

Purification and characterization of a hyperthermostable Mn-superoxide dismutase from *Thermus thermophilus* HB27

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Abstract Thermostable Mn-dependent catalases are promising enzymes in biotechnological applications. In the present study, a Mn-containing superoxide dismutase of the hyperthermophilic *Thermus thermophilus* HB27 had been purified and characterized by a two-stage ultrafiltration process after being expressed in *E. coli*. The enzyme was highly stable at 90°C and retained 57% activity after heat treatment at 100°C for 1 h. The native form of the enzyme was determined as a homotetramer by analytical size exclusion chromatography and sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The final purified enzyme had an isoelectric point of 6.2 and a high α -helical content of 70%, consistent with the theoretical values. This showed that the purified SOD folded with a reasonable secondary structure.

Keywords Superoxide dismutase · *Thermus thermophilus* · Purification · Enzyme characterization

Abbreviations

Mn-SOD	Manganese superoxide dismutase
CD	Circular dichroism
IEF	Isoelectric focusing
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
IgG	Immunoglobulin G
BSA	Bovine serum albumin
PES	Polyethersulfone
MWCO	Molecular weight cut-off
RC	Regenerated cellulose
PCR	Polymerase chain reaction
pI	Isoelectric point
<i>E. coli</i>	<i>Escherichia coli</i>

Introduction

Superoxide dismutase (SOD, EC 1.15.1.1), which catalyzes the dismutation of the superoxide anion (O_2^-) into hydrogen peroxide and molecular oxygen, is one of the most important metalloenzymes in the first line of defense against oxidative stress (McCord and Fridovich 1969; Michalski 1996; Sharma et al. 2005). Due to its antioxidative effects, SOD has been widely applied in medical treatments, and in the cosmetic (Neuilly-sur-Seine et al. 1978), food (Kumar et al. 2006), agricultural (Zhang et al. 2004) and chemical industries.

In industry, a major requirement for commercial SOD is the thermal stability, because thermal denaturation is a common cause of enzyme inactivation. In recent years, there has been increasing interest in SOD of thermophiles, which are expected to produce thermostable SOD (Li et al. 2005). It has been reported that SODs were isolated from

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hyperthermophiles of the genera *Aquifex* (Lim et al. 1997), *Aeropyrum* (Yamano et al. 1999), *Sulfolobus* (Yamano and Maruyama 1999) and *Pyrobaculum* (Whittaker and Whittaker 2000). *Thermus thermophilus* HB27 is an extremely thermophilic, halotolerant bacterium, which was originally isolated from a natural thermal environment in Japan. This organism has considerable biotechnological potential; many thermostable proteins isolated from members of the genus *Thermus* are indispensable in research and in industrial applications (Henne et al. 2004). However, little attention has focused on Mn-SOD from *T. thermophilus*.

In this work, a hyperthermostable Mn-SOD from *T. thermophilus* HB27 was expressed in *E. coli* and purified by a membrane-based process. The resulting Mn-SOD product was then analyzed by circular dichroism (CD), isoelectric focusing (IEF) and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) to confirm its molecular secondary structure, isoelectric point, and molecular weight.

Materials and methods

Materials

Ferritin (Cat. No. 96701), immunoglobulin G (IgG, Cat. No. 15506), bovine serum albumin (BSA, Cat. No. A-4503), ribonuclease A (Cat. No. R6513) were obtained from Sigma and used as supplied. Polyethersulfone (PES) ultrafiltration membrane (50 kDa molecular weight cut-off (MWCO), Cat. No. PBQK09005) and regenerated cellulose (RC) ultrafiltration membrane (100 kDa MWCO, Cat. No. 14451) were purchased from Millipore. Restriction enzyme and polymerase were obtained from TaKaRa (Dalian, China). Primer synthesis and DNA sequencing were done in Sangon (Shanghai, China). Kits and other reagents used in molecular manipulations were obtained from Intron Company. The other chemicals used in the preparation were all purchased from Sinopharm Chemical Reagent Corporation and were of analytical or higher grade.

