

Original Research Article

Expression, Purification, Characterization and *In Vitro* Activity of Recombinant Mouse Cu/Zn-Binding Superoxide Dismutase (mSOD1)

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Abstract

Purpose: To express, purify and characterize recombinant mouse Cu/Zn-binding superoxide dismutase (mSOD1), and investigate its activity *in vitro*.

Methods: The protein, mSOD1, was expressed after induction with isopropyl-beta-D-thiogalactopyranoside (IPTG). The target protein was purified by Ni-NTA affinity chromatography. The identity of the recombinant protein was confirmed by Western-blot and peptide mass fingerprinting (PMF) analysis. Protein activity *in vitro* was investigated by SOD activity assay kit and DNA damage protective assay.

Results: mSOD1 protein was expressed with a final yield of about 60 mg of pure protein per liter of culture medium. After purification, the target protein was > 95 %. The identity of the recombinant protein was confirmed. SOD activity assay showed that the highest activity of the mSOD1 was 3789.0 ± 80.5 U/mg. The present work showed that mSOD1 was effective in protecting DNA from oxidative damage.

Conclusion: High purity recombinant mSOD1 was obtained and characterized, and had high activity *in vitro*. The study indicates that the mSOD1 may serve as a potential therapeutic agent for those diseases caused by oxidative stress.

Keywords: Cu/Zn-binding Superoxide dismutase, Expression, Purification, Metal ions, DNA damage

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INTRODUCTION

Superoxide dismutase (SOD; EC 1.15.1.1) is a ubiquitous enzyme, which catalyzes the dismutation of the superoxide radical into hydrogen peroxide and molecular oxygen ($2O_2^- + 2H^+ \rightarrow H_2O_2$), and plays a central role in protecting against oxidative stress. In the last few years, SOD has been widely used in prevention of oncogenesis and tumor promotion, as well as

protection against reperfusion damages of ischemic tissue [1]. According to the metal co-factor present at the catalytic center, multiple SOD isoforms are classified into four types, copper/zinc (Cu/Zn-SOD), nickel (Ni-SOD), manganese (Mn-SOD) and iron (Fe-SOD) forms. Cu/Zn-SOD is generally found in the cytoplasm, chloroplasts, nuclear compartments, and lysosomes of mammalian cells [2, 3], Mn-SOD in mitochondria, whereas Fe-SOD is present within

the chloroplasts of some plants [4]. Ni-dependent enzymes have only been described from *Streptomyces* species [5].

Molecular cloning and expression of SOD1 have been reported in various prokaryotic and eukaryotic species, for example, green bamboo [6], Black Porgy [7] and sweet potato [8]. However, no data have been published on SOD1 sequences from mouse expressed with a modified pET-15b vector in *E. coli* Rosetta-gami cells. Furthermore, there has been no report on expression process optimization and productivity estimation of mouse SOD1. Although, the activity of mSOD1 *in vivo* has been related to amyotrophic lateral sclerosis [9,10], oxidative stress [11], reactive oxygen species (ROS) [12] and cerebral ischemia [13], most of these studies involved the use of SOD1 cDNA transfection. Due to the absence of mSOD1 expression, there are no reports on mSOD1 activity *in vitro*, especially, the effect of metal ions on the mSOD1 activity. In addition, the DNA damage protective activity of mSOD1 *in vitro* has not been investigated.

EXPERIMENTAL

Reagents

Sma I, CIAP, T₄ DNA ligase and protein marker were purchased from Takara (Osaka, Japan). KOD-plus are from Toybo (Osaka, Japan). *E. coli* Rosetta-gami strain was from Novagen (Darmstadt, Germany). Mouse anti-His monoclonal antibody was from TIANGEN (Beijing, China). Horseradish peroxidase (HRP) labeled goat anti-mouse IgGs were purchased from Proteintech (Chicago, USA). Ni-NTA resin was from QIAGEN (Hilden, German). SOD activity assay kit was from Jiancheng Bioengineering Institute (Nanjing, China). The other reagents used in this study were of analytical grade and are commercially available.

Construction of expression vector

Primers (forward: 5'-ATGGCGATGAAAGCGG TG TG-3', reverse:5'- TTACTGCGCAATCC CAATCAC -3') were designed based on the sequence of mSOD1 (Genbank Accession NM_011434.1). The coding sequence of mSOD1 was amplified by PCR, using template cDNA of mouse liver. The amplified PCR product was ligated into a modified pET15b (*Sma* I) expression vector [14], resulting in the recombinant vector pET15b-mSOD1. The recombinant plasmid constructs were confirmed by direct sequencing (Yinjun, Shanghai, China).

