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ORIGINAL PAPER

Cloning and characterization of a thermostable superoxide dismutase from the thermophilic bacterium *Rhodothermus* sp. XMH10

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Abstract A superoxide dismutase (SOD) gene was cloned from the thermophilic bacterium *Rhodothermus* sp. XMH10 for the first time and highly expressed in *Escherichia coli*. The *Rhodothermus* sp. XMH10 SOD (RhSOD) gene encodes 209 amino acids with a putative molecular weight of 23.6 kDa and a p*I* value of 5.53. The recombinant RhSOD was detected to be an iron type SOD and existed as a dimer on its natural status. Experiments revealed that this RhSOD showed high activity at 50–70 °C and pH 5.0. Compared to SODs from other thermophiles, it was highly thermostable, maintaining more than 90% of its activity after incubation at 70 °C for 12 h, only totally inactivated after more than 4-h incubation at 80 °C. It also showed much higher resistance to KCN, NaN_3 and H_2O_2 as compared to other SODs.

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Keywords Inhibitor · *Rhodothermus* · Superoxide dismutase · Thermophile · Thermostability

Abbreviations

RhSOD	Superoxide dismutase cloned from *Rhodothermus* sp. XMH10
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
PMS	Phenazine methosulfate

Introduction

Superoxide dismutases (SODs), a kind of metalloenzymes, have the capability to convert superoxide radicals to oxygen and hydrogen peroxide [3, 12]. According to the metal located at the active site of the enzyme, SODs could be divided into three types: iron-containing SOD (FeSOD), manganese-containing SOD (MnSOD), and copper zinc-containing SOD (CuZnSOD) [14, 15], each with different sensitivity to KCN, NaN_3 and H_2O_2 [18]. There are also some novel SODs discovered in the past decades, such as cambialistic SOD and nickel SOD (NiSOD) [25], the former could function well either with iron or manganese at its active site [10, 14].

SODs exist in most aerobic bacteria in the form of dimer or tetramer [1, 20, 21] and found to be mainly responsible for the oxidative stress resistance, controlling the oxygen concentration at an appropriate level [5, 13, 27]. SODs have also been proved to be involved in acid tolerance in *Staphylococcus aureus* [4]. Periplasmic CuZnSODs have been found to function as important virulence factors in *Neisseria meningitides* [24].

During these years, some SODs from hyperthermophiles like the archaeon *Sulfolobus solfataricus* [23], *Aquifex*

pyrophilus [8], and from thermophiles like *Thermus aquaticus* [19], *Thermothrix* sp. [20], have been investigated due to their potential application in industry. The thermostability is one of the most important properties that have been discussed since thermal denaturation is a common cause of enzyme inactivation in industry [7]. In our experiments, the SOD gene was cloned from a thermophilc bacterium *Rhodothermus* sp. XMH10, isolated from a hot spring in Jimei, Xiamen, China. The genus of *Rhodothermus*, existing in various environmental conditions, has been the subject of many studies in recent years. Many *R. marinus* enzymes were identified searching for thermostable enzymes with biotechnological potentials [2]. Compared to the SODs from other thermophiles, this recombinant superoxide dismutase cloned from *Rhodothermus* sp. XMH10 (RhSOD) shows unique characteristics in its thermostability and resistance to SOD inhibitors such as KCN, NaN_3 and H_2O_2.

Materials and methods

Bacterial strains and plasmids

The thermophilic bacteria were isolated from hot spring in Jimei, Xiamen, China, and identified as *Rhodothermus* sp. XMH10 in our previous work (unpublished data). The pMD18-T vector was purchased from Takara. The expression vector pET-His and *Escherichia coli* strain BL21(DE3)plysS were purchased from Gene Power Lab Ltd. Shenzhen office.

Cloning strategy of RhSOD

(1) SOD fragment was amplified using two degenerate primers: SODF: 5′-CSTAYGANGCNYTNSARCC-3′ and SODR: 5′-ADRTARTANGCRTGYTCCCA-3′. The primers were designed according to two amino-acid-conservative areas of SOD genes from several thermophiles (amino acid sequences: AYDALEP and WEHAYYL). The fragment was cloned following a touch-down PCR procedure with denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 56 °C for 30 s, and extension at 72 °C for 40 s. In every cycle, the annealing temperature decreased by 0.06 °C. (2) Based on the obtained SOD fragment sequence, inverse PCR [16] was carried out to clone the upstream and downstream sequences of RhSOD. Two pairs of primers were designed following the obtained sequence as follows:

