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RESEARCH PAPER

Expression and Purification of Soluble Recombinant Human Endostatin in *Escherichia coli*

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Abstract Endostatin, a 20 kDa C-terminal fragment of collagen XVIII, is a specific inhibitor of endothelial cell proliferation and angiogenesis. In the present study, we produced soluble and biologically active recombinant human endostatin (rhEndostatin) in *Escherichia coli* by expressing *via* fusion with solubility-promoting peptides and optimizing the expression conditions. The rhEndostatin was expressed *via* fusion with glutathione S-transferase (GST) and NusA protein, respectively. It revealed that NusA protein enhanced the production of soluble rhEndostatin; but GST didn't. By optimizing the expression conditions, the production of soluble NusA-rhEndostatin fusion protein was about 50% of total cellular proteins and about 90% of the products appeared in the cellular supernatant fraction. The soluble NusA-rhEndostatin fusion protein was purified by one-step hydrophobic interaction chromatography and NusA was removed by thrombin. Then rhEndostatin was purified by affinity chromatography and gel filtration chromatography. As a result, a simple and economical purification procedure for rhEndostatin isolation was obtained. The biological activity of the rhEndostatin was demonstrated *in vitro* using a human vascular endothelial cells (HuVECs) proliferation assay. Our study provides a feasible and convenient approach to produce soluble and biologically active rhEndostatin.

Keywords: Human endostatin, soluble expression, *Escherichia coli*, purification procedure

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1. Introduction

Endostatin was initially isolated as a 20 kDa protein from the conditioned cell culture media of a hemangioendothelioma cell line by O'Reilly in 1997 [1]. It has 184 amino acid residues, including 4 cysteine residues and 40% hydrophobic residues with high hydrophobic property. Endostatin has a zinc binding site and a heparin binding site, which is essential for its molecular stability and biological activity [2-4].

Endostatin can inhibit specifically proliferation of vascular endothelial cells and potently interferes with angiogenesis and tumor. Repeated administration of endostatin regressed tumors to "dormant" state [5,6]. Therefore, endostatin was deemed as a new potential anti-tumor drug.

Recombinant endostatin had been produced by many types of expression systems, such as mammalian cell [7], yeast (*Pichia pastoris*) [8], and *E. coli* [9-11] systems. However, the mammalian cell and the yeast expression systems are more expensive and time-consuming than the *E. coli* system. The *E. coli* expression system with its ability of rapid growth, well-characterized genetic background, high expression level, and cheapness, is widely used for the production of proteins on an industrial scale. One significant problem, however, is that heterologous proteins often cannot be correctly folded and deposited as

insoluble inclusion bodies and the refolded yields of proteins are usually low. Because of its high hydrophobic property, recombinant endostatin is more likely to form inclusion bodies than other proteins [9]. During *in vitro* renaturation or refolding process, the recovery of soluble and active endostatin was very low [11]. Therefore, it is necessary to express recombinant human endostatin in a soluble form by *E. coli*.

In the present study, we produced soluble and biologically active recombinant human endostatin (rhEndostatin) in *E. coli* by expressing *via* fusion with a solubility-promoting peptide, NusA and optimizing the expression conditions. On the side, we also developed a simple, economic purification procedure for rhEndostatin isolation to produce the recombinant human endostatin on an industrial scale. The biological activity of rhEndostatin was demonstrated *in vitro* using a HuVEC cell proliferation assay.

2. Materials and Methods

2.1. Chemicals, enzymes, and materials

The restriction endonucleases (*EcoR* I and *Sal* I), *Taq* DNA polymerase, and T4 DNA ligase were purchased from Takara (Dalian, China). *E. coli* JM83, *E. coli* Orogami (DE3), and the expression vector pET43.1a were purchased from Novagen (China). The expression vector pGEX-4T-1 was kindly provided by Dr. Ma ying-yuan (Institute of biological engineering, East China University of Science and Technology). Isopropylthio-D-galactoside (IPTG), Ampicillin, EDTA, thrombin, bFGF (basic fibroblast growth factor), DMEM Medium, and fetal calf serum (FCS) were purchased from Sigma. HuVEC cell lines were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences. *E. coli* strain JM83 was used for the transformation and propagation of recombinant plasmids. *E. coli* strain origami (DE3) with glutathione reductase (*gor*) and thioredoxin reductase (*trxB*) mutations, which greatly facilitated disulfide bond formation in the cytoplasm, was used as host to express recombinant proteins. The ether-650M column (Toyopearl HIC resin) was purchased from Dayou Chromatography Technicao Service Inc. (Shanghai). The heparin-Sepharose CL 6B column and preparative Superdex G-75 were purchased from GE (formerly Amersham bioscience).

