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Original Article

An Approach for Recombinant Epidermal Growth Factor Purification Using an Elastin-Like Protein Tag

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Abstract

Introduction: Wide applications in research, clinical and cosmetic industry of human epidermal growth factor (hEGF) has made it as a research interest target. Its production in different expression systems have shown several limitations.

Materials and Methods: Recombinant expression of hEGF in *Escherichia coli* is always accompanied with inclusion body formation. In order to evaluate a chromatogram independent purification approach for recombinant hEGF production in soluble form, the hEGF gene was fused to an elastin-like protein (ELP) and was expressed in *E. coli* BL21 (DE3) using pET26b expression vector for secreting the product into periplasmic space.

Results: Periplasmic protein content analysis confirmed that the recombinant protein is secreted into periplasm. Purification process was done by using 0.4M ammonium sulfate in two cycles of inverse transition cycling (ITC). After two cycles of purification, the purity reached more than 95%. Western blotting analysis with monoclonal anti-EGF anti body have confirmed the accuracy of EGF. The biological activity of the purified protein was investigated on the NIH-3T3 cell line and results indicated that EGF-induced proliferation in treated cells.

Conclusions: Results revealed that the periplasmic expression is a suitable approach to produce soluble recombinant hEGF. By using of ELP fused to EGF, the purification process was stablished without applying chromatography which will result in decreasing final costs. This study introduced a new economic and efficient approach for the production and purification of recombinant hEGF.

Keywords: hEGF, Inverse Phase Transition, Periplasm, Protein Purification

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Introduction

Human epidermal growth factor (hEGF) is a single-chain polypeptide with 53 amino acid residues and three disulfide bonds in its structure.[1] High mitogenic effects of EGF on different cell types and its growth regulatory impact on in-situ and in-vivo conditions have made it as a research interest and a high demand protein.[2] Due to low amounts of EGF recovery from natural sources, the recombinant form of the product is much more available, and the microbial synthesis of EGF has been reported in several studies.[3]

EGF have been expressed in various expression systems in different conditions including: recovering from *Escherichia coli* inclusion bodies,[3-5] fused with different tags,[6] secreted into periplasm and medium,[2] which is at the same time a cost effective production.

Purification is the most costly step in protein production. Due to their substantial costs, chromatography-based methods are not well suited for large scale purification.[7] Therefore researchers are pursuing new, reliable, simple and economic methods for protein purification.[8] For this goal, one potential method is using elastin-like protein (ELP) as a purification tag, which is an artificial polymer made of a repeating penta-peptide unit consisting of Val-Pro-Gly-Xaa-Gly amino acids, where X could be any amino acid except Pro.[9] The number of repetitive units can be various from 10 to 250.[10] Briefly, ELPs can undergo a sharp and reversible phase transition (inverse transition cycling, ITC) from soluble to insoluble state by reaching the Transition temperature (Tt). In temperatures below Tt, ELPs are soluble, by increasing temperature over Tt, ELPs gradually become insoluble and form insoluble aggregates. This phase transition is reversible and they will get soluble again by decreasing the temperature.[11] By fusing ELP to a target protein, it can be easily purified in a few phase transition cycles (ITC) and subsequent centrifugations.

The aim of this study was to evaluate a chromatography-independent approach for producing EGF in *E. coli* by its fusion to an ELP tag.

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Material and Methods

Plasmid Construction and Expression

In order to purify mature parts of the EGF (accession number: AAA72563.1), its N-terminal was fused to an ELP tag with 50 repeats of V-P-G-V-G unit with an intein between them at DNA levels.[12] The target sequence was optimized to *E. coli* codon template and was then synthesized and cloned in pUC19 plasmid as pUC19-ELP-EGF. The ELP-EGF chimeric gene was amplified by using two specific primers (F-p26: ATGG***CCATGG***GTCCGCGGGTCCCTGGC and R-EGF: ATA***CTCGAG***TCATTAGCGCAGTTCCCACCAT, italic and bolded are *Nco*I and *Xho*I restriction site, respectively) and inserted into pET26b between *Nco*I and *Xho*I restriction sites which resulted in pET26-ELP-EGF construct. Obtained clones were confirmed with polymerase chain reaction (PCR), enzymatic digestion and sequencing.

