S20-029

Isozymes of superoxide dismutase from *Heliobacillus mobilis*

W Ittarat, H Sakurai

Department of Biology, School of Education and Division of Pure & Applied Physics, Graduate School of Science & Engineering, Waseda University, Nishiwaseda, Shinjuku, Tokyo 169-8050, Japan. Fax: +81-3-3207-9694
sakurai@mn.waseda.ac.jp

Keywords: heliobacteria, superoxide dismutase, oxidative stress, Mn-SOD

Introduction

Heliobacteria are relatively recently discovered phototrophic Gram-positive bacteria which require anaerobic conditions for growth. They usually live in soils and mud, and they must confront danger of occasional exposure to oxygen. Superoxide dismutases (SODs) are metallo-enzymes that catalyse the dismutation of superoxide (perhydroxyl radical, the undissociated from), the first reactive oxygen species formed in the metabolism of dioxygen. Generally, SODs have been categorized into three classes according to their metal contents: CuZn-SOD, Mn-SOD and Fe-SOD. Prokaryotes, bacteria and blue green algae contain either one of Mn-SOD and Fe-SOD, or both. Anaerobic photosynthetic bacteria studied thus far (green sulfur bacteria and purple bacteria) contain only Fe-SOD. Gram-positive bacteria usually contain Mn-SOD. We partially purified SOD from *H. mobilis* and studied induction kinetics of SOD activities under oxidative stress conditions

Materials and methods

H. mobilis cells were cultured under strictly anaerobic conditions at about 40^{o}C under fluorescent light of about 1000 lux and allowed to grow until nearly the late logarithmic growth phase (yield: about 4 g (fresh weight)/l). The cells were suspended in 50 mM K-phosphate (pH 7.8), 0.5 mM EDTA, 1 mM phenymethylsulfonyl fluoride (PMSF), 1 mM *p*-aminobenzamidine-2HCl, 1 mM 6-amino-caproic acid, and the suspension was sonicated with 6 cycles of 1 min burst followed by 1-min interval. The cell lysate was cleared by centrifugation at 50,000 x g for 60 min and the supernatant was saved. The supernatant was fractionated with ammonium sulfate between 35%-80% saturation and the precipitate obtained was dissolved in and dialyzed against 10 mM K-phosphate (pH 7.8) containing 0.1 mM EDTA.

SOD activity was measured by the xanthine oxidase/cytochrome *c* method (McCord and Fridovich, 1969). The rate of cytochrome *c* reduction was spectrophotometrically followed at 550 nm in 3 ml of the assay mixture. One unit of SOD is defined as the amount of enzyme required to inhibit the rate of cyt *c* reduction by 50%. Protein was determined by Bio-Rad protein assay (BSA as the standard).