1: 5′-TGTTCTGCAGCTCAGGGTAGCCCT-3′;
   5′-TCTATTCGACGCCGAACCAGGAC-3′
2: 5′-GCAGCAGTTCTTCGATCGACTTGTT-3′;
   5′-ACGAAAACGGCAAGCTCCAGGTCTA-3′

To clone the upstream sequence, genome DNA of the bacteria was digested by *Afa*I (*EcoR*V was used to clone the downstream sequence). The genome DNA fragments were then cyclized overnight at 16 °C in a ligation system of 100 μL containing the genome DNA fragments of approximate 4 μg, T4 DNA ligase and the ligase buffer. The cyclized DNA was purified by Cycle-Pure Kit (OMEGA bio-tek) and used as the template for inverse PCR.

Expression and purification of recombinant RhSOD

Two PCR primers based on the obtained SOD gene sequence were synthesized as follows:

SODF 5′-GGATCCATGGCTTTCACGCTGCC-3′
SODR 5′-GAATTCTCAGGCCGCCGCGAC-3′

The PCR-amplified fragment digested with *Bam*HI and *Eco*RI was then ligated to the similarly digested pET-His vector. The ligation mix was transformed into *E. coli* BL21(DE3)plysS. The bacteria were then grown in LB media containing ampicillin (100 μg/mL) at 37 °C till logarithmic phase was achieved. Expression of the SOD gene was induced by the addition of 0.5 mM isopropyl *β*-D-thiogalactopyranoside (IPTG) to the media, with the bacteria growing at 37 °C for another 4 h. The recombinant RhSOD was then purified using Ni-NTA Purification System (Invitrogen) under native conditions following the manufacture's instruction.

Crude enzyme preparation

The bacteria of *Rhodothermus* sp. XMH10 was grown in 200 mL of culture medium (Yeast extract 2 g/L, Tryptone 2 g/L, PIPES 6.05 g/L, pH 7.2) at 75 °C in a laboratory shaker until OD600 came to about 1.5. The cells were collected by centrifugation (8,000*g*, 10 min), washed once with phosphate buffer (0.01 M, pH 7.2), and centrifuged again to get the precipitate. The precipitate was then suspended in 10 ml of 0.01 M PB, pH 7.2 containing 1 mM EDTA and ruptured with the ultrasonic disintegrator. The suspension was centrifuged (12,000*g*, 15 min) and the resulting clear supernatant was used.

The cell-free extract was then treated with solid $(NH_4)_2SO_4$ in two steps. First, solid $(NH_4)_2SO_4$ was added to the supernatant to 60% saturation in an ice bath, then the mixture was stirred for 15 min, and left at 4 °C for 60 min. The precipitate was removed by centrifugation. In the second step, the supernatant was treated with solid $(NH_4)_2SO_4$ to 80% saturation, stirred for 15 min, and left at 4 °C for 60 min. The precipitate with SOD activity was centrifuged at 8,000*g* for 30 min and then dissolved in a minimal volume of phosphate buffer saline (PBS), pH 7.2 and dialyzed at 4 °C against the same buffer.

Superoxide dismutase assay

SOD activity was assayed based on its ability to inhibit the reduction of nitroblue tetrazolium (NBT) [22]. One unit of SOD activity was defined as the amount of enzyme which causes 50% of maximum inhibition of NBT reduction [20, 22]. The SOD will show a grey band under the blue background in the nondenaturing PAGE gel after staining in the PMS-MTT solution in daylight for half an hour [9]. The protein concentration was determined using the Coomassie (Bradford) Protein Assay Kit (Pierce).

Molecular weight

The purified recombinant RhSOD was subjected to gel filtration using a Superose G-75 column (1 cm × 100 cm) by FPLC (Amersham AKTA). The elution volume of standard proteins of BSA (66 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.4 kDa) (5 mg each) were first detected with the flow rate of 0.3 mL/min, followed by gel filtration of recombinant RhSOD under the same condition. The molecular weight curve of standard proteins was thus constructed based on the data above and the molecular weight of RhSOD was calculated.

Inhibition test

The crude SOD enzyme and purified recombinant RhSOD was preincubated with different concentrations of KCN, NaN_3 and H_2O_2 at room temperature for 1 h, and electrophoresed on nondenaturing PAGE gel. After a brief wash in distilled water, the gel was stained in PMS-MTT solution to detect the SOD activity.

