Characterization of Monofunctional Catalase KatA from Radioresistant Bacterium \textit{Deinococcus radiodurans}

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Catalase plays a key role in protecting cells against toxic reactive oxygen species. Here we report on the cloning, purification and characterization of a catalase (KatA, DR1998) from the extremely radioresistant bacterium \textit{Deinococcus radiodurans}. The size of purified \textit{D. radiodurans} KatA monomer was 65 kDa while gel filtration revealed that the size of the enzyme was 240 kDa, suggesting that KatA formed a homotrimer in solution. Purified KatA displayed a final specific activity of 68,800 U/mg of protein. The catalase activity of KatA was inhibited by sodium azide, sodium cyanide and 3-amino-1,2,4-triazole. The absorption spectrum of KatA exhibited a Soret band at 408 nm. The position of the spectral peak remained unchanged following reduction of KatA with dithionite. No peroxidase activity was found for KatA. These results demonstrate that \textit{D. radiodurans} KatA is a typical monofunctional heme-containing catalase. The stability of KatA with respect to \textit{H}_2\textit{O}_2\textit{O}_2 stress was superior to that of commercially available \textit{Aspergillus niger} and bovine liver catalases. The relative abundance of KatA in cells in addition to the \textit{H}_2\textit{O}_2\textit{O}_2 resistance property may play a role in the survival strategy of \textit{D. radiodurans} against oxidative damage.

[Key words: antioxidant, catalase, \textit{Deinococcus radiodurans}, gene expression, hydrogen peroxide resistance]

Hydrogen peroxide (\(\text{H}_2\text{O}_2\)) and other reactive oxygen species (ROS) such as superoxide anion (\(\text{O}_2^-\)) are produced in aerobes from the partial reduction of molecular oxygen (1). In the Fenton reaction, \(\text{H}_2\text{O}_2\) readily reacts with available transition metals such as ferrous iron to form the hydroxyl radical (OH·), a highly reactive and toxic oxidant (2). ROS including \(\text{H}_2\text{O}_2\) and other reactive oxygen species (ROS) are produced in aerobes from the partial reduction of molecular oxygen (1). In the Fenton reaction, \(\text{H}_2\text{O}_2\) readily reacts with available transition metals such as ferrous iron to form the hydroxyl radical (OH·), a highly reactive and toxic oxidant (2). The catalase activity of KatA was inhibited by sodium azide, sodium cyanide and 3-amino-1,2,4-triazole. The absorption spectrum of KatA exhibited a Soret band at 408 nm. The position of the spectral peak remained unchanged following reduction of KatA with dithionite. No peroxidase activity was found for KatA. These results demonstrate that \textit{D. radiodurans} KatA is a typical monofunctional heme-containing catalase. The stability of KatA with respect to \(\text{H}_2\text{O}_2\) stress was superior to that of commercially available \textit{Aspergillus niger} and bovine liver catalases. The relative abundance of KatA in cells in addition to the \(\text{H}_2\text{O}_2\) resistance property may play a role in the survival strategy of \textit{D. radiodurans} against oxidative damage.

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(12). Overexpression of the *D. radiodurans* recX gene, which encodes a negative regulator for recombinase A (RecA), suppressed radioreistance and catalase activity (13, 14). These results suggest that catalase activity is involved in the radioreistance of this bacterium.

Although the purification and characterization of a protein with SOD activity from *D. radiodurans* has been reported (15), the biochemical property of the *D. radiodurans* catalase had not yet been investigated. Since catalases, together with SOD, play a key role in protecting cells against toxic ROS, here we report on the cloning, purification and characterization of a catalase (KatA) from *D. radiodurans*.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions** *D. radiodurans* strain KR, (16) was grown at 30°C in TGY broth containing 0.5% (wt/vol) tryptone–peptone, 0.1% (wt/vol) glucose and 0.3% (wt/vol) yeast extract. *E. coli* strain JM109 (Toyobo, Osaka) was grown in Luria–Bertani (LB) broth containing 1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract and 0.5% NaCl or tryptone yeast extract (TY) broth containing 1% (wt/vol) tryptone, 0.5% (wt/vol) yeast extract and 0.8% NaCl. Solid media contained 1.5% (wt/vol) agar. Fifty micromolars of ampicillin per ml was supplemented in the medium when necessary.

