Purification and Characterization of a Novel Thermo-Alkali-Stable Catalase from *Thermus brockianus*

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A novel thermo-alkali-stable catalase from *Thermus brockianus* was purified and characterized. The protein was purified from a *T. brockianus* cell extract in a three-step procedure that resulted in 65-fold purification to a specific activity of 5300 U/mg. The enzyme consisted of four identical subunits of 42.5 kDa as determined by SDS−PAGE and a total molecular mass measured by gel filtration of 178 kDa. The catalase was active over a temperature range from 30 to 94 °C and a pH range from 6 to 10, with optimum activity occurring at 90 °C and pH 8. At pH 8, the enzyme was extremely stable at elevated temperatures with half-lives of 330 h at 80 °C and 3 h at 90 °C. The enzyme also demonstrated excellent stability at 70 °C and alkaline pH with measured half-lives of 510 h and 360 h at pHs of 9 and 10, respectively. The enzyme had an unusual pyridine hemochrome spectrum and appears to utilize eight molecules of heme *c* per tetramer rather than protoheme IX present in the majority of catalases studied to date. The absorption spectrum suggested that the heme iron of the catalase was in a 6-coordinate low spin state rather than the typical 5-coordinate high spin state. A K_m of 35.5 mM and a V_{max} of 20.3 mM/min·mg protein for hydrogen peroxide was measured, and the enzyme was not inhibited by hydrogen peroxide at concentrations up to 450 mM. The enzyme was strongly inhibited by cyanide and the traditional catalase inhibitor 3-amino-1,2,4-triazole. The enzyme also showed no peroxidase activity to peroxidase substrates *o*-dianisidine and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), a trait of typical monofunctional catalases. However, unlike traditional monofunctional catalases, the *T. brockianus* catalase was easily reduced by dithionite, a characteristic of catalase-peroxidases. The above properties indicate that this catalase has potential for applications in industrial bleaching processes to remove residual hydrogen peroxide from process streams.

Introduction

There has been growing interest in recent years in utilizing hydrogen peroxide in industrial sectors such as food, dairy, textiles, and pulp and paper (*1*) as a more environmentally friendly alternative to existing chemical treatments. As use of hydrogen peroxide in industrial settings grows, there will also be a need to remove hydrogen peroxide from process streams because it can interfere with subsequent process steps (*2*). An example of hydrogen peroxide use is in the textile industry for bleaching of fabrics (*3*), where it has been shown that hydrogen peroxide interferes with subsequent dyeing steps and must be removed (*2*). Current methods to remove the hydrogen peroxide either utilize extensive washing and result in the generation of large volumes of wastewater (*4*) or utilize chemical treatments such as sodium bisulfite or hydrosulfite to reduce hydrogen peroxide and lead to high salt levels in the process streams (*5*). It has been proposed recently that the enzyme catalase be used to remove residual hydrogen peroxide from the bleach water so that it can be reused in the subsequent dye steps (*6*). However, since the hydrogen peroxide bleaching step occurs at elevated temperatures and pH (> 60 °C and pH 9), commercially available catalases that are optimally active at 20−50 °C and at neutral pH require that the temperature and pH be adjusted prior to their use (*7*). Availability of a catalase enzyme able to operate at higher temperatures and pHs would be attractive for the above applications.

Catalase is a ubiquitous enzyme found in aerobic organisms. It efficiently catalyzes the decomposition of hydrogen peroxide to oxygen and water and, together with other enzyme systems, protects cells against the harmful effects of reactive oxygen species such as superoxide anions, hydrogen peroxide, and hydroxyl radicals (*8*−10). Three general classes of catalases have been described in the literature: the typical or monofunctional catalases (*11*); the catalase-peroxidases that have a peroxidative activity as well as the catalatic activity (*11*); and the Mn-catalases or pseudocatalases (*12*−*14*). Typical catalases have been isolated from numerous animals, plants, and microorganisms and have similar properties regardless of the source. These enzymes have four subunits of equal size with a typical combined molecular mass of 225,000−270,000 kDa and characteristically have four protoheme IX prosthetic groups per tetrameric molecule. These enzymes also have a broad pH activity range from 4 to 10, are specifically inhibited by 3-amino-1,2,4-triazole, and are resistant to reduction by dithionite

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(*11*, *15*, *16*). The catalase-peroxidase enzyme was first isolated from *Escherichia coli* in 1979 (*17*). These enzymes are typically dimers or tetramers with a subunit size of approximately 80 kDa and, in contrast to the typical catalases, have low heme content with only 1−2 hemes per enzyme molecule (*18*). Additionally, the catalase-peroxidases have a sharp pH optimum, are not inhibited by 3-amino-1,2,4-triazole, are sensitive to hydrogen peroxide concentration, and are readily reduced by dithionite (*18*, *19*). Sequence analysis of the two groups of enzymes has shown that they are not related, and on the basis of sequence similarity, the catalase-peroxidases are grouped in class I of the superfamily of plant, fungal, and bacterial peroxidases (*20*). Both catalase and catalase-peroxidases are strongly inhibited by cyanide and azide, classic heme protein inhibitors. The Mn-catalases, in contrast to the other two catalase groups, do not utilize a heme prosthetic group in their active site and instead use manganese ions. These enzymes are, therefore, insensitive to the heme poisons, cyanide, and azide (*12*, *13*). The pseudocatalases have subunit sizes ranging from 28 to 35 kDa and are typically hexameric (*13*, *14*), although a tetrameric pseudocatalase enzyme was described from *Thermoleophilum album* (*12*).

