Purification and characterization of spore-specific catalase-2 from *Bacillus subtilis*

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Catalase-2, the catalase found in spores of *Bacillus subtilis*, has been purified to homogeneity from a nonsporulating strain. The apparent native molecular weight is 504 000. The enzyme appears to be composed of six identical protomers with a molecular weight of 81 000 each. The amino acid composition is similar to the composition of other catalases. Like most catalases, catalase-2 exhibits a broad pH optimum from pH 4 to pH 12 and is sensitive to cyanide, azide, thiol reagents, and amino triazole. The apparent K_m for H_2O_2 is 78 mM. The enzyme exhibits extreme stability, losing activity only slowly at 93°C and remaining active in 1% SDS – 7 M urea. The green-colored enzyme exhibits a spectrum like heme *d* with a Soret absorption at 403 nm and a molar absorptivity consistent with one heme per subunit. The heme cannot be extracted with acetone–HCl or ether, suggesting that it is covalently bound to the protein.

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À partir d'une souche non sporulante, nous avons purifié jusqu'à homogénéité la catalase-2, présente dans les spores de *Bacillus subtilis*. Son poids moléculaire natif apparent est de 504 000. L'enzyme serait composée de six protomères identiques ayant chacun un poids moléculaire de 81 000. La composition en acides aminés est semblable à celle des autres catalases. Comme la plupart des catalases, la catalase-2 a un pH optimum allant de 4 à 12 et elle est sensible au cyanure, à l'azide, aux réactifs thiols et à l'aminotriazole. Le K_m apparent pour H_2O_2 est de 78 mM. L'enzyme manifeste une très grande stabilité; elle perd son activité lentement à 93°C et elle demeure active dans le mélange SDS 1%–urée 7 M. L'enzyme colorée en vert montre un spectre semblable à celui de l'hème *d* avec une bande de Soret à 403 nm et une absorptivité molaire compatible avec un groupe hème par sous-unité. L'hème ne peut être extrait avec l'acétone–HCl ou l'éther, preuve qu'il est lié de façon covalente à la protéine.
[Traduit par la revue]

Introduction

Catalases (EC 1.11.1.6) have been purified and characterized from a large number of microbial sources. Most of those characterized, including catalases from *Saccharomyces cerevisiae* (Seah and Kaplan 1973), *Proteus mirabilis* (Jouve et al. 1983, 1984), *Rhodopseudomonas* (*Rhodobacter*) *spheroides* (Clayton 1959), and *Micrococcus luteus* (Herbert and Pinsent 1948), resemble bovine liver catalase (Deisseroth and Dounce 1970) in being tetramers of identical 62 000-Da subunits with one protoheme IX group per subunit. Other catalases, including those from *Neurospora crassa* (Jacob and Orme-Johnson 1979*a*, 1979*b*), *Escherichia coli* (Claiborne and Fridovich 1979; Loewen and Switala 1986), *Rhodopseudomonas* (*Rhodobacter*) *capsulata* (Hochman and Shemesh 1987), and *S. cerevisiae* (Seah et al. 1973), have been found to differ in such properties as size of subunits, structure of heme groups, and multimeric composition. In addition, a nonheme but manganese-containing catalase has been isolated from *Lactobacillus plantarum* (Kono and Fridovich 1983) and *Thermoleophilum album* (Allgood and Perry 1986).

Not only do several different forms of catalase with diverse physical structures exist, but some organisms including *E. coli* (Claiborne and Fridovich 1979; Claiborne et al. 1979), *S. cerevisiae* (Seah et al. 1973; Seah and Kaplan 1973), and maize (Scandalios et al. 1980) produce more than one catalase. Recently, we have found that *Bacillus subtilis* produces two main species of catalase (Loewen and Switala 1987*a*). Catalase-1 is produced in vegetative cells and catalase-2 is the exclusive catalase in spores. The uniqueness of the two activities has been confirmed by a combination of biochemical and genetic data. As a first step in the physical characterization of the genes encoding the catalases, we have undertaken the purification and characterization of these two enzymes. Catalase-1 has been purified to homogeneity and characterized as a hexamer of identical subunits with a molecular weight of 65 000 and with one protoheme IX per subunit (Loewen and Switala 1987*b*). This report describes the purification of catalase-2, the enzyme normally present in spores of *B. subtilis*, and its characterization from a nonsporulating strain (UM1001) where it is produced intracellularly.