**Construction of expression plasmid pMH1** *D. radiodurans* genomic DNA was isolated as previously described (16). Briefly, *D. radiodurans* cells from 24-h culture were harvested, washed in NE solution containing 0.1 M NaCl and 0.1 M EDTA (pH 8), in butanol-saturated NE solution to facilitate the lysis of the cell walls, and again in NE solution. The washed cells were incubated in NE solution containing 10 mg/ml of lysozyme (Sigma, St. Louis, MO, USA) at 37°C for 1 h. Subsequently, the genomic DNA was isolated and purified by the method of Saiito and Miura (17). A set of 20-mer oligonucleotide probes, 5′-GA(C/T)GA(A/G)AA (C/T)AA(A/C)AA(A/G)GG(G/C)GT-3′, was used to clone the *katA* gene from *D. radiodurans* genomic DNA. Southern blot hybridization was performed as previously described (16). Briefly, the mixed oligonucleotide probes were labeled with digoxigenin using a DIG-ULS labeling kit (Kreatech Diagnostics, Amsterdam, Netherlands). DNA fragments after electrophoresis were transferred from agarose gels to positively charged nylon membrane (Roche, Basel, Switzerland) with alkaline solution (0.4 N NaOH, 1 M NaCl) for 24 h. DNA hybridization signals were detected as recommended by the supplier after stringency washing (two successive washes at room temperature for 5 min in 30 mM sodium citrate plus 0.3 M NaCl [2× SSC] and 0.1% sodium dodecyl sulfate [SDS], followed by two successive washes at 68°C for 15 min in 0.5× SSC and 0.1% SDS). A 5.3-kb *Nsp* fragment containing the *katA* gene was inserted into the *SphI* site of pUC19 (Takara Bio, Otsu). The resulting plasmid, pKD1, was digested with Sau3A and then subcloned into the HincII site of pUC118 (Takara Bio), yielding plasmid pMH1. In an effort to position the *katA* gene under control of the lacZ promoter, pMH1 was digested with *KpnI* and *NcoI*, blunted with T4 DNA polymerase (Takara Bio) and then self-ligated, yielding the expression plasmid pMH11. DNA sequencing was performed using an ABI PRISM 377 DNA Sequencer (Applied Biosystems, Foster City, CA, USA).

**E. coli** *H₂O₂* survival assay *E. coli* preculture grown at 30°C in LB broth was inoculated in fresh LB broth to an initial OD₆₀₀ of 0.1. Thirty percent *H₂O₂* was added to a final concentration of 100 mM and the sample was incubated at room temperature. Following incubation for the indicated amount of time, the reaction was stopped by the addition of 100 µg/ml of bovine liver catalase to the culture. The number of colony-forming units was determined by plating serial dilutions of the culture onto TY agar plates followed by counting after 24 h growth at 37°C.

**Protein purification** *E. coli* strain JM109 carrying pMH11 (1/1) was cultured at 30°C in LB broth containing 50 µg/ml of ampicillin. When the optical density at 600 nm reached approximately 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM. Growth was continued for an additional 4 h, after which time, cells were harvested by centrifugation and then washed with Buffer S consisting of 10 mM potassium phosphate buffer (pH 7) and 0.01% 2-mercaptoethanol. The cell pellet was stored at −20°C until further use.

