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Biochemical characterization of a catalase from *Antrodia camphorata*: Expression in *Escherichia coli* and enzyme properties

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ABSTRACT. Catalase plays important roles in antioxidation and cell signaling. One cDNA (1794 bp, DQ021914) encoding the putative catalase was cloned from *Antrodia camphorata*. The deduced amino acid sequence is conserved among the reported catalases. To characterize the *A. camphorata* catalase, the coding region was subcloned into a vector pET-20b(+) and transformed into *E. coli*. The recombinant 6His-tagged catalase was expressed and purified by Ni^{2+}-nitrilotriacetic acid sepharose. The purified enzyme showed one band by 10% SDS-PAGE. The enzyme retained 50% activity at 60°C for 14 min. The enzyme was active under a broad pH range from 7.8 to 11.2. The enzyme showed 67% activity after 4 h of incubation at 37°C with trypsin. It was also proven able to protect intact supercoiled plasmid DNA from ·OH-induced nicking. Study of the enzyme's properties may prove beneficial for future applications in medicine or health food.

Keywords: *Antrodia camphorata*; Catalase; Expression.

INTRODUCTION

Medicinal mushrooms have a long history of use in folk medicine in Taiwan. *Antrodia camphorata* (*A. camphorata*) is a unique medicinal mushroom species found only in the forests of Taiwan which traditionally has been used as a remedy for drug intoxication, abdominal pain, and cancer. *Antrodia camphorata* has been shown to exhibit antioxidative (Song and Yen, 2002), vasorelaxative (Wang et al., 2003), and anti-inflammatory (Shen et al., 2004) effects. Although *A. camphorata* shows physiological activities with great potential for medical applications, few scientific studies of it have appeared. Recently, we established an EST (expressed sequence tag) from fruiting bodies of *A. camphorata* in order to search for physiologically active components for medicinal use.

Catalase (E.C. 1.11.1.6; H_2O_2 : H_2O_2-oxidoreductase) is one of the important antioxidative enzymes with a heme structure that can catalyze the decomposition of 2 H_2O_2 to 2 H_2O and O_2. It is found in most aerobic organisms, including prokaryotes and eukaryotes (Kashiwagi et al., 1997; Klotz et al., 1997), and it protects cells against the toxic effects of reactive oxygen species. Catalase is usually formed by four identical subunits (Wu and Shah, 1995) of 50-60 kDa (Klotz et al., 1997; Garcia et al., 2000). Though some catalase sequences have been reported, many queries regarding catalase function and structure remain unsolved. Catalases from mammals have a binding site for NADPH, but its function is not entirely known (Kirkman et al., 1999). Catalases from *E. coli*-like hydroperoxidase do not bind to NADPH but have an extra "flavodoxin-like" domain of unknown function (Bravo et al., 1997).

There are three families of catalases: (1) A novel manganese catalase that uses Mn as a cofactor in the active center without a heme structure (Barynin et al., 2001). It is 170-210 kDa in size with a subunit of 50-65 kDa and has been identified in bacteria, plants, fungi, and animals. It may form unusual oligomeric structures. (2) Bifunctional catalases with molecular masses that vary from 120-340 kDa. In general, they are homodimers (Obinger et al., 1997; Nagy et al., 1997). These catalases have a heme structure that contains both catalase and peroxidase activity, and are found mostly in fungi (Fraaije et al., 1996). (3) The typical monofuctional catalase, most of which are homotetramers, 200-400 kDa in size with four prosthetic haem groups (Hirasawa et al., 1989; Sheptovitsky and Brudvig, 1996).

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Lots of fungi have catalases that have been identified and showed different regulation (Navarro et al., 1996; Kawasaki et al., 1997), but no catalases have been reported from *A. camphorata.* Previously, we cloned and characterized a themostable superoxide dismutase (Liau et al., 2007), a 2-Cys peroxiredoxin (Huang et al., 2007), and a 1-Cys peroxiredoxin (Wen et al., 2007) based on the established EST from *A. camphorata.* Gems and McElwee reported that the pro-longevity genes include those encoding antioxidant enzymes that can restore misfolded proteins to their active conformations (Gems and McElwee, 2005). We continue to search for antioxidant enzymes in *A. camphorata* with potential anti-oxidative and anti-inflammation applications. Here, we report the cloning of an antioxidant enzyme, catalase cDNA (Ac-catalase), from *A. camphorata* on the basis of EST. Understanding the properties of this Ac-catalase would be beneficial for its applications in medicine or as a health food. Thus, the coding region of the Ac-catalase gene was introduced into an *E. coli* expression system. The active enzyme was purified and its properties studied.

