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Purification and Characterization of a Catalase from Photosynthetic Bacterium *Rhodospirillum rubrum* S1 Grown under Anaerobic Conditions

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The photosynthetic bacterium, *Rhodospirillum rubrum* S1, when grown under anaerobic conditions, generated three different types of catalases. In this study, we purified and characterized the highest molecular weight catalase from the three catalases. The total specific catalase activity of the crude cell extracts was 88 U/mg. After the completion of the final purification step, the specific activity of the purified catalase was 1,256 U/mg. The purified catalase evidenced an estimated molecular mass of 318 kDa, consisting of four identical subunits, each of 79 kDa. The purified enzyme exhibited an apparent Km value of 30.4 mM and a *Vmax* of 2,564 U against hydrogen peroxide. The enzyme also exhibited a broad optimal pH (5.0~9.0), and remained stable over a broad temperature range (20°C~60°C). It maintained 90% activity against organic solvents (ethanol/chloroform) known hydroperoxidase inhibitors, and exhibited no detectable peroxidase activity. The catalase activity of the purified enzyme was reduced to 19% of full activity as the result of the administration of 10 mM 3-amino-1,2,4-triazole, a heme-containing catalase inhibitor. Sodium cyanide, sodium azide, and hydroxylamine, all of which are known heme protein inhibitors, inhibited catalase activity by 50% at concentrations of 11.5 μM, 0.52 μM, and 0.11 μM, respectively. In accordance with these findings, the enzyme was identified as a type of monofunctional catalase.

***Keywords*:** catalase, photosynthetic bacterium, *Rhodospirillum rubrum* S1

Oxygen is a crucial element for the survival of all aerobic microorganisms, and also to facultative anaerobes. However, during cellular metabolic processes in which aerobes engage, the cells inevitably produce reactive oxygen species (ROS), including superoxide, hydroxyl, and perhydroxyl radicals, which have been implicated in the chemical modification of most cellular constituents, such as nucleic acids, proteins, lipids. Such chemical modifications of cellular constituents induce a variety of deleterious effects, including certain metabolic defects, aging mutagenesis, and even cell death (Gutteridge and Holliwell, 2000). Therefore, most ROS are considered to be highly toxic to biological systems, and must be removed before accumulating to harmful levels (Levine *et al*., 1996).

ROS can be effectively removed via the activity of several innate enzymes, including catalase, peroxidase, and superoxide dismutase (SOD), as well as small molecules, such as glutathione (Almiron *et al*., 1992). Among these, catalase is considered to be a representative constituent of a defense system against certain types of ROS. As expected, the majority of catalases have been detected in aerobic organisms, but some catalases have also been located in facultative anaerobes, and even in a few strict anaerobes (Hicks, 1995; Hochman and Goldberg, 1991; Rocha and Smith, 1995; 1997). Two major classes of bacterial catalases have thus far been characterized (Almiron *et al*., 1992)– monofunctional catalase (HP II), and bifunctional catalase-peroxidase (HP I), which, thus far, has been detected only in bacteria (Hochman and Goldberg, 1991; Miller *et al*., 1997). However, little information is currently available regarding catalases harbored by anaerobic organisms (Loewen, 1992). In anaerobic organisms, the generation of reactive oxygen species is normally not considered to be a problem, due to the absence of oxygen in these species. The lack of protective mechanisms against oxygen toxicity in anaerobic bacteria has traditionally been regarded as

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the primary explanation for their sensitivity to oxygen exposure (Morris, 1980). A host of studies have demonstrated that the anaerobic bacteria are not uniformly sensitive to oxygen, however, and a broad range of oxygen tolerance has been demonstrated from species to species (Morris, 1980; Finegold and George, 1989). It is believed that in some anaerobic bacteria, just as in aerobic organisms, superoxide dismutase and catalase play roles in the detoxification of oxygen by-products (Morris, 1980).

Rhodospirillum rubrum, a nonsulfur anoxygenic photosynthetic bacterium, requires oxygen for its aerobic growth via respiration. However, by way of contrast, this organism also requires strict anaerobic conditions for growth via photosynthesis or fermentation (Uffen and Wolfe, 1970). Therefore, *R. rubrum,* owing to its capabilities for both aerobic and anaerobic growth, is considered a very useful microorganism for studies regarding catalase expression patterns occurring in response to oxidative stresses.

