Soluble Expression of a Human MnSOD and Hirudin Fusion Protein in *Escherichia coli*, and Its Effects on Metastasis and Invasion of 95-D Cells

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Introduction

Superoxide dismutases (SODs) are an array of ubiquitous metalloenzymes in all oxygen-respiring organisms, which are essential enzymes that eliminate superoxide radicals (O²⁻) and thus defend cells from being damaged by reactive oxygen species (ROS) [3, 30]. It is known that oxidative damage can peroxidize denatured proteins and unsaturated bonds of membrane lipids, and attack nucleic acids subsequently [43]. This mechanism may form the molecular basis of numerous diseases, such as cardiovascular alterations, the inflammation process, and carcinogenesis.

Manganese superoxide dismutase (MnSOD) is a vital enzyme that protects cells from free radicals through eliminating superoxide radicals (O²⁻). Hirudin, a kind of small active peptide molecule, is one of the strongest anticoagulants that can effectively cure thrombus diseases. In this study, we fused Hirudin to the C terminus of human MnSOD with the GGGGS linker to generate a novel dual-feature fusion protein, denoted as hMnSOD-Hirudin. The hMnSOD-Hirudin gene fragment was cloned into the pET15b (SmaI, CIAP) vector, forming a recombinant pET15b-hMnSOD-Hirudin plasmid, and then was transferred into *Escherichia coli* strain Rosetta-gami for expression. SDS-PAGE was used to detect the fusion protein, which was expected to be about 30 kDa upon IPTG induction. Furthermore, the hMnSOD-Hirudin protein was heavily detected as a soluble form in the supernatant. The purification rate observed after Ni NTA affinity chromatography was above 95%. The hMnSOD-Hirudin protein yield reached 67.25 mg per liter of bacterial culture. The identity of the purified protein was confirmed by western blotting. The hMnSOD-Hirudin protein activity assay evinced that the antioxidation activity of the hMnSOD-Hirudin protein obtained was 2,444.0 ± 96.0 U/mg, and the anticoagulant activity of the hMnSOD-Hirudin protein was 599.0 ± 35.0 ATU/mg. In addition, in vitro bioactivity assay showed that the hMnSOD-Hirudin protein had no or little cytotoxicity in H9c2, HK-2, and H9 (human CD⁴⁺, T cell) cell lines. Transwell migration assay and invasion assay showed that the hMnSOD-Hirudin protein could suppress human lung cancer 95-D cell metastasis and invasion in vitro.

**Keywords:** hMnSOD-Hirudin, soluble expression, purification, DNA protection, metastasis, invasion
SODs have been proved both clinically and preclinically to resist a number of diseases, such as myocardial oxidative damage, ischemic-reperfusion injury, thrombosis-related diseases, cancer, and inflammation [5, 8, 11, 21, 29, 38, 44]. Based on the metal cofactor in the active site and cellular localization, SOD can be identified and characterized by three distinct isoforms in mammals: Cu, ZnSOD, MnSOD, and ECSOD [36]. In the three distinct SOD isoforms, MnSOD is the unique SOD that has turned out to be key to the survival of organisms [4].

Hirudin is one of the most potent anticoagulant peptides (~7 kDa) ever found [28]. Hirudin can efficiently block the thrombin-mediated translation of fibrinogen into fibrin in the blood clotting reaction; therefore, it suppresses coagulation [17, 22]. Many researchers suggested that Hirudin is effective against many diseases, such as thrombosis-related diseases, kidney disease, cancer, and inflammation [1, 12, 18, 19, 27, 35]. Owing to the limited acquisition of natural Hirudin, numerous ways have been used since the 1990s to produce an abundant amount of recombinant Hirudin or its analog. The United States Food and Drug Administration has approved two Hirudin variants and a Hirudin analog to be marketed as anticoagulant drugs [13, 41].

