction. The active fractions were pooled and saved for the further analyses.

Homogeneity of the Enzyme

The homogeneity of the finally purified enzyme was examined by polyacrylamide disc gel electrophoresis. As shown in Fig. 1, the electrophoresis of the purified *P. polycolor* SOD gives a single discrete band by protein staining (right), corresponding to the position of the activity band(left). The purification procedures appeared not to modify the enzyme in that the location of the purified enzyme on a polyacrylamide disc gel after electrophore-

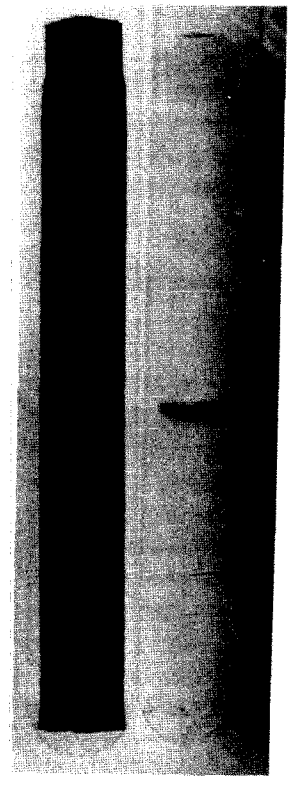

Fig. 1. Polyacrylamide disc gel electrophoresis of purified enzyme.

Purified enzyme (5 μg in left and 20 μg in right) was electrophoresed at pH 7.5. The direction of migration is from cathod to anode. The achromatic zone indicates the enzyme activity (left) and protein was stained by Amido Black (right).

Table 2. Purification of *Pseudomonas polycolor* IFO 3918 SOD

Purification stage	Volume (ml)	Total protein ($O.D._{280\,nm} \times ml$)	Total activity (units)	Specific activity (units/$O.D._{280\,nm} \times ml$)	Yield (%)
Supernatant from sonicator	420	6,040	12,800	2.1	100
40%-80% $(NH_4)_2SO_4$ fractionation	30	1,986	12,600	6.3	98
1st DEAE-Sephadex	236	264	4,933	18.7	39
2nd DEAE-Sephadex	72	73	2,569	35.2	20
Phenyl-Toyopearl 650 M	30	31	1,214	39.2	10
Sephadex G-100	42	8	774	96.8	6

sis coincided with that of the crude extract from the cells. Table 2 is a summary of the purification procedure. About 8 mg of purified enzyme was obtained with a specific activity of 96.8 units/mg and about 46-fold purification from cell-free extract was achieved with a recovery yield of about 6%.

Properties of the Purified Enzyme

Molecular Weight: The molecular weight of SOD was estimated to be approximately 20,000 by comparison with those of marker proteins on SDS-polyacrylamide gel electrophoresis. On the other hand, the molecular weight of the enzyme was found to be about 40,000 by gel filtration on a Sephadex G-100 column. The results are shown in Fig. 2-A and 2-B. These results suggest that the native enzyme is a dimer consisting of two subunits of equal size.

Effects of pH and Temperature on SOD Stability: The pH dependence of the enzyme stability was measured from the residual activity after 40 hr of preincubation at various pHs and at the constant temperature of 25℃. The purified enzyme remained comparatively stable at alkaline pH, pH 9.0~11.0, but rapidly became inactive below pH 9.0 as shown in Fig. 3-A. The thermostability of the enzyme at pH 9.0 was measured by 20 min of preincubation of the enzyme at various temperatures before the enzyme assay. The result is shown in Fig. 3-B. *P. polycolor* SOD was stable up to 45℃ with about 80% relative activity but rapidly became inactive at temperatures above that.

Effects of SOD Selective Inhibitors on the Enzyme Activity: The effects of some compounds known as selective inhibitors on SOD activity were investigated. The enzyme was incubated with each compound for 5 min at 20℃ at final concentrations of either 1.0 mM or 5.0 mM before the substrate was added. The relative activity is represented as the percentage of the activity of a control and the result is shown in Table 3. *P. polycolor* SOD was insensitive to cyanide and fluoride, but 1.0 mM H_2O_2 and 5 mM NaN_3 inhibited the enzyme about 50% and 60%, respectively. These results suggest that *P. polycolor* SOD may contain iron.

