EXPERIMENTAL STUDY

High expression level of human epidermal growth factor (hEGF) using a well-designed fusion protein-tagged construct in *E. coli*

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ABSTRACT

AIM: The study was aimed at design a good fusion construct that would successfully express the recombinant proteins and produce peptides in *Escherichia coli*. Two different constructs including human epidermal growth factor (hEGF) gene were designed to obtain an efficient expression level of hEGF. The hEGF sequence was inserted in pET32a vector containing thioredoxin (Trx) sequence and modified pET15b vector containing intein and elastin-like polypeptide (ELP).

METHODS: The vectors were transformed into *E. coli* TOP10F for multiplication and further into *E. coli* BL21 (DE3) to express protein. The hEGF expression was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG) while the expression levels were evaluated by SDS-PAGE and western blotting and compared by ImageJ analysis, BCA and Elisa assays.

RESULTS: The expression level after 2 hours of IPTG induction was significantly higher than after other induction times. ImageJ, BCA and Elisa analyses demonstrated that the Trx presence enhanced protein expression significantly when compared to ELP-intein-based construct.

CONCLUSION: The pET32a-Trx-hEGF construct had a higher expression than pET15b-ELP-intein-hEGF. Overall, considering Trx, the fusion protein in construct design can make it suitable to significantly express hEGF compared to ELP-intein while its combination with ELP-intein may improve the expression of the ELP-intein construct (Tab. 2, Fig. 7, Ref. 34). Text in PDF www.elis.sk.

KEY WORDS: human epidermal growth factor (hEGF), thioredoxin (Trx), elastin-like polypeptide (ELP), intein, expression.

Introduction

Human epidermal growth factor is one of the most important growth factors used to induce migration, adhesion, and differentiation of various cells in tissue engineering and regenerative medicine (1). In addition, it can expedite the healing of diabetic, dermal and corneal wounds, and induce the growth of cells in different origins (2, 3). Consequently, an industrial production of hEGF in large-scale is necessary in support of medicine and other fields and its market demands have become enormous in recent years. In 1962, Cohen isolated EGF for the first time from mouse sub-maxillary gland (4) and later in 1975, he isolated the latter growth factor from human urine (5). The hEGF gene is located in chromosome 4 and its expression leads to a single-stranded polypeptide consisting of 53 amino acids (6.5 kDa) and three disulfide bands (6, 7). According to the increasing demand in the fields of medicine, nutrition industry, animal husbandry and other technologies for recombinant proteins, researchers have undergone tremendous progress to increase and optimize recombinant productions. However, the production and purification of recombinant proteins are expensive at industrial and semi-industrial levels. Therefore, it is necessary to find new ways to reduce the production costs by using recombinant DNA technology. Using simple and reliable methods to purify protein is one of the most important considerations to produce recombinant proteins (8).

*E. coli* as a work horse for ongoing research has some benefits such as low cost, easy technique, high growth rate and great capacity to express recombinant proteins (9). Considering these general benefits of *E. coli* and overexpression of recombinant proteins in *E. coli* BL21 (DE3), some studies have used it to express recombinant fusion proteins (10) and antibodies (11). Different vectors have been employed to express hEGF in *E. coli* (12, 13). Some problems related to overexpression of cloned gene products in *E. coli* can be overcome by inserting fusion proteins at N- or C-terminal of the target protein (14, 15). Thioredoxin (Trx) fusion protein causes oxidation-reduction of disulfide bonds in many target protein mol-

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ecules and plays an important role in the structural stability and activity of the protein (16, 17). Consequently, adding Trx before the target protein can enhance protein expression.

Elastin-like polypeptides (ELPs) consisting of 20–330 repeated units (Val-Pro-Gly-X-Gly, X not Pro) can undergo reversible phase transition, at the upper phase of which they become insoluble (18). The transition temperature directly depends on the type of X amino acids, chain length and salt concentration (19). Proteins and peptides fused to ELPs have similar temperature-responsive behavior (18, 20) and protein-ELP constructs allow purification of proteins from cell lysis without using chromatography columns (21). This method can be very efficient and cost-effective but ELP separation from target proteins by proteases is a major limitation. Proteases do not act specifically, and high temperature is required to activate them. This has probably a devastating effect on the stability and activity of the recombinant proteins (22).