The ROS level, which is much higher in tumor cells in comparison with normal cells, is related to cancer survival, metastasis, development, and progression [9, 42]. Moreover, high levels of ROS may be beneficial to cancer proliferation and metastasis [37]. The ROS disruption, which cancer cells are more sensitive to compared with their normal counterparts, is often adopted in cancer therapy [7]. SODs are essential enzymes that eliminate superoxide radicals (O\(^2^\)) and catalyze a dismutation reaction that converts the superoxide radicals into \(O_2\) and \(H_2O_2\). Without SOD, superoxide radicals may initiate a radical chain reaction to give rise to other various ROS and in turn cause a number of pathologic processes. Tumor cells also generate lots of thrombin, which causes the formation of thrombosis. Studies have shown that the pro-coagulant activity of malignant cancer cells was closely associated with its metastatic potential. Thrombin, a tumor cell mitosis-promoting agent, not only promotes the metastatic phenotype of tumor cells by enhancing the proliferative response of cancer cells to epidermal growth factor, insulin, and transferrin, but also strengthens the tumorigenesis potential of healthy cells [40, 49]. The relationship between thrombosis and metastasis has been well disclosed by platelet and fibrin deposition [14]. As evidenced by animal experiments, tumor growth and metastasis in vitro [49] and in vivo [32, 33] can be promoted by exogenous thrombin. The generation of host thrombin plays a significant role during tumor growth and spontaneous metastasis [19]. It is proposed that Hirudin, a highly potent and specific thrombin inhibitor, offers a promising anticarcinogenic effect, and therefore, the hMnSOD-Hirudin fusion protein may enhance synergies in cancer diseases via a DNA recombination technique.

**Materials and Methods**

**Reagents**

Smal, DL, 2000 DNA Marker, Taq DNA Polymerase High Fidelity Kit, CIAP, T4 DNA ligase, protein marker, and DL 5000 DNA Marker were obtained from Takara (Japan). Isopropyl-\(\beta\)-D-thiogalactopyranoside (IPTG) and imidazole were obtained from Takara (China). \(E.\ coli\) strain Rosetta-gami was obtained from Novagen (Germany). Mouse anti-His monoclonal antibody and mouse anti-SOD2 monoclonal antibody were from Tiangen (China). Horseradish peroxidase (HRP)-labeled goat anti-mouse IgG was obtained from Proteintech (USA). Ni-NTA resin was obtained from Qiagen (Germany). The SOD activity assay kit, BCA kit, bovine fibrinogen, thrombin, and commercial hirudin were purchased from Biyuntian (China). The other agents applied in this work were of analytical grade or better.

**Cell Culture**

Human lung cancer 95-D cells, human kidney proximal tubular epithelial cells (HK-2), rat H9c2 cells, and human CD4+, T cells (H9) (Shanghai Cell Bank, China) were separately cultured in RPMI-1640 (Gibco, USA) and DMEM (Gibco) with the addition of 10% fetal bovine serum (Gibco), penicillin (100 U/ml), and streptomycin (100 mg/ml). The cells were grown in a 37°C incubator under 5% (v/v) CO\(_2\). Cells were subcultured every 2–3 days.

**Construction of the Expression Vector**

pET15b-hMnSOD was used as a template for the coding region of putative mature hMnSOD for PCR amplification. The PCR primers hMnSODFP (5’ AAGCACAAGCTCCCCGACCT 3’) and hMnSOD-linker-RP (5’ GCCACCCACCCACCTTITTTTGCAACG CAT GTATCTTT 3’) were devised according to the sequence of hMnSOD gene (GenBank Accession ID: NM_000636.2). The procedures of PCR amplification were as follows: 95°C for 5 min; 32 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 45 sec; and 72°C for 5 min. According to the amino acid sequence of Hirudin (GenBank Accession CAA02181.1), it has Leu, Pro, Thr, Gln, Lys, and Glu, which all exist in at least two codons. Based on the common codon of \(E.\ coli\), we optimized those amino acid’s codons to the common codon of \(E.\ coli\). The rare codons changed to the common \(E.\ coli\) codons were used to improve the quantity of protein expression [25, 48]. The optimized Hirudin’s nucleotide sequence and the PCR primers linker-HirudinFP (5’ AAAAGG GTGCGTTTGCTACCAGCTTACCCGACTGCAAGCCAAG 3’) and HirudinRP (5’ TTCCGGTGCAAGTTGCTAGGTCAT 3’) were...
synthesized by Invitrogen (China). The underlined bases are the linker). The PCR products were identified with 1% agarose gel electrophoresis. Hirudin and hMnSOD genes were then connected with a polypeptide linker (GGGGS) by overlap-PCR for constructing the chain of hMnSOD-Hirudin fusion genes, using the PCR products of hMnSOD and Hirudin genes as the template with hMnSODFP and HirudinRP as primers.

