Purification and Characterization of Catalase-3 of *Deinococcus radiophilus*

In-Jeong Lee and Young Nam Lee*

Department of Microbiology, College of Natural Sciences, Chungbuk National University, Cheongju 360-763, Korea

(Received July 5, 1995/Accepted August 24, 1995)

***Deinococcus radiophilus*, an UV resistant bacterium seemed to contain three isoenzymes of catalase. Among them, the smallest and most abundant species in cell-free extract, catalase-3, which also exhibited peroxidase activity was purified to electrophoretic homogeneity (145-fold purification) by chromatographic procedures. Its molecular weight was 155 kDa composed of four 38 kDa subunits. The K_m value of catalase-3 for H_2O_2 was approximately 0.5 mM. This enzyme showed a typical ferric heme spectrum with maximum absorption at 405 nm. Upon binding to cyanide, the 405 nm peak shifted to 420 nm. Catalase-3 was very sensitive to inhibitors of heme proteins, such as cyanide, azide and hydroxylamine. A ratio of A_{405}/A_{280} was 0.5. Catalase-3 was active over a wide range of pH, between pH 7 and 10. The enzyme was rather heat-labile and partially sensitive to ethanol-chloroform treatment, but resistant to 3-amino-1,2,4-triazole. Catalase-3 of *D. radiophilus*, which is a bifunctional catalatic-peroxidatic enzyme seemed to share certain molecular properties with the typical catalase and the catalase-peroxidase along with its own unique features.**

Key words: ***Deinococcus radiophilus*, UV resistant, catalase, isoenzymes, reactive oxygen species**

The reactive oxygen species such as peroxide, superoxide anions, hydroxyl and hydroperoxyl radicals produced during aerobic respiration and UV irradiation, could modify the essential cellular components of lipids, proteins and nucleic acid and cause a great damage to cells leading to a hazardous state. However, the primary protection of cells from the reactive intermediates are achieved by the scavenger enzymes such as catalase, peroxidase and superoxide dismutase and a number of antioxidants, such as glutathione (4). The secondary protection includes correction of the modified components to the normal states by various repairing enzymes (7).

Catalases ubiquitously occurred in all aerobic organisms including animals, plants and microbes, destroy hydrogen peroxide to oxygen and water, whereas peroxidase removes hydrogen peroxide by consuming it in oxidation of a variety of organic compounds. Catalases of eucaryotic as well as procaryotic cells have been extensively studied.

Genus *Deinococcus*, naturally occurring obligately aerobic, Gram-positive coccus is known to be highly resistant to UV and ionizing radiation (16). Although interests on its radioresistance were paid with respect to DNA repairing system (9), roles of scavenging enzymes of the reactive oxygen intermediates were poorly understood. As the steps to investigation on scavengers of the reactive oxygen species in the UV resistant bacteria continued, we studied biochemical and physical properties of purified catalase-3, which is the most abundant in the crude cell-free extract and the smallest among three isocatalases of *D. radiophilus*.

* To whom correspondence should be addressed.

Materials and Methods

Chemicals, enzyme and bacterial culture

General chemicals including hydrogen peroxide, DEAE-cellulose, phenyl-Sepharose CL-4B, diaminobenzidine and horseradish peroxidase were purchased from Sigma Chem Co. Trypton, yeast extract and agar were obtained from Difco Laboratories. The purchased *Deinococcus radiophilus* ATCC 27603 was cultured in TYGM medium (1% trypton, 0.5% yeast extract, 0.2% glucose and 0.2% L-methionine) at 30℃ for 3 days with moderate aeration of 150 rpm.

Activity assay of catalase

Catalase (hydrogen peroxide : hydrogen peroxide oxidoreductase, EC 1.11.1.6) activity was measured by monitoring the decrease in absorbance at 240 nm due to decomposition of H_2O_2 in 50 mM potassium phosphate buffer pH 7.0 at 25℃ (1). One unit of enzyme activity was defined as the amount of enzyme decomposing one μmol of H_2O_2 (ε=43.6 $M^{-1}cm^{-1}$) under the standard assay condition. Activity staining of catalase was performed on polyacrylamide gel using horseradish peroxidase and diaminobenzidine as an electron donor by as described by Clare *et al.* (6). The peroxidase activity staining was conducted in the same way without horseradish peroxidase. Protein was quantitated by Lowry method (14) or during chromatographic procedures by monitoring absorbance at 280 nm.