MATERIALS AND METHODS

Antrodia camphorata

Fruiting bodies of *A. camphorata* which had grown in the hay of *C. kanehirai* were obtained from Asian-Bio Company (http://www.asian-bio.com/).

Total RNA preparation and cDNA synthesis

Total RNA was prepared from fresh fruiting bodies (wet weight 5 g) using Straight A's mRNA Isolation System (Novagen, USA). The total RNA (22 μg) was obtained. Three μg of the mRNA was used for cDNA synthesis using a ZAP-cDNA kit from Stratagene (La Jolla, CA).

Isolation of Ac-catalase cDNA

We previously established an EST database from fruiting bodies of *A. camphorata* and sequenced all clones with insert sizes greater than 0.2 kb (data not shown). The identity of a catalase cDNA clone was assigned by comparing the inferred amino acid sequence in various databases using the basic local alignment search tool (BLAST).

Subcloning of Ac-catalase cDNA

The coding region of the Ac-catalase cDNA was amplified using two gene-specific primers. The 5′ upstream primer contains the *Nde*I recognition site (5′ CAT ATG CCC TCT AAA CAG GTT T 3′) and the 3′ downstream primer contains the *Not*I recognition site (5′ GCG GCC GCA GCG AAA GCC TTG AAA CGA 3′). Using 0.1 μg of *A. camphorata* cDNA as a template, and 10 pmole of each 5′ upstream and 3′ downstream primers, a 1.5 kb fragment was amplified by PCR. The fragment was ligated into pCR4.0 and transformed into *E. coli* TOPO10. Plasmid DNA was isolated from the clone and digested with *Nde*I and *Not*I. The digestion products were separated on a 0.8% agarose gel. The 1.5 kb insert DNA was gel purified and subcloned into *Nde*I and *Not*I sites of pET-20b(+) vector (Novagen, USA). The recombinant DNA was then transformed into *E. coli* BL21(DE3)pLysS.

Expression and purification of the recombinant Ac-catalase

The transformed *E. coli* containing the Ac-catalase was grown at 30°C in 200 mL of Luria Bertani medium containing 50 μg/mL ampicillin until A_{600} reached 0.6. Protein expression was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The culture was incubated for an additional 4 h at 80 rpm, and then the bacterial cells were harvested by centrifugation. Cells were suspended in 2 mL of PBS containing 1% glycerol and 1 g glass beads. The content was vortexed for 5 min and centrifuged at 10,000 *g* for 10 min. The extraction procedure was repeated thrice, and the supernatants were combined. The Ac-catalase was purified by Ni-NTA affinity chromatography as per the manufacturer's instruction (Qiagen) and then dialyzed as described before (Ken et al., 2005). The dialyzed sample was either used directly for analysis or stored at -20°C until use.

Protein concentration measurement

Protein concentration was determined by a Bio-Rad Protein Assay Kit (Richmond, CA) using bovine serum albumin as a reference standard.

Ac-catalase activity assay (ferrithiocyanate assay)

The recombinant Ac-catalase (0.17 μg protein) was incubated in 45-47 μL buffer (1 mM DTT in 0.33 × PBS containing 5% glycerol) for 2 min at room temperature. The reaction was initiated by addition of 3-5 μL 1 mM H_2O_2 (60-100 μM). At 0 and 10 min reaction times, 50 μL of the reaction mixture was taken, and 20 μL of 26% trichloroacetic acid was added to stop the reaction. The peroxidase activity was determined by following the disappearance of the peroxide substrate (the total peroxide, at the beginning of the reaction minus the remaining amount of the 10 min). The remaining peroxide content was determined as a red-colored ferrithiocyanate complex formed by addition of 20 μL 10 mM $Fe(II)(NH_4)(SO_4)_2$ and 10 μL 2.5 M KSCN to the 70 μL reaction mixture, which was quantified by measurement of the absorbance at 475 nm (Thurman et al., 1972).

