【SII-4】

Reactive Oxygen species (ROS) scavenging enzymes in *Deinococcus radiophilus*, an UV resistant bacteria

Young Nam LEE
Division of Life Sciences, Chungbuk National University,
Cheongju, Chungbuk, Rep. of Korea, 361-763

Introduction

The excess solar UV light is detrimental to all kinds of life form in various ways. Recently, all creatures on earth have received more UV because of thinnerization of strastopheric ozone layer by increase of ozone depleting substances in air. There has been a great alert on over-exposure of living thing to UV which imposes an environmental impact as well as global-wide health problems. Thus, World Health Organization (WHO) and United Nations Environmental programme (UNEP) lanched *Intersun Project* in 1995 and asking more efforts of scientists to conduct researches on minimizing UV hazards.

The lethal effect of UV is mediated through either cellular DNA damage occurred by UV absorption of pyrimidine bases or oxidation activity of reactive oxygen species (ROS_s) generated by UV irradiation. However, cells protect themselves from UV damage with ROS_s scavenging systems including enzymes of catalase-peroxidase, superoxide dismutase, G6PDHase etc. and antioxidant chemicals. Repairing of modified cellular components would be indispensible part of protective machinery (10).

The obligate aerobic *Deinococcus* are extremely resistant to lethal effect of UV- and ionizing-radiations as well as to oxidative stress(1,2,17,18). Extreme UV-resistance of these bacteria would probably be due to an efficient repairing machinery of damaged DNA along with powerful scavenging activity of cells toward various reactive oxygen species (ROSs) generated by UV irradiation. ROS scavenging systems include a number of enzymes such as hydroperoxidase (catalase), peroxidase, superoxide dismutase (SOD), glucose-6-phosphate dehydrogenase etc and some antioxidants substances such as glutathione, ascorbic acid, beta carotene etc.

Although a mechanism of UV resistance of *Deinococcus* has been studied with regards to the genes involved in repairing of damaged DNA(3,8,11,15), a little attention was paid to roles of ROS scavenging enzymes in UV resistance. Therefore, we have undertaken studies on key enzymes in the scavenging toxic oxidants, SODs and hydroperoxidases in the UV resistant *Deinocooccus* for years.

Materials and Methods

1. Bacterial strains and medium *Deinococcus radiodurans* ATCC 13939, *D. radiophilus* ATCC 27603, *D. grandis* ATCC 43672, *D. proteolyticus* ATCC 35074 ,and *D. radiopugnans* ATCC 19172 were cultured in TYGM medium (1% tryptone, 0.5% yeast extract, 0.2% glucose, and 0.2% L-methionine) at 30°C with continuous aeration at 150 rpm until the cultures reached the stationary growth phase (19,21).

2. Eelectrophoretic resolution and activity staining of enzymes. Proteins in cell free-extract prepared with cells in stationary phase by ultrasonic disruption (19) were resolved by 10.0% nondenaturing polyacrylamide gel electrophoresis (PAGE) in Tris-glycine buffer (13). Molecular markers for gel electrophoresis were bovine serum albumin (132 kDa, 66 kDa) and bovine milk α-lactalbumin (14.2 kDa). Visualization of enzyme bands resolved on polyacrylamide gel was made by methods reported by Wayne and Diaz for catalase and peroxidase (20), Chou and Tan for SOD(4, 9) and Lee and Lessie for glucose 6-phosphate dehydrogenase (14) . Density of enzyme bands was measured by densitometry (Personal Densitometry SI, Molecular Dynamics, NY). Distinction between FeSOD and MnSOD bands was made by soaking the gels for 60 min at room temperature in a solution of either 20 mM H_2O_2, or 5 mM KCN and 50 mM potassium phosphate (pH 7.8) prior to activity staining for SOD.

3. Assay of catalase, peroxidase, and SOD activity. Catalase activity was measured by method of Beer and Sizer (5) and peroxidase activity was measured by guaiacol autooxidation method. SOD activity was measured by pyrogallol autooxidation (16) or inhibition of NBT reduction by superoxide radicals photochemically generated from riboflavin in presence of SOD enzyme. Protein concentration was determined by the Bradford method (7).

4. Purification of catalases and SOD. Cell-free extract was prepared by ultrasonic disruption. Enzymes were purified by steps of salt fractionation, ion exchange chromatography and gel filtration (6).

