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Cloning and Characterization of Monofunctional Catalase from Photosynthetic Bacterium *Rhodospirillum rubrum* S1

LEE, DONG-HEON[1,2], DUCK-CHUL OH[1], YOU-SUNG OH[1], JULIANA C. MALINVERNI[3], JEROME J. KUKOR[3], AND HYUNG-YEEL KAHNG[2]*

[1]*Department of Life Science, Cheju National University, Jeju 690-756, Korea*
[2]*Department of Environmental Education, Sunchon National University, Sunchon 540-742, Korea*
[3]*Biotechnology Center for Agriculture and the Environment, Rutgers–The State University of New Jersey, 316 Foran Hall, Cook College Campus, 59 Dudley Road, New Brunswick, NJ 08901, U.S.A.*

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Abstract In this study, an approx. 2.5-kb gene fragment including the catalase gene from *Rhodospirillum rubrum* S1 was cloned and characterized. The determination of the complete nucleotide sequence revealed that the cloned DNA fragment was organized into three open reading frames, designated as ORF1, catalase, and ORF3 in that order. The catalase gene consisted of 1,455 nucleotides and 484 amino acids, including the initiation and stop codons, and was located 326 bp upstream in the opposite direction of ORF1. The catalase was overproduced in *Escherichia coli* UM255, a catalase-deficient mutant, and then purified for the biochemical characterization of the enzyme. The purified catalase had an estimated molecular mass of 189 kDa, consisting of four identical subunits of 61 kDa. The enzyme exhibited activity over a broad pH range from pH 5.0 to pH 11.0 and temperature range from 20°C to 60°C. The catalase activity was inhibited by 3-amino-1,2,4-triazole, cyanide, azide, and hydroxylamine. The enzyme's K_m value and V_{max} of the catalase for H_2O_2 were 21.8 mM and 39,960 U/mg, respectively. Spectrophotometric analysis revealed that the ratio of A_{406} to A_{280} for the catalase was 0.97, indicating the presence of a ferric component. The absorption spectrum of catalase-4 exhibited a Soret band at 406 nm, which is typical of a heme-containing catalase. Treatment of the enzyme with dithionite did not alter the spectral shape and revealed no peroxidase activity. The combined results of the gene sequence and biochemical characterization proved that the catalase cloned from strain S1 in this study was a typical monofunctional catalase, which differed from the other types of catalases found in strain S1.

Keywords: *Rhodospirillum rubrum*, monofunctional catalase, overexpression, enzyme purification

*Corresponding author
Phone: 82-61-750-3385; Fax: 82-61-750-3385;
E-mail: kahng@sunchon.ac.kr

Molecular oxygen (O_2) is a key factor for the growth of aerobic and facultative anaerobic organisms. However, it can also be transformed into reactive oxygen species (ROS), such as a superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), or hydroxyl radical (OH^-), which in turn react with nucleic acids, proteins, lipids, or carbohydrates [5, 8], resulting in the induction of mutation, reduction of growth rates, or even cellular death. Thus, aerobic and facultative anaerobic organisms possess several defense mechanisms against ROS [5, 8, 11, 16]. For example, catalase plays a crucial role in defense mechanisms against oxidative stress caused by H_2O_2, through degrading H_2O_2 into water and oxygen or oxidizing it into other substances with water production. Catalase is an oxidoreductase and can be classified into three groups, typical catalase (monofunctional), catalase-peroxidase (bifunctional), and non-heme manganese catalase, according to its enzymatic characteristics [32]. Typical catalase has been found in animals, plants, and microorganisms and consists of four subunits with an identical size. Each subunit has one ferric heme prosthetic group (protoporphyrin IX), and its molecular mass ranges from 225 kDa to 340 kDa [32]. Catalase-peroxidase has only been found in microorganisms and is much more variable in size, as well as being more unstable and sensitive to changes in environmental factors, such as temperature and pH, than typical catalase [6, 7, 32]. Finally, manganese catalase is often found in lactic acid and thermophilic bacteria, and has a manganese substitute instead of the heme in typical catalase [9, 14]. Thus, all three types of catalases have been found in microorganisms, where their expression patterns and biological functions vary according to the microorganism. In *E. coli*, two types of catalases, HPI (*katG*) and HPII (*katE*), have been found, which are bifunctional and monofunctional, respectively. HPII is a monofunctional enzyme consisting of four subunits, each

of which has one heme d group and is identical in molecular weight [3, 18, 19].

The nonsulfur photosynthetic bacterium *Rhodospirillum rubrum* can grow by anaerobic photosynthesis or aerobic respiration according to the presence or absence of light. Moreover, it can even grow under anaerobic conditions by fermentation [12, 24, 27, 28]. A previous study reported that *R. rubrum* S1 has at least three catalase isoforms, each of which exhibits specific expression patterns depending on whether oxygen is present [17]. Furthermore, catalase-2 from *R. rubrum* S1 was better expressed under anaerobic conditions than aerobic conditions. A recent report confirmed that catalase-2 expressed under anaerobic conditions in strain S1 was a large monofunctional catalase [10].

