Purification and characterization of catalase-1 from *Bacillus subtilis*

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The catalase activity produced in vegetative *Bacillus subtilis*, catalase-1, has been purified to homogeneity. The apparent native molecular weight was determined to be 395 000. Only one subunit type with a molecular weight of 65 000 was present, suggesting a hexamer structure for the enzyme. In other respects, catalase-1 was a typical catalase. Protoheme IX was identified as the heme component on the basis of the spectra of the enzyme and of the isolated hemochromogen. The ratio of protoheme/subunit was 1. The enzyme remained active over a broad pH range of 5–11 and was only slowly inactivated at 65°C. It was inhibited by cyanide, azide, and various sulfhydryl compounds. The apparent K_m for hydrogen peroxide was 40.1 m*M*. The amino acid composition was typical of other catalases in having relatively low amounts of tryptophan and cysteine.

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Nous avons purifié jusqu'à homogénéité la catalase-1, l'activité catalasique manifestée par *Bacillus subtilis*, une bactérie végétative. Le poids moléculaire apparent de l'enzyme native est de 395 000. La présence d'un seul type de sous-unité avec un poids moléculaire de 65 000 suggère une structure hexamère pour cette enzyme. À d'autres égards, la catalase-1 est une catalase typique. Sur la base des spectres de l'enzyme et de l'hémochromogène isolé, nous avons identifié la portion hème comme étant le protohème IX. Le rapport entre le protohème et la sous-unité est de 1. L'enzyme demeure active à des pH allant de 5 à 11 et elle est lentement inactivée à 65°C. Elle est inhibée par le cyanure, l'azide et divers composés sulphydrylés. Le K_m apparent pour le peroxyde d'hydrogène est de 40,1 m*M*. La composition en acides aminés est typique des autres catalases en ayant des quantités relativement faibles de tryptophane et de cystéine.

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Introduction

Several organisms including *Escherichia coli* (Claiborne and Fridovich 1979; Claiborne *et al.* 1979), yeast (Seah *et al.* 1973; Seah and Kaplan 1973) and maize (Scandalios *et al.* 1980) produce more than one catalase (EC 1.11.1.6), whereas others, including *Proteus mirabilis* (Jouve *et al.* 1983, 1984) and *Neurospora crassa* (Jacob and Orme-Johnson 1979), produce just one catalase. Several of these enzymes have been purified and characterized, revealing that catalase-T from yeast (Seah and Kaplan 1973) and the catalases from *P. mirabilis* (Jouve *et al.* 1983, 1984), *Rhodopseudomonas spheroides* (Clayton 1959), and *Micrococcus lysodeikticus* (Herbert and Pinsent 1948) all closely resembled the enzyme from bovine liver (Deisseroth and Dounce 1970) in size, heme content, and catalytic properties. Other catalases were found to have different properties including catalase-A from yeast with a smaller subunit size (Seah *et al.* 1973), the catalase from *N. crassa* with larger subunits and a heme-*d*-like component (Jacob and Orme-Johnson 1979), catalase HPI from *E. coli* with larger subunits and half the normal heme content (Claiborne and Fridovich 1979), and catalase HPII also from *E. coli* with a heme-*d*-like group, larger subunits, and a hexameric structure (Loewen and Switala 1986).

Recently *Bacillus subtilis* was found to produce two main species of catalase (Loewen and Switala 1987). One of the main activity bands visualized on polyacrylamide gels, labelled catalase-1, was produced in vegetative cells. The second main activity, labelled catalase-2, was a series of isoenzyme bands with similar molecular weights that were the exclusive activity in purified spores. A combination of biochemical data including different molecular weights, electrophoretic mobilities, apparent K_ms for H_2O_2, and genetic data, including the isolation of mutants selectively lacking one or the other activity, was used to confirm the uniqueness of the two catalase activities.

A comprehensive study of catalase gene expression in *B. subtilis* is being undertaken, including the mapping and cloning of the genes encoding the two catalases. As a first step in this study, the enzymes must be physically characterized to relate them to previously studied enzymes and to provide a reference for the physical characterization of the genes. This report describes the purification of catalase-1, the enzyme present in vegetative *B. subtilis*, and its characterization. Catalase-1 was found to be similar to bovine catalase in many respects but differed in having a hexameric structure.

ABBREVIATIONS: LB, Luria-Bertani broth; SDS, sodium dodecyl sulfate; 2 × SG, modified Schaeffer medium.

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TABLE 1. Purification of catalase-1 from 82 g of cell paste of vegetative *B. subtilis* 168

	Vol. (mL)	Total protein (mg)	Total units $\times 10^{-5}$	Yield (%)	Specific activity ($U \cdot mg^{-1}$)	Purification (*n*-fold)
1. Crude extract	440	5980	32.3	100	540	1.0
2. 2.5% streptomycin sulfate	425	4100	21.2	66	518	1.0
3. $(NH_4)_2SO_4$ fractionation I						
40–50% pellet	38	932	47.50	147	5 096	9.4
50–60% pellet	29	901	19.8	61	2 197	4.1
4. $(NH_4)_2SO_4$ fractionation II of 50% pellet in step 3						
30–40% pellet	15	288	36.7	114	12 738	23.6
40–50% pellet	15	317	20.1	62	6 566	12.2
5. DEAE-Sephadex A-50	5	24.8	21.7	67	87 542	162.1
6. Bio-Gel A-1.5m	24.5	10.5	12.8	40	122 014	226.0
7. Bio-Gel HTP	1.8	3.3	10.2	32	172 037	318.6

Materials and methods

Materials

Bactotryptone and yeast extract were obtained from Gibco. DEAE-Sephadex A-50 and the most common biochemicals were obtained from Sigma. Sepharose 4B was obtained from Pharmacia. Bio-Gel A-1.5m, Bio-Gel HTP, and acrylamide were obtained from Bio-Rad. Ultrapure guanidine hydrochloride was obtained from BRL.