5. Characterization of *D. radiophilus* catalases and SOD. Molecular weight determination of purified enzymes was determined by both PAGE and gel filtration. Subunit structure of the enzymes was studied by either SDS-PAGE or Urea-SDS PAGE. Isoelectric point of enzymes, pH dependency and stability, optimal temperature of enzyme activity and thermostability, effect of a number of chemicals or metal ions were investigated (6).

Results and Discussion

1. Profiles of ROS scavenging enzymes in the mesophilic Deinococcus species. Electrophoretic resolution of catalase, peroxidase, superoxide dismutase (SOD) and glucose-6-phoshate dehydrogenase in the highly UV resistant bacteria, *Deinocccus* species revealed multiple forms with dissimilar electrophoretic properties and with different cofactors

as seen in Fig 1. Activities of scavenging enzymes of reactive oxygen species in the mesophilic *Deinococcus* varied depending upon bacterial species (Fig. 2). The unique profiles of catalase/peroxidase, SODs and G6PDHase of each mesophilic *Deinococcus* species, multiplicity and different cofactors, would be valuable in identifying *Deinococcus* species.

2. Characterization of bifunctional catalases of *D. radiophilus*. *D. radiophilus* posseses three isocatalases, one monofunctional catalase and two of bifunctional catalase-peroxidases. The bifunctional catalases were purified to electrophoretic homogeneity and characterized their properties (Table 1). They are different in molecular structure, pI, substrate affinity and some other properties. The physiological properties of two bifunctional catalases were investigated at different environmental stresses such as H_2O_2 treatment, UV irradiation. AS shown in Fig. 3, production of these two bifunctional catalases varied depending upon internal and external oxidative stress and UV irradiation, that is, production of catalase-3 is induced by environmental stresses whereas production of catalase-2 seems to be unchanged.

3. Purification of *D. radiophilus* SOD. The SOD has been purified from cell-free sonic extract by steps of ammonium sulfate fractionation and Superdex G-75 gel filtration. We assumed that *D. radiophilus* SOD was MnSOD, since it was insensitive to CN^-, N_3^-, and H_2O_2. The molecular weight of the purified enzyme estimated by gel filtration and polyacrylamide gel electrophoresis turned out to be 50.7 kDa and 47.1 kDa, respectively. The SOD seemed to be hetero-dimeric protein composed of 26.5 and 23.1 kDa subunits (Fig. 4). Electrofocusing of the purified SOD also yielded two peptides, one with SOD activity whose pI was 3.8 and pI of peptide with no activity was 4.1 The enzyme was stable at pH 5.0～11.0, however, quite unstable below pH 5.0. SOD was thermostable up to 50℃ but slightly decrease in activity at 60℃. This enzyme was rather insensitive to SDS, but sensitive to EDTA.

Related papers:

Lee, I. J. and Lee, Y. N. (1995) Purification and characterization of catalase-3 of *Deinococcus radiophilus* ATCC 27603. *J. Microbiol.* **33**, 239-243.

Oh, K. A. and Lee, Y. N. (1998) Purification and characterization of catalase-2 of *Deinococcus radiophilus* ATCC 27603. *J. Biochem. Mol. Biol.* **31**, 144-148.

Soung, N. K. and Lee, Y. N. (2000) Iso-catalase profiles of *Deinococcus* spp. Biochem. Mol. Biol. **33**, 412-416.

Yun, E. J. and Lee, Y. N. (2000) Production of two different catalase-peroxidases by *Deinococcus radiophilus. FEMS Microbiol. Letters* **184**, 155-159.

Yun, Y. S. and Lee, Y. N. (2001) Superoxide Dismutase Profiles in the Mesophilic *Deinococcus* Species. *J. of Microbiol.* **39**, 232-235

