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Production of Bioactive Human Hemangiopoietin in *Escherichia coli*

Jie Zhang, Jian-Feng Li, and Shuang-Quan Zhang*

Jiangsu Province Key Laboratory for Molecular and Medical Biotechnology, Life Science College, Nanjing Normal University, Nanjing, 210046, Jiangsu, P. R. China; fax: (8625)8589-1053; E-mail: shuangquanz@yahoo.com

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Abstract—To devise an efficient approach for production of human hemangiopoietin (hHAPO), the gene of hHAPO was synthesized and subcloned into the pSUMO vector with a SUMO tag at the N-terminus. The expression construct was then transformed into the expression strain *E. coli* BL21(DE3). The fusion protein was expressed in soluble form and identified by SDS-PAGE and Western blotting. The fusion protein was purified to 90% purity by metal chelate chromatography with a yield of 45 mg per liter fermentation culture. The SUMO tag was removed by cleavage with SUMO protease at room temperature for 1 h, and the hHAPO was then re-purified by the metal chelate chromatography. Finally, about 21 mg hHAPO was obtained from 1 liter of fermentation culture with no less than 95% purity. The recombinant hHAPO significantly stimulated the proliferation of human umbilical vein endothelial cells.

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Stem cell factor, interleukin 3, granulocyte-macrophage-colony stimulating factor, granulocyte-colony stimulating factor, and macrophage-colony stimulating factor are members of a group of glycoproteins called hematopoietic cytokines. These cytokines regulate the growth and differentiation of hematopoietic progenitor cells and functionally activate mature neutrophils and macrophages.

Hemangiopoietin (HAPO) is a novel growth factor for the hematopoietic and endothelial cell lineages. Human hemangiopoietin (hHAPO) was initially obtained from the urinary extracts of patients with aplastic anemia [1]. Recombinant HAPO has been shown to significantly stimulate the proliferation and hematopoietic and/or endothelial differentiation of human bone marrow mononuclear cells [1]. Recombinant hHAPO also inhibits cell death by apoptosis of MO7e cells through the phosphatidylinositol-3-kinase/AKT pathway [2]. hHAPO is a promising candidate for a new hematopoietic medicine.

Abbreviations: (h)HAPO, (human) hemangiopoietin; HUVEC, human umbilical vein endothelial cells; IPTG, isopropyl-β-D-1-thiogalactopyranoside; SUMO, small ubiquitin-related modifier.

* To whom correspondence should be addressed.

The expression of heterologous genes in bacteria is by far the simplest and least expensive means available for research or commercial purposes. However, heterologous production of hHAPO is complicated by the fact that the protein contains seven disulfide bridges, and heterologous gene products often fail to attain their correct three-dimensional conformation or are simply expressed very poorly in *Escherichia coli*.

Several major protein fusion technologies have been developed to improve expression and purification of heterologous recombinant proteins in bacterial cells. These include maltose binding protein, glutathione-S-transferase, and thioredoxin gene fusion systems. All of these conventional methods have shortcomings, primarily inefficient expression and/or inconsistent cleavage [3-5]. Small ubiquitin-related modifier (SUMO) is an ubiquitin-related protein that functions by covalent attachment to other proteins. It is known that SUMO, fused at the N-terminus with other proteins, can fold and protect the protein by its chaperoning properties, making it a useful tag for heterologous expression. These advantages include the manner in which protein expression is enhanced, proteolytic degradation of the target protein is decreased, protein folding and solubility are increased, and purification and detection are simplified. The main disadvantage of fusion technology, cleaving the protein fusion, is also covered [6].

We have developed a novel SUMO-fusion system that provides increased levels of expression of heterologous proteins in *E. coli* and allows rapid purification of proteins of interest. We report here the application of the SUMO-fusion technology to the expression and purification of hHAPO protein.

