Thermostable Peroxidase from *Bacillus stearothermophilus*

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A peroxidase from *Bacillus stearothermophilus* was purified to homogeneity. The enzyme (M_r 175000) was composed of two subunits of equal size, and showed a Soret band at 406 nm. On reduction with sodium dithionite, absorption at 434 nm and 558 nm was observed. The spectrum of reduced pyridine haemochrome showed peaks at 418, 526 and 557 nm; the reduced *minus* oxidized spectrum of pyridine haemochrome showed peaks of 418, 524 and 556 nm with a trough at 452 nm. These results indicate that the enzyme contained protohaem IX as a prosthetic group. The optimum pH was about 6 and the apparent optimum temperature was 70 °C. The enzyme was relatively stable up to 70 °C; at 30 °C it was stable for a month. The enzyme had peroxidase activity toward a mixture of 2,4-dichlorophenol and 4-aminoantipyrine with a K_m for H_2O_2 of 1.3 mM. It also acted as a catalase with a K_m for H_2O_2 of 7.5 mM.

INTRODUCTION

Peroxidases are important enzymes; widely used in the clinical field for the colorimetric measurement of biological materials. Peroxidases catalyse the oxidation of a large number of aromatic compounds such as phenol, hydroquinones and hydroquinoid amines, especially benzidine derivatives. Typical peroxidases are haemoproteins and occur in animals, plants, fungi and bacteria. The longest known and best studied peroxidase is horseradish peroxidase (Welinder, 1979), but bacterial peroxidases have attracted comparatively little attention.

So far, microbial peroxidases from *Pseudomonas fluorescens* (cytochrome *c* peroxidase) (Lenhoff & Kaplan, 1956), *Streptococcus faecalis* (NADH peroxidase) (Dolin, 1957), *Escherichia coli* (*o*-dianisidine peroxidase, HP-I) (Claiborne & Fridovich, 1979), *Halobacterium halobium* (Fukumori *et al.*, 1985), *Rhodopseudomonas capsulata* (Hochman & Shemesh, 1987), *Pellicularia filamentosa* (Ichikawa *et al.*, 1981), and *Saccharomyces cerevisiae* (cytochrome *c* peroxidase) (Finzel *et al.*, 1984) have been isolated, but there is little information about peroxidases from thermophilic bacteria. Obviously, a thermostable enzyme will provide us with important information about thermal resistance in catalase–peroxidase and will be suitable for practical purposes. In this study, the thermostable peroxidase from a thermophilic bacterium, *Bacillus stearothermophilus*, was purified and some of its characteristics were studied.

METHODS

Cell culture. *B. stearothermophilus* IAM 11001 (ATCC 8005) was grown in LB (Luria–Bertani) broth containing (l^{-1}) 10 g tryptone, 5 g yeast extract and 10 g NaCl, pH 7.3. A loopful of stock culture was inoculated into 100 ml LB broth and incubated at 55 °C with shaking for 12 h. Part of this culture was transferred to 6 l of medium (1% inoculum) and incubated under the same conditions for 18 h.

Purification of peroxidase. All the purification steps were done at room temperature unless otherwise indicated. Cells from 6 l of culture were harvested by centrifugation (3000 *g*, 10 min), suspended in 200 ml 100 mM-sodium/potassium phosphate buffer, pH 7, and disrupted by sonication at 20 kHz for 10 min. The lysate was centrifuged at 3000 *g* for 10 min. Solid ammonium sulphate was added to 180 ml of the supernatant to 35% saturation; the supernatant was then centrifuged at 8000 *g* for 10 min. Ammonium sulphate was further added to

55% saturation and the resulting precipitate was collected by centrifugation under the same conditions. The precipitate was dissolved in 65 ml 20 mM-sodium/potassium phosphate buffer, pH 7, and dialysed against the same buffer for 18 h at 4 °C. The dialysed enzyme was put onto a DEAE-Sephadex A50 column (5.8 × 60 cm), which had been equilibrated with 20 mM-phosphate buffer, pH 7, and eluted with 2 l of a 0 to 0.5 M-NaCl linear gradient in phosphate buffer. Fractions containing peroxidase activity were pooled, concentrated by ultrafiltration, and put on a second DEAE-Sephadex A50 column; the enzyme was then eluted in the same manner. The enzyme fractions were pooled, concentrated by ultrafiltration, adjusted to 4% saturation with ammonium sulphate, and adsorbed on a column (2.6 × 60 cm) of Phenyl-Sepharose CL-4B, which had been equilibrated with 20 mM-phosphate buffer, pH 7, containing 4% saturation ammonium sulphate. The peroxidase activity was then eluted with 2 l of a decreasing linear gradient of ammonium sulphate (3.75 to 0.9% in phosphate buffer). Fractions containing peroxidase activity were pooled, concentrated to 2 ml, and put onto a column (2.5 × 70 cm) of Sephadex G-200 previously equilibrated with 20 mM-phosphate buffer, pH 7. Fractions showing high specific enzyme activity were pooled and further purified by HPLC on a DEAE-5PW column equilibrated with 20 mM-phosphate buffer, pH 7, and eluted with a 0 to 0.5 M-NaCl linear gradient in the same buffer.

