21 卷 1 期
2005 年 1 月

生 物 工 程 学 报
Chinese Journal of Biotechnology

Vol.21 No.1
January 2005

Purification and Characterization of a Monofunctional Catalase from an Alkaliphilic *Bacillus* sp. F26

嗜碱芽孢杆菌 *Bacillus* sp. F26 过氧化氢酶的分离纯化及性质研究

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摘 要 从一株低度嗜盐、兼性嗜碱芽孢杆菌 *Bacillus* sp. F26 中纯化得到一种碱性过氧化氢酶,并对该酶进行了性质研究。纯化过程经硫酸铵沉淀、阴离子交换层析、凝胶过滤层析及疏水层析四步最终获得电泳纯的目标酶(纯化 58.5 倍)。该过氧化氢酶的分子量为 140kD,由两个大小相同的亚基组成。天然酶分子在 408 nm 处显示特征吸收峰(Soret band)。吡啶血色素光谱显示了酶分子以原卟啉Ⅸ(protoheme Ⅸ)作为辅基。计算获得酶的表观米氏常数为 32.5 mmol/L。该过氧化氢酶不受连二亚硫酸钠的还原作用影响,但被氰化物、叠氮化物和 3-氨基-1,2,4-三唑(单功能过氧化氢酶的专一抑制剂)强烈抑制。以邻联茴香胺、邻苯二胺和二氨基联苯胺作为电子供体测定酶活时,该酶不显示过氧化物酶活性。同时,酶的 N-端序列比对结果说明,该过氧化氢酶与单功能过氧化氢酶亚群有一定的相似性,而与双功能过氧化氢酶亚群及猛过氧化氢酶亚群均没有同源性。因此,本文将纯化的碱性过氧化氢酶定性为单功能过氧化氢酶。此外,该酶具有热敏感的特点,且酶活在 pH 5~9 的范围内不受 pH 影响,此后,活性随着 pH 的升高而升高,并在 pH 11 处有明显的酶活高峰。20 ℃、pH 11 条件下的酶活半衰期达 49 h。在 pH 11 的高碱条件下表现出最高活力和一定的稳定性,这在已报道的过氧化氢酶中还未见描述。同时,该酶也显示了良好的盐碱稳定性,0.5 mol/L NaCl、pH 10.5 条件下的酶活半衰期达 90 h。另一方面,本文所研究的过氧化氢酶是第一个来源于嗜碱微生物的同源二聚体单功能过氧化氢酶,也是第一个来源于天然碱湖的单功能过氧化氢酶,它能部分地反映出细胞抗氧化体系对相应环境的适应情况。

关键词 过氧化氢酶, *Bacillus*

中图分类号 Q814.1 **文献标识码** A **文章编号** 1000-3061(2005)01-0071-07

Abstract An alkaline catalase has been purified and characterized from a slightly halophilic and alkaliphilic bacterium *Bacillus* sp. F26. The purification was performed with a four step procedure consisting of ammonium sulfate precipitation, ion exchange, gel filtration and hydrophobic interaction chromatography, and finally achieved a 58.5-fold-purifying over the crude extract. The purified catalase was composed of two identical subunits with a native molecular mass of 140 kD. The native enzyme showed the

Received: May 24, 2004; Accepted: June 16, 2004.

This work was supported by CAS Innovation Program; the Ministry of Science and Technology of China and by the National Basic Research Program of China (No. 2003CB716001). We thank Pro. W.D. Grant for helpful advice.

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中国科学院知识创新工程项目,国家"973"计划(No.2003CB716001).

typical Soret band appearing at 408 nm. The pyridine hemochrome spectrum indicated the presence of protoheme Ⅸ as the prosthetic group. The apparent K_m value for enzyme activity on H_2O_2 was calculated to be 32.5 mmol/L. The activity of this catalase was not reduced by dithionite but was strongly inhibited by cyanide, azide, and 3-amino-1,2,4-triazole (the specific inhibitor of monofunctional catalase). No peroxidase activity of this enzyme was detected when using *o*-dianisidine, diaminobenzidine (DAB) and *p*-phenylenediamine as electron donor. Moreover, the N-terminal sequence of this catalase exhibited substantial similarity to the monofunctional catalase subgroup rather than catalase-peroxidase or Mn-catalase one. Therefore, we characterize the purified catalase as a monofunctional catalase. Besides, this monofunctional catalase was thermosensitive and its activity exhibited pH-independent over pH 5 ~ 9 but showed a sharp maximum at pH 11. An activity half-life of approximately 49 h was measured when the enzyme was incubated at 20 ℃ and pH 11. To our knowledge, pH 11 is the most alkaline condition for optimum catalysis and enzyme stability among the catalases reported up to now. Furthermore, this monofunctional catalase also showed excellent halo-alkali-stability with a half-life of approximately 90 h at 0.5 mol/L NaCl and pH 10.5. On the other hand, so far as we know, the characterized catalase is the first dimeric monofunctional catalase from alkaliphiles and is also the first monofunctional catalase derived from a natural soda lake, which could partially reflect the oxidative stress response in the corresponding environment.