MATERIALS AND METHODS

The linearized pSUMO vector with *Bsa*I and *Bam*HI restriction sites and T7 promotor and kanamycin resistance was purchased from LifeSensors (USA). The Ni-NTA resin was from Qiagen (USA). Other chemicals used in this study were of analytical or higher grade.

Construction of SUMO-hHAPO expression vector. The hHAPO gene was synthesized and cloned into the pSUMO vector using *Bsa*I and *Bam*HI. The construct was verified by DNA sequencing to ensure 100% identity with the expected nucleotide sequence and in-frame orientation.

Expression and characterization of SUMO fusion protein. The constructed recombinant plasmid pSUMO-hHAPO was transformed into competent *E. coli* BL21(DE3). Three colonies were picked and cultured in 3 ml Luria–Bertani (LB) medium with vigorous shaking (220 rpm) at 37°C to a density of ~0.6 absorbance unit at 600 nm. Isopropyl-β-D-1-thiogalactopyranoside (IPTG) (0.5 mM) was then added to induce the expression of the recombinant protein at 37°C for 4 h. The cells were harvested by centrifugation at 7000*g* for 5 min at 4°C and immediately frozen at −20°C until further processing. The BugBuster Master Mix Protein Extraction Reagent (Novagen, USA) was used to extract the expressed proteins from bacterial lysates for solubility of the expressed protein assay. The extraction was carried out according to the manufacturer's instructions.

SDS-PAGE and Western blot. SDS-PAGE analysis was performed according to Laemmli [7] using 15% polyacrylamide gels. Samples were mixed with equivalent sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.005% bromophenol blue, and 10% 2-mercaptoethanol). Gels were stained with Coomassie brilliant blue R-250. For Western blotting, the protein was separated on a 15% polyacrylamide gel under reducing conditions and then transferred to a PVDF membrane, essentially as described previously [8]. After blocking with 5% nonfat dry milk, the membrane was incubated with a 1 : 1000 dilution of mouse anti-His_6 IgG at 30°C for 1 h and then washed three times for 5 min with Tris-buffered saline-Tween-20 (TBST) (50 mM Tris-HCl, 150 mM NaCl, and 0.5% Tween-20, pH 7.6) and incubated with 1 : 1000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibodies at 30°C for 1 h. After three washes for 5 min with TBST and two washes for 2 min with deionized water, specific binding was detected with 3,3′,5,5′-tetramethylbenzidine chemiluminescence as a staining substrate.

Large-scale expression of recombinant protein in batch fermentation. Fermentations were performed in a manner similar to that described previously [9]. Briefly, a single clone of *E. coli* BL21(DE3) harboring the pSUMO-hHAPO vector was inoculated into 10 ml LB medium with 30 mg/liter kanamycin in a 50 ml flask. The seed culture was incubated at 37°C and 220 rpm on a rotary shaker overnight. These cells were then used to inoculate 200 ml 2XYT medium with 30 mg/liter kanamycin in a 500 ml flask, which was incubated at 37°C and 220 rpm on a rotary shaker for 4 h and designated as the secondary seed culture. The production medium consisted of (g/liter): yeast extract, 10; tryptone, 10; glucose, 10; $K_2HPO_4 \cdot 3H_2O$, 4; $Na_2HPO_4 \cdot 12H_2O$, 7; $(NH_4)_2SO_4$, 1.2; KH_2PO_4, 2; and 3 ml/liter 50% (v/v) antifoam. A 5-liter bioreactor (BioXin, China) containing 3 liters production medium was autoclaved at 121°C for 30 min. The secondary seed cultures were then inoculated at an inoculum size of 5% (v/v) of culture medium. The trace element solution contained (mg/liter): $MnSO_4 \cdot 5H_2O$, 1; $CaCl_2 \cdot 6H_2O$, 4; $Na_2MoO_4 \cdot 2H_2O$, 2; $ZnCl_2$, 2; $CuSO_4 \cdot 5H_2O$, 1; H_3BO_4, 0.5; $FeSO_4 \cdot 7H_2O$, 20; $CaCl_2 \cdot 2H_2O$, 20; and $MgSO_4 \cdot 7H_2O$, 300. The fermentation was maintained at 37°C with an initial agitation rate of 300 rpm. The dissolved oxygen was controlled at 50% air saturation by an agitation cascade between 300 and 800 rpm. The cultivation pH was kept at 7.2 by the addition of NH_4OH or H_2SO_4. After incubating for 5 h, the cultures were induced by 0.5 mM IPTG for target protein expression. Cells were collected by centrifugation (7000*g*, 5 min) after inducing for 3 h, and the cell pellet was frozen at −80°C.