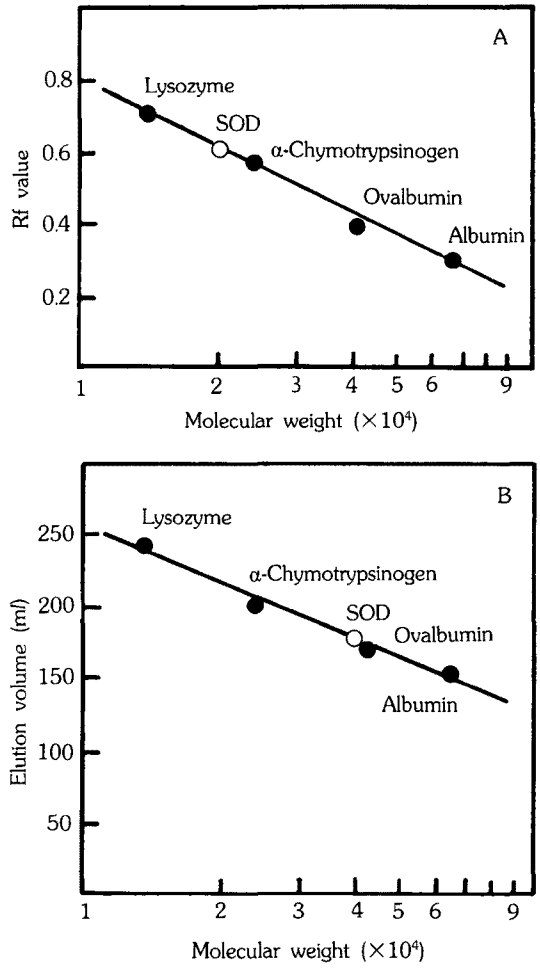

Fig. 2. Determination of molecular weight of SOD.
(A) Determination of subunit molecular weight of SOD by SDS polyacrylamide disc gel electrophoresis.
(B) Determination of molecular weight of SOD by gel filtration on Sephadex G-100.

DISCUSSION

SODs are metalloenzymes and three metallo-forms of SODs have been observed until now (15, 18). The

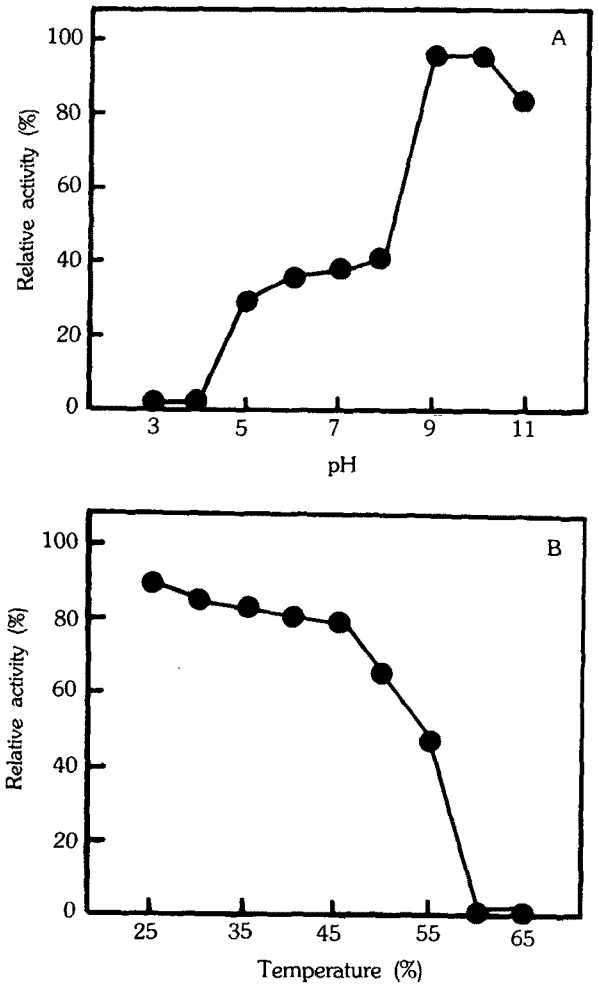

Fig. 3. Effect of pH (A) and temperature (B) on the enzyme activity.

