Cloning, Expression, and Characterization of Thermostable Manganese Superoxide Dismutase from *Thermoascus aurantiacus* var. *levisporus*

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A superoxide dismutase (SOD) gene of *Thermoascus aurantiacus* var. *levisporus*, a thermophilic fungus, was cloned, sequenced, and expressed in *Pichia pastoris* and its gene product was characterized. The coding sequence predicted a 231 residues protein with a unique 35 amino acids extension at the N-terminus indicating a mitochondrial-targeting sequence. The content of Mn was 2.46 μg/mg of protein and Fe was not detected in the purified enzyme. The enzyme was found to be inhibited by NaN_3, but not by KCN or H_2O_2. These results suggested that the SOD in *Thermoascus aurantiacus* var. *levisporus* was the manganese superoxide dismutase type. In comparison with other MnSODs, all manganese-binding sites were also conserved in the sequence (H88, H136, D222, H226). The molecular mass of a single band of the enzyme was estimated to be 21.7 kDa. The protein was expressed in tetramer form with molecular weight of 68.0 kDa. The activity of purified protein was 2,324 U/mg. The optimum temperature of the enzyme was 55°C and it exhibited maximal activity at pH 7.5. The enzyme was thermostable at 50 and 60°C and the half-life at 80°C was approximately 40 min.

Keywords: thermostable, MnSOD, cloning, expression, *Thermoascus aurantiacus*

Reactive oxygen species (ROS) and reactive oxygen intermediates (ROI) are formed in the body as natural products of oxidative metabolism. During defense reactions and in physiological conditions that result in oxidative stress ROS and ROI can increase and may be toxic to cells (Mezes *et al.*, 1997). Cells have developed multilayered interdependent antioxidant system as a defense against oxidative injury. Superoxide dismutase (SOD), catalase, glutathione peroxidase, glutathione reductase, glutaredoxin are the enzymes that play a major role in the dismutation of the ROS and ROI (Bell and Smith, 1994; Rudneva, 1999). SOD detoxifies superoxide radicals by converting them to hydrogen peroxide and oxygen. Hydrogen peroxide is then transformed to water and oxygen by catalase, resulting in innocuous compounds to the cell (Bendich, 1993; Pipe *et al.,* 1993).

Various SOD enzymes have been characterized that employ a metal cofactor for catalysis. Three types of SOD have been identified in eukaryotes and differ by the metallic ions present at the active site: copper zinc SOD (Cu/ZnSOD), manganese SOD (MnSOD), and iron SOD (FeSOD) (Halliwell and Gutteridge, 1999; Plantivaux *et al.*, 2004). There are two types of MnSOD known as mitochondrial MnSOD (mMnSOD) and cytosolic MnSOD (cMnSOD). The former is translocated to the mitochondria by recognition of the N-terminal signal peptide by a mitochondrial translocon. The latter lacks a mitochondrial transit peptide and is retained in cytosol (Brouwer *et al.*, 1997; Brouwer *et al.*, 2003).

Among the said SODs, mMnSOD is particularly important as it is located in mitochondria and represents the first line of defense against superoxide radicals produced as by products of oxidative phosphorylation (Beyer *et al.*, 1991). And it is thought to be a major scavenger of damaging ROS and ROI metabolites in the mitochondrial matrix (Ken *et al.*, 2005).

SODs have recently found application in gene therapy used for cardiovascular diseases as well as in the pharmaceutical and cosmetic industry. SODs are currently of great interest as potential therapeutic treatments for oxidative damage. It has been shown that SOD might be beneficial in the treatment of postischaemic reperfusion injury, rheumatoid and osteoarthritis, brain trauma and influenza-induced lung pneumonitis. SODs have been proposed to be clinically useful for a wide variety of applications, including prevention of oncogenesis, tumour promotion, tumour invasiveness and reduction of the cytotoxic and cardiotoxic effects of anticancer drugs (Trotti, 1997; Zhong *et al*., 1997; Mates *et al.*, 2000; Angelova *et al.*, 2001).