Purification of His_6-SUMO fusion protein. The pellet from 200 ml culture was resuspended in 20 ml binding buffer (20 mM Tris-HCl, 500 mM NaCl, 10 mM imidazole-HCl, and 15 mM phenylmethylsulfonyl fluoride, pH 8.0) and lysed on ice by sonication at 400 W for 100 cycles (4 sec working, 8 sec resting). The supernatant of the cell lysate resulting from centrifugation at 12,000*g* at 4°C for 20 min was applied to a Ni^{2+}-chelating column. After extensive washing with binding buffer, the fusion protein was eluted with five column volumes of elution buffer (20 mM Tris-HCl, 500 mM NaCl, and 250 mM imidazole-HCl, pH 8.0). The peak fractions containing the fusion protein were pooled and dialyzed overnight at 4°C against 20 mM Tris-HCl buffer (pH 8.0).

Cleavage of SUMO-fusion and purification of hHAPO. The fusion protein was reacted with 1 U of SUMO protease per 50 μg fusion protein at room temperature for 1 h. Since both SUMO and SUMO protease had His_6 tag, but hHAPO proteins did not, the cleaved SUMO-fusion samples could be reapplied to the nickel column to obtain the purified hHAPO by subtracting the His_6-tagged proteins. Briefly, after the SUMO-fusions were cleaved by the SUMO protease, the sample was

loaded onto a column with Ni-NTA resin. Most of the hHAPO protein without His_6 tags was eluted in the flow-through (unbound) fractions, and the rest was recovered by washing the resin with binding buffer. The eluted and washed proteins appearing in fractions with high values of A_{280} were pooled as the final purified sample. The purified proteins were checked on SDS gels, and the samples were stored at −80°C for activity assay.

Mass spectrometry analysis. The sample of purified hHAPO was analyzed by MALDI-TOF mass spectrometry on a time-of-flight Ultraflex II mass spectrometer (Bruker Daltonics, Germany).

Cell proliferation assays. Cell proliferation was measured using a Cell-Counting Kit-8 (CCK-8) (Dojindo, USA) according to the manufacturer's instructions. Briefly, human umbilical vein endothelial cells (HUVEC) were routinely grown in complete M199 medium containing 10% fetal bovine serum (FBS) supplemented with 1% ECGS (endothelial cell growth supplement) (v/v) (ScienCell Research Laboratories). The HUVEC cells were prepared by placing 20,000 cells into each well of a 24-well plate with medium M199 containing 10% FBS in triplicate and allowed to adhere overnight at 37°C in a CO_2 chamber. Thereafter, the medium was replaced by M199 medium, and different concentrations of hHAPO were added to each well. After 72 h, 10 µl of CCK-8 was added to each well and incubated at 37°C for 4 h. Cell viability was determined by absorbance measurement at 450 nm.

RESULTS AND DISCUSSION

Construction and pilot expression of SUMO fusion protein. hHAPO gene was synthesized and subcloned into the pSUMO expression vector with the SUMO fusion tag at the N-terminus. The correctness of the recombinant pSUMO-hHAPO was verified by DNA sequencing. The correct construct was transformed into the expression host *E. coli* L21(DE3) and subjected to a pilot expression test. As shown in Fig. 1, there was an obvious protein band after IPTG induction that could be detected by anti-His_6 antibody according to Western blot. Colony 2 was chosen for the induction experiment, and the fusion protein was efficiently produced in a soluble form induced by 0.5 mM IPTG at 37°C.