All subsequent steps were performed at 4°C. The cell pellet (5 g) was resuspended in Buffer S, disrupted by sonication (120 W, 15 min), centrifuged at 37,000 × g for 60 min to remove cell debris, and the supernatant (20 ml) subsequently applied to a DEAE-Toyopearl column (2.6×10 cm; Tosoh, Tokyo) equilibrated with Buffer S. The column was washed with Buffer S containing 100 mM NaCl and then eluted with Buffer S containing 150 mM NaCl at a flow rate of 2 ml/min. Fractions containing high catalase activity were pooled, concentrated to 15 ml at 4000 × g using an Amicon Ultra-15 centrifugal filter device (Millipore, Billerica, MA, USA), and then applied to a Ceramic Hydroxypatite Type-1 column (2.6×3 cm; Bio-Rad Laboratories, Hercules, CA, USA) equilibrated with 10 mM potassium phosphate buffer (pH 7). The column was eluted with a linear gradient of 10 to 100 mM potassium phosphate buffer (pH 7) at a flow rate of 1.5 ml/min. Fractions corresponding to peak catalase activity were pooled, concentrated to 8 ml at 8000 × g using an Amicon YM10 unit (Millipore) and then applied to a Sephacryl S-300 HR column (2.6×100 cm; GE Healthcare Bio-Science Corp., Piscatway, NJ, USA) equilibrated with Buffer S containing 150 mM NaCl. The column was eluted with the same buffer at a flow rate of 1 ml/min and fractions corresponding to peak catalase activity were pooled. Following concentration using an Amicon YM10 unit, the pooled fractions (5 ml) were applied to a Ceramic Hydroxypatite Type-1 column (2.6×3 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7). The column was washed with 50 mM potassium phosphate buffer (pH 7) and then eluted with 100 mM potassium phosphate buffer (pH 7) at a flow rate of 1.5 ml/min. Fractions containing high catalase activity were collected, concentrated to 2 ml using an Amicon YM10 unit and then used for the subsequent biochemical experiments.

**Enzyme assay and protein concentration determination** Catalase activity was determined spectrophotometrically by monitoring the decrease in absorbance at 240 nm resulting from the disappearance of *H₂O₂* (ε₂₄₀=43.6 M⁻¹ cm⁻¹) (18). One unit (U) of catalase activity was defined as that activity required to destroy 1 µmol *H₂O₂* per min. *Aspergillus niger* catalase and bovine liver catalase were obtained from Amano Enzyme (Nagoya) and Sigma, respectively, and used as references. The peroxidase activity of the catalase was examined by measuring the rate of oxidation of 0.25 mM o-dianisidine (ε₄₆₀=11.3 M⁻¹ cm⁻¹) in the presence of 10 mM *H₂O₂* (18). Reactions were monitored spectrophotometrically at 460 nm. The protein concentration was determined using a Bio-Rad Protein Assay kit with bovine serum albumin as the standard. All assays were carried out at room temperature unless otherwise indicated.

**RESULTS**

**Cloning of the *D. radiodurans* katA gene** In an effort to clone the *D. radiodurans* katA gene, the sequence composed of DENNKGV, the N-terminal amino acid sequence of the catalase (11, 19), was used to design a set of mixed
was subcloned into pUC118, digested with E. coli resulting expression plasmid, pMH11, was introduced into blunted with T4 DNA polymerase and then self-ligated. The sequence (20), and that it contained the entire corresponding region of the published (DR1998). In order to position the sequence of the 5.3-kb KatA fragment derived from D. radiodurans genomic DNA. This fragment was cloned into the Spal site of pUC19, yielding plasmid pKDR1. DNA sequencing analysis revealed that the sequence of the 5.3-kb NsiI region was identical to the corresponding region of the published D. radiodurans genome sequence (20), and that it contained the entire katA gene (DR1998). In order to position the katA gene under control of the lacZ promoter, a 2.6-kb Smal fragment of pKDR1 was subcloned into pUC118, digested with Kpnl and Ncol, blunted with T4 DNA polymerase and then self-ligated. The resulting expression plasmid, pMH11, was introduced into E. coli strain JM109.