A few thermostable versions of a monofunctional catalase (*21*), catalase-peroxidases (*18*, *19*, *22*, *23*), and Mn-catalases (*12*, *13*) have been described. In addition to having activity at higher temperatures, the thermostable versions exhibit properties similar to those of their mesophilic counterparts. Many of the reported enzymes exhibited low thermal stability at temperatures above 60 °C, several were rapidly inactivated in the presence of hydrogen peroxide, and most of the enzymes had low activity and stability at elevated temperature and pH, making them unsuitable for industrial applications. This paper describes the isolation, purification, and characterization of an extremely thermo-alkali-stable catalase enzyme from *Thermus brockianus*.

Materials and Methods

Microorganism and Culture Conditions. Hot spring LNN2 in Yellowstone National Park, USA with an average temperature of 70 °C and an average pH of 7 was selected as a sampling site for this study. The GPS coordinates for this site were $x = 515923.1013974$ and $y = 4931375.3306555$ measured on a Tremble GPS Pathfinder unit differentially corrected using the Idaho Falls, ID base station as the reference. Specifics of the GPS unit include Datum = NAD83, PDOP mask = 6.0, minimum satellites = 4. Water, sediment, and fungal mat samples from the spring were collected in sterile 50-mL centrifuge tubes and were maintained at approximately 70−80 °C until they could be processed (typically 4−6 h later). Samples were inoculated into a minimal medium (4.2 g/L sodium lactate, 10 mM NH_4Cl, 5.2 mM K_2HPO_4, 0.8 mM $MgSO_4 \cdot 7H_2O$, 1.74 mM Na_2SO_4, 25 mg/L $MgCl_2$, 6.6 mg/L $CaCl_2$, 2 mg/L $MnSO_4$, 0.5 mg/L $ZnSO_4$, 0.5 mg/L boric acid, 5 mg/L $FeCl_3$, 0.15 mg/L $CuSO_4$, 0.025 mg/L $NaMoO_4$, 0.05 mg/L $CoNO_3$, 0.02 mg/L $NiCl_2$, 0.08 mg/L pyridoxine hydrochloride, 0.01 mg/L folic acid, 0.1 mg/L thiamine hydrochloride, 0.04 mg/L riboflavin, 0.08 mg/L nicotinamide, 0.08 mg/L *p*-aminobenzoate, 0.01 mg/L biotin, 0.0004 mg/L cyanocobalamin, 0.08 mg/L D-pantothenic acid·Ca, 0.02 mg/L myo-inositol, 0.05 mg/L choline bromide, 0.02 mg/L monosodium orotic acid, and 0.1 mg/L spermidine) utilizing lactate as the primary carbon source and incubated in 100-mL serum vials at 70 °C on a rotary shaker at 150 rpm. Oxygen levels were tested daily by gas chromatography (*24*), and the headspace was flushed with air when oxygen levels fell below 5% (initial oxygen levels at 21%). Growth was assumed when the cultures became cloudy in appearance, after which cultures were streaked onto agar plates and maintained at 70 °C until growth occurred. Individual colonies were tested for catalase activity by suspending colonies in a drop of 3% hydrogen peroxide and examining for evolution of bubbles (*25*). The isolate showing the highest catalase activity was selected for further characterization. Microscopic examination of the isolate showed a non-spore-forming, rod-shaped organism. The organism formed diffuse light yellow colonies on agar and was found to be Gram-negative. Sequence analysis (16S rRNA) of this organism (MIDI Labs; Newark, DE) identified it as a 100% match to *Thermus brockianus*.

Catalase Purification from *Thermus brockianus*. *Thermus brockianus* was cultured to stationary phase at 70 °C using the medium described above in a 100-L B.Braun UE-100D fermentor. The fermentor was run with an impellor speed of 260 rpm, the pH was maintained at 7.2, and an aeration rate of 30 L/min air provided between 80% and 100% oxygen saturation (at 70 °C) to the culture. The culture took approximately 100 h to reach stationary phase with a final OD_{600} of 0.38. The cells were collected by centrifugation, resuspended in 20 mM Tris buffer, pH 8 with protease inhibitor (Sigma Aldrich, St. Louis, MO), and disrupted by sonication. Cell debris was removed by centrifugation (34,000*g* for 45 min) and the supernatant was collected.

The crude cell extract was filtered through a 0.2-μm filter and applied to a DEAE ion-exchange column (Amersham Biosciences, Piscataway, NJ) equilibrated with 20 mM Tris buffer, pH 8. The enzyme was eluted with a linear gradient from 0 to 500 mM ammonium sulfate in 100 mM Tris buffer, pH 8. The fractions with catalase activity (using the assay described in the next section) were pooled, and the ammonium sulfate concentration of the sample was adjusted to 1.0 M. The sample was then applied to a HiTrap Phenyl Sepharose High Performance hydrophobic interaction column (Amersham Biosciences, Piscataway, NJ) equilibrated with 100 mM Tris buffer, pH 8, containing 1 M ammonium sulfate. A decreasing linear elution gradient of ammonium sulfate from 1 M to 0 was used to elute the enzyme. Active catalase fractions were pooled and applied to a Sephacryl S-300 HR gel filtration column (Amersham Biosciences, Piscataway, NJ) for the final purification step. The enzyme was eluted with 100 mM Tris buffer, pH 8, containing 0.15 M sodium chloride. The effectiveness of each purification step was determined by SDS−PAGE using a 12% (w/v) acrylamide gel (*26*). Protein concentrations were determined using the DC protein assay (Biorad; Hercules, CA) with bovine serum albumin as a standard.