Atomic absorption spectrophotometry

To confirm the metal content of the RhSOD, atomic absorption spectrum was carried out. RhSOD was obtained with the LB media supplemented with and without 10 μM $FeSO_4$ and 10 μM $MnCl_2$ when the inducement was initiated. The purified RhSODs were then introduced to absorption spectrum to assay the Fe and Mn contents.

Effect of pH on RhSOD activity

The enzyme was diluted in the relevant universal buffer with the ratio of 10:1 between the buffer and the enzyme. Effect of pH on RhSOD activity was then measured after incubating the enzyme in the universal buffer [17] with pH ranging from 2 to 12 at 50 °C for 30 min. The remaining activity was detected using the NBT test under standard conditions and calculated as the percentage of the maximum SOD activity.

Effect of temperature on RhSOD activity

The enzyme was diluted in the universal buffer of pH 5 with the ratio of 10:1 between the buffer and the enzyme. Effect of temperature on RhSOD activity was detected after incubating the enzyme at different temperatures (40, 50, 60, 70, 80 and 90 °C) for 30 min. The remaining activity was tested under standard conditions and calculated as the percentage of maximum SOD activity.

Thermal stability

The enzyme was diluted in the universal buffer of pH 5 with the ratio of 10:1 between the buffer and the enzyme. The enzyme was incubated at 50, 60, 70, 80, and 90 °C, separately, for up to 12 h. Samples were removed at certain time intervals and the remaining activity was assayed under standard conditions.

The nucleotide sequence reported in this paper has been deposited in the GenBank database under the accession number DQ286579.

Results

Cloning and identification of RhSOD and sequence analysis

The RhSOD gene of the thermophilic bacterium *Rhodothermus* sp. XMH10 was cloned in our experiment. The whole nucleotide sequence we cloned is 1,248 bp in length, of which the RhSOD gene starts at the position of 487 and ends at 1116. The coding sequence of RhSOD has the length of 627 bp, encoding 209 amino acids with a putative molecular weight of 23.6 kDa and p*I* value of 5.53. The deduced amino acid sequence shares the highest similarity of 75% with a SOD from *Geobacillus kaustophilus* by NCBI blast search. The alignment of RhSOD amino acid sequence with several other SODs from mesophiles and thermophiles shows that there are several highly conserved domains in RhSOD amino acid sequence like ALEP, H KHH, FGSGW and DVWEHAYYL (Fig. 1). Besides, the amino acid analysis showed that RhSOD was enriched with some charged residues. The number of charged residues—lysine, arginine and glutamic acid (total 35)—of RhSOD was much higher than that of *E. coli* SOD (total 24).

Expression and purification of recombinant RhSOD

RhSOD gene was highly expressed in *E. coli*, and the recombinant RhSOD was purified using the Ni-NTA Purification System (Fig. 2). The monomeric molecular weight of the recombinant RhSOD was found to be approximately

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RhSOD   ..MAFTLPPLPYPYDALEPYVDAQTMEIHHTKHHQGYVNN    38
Gk      ..MPFELPALPYPYDALEPHIDKETMNIHHTKHHNTYVTN    38
Ta      MPYPFKLPELGYPYEALEPHIDARTMEIHHQKHHGAYVTN    40
Tt      MPYPFKLPDLGYPYEALEPHIDAKTMEIHHQKHHGAYVTN    40
St      ..MAIILPDLPYAYDALEPYIDAETMTLHHDKHHATYVAN    38
Bs      ..MAYELPELPYAYDALEPHIDKETMTIHHTKHHNTYVTN    38
Sa      ..MAFKLPNLPYAYDALEPYIDQRTMEFHHDKHHNTYVTK    38
                        ALEP        HH--KHH

RhSOD   LNKALEGYPELQNKSIEELLRGINEIPEAIRTAVRNNGGG    78
Gk      LNAALEGHPDLQNKSLEELLSNLEALPESIRTAVRNNGGG    78
Ta      LNAALEKYPYLQGAEVETLLRHLTALPADIQAAVRNNGGG    80
Tt      LNAALEKYPYLHGVEVEVLLRHLAALPQDIQTAVRNNGGG    80
St      ANAALEKHPEIG.EDLEALLADVEKIPADIRQALINNGGG    77
Bs      LNKAVEGNTALANKSVEELVADLDSVPENIRTAVRNNGGG    78
Sa      LNATVEG.TELEHQSLADMIANLDKVPEAMRMSVRNNGGG    77

