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Expression and Characterization of an Iron-Containing Superoxide Dismutase from *Burkholderia pseudomallei*

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A superoxide dismutase (SOD) gene from *Burkholderia pseudomallei*, the causative agent of melioidosis, was cloned and expressed in *Escherichia coli*, and its product was functionally and physically characterized. The gene has an open-reading frame of 579 bp. The deduced amino acid sequence has 192 residues with a calculated molecular mass of ~22 kDa. Sequence comparison with other bacterial SODs showed that the protein contains typical metal-binding motifs and other Fe-SOD-conserved residues. The sequence has substantial similarity with other bacterial Fe-SOD sequences. The enzymatic activity of the expressed protein was inhibited by hydrogen peroxide but not by sodium azide or potassium cyanide, attributes that indeed are characteristic of typical bacterial Fe-SODs. Western blotting with antiserum against the recombinant Fe-SOD revealed that it is expressed in *B. pseudomallei*. Transformed *E. coli* that expressed the Fe-SOD had significantly increased SOD activity and was highly tolerant to paraquat-mediated replication inhibition, compared to transformed cells carrying an empty vector. Our results provide a basis for further biochemical characterization of the enzyme and elucidation of its role in the pathogenesis of *B. pseudomallei*.

***Keywords*:** *Burkholderia pseudomallei*, Fe-superoxide dismutase, melioidosis

Introduction

Burkholderia pseudomallei is a Gram-negative, opportunistic bacterium that causes acute and fatal septicemic melioidosis in humans. It is found mainly in soils of southeast Asia and northern Australia (Dance, 2000). Human infection usually occurs via skin abrasions or inhalation of contaminated soil or water. The bacterium disseminates from the site of infection to virtually all other tissues and can remain dormant for many years (Loprasert *et al*., 2000).

B. pseudomallei survives and multiplies intracellularly in phagocytic cells such as monocytes and macrophages (Fukuhara *et al*., 1995; Jones *et al*., 1996). Activated phagocytic cells undergo an oxygen-dependent defense mechanism, involving increased oxygen consumption followed by the generation of highly reactive oxygen intermediates, e.g., the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical, to eliminate the ingested pathogens (Fridovich, 1978). These reactive oxygen species (ROS) cause oxidative damage in *B. pseudomallei* that result in DNA mutation, protein denaturation and membrane lipid peroxidation (Badwey and Karnovsky, 1980; Imlay and Linn, 1988; Radi *et al*., 1991).

To protect against host' immune reactions, bacteria detoxify ROS by producing antioxidant enzymes, including catalase, catalase-peroxidase, and superoxide dismutase (SOD). In particular, SODs are among the primary lines of bacterial defense against ROS and catalyze the decomposition of O_2^- to yield H_2O_2 and molecular oxygen (Fridovich, 1986). SODs are classified as containing either copper/zinc, manganese (Mn-SODs), nickel, or iron (Fe-SODs) according to the metal ion required for activity (McCord and Fridovich, 1969). Mn-SODs and Fe-SODs are regulated differently but have overlapping roles in protecting bacteria from O_2^- adventitiously generated inside the cell under aerobic conditions (Hassett *et al*., 1995). In contrast, copper/zinc-SODs are not required for bacterial growth under laboratory conditions and do not seem to be involved in intracellular detoxification of O_2^- (Farrant *et al*., 1997; Sansone *et al*., 2002).

As is the case during infection by other types of bacteria, *B. pseudomallei* may be exposed to substantial levels of ROS during tissue invasion and upon contact with macrophages. The bacterium may, therefore, possess antioxidant enzymes to prevent oxidative killing by macrophages. However, until this report, no *B. pseudomallei* Fe-SOD had been identified. For the study reported herein, we identified and characterized Fe-SOD from a *B. pseudomallei* strain that had been isolated from a Korean melioidosis patient (Shin *et al*., 2011). We expressed the *B. pseudomallei* Fe-SOD gene in *Escherichia coli* and characterized biochemical properties of the enzyme.

