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Cloning, Expression, and Cost Effective Purification of Authentic Human Epidermal Growth Factor With High Activity

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Abstract

Background: Epidermal growth factor (EGF) plays a fundamental role in the healing of wounds relating to skin damage, the cornea, and the gastrointestinal tract.

Objectives: The aim of this study is the cloning, expression, and purification of recombinant human EGF (rhEGF), and an assessment of its activity.

Materials and Methods: In the present experimental study, a synthetic pET28a (+) -*hEGF* construct was prepared. In order to ligate *hEGF* into pET24a (+), the PCR technique was performed, using special primers that possess restriction enzyme sites, which are also located in appropriate sites in pET24a (+). After transferring this construct into *E. coli* cells, protein expression was performed under standard conditions. Protein solubilization was done by urea. hEGF purification and refolding were carried out using gradient dialysis against the urea. We used RP-HPLC to compare between rhEGF and commercial rhEGF as a control. Finally, an MTT assay was performed to assess the viability of the NIH 3T3 cells treated with various concentrations of rhEGF.

Results: Dialysis after urea solubilization caused precipitation of unwanted proteins, resulting in achievement of purified EGF with > 90% purity, without the need for expensive and time-consuming process. The MTT assay results showed that our rhEGF activate significantly higher proliferation of NIH 3T3 cells in comparison to the control (P-values were < 0.0001), in total concentrations and times evaluated

Conclusions: Via our purification protocol, a sufficient amount of bioactive recombinant human epidermal growth factor was obtained in just a few affordable steps, with superlative purity.

Keywords: Epidermal Growth Factor, MTT, NIH 3T3 Cells

1. Background

Epidermal growth factor (EGF) is a thermo stable 53 amino acid residue polypeptide (molecular weight 6045) with a single chain and three intramolecular disulfide bonds, which was isolated from adult male mouse submaxillary glands by Cohen in 1962 (1-3). It is so named because of its potent stimulating effect on epidermal proliferation and keratinization (2, 4-6). The human EGF (urogastron) was isolated and identified by Gregory in 1975. It possesses molecular weight and an amino acid sequence that is identical to that of mouse EGF, with the exception of 16 amino acids that have equal biological activity and potential for binding to the same membrane receptors (7). With regards to the essential role of EGF as a mitogen in the proliferation of a variety of cells in vivo and in vitro, it is widely used in clinical and cosmetic fields (4). In fact, EGF first binds with the cell surface epidermal growth factor receptor and then activates tyrosine kinase in the receptor cytoplasmic domain after dimerization of the receptor. In this way, a signal transduction cascade is initiated, resulting in DNA synthesis and cell proliferation (8, 9). Since 1975, several unsuccessful attempts to extract a sufficient amount of hEGF from urine have been reported (10). Since 1988, scientists have used recombinant DNA technology to produce hEGF efficiently (11). Following this development, a variety of hosts (prokaryotic and eukaryotic), vectors, and purification methods were used, and investigators performed a growth-stimulating activity assessment of recombinant hEGF. Each of these researches has possessed numerous challenges, benefits, and drawbacks.

2. Objectives

The aims of the present investigation are: 1) cloning and expression of the human epidermal growth factor

in *E. coli* and purification of the hEGF protein with an authentic amino acid sequence, and 2) assessment of the growth-stimulating activity of hEGF in the NIH 3T3 cell line.

3. Materials and Methods

3.1. Reagents

All chemicals were purchased from Merck (USA) or Sigma (USA). *Escherichia coli* strain BL21 (DE3, star) and the plasmid vectors pET28a (+) and pET24a (+) were obtained from Novagen (USA). Restriction enzymes and *pfu* DNA polymerase were from Fermentas (Lithuania). Commercial recombinant human EGF was obtained from PeproTech (USA). Mouse anti-hEGF monoclonal antibody and standard polyclonal mouse anti-horse HRP conjugates, as anti-hEGF antibody were from Sigma (USA).