High yield expression by fermentation by IPTG induction. Colony 2 was grown in a 5-liter reactor and induced with IPTG under the above conditions. The bioreactor batch yielded 15 g/liter of wet biomass when A_{600} was approximately 14, over 5-fold higher than that achieved in LB medium. Total soluble protein was 1175 mg/liter as tested by the BSA method. The fusion protein was expressed in soluble form.

Purification of SUMO-hHAPO fusion protein. As described above, Ni-NTA resin was used for fusion protein purification. Most of the proteins without His_6 tags were eluted in the flow-through and washed out by using the washing buffer containing 10 mM imidazole. Finally, the fusion proteins were efficiently eluted with elution buffer containing 250 mM imidazole with more than 90% purity (Fig. 2), and about 45 mg fusion protein per liter of the fermentation culture could be obtained.

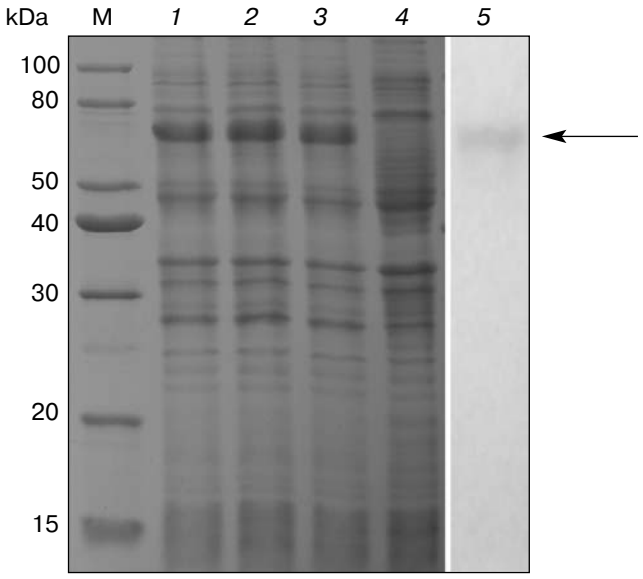

Fig. 1. Analysis of expressed fusion protein by SDS-PAGE and Western blotting. Lanes: M, molecular mass markers; *1-3*) colonies 1-3 induced by 0.5 mM IPTG; *4*) negative control; *5*) Western blot of total protein from colony 2. Arrow indicates the expressed fusion protein.

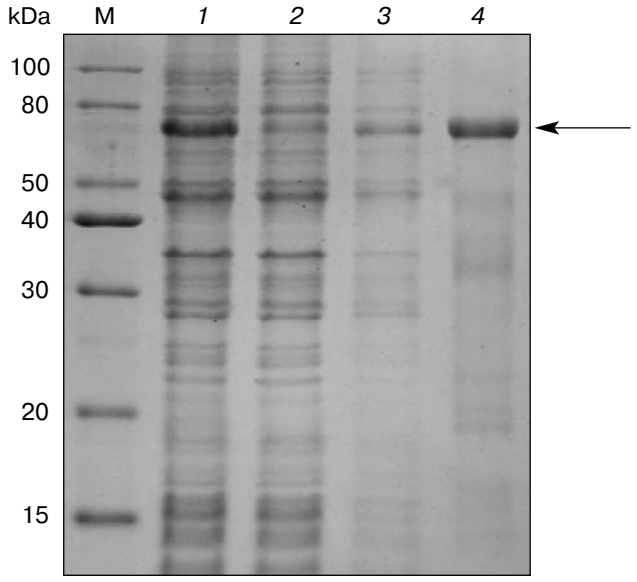

Fig. 2. Purification of SUMO-hHAPO by Ni-NTA. Lanes: M, molecular weight marker; *1*) supernatant; *2*) flow-through; *3*) wash; *4*) elution. Arrow indicates the purified fusion protein.