Materials and Methods

Strain and culture condition

The *B. pseudomallei* 1106a was isolated from a melioidosis

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patient in Korea (Shin *et al.*, 2011) and maintained in our laboratory by serial cultivation in 3.7% brain heart infusion broth (Difco, USA).

Cloning of the Fe-SOD gene

The full-length Fe-SOD gene was amplified from *B. pseudomallei* genomic DNA with the use of primer pairs that corresponded to regions of its open-reading frame (accession number YP001065214). The forward primer was 5′-CACC ATGGCTCATACGCTC-3′, and the reverse primer was 5′-TTACGCGAAGTTCTTCGC-3′. The gene was amplified by PCR using the program: 94°C for 4 min, 35 cycles at 94°C for 30 sec, 58°C for 30 sec, 72°C for 1 min, followed by an extension at 72°C for 10 min. The product was electrophoresed through a 1.2% agarose gel, extracted from the gel with QIAquick Gel Extraction kit reagents (QIAGEN, USA), and ligated into a pENTR/D-TOPO vector (Invitrogen, USA), which was transformed into competent *E. coli* Top10 (Invitrogen). The cloned gene was then sequenced. The nucleotide sequence of the cloned gene was determined by using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystem, UK) and ABI automated DNA sequencer, according to the manufacturer's instructions.

Expression and purification of SOD

The *B. pseudomallei* SOD gene was ligated to an upstream $(His)_6$ sequence in a pDEST17 expression vector (Invitrogen) that contained an isopropyl-β-D-thiogalactoside (IPTG)-nducible *tac* promoter and the T7 promoter transcription-ranslation system (pDEST17-Fe-SOD). The SOD was expressed in *E. coli* BL-21(DE3) cells (Invitrogen) that had been transformed with pDEST17-Fe-SOD by first culturing the cells on LB agar that contained 100 μg/ml ampicillin. Twenty ml of LB media containing 100 μg/ml ampicillin were inoculted and grown at 37°C with shaking until an OD_{600} of 0.6. IPTG was added to a final concentration of 1 mM and the recombinant protein was purified with Ni-NTA resin (Invitrogen). After loading the lysate onto the resin, the resin was washed with 50 mM sodium phosphate, pH 8.0, containing 300 mM NaCl and 10 mM imidazole, and then the recombinant protein was eluted with the same buffer but containing 250 mM imidazole.

Enzyme activity assay

SOD activity was measured using SOD Assay kit reagents (Dojindo Molecular Technologies, USA) according to the manufacturer's protocol. For this assay, O_2^- is generated by xanthine oxidase and reacts with a colorless water-soluble tetrazolium salt to generate the yellow, water-soluble tetrazolium-1 formazan that is observed spectrophotometrically at 450 nm. Color formation is inhibited if O_2^- is degraded by SOD. Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Metal cofactor identification

To determine the type of SOD, the enzyme (20 μg) was mixed with potassium cyanide (KCN, 5 or 10 mM), sodium azide (NaN_3, 5 or 10 mM), or H_2O_2 (5 or 10 mM) and incubated at 37°C for 30 min. After incubation, the SOD activity was assayed in solution as describe above and compared with the activity of a control sample that did not contain an inhibitor.

Optimal pH and thermal stability assays

To determine the optimal pH for SOD activity, the purified enzyme was assayed in 50 mM sodium phosphate, pH 7.0, or 50 mM Tris-HCl, pH 8.0–9.0, or 50 mM glycine-NaOH, pH 10.0–12.0, instead of the assay solution described above. After a 30-min incubation at 37°C, SOD activity was measured. To determine its thermal stability, the enzyme was incubated at 37, 50, 70, and 100°C for various times, and the residual enzyme activity was then measured.

