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Purification and biological activity assessment: Comparison between two recombinant human epidermal growth factors with different molecular weights

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ABSTRACT

Considering its physiological roles in repair of skin damages, cornea and gastrointestinal tracks, epidermal growth factor has attracted scientist's attention among other members of the growth factor family. Many investigations were done for recombinant production of this protein and all of them were focused on the methods of its purification and biological activity assessment. In this study, human epidermal coding gene was prepared as a synthetic construct between T7-tag and His_6-tag in pET21a(+). After designing special primers for this gene, it was amplified by PCR technique and in order to making sticky ends, enzymatic digestions were done and then PCR product was ligated in pET28a(+). After transforming these construct into host cells, protein expression for both of them were done under standard condition. Protein solublization was performed by guanidine hydrochloride. hEGF purification from pET21(+)-EGF was done by affinity chromatography and to wash recombinant hEGF from pET28(+)-EGF, sodium deoxycholat salt and passing them from Amicon filters was used. Significant comparison between produced cases and standard sample were performed by RP-HPLC and ultimately, biological activity assessment for them was performed by MTT standard test. Results either in purification method or in biological activity assessment were benefited to recombinant hEGF protein produced in the present study.

Keywords: epidermal growth factor, gene cloning, recombinant protein expression, protein purification, biological activity assessment.

INTRODUCTION

In 1960, Stanly Cohn extracted a thermal resistance polypeptide from mouse sub-maxillary gland that was responsible for primary differentiations and teeth growth of newborn mice. The molecular weight of this protein was 6.12 k.Da and was named epidermal growth factor because of its stimulating potential effect on proliferation and keratinization of epidermal tissue [1]. Human epidermal growth factor (urogastrone) was identified and isolated by Gerigory in 1975 [2]. With regard to stimulating effect of EGF on proliferation of epithelial cells in culture and possibilities of applications in medicine to repair damaged tissue like skin and cornea, as well as recognition of this inestimable factor, a lot of efforts were done to extract it from human blood and urine [3]. From 1988 due to low abundance of hEGF in natural sources, investigators used recombinant DNA technology techniques to produce it in transgenic organisms with high level of protein expression [4].

First study for recombinant production of hEGF was done by Engler *et al* [5]. To date researchers used different methods for achievement to purified recombinant hEGF. Predominant method for purification of hEGF with precise and without any fusion sequences was RP-HPLC [7, 8]. Investigators believe that many troubles in purification and solubilization of hEGF will be solved by adding a signal peptide to hEGF coding gene. So to attain these purposes several studies were done such as gene fusion, beta lactamase [8], intein [9], thioredoxine [10] and his_6-tag [11]. In this study, the production of hEGF with two different molecular weights in *E.coli* was done and the chosen protein purification method for one of them is affinity chromatography and for another is a novel one.

MATERIALS AND METHODS

2-1.Bacterial strain, plasmid and gene

Escherichia coli strain BL21 (DE3) and plasmid vectors, pET21a(+) and pET28a(+), were purchased from Novagene (USA). hEGF gene codons were optimized according to host codon preference by manufacturer and then was prepared as a synthetic construct in pET21a(+). To maintain the sequence of T7 tag at the Amino terminal and His_6-tag at carboxyl terminal of EGF synthesized, coding gene was cloned between *Bam*HI and *Hin*dIII restriction sites. Then this plasmid was used as a template to amplify the fragment coding hEGF. Following primers were used in the amplification protocol: The forward primer has an *Nco*I restriction site and a methionine codon for translation initiation. The reverse primer was designed to include *Hin*dIII restriction site after stop codon.

Forward primer: 5' AATCTCATCCATGGGCAACTCTGACTCTGAAT3' (62°C)
Reverse primer: 5' CGATGAGCAAGCTTTTAACGCAGTTCCCAc 3' (63°C)

PCR reactions were carried out using *pfu* DNA polymerase from Fermentas (Lithuania). The PCR product was cloned into *Nco*I and *Hin*dIII restriction sites of pET28a(+) vector. Then the recombinant construct was transformed into *E. coli* competent cells prepared with chemical method. The gene of interest in recombinant cells was sequenced to confirm presence of any base deletion or substitution.