ABBREVIATIONS: LB, Luria-Bertani broth.

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Materials and methods

Materials

Bactotryptone and yeast extract were obtained from Gibco,

TABLE 1. Purification of catalase-2 from 175 g of cell paste of *B. subtilis* UM1001

	Vol. (mL)	Total protein (mg)	Total activity units	Yield (%)	Specific activity ($U^1 \cdot mg^{-1}$)	Purification (*n*-fold)
1. Crude extract	785	13 689	235 500	100	17	1.0
2. 2.5% streptomycin sulfate	740	9 768	214 600	91	22	1.3
3. $(NH_4)_2SO_4$ fractionation I 50–65% pellet	75	2 474	190 000	81	77	4.5
4. $(NH_4)_2SO_4$ fractionation II of step 3						
0–40% pellet	33	577	42 400	18	74	4.4
40–50% pellet	36	977	88 000	37	90	5.3
5. $(NH_4)_2SO_4$ fractionation III of combined fractions in step 4						
45–50% pellet	18	341	62 000	26	182	10.7
50–55% pellet	18	289	44 000	19	152	8.9
6. DEAE-Sephadex A-50 I of combined fractions in step 5	230	30.3	54 900	23	1 810	106
7. DEAE-Sephadex A-50 II	113	17.0	56 500	24	3 333	196
8. Bio-Gel A-1.5 m	37	3.7	43 500	18	11 757	692
9. Bio-Gel HTP	1	0.5	25 600	11	51 200	3 012

Burlington, Ont. DEAE-Sephadex A-50 and the most common biochemicals were obtained from Sigma Chemical Co., St. Louis, MO. Sepharose 4B was obtained from Pharmacia, Uppsala, Sweden. Bio-Gel A-1.5 m, Bio-Gel HTP, and acrylamide were obtained from Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ont.

Growth conditions

A preculture of *B. subtilis* UM1001 (Loewen and Switala 1987*a*) inoculated from a frozen stock 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 of 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, we calculated that 100 Klett units represented 0.15 mg/mL (dry cell weight). From 24 to 44 h, the catalase levels were monitored and when the levels stopped increasing, the cells were harvested. The normal yield was 6 g cell paste/L of medium.

Purification of catalase-2

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) and catalase-1 of *B. subtilis* (Loewen and Switala 1987*b*). 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 25% streptomycin sulfate precipitation was carried out. Ammonium sulfate precipitations were repeated twice to maximize the enzyme activity (Table 1). The protein pellets with a specific activity greater than 150 u/mg were resuspended in 50 mM potassium phosphate (pH 7.0), dialyzed for 2 h against 4 L of the same buffer, and applied to a 2.8 × 55 cm column of DEAE-Sephadex A-50 equilibrated in the same buffer. After the column was washed until the A_{280} was less than 0.05, catalase was eluted with a linear gradient prepared by mixing 50 mM potassium phosphate (pH 7.0) with 0.5 M NaCl in the same buffer. The peak of catalase activity was pooled, dialyzed for 2 h against 50 mM potassium phosphate (pH 7.0), and the fractionation repeated on a 2.3 × 17 cm column. The peak of catalase activity was pooled, concentrated by ultrafiltration, and loaded on a 2.2 × 100 cm column of Bio-Gel A-1.5 m equilibrated with 50 mM 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 50 mM potassium phosphate (pH 7.0). The column was eluted with a linear gradient prepared by mixing equal volumes of 50 and 400 mM potassium phosphate (pH 7.0). The fractions containing the peak of catalase were pooled, concentrated by ultrafiltration, dialyzed for 4 h against 1 L of 50% glycerol in 50 mM sodium phosphate (pH 7.0), and stored at −20°C. Some preparations at this stage contained a small amount of a smaller 45-kDa protein as determined on SDS polyacrylamide gels. This contaminant could be removed by adding SDS and urea to final concentrations of 1% and 7 M, respectively, and by separating the mixture on a 2.2 × 100 cm column of Bio-Gel A-1.5 m equilibrated with 50 mM potassium phosphate (pH 7.0) prior to storage, as above. The results from the purification of catalase-2 from *B. subtilis* are summarized in Table 1.