Key words catalase, *Bacillus*

Aerobic organisms generate oxygen free radicals including superoxide radical, H_2O_2 and hydroxyl radical as a result of oxygen metabolism, which are highly harmful to DNA, lipids and proteins[1]. Catalase (CAT), as a member of oxidative stress response system in cells, catalyzes the conversion of H_2O_2 to O_2 and H_2O, and thus it is present in all aerobic organisms and in aerotolerant anaerobic organisms as a protective enzyme[2]. So far, three subgroups of catalases have been described; they are monofunctional catalase (or typical catalase), catalase-peroxidase (CP) and Mn-catalase (or pseudocatalase), each of which shows distinct physicochemical properties and catalytic properties.

To reduce the negative impact on the environment, the usage of hydrogen peroxide in industrial processes including the food, dairy, textile, pulp and paper industries, as a more environmentally friendly bleaching or sterilizing reagent, is more and more attractive[3]. Therefore, an easy and efficient way of decomposing H_2O_2 by catalases to avoid its interference with subsequent steps is also drawing more and more attention[4-6]. Since hydrogen peroxide is more active under alkaline conditions, most industrial processes involving its use proceed at high pH, so alkaline catalases become industrially important enzymes.

Several catalases have been purified from alkaliphiles in the past thirty years[4,7-10]. They were characterized to be either typical catalases or catalase-peroxidases. For alkaline monofunctional catalases, they showed the common characteristics of the group: homotetramers, protoheme Ⅸ as prosthetic group, specifically inhibited by 3-amino-1,2,4-triazole (3-AT), resistant to reduction with dithionite and pH-independent over a broad pH range.

Previously, an alkaliphilic *Bacillus* strain F26 was isolated from Haoji Soda Lake in our laboratory[11]. This strain was found to possess multiple catalases whose expression was growth-dependent. An alkaline monofunctional catalase was purified from these isozymes and was found to possess the unique properties of a sharp maximum activity at pH 11 and an uncommon structure of homodimer, which are distinct from the common characteristics of the monofunctional catalase group. In this paper, we describe the purification and characterization of this catalase.

1 MATERIALS AND METHODS

1.1 Microorganism and Culture Conditions

Bacillus sp. F26, a slightly halophilic alkaliphile, was isolated from Haoji Soda Lake located in the Hulunbeir area of Inner Mongolia Autonomous Region of China (48°23′ N and 118°28′E)[11]. This strain was cultivated aerobically up to the late-logarithmic-growth phase at 37℃ in modified Horikoshi Ⅰ medium containing (per liter of distilled water) 10 g of Glucose, 5 g of polypeptone (BBI), 5 g of yeast extract (Oxoid), 1 g of KH_2PO_4, 0.2 g of $MgCl_2$, 50 g of NaCl and 10 g of Na_2CO_3 (pH 9.8).

1.2 Purification of Catalase from *Bacillus* sp. F26

The harvested cells (approximately 10 g of wet cells) were washed once with buffer A (50 mmol/L phosphate buffer, pH 7.0), then resuspended in the same buffer and disrupted by sonication at 0℃. Cell debris was removed by centrifugation (10000 g for 40 min) and the supernatant was collected as crude cell extract.

The crude extract was subjected to ammonium sulfate precipitation at 50% saturation. The precipitated proteins were obtained by centrifugation (10000 g for 40 min) and dissolved in buffer A. This solution was then dialyzed against the same buffer for 20 h, followed by a concentration treatment with PEG-20000 to a final volume of 15 mL. The resulting sample was loaded on a DEAE Sepharose CL-6B Column (1.6 cm × 19 cm) (Fluka), pre-equilibrated with buffer A, and eluted with a increasing linear gradient elution of 0 mol/L to 0.8 mol/L NaCl in the same buffer. All fractions with catalase activity were pooled, dialyzed and highly concentrated with the methods described above. The sample was then applied to a Bio Gel P-300 column (1.6 cm × 80 cm) (Bio-Rad), pre-equilibrated with buffer A. The eluants containing catalase were combined and ammonium sulfate was added to a final concentration of 0.5 mol/L. The treated sample was fi-