Ac-catalase activity assay on the 10% native PAGE

Duplicate samples containing the Ac-catalase were electrophoresed on a 10% native gel for 2.5 h at 100 V. The duplicate lanes were sliced into two parts. One part was stained for catalase activity, the other for protein staining. For catalase activity staining, the gel was

incubated in 0.005% (v/v) hydrogen peroxide for 10 min, then rinsed with water, followed by an immersion in 1% (w/v) $FeCl_3$ and 1% (w/v) $K_3Fe(CN)_6$ with gentle shaking, the gel became uniformly green except at the position of Ac-catalase, which showed achromatic zones which revealed that catalase had prevented the formation of the insoluble green precipitation.

Enzyme characterization

The enzyme sample was tested for stability under various conditions. Aliquots of the Ac-catalase sample were treated as follows: (1) *Thermal stability*. Enzyme sample was heated to 60°C for 2, 4, 8 or 16 min. (2) *pH stability*. Enzyme sample was adjusted to desired pH by adding a half volume of buffer at different pHs: 0.2 M citrate buffer (pH 2.2, 5.4), 0.2 M Tris-HCl buffer (pH 7.8, or 9.0), or 0.2 M glycine-NaOH buffer (pH 10.4, or 11.2). Each sample was incubated at 37°C for 1 h. (3) *SDS effect*. SDS, a denaturing reagent, was added to the enzyme sample to the levels of 0.5, 1, or 2% and incubated at 37°C for 0.5 h. (4) *Imidazole effect*. During protein purification, the Ac-catalase enzyme was eluted with imidazole. Therefore, the effect of imidazole on protein activity was examined. Imidazole was added to the enzyme sample to the levels of 0.2, 0.4, or 0.8 M and incubated at 37 °C for 1 h. (5) *Proteolytic susceptibility.* The enzyme was incubated with one-tenth its weight of trypsin or chymotrypsin at pH 8.0, 37°C for a period of 60, 120 or 240 min. In the chymotrypsin digestion, $CaCl_2$ was added to 5 mM. Aliquots were removed at various time intervals for analysis. After each treatment, two-thirds of the sample was electrophoresed onto a 10% native polyacrylamide gel electrophoresis (PAGE) to determine any changes in protein levels. The other one-third of the sample was used for ferrithiocyanate assay to determine any changes in enzyme activity.

Thiol mixed-function oxidation (MFO) assay

Ac-catalase-dependent inactivation of DNA cleavage evaluated by MFO assay (Kwon et al., 1993). A reaction mixture (15 µL) containing 40 µM $FeCl_3$, 10 mM dithiothreitol (DTT), 25 mM HEPES (pH 7.0), and 0.69 µg of pUC19 plasmid DNA was incubated with or without the Ac-catalase protein (0.17 or 0.34 µg) at 25°C for 0.5 or 1 h. After incubation, nicking of the supercoiled plasmids by the MFO was evaluated on 1% agarose gels stained with ethidium bromide.

RESULTS AND DISCUSSION

Cloning and characterization of a cDNA encoding Ac-catalase

By sequencing about 20,000 *A. camphorata* cDNA clones, these nucleotide sequences and the inferred amino acid sequences were compared to the NCBI (www.ncbi.nlm.nih.gov) data banks by using the FASTN and FASTP programs. We found a putative Ac-catalase cDNA clone was identified by sequence homology to the published