Enzyme Assay. Catalase activity was determined spectrophotometrically by monitoring the decrease in absorbance at 240 nm caused by the disappearance of hydrogen peroxide (*27*). The assay was initiated by addition of enzyme solution to 20 mM hydrogen peroxide in 20 mM Tris buffer, pH 8 and was conducted at 70 °C unless otherwise specified. The buffer pH was adjusted to 8 at 70 °C. The initial absorbance change (typically the first 30 s) was used to calculate the rate of hydrogen peroxide decomposition. The molar absorption coefficient for hydrogen peroxide at 240 nm was assumed to be 43.6 $M^{-1}\ cm^{-1}$ (*28*) and one unit (U) of catalase activity was defined as the amount of enzyme required to degrade 1 μmol of hydrogen peroxide per minute. Peroxidase activity of the catalase enzyme was tested using *o*-dianisidine

(0.5 mM) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (10 mM) as substrates with the hydrogen peroxide concentration at 1 mM. The reactions were monitored spectrophotometrically at 460 and 420 nm, respectively. The substrates were dissolved in 20 mM Tris buffer, pH 8.0, and the assays were conducted at 70 °C.

Molecular Mass Determination. The molecular mass of the purified catalase was estimated via gel filtration under native nondenaturing conditions using molecular mass standards (Amersham Biosciences; Piscataway, NJ) under the same conditions as described above. The subunit size of the catalase was estimated from SDS−PAGE gel electrophoresis on a 12% acrylamide gel using molecular mass standards obtained from Bio-Rad Laboratories (Hercules, CA). Proteins were visualized on the gel using SimplyBlue SafeStain (Invitrogen Corp.; Carlsbad, CA).

Enzyme Kinetics. The Michaelis−Menten constants for the enzyme were determined using the standard assay with hydrogen peroxide concentrations ranging from 3 to 450 mM. The constants were calculated by fitting the Michaelis−Menten equation to a plot of reaction velocity versus substrate concentration using nonlinear analysis (GraFit Version 4, Erithacus Software Limited, Horley Surrey, U.K.). Irreversible inhibition of the catalase enzyme was tested using 40 mM 3-amino-1,2,4-triazole and 1 mM cyanide. The enzyme was assayed as above but preincubated with the inhibitor for 5 min prior to assay.

Optimum Temperature, Optimum pH, and Stability. The optimum temperature for enzyme activity was determined by assaying the enzyme as described above at temperatures ranging from 20 to 94 °C. For determination of pH response, buffers in the pH range from 4 to 11 were used: 50 mM sodium citrate (pH 4−6), 50 mM potassium phosphate (pH 7), 50 mM Tris (pH 8−9), and 50 mM glycine (pH 10−11). The assays were conducted at 70 °C with 20 mM hydrogen peroxide in the appropriate buffer, and the pH of the buffers was adjusted to the correct value at that temperature. The enzyme assay was conducted as described above. Temperature stability of the catalase enzyme was examined by incubating a 0.1 mg/mL enzyme solution at 80 or 90 °C and periodically removing samples. A mineral oil overlay was placed on top of the enzyme solution to prevent evaporation. Enzyme stability as a function of pH was assessed using a 1 mg/mL solution of catalase enzyme in the appropriate pH 9, 10, or 11 buffers, incubating at 70 °C, and periodically removing samples. The catalase activity of samples collected during the temperature and pH stability studies was determined at 70 °C as described in Enzyme Assay above.

Enzyme Isoelectric Point. The isoelectric point of the enzyme was determined using a model 111 Mini Isoelectric Focusing Cell from Bio-Rad Laboratories (Hercules, CA). A 5% (w/v) acrylamide gel was focused for 15 min at 100 V, 15 min at 200 V, and 60 min at 450 V. After focusing was complete, the gel was removed from the cell and cut in half. Proteins were visualized on one-half of the gel by staining with SimplyBlue SafeStain (Invitrogen Corp.; Carlsbad, CA). On the other half of the gel, hydrogen peroxide solution was added to locate the catalase activity indicated by the evolution of bubbles. The single band of catalase identified was compared to pI standards ranging from 4.45 to 9.6 (Biorad; Hercules, CA).

Enzyme Spectral Characteristics. The absorption spectra of the native enzyme, enzyme reduced with 1 mM sodium dithionite, and enzyme treated with 10 mM KCN were measured. The protoheme type and content were determined through the formation of a pyridine hemochrome as described by Falk (*29*). All spectra were measured at both 22 and 70 °C to examine possible conformational changes by the enzyme at those temperatures. The molar absorption coefficient for the pyridine hemochrome was assumed to be 191.5 mM^{-1} cm^{-1} (*29*).