The conditions of overlap-PCR amplification were as follows: Step 1, 95°C for 5 min; 5 cycles of 95°C for 30 sec, 52°C for 30 sec, and 72°C for 45 sec; and 72°C for 7 min. Step 2, add 2 μl hMnSODFP and 2 μl HirudinRP into the reaction products from step 1, then 95°C for 5 min; 32 cycles of 95°C for 30 sec, 52°C for 30 sec, and 72°C for 45 sec; and 72°C for 7 min. The overlapping PCR products were identified with 1% agarose gel electrophoresis. Hirudin and hMnSOD genes were then connected by PCR amplification using the Pfu DNA Polymerase and linked a linker. The PCR products were identified with 1% agarose gel electrophoresis (1% agarose gel), and the band was excised and purified with a gel purification kit (Tiangen). The purified fragment of the hMnSOD-Hirudin gene was amplified by PCR amplification using the Pfu DNA Polymerase and linked with the pET15b (Smal, CiaP) expression vector [47]. The pET15b-hMnSOD-Hirudin recombinant plasmid was verified by gene sequencing (Invitrogen), then transfected into E. coli strain Rosetta-gami.

Expression and Purification of the hMnSOD-Hirudin Protein

The E. coli Rosetta-gami strains containing pET15b-hMnSOD-Hirudin recombinant plasmid were selected from a single strain colony and were grown overnight on an oscillator (37°C, 220 rpm) in 5 ml of Luria-Bertani (LB) medium supplemented with 100 μg/ml ampicillin. Then, a 2 ml culture mixture of strains was inoculated to 200 ml of fresh LB medium with 100 μg/ml ampicillin for continuous shaking culture, until the OD 600 value reached 0.4–0.6. It was then treated for a further 18 h (24°C, 220 rpm) with IPTG (0.6 mM) to induce hMnSOD-Hirudin fusion protein production. The bacteria were then centrifuged (10 min, 5,000 x g at 4°C) and then resuspended in bacterial lysate (1 mM PMSF, 10 mg/ml Lysozyme, 25 mM NaCl, 20 mM Tris-HCl, pH 7.4, and 0.1% Triton X-100). The bacteria were then sonicated for cell breakage (50 short bursts, about 6 sec each, keeping the bacterial suspension cool on ice during each burst). The lysates were centrifuged at 4°C (20,800 x g, 15 min). The supernatant and precipitate were analyzed by SDS-PAGE on 12% gels according to Laemmli [24]. The supernatant was filtered out and purified by Ni-NTA affinity chromatography (Ni-NTA AC) according to the Qiagen manual.

The fusion protein was eluted and purified via the rising the imidazole concentration (30–300 mM) [26]. To remove the imidazole, the purified hMnSOD-Hirudin fusion protein was dialyzed with dialsyate (20 mM sodium phosphate, 500 mM NaCl, 10% glycerol, pH 7.4) overnight [47].

Western Blot Analysis

The clarified supernatants were subjected to 12% SDS-PAGE, then electroblotting onto a PVDF membrane under a 200 V electric current for a 40 min duration. The membrane was then blocked with blocking reagent (Tris-buffered saline, 0.1% Tween 20, and 5% non-fat milk) at 4°C overnight. The blocked PVDF membrane was rinsed in TBST (four times, 5 min each). One group was hybridized with mouse anti-His IgG (diluted to 1:1,000) and another group was incubated with mouse anti-SOD2 IgG (diluted to 1:1,000) for 2 h at 25°C. The PVDF membranes were then washed in TBST (three times, 15 min each) and incubated with HRP-labeled goat anti-mouse IgG (diluted to 1:4,000) at 37°C for 1 h. The PVDF membrane was then rinsed three times with TBST, visualized by enhanced chemiluminescence with detection reagent, and then exposed to an autoradiography film.

