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SHORT COMMUNICATION

Expression, purification, and characterization of recombinant human hypoxia inducible factor 1α in *E.coli*

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Abstract The Hypoxia-inducible factor 1 (HIF-1) heterodimeric transcription factor which is composed of a regulated α subunit and a constitutively expressed β subunit is a critical regulatory factor that orchestrate the cellular responses to hypoxia. It plays an important role in the pathology of many human diseases. The oxygen-sensing and transactivating functions of HIF-1 are contained within the α subunit, which is up-regulated in most human cancers. To study the function of HIF-1α more intensively, we designed a system to express and purify functional recombinant human HIF-1α (rhHIF-1α) protein from *E. coli*. The expressed His-tagged rhHIF-1α which forms inclusion bodies at 28°C was washed, dissolved and refolded firstly. Then the refolding His-tagged rhHIF-1α fusion protein was purified by diethylaminoethyl ion-exchange chromatography. After cleaved by thrombin, rhHIF-1α was thoroughly separated from tags by using Ni–NTA affinity chromatography, which was confirmed by SDS–PAGE and western blotting analysis. Thereafter, rhHIF-1α was further purified by using molecular sieve and its function was identified by electrophoretic mobility shift assay and the result indicated that the purified rhHIF-1α has DNA binding activity. The present expression and purification procedure enabled us to obtain biologically active rhHIF-1α from *E. coli* for in vitro function studies.

Keywords rhHIF-1α · Inclusion body · Protein purification · Hypoxic response element · Electrophoretic mobility shift assay

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Introduction

The hypoxia-inducible factor-1 (HIF-1) is recognized as the master regulator of the hypoxic response, activating the transcription of >100 genes crucial for adaptation to hypoxia (Semenza and Wang 1992). It induces the expression of proteins controlling glucose metabolism, cell proliferation, and vascularization, and thus plays a key role in the pathology of cancer, heart disease, and stroke (Semenza 2000; Blagosklonny 2001). The HIF-1 transcription factor is composed of the regulated α subunit and the constitutively expressed β subunit, the aryl hydrocarbon receptor nuclear translocator (Wang et al. 1995). The oxygen-sensing and transactivating functions of HIF-1 are contained within the α subunit, which is up-regulated in most human cancers (Zhong et al. 1999). In mice, deletion of *hif-1α* results in neural and cardiovascular developmental arrest and embryonic death, underscoring the critical role of HIF-1α (Iyer et al. 1998).

Several steps in the signal transduction mechanism by which low cellular oxygen concentrations are translated into HIF-1α-mediated transcription of hypoxic response genes have been delineated (Semenza 2001a, b). Under normoxic conditions, HIF-1α becomes hydroxylated at a proline in the oxygen-dependent degradation domain, associates with the von Hippel-Lindau tumor suppressor protein, and is rapidly degraded in the cytoplasm via the

ubiquitin–proteasome pathway (Ivan et al. 2001; Jaakkola et al. 2001). Under hypoxic conditions, HIF-1α protein accumulates and translocates into the nucleus, where it forms an active complex with HIF-1β. The HIF-1α and β complex recruits the general co-activator cAMP response element binding protein (CBP)/p300 to bind hypoxia-responsive elements (HREs) of target genes, such as erythropoietin (EPO), vascular endothelial growth factor (VEGF) and inducible nitric oxide synthase (iNOS), and promotes transcription of them (Carmeliet et al. 1998; Iyer et al. 1998; Ryan et al. 1998).

The involvement of HIF-1 in pathophysiological conditions and its potential value as a therapeutic target has prompted people to understand its functions (Welsh et al. 2006; Yu et al. 2008; Zhao et al. 2009). For this aim, purified HIF-1α protein and its antibody are needed. Although to obtain recombinant proteins in prokaryotes is an economic, highly efficient and widely used technique, there are still many uncertain factors to certain protein. There are few reports about expression and purification of such big protein as rhHIF-1α presumably due to low expression efficiency and high degradation rate of this protein. Thus, a stable, biologically active full length rhHIF-1α are needed for further functional studies. Here, we designed a system to express, purify and characterize the functional recombinant human HIF-1α (rhHIF-1α) protein from *E. coli*. We expressed the rhHIF-1α in *E. coli* which was presented as inclusion bodies, and purified it through diethylaminoethyl (DEAE) ion-exchange, thrombin cleavage, and molecular sieve sequentially. Finally, the DNA-binding activity of the purified rhHIF-1α was tested by electrophoretic mobility shift assay (EMSA).