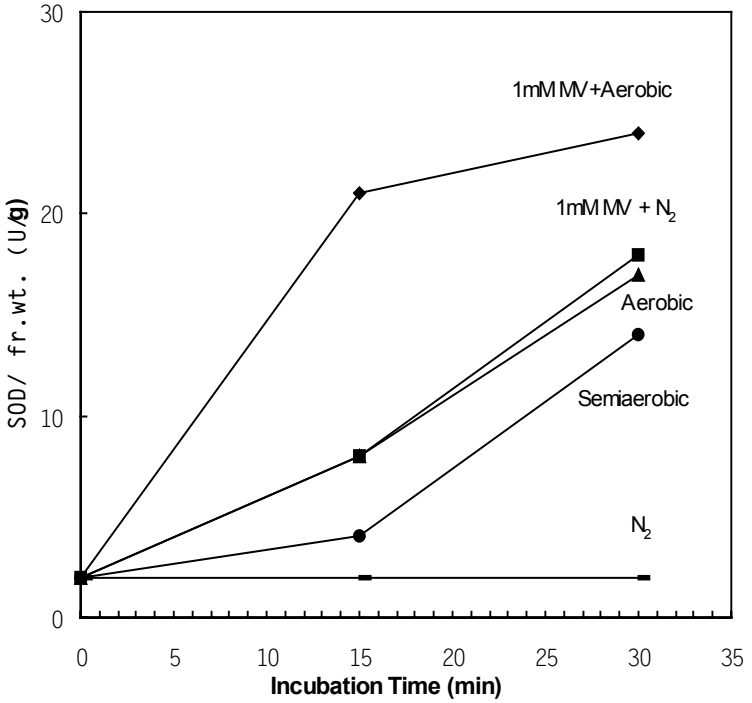

Fig. 1. Induction of SOD activity under oxidative stress conditions. The cultures were incubated under the following conditions: anaerobic (gas phase N_2), semiaerobic (air, open cap), aerobic (air, open cap with vigorous stirring) either in the presence or absence of 1 mM MV (methyl viologen) At the times indicated, the cells were harvested and the SOD activities in the supernatants were measured.

Results

Changes in SOD activity levels were studied when *H. mobilis* cells were transferred to various culture regimes (Fig.1). The results indicated that SOD activity was induced to moderate levels in cells cultured under semiaerobic conditions. In the presence of oxygen with stirring, SOD activity rapidly increased 4 and 8-fold in 15 and 30 min, respectively. When 1 mM MV was added in addition, the total activity increased 10 and 12-fold in 15 and 30 min, respectively.

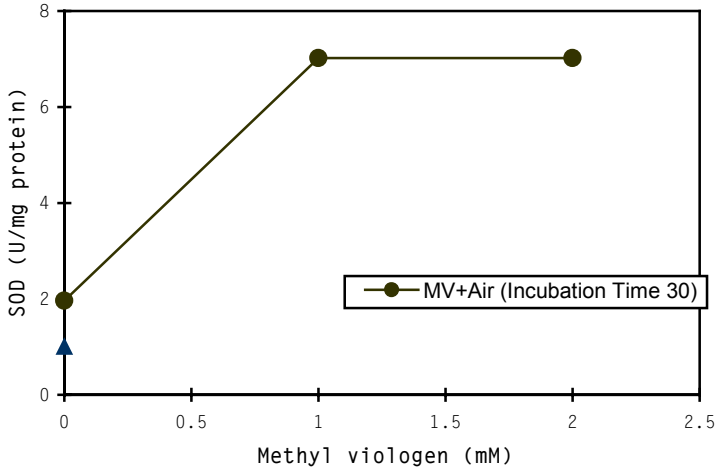

Fig. 2. Effects of MV concentration on induction of SOD activities. Cells were grow anaerobically at 40^{o} C until the cultures reached nearly the late logarithmic growth phase and challenged with the indicated concentrations of MV under air. After incubation for 30 min, SOD activities were measured.

The effects of MV concentration on induction of SOD activities revealed that the effect is saturated at 1 mM MV (Fig.2).

Table 1 Effects of hydrogen peroxide on susceptibility of SOD activities in extracts from *Chalamydomonas renihardtii, Chlorobium tepidum* and *Heliobacillus mobilis*

Fractions	Remaining SOD activity (%)				
	Incubation time				
	0min	15min	30min	60min	120min
Heliobacillus mobilis					
Whole cell fraction (Aerobic + MV 0.2mM)	100	100	100	100	100
Whole cell fraction(anaerobic)	100	100	100	100	100
Unbound DE 23	100	100	100	100	100
Bound DE 23	100	100	100	100	100
Chlorobium tepidum					
Whole cell extract	100	40	17	14	9
Chalamydoonas renihardtii					
Whole cell extract	100	46	14	11	15

Each sample containing 1-4 units of SOD was incubated with 0.9 mM H_2O_2 in 1 mM KCN and 50 mM phosphate buffer (pH 7.8) at 4°C and the activities were determined at the times indicated (Table 1). The results were compatible with the reports that *Chalamydomonas* contains Mn-SOD and Fe-SOD (Sakurai et al. 1993) and *Chlorobium* Fe-SOD (Kanematsu et al. 1978). From the insensitivity to hydrogen peroxide, heliobacterial SODs are deduced to be Mn-SODs.

Purification

The dialyzed enzyme was purified by a column of DEAE 23 cellulose (3.5 x 30 cm, Whatman) equilibrated with 10 mM potassium phosphate buffer (pH 7.8) and 0.1 mM EDTA.

