

Research Article



## Cloning and Expression of Recombinant Human Endostatin in Periplasm of *Escherichia coli* Expression System

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### Abstract

**Purpose:** Recombinant human endostatin (rhEs) is an angiogenesis inhibitor which is used as a specific drug in the treatment of non-small-cell lung cancer. In the current research, we developed an efficient method for expressing soluble form of the rhEs protein in the periplasmic space of *Escherichia coli* via fusing with pelB signal peptide.

**Methods:** The human endostatin (hEs) gene was amplified using synthetic (hEs) gene as a template; then, cloned and expressed under T7 lac promoter. IPTG was used as an inducer for rhEs expression. Next, the osmotic shock was used to extraction of protein from the periplasmic space. The presence of rhEs in the periplasmic space was approved by SDS-PAGE and Western blotting.

**Results:** The results show the applicability of pelB fusion protein system usage for secreting rhEs in the periplasm of *E. coli* in the laboratory scale. The rhEs represents approximately 35 % (0.83mg/l) of the total cell protein.

**Conclusion:** The present study apparently is the first report of codon-optimized rhEs expression as a fusion with pelB signal peptide. The results presented the successful secretion of soluble rhEs to the periplasmic space.

### Introduction

Endostatin (Es), an angiogenesis inhibitor, is firstly identified by O'Reilly et al. It is a 20 kDa protein generated from the non-collagenous carboxyl-terminal end of collagen XVIII by proteolysis from large precursor proteins.<sup>1</sup> Es has a compact structure composed of two heparin-binding sites, two disulfide bonds, and a zinc binding site at the N-terminus. The disulfide bonds are essential for the compactness, stability, and activity of Es.<sup>2</sup>

Recombinant human Endostatin (rhEs) is a specific drug for treatment of non-small-cell lung cancer that was approved by the State FDA in China. The main advantage of rhEs administration as an angiogenesis inhibitor is the lack of toxicity and drug resistance that makes it a broad-spectrum anti-angiogenesis and tumor suppressing agent in a variety of cancers. Meanwhile, as a protein, its clinical application has some problems such as insolubility, instability, high price and need for high dosage.<sup>3</sup>

The rhEs has been expressed by eukaryotic and prokaryotic expression systems such as yeast, mammalian cell, insect cell, baculovirus, and *Escherichia coli* (*E. coli*).<sup>4-6</sup> *E. coli* expression system has many advantages including, rapid growth, high expression level, and well-characterized genetic background, a large number of mutant host strains and expression vectors and cheap cultivation. Thus, it is widely used for recombinant protein production.<sup>4,7,8</sup>

However, a significant disadvantage of this system is that heterologous proteins are often folded incorrectly (especially in proteins with disulfide bonds) and deposited as insoluble inclusion bodies in the cytoplasm.<sup>9</sup> Also, as hydrophobic protein, expression of rhEs in *E. coli* produces insoluble inclusion bodies in the cytoplasm.<sup>10,11</sup> The expression of proteins in periplasm via fusing with signal peptide in N-terminal residue, is one of the common methods for avoidance of inclusion bodies in the cytoplasm.<sup>12</sup> In the most cases, the periplasmic expressions of recombinant proteins facilitate downstream process, including, folding and in-vivo stability as well as production of soluble and bioactive proteins at a reduced process cost. A variety of methods have been reported to solve these obstacles in rhEs production.<sup>13-16</sup> Regarding the advantages of periplasmic expression of recombinant proteins and clinical importance of rhEs, the aim of this research was to express recombinant rhEs in the periplasmic space of *E. coli*.

### Material and Methods

#### Reagents

Enzymes including, NcoI, XhoI, T4DNA ligase, Pfu DNA polymerase, Gene Ruler DNA Ladder Mix (Cat. No. SM0334) and PageRuler Unstained Protein (Cat. No.

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SM0661) were obtained from Thermo Scientific (USA). Mini Plus™ -Plasmid DNA Extraction and Viogene® Gel/PCR DNA Isolation System kits were purchased from Viogene (New Taipei City, Taiwan). PCR reagents were purchased from Cinnagen (Tehran, Iran). Agar, tryptone, yeast extract, kanamycin, ampicillin and IPTG were obtained from Sigma-Aldrich (St. Louis, MO, USA). Prestained Protein Ladder was from Sinaclon (Tehran, Iran). All chemicals used in SDS-PAGE were purchased from Merck (Darmstadt, Germany). Endostatin Human ELISA kit was obtained from Abcam (Cambridge, UK).

