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ORIGINAL PAPER

Fe-superoxide dismutase and 2-hydroxy-1,4-benzoquinone reductase preclude the auto-oxidation step in 4-aminophenol metabolism by *Burkholderia* sp. strain AK-5

Shinji Takenaka · Jyun Koshiya · Susumu Okugawa · Akiko Takata · Shuichiro Murakami · Kenji Aoki

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Abstract *Burkholderia* sp. strain AK-5 converts 4-aminophenol to maleylacetic acid via 1,2,4-trihydroxybenzene, which is unstable in vitro and non-enzymatically auto-oxidized to 2-hydroxy-1,4-benzoquinone. Crude extract of strain AK-5 retarded the auto-oxidation and reduced the substrate analogue, 2,6-dimethoxy-1,4-benzoquinone, in the presence of NADH. The two enzymes responsible were purified to homogeneity. The deduced amino acid sequence of the enzyme that inhibited the auto-oxidation showed a high level of identity to sequences of iron-containing superoxide dismutases (Fe-SODs) and contained a conserved metal-ion-binding site; the purified enzyme showed superoxide dismutase activity and contained 1 mol of Fe per mol of enzyme, identifying it as Fe-SOD. Among three type SODs tested, Fe-SOD purified here inhibited the auto-oxidation most efficiently. The other purified enzyme showed a broad substrate specificity toward benzoquinones, including 2-hydroxy-1,4-benzoquinone, converting them to the corresponding 1,4-benzenediols; the enzyme was identified as 2-hydroxy-1,4-benzoquinone reductase. The deduced amino acid sequence did not show a high level of identity to that of benzoquinone reductases from bacteria and fungi that degrade chlorinated phenols or nitrophenols. The indirect role of Fe-SOD in 1,2,4-trihydroxybenzene metabolism is probably to scavenge and detoxify reactive species that promote the auto-oxidation of 1,2,4-trihydroxybenzene in vivo. The direct role of benzoquinone reductase would be to convert the auto-oxidation product back to 1,2,4-trihydroxybenzene. These two enzymes together with 1,2,4-trihydroxybenzene 1,2-dioxygenase convert 1,2,4-trihydroxybenzene to maleylacetic acid.

Keywords 4-Aminophenol · *Burkholderia* · 1,2,4-Trihydroxybenzene · 2-Hydroxy-1,4-benzoquinone reductase · Fe-superoxide dismutase

Abbreviations

SOD	Superoxide dismutase
Fe-SOD	Fe-containing superoxide dismutase
Benzoquinone reductase	2-Hydroxy-1,4-benzoquinone reductase

S. Takenaka (✉) · J. Koshiya · S. Okugawa · A. Takata
Department of Applied Biological Chemistry, Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodai, Nada-ku, Kobe 657-8501, Japan
e-mail: hakko3@kobe-u.ac.jp

S. Murakami
Department of Bioscience, Biotechnology and Agrochemistry, Faculty of Agriculture, Meiji University, Tama-ku, Kawasaki 214-8571, Japan

K. Aoki
Department of Nutritional Management, Faculty of Nutritional Science, Sagami Women's University, Bunkyo, Sagamihara 228-8533, Japan

Introduction

4-Aminophenol is an intermediate in the biodegradation of hydroxyacetanilide and azo dyes (Hart and Orr 1975; Tan et al. 1999). It is highly toxic and mutagenic and induces DNA cleavage in mouse and human lymphoma cells (Majeska and Holden 1995; Yoshida et al. 1998). 4-Aminophenol is degraded by only a few bacterial species (Takenaka et al. 2003; Ahmed et al. 2001). *Burkholderia* sp. strain AK-5 metabolizes 4-aminophenol to maleylacetic acid via 1,2,4-trihydroxybenzene (Fig. 1, Takenaka et al. 2003). 1,2,4-Trihydroxybenzene is labile and in vitro is immediately converted non-enzymatically to the auto-oxidation product 2-hydroxy-1,4-benzoquinone by O_2 (Zaborina et al. 1999; Bohuslavek et al. 2005). Because 2-hydroxy-1,4-benzoquinone is not part of the pathway to maleylacetic acid, the pathway can only proceed if the auto-oxidation is avoided or if 2-hydroxy-1,4-benzoquinone is converted back to 1,2,4-trihydroxybenzene by benzoquinone reductase. Cell extract of *Rhodococcus* sp. BPG-8 are able to carry out both reactions, but the enzymes responsible have not been conclusively identified; the auto-oxidation is retarded and the free radical chain reaction between the superoxide radical and 1,2,4-trihydroxybenzene is terminated, possibly through the action of SOD and catalase, and 2-hydroxy-1,4-benzoquinone is reduced, possibly by NAD(P)H-dependent reductase (Armstrong et al. 1993). A reductase that reduces the auto-oxidation product back to 1,2,4-trihydroxybenzene has been identified in *Burkholderia cepacia* AC1100 (Zaborina et al. 1998) and *Cupriavidus necator* JMP134 (Belchik and Xun 2008).