Assay of Protein Concentration and Activity of the hMnSOD-Hirudin Protein

The BCA method was used to determine the concentration of hMnSOD-Hirudin fusion protein; bovine serum albumin was used as the standard.

The hMnSOD-Hirudin fusion protein was then divided into a series of groups (200 μl of 32 nM/ml of the hMnSOD-Hirudin protein each group). One group was left intact and the other group was respectively treated with the following equimolar metal ions (Fe²⁺, Na⁺, Zn²⁺, K⁺, Fe³⁺, Cu²⁺, Mn²⁺) [31]. The SOD activity assay kit was utilized to assay the antioxidation activity of the recombinant hMnSOD-Hirudin protein (Jiancheng, China). The determination was based on the inhibitory effect of the enzyme on the oxidation of hydroxylamine catalyzed by the xanthine-xanthine oxidase system [34]. One unit of antioxidation activity was defined as the quantity of SOD that inhibited 50% of the oxidation of pyrogallol and it was represented as U/mg protein. The diluted bovine Cu/Zn SOD (1,000 U/ml) purchased from Sigma was adopted as the standards, equimolar intact hMnSOD and Mn²⁺ were used as the positive control, and 0.01 M phosphate-buffered saline (PBS, pH = 7.4) was used as the negative control. Anticoagulant activity of the hMnSOD-Hirudin protein was measured with the thrombin titration method. The anticoagulant activity of the test samples was determined based on its thrombin neutralizing activity and represented as antithrombin unit (ATU). The sample that neutralizes one NIH unit of thrombin is equal to 1 ATU [20].

Commercial rHirudin (diluted to 500 ATU/ml) was used as the standard, equimolar intact Hirudin was used as the positive control, and 0.01 M PBS (pH = 7.4) was used as the negative control.

DNA Oxidation Damage Protective Assay of the hMnSOD-Hirudin Protein

DNA oxidation damage protective activity of the hMnSOD-Hirudin protein was proved using supercoiled pUC-19 DNA. The hMnSOD-Hirudin protein (0–5 nM/ml) was incubated with 5 μl of DNA (0.5 μg) in a total volume of 10 μl for 10 min at room temperature. Then, 10 μl of Fenton’s reagent (80 μM FeCl₃, 50 μM ascorbic acid, and 30 mM H₂O₂) was added to the mixture of the hMnSOD-Hirudin protein and pUC-19 DNA. The mixture was incubated at 37°C for 30 min, and subsequently analyzed by 0.8% agarose gel electrophoresis [46].
MTT Cytotoxicity Assay

The H9c2 cells, HK-2 cells, and H9 cells were grown for 24 h and then incubated with the hMnSOD-Hirudin fusion protein at serial concentrations (0, 20, 40, 60, 80, and 100 μg/ml) for 24 h. Then, the growth inhibitory effect of the hMnSOD-Hirudin protein toward the H9c2 cells, HK-2 cells, and H9 cells was evaluated by MTT assay [6]. Equal mass concentrations of cis-diaminedichloroplatinum were used as the positive control.

Cell Migration and Invasion Assays

The 95-D cells were separately incubated with the hMnSOD-Hirudin (2.5 and 5 nM/ml), hMnSOD (2.5 nM/ml), and Hirudin protein (2.5 nM/ml) and 0.01 M PBS (pH = 7.4). For the migration assays, the nine groups of infected 95-D cells (1 × 10^4) were plated into the upper well of the transwell plates (Corning, USA). For the invasion assays, infected 95-D cells (2 × 10^4) were seeded into the upper well of the transwell plates coated with 0.1% matrigel (Corning). In both assays, 95-D cells were incubated in 200 μl of serum-free RPMI 1640 medium and 600 μl of 10% FBS/RPMI 1640 medium in the lower well, serving as a chemottractant. After 36 h of incubation, the 95-D cells on the upper surface were carefully wiped out with cotton wool, whereas the migrated or invaded 95-D cells on the lower surface were fixed in 4% paraformaldehyde for 15 min. Cells were stained with DAPI (1 mg/ml) for 5 min in the dark and washed with 0.01 M PBS three times. Cells were visualized under a fluorescent microscope, and three random fields were captured at ×10 magnification (n = 3).

