Cloning, purification and enzymatic characterization of recombinant human superoxide dismutase 1 (hSOD1) expressed in Escherichia coli

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Superoxide dismutase 1 (SOD1) is a metalloenzyme that catalyzes the disproportionation of superoxide into molecular oxygen and hydrogen peroxide. In this study, the human SOD1 (hSOD1) gene was cloned, expressed and purified. The hSOD1 gene was amplified from a pool of Bxpc3 cell cDNAs by PCR and cloned into expression vector pET-28a (+). The recombinant soluble hSOD1 was expressed in E. coli BL21 (DE3) at 37 °C and purified using nickel column affinity chromatography. Soluble hSOD1 was produced with a yield of 5.9 μg/mL medium. As metal ions can have a certain influence on protein structure and activity, we researched the influences of different concentrations of Cu2+ and Zn2+ on hSOD1 activity at induction and the time of activity detection. The results implied that Cu2+ and Zn2+ do not enhance SOD1 expression and solubility; they can, however, improve the catalytic activity at induction. Meanwhile, Cu2+ and Zn2+ also enhanced the enzyme activity at the time of detection. Furthermore, most other bivalent cations had the potential to replace Zn2+ and Cu2+, and also improved enzyme activity at the time of detection.

Key words: superoxide dismutase 1, Escherichia coli, soluble expression, metal ions, catalytic activity

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Abbreviations: hSOD1, human superoxide dismutase 1; GSH-Px, glutathione peroxidase; IPTG, isopropyl-β-D-thiogalactoside

INTRODUCTION

The antioxidant defense system is extremely important as it enables living organisms to scavenge free radicals produced during normal metabolism. Several antioxidant enzymes are known to reduce oxidative damage, such as superoxide dismutase (SOD), glutathione reductase, catalase (CAT) and glutathione peroxidase (GSH-Px) (Shih et al., 2006; Geraghty et al., 2016). Although all of them exhibit definite antioxidative action, SOD is considered the first enzyme in the defense against oxidative stress produced during normal metabolism (Johnson, 2002).

SODs are a major antioxidant enzyme family whose members can convert superoxide radicals to hydrogen peroxide which is further catalyzed to form H2O and O2 (Klicic et al., 2014; Vats et al., 2015). Three forms of SOD are present in humans, in all other mammals, and most chordates. SOD1 is expressed in the intermembrane space of mitochondria, the nucleus, and the cytoplasm. SOD2 is located in the mitochondrial matrix, while SOD3 is secreted into the extracellular space (Hole et al., 2011). SOD1 is a homodimer consisting of two 16-kDa subunits found in the cytoplasm and nucleus of the cell. SOD2 is mitochondrial and the human enzyme has manganese (Mn) in its reactive center and active site, which functions as a metal cofactor (Ghérim, 2016).

Heterologous expression of SOD1 has been conducted in many expression systems including E. coli (Hartman et al., 1986; Zhang et al., 2017), yeast (Yoo et al., 1999; Wu et al., 2009), baculovirus systems (Fujii et al., 1995; Hayward et al., 2002) and plant cells (Park et al., 2002). The recombinant hSOD1 proteins are in all cases expressed in the cytosol, and in E. coli yields are equivalent to at least 10% of the total bacterial protein, and in many cases much more (Ahl et al., 2004). However, the most common problem has been that the protein produced is Cu2+- and Zn2+-deficient at active site resulting in low solubility and enzyme activity. Metal reconstitution in vitro is a method of incorporating Cu2+ into the apoenzyme (apo-hSOD1). The addition of Cu2+ into the E. coli culture was reported to improve Cu2+ incorporation; however, the production of SOD1 with a full Cu2+ complement was still a complication.

SOD1 is a metalloenzyme containing one copper ion and one zinc ion per molecule. Many early investigations of SOD1 focused on the metal-binding properties of the enzyme. The structural integrity of SOD1 depends critically on the correct coordination of zinc and copper (Nordlund et al., 2009). Banci et al. found by in-cell NMR that hSOD1 needs to bind one Zn2+ ion and one catalytic Cu2+ ion per molecule and to form an intramolecular disulfide bridge before it exerts its catalytic function (Banci et al., 2011). Defective metal binding or decreased affinity for zinc and copper is a feature of many SOD1 mutants and has been suggested to play a role in the pathogenic mechanism of amyotrophic lateral sclerosis (ALS) (Sangwan et al., 2017). However, excess cofactors can create toxicity, i.e. zinc toxicity may be due to the binding of zinc to inappropriate sites that inhibit enzyme function or to the displacement of other metal ions from the active sites of enzymes (Wu et al., 2009).