2.2. Construction of expression plasmids

The plasmid pMD-hES containing the human endostatin cDNA had been constructed previously by our laboratory. The plasmid pMD-hES was used as template to obtain the human endostatin gene (*hES*) by PCR. The forward primer was 5'-GC**GAATTC**CATAGCCATCGTGATTTC-3', in which the restriction enzyme site *EcoR* I was represented in bold and followed by the sequence encoding the N-terminus of rhEndostatin. The reverse primer was 5'-CG**GTCGAC**CTACTTGGAGGCAGTCAT-3', which was designed to add a translation stop codon (CTA) after the C-terminus of rhEndostatin, and the restriction enzyme site *Sal* I (in bold) was added for the cloning convenience. The amplified PCR fragment about 550 bp was cloned into *EcoR* I and *Sal* I sites of pGEX-4T-1 or pET43.1a to obtain the expression plasmids pGEX-hES or pET-hES. The construct was transformed into *E. coli* JM83 and checked by complete DNA sequencing of the inserted fragment.

2.3. Expression of recombinant proteins

Recombinant plasmids pGEX-hES or pET-hES were transformed into origami (DE3) to yield expression strains origami (DE3) (pGEX-hES) or origami (DE3) (pET-hES). The expression strains were cultured in LB medium with 100 mg/L ampicillin at 37°C until the OD_{600} reached 0.5, at which IPTG was added to a final concentration of 1 mM. Five hours later, the cells were harvested by centrifugation with 12,000 × g for 10 min at 4°C.

In order to obtain more soluble active recombinant protein, the recombinant strain, harboring the plasmid pET-hES or pGEX-hES, was induced with different concentrations of IPTG (1, 0.5, and 0.1 mM) at different temperatures (37, 30, and 25°C) for different induction durations (5, 7, 9, and 11 h).

In order to yield enough expression supernatant for purification of recombinant proteins, a single bacterial colony carrying pET-hES was used to inoculate an overnight preculture in LB medium with 100 mg/L ampicillin at 37°C. This preculture was used to inoculate 1 L of fresh LB medium with 100 mg/L ampicillin. Approximately 4 h later, the OD_{600} of the bacterial broth reached 0.5. The flasks were then transferred to a 25°C incubator for 30 min and 0.1 mM IPTG was added to initiate a 9 h induction.

2.4. Protein expression analysis

The above harvested cells were resuspended in the buffer B containing 50 mM, Tris·Cl; 150 mM, NaCl; 5 mM, EDTA; and pH 8.0, then were disrupted by sonication (for small-scale processes) or using the French press (for larger-scale processes), centrifuged, and separated into soluble and insoluble fractions. Both the fractions were analyzed by 10 or 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The Coomassie brilliant blue-stained SDS-PAGE was scanned with an UVP White/Ultraviolet trans-illuminator, and analyzed with software Grab-it 2.5 and Gel-work (UVP) for estimating the purity of recombinant protein and rhEndostatin. The protein estimation was

determined by the Bradford (Biorad) method using BSA as standard [12].

2.5. Purification of recombinant proteins

The soluble NusA-rhEndostatin fusion protein was purified by hydrophobic interaction chromatography (HIC). In order to increase the hydrophobic ability of recombinant protein, ammonium sulfate was added slowly to the above expression supernatant with stirring on ice to give a final concentration of 1 M. After centrifugation with 12,000 × g for 10 min at 4°C, the supernatant containing recombinant protein was applied at 2 mL/min to an ether-650M (Toyopearl HIC resin) column (18 × 100 mm) and equilibrated with buffer A (buffer B containing 1 M ammonium sulfate). The protein fractions were eluted at 2 mL/min with a linear gradient of 0~100% B within 60 min and protein contents in gradient fractions were measured by absorbance at A_{280} with an on-line preparative UV detector. Fractions containing recombinant protein (NusA-rhEndostatin fusion protein) were pooled for the next purification step.

