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Purification of three catalase isozymes from facultatively alkaliphilic *Bacillus firmus* OF4

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Abstract

Cell extracts of facultatively alkaliphilic *B. firmus* OF4 were assayed for catalase activity and their catalase isozyme content was analyzed on native polyacrylamide gels stained for catalase activity. pH-10.5-grown cells had about twice the specific catalase activity of pH-7.5-grown cells. The higher activity, however, did not confer resistance to exogenous hydrogen peroxide challenge relative to pH-7.5-grown cells and, in fact, the pH-10.5-grown cells were much more sensitive to the challenge. Electrophoresis resolved three catalase isozymes in cell extracts. The isozymes, labeled I–III in order of decreasing electrophoretic mobility, were purified and their Nterminal amino acid sequences were obtained. Isozyme III corresponded to the product of a cloned gene fragment that had been shown to possess substantial sequence similarity to the KatE (HP-II) catalase of *E. coli* (Quirk, P.G., Krulwich, T.A. and Hicks, D.B. (1993) Biophys. J. 64, 164A) and which had similar biochemical properties to HP-II, i.e., it was a chlorin-containing enzyme expressed only in stationary phase. Isozyme II, a protoheme enzyme, was responsible for the higher activity of alkaline-grown cells and was induced in cells treated with hydrogen peroxide or ascorbate. It showed sequence similarity to katA of *Bacillus subtilis* (Bol, D. and Yasbin, R. (1991) Gene 109, 31–37). Isozyme I was the only isozyme that exhibited detectable levels of peroxidase activity in addition to catalase activity, resembling a catalase enzyme purified from a different alkaliphile, *Bacillus* YN-2000 (Yumoto, I., Fukumori, Y. and Yamanaka, T. (1990) J. Biochem. 108, 583–587), to which it showed some sequence similarity.

Keywords: Catalase; Hydrogen peroxide; Alkaliphile; pH

1. Introduction

The high concentration of cytochromes that are found in alkaliphilic *Bacillus* species appears to be related to the bioenergetic demands of growth at very alkaline pH [1]. One of the cytochrome complexes that may specifically be involved in energy coupling at high pH is cytochrome caa_3, whose concentration is up-regulated 2–3-fold when facultatively alkaliphilic *B. firmus* OF4 is grown at pH 10.5 compared to pH 7.5 [2]. In the course of cloning the *cta* genes encoding the subunits of cytochrome caa_3 using mixed oligonucleotides, one of the clones that was obtained contained an incomplete reading frame that was predicted to encode a protein that was 67% identical to the KatE (HP-II) catalase of *Escherichia coli* in the first 449 amino acids [3] [1]. In order to determine whether *B. firmus* OF4 indeed expressed such a protein, purification of enzymes from *B. firmus* OF4 catalyzing hydrogen peroxide degradation was carried out. As part of that effort, catalase isozymes in crude extracts from cells grown at pH 7.5 and 10.5 were resolved by nondenaturing polyacrylamide gel electrophoresis. This allowed the identification of three distinct catalase isozymes and, coupled with catalase activity assays, the determination that there was a higher specific catalase activity at high pH that was due to an increase in one of the isozymes. The three catalase isozymes were purified and the N-terminal amino acid sequence was determined for each. Possible relationships of the isozymes with other catalases are discussed and the sensitivity of cells to exogenous H_2O_2 was determined in light of the higher activity in alkaline-grown cells.

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); PMSF, phenylmethylsulfonyl fluoride.

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[1] The nucleotide sequence of the catalase gene fragment and the upstream consensus *fur* sequence are deposited in the GenBank™/EMBL Data Bank with accession number LO2551.

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2. Materials and methods

2.1. Growth of Bacillus firmus OF4

B. firmus OF4 was grown in a 30° C incubator with shaking at 200 rpm. The medium contained 100 mM phosphate buffer (growth pH 7.5) or 100 mM carbonate buffer (growth pH 10.5), which minimized pH changes during growth, especially the early exponential growth phase [4]. Sodium D,L-malate (50 mM) was the energy and carbon source, and the medium also contained 0.1% yeast extract and 0.1% trace salt solution [4].

2.2. Effect of hydrogen peroxide on cell viability

B. firmus OF4 was cultured in 50 ml volumes in 250 ml flasks. When the A_{600} reached 0.4, the culture was divided into two halves, pelleted and suspended in 25 ml of growth medium at either pH 7.5 or pH 10.5. The cell suspension was pipetted into the appropriate number of 15 ml conical tubes, 1 ml/tube, and to each tube was added 10 μl of a 100× stock of H_2O_2 or 10 μl of H_2O (control). The tubes were put back in the incubator and shaken for 15 min and the treatment was terminated by rapid dilution into complete growth medium and immediate plating. The pH of the plates was identical to the pH of the H_2O_2 treatment; colonies were scored after overnight incubation at 30° C.