The cleavage on internal proteins (intein) and coupling two sequences of extein to each other are general processes on the cellular proteins after translation. Since this discovery, two hundred inteins with conserved sequences and lengths of 100–800 amino acids have been identified and studied (23). By using genetic engineering, different kinds of inteins have been made which can do the cleavage from one side (23, 24); the cleavage between intein and the desired protein can be induced under suitable thermal and chemical (pH or thiol compounds) conditions without using chromatography column and protease system (25). In this study, we designed two constructs including pET32a-Trx and modified pET15b by inserting ELP and intein to compare and introduce a suitable construct for hEGF expression in E. coli BL21. An appropriate biotechnological construct can be used for hEGF high industrial production in the future.

Materials and methods

Materials

The prokaryotic plasmid pET32a and pET15b obtained from Merck Millipore (USA). Restriction enzymes (NdeI, BamHI and XhoI), T4 DNA Ligase, DNA markers, and protein markers were purchased from TransGen Biotech (China). The colony host TOP10F, expression host BL21 (DE3), and Amicon Ultra-15 Centrifugal Filter Units were obtained from Merck Millipore (USA). Small plasmid extraction kit and Gel and PCR purification mini kit were from Yekta Tajhiz Azma (Iran). Sequencing and primer synthesis were done by Gene Fanavaran (Iran). Tris, acrylamide, bis acrylamide, ammonium persulfate, and tetramethylethylenediamine (TEMED) were purchased from Sigma-Aldrich chemical company (USA). His-tag antibody and anti-mouse HRP were from TransGen Biotech (China). BCA assay kit was purchased from Thermo-Scientific (USA) and Human EGF Standard ABTS ELISA Development Kit was obtained from PeproTech (USA).

Preparation of pET15b Construct

Figure 1 illustrates various steps of the study. ELP, intein and hEGF were cloned in pET15b according to previous study (26). Briefly, the hEGF encoding sequence was obtained from the NCBI gene bank and synthesized by Gene Fanavaran Co. The intein gene used in the pET15b construct was obtained from New England Biolabs (pTEXB1). The ELP sequence was designed to have transition temperature of about 20 °C in the presence of 0.4 M sulfate amine to get precipitation. Then, the primers were designed to replicate ELP-intein-hEGF gene and get final sequence consisting of ELP (1135 bp), intein (591 bp) and hEGF (214 bp) as target protein (Fig. 2A). Finally, the ELP-intein-hEGF sequence was cloned in pUC57 vector.

![Fig. 1. Various steps of the study from design of recombinant vectors to quantitative evaluation of expressed target protein.](image1)

![Fig. 2. Structure of (A) pET15b-ELP-intein-hEGF and (B) pET32a-Trx-hEGF constructs.](image2)
The designed ELP-intein-hEGF sequence has one restriction site for NdeI in the beginning of ELP sequence and other for BamHI at the end of the hEGF gene. After digesting the construct from pUC57 by BamHI and NdeI enzymes, the required mass of ELP-intein-hEGF was calculated by Eq.1 (27) and the inserted gene was ligated in pET15b using T4 ligase.

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V_v = \frac{T}{V_i I_1} + I_v \quad \text{→} \quad V_i I_v = T - V_v \quad \text{(Eq.1)}
\]

in which \( V_v \) and \( I_v \) are vector and insert concentration, \( V_i \) and \( I_i \) are vector and insert length, \( I_r \) is insert-to-vector ratio. Also, \( T, Vv \) and \( v \) are total DNA solution, vector and insert respectively.

To clone the recombinant pET15b, TOP10F became a competent cell using calcium chloride on ice while recombinant vector transformed in it by thermal shock. Then, the transgenic strain was cultured overnight on ampicillin-containing agar plate at 37 °C. After clone selection and plasmid extraction, the presence of ELP-intein-hEGF sequence in pET15b was confirmed by digestion using NdeI and BamHI enzymes. Moreover, PCR method was used to prove the existence of intein, hEGF and intein-hEGF sequences in pET15b by employing designed primers (Tab. 1). Finally, the products of digestion and PCR were electrophoresed on 1 % agarose gel.

**Preparation of pET32a construct**

To make the pET32a construct, hEGF gene was amplified from the ELP-intein-hEGF construct by PCR technique and its primers were designed in such a way that the final construct consisted of Trx in N-terminal and hEGF in C-terminal (Fig. 2B). NEB cutter v2 online software was used to find the selective restriction enzyme sites in the desired gene and good primer design. Digested pET32a and PCR product were electrophoresed on 1 % agarose gel and the vector and hEGF were extracted from agarose gel by Gel and PCR purification mini kit. The DNA ligase enzyme was used to ligate hEGF fragment and pET32a at 4 °C for 16 hours. After clone selection and plasmid extraction, same as pET15b, the presence of hEGF was confirmed in pET32a by PCR.