Molecular weight analysis

Five methods were used. The sedimentation equilibrium method of Yphantis (Yphantis 1964; Chervenka 1969) w

used in a Beckman model E ultracentrifuge at 20°C, with a calculated partial specific volume of 0.732 mL/g (Chervenka 1969). The method of Hedrick and Smith (1968) was also used; the protein was electrophoresed on gels with acrylamide concentrations from 6 to 12%, as described by Davis (1964) but with the following modifications: Tris–HCl (pH 8.1) and 15 × 15 cm slab gels. The gels were stained for protein or catalase activity by the method of Clare et al. (1984) but using 20 mM H_2O_2 for better contrast, and for peroxidase activity by the method of Gregory and Fridovich (1974). Gel filtration was performed using either a 0.7 × 110 cm column of Sepharose 4B equilibrated with 10 mM Tris – 70 mM glycine (pH 8.1), or a 0.7 × 110 cm column of Bio-Gel A-1.5 m equilibrated with 50 mM potassium phosphate (pH 7.0). The fourth method involved chemical cross-linking of enzyme subunits using dimethyl suberimidate (Davies and Stark 1970). After incubation with 10 mg/mL of dimethyl suberimidate for 24 h at 20°C, SDS, urea, and 2-mercaptoethanol were added to final concentrations of 0.5%, 3.5 M, and 70 mM, respectively. After boiling for 2 min, the mixture was separated by electrophoresis on a polyacrylamide slab gel, as described by Laemmli (1970) and Weber et al. (1972), and the resolved protein was stained with Coomassie blue.

For denatured molecular weight analyses, samples were denatured and separated 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).

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 DU-8 spectrophotometer. Unsuccessful attempts were made to extract the heme using published procedures (Fuhrop and Smith 1975; Morell and Crezy 1963; Newton et al. 1965).

Amino acid analysis

Samples containing 1 nmol of catalase-2 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 and methionine determinations, 1 nmol of catalase-2 subunit was oxidized for 20 h at 5°C in performic 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 an LKB 4151 Alpha Plus amino acid analyzer operated by the Department of Animal Science, University of Manitoba.

Results and discussion

Optimization of catalase-2 levels

Catalase-2 was present at very low levels relative to catalase-1 in wild type cells, and the chromatographic properties of catalase-1 and catalase-2 were so similar that we were unsuccessful in separating catalase-1 and catalase-2 preparations from wild type cells. Purified spores contained only catalase-2, but in low levels, and very poor yields of enzyme were achieved from the disruption of spores. Whether this was from inactivation of the enzyme or loss of the enzyme in cell debris was not determined. These problems were circumvented by using a *katA*-containing mutant, UM1001, that produced catalase-2 but no catalase-1. Because of the low levels of catalase-2, attempts were made to optimize the amount of enzyme present in the cell. Final levels around 100 u/mg dry cell weight were achieved by letting the cultures grow for 44 h, but even this optimization resulted in almost 10-fold less catalase-2 than the optimum level of catalase-1 in wild type cells (Loewen and Switala 1987*b*). A second advantage of using UM1001 was that it was a nonsporulating strain in which catalase-2 accumulated in the cell and was not packaged into spores. Thus, the catalase was released by a simple passage through the French press rather than the more laborious spore disruption procedures. A third advantage of using UM1001 was that proteolytic activity was reduced, possibly a result of the defect in sporulation, and this resulted in catalase-2 accumulating as a single activity band rather than the series of isoenzyme bands observed in the wild type strain (Loewen and Switala 1987*a*). That the enzyme in UM1001 was the same as that isolated from spores was confirmed by the similar electrophoretic mobilities and apparent molecular weights determined on nondenaturing polyacrylamide gels. The processing of catalase-2 into multiple bands, probably involving a proteolytic degradation, appears to occur in sporulation. The relationship between this single band of catalase-2 and the multiple bands in spores was investigated using sporulation-defective mutants with blocks at the various stages of sporulation. If sporulation was blocked at an early stage, only a single upper band of catalase-2 was present, but if sporulation was blocked at stage V or later, the single band was replaced by the multiple bands of catalase-2 (data not shown).