nally applied to an Octyl Sepharose CL-4B Column (1 cm × 13 cm) (Fluka), pre-equilibrated with buffer A containing 0.5 mol/L $(NH_4)_2SO_4$. It was found that the target catalase bound to octyl groups with a strong hydrophobic interaction. So it became highly effective to elute with a two-step elution: washing the column with buffer A followed by a decreasing linear gradient elution of 50 mmol/L to 5 mmol/L phosphate buffer [pH 7.0, both without $(NH_4)_2SO_4$]. Active fractions were collected, concentrated by ultrafiltration (Millipore) and stored at −20℃. The effectiveness of each purification step was determined with both SDS-PAGE and Native PAGE.

1.3 Enzyme Assay

Catalase activity was determined spectrophotometrically (Beckman DU-800 UV-Vis spectrophotometer) by monitoring the decrease in absorption at 240 nm caused by the disappearance of hydrogen peroxide at 30℃[12]. The reaction mixture included 1 μL enzyme solution, 199 μL 50 mmol/L $NaHCO_3$-Na_2CO_3 buffer (pH 11) and 100 μL 30 mmol/L H_2O_2 in the same buffer unless otherwise stated. The assay was initiated by addition of the substrate and followed for 1 min, using the initial linear range of the reaction (20 ~ 30 s) to estimate activity. The concentration of H_2O_2 was calculated from the molar extinction coefficient (43.6 $mol/L^{-1} \cdot cm^{-1}$). 1 unit of catalase activity was defined as 1 μmol of H_2O_2 decomposed per min.

Peroxidase activity of the enzyme was also tested spectrophotometrically at 30℃ using different substrates in buffer A. The oxidation of *o*-dianisidine (0.5 mmol/L, with 1 mmol/L H_2O_2), diaminobenzidine (0.5 mmol/L, with 3 mmol/L H_2O_2) and *p*-phenylenediamine (0.3 mmol/L, with 3 mmol/L H_2O_2) was monitored at 460 nm, 465 nm and 485 nm respectively. For comparative purposes, bovine liver catalase (Sigma) was assayed similarly.

Protein concentration was determined by the method of Bradford using the Bio-Rad protein assay dye reagent concentrate with bovine serum albumin as standard.

1.4 Electrophoresis

SDS-PAGE was carried out in 12% polyacrylamide gels with Mini-Protein 3 Cell (Bio-Rad), using low molecular weight marker (Pharmacia) as standard to determine the subunit size of the enzyme. Proteins on the gel were stained with Coomassie Brilliant Blue R-250. Native PAGE was performed in 8% polyacrylamide gels, followed by both protein staining and activity staining (ferricyanide-negative stain with 0.03% (*V*/*V*) H_2O_2)[13].

1.5 Molecular weight estimation

Gel filtration chromatography was used to estimate the native molecular mass of the purified catalase. The method was performed at a flow rate of 0.4 mL/min on AKTA FPLC P-920 system with a Superdex 200 HR 10/30 column (Pharmacia). The system was equilibrated with buffer A (containing 0.15 mol/L NaCl) and high molecular weight gel filtration calibration marker (Pharmacia) was used as standard.

1.6 Spectral characterization

The absorption spectra of the native enzyme, enzyme reduced with 1 mmol/L sodium dithionite and enzyme treated with 10 mmol/L KCN were measured in buffer A at 30℃ using the Beckman DU-800 UV-Vis spectrophotometer. The pyridine hemochrome spectrum was determined according to the description of Falk in a solution of 17.5% (*V*/*V*) pyridine and 0.075mol/L NaOH[14].

2 RESULTS

2.1 Microorganism

Bacillus sp. F26 was a Gram-negative, rod-shaped organism. Its growth could be detected over the pH range of 7 ~ 10, temperature range of 11 ~ 40℃ and NaCl concentrations of 1% ~ 12% (*W*/*V*). The optimal conditions for growth were at pH 9.5, 34 ℃ and 3% (*W*/*V*) NaCl. The 16S rDNA sequence of strain F26 had 99% similarity to both *Bacillus gibsonii* (DSM 8722) and *Bacillus horikoshii* (DSM 8719).