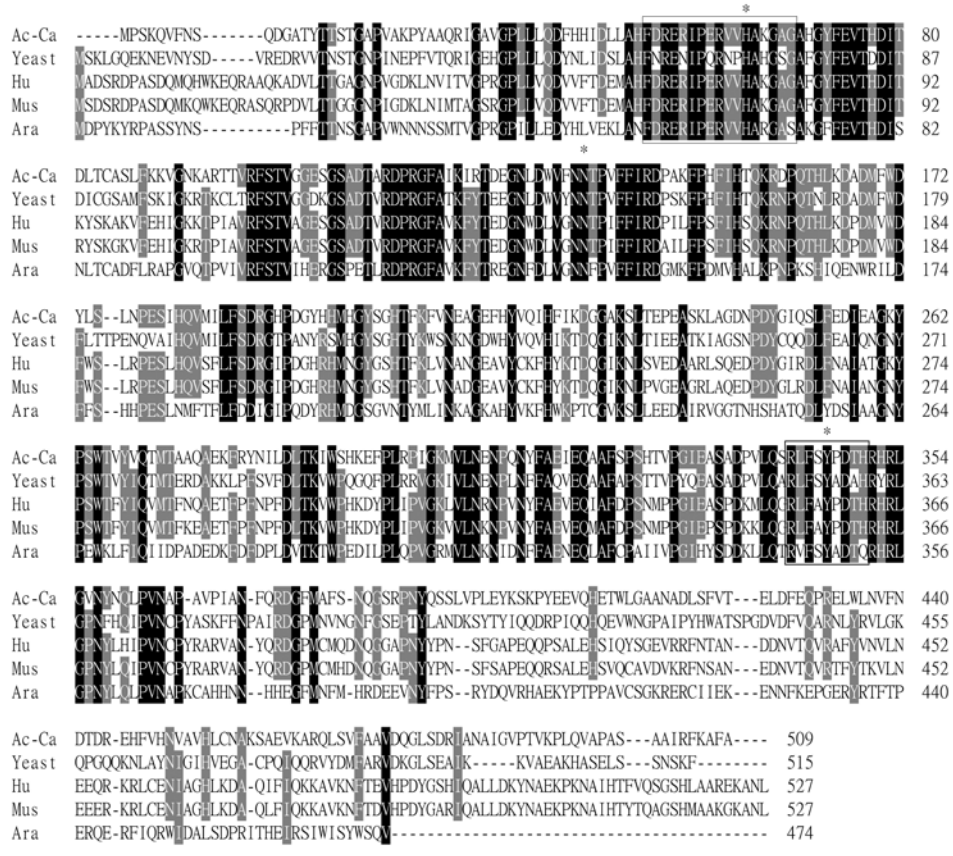

Figure 1. Optimal alignment of the amino acid sequences of Ac-catalase with selected sequences. The catalytic amino acids of Ac-catalase, including H^{63}, N^{137}, and Y^{346}, are indicated with asterisks. The catalytic domain was between F^{52} to G^{68}, and the haem-binding domain was between R^{342} to H^{350}. Both are boxed. Ac-catalase (this study), yeast (*Saccharomyces cerevisiae*), Hu (*Homo sapiens*), Mus (*Mus musculus*), and Ara (*Arabidopsis thaliana*). Identical amino acids in all sequences are shaded black, and conservative replacements are shaded gray.

catalases in NCBI data bank. The coding region of Ac-catalase cDNA was 1,527 bp that encodes a protein of 509 amino acid residues with calculated molecular mass of 57 kDa (EMBL accession no. **DQ021914**). Figure 1 shows the optimal alignment of the amino acid sequences of Ac-catalase with four selected sequences. This Ac-catalase shared 55% identity and 71% similarity with yeast catalase (*Saccharomyces cerevisiae,* accession no. **CAA31443.1)**, shared 54% identity and 67% similarity with human catalase (*Homo sapiens*, **NP001743.1**), shared 54% identity and 66% similarity with mouse catalase (*Mus musculus*, **NP033934.1**), shared 47% identity and 64% similarity with *Arabidopsis thaliana* catalase (**NP001031791.1**) by using the BLAST 2 SEQUENCES program (Tatusova and Madden, 1999). All the catalytic amino acids, including H^{63}, N^{137} and Y^{346}, are identified in each selected sequence and are indicated by an asterisk in Figure 1. The active site was between F^{52} to G^{68}, and the haem-binding site was between R^{342} to H^{350}. Both are also highly conserved and boxed in Figure 1. As compared with yeast catalase, the crystal structure of which was determined by X-ray diffraction (Berthet et al., 1997), the amino acids from Ala^{50} to Tyr^{390} of this Ac-catalase appear conserved as yeast catalase. This conserved core was identified to be a typical catalase because it contains the essential distal catalytic domain and the proximal haem-ligand domain as described in a previous report (Zamocky and Koller, 1999).