Purification

The same basic protocol that was employed for the purification of other bacterial catalases from both *E. coli* and *B. subtilis* was used to purify catalase-2. The only significant modification arose when some preparations were found to contain small amounts (2–5%) of a smaller protein of molecular weight 45 000, which could be removed by treatment with 1% SDS – 7 M urea just prior to a second gel-filtration step on Agarose. The enzyme was sufficiently stable that it remained active in 1% SDS–7 M urea. Treating the preparation with SDS–urea prior to the first agarose gel filtration step did not have the same effect and the presence of large amounts of extraneous protein caused precipitation on the agarose column with correspondingly lower yields.

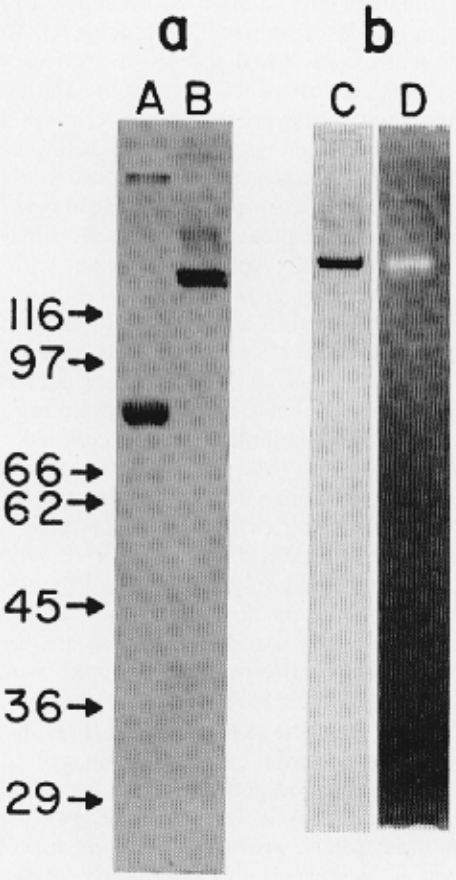

FIG. 1. Electrophoresis of purified catalase-1 from *B. subtilis* UM1001 on an 8% SDS–polyacrylamide gel (*a*) and on 8.5% nondenaturing polyacrylamide gels (*b*). In (*a*), 20 μg of protein was electrophoresed with (lane A) or without (lane B) boiling in a sample buffer containing 1% SDS, 7 M urea, 0.14 M 2-mercaptoethanol, and 0.1 M sodium phosphate (pH 7.0); the gel was subsequently stained for protein. The numbers indicate the location of the following molecular weight standards ($\times 10^{-3}$ from the top down): β-galactosidase, phosphorylase B, bovine plasma albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase, and carbonic anhydrase. In (*b*), lane C shows 20 μg of protein electrophoresed and stained for protein while lane D shows 1.5 U or 30 ng of protein electrophoresed and stained for catalase activity.

Therefore, this step was used only when necessary and only as the final step, at which point there was no precipitation and minimal loss of activity. The final preparation migrated as a single band of protein on denaturing SDS acrylamide gels, corresponding to a molecular weight of 81 000 (Fig. 1*a*), and as a single band of protein on nondenaturing polyacrylamide gels, coincident with the band of catalase activity (Fig. 1*b*).