Materials and methods

Reagents

Restriction enzymes were obtained from New England Biolabs. All other reagents were purchased from Promega, Sigma, Fluka or Bio-Rad respectively. Mouse anti-His antibody and mouse anti-HIF-1α were from Santa Cruz Biotechnology. γ-^{32}P-ATP was from Beijing Free Biotechnology.

Plasmid construction and rhHIF-1α fusion protein expression

The pET-32a (+) vector, a kind gift from Dr. Hong Tang's Laboratory, was designed for cloning and high-level expression of peptide sequences fused with the 109aa Trx-tag thioredoxin protein. Human *hif-1α* was subcloned into pET-32a (+) vector at *Kpn* I/*EcoR* V site. Briefly, plasmids pET-30a-hif-1α (Yu et al. 2001) and pET-32a (+) were digested with *Kpn* I and *EcoR* V simultaneously. DNA fragments were separated, ligated using T4 DNA ligase and transformed into TOP10. Positive clones were identified by restriction endonuclease digestion and further confirmed by DNA sequencing (Bio-Asia, China). The resulting plasmids were transformed into *E. coli* expression strain BL21 (DE3).

BL21 (DE3) *E. coli* cells harboring pET-32a-hif-1α were grown overnight at 37°C in 3 ml LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl). The *E. coli* cells were transferred into 500 ml 2 × YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) the following day and grown at 28°C or 37°C to OD600 = 0.4, whereupon isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture at the final concentration of 0.5 mM to induce the expression of rhHIF-1α fusion protein (there after named His-rhHIF-1α). Then, the cultures grown for another 4 h at 28°C or 37°C were harvested and washed with 1 × PBS for 3 times. For fermentation, 200 ml cultures were transferred into a fermentor (New Brunswick Scientific Co., Inc., BioFlo 310) and grown at 28°C with 40% oxygen concentration. The cultures were induced with 1 mM IPTG when the OD_{600} = 1.0, and the oxygen concentration was adjusted to 5%. Four hours later, The IPTG-induced bacterial cells were collected and resuspended in extraction buffer (25 mM Tris–HCl, pH 8.0).

Purification and characterization of rhHIF-1α

The expressed proteins were extracted out from the cells by sonication and centrifuged to sedimentate insoluble materials such as inclusion bodies. The inclusion bodies were washed three times with 25 mM Tris–HCl, pH 8.0 containing 2 M urea, 0.5% Triton X-100, 1 mM EDTA-Na, 1 mM mercaptoethanol to remove the contaminating proteins and judged by SDS–PAGE. The inclusion bodies were solubilized in a 25 mM Tris–HCl, pH 8.0 containing 6 M guanidine hydrochloride, 1 mM EDTA-Na, 1 mM mercaptoethanol and diluted to 0.5–0.8 mg/ml in the refolding buffer containing 25 mM Tris–HCl, pH 8.0, 1.5 M guanidine hydrochloride, 1 mM EDTA-Na, 1 mM mercaptoethanol at 4°C. After filtered with 0.22 μm pore diameter membrane at 16°C, the refolding His-rhHIF-1α was added to a DEAE Sepharose fast flow column (Pharmacia) prewashed twice with 25 mM Tris–HCl, pH 8.0 (stress ≤0.3 MPa; flow-rate ≤0.5 ml/min). The column was washed with the same buffer twice and eluted linearly with 0–1 M NaCl solution after His-rhHIF-1α loading was completed. The eluate was collected in batches and protein samples were judged by electrophoresis with SDS–PAGE.