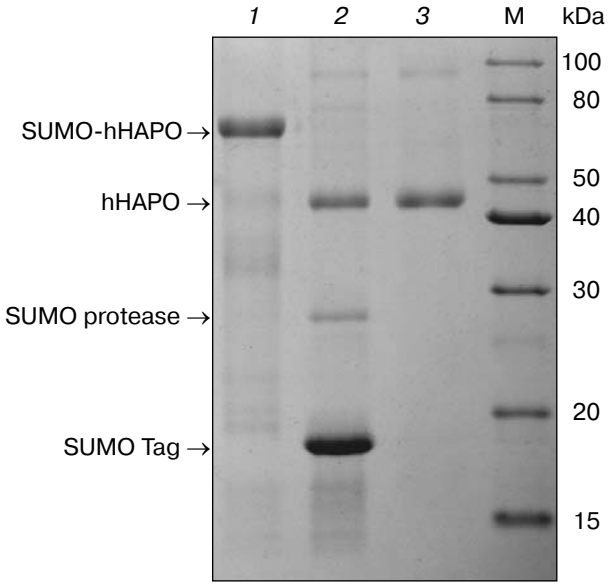

Fig. 3. SDS-PAGE analysis of fusion protein digestion by SUMO protease and hHAPO purification. Lanes: M, molecular weight markers; *1*) purified fusion protein; *2*) mixture of fusion protein by SUMO protease digestion; *3*) Ni-NTA-purified hHAPO.

Purification of hHAPO. The SUMO-hHAPO protein (50 μg) was competently cleaved after incubation with SUMO protease (1 U) at room temperature for 1 h, as confirmed by checking the proteins on a 15% gel in the presence of SDS. After the cleaved sample was reapplied to a Ni-NTA column to remove His_6-tagged SUMO and SUMO protease, finally purified hHAPO was obtained, and 15% gel results indicated that hHAPO had been purified successfully to more than 95% purity (Fig. 3). The purified hHAPO was filtered through a 0.22 μm filter membrane and stored at −80°C for activity assay. Finally, the purified recombinant hHAPO was produced at a yield of 21 mg/liter culture.

Mass spectrometry analysis. As shown in Fig. 4, the actual molecular mass of hHAPO determined by MALDI-TOF mass spectrometry was 31,664.71 Da, which matched the calculated molecular weight of hHAPO.

Effect of hHAPO on HUVEC cells. HUVECs were used to evaluate the biological activity of the recombinant hHAPO. The results presented in Fig. 5 showed that the cell number increased after the addition of hHAPO, and hHAPO significantly stimulated the proliferation of HUVECs in a dose-dependent manner. Thus, the recombinant hHAPO was proved to be able to stimulate the proliferation of HUVECs.

Expressing recombinant cytokine proteins, especially proteins with disulfide bridges, is quite an arduous task because they often fold incorrectly and aggregate, leading to either rapid degradation or to the accumulation of inclusion bodies when they are expressed in *E. coli*. Fortunately, these problems are somewhat alleviated when the desired protein is expressed with a fusion partner. However, in the past, traditional fusion systems have given variable results of expression and have faced major problems attributed to either the inefficient cleavage of the fusion protein or cleavage within the target protein,