Table 1. Purification Procedure for Catalase from *T. brockianus*

	total activity (U)	total protein (mg)	specific activity (U/mg)	yield (%)	purification (fold)
cell extract	139 200	1 700	82	100	1.0
ion exchange	25 440	153	166	18	2.0
hydrophobic interaction	1 440	2.4	600	1.0	7.3
gel filtration	1 160	0.22	5 320	0.8	65

Results and Discussion

Catalase Purification. A three-step purification procedure consisting of ion exchange, hydrophobic interaction, and gel filtration chromatography was developed to obtain a highly purified catalase from *T. brockianus*. The effectiveness of each purification step is given in Table 1 and Figure 1. The three-step procedure described here resulted in 1160 total units of catalase activity, 65-fold purification of the crude cell extract, and a specific catalase activity of 5300 U/mg protein with a yield of 0.8%. The 65-fold purification achieved in this procedure is comparable to that obtained with other bacterial catalases. A survey of catalase literature showed a range of purification levels achieved from 40-fold for a catalase from *Escherichia coli* (*17*) to 400-fold for a catalase from *Streptomyces coelicolor* (*15*). While the yield from this purification method was low compared to those of other published procedures, this method had the advantages of being rapid and yielding a very pure catalase enzyme as evidenced by the presence of a single band after the gel filtration step (Figure 1, Lane 5). Since the goal of this study was to assess the properties of the *T. brockianus* catalase for possible industrial applications, the yield obtained from the above procedure provided sufficient material for that purpose.

Physical and Chemical Characterization of *T. brockianus* Catalase. SDS−PAGE of the purified catalase enzyme showed a single band corresponding to a subunit size at 42.5 kDa, and the gel filtration results showed an approximate native protein molecular mass of 178 kDa, indicative of an enzyme with four identical subunits. The subunit and native enzyme sizes for this enzyme are significantly smaller than those reported for other tetrameric catalase enzymes (i.e., *Bacillus* sp. with 70.5 and 282 kDa, *E. coli* with 84.3 and 337 kDa, *Rhodobacter capsulatus* with 59 and 236 kDa, and *Neurospora crassa* with 80 and 320 kDa subunit and native molecular mass, respectively) (*30*).

The isoelectric point of this catalase was 4.7. Isoelectric points were not reported for the above catalases; however, the measured isoelectric point was comparable to those reported for catalases and catalase-peroxidases from *Halobacterium halobium* of 4.0 (*31*), *Thermoascus aurantiacus* of 4.5 (*21*), *Vitreoscilla* sp. of 5.0 and 5.2 (*32*), and *Anacystis nidulans* of 4.7 (*33*).

The activity of this catalase as a function of temperature and pH is shown in Figures 2 and 3. The enzyme had virtually no activity at 20 °C, and activity increased with increasing temperature up to the maximum activity

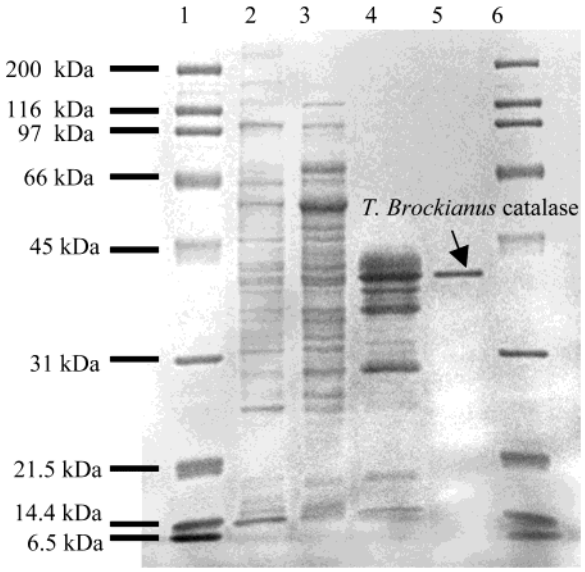

Figure 1. SDS−PAGE of catalase containing fractions after each purification step: (Lane 1) molecular mass standards; (Lane 2) cellular extract; (Lane 3) DEAE ion exchange; (Lane 4) hydrophobic interaction; (Lane 5) gel filtration; and (Lane 6) molecular mass standards.

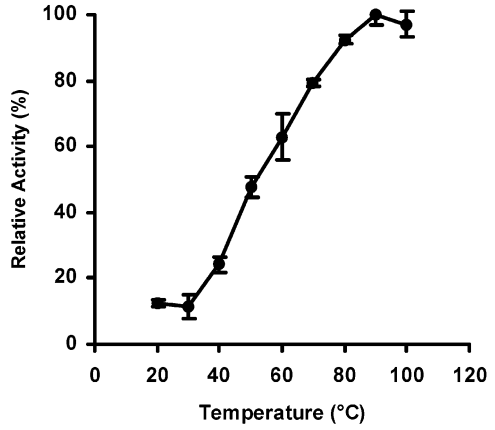

Figure 2. Catalase activity as a function of temperature. Error bars represent one standard deviation from triplicate measurements.

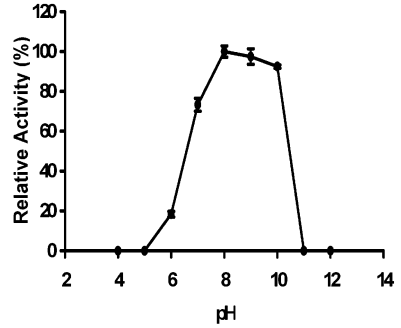

Figure 3. Catalase activity as a function of pH. Error bars represent one standard deviation from triplicate measurements.

at 90 °C. This activity is similar to that reported for a thermostable Mn-catalase from a *Thermus* sp. whose maximum activity occurred at 85 °C and was active over the temperature range of 40−90 °C (*13*). A Mn-catalase from *Thermoleophilum album* had activity over the range of 25−60 °C with an optimum temperature for activity at 35 °C (*12*), and a heme-catalase from *Thermoascus aurantiacus* had activity of the range of 30−90 °C with optimum activity at 70 °C (*21*).