2.2 Catalase Purification

A four-step procedure consisting of ammonium sulfate precipitation, ion exchange, gel filtration and hydrophobic interaction chromatography was developed to obtain a purified catalase from F26. The purification is summarized in Table 1, and the analysis of protein components in crude extract and fractions containing catalase by PAGE is shown in Figure 1. It indicates (Fig. 1A, Lane 1) that more than one catalase is present in the crude extract. To confirm that *Bacillus* sp. F26 yields multiple catalases, different crude extracts obtained from cells growing for different time (2 h to 30 h) were visualized on Native PAGE with activity staining (Fig. 2). Five activity bands (marked in Fig. 2) were seen on the gel, corresponding to different growth phases. It has been previously reported that growth-dependent expression of multiple catalases also exists in some organisms, such as catalase 1 – 3 in *Bacillus Subtilis*[15] and Cat 1 ~ 6 in *Streptomyces coelicolor*[16].

Table 1 Purification of *Bacillus* sp. F26 catalase

	Total activity /u	Total protein /mg	Specific activity /(u/mg)	Yield /%	Purification (-fold)
Cell extract	107340	441	243.4	100	1
$(NH_4)_2SO_4$ precipitation	81433.5	147.4	552.3	75.9	2.3
Ion exchange	8672.1	4.8	1808.6	8.1	7.4
Gel filtration	8452.8	2.6	3238.6	7.9	13.3
Hydrophobic interaction	2848.8	0.2	14244	2.6	58.5

In this study, the marked catalase (see in Fig. 2) was present throughout all the growth phases with relatively high level expression. This catalase was thus considered to play a more important role in the cell's oxidative stress response system and was selected as the target protein for further purification. As Table 1 shows, the four-step procedure achieved a 58.5-fold purification over crude extract and a yield of 2.6 %, which are relatively low compared to some other published procedures. High similarity among the multiple catalases may be a significant reason, since they showed such close electrophoretic mobilities on native gel (Fig. 2), and in addition, partially-overlapped peaks also appeared during the course of elution especially from ion exchange and gel filtration columns (data not shown).

SDS-PAGE revealed the final presence of a single band at an apparent molecular mass of 74kD (Fig. 1B, Lane 5). With the Native PAGE, the CBB staining protein also ap-

peared as a single band, and its position completely corresponded to the catalase activity (Fig. 1A, Lane 5 and Lane 6). Gel filtration chromatography indicated an approximate molecular mass of the native enzyme of 140kD. Taken together with the subunit size, it is therefore proposed that the catalase is a homodimer.

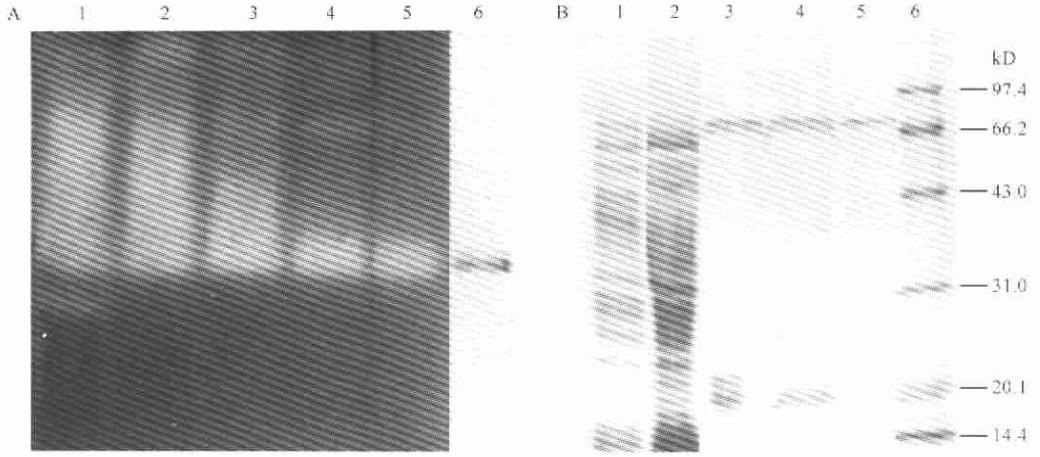

Fig. 1 Analysis of protein components in crude extract and catalase containing fractions after each purification step with PAGE
(Lane 1) crude extract; (Lane 2) ammonium sulfate precipitation; (Lane 3) DEAE ion exchange; (Lane 4) gel filtration; (Lane 5) hydrophobic interaction; and (Lane 6A) same as lane 5A except staining with CBB; (Lane 6B) low molecular weight markers.

