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Purification and properties of mangano-superoxide dismutase from a strain of alkaliphilic *Bacillus*

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Abstract A mangano-superoxide dismutase (EC 1.15.1.1) was purified to homogeneity from a strain of alkaliphilic *Bacillus* for the first time. The purified protein, with an isoelectric point of pH 4.5, had a molecular mass of approximately 50 kDa and consisted of two identical subunits (25 kDa). The N-terminal amino acid sequence was Ala-Tyr-Lys-Leu-Pro-Glu-Leu-Pro-Tyr-Ala-Ala-Asn-Ala-Leu-Glu-Pro-His-Ile-Asp-Glu-Ala. The optimum pH and temperature for the reaction were 7.5 and 35°C, respectively. The properties of the superoxide dismutase were compared with those of the enzyme from thermophilic *Bacillus stearothermophilus*.

Key words Superoxide dismutase · Purification · Alkaliphile · *Bacillus*

Introduction

Alkaliphilic *Bacillus* spp. produce a variety of extracellular, alkaline enzymes, and the industrial use of these enzymes has been recognized mainly in terms of their applications for use in detergents (Horikoshi and Akiba 1982; Horikoshi 1996). We have found and characterized many alkaline enzymes, suitable for use in detergents, from alkaliphilic bacilli such as cellulase (Yoshimatsu et al. 1990), protease (Kobayashi et al. 1995), α-amylase (Igarashi et al. 1996), pullulanase (Ara et al. 1992), isoamylase (Ara et al. 1993), and amylopullulanase (Ara et al. 1995; Hatada et al. 1996). In addition, we have purified and characterized some intracellular enzymes, such as isocitrate dehydrogenase (Shikata et al. 1988) and glutamate dehydrogenase (Koike et al. 1996) from some of these organisms. Recently, we detected mangano-superoxide dismutase (Mn-SOD) activities universally in these alkaliphiles. SODs (EC 1.15.1.1) are a class of metalloenzymes of two main types, the eukaryotic type containing Cu + Zn and the prokaryotic type with Fe or Mn ions at the active site, which dismute superoxide radicals into hydrogen peroxide and molecular oxygen (Fridovich 1975) and are of great interest as potential therapeutic treatments for oxidative damage (Beck et al. 1988).

We report here the purification and characterization of Mn-SOD from a strain of alkaliphilic *Bacillus* for the first time. The purified SOD from the alkaliphile is also compared with the *Bacillus stearothermophilus* enzyme, which is a typical prokaryotic Mn-SOD (Bridgen et al. 1976; Block and Walker 1980).

Materials and methods

Bacterial strain and growth conditions

Bacillus sp. KSM-K16, which produces a high-alkaline protease (Kobayashi et al. 1995), was used. A cell paste was obtained after growth at 30°C for 18 h in the same medium as described elsewhere (Koike et al. 1996), except that $MnSO_4 \cdot 4–6H_2O$ was added at 0.01% (w/v) to ensure high productivity of SOD.

Enzyme assays

SOD assays, a modification of the method of McCord and Fridovich (1969), were performed in 3.0 ml of 50 mM potassium phosphate buffer (pH 7.8) in a cuvette thermostatted at 30°C. The reaction mixture contained 10 μM cytochrome *c* (horse heart prepared without using trichloroacetate; Sigma, St Louis, Mo, USA), 50 μM xanthine, and sufficient xanthine oxidase (buttermilk; Wako Pure Chemicals, Osaka, Japan) to produce a rate of reduction of cytochrome *c* at 550 nm of approximately 0.024–0.030 absorbance units/

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min. One unit of SOD activity was defined as the amount of protein required to produce 50% inhibition of the rate of reduction of cytochrome *c* under the conditions of the assay. Specific activity was expressed as the units of SOD activity per mg of protein. Protein was determined by the Folin method (Lowry et al. 1951), using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard protein.