Accordingly, in the present study, the catalase gene from *R. rubrum* S1 expressed under aerobic conditions was cloned and characterized on the gene and protein levels, resulting in the elucidation of a small subunit of a monofunctional catalase. The cloned catalase gene was then overexpressed in *E. coli* UM255 and purified, followed by biochemical characterization.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

The *Rhodospirillum rubrum* S1 was grown with light in one-liter bottles that were tightly capped and filled to the top with a basal medium supplemented with 0.3% malate as the sole carbon source, under a light of 2,000 lux at 30°C [2]. The *Escherichia coli* strains JM109 and UM255 were grown at 37°C in a Luria-Bertani (LB) medium. Ampicillin was used at a concentration of 100 mg/ml. Strain UM255, a catalase-deficient mutant, was obtained from Dr. P. C. Loewen, Head of the Department of Microbiology, University of Manitoba, Canada.

General Recombinant DNA Techniques

The genomic DNA from *R. rubrum* S1 was isolated using a CTBA miniprep procedure [20], whereas the plasmid DNA was purified from the *E. coli* strains using a Wizard SV miniprep DNA purification system (Promega Co., Madison, WI, U.S.A.). The DNA fragment was recovered from the agarose gels using a GENECLEAN II Kit (BIO 101 Inc.). The Southern blotting and other general DNA manipulation procedures were carried out as described by Sambrook *et al.* [23]. For the hybridization, a nonradioactively labeled method was used. The digoxigenin (DIG)-labeled nucleotide and DIG luminescent detection kit were purchased from Roche Molecular Biochemicals (Mannheim, Germany) and used according to the manufacturer's instructions, and the nucleotides were sequenced using an ABI Prism 310 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, U.S.A.)

PCR and Cloning of Catalase Gene from *R. rubrum*

Degenerate oligonucleotide primers corresponding to most highly conserved regions PERVVHA and VGNNTPV of the monofunctional catalase from prokaryotic cells were designed and synthesized by Bioneer Co., Korea. The genomic DNA of *R. rubrum* S1 was used as the template. The PCR conditions were as follows: an initial denaturation at 95°C for 5 min, followed by 33 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min, plus an additional extension step at 72°C for 10 min. The PCR products were size-fractionated on a 2% (w/v) agarose gel and the band corresponding to the expected size 240 bp was cut out. The DNA fragment was isolated from the agarose gel using a GENECLEAN II Kit (BIO 101 Inc.), cloned into a pGEM-T easy vector (Promega), and sequenced using an ABI Prism 310 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, U.S.A.) with T7 forward and Sp6 reverse sequencing primers.

To screen the full-size DNA fragment including the catalase gene, chromosomal DNA fragments digested with BamHI, EcoRI, HindIII, PstI, and/or SalI restriction enzymes were used for Southern blotting with the catalase gene probe labeled with digoxigenin. The hybridization was performed in accordance with the instructions of Roche. The DNA fragments from the hybridized signals in each lane were recovered from the gel using a GENECLEAN II Kit (BIO 101 Inc.) and subcloned into a pBluescript SK(+) vector.

Complementation of Catalase-deficient Mutant *E. coli*

The catalase gene library was introduced into *E. coli* UM255 by electroporation using a Bio-Rad Gene Pulser with the voltage and capacitance set at 2.5 kV and 125 μFd, respectively. The resulting mixture was then spread onto an LB plate containing ampicillin. The catalase activity of an ampicillin-resistant colony was confirmed by visual identification of bubble formation when 3% hydrogen peroxide was dropped on the individual colonies. Potential catalase-positive colonies were immediately streaked onto a new plate.

Preparation of Crude Enzyme Extracts

The transformed *E. coli* UM255 colonies were harvested in the logarithmic growth phase by centrifugation, washed three times with a 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, and resuspended in the same buffer. The cells were then disrupted by sonification on ice (Bandelin Sonoplus HD 2070, Germany) based on four 30-s cycles at 20% maximum power, and then centrifuged at 12,000 ×*g* for 30 min. The supernatant was used as the crude enzyme extracts.

Catalase Activity Assay

The catalase activity was measured spectrophotometrically by monitoring the decrease at A_{240} resulting from the

elimination of H_2O_2, using a UV visible spectrophotometer (UV-1601, Shimadzu, Japan). The extinction coefficient (ε) for H_2O_2 at 240 nm was 43.6 M/cm. The standard reaction mixture for the assay contained a 50 mM potassium phosphate buffer (pH 7.0), 10 mM H_2O_2, and 100 μl of the catalase solution making a total volume of 3.0 ml. The reaction was performed at 25°C. The amount of enzyme activity that decomposed 1 μmol of H_2O_2 per min was defined as 1 unit (U) of activity [1]. The protein concentrations were determined using a micro-BCA protein assay [25] using bovine serum albumin as the standard.