RhSOD   HANHSLFWTIMKPNGGGEPTGELAEAIKATFGSFEAFKEK    118
Gk      HANHSLFWTILSPNGGGEPTGELAEAINKKFGSFTAFKDE    118
Ta      HLNHSLFWRLLTPGGAKEPVGELKKAIDEQFGGFAALKEK    120
Tt      HLNHSLFWRLLTPGGAKEPVGELKKAIDEQFGGFQALKEK    120
St      HLNHALFWELLSPE.KQEPTAEVAAAINEAFGSFEAFQEV    116
Bs      HANHKLFWTLLSPNGGGEPTGALAEEINSVFGSFDKFKEQ    118
Sa      HFNHSLFWEILSPN..SEEKGGVIDDIKAQWGTLDEFKNE    115

RhSOD   FSAEAAGRFGSGWAWLVVDENGKLQVYSTPNQDSPYMQGH    158
Gk      FSKAAAGRFGSGWAWLVVNN.GELEITSTPNQDSPIMEGK    157
Ta      LTQAAMGRFGSGWAWLVKDPFGKLHVISTANQDNPVMGGF    160
Tt      LTQAAMGRFGSGWAWLVKDPFGKLHVLSTPNQDNPVMEGF    160
St      FTTAATTRFGSGWAWLVVNAEGKLEVVSTPNQDTPISDGK    156
Bs      FAAAAAGRFGSGWAWLVVNN.GKLEITSTPNQDSPLSEGK    157
Sa      FANKATTLFGSGWTWLVVND.GKLEIVTTPNQDNPLTEGK    154
                  FGSGW

RhSOD   TPILGLDVWEHAYYLKYQNRRAEYIQNWWNVVNWDQVAQY    198
Gk      TPILGLDVWEHAYYLKYQNRRPEYIAAFWNIVNWDEVAKR    197
Ta      APIVGIDVWEHAYYLKYQNRRADYLQAIWNVLNWDVAEEI    200
Tt      TPIVGIDVWEHAYYLKYQNRRADYLQAIWNVLNWDVAEEF    200
St      KPILALDVWEHAYYLKYRNVRPNYIKAFFEIINWNKVAEL    196
Bs      TPILGLDVWEHAYYLNYQNRRPDYISAFWNVVNWDEVARL    197
Sa      TPILLFDVWEHAYYLKYQNRRPDYMTAFWNIVNWKKVDEL    194
              DVWEHAYYL

RhSOD   YKEALAKVAAA.....                            209
Gk      YSEAKAK.....                                204
Ta      YKGA.....                                   204
Tt      FKKA.....                                   204
St      YAEAK.....                                  201
Bs      YSDENNGTNKVLIMGPCFFMSSFYISRF                225
Sa      YQAAK.....                                  199
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Fig. 1 Multiple alignment of SOD amino acid sequences from *Rhodothermus* sp. XMH10 and other mesophiles and thermophiles. SOD amino acid sequences from RhSOD (*Rhodothermus* sp. XMH10), Gk (*Geobacillus kaustophilus*), Ta (*Thermus aquaticus*), Tt (*Thermus thermophilus*), St (*Streptococcus thermophilus*), Bs (*Bacillus subtilis*) and Sa (*Staphylococcus aureus* subsp.) are aligned. The *solid boxes* indicate conserved domains

25 kDa. Since SODs often function in the form of dimer or tetramer, we analyzed the molecular weight of native recombinant RhSOD by gel filtration. The molecular weight of native recombinant SOD was detected to be 52 kDa, indicating that the RhSOD formed a dimer (Fig. 3).

Inhibition test

SODs are generally divided into three types by their metal content at the active site and each type of SOD is sensitive