Electrophoresis

Nondenaturing polyacrylamide gel electrophoresis (PAGE), sodium dodecylsulfate-PAGE (SDS-PAGE), and isoelectric focusing PAGE for purified enzyme were performed as described previously (Koike et al. 1996). The procedure used for activity staining of SOD, after nondenaturing PAGE, was essentially similar to the photochemical method of Beauchamp and Fridovich (1971).

Determination of N-terminal amino acid sequence

The purified enzyme was blotted to a polyvinylidene membrane. The N-terminal amino acid residues of the sample were determined directly by Edman degradation with an automated protein sequencer (model 477A; Applied Biosystems, Foster City, CA, USA).

Enzyme purification

The purification of SOD was done at 4°C as follows. Harvested cells (50 g) were suspended in 100 ml of 20 mM potassium phosphate buffer (pH 7.5). The cell suspension was treated with lysozyme (2 mg/ml; Sigma, St. Louis, MO, USA) in the presence of 2 mM $MgCl_2$ at 37°C for 30 min. The lysate obtained was further disrupted by sonication for 10 min on ice. The sonicate was centrifuged at 11 000 × *g* for 15 min and the whole resultant supernatant (140 ml) was used as the cell-free extract. The crude enzyme was prepared by treating the cell-free extract with solid $(NH_4)_2SO_4$ and isolating, by centrifugation, the fraction that did not precipitate even at 80% saturation. The supernatant fraction was directly applied to a column of Butyl-Toyopearl (2.6 cm × 13 cm; Tosoh, Tokyo, Japan) that had been equilibrated with 20 mM Tris/HCl buffer containing 2.8 M $(NH_4)_2SO_4$ (pH 7.5). The column was washed with 140 ml of the equilibration buffer, proteins were eluted with a 360-ml linear gradient from 2.8 to 0.7 M $(NH_4)_2SO_4$ in 20 mM Tris/HCl buffer (pH 8.0), and 7-ml fractions were collected.

The active fractions eluted at around 1 M $(NH_4)_2SO_4$ were combined and dialyzed against a large volume of 20 mM phosphate buffer (pH 8.0). The retentate was then put on a column of DEAE-Sepharose FF (1.6 cm × 20 cm; Pharmacia, Uppsala, Sweden) equilibrated with 20 mM phosphate buffer (pH 8.0) and unadsorbed proteins were eluted with 100 ml of the same buffer. Elution of SOD was done with a 200-ml linear gradient from 20 to 100 mM phosphate buffer (pH 8.0) at a flow rate of 0.75 ml/min per cm^2, and 3-ml fractions were collected. The active fractions were eluted at 70 mM phosphate. They were combined and concentrated by ultrafiltration on a PM-10 membrane (10 kDa cutoff; Amicon, Denvers, MA, USA), and the concentrate was used as the final preparation of purified enzyme.

Results and discussion

Purification of SOD

The result of a typical purification procedure is summarized in Table 1. The degree of purification of the final preparation of enzyme was examined by nondenaturing PAGE (Fig. 1a) and SDS-PAGE (Fig. 1c). Only one protein band could be detected by both PAGE techniques. The sharp protein band on the nondenaturing PAGE coincided exactly with a single activity band seen by an activity staining technique (Fig. 1b).

Absorption spectra

The purified enzyme was a reddish-purple protein, which is characteristic of Mn-SODs. The enzyme had a broad absorption band with a maximum at 470 nm and a shoulder near 600 nm, in addition to protein band at 282 nm, as shown in Fig. 2, and these spectra are reminiscent of the comparable spectra for other Mn-SODs (Vance and Keele 1972; Block et al. 1976). The absorption coefficients ($A^{\%}_{cm}$) of the purified enzyme was 13.7 at 282 nm.

Table 1. Summary of the purification of Mn-SOD from *Bacillus* sp. KSM-K16

Step	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Cell-free extract	44 800	1736	26	100	1
Treatment with $(NH_4)_2SO_4$	22 400	112	200	50	7.7
Butyl-Toyopearl	3000	17	177	7	6.8
DEAE-Sepharose	2520	11	229	5	8.8

Mn-SOD, mangano-superoxide dismutase.

