Cloning and expression of recombinant human superoxide dismutase 1 (hSOD1) in Bacillus subtilis 1012

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Abstract

As an important member of the antioxidant defense system *in vivo*, human Cu, Zn superoxide dismutase (hSOD1) has become a potential therapeutic agent against host diseases, and new strategies were developed to achieve high-yield SOD1 by using efficient production systems, like *E. coli*, yeast, and other eukaryotic expression systems. However, the above systems have their own limitations, such as the formation of inclusion bodies, endotoxins production, and low secretion efficiency. In this study, the hSOD1 coding sequence gene was amplified from human HepG2 cells, cloned into a pHT43-His expression vector, and transformed into *Bacillus subtilis* system. After the optimization with different media, temperatures, and inducer (IPTG) concentrations, the recombinant hSOD1 was successfully expressed in *Bacillus subtilis 1012*. The results show that hSOD1 was produced as a soluble form in Super rich medium with 0.2 mM of IPTG at 37°C after the induction for 24 h. Besides, 20 g/L of lactose also displayed the same inductive effect on hSOD1 expression as that by 0.2 mM of IPTG. Finally, hSOD1 was efficiently purified by nickel affinity chromatography, and the specific activity of hSOD1 was determined to be 1625 U/mg in the presence of 800 μM of Cu^{2+} and 20 μM of Zn^{2+}. Collectively, *Bacillus subtilis 1012* can not only overcome the shortcomings of *E. coli* and yeast expression systems, but also be directly used for disease treatment as a healthy probiotic. These advantages provided a possibility for the application of oral engineering bacteria 1012-hSOD1 to the clinical treatment in the future.

1. Introduction

Aerobic metabolism usually produces excessive reactive oxygen species (ROS). To maintain the physiological homeostasis, organisms will activate the antioxidant defense system to protect cells against ROS-induced damage in aerobic metabolism. In a previous report, superoxide dismutase (SOD) was proved to be the first line of *in vivo* defense against ROS(Yang et al., 2020). SOD catalyzes the dismutation of superoxide radicals to produce oxygen and hydrogen peroxide(Nguyen et al., 2020). If the organism has a low SOD level, ROS will accumulate in abundance, leading to DNA strand breakage, protein damage, or even membrane lipid peroxidation (Kim et al., 2017).

All mammals possess three members of SOD with tightly regulated localization patterns. Of these, there are two copper/zinc superoxide dismutases (Cu, Zn-SODs), i.e., SOD1 and SOD3. SOD1 exists in the cytosol, mitochondrial inter-membrane space, and nucleus, while SOD3 is an extracellular dismutase. Both SODs play a pivotal role in the antioxidant defense system. The manganese-containing superoxide dismutase (MnSOD, SOD2) localizes in the mitochondrial matrix. It is beneficial in the occurrence of many diseases like atherosclerosis (Eleutherio et al., 2021). Thus, SOD is a crucial contributor to alleviating the harmful effects of ROS. However, the physiological level of SOD is too low to meet the demand of clinical applications from living organisms.

For decades, researchers have developed new strategies have to achieve high-yield SOD by using efficient production systems like engineering bacterial systems. In previous years, heterologous expression systems of human SOD (hSOD) were constructed, and *E coli* was the most commonly used one. To date,

the recombinant hSOD1 has been expressed in the cytosol of *E. coli strain A1645* and *E.coli BL21 (DE3)* (Hartman et al., 1986; Lin et al., 2018; Yang et al., 2020). Also, hSOD1 was produced in eukaryotic cells, such as *Pichia pastoris* (Park et al., 2002; Wu et al., 2009), insect cells (Hayward et al., 2002), plant cells (Park et al. 2002), and mammary glands of transgenic animals (Lu et al., 2018). Besides, the full-length hSOD2 recombinant protein was obtained in *E.coli Rosetta-gami*, *BL21 (DE3)*, and mammalian cells (Hosoki et al., 2012; Pan et al., 2017). Also, hSOD3 was expressed in *E.coli* (Bae et al., 2013), *Pichia pastoris* (Chen et al., 2006), insect cells (He et al., 2002), and Chinese hamster ovary cells (Tibell et al., 1987). However, the above systems have their limitations in protein production. For example, the inclusion bodies and endotoxins formed in *E.coli* expression system increased the production cost and thus limited its application to the large-scale expression of heterologous proteins (K. Zhang et al., 2017). Although the yeast is a perfect expression system for the post-translational protein modification and secretion compared to *E.coli*, it has a relatively low secretion efficiency for the lack of a strong and strictly regulated promoter.

As a “generally Recognized as Safe” (GRAS) organism, *Bacillus subtilis* is nonpathogenic and has developed into an attractive host, with no apparent bias of codon usage, especially in the secretion of extracellular functional proteins into the culture medium. This can simplify the purification process of protein and provide a correctly folded and soluble heterologous protein (Gu et al., 2018; Huang et al., 2017; Niu et al., 2018). Collectively, *Bacillus subtilis* can effectively overcome the shortcomings of *E.coli* and yeast expression systems. Nowadays, about 60% of commercial enzymes are produced from *Bacillus subtilis* (Huang et al., 2017). Although hSODs have been expressed in *Escherichia coli* and *Pichia pastoris*, there is no report about their expression in *Bacillus subtilis*.

This study cloned the encoding human *sod1* gene into pHT43-His and transformed it into *Bacillus subtilis 1012.* We investigated the expression level of recombinant hSOD1 with different media, temperatures, and inducers. Further, we examined the effect of Cu^{2+} and Zn^{2+} on the enzymatic activity of purified hSOD1.