3.2. Recombinant hEGF-pET24a (+) Construct

In an earlier research in our lab (12), a pET28a (+) -*hEGF* construct, in which *hEGF* consisted of 165 bp (encompassing 55 amino acids including two additional amino acids), was prepared. This *hEGF* gene was optimized by manufacture, according to host codon preference. This plasmid was used as a template to amplify the fragment that coded authentic *hEGF*. The forward primer had an *Nde*I restriction site containing an initiator methionine codon, and the reverse primer was designed to include an *EcoR*I restriction site and stop codon. The flanking sequence was added on the 5' end of the recognition sites in order to cleave efficiently. The designed primers, using OLIGO software v. 7, are as follows:

Forward primer: 5'-GAATCAACATATGAACTCTGACTCTGAATGC-3' Tm = 61.1°C

Reverse primer: 5'-GTATCATGAATTCTTACGCAGTTCCCACCA-3' Tm = 61°C

The first and stop codons in the oligonucleotides are indicated in the bold and restriction sites that are underlined. PCR reactions were carried out using *Pfu* DNA polymerase. The PCR products were resolved by electrophoresis on a 3% agarose gel; following this, imaging and gel staining with ethidium bromide were performed. The PCR product was purified using a PCR product purification kit (Kawsar Biotech Company, Iran) and digested with *Nde*I and *Eco*RI. The pET24a (+) was digested with the same restriction endonucleases. Upon digestion conformity, both digested products were ligated at 16 °C for 4 hours using a ligation kit (Takara, Japan).

3.3. Host Transformation

E. coli BL21 star (DE3) competent cells were prepared by a chemical method (13). In brief, this method is based on the use of cool shock with $MgCl_2$ and $CaCl_2$ solutions. In the next step, these cells were transfected by expression vector pET24a (+) -*hEGF*. 1 mL LB broth was then added to the mixture, which was incubated at 37°C for 1 hour. This mixture was spread on LB agar with 80 μg/mL kanamycin (selective medium) to grow overnight at 37°C. Several colonies were randomly picked and individually placed in LB broth with 80 μg/mL kanamycin, and incubated overnight at 37°C in a shaking incubator. pET24a (+), with inserted *hEGF* gene, was extracted using a plasmid mini extraction kit (Bioneer, Korea). Three methods were utilized to test for successful gene insertion, as follows: 1) restriction endonuclease digestion followed by gel electrophoresis; 2) PCR with the same primers as used previously; and 3) sequencing using pET vectors' universal primers to confirm presence of any base deletion, insertion, or substitution.

3.4. Expression of Recombinant hEGF Protein

Several positive colonies were selected and grown in LB broth containing kanamycin at 37°C. When optical density at 600 nm reached 0.6, the cells were induced with 1.0 mM IPTG, grown for an additional 5 hours at 37°C, and 1 mL was harvested by centrifugation at 5,000 rpm for 10 minutes. Pellets were suspended in a lysis buffer (100 mM NaH_2Po_4, 10 mM Tris-HCl, pH 8) for an hour, and disrupted by sonication (6 times for 10 seconds each time). 10 μL of SDS-PAGE sample buffer was then added and heated in a water bath at 100°C for 5 minutes. In the next step, we performed electrophoresis on Tricine SDS-PAGE polyacrylamide 16% gel. The gel was stained with Coomassie brilliant blue R250 (14).

3.5. Immunoblot Analysis of the Recombinant hEGF

The recombinant hEGF was confirmed by western blot. This peptide was first resolved on Tricin SDS PAGE, and then transferred onto a nitrocellulose membrane (using the Bio-Rad Protean II system). The membrane was incubated overnight with 3% BSA (bovine serum albumin)/PBS (phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, and 4.3 mM Na_2HPO_4 7 H_2O, pH 7.3) as a blocking buffer. The membrane was then incubated for 1 hour in a 1:1000 dilution of a mouse anti-hEGF monoclonal antibody in the PBST (PBS contained 0.05% Tween). The protein bands were visualized in the presence of DAB (diaminobenzidine) following incubation, with 1:1000 dilution of the standard polyclonal mouse anti-horse HRP conjugate as an anti-hEGF antibody. After each stage, washing was performed 3 times using PBST (15).

3.6. Recombinant hEGF Purification

Recombinant hEGF was expressed as an inclusion body in *E. coli* cells. At first, purification of inclusion bodies was performed using detergent washing with sodium deoxicholate (16), as shown in Figure 1. Urea (8 molar) was used for solubilization of inclusion bodies; dialysis against urea (4 M, 2 M, 1 M, and 0 M) was then performed.