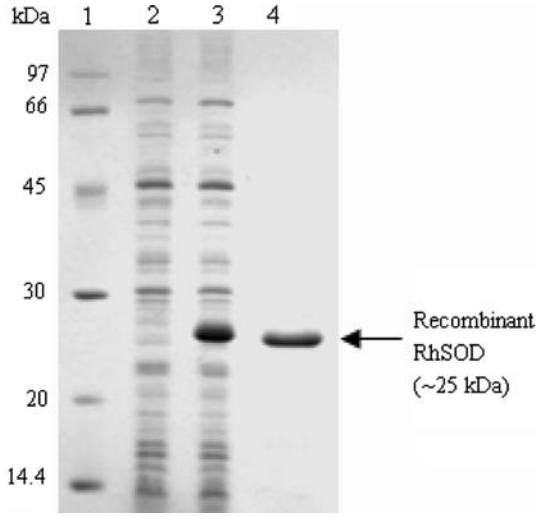

Fig. 2 SDS-PAGE gel analysis of the recombinant RhSOD expressed in *E. coli* BL21(DE3)plysS. *Lane 1*: molecular weight marker, *lane 2*: total cell protein extracts of *E. coli* BL21(DE3)plysS containing pET-His expression vector induced by IPTG for 4 h, *lane 3*: total cell protein extracts of *E. coli* BL21(DE3)plysS containing recombinant pET-His expression vector induced by IPTG for 4 h, *lane 4*: approximate 4 μg of the purified recombinant RhSOD

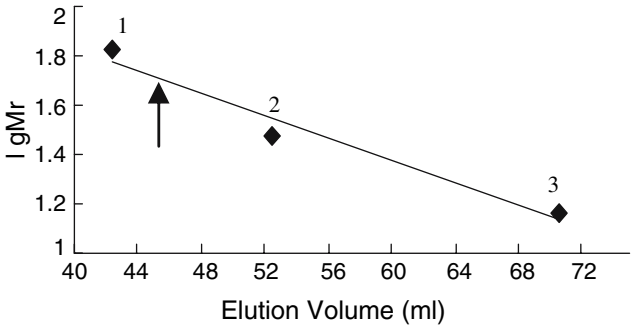

Fig. 3 Determination of the molecular weight of RhSOD. Gel filtration was performed on a column (1 cm × 100 cm) of Sepharose G-75. Standard proteins: (1) BSA (66 kDa), (2) carbonic anhydrase (30 kDa), (3) lysozyme (14.4 kDa). The *arrow* indicates the elution volume of *Rhodothermus* sp. SOD

to different inhibitors [18]. Generally, 1 mM KCN can inhibit the activity of CuZnSOD but not the other two, while 5 mM H_2O_2 can inhibit the activity of FeSOD and CuZnSOD. Addition of 10 mM NaN_3, which can inhibit all three SODs activity, is used to detect the type of MnSOD when the SOD is inhibited neither by KCN nor by H_2O_2 [11].

The RhSOD activity was not inhibited by 1 mM KCN, 10 mM H_2O_2 or 10 mM NaN_3, while the control SOD (Sangon) could be completely inhibited by 10 mM H_2O_2 (Data not shown). When increasing the concentration of H_2O_2 to 250 mM, the RhSOD was inactivated. However, the RhSOD activity was not inhibited after incubating it 250 mM KCN or NaN_3. When incubating with NaN_3, the

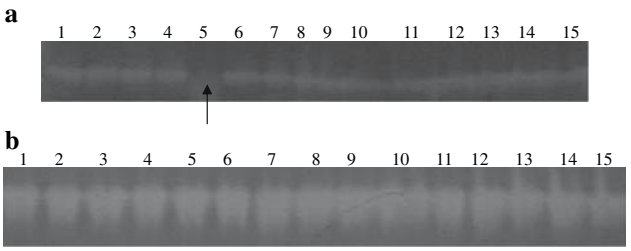

Fig. 4 Detection of RhSOD (approximate 4 μg in each lane) and crude SOD enzyme (approximate 5 μg in each lane) activity by nondenaturing PAGE gel. **a** *Lanes 1–5*: RhSOD preincubated in water, 5 mM, 10 mM, 100 mM and 0.25 M H_2O_2, respectively. The *arrow* showed the inhibited RhSOD by 0.25 M H_2O_2; *lanes 6–10*: RhSOD preincubated in water, 10 mM, 100 mM, 0.5 M and 1 M NaN_3, respectively; *lanes 11–15*: RhSOD preincubated in water, 1 mM, 10 mM, 100 mM, and 0.25 M KCN, respectively. **b** *Lanes 1–5*: crude SOD enzyme preincubated in water, 5 mM, 10 mM, 100 mM and 0.25 M H_2O_2, respectively; *lanes 6–10*: crude SOD enzyme preincubated in water, 1 mM, 10 mM, 100 mM, and 0.25 M KCN, respectively; *lanes 11–15*: crude SOD enzyme preincubated in water, 10 mM, 100 mM, 0.5 M and 1 M NaN_3, respectively

RhSOD activity was not inhibited completely even the concentration of NaN_3 reached up to 1 M (Fig. 4a). These data implied that RhSOD was highly resistant to regular inhibitors. Compared to the RhSOD, the crude SOD enzyme showed even higher resistance. Its inhibition test by KCN and NaN_3 showed similar results to that of RhSOD, but the original SOD enzyme cannot be inhibited even processed with 250 mM H_2O_2 (Fig. 4b).