The pET26-ELP-EGF was transformed to *E. coli* Bl21 (DE3) and then temperature, time and IPTG concentration were optimized for expression. Protein expression was induced by using 0.4 mM IPTG (final concentration) at 25°C for 16-18 hours in 2X LB broth (NaCl 20, tryptone 20 and yeast extract 10 g/L) by incubating in an incubator with 160 rpm.

Protein Extraction and Purification

For extracting periplasmic proteins, cells were harvested by centrifugation at 6000g for 20 minutes in 4°C. Pellet was suspended in TES buffer (Tris 30 mM, pH 8, Sucrose 20%, EDTA 2 mM) and incubated on ice for 20 minutes and was centrifuged according to the above mentioned conditions. Then, cells were suspended again in $MgSO_4$ (5 mM) and incubated on ice for 30 minutes. After on, cells subjected to centrifugation and supernatant were collected as periplasmic protein contents.

Thermal Shift Test

For the ELP tag, the transition temperature (Tt) was calculated by Thermal-Shift assay method using the SYPRO® Orange stain in a real-time thermo cycler. In this method SYPRO® Orange stain binds to hydrophobic area in protein and make signal which is measured in real time mode. Therefore when ELP undergoes phase transition in this test, bind more SYPRO® Orange and produce more signal. The 5 µM of the purified hybrid protein was subjected to increasing temperatures (10 to 100°C, increasing rate: 1°C per minute) in three different conditions (1- using $(NH_4)_2SO_4$ 0.4 M, 2- using NaCl 1.5 M and 3- no salt). Salt concentrations were selected according to Fong et al.'s protocol. For each condition, three repeats were analyzed and PBS buffer was used as a negative control.

Inverse Phase Transition

After Tt determination, 4 mg of periplasmic protein was subjected to purification by the inverse phase transition cycle. For this purpose, three different conditions were used: using $(NH_4)_2SO_4$ (0.4 M) and incubating at 30°C, using NaCl (1.5 M) and incubating at 37°C and finally using no salt and incubating at 50°C (TM). Each sample was incubated at respective temperatures for 6 minutes to induce ELP aggregation and followed by centrifugation at 17 100 g for 6 minutes (called hot spin) and supernatant, was discarded. Pellet was dissolved in cold PBS (pH 8) and incubated on ice for 10 minutes (ITC1). Samples were centrifuged at 17 100 g at 4°C for 6 minutes (cold spin) and supernatant was transferred to a new micro-tube and subjected to another ITC for a hot spin. After two cycles of purification, samples from each step were analyzed on 15 % Tris-Tricin SDS-Page.[13]

Size-Exclusion Chromatography

Size exclusion chromatography was used for the purity assay of the purified protein by using HiLoad® 16/600 Superdex® 200 prep grade column (GE Healthcare Life Science) with 120 mL volume. The sample volume was 1 ml and the used buffer was Tris 25 mM, pH 7.4 and NaCl 150 mM. After column equilibration with buffer, the sample was leaded on column with 1 mL/min rate and proteins were detected in 280 nm. Identified peaks were analyzed on SDS-Page.

Western Blotting

To confirm the accuracy of the produced protein, western blotting analysis was carried out. The peptide was detected by a mouse anti-EGF monoclonal antibody (MAB5256, R&D systems) as a primary antibody which was detected by a Peroxidases-labeled goat-anti-mouse IgG (Calbiochem- Cat no: 401215). As a secondary antibody, bands appeared by incubating the membrane in 3,3',5,5'-tetramethylbenzidine (TMB). The periplasmic protein of non-transformed *E. coli* BL21 and recombinant human EGF (rhEGF) were used as negative and positive controls, respectively.