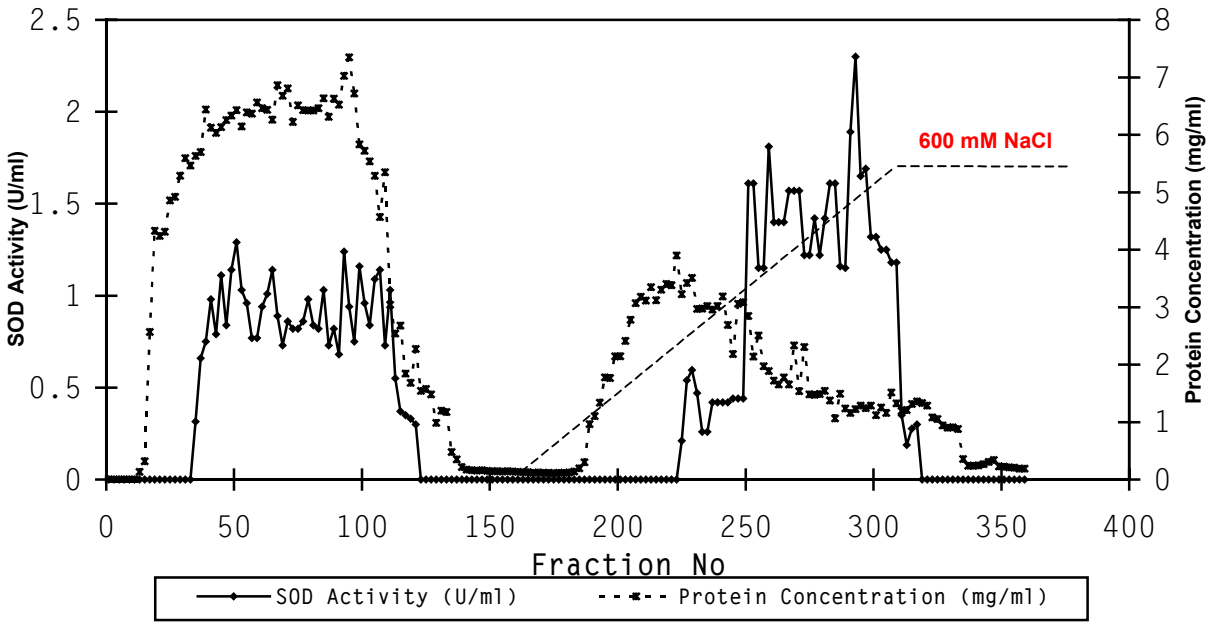

Fig. 3. Elution pattern of SOD activity from a DEAE column. The isozymes of SOD were eluted and separated from each other by eluting with increasing concentrations of KCl gradient (0 to 600 mM) in 10 mM potassium phosphate (pH 7.8)-0.1 mM EDTA. The isozymes were found in the unbound fractions as well as in the bound fractions.