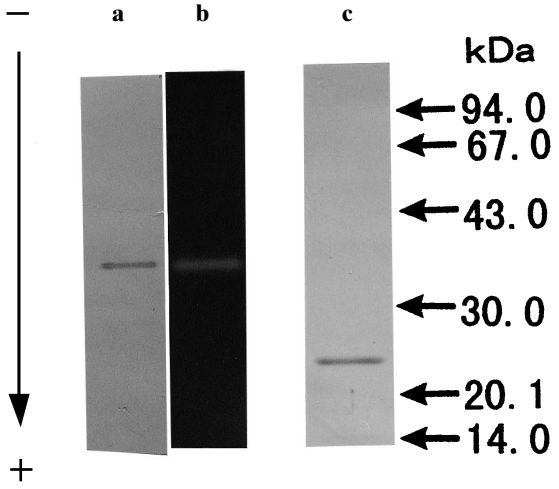

Fig. 1. Nondenaturing polyacrylamide gel electrophoresis (PAGE) (*a*), activity staining PAGE (*b*), and sodium dodecyl sulfate (SDS)-PAGE (*c*) of the purified preparation of superoxide dismutase (SOD) from *Bacillus* sp. KSM-K16. Concentrations of acrylamide were 12% for nondenaturing (*a*) and activity staining PAGE (*b*), and 12.5% for SDS-PAGE (*c*). Proteins were stained with Quick Coomassie Brilliant Blue dye (Wako Pure Chemicals) (**a**, **c**) and activity staining was done according to Beauchamp and Fridorich (1971) (**b**). *Arrows* on the right of **c** show the positions of marker proteins (expressed in kDa, Pharmacia) that include rabbit muscle phosphorylase *b* (94), bovine serum albumin (67), egg white albumin (43), bovine erythrocyte carbonic anhydrase (30), soybean trypsin inhibitor (20.1), and bovine milk α-lactalbumin B (14)

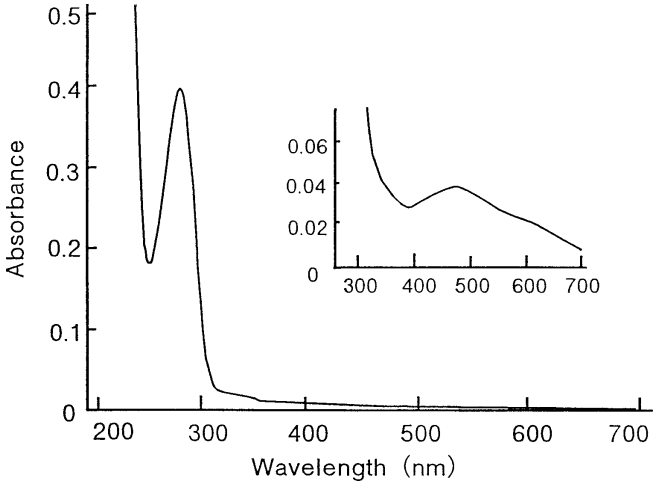

Fig. 2. Absorption spectra of the purified SOD in the ultraviolet and the visible. Spectra were recorded with a Beckman DU spectrophotometer. The enzyme was at 0.3 mg/ml in 50 mM potassium phosphate buffer (pH 8.0)

N-terminal amino acid sequence

The N-terminal amino acid sequence of the purified enzyme, determined by Edman sequencing, was Ala-Tyr-Lys-Leu-Pro-Glu-Leu-Pro-Tyr-Ala-Ala-Asn-Ala-Leu-Glu-Pro-His-Ile-Asp-Glu-Ala. The sequence showed a high homology to those of Mn-SODs from thermophilic bacteria, such as *Bacillus stearothermophilus* (12 out of 21 residues) (Parker and Blake 1988a,b) and *B. caldotenax* (12 out of 21 residues) (Chambers et al. 1992). The sequence was also similar to those of Mn-SODs from a gram-negative *Escherichia coli* (13 out of 21 residues) (Parker and Blake 1988b) and an oral *Streptococcus mutans* (13 out of 21 residues) (Nakamura 1992), as shown in Fig. 3.