Biological Activity Assay

The NIH 3T3 cell lines (ACC 59, DMSZ) were used for evaluating the rhEGF activity. Cells were plated in 96 µL plates in DMEM media with 10% FCS and 1% penicillin/ streptomycin and were incubated in 37°C and 80-90 RH. After 24 hours, media was exchanged by fresh DMEM media with 1% FCS and incubated for an extra 24 hours. Then, media was discarded and fresh DMEM media consisting of different concentration of rhEGF was added to the wells and plate were incubated in 37°C and 80-90 RH. After 72 hours, 100 microliters/well of working solution of Cell Titer-Blue® (Promega, Cat. G8080) was added and after 2 hours, fluorescence was recorded at 560/590 nm. The PBS and commercial EGF (Sigma cat. E4127) were used along with the negative and positive control. Tests were done by three replicates. Results were expressed as proliferation rate to the control.

Results

Plasmid Construction and Expression

Amplifying ELP-EGF chimeric gene in PCR by using specific primers showed a band about 1.4 kb in size on agarose gel (Figure 1b, left). In order to target ELP-EGF protein into periplasmic space, the full recombinant gene was inserted between the *Nco*I and *Xho*I restriction enzymes sites of the pET26b plasmid next to PelB signal (Figure 1a). This resulted

in recombinant plasmid pET26-ELP-EGF. Its digestion by *Nco*I and *Xho*I restriction enzymes confirmed an insert with about 1.4 kb (Figure 1b, right) which imply the right insertion of the target in pET26b plasmid. Sequencing analysis of the constructed plasmid have also shown the accuracy of the amplified fragment. Subsequently, pET26b-ELP-EGF was successfully transferred to an *E. coli* BL21 (DE3) strain for expression.

In order to express the ELP-EGF protein, a single colony of BL21 (DE3) carrying pET26b-ELP-EGF plasmid was cultured for 16 hours, and its expression induced by IPTG, after that

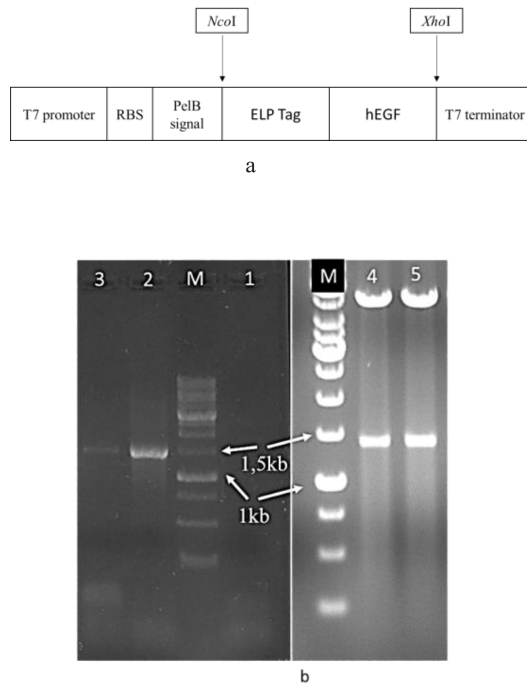

Figure 1. (**a**) Overall Schematic of pET26b-ELP-EGF Construct Expressed in *E. coli* BL21. (**b**) Analysis of PCR Product and Restrict Digestion of pET26b-ELP-EGF on 1% Agarose Gel. Lane 1) Negative control for PCR; Lane 2 and 3) PCR products with specific primers; Lane 4 and 5) digestion results for two pET26b-ELP-EGF clones with *Nco*I and *Xho*I; M: 1kb molecular marker.

cells were harvested and periplasmic proteins were extracted by the osmotic shock method. Subsequent protein analysis on SDS-Page electrophoresis revealed a band around 46 kDa which was absent in control and non-induced ones (Figure 2a).

Thermal Shift and Inverse Phase Transition

Thermal-shift test on ELP-EGF protein showed that ELPs, in no salt conditions, start to phase transition in temperatures higher than 45°C, while by adding NaCl, they start to change at around 30°C. However, this was around 20 °C when ammonium sulfate was used (Figure 2b). Therefore, the minimal Tt was obtained when 0.4 M sulfate was used.