SODs are widely distributed from a wide range of organisms including bacteria, fungi, plants, and animals (Bannister *et al*., 1987). In recent years, there has been increasing interest in SODs from thermophiles, which were expected to produce thermostable SODs. In industry, a major requirement for commercial SODs is thermal stability, because thermal denaturation is a common cause of enzyme inactivation. It has been reported that SODs were isolated from hyperthermophiles of the genera *Sulfolobus*, *Aquifex*, *Pyrophilus*, *Pyrobaculum*, and *Aeropyrum* (Lim *et al.,* 1997; Knapp *et al.,* 1999; Yamano and Maruyama, 1999; Amo *et al.,* 2003).

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Table 1. The primers for PCR used in the study

Primer name	Sequence (5'-3')	Purpose
MnS	5'-AASCACCACCARACYTAYGT-3'	first partial fragment
MnX	5'-AASCACCACCARACYTAYGT-3'	first partial fragment
EMS	5'-CGGCGGCTACGACAAGTT-3'	3'-RACE
M13 M4	5'-GTTTTCCCAGTCACGAC-3'	3'-RACE
MNX-RT	5'-CGGCCTTCCAGTTGACGACCTCCC-3'	5'-RACE
MX51	5'-TTGACGAGCCACGCCCACCC-3'	5'-RACE
MX52	5'-GCCGTTGAAGTTGATGGCGGGTTT-3'	5'-RACE
Oligo d(T)-anchor primer	5'-GACCACGCGTATCGATGTCGACTTTTTTTTTTTTTTTTTV-3' (V = A, C or G)	5'-RACE
EMN5	5'-ATGGCCGCTTCCCTCGTT-3'	ORF cDNA
EMN3	5'-TTAAGCGGCGAAGCGCTT-3'	ORF cDNA
EXP5	5'-CC<u>GAATTC</u>AAGGCGACTCTCCCTGAC-3'	expression
EXP3	5'-GG<u>GCGGCCGC</u>TTAAGCGGCGAAGCGCTT-3'	expression

In thermophilic fungi, a few studies were reported, such as *Thermomyces lanuginosus* MnSOD (Li *et al.*, 2005) and *Thermoascus aurantiacus* var. *levisporus* Cu/ZnSOD (E *et al.*, 2007). In the present study, we report cloning and sequencing of a new MnSOD gene from the thermophilic fungus *Thermoascus aurantiacus* var. *levisporus* and its expression in *Pichia pastoris*. Some characterization of the recombinant enzyme is also shown.

Materials and Methods

Strains, plasmids, and culture media
Thermoascus aurantiacus var. *levisporus* was isolated and preserved by our laboratory. The fungus was grown in shake cultures at 50°C in 250 ml flasks with 50 ml liquid medium (E *et al.*, 2007). After incubation for 2 days, mycelium was harvested by filtration through quantitative filter paper and washed exhaustively with distilled water, then and the mycelia were frozen immediately in liquid nitrogen and stored at -80°C before use. *Escherichia coli* DH5α and JM109 were used for cloning and nucleotide sequencing. *P. pastoris* GS115 and the plasmid pPIC9K were from Invitrogen. *P. pastoris* GS115 was used as a host for expression of SOD. The plasmid pMD18-T was from TaKaRa. Recombinant plasmids were constructed from pMD18-T for subcloning and sequencing and from pPIC9K for expression of the SOD. All media were prepared using Invitrogen protocols.

cDNA cloning
The total RNA was obtained from the mycelia of *T. aurantiacus* var. *levisporus* using TRIZOL reagent (Gibco BRL) according to the RNA PCR kit 3.0 instruction (TaKaRa). Full-length MnSOD cDNA of *T. aurantiacus* var. *levisporus* was obtained by the procedures of reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) method. Multiple alignments and phylogenetic comparisons of MnSOD amino acid sequences of *T. aurantiacus* with other fungi were performed. The first partial fragment sequence of the *sod* gene was obtained by RT-PCR. Degenerated oligonucleotide primers for PCR were synthesized based on two highly conserved amino acid sequences NHHNTY and WEHAYY, corresponding to the other fungal MnSOD. Amplification primers for *T. aur-*