Peroxidase assay. Enzyme activity was measured using 2,4-dichlorophenol/4-aminoantipyrine as substrates (Emerson, 1943). The enzyme (10 µl) was added to 3 ml of a substrate mixture containing 4.1 mM-2,4-dichlorophenol, 0.67 mM-4-aminoantipyrine, 2.9 mM-H₂O₂ and 33 mM-sodium/potassium phosphate buffer, pH 6. The activity was assayed using a Hitachi 220A spectrophotometer with a thermostatted cell compartment at 50 °C. The increase in absorbance at 500 nm was continuously recorded and the catalytic activity was calculated using $\epsilon_{500} = 1.36 \times 10^4 \text{ l mol}^{-1} \text{ cm}^{-1}$ (Tamaoku *et al.*, 1982).

Catalase assay. Catalase was assayed spectrophotometrically by measuring the decrease in absorbance at 240 nm as described by Aebi (1983), using $\epsilon_{240} = 43.6 \text{ l mol}^{-1} \text{ cm}^{-1}$ (Hildebrandt & Roots, 1975).

Protein concentration. This was determined from the absorbance at 280 nm.

Gel electrophoresis. Electrophoresis on 7.5% (w/v) polyacrylamide gels was done as described by Davis (1964). After electrophoresis, protein was stained with Coomassie Brilliant Blue R250. Peroxidase activity was detected by incubating the gel at room temperature with 10 mM-sodium/potassium phosphate buffer (pH 6) containing 40 mM-pyrogallol and 7.4 mM-H₂O₂ for 3 min. The catalase band was detected as described by Gregory & Fridovich (1974), but pyrogallol was used instead of diaminobenzidine. SDS-PAGE in a 10% gel was done according to Weber & Osborn (1969).

***M_r* measurements.** These were done by HPLC (TSK-G3000SW column) using the following proteins as standards: cytochrome *c* (12400), adenylate kinase (32000), enolase (67000), lactate dehydrogenase (142000) and glutamate dehydrogenase (290000). The subunit *M_r* was measured by SDS-PAGE with the following standard proteins: phosphorylase b (94000), bovine serum albumin (67000), ovalbumin (43000), carbonic anhydrase (30000), soy bean trypsin inhibitor (20000) and α -lactalbumin (14400).

Spectroscopy. Absorbance was recorded using a Hitachi 220A spectrophotometer. The native enzyme was scanned from 250 to 700 nm, reduced by adding 1 mg sodium dithionite, and then scanned again. Haem was measured as the pyridine haemochromogen as described by Falk (1964a).

Chemicals. DEAE-Sephadex, Phenyl-Sepharose CL-48 and Sephadex G-200 were obtained from Pharmacia. 2,4-Dichlorophenol, guaiacol and H₂O₂ peroxide were from Nakarai (Japan). 4-Aminoantipyrine, catechol, pyrogallol, pyridine and sodium dithionite were from Wako (Japan).

RESULTS AND DISCUSSION

Purification. Cell-free extract from a 6 l culture of *B. stearothermophilus* IAM 11001 was processed by ammonium sulphate fractionation and separation and through a series of chromatography columns as indicated in Table 1. The enzyme was purified 120-fold from the crude extract to a specific activity of 18 units (mg protein)⁻¹ with 10% recovery of activity. The absorption coefficient ($A_{280 \text{ nm}}^{1\%}$) of the lyophilized enzyme was 16.2.

Homogeneity. The purified enzyme preparation moved as a single protein component in PAGE; it had both peroxidase and catalase activity (Fig. 1). Peroxidases from *E. coli* (HP-I) (Claiborne & Fridovich, 1979), *H. halobium* (Fukumori *et al.*, 1985) and *R. capsulata* (Hochman & Shemesh, 1987) also had both peroxidase and catalase activity. The *M_r* of the enzyme was 175000 by HPLC but only 86000 by SDS-PAGE, indicating that the peroxidase is a dimer consisting of two homologous subunits, with a subunit *M_r* similar to that of *E. coli* peroxidase. However, *E. coli* peroxidase is a tetramer. The catalase from *Comamonas compransor*, which also shows peroxidase activity, has been reported to be a dimer composed of two identical subunits of *M_r* 75000 (Nies & Schlegel, 1982).