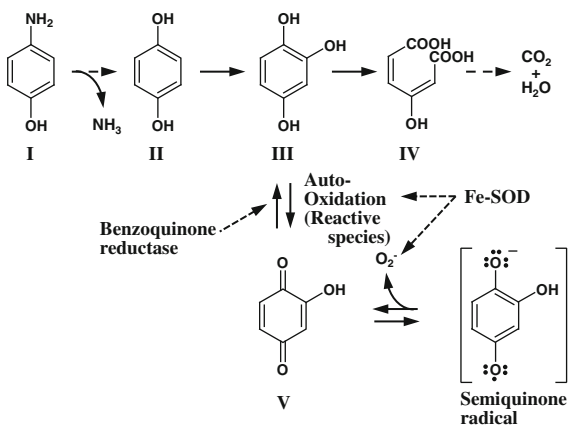

Fig. 1 Proposed pathway of 4-aminophenol metabolism in *Burkholderia* sp. strain AK-5 (adapted from Takenaka et al. 2003). The proposed conversion of the auto-oxidation product 2-hydroxy-1,4-benzoquinone back to the pathway intermediate 1,2,4-trihydroxybenzene by NADH-dependent benzoquinone reductase is indicated. Fe-SOD inhibits the auto-oxidation of 1,2,4-trihydroxybenzene by scavenging reactive species. *I* 4-aminophenol, *II* 1,4-benzenediol, *III* 1,2,4-trihydroxybenzene, *IV* maleylacetic acid, *V* 2-hydroxy-1,4-benzoquinone

The benzoquinone reductases from *B. cepacia* AC1100 (Zaborina et al. 1998) and *C. necator* JMP134 (Belchik and Xun 2008) are involved in trichlorophenol degradation and have been characterized. WrbA from *Escherichia coli*, earlier postulated to be a tryptophan repressor-binding protein, was later identified as an NAD(P)H:quinone oxidoreductase by sequence analysis and biochemical characterization (Patridge and Ferry 2006). An oxidoreductase functions as an oxidative stress protein in archaea (Patridge and Ferry 2006). It has been proposed that these reductases play a role in quinone detoxification by two-electron reduction and in the production of oxide radicals to attack wood via the Fenton reaction (Brock et al. 1995; Jensen et al. 2002; Zaborina et al. 1998).

During our purification of 1,2,4-trihydroxybenzene 1,2-dioxygenase from *Burkholderia* sp. AK-5 (Takenaka et al. 2003), we hypothesized that another set of enzymes involved in 4-aminophenol metabolism inhibits the auto-oxidation directly or indirectly. Here we report the purification and characterization of the enzymes involved in the metabolism of 1,2,4-trihydroxybenzene and their function in the metabolism of 4-aminophenol.

Materials and methods

Bacterial strains, plasmids, and culture conditions

Burkholderia sp. strain AK-5 was cultivated in basal medium (pH 5.5) containing 4-aminophenol as the sole carbon, nitrogen, and energy source as described previously (Takenaka et al. 2003). To analyze the synthesis of the two enzymes reported here, cells were grown in modified basal medium: 0.15% (w/v) NH_4NO_3 as sole nitrogen source and either 1.0% (w/v) sugar, 1.0% (w/v) amino acid, or 1.0% (w/v)

Table 1 Activity of SOD-like protein (Fe-SOD) and benzoquinone reductase (2-hydroxy-1,4-benzoquinone reductase) in cell extract of *Burkholderia* sp. strain AK-5 grown on various carbon sources

Growth substrate	Specific activity (U mg^{-1})[a]	
	SOD-like protein	Benzoquinone reductase
4-Aminophenol	0.72 ± 0.036	0.37 ± 0.017
D-Glucose	0.49 ± 0.025	0.21 ± 0.011
D-Fructose	0.48 ± 0.027	0.23 ± 0.016
D-Mannitol	0.33 ± 0.016	0.19 ± 0.018
Fumaric acid	0.31 ± 0.012	0.21 ± 0.010
D,L-Malic acid	0.31 ± 0.012	0.14 ± 0.006
Disodium succinate	0.19 ± 0.11	0.13 ± 0.007
L-Leucine	0.27 ± 0.062	0.15 ± 0.009
L-Proline	0.52 ± 0.037	0.18 ± 0.012
L-Phenylalanine	0.43 ± 0.011	0.16 ± 0.020

[a] Strain AK-5 was cultivated in each test medium in triplicate. Cells (1.0 g wet wt) were suspended in 20 mM Tris–HCl buffer (pH 8.0) containing 10 μM FMN. Crude extract (10 ml) was dialyzed against the buffer and then applied to a DE52 cellulose column (1.6 × 12.0 cm), and proteins were eluted with a linear gradient (0 to 0.25 M) of NaCl. The specific activity of enzyme formed was quantified as described in section *Enzyme assays*. Each value is the mean ± SD for three experiments

organic acid (Table 1) as sole carbon and energy source instead of 4-aminophenol. Modified basal medium (7 ml) in a test tube was inoculated with strain AK-5 and incubated at 30°C with shaking for 1 day; 3 ml was then used to inoculate 67 ml of the same medium in a 500-ml flask, which was then incubated until the mid- or late-exponential growth phase ($OD_{660\ nm}$ of 1.5–1.7). *Escherichia coli* XL-1 Blue was used for plasmid maintenance and construction. pBluescript KS(+) and pGEM-T easy vector system were used for cloning. *E. coli* was cultivated in LB medium supplemented, if necessary, with ampicillin (100 μg ml^{-1}), isopropyl-β-D-thiogalactopyranoside (0.2 mM), and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (0.04%); cultures were incubated at 37°C with shaking.