Antibody production

Polyclonal antibodies were produced by immunizing six BALB/c mice with purified recombinant *B. pseudomallei* Fe-SOD. Mice were injected intraperitoneally with Fe-SOD (200 μg/ml) emulsified with Freund's complete adjuvant. Animals received booster shots 2 and 4 weeks later with Fe-SOD (200 μg/ml) emulsified with Freund's incomplete adjuvant. Sera were collected 6 weeks after the initial immunization. The antibodies were purified by HiTrap Protein G HP-Sepharose (GE Healthcare, Sweden). The specificity of the antibodies was assessed by a *B. pseudomallei* Fe-SOD western blotting.

SDS-PAGE, Western blotting and protein concentration determination

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (1970). All gels were first stained with Coomassie Brilliant Blue and then destained. Cell lysates from non-transformed *E. coli* and from *B. pseudomallei*, and the purified Fe-SOD were electrophoretically separated and then transferred to a 0.2-μm nitrocellulose membrane (Bio-Rad Laboratories, USA) (Towbin *et al.*, 1979). After transfer, the membrane was blocked in phosphate-buffered saline containing 0.05% Tween 20 (PBST) supplemented with 3% skim milk for 1 h at room temperature and then incubated with the antiserum (1:1,000 dilution, 10 μg in 1 ml) raised against Fe-SOD for 2 h at room temperature. After three washes with PBST, the membrane was incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:1,000; Abcam, UK) for 2 h at room temperature. After three washes with PBST, the blots were developed with enhanced chemiluminescence substrate (Pierce, USA) and exposed to X-ray film (Kodak XAR5; Eastman Kodak, USA). Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Response of *E. coli* to oxidative stress

E. coli was used to determine if, when transformed with the Fe-SOD gene, the cells could resist oxidative stress generated by paraquat. Overnight cultures of *E. coli* BL21 (DE3) transformed with an empty pDEST17 or with pDEST17-Fe-SOD

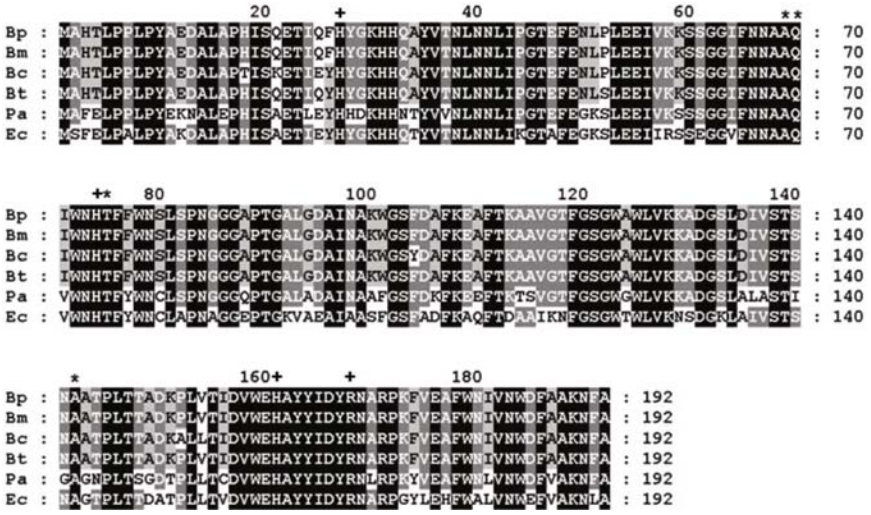

Fig. 1. Alignment of the deduced *B. pseudomallei* Fe-SOD amino acid sequence with those of other bacterial Fe-SODs. Bm, *Burkholderia mallei* (ATCC23344); Bt, *Burkholderia thailandensis* (E264); Bc, *Burkholderia cenocepacia* (AU1054); Pa, *Pseudomonas aeruginosa* (PA01); and Ec, *E. coli* O157:H7 (EDL933). The putative metal-binding residues that are conserved in Fe-SODs are marked with +. Residues that distinguish between Fe-SODs and Mn-SODs are indicated by *. Gaps were introduced to maximize the alignments.

were grown in LB medium, 100 μg/ml ampicillin with shaking at 37°C. When the cultures reached an A_{600} of 0.25, then 1, 2.5, or 5 μM paraquat was added and the cells were incubated for an additional 12 h before measuring the A_{600} again. Cell viability was also measured at different times after addition of 2.5 μM paraquat.