2. Materials And Methods

2.1 Bacterial strains and plasmids

E.coli DH5α (Tsingke Biotechnology Co., Ltd., Beijing, China) was used for plasmid construction and molecular cloning, and the host strain *Bacillus subtilis 1012* (Lab collection) was applied for hSOD1 expression. The *E.coli-B.subtilis* shuttle plasmid pHT43-His (Miaoling Biological Technology Co., Ltd., Wuhan, China) was constructed as a vector for extracellular expression of hSOD1. The cells were grown at 37°C for 12 h in LB, Super Rich, or 2× YT medium (100 μg/mL Ampicillin for *E.coli*; 5 μg/mL Chloramphenicol (Cm) for *Bacillus subtilis*).

2.2 Cloning of *sod1* gene

The human RNA was extracted from HepG2 cells using Trizol (Summer Biotechnology Co., Ltd, Beijing, China). cDNAs were synthesized with a Transcriptor first-strand cDNA synthesis kit (Summer Biotechnology Co., Ltd, Beijing, China). The coding sequence of the *hsod1* gene (GenBank accession number, CR541742.1) was amplified using a pair of SOD1 primers: 5'-ATGGCGACGAAGGCCGTGTG-3' (F); 5'-TTGGGCGATCCCAATTACAC-3' (R).

PCR reaction was performed in 50 μL reaction solution consisting of 2 μL of cDNA, 1 μL each of the forward or reverse primer, 5 μL of dNTP (2 mM), 5 μL of 10× PCR Buffer for KOD-Plus-Neo, 3 μL of $MgSO_4$ (25 mM), 1 μL of KOD-Plus-Neo Polymerase (Toyobo, Osaka, Japan), and 32 μL of ddH_2O. The PCR condition was as followed: 94°C for 2 min; 35 cycles of 94°C for 15 s, 55°C for 30 s, and 68°C for 25 s. The PCR product was purified using a Gel Extraction Kit (Omega Bio-tek, Guangzhou, China) according to the manufacturer's instruction. After the fragment was cloned to the pClone007 Blunt Simple Vector (Tsingke Biotechnology Co., Ltd., Beijing, China) and transformed into *E.coli DH5α*, the positive colony was sequenced (Tsingke Biotechnology Co., Ltd., Beijing, China).

2.3 Construction of expression plasmid

The *hsod1* gene (containing the vector sequence) was amplified with KOD-Plus-Neo (Toyobo, Osaka, Japan) using the following primer: SOD1-pHT43-His-F: ACATCAGCCGTAGGATCCATGGGATCCATGGCGACGAAGGCCGTGT; SOD1-pHT43-His-R: TAGCGGCCGCATGGATCCTTGGGCGATCCCAATTACAC. The pHT43-His plasmid was digested and linearized with BamHI and AP (Alkaline Phosphatase) by MonClone™ Fast AP (Monad Biotech Co., Ltd, Wuhan, China). The purified gene product was inserted into a restricted pHT43-His using the MonClone™ Hi-Fusion Cloning Mix V2 (Monad Biotech Co., Ltd, Wuhan, China). The constructed plasmid was transformed into *E.coli DH5α* competent cells, and the positive transformants were screened by colony PCR and sequenced.

2.4 Preparation and transformation of competent *Bacillus subtilis 1012*

Bacillus subtilis 1012 was inoculated on LB plate and incubated at 37°C overnight. Then, the host strain was transferred with a loop into 2 mL of LB medium and cultured under 180 rpm of shaking at 28°C overnight. Next, 50 μL of the bacterial culture was inoculated in 5 mL of SP I medium under 180 rpm of shaking at 37°C. The growth curve was measured every 30 min until the plateau phase. After that, 0.5 mL of the culture was added to 4.5 mL of SP II medium and incubated under 100 rpm of shaking at 37°C for 90 min. And 50 μL of EGTA (100 mM) was added to the culture medium and incubated under 100 rpm of shaking at 37°C for 10 min. Finally, the culture was dispensed with 300 μL for each tube (Brockmeier et al., 2006; Murayama et al., 2004).

For the bacterial transformation, 1 μg of pHT43-His and pHT43-His-hSOD1 were mixed with *Bacillus subtilis 1012* competent cells and cultured under 100 rpm of shaking at 30°C for 90 min. Then, the bacterial culture was inoculated on LB plates containing 5 μg/mL Chloramphenicol (Cm) at 37°C.

2.5 Expression, preparation, and separation of hSOD1 fusion protein in Bacillus subtilis 1012

The *Bacillus subtilis 1012* containing pHT43-His-*hsod1* was cultured in 10 mL of LB medium (5 μg/mL Cm) under 180 rpm of shaking at 37°C overnight. After the bacterial culture was inoculated to Super Rich (25 g/L of Tryptone, 20 g/L of Yeast extraction, 3 g/L of K_2HPO_4, and 30 g/L of Glucose) (1:100, v/v), different concentrations of IPTG (Isopropylb-D-thiogalatopyranoside) (0.2–1 mM) and 20 g/L lactose were added to induce protein expression at 37°C until a 0.6–0.8 of OD_{600} was obtained. Next, different temperatures (16°C, 25°C, 30°C, 37°C, and 40°C) were used to optimize the induction conditions further. After the incubation for 12 h, 18 h, 24 h, 30 h, and 36 h, 1 mL of culture medium was withdrawn and centrifuged at 4°C, 13000 rpm for 10 min. And 20 μL of cellular supernatant was denatured in 5 μL of 5× denaturing buffer (60 mmol/L of Tris-HCl, 25% glycerol, 2% SDS, 0.1% bromophenol blue) at 95°C for 5 min.