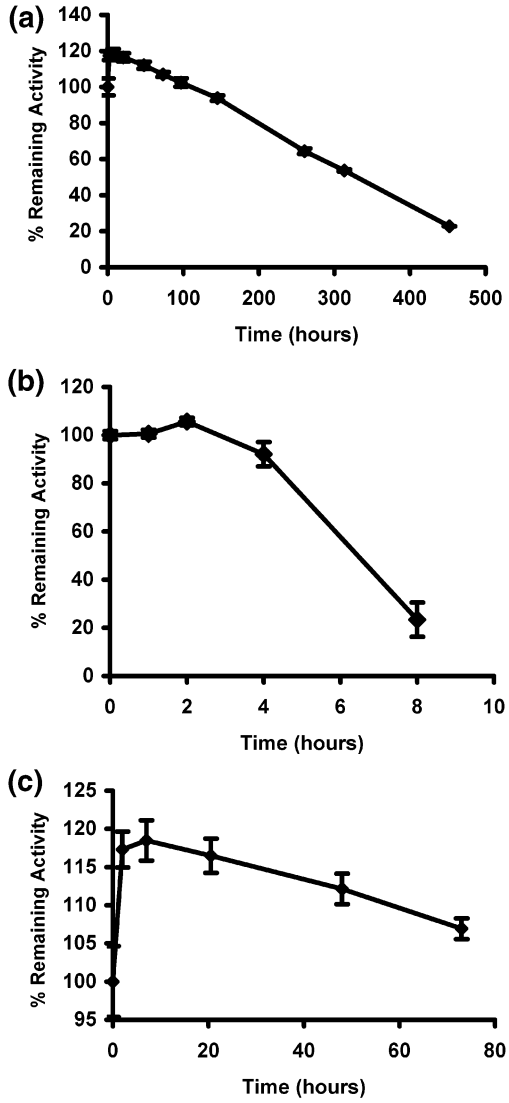

Figure 4. Temperature stability of *T. brockianus* catalase enzyme incubated at (a) 80 °C or (b) 90 °C; and (c) initial activation of catalase activity at 80 °C. Error bars represent one standard deviation from triplicate measurements.

The *T. brockianus* catalase had activity over a broad pH range of 6−10, with the maximum activity at pH 8. This pH range of activity is slightly smaller but typical of monofunctional catalases whose activities usually range from 4 to 10 (*30*). This is in contrast to catalase-peroxidases that have sharp pH optima (*30*).

Stability of the *T. brockianus* catalase was tested at both 80 and 90 °C (Figure 4a and b) at the optimum pH for activity of 8. An unexpected activation effect of the catalase from *T. brockianus* was observed during the stability studies (Figure 4c). At 80 °C, the activity increased approximately 20% over the initial activity in the first 7 h of incubation, and at 90 °C, a 5% increase in activity over the first 2 h of incubation was observed. The reason for this may be an artifact of the storage conditions for the enzyme. Prior to the stability studies, enzyme was stored at 4 °C and was subsequently removed from the refrigerator and assayed at 70 °C to obtain the initial activity. While at 4 °C, the enzyme was apparently configured into a less active state that was maintained during the initial assay. Although the enzyme was heated to 70 °C for 3 min prior to assay, this did not appear to be enough time to reactivate the enzyme to the more active state. When the enzyme was

incubated at elevated temperatures, the enzyme configuration gradually changed to the more active state, such that subsequent assays of activity showed higher activity levels present. This reactivation was temperature-dependent since the activity took longer to peak at 80 °C (7 h, Figure 4c) compared to 90 °C (2 h, Figure 4b).

Activation of mesophilic catalase enzymes from *Rhodospirillum rubrum* and *Micrococcus luteus* has also been reported with activations of 88% and 55% above the initial activity, respectively, after 5 min of incubation at 50 °C (*16*). The authors attributed this effect to a reversible conformation change in the enzyme. The effect was also determined to be temperature-dependent, with the amount of activation increasing with increasing temperature up to 50 °C and then decreasing with further increases in temperature. The activation effect was much more rapid in the mesophilic catalases, with activation being observed after 5 min of incubation and starting to decline after 15 min of incubation compared to the 2−7 h required for the activation effect to peak in the *T. brockianus* catalase. This may be due to the physical nature of thermostable enzymes since they tend to be more rigid than their mesophilic counterparts (*34*) and may take longer to reconfigure to the higher activity level. The authors also observed that the activation effect did not occur in catalase-peroxidase enzymes from *E. coli* and *Rhodopseudomonas capsulata*.

Because of the activation effect observed, the *T. brockianus* catalase half-lives at 80 and 90 °C were calculated using only the data obtained after the full activation had occurred. The enzyme was found to be very stable at 80 °C, with an activity half-life of approximately 330 h (14 days). At 90 °C, the catalase was less stable, with an activity half-life of about 3 h.

By comparison, the heme-catalase from *T. aurantiacus* also had a half-life of about 3 h at 85 °C (*21*), and the Mn-catalase from *T. album* lost 10% of its activity after 1 h of incubation at 80 °C and 7% of its activity after 24 h of incubation at 60 °C (*12*). The *T. brockianus* catalase was also extremely stable when stored in 20 mM Tris, pH 8 at 4 °C, with no apparent loss of activity after 2 years of storage (data not shown).