Statistical Analysis

All treatments were duplicated 3 times. Experimental data were expressed as the mean ± SEM. Statistical analyses were subjected to one-way ANOVA followed by the Newman-Keuls multiple comparison test to evaluate statistical significance, and p < 0.05 was considered significant.

Results

Cloning of the hMnSOD-Hirudin Gene and Construction of the Expression Vector

The hMnSOD-Hirudin gene fragment contained an 807 bp open reading frame and ended with the stop codon TGA (Fig. S1A). It encodes a putative 269 amino acid protein with an estimated molecular mass of about 30 kDa and a pI equal to 5.53. The hMnSOD, Hirudin, and recombinant of hMnSOD-Hirudin gene PCR products were characterized by 1% agarose gel electrophoresis (Fig. S1B). The recombinant plasmid was identified by 1% agarose gel electrophoresis (Fig. S1C) and verified by gene sequencing. Sequence analysis showed that the fragment of the hMnSOD-Hirudin gene was successfully inserted in-frame to the upper stream of the vector-derived His-tag coding sequence (Fig. S1D). The pET15b-hMnSOD-Hirudin recombinant plasmid construct was supposed to be able to express the hMnSOD-Hirudin fusion protein with a His-tag. With this His-tag, the expressed hMnSOD-Hirudin fusion protein could be easily purified by Ni-NTA AC.

Expression and Purification of Recombinant hMnSOD-Hirudin Protein

After induction with IPTG, E. coli strain Rosetta-gami transformed with pET15b- hMnSOD-Hirudin recombinant plasmid expressed a fusion protein of about 30 kDa (His6-tagged hMnSOD-Hirudin protein) as shown in (Fig. 1A). After a series of expression condition optimizations, the optimal expression of hMnSOD-Hirudin fusion protein in soluble type was observed by induction with 0.6 mM IPTG at 22°C for 20 h. The expressed hMnSOD-Hirudin in soluble form immensely helped its purification. Ni-NTA AC was applied to purify the expressed fusion protein. The SDS-PAGE gel showed that the hMnSOD-Hirudin fusion protein appeared as nearly a single band of approximately 30 kDa after Ni-NTA AC purification (Fig. 1B). The hMnSOD-Hirudin protein was verified by western blotting with anti-SOD2 and anti-His antibodies (Fig. 1C). The final expression quantity of the hMnSOD-Hirudin protein was approximately 67.25 mg/l of cultures.

Protein Concentration and Enzyme Activity

The BCA method indicated that the hMnSOD-Hirudin protein concentration reached 3.42 mg/ml. To determine the activity of hMnSOD-Hirudin protein and the influences of metal ions on the antioxidation activity of the hMnSOD-Hirudin protein, the hMnSOD-Hirudin protein was respectively treated with diverse equimolar metal ions (Na^+, Fe^{2+}, K^+, Fe^{3+}, Zn^{2+}, Cu^{2+}, Mn^{2+}). A SOD activity assay kit was used to detect the antioxidation activity of the hMnSOD-Hirudin protein.

The antioxidation activities of the various reaction mixtures were determined. The data show that the antioxidation activity of the hMnSOD-Hirudin protein was greater than that of the hMnSOD protein and the hMnSOD-Hirudin protein was more resistant to the antioxidation activity of Na^+, Fe^{2+}, K^+, Fe^{3+}, Zn^{2+}, Cu^{2+}, Mn^{2+}). It is interesting that the mixture of the hMnSOD-Hirudin protein with Mn^{2+} group and the hMnSOD protein with Mn^{2+} group showed a higher antioxidation activity, but there was almost no significant difference in antioxidation activity among the other groups (Fig. 2A). However, there was no significant difference in antioxidation activity among all groups (Fig. 2B). Thus, the highest antioxidation activity of the hMnSOD-Hirudin protein obtained was 2,444.0 ± 96.0 U/ml (about 2,444.0 ± 96.0 U/mg), and the
anticoagulant activity of the hMnSOD-Hirudin protein was 599.0 ± 35.0 ATU/ml (about 599.0 ± 35.0 ATU/mg).