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9. Chou, F. I. and Tan, S. T. (1990) Manganese (II) induces cell division and increases in superoxide dismutase and catalase activities in an aging Deinococcal culture. *J. Bacterio.* **172**, 2029-2035.
10. Demple, B. and Harrison, L. (1994) Repair of oxidative damage to DNA. *Ann. Rev. Biochem.* **63**, 915-948.
11. Gutman, P. D., Carroll, J. D., Masters, C. I. and Minton, K. W. (1994) Sequencing, targeted mutagenesis and expression of *recA* gene required for the extreme radioresistance of *Deinococcus radiodurans*. *Gene* **141**, 31-37.
12. Halliwell, B. and Gutteridge, J. M. C. (1999) *Free Radicals in Biology and Medicine* (3rd ed.), pp. 105-350. Oxford Univ. Press, Oxford, UK.
13. 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.
14. Lee, Y. N. and Lessie, T. G. (1974) Purification and characterization of the two 6-phosphogluconate dehydrogenase species from Pseudomonas multivorans. *J. Bacteriol.* **120**, 1043-1057.
15. Makarova, K. S., Aravind, L., Wolf, Y. I., Tatusov, R. L., Minton, K. W., Koonin, E. V., and Daly, M. J. (2001) Genome of the extremely radiation resistant bacteria, *Deinococcus radiodurans* viewed from the perspective of comparative genomics. *Microbiol.*

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Resolution of catalases/peroxidases by PAGE

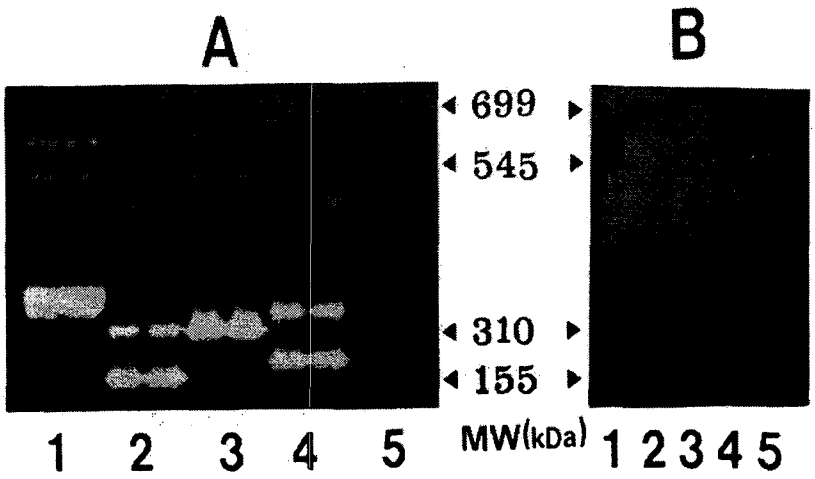

Fig.1-1. Electrophoretic profiles of Catalase(A) / Peroxidases (B) of *Deinococcus* spp.

Proteins in cell-free extracts of *Deinococcus* were resolved on 8.5% of polyacrylamide. Each well was loaded with 10 μg of protein. 1, *D. radiodurans* 2, *D. radiophilus* 3, *D. grandis* 4, *D. proteolyticus* 5, *D. radiopugnans.* Gel size was 8×7 cm.

Resolution of SODs by PAGE

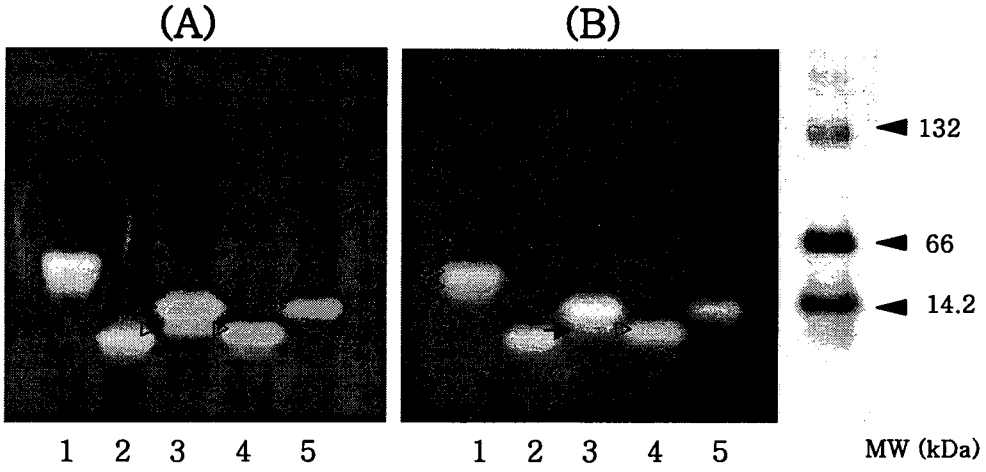

Fig. 1-2. Electrophoretic profiles of SOD of *Deinococcus* spp.