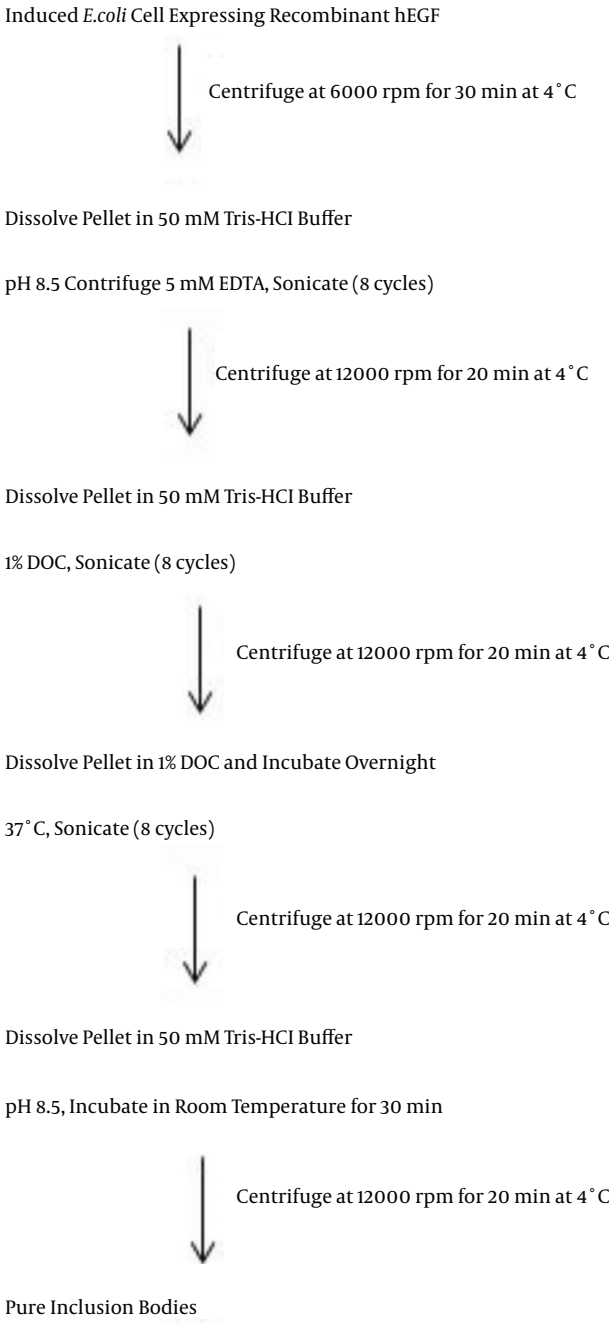


Figure 1. The Scheme for Purification of Inclusion Bodies From *E. coli* Cells Using Detergent Washing

3.7. Reverse Phase-High Performance Liquid Chromatography (RP-HPLC)

RP-HPLC analysis was performed in order to carry out a significant comparison between this recombinant hEGF and commercial recombinant hEGF (PeproTech, USA), as a standard assessment of rhEGF purity. A Kromasil C_{18} column of 250 × 4.6 mm, with a mobile phase containing buffer A: 0.5% trifluoroacetic acid in 20% acetonitrile and buffer B: 0.5% trifluoroacetic acid in 80% acetonitrile, was used. The flow rate was 2 mL/min. The column was kept at room temperature, and absorbance was read at 280 nm. The obtained profile was compared with the standard.

3.8. Mitogenic Activity Assessment

A MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide) test, with an NIH 3T3 cell line (mouse embryonic fibroblast cells), was used for the biological activity assessment. This assay is dependent on the reduction of the tetrazolium salt MTT by the mitochondrial dehydrogenase of viable cells to form a purple insoluble formazan product. First, the necessary sub-culturing process was carried out using 0.0125% Trypsin and 0.02% EDTA in Mg^{2+} and Ca^{2+} free PBS in incubation at 37°C and CO_2 (5%) in 90% DMEM with 10% calf serum. A pilot experiment was performed using a cell concentration of 1×10^4 cells/well in a 96-well microculture plate. The expressed rhEGF and commercial EGF were added to the wells (100 μL) in 4 different concentrations of EGF (1, 10, 100, and 500 ng/mL) (12, 17). Control experiments were carried out using the complete growth culture medium without rhEGF. The culture plates was incubated at 37°C in a CO_2 (5%) incubator for 24 and 72 hours. About 100 μL of the MTT solution (5 mg/mL in PBS, pH 7.4) was added to all wells, and the plates were incubated at 37°C in a CO_2 incubator. After 4 hours, 150 μL of dimethylsulfoxide (DMSO) was added to all wells to dissolve the formazan crystals, and optical density (OD) was measured at a wave length of 570 nm. The cell viability in each concentration of rhEGF was expressed as a percentage, relative to the control results. Three independent experiments were performed.