Polyacrylamide Gel Electrophoresis (PAGE)

The nondenaturing PAGE and denaturing sodium dodecyl sulfate (SDS)-PAGE were performed as described by Laemmli [15]. After the electrophoresis, proteins were detected by staining the gel with Coomassie brilliant blue. Visualization of catalase activity on the nondenaturing polyacrylamide gel was performed using the ferric chloride–potassium ferric cyanide double-staining method [30], where the gels were initially incubated with 5 mM hydrogen peroxide, followed by staining with a freshly prepared mixture of 2% ferric chloride and 2% potassium ferric cyanide.

Protein Purification

A crude lysate in a 50 mM potassium phosphate buffer (pH 7.0) was prepared with the bacterial pellet using ultrasonification. Ammonium sulfate precipitation of the crude enzyme extracts of *E. coli* UM255 (pCAT025) was performed within the range of 30%–70% saturation. The precipitate was then collected by centrifugation (12,000 ×*g* for 30 min), resuspended in a 50 mM potassium phosphate buffer (pH 7.0), and dialyzed against the same buffer overnight. The following procedures were then carried out at 4°C: the catalase was purified to electrophoretic homogeneity from the dialyzed preparation of ammonium sulfate by successive chromatography on phenyl-Sepharose CL-4B, followed by preparative gel electrophoresis on a 7.5% polyacrylamide gel, as previously described [10]. The purified enzyme was then collected and stored at −20°C.

Molecular Mass Determination

The molecular mass of the native enzyme was determined by gel filtration using a Superose-12HR 10/30 column (Amersham Pharmacia Co.) equilibrated with a 50 mM potassium phosphate buffer (pH 7.0). For the molecular mass standards, the following proteins were used: ferritin (386 kDa), catalase (209 kDa), aldolase (179 kDa), bovin serum albumin (66 kDa), and carbonic anhydrase (29.4 kDa). The molecular mass of the subunit was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 12% acrylamide gel according to the method of Laemmli [15]. The molecular mass standards for the SDS-PAGE were Bio-Rad low-range markers (Hercules, CA, U.S.A.).

Characterization of Catalase

To investigate the effect of pH, the purified catalase was incubated for 10 min at room temperature prior to initiating the reaction. Thereafter, the catalase activity was measured within a range of pH 3.0–11.0. Different buffer systems were used according to pH range: 50 mM citrate-phosphate buffer for pH 3.0–6.0, 50 mM potassium phosphate buffer for pH 6.0–7.0, 50 mM Tris-HCl buffer for pH 7.0–9.0, and 50 mM carbonate buffer for pH 9.0–11.0 [26]. For the effect of temperature, the standard reaction mixtures were assayed for catalase activity at different temperatures ranging from 20°C to 70°C [10].

For the inhibitor test, the enzyme solution was mixed with different concentrations of metal inhibitors, such as sodium cyanide, sodium azide, and hydroxylamine [10]. After incubating the reaction mixtures for 2 min at 25°C, the enzyme activity was assayed. To evaluate the effect of chemical inhibitors on the catalase activity, a mixture of the enzyme solution and organic solvents (enzyme solution:ethanol:chloroform=10:5:3) was vortexed for 10 min at room temperature, and then the activity was measured [21]. The same procedure was performed after incubating the enzyme solution with 10 mM 3-amino-1,2,4-triazole for 10 min [27]. Finally, the absorption spectrum of the purified enzyme in a 10 mM phosphate buffer, pH 7.0, was measured between 250 nm and 800 nm (Shimadzu UV-1601, UV/Visible Spectrophotometer, Japan).

RESULTS AND DISCUSSION

Cloning of Catalase Gene from *R. rubrum* S1

The PCR amplification with a set of degenerate primers and the total genomic DNA from *R. rubrum* S1 produced an approx. 245-bp PCR product. A sequence analysis then revealed the PCR product to be a portion of the monofunctional catalase. To clone the full-length catalase gene, Southern hybridization was performed using the total genomic DNA from *R. rubrum* S1 digested by restriction enzymes and the catalase DNA fragment as a probe. Strong signals were obtained in BamHI DNA fragments of approx. 20 kb and 6.5 kb, EcoRI fragments of approx. 7.4 kb and 1.0 kb, and HindIII fragments of approx. 8.5 kb and 6.0 kb (Fig. 1), suggesting the possibility that *R. rubrum* S1 has at least two copies of the catalase gene, as two catalase genes were found in the chromosome from *R. rubrum* ATCC 11170 (Accession No. CP000230). The strong fragments, i.e., the BamHI fragment (6.5 kb), EcoRI fragment (7.4 kb), and HindIII fragment (6.0 kb), were