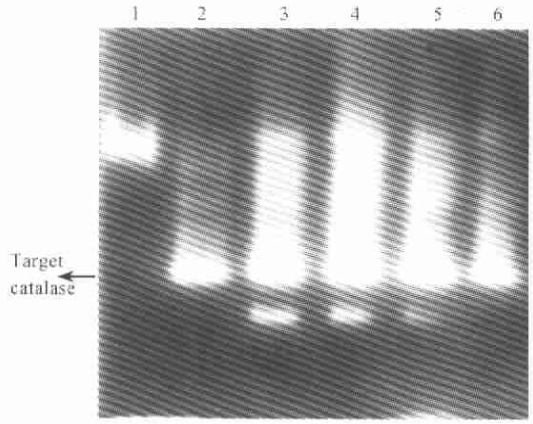

Fig. 2 Expression of multiple catalases in F26 at different growth phases
(Lane 1) bovine liver catalase as control; (Lane 2) grown for 2h; (Lane 3) grown for 6h; (Lane 4) grown for 12h; (Lane 5) grown for 24h; (Lane 6) grown for 30h.

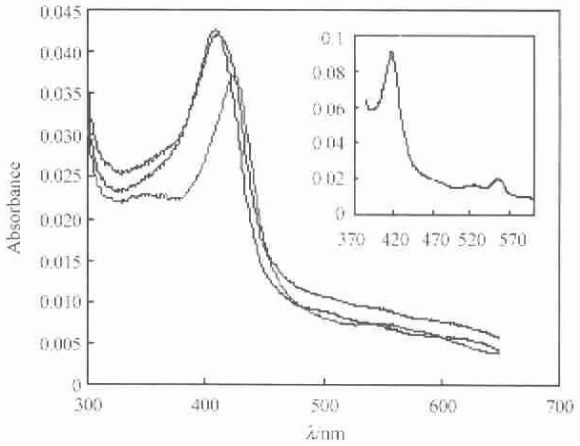

Fig. 3 Spectroscopic analysis of *Bacillus* sp. F26 catalase
(recorded against a blank of identical buffer): Native enzyme (curve 1); treated with dithionite (curve 2); treated with KCN (curve 3). The inset shows the pyridine hemochrome spectrum of *Bacillus* sp. F26 catalase.

2.3 Spectroscopic properties

The absorption spectrum of the native catalase from F26 is shown in Figure 3. A Soret band was present at 408 nm (curve 1), which indicated that this enzyme is a heme catalase rather than a Mn-catalase[17,18]. Furthermore, the Soret absorption of this catalase is slightly red-shifted compared to the more typical 406 nm for other monofunctional catalases[8, 19-20]. The Reinheitzal number (A_{408}/A_{280}) was calculated to be 0.54, which is low for typical catalases that usually exhibit ratios of approximately 1[21]. This result indicated a low heme content of the protein.

Treatment of the enzyme with 1 mmol/L sodium dithionite did not alter the spectral shape but a small shift of Soret peak to 412 nm could be discerned (curve 2). Recently, a monofunctional catalase from *T. brockianus* was also reported to show a red shift of 9 nm when treated with dithionite[3]. This may suggest that the catalase purified in our laboratory is a monofunctional catalase. The presence of 10 mmol/L KCN shifted the Soret peak to 424 nm (curve 3), indicating the distal 6-coordinate position of the heme iron was occupied by the cyanide and thus a transition from high spin to low spin state was shown.

The prosthetic group of the native enzyme was determined as pyridine hemochrome. As Figure 3 (inset) showed, three absorption peaks typically appeared at 418 nm, 525 nm and 555nm, which indicated the presence of protoheme Ⅸ. The protoheme content was estimated from the absorption at α-band (555 nm) and an ε value of 34.4 L/(mmol·cm) to be 1.01 molecules per dimer. This level is a little low but consistent with the Rz ratio.

2.4 Catalytic Properties

The influence of hydrogen peroxide concentration on its decomposition was examined according to the standard assay described in Materials and Methods (except that the concentration of substrate was increased by degrees). Results are given in Figure 4, which reveals that the activity of the enzyme proportionally increased with respect to the substrate concentration up to 70 mmol/L H_2O_2. However, higher concentrations caused the rapid inactivation of the catalase. It is

known that catalases do not show true Michaelis-Menten behavior and the apparent saturation observed for its activity could actually be a result of the inhibitory effect of H_2O_2 at high concentration[22]. On the other hand, however, in the range of low concentrations, the catalytic behavior follows first-order reaction kinetics and thus Michaelis-Menten equation could be applied. In this work, the concentration dependence below 50 mmol/L H_2O_2 fitted the Michaelis-Menten equation, and the apparent K_m and V_{max} values could be therefore deduced from the Lineweaver-Burk plot to be 32.5 mmol/L and 12 ($u \cdot mg^{-1}$), respectively.

