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# Gene cloning and biochemical characterization of a catalase from *Gluconobacter oxydans*

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***Gluconobacter oxydans*** **has a large number of membrane-bound dehydrogenases linked to the respiratory chain that catalyze incomplete oxidation of a wide range of organic compounds by oxidative fermentation. Because the respiratory chain is a primary site of reactive oxygen species (ROS) production, the bacterium is expected to have a high capacity to detoxify nascent ROS. In the present study, a gene that encodes a catalase of *G. oxydans*, which might act as a potential scavenger of $H_2O_2$, was cloned, and the expression product (termed rGoxCat) was characterized biochemically. rGoxCat is a heme *b*-containing tetrameric protein (molecular mass, 320 kDa) consisting of identical subunits. The recombinant enzyme displayed a strong catalase activity with a $k_{cat}$ of $6.28\times10^4$ $s^{-1}$ and a $K_m$ for $H_2O_2$ of 61 mM; however, rGoxCat exhibited no peroxidase activity. These results, along with the phylogenetic position of the enzyme, provide conclusive evidence that rGoxCat is a monofunctional, large-subunit catalase. The enzyme was most stable in the pH range of 4–9, and greater than 60% of the original activity was retained after treatment at pH 3.0 and 40°C for 1 h. Moreover, the enzyme exhibited excellent thermostability for a catalase from a mesophilic organism, retaining full activity after incubation for 30 min at 70°C. The observed catalytic properties of rGoxCat, as well as its stability in a slightly acidic environment, are consistent with its role in the elimination of nascent $H_2O_2$ in a bacterium that produces a large amount of organic acid via oxidative fermentation.**



[**Keywords:** Reactive oxygen species; Gluconobacter oxydans; Catalase; Peroxidase; Monofunctional; Hydrogen peroxide]

*Gluconobacter oxydans* is a gram-negative, obligately aerobic, rod-shaped acidophilic bacterium that belongs to the family Acetobacteriaceae (1). This bacterium is capable of catalyzing incomplete oxidation of a wide range of organic compounds, such as carbohydrates and alcohols, in a process that is referred to as oxidative fermentation (1,2). Because the oxidative fermentation of *G. oxydans* is generally regio- and stereo-selective, it is used in the production of industrially important compounds, such as L-sorbose, 6-amino-L-sorbose, D-gluconic acid, ketogluconic acids, and dihydroxyacetone.

A variety of membrane-bound dehydrogenases are involved in the oxidative fermentation that produces these useful compounds (1,2). Many of these enzymes, such as glucose-, fructose-, alcohol-, aldehyde-, sorbitol-, and glycerol-dehydrogenases, are pyrroloquinolinquinone-dependent enzymes, and some of them also possess heme *c*-containing subunits. These dehydrogenases are linked to the respiratory chain, which transfers electrons via ubiquinone to a $bo_3$-type quinol oxidase. Thus, the reducing equivalents that are derived from the oxidative processes are ultimately transferred to oxygen (1,2).

The electron transport chain is a primary site of reactive oxygen species (ROS) generation. Such ROS include superoxide anion radicals ($O_2\cdot^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals ($OH\cdot$) (3). The electron transport chain occasionally leaks free radicals in the form of semiquinone radicals, and the nonspecific interaction of these radicals with molecular oxygen results in the formation of $O_2\cdot^-$, which is a strong free-radical initiator. Because ROS are generally cytotoxic, cells have to protect themselves from damage by nascent ROS (4). In living systems, superoxide dismutase removes $O_2\cdot^-$, and produces molecular oxygen and $H_2O_2$. In turn, $H_2O_2$ serves as a source of $OH\cdot$. Catalase [EC 1.11.1.6] detoxifies $H_2O_2$ by decomposing it into molecular oxygen and water:

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

Because *G. oxydans* has many membrane-bound dehydrogenases that are linked to the respiratory chain, this bacterium is expected to have a high capacity for detoxification of nascent ROS. For example, crude extracts of *G. oxydans* cells exhibit a high catalase activity. However, the molecular properties of the enzymes that are involved in the removal of ROS, including catalase, have not been described in detail.