The pET15b-mSOD1 plasmid was transformed into *E. coli* Rosetta-gami strain for expression.

Protein expression and purification

A single colony of *E. coli* Rosetta-gami cells bearing pET15b-mSOD1 plasmid was inoculated into 5 ml LB medium containing 100 µg/ml ampicillin. After grown at 37 °C with shaking at 220 rpm for 12 h, the culture (2 ml) was then inoculated into 200 ml LB medium and grown at 37 °C. At an OD₆₀₀ of 0.6 - 0.8, the culture was induced by adding IPTG to a final concentration of 0.5 mM, and incubation was continued for an additional 10 h at 30 °C. Cells were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris-HCl, 0.2 mM EDTA, 100 mM NaCl, 1 % Triton X-100, 10 mg/ml lysozyme, pH 8.0). The cells were disrupted by sonication on ice. The cell suspension was centrifuged at 12,000 rpm for 15 min at 4 °C.

The supernatant of lysate containing target protein was purified with Ni-NTA affinity chromatography [14]. After filtering with a 0.22 µm filter, the supernatant was loaded onto a Ni-NTA-His bind column equilibrated with buffer A (20 mM sodium phosphate, 500 mM NaCl, 10 mM imidazole, pH 7.4) at 4 °C. The column was washed with 10 column volumes of buffer B (20mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4). The mSOD1 was eluted with buffer C (20 mM sodium phosphate, 500 mM NaCl, 250 mM imidazole, pH 7.4). The protein-containing fractions were pooled and dialyzed overnight against a buffer containing 20 mM sodium phosphate, 500 mM NaCl (pH 7.4) to remove imidazole [14]. Protein concentration was determined by bicinchoninic acid (BCA) method, using bovine serum albumin (BSA) as standard. The dilution was freeze-dried and the final product stored at -80 °C.

SDS-PAGE and Western blot

SDS-PAGE was performed in a 12 % w/v polyacrylamide gel. Protein samples (32 µl) were each mixed with 8 µl of 5×SDS-PAGE loading buffer (250 mM Tris-HCl, 10 % SDS, 0.5 % bromophenol blue, 50 % glycerol, 5 % β-mercaptoethanol, pH6.8), and boiled at 100°C for 5 min. The samples were loaded on a 12 % SDS-polyacrylamide gel. The gels were stained with Coomassie Brilliant Blue R-250 and destained with the destain solution (2.5 % methanol, 10 % acetic acid).

For Western blot, proteins were transferred onto a PVDF membrane after separation by 12 % acrylamide gels. The membrane was blocked

with 5 % nonfat dry milk (NFDM) in Tris-buffered saline plus 0.1 % tween 20 (TBST) overnight at 4 °C. The blocked membrane was washed twice for 5 min in TBST and incubated with mouse anti-His IgG (1:1000) for 2 h at 37 °C. The membrane was washed three for 5 min in TBST. After washing, the membrane was incubated with HRP-labeled goat anti-mouse IgG (1:1000) for 1.5 h at 37 °C. The membrane was washed again with PBST. After a final wash of the membrane with TBST, the bands were revealed in films by ECL staining [15].

Mass spectrometry

The putative protein was identified by matrix-assisted laser desorption/ionization time-of-flight mass (MALDI-TOF-MS) using an ABI 4800 plus MALDI-TOF/TOF Analyzer (ABI, USA). Sample was digested by sequencing grade modified trypsin (Promega), and then analyzed by the MALDI-TOF-MS using the ABI 4800 plus MALDI-TOF/TOF Analyzer. The result was searched against IPI database (V3.36) by GPS Explore software (V3.6, ABI, USA) and MASCOT (V2.1, Matrix Science, London, UK) [14, 16].

Effects of metal ions on the activity of mSOD1 protein

mSOD1 proteins were treated with various metal ions (Na^+ , K^+ , Fe^{2+} , Fe^{3+} , Zn^{2+} , Cu^{2+}) for 10, 20, 30 and 40 min respectively (concentration of protein is 10 mg/L, concentration of ion is 5 mM). mSOD1 proteins were treated with Zn^{2+} and Cu^{2+} of different ions concentration (concentration of ion: 0.05, 0.5, 5 and 50 mM, respectively). mSOD1 proteins were treated with different temperature (25, 30, 37 and 50 °C) [17]. SOD activity assay kit was used to detect the activity of mSOD1.