Stability of the *T. brockianus* catalase was also measured at alkaline pHs ranging from 9 to 11 at 70 °C. The enzyme had half-lives of 510 and 360 h (21 and 15 days) at pH 9 and 10, respectively. In contrast, a thermo-alkalistable catalase purified from *Bacillus* sp. SF for potential treatment of textile bleaching effluents had half-lives of only 38 and 4 h when incubated at pH 9 and 10 and 60 °C, respectively (*3*). Another thermostable catalase from *T. aurantiacus* maintained 100% of its initial activity when incubated for 1 h at pH 9 and 80 °C but retained only 40% of initial activity when incubated in a pH 10 buffer (*21*). At pH 11 and 70 °C, the stability of the *T. brockianus* catalase was drastically reduced, with complete loss of activity after 30 min.

The rate of hydrogen peroxide decomposition as a function of hydrogen peroxide concentration is given in Figure 5 for the *T. brockianus* catalase. Nonlinear curve fitting of the data to the Michaelis−Menten equation yielded a K_m of 35.5 mM and a V_{max} of 20.3 mM/min·mg protein, which corresponds to a turnover number (k_{cat}) of 3.6×10^5 min^{-1} and a catalytic efficiency (k_{cat}/K_m) ratio of 1.7×10^5 s^{-1} M^{-1}. The turnover number was calculated assuming four active centers per catalase molecule. A comparison of kinetic parameters of catalase enzymes from various sources is given in Table 2. The K_m value for the *T. brockianus* catalase is lower than that reported for the thermostable catalase from *T. aurantiacus* but

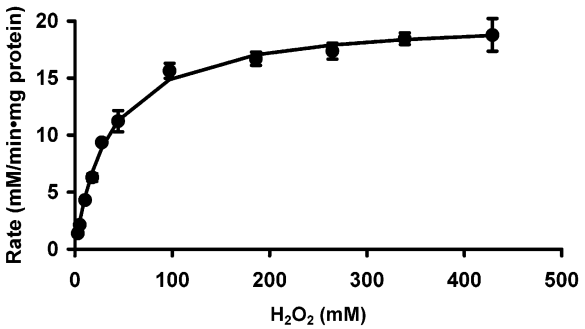

Figure 5. Rate of hydrogen peroxide decomposition as a function of hydrogen peroxide concentration. Solid line represents a nonlinear fit of V_m and K_{max} to the Michaelis−Menton equation. Error bars represent one standard deviation from triplicate measurements.

higher than the K_m values reported for most other catalases. The turnover number of the *T. brockianus* catalase was an order of magnitude lower than the turnover numbers of the thermostable catalase from *T. aurantiacus,* the catalase from *Vitreoscilla* sp., and the catalase-peroxidase from *E. coli,* whereas the reported turnover numbers for catalase-peroxidases from *M. tuberculosis* and *A. nidulans* were of the same order of magnitude as for the *T. brockianus* catalase. The catalytic efficiency of the *T. brockianus* catalase was 1 order of magnitude lower than those reported for *M. tuberculosis* and *A. nidulans*, and the *Vitreoscilla* sp. catalase had a catalytic efficiency 2 orders of magnitude higher. In contrast, beef liver catalase, the most efficient catalase enzyme reported, has a catalytic efficiency 3 orders of magnitude higher than that of the *T. brockianus* catalase (*35*).

In contrast to many catalases that do not show true Michaelis−Menten behavior (i.e., saturation at high substrate levels) because of inactivation/inhibition of the enzyme by hydrogen peroxide (*35*) at fairly low concentrations (Table 2), the *T. brockianus* catalase demonstrated saturation kinetics at hydrogen peroxide concentrations above 50 mM. There was no apparent substrate inhibition/inactivation of the catalase enzyme at hydrogen peroxide concentrations up to 450 mM, the limit of the spectrophotometric assay. This may be a result of the thermostability of the *T. brockianus* catalase, although the catalases from *T. aurantiacus* and *T. album* both show substantial substrate inhibition at 60 and 20 mM hydrogen peroxide, respectively. The *T. brockianus* catalase was completely inhibited by 40 mM 3-amino-1,2,4-triazole. Since this compound is a classic inhibitor of monofunctional catalases while catalase-peroxidases are insensitive to it (*30*), this classifies the *T. brockianus* catalase as monofunctional. This characterization was confirmed when the enzyme did not demonstrate peroxidase activity using peroxidase substrates *o*-dianisidine and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid). Another common catalase and catalase-peroxidase inhibitor, potassium cyanide, also inhibited the *T. brockianus* catalase, with 91% inhibition of activity at 1 mM concentration.

