Characterization of the major catalase from *Streptomyces coelicolor* ATCC 10147

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***Streptomyces coelicolor* ATCC 10147 produced catalases whose electrophoretic mobility varied depending on the growth phase in liquid culture.**

Polyacrylamide gel electrophoresis of cell extracts resulted in six catalase activity bands, which were designated Cat1 to Cat6. Of these, Cat4 appeared during all growth phases, whereas Cat1 appeared only during the stationary phase. Catalase-deficient mutants were screened by the H₂O₂ bubbling test following NTG mutagenesis. In all the non-bubbling mutants tested, the Cat4 activity band significantly decreased or disappeared, suggesting that Cat4 is the major catalase. Cat4 was purified to electrophoretic homogeneity and some of its properties analysed. The enzyme has a native molecular mass of 225 kDa, as determined by gel permeation column chromatography, and consists of four identical subunits of 57 kDa, as determined by SDS-PAGE. The enzyme contains 2.6 molecules of protohaem IX per tetramer, as indicated by the absorption spectrum. It was not reducible by sodium dithionite and exhibited no peroxidase activity with o-dianisidine as the substrate. All these characteristics, as well as inhibitor studies, indicate that the major vegetative catalase in *S. coelicolor*, unlike *E. coli* vegetative catalase, is a member of the typical monofunctional catalases found in eukaryotes and some bacteria.

Keywords: *Streptomyces coelicolor*, catalase

INTRODUCTION

All aerobic organisms have evolved specific enzyme systems to neutralize potentially lethal reactive oxygen species, including the superoxide anion, hydrogen peroxide and the hydroxyl radical as well as singlet oxygen (Cadenas, 1989; Morgan *et al.*, 1986; Halliwell, 1990). Among these systems two types of enzyme exist to remove hydrogen peroxide within cells: catalases and peroxidases. The former catalyse electron pair transitions in which H₂O₂ is decomposed to O₂ and H₂O, whereas the latter catalyse single electron transfers resulting in the oxidation of various organic compounds by H₂O₂ (Deisseroth & Dounce, 1970; Dunford & Stillman, 1976).

Most of the catalases characterized so far can be classified as belonging to one of two types: typical catalases and bifunctional catalase-peroxidases. The typical catalases, which are commonly isolated from animals (Dunford & Stillman, 1976; Schonbaum & Chance, 1976), plants (Esaka & Asahi, 1982) and micro-organisms (Herbert & Pinsent, 1948; Clayton, 1959), resemble each other very closely; they are composed of four subunits of equal size, containing 2.5–4 haemin prosthetic groups per tetramer with a combined molecular mass in the range

225–270 kDa, show a broad optimum pH in the range of 5–10, are resistant to treatment with ethanol/chloroform, and are specifically inhibited by 3-amino-1,2,4-triazol (Margoliash *et al.*, 1960). Bifunctional catalase-peroxidases, which until now have been detected only in bacteria (Diaz & Wayne, 1974; Claiborne & Fridovich, 1979; Nies & Schlegel, 1982; Loewen *et al.*, 1985a; Hochman & Shemesh, 1987; Nadler *et al.*, 1986), share several properties which distinguish them from the typical catalases; their catalytic activity is pH-dependent with a pH optimum at 6–6.5, they are more sensitive to temperature, ethanol/chloroform and H₂O₂ than the typical catalases and are insensitive to 3-amino-1,2,4-triazol (Goldberg & Hochman, 1989a; Hochman *et al.*, 1992).

While some organisms produce only one type of catalase, more than one catalase has been shown to be present in organisms such as *Escherichia coli* (Claiborne & Fridovich, 1979; Claiborne *et al.*, 1979), *Saccharomyces cerevisiae* (Seah *et al.*, 1973; Seah & Kaplan, 1973), *Bacillus subtilis* (Loewen & Switala, 1987b, c) and *Klebsiella pneumoniae* (Goldberg & Hochman, 1989a, b).