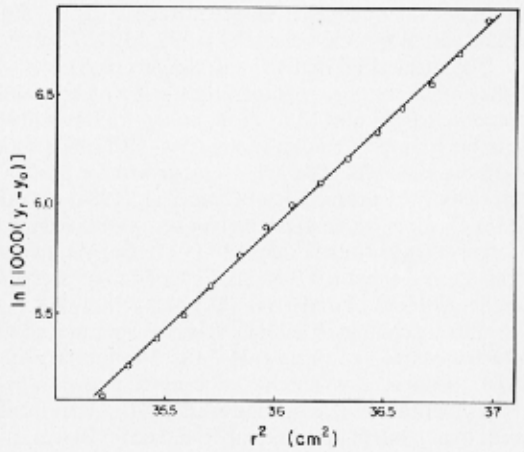

FIG. 2. Determination of the nondenatured molecular weight of catalase-1 by sedimentation equilibrium at 5600 rpm. The apparent molecular weight was calculated as outlined by Chervenka (1969): r, distance from center of rotor; y_r, vertical displacement of fringes at distance r; y_0, vertical displacement of fringes at miniscus.

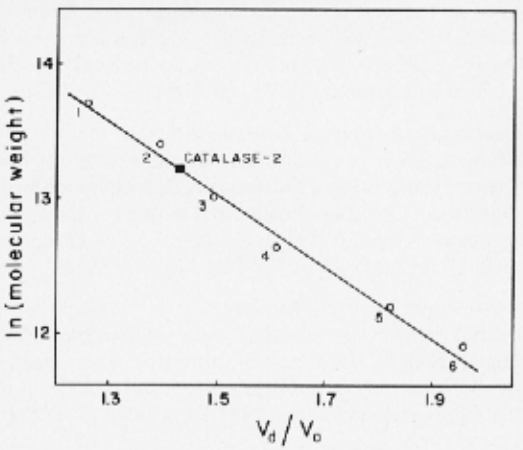

FIG. 3. Determination of the nondenatured molecular weight of catalase-2 by elution from Sepharose 4B equilibrated with Tris–glycine buffer. The standards used were as follows: **1**, apoferritin (dimer), 886 000; **2**, thyroglobulin, 669 000; **3**, apoferritin (monomer), 443 000; **4**, alcohol dehydrogenase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

Nondenatured size determination

Four independent methods were used to determine the nondenatured size of catalase-2. The sedimentation equilibrium method revealed an apparent molecular weight of 504 000 ± 10 000 (Fig. 2). 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

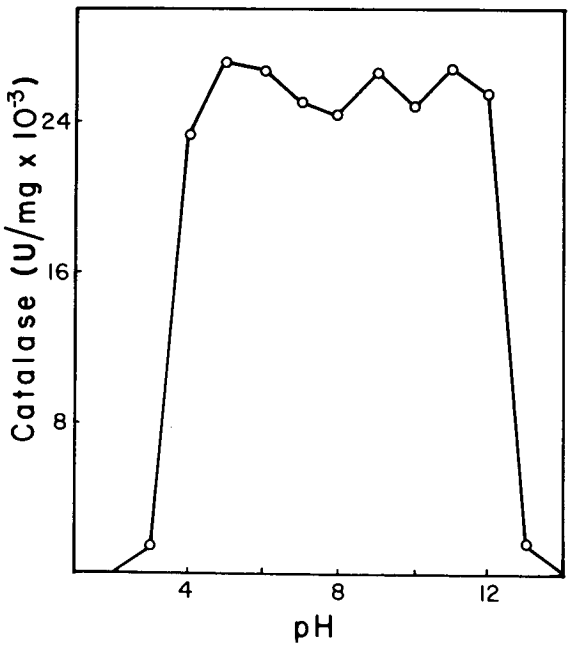

FIG. 4. Activity of purified catalase-2 as a function of pH. The enzyme was incubated in 50 mM potassium phosphate at the various pH values for 1 min prior to commencing the assay by adding 60 mM H_2O_2.

