Extremophiles (2001) 5:423–429
Digital Object Identifier (DOI) 10.1007/s007920100218

ORIGINAL PAPER

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A catalase-peroxidase from a newly isolated thermoalkaliphilic *Bacillus* sp. with potential for the treatment of textile bleaching effluents

Received: March 14, 2001 / Accepted: June 8, 2001 / Published online: October 16, 2001

Abstract A new thermoalkaliphilic bacterium was isolated from a textile wastewater drain and identified as a new *Bacillus* sp. (*Bacillus* SF). Because of its high pH stability and thermostability, a catalase-peroxidase (CP) from this strain has potential for the treatment of textile bleaching effluents. The CP from *Bacillus* SF was purified to more than 70.3-fold homogeneity using fractionated ammonium sulfate precipitation, hydrophobic interaction, and anion-exchange and gel-filtration chromatography. The native CP had a molecular mass of 165 kDa and was composed of two identical subunits. The isoelectric point of the protein was at pH 6.0. Peptide mass mapping using matrix-assisted laser desorption ionization–mass spectrometry showed a homology between the CP from *Bacillus* SF and the CP from *Bacillus stearothermophilus*. The apparent K_m value of the catalase activity for H_2O_2 was 2.6 mM and the k_{cat} value was 11,475 s^{-1}. The enzyme showed high catalase activity and an appreciable peroxidase activity with guaiacol and *o*-dianisidine. The enzyme was stable at high pH, with a half-life of 104 h at pH 10 and 25°C and 14 h at 50°C. The enzyme was inhibited by azide and cyanide, in a competitive manner, but not by the catalase-specific inhibitor 3-amino-1,2,4-triazole.

Key words Thermostable · Alkalistable · *Bacillus* sp. · Catalase · Peroxidase · Textile bleaching

Communicated by G. Antranikian

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Introduction

The application of catalases for the elimination of hydrogen peroxide in textile bleaching effluents has been suggested recently (Tzanov et al. 2001a, b). This process would allow recycling of the water used for dyeing, for example, as many dyes would be oxidized and decolorized by hydrogen peroxide. Consequently, the enormous water consumption (100 l/kg of fabric) of the textile processing industry could be reduced (Hillenbrand 1999). The application of catalases for this purpose has the advantage over the use of chemicals such as sodium bisulfite or hydrosulfite in that they are only needed in catalytic quantities, avoiding unfavorable high salt concentrations in the process. Several companies have recently marketed their catalases for the treatment of bleaching baths; however, most commercial catalases would hardly withstand the conditions used during textile bleaching. Therefore, new thermoalkalistable enzymes acting at temperatures above 50°C and pH values above 9 are required. We have isolated several thermoalkaliphilic bacteria from textile processing effluents, and a catalase preparation of a new *Bacillus* sp. (*Bacillus* SF) has shown promising stabilities at high pH and temperature (Paar et al. 2001).

Catalase (EC 1.11.1.6) is an enzyme, present in all aerobic cells, that decomposes hydrogen peroxide to molecular oxygen and water. Its main function is to protect cells from the toxic effect of hydrogen peroxide. In eukaryotic organisms and in some prokaryotes, catalase is a molecule composed of four identical subunits. Each of the subunits binds one protoheme IX group (Murthy et al. 1981; von Ossowski et al. 1993). This enzyme can also act as a peroxidase (EC 1.11.1.7) for which several organic substances can act as a hydrogen donor. Therefore, about 20 years ago, a new class of bacterial enzymes, the catalase-peroxidases (CP), was identified. These enzymes are considered to be ancestral forms of catalase or peroxidase in evolutionary flow (Youn et al. 1995). It has been proposed that CPs may act as catalases because the substrates for peroxidatic activity are not found in cytoplasm (Claiborne and Fridovich 1979).