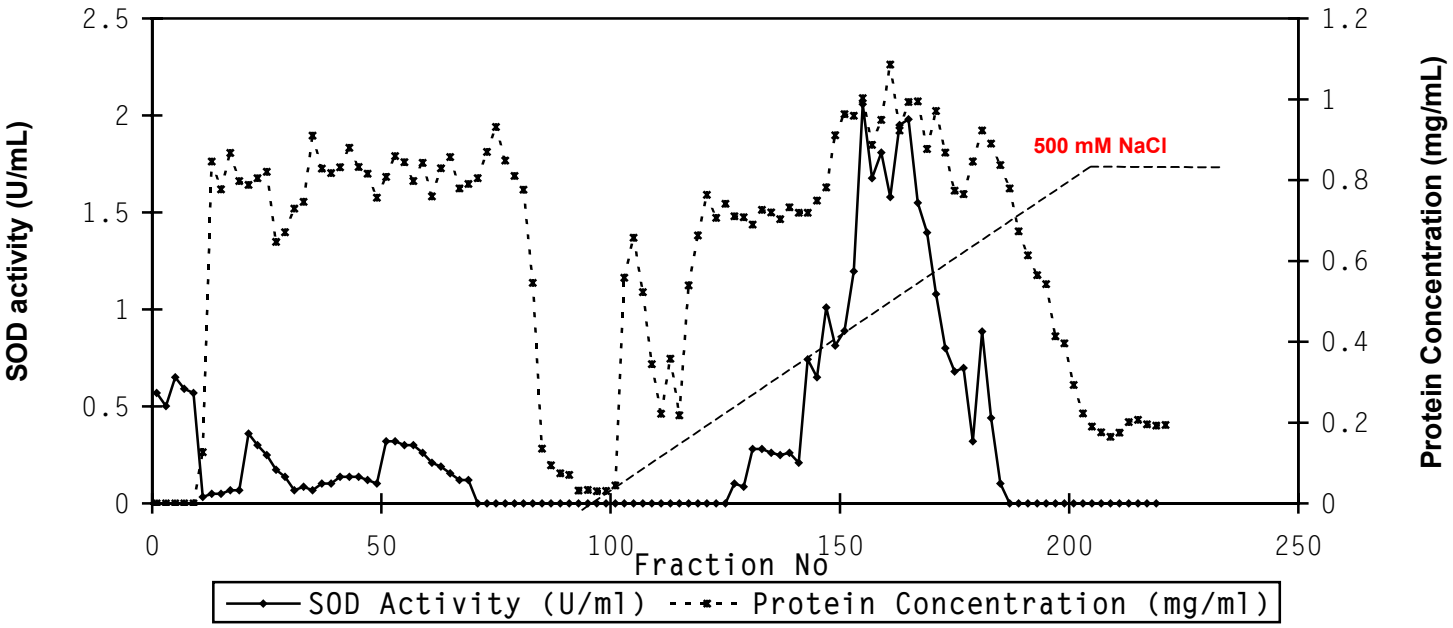

Fig. 4. Elution pattern of SOD activity from a Q-Sepharose column. The unbound fractions from a DEAE column were dialyzed against 10 mM Tris-Cl (pH 7.8)- 0.1 mM EDTA and applied to a Q Sepharose column (2.5 x 30 cm, Pharmacia) equilibrated with 10 mM potassium phosphate (pH 7.8)-0.1 mM EDTA. SOD was eluted with a linear concentration gradient of NaCl (0 to 500 mM).

SOD-active fractions were separately pooled in two (unbound and bound). Each peak fraction was concentrated with an Amicon PM10 to 5 ml and applied to a Sephacryl S-200 column (2.6 x 60 cm, Pharmacia) equilibrated with 10 mM potassium phosphate (pH 7.8), 0.1 mM EDTA and 0.1 M KCl. The SOD in the unbound fractions from a DEAE column was eluted with the same buffer, dialyzed against 10 mM potassium phosphate (pH 7.8)-0.1 mM EDTA and applied to a hydroxyapatite column (2.5 x 5 cm, Bio-Gel HTP, BioRad) equilibrated with 10 mM potassium phosphate (pH 7.8)-0.1 mM EDTA. SOD was not adsorbed to the column, eluted with the equilibration buffer and then dialyzed against 10 mM Tris-Cl (pH 7.8)-0.1 mM EDTA. The SOD was then adsorbed to a column of Mono Q (HR 5/5, Pharmacia) equilibrated with 10 mM Tris-Cl (pH 7.8)-0.1 mM EDTA. SOD was eluted with a linear concentration gradient of NaCl (0 to 500 mM). The peak fractions of SOD were combined and concentrated with a Centricon-10 (Amicon) and applied to a Superdex 75 column (1 x 30 cm, Pharmacia) equilibrated with10 mM Tris-Cl (pH 7.8), 0.1 mM EDTA-0.1 mM NaCl yielding purified SOD.

Conclusion

Lysate from *H. mobilis* cells contains two SOD isozymes. From insensitivity to hydrogen peroxide, both are deduced to be Mn-SODs. When cells were exposed to oxidative stress, SOD activity rose about 10 fold in 15 min.

Acknowledgments

This work was supported in part by Scholarship from the Ministry of Education, Science and Culture, Japan to W.I. and Grant from Iwaki Scholarship Foundation to H.S.

References

Kanematsu S, Asada K (1978) *FEBS Lett.* **91**, 94-98.
McCord JM, Fridovich I (1969) *J. Biol. Chem.***244**, 6049-6055
Sakurai H, Kusumoto N, Kitayama K, Togasaki RK (1993) *Plant Cell Physiol* **34**, 1133–1137.