antiacus var. *levisporus* MnSOD are shown in Table 1. Briefly, first fragment of partial the *sod* gene was obtained using primer MnS and MnX, then SMART-RACE was performed using the 5'/3' RACE-Kit, 2nd Generation (Roche Applied Science) to make a full-length *sod* cDNA. The specific primers for RACE-PCR were designed on the basis of the gene fragment sequence obtained above. For 3'-RACE, the reverse transcription was performed by M13M4 primer. PCR for 3'-RACE were carried out with the primer M13M4 and EMS (Table 1). For 5'-RACE, the first-strand cDNA was performed with MNX-RT primer by 5'-RACE system. The first-strand cDNA was tailed at the 5'-end by terminal transferase TdT and dATP. The primer set consisted of MX51 and Oligo d(T)-anchor primer for the first-run PCR, and MX52 and PCR Anchor primer for the second-run PCR (Fig. 1).

Analysis of nucleotide and amino acid sequence
In order to search for nucleotide and protein sequences similar to the *T. aurantiacus* var. *levisporus* MnSOD, the NCBI BLAST program (http://www.ncbi.nlm.nih.gov) was used. The deduced amino acid sequences of MnSOD cDNA were analyzed using ExPASy search program (http://au.expasy.org/tools/). The signal peptide was predicted by Signal P 3.0 program (http://www.cbs.dtu.dk/services/Signal P). The MITOPROT was used to see if the extend sequence contains any targeting sequence (http://ihg.gsf.de/ihg/mitoprot.html).

Cloning the coding sequence of the SOD into expression vector
The coding region of the *sod* cDNA was amplified by PCR, using *T. aurantiacus* var. *levisporus* cDNA as templates. And the pair of primers was designed for cloning the coding sequence of the the SOD into expression vector based on the restriction enzyme sites of the *T. aurantiacus* var. *levisporus sod* sequence. The 5' upstream primer contains *Eco*RI (CCGAATTCAAGGCGACTCTCCCTGAC) recognition site and 3' downstream primer contains *Not*I (GGGCGGCCGCTTAAGCGGCGAAGCGCTT) recognition site. The amplified product (0.6 kb) was cloned into pUC18 and transformed into *E. coli* DH5α. Plasmid DNA was isolated from a positive clone and digested with *Eco*RI and *Not*I, and then ligated to pPIC9K (also digested by *Eco*RI and *Not*I). The expressin
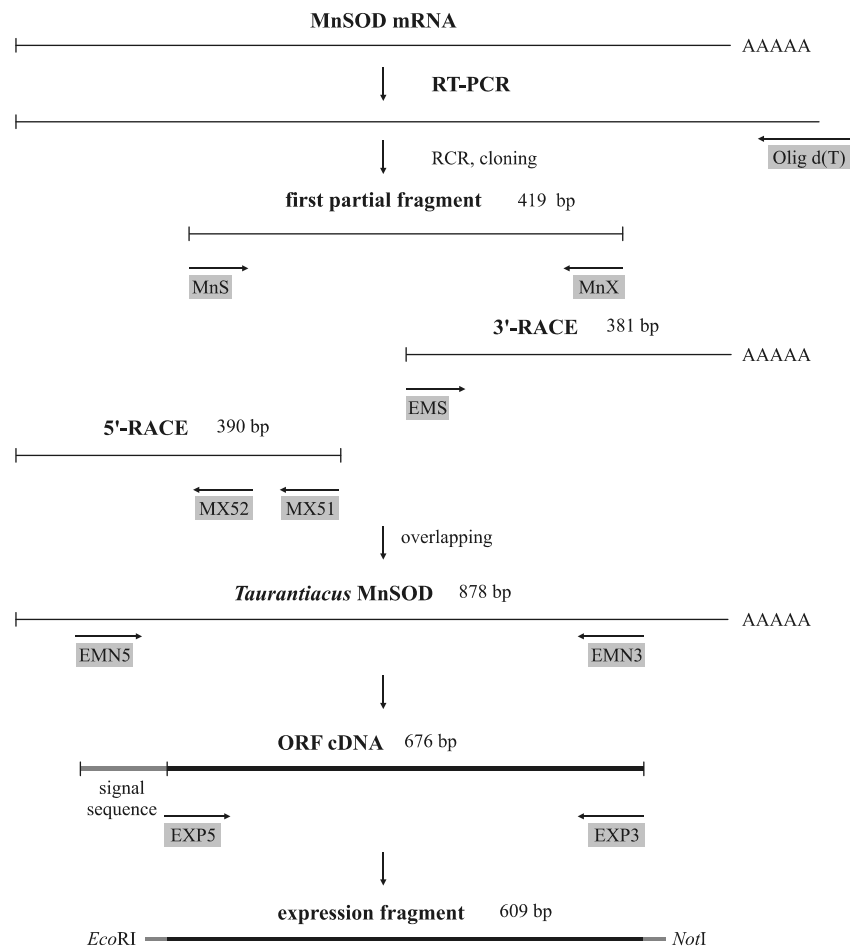
Fig. 1. Schematic diagram showing the relative positions of *T. aurantiacus* var. *levisporus* MnSOD. Primer MnS and MnX are degenerate primers. Primer MnX51, MnX52, EMN5, EMN3, EXP5, EXP3 are specific primers.