Analysis of purification steps on SDS-Page in three conditions (0.4 M $(NH_4)_2SO_4$ with incubating at 30°C, 1.5 M NaCl with incubating at 37°C and using no salt and incubating at 50°C (TM)) revealed that minimum loosing of target protein took place in the first condition (Figure 3b). Actually, from 4 mg of periplasmic protein, an amount of 0.41 mg was recovered by $(NH_4)_2SO_4$ after the first ITC (Figure 3b, A1) while this was 0.39 and 0.12 mg for NaCl and TM, respectively (Figure 3b, B1 and C1). The amount of purified protein after the second ITC was 0.35 mg for $(NH_4)_2SO_4$. In the other words, 85% of the protein from ITC1was recovered in ITC2 by ammonium sulfate, but this amount was 80% and 41% for NaCl and TM, respectively (Figure 3b, B3 and B3, Table 1).

Size-Exclusion Chromatography

Size exclusion chromatography was done to assess the purity of the purified recombinant protein. Results showed that two cycles of purification with an inverse phase transition cycle resulted in more than 95% purity of the hybrid protein (Figure 4).

Western blotting analysis of periplasmic and purified ELP-EGF hybrid protein with a monoclonal anti-EGF antibody (Figure 3b) showed a single band corresponding to the ELP-EGF hybrid protein size. Regarding that EGF is located at C terminus of the fused protein, its detection by a specific

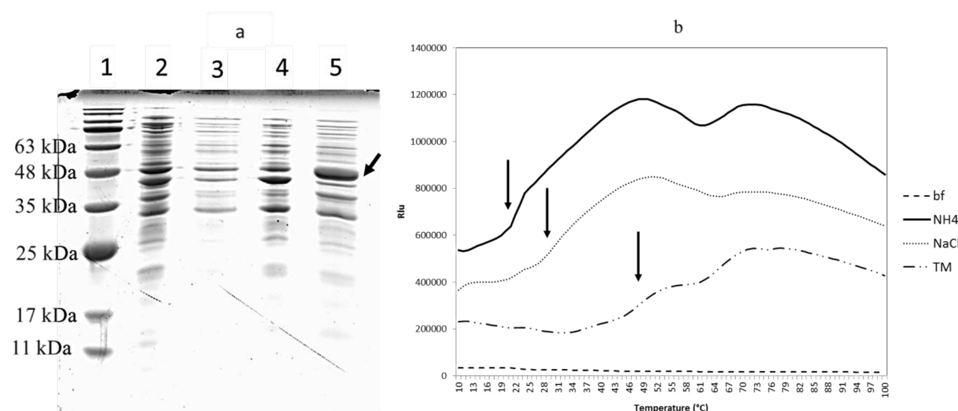

Figure 2. (**a**) SDS-Page Analysis of Expression of pET26b-ELP-EGF in E. coli BL21. Lane 1) Molecular Marker; Lane 2) Un-induced pET26b Without insert; Lane 3) Induced pET26b Without Insert; Lane 4) Un-induced pET26b-ELP-EGF; Lane 5) Induced pET26b-ELP-EGF. (**b**) Thermal-shift Test for Determining Tt in Three Condition. bf: buffer without protein. NH4: purified protein with 0.4 M ammonium sulfate. NaCl: purified protein with 1.5 M sodium chloride. TM: purified protein with temperature without adding salt. Arrows indicating phase transition starting in each sample.

Table 1. Measurement of Protein Amount (mg) in Each Step/Method During Purification of the Recombinant Protein With ITC

Step	Method		
	Ammonium Sulfate	Sodium Chloride	Temperature
Start	4	4	4
ITC_1 pellet	0.41	0.39	0.12
ITC_2 pellet	0.35	0.31	0.05

antibody implies a correct sequence of the ELP-EGF protein. The ELP-EGF activity was also successfully evaluated on the NIH-3T3 cell line and it showed a significant effect on the proliferation rate of the treated cells compared with the control (Figurer 5).