Purification of catalase-3

Crude lysate in 50 mM potassium phosphate buffer, pH 7.0 was prepared with bacterial pellet obtained from 3-liter culture in TYGM medium by ultrasonification, followed by centrifugation. The lysate was subjected to ammonium sulfate fractionation (30~65% saturation). The most abundant and smallest catalase among three isozymes, catalase-3 was purified to electrophoretic homogeneity by successive chromatography of DEAE-cellulose, phenyl-Sepharose CL-4B followed by gel filtration of Sephadex G-200 and hydroxyapatite column chromatography from the dialysed preparation of ammonium sulfate fraction (12).

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 8.0% gel by the Laemmli method (11). Nondenatured polyacrylamide gel electrophoresis was carried out on 8.0% gel slabs in 25 mM Tris-glycine buffer, pH 8.8 (2).

Molecular weight determination of catalase-3

The molecular weight of the purified catalase-3 was determined by gel filtration of Sephadex G-200 in 50 mM potassium phosphate buffer, pH 7.0 (17).

Kinetics of *D. radiophilus* catalase-3

Substrate affinity (K_m value), optimal pH and temperature for enzyme activity, thermostability, pH stability and effects of inhibitors were investigated as described by Price and Stevens (17).

Spectral analysis of catalase-3

The absorption spectrum of the purified catalase-3 in 10 mM Hepes buffer, pH 7.0, was measured between 260 nm and 700 nm.

Results and Discussion

Catalase is ubiquitous in all aerobic organisms. There are two types of catalases; typical monofunctional catalase and bifunctional catalase-peroxidase, although, an occurrence of an atypical catalase was reported in a few procaryotes (8). Animal cells predominantly possess the typical catalase whereas plant and microorganims own either types or both. The properties of monofunctional typical catalase differs from those of the bifunctional catalase. The typical catalase shows broad pH optima in a range of 4-10 and is thermostable, and is insensitive to organic solvents, but sensitive to 3-amino 1,2,4 triazole (AZT) treatment. However, the bifunctional catalase shows narrow pH optima, near neutral pH 6-8 and is heat-labile, and is sensitive to organic solvents and insensitive to AZT (5, 10, 18, 19). The K_m values of the typical catalases are reported one order magnitude higher than those of the bifunctional catalases (5, 10, 18, 19).

Cell-free extract of *D. radiophilus* ATCC 27603 revealed three forms of catalase with different electrophoretic mobility (Fig. 1). Two of them, catalase-2 and catalase-3, turned out to be bifunctional catalase-peroxidase. The occurrence of multiple bifunctional catalase in one organism is rarely reported (13). The smallest and most abundant species designated catalase-3 was purified to

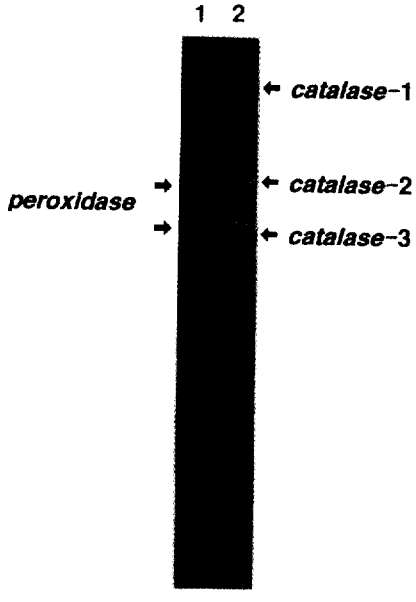

Fig. 1. Activity staining of catalase and peroxidase of the cell-free extract prepared from *D. radiophilus*. Cell-free extract prepared by ultrasonication was resolved on 8.0% polyacrylamide gel by electrophoresis. Bands of peroxidase (lane 1) and catalase (lane 2) were visualized by activity staining.