Although they show high catalase activity, these bifunctional enzymes have little sequence homology with typical

heme-containing monofunctional catalases but have high homology with fungal cytochrome *c* peroxidase and plant ascorbate peroxidase. Thus, they have been recognized as a part of the class I of the superfamily of plant, fungal, and bacterial peroxidases (Welinder 1992). CP is distinct from typical catalases because it is reduced by dithionite; like peroxidase, it is not inhibited by the catalase-specific inhibitor 3-amino-1,2,4-triazole but is inactivated by hydrogen peroxide, and it possesses a narrow pH range for its maximal activity (Regelsberger et al. 1999b; Marcinkeviciene et al. 1995; Youn et al. 1995). The most interesting feature of bifunctional catalase-peroxidase is the overwhelming catalase activity with k_{cat}/K_m values comparable with monofunctional catalases (Regelsberger et al. 2000). Until now, CP have been isolated and characterized from a variety of different bacteria including *Deinococcus radiophilus* (Yun and Lee 2000), *Escherichia coli* (Claiborne and Fridovich 1979), *Septoria tritici* (Levy et al. 1992), *Bacillus stearothermophilus* (Loprasert et al. 1988), *Klebsiella pneumonia* (Hochman and Goldberg 1991), *Rhodobacter capsulatus* (Levy et al. 1992; Forkl et al. 1993), *Streptomyces* sp. (Youn et al. 1995), *Mycobacterium smegmatis* (Marcinkeviciene et al. 1995), *Mycobacterium tuberculosis* (Nagy et al. 1997; Johnsson et al. 1997), and *Synechocystis* sp. (Regelsberger et al. 1999b). However, only very little information is available in the literature on thermostability and pH stability of catalase-peroxidases. In this article, we describe the purification of a thermoalkalistable catalase-peroxidase from this *Bacillus* sp. and compare the properties of this enzyme to those of other known catalase-peroxidases.

Materials and methods

Screening and cultivation

A sample (1 ml) from a wastewater drain from a textile finishing company was added to 50 ml Standard I nutrient broth (Merck, Darmstadt, Germany), buffered with 50 mM $NaHCO_3/Na_2CO_3$ at pH 10.0, and incubated in 100-ml Erlenmeyer flasks with chicanes in a rotary shaker at 60°C and 160 rpm. Growth was monitored under the microscope, and microorganisms (1 ml) were transferred to fresh culture medium after 2 days. To isolate various strains, a dilution row was spread on Standard I nutrient agar (Merck) buffered with 50 mM $NaHCO_3/Na_2CO_3$ (pH 10). Agar plates were incubated at 60°C and constant humidity, and the isolated strain was deposited and identified by the German culture collection DSMZ (Braunschweig, Germany).

Bacillus SF was grown in a medium containing 8 g l^{-1} yeast extract, 8 g l^{-1} extract from meat (Merck), and 1 g l^{-1} KH_2PO_4 at 65°C and pH 10.0, buffered with 50 mM $NaHCO_3/Na_2CO_3$. Then, 1% (w/w) of a trace element solution (2.5 g l^{-1} Na_2EDTA, 0.1 g l^{-1} $ZnSO_4{\cdot}7H_2O$, 0.030 g l^{-1} $MnCl_2{\cdot}4H_2O$, 0.3 g l^{-1} H_3BO_3, 0.2 g l^{-1} $CaCl_2{\cdot}6H_2O$, 0.01 g l^{-1} $CuCl_2{\cdot}2H_2O$, 0.02 g l^{-1} $NiCl_2{\cdot}6H_2O$, 0.9 g l^{-1} $Na_2MoO_4{\cdot}2H_2O$, 0.03 g l^{-1} $Na_2SO_3{\cdot}5H_2O$, and 1 g l^{-1} $FeSO_4{\cdot}7H_2O$) was added to the incubation mixture.

Purification of the catalase-peroxidase from *Bacillus* SF

Cells were harvested at the end of the exponential phase of growth, centrifuged (3,000 *g*, 15 min), and the pellet was suspended with phosphate buffer (50 mM, pH 7.0). Cell disruption was carried out using sonication (Sonoplus HD 70; Bandelin, Berlin, Germany) monitoring progress under the microscope. Cell debris was removed by centrifugation (6,500 *g*, 20 min) and the remaining supernatant was used for purification.