Each well was loaded with 50 μg of protein in *Deinococcus* cell-free extracts obtained from stationary culture. Activity staining of SOD on gel (10%) prior to H_2O_2 treatment (A) and activity staining of SOD after H_2O_2 treatment (B). Treatment of H_2O_2 caused inactivation of FeSOD. KCN treatment prior to activity staining caused no change of Deinococcal SOD profiles (Data not shown). This indicates that Deinococcal SODs are not CuZnSOD. 1. *D. radiodurans* 2. *D. radiophilus* 3. *D. grandis* 4. *D. proteolyticus* 5. *D. radiopugnans*. Symbols (▷, ➡) for SOD activity bands. ➡ for SOD band whose intensity was reduced after H_2O_2 treatment.

Resolution of G6PDHases by PAGE

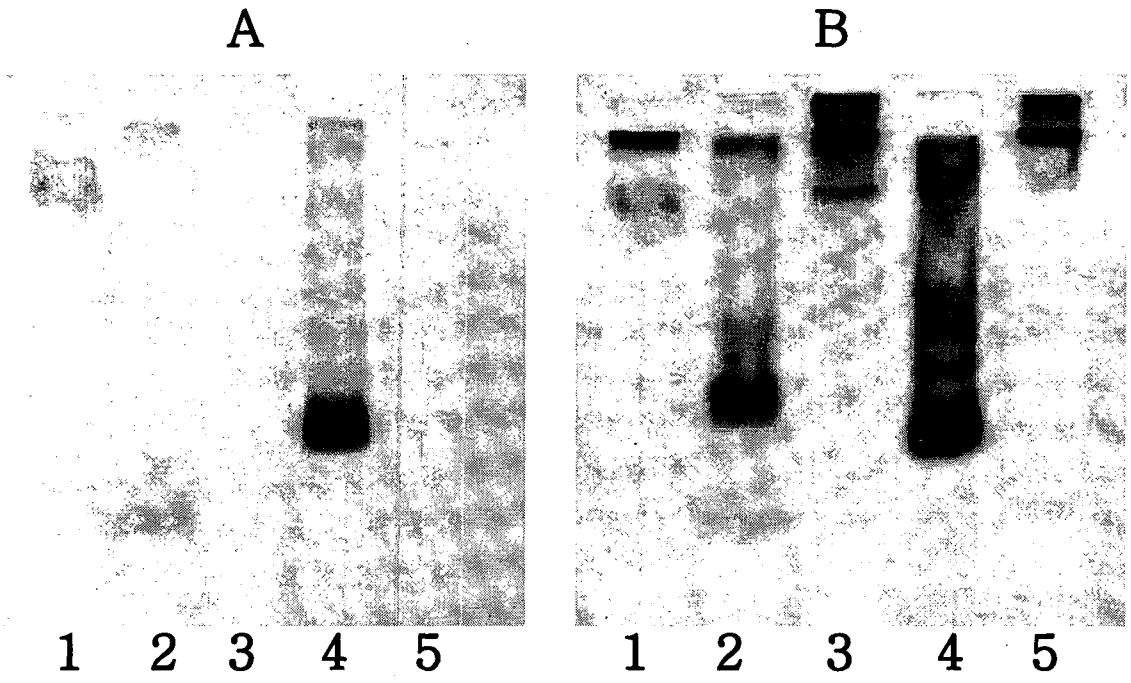

Fig.1-3. G6PDHases of *Deinococcus* spp.
(A) NAD (B) NADP

Proteins in cell-free extracts of *Deinococcus* were resolved on 10 % of polyacrylamide. Each well was loaded with 200 μg of protein. 1, *D. radiodurans* 2, *D. radiophilus* 3, *D. grandis* 4, *D. proteolyticus* 5, *D. radiopugnans.* Gel size was 8×7 cm.