In our previous study (Lim *et al*., 2001), we determined that *Rhodospirillum rubrum* S1 generates 5 distinct catalases, which were designated Cat1, Cat2, Cat3, Cat4, and Cat5, in order of molecular weight, under aerobic conditions. Three of these 5 catalases (Cat 2,3,4) were also generated under anaerobic conditions. We discovered a few interesting things. First, the expression of one catalase, Cat2, remained constant, regardless of the carbon sources used (data not shown). Second, the quantitative expression of Cat2 under anaerobic culture conditions was far in excess of what was observed under aerobic conditions. In accordance with these results, we assumed that the Cat2 of *R. rubrum* S1 may perform an important role with regard to the survival and proliferation of cells, functioning as a house-keeping enzyme against various deleterious environments, such as accidental exposure to oxygen, particularly under anaerobic conditions. In this study, we have, for the first time, purified and characterized a catalase, Cat2, from *R. rubrum* S1 grown under anaerobic conditions.

Materials and Methods

Bacterial strain and culture conditions

Rhodospirillum rubrum S1 (ATCC 11170) was used in this study. Cells, anaerobically pre-grown to logarithmic phase on basal medium supplemented with 0.3% malate as a sole carbon source (Bose *et al*., 1962) under a 2,000 Lux light at 30°C, were transferred to 2.5 L transparent culture bottle, and cultured under conditions identical to that of the preculture.

Preparation of crude enzyme extract

Cells were harvested via centrifugation at the logarithmic growth phase, washed three times in 50 mM potassium phosphate buffer (pH 7.2) containing 1 mM EDTA, then resuspended in the same buffer. The following procedures were conducted at 4°C. The cells were disrupted using a sonicator (Bandelin Sonoplus HD 2070, Germany), then centrifuged for 1 hour at 16,000×g. The final supernatant was then used as crude enzyme extract.

Activity assay

Catalase activity was evaluated spectrophotometrically by monitoring the decrease at A_{240} resultant 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 $M^{-1}cm^{-1}$. The standard reaction mixture for the assay contained 50 mM potassium phosphate buffer (pH 7.2), 20 mM H_2O_2, and 20 μL of catalase solution, in a total volume of 3.0 mL. The reaction was conducted at 25°C. The amount of enzyme activity required to decompose 1 μmole of H_2O_2 per min was defined as 1 unit (U) of activity (Beers and Sizer, 1952). Protein concentrations were determined via a micro-BCA protein assay (Smith *et al*., 1985) using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis (PAGE)

Non-denaturing PAGE and denaturing sodium dodecyl sulfate (SDS)-PAGE were conducted according to the method developed by Laemmli (1970). After electrophoresis, the proteins were detected via the staining of the gel with Coomassie's brilliant blue. Catalase activity on the non-denaturing polyacrylamide gels was visualized via ferric chloride-potassium ferric cyanide double staining (Wayne and Diaz, 1986). Using this staining method, 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.

Purification procedure

The ammonium sulfate precipitation of the crude *Rhodospirillum rubrum* S1 cell extracts was conducted in a saturation range of 30~70%. The precipitate was then collected via centrifugation (16,000 × g for 1h) and resuspended in 50 mM potassium phosphate buffer (pH 7.2). The resuspended solution was then dialyzed overnight, against the same buffer. The following procedures were conducted at 4°C. The dialyzate was loaded onto a DEAE-cellulose anion exchange column which had been equilibrated with the same buffer. The adsorbed protein was eluted with a 400 mL linear 0～1 M NaCl gradient. Fractions exhibiting catalase activity were pooled and concentrated using 70% ammonium sulfate. The concentrated

samples were then dialyzed for 12 hours with 50 mM potassium phosphate buffer. After dialysis, the dialyzates were loaded onto a Sephadex G-200 gel filtration column, which had been equilibrated with 50 mM potassium phosphate buffer. The concentrated sample was then purified further via 7.5% non-denaturing polyacrylamide gel electrophoresis (PAGE), followed by electroelution at 40 mA of constant current with a Prep-Cell 491 (Bio-Rad Laboratories, USA).

Molecular weight determination

The molecular weight of the native enzyme was determined via gel filtration using a Superose-12HR 10/30 column (Amersham Pharmacia Co.), equilibrated with 50 mM potassium phosphate buffer (pH 7.4). The following proteins were used as molecular weight standards: ferritin (386 kDa), catalase (209 kDa), aldolase (179 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29.4 kDa). The molecular weights of the subunits were then determined via sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% acrylamide gel, in accordance with Laemmli's method (1970). Bio-Rad low range markers were used as molecular weight standards for SDS-PAGE (Bio-Rad, USA).