Growth conditions

A preculture of *B. subtilis* 168 was grown overnight with shaking at 37°C in LB medium (Miller 1974) containing 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per litre. Batches of 500 mL LB medium in 2-L erlenmeyer flasks were inoculated with 10 mL of the preculture and grown with shaking at 37°C. Growth was monitored using a Klett-Summerson colorimeter with a blue filter. By weighing culture samples after drying at 100°C and correcting for LB medium weight, it was calculated that 100 Klett units represented 0.15 $mg \cdot mL^{-1}$ (dry cell weight). When the cell density reached approximately 600 Klett units, four additions of H_2O_2 were made at 10-min intervals, resulting in the H_2O_2 concentration being raised by 0.1 m*M* at each addition. Catalase levels were monitored (see below) and when maximum specific activity was reached, the cells were harvested. The normal yield was 5 g cell paste $\cdot L^{-1}$ of medium.

Purification of catalase-1

The procedure followed was essentially that of Claiborne and Fridovich (1979), as applied to the purification of catalase HPII of *E. coli* (Loewen and Switala 1986). All manipulations were carried out at 5°C. The crude extract was prepared using the Aminco French Press at 20 000 psi (1 psi = 6.895 kPa) and a 2.5% streptomycin sulfate precipitation was carried out. Repeated ammonium sulfate precipitations were carried out to maximize the enzyme specific activity at this stage. The first fractionation was carried out at 30, 40, 50, and 60% saturation with solid ammonium sulfate. At each step, the solution was stirred gently for 30 min to ensure complete dissolution and precipitation. The precipitate was collected by centrifugation and additional ammonium sulfate was added to the supernatant. Most catalase-1 was found in the 50 and 60% pellets and, following dissolution, the 50% pellet was fractionated a second time with ammonium sulfate, resulting in most catalase being found in the 40 and 50% pellets. The 40% pellet was used in the subsequent purification steps as described below. The other pellets, including the 60% pellet from the first fractionation and the 50% pellet from the second fractionation, could be dissolved and subjected to additional ammonium sulfate fractionations to raise the specific activity prior to being subjected to column chromatography (data not shown).

The 40% pellet was dissolved, dialyzed for 2 h against 4 L of 50 m*M* potassium phosphate (pH 7.0), and charged on a 2.8 × 55 cm column of DEAE-Sephadex A-50 equilibrated in the same buffer. The column was washed until the absorbance of the eluate at 280 nm had dropped below 0.05. The catalase was eluted with a linear gradient prepared by mixing 50 m*M* potassium phosphate (pH 7.0) with 0.5 *M* NaCl in the same buffer. The peak of catalase activity was pooled, concentrated by ultrafiltration (Amicon), and loaded on a 2.2 × 100 cm column of Bio-Gel A-1.5m equilibrated with 5 m*M* potassium phosphate (pH 7.0). The peak of catalase activity was pooled and immediately applied, without further concentration or dialysis, to a 2.2 × 10 cm column of Bio-Gel HTP equilibrated with 5 m*M* potassium phosphate (pH 7.0). The column was eluted with a linear gradient prepared by mixing equal volumes of 5 and 400 m*M* potassium phosphate (pH 7.0). The catalase-1-containing eluate was concentrated by ultrafiltration, dialyzed for 4 h against 1 L of 50 m*M* sodium phosphate (pH 7.0), and stored at −20°C. A summary of the results from the purification of catalase-1 from *B. subtilis* 168 is contained in Table 1.

Native molecular weight analysis

Three independent methods were used. The sedimentation equilibrium method of Yphantis (Yphantis 1964; Chervenka 1969) in a Beckman model E ultracentrifuge at 20°C was used. Samples were dialyzed against 50 m*M* potassium phosphate (pH 7.0). The dialyzed samples were run at 0.2, 0.5, and 0.8 $mg \cdot mL^{-1}$ in a six-sector cell, with dialyzate used as reference. A partial specific volume of 0.73 $mL \cdot g^{-1}$ was calculated based on the amino acid composition (Chervenka 1969).

The method of Hedrick and Smith (1968) was also used, in which the protein was electrophoresed as described by Davis (1964), except in pH 8.1 Tris–HCl and on 15 × 15 cm slab gels, on a series of gels of various acrylamide concentrations from 6 to 12%. The gels were stained for protein with Coomassie brilliant blue dye or for catalase as described below. The R_f of the protein relative to the bromphenol blue tracking dye was determined for each different percentage gel. The retardation coefficient for each protein was determined from the slope of a graph of 100 log $100R_f$ against acrylamide concentration. The logarithm of the retardation coefficient was then plotted against the logarithm of the protein molecular weight.

Lastly, a gel filtration method was used. A 0.7 × 110 cm column of Sepharose 4B was equilibrated with 10 m*M* Tris – 70 m*M* glycine (pH 8.1). The elution volumes V_d were determined by absorbance at 280 nm or by assaying for catalase activity, and blue dextran was used to determine the void volume V_0. A 0.7 × 110 cm column of Bio-Gel A-1.5m equilibrated with 50 m*M* potassium phosphate (pH 7.0) was also used.