2-2. Expression and Induction of Recombinant hEGF protein

Recombinant host cells which have pET21(+)-GEF and pET28(+)-EGF were grown on a selective LB-agar medium. Individual colonies were obtained from LB agar plates and inoculated into 5 ml LB medium containing 50 µg/ml ampicillin antibiotic (for pET21-GEF recombinant cells) and 50 µg/ml kanamycin antibiotic (for pET28-EGF recombinant cells) and then incubated in the orbital shaker for overnight at 37°C. In the following day, 50 µl of each recombinant cell culture was inoculated into distinctive 5 ml LB medium containing relative antibiotics and grown at 37°C until their OD_{600} reached to 0.6. The cells were then induced with 1.0 mM IPTG and grown for additional 5 hours at 37°C and harvested by centrifugation at 5,000 rpm for 10 minute. Cells resuspended in lysis buffer (100 mM NaH_2Po_4, 10 mM Tris–HCl, pH 8) for an hour and lyzed by sonication (6 times for 10 seconds each) and then electrophoresed on Tricine SDS-PAGE polyacrylamide 16% gel.

2-3. Protein purification

Protein purification method for pET21(+)-EGF recombinant protein was affinity chromatography method containing Ni-NTA resin because this recombinant protein has His_6-tag at the carboxyl terminal. Quite novel and invented method was performed for purification of pET28(+)-EGF recombinant protein using sodium deoxycholate salt and Amicon filters with 50 and 10 kDa pore sizes because this recombinant protein did not has any tag to use affinity chromatography methods. Several stages such as incubating, protein washing using sodium deoxycholate salt ,and finally passing total solubilized protein from the filters were performed and quite purified hEGF recombinant protein was achieved.

2-4. Western blotting & RP-HPLC analysis

Two recombinant hEGF proteins were first electrophoresed on a 18% Tris-glycine SDS polyacrylamide gel and then transferred to nitrocellulose membrane (Qiagen,USA) using Bio-Rad Protean II system and transfer buffer (39 mM glycine, 48 mM Tris–base, 0.037% SDS, and 20% methanol). The membrane was incubated in the blocking buffer of 3% BSA (bovine serum albumin)/PBS (phosphate-buffered saline) (PBS, 137 mM NaCl, 2.7 mM KCl, and 4.3 mM Na_2HPO_4 $7H_2O$, pH 7.3) with gentle shaking for 1 h at room temperature. After decanting and discarding the blocking buffer, the membrane was incubated in a 1:5000 dilution of a mouse anti-hEGF monoclonal antibody (Sigma,U.S.A.) in the PBST (PBS containing 0.05% Tween) with gentle shaking for 1 h at room temperature. After washing the membrane with PBST for three times, each time for 5 min, blots incubated with a 1:10,000 dilution of

the standard polyclonal mouse anti-horse HRP conjugate as anti-hEGF antibody (Sigma,USA). The blot was washed three times in PBST and stained with HRP staining solution, DAB (Diaminobenzidine) (Sigma,USA). Chromogenic reaction was stopped by rinsing the membrane twice with water.

RP-HPLC analysis was performed to do significant comparison between these recombinant hEGF with standard recombinant hEGF (Peprotech, USA) and assessment of their purity.

2-5. Biological activity assessment

Finally, to ensure that these recombinants hEGF proteins have biological activity, NIH-3T3 cell line for MTT standard test was used. The assay was performed using BALB/C 3T3 cells (mouse fibroblast cell line). Seed preparation followed by necessary incubation periods at 37°C, CO_2(5%) in 90% DMEM with 10% calf serum and several subculturing process. Subculturing process was carried out using 0.0125% Trypsin and 0.02% EDTA in Ca^{2+}, Mg^{2+} free PBS. Cells (1×10^5) were prepared in basal medium and 50 µl was added to each well of 96-well micro culture plates. The samples and standard EGF were added to the wells (50 µl) in 4 different concentrations (1, 10, 100 and 1000 ng). Incubation at 37°C and 5% CO_2 for 24, 48, and 72 h was followed by addition of methylthiazolyldiphenyl-tetrazolium bromide (MTT) solution in PBS and then plates were incubated for additional 4 h. Subsequently, 100 µl of SDS(10%) and 0.01 N HCl were added to each well and the plates were incubated at 37°C and CO_2(5%) for 24 h. After the incubation, sample's absorbance was measured at 570 nm in three days after incubation of recombinant hEGF proteins.