In this research, the open reading frame (ORF) of hSOD1 was cloned and the recombinant enzyme was expressed in E. coli BL21 (DE3). The antioxidative activity of the recombinant hSOD1 protein was detected. Because the zinc and copper cofactors are involved in the stable structure and high activity of eukaryotic SOD1 (Leitch et al., 2009; Girotto et al., 2014; Lin et al., 2015), we investigated the influences of Cu2+ and Zn2+ on...
hSOD1 activity at induction. Meanwhile, we also studied the effects of Cu\textsuperscript{2+} and Zn\textsuperscript{2+} on hSOD1 enzymatic activity. Furthermore, we examined the effects of substitutions of metal ions on hSOD1 activity at the time of detection.

**MATERIALS AND METHODS**

**Materials.** TRIZol (Sangon Biotech, China), random hexamers (50 ng/mL), dNTP Mix (10 mM each), 0.1 M DTT, 5× first strand buffer, reverse transcriptase M-MLV (Rnase H-), rTaq polymerase, restriction enzymes, T4 DNA ligase, DNA markers and protein markers were purchased from TaKaRa (Dalian, China). The expression vector pET-28a (+) and E. coli strain DH5α, BL21 were obtained in our lab. All chemicals were all from Sigma (St. Louis, MO, USA) or a domestic provider in China if not stated otherwise.

**Construction of an expression vector containing the hSOD1 gene.** The hSOD1 specific primers, forward primer (5′ CCAGCTCTGGATGGCAAGGGCC-CCGTG 3′) with a HindIII site (underlined), and reverse primer (5′ CCGTGGAGCC-CTTGTTATTGGGCGATCTCCAA 3′) with an XhoI site (underlined) were designed to amplify the hSOD1 gene (GenBank accession number CR541742.1). cDNA of Bspce3 cell lines served as a template for amplifying the hSOD1 gene with PCR. Following this, the PCR product was digested using two kinds of restriction endonucleases (HindIII and XhoI). Finally, the digested product was cloned into expression vector pET-28a (+) between the HindIII and XhoI restriction sites. The recombinant plasmid was verified by DNA sequencing.

**Expression and purification of the recombinant protein.** The constructed recombinant expression plasmids were transformed into E. coli BL21 (DE3) for protein production. The freshly transformed colony was cultured in LB medium supplemented with kanamycin (50 μg/mL). Expression of the recombinant protein was induced by 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 6 h at 37°C. Cells were harvested by centrifugation (4500 × g, 4°C, 15 min). The cell pellet was resuspended in Tris-HCl (50 mM, pH 8.0), and then incubated with 1 mg/mL lysozyme on ice for 30 min. Finally, the suspension was lysed by sonication, and the precipitate and supernatant were separated by centrifugation twice at 8000 × g and 4°C for 20 min.

The supernatant was loaded onto a Ni-NTA affinity column (GenScript, Nanjing, China). Purification conditions followed the manufacturer’s instructions. After washing the column with 50 mM and 100 mM imidazole, the fusion protein was eluted with 400 mM imidazole. The eluted protein was dialyzed against dialysis buffer (50 mM Tris-HCl, pH 8.0) at 4°C to remove imidazole. The protein concentration was detected by the BCA Protein Assay Kit (Pierce, Bonn, Germany).

**Western blotting.** Western blotting was performed according to the method described by Zhou and coworkers (Zhou et al., 2017). Proteins were resolved on a 15% SDS-PAGE gel and electro-transferred onto a PVDF membrane. The membrane was subsequently blocked and incubated with mouse anti His-tag antibody (1:1000 dilution) at 4°C overnight, followed by goat anti-mouse IgG (1:2000 dilution) for 2 h. Lastly, the bands were visualized with ECL Western Blotting Substrate (Vazyme, Nanjing, China).