Enzyme purification

All steps of the enzyme purification were carried out at 0–4°C. All centrifugations were at 20,000×*g* and for 10 min. The enzyme purity was checked by SDS-PAGE.

(i) SOD-like protein (Fe-SOD)

Cells (13 g wet wt) were suspended in 20 mM Tris–HCl buffer (pH 8.0) (buffer A). Cell extract (fraction 1, 130 ml) was prepared with an ultrasonic oscillator and treated with streptomycin sulfate (fraction 2, 137 ml) as described previously (Aoki et al. 1997). Fraction 2 was fractionated with ammonium sulfate (55–75% saturation). After centrifugation, the pelleted precipitate was dissolved in buffer A. The solution was dialyzed against buffer A (fraction 3, 34 ml). Fraction 3 was applied to a DE52 cellulose column (1.6 × 17.5 cm), and proteins were eluted with a linear gradient (0 to 0.2 M NaCl). The active fractions (fraction 4, 24 ml) were applied to a DEAE-Cellulofine A-800 column (1.6 × 12 cm), and proteins were eluted with a linear gradient (0 to 0.2 M) of NaCl. The active fractions (fraction 5, 15 ml) was applied to a Phenyl-Cellulofine column (1.6 × 7.5 cm), and proteins were eluted with a linear gradient (1.0 to 0 M) of $(NH_4)_2SO_4$. The active fractions were pooled (fraction 6, 12 ml).

(ii) Benzoquinone reductase (2-hydroxy-1,4-benzoquinone reductase)

Cells (15.3 g wet wt) were suspended in 20 mM Tris–HCl buffer (pH 8.0) containing 10 μM FMN to stabilize benzoquinone reductase (buffer B). Cell extract (fraction 1, 150 ml) was treated with streptomycin sulfate (fraction 2, 137 ml). Fraction 2 was fractionated with ammonium sulfate (45–65% saturation). After centrifugation, the pelleted precipitate was dissolved in buffer B. The solution was dialyzed against buffer B (fraction 3, 26 ml). Fraction 3 was applied to a DE52 cellulose column (1.6 × 17.5 cm), and proteins were eluted with a linear gradient (0 to 0.25 M NaCl). The active fractions (fraction 4, 48 ml) were applied to a DEAE-Cellulofine A-800 column (2.2 × 13 cm), and proteins were eluted with a linear gradient (0 to 0.25 M) of NaCl at a flow rate of 40 ml h^{-1}. The active fractions (fraction 5, 30 ml) were applied to a Phenyl-Cellulofine column (1.6 × 14 cm), and proteins were eluted with a linear gradient (0.5 to 0 M) of $(NH_4)_2SO_4$. The active fractions were pooled (fraction 6, 33 ml).

Enzyme assays

(i) SOD-like protein (Fe-SOD)

1,2,4-Trihydroxybenzene was non-enzymatically converted to 2-hydroxy-1,4-benzoquinone (Fig. 2a). The enzyme fraction reported here retarded the auto-oxidation of 1,2,4-trihydroxybenzene. In the assay, the auto-oxidation was monitored by following the formation of benzoquinone at 260 nm using the molar extinction coefficient of 4.5×10^3 M^{-1} cm^{-1}. The reaction mixture contained 2.8 ml of 100 mM sodium–potassium phosphate buffer (pH 7.5), 0.05 ml of enzyme solution, and 0.05 ml of 10 mM freshly prepared 1,2,4-trihydroxybenzene to start the reaction and was incubated for 10 min at 30°C. One unit of enzyme activity was defined as the amount of enzyme that inhibits the formation of 1 μmol of 2-hydroxy-1,4-benzoquinone per min.

(ii) Benzoquinone reductase (2-hydroxy-1,4-benzoquinone reductase)

The reductase activity was assayed using 2,6-dimethoxy-1,4-benzoquinone and monitoring the oxidation of NADH at 340 nm according to Brock et al. (1995) and Zaborina et al. (1998). The molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1} at 340 nm for NADH was used. One unit of enzyme activity was defined as the amount of enzyme required to consume 1 μmol of NADH per min.

(iii) Superoxide dismutase

The activity was determined using the SOD assay kit WST (Dojindo, Tokyo, Japan) according to Ukeda et al. (2002) and the manufacturer's instructions. SOD from bovine erythrocytes (Sigma, St. Louis, Mo) was used for the standard curve.