Denatured molecular weight analysis

Samples were mixed with an equal volume of sample buffer containing 0.1 *M* sodium phosphate (pH 7.0), 1% SDS, 0.14 *M* 2-mercaptoethanol, and 7 *M* urea and boiled for 2 min. This solution was loaded on a discontinuous 15 × 15 cm slab gel composed of a 4% stacking gel at pH 6.8 and an 8% running gel at pH 8.8, with both layers containing 0.1% SDS as described by Laemmli (1970) and Weber *et al.* (1972). Electrophoresis was at 2 mA·cm^{-1} of gel length until the bromphenol blue marker dye reached the bottom of the gel, after which the gel was stained with Coomassie brilliant blue.

Visualization of catalase and peroxidase activity on polyacrylamide gels

Following electrophoresis on nondenaturing 8.5% polyacrylamide gels as described above, peroxidase activity was visualized by the method of Gregory and Fridovich (1974) and catalase was stained by the method of Clare *et al.* (1984), but using 20 m*M* H_2O_2 for better contrast.

Catalase assay and protein determination

Catalase activity was determined by the method of Rorth and Jensen (1967) using a Gilson oxygraph equipped with a Clark electrode. One unit of catalase is defined as the amount that decomposes 1 μmol of H_2O_2 in 1 min at 37°C. Protein was estimated by the methods outlined by Layne (1957).

Hemochromogen characterization

Optical spectra were obtained using a Beckman DU8 spectrophotometer. Following extraction with acetone–HCl and then diethyl ether, the iron content of the ether-extracted hemochromogen was assayed by the method of Suzuki and Silver (1966).

Amino acid analysis

Samples containing 1 nmol of catalase-1 subunit were mixed with constant boiling HCl, sealed in a glass tube, and incubated at 110°C for 24, 48, and 72 h. For cysteic acid determination, 1 nmol of catalase-1 was oxidized for 20 h at 5°C in peformic acid (prepared by mixing 0.2 mL of 30% H_2O_2 with 1.8 mL of 90% formic acid), followed by lyophilization and hydrolysis. The amino acid analyses were performed on a LKB 4151 Alpha Plus amino acid analyzer operated by the Department of Animal Science, University of Manitoba.

TABLE 2. Catalase levels in *B. subtilis* 168

Medium	Klett units	Catalase (U·mg dry cell weight^{-1})
2 × SG	400	11.1
2 × SG	600	10.4
LB	400	73.1
LB	600	273.3
LB[a]	680	406.2

[a] At Klett of 600 and at three subsequent 10-min intervals, H_2O_2 was added to a final concentration of 0.1 m*M*. At 40 min after the first addition, the Klett value and catalase activity were determined.

Results

Growth conditions

During growth in 2 × SG sporulation medium, *B. subtilis* was found to produce more than one catalase (Loewen and Switala 1987). If growth was restricted to log phase, only catalase-1 was present in extracts, but the levels were relatively low. In an attempt to delay the sporulation-specific catalases and to increase the amount of catalase-1 present, growth in LB medium which does not allow efficient sporulation was investigated. It was found that cells grown into late log phase produced only catalase-1 and that the levels were higher than in sporulation medium. The yield of catalase-1 could be further optimized by adding H_2O_2 four times at 10-min intervals to a final concentration each time of 0.1 m*M*. Hydrogen peroxide has been shown to specifically induce catalase-1 in mid-log cells and there was a similar induction in late log cells, although the extent of induction in late log cells was not as great because the levels were already elevated (Table 2). This procedure of inducing catalase synthesis in late log cells just prior to harvesting yielded up to 5 g cell paste·L^{-1} of medium, exhibiting 400 U·mg dry cell weight^{-1}. Visualization of catalase activities in crude extracts of the cell paste followed electrophoretic separation on polyacrylamide gels revealed only catalase-1.

Purification

The procedure of Claiborne and Fridovich (1979) developed for the purification of catalase HPI from *E. coli* and adapted for the purification of catalase HPII (Loewen and Switala 1986) was used, as described in Materials and methods. As was found for catalase HPII, a repetition of the ammonium sulfate precipitation step was important in achieving a 20-fold purification prior to column chromatography. In part, this large degree of purification was the result of removing an inhibitory

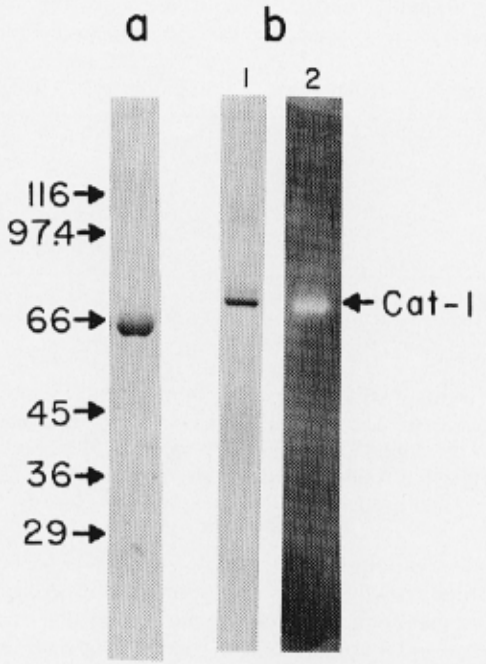

FIG. 1. Electrophoresis of purified catalase-1 (Cat-1) from *B. subtilis* 168 on an 8% SDS–polyacrylamide gel (*a*) and 8.5% nondenaturing polyacrylamide gels (*b*). In *a*, 25 μg of protein was electrophoresed following denaturation and the gel was stained for protein. The numbers indicate the location of the following molecular weight standards ($\times 10^{-3}$): β-galactosidase, phosphorylase B, bovine plasma albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, carbonic anhydrase. In *b*, 15 μg of protein was electrophoresed in lane *1* and stained for protein, while 1.5 U or 8 ng of protein was electrophoresed in lane *2* and stained for catalase activity.