RESULTS

3-1. Gene cloning

hEGF coding gene was prepared as synthetic construct in pET21a(+). This construct was used as a template and recombinant hEGF gene was amplified by PCR. PCR products were electrophoresed on 1% agarose gel (figure 1).

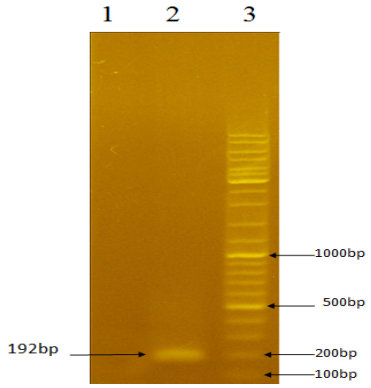

Figure1: The result of amplification of recombinant hEGF gene on 1% agarose gel. Lane1: Negative control for PCR. Lane2: PCR product amplified by *Pfu* DNA polymerase. Lane3: DNA size marker.

Enzymatic digestion for cloning PCR products into pET28a(+) vectors was done and after purification of digested pET28a(+) vectors by commercial kit, ligation reaction was performed by T4 DNA ligase. Recombinant vectors (pET21a(+)-EGF & pET28a(+)-EGF) were transferred into host competent cells. Plasmid extraction was carried out by alkaline lysis method and to cloning verification, enzymatic digestion was done by *Nco*I and *Hin*dIII restriction enzymes (figure 2).

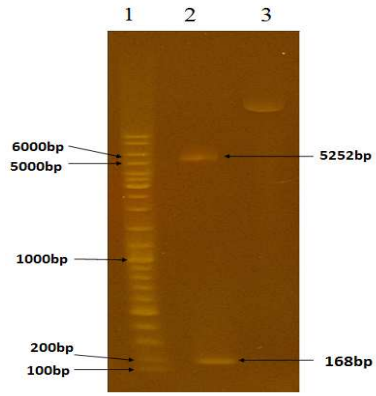

Figure 2: Cloning verification of *hEGF* gene in pET28a(+) between *Nco*I and *Hin*dIII restriction sites. Lane1: DNA size marker. Lane2: Cleaved segment and linear vector after digestion. Lane3: Uncleaved pET28a(+)-EGF construct.

3-2. Protein expression & purification analysis:

Induction of protein expression was performed by adding IPTG (1mM) to mediums of two recombinant host cells. With regard to 6 cysteine residues in hEGF's amino acid sequence made 3 disulphids bond in hEGF structure, soluble expression of hEGF in *E. coli* cells was approximately impossible (figure3).

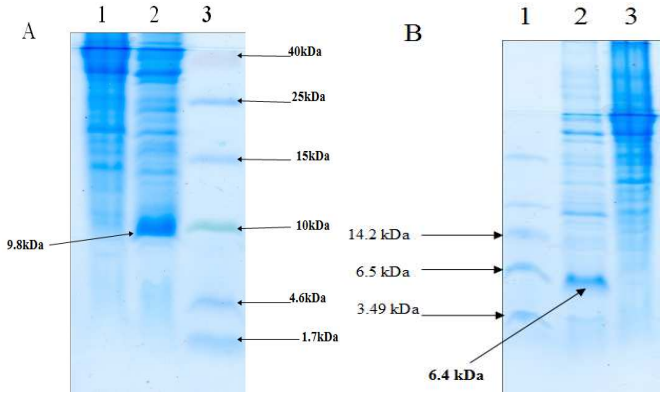

Figure3: Expression analysis on Tricine SDS-PAGE 16%. A) Recombinant hEGF protein expressed in recombinant host cells containing pET21a(+)-hEGF construct. Lane1: Uninduced cells. Lane2: Induced host cells with IPTG (1mM). The 9.8 kDa band shown is recombinant hEGF protein with fused signal(His_6-tag). Lane3: Protein size marker. B) Recombinant hEGF protein expressed in recombinant host containing pET28a(+)-hEGF construct. Lane1: Protein size marker. Lane2: Induced host cells with IPTG (1mM). The 6.4 kDa band shown is recombinant hEGF protein without fused signal (His_6-tag). Lane 3: Uninduced cells.