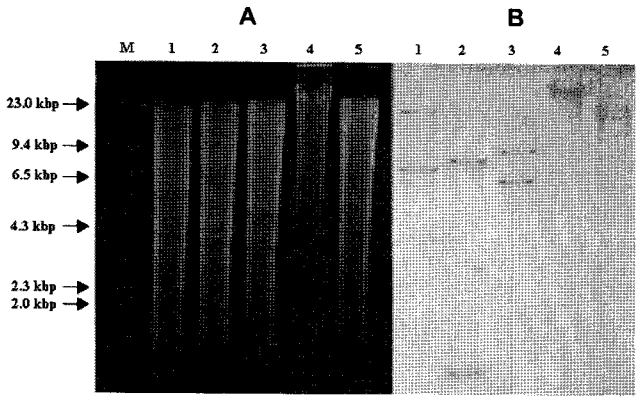

Fig. 1. Southern hybridization of catalase gene using total genomic DNA from *Rhodospirillum rubrum* S1.
The DNA fragments digested with several restriction enzymes were hybridized with the catalase DNA fragment obtained from the PCR amplification. The electrophoresis pattern of the total genomic DNA from *R. rubrum* S1 digested with several restriction enzymes is shown in the left panel (**A**), whereas the signals indicating the catalase gene based on Southern hybridization with the catalase gene probe labeled with a Dig DNA labeling kit are shown in the right panel (**B**). Lanes; M, λ-HindIII DNA size marker; 1, DNA digested with BamHI, 2, DNA digested with EcoRI; 3, DNA digested with HindIII; 4. DNA digested with PstI; 5, DNA digested with SalI.

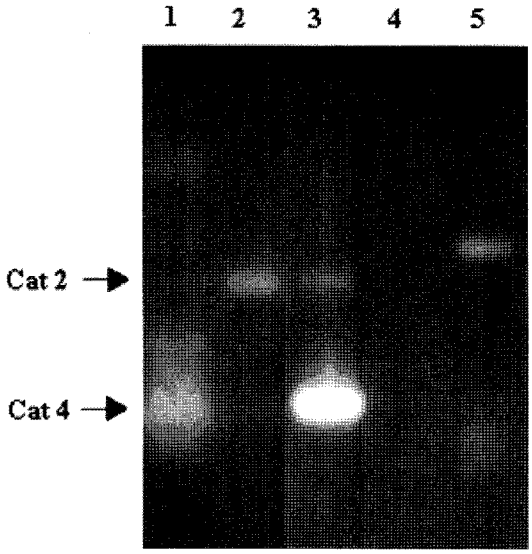

Fig. 2. Expression profiles of catalase genes from *Rhodospirillum rubrum* S1 and other bacterial strains according to a catalase activity test.
The nondenaturing PAGE was carried out using cell lysates from the photosynthetic bacterium *R. rubrum* S1 and *Escherichia coli* grown under different conditions. Lane 1, aerobically grown cells of *R. rubrum* S1; 2, anaerobically grown cells of *R. rubrum* S1; 3, aerobically grown cells of *E. coli* UM255 harboring recombinant plasmid pSCAT02 containing the catalase gene from strain S1; 4. *E. coli* UM255 (HP I−, HP I−; pBluescript SK+); 5, *E. coli* JM109 (HP I+, HP I+).

then cloned into a pBluescrip SK(+) vector, followed by transformation into *E. coli* UM255 that has mutated gene loci for *katE* and *katG*. The transformant containing the catalase gene was selected using 3% H_2O_2, and designated pSCAT02. The restriction digestion pattern proved that the recombinant plasmid, pSCAT02, included the 7.4 kb EcoRI fragment obtained from Southern hybridization. Activity staining of the cell lysate from *E. coli* UM255 harboring pSCAT02 revealed that the catalase gene inserted into pSCAT02 corresponded to that found in a catalase from *R. rubrum* S1 (Fig. 2). *R. rubrum* S1 has at least three types of catalases, and their expression levels differ according to the presence or absence of oxygen [17]. This study found that the catalase band from *E. coli* UM255 harboring pSCAT02 corresponded to the catalase band designated as Cat 4 in *R. rubrum* S1 (Fig. 2). The catalase gene from *R. rubrum* S1 was strongly expressed in *E. coli* under aerobic conditions without IPTG, which stimulates expression of the *lac* promoter. As such, this fact suggests that the S1 catalase gene inserted into pSCAT02 was expressed under the control of the cloned gene promoter, independently from the *lac* promoter. Unexpectedly, an upper weak band in lane 3 of Fig. 2 was observed even though pSCAT02 has a catalase gene, thereby warranting further study for clarification.