Results

Cloning and sequencing

We obtained the SOD gene from *B. pseudomallei* using PCR amplification, which yielded a product with an open-reading frame of 579 bp (192 amino acids). An alignment of the sequence with those of other bacterial Fe-SODs showed substantial similarity among the sequences (Fig. 1). After introducing appropriate gaps, the sequence of the *B. pseudomallei* Fe-SOD was found to have the following sequence identities to Fe-SODs from other bacteria: *Burkholderia mallei* (ATCC23344), 100%; *Burkholderia thailandensis* (E264), 98%; *Burkholderia cenocepacia* (AU1054), 95%; *Pseudomonas aeruginosa* (PA01), 75%; and *E. coli* O157:H7 (EDL933), 68%. All residues known to be responsible for binding Fe in previously characterized SODs (His27, His74, His161, and Arg168) are also present in the *B. pseudomallei* Fe-SOD, and there are also signature residues (Ala69, Gln70, Tyr75, and Ala142) that discriminate between Fe-SODs and Mn-SODs (Parker and Blake, 1988).

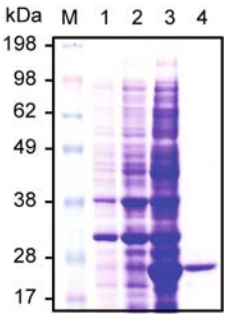

Fig. 2. Expression and purification of the *B. pseudomallei* recombinant Fe-SOD produced in *E. coli*. Expressed proteins were separated by SDS-PAGE and stained with Coomassie Blue. Lanes: 1, uninduced *E. coli* lysate; 2, IPTG-induced *E. coli* lysate (empty pDEST17); 3, IPTG-induced *E. coli* lysate (pDEST17-Fe-SOD); 4, purified recombinant *B. pseudomallei* Fe-SOD.

Expression and characterization

Fe-SOD was expressed in *E. coli* and purified using Ni-NTA affinity chromatography. Expressed protein was ~25 kDa (Fig. 2) corresponding to the sum of each predicted molecular weight of the protein (~22 kDa) and the $(His)_6$ tag (~2.6 kDa). SOD activity was inhibited by H_2O_2 but not by KCN or NaN_3 (Table 1). This result confirmed that the expressed protein is Fe-SOD. The enzyme was active over a broad range of pH (7.0–12.0) and showed maximum activity at pH 8.0 (Fig. 3A). It was stable at 37°C, retaining more than 80% of its activity for at least 48 h (Fig. 3B). However, it was very unstable at temperatures over 70°C. A single band of ~22 kDa was detected on the results of the western blot analysis using *B. pseudomallei* lysate and a single band of ~25 kDa was detected on the results of the western blot analysis using recombinant Fe-SOD. However, the corresponding band was absent when *E. coli* lysate was applied as a control (Fig. 4).

Response of *E. coli* to oxidative stress

To compare the effects of O_2^- produced by paraquat on the viability of *E. coli* transformed with pDEST-Fe-SOD, we cultured both the Fe-SOD-transformed strain and control strain with empty vector in the presence of paraquat. Within the range of paraquat concentrations tested, the cell viability of the transformed strain with pDEST-Fe-SOD was significantly higher than that of cells transformed with an empty pDEST as a control (Fig. 5A). The effect of paraquat on cell viability was also assessed over time. As expected, Fe-SOD-expressing cells were more resistant to paraquat at the con-

Table 1. Sensitivity of *B. pseudomallei* SOD to various SOD inhibitors

Inhibitor		Residual activity (%)
Control[a]		100.0
KCN	5 mM	95.3
	10 mM	89.4
NaN_3	5 mM	100.0
	10 mM	94.9
H_2O_2	5 mM	51.2
	10 mM	32.4