Proteins solubilization was performed by guanidine hydrochloride (6M) and solubilization was completely done (results not shown).

Verification of both recombinant hEGF proteins with different molecular weights were done by Western blotting technique (figure4).

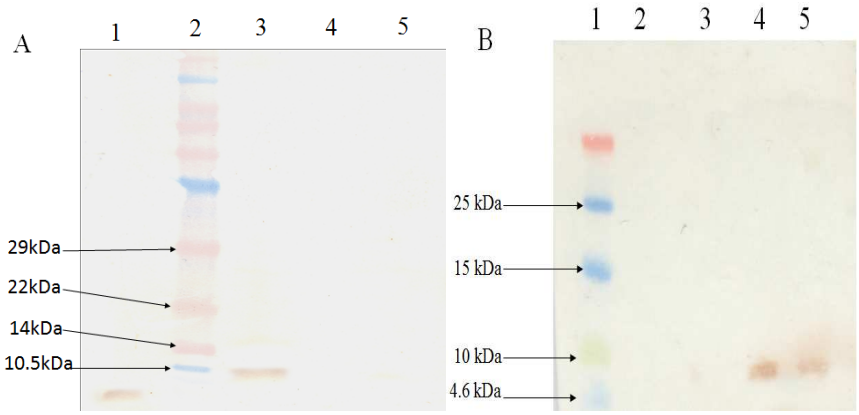

Figure 4: Verification of both recombinant hEGF proteins by Western blotting technique. A) The Western blot results for recombinant hEGF protein from pET21a(+)-hEGF construct. Lane1: Positive control (commercial hEGF protein). Lane2: Protein size marker. Lane3: The 9.8 kDa band confirming recombinant hEGF protein. Lane 4: Uninduced recombinant *E.coli* cells. Lane5: Negative control(BSA). B) The Western blot results for recombinant hEGF protein from pET28a(+)-hEGF construct. Lane1: Protein size marker. Lane2: Negative control(BSA). Lane3: Uninduced recombinant *E.coli* cells. Lane 4: Positive control. Lane 5: The 6.4 kDa band confirming recombinant hEGF protein.

After solubilizing inclusion bodies with guanidine hydrochloride, dialysis was done to remove reducting agents and then purification process for proteins from pET21a(+)-EGF construct was performed by a Ni–NTA affinity chromatography. The purified recombinant protein was analyzed by Tricine SDS–PAGE and a 9.8 KDa band corresponding to recombinant protein was obtained (figure 5).

For protein purification produced by pET28a(+)-EGF construct, a novel and invented method was used. The details of this method were described in figure 6 legends.

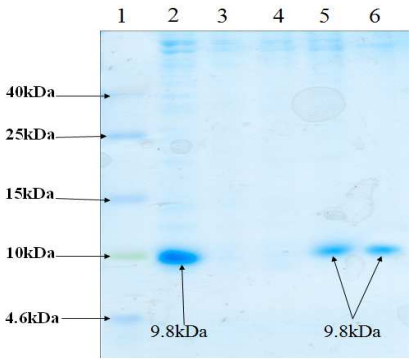

Figure5: Purification of recombinant hEGF protein expressed from pET21a(+)-hEGF construct. Lane1: Protein size marker. Lane2: Renaturated samples before passing from the column. Lane 3-5: Samples were washed by imidazole buffers (40, 170,and 250mM) and MES buffer, respectively.

As shown in figure7, negligible differences between the produced recombinant hEGF with the molecular weight of 6.4 kDa and standard hEGF were resulted from those chromatogram alignments but these differences were more for alignment between produced recombinant hEGF with the molecular weight of 9.8 kDa and standard hEGF.