### Bacterial strains, plasmids, Cell lines, media

The *E. coli* strains DH5 $\alpha$  and BL21 (DE3) (Pasteur Institute, Iran) were used as cloning and expression hosts respectively, pET26b (+) as expression vector was obtained from Pasteur Institute of Iran. Clone JET™ PCR Cloning Kit was from Thermo Scientific Company. Primers were from Shine Gene Company (Shanghai, China). In addition, the interest synthetic construct prepared from Shine Gene Company (Shanghai, China) as cloned sequence in pUC57 plasmid.

### Construction of synthetic gene and amplification

The human Endostatin coding sequence from human collagen XVIIIa gene (GenBank accession No. AF184060.1) was taken for codon optimization. Gene optimization and synthesis was done by Shine Gene Bio-Technologies Company (Shanghai, China). This construct was sub cloned in pUC57 including these sequences; NdeI restriction site, alkaline phosphate signal peptide coding sequence (GenBank accession No. M 13763.1), hEs coding sequence and XhoI restriction site.

Further, the hEs gene was amplified using pUC57-phoA sp-hEs as a template. PCR was performed using the Eppendorf thermal cycler with a set of primers in a total volume of 40  $\mu$ L. The reaction mixture contained 4  $\mu$ L of 10  $\times$  PCR buffer, 4  $\mu$ L MgCl<sub>2</sub> (20 mM), 0.7  $\mu$ L for each dNTP (2.5mM), 1.5  $\mu$ L plasmid DNA, 0.6  $\mu$ L of each primer and 1  $\mu$ L of Pfu DNA polymerase (5 units/  $\mu$ L). The program for PCR was one cycle at 95°C for 5 minutes, 30 cycles with denaturation at 95°C for 35 seconds, annealing at 60°C for 40 s, then 1 minute at 72°C and final extension at 72°C for 7 minutes. The amplification products were electrophoresed on the 1% agarose gel and visualized after SYBR Green I staining. The sequences of primers were hEs F: TCATCACCACCATGGCGCACTCTCACCG and hEs R: GATCCGATAATTTGGCTCGAGTCA. The Unique NcoI and XhoI sites (underlined bases) were added to amplified hEs synthetic gene, using these primers. In addition, for avoiding of frame shifting during the translation of hEs transcript, two nucleotides (CG, Bolded bases) were added after the NcoI site.

### Constructing of the hEs expression vector

The NcoI-hEs-XhoI amplified fragments were purified with Viogene® Gel/PCR DNA Isolation kit and then

ligated into pJET1.2/blunt cloning vector, using Clone JET™ PCR Cloning Kit (Thermo Scientific Company) following manufacturer's instructions. The *E. coli* DH5 $\alpha$  competent cells were transformed by calcium chloride method.<sup>17</sup> With the recombinant plasmid (pJET-hEs) for plasmid amplification and selected on LB agar with 100  $\mu$ g /ml ampicillin. For confirmation of successful transformation, a single colony of transformants was grown in 3 of ml LB medium containing ampicillin, followed by colony PCR. For further confirmation, clones containing recombinant vectors were analyzed by sequencing.

Further, the pJET-hEs and the pET26b (+) vector were double-digested with the NcoI and the XhoI restriction enzymes. Following, the hEs gene was inserted just after a pelB leader sequence in the expression vector pET26b (+). The recombinant vectors were transformed into the competent *E. coli* BL21 (DE3) by calcium chloride method.<sup>17</sup> Transformants were selected on LB agar containing kanamycin (50  $\mu$ g /ml). Then, the clones containing hEs gene were confirmed by colony PCR. Subsequently, the accuracy of the inserted fragment in positive clone was confirmed by DNA sequencing. The bacterial stocks were kept at -70 °C in 20 % (v/v) glycerol for long-term usage. The detail of construction steps is demonstrated in Figure 1.

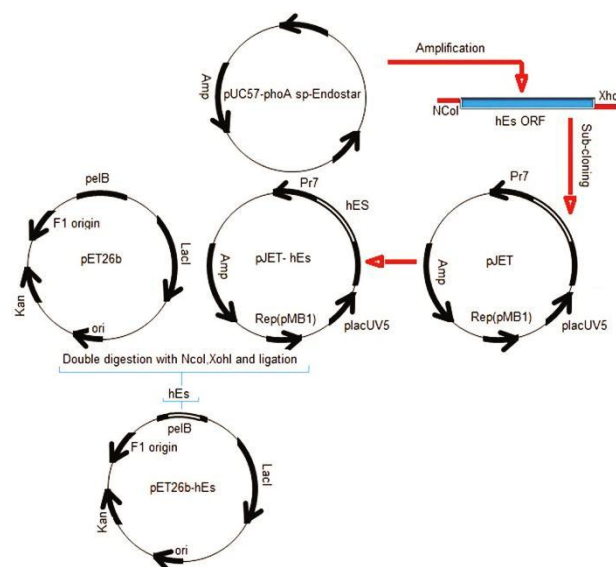


Figure 1. Construction of the expression vector (pET26b-hEs).

