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An Efficient Method for the Expression and Reconstitution of Thermostable Mn/Fe Superoxide Dismutase from *Aeropyrum pernix* K1

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The gene APE0743 encoding the superoxide dismutase (*Ap*SOD) of a hyperthermophilic archaeon *Aeropyrum pernix* K1 was cloned and overexpressed as a GST fusion protein at a high level in *Escherichia coli*. The expressed protein was simply purified by the process of glutathione affinity chromatography and thrombin treatment. The *Ap*SOD was a homodimer of 25 kDa subunits and a cambialistic SOD, which was active with either Fe(II) or Mn(II) as a cofactor. The *Ap*SOD was highly stable against high temperature. This thermostable *Ap*SOD is expected to be applicable as a useful biocatalyst for medicine and bioindustrial processes.

Keywords: *Aeropyrum pernix*, APE0743, superoxide dismutase, GST tag, reconstitution, thermostable enzyme

Superoxide dismutases (SODs) are a ubiquitous class of antioxidant defense metalloenzymes that disproportionate superoxide radical ions into dioxygen and hydrogen peroxide.

$$2\ O_2^{\cdot-} + 2H^+ \rightarrow O_2 + H_2O_2 \quad (1)$$

It is generally accepted that in all SODs, the metal ion (M) catalyzes dismutation of the superoxide radical through a cyclic redox mechanism.

$$M(III)\text{-}SOD + O_2^{\cdot-} \rightarrow M(II)\text{-}SOD + O_2 \quad (2)$$
(oxidative reaction)

$$M(II)\text{-}SOD + O_2^{\cdot-} + 2H^+ \rightarrow M(III)\text{-}SOD + H_2O_2 \quad (3)$$
(reductive reaction)

Four classes of SODs are distinguished by the metal prosthetic group: Cu/Zn, Fe, Mn, and Ni [13, 16]. Fe- and Mn-SODs are assumed to have a common evolutionary origin due to the similarity in their amino acid sequences and three-dimensional protein structures [7]. They are unequally distributed throughout the living organisms and are located in different cellular compartments [3]. The active site manganese ion is complexed by the side chains of histidine and aspartic acid residues, forming a trigonal pyramidal coordination polyhedron. Studies on the closely related Fe-SODs suggest that this arrangement of protein ligands is independent of the presence of the metal ion, being nearly identical for holo- and apoenzymes. In spite of these similarities, metal-reconstitution studies have shown, that most Fe- and Mn-SODs have strict metal binding specificities. Fe- and Mn-SODs from some organisms such as *Escherichia coli* exhibited almost absolute metal specificity [1], whereas other enzymes such as cambialistic SOD from *Propionibacterium shermanii* were active with either metal [10].

Aeropyrum pernix K1 is a strict aerobic hyperthermophilic archaeon that grows optimally at 95°C [8]. The thermostable enzymes from this archaeon are expected to be useful for a variety of industrial applications. In particular, the thermostable SOD may be useful as a cofactor in antiaging cosmetics and in protein therapy for various disorders related to the antioxidant enzymes or reactive oxygen species [8]. In the genome sequence data of *A. pernix* K1, we found that the open reading frame APE0743, composed of 645 base pairs, encodes a protein homologous with SODs from *Methanothermobacter thermautotrophicus*, *Pyrobaculum aerophilum*, and *Sulfolobus acidocaldarius* (approximately 50–70% similarity) as judged by homolog searches in the NCBI database (Fig. 1). To effectively overexpress and purify the APE0743 gene product from *Aeropyrum pernix* K1 (*Ap*SOD), a recombinant plasmid was designed using the expression vector pGEX-KG containing glutathione *S*-transferase (GST) as the fusion partner. The APE0743 gene was amplified by polymerase chain reaction (PCR) using cDNA as the template with the following PCR primers: 5'-GAATTCTAGTGAGCTTTAAGAGGTACG-3'