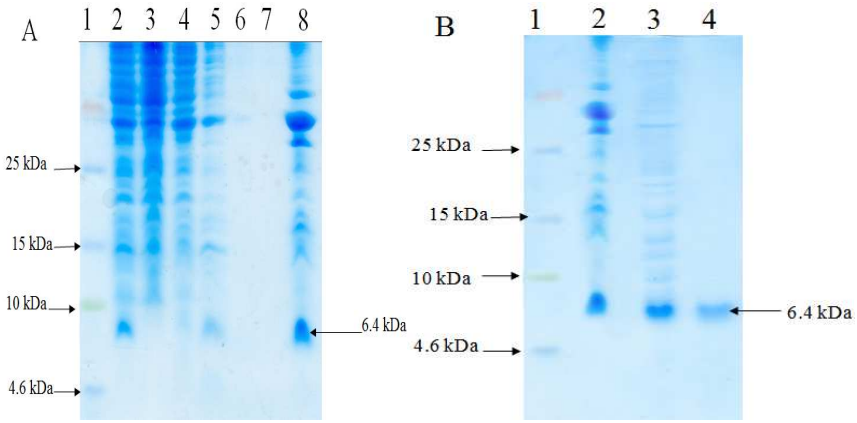

Figure 6: Novel purification method for recombinant hEGF protein with the molecular weight of 6.4 kDa. Inclusion bodies stepwise purification manner. A) Lane 1: Protein size marker. Lane 2: Total proteins of *E.coli* BL21(DE3) cells containing pET28a(+)-EGF recombinant vector. Lane 3-7: Supernatant solutions from cell sediments washed with Tris-Hcl pH=8.5 containing 5 mM EDTA and 1 mM PMSF, Tris-HCl pH= 8.5 containing 1% sodium deoxycholate salt (DOC), 1% sodium deoxycholatesalt, Tris-Hcl pH= 8.5 and PBS, respectively. Lane 8: final cell sediments. B) lane 1: Protein size marker. Lane 2: Sample purified from inclusion bodies. Lane 3,4: Samples passed from Amicon filters with 50 and 10 kDa pore size respectively.

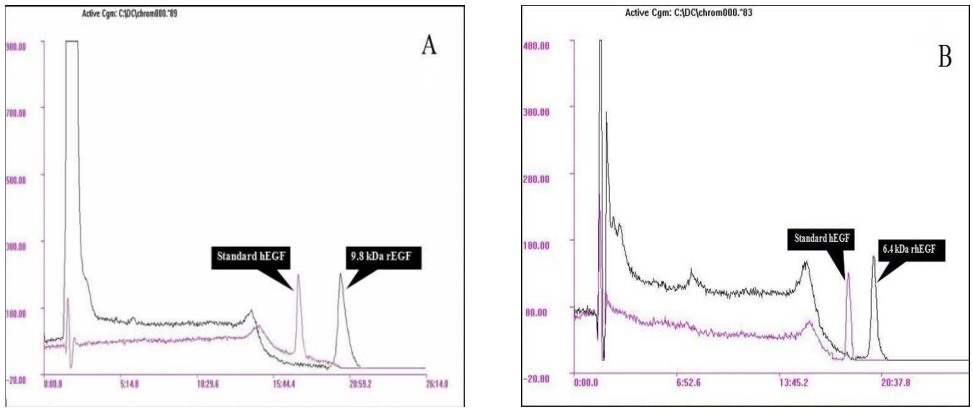

Figure7: The biologically active and pure protein analyzed by HPLC, using kromasil C-18 column (300 mm-4.6 mm), with a mobile phase containing 0.1% trifluoroacetic acid in water and acetonitrile (0–80%) shown a main single peak. A) Chromatogram's alignment between standard EGF protein and recombinant hEGF protein with the molecular weight of 9.8 kDa. B) Chromatogram's alignment between standard EGF and recombinant hEGF protein with the molecular weight of 6.4 kDa.