Table 1. Purification of *D. radiophilus* catalase-3.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Activity recovery (%)
Crude extract	3,380	576,100	170	1	100
$(NH_4)_2SO_4$ precipitation (30~65%)	227	495,400	2,200	13	86
DEAE-cellulose	82	275,000	3,400	20	48
Phenyl-Sepharose CL-4B	13.4	135,400	10,100	59	24
Sephadex G-200	4.5	75,600	16,800	99	13
Hydroxyapatite	1.1	27,100	24,600	145	4.7

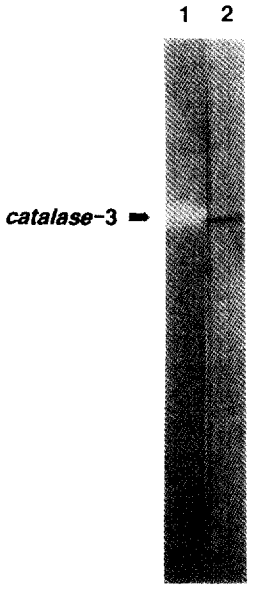

Fig. 2. The purified *D. radiophilus* catalase-3 from hydroxyapatite column chromatography. Electrophoresis was performed on nondenaturing 8.0% polyacrylamide gel. Bands of catalase (lane 1) and peroxidase (lane 2) were visualized by activity staining.

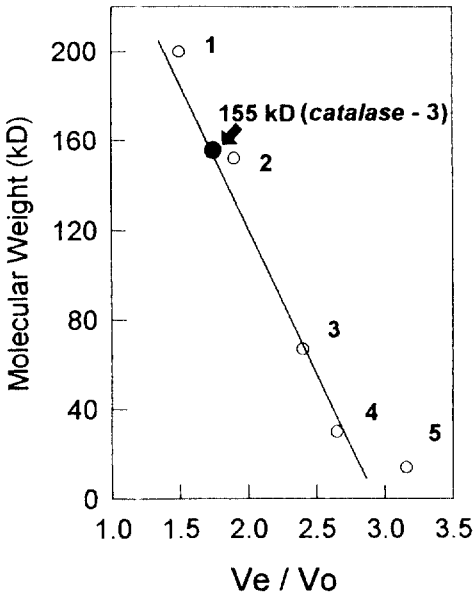

Fig. 3. Determination of molecular weight of the purified *D. radiophilus* catalase-3 by gel filtration on Sephadex G-200 column. The standard size markers are as follows: 1 (α-amylase, 200,000), 2 (alcohol dehydrogenase, 150,000), 3 (albumin, 66,000), 4 (carbonic anhydrase, 29,000) and 5 (cytochrome c, 12,400).

electrophoretic homogeneity by chromatographic procedures (Table 1, Fig. 2). The molecular weight of *D. radiophilus* catalase-3 was 155 kDa composed of four 38 kDa subunit of an equal size (Fig. 3, Fig. 4). This catalase-3 was much smaller compared with catalase-peroxidase from those of either *E. coli* (5), *Rhodopseudomonas capsulata* (10), *Bacillus* spp. (19) or *Halobacterium halobium* (3), but similar to 160 kDa catalase of *Mycobacterium tuberculosis* (20). The apparent K_m of catalase-3 for hydrogen peroxide was 0.5 mM. Therefore one may assume that *D. radiophilus* catalase-3 scavenges efficiently hydrogen peroxide relative to other procaryotic catalases, for examples, *E. coli* HPI (3.9 mM) (5), *R. capsulata* catalase (4.2 mM) (10) and *Streptomyces cyaneus* (2.07 mM) (15). The Deinococcal catalase-3 seems to be highly sensitive to cyanide, azide and hydroxylamine, and their concentrations of 50% inhibition were 8.1×10^{-6} M, 2.9×10^{-7} M and 2.7×10^{-7} M, respectively, which were similar to those of a typical catalase of Baker's yeast (19).