Atomic absorption spectrophotometry

Since the method with low concentration of inhibitors could not suggest the metal content of RhSOD, we confirmed it through the method of atomic absorption spectrum. When supplemented with and without 10 μM $FeSO_4$ and 10 μM $MnCl_2$ in the LB media, similar metal quantities of the purified RhSOD were detected. In both conditions, every subunit of RhSOD was calculated to contain approximate 0.37 iron atom and 0.03 manganese atom (Table 1). Considering that 10 μM $FeSO_4$ and 10 μM $MnCl_2$ were enough for iron and manganese need of RhSOD, this data shown that RhSOD was an iron type SOD and one RhSOD dimer contained one iron atom (2 × 0.37).

Table 1 Atomic absorption spectrophotometry for metal content

	Metal free		Metal supplemented	
	Fe^{2+}	Mn^{2+}	Fe^{2+}	Mn^{2+}
Metal concentration (μg/L)	338	23.2	61.5	<5
Metal quantity (/SOD)	0.37	0.03	0.37	0.03
SOD concentration (μg/mL)	410		75	

Effect of pH on RhSOD activity

The purified recombinant RhSOD showed high enzyme activity between pH 4.0 and 5.0 and had the highest activity at pH 5.0. When the pH was lower than 4, the activity decreased sharply, and the enzyme was totally inactivated under the condition of pH 2. On the other hand, the RhSOD activity kept stable during the pH range of 6.0–12.0 and still held around 70% enzyme activity under the condition of pH 12.0 (Fig. 5).

Effect of temperatures on RhSOD activity

The purified enzyme activity kept stable during the temperature range of 40–70 °C. When the enzyme was incubated at 80 °C for 30 min, the activity decreases sharply, retaining only around 40% activity of the maximum. It held approximate 10% activity after incubation at 90 °C for 30 min, and was totally inactivated at 100 °C for 30 min (Fig. 6).

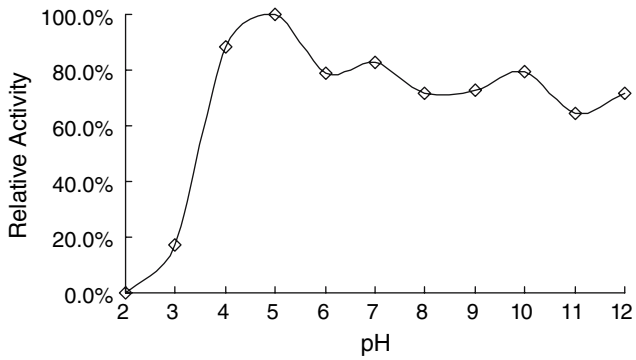

Fig. 5 Effect of pH on SOD activity. The recombinant SOD was incubated in the universal buffer with pH ranging from 2 to 12 at 50 °C for 30 min. The remaining activity was detected under standard conditions and calculated as the percentage of the maximum SOD activity

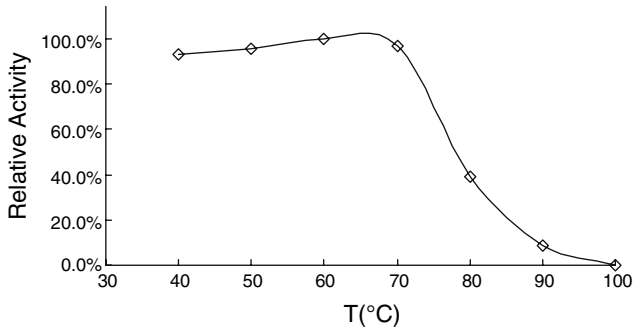

Fig. 6 Effect of temperature on SOD activity. The recombinant SOD was incubated at different temperatures (40–90 °C) for half an hour. The remaining activity was tested under standard conditions and calculated as the percentage of maximum SOD activity

Thermal stability

To test the thermal stability of recombinant RhSOD, the enzyme was incubated at 50, 60, 70, 80, and 90 °C, separately, for up to 12 h. As shown in Fig. 7, the RhSOD activity increased when incubating at 50 and 60 °C for 12 h. Over 90% enzyme activity of the maximum was remained after incubation at 70 °C for 12 h. However, SOD activity declined rapidly when the temperature reached more than 80 °C. Only approximate 40% enzyme activity remained after half-hour incubation at 80 °C and about 15% at 90 °C. The RhSOD activity was totally lost when incubating for more than 4 h at 80 °C or 1 h at 90 °C. The results indicated that RhSOD was highly thermostable.