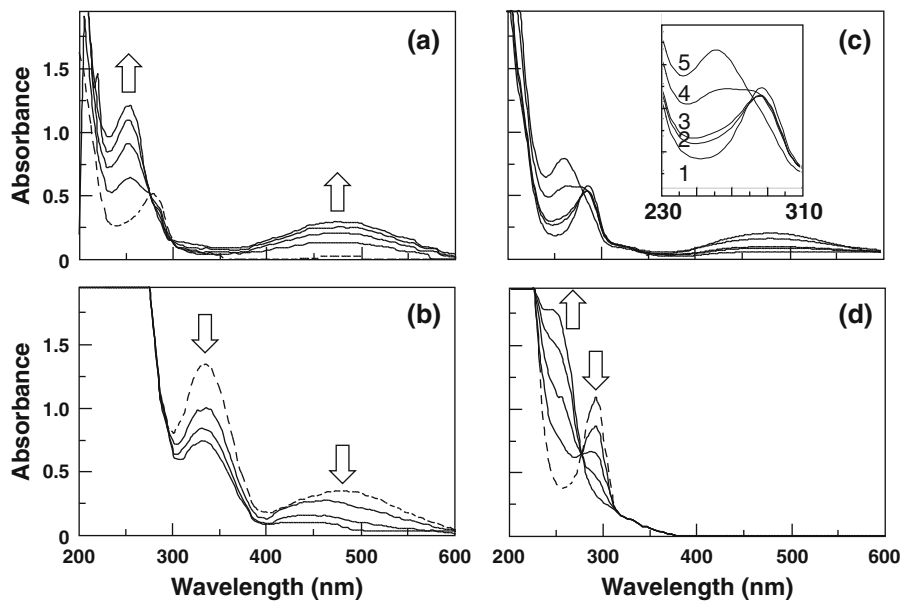

Fig. 2 Spectral changes of the reaction products from 1,2,4-trihydroxybenzene. The reaction mixtures consisted of **a** 2.8 ml of 100 mM sodium–potassium phosphate buffer (pH 7.5), 0.1 ml of 2.5 mM 1,2,4-trihydroxybenzene, and 0.1 ml of 20 mM Tris–HCl buffer (pH 8.0) and **b** 2.8 ml of 100 mM sodium–potassium phosphate buffer (pH 7.5), 0.1 ml of 2.5 mM 2-hydroxy-1,4-benzoquinone, 0.1 ml of purified benzoquinone reductase (37 μg ml^{-1}), and 0.15 ml of 6 mM NADH. The reaction mixture (**c**) consisted of 2.7 ml of 100 mM sodium–potassium phosphate buffer (pH 7.5), 0.1 ml of 2.5 mM 1,2,4-trihydroxybenzene, 0.1 ml of purified SOD-like protein (11 μg ml^{-1}, *line 1*), commercial Fe-SOD (11 μg ml^{-1}, *line 2*), Mn-SOD (11 μg ml^{-1}, *line 3*), or Cu–Zn-SOD (11 μg ml^{-1}, *line 4*). The samples were scanned after addition of 1,2,4-trihydroxybenzene (*line 5*) and incubation for 10 min. The reaction mixture (**d**) consisted of 2.8 ml of 100 mM sodium–potassium phosphate buffer (pH 7.5), 0.1 ml of 5 mM 1,2,4-trihydroxybenzene, and 0.1 ml of purified SOD-like protein (Fe-SOD, 37 μg ml^{-1}), 0.1 ml of partially purified 1,2,4-trihydroxybenzene 1,2-dioxygenase (1.8 U ml^{-1}). Each sample was scanned at 2-min intervals with a Hitachi U-2800 spectrophotometer. The spectrum (initial line) is shown with a *dotted line*. The directions of spectral changes are indicated by *arrows*

Characterization of the purified enzymes

The pH and thermal stabilities of the purified SOD-like protein (0.2 to 25 μg) and benzoquinone reductase (0.5 to 20 μg) and the effect of various compounds on their activities were analyzed. The effect of metal salts and chelating and sulfhydryl agents on SOD-like protein activity with 1,2,4-trihydroxybenzene as substrate and the benzoquinone reductase activity with 2,6-dimethoxy-1,4-benzoquinone as substrate was tested using methods described previously (Takenaka et al. 2003). The effects of H_2O_2, KCN, and NaN_3 on SOD activity was tested using the SOD assay kit WST described above. Each assay was carried out in triplicate.

Determination of NH_2-terminal and internal amino acid sequences

To determine the NH_2-terminal amino acid sequences, each of the two purified enzymes reported here (100 μg) was electroblotted onto Clear Blot Membrane-P (ATTO, Tokyo) according to the manufacturer's instructions. The enzyme (200 μg) was hydrolyzed by a lysylendo-peptidase, and the peptides formed were fractionated by reversed-phase HPLC on a Cosmosil 5C18 AR-II column (4.6×150 mm; Nacalai Tesque, Kyoto). The HPLC column was equilibrated with 5 mM trifluoroacetic acid in H_2O; peptides were separated with a linear gradient of acetonitrile at 0.8% per min at a flow rate of 0.50 ml min^{-1}. The NH_2-terminal and internal amino acid sequences were identified with a Shimazu PPSQ-10 protein sequencer.

DNA manipulations

All procedures followed standard methods or manufacturer's instructions. Amplified DNA fragments were purified using a MonoFas gel extraction kit (GL Science, Tokyo, Japan) and ligated into a pGEM-T Easy vector system (Promega, Madison, WI, USA).

Amplification of the ORF gene encoding the SOD-like protein

To amplify part of ORF encoding the SOD-like protein, we synthesized the following degenerate oligonucleotide primers deduced from the NH_2-terminal amino acid sequence (PLPFDKNA) and the internal amino acid sequence (WNIANWDF): forward primer, SOD_F_2 primer (5′-CCIYTICCITT YGAYAARAAYGC-3′); and reverse primer, SOD_R_4 primer (5′-RAARTCCCARTTIGCDTARTTC C-3'), where R is A or G, Y is C or T, and I is inosine. The amplified fragment was then used as a probe for southern hybridization.