For Western blot analysis, 20 μL of the denatured sample was separated on a 15% SDS-PAGE. After the electrophoresis, the gels were visualized by Coomassie brilliant blue R-250 staining and then transferred to polyvinylidene difluoride (PVDF) membranes. A mouse anti His-Tag mAb (1:2000, Cat #AE003) (ABclonal, Wuhan, China) was used to detect the FLAG-tagged recombinant hSOD1. The goat anti-mouse second antibody (1:5000) (Cell Signaling Technology, MA, USA) was used to detect the native form of hSOD1 with His-tag. The 15% Native PAGE was run at 100 V for 3 h at 4°C to avoid protein denaturation.

2.6 Enzyme activity assay of hSOD1 in *Bacillus subtilis 1012*

The activity of purified hSOD1 was detected *in vitro* using a Total Superoxide Dismutase Assay Kit with WST-8 (Beyotime Institute of Biotechnology, Shanghai, China), based on measuring the color of a water-soluble formazan dye. In brief, 20 μL of hSOD1, 160 μL of WST-8/enzyme working solution, and 20 μL of reaction starting solution were mixed and incubated at 37°C for 30 min. After the reaction was stopped, the absorbance was detected at 450 nm. One unit of SOD was defined as the enzyme amount in 20 μL of the sample that inhibited the reduction reaction between WST-8 and superoxide anion by 50%.

2.7 Effects of Cu^{2+} and Zn^{2+} on purified hSOD1

To determine the effects of Cu^{2+} and Zn^{2+} on hSOD1 activity, we added different concentrations of Cu^{2+}/Zn^{2+} (0/0 μM、200/0 μM、0/5 μM、200/5 μM、800/20 μM) to purified hSOD1 solution. The activity of hSOD1 was measured using a Total Superoxide Dismutase Assay Kit with WST-8 as the above mentioned. The purified hSOD1 with Cu^{2+}/Zn^{2+} (0/0 μM) was used as a control.

3. Results

3.1 Growth of recombinant 1012-hSOD1 strain in different culture media

To explore the effects of media and bacterial inoculum on the growth of recombinant 1012-hSOD1 strain, we cultured the bacteria in three different media containing 1% or 2% inoculum at 37°C. The OD_{600} was measured at other time points. As shown in Fig. 1, either 1% or 2% inoculum led to faster growth of 1012-hSOD1 strain in Super rich media than LB or 2× YT media (Fig. 1a and 1b). There was no difference in the bacterial growth between LB and 2× YT media with 1% and 2% inoculum (Fig. 1c and 1d). We observed a faster growth rate at 2% inoculum for the Super rich medium than 1% inoculum (Fig. 1e). Thus, the Super rich media with 2% inoculum was chosen for the recombinant hSOD1protein expression.

3.2 Secretory expression of hSOD1 in Bacillus subtilis 1012 using different IPTG concentrations and temperatures

The recombinant protein expression level is usually affected by IPTG concentration and induction temperature. IPTG is an efficient inducer for the expression system with lac promoter, which can't be metabolized by bacteria and is stable. Therefore, the effect of different concentrations of IPTG on hSOD1 expression was investigated at 37°C to obtain a high yield of hSOD1. The 1012-hSOD1 strain samples were separately collected at 6, 12, 24, 36, 48 and 60 h after a series of concentrations of IPTG (0.2, 0.4, 0.6, 0.8 and 1 mM) were added. It was indicated that the highest yield of hSOD1 was obtained after the induction for 24 h (Fig. 2a-2e). Further, the expression yields of hSOD1 were compared after 24 h induction with different concentrations of IPTG, and the optimal concentration of IPTG was determined to be 0.2 mM (Fig. 2f). IPTG is an efficient inducer for the expression system with lac promoter, which can't be metabolized by bacteria and is stable. Therefore, the effect of different concentrations of IPTG on hSOD1 expression was investigated at 37°C to obtain a high yield of hSOD1. The 1012-hSOD1 strain samples were separately collected at 6, 12, 24, 36, 48 and 60 h after a series of concentrations of IPTG (0.2, 0.4, 0.6, 0.8 and 1 mM) were added. It was indicated that the highest yield of hSOD1 was obtained after the induction for 24 h (Fig. 2a-2e). Further, the expression yields of hSOD1 were compared after 24 h induction with different concentrations of IPTG, and the optimal concentration of IPTG was determined to be 0.2 mM (Fig. 2f).

Since the induction temperature is vital to enhance recombinant protein expression, the engineered strain 1012-hSOD1 was induced by 0.2 mM IPTG for 24 h at 16, 25, 30, 37, and 40°C, respectively. As suggested in Fig. 3, the highest yield of hSOD1 was achieved at 37°C after the induction for 24 h.

3.3 Secretory expression of hSOD1 in Bacillus subtilis 1012 induced by lactose

Although IPTG is widely used to induce recombinant proteins, it is relatively expensive and toxic to humans. Hence, IPTG is not suitable for the large-scale preparation of drugs. To find a safe and low-cost

production system for the expression of hSOD1, we chose lactose as an inducer to produce recombinant 1012-hSOD1. To explore the peak level of hSOD1 by lactose (20 g/L), we compared the product contents at 37°C for different induction times. As revealed in Fig. 4a, the yield of hSOD1 reached a maximal level after the induction for 24 h. Noticeably, 20 g/L of lactose almost displayed the same inductive effect on hSOD1 expression as that by 0.2 mM of IPTG (Fig. 4b). It was clear that lactose had a high potential to replace IPTG as an inducer for hSOD1 expression.

