Journal of Biochemistry and Molecular Biology, Vol. 31, No. 2, March 1998, pp. 144–148

JOURNAL OF
Biochemistry
and
Molecular Biology

Purification and Characterization of Catalase-2 from *Deinococcus radiophilus*

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Received 11 October 1997

A bifunctional catalase-peroxidase, designated catalase-2, of a UV resistant *Deinococcus radiophilus* was purified to electrophoretic homogeneity by both chromatographic and electrophoretic methods. Its molecular weight was 310 kDa and composed of a tetramer of 80 kDa subunits. The catalase-2 exerted its optimal activity at 30°C and around pH 9. Its K_m value for H_2O_2 was about 10 mM. It showed the typical ferric heme spectrum with maximum absorption at 403 nm which shifted to 419 nm in the presence of cyanide. The ratio of A_{403}/A_{280} was 0.48. Fifty percent inhibition of the enzyme activity was observed at 4.6×10^{-6}, 7.7×10^{-6}, and 3.0×10^{-7} M of NaCN, NaN_3, and NH_2OH, respectively. The enzyme was thermostable and not sensitive to 3-amino-1,2,4-triazole. Treatment of the enzyme with ethanol-chloroform caused a partial loss (30%) of its activity. The catalase-2 was distinct from the Deinococcal bifunctional catalase-3 in a number of properties, particularly in its molecular structure and substrate affinity.

Keywords: Bifunctional catalase-peroxidase, *Deinococcus radiophilus*, Purification, UV resistance.

Introduction

Catalase (hydrogen peroxide:hydrogen peroxide oxidoreductase, EC 1.11.1.6) is an essential enzyme in all aerobics for the protection from the toxicity of hydrogen peroxide generated in various cellular reactions. Reactive oxygen species such as peroxides, superoxides, hydroxyl, and hydroperoxyl radicals are generated in cells by aerobic respiration and some environmental stress, for example, UV and ionizing radiations, cigarette smoking, and certain redox active drugs (Hassan and Fridovich, 1979). These reactive oxygen species exert deleterious effects on cells by oxidizing the essential cellular constituents, membrane lipids, proteins, and nucleic acids (Cadenas, 1989). However, cells operate various defense systems for the protection from the offending toxic oxidants; primary defenses with the aid of various antioxidant enzymes, such as catalases, superoxide dismutases, peroxidases, and/or antioxidant components, and secondary defenses with repairing enzymes of the damaged cellular constituents (Stortz *et al.*, 1990).

The genus *Deinococcus*, an obligate aerobic, gram-positive coccus, is known to be extraordinarily resistant to ultraviolet and ionizing radiation such as γ-radiation. The bacterium has a number of peculiar features, such as a thick cell wall of several distinct layers, an outer membrane constituting lipids and protein, and cell-bound carotenoid pigments (Murray, 1986). Although it could be speculated that the unusual radio-resistance is attributed to its morphological charactersitics, one can assume that operation of the efficient defense systems against reactive oxygen species and the repair systems of damaged cellular components may also be involved.

In *Deinococcus*, although the mechanism of UV resistance has been studied with regard to the repairing enzymes of damaged DNA (Gutman *et al.*, 1993; Mun *et al.*, 1994), little attention has been paid to the roles of the antioxidant scavenging systems. Interestingly, *Deinococcus radiophilus* was found to possess three species of catalases, designated catalase-1, catalase-2, and catalase-3 (Lee and Lee, 1995). These catalase isoenzymes showed different electrophoretic behavior on native polyacrylamide gel. Both catalase-2 and catalase-3 were the bifunctional enzymes exhibiting not only catalase activity but also peroxidase activity whereas catalase-1 is a monofunctional catalase. Studies on the properties of catalase-3 were reported previously (Lee and Lee, 1995). This paper describes the purification and characterization of the Deinococcal catalase-2.