Chromosomal DNA was partially digested with several restriction enzymes, separated on a 1% (w/v) agarose gel, and blotted onto a Hybond-N+ membrane (GE Healthcare UK Ltd, Buckinghamshire, England) using a VacueGene XL vacuum blotting system (GE Healthcare). The amplified fragment was labelled with an AlkPhos direct-labelling module (GE Healthcare) and was used as a probe. DNA fragments that hybridized with the probe were detected using a CDP-Star detection reagent (GE Healthcare) according to the manufacturer's instructions. Fragments of 1.6 kb generated with *Mul*I hybridized to the probe. The *Mul*I fragment that hybridized with the 540-bp fragment was amplified by inverse PCR.

The *Mul*I-digested DNA fragments were extracted with phenol–chloroform–isoamyl alcohol, ethanol precipitated, and then self-ligated with T4 DNA ligase. The ligated DNA was used as a template for inverse PCR (Triglia et al. 1988) to amplify the SOD-like protein ORF fragment using the following degenerate oligonucleotide primers based on the sequence of the amplified fragment: SOD_inverse_1 sense primer (5′-GCGTTGATGGCGTCAGCC-3′) and SOD_inverse_2 anti-sense primer (5′-AAGTT-CAAGGAAGAGTTCGCG-3′).

The entire ORF of the SOD-like protein was amplified by PCR using the primers SOD_F_HindIII (5′-AAAGG AAGCTTGCATCATGG-3′) and SOD_R_SacI (5′-AC ACGGAGCTCGTCAAACCC-3′) and high fidelity KOD plus DNA polymerase (Toyobo) to reduce mispriming of the amplified nucleotide.

Amplification of the ORF encoding benzoquinone reductase

To amplify part of the reductase ORF, we synthesized the following degenerate oligonucleotide primers based on the deduced NH_2-terminal amino acid sequence (KIAVVVG) and the deduced internal amino acid sequence (KYAALVX): forward primer, Reductase II_F primer (5′-AARATHGCIGTIGTIGTIG G-3′); and reverse primer, Reductase II_R1 primer

(5′-ACDATICCIGTIARRTAYTT-3′). This fragment was then used as a probe for Southern hybridization and colony hybridization.

Digestion of chromosomal DNA from strain AK-5, blotting on the membrane, and labeling the 520-bp amplified fragment were carried out as described above. Fragments of 3.5 kb generated with *Sal*I hybridized to the probe.

To clone the benzoquinone reductase ORF, genomic DNA from strain AK-5 was digested with *Sac*I, and the fragments were separated by electrophoresis on a 1% (w/v) agarose gel. The digested 3.0–4.0-kb DNA fragments were cut out from the agarose gel, purified with the gel extraction kit, and ligated to *Sac*I-predigested pBluescript KS(+) vector. The ligation products were introduced into *E. coli* XL-1 Blue cells by transformation. One positive clone, strain 8-7B, was identified among 1,056 transformants. The ORF encoding the benzoquinone reductase was sequenced.

Analytical methods

UV absorption spectra of reaction products were recorded with a Hitachi U-2800 spectrophotometer. The apparent molecular mass of the native enzyme was determined by gel filtration on Cellulofine GCL-1000 sf. The molecular mass of the enzyme subunits was determined using SDS-PAGE. Protein concentrations were measured using the method of Lowry et al. (1951). The enzymatic reaction product formed by benzoquinone reductase was derivatized with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide and was analyzed as described previously (Takenaka et al. 2003). Iron in the SOD-like protein (520 μg) was measured by inductively coupled plasma spectroscopy using a Shimadzu ICP 1000 III atomic emission spectrometer. SOD activity staining on native polyacrylamide gels was carried out with nitro blue tetrazolium and riboflavin, following the method of Beauchamp and Fridovich (1971). O_2^- was produced by riboflavin under light; SOD reduction of nitro blue tetrazolium with scavenged O_2^- formed a clear zone on the gel.

Chemicals

4-Aminophenol, 1,2,4-trihydroxybenzene (hydroxyhydroquinone), 1,4-benzoquinone, 2-methoxy-1,4-benzoquinone, 2,6-dimethoxy-1,4-benzoquinone, *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide, and lysyl-endo-peptidase were purchased from Wako Pure Chemicals (Osaka, Japan). Fe-SOD (Sigma, product number: S5639) and Mn-SOD (Sigma, product number: S5389) from *Escherichia coli* and Cu–Zn SOD (Sigma, product number: S5395) from bovine erythrocytes were from Sigma-Aldrich (St. Louis, Mo.). Partially purified 1,2,4-trihydroxybenzene 1,2-dioxygenase from strain AK-5 was prepared as described previously (Takenaka et al. 2003).

Nucleotide sequence accession number

The nucleotide sequences of the ORFs encoding Fe-SOD (SOD-like protein) and 2-hydroxy-1,4-benzoquinone reductase (benzoquinone reductase) have been submitted to the DNA Data Bank of Japan (DDBJ) with the accession number AB518002 and AB518003, respectively.

Results and discussion

Synthesis of SOD-like protein and benzoquinone reductase by *Burkholderia* sp. strain AK-5 grown on various carbon sources

Cell extract of 4-aminophenol-grown cells showed NAD(P)H-dependent quinone reductase activity toward 2,6-dimethoxy-1,4-benzoquinone. The peak of NADH-dependent reductase activity eluted from a DE52-cellulose anion-exchange column at 0.20 M NaCl. Each fraction separated by DE52 was then incubated with 1,2,4-trihydroxybenzene instead of 2,6-dimethoxy-1,4-benzoquinone as substrate. Although 1,2,4-trihydroxybenzene was non-enzymatically oxidized in most of the reaction mixtures (Fig. 2a), another fraction eluted at 0.077 M NaCl inhibited the auto-oxidation without NAD(P)H was found out. Analysis of the absorption spectra indicated that the inhibitory activity without cofactor was mainly due to SOD. Therefore, the enzymes in these two fractions were designated SOD-like protein (fraction eluting at 0.077 M NaCl) and benzoquinone reductase (fraction eluting at 0.20 M NaCl), respectively.