### Expression of rhEs

A single colony of *E. coli* BL21 (pET-hEs) was inoculated in 5 ml of LB medium that supplemented with kanamycin (50 $\mu$ g/ml) and incubated overnight at 37 °C in a shaking (180 rpm) incubator. Then, 2 ml of starter culture was transferred into 500 ml Erlenmeyer flask with 50 ml of the same broth containing kanamycin (50 $\mu$ g/ml) and was incubated in a shaker incubator (37°C, 180rpm). The cell growth rate was checked during cultivation using optical density measurements at 600 nm every one hour. In following, the cells were induced with 0.5 mM IPTG at OD600 of 0.6. A part of

the cultivation was used as negative control without adding IPTG. After 7 hours of additional growth at 37 °C, cells were harvested through centrifugation at 8000 g for 15 min at 4 °C.

### Isolation of the periplasmic rhEs

The isolation of the rhEs, which was expressed in the periplasm, was done by preparing cell fractions according to the literature.<sup>18,19</sup> Briefly, the culture was centrifuged at 8000g for 15 minutes at 4 °C. Then, the pellet was suspended in 10ml of buffer (50 mM Tris-HCl, 5 mM MgSO<sub>4</sub>, 18% sucrose, 0.1 mM EDTA, pH 8.0) and stirred slowly with magnetic stirrer at 4 °C for 10 minutes to mix thoroughly. The mixture was centrifuged at 10000 g for 15 minutes and the supernatant was collected as the periplasmic fraction. The total soluble protein was analyzed by the Bradford method (Quick Start™ Bradford Protein Assay, BioRad) using serum bovine albumin as a standard.<sup>20</sup>

### Purification

The supernatant contains rhEs was applied for purification. The crude supernatant containing soluble rhEs was concentrated using an Amicon Ultra centrifugal filter (Ultra-15, MWCO 10 kDa, Z706345 Sigma). Concentrated sample was loaded onto a 1mL SP Sepharose (GE Healthcare Life Science) column that had been equilibrated with 50 mM Tris-HCl, 5 mM MgSO<sub>4</sub>, 0.1 mM EDTA, pH 6.0 at a flow rate of 0.5 mL/min. The column was washed with 10 mL of the equilibration buffer. The bound rhEs eluted sequentially with different concentrations of NaCl (100 to 1000mM) in Tris buffer. The peak fraction containing the rhEs was collected, pooled and desalted by dialyzing (overnight) at 4°C against 10mmol/L Tris buffer (pH 6.0). The dialyzed sample was then loaded onto a HiPrep 16/60 Sephacryl S-100 HR (GE Healthcare Life Science) equilibrated with Phosphate buffer for further purification. All chromatography was performed at 4°C. Also, Endostatin Human ELISA kit was used to obtain rhEs concentrations, following manufacturer's instructions.

### SDS-PAGE and Western blot analysis

The expressed and purified rhEs was checked using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reduced conditions as described by Laemmli.<sup>21</sup> Briefly, about 10-20 µl of samples were homogenized in SDS sample loading buffer. The total polyacrylamide concentration was 12% for separating gel and 4% for stacking gel. Protein bands were imaged using Coomassie Brilliant Blue R250. In addition, western blot analysis was applied for further identification of produced rhEs. Briefly, Proteins were transferred onto a polyvinylidene fluoride (PVDF) membrane. The membranes were blocked with blocking buffer for 2 h at 37°C and incubated with rabbit anti-endostatin polyclonal antibody (ab3453) overnight at 37°C. After four times washing with phosphate-buffered saline (PBS), the membranes were incubated with Goat

Anti-Rabbit IgG H&L (HRP) (ab6721) for 1 h at room temperature and followed by three times washing with phosphate-buffered saline (PBS). Proteins were visualized by incubation with ECL Western Blotting Substrate (ab65623).