Spectral Characterization of *T. brockianus* Catalase. The absorption spectra of native catalase, catalase treated with 1 mM sodium dithionite, and catalase treated with 10 mM KCN are shown in Figure 6. The catalase had virtually no absorbance at 280 nm, suggesting that the enzyme has few aromatic amino acids. The catalase showed a strong Soret peak at 410 nm and a peak at 534 nm with a shoulder occurring from 560 to

Table 2. Comparison of Kinetic Parameters of Catalase and Catalase-Peroxidases from Various Organisms

source	K_m (mM)	V_{max} (mM min^{-1} mg^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	H_2O_2 inhibition (mM)
Thermoascus aurantiacus (*21*)	48	nr[a]	6.4×10^6	2.2×10^6	60
Thermoleophilum album (*12*)	15	2.3	nr	nr	20
Bacillus sp. (*30*)	6.8	nr	nr	nr	30
Escherichia coli (*30*)	3.9	nr	9.8×10^5	nr	nr
Rhodobacter capsulatus (*30*)	4.2	nr	nr	nr	nr
Neurospora crassa (*30*)	25	nr	nr	4.57×10^6	none[b]
Vitreoscilla sp. (*32*)	16	nr	1.60×10^6	2.70×10^7	nr
Mycobacterium tuberculosis (*36*)	5.2	nr	6.06×10^5	1.95×10^6	nr
Anacystis nidulans (*33*)	4.3	nr	4.30×10^5	1.66×10^6	10
Thermus brockianus (this study)	35.5	20.3	3.60×10^5	1.70×10^5	none[c]

[a] nr: not reported. [b] No inhibition was observed for H_2O_2 concentrations up to 200 mM. [c] No inhibition was observed for H_2O_2 concentrations up to 450 mM.

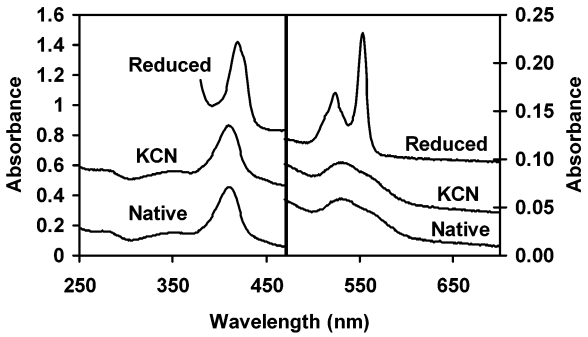

Figure 6. Absorption spectra of native enzyme, enzyme treated with 1 mM sodium dithionite, and enzyme treated with 10 mM KCN.

570 nm. This provides evidence that the *T. brockianus* catalase is a heme catalase rather than a Mn-catalase as have been described in other *Thermus* species (*13*). Absorbance spectra of Mn-catalases completely lack the Soret peak (*13*). The Soret peak for the *T. brockianus* is red shifted compared to the more typical 406 nm Soret peak for other catalases (*30*, *31*, *32*) although a Soret peak at 408 nm has been reported (*36*).

The *T. brockianus* catalase lacks the typical heme charge-transfer bands at 505 and 624 nm that are distinctive of high spin ferric heme proteins (*36*, *37*) and instead has a broad peak centered at 534 nm with a shoulder from 560 to 570 nm that is more typical of heme protein spectra in a low spin configuration (*37*). Crystal structures solved for catalases from a variety of organisms indicate that the heme iron is 5-coordinate in the native resting state with positions 1−4 occupied by the four pyrrole nitrogens of the heme group, position 5 on the proximal side of the heme occupied with the amino acid tyrosine and the 6 position on the distal side of the heme vacant (*37*, *38*). The distal side of the heme is where the catalatic reaction occurs. In the resting state, the absence of a ligand in the 6 position allows the electrons of the iron to be unpaired, resulting in a high spin state. In the presence of a ligand such as cyanide, the heme iron becomes 6-coordinate with a strong ligand field resulting in only one unpaired electron in the heme iron and a corresponding low spin state (*37*). Since the *T. brockianus* catalase in the native resting state has a spectrum typical of a low spin state, we hypothesize that the distal 6-coordinate position of this enzyme is filled with a ligand that results in the low spin state. Results obtained from site-directed mutagenesis of the proximal His/Trp/Asp of a catalase-peroxidase from the cyanobacteria *Synechocystis* supports this assertion. Mutants with a 6-coordinate low spin heme state were indicated by a slight red shifting of the Soret peak from 406 nm to 410−416 nm, a peak at about 530 nm, and either an absent or weak peak at 630 nm (*39*). These alterations of the absorbance spectra are very similar to the spectrum obtained for the *T. brockianus* catalase. A 6-coordinate heme iron may also explain the relatively lower activity of the *T. brockianus* catalase compared to that of other catalases, since the 6-coordinate mutants generated by Jakopitsch et al. (*39*) were much less active than the wild-type enzyme. The spectrum obtained in the presence of 10 mM KCN also supports the 6-coordinate heme hypothesis since the spectrum obtained was identical to the native enzyme with no shift in the Soret peak and no changes in the minor peak at 534 nm or the 560−570 shoulder. Catalases with a vacant distal heme position exhibit a Soret peak shift of approximately 15−20 nm when cyanide binds in that position. Since this shift did not occur, the cyanide is apparently blocked from binding at this site as would occur if the site were already occupied. Similarly, α and β bands at 555 nm and 580−590 nm that are seen when cyanide binds to the distal heme position were not observed in the *T. brockianus* cyanide spectrum (*37*). This result was unexpected because this catalase was strongly inhibited by cyanide. It is widely accepted that cyanide acts to inhibit catalases through binding in the distal heme position, which blocks the active site of the enzyme (*38*). If cyanide is not binding in this location for the *T. brockianus* catalase, then cyanide inhibition of the enzyme must occur through some other mechanism. There are heme proteins that do possess 6-coordinate heme iron, an example is cytochrome *c* peroxidase that has a heme *c* with thiolate and imidazole groups in the 5- and 6-coordinate positions (*37*); however, there have been no previous reports of a naturally occurring 6-coordinate catalase enzyme.