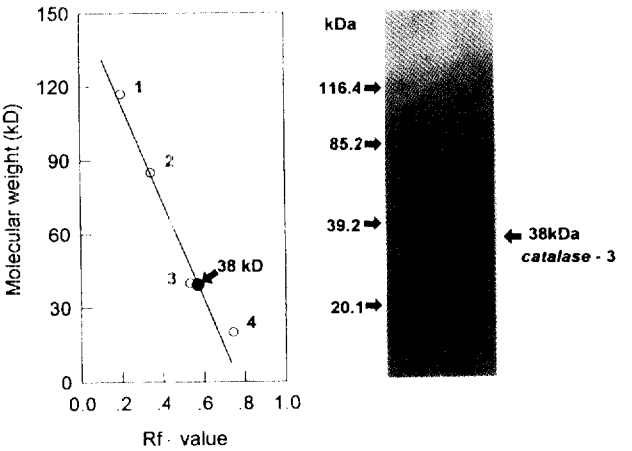

Fig. 4. SDS-polyacrylamide gel electrophoresis of the purified *D. radiophilus* catalase-3. Electrophoresis was performed on 8.0% polyacrylamide gel containing 0.2% SDS with the size markers, 1 (β-galactosidase, 116,400), 2 (fructose-6-phosphate kinase, 85,200), 3 (aldolase, 39,200) and 4 (trypsin inhibitor, 20,100).

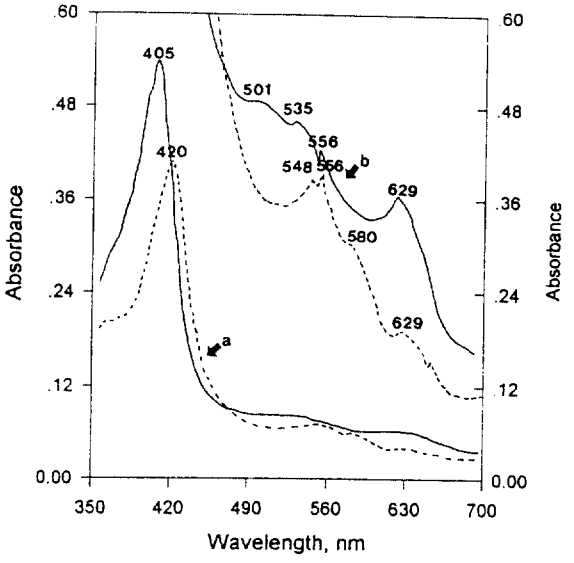

Fig. 5. Absorption spectrum of *D. radiophilus* catalase-3. The purified enzyme (1.1 mg/ml) was dissolved in 10 mM Hepes buffer, pH. 7.0. Scanning was performed from 350 to 700 nm with/without 10 mM NaCN (— without NaCN,····· with NaCN). a: absorbance scale 0.0~0.6, b: absorbance scale 0.0~0.1 for detail of spectrum between 450 and 700 nm.

Interestingly, these values are much lower than those of procaryotic catalase-peroxidase, for example, values of *R. capsulata* catalase-peroxidase reported are, 2.2×10^{-5} M, 1.5×10^{-4} M and 2.2×10^{-6} M, respectively (10). The Deinococcal catalase-3 was active at broad range of alkaline pH 7~10, (optimum pH 9~10), whereas catalase-peroxidases from other procaryotic sources show a sharp pH dependency around pH 6~8. The catalase-3 activity was maintained 80% at 5 mM AZT. Eighty% and 40% of inactivation of catalase-3 activity were occured at 45 min incubation at 50℃ and by treatment of ethanol/chloroform, respectively, thus it was thermolabile and partially sensitive to organic solvents.

The Deinococcal catalase-3 showed maximum absorption at 405 nm and shoulders at 501, 535, 556 and 629 nm. Treatment of the enzyme with NaCN caused a shift of 405 nm peak to 420 nm with the abolishment of the 501 and 535 nm shoulders along with the appearance of 548 nm peak (Fig. 5). It suggested that the prosthetic group of the catalase-3 is protoporphyrin IX with ferric heme like other catalases. The catalase-3 has relatively low A_{405}/A_{280} ratio of 0.5 like other procaryotic bifunctional calatases (0.55 for *E. coli* PHI and 0.4 for *R. capsulata*) (5, 10) in contrast to the ratio of the typical catalase, 0.93 for Baker's yeast (19).