## Results

### Amplification of the hEs gene

The wild type hEs gene has 31 rare codons including: 3CGG, 3AGG for Arginine, 8CCC for proline, 4 TCC, 2 TCA, 1 AGT, 4TCG for serine and 6 GGG for glycine. These codons were replaced with preferred *E. coli* codons in synthetic hEs. A summary of codon usage in wild type and codon optimized synthetic hEs gene is shown in Table 1. CAI applied to estimate the adaptation of codon optimized hEs to *E. coli* codons, was 0.878. The optimized gene had 75.8% of identity with respect to the wild type hEs gene. The construct analyzing showed that there is no cryptic splice donor, cryptic splice acceptor, prokaryotic ribosome binding site, RNA destabilizes sequence and shin-dalgarn sequence. Additionally, restriction analyses showed no restriction sites that may interfere with cloning procedure.

Subsequently, the hEs was amplified using specific primers and pUC57- phoA sp- hEs as a template that showed a 592bp band on 1% agarose gel (Figure 2). This fragment contains hEs coding sequence (555bp), NcoI and XhoI restriction sites.

The PCR product was inserted into pJET1.2/blunt cloning vector (between the nucleotides 371 and 372) and transformed into competent cells of *E. coli* DH5a for propagation (as mentioned in Material and Methods). White- red screening method confirmed the authenticity of transformation. Following, colonies containing recombinant plasmids were confirmed by colony PCR (data are not shown). Also, the positive clones were further confirmed by DNA sequencing (ShineGene Bio-Technologies, Inc., China).

### Construction of the pET26b-rhEs expression vector

The plasmid pET26b (+) was used to create expression vectors that targets rhEs into the *E. coli* periplasm. The protein-coding sequence of the hEs gene was placed between the NcoI and XhoI sites of the pET26 (+). The recombinant pET26b (+) - hEs contained a T7 promoter, Lac operon, N-terminal pelB signal sequence, multiple cloning sites, rhEs encoding gene and T7 terminator. Following the transformation of *E. coli* BL21 (DE3), the positive clones were selected on LB agar containing kanamycin (50µg/ml). The transformed colonies that contain recombinant pET26b (+) - hEs plasmids were PCR positive against the hEs gene specific primers (Figure 3). The correct orientation of the hEs gene in the pET26b (+) - hEs plasmid was confirmed by double digestion with NcoI and XhoI restriction enzymes. Digestion of the pET26b (+) - hEs plasmid using restriction enzymes (Xho I, Nco I) produced two expected fragments (data are not shown). Digestion results showed the hEs gene had been inserted into the

pET26b+ plasmid. Sequencing analysis of the recombinant pET26b (+) - hEs plasmid with the mentioned primers established that there were no amplification mistakes and frame shift in sequence of the

cloned hEs gene. Also, the coding sequence of hEs was placed correctly in downstream of peIB signal sequence at the 5' region and the two stop codons placed at the 3' end of gene before the XhoI site.