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Materials and Methods

Bacterial culture, chemicals, and enzymes *Deinococcus radiophilus* ATCC 27603 was cultured in modified TYMG medium (1% trypton, 0.5% yeast extract, 0.2% glucose and 0.2% methionine, Difco Lab., Detroit, USA). The incubation was carried out at 30°C for 3 days with continuous aeration at 150 rpm. General chemicals including hydrogen peroxide, diaminobenzidine, horseradish peroxidase, and materials for chromatography such as DEAE-Cellulose, were purchased from Sigma Chemicals Co. (St. Louis, USA).

Catalase assay and protein quantification Catalase activity was measured spectrophotometrically by monitoring the decrease in absorbance at 240 nm due to decomposition of hydrogen peroxide in 50 mM potassium phosphate buffer, pH 7.0 at 25°C (Beers and Sizer, 1951). One unit of catalase activity was defined as the disappearance of 1 μmol of hydrogen peroxide ($\varepsilon = 0.041\ mM^{-1}\ cm^{-1}$) per min. Protein concentration was determined by the method of Bradford (1976) and the relative quantification of protein during chromatographic procedures was made by monitoring absorbance at 280 nm. Visualization of catalase activity on a nondenaturing gel was performed with the system of horseradish peroxidase and diaminobenzidine as an electron donor by the method of Clare *et al.* (1984). The peroxidase activity staining was done in the same way without horseradish peroxidase.

Purification of catalase-2 The cell pellet, prepared from a 3-litre culture in TYGM medium, was suspended in 50 mM potassium phosphate buffer, pH 7.0 and disrupted by ultrasonication for 30 min with occasional coolings (Fisher Sonic Dismenbrator Model 300, Fisher Scientific Co., New Hampshire, USA). The cell-free lysate was then obtained by centrifugation at 16,000 × *g* for 1 h followed by ammonium sulfate fractionation (30–65% saturation). The catalase-2 was purified to electrophoretic homogeneity from the dialyzed preparation of ammonium sulfate by successive chromatography on DEAE-cellulose and phenyl-Sepharose CL-4B, followed by preparative gel electrophoresis on 7.0% polyacrylamide gel.

Molecular weight determination The molecular weight of the purified catalase-2 was determined by polyacrylamide gel electrophoresis according to the method of Hedrick and Smith (1968). The molecular weight of the denatured enzyme was measured by 0.2% SDS–6.5% polyacrylamide gel electrophoresis (Laemmli, 1970).

Kinetics of catalase-2 Substrate affinity (K_m value), optimal pH and temperature for enzyme activity, thermostability, pH stability, and effects of inhibitors were studied as previously described (Price and Steves, 1988). Since catalase is known to be inactivated by excessive hydrogen peroxide, kinetic studies were done with relatively low concentations of hydrogen peroxide (0–60 mM). The concentrations of inhibitors were varied from 0–50 μM depending on the heme inhibitors used and from 0–20 mM of 3-amino-1,2,4-triazole.

Spectral analysis of catalase-2 The absorption spectrum of the purified enzyme in 10 mM Hepes buffer, pH 7.0 was measured between 260 nm and 700 nm (Beckman DU-65, UV/Visible Spectrophotometer, Fullerton, USA).

Results and Discussion

Deinococcus radiophilus has been found to possess three species of catalases with different molecular weights. Thus, the occurrence of multiple catalases has drawn our curiosity about their physiological roles and properties. Previously, the smallest catalase-3 of *D. radiophilus* has been purified and characterized (Lee and Lee,1995).

D. radiophilus catalase-2 was purified to 95-fold as depicted in Table 1. The purified catalase-2 exhibited peroxidase activity like catalase-3 (Fig. 1). A number of microorganims are reported to have multiple species of catalases, i.e., monofunctional typical catalases and bifunctional catalase-peroxidases (Claiborne and Fridovich, 1979; Loewen and Switala, 1987; Goldberg and Hochman, 1989; Kim *et al.*, 1992, Klotz and Hutchson, 1992; Shin *et al.*, 1994). However, few have been found to posses multiple forms of the bifunctional catalase-peroxidase (Brown-Peterson and Salin, 1993). The molecular weight of *D. radiophilus* catalase-2 was 310 kDa with a tetramer of 80 kDa subunits (Fig. 2, Fig. 3). This is quite distinct from Deinococcal catalase-3, the tetramer molecular weight of which is 155 kDa with 39 kDa subunits (Lee and Lee, 1995).

