Expression and Purification of Recombinant Superoxide Dismutase (PaSOD) from Psychromonas arctica in Escherichia coli

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The psychrophilic bacteria Psychromonas arctica survives at subzero temperatures by having adapted several protective mechanisms against freezing and oxidative stresses. Many reactive oxygen species are likely generated in P. arctica as a result of reduced metabolic turnover rates. A previous study identified the pasod gene for superoxide dismutase from P. arctica using a series of PCR amplifications. Here, upon cloning into a His-tag fused plasmid, the sod gene from P. arctica (pasod) was successfully expressed by IPTG induction. His-tagged PaSOD was subsequently purified by Ni²⁺-NTA affinity chromatography. The purified PaSOD exhibited a higher SOD activity than that of Escherichia coli (EcSOD) at all temperatures. The difference in activity between PaSOD and EcSOD becomes even more significant at 4 °C, indicating that PaSOD plays a functional role in the cold adaptation of P. arctica in the Arctic.

Key Words: Psychrophilic, Superoxide dismutase, Cold-adaptation, Chaperone
Experimental Section

**Bacterial Stains and Chemicals.** KOPRI22215 (donated by KOPRI, Korea Polar Research Institute) was identified as *Psychromonas arctica* from its 16S rDNA gene sequence (personal communication). *P. arctica* was cultured at 15 °C in Difco™ marine broth medium (Difco, Grand Island, NY). Cell growth of BL21(DE3) transformants grown in LB medium (trypsyn 10 g, yeast extract 5 g, NaCl 10 g per liter) with appropriate antibiotics was monitored by measuring optical densities at 600 nm (OD$_{600nm}$). The total genomic DNA was extracted from collected cells using a Cell DNA isolation kit (GeneAll, Seoul, Korea). IPTG (isopropyl β-D-1-thiogalactopyranoside) and NBT (nitro blue tetrazolium) were obtained from Sigma-Aldrich (St. Louis, MO). Amicon filters were acquired from Millipore (Billerica, MA) and Ni$^{2+}$-NTA chelating agarose CL-6B was purchased from Pepton (Daejeon, Korea).

**Construction and Expression of PaSOD and EcSOD Plasmids.** The *pasod* gene was amplified in its entirety using the corresponding primers containing NdeI and BamH1 sites at each end [forward F1: 5’-CAT ATG GCT TTT GTA TCA CCA GCA TTA CC-3’; reverse R1: 5’-GGAG TCC CCA TTA TAG GAC GAC TGC AGC AAA-3’]. After cloning into a pBluescript II vector (Stratagene, La Jolla, CA), the Ndel- and BamH1-digested fragments were ligated into the pET28a vector, which had been previously cut using the same enzymes, to provide PaSOD/pET28a. To overexpress PaSOD in *E. coli*, BL21(DE3) transformed with PaSOD/pET28a was cultured at 28 °C in LB$_{30}$ to an OD$_{600nm}$ of 0.5, at which point IPTG was added to a final concentration of 0.5 mM. After a 5-h incubation with IPTG, cells were harvested and frozen at −70 °C.

Cloning and expression of the corresponding *E. coli* SODs were performed similarly. Genomic DNA was purified by a Cell DNA isolation kit (Exgene Cell SV, GeneAll) from *E. coli* strain DH5 that had been cultured overnight in LB media. The * sod* gene from *E. coli* genomic DNA was amplified via PCR using F2 and R2 primers for Fe-type SOD (EcFeSOD) and F3 and R3 primers for Mn-type EcSOD (EcMnSOD). The sequences of primers were as follows: F2, 5’-AGG AGA GCT AGC ATG TCA TTC GAA TTA-3’; R2, 5’-CAT TTG GGA TTC GAT ATT ATG CAG CGA-3’; F3, 5’-CTG GAG ATG CAT ATG AGC TAT ACC CTC-3’; and R3, 5’-GCC TCA TTG CAG CAG GAT CCA AAT GAT-3’.

The annealing temperatures were 58 °C and 56 °C, respectively.