The restriction digestion of pSCAT02 with several restriction enzymes produced several fragments, such as EcoRI-BamHI (2.3 kb), BamHI-EcoRI (5.1 kb), EcoRI-HindIII (6.3 kb), HindIII-EcoRI (1.1 kb), NotI-EcoRI (2.5 kb), and BamHI-NotI (2.4 kb). These DNA fragments were subcloned into a pBluescrip SK(+) vector, and the recombinant plasmids transformed into *E. coli* UM255. The transformants were then used for a catalase activity test using 3% H_2O_2. The subclone harboring the 2.5 kb NotI-EcoRI DNA fragment, designated as pSCAT025, was confirmed to include the catalase gene, based on bubble formation (Fig. 3), and thus used for a sequence analysis.

Analysis of Catalase Gene Sequences

The determination of the nucleotide sequence of the pSCAT025 catalase gene revealed that the insert DNA consisted of three open reading frames (ORF1, catalase, and ORF3 in that order), and was located 326 bp upstream in the opposite direction of ORF1. The catalase gene consisted of 1,455 bp and 484 amino acids, including the initiation codon and stop codon. The ribosomal binding site (GGAGA) was located 6 bp upstream of the initiation codon (ATG). The molecular mass of the catalase gene deduced from the amino acid sequences was 54,316 Da, and the isoelectric point was 6.468, which are characteristic of monofunctional catalases from bacteria and eukaryotic organisms. Bacterial monofunctional catalases are divided into small and large monofunctional catalases that are approx. 55–69 kDa and 79–84 kDa in size, respectively. In addition to an active site, catalase also has an NADPH and water molecule (H_2O) binding site, which is capable of dividing a monofunctional catalase into a small monofunctional

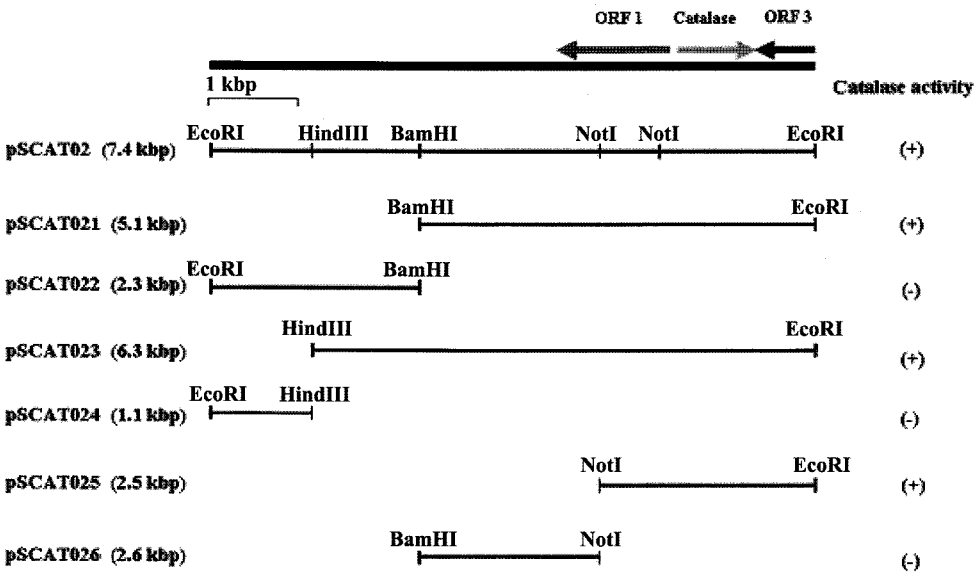

Fig. 3. Restriction enzyme map for the catalase gene cluster from *Rhodospirillum rubrum* S1.
Catalase activity was determined by a bubble test using hydrogen peroxide.

catalase (typical catalase) and large monofunctional catalase (atypical catalase) [32]. Among the amino acid residues of the bovine liver catalase site to which NADPH and water molecule bind, His304 includes a binding site for the pyrophosphate group of NADPH. Moreover, His214, Lys236, and Try214, which are all binding sites for water molecules, are dominantly conserved in typical catalase [32]. An analysis of the amino acids of the catalase revealed that the active sites (H53, S92, N126), binding sites of the distal region of heme (V94, T116, F131), proximal site of heme (Y336, R343), and NADPH binding site (His263, His193, Lys195, and Try173) were all well conserved, confirming that the cloned catalase from *R. rubrum* S1 was a small monofunctional catalase.

A comparative analysis of the amino acids revealed that the cloned catalase shared a 100% similarity with a catalase of *Rhodospirillum rubrum* ATCC 11170 (YP_427576), 78% with Kat A of *Rhodobacter sphaeroides*, 75% with KatA of *Sinorhizobium meliloti*, 74% with KatE of *Brucella melitensis*, 63% with Kat A of *Pseudomonas putida*, 62% with KatA of *Neisseria meningitidis* MC58 and KatE of *Haemophilus influenzae* KW20, 62% with catalase KatE of *Desulfovibrio vulgaris*, and 61% with KatE of *Proteus mirabilis*.