The cell lysate was subjected to fractionation by ammonium sulfate precipitation at 40% saturation to remove impurities, followed by 70% saturation in a second step to precipitate the CP. The precipitated proteins were collected by centrifugation, and the pellet was dissolved in phosphate buffer (50 mM, pH 7.0) with 1.0 M $(NH_4)_2SO_4$. The solution was applied to a Phenyl Sepharose High Performance column (Pharmacia, Uppsala, Sweden) equilibrated with 1.0 M $(NH_4)_2SO_4$ in phosphate buffer (as described earlier). Fractions containing protein were eluted with a linear gradient of 1.0–0 M $(NH_4)_2SO_4$. Catalase active fractions were pooled and loaded onto a UNO Q6 column (Bio-Rad, Hercules, CA, USA) equilibrated with phosphate buffer (50 mM, pH 7.0). After adsorption, proteins were eluted with a linear gradient of 0–1 M NaCl in the same buffer; the catalase was eluted at about 0.25 M NaCl. Active fractions were pooled, concentrated in an ultrafiltration cell (Sartorius, Epsom, UK) using a membrane with a 100-kDa cutoff, and subjected to a Superdex 200 HR 10/30 column (Pharmacia) previously equilibrated with phosphate buffer (50 mM, pH 7.0) containing 0.15 M NaCl. The column was run with the same buffer, and active fractions were collected and stored at –20°C until further use. The chromatographic purification of the enzyme was performed with the FPLC system AEKTA from Pharmacia.

Protein characterization

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) was carried out in 12% polyacrylamide gels using broad molecular weight markers from Pharmacia as standards. Proteins on the gel were stained with Coomassie brilliant blue. Native PAGE was performed in 10% polyacrylamide gels that were stained for catalase activity as described previously (Harris and Hopkinson 1976) using 0.001% H_2O_2. Peroxidase activity was determined according to the modified method of Graham et al. (1964) using guaiacol or *o*-dianisidine as a substrate. Isoelectric focusing (IEF) was carried out with the Mini-PROTEAN 3 cell from Bio-Rad using IEF Ready Gels (pH 5–8; Bio-Rad), and protein was located by Coomassie brilliant blue staining.

Amino acid sequence analysis

For internal amino acid sequence analysis, using matrix-assisted laser desorption ionization–mass spectrometry (MALDI-MS), the excised gel band of catalase-peroxidase

from *Bacillus* SF was digested with endoproteinase Lys-C, separated by high-performance liquid chromatography (HPLC) with spectrometric mass determination, and the amino acid sequence of peptides was determined as described previously (Eckerskorn and Lottspeich 1989). For direct N-terminal amino acid sequencing of protein, the gel band was blotted onto poly(vinylidene difluoride) membranes (ProBlottTM PVDF-membrane; Applied Biosystems, Weiterstadt, Germany) by the "semidry" method, as described previously (Eckerskorn et al. 1988). The blotted polypeptides were stained with Coomassie blue and excised, and the N-terminal amino acid sequence was determined in a protein sequencer model 477 A (Applied Biosystems).

Peptide mass mapping

The proteolytic digest pattern derived from the purified catalase-peroxidase was compared with virtual digest of the CP of *B. stearothermophilus* using the Peptide Mapping software, ProFound, available via the PROWL World Wide Web server, at the URL address (http://129.85.19.192/profound_bin/WebProFound.exe).

Enzyme assay

Catalase activity was monitored spectrophotometrically. The assay mixture contained 100 µl enzyme preparation and 900 µl phosphate buffer (50 mM, pH 7.0). The reaction was initiated by addition of 1,000 µl 26 mM H_2O_2 solution monitoring the decrease in absorbance at 240 nm at room temperature. The linear range of the reaction (30 s) was used to calculate the rate of the reaction. One unit of enzyme activity was defined as 1 µmol H_2O_2 decomposed per minute. Kinetic parameters (mean SD for three assays) of the CP were determined using the standard assay with different substrate concentrations. K_m and v_{max} were calculated by nonlinear analysis using the program Origin, version 4.10. Inhibition of the *Bacillus* SF catalase-peroxidase by 3-amino-1,2,4-triazol, azide, and cyanide was measured as already described, except that the enzyme was preincubated for 5 min at 20°C with the inhibitor. Peroxidase activity was assayed in an incubation mixture containing 1 mM peroxoacetic acid and 1 mM of either guaiacol or *o*-dianisidine in phosphate buffer (50 mM, pH 7.0). The reaction was monitored spectrophotometrically as described previously (Cendrin et al. 1994; Hochman and Goldberg 1991).

Other procedures

The pH optimum of the CP was determined in 50 mM potassium phosphate buffer (pH 6.0–8.5) and 50 mM $NaHCO_3$/Na_2CO_3 for pH ≥ 9. To determine enzyme stabilities, 1 ml of enzyme preparation was diluted with 9 ml buffer in test tubes that were shaken at 50 rpm in a water bath. Samples were withdrawn at various time intervals, and remaining enzyme activity was measured as already described.