Expression and purification of the recombinant Ac-catalase

The coding region of Ac-catalase (1.5 kb) was amplified by PCR and subcloned into an expression vector, pET-20b(+) as described in Materials and Methods.

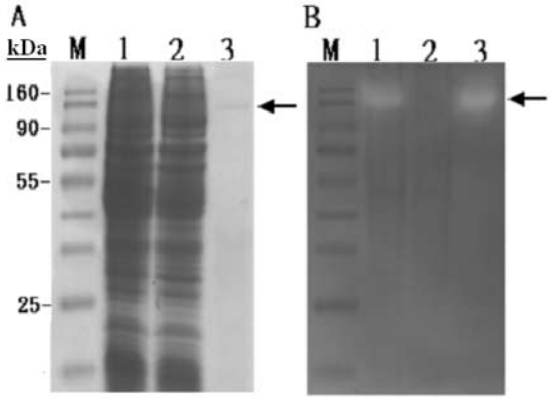

Figure 2. Expression and purification of recombinant Ac-catalase in *E. coli*. Fifteen μL (loading buffer with mercaptoethanol and without boiling) of each fraction was loaded into each lane of the 10% SDS-PAGE. Lane 1, crude extract from *E. coli* expressing Ac-catalase; 2, flow-through proteins from the Ni-NTA column; 3, purified Ac-catalase eluted from Ni-NTA column. A, Coomassie blue-stained; B, Activity stained. Molecular masses (in kDa) of standards are shown at left. Arrow indicates the target protein.

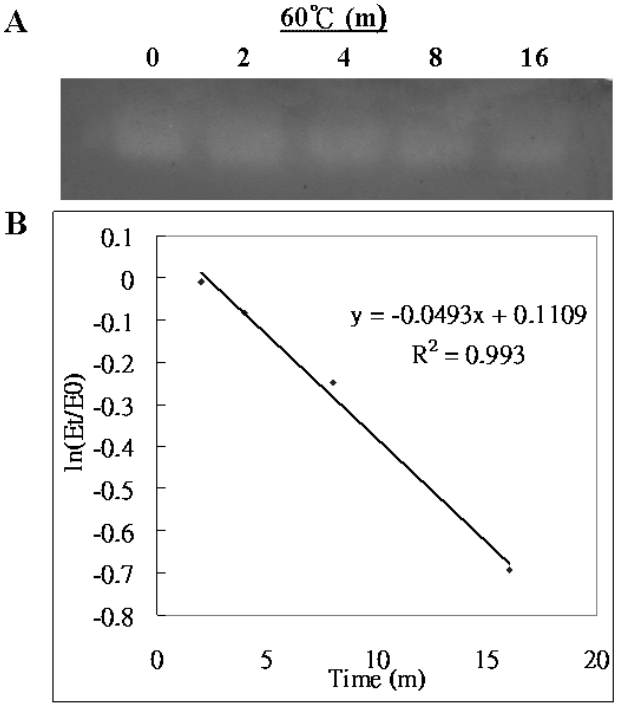

Figure 3. Effect of temperature on the purified Ac-catalase. The enzyme sample was heated at 60°C for various time intervals. Aliquots of the sample were taken at 0, 2, 4, 8 or 16 min and analyzed by a 10 % native-PAGE or assayed for enzyme activity. A, Staining for protein (0.82 μg protein/ lane) after separation on a 10% native-PAGE; B, Plot of thermal inactivation kinetics. Ac-catalase activity assay (0.17 μg protein/ time interval). E_0 and E_t are original activity and residual activity after being heated for different time intervals. Data are means of three experiments.

Positive clones were verified by DNA sequence analysis. The recombinant Ac-catalase was expressed, and the proteins were analyzed by a 10% SDS-PAGE in the presence a reducing agent without boiling (Figure 2). The recombinant Ac-catalase was expressed as a 6His-tagged fusion protein and was purified by affinity chromatography with nickel chelating Sepharose. A band with a molecular mass of ~114 kDa (expected size of Ac-catalase dimer) was detected in Ni-NTA eluted fractions by SDS-PAGE (Figure 2A, B, lane 3). The Ni-NTA eluted fractions containing pure protein were pooled and characterized further. The yield of the purified 6His-tagged Ac-catalase was 80 μg from 200 mL of culture. Functional Ac-catalase was detected by activity assay as described below.