DNA nicking induced by hydroxyl radical

DNA damage protective activity of mSOD1 was performed using super coiled pUC-19 DNA. The mSOD1 protein (5 - 60 µg/ml) was added to 6 µl DNA (0.5 µg) in a final volume of 10 µl. Experiments were conducted at room temperature for 10 min. Then, 10 µl of Fenton's reagent (30 mM H_2O_2 , 50 µM ascorbic acid and 80 µM of FeCl_3) was added to the mixture of the mSOD1 and pUC-19 DNA. The final volume of mixture was made up to 20 µl and incubated for 30 min at 37 °C. The DNA was analyzed on 1 % agarose gel using ethidium bromide staining and photographed in Gel Doc[18,19].

Statistical analysis

The experimental results were processed using Statistical Package for Sciences and Society (SPSS 12.0) (SPSS Inc, Chicago, IL, USA) and expressed as mean \pm SD (standard deviation) of triplicate measurements. The data were subjected to one-way analysis of variance (ANOVA) and the significance of differences between means was calculated. $p < 0.01$ was considered highly significant. The software used was SigmaPlot 12 (Systat Software Inc).

RESULTS

Construction of expression vector

The fragment was inserted into pET15b (*Sma* I) vector, achieving recombinant plasmid pET15b-mSOD1. Expected sizes of the recombinant plasmid constructs was confirmed by direct sequencing. The sequencing result indicated that the coding region of the mSOD1 gene was fused in-frame to upstream of the vector-derived His tag coding sequence successfully. Integration of DNA fragment amplifier and sequence analysis indicated that it encoded a mature mouse Cu/Zn-binding superoxide dismutase (mSOD1) protein with an estimated molecular mass of 20 kDa.

Expression and purification of recombinant mSOD1 protein

Cells were grown under several conditions of temperature and IPTG concentration to optimize protein expression. At 37 °C, some protein was localized in the inclusion body fraction while almost all mSOD1 protein was obtained in the soluble fraction in cultures grown at 30 °C and induced by adding IPTG to a final concentration of 0.5 mM[20]. Cells were harvested by centrifugation. As shown in Fig 1A, an obvious band about 20 kD of mSOD1 was visualized in the supernatant. The mSOD1 protein was almost in the supernatant of the lysis. According to the quantitative analysis, the percentage of the target protein in the supernatant of the lysis was about 40 %.

Availability of the expressed mSOD1 in the soluble fraction greatly facilitated its isolation and purification. In SDS-PAGE analysis, the protein migrated according to its predicted molecular mass. The purity was $> 95\%$ (Fig 1B). In addition, the process of purification was short and straightforward, because we purified soluble mSOD1 material directly after cell lysis without protein denaturing and refolding.

The identity of purified target protein was confirmed by Western blot detection with anti-His tag antibody (Fig 1C).

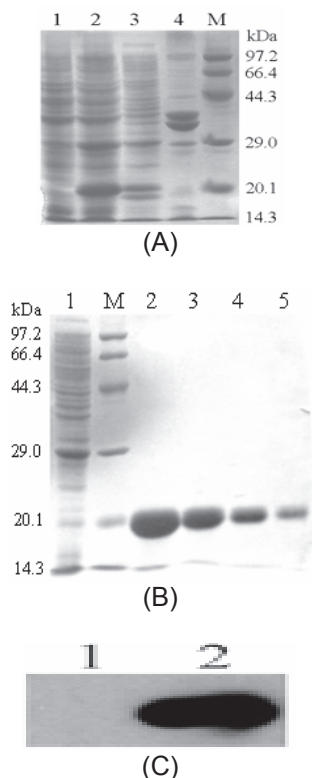


Figure 1: Expression and purification of mSOD1 protein; (A). Lanes 1, extract from the uninduced cell ; Lanes 2, extract from cells after IPTG induction; Lanes 3 and 4, supernatant and cell debris, respectively, obtained after centrifugation of the sonicated cells; M, Protein marker. (B) Lanes 1, flow-through; Lanes 2-5, four serial elution fractions; M, Protein marker (C) Western blot detection of fusion protein with anti-His mAb. Lanes 1, cell lysate prior to induction; Lane 2, the sample after Ni-NTA affinity chromatography.

It showed that the finally expression quantity of mSOD1 was approximately 60 mg target protein per liter of culture. The Table 1 summarizes the yield of mSOD1 during purification.

Table 1: Purification summary of mSOD1 protein from culture

Purification step	Total protein ^a (mg)	Purity (%)	mSOD1 (mg)	Yield ^b (%)
Soluble lysate	40.68	40.6	16.52	100
Ni-NTA affinity chromatography	12.53	95	11.90	72.03

^a Wet weight cells (1.08 g) from 200ml culture were lysed using sonication; ^b Protein yield was calculated using the amount of mSOD1 after concentration and mSOD1 amount in soluble lysate.