**Table 1.** Comparison of codon usage for *E. coli* in wild and optimized hEs gene based on Ikemura classification.

Codons (Number)	<sup>a</sup> <i>E. coli</i> Kazusa (%)	<sup>b</sup> <i>E. coli</i> high exp (%)	Wt.hEs (%)	Opt.hEs (%)	Codons (Number)	<sup>a</sup> <i>E. coli</i> Kazusa (%)	<sup>b</sup> <i>E. coli</i> high exp. (%)	Wt.hEs (%)	Opt.hEs (%)
<b>Ala (19)</b>	-	-	-	-	<b>Pro (10)</b>	-	-	-	-
GCC	23.87	16.14	57.89	0.00	*CCC	5.63	1.63	80	0
GCG	27.99	32.30	21.05	5.26	CCG	19.35	71.89	20	100
GCT	17.36	27.54	10.52	84.21	<b>Ser (20)</b>	-	-	-	-
GCA	21.60	24.01	10.52	10.52	AGC	15	24.33	40	0
<b>Arg (15)</b>	-	-	-	-	*TCG	8.52	7.39	20	0
CGC	18.38	32.97	37.5	0	*TCC	9.29	26.56	20	0
*CGG	6.49	0.80	20	0.00	*TCA	9.94	4.79	10	0
*AGG	2.56	0.29	20	0	*TCT	10.94	32.41	5	100
CGT	18.92	64.25	6.66	100	*AGT	10.73	4.52	5	0
<b>Asp (8)</b>	-	-	-	-	<b>Trp (4)</b>	-	-	-	-
GAC	18.83	46.05	100	12.5	TGG	13.78	100.00	100	100
GAT	32.38	53.95	0	87.5	<b>Val (9)</b>	-	-	-	-
<b>Asn (4)</b>	-	-	-	-	GTG	23.47	26.81	66.66	0
AAC	21.20	82.75	100	100	GTC	14.04	13.45	22.22	0
<b>Cys (4)</b>	-	-	-	-	GTT	20.04	39.77	11.11	100
TGC	5.99	61.15	75	100	<b>Lys (5)</b>	-	-	-	-
TGT	5.35	38.85	25	0	AAG	13.05	21.45	100	0
<b>Gly (16)</b>	-	-	-	-	AAA	35.60	78.55	0	100
GGC	25.66	42.83	56.25	0	<b>Thr (7)</b>	-	-	-	-
*GGG	11.58	4.36	37.5	0	ACC	21.39	53.60	42.86	100
GGT	24.93	50.84	6.25	93.75	ACG	13.76	12.65	42.86	0
*GGA	1.61	1.97	0	6.25	ACT	11.02	29.08	14.28	0
<b>Glu (7)</b>	-	-	-	-	<b>Phe (10)</b>	-	-	-	-
GAG	18.80	24.65	100	85.72	TTC	15.62	70.92	80	90
GAA	18.83	75.35	0	14.28	TTT	22.46	29.08	20	10
<b>Gln (8)</b>	-	-	-	-	<b>Ile (6)</b>	-	-	-	-
CAG	28.12	81.35	100	100	ATC	22.69	65.94	83.33	100
<b>Tyr (3)</b>	-	-	-	-	ATT	29.67	33.49	16.67	0
TAC	12.01	64.77	100	100	<b>Met (3)</b>	-	-	-	-
<b>Leu (20)</b>	-	-	-	-	ATG	25.95	100.00	100	100
CTG	46.4	76.67	75	100	<b>Stop</b>	-	-	-	-
CTC	10.08	8.03	25	0	TAA	1.99	-	0	50
<b>His (7)</b>	-	-	-	-	TGA	1.04	-	0	50
CAC	8.82	70.23	71.42	100	TAG	0.29	-	100	0
CAT	12.47	29.77	28.58	0	<b>GC%</b>	-	-	68%	55%

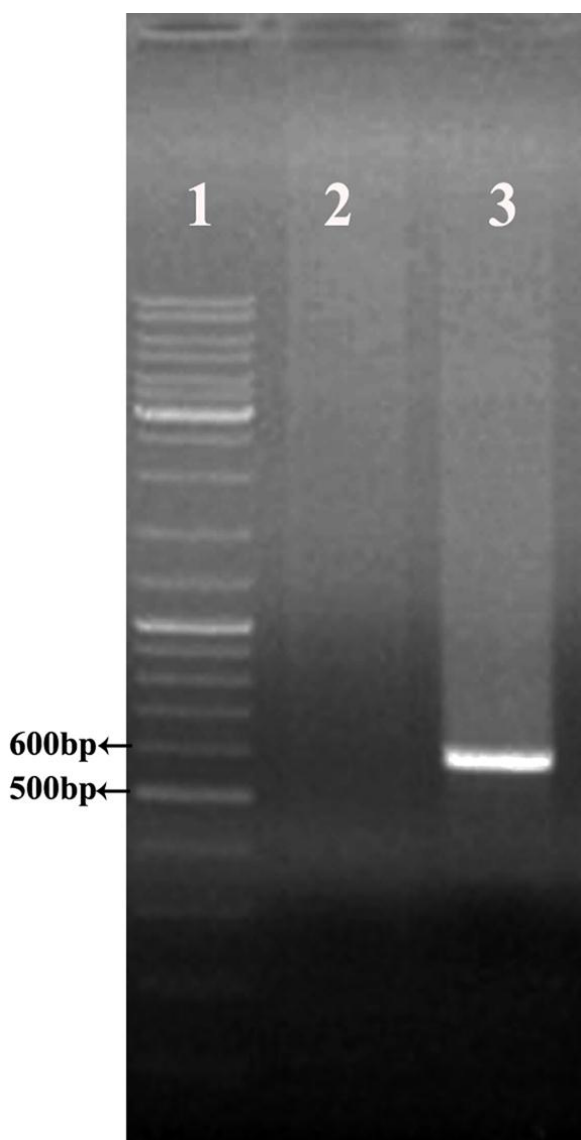
Codons with low frequency (<10%) are highlighted in yellow (light) and the most preferred codon for each amino acid is highlighted in gray (dark). The optimized hEs gene has lower preferred codons with low frequency than native gene. For gene optimization, rare codons frequency can cause translation error (3.94%) were set to 0.18 and the GC content reduced from 68 % to 55 %, closer to the average GC content of other highly expressed genes in *E. coli*.