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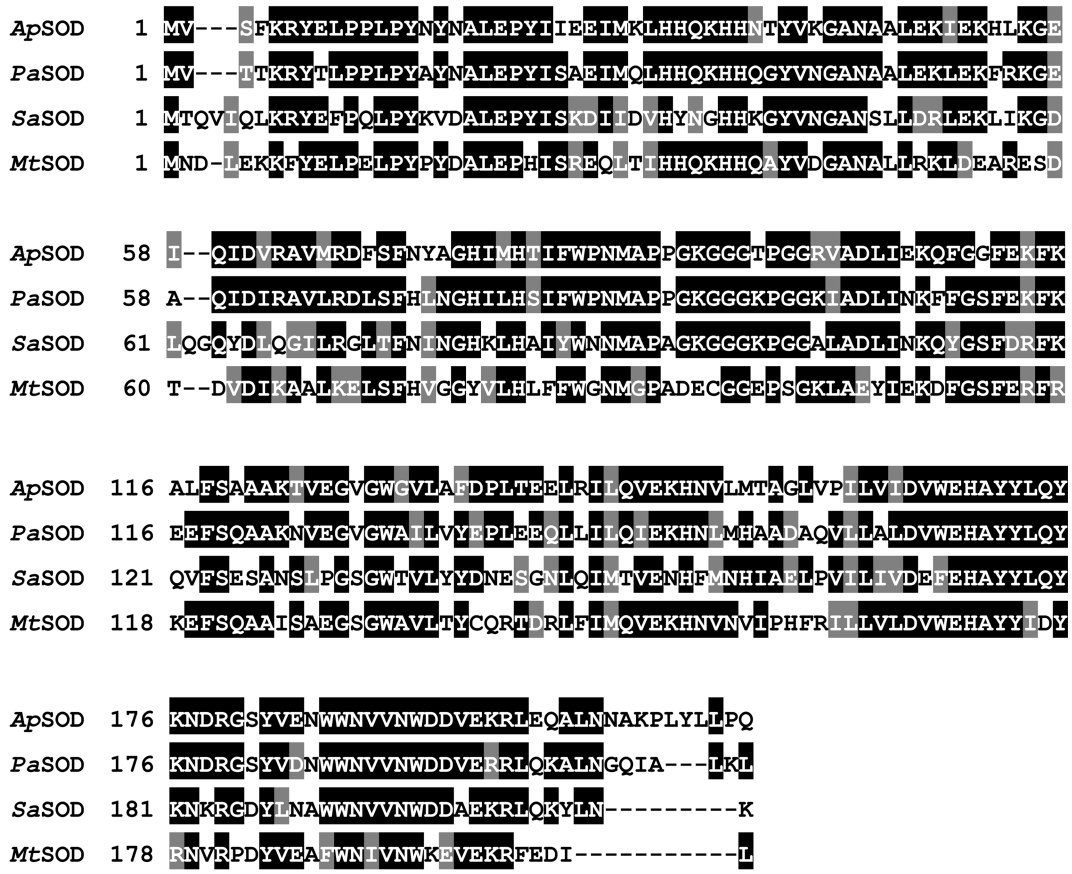

Fig. 1. Aligned amino acid sequences of *Ap*SOD, *Pa*SOD, *Sa*SOD, and *Mt*SOD.
Abbreviations of the sources of the enzymes: *Ap*SOD, superoxide dismutase from *Aeropyrum pernix* (GenBank Accession No. NP_147461); *Pa*SOD, superoxide dismutase from *Pyrobaculum aerophilum* (NP_558493); *Sa*SOD, superoxide dismutase from *Sulfolobus acidocaldarius* (YP_254907); *Mt*SOD, superoxide dismutase from *Methanothermobacter thermautotrophicus* (NP_275303). The sequences have been aligned with dashes indicating gaps. The consensus identical (black) or similar (gray) amino acids are shaded.

(upper primer, containing an *Eco*RI recognition site as underlined) and 5'-AAGCTTCTACTGGGGGAGCAGG-TAGAG-3' (lower primer, containing a *Hin*dIII recognition site as underlined). The PCR product was cloned into expression vector pGEX-KG using *Eco*RI and *Hin*dIII digestion. The resulting vector was designated as pGEX-*Ap*SOD and used to transform the *E. coli* strain BL21 (DE3). The colony containing the appropriate insert was identified by DNA sequencing and grown in LB broth containing ampicillin (25.0 μg/ml) at 37°C and induced at OD_{600}=0.9–1.0 with 0.4 mM IPTG for 8 h.