After purified with ion-exchange chromatography, the His-rhHIF-1α was cleaved by thrombin for 20 h at 4°C to

remove the tags according to the GST Gene Fusion System Guideline (Pharmacia). The solution was then added to a 1.5 ml His-tag affinity chromatography column (Pharmacia) according to the manufacturer's protocols. The gleaned solution was dialyzed in order to be changed to a more suitable buffer, and then judged by SDS–PAGE and western blotting analysis, which uses anti-His-tag or anti-HIF-1α monoclonal antibody. The cleaved rhHIF-1α was concentrated by a 30 kDa ultrafiltration tube (Millipore) and filtrated at 16°C. The sample was then added to a prewashed Ready-Sepharose-200 column (Pharmacia) and eluted with 25 mM Tris–HCl, pH 8.0 (stress ≤0.3 MPa, flow-rate ≤0.5 ml/min). The eluate was gleaned in batches overnight and protein samples were judged by electrophoresis with SDS–PAGE and the absorbance were read (Ultraspec 3000, Pharmacia Biotech, Cambridge, UK) at 280 nm. Protein samples were preserved at −80°C with 16% ultra pure glycerin (Sigma) for further usage.

Assay for bioactivity of rhHIF-1α

The sequences of hypoxia-responsive element (HRE) probes were 5′- ggcgcgtgctacgtgatcaggccccccagc- 3′ (Top) and 5′-gctgggggggcctgatcacgtagcacgcgcc- 3′ (Bottom) including the core sequence of "acgtg", which were synthesized by *Sbs China* (Semenza 1999; Narravula and Colgan 2001). The probes were end labeled with γ-^{32}P by T4 polynucleotide kinase in the following reaction mixture: "upper strand" oligonucleotide (1.5×10^{-5} M), 1 μl; T4 kinase buffer, 2 μl; T4 kinase, 1 μl; γ-^{32}P-ATP(3000 Ci/mmol), 10 μl; MilliQ water, 6 μl. The reaction was run for 1 h at 37°C followed by 10 min at 68°C, then was allowed to cool to room temperature. The labeled oligo was annealed with the lower strand in the following reaction mixture: γ-^{32}P labeled template oligonucleotide, 20 μl; 5 × annealing buffer (50 mM Tris, pH 8.0; 5 mM EDTA, pH 8.0; 250 mM NaCl), 10 μl; "upper strand" oligonucleotide (1.5×10^{-5} M), 4 μl; "lower strand" oligonucleotide (1.5×10^{-5} M), 5 μl; MilliQ water, 11 μl. The reaction was run for 5 min at 95°C, then put into 70°C water bath with power shut off and was allowed to cool to room temperature. The double DNA strains were extracted with standard phenol/chloroform technique at the end of the reaction.

The rhHIF-1α protein sample was adjusted to a concentration of 50 μg/ml. Five 1.5 ml eppendorf tubes were prepared with 0 ng, 2 ng, 5 ng, 10 ng, 50 ng rhHIF-1α per tube respectively and 1 μl γ-^{32}P labeled probe (10^4cpm). Add 4 μl fresh 5 × binding buffer (50 mM Tris HCl, pH 8.0, 750 mM KCl, 2.5 mM EDTA, 0.5% Triton-X 100, 62.5% glycerol, 1 mM DTT) into each tube and bring the total volume to 20 μl with ddH_2O. The protein and DNA reactions were conducted in a 37°C water bath for 10 min and the results were judged by fresh made 4% non-denaturing polyacrylamide gel electrophoresis (Native-PAGE). The electrophoresis conditions were 200 V in 0.2 × TBE buffer at 37°C. When the electrophoresis finished, the gel was dried, put into a cassette (Pierce) accompanied by 3–5 leafs of X-sensitization film and then fixed and placed at −80°C. One leaf was taken out per day and was undergone photographic developing.

Results and discussion

Expression of rhHIF-1α fusion protein

E. coli BL21 (DE3) is a prokaryote host specifically designed for the pET vector system, and yields a high quality product under the control of T7 promotor (Huxtable