component, a conclusion supported by the presence of more total activity following the fractionation than was present in the crude extract. The purification and yields of catalase-1 at each step in the protocol are outlined in Table 1. The final 319-fold purification compared favorably with the purification of other bacterial catalases (Claiborne *et al.* 1979; Clayton 1959; Herbert and Pinsent 1948; Jouve *et al.* 1983; Loewen and Switala 1986). The observed specific activity of 172 000 U·mg protein^{-1} was 100-, 10-, and 2-fold higher than the activities reported for catalase HPI (Claiborne and Fridovich 1979), catalase HPII (Loewen and Switala 1986) and the catalase from *P. mirabilis* (Jouve *et al.* 1983), respectively. It was also possible to isolate homogeneous batches of catalase-1 with significantly lower specific activity (e.g., 68 100 U·mg protein^{-1}). The reason for this reduced activity in some batches will be discussed below. Electrophoresis of catalase-1 on polyacrylamide gels revealed, under denaturing conditions, a single predominant band of protein (Fig. 1*a*)

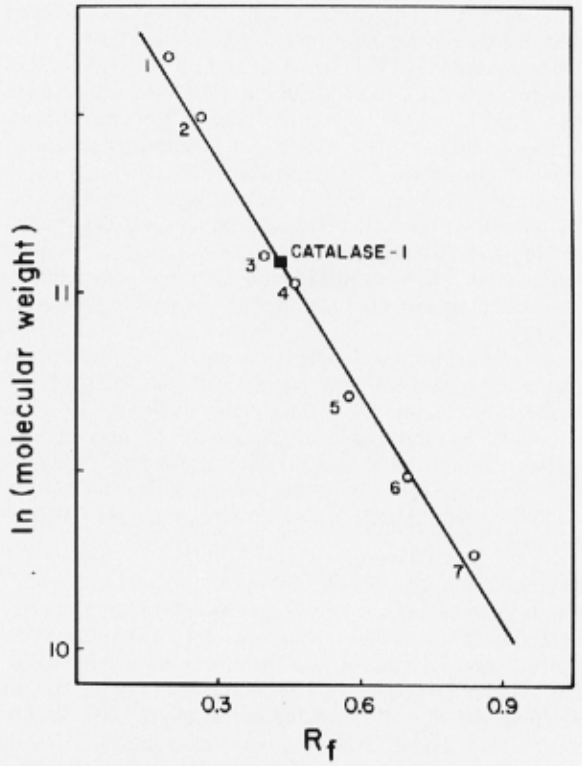

FIG. 2. Determination of the subunit molecular weight of purified catalase-1 from a denaturing SDS–polyacrylamide gel. Purified enzyme was electrophoresed as shown in Fig. 1*a* alongside standards and the R_f values relative to the bromophenol blue tracking gel were compared. The standards used were as follows: *1*, β-galactosidase; *2*, phosphorylase B; *3*, bovine plasma albumin; *4*, bovine catalase; *5*, egg albumin; *6*, glyceraldehyde-3-phosphate dehydrogenase; *7*, carbonic anhydrase. The molecular weight determined for catalase-1 was 65 000 ± 1000.

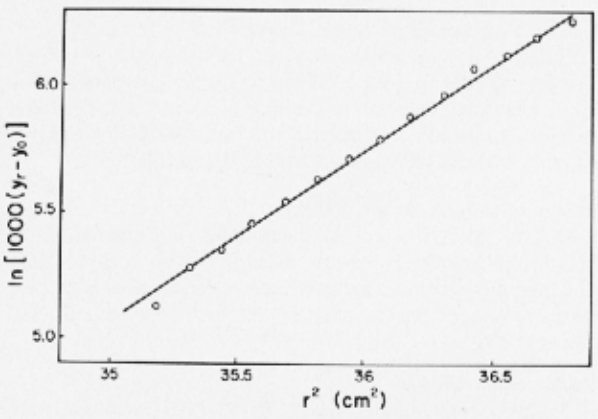

FIG. 3. Determination of the nondenatured molecular weight of catalase-1 by sedimentation equilibrium at 5600 rpm. The apparent molecular weight of 392 000 was calculated as outlined by Chervenka (1969).