**Analyzing molecular weight of proteins**

Protein weight was analyzed by CLC main workbench v5.5 (CLC bio) to translate nucleotide sequences to amino acids and Protein Molecular Weight online software (28). As a result, the molecular weights of expressed recombinant protein in pET32a and pET15b were 21 kDa and 61 kDa, respectively.

**Expression of the proteins and purification by Ni-NTA**

The constructs were extracted by plasmid extraction kit, transformed into expression host BL21 (DE3), and cultured on a solid medium containing ampicillin antibiotic. The intended clone was selected by PCR and then cultivated in 5 ml of LB medium for 16 hours at 37 °C while being shaken at 200 rpm. Then, 50 μl of cultured clone was transferred to 100 ml of the traffic culture (rich media). After reaching OD = 0.6 at 600 nm, IPTG was added at 0.5 mM concentration, and the culture was incubated for 16 hours at 37 °C while being shaken at 200 rpm. Samples were taken after 2, 4, 6 and 16 hours of induction to determine the maximum expression of each construct. The samples before and after induction were studied by SDS-PAGE and SDS-PAGE images of two different constructs were imported to ImageJ software to measure the expression qualitatively (29). Also, protein expression was verified by western blot technique using mouse Anti-His antibody (1:3000 dilution in PBS containing 0.05 % Tween20) and anti IgG-conjugated HRP secondary antibody (1:2000 dilution in PBS) for one hour at 25 °C. Finally, the target band was detected by 3,3’-diaminobenzidine (DAB).

**Quantitative measurements by BCA and Elisa assay**

To measure the protein amount quantitatively, unfavorable proteins had to be removed from expressed recombinant proteins as much as possible. The bacterial culture was centrifuged, the cell pellet was lysed by sonication on ice and centrifuged for 20 min at 15000 × g (30). After transferring supernatant including soluble proteins (Sup1) to other tube, the pellet was resuspended in NT solution, mixed with Sup1 and loaded on nickel resin for purification. Both constructs had His-tag at N-terminal to increase affinity with nickel resin. After incubation for 2 h at 4 °C, the Ni-NTA column was washed twice by NT solution to remove unbound proteins. Afterwards, elute buffer (200 mM imidazole in NT solution) was added to resin, the column was centrifuged and the flow-through mixed with NT solution was filtered using Amicon filter.

BCA assay was used to determine the recombinant protein concentration because of its high accuracy and easy execution. To specify the concentration of ELP-intein-hEGF and Trx-hEGF proteins, a protein standard curve in the range of 25–2000 μg/ml

**Tab. 1. Intein and hEGF primers for PCR reaction.**

| Name of Primer | Primers (5’------3’) |
|----------------|----------------------|
| F-intein       | ATGCCACGCAGGGCCAGGCATAGCAACAAATAACAAATAGCCTGCATTACCGGTGATG |
| R-intein       | GTTCTCGAGTTGTAGAGAAACAAAACCGT |
| F-hEGF         | AAGGATCACAAGCAAGAAACCCCCTGCGT |
| R-hEGF         | ATCTCGAGTTATCATCTCAGTTCGCCACCAC |
was prepared. Each sample was analyzed by BCA assay kit according to manufacturer’s instruction and the absorbance was measured at 562 nm by spectrophotometer (PowerWave XS, BioTek). Also, the amount of hEGF in the samples containing ELP-intein-hEGF and Trx-hEGF proteins was quantified using hEGF ELISA assay kit. The preparation of the 96-well plate and samples as well as examination were done according to the instruction of kit’s manufacturer.

**Statistical analysis**

A one-way ANOVA on Ranks Tukey’s test post hoc (α =0.05) (GraphPad Prism v7.0) was carried out to assess the significant effect of IPTG induction time on protein expression in each designed construct. Also, to compare effect of construct design on protein expression after 2 hours of IPTG induction, Student’s t-test (α = 0.05) was done on ImageJ, BCA and Elisa data.