3.4 Purification and enzyme activity of recombinant hSOD1

Next, we purified hSOD1 using a Nickel column, which was performed by chromatography (chromatographic) system on the AKTA pure 25 (GE Healthcare Life Sciences, Uppsala, Sweden). After the elution and desalination, the recombinant protein was collected and detected by SDS-PAGE (Fig. 5).

As a metalloenzyme, hSOD1 needs Cu^{2+} and Zn^{2+} to maintain its structure and enzymatic activity. The lack of Cu^{2+} and Zn^{2+} will reduce its activity and increase the degradation. Therefore, we chose different concentrations of Cu^{2+} and Zn^{2+} to detect their effects on the enzymatic activity of purified hSOD1. As shown in Fig. 6, a 51.8-fold increase in the specific activity of hSOD1 was observed after the supplementation of Cu^{2+} (200 μM) alone, whereas 5 μM of Zn^{2+} alone slightly increased the SOD activity. Interestingly, the hSOD1 activity elevated about 160-fold after the supplementation with Cu^{2+} (200 μM) plus Zn^{2+} (5 μM) compared to that of the control. And the highest activity was obtained after the combined use of 800 μM of Cu^{2+} plus 20 μM of Zn^{2+}, which induced a 1300-fold increase in enzymatic activity (Fig. 6).

Finally, 36 mg of hSOD1 was purified from 1 L of Super rich medium by nickel affinity chromatography. The specific activity of purified hSOD1 was determined to be 1625 U/mg in the presence of 800 μM of Cu^{2+} and 20 μM of Zn^{2+}.

4. Discussion

The occurrence and development of many diseases are related to the deficiency or false folding of SOD1, such as amyotrophic lateral sclerosis, inflammatory bowel diseases, and lung cancer (Dziabowska-Grabias et al., 2021; Proctor et al., 2016; Wang et al., 2021). In addition to diminishing oxidative stress and ROS generation, SOD1 can inhibit the activation of endothelial cells and regulate the expression of adhesion molecules (Carroll et al., 2007; Segui et al., 2004). Recently, SOD1 was also reported to facilitate cytoprotective pathways by activating gene transcription and plays a physiological role in regulating signal transductions involving ROS, indicating a high potential in disease treatment (Trist et al., 2021). These biological activities make SOD1 become a potential therapeutic agent against host diseases. In this study, we successfully constructed the *Bacillus subtilis* expression system which the recombinant human SOD1 was produced with good enzymatic activity.

The *Bacillus subtilis* has been broadly applied to the expression of agricultural, medical, and industrial products. To gain an endotoxin-free hSOD1, we produced a gram-positive expression system based on the host *Bacillus subtilis 1012* and the *E.coli-B.subtilis* shuttle vector pHT43-His, which had a strong IPTG-inducible Pgrac promoter with lac operator. Therefore, the recombinant hSOD1 could be expressed by IPTG or lactose as inducers. (Tran et al., 2020). In this study, lactose had similar inductive activity to that of IPTG in the expression of hSOD1 (Fig. 4b). Lactose is a safer and cheaper inducer than IPTG, and the heterogeneous hSOD1 can be directly obtained from the supernatant of culture medium. Thus, our detection system not only saves the purification cost but avoids the possible activity loss in the purification. These advantages may lay a foundation for the industrial production of hSOD1 in the future.

The *sod1* gene is located on chromosome 21q22.11 and encodes Cu/Zn superoxide dismutase (Abati et al., 2020). Human SOD1 protein forms a homodimer, and each monomer binds Cu^{2+} and Zn^{2+} to harbor a disulfide. Both metal cofactors are necessary for catalyzing one-electron oxidation followed by one-electron reduction of two $O_2^{\bullet-}$ anions to affect disproportionation (Griess et al., 2017). SOD1 exerts its catalytic function only when it binds a Cu^{2+} and a Zn^{2+} per molecule to form an intramolecular disulfide bridge (Banci et al., 2011). In this study, we purified the hSOD1 and detected the highest activity when the 800 μM of Cu^{2+} and 20 μM of Zn^{2+} were used for catalytic reaction (Fig. 5 and Fig. 6). By comparing the optimal catalytic concentrations between Cu^{2+} and Zn^{2+}, we observed that Cu^{2+} played a predominant role in hSOD1 activity, and a minor contribution was made by Zn^{2+} in the hSOD1-mediated catalysis, in consistence with a previous report (Li et al., 2010). On the other hand, the synergistic effect of Cu^{2+} and Zn^{2+} is essential for the exertion of hSOD1 activity (Fig. 6).

Since *E.coli* is a conditional pathogen, the products from *E.coli* expression systems must be extracted and purified before applying to human health. Different from *E.coli*, *Bacillus subtilis* is a safe probiotic in mammals. Both the bacterium itself and its expression products can be directly used for disease treatment. When we apply genetically engineered *Bacillus subtilis* with protein expression ability to the host, it will play the role of drug therapy and have the function of prebiotic. For instance, it was suggested that SOD1 produced from genetically engineered lactic acid bacteria could be applied to treat ROS-induced gastrointestinal pathologies (J. Zhang et al., 2013). In another study, a catalase- or SOD-producing lactic acid bacteria increased the degradation of H_2O_2 and reduced the severity of colitis (LeBlanc et al., 2011). In our experiment, the highest yield of hSOD1 was achieved when the induction temperature was set at 37°C, which is close to body temperature. Besides, the hSOD1 expression in *Bacillus subtilis* can be easily induced by lactose. These advantages provided a possibility for the application of oral engineering bacteria 1012-hSOD1 and lactose to the clinical treatment of gastrointestinal diseases.