After purification, the NusA-rhEndostatin fusion protein was digested by thrombin and was applied to a heparin-Sepharose CL 6B column that had been equilibrated with buffer B. The column was equilibrated with buffer B and then eluted sequentially with 0.2, 0.4, and 0.6 M NaCl in buffer B. The 0.4 M NaCl eluted fraction was loaded on a gel filtration column (preparative Superdex G-75 by Amersham), previously equilibrated with buffer B.

2.6. Cell cycle analysis

The activity of rhEndostatin was detected on human vascular endothelial cells (HuVECs) by cell cycle analysis. HuVECs were maintained in DMEM supplemented with 10% FCS until 50~60% confluence. The medium was replaced with fresh DMEM containing 2% FCS and 5 ng/mL of basic fibroblast growth factor (bFGF) with or without rhEndostatin. After 72 h incubation at 37°C in 5% CO_2, the cells were trypsinized, washed gently with PBS, and then fixed in 70% ice-cold ethanol for 30 min. Cells were collected by centrifugation, and RNase was added to final concentration of 50 μg/mL and incubated at 37°C for 1 h, followed by staining with 5 mg/mL propidium iodide. Cells were assessed with a FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA).

3. Results

3.1. Expression of recombinant proteins

E. coli strain origami (DE3) was used as host for the expression of recombinant endostatin. The host strains, harboring the plasmid pGEX-hES or pET-hES, were cultured in LB with 100 mg/L ampicillin at 37°C. The results revealed that GST tag showed poor soluble protein production with the majority of the production found in the insoluble fraction (Fig. 1A) and NusA tag could enhance the production of soluble rhEndostatin, about 47% of the produced rhEndostatin existed in the soluble form (Fig. 1B).

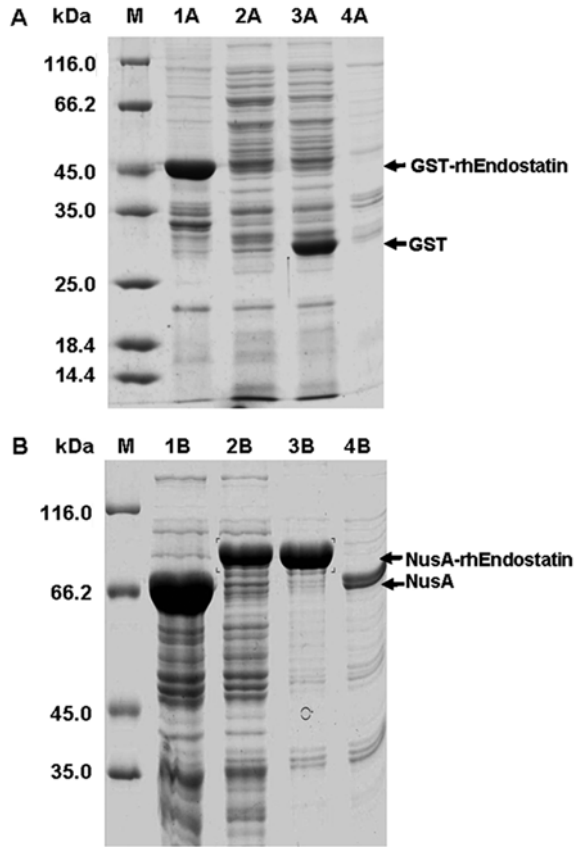

Fig. 1. Effects of different fusion tags on the solubility of rhEndostatin expressed by *E. coli* corigami (DE3) based strains harboring (A) pGEX-hES or (B) pET-hES. The expression was induced with 1.0 mM IPTG at 37°C for 5 h. The expressing cells were separated into soluble (supernatant) and insoluble (precipitation) fractions and analyzed by SDS-PAGE (12% for A and 10% for B). Lane 1A, precipitation from crushed cells of origami (pGEX-hES); Lane 2A, supernatant from crushed cells of origami (pGEX-hES); Lane 3A, supernatant from crushed cells of origami (pGEX-4T-1); Lane 4A, precipitation from crushed cells of origami (pGEX-4T-1); Lane 1B, supernatant from crushed cells of origami (pET43.1a); Lane 2B, supernatant from crushed cells of origami (pET-hES); Lane 3B, precipitation from crushed cells of origami (pET-hES); Lane 4B, precipitation from crushed cells of origami (pET43.1a); and Lane M, protein marker. The protein bands for GST-rhEndostatin, GST, NusA-rhEndostatin, and NusA are indicated by arrows.