3.9. Statistical Analysis

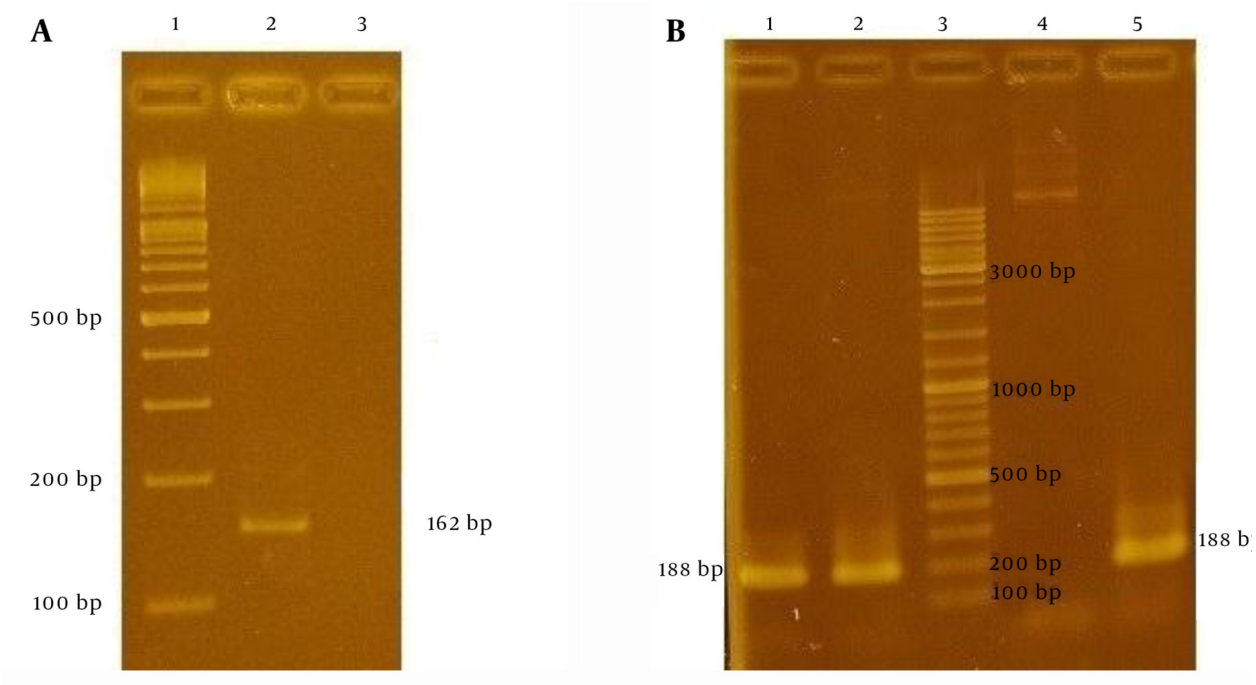
Statistical analysis was performed by one-way ANOVA, and Tukey's multiple comparison test was applied for evaluation of differences between the groups, using GraphPad Prism v6 software. The statistical operations included Mean (M), Standard Deviation (SD), Standard Error of mean (SEM), and confidence intervals (95% CI).

4. Results

4.1. hEGF Cloning and Expression

To obtain the *hEGF* gene from pET28a (+) plasmid, a PCR reaction was performed using the specific primers described earlier. The PCR products were electrophoresed on 3% agarose gel (Figure 2A).

Figure 2. The Amplification Result of *hEGF* Gene (162 bp) Within pET28a (+) -*hEGF* Construct on 1.5% Agarose Gel



Lane 1, DNA size marker; Lane 2, PCR product (188 bp); Lane 3, PCR negative control; B, the result of colony screening; Lanes 1 and 2, extracted plasmid amplified with the aforementioned primers; Lane 3, DNA size marker; Lane 4, Negative control of PCR; Lane 5, PCR positive control.