The induced cells were centrifuged and completely lysed by sonication. The expressed protein was purified according to the manufacturer's protocol. The protein was either eluted from glutathione-Sepharose beads using 10 mM reduced glutathione or after digestion (on column) with 1 U thrombin/100 μg protein (Amersham Pharmacia Biotech, Uppsala, Sweden) for 30 min at 37°C. The eluted protein was subjected to dialysis for 24 h at 4°C with three successive changes against 20 mM potassium phosphate buffer. The protein was then concentrated using a MWCO 5 k Amicon Ultra Centrifugal Filter (Millipore, Billercia, MA, U.S.A.). The recombinant *Ap*SOD apoenzyme was efficiently purified 20-fold with a yield of 38% by GSH-Sepharose affinity chromatography and thrombin treatment. The purified *Ap*SOD apoenzyme gave a single band on electrophoresis in the presence of SDS, as shown in Fig. 2A.

The enzyme's quaternary structure was analyzed by SDS–PAGE and gel filtration. Comparison of the relative mobility of the *Ap*SOD with a standard protein indicated that the molecular mass of the *Ap*SOD was approximately 25 kDa by SDS–PAGE analysis (Fig. 2A). However, gel filtration analysis under nondenaturing conditions showed that the *Ap*SOD had a molecular mass of approximately 56 kDa (Fig. 2B). These results indicated that the *Ap*SOD is a homodimer of 25 kDa subunits.

The recombinant *Ap*SOD produced in *E. coli* expressed as an apoprotein. This apoprotein showed no SOD activity. To determine the metal specificity of the recombinant *Ap*SOD, metal-reconstitution experiments were carried out [10]. The recombinant *Ap*SOD apoprotein was activated with $Fe(NH_4)_2(SO_4)_2$ and $MnSO_4$ salts at elevated temperature,