In a previous study, Lee *et al.* (1993) have shown that *Streptomyces coelicolor* ATCC 10147 became resistant to

killing by hydrogen peroxide (H_2O_2) when pretreated with non-lethal concentrations of H_2O_2 , and that the specific activities of catalase, peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase increased. The H_2O_2 -resistant mutants produced 2–8-fold more catalase activity than the wild-type, suggesting that catalase plays an important role in oxidative defence in this organism. In this paper, we describe an investigation of the catalases present in *S. coelicolor* and report on the purification and characterization of the major vegetative catalase.

Media and culture conditions. Growth and maintenance of *S. coelicolor* ATCC 10147 were essentially as described by Hopwood *et al.* (1985). Pre-germinated spores or seed-culture of *S. coelicolor* were inoculated in minimal medium and grown at 30 °C with vigorous shaking. The growth phases of the culture were determined by measuring OD_{640} , the weight of dried mycelium, or DNA content (Baserga, 1989; Lee, 1994).

Mutagenesis with NTG and screening for catalase-deficient mutants. Spores were treated with NTG (1 or 3 mg ml⁻¹) to a survival ratio of 0.1–1.0% and plated on Bennet's medium (Jones, 1949). Catalase activity was screened by applying a drop of 30% (v/v) H_2O_2 with a syringe to the edge of each colony. Catalase-deficient cells failed to evolve oxygen bubbles and were selected and immediately restreaked on fresh Bennet's medium.

Catalase activity assays. Catalase activity was measured spectrophotometrically by following the rate of decrease in absorbance at 240 nm caused by the disappearance of H_2O_2 (Beers & Sizer, 1952). The absorption coefficient at 240 nm for H_2O_2 was taken to be 43.6 M⁻¹ cm⁻¹ (Hildebrandt & Roots, 1975). The reaction mixture contained 16 mM H_2O_2 and an appropriate amount of enzyme in 50 mM potassium phosphate buffer (pH 7.0). The reaction was run at 30 °C for 1 min, and only the initial linear rate was taken to estimate activity. One unit of activity was defined as the disappearance of 1 μmol H_2O_2 min⁻¹.

Catalase activity staining. Native catalases were electrophoretically separated on 7% polyacrylamide gel according to Laemmli (1970). Staining for catalase activity was done by the method of Clare *et al.* (1984): the gel was soaked for 45 min in 50 mM potassium phosphate buffer (pH 7.0) containing 50 μg horseradish peroxidase ml⁻¹, followed by addition of 5 mM H_2O_2 for 10 min. The gel was washed twice with distilled water, and then was soaked in 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mg diaminobenzidine ml⁻¹ to develop the background brown colour. No colour would appear in the area where catalase depleted H_2O_2 .

Purification of the major catalase from *S. coelicolor*. All the purification steps were carried out at 4 °C or on ice. Mycelium cultured for 48 h was harvested and washed twice with 10 mM potassium phosphate buffer (pH 6.2). The washed mycelium was suspended in 50 mM potassium phosphate buffer (pH 6.2) and disrupted by abrasion with glass beads in a bead beater (Biospec). Cell debris was removed and the lysate was precipitated by adding ammonium sulfate to 70% saturation. The precipitates were dissolved in 50 mM potassium phosphate buffer (pH 6.2) and applied to a column of Sepharose CL-4B equilibrated with 50 mM potassium phosphate buffer (pH 6.2). The fractions showing catalase activity were pooled and immediately applied, without further concentration or dialysis, to a column of DEAE-Sepharose CL-6B equilibrated with

50 mM potassium phosphate buffer (pH 6.2). The column was washed with 50 mM potassium phosphate buffer (pH 6.2) and then eluted with a linear gradient of NaCl (0–0.6 M). The fractions containing catalase activity were pooled and ammonium sulfate was added to a final concentration of 1 M. This was then applied to a Phenyl-Sepharose CL-4B column equilibrated with 0.5 M ammonium sulfate in 10 mM potassium phosphate buffer (pH 6.8). The column was washed with equilibration buffer and eluted with a descending gradient of 0.5–0 M ammonium sulfate. The eluate containing catalase was concentrated by ultrafiltration and stored at 4 °C for the subsequent analyses.