Characterization of the purified Ac-catalase

To examine the heat stability, the purified Ac-catalase was heat-treated as described in Materials and Methods and then analyzed by 10% native gel or activity assay. The enzyme appeared to be heat stable. Approximately 50% of the Ac-catalase activity was lost at 60°C for 14 min (Figure 3). The Ac-catalase was stable in a broad range of pH from 7.8 to 11.2 as shown in Figure 4A. The enzyme retained 20% activity in 2% SDS (Figure 4B). The enzyme retained about 38% activity in 0.8 M imidazole (Figure 4C). The

enzyme showed 70% activity after 4 h of incubation at 37 °C with one-tenth its weight of trypsin (Figure 4D). The enzyme showed 20% activity after 3 h of incubation at 37° C with chymotrypsin (data not shown).

The Ac-catalase as shown in Figure 2 contains 44 potential trypsin cleavage sites and 53 potential chymotrypsin-high specificity (C-term to [FYW], not before P) cleavage sites. However, the enzyme appeared susceptible to chymotrypsin but resistant to digestion by trypsin, even at a high enzyme/substrate (w/w) ratio of 1/10 (Figure 4D).

To test peroxidase activity of the Ac-catalase, we used the assay of plasmid DNA protection against ROS generated by the Fenton reaction induced by this metal-catalyzed system. In the presence of Ac-catalase, the alteration of the plasmid DNA from the supercoiled to the nicked form was prevented. The inhibition was dose-dependent (Figure 5). The assay employs a system that generates hydroxyl radicals to damage DNA. In the absence of Ac-catalase, the hydroxyl radicals produced in the system caused nicking of the DNA, as evidenced by a shift in gel mobility of the supercoiled plasmid. The addition of purified Ac-catalase to the MFO system at concentrations of 0.17 μg and 0.34 μg prevented nicking of the supercoiled DNA, demonstrating antioxidant activity of Ac-catalase.

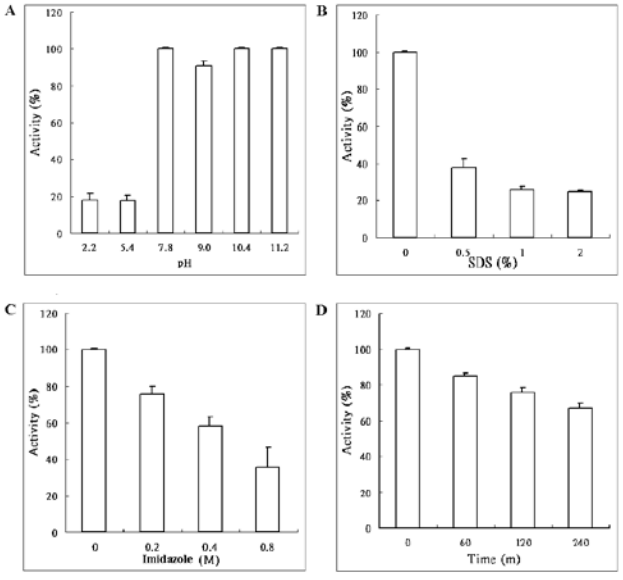

Figure 4. Effect of pH, SDS, imidazole and trypsin on the purified Ac-catalase. A, The enzyme samples were incubated with different pH buffer at 37°C for 1 h and then assayed for Ac-catalase activity (0.17 μg protein/ pH point); B, The enzyme samples were incubated with various concentration of SDS at 37°C for 30 min and then checked for activity (0.17 μg protein/ SDS level); C, The enzyme samples were incubated with various concentration of imidazole at 37°C for 1 h and then checked for Ac-catalase activity (0.17 μg protein / imidazole level); D, The enzyme samples were incubated with trypsin at 37°C for various times and then checked for Ac-catalase activity (0.17 μg protein / time interval). Data are means of three experiments.