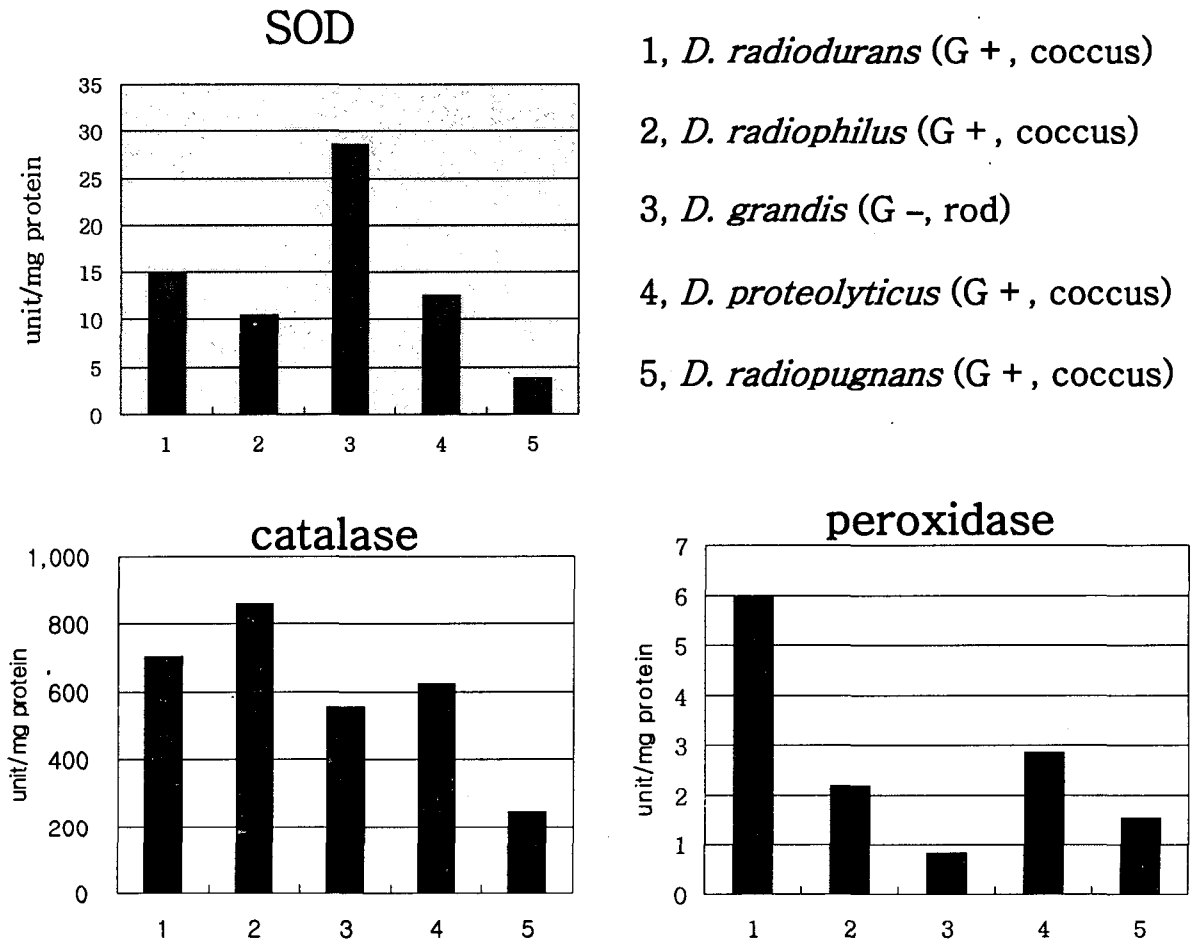

Fig. 2. Enzyme activities of *Deinococcus* species.

Enzyme assays were done with cell-free extracts prepared with stationary culture.