In summary, we established a *Bacillus subtilis* system with hSOD1 activity for the first time. In this study, 36 mg of hSOD1 was purified from 1 L of Super rich medium, and the activity of purified hSOD1 was determined to be 1625 U/mg with 800 μM of Cu^{2+} and 20 μM of Zn^{2+}. In addition, we found that lactose

can replace IPTG as an inducer to produce hSOD1 in our expression system. Our studies shed light on the industrial production and clinical application of hSD1 in the future.

Declarations

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Competing Interests

All the authors declare that they have no conflict of interest in the manuscript.

Author Contributions

Hongtao Liu and Zhigang Zhang designed the study. Mingzhu Yin, Nian Wang, Qiqi Wang, Hui Xia, Xue Cheng, and Haiming Hu were responsible for the acquisition of data. Mingzhu Yin and Nian Wang interpreted the experimental data. Mingzhu Yin and Hongtao Liu were the major contributors in drafting and revising the manuscript. All authors have read and approved the final manuscript.

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Figures

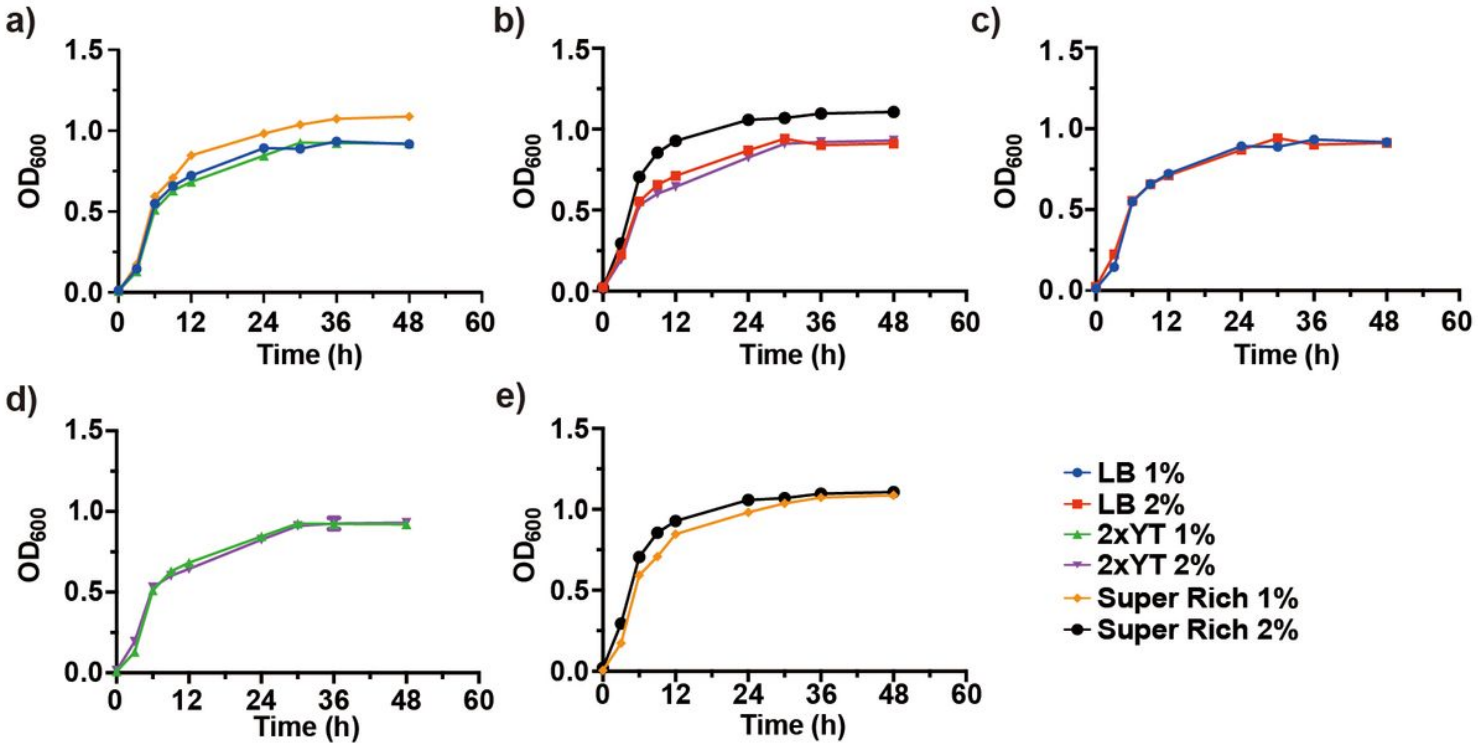

Figure 1

Growth curve of recombinant pHT43-His-hSOD1 strain in different media at 1% and 2% inoculum. a) OD_{600} of 1012-hSOD1 strain cultured in Super rich, LB, or 2× YT media with 1% inoculum. b) OD_{600} of 1012-hSOD1 strain in Super rich, LB, and 2× YT media with 2% inoculum. c) OD_{600} of 1012-hSOD1 strain in LB medium at 1% and 2% inoculum. d) OD_{600} of 1012-hSOD1 strain in 2× YT medium at 1% and 2% inoculum. e) OD_{600} of 1012-hSOD1 strain in Super rich medium at 1% and 2% inoculum.