Effects of pH, temperature, and inhibitors

In order to evaluate the effects of pH, the purified catalase was incubated for 10 min at 30°C, prior to the initiation of the reaction. Catalase activity was measured in a pH range of 3.0~11.0. Different buffer systems were employed in accordance with respective pH ranges: 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 (Terzenbach and Blaut, 1998). To characterize the effects of temperature, standard reaction mixtures were assayed for catalase activity at different temperatures, in a range of 20°C~80°C (Lee and Lee, 1995).

For the heme inhibitor test, the enzyme solution was mixed with different concentrations of metal inhibitors, including sodium cyanide (0~20 μM), sodium azide (0~0.8 μM) and hydroxylamine (0~0.8 μM). After 2 minutes of incubation of the reaction mixtures at 25°C, the enzyme activity was determined. In order to evaluate the effects of the organic inhibitors on catalase activity, the mixtures of enzyme solution and organic solvents (enzyme solution : ethanol : chloroform = 10 : 5 : 3) were vortexed for 10 min at room temperature, followed by measurements of activity. The same was done with enzyme solution, incubated with 10 mM 3-amino-1,2,4-triazole, which inhibits catalase activity via 10 minutes of covalent binding to the heme-containing active center (Lim *et al*., 2001; Ueda *et al*., 2003).

Results and Discussion

Purification of catalase

In a previous study (Lim *et al*., 2001), we reported that the photosynthetic bacterium, *Rhodospirillum rubrum* S1, harbors three catalases, which are expressed under both aerobic and anaerobic culture conditions. The highest molecular weight catalase (Cat2) among these three catalases was expressed more prominently in anaerobic cultures than in aerobic cultures. Thus, we purified the Cat2 catalase from anaerobically grown cells, and conducted an investigation into its characteristics.

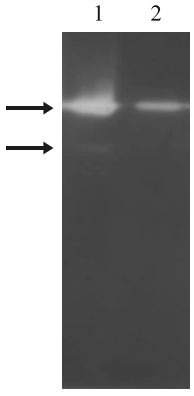

Fig. 1. Activity staining on 7% non-denaturing PAGE of catalases from *Rhodospirillum rubrum* S1 grown under anaerobic conditions. Arrows indicate catalase bands. Lane 1, crude cell extracts. The third catalase is not shown, probably due to very low levels of expression. Lane 2, purified catalase (Cat 2).

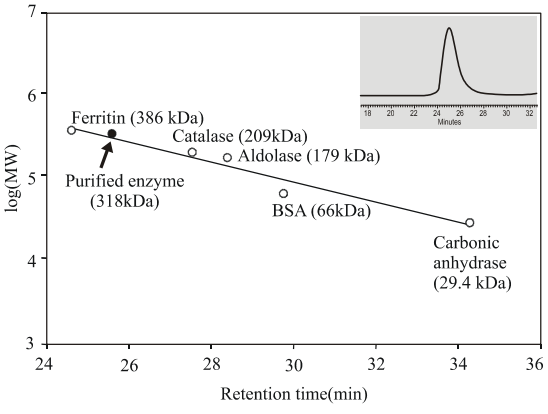

Fig. 2. Determination of molecular weight of native catalase isolated from *Rhodospirillum rubrum* S1, via gel filtration HPLC. ○, Mr standards; ferritin (386 kDa), catalase (209 kDa), aldolase (179 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29.4 kDa), ●, purified catalase enzyme.