**Purification of PaSOD by Ni$^{2+}$-Affinity Chromatography.** Bacterial cells were lysed on ice by incubating with binding buffer [50 mM Tris-Cl (pH 7.9), 250 mM NaCl, 8 mM imidazole] for 30 min. After sonication, protein extracts were collected by centrifugation at 4 °C for 20 min, separated by 10% SDS-PAGE, and examined by Coomassie blue staining. His-tagged PaSOD induced by IPTG was further purified by Ni$^{2+}$-NTA chromatography. After loading a protein extract sample, the column was washed with ten volumes of binding buffer [50 mM Tris-Cl (pH 7.9), 250 mM NaCl, 8 mM imidazole], six volumes of washing buffer [50 mM Tris-Cl (pH 7.9), 250 mM NaCl, 20 mM imidazole], and finally six volumes of elution buffer [50 mM Tris-Cl (pH 7.9), 250 mM NaCl, 800 mM imidazole]. The overexpressed PaSOD was obtained with successive buffer exchange using Amicon-10 filtration (Millipore). The concentration of PaSOD was measured by the Bradford method using bovine serum albumin as a standard and the purity of PaSOD was confirmed by 10% SDS-PAGE.

**Identification of SOD Activity on Native PAGE Gels.** The purified SODs were separated on native polyacrylamide gels (PAGE) for SOD activity staining as follows. Native gels without SDS and β-mercaptoethanol were prepared with 10% polyacrylamide and run at 120 V for 1 h. The native gels were incubated in 1.23 mM NBT for 15 min followed by incubation in 100 mM phosphate buffer (pH 7.0) containing 0.028 mM riboflavin and 28 mM TEMED with gentle shaking at 75 rpm in the dark. After brief washing, the gels were illuminated with a fluorescent lamp for 15 min.

**The Specific SOD Activity of Expressed SODs.** The specific activity of superoxide dismutase was measured spectrophotometrically with a SOD assay kit with WST (Dojindo Molecular Technologies, Inc., Tokyo, Japan). In principle, the reduction of WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) inhibition by the superoxide anion is correlated with the inhibition of xanthine oxidase by the SOD enzyme. The IC$_{50}$ value, which indicates the concentration of SOD at which 50% inhibition activity was observed, was determined according to the expression below by measuring the OD of the solution at 450 nm in a 96-well plate following a 20-min incubation at 37 °C. One unit of SOD is defined as the amount of the enzyme in 20 μL sample solution at IC$_{50}$. The SOD activity was calculated using the following equation.

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\text{SOD activity (inhibition rate %)} = \frac{(A_{\text{blank1}} - A_{\text{blank3}}) - (A_{\text{sample}} - A_{\text{blank2}})}{(A_{\text{blank1}} - A_{\text{blank3}})} \times 100
\]

**Results and Discussions**

Cloning and Purification of PaSOD and EcSOD. The *pasod* gene is 594 bp in length and expected to encode...
PaSOD with 197 amino acids. To purify the expressed PaSOD in a one-step process using affinity chromatography, we developed a His-tagging system using the pET28a vector for PaSOD cloning. After amplification using two primers containing NdeI and BamH1 restriction sites, the *pasod* gene was successfully cloned into the pET28a vector, yielding PaSOD/pET28a. Overexpression of PaSOD in BL21(DE3) was carried out in the presence of 0.5 mM IPTG and examined by 10% SDS-PAGE and Coomassie blue staining (Fig. 1). Most expressed PaSOD proteins remained in the pellet forming inclusion body. We have tried to resolubilize the protein by on-column refolding but failed. The soluble fraction of His-tagged PaSOD (lane 7 in Fig. 1) was further purified by NTA-chromatography to provide pure PaSOD (lane 8 in Fig. 1).

Cloning of *E. coli* SOD in pET28a was also carried out by PCR amplification of the *E. coli* genomic DNA with the appropriate primers designed in this study. Induction of EcMnSOD and EcFeSOD by IPTG was confirmed by 10% SDS-PAGE analysis (Fig. 2). The molecular mass of each enzyme was estimated as 23 kDa for EcMnSOD (206 aa) and 21 kDa for EcFeSOD (193 aa).