Strain AK-5 grew well on the carbohydrates tested, and SOD-like protein and benzoquinone reductase

activities were detected in all cell extracts; the activities were highest in extract of cells grown on 4-aminophenol (Table 1). Quantitative proteomics analysis has indicated that four oxidative stress responsible proteins, including SodB (Fe-SOD), are up-regulated by the addition of phenol to the succinate medium (Santos et al. 2004). Higher specific activities of benzoquinone reductase have also been observed when *P. chrysosporium* OGC101 (Akileswaran et al. 1999), *G. trabeum* ATCC 11539 (Jensen et al. 2002), and *P. chrysosporium* ATCC 24725 (Constam et al. 1991) are grown in the presence of substrates or their precursors.

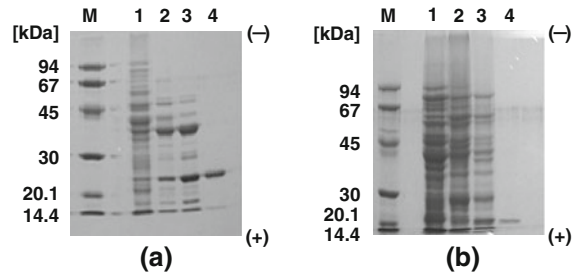

Fig. 3 SDS-PAGE of **a** the purified SOD-like protein (Fe-SOD) and **b** the purified benzoquinone reductase (2-hydroxy-1,4-benzoquinone reductase). Lanes: *M* protein size markers (electrophoresis calibration kit LMW, GE healthcare), *1* fraction 3 (ammonium sulfate), *2* fraction 4 (DE52 fraction), *3* fraction 5 (DEAE-Cellulofine A-800 fraction), and *4* fraction 6 (Phenyl-Cellulofine fraction, 10 μg of purified enzyme)

Purification and molecular properties of the SOD-like protein and benzoquinone reductase

The SOD-like protein was purified (Table 2) to form a single protein band on both native-PAGE (data not shown) and SDS-PAGE (Fig. 3a). We tested the purified protein for superoxide radical scavenging activity, which can be observed as achromatic zones on otherwise uniformly blue gels; activity was detected in polyacrylamide gels lacking SDS at the position where the purified enzyme occurred (data not shown). The molecular mass was determined to be 28 kDa by gel filtration and 23 kDa by SDS-PAGE, which indicated that the enzyme is a monomer.

The benzoquinone reductase was purified (Table 3) to form a single protein band on both native-PAGE (data not shown) and SDS-PAGE (Fig. 3b). NADH-dependent enzymes in the crude extract interfered with the measurement of reductase activity, which explains the large increase in total activity after the initial purification step. The molecular mass was determined to be 68 kDa by gel filtration and 18 kDa by SDS-PAGE, which indicated that the enzyme is a tetramer.

In the mixture lacking enzyme, the maximal absorption peak at 280 nm of 1,2,4-trihydroxybenzene in sodium–potassium phosphate buffer (pH 7.5) gradually decreased over time, and absorption peaks at 260 and at 473 nm derived from the auto-oxidation product 2-hydroxy-1,4-benzoquinone gradually increased (Fig. 2a). Similar spectral changes during non-enzymatic oxidation of hydroxyquinol (1,2,4-trihydroxybenzene) have been reported by Chapman and Ribbons (1976). In our study, the auto-oxidation was inhibited almost completely in the presence of purified SOD-like protein, and the maximal absorption peak at 473 nm derived from

Table 2 Purification of SOD-like protein (Fe-SOD) from *Burkholderia* sp. strain AK-5

Purification step[a]	Total activity[b] (U)	Total protein (mg)	Specific activity (U mg^{-1})	Recovery (%)
1: Cell extract	–[c]	1,700	–	–
2: Streptomycin sulfate	–	1,600	–	–
3: Ammonium sulfate	150	100	1.5	100
4: DE52	40	6.5	6.2	27
5: DEAE-Cellulofine A-800	12	1.2	10	8.0
6: Phenyl-Cellulofine	5.8	0.31	19	3.9

[a] The activities and protein contents of the fractions pooled after each step were determined. See the text for details

[b] The auto-oxidation of 1,2,4-trihydroxybenzene was monitored by following the formation of 2-hydroxy-1,4-benzoquinone at 260 nm, and the enzyme activity was calculated (see the text for details)

[c] Fractions contained 1,2,4-trihydroxybenzene 1,2-dioxygenase, which interfered with the measurements of the enzyme activity

Table 3 Purification of benzoquinone reductase (2-hydroxy-1,4-benzoquinone reductase) from *Burkholderia* sp. strain AK-5

Purification step[a]	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Recovery (%)
1: Cell extract	9.1	2,000	0.0046	100
2: Streptomycin sulfate	8.2	1,700	0.0048	90
3: Ammonium sulfate	0.75	350	0.0021	8.2
4: DE52	110	25	4.4	1,200
5: DEAE-Cellulofine A-800	57	5.2	11	630
6: Phenyl-Cellulofine	24	0.24	100	260

[a] The activities and protein contents of the fractions pooled after each step were determined. See the text for details

2-hydroxy-1,4-benzoquinone gradually decreased when purified benzoquinone reductase and cofactor were added (Fig. 2b).