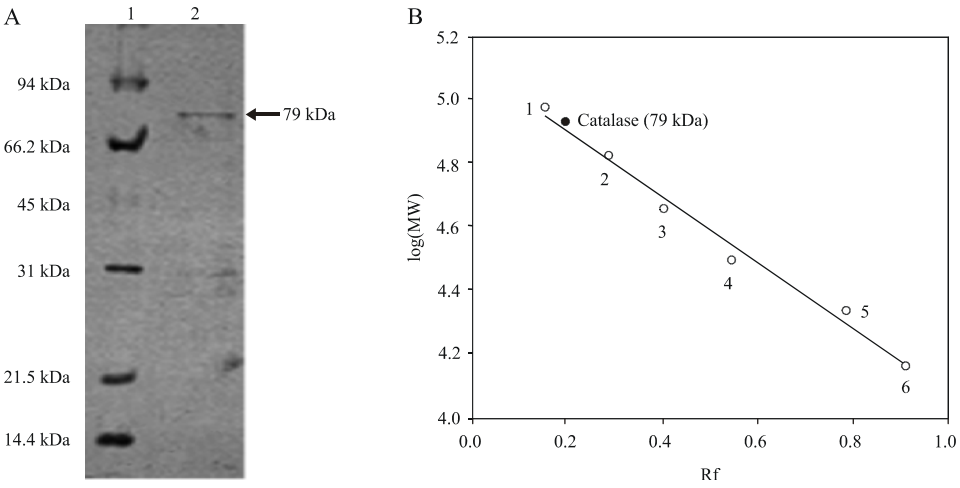

Fig. 3. SDS-polyacrylamide gel electrophoresis (A) and molecular weight determination of subunit (B) of the purified catalase from *R. rubrum* S1. A, 12% SDS-PAGE of purified catalase. The gel was stained with silver. Lane 1, 1. phosphorylase b (94 kDa), 2. bovine serum albumin (66.2 kDa), 3. ovalbumin (45 kDa), 4. carbonic anhydrase (31 kDa), 5. soybean trypsin inhibitor (21.5 kDa), 6. lysozyme (14.4 kDa). Lane 2, purified catalase (arrow). B, Plot of log molecular weight as a function of relative mobility. ○, Mr standards; ●, catalase subunit.

The highest molecular weight catalase (Cat 2) was purified via several purification steps, as described above. The specific activity of catalase in the crude cell extracts of the anaerobically-grown *R. rubrum* S1 was 88 U/mg. After the final purification step, this value reached 1,256 U/mg. Non-denaturing PAGE of the purified enzyme revealed exactly one single band, and the catalase activity of the purified protein was clearly revealed by the activity staining (Fig. 1). The purified enzyme migrated as a single band during electrophoresis under denaturing conditions, indicating that the single pure protein had been isolated after the final purification step (Fig. 3-A).

Molecular weight determination of purified catalase

The molecular weight of the native enzyme was estimated as 318 kDa, according to the results of gel filtration using Superose-12HR (Fig. 2). The subunit molecular weight was estimated, via SDS-PAGE, to be 79 kDa , which suggests that the purified catalase was composed of four identical subunits (Fig. 2., Fig. 3-A,B). The M.W of the catalase of *R. rubrum* S1 is not unusual, considering that the M.W. of most bacteria are found in a range of approximately 200 kDa to 300 kDa, although some exceptions exist (Lim *et al.,* 2001). This is similar to other large monofunctional catalases, such as *E. coli* HPII with 84.3 kDa and 337 kDa subunits, and *Neurospora crassa* Cat-1, with 80 kDa and 320 kDa subunits (Loewen *et al.,* 2000; Michán *et al.,* 2002).

Enzyme Kinetics

A Lineweaver-Burk plot of the purified catalase from the anaerobically-grown *R. rubrum* S1 revealed a Km value of approximately 30.4 mM, and a *Vmax* for hydrogen peroxide of 2,564 U/mg (Fig. 4). This is a lower value than is typically observed for other catalases. For example, the Km values of *Brucella abortus*, *Listeria seeligeri*, and *Pseudomonas aeruginosa* were 84 mM, 49 mM, and 38 mM (Switala and Loewen, 2002), respectively. This result, then, indicates that catalase purified from anaerobically grown *R. rubrum* S1 is more efficient with regard to the scavenging of hydrogen peroxide than are typical catalases isolated from other bacteria.

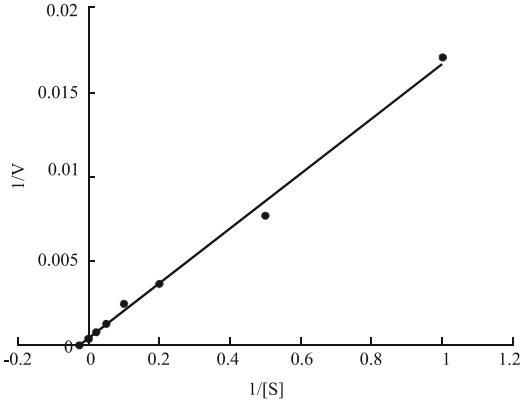

Fig. 4. Lineweaver-Burk plot of the reaction velocity of a catalase purified from anaerobically grown *Rhodospirillum rubrum* S1. The enzyme assays were conducted at various hydrogen peroxide concentrations under standard assay conditions, as described in the Materials and Methods section.