In the present study, we searched the genome sequence of *G. oxydans* (1) for genes that encode catalases, which are potential scavengers of $H_2O_2$ in *G. oxydans* cells. The *G. oxydans* genome contained one putative catalase gene, *gox1138*. We heterologously expressed *gox1138* as a catalytically active protein (termed rGoxCat) in *Escherichia coli* cells. Herein, we report on the cloning, expression, and biochemical characterization of rGoxCat.

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## MATERIALS AND METHODS

**Materials** The restriction enzymes NdeI and BamHI were purchased from Takara Bio, Shiga, Japan. The His-Trap HP was purchased from GE Healthcare Japan, Tokyo.

**Bacterial strains and plasmids** *G. oxydans* JCM7642 was obtained from the Japan Collection of Microorganisms, Wako, Saitama, Japan. Cells of the JCM7642 strain were grown at 30°C with shaking for 3 days in medium 1, which contained, per liter, 20 g of sodium gluconate, 3 g of D-glucose, 3 g of yeast extract, and 2 g of peptone. *E. coli* strains DH5α and BL21(DE3), and the pMD20-T and pET-15b plasmids were purchased from Takara Bio.

**Molecular cloning of the *gox1138* gene** The DNA fragment that encodes the full-length *gox1138* gene was amplified by PCR using the genomic DNA of *G. oxydans* JCM7642 as a template and the primers 5′-TACCATATGAGCACGGACAAACG-3′ and 5′-TACGGATCCTCAGACAGCG TGGGTTC-3′, in which the NdeI and BamHI sites, respectively, are underlined. The amplified fragment was ligated with NdeI/BamHI-digested pMD20-T. *E. coli* DH5α cells were transformed with the resulting plasmid, and the transformant cells were grown in an LB agar medium containing 50 μg/mL ampicillin, 100 μM isopropyl-β-D-thiogalactopyranoside, and 80 μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Positive colonies were selected by means of blue/white selection. The transformant cells were cultured in a 2× YT medium, which contained, per liter, 16 g of tryptone, 10 g of yeast extract, 5 g of NaCl, and 50 mg ampicillin. The recombinant pMD20-T that contained the *gox1138* gene was purified from the cultured cells by use of a GenElute Plasmid Miniprep Kit (Qiagen). The nucleotide sequence of the plasmid was confirmed using a CEQ2000XL DNA Analysis System (Beckman Coulter, Fullerton, CA, USA). The GENETYX program (ver. 5.0; Software Development, Tokyo) was used for the analysis of nucleotide and to deduce amino acid sequences.

**Construction of the expression vector and over-expression** The recombinant pMD20-T that contained the *gox1138* gene was digested with NdeI and BamHI, and the resulting 2-kbp fragment was ligated with the NdeI/BamHI-digested pET-15b. *E. coli* BL21(DE3) cells were transformed with the resulting plasmid (termed pET-gox1138). The transformant cells that contained pET-gox1138 were grown in an LB agar medium containing 50 μg/mL ampicillin. The culture (500 μL) was then used to inoculate 1 L of a ZYP-5052 medium in a 3-L culture flask. The ZYP-5052 medium contained, per liter, 10.0 g of NZ Amine Type A (Sheffield Chemical Co., San Ramon, CA, USA), 5.0 g of yeast extract, 25 mg of $MgSO_4 \cdot 7H_2O$, 3.3 g of $(NH_4)_2SO_4$, 6.8 g of $KH_2PO_4$, 7.1 g of $Na_2HPO_4$, 5.0 g of glycerol, 0.5 g of D-glucose, and 2.0 g of α-lactose. The transformant cells were grown at 20°C for 30 h with shaking. The cells were collected by centrifugation at 3000 × *g* for 10 min and were stored at −20°C until their use for enzyme purification.