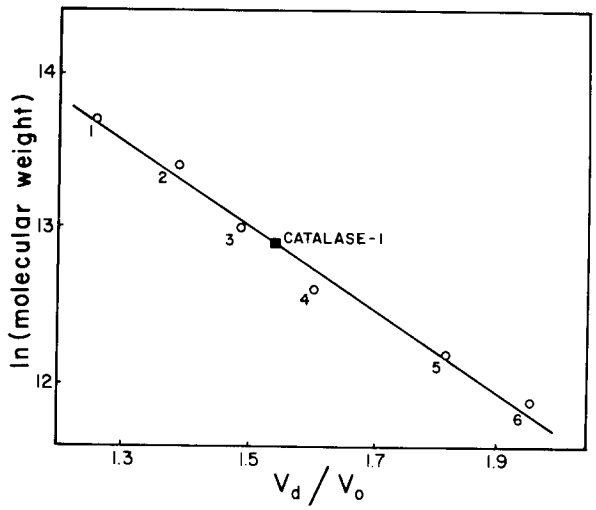

FIG. 4. Determination of the nondenatured molecular weight of catalase-1 by elution from Sepharose 4B equilibrated with Tris–glycine buffer. The standards used were as follows: *1*, apoferritin (dimer), 886 000; *2*, thyroglobulin (tetramer), 669 000; *3*, apoferritin (monomer), 443 000; *4*, alcohol dehydrogenase (dimer), 300 000; *5*, α-amylase, 200 000; *6*, alcohol dehydrogenase (monomer), 150 000. The molecular weight determined for catalase-1 was 400 000 ± 15 000.

and, under nondenaturing conditions, also a single band of protein that migrated coincident with the band of catalase activity (Fig. 1*b*). The purified enzyme exhibited no detectable peroxidase activity.

Subunit size determination

A number of proteins of known molecular weights were electrophoresed alongside catalase-1 on denaturing SDS–polyacrylamide gels (Fig. 1*a*) and the R_f values relative to bromphenol blue dye were compared (Fig. 2). An apparent subunit size of 65 000 was determined, which was very similar to the subunit size of most other catalases.

Nondenatured size determination

Three independent methods were used to determine the nondenatured size of catalase-1. The sedimentation equilibrium method revealed an apparent molecular weight of 392 000 (Fig. 3). Similar molecular weights were obtained at two different rotor speeds, 5600 and 6000 rpm, and at different enzyme concentrations. A second method involving gel filtration on Sepharose 4B revealed an apparent molecular weight of 400 000 (Fig. 4). Gel filtration on Bio-Gel A-1.5m yielded a similar value. A third method correlated the rate of migration with molecular weight on a series of different percentage polyacrylamide gels, revealing an apparent molecular weight of 205 000 (Fig. 5), which was approximately half the size determined by the other two techniques. A similar observation was made for both catalases, HPI and HPII, from *E. coli*, for which the migratory rates on gels indicated a size that was half of what was observed on columns or during centrifugation (Loewen and Switala 1986). A possible explanation proposed was that the Tris–glycine buffer being somewhat chaotropic

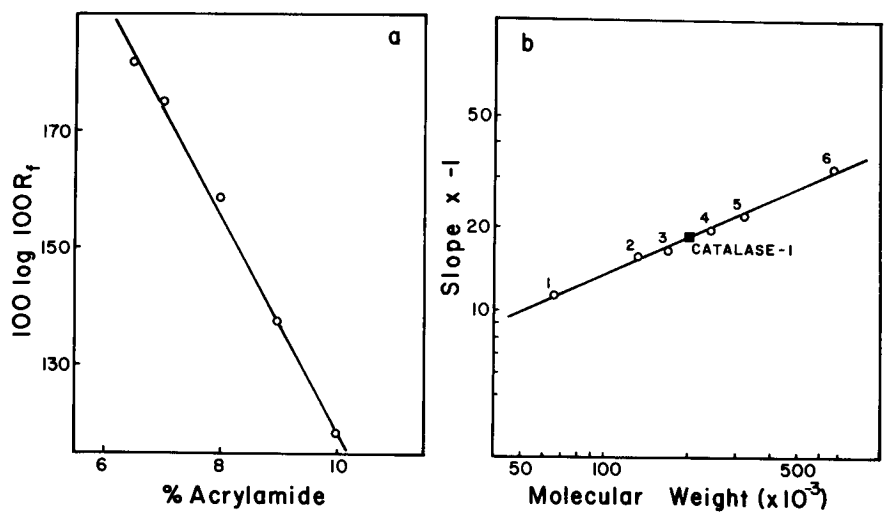

FIG. 5. Determination of the nondenatured molecular weight of purified catalase-1 by electrophoresis on native polyacrylamide gels. (*a*) Effect of different acrylamide concentrations on the mobility of purified catalase-1. (*b*) Determination of the molecular weight of catalase-1 from the slope determined in *a*. The proteins used as molecular weight standards were as follows: *1*, bovine plasma albumin (monomer); *2*, bovine plasma albumin (dimer); *3*, catalase HPI, 170 000; *4*, bovine catalase, 240 000; *5*, thyroglobulin (dimer), 335 000; *6*, thyroglobulin (tetramer). The molecular weight determined for catalase-1 was 205 000 ± 5000.