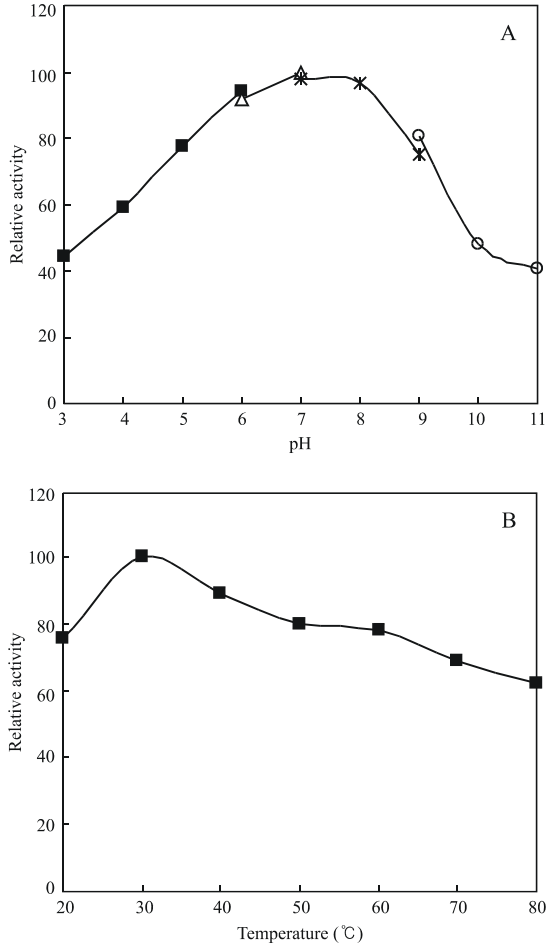

Fig. 5. Effects of pH and temperature on the activity of a catalase purified from *R. rubrum* S1. (A) Purified catalase was incubated for 10 minutes 30°C prior to the initiation of the reaction. Different buffer systems were utilized according to the pH ranges; 50 mM citrate-phosphate buffer for pH 3.0-6.0, 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 9.0-10.0. (B) The purified catalase was incubated for 10 minutes at the indicated temperatures, prior to the initiation of the reaction.

Effects of pH and temperature on catalase activity

The catalase purified from the anaerobically-grown *R. rubrum* S1 evidenced a broad optimum pH range, from 5.0 to 9.0. Optimal activity was observed at neutral pH, a finding which is consistent with other reports (Nader *et al*., 1986). The enzyme evidenced an activity of approximately 40% at pH values below 3.0 or in excess of 11.0 (Fig. 5-A). With regard to the effects of temperature on catalase activity, like most monofunctional catalases, catalase activity did not appear to be greatly influenced by temperature (Switala and Loewen, 2002). The optimum temperature for enzymatic activity was found to be approximately 30°C (Fig. 5-B). The enzyme evidenced a relative activity of approximately 70% in a temperature range spanning up to 60°C (from 20°C to 80°C).