2.3. Induction of catalase activity and preparation of cell extracts

Cells grown to an A_{600} of 0.4 to 0.5 in separate 50 ml cultures were pooled and divided into 40 ml portions and treated with 0.4 ml of water or 0.4 ml of 100× stocks of H_2O_2 or sodium ascorbate. The cultures were immediately put back in the shaker. For the H_2O_2 treatments, the same additions were made at 10 and 20 min. For example, three additions of a stock 10 mM H_2O_2 solution were made to yield a nominal final concentration of 300 μM H_2O_2 (although the actual H_2O_2 concentration may have changed during the incubation). This was similar to the protocol described by Loewen and Switala [5]. The cultures were removed at 60 min, pelleted, and washed once with 40 ml of the suspension buffer (50 mM Tris-SO_4 (pH 8), 5 mM $MgCl_2$, 0.1 mM PMSF). The pellet was suspended in 1 ml of the suspension buffer and put in a 15 ml glass conical tube supplemented with glass beads. The suspension was sonicated in an ice-bath twice for 1 min. Unbroken cells and debris were removed by centrifugation in a microfuge.

2.4. Catalase assay

The standard catalase assay contained 50 mM Mes-NaOH (pH 6.5), 30 mM H_2O_2 and sample in a 1 ml volume. The reaction was initiated by the addition of the sample and activity was detected by the decrease in absorbance at 240 nm at room temperature (approx. 25° C). The linear portion of the reaction, usually 20–30 s, was used to calculate the rate of the reaction. An extinction coefficient of 43.6 $M^{-1}\,cm^{-1}$ at 240 nm was assumed for H_2O_2. One unit of activity was defined as 1 μmol H_2O_2 decomposed per min at room temperature. The apparent K_m for H_2O_2 was estimated by double-reciprocal plots ($1/v$ vs. $1/[H_2O_2]$), with the assays carried out at the pH optimum for the particular isozyme (isozyme I, pH 8.0; isozyme II, pH 10.0). The apparent K_m of isozyme III at pH 5.5 was too high to be determined by the spectrophotometric assay.

2.5. Peroxidase assay

Peroxidase activity was monitored using either guaiacol or ABTS as substrates. The reaction mixtures contained, in a volume of 1 ml, 50 mM Mes-NaOH (pH 6), 11 mM H_2O_2, and either 30 mM guaiacol or 10 mM ABTS. The increase in absorbance at 470 nm (guaiacol) or 420 nm (ABTS) was monitored after addition of sample. For comparative purposes, horseradish peroxidase (Sigma P 6782) was assayed similarly, except that the H_2O_2 concentration was 1 mM. Only isozyme I showed detectable levels of peroxidase activities under these conditions. For guaiacol, the activity of isozyme I was 16.0 and for ABTS the activity was 138.4, with the activity being expressed as $\Delta A\,min^{-1}\,(mg\ protein)^{-1}$. At pH 9, the activity was less than 33% that measured at pH 6. These activities were less than 1% that of horseradish peroxidase when expressed as $\Delta A\,min^{-1}\,mg^{-1}$ horseradish peroxidase.

2.6. Native and SDS polyacrylamide gel electrophoresis

The buffer system was the Schagger and von Jagow Tricine formulation [6]. SDS polyacrylamide gels were cast in either the Bio-Rad mini apparatus or the LKB full-sized format. These were stained by a sensitive Coomassie Brilliant Blue G procedure. Molecular weights were estimated with the broad range standards purchased from Bio-Rad (isozymes I and III) or Bio-Rad's low molecular weight standards (isozyme II). Native 5% polyacrylamide gels (with a 4% T stacking gel) were cast only in the full-sized format. These were run for 1 h at 50 V and 5 h at 100 V until the Bromophenol blue front had run off the gel for about 30–45 min. The gels were stained for catalase activity by the procedure of Clare et al. [7], using 10 mM H_2O_2.

2.7. N-terminal amino acid sequence determination

Each purified catalase isozyme was resolved in the mini SDS 10% polyacrylamide gel system and transferred to a PVDF membrane using a standard buffer formulation (20% methanol, 25 mM Tris, 192 mM glycine). After very brief

Coomassie brilliant blue R staining and destaining, the membrane was equilibrated with 50% methanol, HPLC grade, and air-dried. The N-terminal amino acid sequence was determined on a Porton Instruments Model 2090E automated gas phase sequencer, using modified Edman degradative chemistry.

2.8. Purification of catalase isozymes: general comments

Unless otherwise noted, all purification steps were done at 4° C, and the buffer for column chromatography was 50 mM Tris-SO_4 (pH 7.0), 0.1 mM PMSF (buffer A). Initial characterization of the pH-dependence of catalase activity in cell extracts indicated that the optimum was pH 6.5. Therefore, that pH was used routinely in enzyme assays during the purification procedures. pH profiles of the individual enzymes indicated that pH 6.5 gave activity that was about 50% (isozyme I) or 75% (isozymes II and III) of the optimal pH, and thus did not materially affect the ability to detect any isozyme by enzyme activity. The expression of different isozymes may be dependent on the extent of aeration, since cells grown in sparged carboys had a mixture of isozymes that did not correspond precisely to that found in cells grown in shaken flasks. As a consequence, some enzyme activity was discarded at different purification steps to remove contaminating isozymes and the yield of particular isozymes was not easily accessible. Yields are given as the % of total initial activity.