Thus, catalase-3 of *Deinococcus radiophilus*, which is a bifunctional catalatic-peroxidatic enzyme seems to share certain molecular properties with the typical catalase and the catalase-peroxidase along with its own unique features.

The abundancy of catalase-3 and its low K_m value for H_2O_2 suggested that efficient scavenging system of H_2O_2 is under operation in *Deinococcus radiophilus*. A correlation between the UV resistance of *D. radiophilus* and the efficient removal of toxic oxidants by catalase-3 can be speculated, but further studies are required to demonstrate relations of antioxidants enzyme system to the unusual UV resistance of the genus *Deinococcus*.

References

1. **Beers, R.F. Jr. and I.W. Sizer,** 1951. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* **159**, 133-142.
2. **Bollag, D.M. and S.J. Edelstein,** 1991. Protein method, p. 1-230. Wiley-Liss, Inc., New York.
3. **Brown-Peterson, N. and M.L. Salin,** 1993. Purification of a catalase-peroxidase from *Halobacterium halobium*: Characterization of some unique properties of the halophilic enzyme. *J. Bacteriol.* **175**, 4197-4202.
4. **Cadenas, E.,** 1989. Biochemistry of oxygen toxicity. *Ann. Rev. Biochem.* **58**, 79-110.
5. **Claiborne, A. and I. Fridovich,** 1979. Purification of *o*-dianisidine peroxidase from *Escherichia coli*. *J. Biol. Chem.* **254**, 4245-4252.
6. **Clare, D.A., M.N. Duong, D. Darr, F. Archibald, and I. Fridovich,** 1984. Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity staining for catalase. *Anal. Biochem.* **140**, 532-537.
7. **Demple, B. and J. Halbrook,** 1983. Inducible repair of oxidative DNA damage in *Escherichia coli*. *Nature* (London) **304**, 466-468.
8. **Goldberg, I. and A. Hochman,** 1989. Three different types of catalases in *Klebsiella pneumoniae*. *Arch. Biochem. Biophys.* **268**, 124-128.
9. **Gutman, P.D., P. Fuchs, L. Ouyang, and K.W. Minton,** 1993. Identification, sequencing and targeted mutagenesis of *Deinococcus radiodurans*. *J. Bacteriol.* **175**, 3581-3590.
10. **Hochman, A. and A. Shemesh,** 1987. Purification and characterization of a catalase-peroxidase from the photosynthetic bacterium *Rhodopseudomonas capsulata*. *J. Biol. Chem.* **262**, 6871-6876.
11. **Laemmli, U.K.,** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* (London) **227**, 680-685.
12. **Lee, I.-J.,** 1994. M.S. Thesis. Chungbuk National Univ., Cheongju.
13. **Loewen, P. and J. Switala,** 1987. Multiple catalases in *Bacillus subtilis*. *J. Bacteriol.* **169**, 3601-3607.
14. **Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall,** 1951. Protein measurement with the folin phenol

reagent. *J. Biol. Chem.* **193**, 265-275.

15. **Mliki, A. and W. Zimmerman,** 1992. Purification and characterization of an intracellular peroxidase from *Streptomyces cyaneus*. *Appl. Environ. Microbiol.* **58**, 916-919.
16. **Murray, R.G.E,** 1986. Family II Deinococcaceae, p. 1035-1043. *In* P.H.A. Sneath, N.S. Mair, M.S. Sharper and J.G. Holt (Eds), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
17. **Price, N.C. and L. Stevens,** 1988. Fundamentals of enzymology, 2nd ed. Oxford Science Publications, Oxford.
18. **Shin, K.J., Y.T. Ro, and Y.M. Kim,** 1994. Catalase in *Acinetobacter* sp. strain JCI DS 3803 growing on glucose. *Kor. J. Microbiol.* **32**, 155-162.
19. **Yumoto, I., Y. Fukumori, and T. Yamanaka,** 1990. Purification and characterization of catalase from a facultative alkalophilic bacillus. *J. Biochem.* **108**, 583-587.
20. **Zhang, Y., B. Heym, B. Allen, D. Young, and S. Cole,** 1992. The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature* (London) **358**, 591-593.