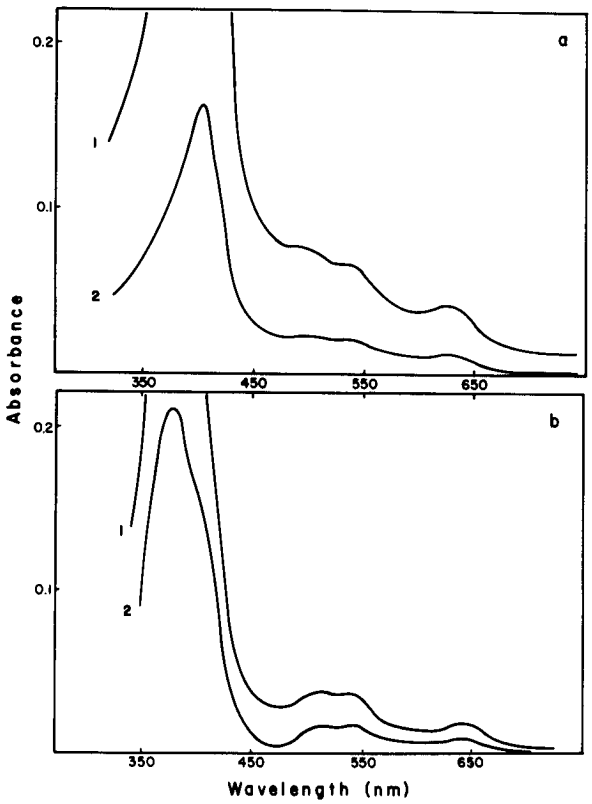

FIG. 6. Optical absorption spectra of purified catalase-1 (*a*) and the hemochromogen extracted in acetone–HCl (*b*). Catalase-1 was present at 9.2×10^{-7} (curve *1*) and 3.2×10^{-7} *M* (curve 2) in 50 m*M* sodium phosphate (pH 7.0) in *a*. In *b*, 1.3 nmol of catalase-1 was extracted with 0.015 *M* HCl in acetone and the spectrum was determined following removal of the precipitated protein (curve *1*) and following dilution by a factor of 2 in the same solution (curve 2).

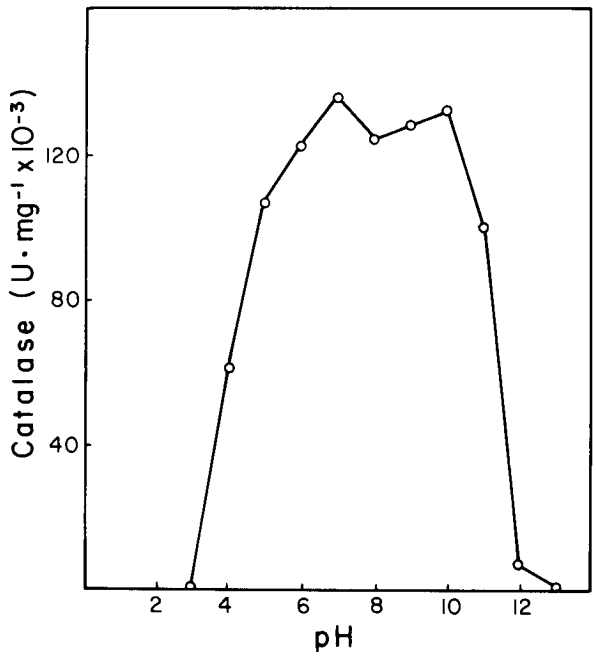

FIG. 7. Determination of the activity of purified catalase-1 as a function of pH. The enzyme was incubated in 50 m*M* potassium phosphate buffer at the various pH values for 1 min prior to commencing the assay by adding 60 m*M* H_2O_2.

caused the enzymes to dissociate to half size, either dimer or trimer. Some other undefined property of electrophoresis may also have contributed to the dissociation. Whether the trimer form of catalase-1 was the active form stained on the gel or there was a reassociation of trimers to form an active hexamer during staining could not be determined.

Heme identification and quantitation

The absorbance spectrum of purified catalase-1 is shown in Fig. 6*a* and is very similar to the spectra of catalases isolated from other sources (Claiborne and Fridovich 1979; Clayton 1959; Deisseroth and Dounce 1970; Jouve *et al.* 1984). The Soret band at 406 nm and smaller peaks at 504, 540, and 627 nm exhibited molar absorptivities of 100.7×10^3, 14.6×10^3, 12.7×10^3, and 8.1×10^3 $M^{-1} \cdot cm^{-1} \cdot subunit^{-1}$, respectively. The molar absorptivity of the Soret band was similar to that reported for other protoheme-IX-containing catalases with one heme per subunit. The absorbance ratio (A_{406}/A_{280}) of homogeneous catalase-1 was 1.0, also consistent with a catalase structure containing one heme per subunit. The spectrum of the isolated heme both in acetone–HCl (Fig. 6*b*) and as the pyridine hemochromogen was identical to the spectra of heme isolated from other catalases (Claiborne and Fridovich 1979; Deisseroth and Dounce 1970; Jouve *et al.* 1984; Smith 1975) and from hemoglobin. It was, therefore, concluded that catalase-1 was similar to most other catalases in containing up to one protoheme IX group per subunit.

To further substantiate the ratio of one heme per subunit, the heme was extracted and subjected to iron quantitation revealing 0.9–1.1 iron atoms per subunit. These values were obtained using diethyl ether extracted heme to exclude any nonheme iron. The preparation of catalase that was homogeneous by gel electrophoresis but had a 60% lower specific activity of 68 100 U·mg protein^{-1} was found to contain 0.7 iron atoms per subunit and exhibited an absorbance ratio (A_{406}/A_{280}) of 0.74. At least a portion of the reduction in activity could therefore be ascribed to the reduced heme content. Preparations of catalase from *P. mirabilis* (Jouve *et al.* 1984) and bovine liver (Deisseroth and Dounce 1970) have also been reported with less than the expected ratio of one heme per subunit.