Bacterial culture and molecular cloning

T. thermophilus HB27 was purchased from American Type Culture Collection (accession number: BAA-163). *T. thermophilus* HB27 strain was grown at 70°C in a rich medium (TM) or a minimal medium (MM), as described previously (Koyama et al. 1986). When necessary, proline and/or uracil (50 µg/ml each) were added to MM. *E. coli* BL21 (DE3) strains were grown in Luria–Bertani (LB) medium. Kanamycin (50 µg/ml) was added to the medium when necessary. The encoding sequence for SOD from *T. thermophilus* HB27 was amplified with the genomic

DNA as template using polymerase chain reaction (PCR). Two specific oligonucleotides containing recognition sequences for *Eco*RI and *Sal*I (underlined parts) at the 5' ends of forward and reverse primers were used in the amplification system to facilitate the subsequent manipulations (forward, 5'-AAGAATTCATGCCGTACCCGTTCAAGCT-3'; reverse, 5'-CTGTCTGACTCAGGCTTTATTGAAGAAC-3'). Amplification was carried out in a TaKaRa PCR thermal cycler with the parameter settings as follows: pre-denaturation 95°C, 3.0 min; denaturation 94°C, 45 s; annealing 50°C, 45 s and extension at 72°C for 1.0 min (30 cycles). After the cycles completed, an extension at 72°C for 10.0 min was added to ensure the completeness of the amplified products. The right-sized PCR product was recovered after agarose electrophoresis and was digested with enzymes *Eco*RI and *Sal*I according to the manufacturer's instruction. Subsequently, the double-digested PCR product was ligated into between the *Eco*RI and *Sal*I sites in plasmid pET-28a⁽⁺⁾ (Stratagene) treated with the same two enzymes. The sequence of SOD gene in the recombinant plasmid (designated as pETSOD) was confirmed by commercial DNA sequencing.

Protein expression and purification

The recombinant pETSOD was transformed into competent cell *E. coli* BL21 (DE3). Expression of the SOD was performed according to the pET system manual (Novagen, Germany). Since the SOD was manganese-containing protein, manganese ion was supplemented (as MnCl₂) to a previously determined initial concentration 2 mM with inducer IPTG (Isopropyl β-D-1-thiogalactopyranoside, 0.01 mM) to start the induced expression. Expression was optimized to be 8 h at 37°C with orbital shaking at a speed of 250 rotations per min. Two hundred milliliters of culture broth was harvested by centrifugation at 10,000g at 4°C for 10 min. Pellets were washed twice and resuspended in 1× TE buffer (10 mM Tris–HCl plus 1 mM EDTA, pH 8.0). Cell disruption was performed in ice bath by an ultrasonic processor. Cell lysates were sampled as total proteins that would be subjected to subsequent SDS–PAGE analysis, enzyme activity assay, and purification. The experimental set-up used for enzyme purification comprised two main parts: a stirred cell ultrafiltration module and an AKTA prime liquid chromatography system (GE Healthcare, USA), as previously described (Liu et al. 2007, 2010). The stirred cell had a working volume of 16.0 mL and was fitted with a membrane disk having an effective diameter of 24 mm. There was a suspended bar impeller with a diameter of 20 mm inside the module, which was magnetically driven by a RCT basic stirrer (IKA, Japan Y.K.). The stirring speed was monitored using a digital photo/contact tachometer (RS 163-5348, RS components). The

AKTA prime liquid chromatography system was used for pumping the buffer through the membrane housed within the stirred cell, as well as for process control and monitoring. Separating SOD from smaller impurity proteins was carried out with 50 kDa PES membranes. The operating conditions were pH 8.0 (sodium phosphate buffer at 50 mM), 1,200 rpm, and 26.5 L/m²h. After the first ultrafiltration step, the permeation was used as feed and the 100 kDa RC membranes were employed under the same conditions for further fractionation of SOD from larger impurity proteins. Each ultrafiltration step lasted for 4 h from injection. The retentate of the second ultrafiltration step was collected and used as the final SOD product for characterization. The two-stage ultrafiltration process was repeated at least three times, and the data reported are the average values. For each ultrafiltration experiment, a fresh membrane disk was used.