**DNA Protection Activity of the hMnSOD-Hirudin Protein**

The agarose gel results showed that the pUC-19 native plasmid DNA without pre-incubation with the hMnSOD-Hirudin protein was almost entirely damaged by Fenton’s reagent. Meanwhile, as hMnSOD-Hirudin’s dose increased, the Fenton’s reagent damage to the nicked DNA bands was abated. As a result, the supercoiled DNA bands increased and exhibited dose-dependency. The pUC-19 DNA was almost not damaged by Fenton’s reagent and was extremely close to the natural pUC-19 DNA when the hMnSOD-Hirudin protein concentration reached 5 nM/ml (Fig. 3). The intact hMnSOD protein and the intact Hirudin protein plasmid DNA without pre-incubation with the hMnSOD-Hirudin protein was almost entirely damaged by Fenton’s reagent. Meanwhile, as hMnSOD-Hirudin’s dose increased, the Fenton’s reagent damage to the nicked DNA bands was abated. As a result, the supercoiled DNA bands increased and exhibited dose-dependency. The pUC-19 DNA was almost not damaged by Fenton’s reagent and was extremely close to the natural pUC-19 DNA when the hMnSOD-Hirudin protein concentration reached 5 nM/ml (Fig. 3). The intact hMnSOD protein and the intact Hirudin protein...
were used as controls. The result suggested that the hMnSOD-Hirudin protein has excellent DNA protection activity.

No Cytotoxicity of the hMnSOD-Hirudin Protein in H9c2, HK-2, and H9 Cell Lines

MTT assay results indicated that 0–100 µg/ml hMnSOD-Hirudin protein exhibited no or little cell toxicity in the H9c2, human HK-2, and H9 cell lines; the cell viability had no significant difference with the control group (Fig. 4).

hMnSOD-Hirudin Protein Suppresses Cancer 95-D Cell Invasion and Metastasis In Vitro

The changes of the invasive and migratory abilities of the 95-D cells were detected by transwell invasion assay and migration assay after being incubated with 5 nM/ml hMnSOD, Hirudin, hMnSOD + Hirudin, and hMnSOD-Hirudin protein, respectively. The migratory ability of 95-D cells respectively decreased about 24%, 26%, 28%, and 48%. Compared with the 2.5 nM/ml hMnSOD-Hirudin...
The migratory ability of 95-D cells of the 5 nM/ml hMnSOD-Hirudin group respectively decreased about 34% and 32% (Fig. 5A), and the invasive ability of 95-D cells respectively decreased about 22%, 24%, 30%, and 49%. Compared with the 2.5 nM/ml hMnSOD-Hirudin group and the 5 nM/ml hMnSOD + Hirudin group, the invasion ability of 95-D cells of the 5 nM/ml hMnSOD-Hirudin

A. Migration assay

- Control
- 2.5 nM/ml hMnSOD-Hirudin
- 5 nM/ml hMnSOD-Hirudin
- 5 nM/ml hMnSOD
- 5 nM/ml Hirudin
- 5 nM/ml (hMnSOD+Hirudin)

B. Invasion assay

- Control
- 2.5 nM/ml hMnSOD-Hirudin
- 5 nM/ml hMnSOD-Hirudin
- 5 nM/ml hMnSOD
- 5 nM/ml Hirudin
- 5 nM/ml (hMnSOD+Hirudin)

Fig. 5. The hMnSOD-Hirudin protein suppresses 95-D human lung cancer cell metastasis and invasion in vitro. Significantly impeded abilities of cell migration (A) and invasion (B) in 95-D cells after being incubated with the hMnSOD-Hirudin (2.5 and 5 nM/ml), hMnSOD (2.5 nM/ml), and Hirudin protein (2.5 nM/ml) and phosphate-buffered saline (0.01 M, pH = 7.4). Results are expressed as the mean ± SD of three independent experiments. Statistically and highly significant differences from the control in each group are indicated as *p < 0.05, **p < 0.01, and ***p < 0.001, respectively.
group respectively decreased about 36% and 31%. (Fig. 5B).