first metallo-form of these contains both copper and zinc, the second manganese, and the third iron. The sole function of SOD is to remove the superoxide anion formed via the univalent reduction of dioxygen and thus protect the cells against oxygen toxicity. A species contains one or two SOD isozymes. Eukaryotes contain two isozymes, Cu, Zn-SOD and Mn-SOD in cytosol and in organelle, respectively. Bacteria contain Mn-SOD and/or Fe-SOD with exceptional presence of bacteriocuprein (bacterial Cu,Zn-SOD) (18). To do comparative studies of Mn- and Fe-SOD in bacteria, particularly the difference between physiological functions of the two isozymes in one species, we tried to screen the species containing both Mn- and Fe-SOD in one species by activity staining of sonicates in electrophoresis gel. Unfortunately however, all the bacteria examined contained only one SOD isozyme type. If other buffer system is used, the minor zones might be resolved. So *Pseudomonas polycolor* was selected from the bacteria examined as it produced relatively a large amount of SOD, presumably only Fe-SOD. Investigators postulated Fe-SOD might be the most primitive class among the three isozymes because of its presence in anaerobic bacteria and other prokaryotes (17, 18). Fe-SODs have not so far been found in mammalian cells, but they have been found in prokaryotes, particularly in pathogens such as *Mycobacterium tuberculosis*, *Nocardia asteroides* (10). And some infective protozoa (24) very often contain Fe-SODs. Therefore an specific inhibitor for the Fe-SOD might provide advantages as an antibacterial pharmaceutical without any damage to hosts. Considerations such as those have prompted a search for inhibitors of Fe-SOD, but such compounds have not yet been found.

Table 3. Effect of specific inhibitors on the enzyme activity

Treatment	Inhibition (%)
1.0 mM NaCN	8
5.0 mM NaCN	0
1.0 mM H_2O_2	50
5.0 mM H_2O_2	–
1.0 mM NaN_3	64
5.0 mM NaN_3	60
1.0 mM NaF	9
5.0 mM NaF	7
None	0

–, unmeasured by inhibition of autoxidation

P. polycolor SOD was similar to the other Fe-SODs isolated from most prokaryotes and eukaryotes when considered on the basis of some analyses performed. The similarities included molecular weight, subunit weight, pH and temperature stability, and effects of specific inhibitors on SOD activity (8, 9, 10, 24, 28). *P. polycolor* SOD was dimeric like most Fe-SODs studied till now, although Fe-SODs from *Mycobacterium tuberculosis*, *Methanobacterium bryantii*, and *Nocardia asteroides* are tetrameric (17, 18). Treating of *P. polycolor* SOD with H_2O_2, which selectively inactivated the Fe-containing enzyme but not Mn-SOD (17, 18), resulted in partial inactivation. The color of the enzyme solution during the final purification step, and the effects of specific inhibitors on SOD activity suggested that *P. polycolor* SOD be an Fe-SOD. Metal analysis and spectrum in visible range would confirm which metal is contained in the SOD as a cofactor.

REFERENCES

1. **Andrews, P.** 1970. Estimation of molecular size and mole-

cular weights of biological compounds by gel filtration. *Methods Biochem. Anal.* **18**: 1-7.