<sup>a</sup> Taken and adapted from <http://www.kazusa.or.jp/codon/>, <sup>b</sup> the genes correspond to genes highly expressed during exponential growth and \*Rare codon.

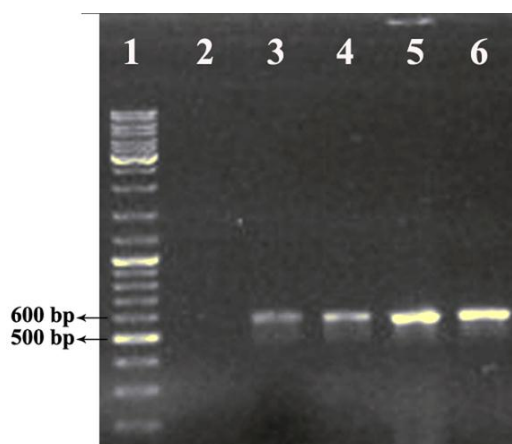
### Expression and analysis of recombinant human Endostatin

In order to analyze the expression of the rhEs protein, a single colony of *E. coli* BL21 (DE3) carrying the pET26b (+)-hEs vector was grown and induced with IPTG. The final concentration of IPTG was 0.5 mM with a 7 h incubation time at 37 °C. Following the induction, total protein was extracted and determined by the Bradford method showing a 2.37 mg/l concentration. Also, the total periplasmic protein of induced and non-induced cells were run and compared on a 12% SDS-

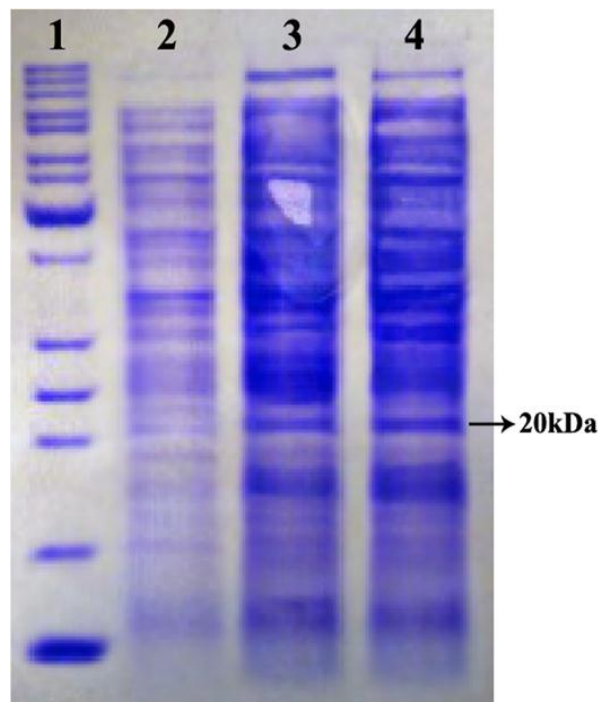
PAGE (Figure 4). The presence of corresponding protein bands approved the expression of the rhEs protein. The result from SDS-PAGE showed that there is a single band of 20 kDa corresponding to hEs (pET26b (+) - hEs). The identity and quantity of the expressed protein was further confirmed by an endostatin specific ELISA test. The rhEs represents about 35 % (0.83mg/l) of the total protein according to ELISA and Bradford results.



**Figure 2.** Amplification of hEs coding sequence using Pfu DNA polymerase. (lane1) Gene Ruler DNA Ladder Mix (Thermo scientific), (lane 2) negative control, (lane 3) amplified ORF of hEs (592 bp).



**Figure 3.** Colony-PCR reaction for randomly selected transformants (four colonies). (lane1) Gene Ruler DNA Ladder Mix (Thermo scientific), (lane 2) negative control, (lane3-6) PCR products at 592 bp representing positive colonies that had pET26b-hEs plasmid.