**Discussion**

Using DNA recombination techniques, we can make different genes or gene fragments fuse together. Then, by piecing different functional proteins together after the expression, we can acquire artificial proteins with new multi-domains. This method has been widely used in the study of many fields, and it has shown to be of high value in targeted drug design or in the production of new cytokines. The linker sequence of the two macromolecules is important for the construction and function of the fusion proteins. According to Robinson and Sauer’s [39] research, the folding stability of a fusion protein is heavily associated with the linker sequence composition. We designed a GGGGS linker sequence based on the literature of Arai et al. [2] and Gustavsson et al. [16]. Our enzyme activity results of the hMnSOD-Hirudin fusion protein indicate that each domain worked independently even after the spatial isolation of the double-functional domains (connected via GGGGS linker peptide). Recombinant pET15b-hMnSOD-Hirudin plasmid was expressed in the Rosetta-gami strain of *E. coli* at a high level as a soluble form and purified via Ni–NTA affinity chromatography purification. Our results indicate that the Rosetta-gami *E. coli* production system is suitable for high-level expression of the hMnSOD-Hirudin protein.

The results indicated not only that the hMnSOD-Hirudin protein has outstanding antioxidation activity but also excellent anticoagulant activity. The metal ion effect on the activity of the hMnSOD-Hirudin protein results showed that the antioxidation activity was activated significantly by Mn²⁺, but the metal ions did not affect the anticoagulant activity. ROS elimination is a usually recognized mechanism of antioxidants inhibiting oxidative DNA lesion. The hMnSOD-Hirudin protein proved to be efficient in safeguarding oxidative DNA lesion. Furthermore, an assay of bioactivity in vitro showed that the hMnSOD-Hirudin protein would not suppress the proliferation of H9c2, HK-2, and H9 cells, and showed little to no cytotoxicity in these three cell lines. Cell migration and invasion are the major features of metastatic tumor cells responsible for most cancer-related deaths [15]. Compared with the control group, transwell migration and invasion assays showed that the hMnSOD protein group, Hirudin protein group, the mixture of hMnSOD and hirudin protein group, as well as the hMnSOD-Hirudin protein group all could significantly suppress 95-D human lung tumor cell line invasion and metastasis in vitro, and the equimolar hMnSOD-Hirudin protein had the best effects on suppressing 95-D human lung cancer cell metastasis and invasion. Besides this, with the increasing concentration of hMnSOD-Hirudin, the ability of hMnSOD-Hirudin to suppress 95-D human lung cancer cell metastasis and invasion was more powerful. The hMnSOD-Hirudin fusion protein exhibited a synergistic effect, compared with either hMnSOD or Hirudin, as this is probably due to the fact that it possesses two catalytic domains. Interestingly, compared with the mixture of hMnSOD and hirudin group, the hMnSOD-Hirudin fusion protein exhibited a better synergistic effect. We speculated that the higher molecular weight of hMnSOD-Hirudin fusion protein could partially account for this result. It could also be that Hirudin is a specific depressor of thrombin, which can target thrombin and lead to a higher level of local hMnSOD-Hirudin fusion protein concentration, but the real reason requires further experimental inquiry and verification. It was suggested that both the MnSOD and Hirudin proteins may have therapeutic benefits toward many diseases, including thrombosis-related diseases, inflammation, and cancer. The synergistic effect of the hMnSOD-Hirudin fusion protein may provide enhanced therapeutic effects in thrombotic diseases, inflammation, and cancer. A stable and purified form of the hMnSOD-Hirudin protein is necessary for further studies. Our work will facilitate further in vivo and in vitro study of hMnSOD-Hirudin’s resistance and novel therapy towards thrombotic diseases, inflammation, and cancer.

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**Table 1. Summary of the hMnSOD-Hirudin fusion protein purification from culture.**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Purity (%)</th>
<th>hMnSOD-Hirudin (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble lysate</td>
<td>53.7</td>
<td>34.9</td>
<td>18.74</td>
<td>100</td>
</tr>
<tr>
<td>Ni-NTA affinity chromatography</td>
<td>14.1</td>
<td>95.4</td>
<td>13.45</td>
<td>71.8</td>
</tr>
</tbody>
</table>

*Wet weight cells (1.14 g) from 200 ml culture were lysed using sonication.*

*Protein yield was calculated using the amount of the hMnSOD-Hirudin protein after concentration and the amount of the hMnSOD-Hirudin protein in soluble lysate.*

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References