Purification of isozyme I

60 liters of cells grown to late exponential/early stationary phase were harvested, washed, and suspended in 50 mM Tris-SO_4 (pH 8), 10 mM $MgSO_4$, 1 mM PMSF, and 5 mM *p*-aminobenzamidine (supplemented with a small amount of DNase) and broken in pre-cooled French Pressure cell at 18000 p.s.i.. Unbroken cells and debris were removed by two low speed centrifugations. It was determined that centrifugation of the crude extract for 2 h at $186000 \times g_{max}$ resulted in the catalase activity fractionating with the supernatant. Prolonged centrifugation, 17 h at $186000 \times g_{max}$, on the other hand, resulted in the catalase activity cosedimenting with the membranes, presumably by rate zonal centrifugation. For convenience, isozyme I was obtained from membranes that were obtained after a 17 h centrifugation run. The vesicle pellet was suspended in 10% glycerol, 10 mM Tris-SO_4 (pH 8), 10 mM EDTA, 1 mM PMSF, and 5 mM *p*-aminobenzamidine, and centrifuged for 3 h at $186000 \times g_{max}$. The supernatant fraction containing the catalase activity was loaded on a 20×2.6 cm column of DEAE-Sepharose CL-6B that had been equilibrated with 10% glycerol, 20 mM Tris-SO_4, 1 mM EDTA, 0.2 mM PMSF, and 1 mM *p*-aminobenzamidine (pH 7.3). The column was washed successively with 4–5 bed volumes of 0, 50, 100 and 200 mM $(NH_4)_2SO_4$ in the same buffer and the catalase activity eluted at 200 mM salt. The active fraction was subjected to ammonium sulfate fractionation and most of the activity precipitated between 65 and 80% saturation. The pellet was brought to 0.75 M $(NH_4)_2SO_4$ in buffer A just prior to loading on a 23×1.6 cm phenyl Sepharose CL-6B column which had been equilibrated with 750 mM $(NH_4)_2SO_4$ in buffer A. The column was washed with 3 bed volumes of 750 mM $(NH_4)_2SO_4$ and a fraction enriched in isozyme I eluted at 600 mM. The enriched fraction was applied to a hydroxyapatite column (7×1.6 cm) and washed successively with 1, 10, and 25 mM potassium phosphate (pH 7). Enzyme activity eluted at 25 mM potassium phosphate. This fraction was concentrated to 1.4 ml and applied to a 45×2.6 cm Sephacryl S-300 gel-filtration column at room temperature. The developing buffer was 50 mM Tris-SO_4 (pH 7.3), 0.1 mM PMSF. Finally, isozyme I was purified by HPLC anion exchange on a 25×1 cm Rainin SynChropak column at room temperature. Solution A was 50 mM Tris-SO_4 (pH 7), and solution B contained 0.4 M $(NH_4)_2SO_4$ in 50 mM Tris-SO_4 (pH 7). PMSF was left out of these solutions. After washing the column with 10% and 20% B, a 70 ml gradient from 20–100% B was applied to the column at a flow rate of 1 ml/min. Isozyme I eluted sharply at about 0.2 M $(NH_4)_2SO_4$. The specific activity of isozyme I was about 4000 U(mg protein)$^{-1}$ at pH 6.5, and 2.6 mg of protein was obtained in the final purification step. This represented a 90-fold purification of the original membrane wash material with a yield of about 5%. The preparation exhibited one major band at 84000 on SDS polyacrylamide gels as well as a minor band that appeared to run at the same size as the monomer of isozyme II, but there was no evidence on native polyacrylamide gels that were stained either enzymatically or by Coomassie for any isozyme II in the purified isozyme I preparation. The Coomassie-stained native gel indicated a minor component with higher mobility than isozyme I.

Purification of isozyme II

16 liters of cells grown to stationary phase were harvested, washed and broken in the French Press as described for isozyme I, except that the buffer was 50 mM Tris-SO_4 (pH 8), 5 mM $MgSO_4$, 0.1 mM PMSF. The crude extract was centrifuged for 90 min at $186000 \times g_{max}$ and the resulting supernatant fraction contained the catalase activity. This fraction was loaded on a DEAE-Sephacel column (10×2.6 cm). After washing the column with 4 bed volumes of 100 mM $(NH_4)_2SO_4$ in buffer A, enzyme activity was eluted with 200 mM $(NH_4)_2SO_4$. Most of the activity was subsequently found to precipitate between 50 and 65% $(NH_4)_2SO_4$ saturation, which was then applied to a phenyl Sepharose CL-6B column (13×1.6 cm) equilibrated with 700 mM $(NH_4)_2SO_4$ in buffer A. The column was washed successively with 700, 600, 500, 400, 300, 200, 100 and 50 mM $(NH_4)_2SO_4$. The bulk of the catalase activity eluted with 100 and 50 mM $(NH_4)_2SO_4$. The salt concentration of the active sample