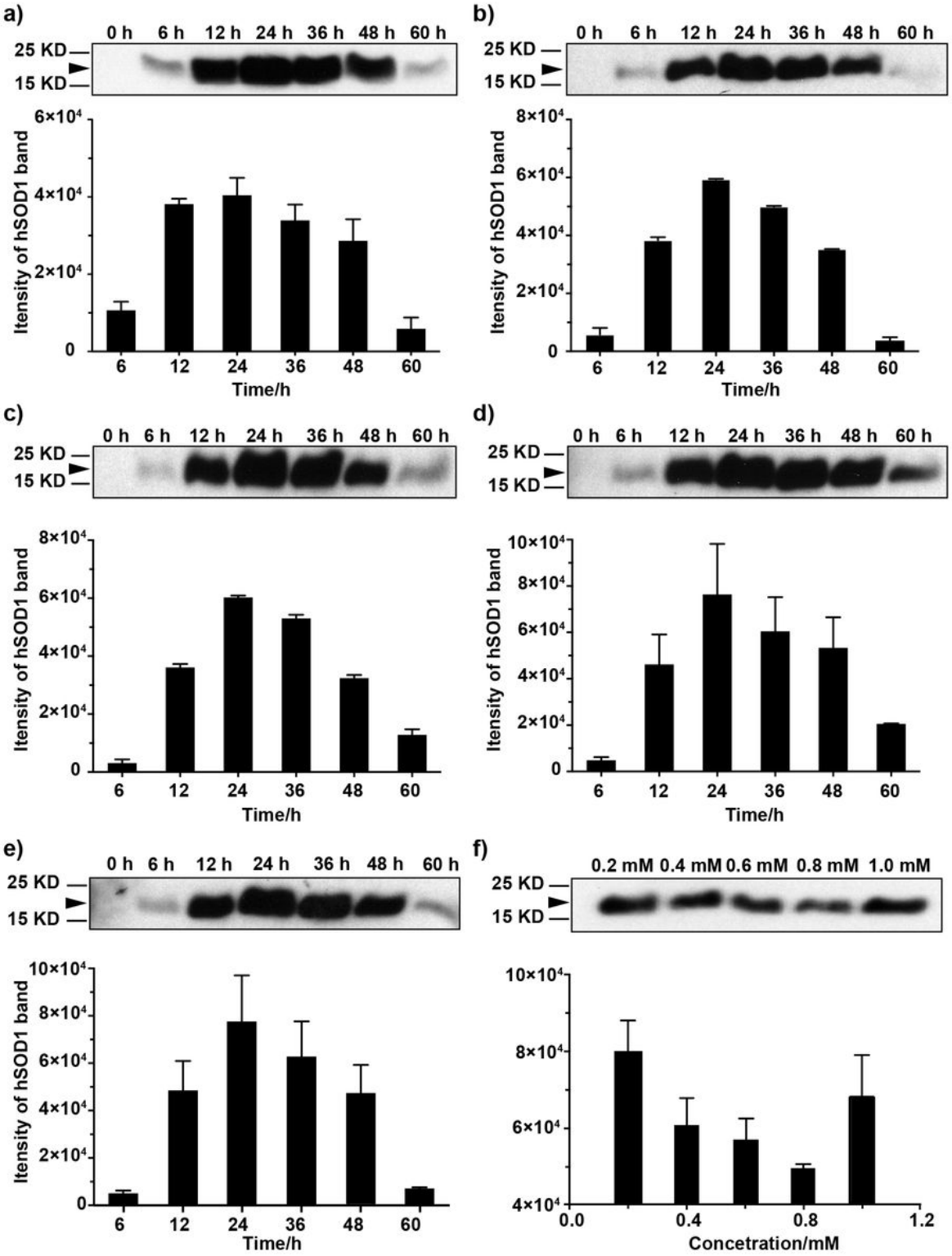

Figure 2

Expression levels of recombinant pHT43-His-hSOD1 at different concentrations of IPTG. a) Protein level of recombinant hSOD1 at 6-60 h induction with 0.2 mM IPTG. b) Protein level of recombinant hSOD1 at 6-60 h induction with 0.4 mM IPTG. c) Protein level of recombinant hSOD1 at 6-60 h induction with 0.6 mM IPTG. d) Protein level of recombinant hSOD1 at 6-60 h induction with 0.8 mM IPTG. e) Protein level of recombinant hSOD1 at 6-60 h induction with 1.0 mM IPTG. f) Protein level of recombinant hSOD1 after 24 h induction with 0.2-1 mM IPTG.