Purification and Characterization of Monofunctional Typical Catalase Gene from *R. rubrum* S1 in *E. coli* UM255

The purification and characterization of the *R. rubrum* S1 catalase overexpressed in *E. coli* through several protein purification steps showed that the specific activity of the purified enzyme was 13,273 U/mg, whereas that of the cell crude extract was 146 U/mg. The enzyme purification yield and fold were 4.2% and 91, respectively (data not

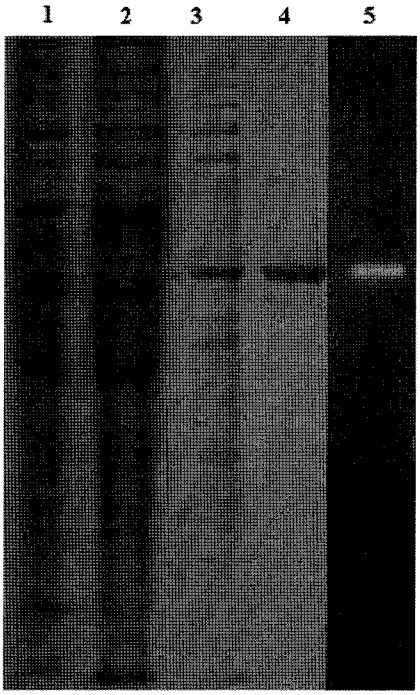

Fig. 4. Nondenatureing polyacryamide gel electrophoresis of *Rhodospirillum rubrum* S1 catalase purified from *Escherichia coli* UM255 by overexpression of the catalase gene in recombinant plasmid pSCAT025.
Lanes 1–4 show total peptide profiles stained with Coomassie Brilliant Blue R-250 at each catalase purification step. Lane 1, crude extract; 2, 30%–70% ammonium sulfate precipitate; 3, phenyl-sepharose fraction; 4, prep-cell electrophoration fraction; 5, catalase band observed after activity staining.

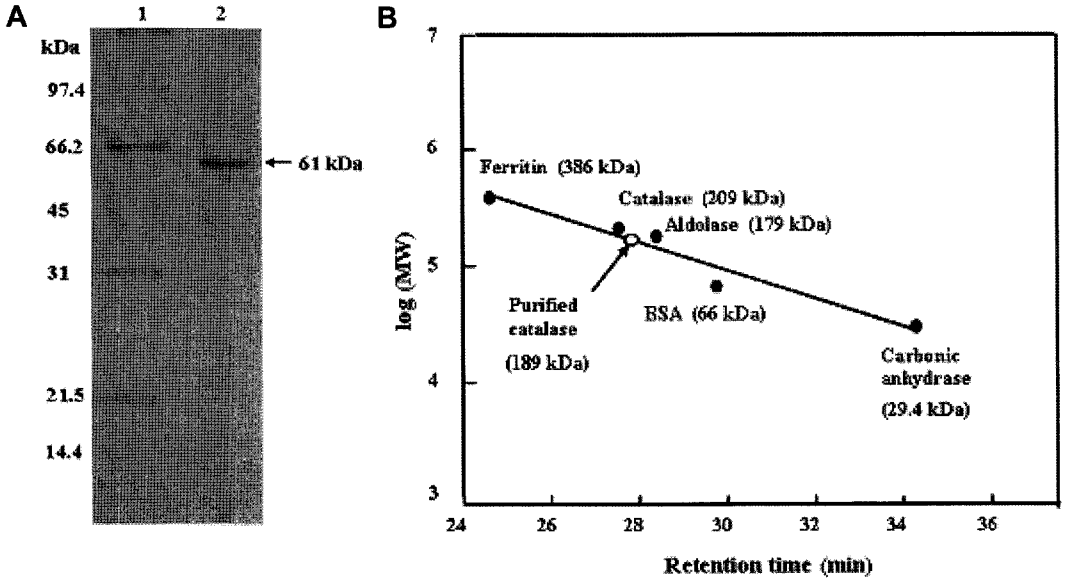

Fig. 5. Determination of molecular mass of *Rhodospirillum rubrum* S1 catalase purified from *Escherichia coli* UM255.
A. Denaturing SDS-PAGE of *R. rubrum* S1 catalase purified from *E. coli* UM255. The protein was stained with Coomassie Brilliant Blue R-250. Lane 1, protein marker consisting of phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa); 2, purified enzyme. **B.** Molecular mass of native *R. rubrum* S1 catalase purified by gel filtration Superose 12HR.

shown). Nondenaturing PAGE of the purified enzyme revealed exactly one single band, and the catalase activity of the purified protein was clearly revealed by activity staining (Fig. 4). However, the purified protein did not exhibit any peroxidase activity. A 12% SDS-polyacrylamide gel analysis revealed that the molecular mass of the subunit of the purified enzyme was approx 61 kDa (Fig. 5A). However, the molecular mass of the subunit size in the SDS-PAGE was not consistent with the 54 kDa deduced from the 484 amino acid sequences of the *R. rubrum* catalase-4 gene product, which may have been because proteins with hemes have a different mobility in SDS-PAGE [22]. The molecular mass of the native catalase determined by Superose 12HR gel filtration chromatography was 189 kDa (Fig. 5B), revealing that the catalase cloned from *R. rubrum* S1 was a typical monofunctional enzyme consisting of four identical subunits.