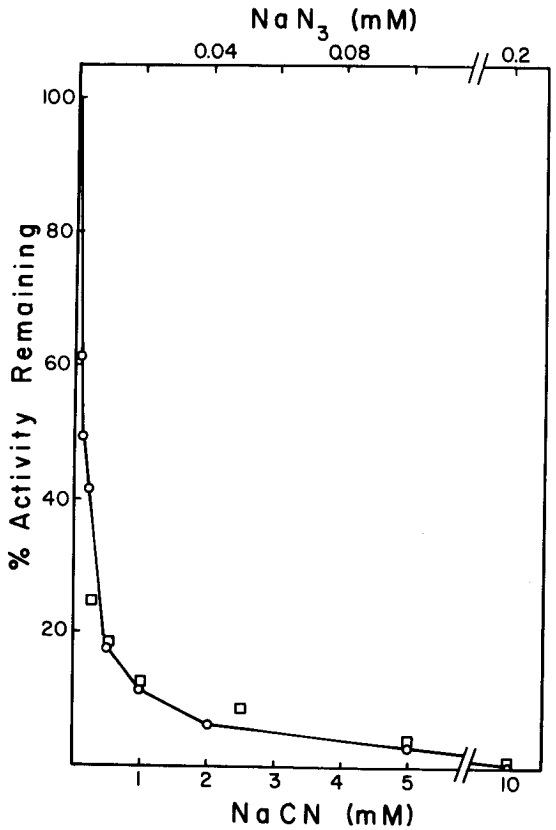

FIG. 8. Determination of the activity of purified catalase-1 in the presence of sodium cyanide (○) and sodium azide (□). The enzyme was incubated with the inhibitor in 50 m*M* sodium phosphate (pH 7.0) at 37°C for 1 min prior to commencing the assay by adding H_2O_2. Data are expressed as the percentage of initial activity remaining.

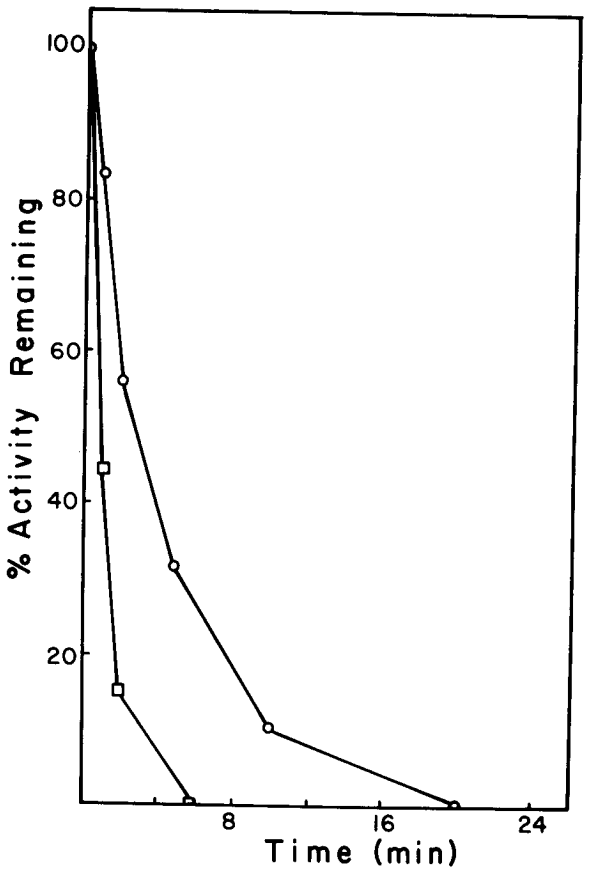

FIG. 9. Determination of the activity of purified catalase-1 (○) and bovine catalase (□) during incubation at 65°C. The enzymes were incubated at 65°C in 50 m*M* sodium phosphate buffer (pH 7.0) and aliquots were removed at various times and assayed. Data are expressed as the percentage of initial activity remaining.

Properties of catalase-1

A number of properties of catalase-1 were investigated as a further means of relating it to other catalases. It exhibited a broad pH optimum with little change in specific activity from pH 5 to 11 (Fig. 7), similar to the broad pH optimum exhibited by catalase HPII of *E. coli* (Loewen and Switala 1986). Catalase-1 was sensitive to both sodium cyanide and sodium azide, experiencing 50% inhibition at 0.1 m*M* cyanide and 0.002 m*M* azide (Fig. 8) and indicating that it was more sensitive to azide but less senstive to cyanide than the catalase from *P. mirabilis* (Jouve *et al.* 1983). Incubation at 65°C resulted in a complete inactivation of catalase-1 only after 20 min, whereas bovine catalase was completely inactivated within 2 min (Fig. 9). Catalase-1 was somewhat more sensitive to thiol reagents than the enzyme from *P. mirabilis* (Jouve *et al.* 1984), although the two catalases were similar in being more sensitive to 2-mercaptoethanol than glutathione or dithiothreitol (Fig. 10). The apparent K_m of catalase-1 for H_2O_2 was determined to be 40.1 m*M*, a value very similar to the 36.6 m*M* determined in crude extracts containing only catalase-1 (Table 3).

Amino acid content

The amino acid content of purified catalase-1 was determined and the results are shown in Table 4. For comparison the amino acid compositions of the catalases from bovine liver and *P. mirabilis* are also included. It can be seen that the compositions are similar, notably in the low cysteine and tryptophan contents, despite the diverse sources of the enzymes.