In order to obtain more soluble active recombinant protein, the recombinant strain, harboring the plasmid pET-hES, was induced with different concentrations of IPTG at different temperatures for different induction durations as described previously. By optimizing the expression condi-

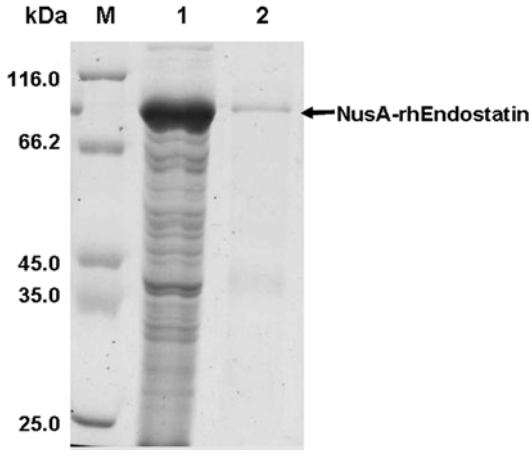

Fig. 2. Coomassie brilliant blue-stained SDS-PAGE (10%) analysis of proteins in origami (pET-hES) expression system under optimum conditions with 0.1 mM IPTG at 25°C for 9 h. Lane 1, supernatant from crushed cells of origami (pET-hES); Lane 2, precipitation from crushed cells of origami (pET-hES); and Lane M, protein marker. The protein band for NusA-rhEndostatin is indicated by an arrow.

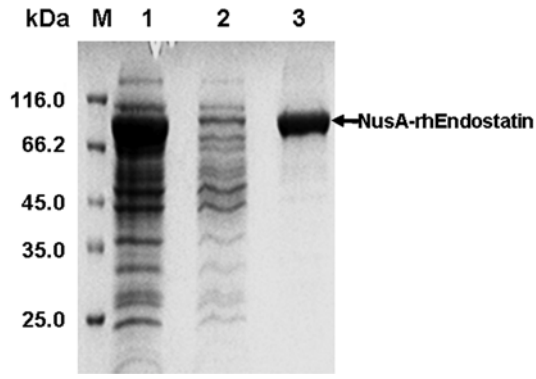

Fig. 3. Coomassie brilliant blue-stained SDS-PAGE (12%) analysis of purified NusA-rhEndostatin fusion protein by an ether-650M (Toyopearl HIC resin) column. Lane 1, supernatant from crushed cells of origami (pET-hES) with 1 M ammonium sulfate; Lane 2, flow through; Lane 3, fraction eluted by decreasing concentration of ammonium sulfate with a linear gradient of 1~0 M within 60 min; and Lane M, protein marker. The protein band for NusA-rhEndostatin is indicated by an arrow.

tions, the production of soluble NusA-rhEndostatin fusion protein increased to 50% of total cellular proteins and about 90% of the products appeared in the cellular supernatant fraction (Fig. 2), when the recombinant strain was induced with 0.1 mM IPTG at 25°C for 9 h. Using the similar strategy to optimize the expression conditions of the recombinant strain harboring the plasmid pGEX-hES, the GST-rhEndostatin fusion protein was still expressed as insoluble (data not shown).

3.2. Purification of recombinant proteins

In our construct, a His6 tag was fused to the N-terminus of endostatin in order to simplify purification. Unfortunately, poor results were obtained with His-taged NusA-rhEndostatin fusion protein in affinity chromatography specific for the His6 tag (data not shown). Therefore, other kinds of chromatography were tried, including anion-exchanged chromatography, cation-exchanged chromatography, and hydrophobic interaction chromatography (HIC). As a result, the NusA-rhEndostatin fusion protein was purified by one-step HIC using an ether-650M (Toyopearl HIC resin) column by optimizing the concentration of ammonium sulfate in loading buffer (Fig. 3).

In order to estimate the yield of the purified rhEndostatin, a 1 L culture of origami (DE3) carrying pET-hES was induced as described previously, and cell pellet was thawed and dissolved in 30 mL buffer B. Cells were disrupted by using the French press (1,000 Psi), necessitating at least 2 passages. The crude extract was then centrifuged twice at 12,000 × g for 10 min at 4°C. The supernatant was diluted in 200 mL buffer A (buffer B containing 1 M ammonium sulfate).