An alternative explanation of the above phenomena is that the enzyme was in an inactive state during measurement of the spectra. The above spectra were taken at 22 °C, a temperature where the enzyme has virtually no activity. The activation phenomena described above also supports the assertion that the enzyme is locked into a nonactive state at lower temperatures. It is possible that the nonactive state of the enzyme is the 6-coordinate heme configuration observed from the spectra. To test this hypothesis, the native and KCN spectra were measured again at 70 °C after a 2-h incubation at 80 °C. These spectra were identical to those obtained at the lower temperature, indicating that the *T. brockianus* catalase is also in a 6-coordinate low spin state while in the active configuration.

The *T. brockianus* catalase was reduced with sodium dithionite, resulting in a shift of the Soret peak to 419 nm, loss of the 534 nm peak, and appearance of peaks at 523 and 553 nm. This behavior was also surprising because monofunctional catalases are generally very resistant to reduction, whereas catalase-peroxidases are

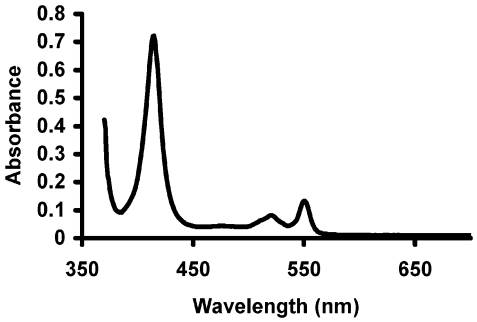

Figure 7. Absorption spectrum of pyridine hemochrome of *T. brockianus* catalase.

easily reduced with dithionite. Although most of the properties of the *T. brockianus* catalase are consistent with monofunctional catalases, it also appears to have at least one property that has only been seen previously in catalase-peroxidase enzymes. This spectrum was also acquired at 70 °C to ensure that the observed effect was not an artifact of the original scan conditions. The same results were obtained at both temperatures. Although no previously reported monofunctional catalases have been shown to have properties of both types of enzymes, there has been one report of a recombinant catalase-peroxidase cloned from the putative *perA* gene of *Archaeoglobus fulgidus* that also had a property previously only seen in monofunctional catalases. This enzyme demonstrated the classic behavior of catalase-peroxidases with both catalatic and peroxidative activity, a sharp pH optimum for activity, rapid inactivation in the presence of hydrogen peroxide, and was easily reducible by dithionite (*18*). However, the enzyme was inhibited 3-amino-1,2,4-triazole, a property previously attributed only to monofunctional catalases.

Treatment of the *T. brockianus* catalase with pyridine/NaOH and sodium dithionite produced a pyridine hemochrome with spectral peaks at 415, 521, and 550 nm (Figure 7). Most reported catalases utilize protoheme IX as the heme group in the enzyme, which have pyridine hemochrome absorption peaks at 418, 526, and 556 nm (*15*). The peaks observed in the *T. brockianus* pyridine hemochrome spectrum are slightly shifted from those peaks. If it is assumed that the *T. brockianus* catalase possesses a protoheme IX and the protoheme content is calculated from the absorption of the pyridine hemochrome peak at 415 nm, a value of 6.7 molecules of protoheme IX per molecule of catalase is obtained. This level of heme is the highest reported for any catalase enzyme, where more typical levels are 2−4 molecules of heme per molecule of catalase (*15*, *30*). This high level is consistent with the uncharacteristically high Reinheitzal number (A_{410}/A_{275}) of 2.8 compared to more typical ratios of 0.5−1.0 (*15*, *30*, *33*).

However, the fact that the *T. brockianus* pyridine hemochrome peaks are shifted from typical protoheme IX peaks suggests that the *T. brockianus* catalase utilizes another type of heme group in its active site. Examination of the literature indicated that the *T. brockianus* spectrum closely resembles the pyridine hemochrome spectra of heme *c* (*19*, *29*, *40*). Although most reported catalases utilize protoheme IX, there are a few reports of other types of hemes such as heme *d* in the HPII catalase from *E. coli* (*41*), a novel heme type in the catalase from *N. crassa* (*30*), and heme *b* in a catalase-peroxidase from *Synechocystis* PCC 6803 (*39*). The presence of heme *c* in the *T. brockianus* catalase may also support the finding of the 6-coordinate heme since it has been shown that cytochrome *c* peroxidase containing a heme *c* group is 6-coordinate (*37*). Using molar absorption coefficient reported by Falk (*29*) for heme *c* of 29.1 mM^{-1} cm^{-1} for the absorption peak at 550 nm, there are approximately eight molecules of heme *c* per molecule of catalase (two per subunit).

Conclusions

The *T. brockianus* catalase isolated and purified in this study showed exceptional stability at elevated temperatures and pH compared to that of many other reported catalase enzymes and has potential applications in the treatment of industrially generated hydrogen peroxide process streams. In addition, this catalase has a number of unusual features compared to those of other reported catalases. The enzyme shares most of the features common to monofunctional catalases such as a broad pH optimum, no peroxidative activity, and inhibition by 3-amino-1,2,4-triazole; yet the enzyme was easily reduced by dithionite, a property previously only observed in catalase-peroxidase enzymes. Other unusual properties of the *T. brockianus* catalase included the presence of heme *c* rather than protoheme IX utilized by most other catalases, the 6-coordinate nature of the heme, and the apparent novel inhibition mechanism by cyanide.

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