Properties of purified SOD-like protein

Purified SOD-like protein was stable for one month in 50 mM sodium–potassium phosphate buffer (pH 7.0). The enzyme (5.0 ± 0.07 U mg^{-1}) maintained more than 80% activity (4.0 ± 0.05 U mg^{-1}) after 10-min incubation at up to 75°C, and showed maximal activity at pH 7.5. Among the metal salts, chelating agents, and sulfhydryl agents tested, the enzyme (5.0 ± 0.07 U mg^{-1}) was only inhibited slightly by 1 mM $AgNO_3$ (4.1 ± 0.03 U mg^{-1}) and 1 mM $NiSO_4 \cdot H_2O$ (4.3 ± 0.10 U mg^{-1}). The addition of *o*-phenanthroline (1 mM) slightly decreased the enzyme activity to 73% (3.65 ± 0.06 U mg^{-1}). The concentrated enzyme solution was light yellow. The UV–visible spectrum of the enzyme solution showed two maxima at 280 and 360 nm, with an A_{280}/A_{360} ratio of 13.5. One unit of SOD that causes 50% inhibition under previously reported conditions (Ukeda et al. 2002) is equivalent to 0.045 μg of commercially pure bovine erythrocyte SOD per ml (Beauchamp & Fridovich, 1971). The purified enzyme (3.4 μg) from strain AK-5 showed 50% inhibition under these conditions. SODs are classified into three categories: Cu, Zn-SOD; Mn-SOD; and Fe-SOD. Cu, Zn-SOD is sensitive to H_2O_2 and KCN (Dos Santos et al. 2000; Fridovich, 1975; Misra and Fridovich, 1978), whereas Fe-SOD is sensitive to H_2O_2 and NaN_3 but not to KCN. Mn-SOD is inhibited by NaN_3 but not by KCN or H_2O_2. The addition of 1 mM H_2O_2, KCN, and NaN_3 decreased the SOD activity of purified SOD-like protein to 60, 98, and 75%, respectively. Furthermore, inductively coupled plasma spectroscopy analysis indicated that the SOD-like protein contained 1 mol Fe per mol protein considering a molecular mass of 23 kDa. All these results indicated that the SOD-like protein is an Fe-SOD.

The NH_2-terminal amino acid sequence (MEHT LPPLPFDKNALADPMMEET) and internal amino acid sequence (ALLTIDVWXHAYYIDYA) of Fe-SOD were determined, and the encoding ORF was cloned. The 579-bp ORF encodes a 193 amino-acid protein and a TAA stop codon. The calculated molecular mass of the predicted translation product is 21,512 Da. Also the entire deduced amino acid sequence had a high level of identity to that of previously reported Fe-SOD from *E. coli* (AP_002278, 67.4% identity with Fe-SOD from strain AK-5), *Photobacterium leiognathi* (P09213, 60.0%), *Bordetella pertussis* (AAC36882, 79.2%), and *Pseudomonas ovalis* (1DT0_A, 68.3%). The amino acid sequence of the Fe-SOD contains amino acids conserved in Fe-SODs, including metal ion binding residues (27-His, 74,-His, 157-Asp, and 161-His) (Stoddard et al. 1990). To our knowledge, this is the first report of a purified Fe-SOD from *Burkholderia*.

Properties of purified benzoquinone reductase

The structure of the enzymatic reaction product from 2-hydroxy-1,4-benzoquinone was analyzed by GC–MS. The mass spectrum of the trimethylsilylated enzyme reaction product (compound III) yielded a molecular ion at *m/z* 342 (M^+, relative intensity 30%). This mass spectrum and the GC retention time (12.1 min) of the trimethylsilylated enzyme reaction product agreed with those of authentic trimethylsilylated 1,2,4-trihydroxybenzene. UV–visible and MS analyses indicated that the purified enzyme converted 2-hydroxy-1,4-benzoquinone to 1,2,4-trihydroxybenzene.

Benzoquinone reductase was stable for 1 month in 50 mM acetate-sodium acetate buffer (pH 3.5–5.5). The enzyme (20 ± 1.7 U mg^{-1}) maintained more than 80% activity (16 ± 2.1 U mg^{-1}) after 10-min incubation at temperatures up to 50°C, and showed maximal activity at pH 5.0. The addition of 1 mM Hg^{2+}, Zn^{2+}, *p*-chloromercuribenzoic acid, 5,5′-dithiobis(2-nitrobenzoic acid), or Tiron decreased the enzyme activity (25 ± 1.9 U mg^{-1}) to 0, 51 (20 ± 1.7 U mg^{-1}), 54 (20 ± 1.7 U mg^{-1}), 55 (20 ± 1.7 U mg^{-1}), and 42% (20 ± 1.7 U mg^{-1}), respectively. The benzoquinone reductase from strain AK-5 reported here showed a broader substrate specificity toward benzoquinones than the enzyme from *P. chrysosporium* OGC101 (Table 4), which attacks *p*- and *o*-quinones (Brock et al. 1995), and that from *B. cepacia* strain AC1100, which attacks hydroxybenzoquinone, methylbenzoquinone, and 5-chlorohydroxybenzoquinone. The enzyme from strain AK-5 reduced benzoquinones to the corresponding hydroquinones. The reductase from *P. chrysosporium* OGC101 differs from that of strain AK-5 in its inhibition pattern; the reductase from strain OGC101 is not inhibited by Zn^{2+}, Cu^{2+}, and Mg^{2+} and is slightly inhibited by EDTA (Brock et al. 1995). These quinone reductases from strains OGC101 and AC1100 have been characterized in detail, but the genes encoding the enzymes have not yet been cloned and analyzed.