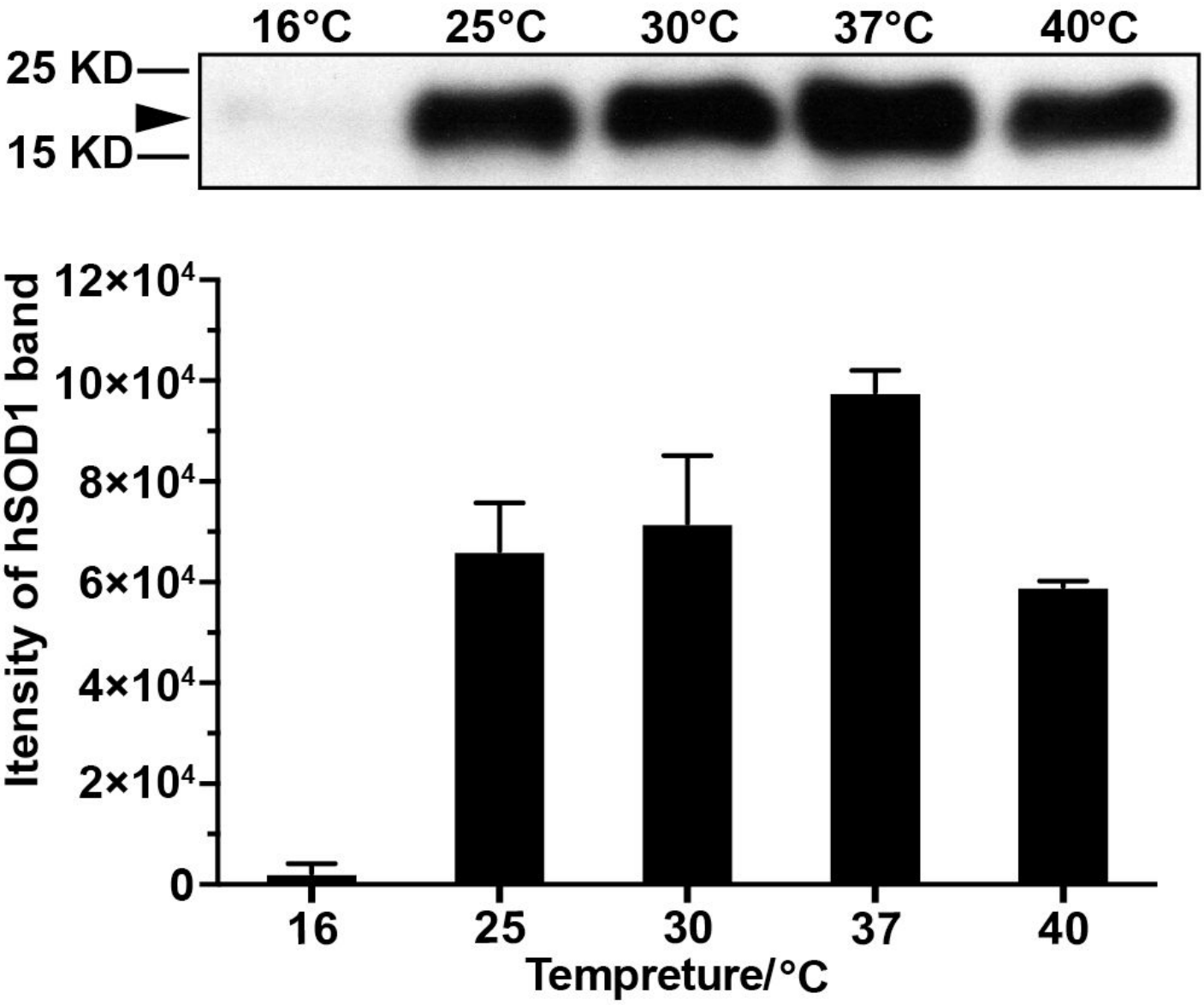

Figure 3

Expression levels of recombinant pHT43-His-hSOD1 at different temperatures after induction of 0.2 mM IPTG for 24 h.