Discussion

RhSOD was characterized to be an iron type SOD in our experiments. Its activity was not affected by H_2O_2 with concentration from 10 to 100 mM, and could only be inhibited as the concentration of H_2O_2 went up to 250 mM. Also, its activity could not be inhibited by 250 mM KCN and even by 1 M NaN_3. These results suggested that this enzyme had high resistance to all the three inhibitors of KCN, H_2O_2 and NaN_3. In most conditions, SODs could be inhibited by low concentration of relevant inhibitors [7, 8, 11], which was why reduction of SOD activity at relatively low levels of all three inhibitors was frequently used as an indicative marker for metal content at the active site. Inhibition of SOD activity at high concentrations of inhibitors and its mechanism were seldom discovered in the past decades. Glutamines (Q69) of FeSODs were found to be involved in the inhibition of FeSODs to their inhibitors [6]. The inhibition experiments were conducted at lower concentrations of hydrogen peroxide and sodium azide, which differed from ours but shown in a certain level the responsibility of the enzyme structure for the high resistance to inhibitors.

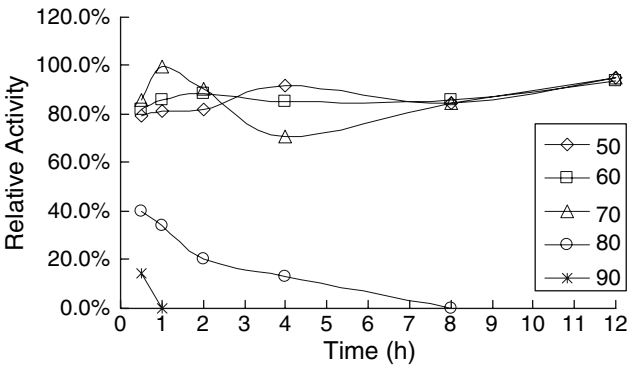

Fig. 7 SOD thermostability. The recombinant SOD was incubated at 50, 60, 70, 80, and 90 °C for up to 12 h. The enzyme was removed at certain time intervals and the remaining activity was assayed under standard conditions and calculated as percentage of maximum SOD activity

The bacterium *Rhodothermus* sp. XMH10 was isolated from hot spring with the environmental pH of 4.0–5.0. The optimal pH value for the RhSOD activity was 4.0–5.0, which was in correspondence with the environmental pH condition of the bacterium habitat. Unexpectedly, this recombinant RhSOD was found to be stable in alkaline environment, retaining about 70% of its maximum activity after incubation at pH 12 for 30 min. It is a specific property of the bacterium *Rhodothermus* sp. XMH10 ever discovered in other bacteria before.

The recombinant RhSOD was highly thermostable as compared to SODs from other bacteria. It retained more than 90% of its activity after 12-h incubation at 70 °C. The CpSOD, a highly thermostable SOD from *Chlamydia pneumoniae*, could hold more than 90% activity only after 1-h incubation at 70 °C [26]. When the temperature went up to 80 °C, the RhSOD could retain its activity for more than 4 h. However, the SODs from *Thermothrix* sp. [20], and *Thermomyces lanuginosus* [7], were totally inactivated only after 1-h incubation at 80 °C. Although the mechanism responsible for protein thermostability is not clear yet, the increased number of charged residues and hydrophobic residues, increased number of ion-pairs, and the increased buried surface area are definitely involved [8, 26]. The number of charged residues of *A. pyrophilus* SOD and thermophilic SODs from *Methanobacterium thermoautotrophicum* and *T. aquaticus* were found much more than *E. coli* SOD [8]. The RhSOD was found to be enriched with some charged residues. The number of charged residues—lysine, arginine and glutamic acid (total 35)—of RhSOD was much higher than that of *E. coli* SOD (total 24), which explains in one way its high thermostability.

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