The sample was loaded on a previously pre-equilibrated column with buffer A and the recombinant protein was eluted in buffer B. Although the eluted fraction contained few contaminants (Fig. 4), the Nus-endostatin fusion protein could be digested by the thrombin directly in buffer B (50 mM, Tris·Cl; 150 mM, NaCl; 5 mM, EDTA; and pH 8.0) without desalting and replacing by other buffer, which could simplify purification process. After the NusA-rhEndostatin fusion protein was digested by the thrombin directly to remove NusA, the recombinant human endo-

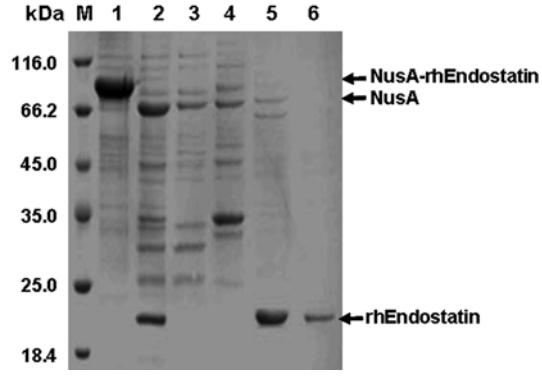

Fig. 4. Coomassie brilliant blue-stained SDS-PAGE (12%) analysis of purified recombinant human endostatin. Lane 1, NusA-rhEndostatin fusion protein purified by an ether-650M (Toyopearl HIC resin) column; Lane 2, NusA-rhEndostatin fusion protein digested by thrombin; Lane 3, flow through from heparin-Sepharose CL 6B column; Lane 4, 0.2 M NaCl eluted protein; Lane 5, 0.4 M NaCl eluted protein; Lane 6, purified protein from a preparative Superdex G-75 column; and Lane M: protein marker. The protein bands for NusA-rhEndostatin, NusA, and rhEndostatin are indicated by arrows.

Table 1. Purification of recombinant human endostatin from 1 L *E. coli* culture

Purification steps	Total Protein (mg)	Purity of fusion protein (%)	Purity of rhEndostatin (%)
Cell extract (supernatant)	285	50	
Ether-650M column	126.5	92	
Thrombin digest	126.5		21.6
Heparin-Sepharose	28.2		80.2
Superdex G-75	28.2		95.3

statin was purified by affinity chromatography using a heparin-Sepharose CL 6B column (5 mL). Most of rhEndostatin could bind tightly to the column. The 0.2 M NaCl elution could remove most of the impurity proteins, the following 0.4 M NaCl contained most of rhEndostatin with a purity of about 80% and the final 0.6 M NaCl could not elute any proteins. The purified protein migrated at M_r 20 kDa in reduced SDS-PAGE (Fig. 4). It was the same as that found in the previous report [8]. The protein fraction that eluted in 0.4 M NaCl solution was pooled and loaded on a gel filtration column (preparative Superdex G-75),

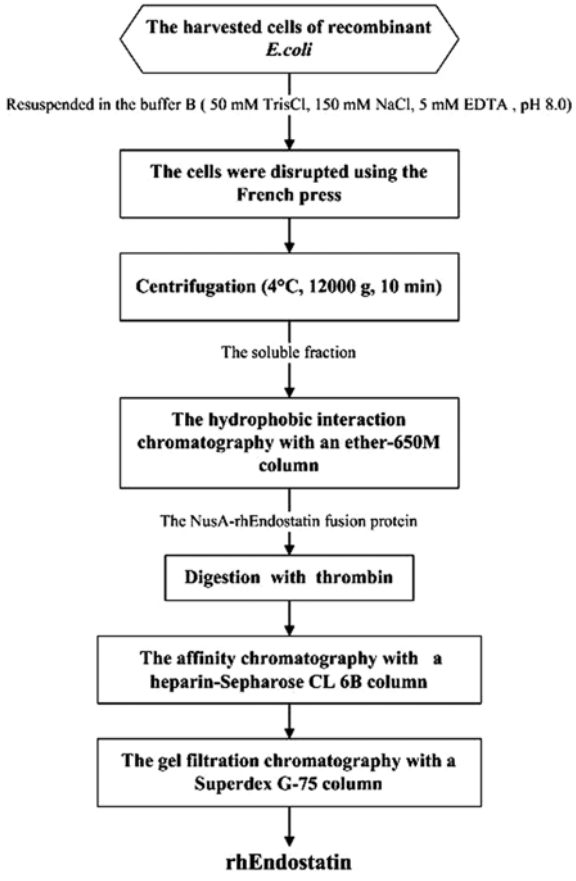

Fig. 5. The purification procedure for rhEndostatin isolation.