Fig. 1. PAGE of purified peroxidase. (a) SDS polyacrylamide (10%, w/v) gel. Lane 1, M_r marker proteins; lane 2, purified peroxidase. (b) Polyacrylamide (non-denatured) (7.5%, w/v) gel. Lane 1, protein staining with Coomassie Brilliant Blue; lane 2, activity staining for peroxidase with pyrogallol. Details are described in Methods.

Table 1. Purification of peroxidase from *B. stearothermophilus*

Step	Total protein (mg)	Total activity (units)	Specific activity [units (mg protein) ⁻¹]	Yield (%)
Crude extract	4100	610	0.15	100
Ammonium sulphate	2700	480	0.18	79
DEAE-Sephadex A-50(I)	260	440	1.6	72
DEAE-Sephadex A-50(II)	170	400	2.3	66
Phenyl-Sepharose CL-4B	40	360	9.9	59
Sephadex G-200	7	120	17.1	20
HPLC-DEAE-5PW	3.3	58	17.7	10

Substrate specificity. Various kinds of hydrogen donors were examined as potential substrates for the peroxidase (Table 2). A mixture of 4-aminoantipyrine and 2,4-dichlorophenol was the best substrate giving the highest enzyme activity; also, the reaction product had the highest absorption peak at 512 nm. 2,4-Dichlorophenol was a better hydrogen donor than phenol, giving three times higher activity. The derivatives of phenol also gave a higher colour intensity than phenol in a study of peroxidase from the fungus *Arthromyces ramosus* (Shinmen *et al.*, 1986). The peroxidase from *B. stearothermophilus* was highly specific for a mixture of 4-aminoantipyrine and 2,4-dichlorophenol (or phenol) among the substrates examined. The activity towards *o*-aminophenol and pyrogallol was low, and activity was negligible towards catechol and guaiacol: this is similar to the substrate specificity of *Pellicularia filamentosa* peroxidase (Ichikawa *et al.*, 1981). Haemoprotein *b*-590 from *E. coli*, which has a close structural and functional relationship to HP-I, a cytochrome *a₁b* preparation (Poole *et al.*, 1986) and the catalase-peroxidase from *R. capsulata* (Hochman & Shemesh, 1987) also showed no detectable peroxidase activity toward guaiacol.

Table 2. *Substrate specificity of peroxidase from B. stearothermophilus*

For substrates 1 and 2, conditions were as described in the Methods. For substrates 3 to 6, the concentration of substrate in the reaction mixture was 0.7 mM; absorbance measured at 500 nm for 1 and 2, 480 nm for 3 and 5, 420 nm for 4, and 430 nm for 6. Increases in absorbance in 1 min were compared and expressed as relative activity. For dichlorophenol + aminoantipyrine 100% relative activity was $18.6 \mu\text{mol min}^{-1} \text{ ml}^{-1}$.

Substrate	Relative activity (%)
1 Dichlorophenol + aminoantipyrine	100
2 Phenol + aminoantipyrine	37
3 <i>o</i> -Aminophenol	1.6
4 Pyrogallol	1.3
5 Catechol	0
6 Guaiacol	0

pH optimum. The peroxidase had an optimum pH at 6.0 both in acetate and in phosphate buffers. At pH 5.5 to 6.5, the enzyme was highly active, but at pH values higher than 7 or lower than 5, activity decreased. Imidazole at concentrations from 10 to 50 mM enhanced the peroxidase activity by about 40%, which is similar to the effect of imidazole on the enzyme from *C. compransoris* (Nies & Schlegel, 1982).

Optimum temperature and stability. The apparent optimum temperature for the peroxidase activity was 70 °C. To check the thermostability of the enzyme, it was incubated in 100 mM-sodium/potassium phosphate buffer, pH 6, for 10 min at various temperatures. Activity was then assayed and expressed as a percentage of the activity of the untreated sample. The peroxidase retained 80% activity after incubation at 70 °C. In contrast, catalase-peroxidase from a mesophile (*E. coli*) and from a photosynthetic bacterium (*R. capsulata*) lost all activity after 5 min at 60 °C (Nadler *et al.*, 1986).