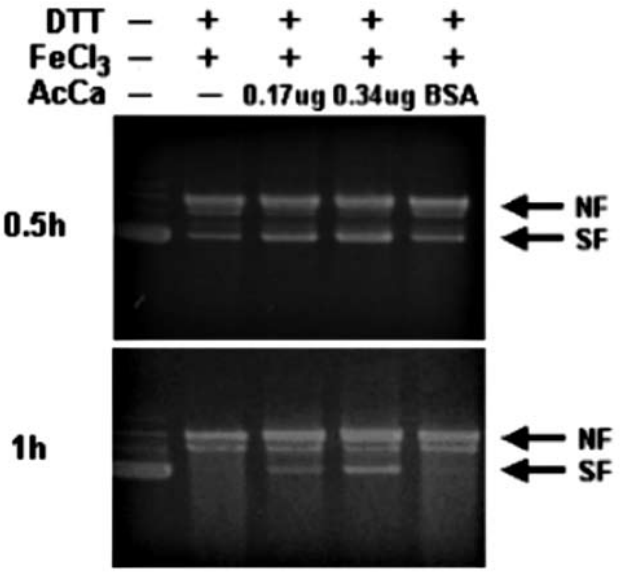

Figure 5. Functional activity of recombinant Ac-catalase. The reaction was performed in a mixture consisting of 40 μM $FeCl_3$, 10 mM DTT, 25 mM HEPES (pH 7.0) and 0.69 μg of pUC19 plasmid DNA. The reaction was stopped after incubation for 0.5 h (A), 1 h (B), and the plasmid DNA was analysed on ethidium bromide stained agarose gel. Lane 1, only DNA; 2, Fe^{3+} + DTT; 3, Fe^{3+} + DTT + 0.17 μg Ac-catalase; 4, Fe^{3+} + DTT + 0.34 μg Ac-catalase; 5, Fe^{3+} + DTT + 0.34 μg BSA. SF, supercoiled form; NF, nicked form.

Heat stability and imidazole effects were tested because the information is useful for developing enzyme purification protocols. Protease tests were useful in understanding the effect of the digestive enzymes on the Ac-catalase and its suitability as health food.

The fruiting body of *A. camphorata* is known in Taiwan for treating cancer and inflammation, yet little is known about its biological effects. This Ac-catalase may be also one of the important physiological components in *A. camphorata* responsible for its medicinal efficacy. We are particularly interested in the antioxidant effects of the *A. camphorata*. To aid us in understanding them, we cloned the antioxidant enzyme catalase from *A. camphorata*, expressed in *E. coli* and characterized it. This enzyme appears to be stable under various conditions. The most significant finding in this paper is that the recombinant Ac-catalase can enzymatically detoxify ·OH. Using a function oxidation (MFO) system to generate ·OH *in vitro*, we have demonstrated the ability of Ac-catalase to protect intact supercoiled plasmid DNA from ·OH-induced nicking. Further investigation is required to evaluate the possible medical applications of Ac-catalase, including the removal of peroxide (especially hydroxyl radicals) from wounded tissue to promote healing or its use as a health food.

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樟芝過氧化氫酶在大腸桿菌中的表現及其生化特性

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過氧化氫酶 (catalase) 在抗氧化與細胞信息傳遞中扮演重要的角色。依據樟芝 (*Antrodia camphorata*) 表現庫序列資訊 (expressed sequence tag database) 選殖出過氧化氫酶 cDNA 序列 (DQ021914)，全長共 1794 個核苷酸，內含轉譯區 1527 個核苷酸，可轉譯出 509 個胺基酸。經序列比較樟芝過氧化氫酶與其他物種的序列有很高的相似性。將其轉譯區選殖入表現載體 pET-20b(+)，以大腸桿菌 *E. coli* BL21(DE3)pLysS 作為表現宿主，經親和性管柱純化可得到具有活性的過氧化氫酶。其特性在 60°C 加熱活性降低一半的時間為 14 分鐘，在鹼性環境下 (pH 7.8 ~ 11.2) 仍然具有相當的活性，以 trypsin 處理 4 小時後仍然有 67% 的活性。此酶具有保護 DNA 防止自由基 (·OH) 攻擊。此酶特性之研究有利於未來開發為醫學及健康食品之應用。

關鍵詞：樟芝；過氧化氫酶；基因表現。