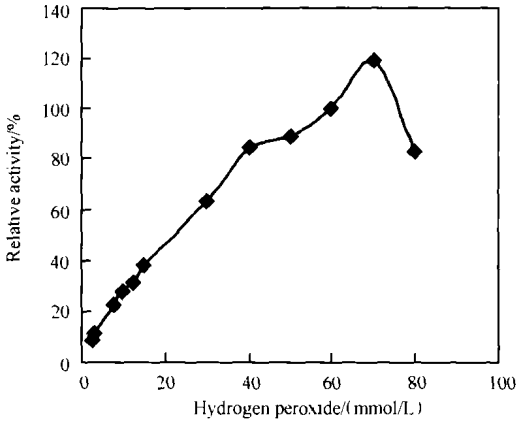

Fig. 4 Effect of H_2O_2 concentration on the catalatic activity of purified F26 catalase.
Each data represents the result derived from triplicate measurements.

Optimum pH conditions for the purified enzyme were determined by assaying catalase activity in the following buffers: 50 mmol/L Na_2HPO_4-citrate buffer (pH 4 ~ 6), 50 mmol/L KH_2PO_4-Na_2HPO_4 buffer (pH 6 ~ 8), 50 mmol/L Tris-HCl buffer (pH 8 ~ 9), 50 mmol/L Na_2CO_3-$NaHCO_3$ buffer (pH 9 ~ 11), 50 mmol/L KCl-NaOH buffer (pH 11 ~ 12.5), with other conditions the same as the standard assay. As Figure 5 shows, the enzyme was active over a broad pH range of 4.5 to 12, which contains a relatively pH-independent range from pH 5 ~ 9 and a sharp activity maximum at pH 11. This pH dependence is quite special compared to that of other monofunctional catalases. It is known that typical catalases characteristically have a broad optimal pH range (i.e. pH independent) while catalase-peroxidases usually exhibit maximal activity at a certain pH. In addition, the high activity appearing at high pH (pH 11 ~ 12) is also unusual. So far as we know, only the catalase-2 from spores of *Bacillus subtilis* is in any way comparable[23].

For determination of the optimum temperature at pH 11, the reaction mixture (without enzyme) was preincubated for 1 min prior to the measurement of activity at each temperature. Results are displayed in Figure 5, which indicates that the catalase activity dramatically decreased at the temperatures higher than 40 ℃, corresponding to the physiological growth limit of strain F26 which also appeared at 40 ℃. The temperature range of 20 ~ 40 ℃ was considered to be optimal since relatively high activity could be observed within this range.

Stability of the catalase from strain F26 was also measured. It was found that at pH 7, incubation of the enzyme at 40℃ for 10 min caused a reduction of its activity by 20%, and after incubation at 50 ℃ for 10 min, only 37% of its activity remained. However, the enzyme's stability at lower temperature and high pH was excellent, no decrease was detected after 60-min-incubation at 15 ~ 30 ℃ and pH 11. Moreover, an activity half-life of approximately 49 h was shown when the enzyme was incubated at 20 ℃ and pH 11. This characteristic of "cold-adapted activity" is rather similar to the catalase from the facultatively psychrophile *Vibrio rumoiensis* S-1[T,19], which also showed maximum activity at 40 ℃ and slight suppression by incubation at 35 ~ 40 ℃.

Though *Bacillus* sp. F26 was identified to be a slight halophile with optimal growth at 0.5 mol/L NaCl and no growth could be observed in the absence of salt, we discovered that its catalase activity in crude extracts needed no NaCl to sustain or activate during purification and storage. As for salt dependence of the purified catalase, the enzymatic activity showed a maximum in the absence of NaCl. 73% of its activity remained at the concentration of 2 mol/L NaCl while higher salt concentrations yielded a dramatic decrease in catalytic activity (data not shown). This phenomenon was similar to a mesohalic catalase from the halophilic archaeon *Halobacterium halobium*[24]. On the other hand, however, our catalase exhibited outstanding halo-alkali-stability with a half-life of approximately 90 h at 0.5 mol/L NaCl and pH 10.5.