**Enzymatic activity assay.** The catalyzing activity of the recombinant hSOD1 was measured using CuZn-SOD and Mn-SOD Assay Kits with WST-8 (Beyotime Biotechnology, Shanghai, China). The assay was based on measuring the color of a formazan dye. According to the manufacturers’ instructions, the WST-8/enzyme working solution and reaction starting solution were prepared beforehand. A preliminary experiment was conducted to find the optimal amount of hSOD1, so that the inhibition percentage of hSOD1 lay between 30% and 70%. A certain amount of hSOD1 (final volume was 20 μL), WST-8/enzyme working solution (160 μL) and reaction starting solution (20 μL) were incubated at 37°C for 30 min. The absorbance at 450 nm was detected. Crude cell lysate (only containing pET-28a (+)) was regarded as a negative control. The activity of hSOD1 was calculated according to the formula in the manufacturers’ instructions.

**The influence of Cu\textsuperscript{2+} and Zn\textsuperscript{2+} on hSOD1 activity at induction.** Overnight cultured bacteria were inoculated into fresh LB medium and shaken at 37°C until the OD\textsubscript{600}=0.4, and the IPTG was added (to a final concentration of 1 mM). Meanwhile, different concentrations of Cu\textsuperscript{2+} (30–1000 μM) and Zn\textsuperscript{2+} (10–100 μM) were also individually injected into the bacterial solution. Bacteria samples with no Cu\textsuperscript{2+} or Zn\textsuperscript{2+} added were seen as the control. After the bacteria were exposed to individual Cu\textsuperscript{2+} (30–1000 μM) or Zn\textsuperscript{2+} (10–100 μM) at induction, we selected the concentrations at which the hSOD1 activity was highest. Then three Cu\textsuperscript{2+} + Zn\textsuperscript{2+} combinations (750 μM Cu\textsuperscript{2+}/15 μM Zn\textsuperscript{2+}; 1,500 μM Cu\textsuperscript{2+}/15 μM Zn\textsuperscript{2+}; and 750 μM Cu\textsuperscript{2+}/30 μM Zn\textsuperscript{2+}) were chosen. The method of detecting hSOD1 activity was mentioned previously.

**Effect of Cu\textsuperscript{2+}, Zn\textsuperscript{2+}, other metal ions and a de-naturant on hSOD1 activity at the time of detection.** To examine the effects of Cu\textsuperscript{2+} and Zn\textsuperscript{2+} on hSOD1 activity, various concentrations of Cu\textsuperscript{2+} (30, 100, 500, 750, 1000, 2000, 5000, 6000 μM) and Zn\textsuperscript{2+} (10, 20, 30, 100, 500, 1000, 2000, 5000, 6000 μM) were added to the purified hSOD1. Meanwhile, a stock solution containing CaCl\textsubscript{2}, MgCl\textsubscript{2}, MnCl\textsubscript{2}, FeCl\textsubscript{3}, CdCl\textsubscript{2}, NiSO\textsubscript{4} and CoCl\textsubscript{2} was added to the purified hSOD1 to achieve 5 mM. Here, the purified hSOD1 was obtained from standard LB medium without supplementation Cu\textsuperscript{2+} and/or Zn\textsuperscript{2+}. As a detergent, the effects of 1%, 5%, 10% SDS on the enzyme were also examined according to the aforementioned methods. The activity of the control enzyme with no metals or detergent was taken as 100%. The results presented are the average of three independent experiments.

**RESULTS AND DISCUSSION**

**Expression and purification of recombinant protein**

The 465-bp DNA fragment of hSOD1 encoding the mature protein was cloned from the Bspce3 cell line cDNA. DNA sequencing confirmed hSOD1 was correctly fused to the N-terminal His-tag, and it also revealed the cloned hSOD1 gene was the same as the published hSOD1 (GenBank accession number CR541742.1). In general, the exogenous recombinant plasmid that was expressed in E. coli was induced at 37°C with 1 mM IPTG (50 μg/mL). Expression of the recombinant protein was induced by 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for 6 h at 37°C. Cells were harvested by centrifugation (4500 × g, 4°C, 15 min). The cell pellet was resuspended in Tris-HCl (50 mM, pH 8.0), and then incubated with 1 mg/mL lysozyme on ice for 30 min. Finally, the suspension was lysed by sonication, and the precipitate and supernatant were separated by centrifugation twice at 8000 × g and 4°C for 20 min.