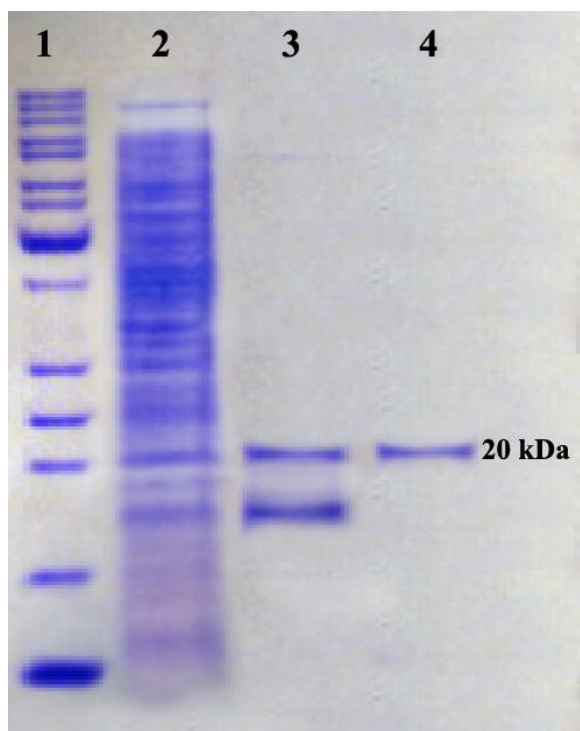


**Figure 4.** Expression analysis of rhEs in periplasmic space using 12% SDS-PAGE under reduced condition. The band about 20 kDa represents the produced rhEs. Lane 1, protein marker is 10 to 200 kDa from down to up; lane 2, total protein before induction with IPTG as a control; lane 3 and 4, total protein after induction with IPTG in recombinant pET26b.

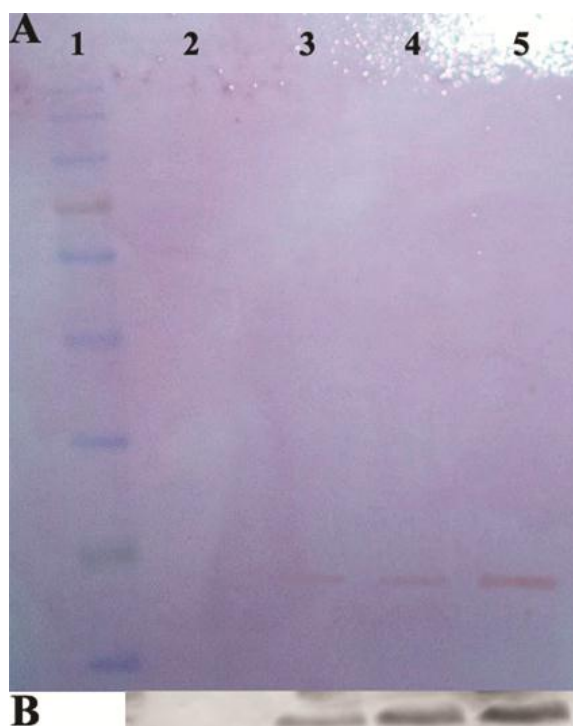
**Purification and identification of rhEs**

After isolating the periplasmic proteins, rhEs was purified using cation exchange chromatography and size exclusion chromatography sequentially. The SP-Sepharose column was used for rhEs purification as the first step of chromatography.

The recombinant protein bound strongly, and washing with the low-salt Phosphate buffer (0.1-0.3 M) removed other *E. coli*-derived proteins. Protein eluted with the 0.4 M NaCl fraction had a maximum amount of rhEs, but was contaminated with another low molecular weight protein (Figure 5). The protein fractions eluted at 0.4 M NaCl were pooled, concentrated and dialyzed against Tris Puffer; pH 6.5. The purified protein was further separated by size exclusion chromatography using a Sephacryl S-100 column. The purified rhEs presented approximately 20 kDa on SDS-PAGE (Figure 5) that was compatible with the theoretical molecular mass, and was found to be immune-reactive when evaluated by Western blot through using rabbit anti-endostatin polyclonal antibody (Figure 6). Finally, the results of SDS-PAGE, Western blot and ELISA assay confirmed the successful secretion of the expressed hEs protein into the periplasm.



**Figure 5.** SDS-PAGE analysis of the purified rhEs. Lane 1, protein marker; lane 2, crude protein; lane 3, rhEs protein purified by SP Sepharose column; lane 4, rhEs protein purified by Sephacryl S-100.



**Figure 6.** Western-blot analysis with specific rabbit anti-endostatin polyclonal antibody. Panel (A) is the ponceaus staining of pvdf membrane. Lane 2 is the control sample; lane 3-5 represent the sample related to periplasmic proteins after induction, cation exchange purified rhEs and size exclusion purified rhEs, respectively. Panel (B) is the immunoblotting image of samples shown in panel A. Lanes 2, 3, 4 and 5 correspond to the lanes 2, 3, 4 and 5 in panel A. Lane 1 is protein marker.