The molecular mass of the purified enzyme was determined by gel filtration chromatography on a Superdex 200 HR 10/30 column (Pharmacia), equilibrated with potassium phosphate buffer (50 mM, pH 7.0) containing 150 mM NaCl, and calibrated with a high molecular weight gel filtration calibration kit from Pharmacia. Protein concentrations were determined by the method of Bradford (1976) (Bio-Rad) using bovine serum albumin as a standard.

UV-visible spectra were recorded on a Hitachi U 2001 spectrophotometer (Hitachi, Tokyo, Japan).

Results

Thermoalkaliphilic *Bacillus* SF

A thermoalkaliphilic bacterium was isolated from textile finishing effluent. The cells were gram positive, catalase positive, non-spore-forming, and rod shaped. Growth occurred at pH values ranging from 7.5 to 10.1, and optimum growth occurred around pH 9.5. The optimum temperature for growth was around 60°C and the upper temperature limit for growth was 65°C. Standard analysis of the cellular fatty acids indicated that the isolated bacterium belongs to a thermophilic *Bacillus* sp., according to the DSMZ database. Similarly, the physiological conditions determined for the organism clearly pointed to an alkalophilic *Bacillus* sp. A sequence analysis of the 16S rRNA gene revealed that the new organism is closely related phylogenetically to members of the genus *Bacillus*.

Purification of catalase-peroxidase

Bacillus SF was cultivated in a 10-l bioreactor, and a crude enzyme preparation was obtained showing catalase activities of 48 U mg^{-1}. Figure 1A indicates that cytosolic fractions of the thermoalkaliphilic *Bacillus* SF contain only one isoform of an enzyme with hydroperoxidase activity because native PAGE gave only single bands of identical migration characteristics detectable with staining procedures for either catalase (Fig. 1A) or peroxidase activity (not shown). Table 1 summarizes the four steps of the purification procedure of this enzyme, yielding a homogenous protein purified approximately 70.3-fold over the crude extract and giving a final recovery of 11.3% of catalase activity. During the various purification steps, both catalase and peroxidase activity were always eluted as a sharp and single peak. SDS-PAGE of the purified enzyme gave a single band at about 82 kDa (Fig. 1B), whereas molecular mass analysis by gel filtration on a Superdex 200 HR 10/30 column gave a molecular mass of about 165 ± 10 kDa (data not shown), indicating that the enzyme is composed of two subunits of identical size. The isoelectric point of the enzyme protein was determined by IEF to be at pH 6.0.

Physical and chemical characterization of the CP from *Bacillus* SF

The temperature optimum for catalase activity at pH 9.5 was observed at 55°C. The activity decreased by 10% at

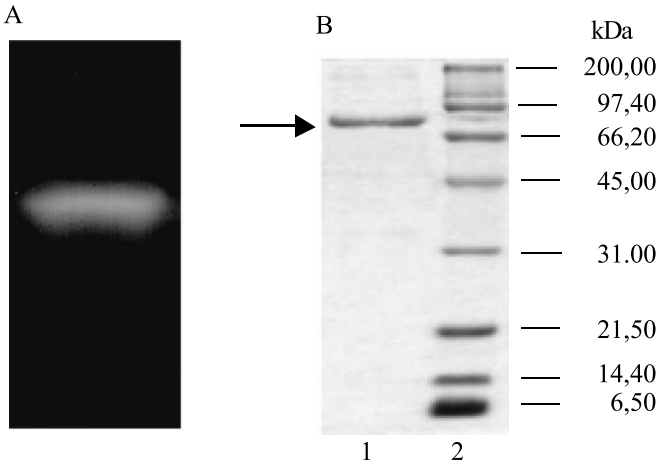

Fig. 1A,B. Native and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of the purified catalase-peroxidase (CP) from *Bacillus* SF. **A** Native 10% polyacrylamide gel stained for catalase activity according to Harris and Hopkinson (1976). **B** Denaturing SDS 12% polyacrylamide gel stained for protein with Coomassie brilliant blue. *Lane 1*, purified CP; *lane 2,* Pharmacia molecular mass standard. *Arrow* indicates application of the catalase