The E. coli transformant carrying pMH11 grew on a TY agar plate containing 10 mM H₂O₂, whereas E. coli strain JM109 without pMH11 did not (data not shown). Figure 1 shows the survival of the transformant following exposure to 100 mM H₂O₂. The pMH11 transformant exhibited higher resistance to H₂O₂ compared with E. coli without the plasmid. This suggests that the pMH11 transformant produced functional KatA protein (a product of the D. radiodurans katA gene) even without IPTG induction.

**Purification of D. radiodurans KatA**

Purification of D. radiodurans KatA generated following induction with IPTG in E. coli carrying pMH11 was accomplished by a four-step chromatographic procedure utilizing DEAE-Toyopearl, 1st Hydroxyapatite, Sephacryl S-300 HR and 2nd Hydroxyapatite columns (Table 1). The procedure resulted in approximately 21-fold purification with 41% yield.

SDS–PAGE analysis indicated the presence of a major protein band of approximately 65 kilodaltons (kDa) (Fig. 2). This is in good agreement with the molecular weight (60,512 Da) deduced from the amino acid sequence of the D. radiodurans katA gene product (20). KatA was purified to greater than 95% purity as determined by SDS–PAGE. Purified KatA displayed a high final specific activity of 68,800 U/mg of protein at 30°C. No peroxidase activity was found for KatA using the o-dianisidine method. The native molecular mass of KatA was estimated to be 240 kDa by gel filtration using Sephacryl S-300 HR (data not shown). This suggests that purified KatA is composed of four identical subunits.

**Effect of inhibitors on activity and spectroscopic properties of the catalase**

Catalase activity was strongly inhibited by sodium azide and sodium cyanide, inhibitors of heme-containing catalase (Table 2). Treatment of D. radiodurans KatA with 3-amino-1,2,4-triazole at 0.1 mM and 1.0 mM resulted in marginal reduction of catalase activity. As shown in Fig. 3, the absorption spectrum of D. radiodurans KatA exhibited a Soret band at 408 nm typical of a heme-containing catalase (21). This peak shifted to 422 nm following the addition of 10 mM sodium cyanide, indicating direct binding of cyanide to the heme moiety. The position of the spectral peak remained unchanged following treat-

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**TABLE 1. Summary of the purification of D. radiodurans catalase KatA from overexpressed E. coli cells**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total activity (U)</th>
<th>Specific activity (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>609</td>
<td>201 × 10⁴</td>
<td>3300</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>DEAE-Toyopearl</td>
<td>86</td>
<td>204 × 10⁴</td>
<td>23700</td>
<td>101</td>
<td>7.2</td>
</tr>
<tr>
<td>1st hydroxyapatite</td>
<td>51</td>
<td>180 × 10⁴</td>
<td>35300</td>
<td>90</td>
<td>10.7</td>
</tr>
<tr>
<td>Sephacryl S-300 HR</td>
<td>26</td>
<td>176 × 10⁴</td>
<td>67700</td>
<td>87</td>
<td>20.5</td>
</tr>
<tr>
<td>2nd hydroxyapatite</td>
<td>12</td>
<td>82.5 × 10⁴</td>
<td>68800</td>
<td>41</td>
<td>20.8</td>
</tr>
</tbody>
</table>

One unit (U) of activity is defined as that activity required to destroy 1 µmol H₂O₂ per min.

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**FIG. 1.** Survival of exponential phase cells of E. coli JM109 (open circles) and E. coli JM109 carrying plasmid pMH11 (closed circles) following exposure to 100 mM H₂O₂. Data represents the mean of at least three independent experiments.

**FIG. 2.** SDS–PAGE analysis of the expression of D. radiodurans KatA in E. coli JM109 cells and its purification. Each protein sample (2 to 3 µg) was mixed with an equal volume of loading buffer containing 2% SDS and 5% 2-mercaptoethanol, and heated at 100°C for 10 min prior to being resolved in a 10% SDS–polyacrylamide gel. The resolved proteins in the gel were stained with Coomassie Brilliant Blue R-250. The position of the 65-kDa KatA band is indicated on the right. Lane 1, Molecular markers; lane 2, crude protein extract from sonicated cells; lane 3, pooled fractions following DEAE-Toyopearl chromatography; lane 4, pooled fractions following 1st Hydroxyapatite chromatography; lane 5, pooled fractions following Sephacryl S-300 chromatography; lane 6, pooled fractions following 2nd Hydroxyapatite chromatography.
ment of the enzyme with 1 mM sodium dithionite.