Molecular mass determination. The molecular mass of the native enzyme was determined by size exclusion on a Superose 6 column equilibrated with 0.25 M NaCl in 10 mM potassium phosphate buffer (pH 6.8). For molecular mass standards, the following proteins were used; ferritin (440 kDa), aldolase (158 kDa), bovine serum albumin (66.2 kDa) and ovalbumin (45 kDa). Subunit molecular mass was determined by SDS-PAGE on a 10% (w/v) acrylamide gel according to the procedure of Laemmli (1970).

Spectrophotometry. Absorption spectra were measured on Hewlett Packard Diode Array Spectrophotometer. The type and content of prosthetic haem group was determined as the pyridine haemochromogen by the method of Falk (1964) using pyridine-dithionite as a blank.

Changes in catalase band patterns at different growth phases

Different catalase activities present in crude extracts of *S. coelicolor* cells grown for various lengths of time in liquid minimal media were separated by electrophoresis on native polyacrylamide gel and visualized by activity staining (Fig. 1). Two catalase bands (Cat2 and Cat4) were present in extracts of mycelia from the mid-exponential growth phase. As growth progressed changes in the catalase band pattern were observed. Mycelia from the late-exponential phase produced four catalase bands (Cat3, Cat4, Cat5 and Cat6), whereas mycelia from the stationary phase produced a Cat1 band, detectable only in this growth phase, in addition to those present in late-exponential phase. The synthesis of Cat1 can be correlated with secondary metabolism. In *S. coelicolor*, secondary metabolism takes place only after rapid cell growth has ceased, and involves the biosynthesis of pigmented antibiotics. Cat1 was detectable only after cells began to produce pigments. Cat1 was also detected in purified spores along with the other catalases, except Cat2 (data not shown).

Growth-dependent expression of multiple catalases has previously been reported in *E. coli* (Loewen *et al.*, 1985b) and *B. subtilis* (Loewen & Switala, 1987a). In *E. coli* two types of catalase (HPI and HPII) were found to be produced. HPI is the major vegetative catalase and was shown to be induced when cells were treated with a sublethal level of H_2O_2 . The expression of HPI is under the control of the *oxyR* gene product (Christman *et al.*, 1985). HPII synthesis is elevated in the stationary phase as well as during growth on TCA cycle intermediates, and

Fig. 1. Expression of multiple catalases with different electrophoretic mobilities in *S. coelicolor* at different growth phases. Cell extracts were obtained from cells grown for 30 h (mid-exponential growth phase; lanes 1 and 2), 48 h (late-exponential phase; lanes 3 and 4), or 10 d (stationary phase; lanes 5 and 6) after inoculation of germinated spores in minimal medium. Cells were either untreated (lanes 1, 3 and 5) or treated with 100 μ M H_2O_2 for 30 min (lanes 2, 4 and 6) before harvest. Cell extracts containing 50 μ g protein were loaded on 7% native polyacrylamide gel. Catalase bands were stained negatively, as described in Methods.

regulated by the stationary-phase-specific regulator RpoS (*katF*) (Loewen & Triggs, 1984). *B. subtilis* produces two main species of catalase; catalase-1 and catalase-2. Catalase-1 is produced in vegetative cells and catalase-2 is produced after cells have entered the stationary phase. Catalase-2 is the exclusive catalytic activity in purified spores. In *S. coelicolor*, Cat4 turned out to be the vegetative catalase present throughout all the growth phases, whereas Cat1 was the stationary-phase-specific enzyme. Except in mid-exponential phase, Cat3, Cat5 and Cat6 bands appeared at all growth phases in varying amounts. Cat3 was more abundant in the exponential phase, whereas Cat5 was more abundant in the stationary phase. The presence of Cat2 was transient and was observed only within a short period of time during the early phase of rapid growth. It is not certain whether each catalase activity band is a discrete enzyme or a variant in terms of charge or oligomerization status, since there have been reports that catalase-2 of *B. subtilis* and catalase-peroxidase of *Rhodopseudomonas capsulata* appear as multiple bands, probably due to limited proteolysis (Loewen & Switala, 1987a; Hochman & Shemesh, 1987). In *S. coelicolor*, however, Cat5 and Cat6 are not the proteolytic products of Cat4, as indicated by Western blotting. Polyclonal antibody raised against purified Cat4 reacted with a single polypeptide band on a blot of cell extracts electrophoresed on SDS-PAGE (data not shown). Whatever the nature of each enzyme band is, *S. coelicolor* Cat4 corresponds to HPI of *E. coli* and catalase-1 of *B. subtilis*, whereas Cat1