was reduced by dilution and concentration by ultrafiltration and subjected to anion HPLC as described for isozyme I. At this point, the sample contained a small amount of contamination, according to SDS-polyacrylamide gel electrophoresis, which was removed by sucrose gradient ultracentrifugation. The gradients consisted of a linear, 39 ml, 10–30% sucrose gradient in 50 mM Tris-SO_4 (pH 7), and were centrifuged for 3 h at 20° C and 190000 × g_{ave} in a VTi50 rotor. The gradients were fractionated from the top using a Buchler auto-densi flow apparatus. The purified preparation had a specific activity of 75000 U/mg at pH 6.5, representing an enrichment of 130-fold over the starting material (supernatant fraction of crude extract), with a yield of 9% and 0.62 mg of protein. According to SDS-polyacrylamide gel electrophoresis, the preparation at this step had one major band with an M_r of 60000, a minor band at about 116000, and a faint band at about 50000. Native polyacrylamide gel electrophoresis indicated that the isozyme II preparation consisted of a single Coomassie-staining band that coincided with the single enzymatic band.

Purification of isozyme III

The ammonium sulfate fraction of the cytoplasmic extract, P_{50-60}, from 90 l of cells grown at pH 10.5 to late exponential/early stationary phase was enriched in isozymes I and III. This fraction, after dialysis against buffer A, was subjected to DEAE-Sepharose CL-6B anion exchange chromatography (17.5 × 2.6 cm). The column was washed with 2 bed volumes of buffer A and 5 bed volumes of 100 mM $(NH_4)_2SO_4$ in buffer A. Catalase activity that eluted at 150 mM $(NH_4)_2SO_4$ was desalted by dilution and ultrafiltration, and was purified further on a second DEAE column (17 × 2.6) in which the catalase activity was eluted by an ammonium sulfate gradient running from 0 to 300 mM $(NH_4)_2SO_4$. The activity eluted off this column between about 110–230 mM $(NH_4)_2SO_4$. Isozymes I and III were then partially resolved by hydrophobic interaction on phenyl Sepharose CL-6B (26 × 1.6 cm). Isozyme I was enriched in the 500 mM $(NH_4)_2SO_4$ wash, while isozyme III eluted at 400 and 300 mM $(NH_4)_2SO_4$. Isozyme III was rechromatographed on phenyl Sepharose; peak fractions eluting at 400 mM $(NH_4)_2SO_4$ were pooled, concentrated and purified further by sucrose density centrifugation as detailed for isozyme II. The specific activity, 8000 U/mg at pH 6.5, was 70-fold that of the P_{50-60} fraction, with a yield of 0.46 mg and 3% of the starting activity. At this stage, isozyme III yielded a single band on SDS polyacrylamide gel electrophoresis with an M_r of 80000 on SDS 8% polyacrylamide gels. Native gel electrophoresis gave single, coincident bands for protein (Coomassie stain) and catalase activity.

2.9. Spectral characterization

Absorption spectra were taken at room temperature on a Perkin-Elmer Model 550 spectrophotomer. Heme was extracted from the native protein by treatment with 9 vols. of acetone/HCl (15 mM HCl in acetone).

2.10. Protein assay

Protein content of samples was estimated by the Lowry method [8], using bovine serum albumin as the standard.

3. Results

3.1. Catalase activity in cell extracts and induction of catalase activity

Crude extracts from cells grown at pH 7.5 and 10.5 to an A_{600} of 0.4–0.5 were assayed for catalase activity. pH-10.5-grown cells had a specific activity of 86.3 U/mg cell extract protein, or 1.85-fold higher than observed in extracts from pH-7.5-grown cells (Table 1). Some variability in catalase activity was observed in different preparations, which may be at least partly attributable to the finding that the specific activity increases during growth (data not shown) such that small differences in cell density may have resulted in differences in catalase activity.

Table 1
Induction of catalase activity by treatment of cells with H_2O_2 or sodium ascorbate [a]

Treatment	Induction (*x*-fold of control) [b]			
	pH 7.5 [c]		pH 10.5 [c]	
Control	1		1	
[specific activity]	[46.6 ± 13.4, $n = 6$] [d]		[86.3 ± 51.5, $n = 8$] [d]	
30 μM H_2O_2	8.3 ± 2.3	($n = 3$)	4.5	($n = 2$)
300 μM H_2O_2	32.5	($n = 2$)	30.7 ± 27.1	($n = 3$)
1500 μM H_2O_2	6.2 ± 5.0	($n = 3$)	1.5 ± 1.9	($n = 3$)
10 mM sodium ascorbate	2.3	($n = 2$)	32.5 ± 16.2	($n = 3$)

[a] See Materials and Methods for details on induction and preparation of cell extracts.
[b] Specific catalase activity of induced cells/specific catalase activity of control cells.
[c] Induction was carried out at the growth pH.
[d] Expressed as units (mg cell extract protein)$^{-1}$.