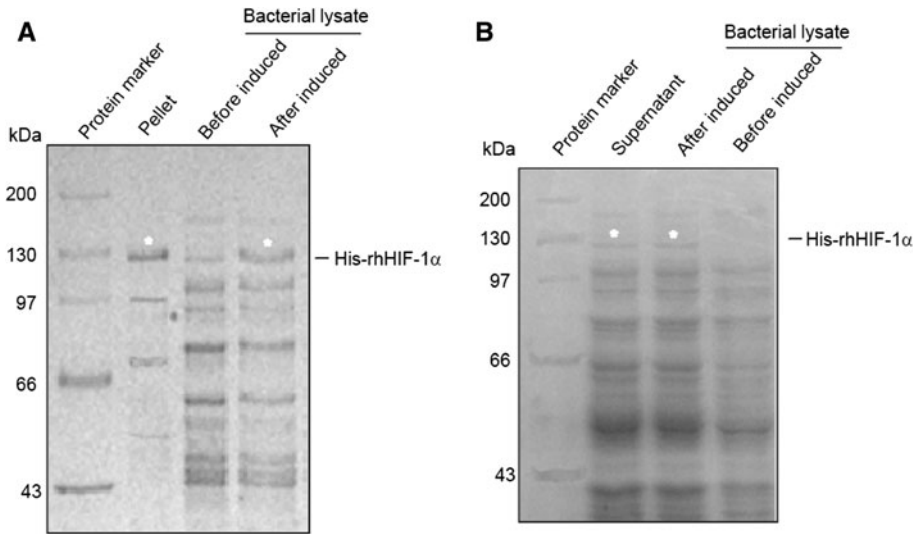

Fig. 1 Expression of His-rhHIF-1α fusion protein at 28°C (**A**) and at 37°C (**B**). Lysates were obtained from *E. coli* BL21 cells transformed with pET-32a-hif-1α with or without 0.5 mM IPTG induction, and were analyzed on 8% SDS–PAGE gels. Please note that the His-rhHIF-1α fusion protein (*asterisk*) was presented as inclusion bodies at 28°C and about 120 kDa

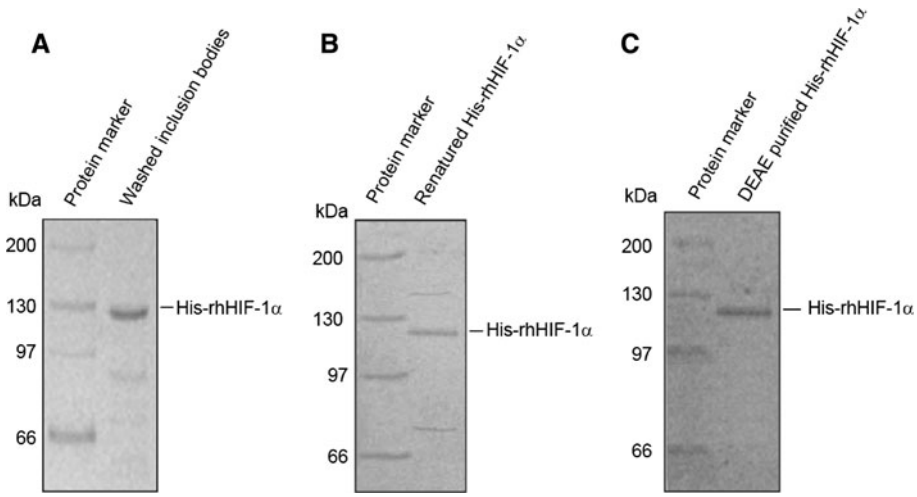

Fig. 2 SDS–PAGE analysis of firstly purified His-rhHIF-1α. **A** Purified inclusion bodies after washed with 2 M urea. The protein samples were analyzed on 8% SDS–PAGE gel. **B** 4% SDS–PAGE analysis of His-rhHIF-1α fusion protein after refolding. **C** 8% SDS–PAGE analysis of His-rhHIF-1α after DEAE ion-exchange

et al. 1998). Proteins expressed by pET-32a (+) vector have Trx-tag, His-tag, thrombin, enterokinase sites and the fusion protein can be cleaved by thrombin or enterokinase to remove tags without affecting the function and structure of protein. We expressed the rhHIF-1α fusion protein by pET-30a (+) vector and pET-32a (+) vector respectively and found that the product of pET-30a (+) vector were soluble but degraded rapidly, while that of pET-32a (+) vector in cryo-temperatures and hypoxic conditions were presented as inclusion bodies (data not shown). So, we chose the pET-32a (+) as our expression vector. The molecular weight of His-rhHIF-1α was about 120 kDa either at 28°C or at 37°C (Fig. 1A, B) due to the Trx-tag and His-tag. Although the protein expressed at 28°C was presented as inclusion bodies, the yield was higher than that expressed at 37°C which was soluble. Moreover, the optimum time for induction was $OD_{600} = 0.4$, and the optimum concentration of IPTG was 0.5 mM (data not shown). $CoCl_2$ and low oxygen concentration promoted not only expression efficiency of the fusion protein but also its stability (data not shown).