**Effect of temperature on catalase activity** Catalase activity was assayed at various temperatures using purified KatA and was compared with that of commercially available bovine liver and A. niger catalases (Fig. 4A). Purified KatA was active over a temperature range from 20°C to 70°C, with optimum activity occurring at 30°C. The optimum assay temperature demonstrating the enzymatic activity of bovine liver and A. niger catalases was 40°C and 50°C to 60°C, respectively. At 70°C, the activity of A. niger, bovine liver and D. radiodurans catalases was reduced to 82%, 45% and 20%, respectively, of each activity at the optimum assay temperature.

The stability of D. radiodurans KatA to heat was examined by incubating the purified enzyme at a fixed temperature for 30 min (Fig. 4B). KatA retained 100% of its initial activity following incubation at 40°C, and 82% of its activity following incubation at 50°C. The activity of KatA decreased significantly to 30% of the initial activity following incubation at 60°C. Bovine liver catalase was less stable at temperatures greater than 40°C and was completely unstable at 60°C. A. niger catalase activity was abolished following incubation at 80°C. These results suggest that D. radiodurans KatA is less heat-labile than bovine liver catalase but more heat-labile than A. niger catalase.

**Response to exogenously added H₂O₂** Many catalases are known to be inhibited or inactivated by the substrate H₂O₂ (22). The susceptibility of D. radiodurans KatA to H₂O₂ stress was investigated by the dialysis method as described in Materials and Methods. As shown in Fig. 5, the residual activity of KatA decreased gradually with increasing H₂O₂ concentration, demonstrating apparent substrate inhibition/inactivation of the enzyme. However, D. radiodurans KatA retained 82% activity following exposure to 100 mM H₂O₂, whereas A. niger and bovine liver catalases retained 70% and 48% activity, respectively. This indicates that the stability of D. radiodurans KatA to H₂O₂ stress was superior to that of A. niger and bovine liver catalases under the experimental conditions used.

**DISCUSSION**

Catalases are classified into three general classes based upon structure and properties, being the monofunctional catalases, the bifunctional catalases that possess both catalase and peroxidase activity, and the non-heme or manganese-containing catalases (23). Typical monofunctional catalases exist as a homotetramer containing four protoheme IX prosthetic groups per tetramer, with a molecular mass of 225 to 270 kDa. These enzymes are specifically inhibited by sodium cyanide and 3-amino-1,2,4-triazole, and are resistant to reduction by sodium dithionite (24).

In this study, we purified and characterized the catalase KatA from D. radiodurans. The size of purified KatA was
65 kDa by SDS–PAGE analysis (Fig. 2) and 240 kDa by gel filtration, suggesting that KatA forms a homotetramer in solution. KatA catalase activity was inhibited by sodium azide, sodium cyanide and 3-amino-1,2,4-triazole, traditional inhibitors of heme-containing catalase (Table 2). The absorption spectrum of KatA exhibited a Soret band at 408 nm. The position of the spectral peak remained unchanged following reduction of KatA with dithionite (Fig. 3). Furthermore, no peroxidase activity was found for KatA. These results demonstrate that D. radiodurans KatA is a typical monofunctional heme-containing catalase. Soung and Lee (25) showed that the catalases from wild-type D. radiodurans (ATCC13939) possessed peroxidase activity as determined by activity staining analysis. We assume that Soung and Lee detected false positive signals for peroxidase activity. In this study, strain KR1 was used, another wild-type isolate of D. radiodurans. Therefore, the reason for this discrepancy might have to do with strain polymorphism since wild-type D. radiodurans isolates have been shown to exhibit a wide variety of differences (26–29).