Influence of inhibitors on the purified catalase was determined by incubating the enzyme in 50 mmol/L Tris-HCl buffer

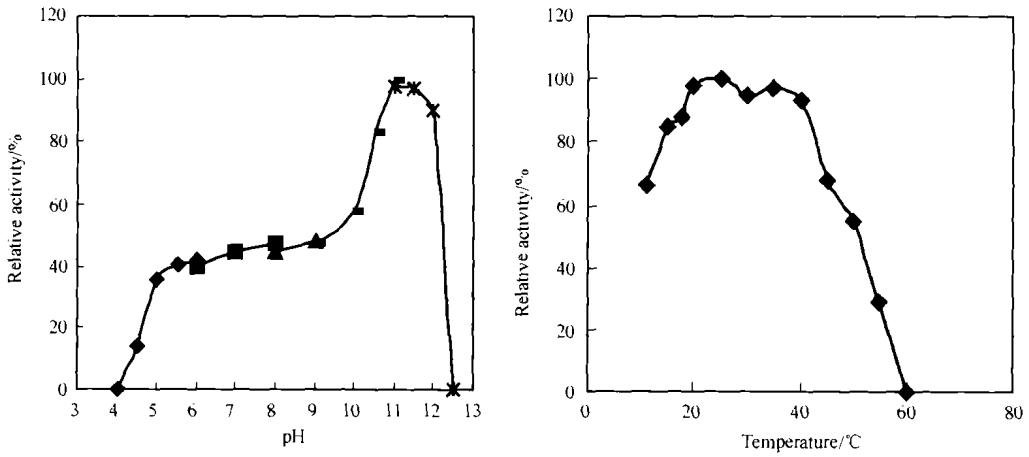

Fig. 5 pH dependence and temperature dependence of the purified F26 catalase activity
Each data represents the result derived from triplicate measurements.

(pH 9.0) containing various inhibitors at 30 ℃ for 30 min, the residual activity being then measured. As Table 2 showed, the enzyme was strongly inhibited by cyanide, azide and hydroxylamine hydrochloride. The specific inhibitor of typical catalase, 3-amino-1,2,4-triazole, also caused loss of the enzyme activity, which supports the deduction that this enzyme is a monofunctional catalase. This conclusion was further confirmed when the enzyme did not exhibit peroxidase activity using *o*-dianisidine, DAB and *p*-phenylenediamine as electron donors.

Table 2 Effect of inhibitors on the activity of F26 catalase

Inhibitors	Final conc./(mmol/L)	Inhibition/%
Hydroxylamine hydrochloride	0.01	63
Sodium azide	0.1	40
	0.5	87.5
KCN	0.01	24
	0.05	95
3-amino-1,2,4-triazole	0.5	11
	1	43.5
	5	83
EDTA	1	21
	5	50

2.5 N-terminal amino acid sequence

The first 10 amino acids were sequenced on a Model 491 ABI protein sequencer to be M-K-K-L-T-T-N-Q-G-Q. The homologous comparison of the N-terminal sequences among catalases showed that this purified enzyme shared substantial similarity to some other monofunctional catalases, especially to those from the genus *Bacillus*, while no homology was shown with catalase-peroxidases or Mn-catalases (Tab. 3).

Table 3 Relationships of N-terminal sequences among monofunctional catalases of different origins (data from GenBank-EMBL/Swiss-Prot databases)

Organisms	Sequences	Sources
B. sp. F26	1 MKK [LT] T NQ G Q	This work
B. firmus OF4 (Isozyme Ⅱ)	1 MKK [L] S T NQ G A	Q9R4M7
B. pseudofirmus	24 GKK [L T] T NQ G L	P30266
B. subtilis (catalase 2)	27 GKKM [T] T NQ G L	P42234
B. halodurans	28 N TK [L T] T NQ G V	Q9KBE8
Listeria monocytogenes	4 RKN [L T] T NQ G V	Q8Y3P9
Lactobacillus sakei	2 T NQ [L T] T N E G Q	P30265
Homo sapiens (human)	24 ADV [L T] T GA G N	P04040
Methanosarcina barkeri (anaerobic archaeon)	6 S K V [L T] T GF G I	O93662

In addition, the comparison also showed the presence of some same residues (shaded in Tab. 3) regardless of the enzyme sources, which are even found in eukaryotic and anaerobic archaeon typical catalases. Thus we conclude that they are conserved amino acids (T and G) existing at the N-terminal of monofunctional catalases[2].