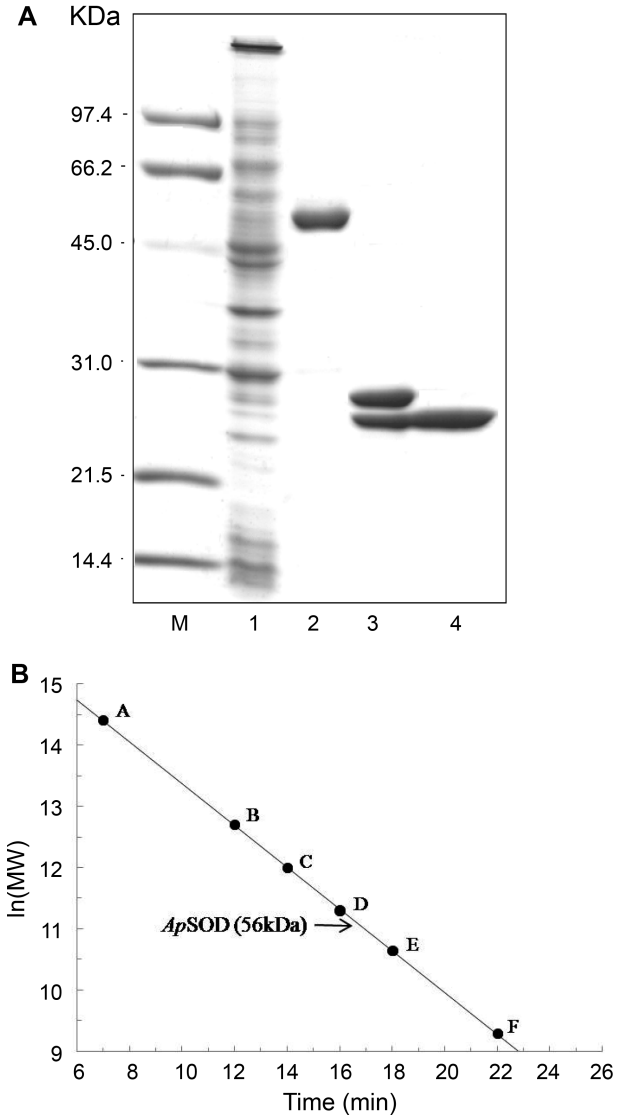

Fig. 2. Molecular mass determination of the recombinant *Ap*SOD.
A. SDS–PAGE analysis at different stages of purification. Aliquots of samples obtained at the various purification steps were analyzed by SDS–PAGE on a 12.5% gel. The molecular mass of the fusion protein was 51 kDa. After thrombin cleavage, GST and SOD were 26 and 25 kDa, respectively. Lane M, protein size markers; lane 1, supernatant of a cell lysate; lane 2, fusion proteins eluted from glutathione-Sepharose beads using 10 mM reduced glutathione; lane 3, proteins (GST and SOD) after digestion with 1 U thrombin/100 μg eluted fusion proteins; lane 4, purified *Ap*SOD. **B**. Molecular mass determination of the native recombinant *Ap*SOD by gel filtration analysis. The X axis is elution time of proteins and the Y axis is plotted by calculating to ln (molecular mass). A, blue dextran (2,000 kDa); B, β-amylase (200 kDa); C, yeast alcohol dehydrogenase (150 kDa); D, bovine serum albumin (66 kDa); E, trypsinogen (24 kDa); F, cytochrome *c* (12.4 kDa).

with maximal activity being obtained by incubation with Fe(II) and Mn(II) ions at 95°C. The time dependence of activation at 65°C and the temperature dependence of activation are shown in Fig. 3. Virtually no activation was observed below 50°C, whereas above that temperature

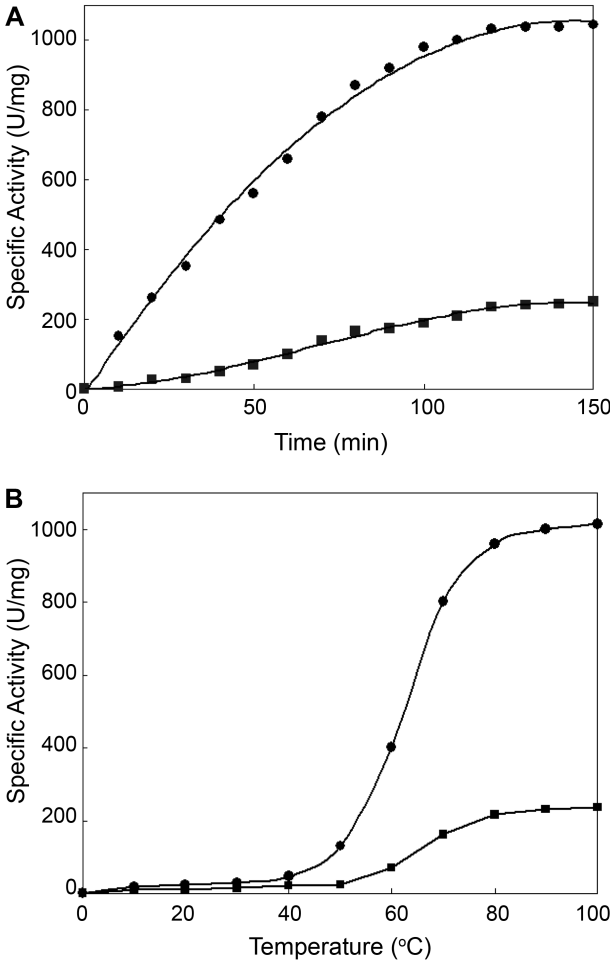

Fig. 3. Activity of the recombinant *Ap*SOD.
A. Time dependence of activation of apoprotein; samples were heated at 95°C for the indicated time in the presence of 10 mM $MnSO_4$ (-●-) under aerobic condition or 10 mM $Fe(NH_4)_2(SO_4)_2$ (-■-) under anaerobic condition. **B**. Temperature dependence of the activity of apoprotein; samples were heated at the indicated temperature for 120 min in the presence of 10 mM $MnSO_4$ (-●-) under aerobic condition or 10 mM $Fe(NH_4)_2(SO_4)_2$ (-■-) under anaerobic condition. Aliquots of the reactions were assayed for SOD activity using the xanthine oxidase/cytochrome *c* inhibition assay at room temperature [2].