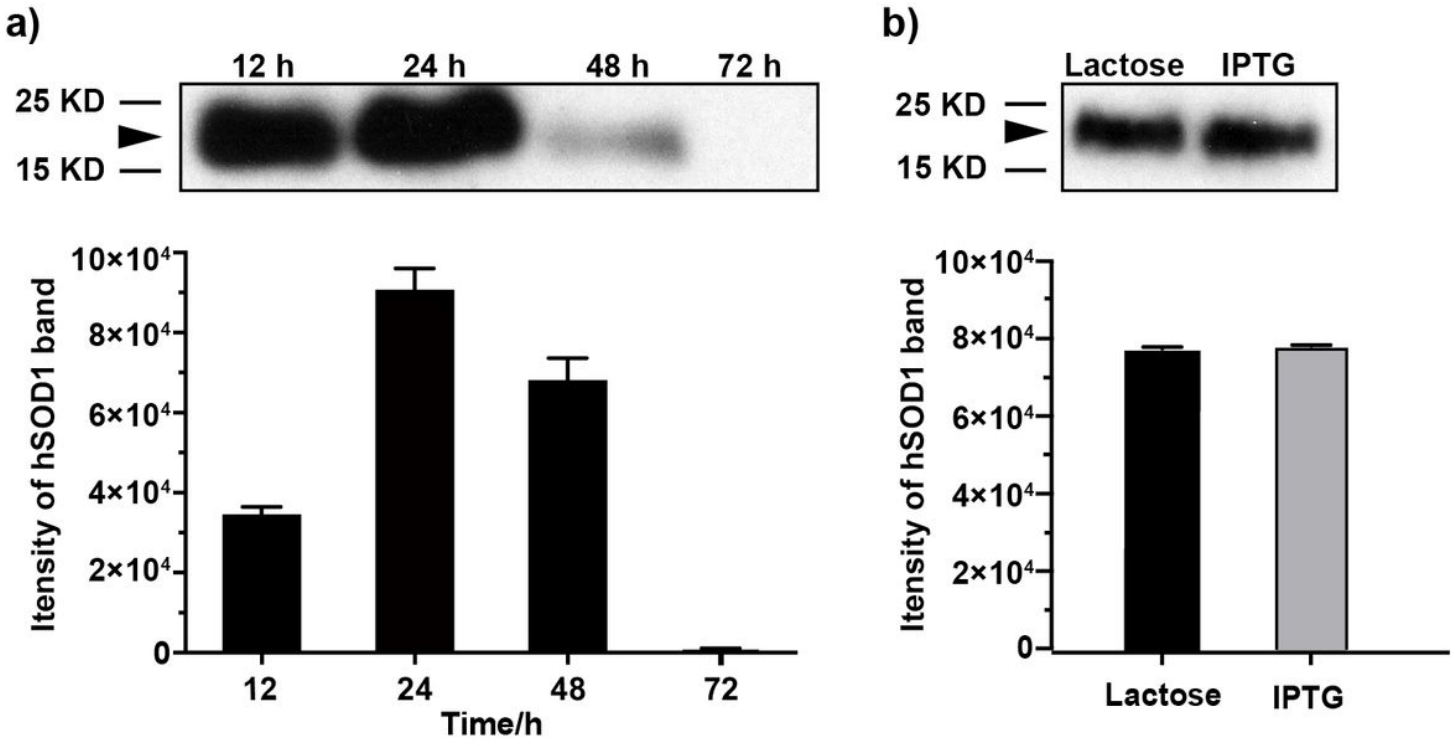

Figure 4

Expression level of recombinant pHT43-His-hSOD1 using Lactose as an inducer. a) Protein level of hSOD1 expression after the induction of Lactose (20 g/L) for different times. b) Protein level of hSOD1 expression after the induction by 20 g/L of Lactose or 0.2 mM of IPTG for 24 h.

Figure 5

SDS-PAGE analysis of purified hSOD1. Lane M: pre-stained protein marker; Lane 1: cellular supernatant; Lane 2: purified hSOD1.

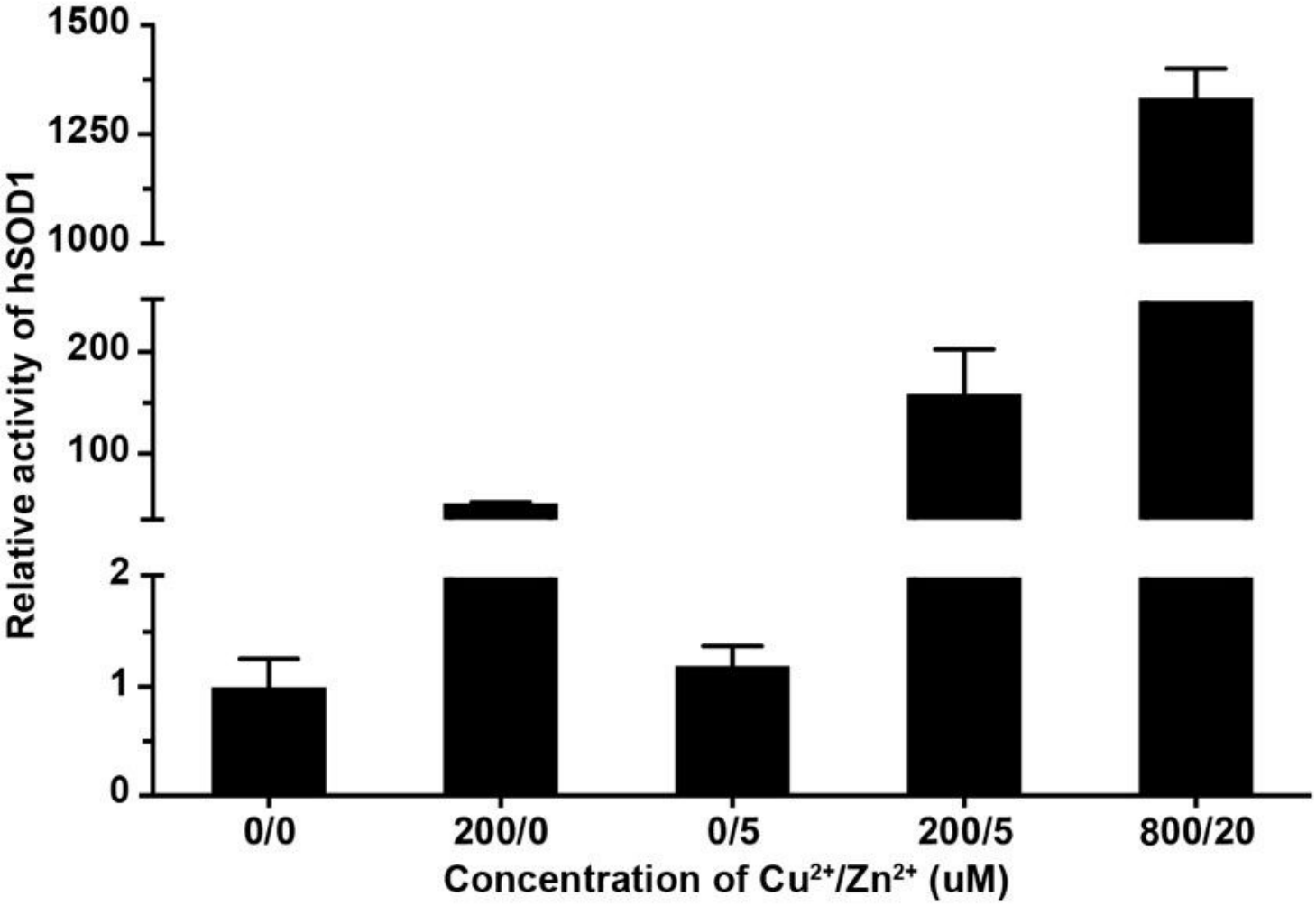

Figure 6

Comparison of hSOD1 activity in presence of different concentrations of Cu^{2+} and Zn^{2+}.