Biochemical Characterization of Catalase Purified in *E. coli* UM255

The catalase activity was assayed at various temperatures and pHs using the purified catalase, designated as catalase-4. The catalase purified from *R. rubrum* S1 exhibited enzyme activity over a broad pH range, from pH 5.0 to 11.0, with the maximum activity at pH 7.5. The enzyme showed approximately 50% activity at pH 5.0, and there was no activity below pH 4.0 (Fig. 6A). As regards the effect of temperature on the catalase activity, the maximal activity was at 30°C. The enzyme activity was maintained within a range of 20°C–55°C, yet reduced to 45% at 60°C

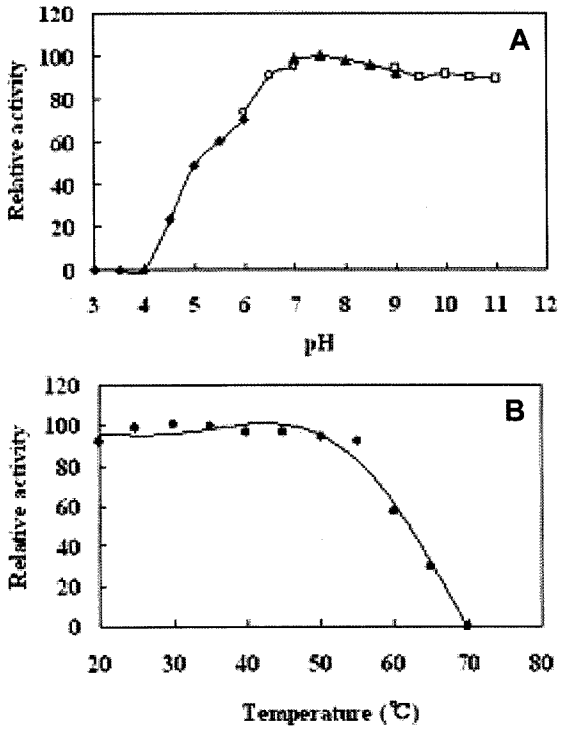

Fig. 6. Effect of pH and temperature on activity of *Rhodospirillum rubrum* S1 catalase purified from *E. coli* UM255.
A. The purified catalase was incubated for 10 min at 25°C prior to incubating the reaction. Different buffer systems were used according to the pH range: 50 mM citrate-phosphate buffer for pH 3.0–6.0, 50 mM potassium phosphate buffer for pH 6.0–7.0, 50 mM Tris-HCl buffer for pH 7.0–9.0, and 50 mM carbonate buffer for 9.0–11.0. **B.** The purified catalase was incubated for 10 min at the indicated temperatures prior to initiating the reaction.

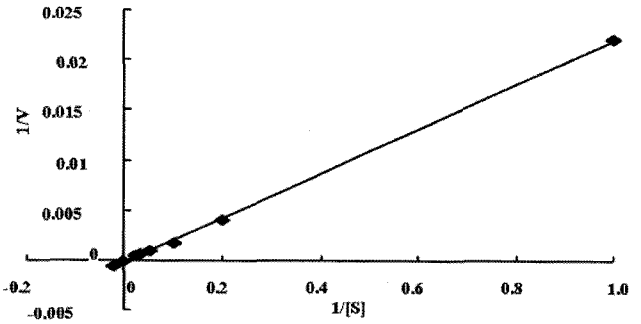

Fig. 7. Lineweaver-Burk plot of reaction velocity of *Rhodospirillum rubrum* S1 catalase purified from *Escherichia coli* UM255.
The enzyme assay was performed at various concentrations of hydrogen peroxide under standard assay conditions, as described in Materials and Methods.