The K_m value of the Deinococcal catalase-2 was about 10 mM (Fig. 4). This value was much higher than the 0.5 mM of the Deinococcal catalase-3 (Lee and Lee,1995). This suggested that catalase-2 is less efficient in scavenging hydrogen peroxide compared with the

Table 1. Purification of *D. radiophilus* catalase-2.

Fraction	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification recovery (n-fold)	Activity (%)
Crude extract	150	1938	213,600	110	1	100
Ammonium sulfate (30–65%)	35	233	15,370	660	6	72
DEAE-cellulose	7	64	93,080	1454	13	44
Phenyl-Sepharose	4.5	12	47,370	3948	35	22
Preparative gel electrophoresis	5.4	0.7	7340	10,485	95	3.4

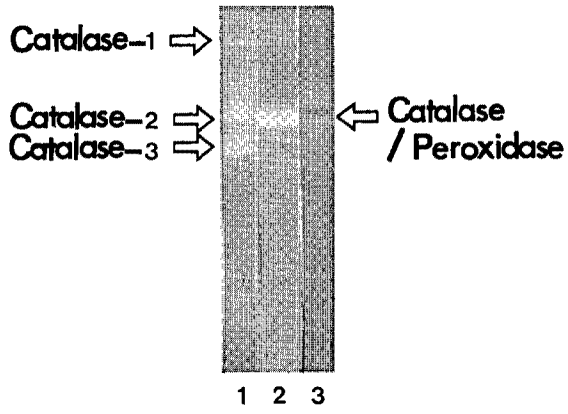

Fig. 1. Activity staining of the purified catalase-2. The enzyme preparation was resolved on 8.0% polyacrylamide gel. Lane 1, activity staining of Deinococcal iso-catalase; lane 2, catalase activity band of purified catalase-2; lane 3, peroxidase activity of purified catalase-3. Catalase and peroxidase activities in the gel were visualized by the staining method of Clare *et al.* (1984).

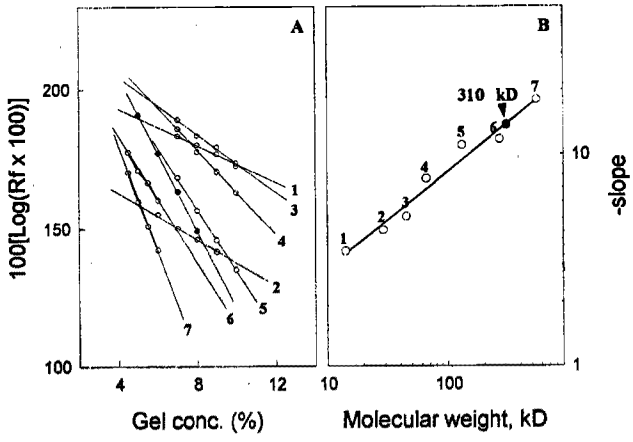

Fig. 2. Determination of the molecular weight of the purified catalase-2 on polyacrylamide gel electrophoresis. The determination was done by the method of Hedrick and Smith (1968). Size markers were 1. bovine α-lactalbumin (14,200), 2. bovine erythrocytes carbonic anhydrase (29,000), 3. chicken egg albumin (45,000), 4 and 5. bovine serum albumin (66,000-monomer, 132,000-dimer, respectively), 6 and 7. Jack bean urease (272,000-trimer, 545,000-hexamer, respectively).