Nonetheless, the pH-dependent difference was consistently observed in the same direction. Other workers have noted a higher catalase content in alkaline vs. neutral grown cells in a different facultatively alkaliphilic *Bacillus* [9].

It was of interest to determine whether the higher activity in pH-10.5-grown cells was already expressed maximally or, as found in other systems [5], could be induced to higher levels. Exponentially-grown cultures of *B. firmus* OF4 were treated with different concentrations of H_2O_2 or with 10 mM sodium ascorbate as detailed in Materials and Methods. For these experiments, induction was carried out at the same pH as the growth pH. These treatments were capable of inducing catalase activity of pH-10.5-grown cells to different levels in a manner that was dependent on agent and concentration (Table 1). Induction was not observed at a high concentration of H_2O_2 (1500 μM) in pH-10.5-grown cells although that concentration did induce pH-7.5-grown cells. Ascorbate was only substantially effective at pH 10.5 (Table 1).

3.2. Hydrogen peroxide toxicity as a function of pH

To determine whether the higher catalase activity of pH-10.5-grown cells protected cells from exogenous H_2O_2, cultures of *B. firmus* OF4, grown at pH 7.5 or 10.5 to early exponential phase, were exposed to different H_2O_2 concentrations. To separate an effect of treatment pH from growth pH on H_2O_2 sensitivity, the cultures were split into two portions, harvested, and suspended in media at pH 7.5 or 10.5. Hydrogen peroxide was added to the cells, incubated for 15 min under aerobic conditions, and immediately plated to determine the percentage of cells that survived the challenge. As indicated in Fig. 1, the pH of the treatment strongly affected the sensitivity of cells to hydrogen peroxide. For example, the H_2O_2 concentration that killed 50% of cells grown at pH 7.5 was about 450 μM when the treatment was carried out at pH 7.5 but was about 250 μM with pH 10.5 treatment. Similarly, there was about a 2-fold difference with respect to treatment pH in the 50% killing concentration with cells grown at pH 10.5. In addition to the sensitizing effect of alkaline treatment pH, it is clear from Fig. 1 that alkaline growth pH increased the toxicity of H_2O_2. The 50% killing concentrations of H_2O_2 at pH 10.5 were about 250 μM H_2O_2 and 100 μM H_2O_2 for pH-7.5- and pH-10.5-grown cells, respectively. These data show that the higher catalase activity of alkaline-grown cells did not protect cells against a H_2O_2 challenge relative to H_2O_2-challenged cells grown at pH 7.5 regardless of the pH of the H_2O_2 treatment.

3.3. Identification of B. firmus OF4 catalase isozymes on native polyacrylamide gels

The catalase isozyme content of *B. firmus* OF4 cell extracts was determined on native polyacrylamide gels that were stained for catalase activity. Three major bands were observed on native gels. Assuming that these bands represented distinct catalase isozymes, protein purification was carried out as detailed in Materials and Methods to isolate each of the isozymes, which were labeled I–III in order of decreasing electrophoretic mobility. The three isozymes were purified and were found to have distinct N-terminal amino acid sequences. The purified proteins yielded the pattern of enzyme staining on native gels shown in Fig. 2 (right). The intensity of enzyme staining appeared to reflect the relative differences in specific activity of the isozymes. At their optimal pH, the ratio of specific activities was approx. 1:20:2 for isozymes I–III, respectively. Coomassie-stained native and SDS polyacrylamide gels of the purified isozymes are shown in Fig. 3. To determine which isozyme(s) was responsible for the higher catalase activity observed in pH-10.5-grown cells, three independent sets of cell extracts prepared from pH-7.5- and pH-10.5-grown cells were subjected to native gel analysis, as shown in the left side of Fig. 2. Isozyme II was clearly amplified in the alkaline-grown cells. In two of the three

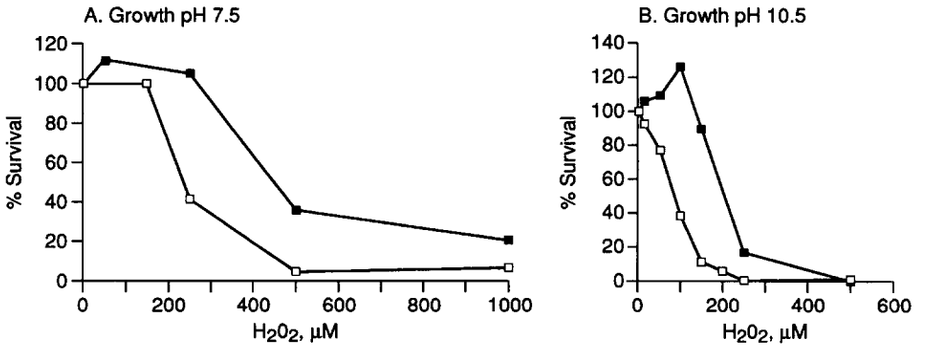

Fig. 1. Sensitivity of *B. firmus* OF4 to exogenous H_2O_2 as a function of the growth pH and/or the H_2O_2 treatment pH. (A) Cells grown to an A_{600} of 0.4 at pH 7.5 were suspended in pH 7.5 (■) or pH 10.5 (□) growth medium at the initial cell density, treated with the indicated concentrations of H_2O_2 or water as a control and plated at the pH equivalent to the treatment pH. (B) Cells grown at pH 10.5 to an A_{600} of 0.4 were treated at pH 7.5 (■) or at pH 10.5 (□); other conditions were identical to (A). The results are the averages of 2 or 3 experiments. Other procedures are described in Materials and methods.