Table 2. Recombinant human endostatin affects HuVEC proliferation through a G1 arrest

Treatment	Cells in different phases (%)		
	G0/G1	S	G2/M
HuVEC + bFGF (Control)	58.8	10.9	30.3
HuVEC + bFGF + 5 μg/mL endostatin	67.1	9.1	23.9
HuVEC + bFGF + 10 μg/mL endostatin	73.6	6.7	19.8

previously equilibrated with buffer B. After being further separated using the preparative Superdex G-75 column, the purity of rhEndostatin in product protein was more than 95% as shown in SDS-PAGE analysis (Fig. 4). A final yield of 18 mg rhEndostatin was achieved from 1 L shaking flask culture of bacteria (Table 1). As a result, a simple and economical purification procedure for rhEndostatin isolation was obtained (Fig. 5).

3.3. Cell cycle analysis

The rhEndostatin was assayed for its inhibitory activity on HuVEC growth stimulated by bFGF. The cell cycle analysis (Table 2 and Fig. 6) showed a great change after treatment with different concentrations of rhEndostatin. The lowest percentage of cells in G0/G1 occurred in the control (58.8%) and the percentage increased with rhEndostatin concentration (up to 73.6%). The percentage of cells in S and G2/M phases decreased with increasing recombinant human endostatin concentration. These results suggest that one mechanism of endostatin effect on endothelial cell proliferation is through a G1 arrest.

4. Discussion

Recombinant proteins expressed in *E. coli* are often produced as aggregates called inclusion bodies. However in many applications, it is desirable to express target proteins in their soluble active forms. In a previous report, the recombinant endostatin expressed in *E. coli* was in the form of inclusion bodies and more than 99% of the protein was precipitated during refolding [1]. This limited its further purification and resulted in its costly preparation. In order to express soluble heterologous proteins in *E. coli*, two approaches have been used. One approach was to co-express molecular chaperones, which aid in protein folding. Xu *et al.* [10] reported that the co-expression of molecular chaperones, GroEL/ES and DnaK-DnaJ-GrpE, helped to decrease the aggregation of rhEndostatin. Another approach was the use of gene fusions. The carrier proteins used success in recent years in producing soluble heterologous proteins by *E. coli* included thioredoxin (TRX)