3 DISCUSSION

A monofunctional catalase has been purified and characterized from a slightly halophilic and alkaliphilic bacterium *Bacillus* sp. F26. The strain was found to express multiple catalases, whose pattern changed as the growth progressed. In the same genus, facultatively alkaliphilic *Bacillus firmus* OF4[9] and *Bacillus subtilis*[15] were previously reported to have multiple catalases. Considering that the catalase-2 from *Bacillus subtilis* appeared as a series of close activity bands on polyacrylamide gel, which was probably due to limited proteolysis, the precise isozyme pattern of *Bacillus* sp. F26 still needs further study.

Properties of the purified catalase are summarized in Table 4. As we have mentioned above, this catalase has an uncommon homodimer structure compared to the typical homotetramer of monofunctional catalases. Only one other dimeric typical catalase has been reported in the literature as far as we know, the KpA from *Klebsiella pneumoniae*[25], with a subunit size of 80kD and a combined molecular mass of 150.2kD. Considerable structure diversity in monofunctional catalases has been realized previously[26], for example, a heterodimer[27], three homohexamers[23,28-29] and even an unusual heterotrimer[30] have been reported, however, our catalase is the first dimeric monofunctional catalase from alkaliphiles.

Table 4 Comparison of the enzymatic properties of F26 catalase with those of other monofunctional catalases

Property	*Vibrio rumoiensis*	*Halomonas* sp. SK1	*Halobacterium halobium*	*Bacillus* sp. F26
Subunit mass (kD)	57.3	68	62	74
Subunits	4	4	4	2
Soret bank /nm	406	n.d.	406	408
Rz ratio	0.93	n.d.	0.3	0.54
K_m for H_2O_2 /(mmol/L)	n.d.	n.d.	60	32.5
pH optima	6 ~ 10	6 ~ 10	6.5 ~ 8.5	11
T optima/℃	40	n.d.	n.d.	20 - 40
Salt resistance /(mol/L)	n.d.	0 - 3.8	0 - 3	0 - 2
pH stability				
1. pH	6 - 10			6 - 11.5
2. T/℃	30			30
3. Incubation		n.d.	n.d.	
Time/min	30			60
4. Residue				
Activity	100%			100%
T stability				
1. pH	7.0	7.4	7.0	7.0
2. T/℃	40	37	45	40
3. Incubation				
Time/min	15	10	5	10
4. Residue				
Activity	88%	64%	88%	80%
Extremophile	psychro-	alkali-	halo-	alkali-
Type	halo-	halo-		

n.d. means not determined

Strain F26 catalase is also unique in its property of high

pH dependence. As has been known, monofunctional catalases from alkaliphiles are pH-independent over a broad pH range similar to their neutralcounterparts. For example, isozyme Ⅱ from facultatively alkaliphilic *Bacillus firmus* OF4 showed a broad optimum pH over 8.0 to 10.5[9], which is alkali-shifted compared to the common activity range of 4 to 10 for typical catalases[3]. However, our catalase had a high optimal pH value of 11, as well as the excellent stability at the same pH, which to our knowledge is the most alkaline condition among the monofunctional catalases reported up to now. Therefore, this property of high pH dependence might make the strain F26 catalase a good model enzyme for elucidating the molecular basis of alkaliphilicity.

On the other hand, the temperature dependence of this enzyme is rather parallel to the *V. rumoiensis* S-1^T catalase which was identified as the first psychrophilic heme-containing enzyme reported[19]. According to Yumoto et al., thermoinstability is one of the most fundamental features of the psychrophilic enzyme; however, more indicators are still needed to be determined to prove that the new purified catalase is psychrophilic. To be more precise, therefore, we prefer to describe it as a "cold-adapted" enzyme.

The salt-resistance of strain F26 catalase should also not be ignored. Its halo-alkali-stability with a half-life of 90 h at 0.5 mol/L NaCl and pH 10.5 may make the enzyme possibly useful in the H_2O_2-using industries.

As far as we know, there is still little information about the catalases from haloalkaliphiles. An alkali- and halo-tolerant bacterium *Halomonas* sp. SK1 has been isolated recently, exhibiting high catalase activity[4]. It was reported that strain SK1 catalase was highly homologous (99%) to the *V. rumoiensis* S-1^T catalase. Moreover, they were both thermosensitive (see in Tab. 4) and to be in accordance, these two distinct strains were both isolated from the drain pool of H_2O_2-using industries, which suggests the influence of the environment on certain functions of different microorganisms. As reported here, *Bacillus* sp. F26 catalase is the first monofunctional catalase derived from a natural soda lake. The multiple catalases existing in the strain could reflect the oxidative stress response in the soda lake environment. At present, the gene encoding the purified catalase is being cloned and expressed for further study.

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