sequence of *sod* gene was ligated to pPIC9K without signal sequence. The DNA manipulations were carried out by using standard procedures (Sambrook *et al*., 1989). The expression plasmid pPIC9K/Mnsod was subsequently confirmed by PCR analysis, restriction analysis and DNA sequencing.

Expression and purification of *T. aurantiacus* var. *levisporus* MnSOD

The *SacI*-linearized recombinant plasmid pPIC9K/Mnsod was transformed into *P. pastoris* GS115 by electroporation with Eppendorf Electroporartor 2510. The transformation and screening transformants were performed according to Pichia Expression System Kit (Invitrogen). The transformants were grown in BMGY at 28°C until A600 reached 1~2. The cells were harvested by centrifugation at 5,000×g for 5 min, and then cultured in BMMY. The secreting expression was induced by supplement of 1% methanol into BMMY, using another not added as the control. Aliquots of each culture were removed at 24 h intervals and induced expression of the recombinant MnSOD was detected by SDS-PAGE (Laemmli, 1970).

The recombinant MnSOD protein was purified with DEAE-Sepharose column and gel filtration on Sephacryl S-100, according to the procedure provided by vendor as the manufacturer's instruction (Sweden). Purity of the recombinant MnSOD was determined by SDS-PAGE (Laemmli, 1970).

Enzyme activity assay and determination of protein concentration

Activity of the enzyme was determined using the method described by Stewart and Bewley (1980). Enzyme required to reduce the reaction by 50% was considered as one enzyme unit. The protein concentration was determined by the method of Lowry *et al.* (1951), with bovine scrum albumin as the standard.

In order to determine the optimal temperature, the reaction was carried out at different temperatures (35~90°C) under standard conditions. To determine the thermal stability, each enzyme sample was heated to 50, 60, 70, 80, and 90°C for 10, 20, 30, 40, 50, and 60 min, and the relative activity was determined under standard conditions. The optimum reaction pH of MnSOD was measured under different pH conditions. The buffers of CH_3COOH-CH_3COONa (pH 4.0 ~6.0), NaH_2PO4-Na_2HPO_4 (pH 6.0~7.0), Tris-HCl (pH 7.0 ~10.0), and Na_2HPO_4-NaOH (pH 10.0~12.0) were used,

and the relative activity was measured. The effect of pH on the enzyme stability was investigated by measuring the remaining activity after incubation for 60 min in different pH buffer (pH 5.0~10.0) under standard conditions.

Metal analysis

Metals of the purified enzyme dissolved in glass distilled, deionized water were analyzed using atomic absorption spectrophotometer (SP9-400, UK).

Enzyme inhibition studies

The inhibitors H_2O_2, KCN, NaN_3 were used to determine the effects on SOD activity. The enzyme was preincubated with each inhibitor at pH 7.5, 37°C for a period of 1 h. The treated samples were electrophoresed onto a 12.5% native

gel to determine the changes in MnSOD activity of each sample (Beauchamp and Fridovich, 1971). And the residual activities of each preincubated samples were measured using NBT method for parallel comparison.