Peptide mass fingerprinting (PMF) of mSOD1 protein

To confirm the identity of the purified recombinant protein, the protein obtained after Ni-exchange chromatography was digested with trypsin and the resulting peptides were analysed by MALDI-TOF MS. A clear match between the mass of six generated peptides and the predicted peptides obtained from the mSOD1 protein sequence was observed (Fig. 2).

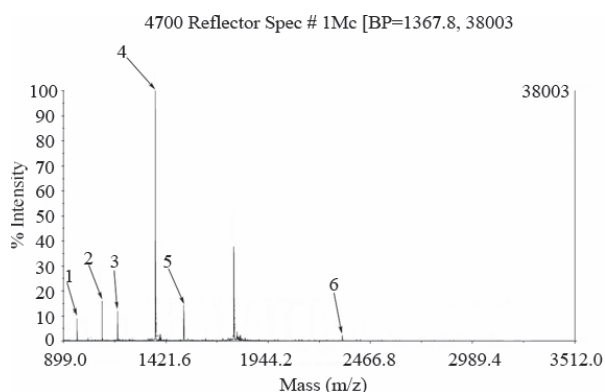


Figure 2: Identification of mSOD1 recombinant proteins by mass spectrometry analysis Peak 1 matched peptides of "HGGPADEER"; peak 2 matched peptides of "KHGGPADEER"; peak 3 matched peptides of "DGVANVSIEDR"; peak 4 matched peptides of "VISLSGEHSIIGR"; peak 5 matched peptides of "GDGPVQGTIHFEQK"; peak 6 matched peptides of "HVGDLGNVTAGKDGVANVSIEDR".

Effect of metal ions on the activity of mSOD1 protein

To determine the effects of metal ions on the activity of mSOD1 protein, mSOD1 was treated with different metal ions at the same concentration (5 mM) in 37 °C. Each reaction mixture was treated for 10, 20, 30 and 40 min. SOD activity assay kit was used to detect the activity of mSOD1. Among the activities of the various reaction mixtures, mSOD1 and Zn²⁺, Cu²⁺ and Zn²⁺/Cu²⁺ exerted greater effects on mSOD1 than the other ions (Na⁺, K⁺, Fe²⁺ and Fe³⁺). The reaction mixture of mSOD1 and Zn²⁺/Cu²⁺ showed the highest activity. The mSOD1 activity was activated significantly by the metal ions of Zn²⁺/Cu²⁺[17]. The efficiency of Zn²⁺/Cu²⁺ for mSOD1 activation was about 10-fold more potent than that of control. In addition, the activities of other reaction mixtures were improved minimally (Fig 3A).

We also found that the activity of mSOD1 improved with increase in metal ion concentration. The activity of mSOD1 was hardly improved when metal ion concentration exceeded 5 mM (Fig 3B) [23]. As shown in Fig

3C, temperature (25 - 50 °C) had a small influence on the activity of mSOD1. Thus, the highest activity of the highest mSOD1 activity obtained was 3789.0 ± 80.5 U/mg, and it was obtained when it was treated with metal ion of Zn²⁺ / Cu²⁺ (5 mM) at 37 °C for 40 min.