518 000 ± 15 000 (Fig. 3). Gel filtration on Bio-Gel A-1.5 m 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 251 000 ± 5000, which was approximately half the size determined by the other two techniques, as observed previously with HPI and HPII from *E. coli* (Loewen and Switala 1986) and catalase-1 from *B. subtilis* (Loewen and Switala 1987*b*). This phenomenon was attributed to a combination of the mild denaturing effect of the Tris–glycine buffer and undefined pressures during electrophoresis. A fourth method involved chemical cross-linking with dimethyl suberimidate, which resulted in the formation of multimers ranging from dimers to poorly resolved pentamers and hexamers, trimers being the predominant cross-linked form.

Properties of catalase-2

Catalase-2 exhibited a broad pH optimum with high activity from pH 4 to pH 12 and approximately 5–10% residual activity at pH 3 and pH 13 (Fig. 4), similar to the activity pattern exhibited by other catalases (Loewen and Switala 1986, 1987*b*). Catalase-2 was sensitive to both potassium cyanide and sodium azide, experiencing a 50% inhibition in 0.01 mM cyanide and 0.06 mM azide (Fig. 5). Therefore, it was 30-fold less sensitive to azide than catalase-1 but more sensitive to cyanide. Incubation at 75°C did not result in inactivation of

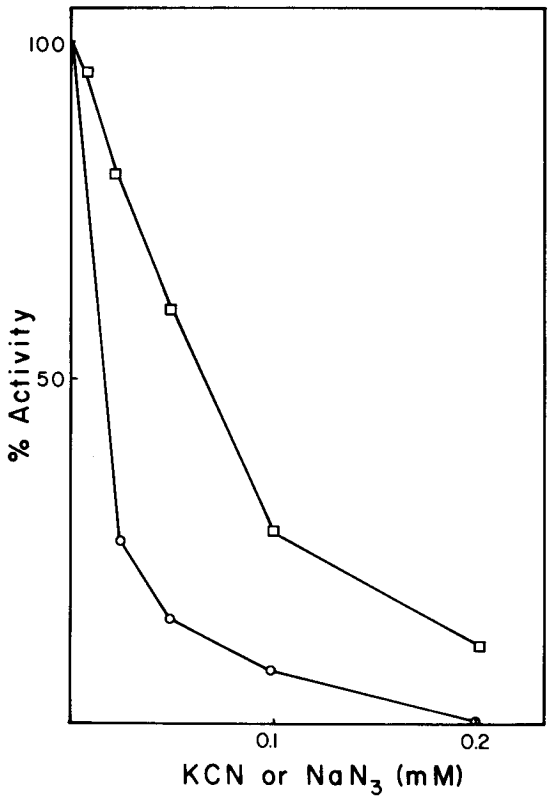

FIG. 5. Activity of purified catalase-2 in the presence of potassium cyanide (○) or sodium azide (□). The enzyme was incubated with the inhibitor in 50 mM sodium phosphate (pH 7.0) at 37°C for 1 min prior to commencing the assay by adding 60 mM H_2O_2. Data are expressed as the percentage of initial activity remaining.

catalase-2 while incubation at 93°C resulted in a 50% inactivation after 4 min, indicating far greater thermal stability than any other catalase thus far. Catalase-2 was less sensitive to thiol reagents than catalase-1 and also differed in being slightly more sensitive to glutathione than to mercaptoethanol (Fig. 6). The concentration effect of H_2O_2 on the reaction rate produced an apparent K_m of catalase-2 for H_2O_2 of 78 mM. Aminotriazole is known to bind to the heme of catalases, preventing decomposition of compound 1. Catalase-2 was more sensitive to aminotriazole than catalase-1 but less sensitive to aminotriazole and ascorbate (Fig. 7). The amino acid composition has been compared with the compositions of other catalases (Table 2) revealing some similarities, particularly in the low cysteine and tryptophan contents.