Fig. 2. Catalase activity in cell extracts from various catalase-deficient mutants. Non-bubbling and slow-bubbling mutants were selected as described in Methods and grown to either exponential (■) or stationary (□) phases. K1-K10, non-bubbling mutants; K11-K14, slow-bubbling mutants; WT, wild-type.

corresponds to HPII of *E. coli* and catalase-2 of *B. subtilis*, in terms of growth-phase-specific expression. To examine the inducibility of each catalase activity band in response to H_2O_2 , mycelia at each growth phase were treated with 100 μ M H_2O_2 for 30 min (Fig. 1, lanes 2, 4 and 6). Cat2 and Cat4 activity bands increased slightly at the mid-exponential phase. However, since quantification by activity staining in the gel was insufficiently accurate, the extent of induction was not estimated.

Isolation and characterization of mutants deficient in catalase activity

To carry out a genetic investigation on the presence of multiple catalase genes in *S. coelicolor*, mutants deficient in catalase activity were isolated. From approximately 5000 surviving colonies, eight failed to evolve oxygen bubbles (non-bubbling mutant) and three evolved bubbles more slowly than the wild-type (slow-bubbling mutants). The catalase activity of these mutants was measured in extracts from cells at exponential and stationary growth phases (Fig. 2). All the mutants contained lower levels of catalase activity compared with the wild-type cells. Catalase activities in non-bubbling mutants were 10–30% those of the wild-type at exponential growth phase and 10–15% at stationary growth phase. In slow-bubbling mutants, catalase activities were 40–60% those of the wild-type at exponential growth phase and about 50% at stationary growth phase. To determine the changes in the pattern of catalase activity bands for these mutants, activity staining of crude extracts was done. Table 1 summarizes the result of gel activity staining of extracts from mutants. In all the non-bubbling mutants, the Cat4 band was significantly weakened or absent at both growth phases. In slow-bubbling mutants, however, the Cat4 band was observed at a similar level as that of the wild-type. In contrast, other catalase bands were weakened. Cat1, which was detected

Table 1. Changes in catalase band patterns of catalase-deficient mutants

Band intensity: + + +, wild-type level; + +, medium; +, weak; —, no activity.

Band	Growth phase*	Non-bubbling								Slow-bubbling		
		K1	K6	K7	K9	K10	K4	K5	K8	K11	K12	K14
Cat1	S	+	++	+	+	+	—	—	—	+	+	+
Cat3	E	++	+	++	++	+++	—	—	—	—	+++	—
	S	+	+	+	+	+	—	—	—	+++	+++	+++
Cat4	E	—	—	—	—	—	+	+	+	+++	+++	+++
	S	—	+	+	+	—	+	+	+	+++	+++	+++
Cat5	E	+	—	+	++	—	+++	+++	—	—	++	—
	S	+	++	++	++	+	+	+	+	—	—	+++
Cat6	E	++	+	++	+++	+	+++	+++	+++	+	+++	+++
	S	+	++	++	+	++	+	++	+	+	++	++

* E, Exponential phase, cultured for 48 h in minimal medium inoculated with germinated spores; S, stationary phase, cultured for 10 d in minimal medium inoculated with germinated spores.

Table 2. Purification of the major catalase from *S. coelicolor*

Purification step	Vol. (ml)	Total protein (mg)	Total activity (units)	Specific activity (units mg ⁻¹)	Recovery (%)
Crude extract	70	359	2.12 × 10 ⁵	591	100
Ammonium sulfate	10	256	1.90 × 10 ⁵	742	90
Sepharose CL-4B	118	63	1.75 × 10 ⁵	2780	83
DEAE Sepharose CL-6B	49	8.9	5.14 × 10 ⁴	5780	24
Phenyl Sepharose CL-4B	8	0.32	4.43 × 10 ⁴	138000	21