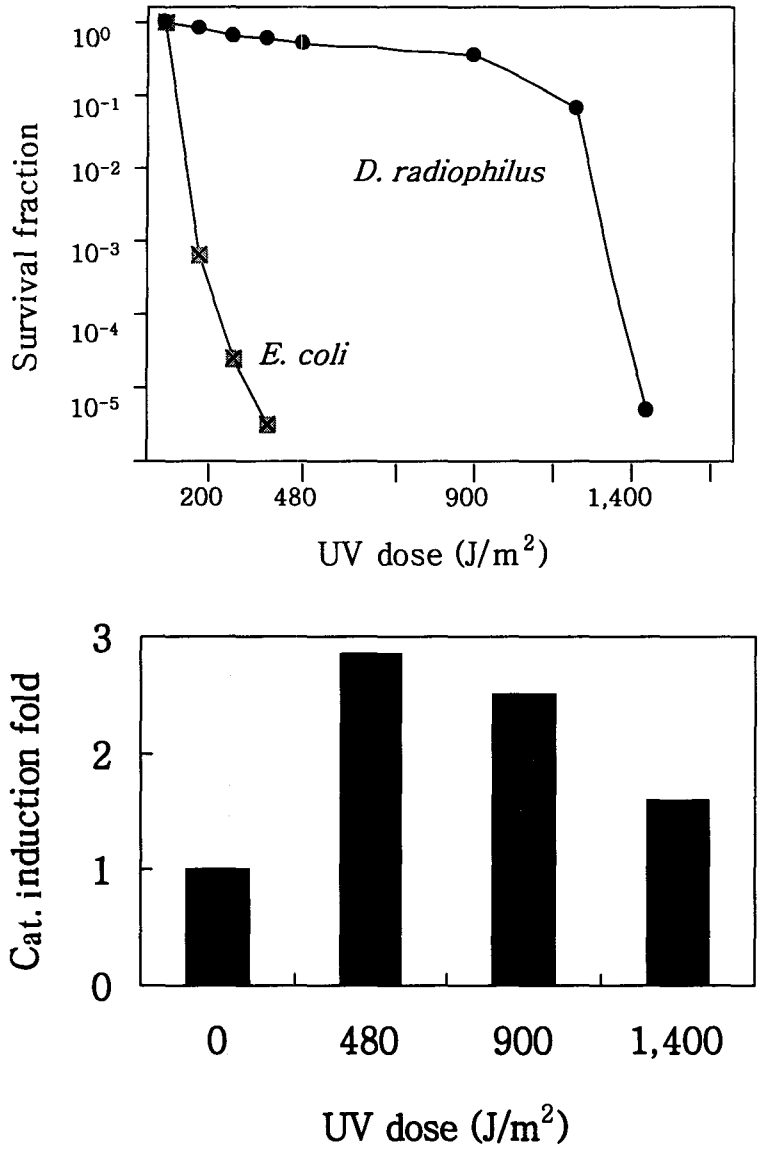

Fig. 3-1. Survival of *D. radiophilus* by UV irradiation

Bacterial cultures of OD_{600}=0.2 were irradiated. Survival fraction was calculated CFUs of irradiated cultures/CFUs of nonirradiated cultures. Specific activity of catalase of UV irradiated cultures was compared with that of nonirradiated cultures.