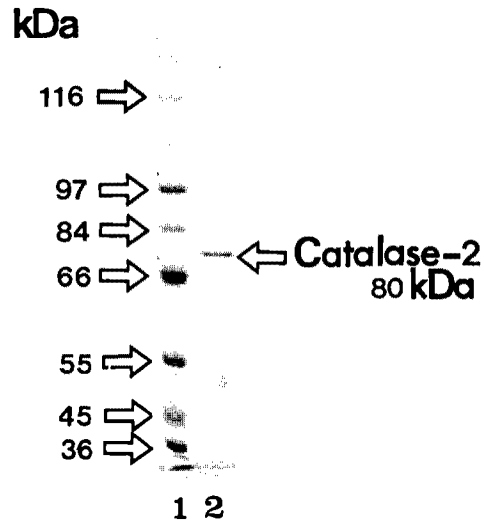

Fig. 3. SDS-polyacrylamide gel electrophoresis of the purified catalase-2. Electrophoresis was performed on 6.5% polyacrylamide gel containing 0.2% SDS (lane 2). Lane 1, size markers.

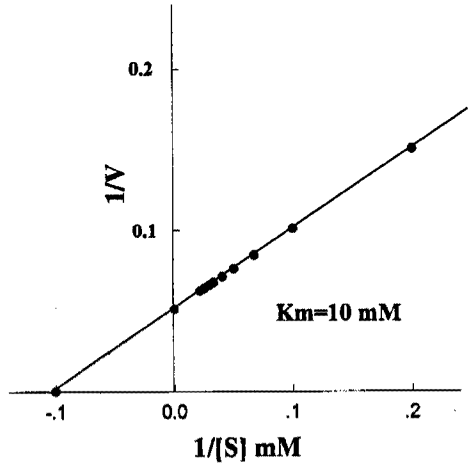

Fig. 4. Lineweaver-Burk plot of the reaction velocity of catalase-2 for hydrogen peroxide. The assay was performed with substrate concentration of 0–60 mM.

Deinococcal catalase-3. Interestingly, the K_m value of the Deinoccocal catalase-2 was higher than other prokaryotic bifunctional catalase-peroxidases, for example, 3.9 mM of *E. coli* HPI (Claiborne and Fridovich, 1979), 4.2 mM of *Rhodopseudomonas capsulata* (Hochman and Shemesh, 1987), and 2.07 mM of *Streptomyces cyaneus* (Mliki and Zimmerman, 1992). The bifunctional catalase-peroxidase is known to be sharply pH dependent and thermolabile for its activity (Nadler *et al.*,1986). However, the Deinococcal catalase-2 was surprisingly stable over the broad pH range of between pH 5 and 12. Its full activity remained even after incubation at pH 12 for 90 min, whereas a sudden decrease of the catalase-3 activity occurred above pH 10 (Lee and Lee, 1995). The catalase-2 was rather thermostable, conserving more than 80% of activity after incubation at 50°C for 90 min. This nature of thermostability and stability over a broad pH range of the catalase-2 was in contrast to that of the catalase-3 (Lee and Lee, 1995). The catalase-2 seemed to be insensitive to organic solvents (ethanol-chloroform treatment), a characteristic which is distinct from other catalase-peroxidases. However, the catalase-2 was insensitive to 3-amino-1,2,4-triazole like other bifunctional catalase-peroxidases. The catalase-2 showed similar sensitivity of the catalase-3 to cyanide and hydroxylamine, but was comparatively less sensitive to azide (Table 2).

Catalases are typical ferric-proteins with maximum

Table 2. Comparison of properties of *D. radiophilus* catalase-2 and catalase-3.

Property	Catalase-2	Catalase-3*
Mol. weight (kDa)	310 (80 × 4)	155 (39 × 4)
K_m for H_2O_2 (mM)	10	0.5
Conc. for 50% inhibition		
NaCN (μM)	4.6	8.1
NaN_3 (μM)	7.7	0.29
NH_2OH (μM)	0.3	0.27

* Data from Lee and Lee (1995).

absorption at 402–406 nm and a series of bands at 550–560, 580–590, and 620–630 nm. The Deinococcal catalase-2 showed a maximum absorption at 403 nm and several shoulders at 557, 587, and 627 nm. The absorption peak at 403 nm was shifted to 419 nm by addition of cyanide (Fig. 5). The catalase-2 had a low A_{403}/A_{280} ratio (0.48) like other prokaryotic bifunctional catalases, i.e., 0.5 for the Deinococcal catalase-3 (Lee and Lee, 1995) and 0.55 for *E. coli* HPI (Claiborne and Fridovich, 1979). However, the ratio of A_{403}/A_{280} of monofunctional catalase was much higher (0.93) than that of the bifunctional catalases (Seah and Kaplan, 1973).