Molecular mass and isoelectric point

An apparent molecular mass of 50 kDa was found for the purified Mn-SOD, as estimated by gel chromatography on a calibrated column of Sephacryl S-200 (1.6 × 85 cm, Bio-Rad) (data not shown). The subunit size was determined to be approximately 25 kDa by SDS-PAGE (Fig. 1c), indicating that the SOD consists of two subunits with an identical molecular mass. The isoelectric point of the native protein was pH 4.5.

Effects of pH and temperature

The enzyme had a pH optimum of 7.5 at 30°C in 70 mM Britton-Robinson buffer, as shown in Fig. 4a. In 50 mM phosphate buffer, the pH optimum was 7.8. As shown in Fig. 4b, the enzyme was stable to incubation at 4°C for 24 h over a range between pH 5.0 and pH 10 in 30 mM Britton-Robinson buffer.

The optimum temperature was around 35°C in 50 mM phosphate buffer (pH 7.8), as shown in Fig. 5a. The stability to heating of the SOD activity was examined after the enzyme had been heated at various temperatures for 10 min at pH 7.8 in 100 mM phosphate buffer. The enzyme was stable up to 55°C and above this temperature it was inactivated moderately (Fig. 5b).

In this study, we also characterized the thermophilic *B. stearothermophilus* Mn-SOD (Seikagaku Kogyo, Tokyo, Japan; code no. 120331) and compared its properties with those of the alkaliphilic *Bacillus* sp. KSM-K16 enzyme, as shown in Figs. 4 and 5. The enzymes resemble each other with respect to their responses to pH and temperature,

	1								10								20
B. stearothermophilus	**P**	**F**	**E**	**L**	**P**	**A**	**L**	**P**	**Y**	**P**	**Y**	**D**	**A**	**L**	**E**	**P**	**H**	**I**	**D**	**K**	**E**
Bacillus sp. KSM-K16	A	Y	K	**L**	**P**	E	**L**	**P**	**Y**	A	A	N	**A**	**L**	**E**	**P**	**H**	**I**	**D**	E	A
B. caldotenax	**P**	**F**	**E**	**L**	**P**	**A**	**L**	**P**	**Y**	**P**	**Y**	**D**	**A**	**L**	**E**	**P**	**H**	**I**	**D**	**K**	**E**
S. mutans	A	I	L	**L**	**P**	D	**L**	**P**	**Y**	A	**Y**	**D**	**A**	**L**	**E**	**P**	**Y**	**I**	**D**	A	**E**
E. coli	S	Y	T	**L**	**P**	S	**L**	**P**	**Y**	A	**Y**	**D**	**A**	**L**	**E**	**P**	**H**	F	**D**	**K**	Q

Fig. 3. Comparison of the N-terminal amino acid sequence of *Bacillus* sp. KSM-K16 Mn-SOD with those of Mn-SODs from other bacteria. Amino acid residues identical to those in the *B. stearothermophilus* sequence are shown by *bold letters*