[a] No inhibitor present

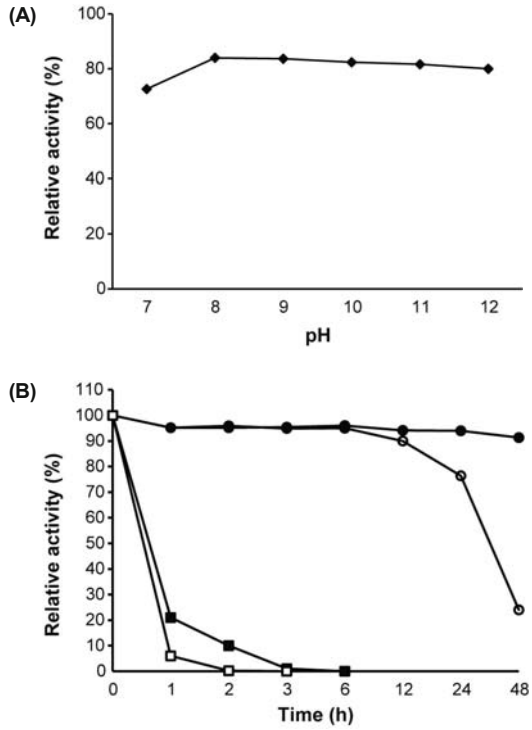

Fig. 3. Characterization of Fe-SOD. (A) Effect of pH on enzyme activity. SOD activity was assayed in 50 mM sodium phosphate, pH 7.0, or 50 mM Tris-HCl, pH 8.0–9.0, or 50 mM glycine-NaOH, pH 10.0–12.0. (B) Thermal stability. Purified Fe-SOD was incubated at various temperatures for the indicated times, and then, the residual enzyme activity was measured. Maximal activity was taken as 100%. 37°C (●), 50°C (○), 70°C (■), and 100°C (□).

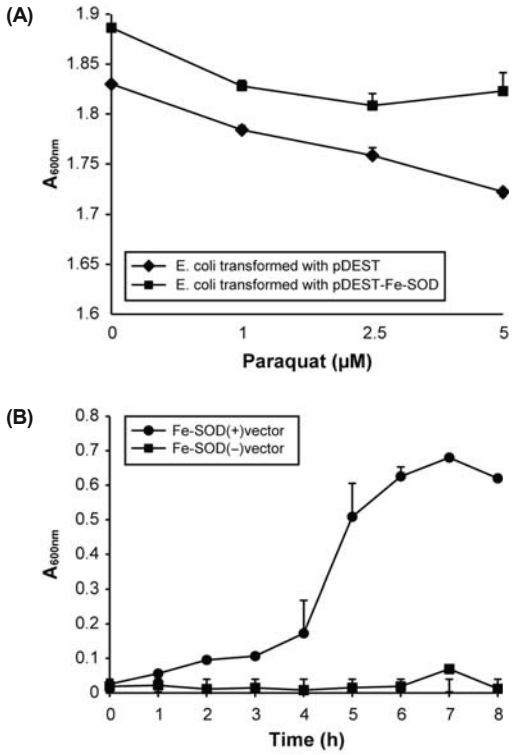

Fig. 5. Effect of paraquat on the viability of transformed and non-transformed *E. coli*. (A) *E. coli* cell density (A_{600}) for cells transformed with pDEST17 (empty vector) or with pDEST17-Fe-SOD after a 12-h incubation with different concentrations of paraquat and (B) in the absence or presence of 2.5 μM paraquat as a function of time. Cells were cultured in liquid LB medium containing 100 μg/ml ampicillin in all experiments.

centration of 2.5 μM, and the largest difference in cell viability of transformed and control strain was observed at 7 h after paraquat treatment (Fig. 5B). The total SOD activity from *E. coli* transformed with pDEST-Fe-SOD was nearly five-fold higher than the activity of the control strain in the presence or absence of paraquat (data not shown).