Results

Cloning and analyzing *T. aurantiacus* var. *levisporus* MnSOD

The full length cDNA sequence was deduced from three overlapping cDNA fragments (that were thoroughly sequenced). The first 419 bp fragment was obtained by RT-PCR amplification using degenerated primers, corresponding to two highly conserved amino acids sequences, NHHNTY and WEHAYY of the MnSOD. The partial sequence was

Fig. 2. Multiple alignment of the deduced amino acid sequence of the mMnSOD from *T. aurantiacus* var. *levisporus* with MnSOD from other fungi. A.F, *Aspergillus flavus* (AAT81154); C.M, *Cordyceps militaris* (AY206454); E.N, *Emericella nidulans* (AY027564); C.A, *Candida albicans* (AAL08560); P.C, *Penicillium chrysogenum* (AF026523); B.G, *Blumeria graminis* (AAL56985).
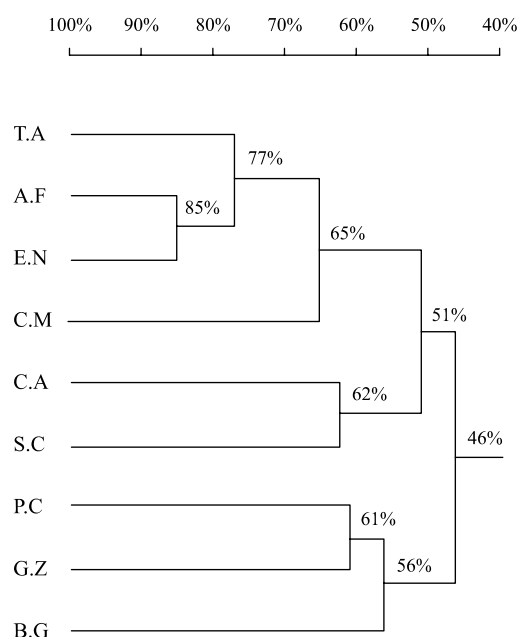
Fig. 3. Homology tree between *T. aurantiacus* var. *levisporus* MnSOD and other MnSODs by dnaman software. A.F, *Aspergillus flavus*; C.M, *Cordyceps militaris*; E.N, *Emericella nidulans*; C.A, *Candida albicans*; P.C, *Penicillium chrysogenum*; B.G, *Blumeria graminis*; G.Z, *Gibberella zeae* PH-1 (EAA69573); S.C, *Saccharomyces cerevisiae* (CAA26092).

72.66% identical to the Mn-*sod* gene of *Paracoccidioides brasiliensis*. The cDNA sequence was extended in the 5'/3' directions by RACE-PCR. A 878 bp full-length sequence of *T. aurantiacus* var. *levisporus* Mn-*sod* was obtained by PCR. Fig. 1 shows the nucleotide and deduced amino acid sequences of Mn-*sod* gene. The *T. aurantiacus* var. *levisporus* Mn-*sod* cDNA (GenBank database, the accession no. EF428323) sequence analysis resulted in an open reading frame of 696 bp. Two short fragments 1~81 bp and 778~878 bp corresponds to 5'UTR and 3'UTR. Signal P 3.0 program analysis revealed that it contained a putative signal peptide of 35 amino acids which was predicted to be a mitochondrial targeting sequence by MITOPROT. We found that the probability of exporting to mitochondria was 0.9922 and the cleaved sequence was MAASLVHPSAARAALRAGASATR TAGLAGTSFVRG. Signal peptide of 32 amino acids for primate MnSOD has been reported (Liau *et al.*, 2007). The function of this presumed signal peptide was reported as trans-location of MnSOD into mitochondria. Multiple alignments of *T. aurantiacus* var. *levisporus* MnSOD with other fungal MnSODs showed high conservation in four putative manganese binding sites (H88, H136, D222, and H226). Furthermore, the signatures of MnSOD (DAWEHAYY, 222~229AA) were observed in *T. aurantiacus* var. *levisporus* MnSOD.