Table 1. Purification of catalase peroxidase from the thermoalkaliphilic *Bacillus* SF

Step	Total protein (mg)	Tota activity (U)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Crude extract	200	9,600	48	1	100
40%–70% $(NH_4)2SO_4$	98	8,540	87.1	1.8	88.8
Phenyl-Sepharose	35	5,600	160	3.3	58.3
UNOQ-6	1.45	1,560	1,076	22.4	16.3
Superdex 200	0.32	1,080	3,375	70.3	11.3

60°C and by 50% at 70°C. The enzyme was active at pH 6.0–10.0; however, a sharp activity maximum appeared at pH 7.0 (25°C). Activity rapidly decreased until it reached 25% at pH 8.0, where it remained almost constant until pH 9.0; at pH 10, only 4% activity remained (Fig. 2).

The CP from *Bacillus* SF was quite stable at pH 10 and 25°C, showing half-lives of at least 4 days (Table 2). Interestingly, at this temperature the CP showed substantially higher stabilities at pH 8 and pH 9 than at pH 7. At 60°C, the CP had the longest half-lives at pH 8.0 and pH 9.0, and its half-life decreased to only 5 h at pH 10.

Catalytic and spectral properties

The visible optical density spectrum of thermoalkaliphilic CP from *Bacillus* SF showed a strong Soret band at 405 nm and a peak at 623 nm. The OD_{405}/OD_{280} ratio of the enzyme preparations was 0.36, which is consistent with one heme per homodimer. On addition of cyanide, the Soret band

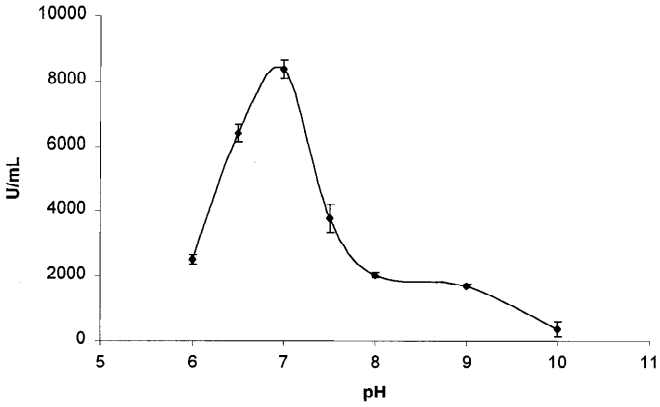

Fig. 2. Effect of pH on the catalase activity of the purified CP from *Bacillus* SF. Catalytic activity was assayed at 25°C as described in Materials and methods

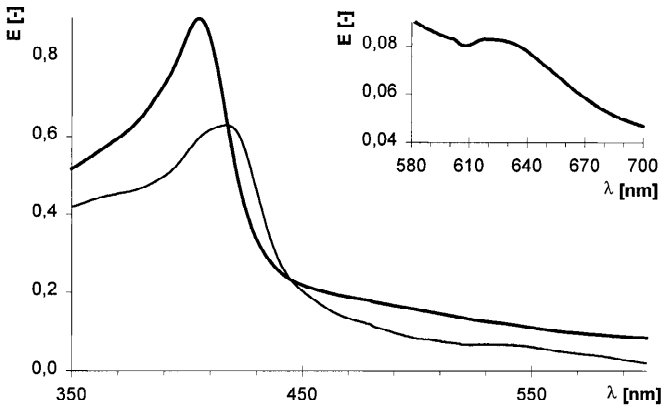

Fig. 3. Absorption spectrum of purified CP from *Bacillus* SF. *Thick line*, 9 μM native enzyme; *thin line*, the cyano-adduct obtained by the addition of 25 mM KCN to the native enzyme. *Inset* is an expanded scale of the visible portion of the spectrum. Experimental conditions were as described in Materials and methods

Table 2. Stability of a catalase peroxidase from *Bacillus* SF

Temperature (°C)	pH			
	7.0	8.0	9.0	10.0
25	190	260	240	104
50	15	35	48	14
60	22	40	38	5

Data are half-life in hours

shifted from 405 to 417 nm, indicating a high to low spin transition of the iron center of the heme (Fig. 3). The catalase-peroxidase was reduced by dithionite, as indicated by two new absorption maxima at 435 and 553 nm.