only during the stationary growth phase, was weakened in all the catalase-deficient mutants. In this analysis, we were not able to correlate any two catalase bands which were reduced simultaneously in all the non-bubbling or slow-bubbling mutants. Instead, different combinations of catalase bands were reduced in each mutant. It is highly unlikely that the enzymes in all the bands are encoded by a single gene since all the non-bubbling mutants contained at least 10 % of catalase activity compared with the wild-type. From a separate study in our laboratory, we found at least two genes which contain homologous sequences with typical eukaryotic catalase (Y. H. Cho, personal communication). The reason for the simultaneous decrease in several catalase bands is not clear at this moment. Considering the mutation frequency, it is not plausible to assume multiple mutations in each mutant. If the expression of different catalases is correlated with growth phases, which are governed by many metabolic factors in cells, mutation in one catalase gene could change the intracellular environment and affect the expression of other genes in a complex regulatory circuit. On the other hand, if several catalase bands are variants in charge and/or oligomerization status, more than one band can be affected by a single mutation. To clarify this, further

genetic study of genes encoding catalase is necessary. Whatever the situation is, the current results clearly indicate that there should be at least two catalase genes in *S. coelicolor* and Cat4, the catalase present in all growth phases, is the major catalase since all the non-bubbling mutants were deficient in its activity.

Purification of the major catalase

The typical purification steps for catalase from crude extracts are summarized in Table 2. This procedure resulted in about 230-fold purification with 21 % yield. Catalase activity was detected as a single broad peak in Sepharose CL-4B eluate fractions, containing Cat3, 4, 5 and 6 activities. When these fractions were run on a DEAE Sepharose CL-6B column, only Cat4 activity was detected in fractions eluted by 0.2–0.3 M NaCl. The purified enzyme preparation showed a single protein band near homogeneity on both native (Fig. 3a) and SDS-polyacrylamide gel (Fig. 3b). Activity staining of the native gel revealed that this protein corresponded to Cat4 (Fig. 3a, lane 2). SDS-PAGE with several molecular mass markers revealed that the enzyme is composed of a single type of subunit with a molecular mass of 57 kDa. This

Fig. 3. (a) Electrophoretogram of purified Cat4 on 7% non-denaturing polyacrylamide gel. Lanes: 1, purified Cat4 stained with Coomassie brilliant blue G; 2, purified Cat4 stained for catalase activity; 3, crude extracts of cells at stationary phase stained for catalase activity. (b) Electrophoretogram of purified Cat4 on SDS-polyacrylamide gel. The gel was stained with Coomassie brilliant blue G. The position of molecular mass markers is indicated on the right.

enzyme was found to contain no significant peroxidase activity when assayed using *o*-dianisidine and diaminobenzidine as substrates. The purified Cat4 was electrophoresed on polyacrylamide gel with pH gradient of 3–10, and the isoelectric point was determined to be 5.1.

Molecular mass determination of the native enzyme

The molecular mass of the purified native enzyme was determined by gel filtration through a Superose 6 column. Compared with several size markers, the molecular mass was estimated to be approximately 225 kDa. Considering the subunit molecular mass, this enzyme appears to be a tetramer composed of four identical subunits.

Spectroscopic properties

The absorption spectrum of the purified catalase exhibited a *soret* peak at 406 nm and an additional minor peak at 610 nm (Fig. 4, spectrum A). Treatment of the enzyme with sodium dithionite did not alter the spectral shape (Fig. 4, spectrum B), indicating that Cat4, like other typical monofunctional catalases from eukaryotic sources, is resistant to reduction. This characteristic is consistent with the above observation that Cat4 lacks peroxidase activity. Treatment of the enzyme with KCN shifted the maximum of the *soret* band to 424 nm and resulted in the appearance of minor peaks at 554 nm and 594 nm (Fig. 4, spectrum C). The efficient formation of a cyanide complex is further evidence for the presence of ferric haem in the

Fig. 4. Absorption spectra of purified Cat4. The spectra of enzyme in 10 mM potassium phosphate buffer (pH 6.8) were recorded against a blank containing buffer only. A, native enzyme; B, enzyme treated with 1 mM sodium dithionite; C, enzyme treated with 10 mM KCN.