**Enzyme assays** Method I—Catalase activity was spectrophotometrically assayed by monitoring the decrease in $H_2O_2$ at 25°C (5), as follows. The standard assay system consisted of 10 μmol of $H_2O_2$, 25 μmol of potassium phosphate buffer (pH 7.0), and enzyme (typically 0.38 μg) in a final volume of 500 μL. The assay mixture, without the enzyme, was pre-incubated at 25°C for 5 min. The reaction was initiated by addition of the enzyme, and changes in absorbance at 240 nm were recorded at 25°C for 1 min using a Hitachi double-beam model U-2000 spectrophotometer equipped with a temperature-controlled cell holder. The published ε value of $H_2O_2$ at 240 nm ($\varepsilon_{240}$, 43.6 $mM^{-1}\,cm^{-1}$) was used in the calculation (6). One unit of catalase activity was defined as the amount of enzyme that decomposed 1 μmol of $H_2O_2$ in 1 min.

Method II—Peroxidase activity was spectrophotometrically assayed by monitoring the oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) at 25°C, as follows (7). The standard assay system consisted of 0.625 μmol of *m*-chloroperoxybenzoic acid, 5 μmol of ABTS, 25 μmol of potassium phosphate buffer (pH 7.0), and 4 μg of enzyme for a final volume of 500 μL. The assay mixture, without the enzyme, was pre-incubated at 25°C for 5 min. The reaction was initiated by addition of the enzyme, and changes in absorbance at 660 nm were recorded at 25°C for 1 min.

Method III—rGoxCat-catalyzed oxidation of ethanol was examined with a reaction system, in which $H_2O_2$ was supplied in situ by means of glucose oxidase-catalyzed oxidation of glucose. The reaction mixture consisted of 11 μmol ethanol, 5 μmol β-D-glucose, 0.7 U glucose oxidase from *Aspergillus niger* (Nacalai Tesque), 25 μmol potassium phosphate buffer (pH 7.0), and the enzyme (typically 8 μg) for a final volume of 500 μL. The reaction mixture, without the enzyme, was pre-incubated at 25°C for 5 min. The reaction was initiated by addition of the enzyme, and the reaction mixture was incubated in tightly sealed tubes at 25°C for up to 16 h. The reaction was arrested by incubation at 98°C for 2 min. Acetaldehyde formed in the reaction mixture was quantified by means of head-space gas chromatography on a INNOWAX 19091N −233 column (0.25 mm × 30 m) using a Varian CP-3800/Tekmar 7000 apparatus.

**Purification of rGoxCat** All procedures were performed at 4°C, unless otherwise stated. Transformant cells were harvested by centrifugation, suspended in buffer A (20 mM potassium phosphate buffer, pH 7.0) containing 20 mM imidazole and disrupted by ultrasonication for 5 min by using a SONIFIER 250 apparatus (Branson Ultrasonics, Danbury, CT, USA; output, 20 kHz; duty cycle, 50%). After centrifugation, the supernatant was filtered through a membrane filter (pore size, 0.2 μm) and applied to a His-Trap column (5 mL, GE Healthcare Japan) equilibrated with buffer A containing 20 mM imidazole. After washing the column with the equilibration buffer, catalase was eluted with buffer A containing 200 mM imidazole. The fractions that contained homogeneous rGoxCat, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were combined and dialyzed against buffer A.

**UV–visible spectroscopy of rGoxCat** The UV–visible absorption spectra were recorded at wavelengths ranging between 200 and 800 nm using a Hitachi double-beam spectrophotometer (model U-2000) at room temperature.

**Determination of heme content** The heme content was determined from the differential absorbance at 555 nm of the different redox spectra, which were obtained using the pyridine hemochromogen method (8). The published $\Delta\varepsilon^{red/ox}{}_{555}$ value of 34.4 $mM^{-1}\,cm^{-1}$ was used in the calculation (9).

**Stability studies** For the thermal stability studies, the enzyme (0.76 μg/mL) was incubated in buffer A at different temperatures (10°C, 20°C, 30°C, 40°C, 50°C, 60°C, 70°C, 80°C, or 90°C) for 30 min. After incubation, the enzyme solutions were immediately chilled on ice. The remaining activity was assayed under standard conditions (see above).