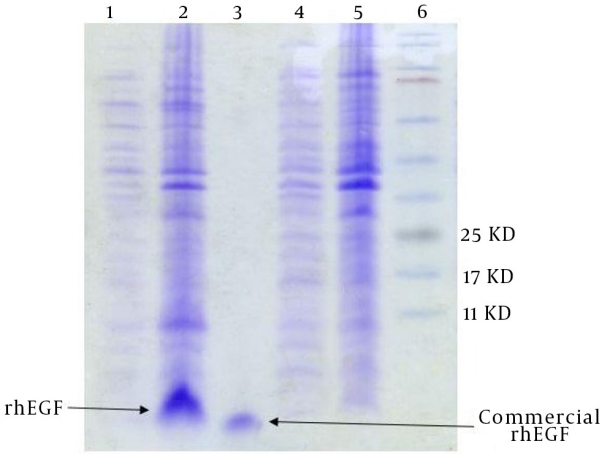
After purification, the PCR product and pET24a (+) vector were digested with *Nde*I and *Eco*RI restriction enzymes. Ligation reaction was performed using T4 DNA ligase, and the recombinant plasmid was transferred into a competent *E. coli* strain. Several colonies were found positive for *hEGF*, inserted using PCR screening (Figure 2B), and verified by restriction enzyme analysis of the plasmid DNA with *Nde*I and *Eco*RI to release the *hEGF* insert (data not shown). In addition, sequencing of pET24a (+) using pET vectors' universal primers confirmed 100% identity (data not shown) of the insert with subcloned hEGF.

After protein expression, Tricin SDS-PAGE 16% electrophoresis of cell lysis indicated that recombinant hEGF was expressed as an inclusion body (Figure 3).

4.2. Recombinant hEGF Purification

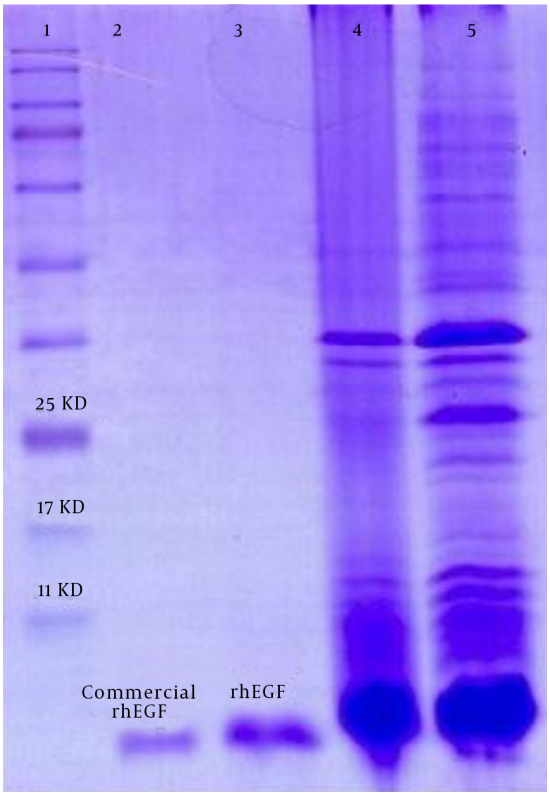
Initially, inclusion bodies were purified by a sodium deoxicholate-based method. The steps of this method are indicated in Figure 1. After inclusion of bodies' solubilization, we carried out dialysis against the urea gradient to remove urea. To facilitate refolding of recombinant proteins obtained from inclusion bodies, 0.1 to 1M arginine was customarily included in the solvents used for refolding the proteins by dialysis (18). After dialysis as shown in Figure 4, a single band of recombinant hEGF was observed in SDS PAGE. In fact, during the dialysis steps, almost all the unwanted proteins and the amount of rhEGF that was precipitated and purified were obtained.

Figure 3. Expression Analysis of rhEGF on 16% Tricin SDS-PAGE



Lanes 1 and 2, Soluble and insoluble intracellular fractions from the cultures of induced host cells; Lane 3, commercial rhEGF as positive control; Lanes 4 and 5, Soluble and insoluble intracellular fractions from the cultures of the uninduced cells; Lane 6, protein marker.

Figure 4. SDS PAGE Banding Pattern Before and After Dialysis



After centrifugation, the pellet and supernatant were electrophoresed. Lane 1, Protein marker; Lane 2, commercial rhEGF; Lane 3, Supernatant after dialysis and centrifuging; Lane 4, Pellet after dialysis without centrifuging; Lane 5, Lysate after processing by 8 M urea, before dialysis.

4.3. RP-HPLC Analysis

The final purified rhEGF was quantified by RP-HPLC. A chromatogram showed a main single peak (Figure 5A). This peak was related to rhEGF, as it completely matched commercial rhEGF. The purity was calculated and estimated at > 99%.

4.4. Western Blotting

Verification of the recombinant hEGF was done by Western blot technique (Figure 5B).