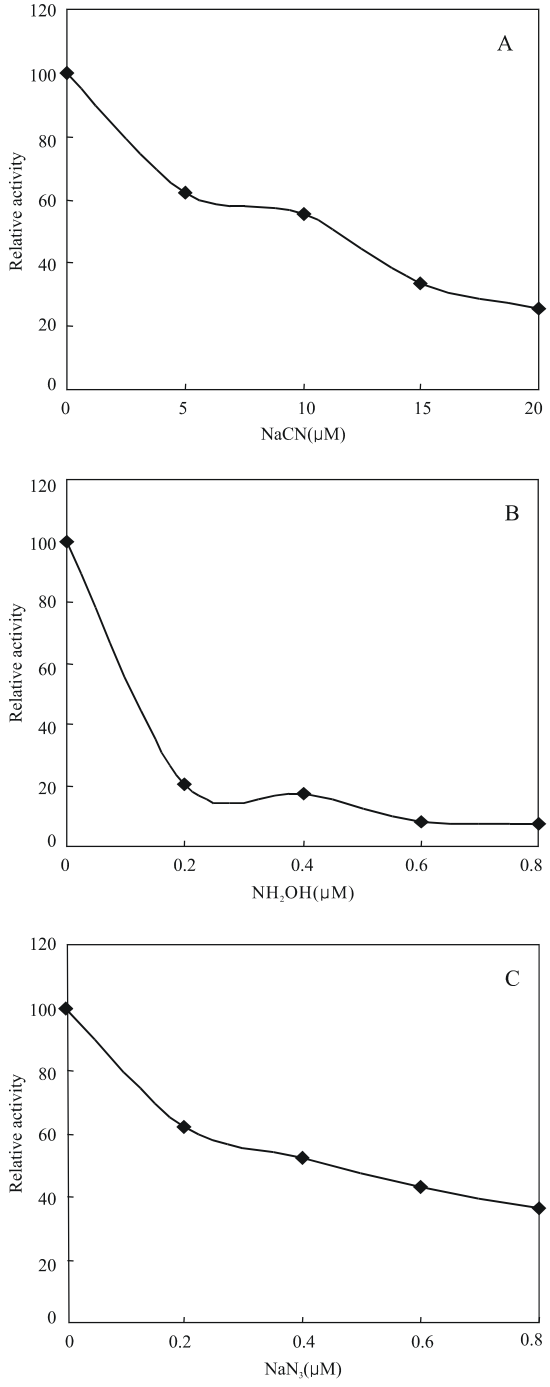

Fig. 6. Effects of heme protein inhibitors on the activity of the catalase purified from *Rhodospirillum rubrum* S1. (A) Effect of NaCN, (B) Effect of NH_2OH, (C) Effect of NaN_3.

Effects of metal and chemical inhibitors on the catalase activity of purified enzyme

Sodium azide, sodium cyanide, and hydroxylamine are well-established heme inhibitors of catalase (Shonbaum and Chance, 1976). We conducted an investigation

into the effects of heme inhibitors on the catalase activity of the purified enzyme (Fig. 6). At a concentration of 11.5 μM, sodium cyanide was shown to inhibit the activity of *R. rubrum* S1 catalase, by 50%. Sodium azide and hydroxylamine inhibited it by 50% at concentrations of 0.52 μM and 0.11 μM, respectively. The results are similar to those of a typical yeast catalase (Yumoto *et al*., 1990), suggesting that purified catalase harbors a heme prosthetic group. By way of contrast, these values are far lower than those measured for catalase-peroxidase (Hochman and Shemesh, 1987).

With regard to the effects of organic solvents, after the enzyme solution had been incubated for 10 minutes with organic solvent at 25°C, only 10% of catalase activity had been lost. This demonstrates the marked resistance of *R. rubrum* S1 catalase to organic solvents (Table 1). Conversely, the catalase activity of the purified enzyme was inhibited by 81%, after a similar 10-minute incubation with 10 mM 3-amino-1,2,4-triazole (Table 1). According to these results, the purified enzyme appears rather insensitive to organic solvents (ethanol/chloroform), but relatively sensitive to 3-amino-1,2,4-triazole. This result is consistent with results gleaned from similar experiments using typical heme-containing catalases (Switala and Loewen, 2002; Ueda *et al*., 2003).

In conclusion, the results obtained from this study collectively demonstrate that *Rhodospirillium rubrum* S1 harbors a typical catalase that is responsible for the scavenging of hydrogen peroxide. This catalase may also be responsible for other important functions relevant to the survival of the organism under anaerobic photosynthetic conditions, although these functions remain a matter of supposition at the present time. Our findings suggest that the expression of the *R. rubrum* catalase gene can be regulated by environmental conditions, including oxygen or light. Further studies of catalase and its molecular mechanisms will augment our current understanding of the anaerobic mechanisms underlying responses to oxidative stress, as well as the regulation of antioxidant enzymes, in the photosynthetic bacterium, *R. rubrum* S1.

Table 1. Effect of organic inhibitors on the activity of catalase purified from *Rhodospirillum rubrum* S1

Inhibitors	Relative catalase activity(%)
Control	100
Organic solvent[a]	89.4
3-amino-1,2,4-triazole[b]	18.6

a : The enzyme solution was mixed with organic solvents to give a final composition, enzyme solution: ethanol: chloroform = 10: 5: 3, and then vortexed for 10 min at room temperature.

b : The enzyme solution was incubated in 10 mM 3-amino-1, 2,4-triazole for 10 min.

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