2. **Beauchamp, C.O., and I. Fridovich.** 1971. Superoxide dismutase: Improved assays and applicable to acrylamide gels. *Anal. Biochem.* **44**: 276-287.
3. **Davis, B.J.** 1964. Disc electrophoresis-II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **121**: 404-427.
4. **Fridovich, I.** 1986. Superoxide dismutase. *Adv. Enzymol.* **58**: 61-97.
5. **Gregory, E.M., and C.H. Dapper.** 1983. Isolation of iron-containing superoxide dismutase from Bacteroides fragilis. *Arch. Biochem. Biophys.* **220**: 293-300.
6. **Gregory, E.M., F.J. Yost, JR., and I. Fridovich.** 1973. Superoxide dismutases of *E. coli*: Intracellular localization and functions. *J. Bacteriol.*, **115**: 987-991.
7. **Hassan, H.M., and I. Fridovich.** 1977. Regulation of the synthesis of superoxide dismutase in *E. coli*. *J. Biol. Chem.* **252**: 7667-7672.
8. **Keele, B.B. JR., McCord, J.M., and I. Fridovich.** 1970. Superoxide dismutase from *E. coli* B. *J. Biol. Chem.* **245**: 6176-6180.
9. **Kim, S.W., S.O. Lee, and T.H. Lee.** 1991. Purification and characterization of superoxide dismutase from Aerobacter aerogenes. *Agric. Biol. Chem.*, **55**, 101-108.
10. **Kirby, T.W., J.R. Lancaster, Jr., and I. Fridovich.** 1981. Isolation characteriation of the iron-containing superoxide dismutase of *Methanobacterium bryantii*. *Arch. Biochem. Biophys.* **210**: 140-148.
11. **Lee, T.H., and S.O. Lee.** 1988. Purification and properties of superoxide dismutase from Bacillus circulans. *Agric. Biol. Chem.* **52**: 1361-1367.
12. **Marklund, S.L.** 1984. Extracellular superoxide dismutase in human tissues and human cell lines. *J. Clin. Invest.* **74**: 1398-1403.
13. **Marklund, S.L.** 1984. Properties of extracellular superoxide dismutase from human lung. *Biochem. J.* **220**: 269-272.
14. **Martin, J.P. JR., and I. Fridovich.** 1981. Evidence for a natural gene transfer from the ponyfish to its bioluminescent bacterial symbiont *Photobacterium leiognathi*. *J. Biol. Chem.* **256**: 6080-6089.
15. **McCord, J.M., and I. Fridovich.** 1969. Superoxide dismutase, an enzymatic function for erythrocuprein. *J. Biol. Chem.* **244**: 6049-6055.
16. **Misra, H.P., and I. Fridovich.** 1972. The role of superoxidre anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J. Biol. Chem.* **247**: 3170-3175.
17. **Oyanagui, Y.** 1981. Superoxide and medicine (in Japanese), Kyoritsu-Shuppan,Tokyo.
18. **Oyanagui, Y.** 1989. SOD and active oxygen modulators, pharmacology and clinical trials (in Japanes), Nihon-Igakukan, Tokyo.
19. **Parker, M.W., and C.C.F. Blake.** 1988. Iron- and manganese-containing superoxide dismutase can be distinguished by analysis of their primary structures. *FEBS Lett.* **229**: 377-382.
20. **Pennington, C.D., and E.M. Gregory.** 1986. Isolation and reconstitution of iron- and manganese-containing superoxide dismutase from *Bacteroides thetaiotaomicron*. *J. Bacteriol.* **166**: 528-532.
21. **Steinman, H.M.** 1985. Bacteriocuprein superoxide dismutase in *Pseudomonas*. *J. Bacteriol.* **162**: 1255-1269.
22. **Steinman, H.M.** 1987. Bacteriocuprein superoxide dismutase of Photobacterium leiognathi. *J. Biol. Chem.* **262**: 1882-1887.
23. **Tally, F.O., B.R. Goldin, N.V. Jacobus, and S.L. Gorbach.** 1977. Superoxide dismutase in anaerobic bacteria of clinical significance. *Infection and Immunity.* **16**: 20-25.
24. **Trant, N.L., S.R. Meshnick, K. Kitchner, J.W. Eaton, and A. Cerami.** 1983. Iron-containing superoxide dismutase from *Crithidia fasciculata*. *J. Biol. Chem.* **258**: 125-130.
25. **Vance, P.G., and B.B. Keele, JR.** 1972. Superoxide dismutase from *Streptococcus mutans*. *J. Biol. Chem.* **247**: 4782-4786.
26. **Weber, K., and Osborn, M.** 1969. *J. Biol. Chem.* **244**: 4406-4412.
27. **Weisiger, R.A., and I. Fridovich.** 1973. Superoxide Dismutase: Organelle Specificity. *J. Biol. Chem.* **248**: 3582-3592.
28. **Yost, F.J. JR., and I. Fridovich.** 1973. An iron-containing superoxide dismutase from *E. coli*. *J. Biol. Chem.* **248**: 4905-4908.

(Received June 21, 1993)