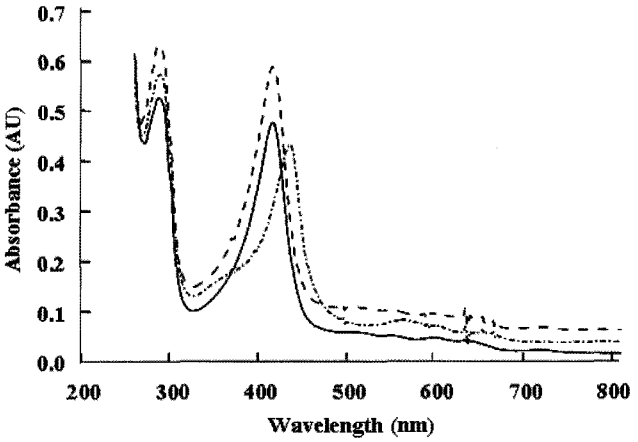

Fig. 8. Absorption spectra of *Rhodospirillum rubrum* S1 catalase purified from *E. coli* UM255.
The enzyme was dissolved in a 10 mM potassium phosphate buffer (pH 7.0). The reduced form (—) was obtained by the addition of 1 mM sodium dithionite to the native form (...), whereas the cyano-adduct (-·-) was obtained by the addition of 30 mM KCN to the native enzyme.

(Fig. 6B). These results reflected the same properties found for monofunctional catalase enzymes from other bacteria.

Based on a Lineweaver-Burk plot, the K_m value and V_{max} of the catalase for H_2O_2 were 21.8 mM and 39,960 U/mg, respectively (Fig. 7). Since the K_m value of *R. rubrum* catalase-4 was lower than that of *R. rubrum* catalase-2 at 30.4 mM [10], this suggests that catalase-4 may be more efficient in scavenging hydrogen peroxide than *R. rubrum* S1 catalase-2, the other monofunctional catalase. As such, catalase-4 may function to remove hydrogen peroxide generated during aerobic respiration metabolism. Generally, the K_m values of monofunctional catalases are known to range from 40 mM to 80 mM, whereas the K_m value of catalase-peroxidases are within a range of 1–10 mM, which is relatively lower [4, 7, 32]. When comparing the K_m values from other bacteria, *Klebsiella pneumoniae* has a typical catalase, KpT, and catalase-peroxidase, KpCP, where the K_m values for H_2O_2 are 50 mM and 6.5 mM, respectively [6]. *B. subtilis* has catalase-1 and catalase-2, both of which are typical catalases, where the K_m values for H_2O_2 are 40.1 mM and 70 mM, respectively [31]. Thus, *R. rubrum* S1 catalase-4 exhibited a lower K_m value than the monofunctional catalases from other bacteria, suggesting that the catalase enzyme purified from *E. coli* harboring the recombinant plasmid pSCAT025 was a typical catalase.

Treatment with inhibitors of catalase, such as NH_2OH, NaCN, and NaN_3, revealed that the catalase activity was reduced to 50% in the presence of 4.4 μM NH_2OH, 8.8 μM NaCN, and 0.4 μM NaN_3 (Table 1). It is also notable that the typical inhibitor, 3-amino-1,2,4-triazole (AT), only reduced the catalase activity to 26%, even in a concentration of 10 mM (data not shown). Therefore, the inhibition effect of AT needs to be determined through further study in concentrations higher than 10 mM. Catalase-4 showed a similar sensitivity as catalase-2 towards cyanide and azide, yet was less sensitive to 3-amino-1,2,4-triazole (AT) and hydroxylamine (Table 1). The addition of an organic solvent to the enzyme solution for 10 min at room temperature resulted in a reduction of the catalase activity

Table 1. Comparison of enzymatic properties of catalase-2 and catalase-4 found in *R. rubrum* S1.

Properties	Catalase-2*	Catalase-4
Molecular mass (kDa)	318 (79 kDa)	189 (61 kDa)
No. of subunits	4	4
pH range	5.0–9.0	6.0–11.0
Temperature range	20–60	20–60
K_m of H_2O_2 (mM)	30.4	21.8
Concentration for 50% inhibition		
NaCN (μM)	11.5	8.8
NaN_3 (μM)	0.52	0.4
NH_2OH (μM)	0.11	4.4
Peroxidase activity	None	None

*Data are from [10].

to 20%, indicating that the catalase clone from *R. rubrum* S1 was a typical monofunctional catalase.

Spectroscopic analysis indicated that the purified catalase had the maximal optical density at 406 nm, suggesting the presence of a ferric heme prosthetic group (Fig. 8). The ratio of A_{406} to A_{280} for the catalase was 0.97, which is similar to the values for most typical tetramer catalases. The native enzyme had a Soret band at 406 nm, which shifted to 427 nm when 10 mM cyanide was added, plus the prosthetic group was not reducible by 1 mM dithionite, both of which are characteristic properties of monofunctional catalases [13, 32]. In conclusion, the genetic and biochemical characterization of the cloned gene from the photosynthetic bacterium *R. rubrum* S1 revealed that the catalase clone was a typical monofunctional catalase.

To clarify the detailed defensive mechanism of strain S1 depending on the presence or absence of oxygen, the other catalases that have already been found in *R. rubrum* S1, although not yet elucidated, need to be studied under various environmental conditions.

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