Discussion

Hosts mostly use immune mechanisms to defend against infection by facultative, intracellular bacteria such as *Salmonella*, *Listeria*, *Legionella*, and *Mycobacterium* species (Kaufmann, 1996). In particular, macrophages kill such pathogens after being stimulated by interferon-γ, which is produced by natural killer cells, γδ T cells, and type-1 helper T cells (Kaufmann, 1996). Interferon-γ-activated macrophages inhibit the intracellular viability of *B. pseudomallei* via reactive nitrogen intermediate- and reactive oxygen intermediate-dependent killing mechanisms (Miyagi *et al.*, 1997). As is the case for many other intracellular bacteria, *B. pseudomallei* are exposed to phagocyte-derived ROS during infection and multiplication. Therefore, detoxification of ROS might be important for the survival of *B. pseudomallei*.

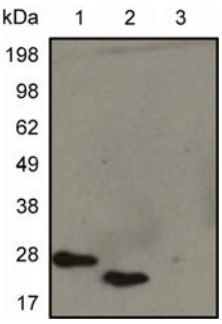

Fig. 4. Western blotting. *B. pseudomallei* lysate, *E. coli* lysate, and purified recombinant Fe-SOD (10 μg each) were separated by SDS-PAGE, Western blotting, and probed with polyclonal antibodies against the recombinant protein. Lanes: 1, purified recombinant Fe-SOD of *B. pseudomallei*; 2, *B. pseudomallei* lysate; 3, *E. coli* lysate.

Two major types of bacterial SODs exist: Mn-SODs, encoded by *sodA*, and Fe-SODs, encoded by *sodB* (Loprasert *et al.*, 2000). Several studies with bacterial pathogens have shown that microbial Fe-SODs contribute to pathogenicity, especially when they are secreted or membrane associated (Pesci *et al.*, 1994; Hess *et al.*, 1997; Clements *et al.*, 1999). In addition, previous studies have identified that *P. aeruginosa* SODs are required for full virulence, and its Fe-SOD is more important than its Mn-SOD during the infection process (Hassett *et al.*, 1995; Onate *et al.*, 1999; Iiyama *et al.*, 2007). Keith and Valvano (2007) provided preliminary information about the roles of SODs in the resistance of *B. cenocepacia* to ROS *in vitro*. Prior to this report, however, the biochemistry of *B. pseudomallei* Fe-SOD had not been characterized. Therefore, we characterized *B. pseudomallei* Fe-SOD, isolated from a Korean melioidosis patient, after

expressing the corresponding gene in *E. coli*. Fe-SOD of the *B. pseudomallei* isolated from a Korean patient showed 99.9% homology with the sequence of GenBank registered *B. pseudomallei*. However, the *B. pseudomallei* in this study is an important pathogen from the first Korean melioidosis isolate since *B. pseudomallei* was registered in the Act on Prevention and Control of Contagious Diseases in 2010.

In *B. pseudomallei*, coordinated regulation of gene expression by ferric ions depends on the regulatory protein Fur (ferric-uptake regulator). Fur is a positive regulator of Fe-SOD and peroxidase activities (Loprasert *et al*., 2000). A Fur mutant decreased Fe-SOD activity, which affected the bacterium's pathogenicity and its survival in macrophages (Loprasert *et al*., 2000). In our study, the effect of paraquat on the viability of *E. coli* transformed with an empty pDEST or pDEST-Fe-SOD was assessed as a function of time. We found that Fe-SOD-producing *E. coli* were significantly more resistant to paraquat as compared with control *E. coli* (Fig. 5B). Comparing Fe-SOD of other organisms, *B. pseudomallei* and columns of the Fe-SOD external environmental factors, such as pH, and showed a strong resistance.

In summary, cloning and expression of *B. pseudomallei* Fe-SOD provided an opportunity to study the biochemistry of the enzyme and its possible role in pathogenesis. Whereas SODs have not yet been reported as effective treatments for melioidosis, their possible use as drug candidates has been proposed. Our findings will allow us to identify potential chemotherapeutic and immunodiagnostic reagents for melioidosis. We are attempting to generate an Fe-SOD null-mutant *B. pseudomallei* strain by eliminating the gene. If we succeed, we can then investigate the biological and pathogenic *in vivo* roles of the enzyme more thoroughly.

Acknowledgements

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