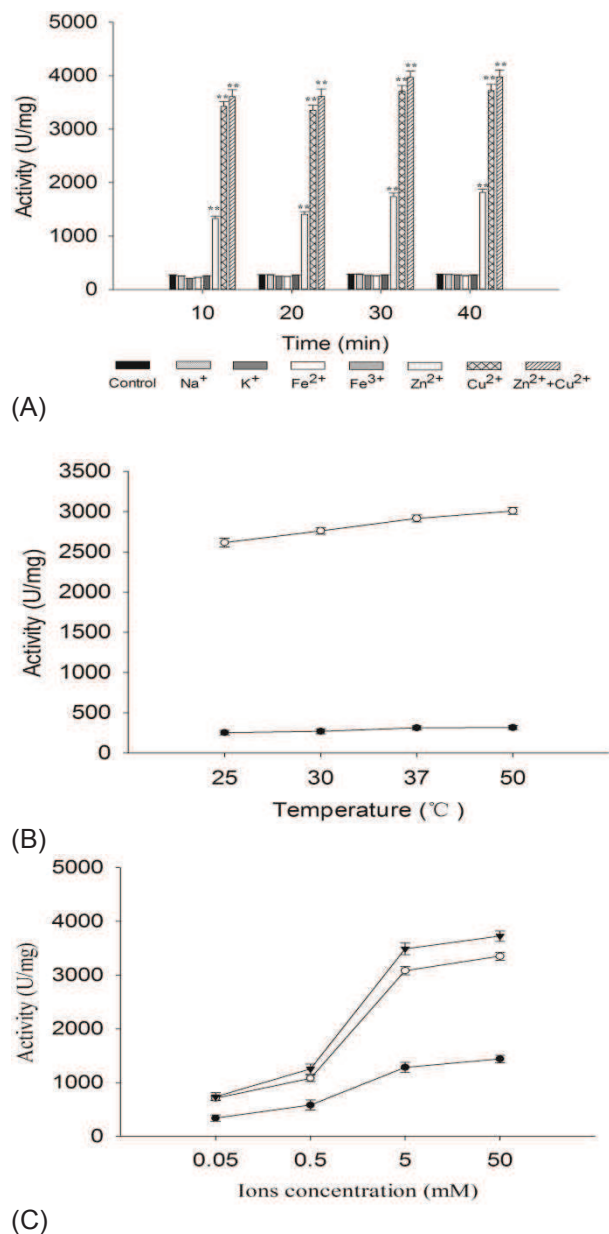


Figure 3: Effects of metal ions on the activity of protein mSOD1. (A) mSOD1s were treated with different ions for different time. The average data from three independent experiments. Statistically significant differences from the control in each group are indicated as ***p* < 0.01. (B) mSOD1s were treated with different ions concentration (○=Zn²⁺ and Cu²⁺, ●=Control). (C) mSOD1s were treated with different temperature (Concentration of mSOD1 is 10 mg/L, and concentration of ion is 5 mM, ▼=Zn²⁺ and Cu²⁺, ○=Cu²⁺, ●=Zn²⁺).

DNA damage protective activity of mSOD1

Gel electrophoresis showed that the protection offered against DNA damage by mSOD1 (5 - 60 µg/ml) was concentration-dependent. At a concentration of 60 µg/ml, protection was optimum and slightly close to that of native pUC-19 DNA (Fig 4). mSOD1 showed effective and damage dependent reduction in the formation of nicked DNA and increased super coiled form of DNA.

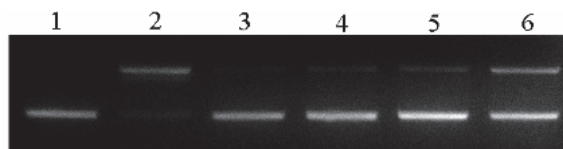


Figure 4: Effect of mSOD1 on oxidative DNA nicking caused by hydroxyl radicals. Lane 1, native pUC-19 DNA; Lane 2, DNA + Fenton's reagent; Lane 3- 6, DNA + Fenton's reagent + mSOD1 (60, 40, 20, 5 mg/L, respectively).

DISCUSSION

mSOD1 is one of the most important enzymes responsible for defense against oxidative damage due to ROS and particularly superoxide anion radicals. mSOD1 is strongly associated with and widely used in amyotrophic lateral sclerosis therapy. For these studies, a stable, purified mSOD1 is necessary. However, its use has been limited by its source. The activity investigation of mSOD1 mainly focused on *in vivo*, and especially related to amyotrophic lateral sclerosis. There are no reports on mSOD1 activity *in vitro*.

Here, we efficiently produced the genetically engineered mSOD1 protein, and also successfully developed and optimized a useful assay for determining the effect of metal ions on the activity of protein mSOD1. The present work showed that mSOD1 was effective in protecting oxidative DNA damage. ROS are highly toxic and might be generated by oxidative stress, such as DNA damage, lipid peroxidation, protein modification and other effects. All these symptoms manifest in various numerous diseases, including cancer, cardiovascular disease, diabetes, atherosclerosis, neurological disorders, chronic inflammation, etc [24]. This study will facilitate further *in vitro* and *in vivo* experimentation on mSOD1 as an antioxidant enzyme defense system against ROS and would be helpful in ameliorating diseases caused by DNA damage. mSOD1 may serve as a novel therapeutic for those diseases where the approach is to suppress oxidative stress.

CONCLUSION

The structural gene of mSOD1 has been cloned in pET-15b vector. High purity recombinant mSOD1 was obtained and characterized. We observed that the mSOD1 activity was significantly activated after treated with the metal ions of $Zn^{2+} + Cu^{2+}$. Furthermore, mSOD1 protein can protect DNA from damage induced by hydroxyl radical.

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#The authors, ZZ and LH, contributed equally to this work.

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