The NH_2-terminal and inner amino acid sequences predicted from the ORF (549 bp) correspond to those of the purified the benzoquinone reductase. The ORF encodes a 183 amino-acid protein and a TAA stop codon. The calculated molecular mass of the predicted translation product is 20,210 Da. The benzoquinone reductase amino acid sequence shows only 38.6% identity with that of NAD(P)H:quinone reductase from *Arabidopsis thaliana* (Sparla et al. 1999, AF145234) and did not show any identity with microbial quinone reductases, such as 6-chlorohydroxyquinone reductase (TcpB) from the 2,4,6-trichlorophenol-assimilating *Cupriavidus nector* JMP134 (Belchik and Xun 2008, AAM55215), the *p*-benzoquinone reductase from the *p*-nitrophenol-assimilating *Pseudomonas* sp. WBC-3 (Zhang et al. 2009, ABU50909), and 1,4-benzoquinone reductase from the chlorinated-phenol-degrading white rot basidiomycete *Phanerochaete chrysosporium* (Akileswaran et al. 1999, AF106939). The benzoquinone reductase from strain WBC-3 plays a critical role in *p*-nitrophenol degradation; the enzyme enhances the activity of the first enzyme in the pathway, *p*-nitrophenol monooxygenase (Zhang et al. 2009). TcpB from *Cupriavidus nector* strain JMP134 reduces 6-chlorohydoxyquinone and can enhance the monooxygenase that acts on 2,4,6-trichlorophenol (Belchik and Xun 2008).

The role of Fe-SOD and 2-hydroxy-1,4-benzoquinone reductase in strain AK-5

Armstrong et al. (1993) previously proposed that SOD and catalase are involved in the metabolism of 1,2,4-trihydroxybenzene by *Rhodococcus* sp. BPG-8 and that a specific reductase converts 2-hydroxy-1,4-benzoquinone back to 1,2,4-trihydroxybenzene based on their results using cell extract and commercial SOD and catalase. In our study, we purified, characterized, and identified the enzymes involved as Fe-SOD and NADH-dependent 2-hydroxy-1,4-benzoquinone reductase. In our assay, commercial Fe-SOD from *E. coli* also effectively inhibited the auto-oxidation, whereas Cu–Zn SOD from bovine erythrocytes and Mn-SOD from *E. coli* did not

Table 4 Substrate specificity of benzoquinone reductase (2-hydroxy-1,4-benzoquinone reductase)

Compound	Relative activity (%)	K_m (mM)	V_{max} (U mg^{-1})
1,4-Benzoquinone	1,200	0.035 ± 0.002	0.83 ± 0.042
2,6-Dimethoxy-1,4-benzoquinone	23	N.D.	N.D.
2-Hydroxy-1,4-benzoquinone	100	0.12 ± 0.008	0.10 ± 0.007
2-Methoxy-1,4-benzoquinone	1,500	0.031 ± 0.001	0.56 ± 0.002
2-Methyl-1,4-benzoquinone	1,600	0.048 ± 0.004	0.79 ± 0.063

Each K_m and V_{max} values is the mean ± SD for three experiments

N.D. not data

(Fig. 2c). The synthesis of Fe-SOD and 2-hydroxy-1,4-benzoquinone reductase reported here was promoted in the presence of 4-aminophenol (Table 1), and might be inducible.

The spectral changes of the reaction products (Fig. 2b, d) indicates that the two enzymes reported here function together with 1,2,4-trihydroxybenzene 1,2-dioxygenase in vivo to convert the 4-aminophenol pathway intermediate 1,2,4-trihydroxybenzene only to maleylacetic acid (Fig. 1), and that they probably play a role in 4-aminophenol metabolism, Fe-SOD indirectly and benzoquinone reductase directly. Fe-SOD from strain AK-5 possibly savages and detoxifies reactive species, including super oxide (O_2^-), that promote the auto-oxidation of 1,2,4-trihydroxybenzene (Fig. 1).

The role of the benzoquinone reductase in vivo and in the metabolic pathway in *Saccharomyces cerevisiae* (Sollner et al. 2007) and in the *p*-nitrophenol-degrading *Pseudomonas* sp. strain WBC-3 (Zhang et al. 2009), respectively, was studied using deletion mutants. We have previously characterized 1,2,4-trihydroxybenzene 1,2-dioxygenase involved in 4-aminophenol metabolism in strain AK-5 (Takenaka et al. 2003) and separated two dioxygenases by hydrophobic chromatography (unpublished data). By disrupting each of the genes encoding Fe-SOD, 2-hydroxy-1,4-benozoquinone reductase, and 1,2,4-trihydroxybenzene 1,2-dioxygenase and comparing the growth of these mutants on 4-aminophenol of these mutants with that of the wild-type strain, the roles of the enzymes in vivo will become clearer.

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