Enzyme activity assay and protein determination

SOD activity was determined by a modified procedure of pyrogallol autooxidation (Gu et al. 2006; Xu et al. 2001). The enzyme solution (20 μ L) was mixed with 4.96 mL of 50 mM Tris–EDTA at pH 8.2. After preincubation for 5 min at 25°C, the reaction was initiated by the addition of 20 μ L of 50 mM pyrogallol solution in 10 mM HCl. The change of absorbance at 325 nm was measured every 30 s for 4 min at 25°C. One unit of SOD activity was defined as the amount of enzyme required to inhibit the autooxidation of pyrogallol to 50%. The protein concentration was measured by the Folin–phenol method (Lowry et al. 1951). All measurements in this experiment were made in triplicate.

Circular dichroism spectroscopy analysis

CD experiments were performed on PMS 450 spectropolarimeter (Biologic, France) with a 1-mm path length cell at 25°C. The CD spectrum was recorded over a wavelength range of 190–250 nm with 1 nm resolution and 5 s of average time. Ultrapure water was used as the blank to correct the baseline. Results were expressed as the molar mean residue ellipticity (θ) at a given wavelength.

Native molecular weight determination

The native molecular weight of SOD was determined using an FPLC system (Amersham Biosciences, GE) equipped with a size exclusion chromatography column Superose 6 10/300 GL (Tricorn, GE Healthcare, Sweden). The elution was 50 mM sodium phosphate buffer (pH 8.0) containing 0.15 M NaCl. The effluent stream was monitored at 280 nm using a UV detector UV–Vis (Unicam UV-2450). Gel filtration chromatography was performed at room

temperature and a gel filtration protein marker comprising ribonuclease A (13.7 kDa), BSA (68 kDa), IgG (150 kDa) and ferritin (440 kDa) was used.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The SDS–PAGE analysis was performed under reducing conditions using NuPAGE 4–10% Bis–Tris mini gels (Invitrogen, USA), following the manufacturer's instructions. Protein bands were detected by staining with Brilliant Blue Coomassie G-250.

Isoelectric focusing

The isoelectric point (*pI*) of SOD was examined with the Rotofor isoelectric focusing cell (Bio-Rad, USA) using Bio-Lyte ampholyte (pH 3.9–9.5, Cat. No. 163-1112) to produce the necessary pH gradient. The SOD sample (18 mL) was loaded into the Rotofor cell and constant power (10 W) was applied with the system cooled to 4°C. Initial voltage was 570 V and a plateau of 1,100 V was reached after 4 h. A total of 20 fractions were collected in separate vials, using a vacuum source attached through plastic tubing to an array of 20 needles. The SOD assay was measured for each of the Rotofor fractions, and pH values determined.

Effect of pH and temperature on activity of the SOD

The influence of pH on SOD activity was determined at 35°C in 50 mM citrate buffer (pH 3.0–5.0), 50 mM sodium phosphate buffer (pH 6.0–7.0), 50 mM Tris–HCl (pH 8.0–9.0), and 50 mM glycine–NaOH (pH 10.0–11.0). All pH values were adjusted at room temperature and activity was estimated as percentage of the maximum. Optimum temperature was determined in 50 mM sodium phosphate buffer (pH 7.8) over a temperature range of 60–120°C for 30 min.

Thermostability

Thermostability of SOD was examined in the range of 70–120°C. The purified enzyme was incubated in 50 mM sodium phosphate buffer (pH 7.8) for 1 h. The remaining enzyme activity was then measured as described above.

Effect of chemicals on SOD activity

The effects of metal ions on SOD were examined by determining the activities after 1 h incubation at 25°C in 50 mM phosphate buffer (pH 7.8) containing various metal ions at 10 mM. The activity assayed in the absence of

metal ions was defined as control. The metal ions tested include $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, ZnCl_2 , KCl , AlCl_3 , NiCl_2 , CaCl_2 , FeCl_2 , $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, HgCl_2 , and MnCl_2 .