Discussion

Several studies have reported that EGF regulates a wide range of biological reactions including cell proliferation, differentiation and migration in in-vivo and in-situ conditions.[6] However, because of having three disulfide bonds in its structure, correct folding of hEGF during its bacterial expression is still a challenge.[4,14] In this study, we established a purification method for EGF by its fusion to a thermal-responsive tag following by expression in periplasmic space of *E. coli*. SDS-Page analysis of periplasmic protein showed that PelB signal at N-terminal of the recombinant protein successfully targeted it into the periplasmic space. This signal peptide derived from *Pectate lysate* B in *Erwinia carotovora* and it has been used for secretion of wide ranges of different proteins in the periplasmic space with good results and minimal needs to co-express chaperonin or strain engineering.[15,16] SYPRO Orange protein stain is able to bind to the hydrophobic area of proteins and produce signals. In a soluble state, hydrophobic parts of proteins are arrayed inside the protein and they are not accessible to bind, therefore the produced signal is low. By increasing the temperature, proteins start to denature and their hydrophobic parts become more available to bind

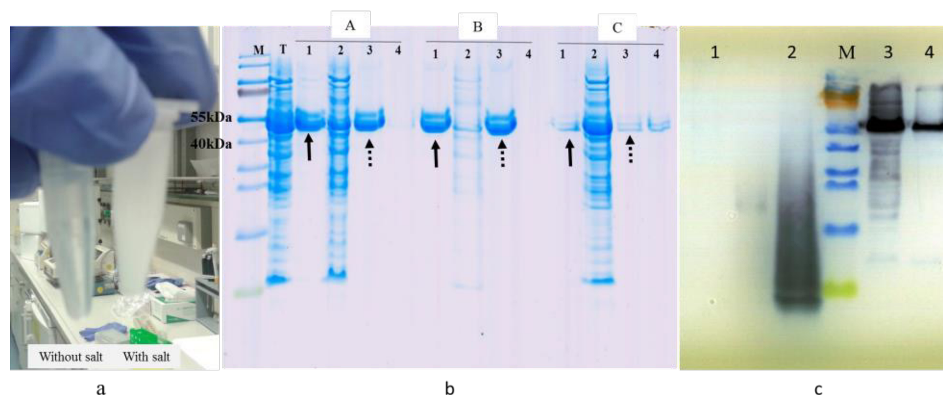

Figure 3. (**a**) Inverse Phase Transition of ELPs in Room Temperature; (**b**) Different Steps of Purification Process of the recombinant Protein With Three Methods: A) Purification with Ammonium sulfate; B) Purification with sodium chloride; C) Purification with temperature. M: Molecular marker, T: Periplasmic protein content (4 mg), Lane 1) Pellet from ITC_1, Lane 2) Supernatant from ITC_1, Lane 3) Pellet for ITC_2, Lane 4) Supernatant from ITC_2. Continues arrows indicating the amount of precipitated protein in ITC_1 from total periplasmic protein and dis-continues arrows indicating the amount of precipitated protein in ITC_2 from ITC_1. (**c**) Western blotting analysis of total periplasmic and purified recombinant protein with monoclonal anti-EGF antibody. Lane 1) Negative control, Lane 2) rhEGF, Lane 3) Periplasmic protein, Lane 4) Purified protein.