Purification of rhHIF-1α

When the *E. coli* was fully lysed, it looked like sheets of milling-sand-shade under microscopic examination but not the original rod-like thallus. Ivory-enamel-like inclusion bodies were clearly seen after centrifugation, and then they were washed and judged by SDS–PAGE (Fig. 2A). Thereafter, the inclusion bodies were solubilized and the denatured fusion protein was refolded by dialysis approach (Fig. 2B). In our experiment, we obtained about 75% protein recovery rate.

Each protein has its specific optimum renaturation concentration (Graeber et al. 1996). In our system, the optimum concentration of His-rhHIF-1α was 0.5–0.8 mg/ml. DNA analysis showed that the theoretical isoelectric point (p*I*) of rhHIF-1α (2481 bp, coding for 827 aa residues) should be 5.83, but in operation, we found the optimum pH of the buffer was 8.0, which was much different from our expectation. We thought that the pH of the buffer was related to pET-32a (+) vector, which expressed proteins having large tags at the same time.

Ion-exchange chromatography and affinity chromatography are the two most frequently used methods to purify proteins (Graeber et al. 1996). Each protein has diverse binding ability to certain ion-exchange chromatography medium. With the addition of Trx-tag, His-tag and S-tag, the pH of His-rhHIF-1α changes considerably, which affects binding to medium and penetration of columns. In our system, the His-rhHIF-1α did not bind the column in DEAE fast flow medium and was all collected in the

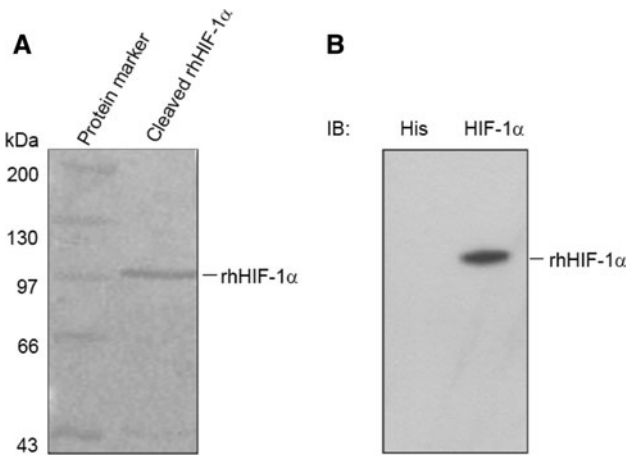

Fig. 3 Purification of rhHIF-1α with thrombin cleavage and Ni–NTA affinity chromatography. **A** SDS–PAGE analysis of rhHIF-1α after thrombin cleavage and Ni–NTA affinity chromatography. **B** Western blot analysis of rhHIF-1α after thrombin cleavage and Ni–NTA affinity chromatography. The membrane was cut lengthwise direction between the two lanes and incubated with anti-His and anti-HIF-1α antibodies respectively. Note the purified rhHIF-1α is about 97 kDa