Fig. 5. Absorption spectrum of pyridine haemochrome of *S. coelicolor* Cat4. Enzyme (1.27 μ M) was incubated in 10 mM potassium phosphate buffer (pH 6.8) containing 0.1 M NaOH, 20% (w/v) pyridine and 1 mM sodium dithionite for 1 min before spectrophotometric measurement.

enzyme and indicates that the haem ligand of Cat4 might be tyrosine phenolate, as in other typical catalases (Reid *et al.*, 1981; Fita & Rossman, 1985). Treatment of the enzyme with pyridine/NaOH and sodium dithionite produced pyridine haemochrome whose major absorption peaks appeared at 418, 526 and 556 nm (Fig. 5). This indicates that Cat4 contains protohaem IX as its haem group. The protohaem content of this enzyme was determined from the absorption of its pyridine hae-

mochromogen at 418.5 nm, on the basis of the relationship, $\epsilon_{418.5} = 191.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ (Falk, 1964). We estimated that there are 2.6 molecules of protohaem IX per tetrameric molecule of Cat4. The haem content as well as the A_{405}/A_{280} ratio of 1.0 again indicates that Cat4 shares many properties with the typical monofunctional catalases.

Inhibitors

The effect of KCN, NaN_3 , hydroxylamine, β -mercaptoethanol and sodium dithionite on the enzyme activity was examined. Treatment for 1 min with 1 mM KCN, 0.1 mM NaN_3 and 0.5 mM hydroxylamine inhibited enzyme activity by 95, 97 and 63 %, respectively. β -Mercaptoethanol and sodium dithionite were moderate inhibitors at 1 mM, inhibiting catalase activity by 36 and 13 %, respectively.

Protein stability

Cat4 retained 100 % of its activity when treated with a mixture of ethanol/chloroform (enzyme solution:95 % ethanol:chloroform = 10:5:3, by vol.) for 10 min with vortexing at room temperature. When the purified enzyme was incubated at various temperatures for 5 min, full activity remained at temperatures up to 40 °C. At 50 °C, however, about 60 % of the activity measured at 30 °C remained, and at 60 °C the enzyme was completely inactivated. Incubation of Cat4 for 2 min in the presence of 8 M urea also caused its complete inactivation. The effect of pH on Cat4 activity was also determined. Catalase activity showed a broad maximum in the range of pH 5.5–9.5.

The major vegetative catalase Cat4 isolated from *S. coelicolor* shares general molecular properties with typical eukaryotic catalases. Bacterial catalases that closely resemble the typical eukaryotic catalase, with four protohaem IX groups associated with a tetramer of subunits of approximately 60 kDa, have been shown to be produced in *Micrococcus lysodeikticus* (Herbert & Pinsent, 1948) and *Proteus mirabilis* (Jouve *et al.*, 1983). Other than these, Cat4 differs in its molecular properties from other bacterial monofunctional catalases. *E. coli* HPII (Loewen & Switala, 1986) and *B. subtilis* catalase-2 (Loewen & Switala, 1987c), both of which are expressed in the stationary phase, contain six haem D-isomer prosthetic groups in a hexameric structure of larger subunits. *E. coli* HPI (Claiborne & Fridovich, 1979) and catalase from *R. capsulata* (Hochman & Shemesh, 1987), *Halobacterium halobium* (Brown-Peterson & Salin, 1993) and facultative alkalophilic *Bacillus* species (Yumoto *et al.*, 1990) are bifunctional catalase-peroxidases. The catalases of *Comamonas compransoris* (Nies & Schlegel, 1982), *Mycobacterium tuberculosis* (Diaz & Wayne, 1974) and *Streptomyces venezuelae* (Knoch *et al.*, 1989) have a molecular mass of 150–160 kDa, and consist of two identical subunits.

We have shown in this paper that among six catalase activities with different electrophoretic mobilities and

growth-specific expression patterns, Cat4 is the major catalase. We compared its molecular properties with other known catalases and found that the enzyme is distinctly different from the vegetative catalase from *E. coli* in both its enzymic characteristics and the makeup of the catalase active centre. The limited information on the vegetative catalase (catalase-1) of *B. subtilis*, suggests that *S. coelicolor* Cat4 has similar properties, except that *B. subtilis* catalase-1 is a hexameric enzyme (Loewen & Switala, 1987b).

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