Results and discussion

Protein purification

The purification of Mn-SOD is summarized in Table 1. Total Mn-SOD activity from the initial soluble cell extract was 40,394.9 U. After the two ultrafiltration steps, the Mn-SOD was purified 3.4-fold, with a yield of 86% to a specific activity of 1,417.5 U/mg of protein. Approximate purity of the resulting Mn-SOD product was 93%, corresponding with the purity of 92.6% determined from SDS-PAGE results (Fig. 1).

Secondary structure analysis by CD spectroscopy

CD is a valuable method to estimate the secondary structure of proteins and to determine whether a protein is correctly folded or not. The far-UV CD spectroscopy from 190 to 250 nm was used to estimate the contents of the secondary structures from the ultrafiltration-purified Mn-SOD. The CD profile displayed a typical alpha-helix curve with ~66% helix content (Fig. 2). This value is very close to the theoretical value of ~70% (<http://www.embl.de/~andrade/k2d/>) (Chang et al. 1997; Yang et al. 1986). The results suggested that the purified Mn-SOD folded with a reasonable secondary structure.

Approximate molecular weight and pI

To determine the molecular weight and molecular structure of the recombinant Mn-SOD, gel filtration chromatography and reducing SDS-PAGE analysis (Fig. 1) were performed. The native molecular weight of Mn-SOD was found to be approximately 96 kDa on Superose 6 10/300 GL column,

while denaturing SDS-PAGE analysis showed a single band near 24 kDa. This indicates that the recombinant Mn-SOD is probably a homo-dimeric protein consisting of four identical subunits. The pI of the enzyme was found to be 6.2, which is close to the theoretical value of 6.4.

Thermostability

The thermostability was investigated by preincubating the enzyme in 50 mM sodium phosphate buffer (pH 7.8) for 1 h. The enzyme was thermostable at 80 and 90°C and retained 57% activity after 60 min at 100°C (Fig. 3). The half-life of the Mn-SOD at 100°C was approximately 70 min and even retained 20% activity after 30 min at 110°C. For comparison of SOD from fungi, *T. thermophilus* Mn-SOD is more thermostable than the SODs from *Thermomyces lanuginosus* (Li et al. 2005), *Aspergillus fumigatus* (Holdom et al. 1995) and *Aspergillus flavus* (Holdom et al. 1996). To our knowledge, the Mn-SOD from *T. thermophilus* HB27 is the most thermostable SOD so far isolated from fungi.

Effect of pH and temperature on SOD activity

The activity of the recombinant Mn-SOD was measured in different buffers with pH ranging from 3.0 to 11.0. As shown in Fig. 4a, Mn-SOD exhibited optimal activity at pH 5.0–7.0. Outside this range, activity was lost rapidly. Optimum pH of the enzyme was 5.0. The effect of temperature on Mn-SOD revealed that the enzyme was optimally active at 80°C (Fig. 4b). As Fig. 4b shows, a slight increase of the enzyme activity occurred when the temperature increased from 60 to 80°C. When the temperature exceeded 100°C, the enzyme activity decreased rapidly.

Effect of chemicals on the activity of SOD

The effect of chemical reagents on Mn-SOD activity was tested (Table 2). Among the cations used, Cu^{2+} , Zn^{2+} ,

Table 1 Purification of manganese-containing superoxide dismutase from *T. thermophilus* HB27

Purification steps	Total protein (mg) ^a	Target protein (mg) ^b	Specific activity (U/mg)	Yield (%) ^c	Approximate purity (%)
Soluble cell extract ^d	438.5	96.5	418.6	100	22
After first ultrafiltration	189	90.7	675.2	94	48
After second ultrafiltration	89.2	83	1417.5	86	93

^a Total protein concentration was measured by Folin–phenol method

^b The amount of target protein in total protein was determined using FPLC system equipped with a size exclusion chromatography column Superose 6 10/300 GL