As the enzyme was thermostable, it might be expected to be stable at lower temperatures. Peroxidase in 100 mM-sodium/potassium phosphate buffer, pH 7, plus 10% (v/v) glycerol was incubated at 30 °C. For the first 2 d the enzyme retained 100% activity; after 5 and 34 d it still retained 81% and 67% respectively, of its original activity. This high stability enabled purification to be done at room temperature and facilitated the study and storage of the enzyme. It is clearly different from horseradish peroxidase, which loses about 10% of its activity per day at 20 °C (Putter & Becker, 1983), or peroxidase from *H. halobium* which has to be kept at a high salt concentration (Fukumori *et al.*, 1985).

Optical spectra and haem content. The absorbance spectrum of the native enzyme had a Soret band at 406 nm (Fig. 2a). After reducing the enzyme with sodium dithionite, the Soret band was observed at 434 nm; a small peak at 558 nm appeared also (Fig. 2a). The Soret band indicated that the peroxidase contains haem. To identify the type of haem, pyridine haemochrome was examined. When the enzyme was reduced in alkaline pyridine, it yielded a haemochromogen whose spectrum was identical with that of protohaem IX. The spectrum of reduced pyridine haemochrome showed peaks at 418, 526 and 557 nm (Fig. 2b). The pattern of reduced *minus* oxidized pyridine haemochrome showed peaks at 418, 524 and 556 nm with a trough at 452 nm (Fig. 2b). The protohaem content was estimated from the spectrum of its pyridine haemochromogen using $\epsilon_{418.5} = 1.92 \times 10^5 \text{ l mol}^{-1} \text{ cm}^{-1}$ (Falk, 1964b). On this basis we assume that there is one molecule of protohaem IX per dimer of peroxidase. This low haem content is consistent with an A_{406}/A_{280} ratio of 0.35. However, the possibility still remains that haem molecules were lost during purification. Protohaem IX is the common type of haem found in peroxidases and catalases from micro-organisms such as *Micrococcus luteus* (Herbert & Pinsent, 1948), *Rhodopseudomonas sphaeroides* (Clayton, 1959), *S. cerevisiae* (Finzel *et al.*, 1984), *C. compransoris* (Nies & Schlegel, 1982) and *E. coli* (Claiborne & Fridovich, 1979).

Kinetics of the peroxidase reaction. The peroxidase from *B. stearothermophilus* had both peroxidase and catalase activity. For the peroxidatic activity with 2,4-dichlorophenol/4-

Fig. 2. (a) Absorption spectra of peroxidase (3 μ M) in 20 mM-sodium/potassium phosphate buffer, pH 7. (Inset, absorption spectrum of peroxidase reduced by sodium dithionite.) (b) Absorption spectra of pyridine haemchromogen derived from 4 μ M-peroxidase). —, Reduced form; ···, oxidized form; ---, reduced minus oxidized.

aminoantipyrine as substrate, the K_m for H_2O_2 was 1.3 mM. Study of the peroxidase reaction was frustrated by the ability of the enzyme to act as a catalase and decompose the H_2O_2 . Therefore, the K_m value would be lower than estimated as the actual concentration of H_2O_2 was reduced by decomposition. The K_m is higher than the value of 0.048 mM for HP-I and/or II (Loewen & Triggs, 1984) and the value of 0.18 mM for haemoprotein *b*-590 (Poole *et al.*, 1986).

Kinetics of the catalase reaction. At H_2O_2 concentrations up to 40 mM, *B. stearothermophilus* hydroperoxidase was not rapidly inactivated and the initial linear rate of H_2O_2 decomposition was used to estimate K_m from a Lineweaver–Burk plot. The K_m was 7.5 mM, which is similar to the values for *C. compransoris* catalase (1.9 mM) (Nies & Schlegel, 1982), *E. coli* HP-I (3.9 mM) (Claiborne & Fridovich, 1979), *E. coli* catalase (4.1 mM) (Loewen & Triggs, 1984), and *E. coli* haemoprotein *b*-590 (11 mM) (Poole *et al.*, 1986).

In conclusion, the peroxidase from *B. stearothermophilus* acts as a hydroperoxidase or catalase–peroxidase enzyme, as implied by its name, that can catalyse both catalase and peroxidase reactions at significant rates. Peroxidase and catalase are enzymes that share a number of structural and physicochemical properties. The prosthetic group is haem, with the haem iron in the ferric high-spin state, which is able to bind fluoride, cyanide or azide (Paul, 1963; Brill, 1966).

There have been few reports of catalase–peroxidases in recent years. The catalase–peroxidase from *B. stearothermophilus* studied here is the first to be isolated from a thermophile. Study of this enzyme should provide significant information on the heat stability of catalase–peroxidase, its molecular mechanisms and a comparative study of its evolution. The enzyme should also be a good candidate for industrial applications.

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