Searches of current nucleotide and protein databases with the BLAST network service revealed that the sequence of *T. aurantiacus* var. *levisporus* MnSOD showed a homology with the MnSOD sequences from other fungi (Fig. 2). Homology tree between this MnSOD and other MnSODs by DNAMAN software (Lynnon Corporation,
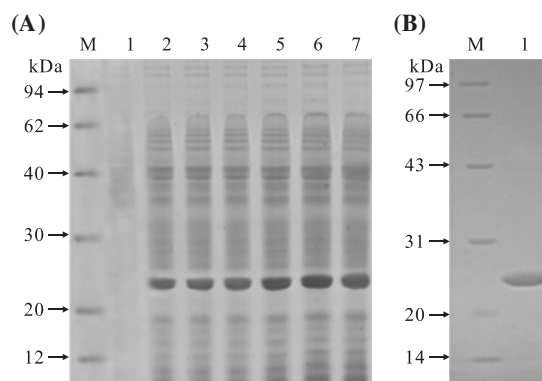

Fig. 4. SDS-PAGE analysis of the recombinant MnSOD from *P. pastoris* and purified protein. (A) Lanes: M, protein marker; 1, P. pastoris GS115 control; 2~7, 2, 3, 4, 5, 6 and 7 day, respectively. (B) Lanes: M, protein marker; 1, Purified SOD visualized by Coomassie brilliant blue staining.

Vaudreuil-Dorion QC, Canada) showed that it shared 77%, 72%, 64%, 48% identity with MnSOD from *Aspergillus flavus* (AAT81154), *Emericella nidulans* (AY027564), *Cordyceps militaries* (AY206454), and *Saccharomyces cerevisiae* (CAA26092), respectively (Fig. 3).

Expression and purification of MnSOD
The cDNA was introduced into the yeast expression system and heterologous expression of MnSOD was under transcriptional control of the AOX1 promoter in *P. pastoris* GS115. The MnSOD was successfully secreting expressed when induced by supplement of methanol. Figure 4 shows SDS-PAGE of the induced recombinant MnSOD from a transformant strain MS18. After seven days induction the transformant strain had the highest activity and the expression level was 0.92 mg/ml. The expressed protein was easily purified with DEAE-Sepharose column protein purification system. Electrophoresis of the enzyme on SDS-PAGE gave a single band with a molecular weight of 21.7 kDa. The molecular mass of the SOD was estimated to be approximately 86.0 kDa by gel filtration on Sephacryl S-100. The results showed that the SOD was composed of four identical subunits of 21.7 kDa each. In enkaryotes, the family of MnSOD is comprised of tetramers of four approximately 21.0 kDa identical subunits generally (Culotta *et al.*, 2006). The purified enzyme showed 2,324 U/mg activity. This value was almost similar to the MnSOD activity reported from *Haliotis discus discus* (Ekanayake *et al.*, 2006), but lower to MnSOD reported from *Tatumella ptyseos* (Ken *et al.*, 2005).