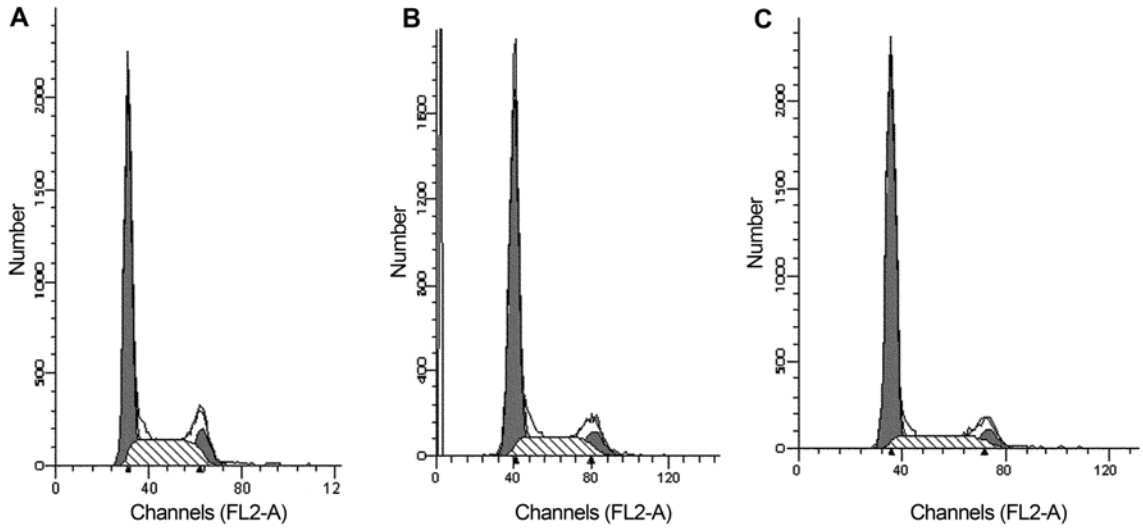

Fig. 6. The cell cycle analysis of HuVECs treated with rhEndostatin. HuVECs were exposed for 72 h to rhEndostatin in DMEM supplemented with 2% FCS and cells were assessed by FACScalibur (Becton Dickinson, Franklin Lakes, NJ, USA). (A) Control without endostatin; (B) cells were treated with 5 μg/mL rhEndostatin; and (C) cells were treated with 10 μg/mL rhEndostatin.

[13], maltose binding protein (MBP) [14], glutathione S-transferase (GST) [15], and NusA [16]. The solubility of the recombinant proteins was dependent on different carrier proteins and the characteristics of target proteins.

In the present study, we have shown that rhEndostatin with biological activity can be expressed in soluble form by *E. coli.* Firstly, GST and NusA were chosen as carriers fused to the N-terminus of rhEndostatin. Unfortunately, GST tag showed poor soluble protein production (Fig. 1A) with the majority of the production found in the insoluble fraction. While the NusA·fusion tag enhanced solubility of rhEndostatin (Fig. 1B). It was reported that greater than 85% of the expressed protein was soluble in tests with each of 4 different NusA fusion proteins [17]. Secondly, the host used in the study was the *trxB*/*gor* mutant origami (DE3), which greatly facilitates disulfide bond formation in the cytoplasm. It was reported that the *trxB*/*gor* mutant strains have the potential to enhance disulfide bond formation, and ultimately solubility and activity to a greater degree than the *trxB* only mutants [18]. Thirdly, the expression conditions were shown to be very important in the study. In general, conditions that decrease the rate of protein synthesis, such as low induction temperature or low inducer concentration, tend to increase the percentage of target protein in soluble form. In the present study, when the induction conditions of recombinant strain was changed from 1 mM IPTG, 37°C, and 5 h to 0.1 mM IPTG, 25°C, and 9 h, the percentage of target protein in soluble form was increased from 47% to 90%. On the other side, growing at 25°C is more convenient since it can save the energy at the room temperature.

We also developed a simple, economic purification process for rhEndostatin isolation. By optimizing the concentration of ammonium sulfate in loading buffer, the NusA-rhEndostatin fusion protein was purified by one-step HIC using an ether-650M (Toyopearl HIC resin) column at 1 M ammonium sulfate in loading buffer. Because the recombinant fusion protein was eluted by buffer B (50 mM, Tris·Cl; 150 mM, NaCl; 5 mM, EDTA; and pH 8.0), it could be digested by thrombin directly without desalting and replacing with other buffer, which reduced the purification steps. Another crucial parameter in our protocol was the use of buffer B (50 mM, Tris·Cl; 150 mM, NaCl; 5 mM, EDTA; and pH 8.0), which enabled us to purify the protein on different chromatography columns. After affinity chromatography, the protein fraction eluted by 0.4 M Nacl in buffer B could be further purified and desalted by gel filtration chromatography, which also made purification process simple. As a result, about 18 mg of rhEndostatin with a purity of SDS-PAGE homogeneity of 95% was finally obtained from 1 L flask culture (Table 1).

The rhEndostatin expressed by the *E. coli* system was in soluble form with a molecular weight of M_r 20 kDa, in agreement with the size obtained from other sources [8,19]. Some studies showed that the activity of endostatin was dependent on the heparin-bonding site of endostatin [20,21]. The recombinant protein could bind to a heparin-sepharose column in the present work, which implied that the recombinant endostatin was folded properly. The results presented in this study offer a new method for expressing and purifying human endostatin in soluble form with biological activity by *E. coli*.

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ing the expression vector pGEX-4T-1. This study was supported by the Fujian Provincial Nature Science Foundation (C0740012), the foundation for innovative Research Team of Jimei University (2006A002) and the Key Research Foundation of Fujian provincial department of Science and Technology, China (2009N0042).

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