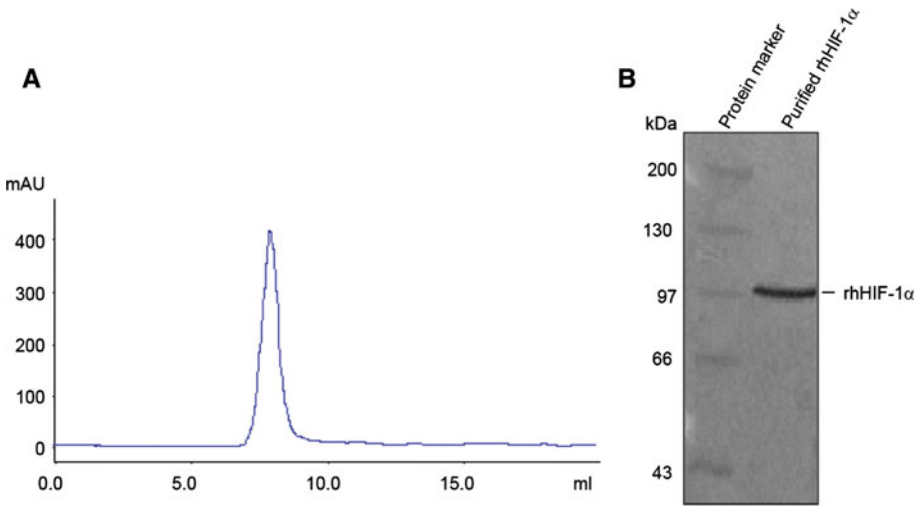

Fig. 4 Purification of rhHIF-1α by Sepharose-200. **A** The absorbance of the eluate protein samples from Sepharose-200 column at 280 nm. Eluant from the Sepharose-200 column showed a single tight and symmetrical peak at 280 nm, which suggested that the pure protein was acquired. **B** SDS–PAGE analysis of purified rhHIF-1α by Sepharose-200

penetrate-through solution (Fig. 2C). Affinity chromatography which is used to separate proteins is based on their different affinities to a ligand. After cleaved by thrombin, rhHIF-1α can not bind the Ni–NTA medium and were purified by Ni–NTA affinity chromatography, which was confirmed by a single clear band about 97 kDa visualized by SDS–PAGE and western blotting analysis (Fig. 3A, B), indicating that all the tags were removed. Thereafter, rhHIF-1α was further purified by Sepharose-200 column and the eluant from the column showed a single tight and symmetrical peak at 280 nm, which suggested that the pure protein was acquired (Fig. 4A). At the same time, SDS–PAGE showed a single band of protein about 97 kDa (Fig. 4B).

Characterization of rhHIF-1α bioactivity

Electrophoretic mobility shift assay (EMSA) is a good technique to detect the DNA–protein interaction since the shift velocity of DNA–protein complex was slower than that of DNA alone. Under hypoxic conditions, The HIF-1α and β complex binds to the HRE of hypoxia-inducible target genes, such as EPO and VEGF, to promote their transcription (Carmeliet et al. 1998; Iyer et al. 1998; Ryan et al. 1998). To test the bioactivity of purified rhHIF-1α, we designed the HRE probe including the core sequence of "acgtg" and labeled it with ^{32}P at the 5′ tip of the template chain (Semenza 1999; Narravula and Colgan 2001). Radiometry of a 1 μl aqueous solution was above 10^4 cpm. After reacted with rhHIF-1α, the DNA–protein complex was then separated by Native-PAGE and analyzed by autoradiography (Fig. 5). The middle clear bands were the HRE-rhHIF-1α complex and the lower bands were free HRE. The results showed that the best dose of rhHIF-1α was 10 ng because reaction saturation was achieved at this point. These data provided a strong support for physical

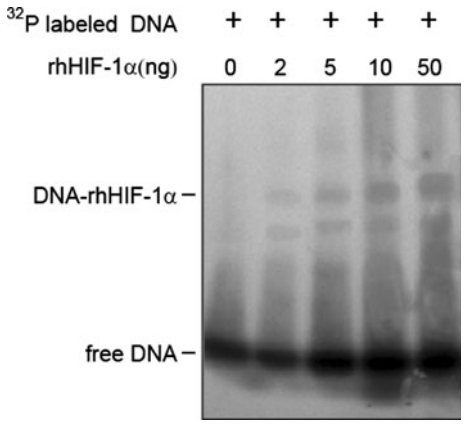

Fig. 5 Function characterization of rhHIF-1α with EMSA. The purified rhHIF-1α was allowed to bind to ^{32}P labeled HRE probes in appropriate binding buffer. The DNA–protein complex was then separated by 4% SDS–PAGE and analyzed by autoradiography. The *middle clear bands* were the HRE-rhHIF-1α complex and the *lower bands* were HRE probe alone

interaction (directly or indirectly) between rhHIF-1α and HRE, implying physical association ability of rhHIF-1α with other factors such as CBP/p300 (Chan et al. 2002). These results indicated that the purified rhHIF-1α has normal DNA-binding activity.

Conclusion

We successfully expressed and purified rhHIF-1α from *E. coli*, and further tested its bioactivity in vitro. The rhHIF-1α was active in vitro after the cleavage of tags. The purified rhHIF-1α could be used for many studies such as HIF-1α antibody production in rabbits. Overproduction and purification system for the rhHIF-1α developed in this study would be invaluable in future studies of the functions of HIF-1.

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