4.5. Biological Activity Assessment

Finally, to ensure that EGF had been properly folded and was therefore functionally active, we tested its ability to induce NIH 3T3 cells proliferation using an MTT assay. Results were recorded on both Day 1 and Day 3, as shown in Figure 6. The means ± SEM of the cell viability (% control) and P-values are shown in Tables 1 and 2, respectively. The P-values of the comparison between the control and the cells treated with 1, 10, 100, and 500 ng/mL rhEGF after Day 1 were < 0.0001, < 0.0001, < 0.0001, and 0.0201 respectively, and after Day 3 they were < 0.0001, < 0.0001, < 0.0001, and < 0.0001 respectively, which indicates that our rhEGF significantly increased cell proliferation. Additionally, as shown in Table 2, EGF obtained from the construct pET24a (+) *–hEGF* in all experiments, except the cells treated with 500 ng/ml on Day 1 and 1 ng/mL on Day 3, were significantly (P-value < 0.0001) more active than commercial EGF at the same concentrations.

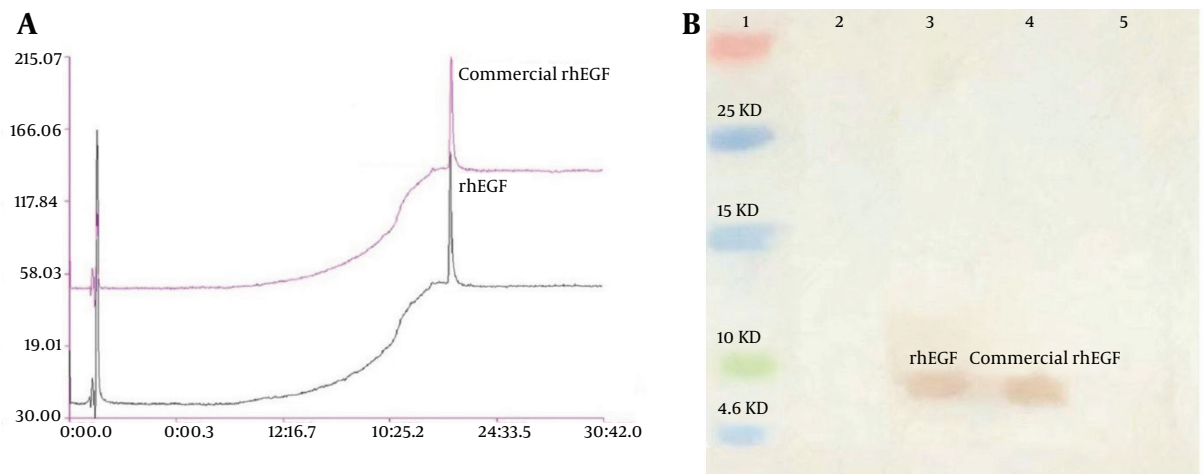
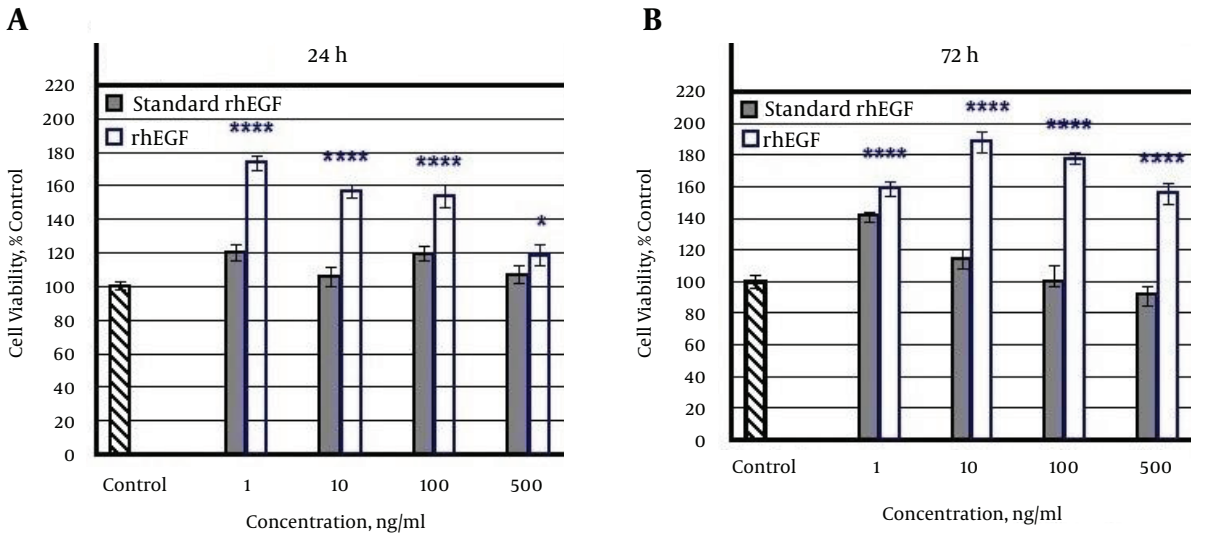