**Results**

**Confirmation of ELP-intein-hEGF (pUC57 and pET15b) and hEGF (pET32a) fragments**

By employing suitable primers for hEGF (F- and R-hEGF), intein (F- and R-Intein), and hEGF-intein (F-hEGF and R-Intein), PCR products with size of 214, 591 and 805 bp were obtained, respectively. Figure 3A shows related bands of hEGF, intein and intein-hEGF obtained by PCR. Also, Figure 3B depicts ELP-intein-hEGF fragment produced by digestion of pUC57 and pET15b. The presence of hEGF fragment in pET32a was confirmed by colony PCR method (Fig. 3C).

**Expression of pET32a-Trx-hEGF and pET15b-ELP-Intein-hEGF in BL21**

After transforming the recombinant vectors (pET32a-Trx-hEGF and pET15b-ELP-Intein-hEGF) in BL21, expression was done. According to electrophoresed gels, no expression bands were visible in pre-induction samples in the 21 kDa and 61 kDa regions for pET32a-Trx-hEGF and pET15b-ELP-Intein-hEGF, respectively. After IPTG induction, a thick and clear band in the mentioned regions appeared according to the protein marker size (Fig. 4). Comparing the expression of the pET32a construct using ImageJ software showed that the expression level after 2 hours of induction by IPTG was higher than that after 4 hours of induction by 14.3 % but it was not significant (p > 0.05). Also, the expression after 2 hours of induction was higher than those after 6 (p < 0.05) and 16 hours of induction (p < 0.0001) by 15.6 % and 167.5 % respectively (Fig. 5A). Extra 2 hours of IPTG induction

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**Fig. 3.** Gel electrophoresis of (A) hEGF, intein and intein-hEGF obtained by PCR in pUC57 (well 2–4) and pET15b (well 5–7) (B) ELP-intein-hEGF fragment produced by digestion of pUC57 (well 2) and pET15b (well 3) and (C) hEGF fragment in pET32a.

**Fig. 4.** The electrophoresed samples from the expression reaction in (A) pET15b and (B) pET32a constructs. Well 2: non-transgenic BL21 strain of recombinant after 4 hours and treated with IPTG; Well 3: BL21 transplanted by recombinant constructs after 4 hours and without treatment with IPTG; Wells 4–7: BL21 transplanted by recombinant pET15b and pET32a treated with IPTG for 2, 4, 6, and 16 hours indicating a protein expression of 61 kDa and 21 kDa.
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(6 hours) had no significant effect on expression in comparison with the induction time of 4 hours. Fig. 5B shows that the expression of another construct (pET15b-ELP-Intein-hEGF) after 2 hours of induction was higher than those after 4, 6, and 16 hours of induction (p < 0.0001) by 65.7 %, 105.1 %, and 352.7 %, respectively. It must be considered that although the expression level in pET15b after 2 hours of IPTG induction was significantly better than other various induction times when compared to pET32a, the overall expression in pET32a was significantly higher than that in pET15b (Fig. 5C). The Table 2 includes the calculated expression difference (in percent) between different IPTG induction times in two constructs. According to ImageJ analysis, the best IPTG induction time for both constructs was 2 hours and caused a 132.3 % higher expression in pET32a-Trx-hEGF in comparison with pET15b-ELP-Intein-hEGF.

**Western blot assays**

Western blot analysis confirmed the expression of His-Trx-hEGF and His-ELP-intein-hEGF proteins in BL21 using anti-his monoclonal antibody which showed reactivity of target protein (Fig. 6).
Tab. 2. Difference in percent expression between different IPTG induction times.

| pET32a   | h=16 | h=6 | h=4 | h=2 | pET15b |
|----------|------|-----|-----|-----|--------|
| h=2      | 167.5| 15.6| 14.3|     | h=2    |
| h=4      | 134.1| 1.2 | 65.7|     | h=4    |
| h=6      | 131.5| 23.8| 105.1|    | h=6    |
| h=16     | 120.7| 173.2|352.7|    | h=16   |

Quantitative measurement of recombinant protein concentration

The result by BCA assay revealed that the concentration of recombinant expressed by pET32a-Trx-hEGF after 2 hours of IPTG induction was significantly higher than that expressed by pET15b-ELP-intein hEGF (p < 0.0001) (Fig. 7) and Trx-hEGF expression was 100 % higher than that of ELP-intein-hEGF.

Also, Elisa assay proved that Trx fusion to hEGF caused an increase in protein expression for pET32a-Trx-hEGF construct by 318.1 % which was significantly higher than that of pET15b-ELP-intein-hEGF (p < 0.001) (Fig. 7).