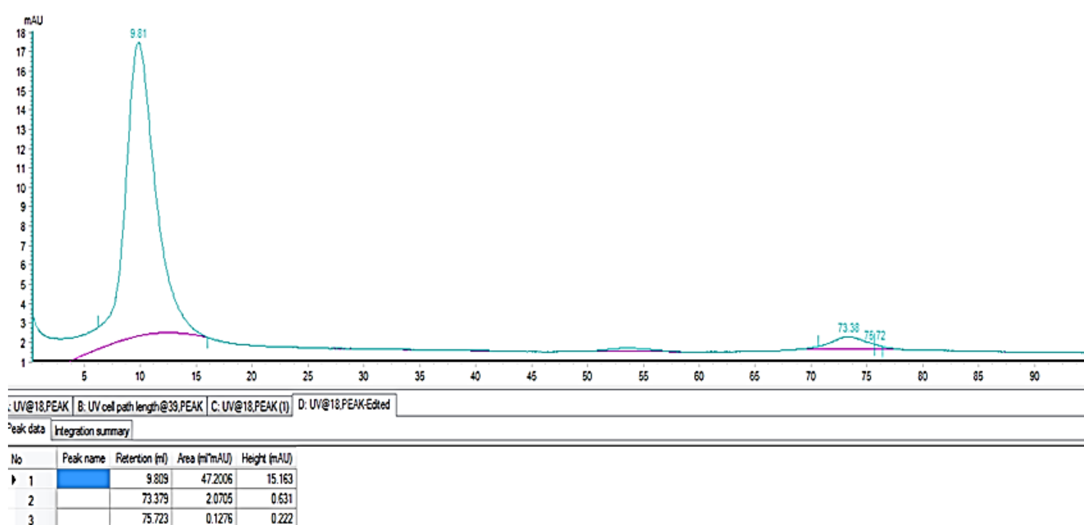

Figure 4. Chromatogram of Size-Exclusion of Purified Protein and Calculated Peak Area.

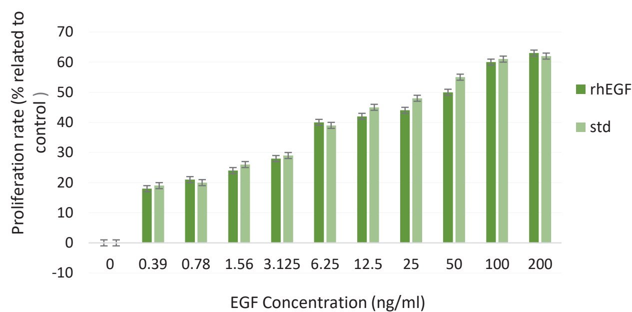

Figure 5. Biological Activity of Purified EGF by Non-chromatographic Approach.

and result in more signals.[17] In case of ELPs, with increasing the temperature, they undergo a phase transition and their hydrophobic area come to the surface and form an insoluble structure.[11] So, in the thermal shift essay, by starting the phase transition, more stain binds to the protein and the signal starts to increase. This temperature could be considered as Tt for ELP in a given condition. Transition temperature is affected by several items including buffer, salt concentration, length and concentration of ELP and physicochemical properties of the target protein.[7,11] The results of the present study revealed that Sodium Chloride and Ammonium Sulfate decrease Tt from temperatures higher than 40°C to 30°C and room temperature range, respectively. In addition, ammonium sulfate resulted in better precipitation of the target protein compared to sodium chloride. Another advantage of using ammonium sulfate instead of sodium chloride is using lower amounts of the salt (0.4M versus 1.5M) which causes less corrosion of equipment and thus reducing costs, especially in industries.[18]

By fusing the target protein to an ELP tag, it could be easily and properly separated from the host proteins in a non-chromatographic way. ELP's efficiency in recombinant protein purification have been highlighted by numerous studies.[19-21] Furthermore, several studies have pointed out to the fact that ELP tags have a sharp positive effect on the expression level and solubility of various fused proteins.[10,22] EGF is a high demand protein and its purification from natural sources is considered complicated and at the same time expensive. Therefore, recombinant protein production in *E. coli* is known to be the fastest, easiest, and an inexpensive technique to produce high amounts of the protein. Application of chromatography for EGF purification lead to an increase in the final price of the product. In the other hand, EGF expression in *E. coli* will form inclusion body which need extra refolding steps. In this study, a thermal responsive tag was fused to EGF and native recombinant protein was recovered from periplasmic space. Two cycles of ITC were carried out to purity the target protein. In addition, the produced protein not only had an acceptable purity degree but was also biologically active. Positive effects of purified EGF fused to ELP on the NIH-3T3 cell proliferation confirmed the fact that this tag has no adverse effects on EGF binding to its receptor. This neutral effect of ELP has also already been observed on erythropoietin and its receptor.[10]

Conclusions

In this study, the purification process for recombinant EGF fused to ELP has been done by the phase transition method and its analysis with size exclusion chromatography confirmed that it is more than 95% pure after two ITC cycles. The produced protein was confirmed by western blotting and it was biologically active and instigated proliferation of NIH-3T3 cells. It seems that this approach is a promising method for producing active and soluble EGF in regards to its costs.

Authors' Contributions

All authors contributed equally to current study.

Conflict of Interest Disclosures

The authors declare that they have no conflict interests.

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