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The apparent molecular mass of hSOD1 is higher than 20.1 kDa, at 25–26 kDa (Hartman et al., 1995). Our data showed that Cu\(^{2+}\) supplementation to the medium to see whether this altered the specific activity of hSOD1 in crude bacterial lysates at induction. Our results showed that supplementation of Cu\(^{2+}\) and Zn\(^{2+}\) increased the specific activity of hSOD1. The highest enzymatic activity was observed when the individual Cu\(^{2+}\) and Zn\(^{2+}\) concentrations were 750 μM and 15 μM (Fig. 2), respectively, and the SOD1 activity declined when the concentrations of Cu\(^{2+}\) and Zn\(^{2+}\) continued to increase which indicated that excess Cu\(^{2+}\) and Zn\(^{2+}\) may be toxic to the cells at induction (Fig. 2). Furthermore, from Fig. 3, the yield of SOD1 did not significantly increase compared to the control.

The highest activity of hSOD1 produced with 750 μM Cu\(^{2+}\) added was 7.7-fold greater than that of the control. This is consistent with a previous observation that supplementation of Cu\(^{2+}\) increases the specific activity of hSOD1 in E. coli (Hartman et al., 1986) and sf21 cells (Fujii et al., 1995). Our data showed that Cu\(^{2+}\) had no obvious effects on improving the solubility (data not shown) or enhancing the yield of SOD1 (Fig. 3A), but given the phenomenon, correctly fold the yield of SOD1 did not. Given this phenomenon, the higher yield of SOD1 did not increase when the concentrations of Cu\(^{2+}\) and Zn\(^{2+}\) were added to the medium to see whether this altered the specific hSOD1 activity in crude bacterial lysates at induction. Our results showed that supplementation of Cu\(^{2+}\) and Zn\(^{2+}\) increased the specific activity of hSOD1. The highest enzymatic activity was observed when the individual Cu\(^{2+}\) and Zn\(^{2+}\) concentrations were 750 μM and 15 μM (Fig. 2), respectively, and the SOD1 activity declined when the concentrations of Cu\(^{2+}\) and Zn\(^{2+}\) continued to increase which indicated that excess Cu\(^{2+}\) and Zn\(^{2+}\) may be toxic to the cells at induction. Furthermore, from Fig. 3, the yield of SOD1 did not significantly increase compared to the control.

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it made a major contribution to the recovery of SOD1 activity (Fig. 2A).

Li and coworkers demonstrated that stoichiometric zinc played an important role in the oxidative refolding of bovine SOD1 by accelerating the oxidative refolding, suppressing aggregation during refolding and helping the protein to form a compact conformation with high protease resistance activity (Li et al., 2010). We expected an increase in the soluble expression levels of SOD1 in E. coli upon supplementation with Cu\(^{2+}\) and Zn\(^{2+}\); however, no significant improvement in hSOD1 solubility was found by inducing at 37 °C compared with samples without supplementation Cu\(^{2+}\) and Zn\(^{2+}\) (data not shown). Meanwhile, a 2.3-fold increase in the specific activity of hSOD1 with 15 μM Zn\(^{2+}\) supplementation was observed. Fujii et al. reported that supplementation with Zn\(^{2+}\) alone did not enhance the SOD activity (Fujii et al., 1995), whereas our data showed that Zn\(^{2+}\) slightly increased SOD activity. Wu and coworkers noted that

SOD1 activity was decreased by about 50% in zinc-limited cells (Wu et al., 2009). Li et al. indicated that copper played a dominant role in SOD1 activity and zinc only made a small contribution to SOD1 activity (Li et al., 2010).

The specific activity of hSOD1 upon supplementation with 750 μM Cu\(^{2+}\)/15 μM Zn\(^{2+}\) (Fig. 2C) was 7.5-fold greater than that of the control, which was approximately equal to 7.7-fold enhancement with supplementation of 750 μM Cu\(^{2+}\) alone. This implied that, when the Cu\(^{2+}\)-binding sites were fully saturated, it was possible that the metallation of Cu\(^{2+}\) site facilitated the metallation of Zn\(^{2+}\) sites or the Zn\(^{2+}\) could be replaced by Cu\(^{2+}\) with full function. Crow and coworkers reported that zinc was more likely to disassociate than copper, because SOD has an approximately 7000-fold lower affinity for zinc than it does for copper (Crow et al., 1997).