Figure 5. A, Chromatogram's alignment between produced rhEGF and commercial rhEGF as standard. A main single peak, observed in pure recombinant hEGF, was exactly the same as that of commercial hEGF; B, Western blotting technique for verification of rhEGF; Lane 1, protein size marker; Lane 2, Uninduced *E. coli* cell lysate; Lane 3, induced *E. coli* cell lysate comprising an rhEGF protein band; Lane 4, Positive control (PeproTech rhEGF as standard); Line 5, Negative control (BSA).

Figure 6. Biological Activity Assessment of rhEGF on NIH 3T3 Cell Line



Growth-promoting activity was monitored by cell proliferation. Diagrams show cell viability after A, 1 day and B, 3 days. Results show that the viability of cells treated by our rhEGF was significantly higher than that of the control. (*P > 0.05; **P > 0.01; ***P > 0.001; ****P > 0.0001).

Table 1. The Means ± SEM of Cell Viability (% Control) in Three Independent MTT Experiments[a]

	No Treatment (Control Group)	Treatment With Our Produced rhEGF, ng/mL				Treatment With Commercial rhEGF, ng/mL			
		1	10	100	500	1	10	100	500
Day 1	102 ± 1.15	173 ± 2.64	157.33 ± 2.18	154 ± 3.78	118.33 ± 3.75	120 ± 2.18	106.66 ± 3.38	119.67 ± 2.60	107.33 ± 2.90
Day 3	103 ± 1.15	157.3 ± 2.84	188.7 ± 4. 09	179.3 ± 4.7	154.7 ± 3.84	142 ± 2.08	114.7 ± 3.18	101.01 ± 5.85	91 ± 2.67

[a]Data are presented as mean ± SEM.

Table 2. Statistical Analysis of MTT Results, P-Values Demonstrated Between Our Produced rhEGF-Treated Cells, Cells Without Additive (as Control), and Commercial rhEGF at Different Times and Concentrations

Concentration (ng/mL) of Our Produced rhEGF	P-Value Compared to Control at the Same Concentration	P-Value Compared to Commercial rhEGF at the Same Concentration
1, Day 1	< 0.0001	< 0.0001
10, Day 1	< 0.0001	< 0.0001
100, Day 1	< 0.0001	< 0.0001
500, Day 1	0.0201	0.2267
1, Day 3	< 0.0001	0.1326
10, Day 3	< 0.0001	< 0.0001
100, Day 3	< 0.0001	< 0.0001
500, Day 3	< 0.0001	< 0.0001

5. Discussion

This study was an experimental study to produce a biologically active form of recombinant human epidermal growth factor via a synthetic construct. EGF is in high demand due to its potential uses in the healing of corneal epithelial wounds, upper respiratory tract, gastric and peptic ulcers, sulfur mustard wounds, and especially diabetic ulcers (19-24). Due to the application of EGF as a precursor of restorative ointments and the exclusive production of this protein, production of recombinant hEGF with an authentic amino acid sequence is an important issue in EGF studies. However, some commercial recombinant hEGF have 1 (initiator Met) or 2 amino acids that are in excess of the 53

original ones in hEGF. In this study, we have expressed biologically active rhEGF containing 54 amino acids (excess amino acid is the first methionine), which is identical to some commercial recombinant hEGF, with high purity in a cost-effective yet time-consuming process in *E. coli*. The viability of cells treated by our rhEGF was significantly higher than that those in the control group.

It was necessary to remove the excess amino acid at the start of the *hEGF* sequence within the previous construct (pET28a (+) -*hEGF*). Therefore, it was crucial to select the correct vector and restriction endonuclease. Finally, pET24a (+), *Nde*I, and *EcoR*I were selected.