In summary, the *Deinococcus radiophilus* catalase-2 and catalase-3 were distinct from each other with respect to their sizes, subunit compositions, and substrate affinities. Such differences between the multiple species of catalases suggest that the enzymes are governed by different genes. The occurrence of multiple species of catalases in *D. radiophilus* leads us to contemplate about their localization in the cell and the physiological roles they play during the growth phase. In fact, independent induction and different localizations of the multiple catalases have been reported in some bacteria (Loewn *et al.*, 1985; Kim *et al.*, 1992; Klotz and Hutchson, 1992; Schnell and Steinman, 1995). A report (Wang and Schellhorn, 1995) on the inducible defense mechanisms of *Deinococcus* spp. against the toxic oxidants, and ionizing and UV radiations, seems to support our speculation that the efficient removal of peroxide by multiple catalases in *D. radiophilus* would protect the cells from radiation toxicity to a certain extent although further extensive studies on this are required.

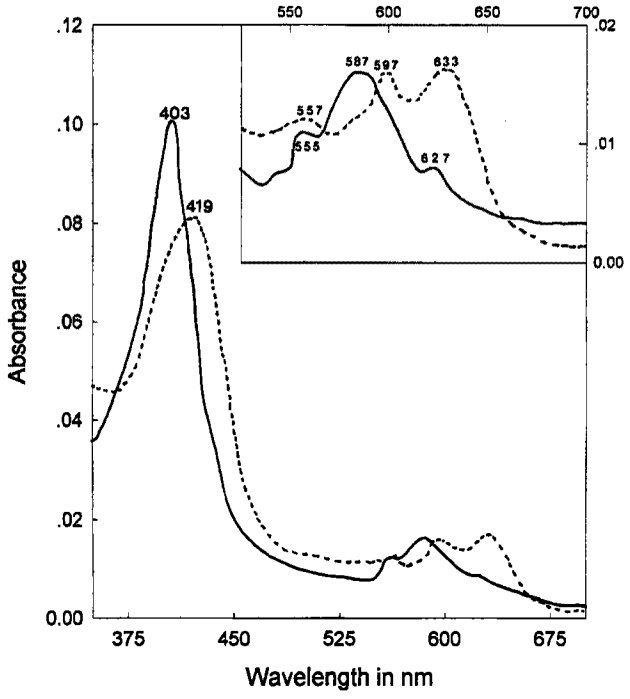

Fig. 5. Absorption spectrum of catalase-2. The purified enzyme (500 μg/ml) was dissolved in 10 mM Hepes buffer, pH 7.0. Scanning was peformed from 350 to 700 nm with (– – –)/without (———) 10 mM NaCN. The insert was for scale-up of spectrum between 525 and 700 nm.

References

Beers, R. F. Jnr. and Sizer, I. W. (1951) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* **159**, 133–140.

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Brown-Peterson, N. and Salin, M. L. (1993) Purification of a catalase-peroxidase from *Halobacterium halobium*: Characterization of some unique properties of the halophilic enzyme. *J. Bacteriol.* **175**, 4197–4202.

Cadenas, E. (1989) Biochemistry of oxygen toxicity. *Annu. Rev. Biochem.* **58**, 79–110.

Claiborne, A. and Fridovich, I. (1979) Purification of *o*-dianisidine peroxidase from *Escherichia coli* B. *J. Biol. Chem.* **254**, 4245–4252.

Clare, D. A., Duong, M. N., Darr, D., Archibald, F., and Fridovich, I. (1984) Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. *Anal. Biochem.* **140**, 532–537.

Goldberg, I. and Hochman, A. (1989) Three different types of catalases in *Klebsiella pneumoniae*. *Arch. Biochem. Biophys.* **268**, 124–128.