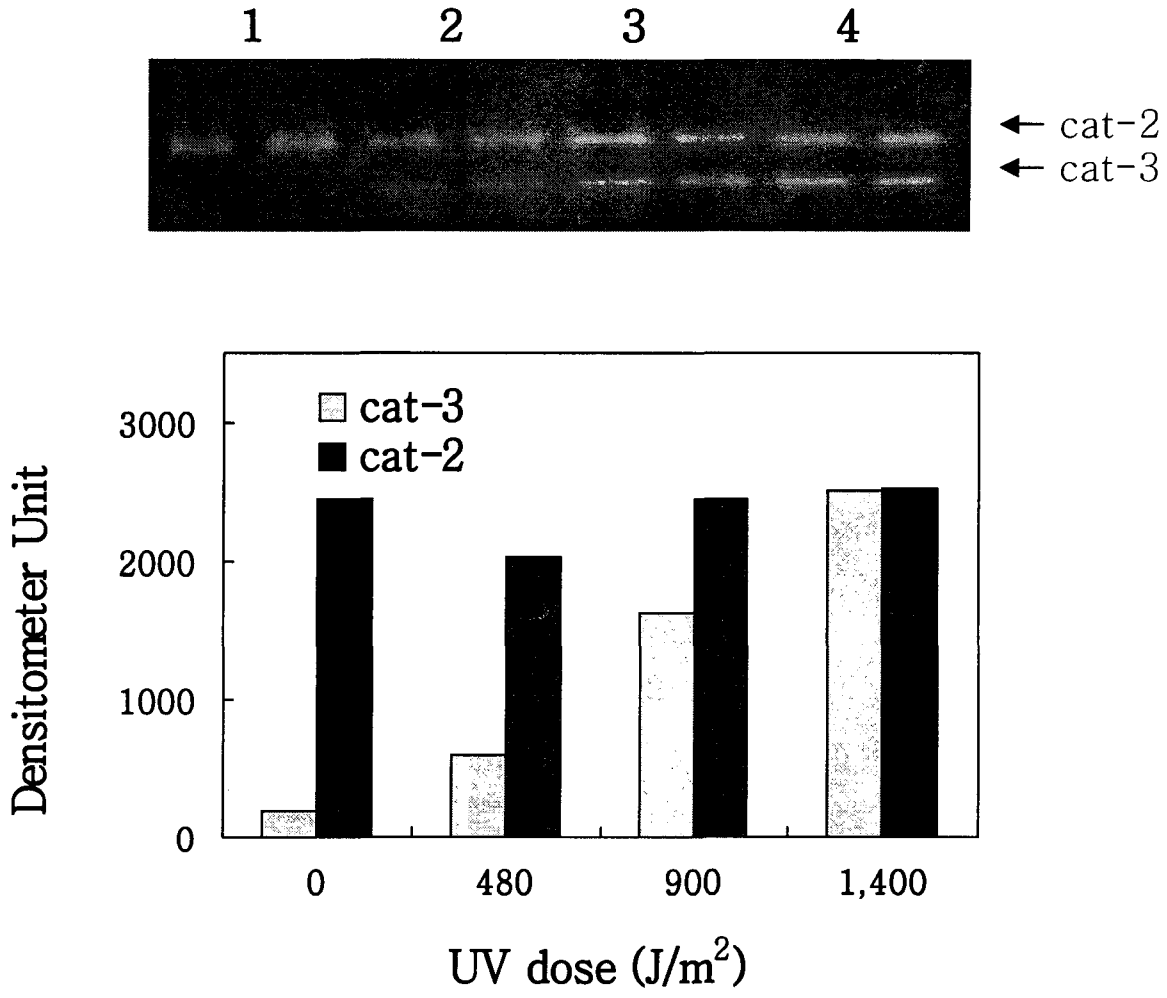

Fig. 3-2. Induction of catalases by UV irradiation

Table 1. Comparison of properties of *D. radiophilus* catalase-2 and catalase-3

	Catalase-2	Catalase-3
Peroxidatic activity	Exist	Exist
Molecular weight (kDa)	310 (80×4)	155 (39 ×4)
pI	5.8-5.9	5.1-5.2
Km for H_2O_2	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
pH optimum [stability]	9-10 [5-12]	9-10 [6-10]
Temperature optimum (°C)	30	30
Inhibition by Et-OH-chloroform (%)	30	40
Inhibition by 1 mM 3-amino-1,2,4-tetrazole (%)	10	20
Absorption peak (Soret, nm)	403	405
A_{403}/A_{280}	0.48	0.55

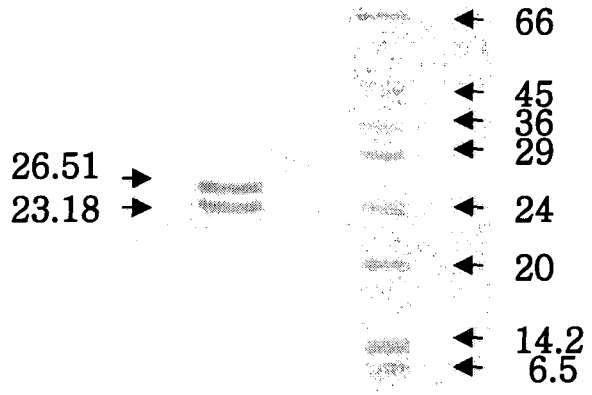

Fig. 4. SDS PAGE of purified *D. radiophilus* SOD

Electrophoresis was performed on 12 % polyacrylamide gel containing 0.4% SDS

1: aprotinin (6,500), 2: bovine α-lactalbumin (14,200), 3: soybean trypsin inhibitor (20,000), 4: bovine trypsinogen (24,000), 5: bovine erythrocytes carbonic anhydrase (29,000), 6: rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (36,000), 7: chicken egg albumin (45.000), 8: bovine serum albumin (66,000).