## Discussion

Recently, numerous studies have revealed the important role of angiogenesis in tumor growth. Therefore, inhibition of angiogenesis with anti-angiogenic agents is an attractive field of medical research. Among the anti-angiogenic agents, Es is an attractive candidate as anti-cancer agent with high efficacy, fewer side effects and no reported drug resistance.<sup>3,5</sup>

Several hosts, including *E. coli*, yeast, mammalian cell and baculovirus were used to produce rhEs.<sup>4,6</sup> Expressing heterologous proteins in *E. coli* system has some advantages to other systems, including, high expression level, rapid growth and low cost.<sup>10,22</sup> However, despite the many advantages of *E. coli* expression system, high-level expression is not easily achieved. The most common preventing factors in the high level protein expression by *E. coli* include codon bias, gene product toxicity, insolubility, mRNA secondary structure and mRNA instability.<sup>23-26</sup> In addition, there are several reports that proposed the use of synthetic genes to increase expression efficiency.<sup>27</sup> Considering the factors involved in the heterologous protein expression, in this study optimized synthetic hEs gene based on the Ikemura classification was applied to amplify hEs gene, followed by cloning and producing in *E. coli* BL21 (DE3).<sup>28</sup>

Another factor that limits the using of *E. coli* expression system is the lack of posttranslational modification. To overcome this problem, expression of recombinant protein in periplasmic space is one of the solutions. Secretory production of recombinant proteins into the periplasm has the following advantages: (a) providing purer product because of having fewer impurities than the cytoplasm (b) providing a more oxidative space than cytoplasm which results in facilitating the disulfide bond formation (c) lesser protease concentration which results in decreasing the degradation of heterologous proteins and the possibility of proper folding of proteins. Wild type signal peptides have been used successfully in *E. coli* for protein translocation into the periplasm.<sup>29,30</sup> There are several reports on the cytoplasmic expression of the hEs gene.<sup>4,5,14,15</sup> However, there is no article on the periplasmic expression of rhEs protein.

Thus, considering the structure of rhEs that has two disulfide bonds, periplasmic secretion strategy has been used for expression of rhEs via fusing with pelB signal peptide. The expression vector pET26b(+) harbors a pelB signal peptide at N-terminal of protein that transports the expressed protein into the periplasmic space.<sup>31</sup> Also, the powerful promoter of pET26b(+) vector allows for over-expression of the cloned gene.<sup>19,32</sup>

Periplasmic secretion of rhEs protein was attained because of the attendance of pelB signal peptide, which directly led the interest protein to periplasmic region.

But the produced soluble rhEs level was very low in comparison with *Du et al.* and *Xu et al.* findings which expressed rhEs in the cytoplasm.<sup>5,15</sup> *Du et al.* studied the effects of glutathione S-transferase (GST) and NusA protein on the solubility of rhEs in *E. coli*. They revealed that NusA protein increased the production of soluble

rhEs (18mg/L); but GST did not effect on the amount of produced rhEs.<sup>5</sup> Also, Xu *et al* produced soluble rhEs in *E. coli* employing DnaK-DnaJ-GrpE as molecular chaperone. The obtained yield of soluble rhEs was about 16mg/L.<sup>15</sup> This difference in the rhEs concentration can be due to the following reasons: (1) expression in the lab scale condition instead of fermenter, (2) choosing pelB as a signal peptide (3) culture condition (4) cultivation medium and (5) smaller space of periplasm than cytoplasmic space. Releasing periplasmic proteins are done with mechanical and physical cell disruption methods. Gao *et al.* reported that the physical cell disruption methods are safe and effective method than mechanical methods. In mechanical method, the selectivity of releasing proteins is low and purification process is difficult.<sup>33</sup> Thus, in this study osmotic shock method was adopted for the releasing rhEs from the periplasm in accordance with Cao *et al.* report.<sup>19</sup> The advantages of this method are low-cost, quickness and high selectivity in the protein releasing process.<sup>33</sup>

### Conclusion

Based on these findings, it is concluded that the designed construct, which pelB signal peptide fused with rhEs, could express rhEs protein into periplasmic space of *E. coli*. Considering the low level expression of the target protein, optimization of culture condition variables and other effective factors seems to be needed.

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### Ethical Issues

Not applicable.

### Conflict of Interest

Authors declare no conflict of interest in this study.

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