Spectral analysis

The spectrum of purified catalase-2 shown in Fig. 8 is similar to the spectra of catalase HPII from *E. coli* (Loewen and Switala 1986) and catalase from *N. crassa*

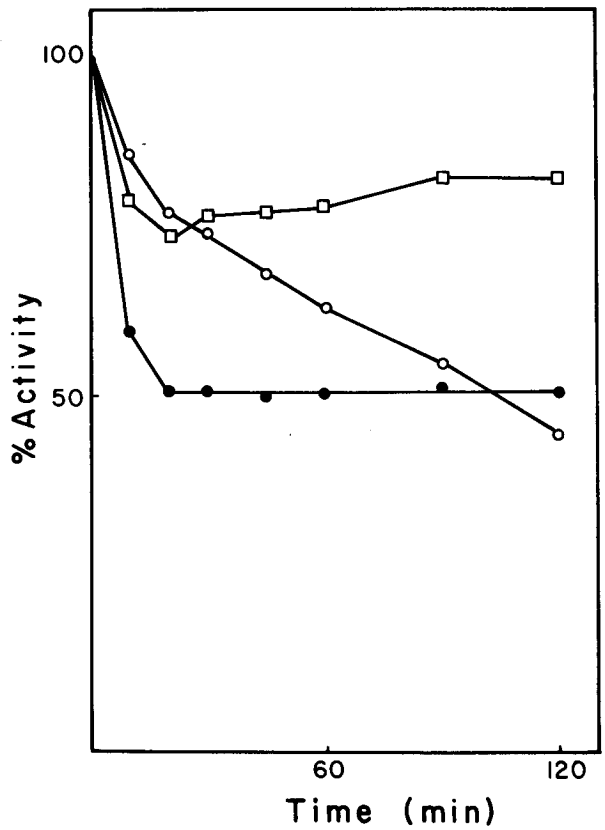

FIG. 6. Activity of purified catalase-2 in the presence of 2-mercaptoethanol (○), glutathione (●), and dithiothreitol (□), all at 5 mM. The enzyme was incubated at 37°C in 50 mM sodium phosphate buffer (pH 7.0) in the presence of the thiol reagent, and aliquots were removed at various times and assayed. Data are expressed as the percentage of initial activity remaining.

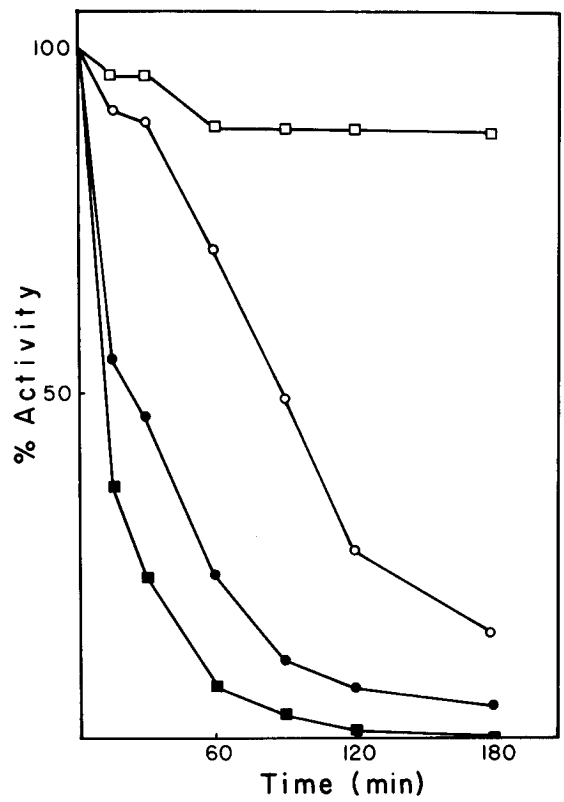

FIG. 7. Determination of the activity of purified catalase-2 and catalase-1 in the presence of aminotriazole. Catalase-2 was incubated with 8 mM aminotriazole (○) and a mixture of 8 mM aminotriazole and 1 mM ascorbic acid (●) for various times at 37°C, and aliquots were removed for assay. Catalase-1 was incubated under the same conditions with aminotriazole (□) and aminotriazole plus ascorbate (■). Data are expressed as the percentage of initial activity remaining.