^c The purification yield was calculated based on the amount of target protein

^d The start material was 0.9 g of *E. coli* BL21 (DE3) bacterial pellets (wet weight) from 200 mL culture

Fig. 1 Elution profile of Mn-SOD on column Superose 6 10/300 GL and SDS-PAGE analysis of Mn-SOD samples. *M* Molecular mass marker, *I* Purified Mn-SOD, *II* Crude enzyme solution from induced cells, *III* Crude enzyme solution from uninduced cells

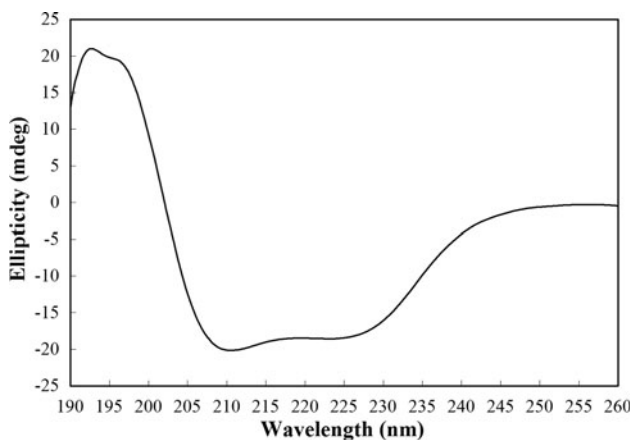
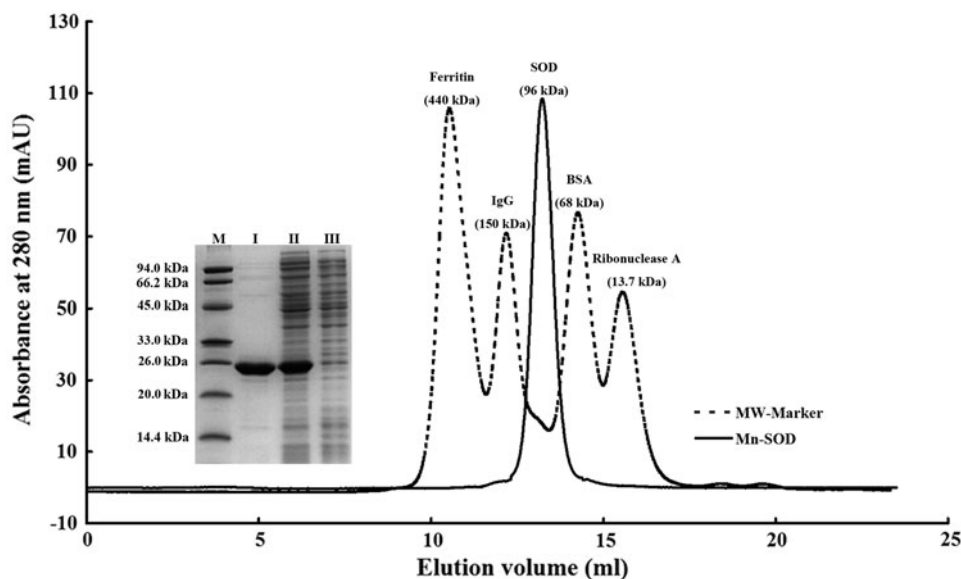


Fig. 2 Circular dichroism spectrum of purified Mn-SOD sample

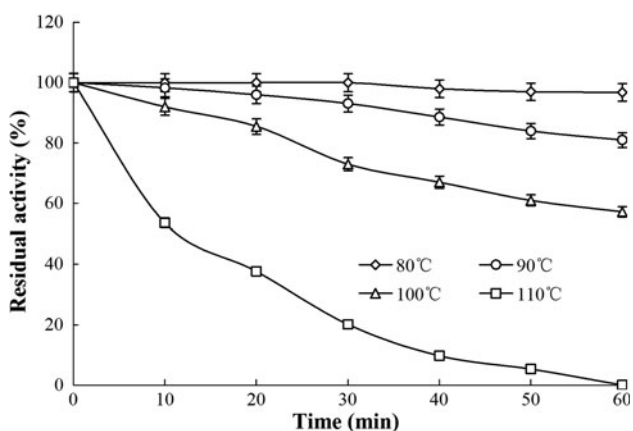


Fig. 3 Kinetics of thermostability of Mn-SOD

Mn²⁺ and Al³⁺ were found to have a stimulating effect on the enzyme activity, whereas Ni²⁺, Hg²⁺, Fe²⁺, and Mg²⁺ were found to be strong inhibitors.