The catalytic activity of the purified enzyme was 3,375 U mg^{-1} protein. The apparent K_m was 2.6 ± 0.1 mM H_2O_2, and the turnover number (k_{cat}) was 11,475 s^{-1}. The observed k_{cat}/K_m was in the same order of magnitude as that from *Escherichia coli* HP1 (Table 3) and reflects the high

Table 3. Kinetic parameters of various catalase-peroxidases

Organism	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})	Reference
Bacillus SF	2.6	11,475	4.41×10^6	This study
Bacillus stearothermophilus	4.4	1,400	0.32×10^6	Kobayashi and Suga (1997)
Alkalophilic *Bacillus*	6.8	n.d.	n.d.	Yumoto et al. (1990)
Rhodobacter capsulatus	4.2	n.d.	n.d.	Hochman and Goldberg (1991)
Mycobacterium smegmatis	1.4	2,380	1.70×10^6	Marcinkeviciene et al. 1995)
Mycobacterium tuberculosis	30	2,300	0.076×10^6	Nagy et al. (1997)
Mycobacterium tuberculosis	5.2	10,140	1.95×10^6	Johnsson et al. (1997)
Anacystis nidulans	4.8	8,850	1.8×10^6	Engleder et al. (2000)
Synechocystis 7942	4.2	26,000	6.19×10^6	Mutsuda et al. (1996)
Synechocystis 6301	4.3	7,200	1.66×10^6	Obinger et al. (1997)
Synechococcus 6803	4.8	3,450	0.72×10^6	Regelsberger et al. (1999a)
Escherichia coli HP1	3.9	16,300	4.17×10^6	Claiborne and Fridovich (1979)

catalytic efficiency of the reaction. The purified enzyme was irreversibly inhibited by high hydrogen peroxide concentrations. Starting at concentrations of 15 mM H_2O_2, progressive inhibition of the enzyme was seen (data not shown). In contrast to monofunctional catalases, 3-amino-1,2,4-triazole did not inhibit the catalase-peroxidase from *Bacillus* SF. However, addition of cyanide and azide caused pronounced inhibition of the enzyme activity. For both azide and cyanide, the inhibition was competitive with a K_I^{EI} of 15 µM and 1.5 µM for the dissociation of the enzyme-inhibitor complex, respectively (calculated from Dixon plots and the method of Eadee–Hofsted). Both guaiacol and *o*-dianisidine functioned as electron donors for *Bacillus* SF catalase-peroxidase after compound I formation with peroxoacetic acid; *o*-dianisidine was the better substrate (data not shown).

Discussion

A thermoalkaliphilic bacterium was isolated from textile finishing effluent and identified as a new representative of the genus *Bacillus*. There are only a few reports on thermoalkaliphilic *Bacillus* species, such as *Bacillus* sp. TAR-1 (Takahashi et al. 2000), *Bacillus thermocatenulatus* (Rua et al. 1997), *Bacillus thermoalcaliphilus* (Sarkar and Upadhyay 1993), or an anaerobic strain, LBS3 (Prowe et al. 1996). Although catalases, which are produced by most aerobic microorganisms, are very well studied enzymes, there are only few reports on thermostable and alkalistable catalases such as those from *Thermoleophilum album* (Allgood and Perry 1986). Similarly, there is only little information in the literature about the thermostability and pH stability of catalase-peroxidases.

At pH 6.5 and 58°C, a catalase-peroxidase from *Synechococcus* PCC 7942 and one from *Mycobacterium tuberculosis* (Mutsuda et al. 1996; Nagy et al. 1997) both showed half-life times of 30 min. A CP from an alkalophilic *Bacillus* sp. had a half-life of 60 min at pH 11 and 60°C (Yumoto et al. 1990), whereas the CP from *Bacillus* SF investigated in this report showed a half-life of 300 min at the same temperature but at pH 10. At room temperature and pH 10, this CP was stable for 104 h. For the closely related CP from *B. stearothermophilus* (see following), an interesting phenomenon was observed. The CP from this organism showed an increase in k_{cat} with a concomitant decrease of K_m after heating. In contrast to these findings, no second active form could be found on thermal activation for the CP of *Bacillus* SF.