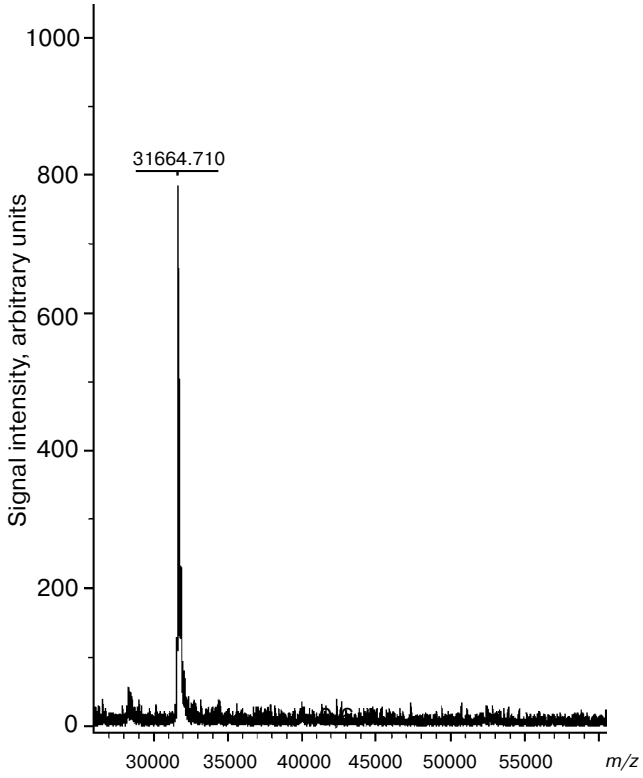

Fig. 4. MALDI-TOF mass spectrometry analysis of hHAPO.

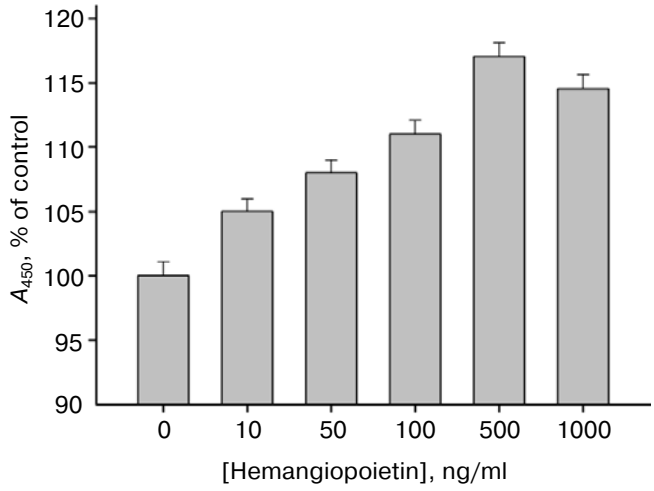

Fig. 5. Bioactivity assay of recombinant hHAPO on HUVEC.

both of which compound the difficulties of purification. SUMO is superior to commonly used fusion tags in enhancing expression and solubility with the distinction of generating recombinant protein with native sequences [10]. We hypothesized that the attachment of a highly stable and compact SUMO structure to the N-terminus of the hHAPO would facilitate correct protein folding and enhance solubility and expression.

In this study, we expressed hHAPO as SUMO fusions in *E. coli* to evaluate the roles of SUMO and SUMO protease on the production of the cytokine. The SUMO fusion protein was successfully expressed in *E. coli* and high expression levels of soluble fusion protein were achieved. Incorporation of His tag and SUMO protease recognition sites into the expression construct facilitated easy purification of hHAPO with native sequences by nickel affinity chromatography. The fusion protein could be purified in one step with a purity of not less than 90%; 45 mg SUMO-hHAPO per liter of fermentation culture was obtained. The fusion protein was then completely cleaved by SUMO protease, which is remarkably robust and highly specific. The hHAPO protein was recovered with 95% purity by purification with nickel affinity chromatography again, and 21 mg hHAPO per liter of fermentation culture was finally obtained. The purified hHAPO has the same molecular mass as native hHAPO. The recombinant hHAPO stimulated the proliferation of HUVECs in a dose-dependent manner. The efficient procedure of expression and purification can be used for the mass production of the protein.

In summary, we have successfully produced untagged hHAPO with high purity and activity. The SUMO fusion technology could be widely applied to the production of a variety of cytokine proteins in *E. coli*.

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