**The Specific SOD Activity of Expressed SODs.** The specific activity of SOD was measured spectroscopically using a xanthine oxidase system (Fig. 3(a)). Even if this method is indirect one including another enzyme, xanthine oxidase, it gave us reliable results with high sensitivity. The effect of temperature on PaSOD activity could be evaluated by comparing with positive control, in our case, EcSODs. To monitor the activity of SODs, IC$_{50}$ values were determined by measuring the solution absorbance at 450 nm. 9 Note that PaSOD exhibited the highest activity of the enzymes tested (Fig. 3(b)). The IC$_{50}$ of PaSOD (0.35 μg/mL) was slightly lower than that of Ec-FeSOD (0.49 μg/mL). EcMnSOD (IC$_{50}$ of 2.68 μg/mL) exhibited a drastically lower activity than that of PaSOD by a factor of seven. To assess the role of PaSOD in cold adaptation, SOD-specific activities were measured as a function of decreasing temperature from 37°C to 4°C (Fig. 3(c)). It should be noted that PaSOD showed higher activities than those of EcFeSOD at all temperatures. PaSOD had the highest SOD activities at 4°C as we expected. However, enhancement of the SOD activity upon decreasing temperature was also found even in mesophilic EcSOD, probably due to the high solubility of oxygen at low temperature. Nonetheless, the difference in activity between PaSOD and EcSOD becomes even more noteworthy at 4°C, indicating that PaSOD plays a functional role in the cold adaptation of *P. arctica* in the Arctic.

**In-gel Assay for Specific Inhibition of SOD.** In order to check the SOD activity in native gel, purified SOD proteins were separated on native PAGE and stained with NBT. We observed that PaSOD displayed a higher SOD activity than EcFeSOD, as demonstrated by twofold serial dilutions (Fig. 4(a)). PaSOD exhibited the SOD activity at the lowest level of 0.063 mg. The inhibition patterns exhibited by several inhibitors can be rather specific depending on the metal cofactor incorporated in the SOD. For example, FeSOD is inhibited by H$_2$O$_2$, but not by cyanide. Cu/ZnSOD can be blocked by both of these compounds while MnSOD is unaffected in the presence of either one. The metal-specific inhibition of PaSOD was characterized using the native gel assay with EcFeSOD as a control (Fig. 4(b)). PaSOD showed a very similar inhibition pattern as that of EcFeSOD and was blocked by H$_2$O$_2$ but not by CN$^-$. This result implies that PaSOD behaves as an Fe-type SOD, which is in a good agreement with homology comparisons of its amino acid sequence.
Although structural data for PaSOD are lacking, the SOD from *Psychromonas ingrahamii* (PiSOD) was categorized as a Fe/Mn SOD with an α-hairpin N-terminal and C-terminal α+β domains with three-stranded antiparallel sheets. Since PaSOD (197 aa) from *P. arctica* displays significant homology with PiSOD (193 aa) by a score of 83 in ClustalW2 analyses (EBI server), PaSOD likely contains similar protein domains. ClustalW2 analyses have also indicated that PaSOD is more homologous with EcFeSOD, with a score of 75, than with EcMnSOD, with a score of 42. This comparison suggests that PaSOD is a Fe-type SOD. SOD inhibition assays focusing on the metal dependence of SOD activity also showed that PaSOD was sensitive to H$_2$O$_2$ inhibition, a further characteristic of Fe-type SODs. Further studies employing atomic absorption spectroscopy may be necessary to confirm the metal content in PaSOD.

The search for a Mn-type PaSOD by PCR amplification with the proper primers has been unsuccessful. At this point, one cannot rule out that Mn- or Ni-type PaSODs may be key factors in the cold-temperature survival of *P. arctica*. Further exploration of other type of *sod* genes from *P. arctica* is in progress.

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