Effect of temperature and pH on MnSOD activity and stability
The effect of temperature and pH on the enzyme activity and stability are shown in Fig. 5. The enzyme had temperature optima at 55°C. The recombinant MnSOD from *T. aurantiacus* was a thermostable SOD. The enzyme was thermostable at 50 and 60°C, and retained 65% activity after 60 min at 70°C. The half-life of the enzyme at 80°C was approximately 40 min and even retained 20% activity after 50 min at 90°C. The enzyme activity was relatively stable in a
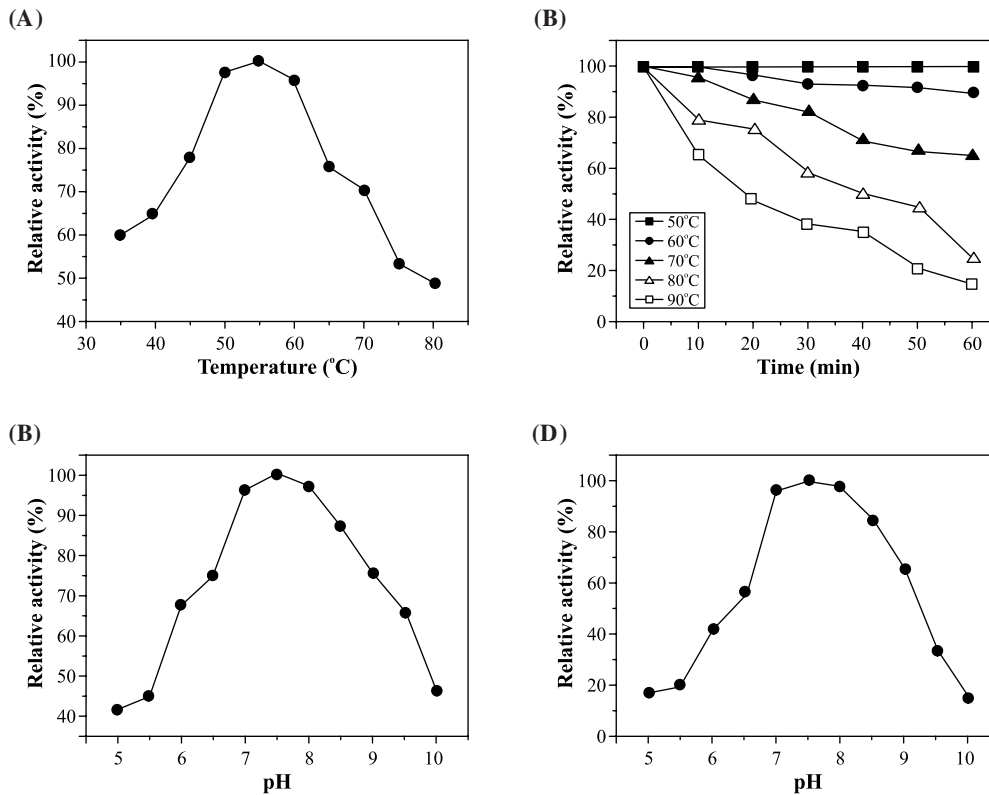
(A)

(B)

(B)

(D)

Fig. 5. Effect of temperature and pH on enzyme activity and stability: (A) temperature-enzyme activity; (B) temperature-enzyme stability; (C) pH-enzyme activity; (D) pH-enzyme stability. Every run was repeated at least three times

broad pH range, from pH 6.5 to 9.0, and the enzyme showed maximum activity at pH 7.5.

Metal analysis
Atomic absorption spectrophotometric analysis showed that the content of Mn was 2.46 μg/mg of protein and Fe was not detected in the purified enzyme. The result suggested that the SOD in *T. aurantiacus* var. *levisporus* is of the MnSOD type.

Inhibitors effect on the enzyme activity
The SOD was found to be inhibited by NaN_3, but not by KCN or H_2O_2. Fig. 6 shows the result of treated samples electrophoresed onto native gel. The inhibition rate of the SOD activity by inhibitors, KCN, H_2O_2, and NaN_3 was 4.3, 3.2, and 89.6%. This was consistent with that the *sod* gene we cloned from *T. aurantiacus* var. *levisporus* was MnSOD.

Discussion

This study reported the cloning and expression of the mMnSOD from *T. aurantiacus* var. *levisporus*. The biologically active form of this MnSOD had been successfully expressed in yeast. We found that the extended N-terminal sequence of the MnSOD contains potential mitochondrial targeting sequence with probability of export to mitochondria 0.9922. This is consistent with the fact that MnSOD we cloned belongs to the mMnSOD, not the cMnSOD.

In eukaryotic cells, mMnSOD is synthesized in the cytosol and imported post-translational into the mitochondrial matrix (Bannister *et al.*, 1987). Mitochondria are the major source of superoxide from complexes I and III of the electron transport chain, and superoxide can damage phospholipids, proteins, and nucleic acids (Kowluru *et al.*, 2006). mMnSOD catalyzes the dismutation of superoxide radicals
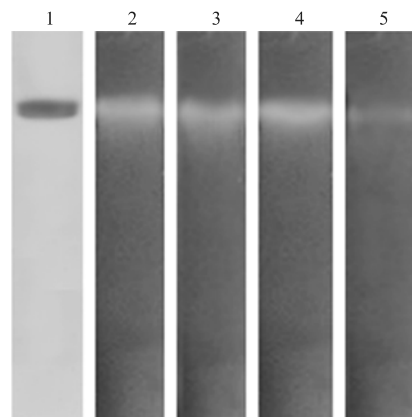