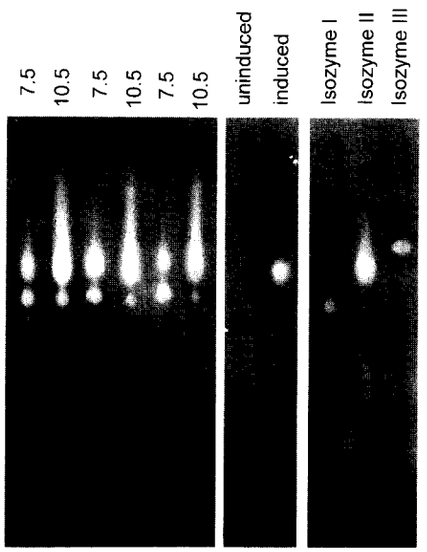

Fig. 2. Identification of catalase isozymes and their expression as a function of growth pH and induction in early exponentially-grown cells. In the left and middle portions of the figure, cell extracts were subjected to native polyacrylamide gel electrophoresis and stained for catalase activity. Left, three independent sets of pH-7.5- and 10.5-grown cells are shown (120 μg of protein per lane). Middle, 130 ng of cell extract protein was loaded in each lane from pH-10.5-grown cells that were either treated with water (uninduced) or sodium ascorbate (induced) as described in Materials and methods. Right, the purified isozymes (0.5 μg) were resolved on native polyacrylamide gels and stained for enzymatic activity.

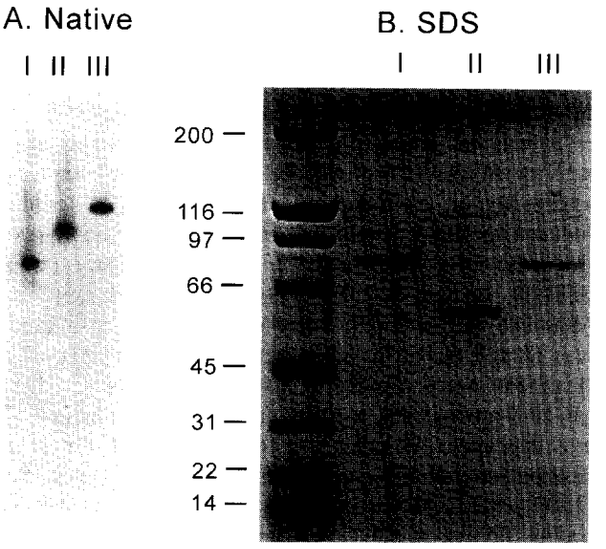

Fig. 3. Native and SDS polyacrylamide gel electrophoresis of the purified catalase isozymes. (A) The isozymes (2 μg) were electrophoresed on a native 5% polyacrylamide gel and stained for protein with Coomassie G-250. (B) The isozymes (1 μg) were resolved on a denaturing SDS 7% polyacrylamide gel and stained for protein with Coomassie G-250. Molecular mass markers are shown in the left margin in kDa.

sets, isozyme I was present at lower levels in pH-10.5-grown cells. In general, pH-10.5-grown cells appeared to have a much higher ratio of isozyme II to isozyme I than pH-7.5-grown cells. Isozyme II was induced by treatment of cells with ascorbate (Fig. 2, middle) or hydrogen peroxide (data not shown). A third isozyme, isozyme III, was only observed in stationary phase cells (data not shown).

3.4. Properties of the individual catalase isozymes

A similarity between isozyme I and a catalase purified from a different alkaliphile, *Bacillus* YN-2000 [9] was deduced on the basis of the N-terminal amino acid sequences obtained for the two proteins (Table 2). The absorption spectrum of isozyme I, shown in Fig. 4, had one major peak at 406 nm, with a minor, broad, peak evident at higher sensitivity (data not shown) centered at approx. 629 nm. The ratio of A_{406} to A_{280} was 0.57, similar to that found for the *Bacillus* YN-2000 catalase [9]. Isozyme I was reducible by dithionite, yielding the reduced vs. air oxidized difference spectrum shown in Fig. 4

Table 2
N-terminal amino acid sequence of *B. firmus* OF4 catalase isozymes: relationship to other catalases

Isozyme or related catalase	N-terminal amino acid sequence														
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
I	M	D	T	Q	N	N	E	[N]	A	[G]	[K]	X	[P]	[F]	T
Bacillus YN-2000 [a]	M	K	S	F	D	S	T	[N]	T	[G]	[K]	H	[P]	[F]	F
II	M	K	[K]	[L]	S	[T]	N	Q	[G]	[A]	[P]	X	Y	[D]	G
Bacillus subtilis [b]		N	[K]	[L]	T	[T]	S	W	[G]	[A]	[P]	V	G	[D]	N
		4	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
III [c]	S	N	E	R	E	M	Q	N	K	K	D	Q	Q	L	E

[a] Ref. [9].
[b] Deduced from nucleotide sequence (positions 4–17) (Ref. [10].)
[c] Identical to predicted sequence (positions 2–16) of a fragment of the gene isolated from *B. firmus* OF4 which has 67% identity in the first 449 amino acids to the HP-II catalase from *E. coli* [12].