For the pH stability studies, the enzyme was incubated at 40°C for 1 h at pH ranging from 2 to 11 using the following buffers: pH 2–4, sodium acetate; pH 4–6, sodium citrate; pH 6–8, potassium phosphate; pH 8–9, Tris-HCl; and, pH 9–10, glycine-NaOH. After incubation, the residual enzyme activity was assayed under standard conditions (see above).

**Effects of pH, temperature, and inhibitors on enzyme activity** To examine the pH-activity profiles of the recombinant enzyme, enzymatic decomposition of $H_2O_2$ was assayed as described above except that the reaction mixtures (500 μL) contained 25 μmol of one of the following buffers: pH 2–4, sodium acetate; pH 4–6, sodium citrate; pH 6–8, potassium phosphate; pH 8–9, Tris-HCl; or, pH 9–10, glycine-NaOH.

To examine the temperature-activity profiles of the recombinant enzyme, the enzymatic decomposition of $H_2O_2$ was assayed as described above except that the temperature of the reaction mixture was maintained at 10–80°C using a temperature-controlled cell holder.

To examine the effects of hemoprotein inhibitors, the enzyme solution was mixed with different concentrations of different inhibitors, including potassium cyanide (0–20 μM), sodium azide (0–50 μM), hydroxylamine (0–1 μM), and 10 mM 3-amino-1,2,4-triazole. After incubation for either 2 min (for potassium cyanide, sodium azide, and hydroxylamine) or 10 min (for 3-amino-1,2,4-triazole) at 25°C, the residual enzyme activity was determined.

**Analytical methods** SDS-PAGE was carried out on a 12% gel according to the Laemmli method (10). The native molecular mass of rGoxCat was estimated by gel filtration on a Superdex 200 10/300 GL column (GE Healthcare), equilibrated with buffer A containing 0.15 M NaCl. For calibration, marker proteins [carbonic anhydrase (molecular mass, 27 kDa), conalbumin (75 kDa), catalase (232 kDa), and ferritin (440 kDa); GE Healthcare] were subjected to chromatography under the same conditions.

## RESULTS AND DISCUSSION

**Identification of GoxCat as a monofunctional, large-subunit catalase** Crude extracts of *G. oxydans* cells (strain JCM7642) exhibited high catalase activity (74.4 U/mg protein) when the cells were grown in medium 1 under the conditions described in the Materials and methods section. The *G. oxydans* genome contained one 2.106-bp, putative catalase gene, *gox1138*, which was predicted to encode a protein (termed GoxCat) comprised of 701 amino acids (calculated molecular mass, 77.5 kDa) (1). Among the biochemically characterized catalases, the GoxCat sequence was the most similar to that of catalase C of *Sinorhizobium meliloti* (68% identity) (11) and catalase HP-II of *E. coli* (51%) (12) (Fig. 1). The sequence of the C-terminal domain of GoxCat was also similar to those of proteins belonging to the type-I glutamine amidotransferase superfamily (13). However, as is also the case for HP-II catalase, the catalytic triad (Cys-His-Asp/Glu) that is essential for glutamine amidotransferase activity was not conserved in the GoxCat sequence.

The *GoxCat* gene was over-expressed in *E. coli* BL21(DE3) cells under control of the T7 promoter to produce a soluble, catalytically active protein (Fig. 2A). The expressed product, i.e., the recombinant GoxCat protein (termed rGoxCat), was purified to homogeneity from a crude extract of *E. coli* cells using $Ni^+$-affinity chromatography. SDS-PAGE of the purified protein showed a single band at a molecular mass of 79.5 kDa (Fig. 2B), which was consistent with the molecular mass calculated from the deduced amino acid sequence. The native molecular mass of the enzyme was estimated to be 320 kDa by gel filtration chromatography (Fig. 2C). These results suggest that rGoxCat is a tetrameric protein made up of identical subunits.