Fig. 6. Detection of SOD from *T. aurantiacus* var. *levisporus* by polyacrylamide gel electrophoresis (PAGE). Lanes: protein staining; 2, activity staining without inhibitors; 3, activity staining with 10 mM H_2O_2; 4, activity staining with 10 mM KCN; 5, activity staining with 10 mM NaN_3.

in mitochondria by converting superoxide to oxygen and hydrogen peroxide which will be eliminated by catalase, and finally prevents the disruption of mitochondrial membrane and the damage of proteins and nucleic acids. So, it can be deduced that mMnSOD might play a more important role than cMnSOD in protecting cells from oxidative stresses since mMnSOD locates in mitochondria while cMnSOD in cytoplasm (Zhang *et al.*, 2007).

In recent years biological functions of MnSOD have aroused increasing attention among researchers. It has been shown that MnSOD is close to senescence, cell impairment, and carcinogenesis. In human, the most promising role of MnSOD is associated with its inhibition of tumorigenicity. Many studies suggest that MnSOD may function as a general tumor suppressor gene (Zhong *et al*., 1997). It is believed that MnSOD will be applied to cancer therapy in the future.

Thermostable enzymes have long been of interest to biochemists. The recombinant MnSOD from *T. aurantiacus* var. *levisporus* exhibited remarkable thermal stability. The activity of a MnSOD from *Haliotis discus discus* was destroyed completely at 70°C after 1 h (Ekanayake *et al*., 2006). The periplasmic Cu/ZnSOD from *E. coli* suffered a reversible inactivation when heated to 70°C for 29 min (Benov *et al*., 1997). A NiSOD of *Streptomyces* species was stable up to 70°C (Youn *et al*., 1996). Taken together, it can be seen that almost all reported SODs were inactivated at 70°C except for SODs from *A. camphorate* (Liau *et al.*, 2007) and hyperthermophilic archaeon such as *Sulfolobus acidocaldarius* (Knapp *et al*., 1999), *Aeropyrum pernix* (Yamano *et al*., 1999) and *S. solfataricus* (Yamano and Maruyama, 1999). For comparison of SOD in fungi, the MnSOD from *T. aurantiacus* var. *levisporus* is more thermostable than Cu/ZnSODs of *Aspergillus flavus*, *A. niger*, *A. nidulans*, *A. terreus* (Holdom *et al.,* 1996), and *A. fumigatus* Cu/ZnSOD (Holdom *et al*., 1995), and MnSOD from *Thermomyces lanuginosus* (Li *et al*., 2005). Our result showed that the *T. aurantiacus* var. *levisporus* MnSOD was one of the most thermostable enzymes among the published SODs in eukaryotic organisms. The high stability of the SOD may make it useful in applications (Gorecki *et al*., 1991). Therefore, this highly stable SOD is more suitable for using in cosmetics. It has been suggested that the enzymes of thermophilic fungi are appreciably thermostable, but less so than those of hyperthermophilic archaea. Since the major enzymatic action occurs at 40~60°C in most operational situations, thermostable enzymes from thermophilic fungi may be better suited than enzymes from hyperthermophiles (Maheshwari *et al*., 2000).

In conclusion, for the first time, we cloned the gene encoding the MnSOD from thermophilic fungus *T. aurantiacus* var. *levisporus* and characterized the recombinant MnSOD expressed in *P. pastoris* cells. The deduced amino acid sequence showed high identity 79% to the sequence of MnSOD from *A. niger*. Based on the sequence analysis, it may be concluded that the putative MnSOD is mitochondrial MnSOD. The specific activity of this recombinant MnSOD was 2324 U/mg, almost similar to the commercially available products (2,000~4,000 U/mg). The recombinant MnSOD is one of the most stable enzymes among the published SODs, and its half-life at 80°C was approximately 40 min. Therefore, this highly stable SOD is suitable for use in cosmetics. The information on this MnSOD may be useful in studies of MnSOD in other fungi.

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