Discussion

The study was aimed at comparing the expression of hEGF by two different constructs. After successful expression by using a suitable bacterial host, each recombinant protein needs to be purified at rational cost level using appropriate purification method. In traditional methods, polyepitides are used to obtain the target recombinant protein. However, they have a non-specific cleavage function as well as low efficiency, and need high temperature to cleave the target recombinant protein (31). HPLC and Ni-NTA or other resins are different methods for purification, but on a larger scale, they have high cost in this step of production due to high column cost for HPLC and high material expenses for resins (8).

The design of the construct is the most important stage of protein production. It can influence the successful expression in host, suitable purification, and production of active native recombinant proteins. All of mentioned qualitative and quantitative improvements can be obtained by inserting tag and protein fusions (32). To decrease the financial expenses at purification step, the ELP tag can be inserted at the N-terminal of construct, which can help to separate the construct from other host proteins without using HPLC column and resins. The ELP can be sedimented under suitable conditions (temperature and salt concentration) (33), which may reduce the costs. From commercial and scientific points of view as well as when considering useful properties of intein such as self-cleavage by pH, and specific cleavage site (23, 24), we designed a construct composed of ELP, intein and target protein (pET15b-ELP-Intein-hEGF) to get the native protein. Furthermore, high recombinant protein expression in host is another important point in research. Low expression level in *E. coli* has several reasons such as poor protein stability, protein toxicity, and rare codons within the gene. To get over the low expression level of long recombinant gene in *E. coli*, different approaches have been chosen by researchers. Utilizing various expression hosts or vectors, using different temperature, employing different concentration of IPTG, making short or removing the sequence from N-terminal of long recombinant protein or gene by bioinformatics and using fusion proteins are main categories of such proposed techniques. With the exception of fusion proteins, other approaches may reduce or remove recombinant protein activity (14, 15). Considering oxidation-reduction function of Trx and its effect on improvement of hEGF expression in *E. coli*, the presence of Trx in pET32a makes it suitable to increase the expression of recombinant protein (13). Hence, another construct consisting of Trx fusion protein and hEGF (pET32a-Trx-hEGF) was designed.

The decrease in expression level of recombinant protein in both constructs after two hours of IPTG induction (Fig. 5 and Tab. 2) can be discussed from the molecular point of view. Similarly to other cells, the proliferation and growth of *E. coli* BL21 takes place via four stages, namely lag phase, logarithmic phase, stationary phase and death phase (34). The amounts of enzymes involved in biosynthesizing amino acids, most proteins of phospho-transferase system, some proteins involved in transportation of amino acids and peptides, and enzymes degrading less favorable substrates increase during the stationary phase in rich media (9). Because each recombinant protein is a non-specific molecule as well as a source of amino acids, it is probable that it is degraded by specific enzymes in *E. coli* BL21 cytoplasm, while resulting in reduction of target protein after two hours of IPTG induction.

The qualitative and quantitative analysis by ImageJ software, BCA and Elisa assays demonstrated truly that the expression level of recombinant protein increased (by 132.3 % for ImageJ, 100 % for BCA and 318.1 % for Elisa) using pET32a-Trx-hEGF construct compared to pET15b-ELP-intein-hEGF construct in BL21 after two hours of IPTG induction. Although pET32a provides suitable conditions for high expression, it needs Ni-NTA resin and peptidases for protein purification. Thus, it is not applicable in terms of cost and pET15b-ELP-intein-hEGF fragment is cheaper and has a simple method of hEGF purification. Overall, considering the fact that the expression of this construct is lower than that of pET32a-Trx-hEGF as well as on account of the effect of Trx on hEGF expression, pET32a-Trx in combination with ELP-intein (pET32a-Trx-ELP-intein-hEGF construct) would have low cost and a simple purification route suitable for producing hEGF at a large scale.

Conclusion

This study was focused on the expression of hEGF by two constructs with different modes of design logic to introduce a suitable inexpensive expression and purification system. One construct (pET15b-ELP-Intein-hEGF) was designed considering ELP-intein sufficiency for easy and cheap purification forgoing its lower expression level. In the other construct (pET32a-Trx-hEGF), Trx fusion protein was used to enhance the expression level. Various analyses demonstrated that Trx-containing construct had a significantly higher expression level than the other. Although further studies are required to investigate the Trx effect on expression improvement of ELP-intein-hEGF in BL21, a combined construct consisting of Trx, ELP, and intein may be suggested to produce hEGF for industrial and research purposes.
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