It has been shown that D. radiodurans exhibits relatively high catalase activity (10). The specific activity of purified D. radiodurans KatA protein was 68,800 U/mg (Table 1). This is not particularly outstanding when compared with the catalase activity of other bacterial species. For example, it was shown that the specific activity of purified catalase from the facultatively psychrophilic bacterium Vibrio ronoletensis was approximately 400,000 U/mg (30). However, in this study we showed that, even under non-induced conditions, E. coli JM109 cells carrying the expression plasmid pMM1111i exhibited higher resistance to H2O2 compared with E. coli without the plasmid (Fig. 1). Moreover, purified D. radiodurans KatA protein was more stable to H2O2 stress than commercially available A. niger and bovine liver catalases (Fig. 5). These results indicate that D. radiodurans KatA possesses relatively superior ability in H2O2 resistance compared to catalases from other species. In this context, it is worth noting that the deduced amino acid sequence of the D. radiodurans KatA exhibited higher similarity to that of KatX, which is the major catalase in dormant spores of B. subtilis than those of the B. subtilis vegetative catalases, KatA and KatE (Fig. 6). B. subtilis KatX has no role in dormant spore resistance to H2O2, but functions in protecting germinating spores from H2O2 (31). Although D. radiodurans does not form spore, the high similarity of the D. radiodurans KatA to the B. subtilis KatX will provide a clue to clarify the structure-function relationship of these enzymes.

Karlin and Mrázek (32) devised an algorithm to estimate the protein abundance in a cell population on the basis of codon usage, and applied the algorithm to predict the gene expression levels of the D. radiodurans genome. Not unexpectedly, according to this prediction D. radiodurans KatA was one of a number of highly-expressed proteins (32). The proteomic analysis of D. radiodurans confirmed the presence of KatA under most culture conditions tested, suggesting constitutive high expression (33). Thus, the abundance of KatA in cells in addition to its H2O2 resistance might play a role in the survival strategy of D. radiodurans against oxidative damage.

Based on experiments showing that dehydration induces DNA double-strand breaks in D. radiodurans and that ionizing radiation-sensitive mutants of this bacterium might also be sensitive to desiccation, Battista and his colleague hypothesized that the ionizing radiation resistance phenotype of D. radiodurans might be a fortuitous consequence of an evolutionary process that permitted this bacterium to cope with commonly encountered environmental stressors such as desiccation (desiccation adaptation hypothesis) (34). Recently, the ionizing radiation-resistant fractions of two soil bacterial communities were investigated by exposing an arid soil from desert area and a nonarid soil from forest area to ionizing radiation (35). The authors of the aforementioned investigation argued that recovery of large numbers of ionizing radiation-resistant bacteria including new species of the genus Deinococcus from arid soil but not from nonarid soil provides ecological support for the desiccation adaptation hypothesis (34). The mechanism that mediates the generation of ROS during desiccation remains poorly understood (36). ROS might be involved in the response of arid soil bacteria to solar UV irradiation (37). D. radiodurans exhibits high resistance to UV light (4, 10). The relative abundance and superior resistance to H2O2 of catalase in D. radiodurans can be rationalized in terms of protection against solar UV-induced oxidative stress. However, it is not necessarily the case for all members of the genus Deinococcus, since two species (D. hohokamensis and D. navajonensis) isolated from desert soil from Arizona lack catalase activity (35). The optimum temperature required for activity of D. radiodurans KatA was 30°C (Fig. 4A), which is in agreement with the optimum growth temperature for this bacterium. D. radiodurans KatA was found not to be particularly thermostable (Fig. 4B). These results suggest that there is no significant relationship between high UV resistance and protein thermostability in this bacterium. This might reflect the evolution of D. radiodurans in the natural habitat. Further comparative study using catalases from other Deinococcus species will help to delineate the physiological role of deinococcal catalases in oxidative stress protection.
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