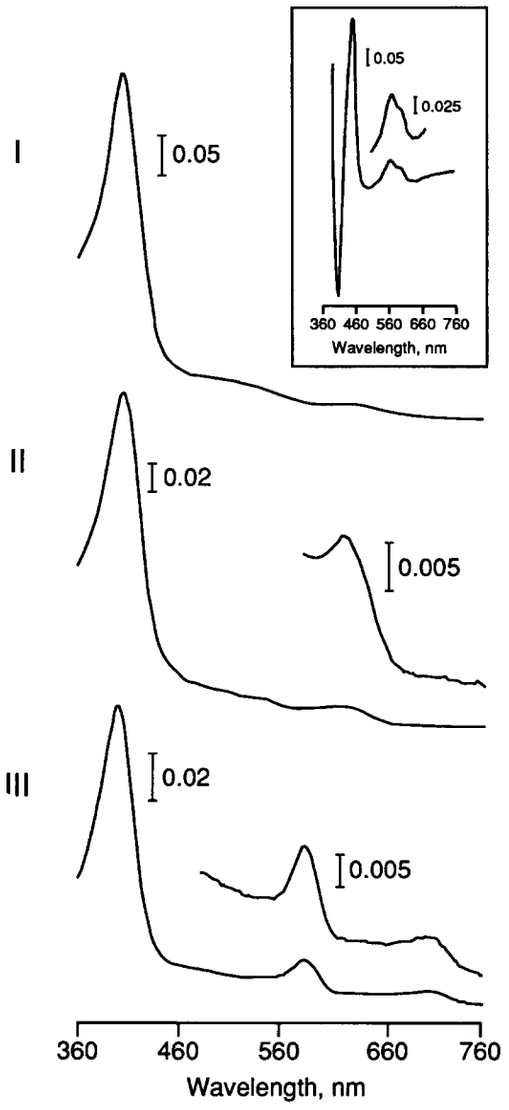

Fig. 4. Absorption spectra of the purified *B. firmus* OF4 catalase isozymes. Spectra were taken against a buffer reference. The inset is a reduced vs. oxidized difference spectrum of isozyme I. The sample cuvette was reduced with dithionite and the reference cuvette was air-oxidized.

(inset). The peaks at 560 and 439 nm and shoulder at 590 nm resembled that reported for the *Bacillus* YN-2000 catalase [9]. The absorption spectrum of the heme moiety extracted from the protein indicated that this isozyme contained protoheme (data not shown). Isozyme I was the only isozyme that catalyzed detectable levels of peroxidase activity with ABTS or guiacol as substrates, resembling the *Bacillus* YN-2000 catalase. Differences in the two catalases included the catalase pH optima and the K_m for H_2O_2. The *B. firmus* OF4 isozyme pH optimum was 2 pH units more alkaline than the pH 6.0 optimum of the *Bacillus* YN-2000 enzyme (data not shown), and the K_m was lower, 2 mM compared to 6.8 mM [9]. The subunit size of the *B. firmus* OF4 isozyme was also larger, about 84 000, compared to 73 000.

Seven of the first 15 amino acids of isozyme II were identical to the deduced amino acid residues in positions 4–17 in the gene designated *katA* encoding a vegetative catalase from *B. subtilis* [10] (Table 2). This latter gene was suggested [10] to encode the catalase enzyme characterized by Loewen and Switala [11] as catalase-1 from *B. subtilis*. The subunit molecular mass of isozyme II, 60 kDa, was similar to the size of the deduced *B. subtilis* product (65.8 kDa). The absorption spectrum showed a major peak at 404 nm (Fig. 4); a small peak was observed at 622 nm with greater sensitivity. The A_{404} to A_{280} ratio was 0.71, lower than the value of 1.0 observed with the *B. subtilis* catalase-1 [11]. Extraction of the heme by acetone/HCl and formation of the pyridine hemochrome gave a spectrum similar to that observed with bovine catalase, suggesting that this isozyme, like isozyme I, contained protoheme (data not shown). In other respects, the *B. firmus* OF4 enzyme resembled *B. subtilis* catalase-1. The K_m for H_2O_2, 36 mM, was similar to that of catalase-1, 40.1 mM, and both enzymes had broad pH optima [11]. However, the *B. firmus* OF4 catalase showed a somewhat more pronounced preference for highly alkaline pH (data not shown).