The completely metal-free apo-hSOD1 has no specific activity (Hartman et al., 1986). Hartman and coworkers...
Table 2. Effects of metal ions and denaturant on the purified hSOD1

<table>
<thead>
<tr>
<th>Metals/denaturant</th>
<th>Concentration</th>
<th>Relative activity (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>1.0 ± 0.00</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>5 mM</td>
<td>1.2 ± 0.20</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>5 mM</td>
<td>1.4 ± 0.31</td>
</tr>
<tr>
<td>FeCl₃</td>
<td>5 mM</td>
<td>1.6 ± 0.25</td>
</tr>
<tr>
<td>CdCl₂</td>
<td>5 mM</td>
<td>3.9 ± 0.38</td>
</tr>
<tr>
<td>NiSO₄₂</td>
<td>5 mM</td>
<td>3.4 ± 0.35</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>5 mM</td>
<td>6.7 ± 0.25</td>
</tr>
<tr>
<td>SDS</td>
<td>1, 5, 10%</td>
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Control activity (1.0) was determined when none of the metal ions were added. Data represent mean ± S.D. for three independent experiments.

suggested that the intracellular concentration of Cu²⁺ in E. coli is insufficient to saturate human CuZnSOD, especially at high expression levels (Hartman et al., 1986). Our results showed that, in LB medium, the addition of 750 μM of Cu²⁺ to the medium apparently raised the intracellular Cu²⁺ concentration to levels that are sufficient to saturate the active sites for the overproduced hSOD1. In contrast, adequate Zn²⁺ was incorporated into hSOD1 when 15 μM of Zn²⁺ was present in the medium.

Effects of Cu²⁺, Zn²⁺, the other metal ions and a denaturant on hSOD1 activity at the time of detection

To study the effects of Cu²⁺ and Zn²⁺ on hSOD1 enzymatic activity, we added various concentrations of Cu²⁺ and Zn²⁺ to the purified hSOD1. The results showed that specific hSOD1 activity significantly increased at concentration of Cu²⁺ ranging from 30 to 6000 μM (Fig. 4A). The specific hSOD1 activity only slightly increased with 0–2000 μM Zn²⁺, but it significantly increased at Zn²⁺ concentrations of 5000 and 6000 μM (Fig. 4B). This indicated that Cu²⁺ and Zn²⁺ also enhanced the enzyme activity at the time of detection.

The effects of the other metal ions and a denaturant on the purified recombinant hSOD1 were studied and are shown in Table 2. hSOD1 exhibited high activity at 5000 μM Cu²⁺ or Zn²⁺ (Fig. 4). Then, we selected 5 mM of metal ions (CaCl₂, MgCl₂, MnCl₂, FeCl₃, CdCl₂, NiSO₄ and CoCl₂) and individually added them to the purified hSOD1. Compared to the control without metals, the activity of hSOD1 was enhanced to varying degrees. The influences of Co²⁺ and Mn²⁺ on the activity of hSOD1 were very significant, in that the activity increased by 6.7- and 16.9-fold compared to the control. hSOD1 exhibited little effect upon the addition of other metal ions (Ca²⁺, Mg²⁺, Fe³⁺, Cd²⁺, and Ni²⁺), whose influence ranged from 1.9- to 5.5-fold. From these results, we determined that all of the divergent metal ions (M²⁺) have the potential to replace Zn²⁺ and Cu²⁺. Furthermore, the activity of hSOD1 was totally inhibited by SDS.

Table 2. Effects of metal ions and denaturant on the purified hSOD1

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Control activity (1.0) was determined when none of the metal ions were added. Data represent mean ± S.D. for three independent experiments.

In this research, we have cloned and expressed a homo Cu/Zn SOD, hSOD1 in E. coli BL21 (DE3). The purified recombinant hSOD1 protein was capable of inhibiting the formation of formazan dye suggesting that the hSOD1 gene encodes a functional superoxide dismutase. Considering that metal cofactors are essential to the structure and activity of hSOD1, hSOD1 activity was measured upon the addition of Cu²⁺ and Zn²⁺ at induction compared to no addition. The results implied that
Cu$^{2+}$ and Zn$^{2+}$ do not enhance SOD1 expression and solubility; however, they can improve the catalytic activity at induction. This adequately showed that the recombinant hSOD1 was well folded in the presence of Cu$^{2+}$ and Zn$^{2+}$, and would be suitable for further functional study. Meanwhile, Cu$^{2+}$ and Zn$^{2+}$ also enhanced the enzyme activity at the time of detection. Furthermore, most other bivalent cations had the potential to replace Zn$^{2+}$ and Cu$^{2+}$, and also improved enzyme activity at the time of detection.

Acknowledgements

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