(Jacob and Orme-Johnson 1979*a*, 1979*b*), with a Soret band at 403 nm and smaller peaks at 588, 640, and 710 nm with associated molar absorptivities of 33.5×10^3, 7.1×10^3, 3.4×10^3, and 3.1×10^3 $M^{-1} \cdot cm^{-1} \cdot subunit^{-1}$. These values are virtually identical to the values calculated for HPII (Loewen and Switala 1986), in which the ratio of one heme per subunit was determined. This suggests that there is also one heme per subunit in catalase-2. The absorbance ratio (A_{403}/A_{280}) of homogeneous catalase-2 was 0.42. Despite the similarity to HPII and *N. crassa* catalases, it was not possible to extract the heme in acetone–HCl or ether, suggesting that the heme was either covalently attached or very tightly bound to the protein. Other proteins with covalently attached hemes include cytochrome *c*, from which the heme can be removed by treatment with mercurous or silver salts (Fuhrop and Smith 1975), and animal peroxidases, from which the heme can be removed by treatment with HBr, alkali, or mercaptoeth-

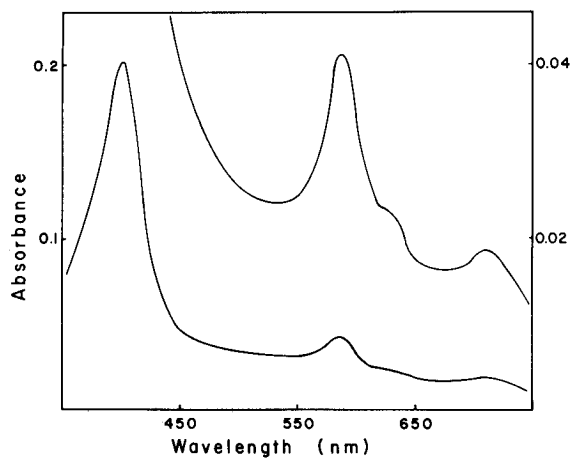

FIG. 8. Absorption spectrum of purified catalase-2. Catalase-2 was present at 1 μM in 50 mM sodium phosphate. For details, see text.

TABLE 2. Amino acid analysis of catalase-2 from *B. subtilis* UM1001 and catalases from bovine liver and *P. mirabilis*

Amino acid	*B. subtilis* UM1001[a]	Bovine liver[b]	*P. mirabilis*[c]
Asp	80	75	69
Thr[d]	42	25	22
Ser[d]	39	25	31
Glu	76	50	61
Pro	34	42	31
Gly	64	40	43
Ala	61	39	42
Cys[e]	5	7	5
Val	60	36	31
Met[f]	38	10	15
Ile	32	20	20
Leu	45	39	36
Tyr	11	22	21
Phe	45	34	37
His	23	23	22
Lys	47	30	32
Arg	42	33	36
Try[g]	7	6	6

[a]Except where otherwise noted, values represent averages of 24, 48, and 72 h of hydrolysis; measured as residues per 81 000 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]Values 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.

anol (Morell and Clezy 1963; Newton et al. 1965; Nichol et al. 1987). Unfortunately, no ether-soluble heme could be isolated from catalase-2 following treatment with any of these reagents. In the case of mercurous chloride, the chromophore remained associated with the protein, whereas with HBr – acetic acid and alkali, the chromophore was destroyed. Boiling the enzyme also destroyed the chromophore.

In summary, catalase-2 from *B. subtilis* is an unusual catalase in several respects, including the apparent covalent attachment of the heme to the protein, the spectral similarity of the heme to heme *d*, the enzyme's stability at high temperatures, and its hexameric structure. The strong association of the heme with the protein would obviously contribute to the enzyme's stability. In addition, the hexameric structure may contribute to the heat stability as two other hexameric catalases, HPII from *E. coli* and catalase-1 from *B. subtilis*, are also more heat stable than normal tetrameric catalases.

Acknowledgement

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