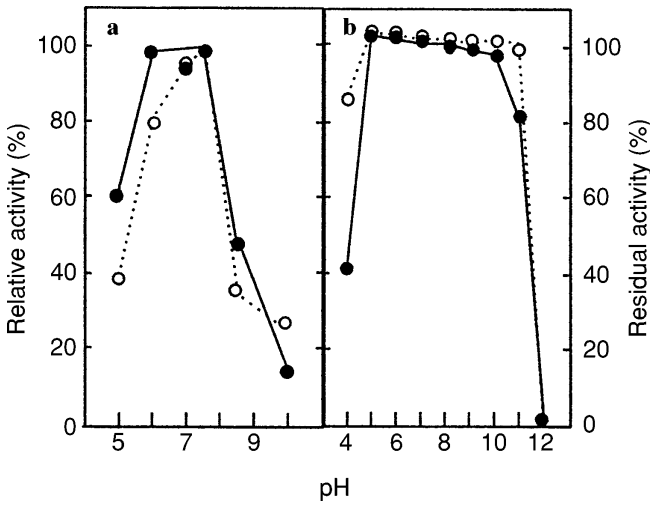

Fig. 4. Effect of pH on the activity and stability of the Mn-SOD from *Bacillus* sp. KSM-K16. **a** The activity of the SOD purified from *Bacillus* sp. KSM-K16 was measured at 30°C in 70 mM Britton-Robinson buffers at the indicated pH (*solid circles*). The commercial *B. stearothermophilus* SOD from Seikagaku Kogyo (code no. 120331) was used as reference enzyme (*open circles*). The activities at different pH are expressed as the percentages of the respective original activity. **b** The stability of the two enzymes was checked by incubation at 4°C for 24 h in 30 mM Britton-Robinson buffers at the indicated pH. Aliquots (0.1 ml) were withdrawn and the remaining SOD activity was then measured at 30°C and at pH 7.8 in 50 mM potassium phosphate buffer. The activities were expressed as the percentage of the respective original activity: *solid circles*, the result with *Bacillus* sp. KSM-K16 enzyme *open circles*, the result with *B. stearothermophilus* enzyme

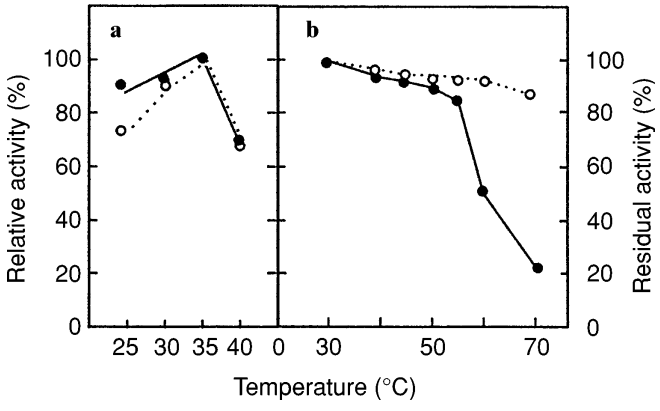

Fig. 5. Effect of temperature on the activity and stability of the Mn-SODs from *Bacillus* sp. KSM-K16 and *B. stearothermophilus*. **a** Initial velocities of the SOD activities of *Bacillus* sp. KSM-K16 enzyme (solid circles) and *B. stearothermophilus* enzyme (open circles) were measured at the indicated temperatures and at pH 7.8 in 50 mM potassium phosphate buffer. The SOD activity at 35°C for each enzyme was taken as 100%. **b** The stability of both enzymes to heating was examined by incubation for 10 min at the indicated temperatures in 0.1 M phosphate buffer (pH 7.8). After heating, the residual SOD activities of *Bacillus* sp. KSM-K16 (solid circles) and *B. stearothermophilus* (open circles) were measured at 30°C and at pH 7.8 in 50 mM potassium phosphate buffer and expressed as the percentage of the respective original activity

including molecular mass and isoelectric point (Tomita et al. 1987) (also see the enzyme data sheet library of Seikagaku Kogyo). These results suggest that Mn-SODs from the alkaliphilic and the thermophilic *Bacillus* strains share a common ancestry, although there is a slight difference in susceptibility to heating. Our work is now directed toward establishing an economical process for the simultaneous large-scale isolation of useful enzymes, including Mn-SOD, isocitrate dehydrogenase (Shikata et al. 1988), and glutamate dehydrogenase (Koike et al. 1996), from cell wastes of alkaliphilic *Bacillus* after the fermentation for the primary purpose of producing alkaline exoenzymes used in detergents.

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