Gutman, P. D., Fuchs, P., Ouyang, L., and Minton, K. W. (1993) Identification, sequencing, and targeted mutagenesis of DNA polymerase gene required for the extreme radioresistance of *Deinococcus radiodurans*. *J. Bacteriol.* **175**, 3581–3590.

Hassen, H. M. and Fridovich, I. (1979) Intracellular production of superoxide radical and of peroxide by redox active compounds. *Arch. Biochem. Biophys.* **196**, 385–395.

Hedrick, J. L. and Smith, A. J. (1968) Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. *Arch. Biochem. Biophys.* **126**, 155–164.

Hochman, A. and Shemesh, A. (1987) Purification and characterization of a catalase peroxidase from the photosynthetic bacterium *Rhodopseudomonas capsulata*. *J. Biol. Chem.* **262**, 6871–6876.

Kim, H. P., Lee, J. S., Hah, Y. C., and Roe, J. H. (1992) Multiple catalases in *Streptomyces coelicolor. Kor. J. Microbiol.* **30**, 291–298.

Klotz, M. G. and Hutchson, S. W. (1992) Multiple periplasmic catalases in phytopathogenic strains of *Pseudomonas syringae. Appl. Environ. Microbiol.* **58**, 2468–2473.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4 . *Nature* **227**, 680–685.

Lee, I-J. and Lee, Y. N. (1995) Purification and characterization of catalase-3 of *Deinococcus radiophilus. Kor. J. Microbiol.* **33**, 239–243.

Loewen, P. and Switala, J. (1987) Multiple catalases in *Bacillus subtilis. J. Bacteriol.* **169**, 3601–3607.

Loewen, P. C., Switala, J., and Triggs-Raine, B. L. (1985) Catalases HPI and HPII in *Escherichia coli* are induced independently. *Arch. Biochem. Biophys.* **243**, 144–149.

Mliki, A. and Zimmerman, W. (1992) Purification and characterization of an intracellular peroxidase from *Streptomyces cyaneus. Appl. Environ. Microbiol.* **58**, 916–919.

Mun, C., Rowe, J. D., Sandigursky, M., Minton, K. W., and Franklin, W. A. (1994) DNA deoxyribophosphodiesterase and an activity that cleaves DNA containing thymine glycol adducts in *Deinococcus radiodurans. Radiation Res.* **138**, 282–285.

Murray, R. G. E. (1986) Family II Deinococcaceae, in *Bergey's Manual of Systematic Bacteriology,* Sneath, P.H.A., Mair, N. S., Sharper, M. S. and Holt, J. G., (eds.), pp. 1035–1043, The Williams & Wilkins Co., Baltimore.

Nadler, V., Goldberg, I., and Hochman, A. (1986) Comparative study of bacterial catalases. *Biochem. Biophys. Acta* **882**, 234–241.

Price, N. C. and Stevens, L. (1988) *Fundamentals of Enzymology,* 2nd ed. pp 1–151. Oxford Science Publications, Oxford.

Schnell, S. and Steinman, H. M. (1995) Function and stationary-phase induction of periplasmic copper-zinc superoxide dismutase and catalase/peroxidase in *Caulobacter crescentus. J. Bacteriol.* **177**, 5924–5929.

Seah, T. C. M. and Kaplan, J. G. (1973) Purification and properties of catalase of baker's yeast. *J. Biol. Chem.* **218**, 2889–2893.

Shin, K. J., Ro, Y. T., and Kim, Y. M. (1994) Catalase in *Acinetobacter* sp. strain JC1 DSM 3803 growing on glucose. *Kor. J. Microbiol.* **32**, 155–162.

Stortz, G., Tartaglia, L. A., Farr, S. B., and Ames, B. N. (1990) Bacterial defense against oxidative stress. *Trends Genet.* **6**, 363–368.

Wang, P. and Schellhorn, H. C. (1995) Induction of resistance to hydrogen peroxide and radiation in *Deinococcus radiodurans. Can. J. Microbiol.* **41**, 170–176.