The N-terminal amino acid sequence of isozyme III was identical to that predicted from the nucleotide sequence of the gene from *B. firmus* OF4 that was described in the

Table 3
Properties of *B. firmus* OF4 catalase isozymes

Isozyme	Subunit mass (kDa)	Type of heme	K_m for H_2O_2 (mM)	pH optima	Absorption maxima (nm) [a]	Inducible by H_2O_2? [b]	Peroxidase activity? [c]	Comments
I	84	protoheme	2	8.0	406, 629	no	yes	may be down-regulated in early exponentially-grown cells at pH 10.5 relative to pH 7.5
II	60	protoheme	36	broad, ~ 8.0–10.5	404, 622	yes	no	up-regulated in early exponentially-grown cells at pH 10.5 relative to pH 7.5
III	80	chlorin	n.d. [d]	broad, < 6.5	402, 584, 705	no	no	expressed only in stationary phase

[a] Major peaks are underlined.
[b] In early exponential growth phase cells.
[c] With either ABTS or guiacol as substrates; see Materials and methods for details.
[d] n.d., not determined (apparent K_m too high to be determined by spectrophometric assay).

introduction. The predicted amino acid sequence of the gene showed 67% identity in the first 449 amino acids with the HP-II catalase from *E. coli* [12], a chlorin-containing catalase whose expression is turned on in stationary phase and is controlled by KatF [13]. The absorption spectrum of isozyme III, shown in Fig. 4, had maxima at 705, 584 and 402 nm. The A_{402}/A_{280} ratio was 0.63, somewhat higher than the value of 0.42 found for the chlorin-containing *B. subtilis* catalase-2 [14]. The absorption peak at 584 nm resembled that found in the chlorin-containing catalases of *E. coli* and *B. subtilis*, catalase-2 (590 and 588 nm, respectively) [14,15]. The heme of isozyme III could be extracted with acetone/HCl, unlike the heme from catalase-2 of *B. subtilis* which appeared to be either covalently or very tightly associated with the apoprotein [14]. The extracted heme exhibited peaks at 599 and 382 nm; pyridine addition shifted those peaks to 598 and 403 nm, respectively (data not shown). These spectral peaks were similar to those exhibited by the heme isolated from HP-II [15]. Thus, the heme of isozyme III was likely a chlorin-type rather than protoheme. Further evidence that isozyme III was related to HP-II was its pattern of expression; isozyme III was only detected in cell extracts from stationary phase cells. The subunit size of III, 80 000, was more similar to the catalase-2 of *B. subtilis*, which was 81 000 [14], than HP-II, 92 000 [15]. Like the latter two chlorin-containing catalases [14,15], isozyme III of *B. firmus* OF4 showed a very broad pH optimum for catalase activity but the alkaliphile enzyme was distinctive in preferring acid pH for highest activity (data not shown).

4. Discussion

A summary of the properties of the three isozymes is given in Table 3. Unlike *E. coli* and *B. subtilis*, which are thought to possess two catalase isozymes, *B. firmus* OF4 clearly expresses at least three isozymes. Three isozymes were also demonstrated in *Klebsiella pneumoniae* [16] but to our knowledge this is the first report of three purified enzymes in a *Bacillus*, as well as a determination of their N-terminal amino acid sequences and their growth phase expression. In *B. firmus* OF4, isozyme II was expressed at higher levels in cells grown at high pH and was induced in cells treated with by H_2O_2 or ascorbate. Whether isozyme II is involved in the oxidative stress response under the conditions used in this study remains to be determined, although it is duly noted that the related catalase gene product from *B. subtilis*, KatA, was found to serve a protective role in similar H_2O_2 challenge experiments [17]. Clearly, the experiments shown in Fig. 1 indicate that, regardless of which isozyme(s) are involved in the oxidative stress response, alkaline growth pH and alkaline treatment pH sensitize cells to exogenous H_2O_2 challenge, in spite of the fact that pH-10.5-grown cells possessed a higher specific catalase activity. A different role for isozyme I may be related to its relatively low K_m for H_2O_2; it might therefore function to remove low levels of metabolically generated H_2O_2. It is also conceivable that the function of this isozyme, which was related to the catalase/peroxidase characterized from alkaliphilic *Bacillus* YN-2000 [9], has more to do with its peroxidatic activity as opposed to its catalatic activity. Another catalase from an alkaliphilic *Bacillus* has been isolated by Kurono and Horikoshi [18], but its N-terminal amino acid sequence has not been published, so that its relationship to this or the other isozymes of *B. firmus* OF4 cannot be assessed. The expression of chlorin-containing isozyme III is interesting in that another putative chlorin-containing enzyme of *B. firmus* OF4, cytochrome *bd* oxidase, was also expressed highly in stationary phase [19]. The genes encoding these two enzymes may be under some sort of common, global regulation related to stationary phase growth. In addition, the gene encoding catalase isozyme III is preceded by a consensus *fur*-binding element, linking it to a discrete system of regulation [20] that is not yet implicated in connection with any of the alkaliphile cytochromes.

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