Inclusion body solubilization process was carried out using 8 M urea. After solubilization, the sample was dialyzed. Through this process, almost all the unwanted proteins and the amount of our recombinant hEGF were aggregated and precipitated. In spite of precipitation of the rhEGF, the decrease in purification efficiency was neglected, because further expensive and time-consuming steps such as RP-HPLC and ion-exchange chromatography were eliminated.

Previously, several attempts have been made to purify recombinant human EGF (25-32). Brewer et al. (26) purified rhEGF using HPLC. Solid phase extraction and HPLC were used for rhEGF purification by Yadwad et al. (27), while Lee et al. (28) purified rhEGF with two refolding steps and an affinity chromatography method from inclusion bodies in *E. coli*, with a yield of 75%. Lee et al. (29) reported soluble rhEGF purification from *E. coli* culture broth by using expanded bed adsorption chromatography, with recovery of 94% of the proteins and 84% purity. Ferrer Soler et al. (30) expressed rhEGF proteins via 3 expressing constructs, comparing their activities by Western blot analysis to show 94% activity for cytoplasmic hEGF and 42.5% and 67.5% activity, respectively, for soluble and insoluble hEGF fused with thioredoxin. Solubilization was done by urea and guanidinum chloride, and purification was done using RP-HPLC and affinity chromatography in separate experiments. Tong et al. (31) reported partial purification properties of rhEGF from recombinant *E. coli* by expanded bed adsorption chromatography. Sharma et al. (32) purified rhEGF using EBA chromatography after solubilization with 8M urea. Final purification was done by ion-exchange chromatography.

Our purification protocol has one major advantage over these earlier investigations: there is no need to use expensive and time-consuming methods such as HPLC or other chromatography-based methods. In fact, during the refolding steps, unwanted proteins were precipitated by using a proper refolding buffer.

In this study, pET24a (+) was applied. Because of its strong and specific promoter, the pET system is the most powerful system yet developed for cloning and expression of recombinant proteins in *E. coli* (33). To date, a variety of host cells have been used for transfection; however, *E. coli* is still the most suitable choice. In fact, the use of *E. coli* for protein expression is well documented due to its advantages of low cost, easy transformation and fermentation, and high protein yields. However, some proteins, such as our recombinant protein, are insoluble and aggregate in inclusion bodies when expressed in *E. coli* cells. A biological assay must be carried out to ensure proper recombinant protein folding. EGF is a growth factor with a mitogenic effect on a variety of cells, including fibroblasts. Therefore, the mitogenic effect of hEGF is the outcome of its proper folding.

However, in some previous investigations (11, 34), EGF purified from inclusion bodies did not possess suitable bioactivity, as a result of misfolding. Here, we have successfully achieved the proper refolding of inclusion body proteins into bioactive forms. Surprisingly, the MTT results demonstrated that at both 24- and 72-hour timespans and in all concentrations, the viability of cells which were treated with our rhEGF was significantly higher than those treated with commercial rhEGF. The MTT results demonstrated that our rhEGF had significantly higher activity compared to commercial (PeproTech) EGF. We think there were two reasons for these results: 1) the commercial EGF did not fresh, and therefore its activity was possibly reduced; and (2) the refolding and purification procedures provided good conditions for maintaining the activity of rhEGF. Although we seem to have identified the causes, we could carry out a comparison and evaluation of the structures of our recombinant protein and the commercial one, in order to be sure. However, our main aim in the future should be to do more research into industrial-scale purification and production of rhEGF, using the protocol presented in this study.

The maximum activity increase observed at 10 ng/mL concentration in 72 hours was 182.5% of the control. RP-HPLC analysis showed that purification was completely successful, and the final protein obtained was almost 100% pure.

5.1. Conclusion

In conclusion, compared to other studies our purification protocol had one great advantage: a sufficient amount of bioactive protein was obtained in only a few steps, without the need for a further expensive and time-consuming process such as RP-HPLC to achieve a final purification with superlative purity.

Our results demonstrated that proper folding of rhEGF resulted in higher proliferation of NIH 3T3 cells, compared with the control. In fact, a cytoplasmic expression system is fairly efficient in producing recombinant hEGF.

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Footnotes

Authors' Contribution: Sara Pouranvari collected the data, carried out the laboratory operations, statistical

analysis, and interpretation of data, and wrote the manuscript. Firouz Ebrahimi designed and supervised the study, contributed to data analysis, and provided technical and material support. Gholamreza Javadi edited the manuscript and provided technical support. Bozorgmehr Maddah contributed material support and helped in editing of the manuscript.

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