The catalase-peroxidase from *Bacillus* SF was found to share a number of structural and spectroscopic properties with other bacterial catalase-peroxidases. This enzyme is a dimer of 82-kDa subunits, containing one ferric protoporphyrin IX prosthetic group, and exhibits a high-spin ferric heme optical spectrum. High-spin to low-spin conversion was observed in the presence of CN^-. Homodimeric or homotetrameric structures are typical for this type of hydroperoxidase. In general, the identical subunits of catalase-peroxidases are 78–85 kDa in size, as has been demonstrated for the subunits in *E. coli* (Claiborne and Fridovich 1979), *Streptomyces* sp. IMSNU-1 (Youn et al. 1995), *Mycobacterium smegmatis* (Marcinkeviciene et al. 1995), *Mycobacterium tuberculosis* (Johnsson et al. 1997), *Rhodobacter capsulatus* (Hochman and Goldberg 1991), and *B. stearothermophilus* (Loprasert et al. 1988). Other heme-containing peroxidases of fungi and plants are in most cases monomeric proteins. The double length of the bacterial peroxidases has been ascribed to gene duplication (Welinder 1992).

The OD_{405}/OD_{280} ratio of the enzyme preparations from *Bacillus* SF was 0.36, which is consistent with one heme per homodimer. It has been reported that catalase-peroxidases found in other bacterial systems also have low heme content. The OD_{405}/OD_{280} ratios per subunit of typical catalase-peroxidases were 0.54 in *Streptomyces* sp. (Youn et al. 1995), 0.56 in *M. smegmatis* (Marcinkeviciene et al. 1995), 0.52 in *E. coli* HPI (Claiborne and Fridovich 1979), 0.3 in *R. capsulatus* (Hochman and Goldberg 1991), and between 0.35 and 0.46 in *Synechocystis* sp. (Regelsberger et al. 1999b).

Additional characteristics shared with other bacterial catalase-peroxidases include the competitive inhibition by cyanide and azide and the lack of inhibition by the catalase inhibitor, 3-amino-1,2,4-triazole (Marcinkeviciene et al. 1995). The linear, competitive nature of the inhibition by

CN^- suggests that the heme iron atom of the catalase-peroxidase is the site of both peroxide and cyanide binding, as is the case for other peroxidases (Dunford 1991). The K_I^{EI} of 1.5 µM measured for the inhibition of the CP from *Bacillus* SF by cyanide was significantly lower than the K_I^{EI} of 18.7 µM reported previously for a CP from *Anacystis nidulans* (Engleder et al. 2000). The absorption spectrum of the *Bacillus* SF catalase-peroxidase obtained after addition of dithionite was comparable with data reported in the literature for other catalase-peroxidases. Dithionite reduction distinguishes the CP from typical catalases, which are not reduced by this agent, whereas typical peroxidases are rapidly reduced by dithionite (Johnsson et al. 1997; Marcinkeviciene et al. 1995; Loprasert et al. 1988).

With a k_{cat} of 11,475 s^{-1}, the catalytic activity of the purified CP from *Bacillus* SF is about in the same order of magnitude as that of other typical catalase-peroxidases (see Table 3). In addition to its catalase activity, the enzyme functions as a peroxidase, oxidizing guaiacol and *o*-dianisidine, for example. In contrast, no activity on guaiacol was previously found for CP from *Synechocystis* sp. and *Klebsiella pneumoniae* (Cendrin et al. 1994; Hochman and Goldberg 1991) whereas *o*-dianisidine seemed to be a substrate for most CP (Johnsson et al. 1997; Marcinkeviciene et al. 1995; Youn et al. 1995; Mutsuda et al. 1996).

The homology of the purified protein with the catalase-peroxidase of *Bacillus stearothermophilus* was determined by peptide mass mapping using MALDI-MS. Thus, in conclusion, physical characterization of the catalase-peroxidase from the alkalithermophilic *Bacillus* SF clearly indicates that this enzyme belongs to the developing class of bacterial enzymes that catalyze both catalase- and peroxidase-like reactions. The low heme content of catalase-peroxidases is in contradiction to sequence alignment models proposing one heme binding per monomer (Welinder 1991). Nevertheless, this OD_{405}/OD_{280} ratio seems to be one of the characteristics of this type of hydroperoxidase. To obtain more information about the structure–function relationship of this enzyme, the determination of the three-dimensional structure of this new thermoalkalistable catalase-peroxidase has been initiated.

Acknowledgments The authors thank the European Commission for financial support of project EW 98 0009.

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