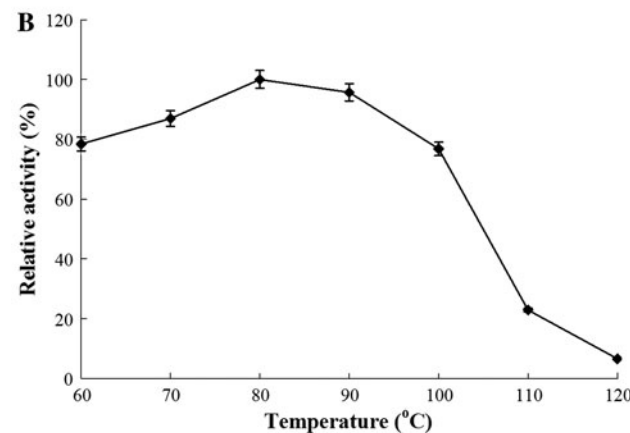
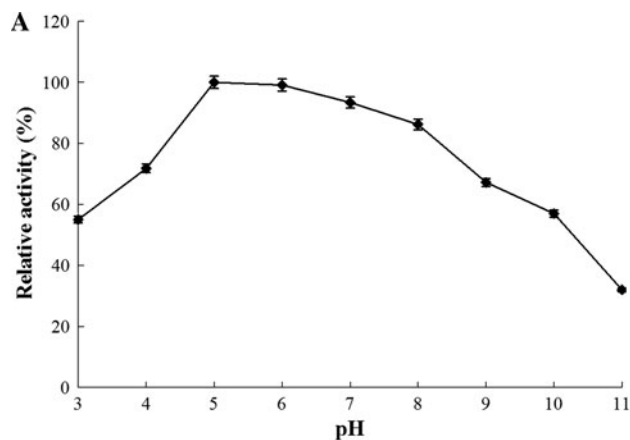


Fig. 4 Effect of pH and temperature on the activity of Mn-SOD

In order to prove the type of the recombinant SOD, the SOD inhibitors KCN and H₂O₂ were used to determine the effect on SOD activity. It was found that the recombinant SOD was inhibited by KCN, but not by H₂O₂. The

Table 2 Effects of various reagents on the activity of manganese-containing superoxide dismutase

Reagents	Relative activity (%)	Reagents	Relative activity (%)
None	100	None	100
K ⁺	86	Zn ²⁺	148
Cu ²⁺	116	Fe ²⁺	57
Ni ²⁺	43	Al ³⁺	110
Hg ²⁺	65	Co ²⁺	75
Mn ²⁺	112	Mg ²⁺	36

Enzyme activities were determined using the standard method of assay described in “Materials and methods” after preincubation of the enzymes in 50 mM phosphate buffer (pH 7.8) containing 10 mM metal ions below compounds at 25°C for 1 h

inhibition of the SOD activity by KCN and H₂O₂ was 3.1 and 98.2%, respectively. These results indicate that the recombinant SOD from *T. thermophilus* HB27 is the Mn-SOD type (Michalski 1996).

Conclusions

Thermal denaturation is still the common cause of SOD inactivation in industrial application. In the present study, a homogeneous recombinant manganese-containing superoxide dismutase, which is the most thermostable SOD so far, from *T. thermophilus* HB27 was obtained by expressing in *E. coli* and purifying with a novel membrane-based process. In addition, the resulting Mn-SOD product was then analyzed by various biotechnical methods to obtain the molecular properties. The present results suggest that this recombinant Mn-SOD may have a wide potential implication for both industrial use and fundamental studies.

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