there was an abrupt increase in the activation rate. The restricted protein dynamics at lower temperature (below 50°C) may prevent the large amplitude motions required for cofactor insertion. Expression of the recombinant *Ap*SOD as apoprotein in *E. coli* and its convenient heat reconstitution have proven valuable in these studies, allowing us to prepare pure Mn and Fe forms of the *Ap*SOD without resorting to harsher denaturation/renaturation procedures.

As shown in Table 1, the Mn-reconstituted *Ap*SOD contained 0.82±0.05 atom of manganese per subunit and exhibited a specific activity of approximately 1,030±40 U/mg. The Fe-reconstituted *Ap*SOD also contained 0.79±0.04 atom of iron per subunit and exhibited a specific activity of approximately 234±15 U/mg. These specific activities of

Table 1. Specific activity and metal contents of reconstitution for apoprotein.

	Specific activity (U/mg)	Metal content (atom/subunit)	
		Mn	Fe
Apoprotein	0	0	0
Mn-reconstituted SOD	1,030±40	0.82±0.05	-
Fe-reconstituted SOD	234±15	-	0.79±0.04

SOD activity was measured using the xanthine oxidase/cytochrome *c* inhibition assay at room temperature [3]. SOD activity unit is defined as the amount of enzyme that inhibits the rate of cytochrome *c* reduction by 50%. The values showed are means±SD, generally based on n≥5.

the Mn- and Fe-reconstituted *Ap*SOD were more active than those obtained by Yamano *et al.* [15]. However, neither Cu nor Zn was bound to the recombinant *Ap*SOD apoprotein and the resultant proteins had no SOD activity under the same experimental conditions for metal reconstitution. These results indicated that the recombinant *Ap*SOD was a cambialistic SOD that was active with either Fe(II) or Mn(II) as a cofactor. Cambialistic SODs are likely to bind Mn in aerobic environments because aqueous Fe is usually limited in aerobic environments at neutral pH. On the other hand, they bound Fe in anaerobic environments because manganese might be easily incorporated into cells because of its solubility [14].

SOD activity was measured using the xanthine oxidase/cytochrome *c* inhibition assay at room temperature [2]. SOD activity unit was defined as the amount of enzyme that inhibits the rate of cytochrome *c* reduction by 50%. The specific activities of the recombinant *Ap*SOD were lower than the reported values for mesophilic eubacterial Fe- and Mn-SODs (Table 1) [12]. These lower activities of the recombinant *Ap*SOD were likely due to the reaction temperature. Generally, SOD activity was measured at 25°C because xanthine oxidase in the assay mixture for the assay of SOD activity was not thermostable. This temperature was probably much lower than the optimum temperature (95°C at pH 7.0) for the *Ap*SOD activity because the optimum growth temperature for *A. pernix* was 95°C [8]. The half-life ($T_{1/2}$) of the *Ap*SOD at 95°C was approximately 8 h. This value was greater than those reported for other thermophilic microorganism SODs such as from *Aquifex pyrophilus* ($T_{1/2}$ at 95°C, 175 min) [6], *Thermoascus aurantiacus* ($T_{1/2}$ at 90°C, 10 min) [11], and *Thermomyces lanuginosus* ($T_{1/2}$ at 80°C, 28 min) [5]. These results indicated that the *Ap*SOD was extremely stable against high temperature.

In conclusion, the recombinant pGEX-*Ap*SOD construct allowed the expression of a sufficient quantity of *A. pernix* SOD in *E. coli* and rapid purification by using affinity chromatography. The recombinant *Ap*SOD was a cambialistic SOD, which was active with either Fe(II) or Mn(II) as a cofactor. The recombinant *Ap*SOD was highly stable against high temperature. In this respect, the recombinant *Ap*SOD showed excellent and competitive characteristics when compared with other available SODs.

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