Discussion

Catalase-1 from *B. subtilis* was similar in many respects to catalases isolated from other sources. For example, one protoheme IX group per subunit was present, a common feature of most catalases. Further-

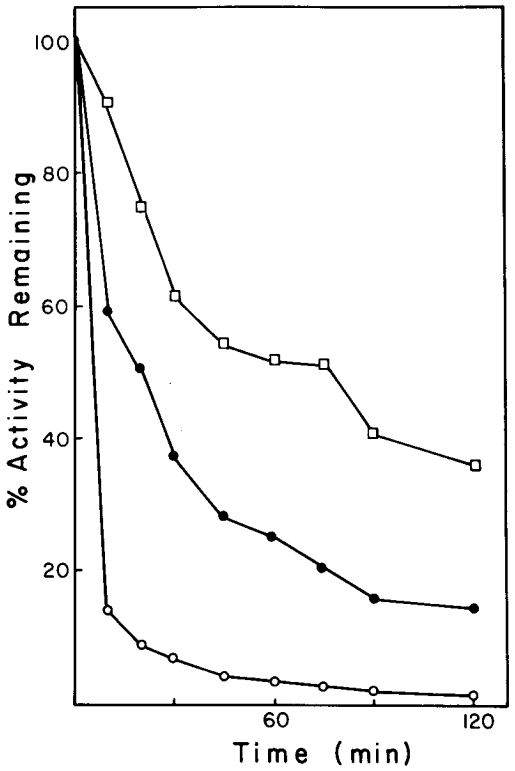

FIG. 10. Determination of the activity of purified catalase-1 in the presence of 2-mercaptoethanol (○), glutathione (●), and dithiothreitol (□), all at 5 m*M*. The enzyme was incubated at 37°C in 50 m*M* sodium phosphate buffer (pH 7.0) in the presence of the thiol reagent and aliquots were removed at various times and assayed. Data are expressed as the percentage of initial activity remaining.

TABLE 3. Variation of reaction velocity with H_2O_2 concentration for determination of the apparent K_m

$[H_2O_2]$ (m*M*)	$[H_2O_2]^{-1}$	$V \times 10^{-3}$ (mol H_2O_2 decomposed · $min^{-1} \cdot mL^{-1}$)	$V^{-1} \times 10^4$
1.5	0.66	4.3	2.32
2	0.5	5.3	1.87
5	0.2	11.7	0.857
10	0.1	22.2	0.450
20	0.05	43.9	0.228
40	0.025	65.3	0.153
60	0.017	67.4	0.148
75	0.013	84.7	0.118
100	0.01	90.3	0.111

more, it was possible to isolate less active preparations with less than the full complement of heme, also a common feature of catalases (Jouve *et al.* 1984; Deisseroth and Dounce 1970). The enzyme was inhibited by cyanide, azide, and sulfhydryl agents, all of which have been shown to inhibit other catalases. The subunit size of 65 000 and amino acid composition were very similar to the size and compositions of most catalase subunits, except for the catalases from *E. coli*. On the other hand, the stability of catalase-1 over a broad pH range and at high temperature was different from the properties of the more common catalases, but was similar to the properties of catalase HPII from *E. coli*. Where catalase-1 differed from most other catalases was in its unusual structure of a hexamer, as opposed to the more usual tetramer structure. Previously the only catalase observed to exist as a hexamer was HPII from *E. coli* (Loewen and Switala 1986). Consequently, the native molecular weight of 390 000, composed of six identical subunits of 65 000, is exceeded only by HPII among the catalases, because of the latter's significantly larger subunit size.

The use of H_2O_2 as a tool to increase the yield of catalase-1 is another illustration of the inducibility of catalase synthesis in *B. subtilis* (Ishida and Sasaki 1981; Loewen and Switala 1987). The mechanism involved in the induction has only been investigated in *E. coli* and involves *oxyR* which modulates the expression of *katG* and several other genes (Christman *et al.* 1985). Whether or not a similar mechanism functions in *B. subtilis* will be determined in future studies.

TABLE 4. Amino acid analysis of catalase-1 from *B. subtilis* 168 and catalases from bovine liver and *P. mirabilis*

Amino acid	*B. subtilis* 168[a]	Bovine liver[b]	*P. mirabilis*[c]
Asp	66.9	75	69
Thr	41.3	25	22
Ser	35.6[d]	25	31
Glu	58.3	50	61
Pro	37.0	42	31
Gly	47.6	40	43
Ala	51.5	39	42
Cys	8.1[e]	7	5
Val	43.8	36	31
Met	18.4[f]	10	15
Ile	27.0	20	20
Leu	36.4	39	36
Tyr	16.1	22	21
Phe	28.8	34	37
His	18.8	23	22
Lys	34.1	30	32
Arg	29.3	33	36
Trp	4.3[g]	6	6

[a]Except where otherwise noted, values represent averages of 24, 48, and 72 h of hydrolysis, measured as residues per 65 000 subunit.
[b]Data from Schroeder *et al.* 1962, measured as residues per 62 000 subunit.
[c]Data from Jouve *et al.* 1984, measured as residues per 62 000 subunit.
[d]This value was extrapolated to zero hydrolysis time to compensate for hydrolytic destruction.
[e]Determined as cysteic acid following performic acid oxidation.
[f]Determined as methionine sulfoxide and methionine sulfone following performic acid oxidation.
[g]Determined after Edelhoch (1967).

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