3-3. Biological activity assessment:

Ultimately the ability of this recombinant hEGF protein in the induction of cell proliferation was assessed on NIH-3T3 cell line. Results were recorded during 3 days as shown in diagrams (figure 8).

Important points were perceived by comparing these results. For example in the first day after treatment, produced samples from this study in 1 ng concentration have more effect on cell proliferation than standard sample even in 10 ng concentration. In 100 and 1000 ng concentrations same results were almost achieved. Results from second and third days were showed that these recombinants hEGF proteins have more influence on cell proliferation than standard hEGF in all concentrations.

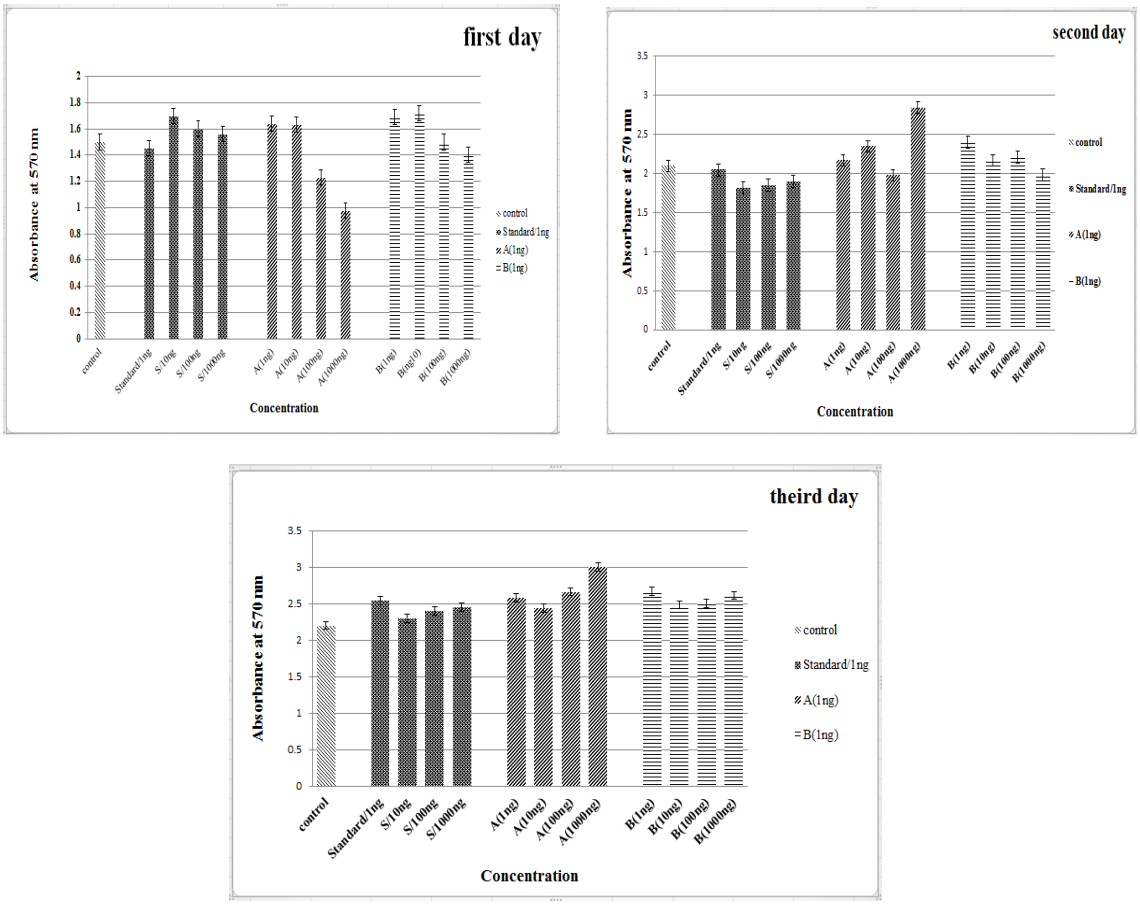

Figure 8: Diagrams for OD absorbance from MTT assay during three sequential days after cell cultures treatment with recombinants hEGF. A (6.4 kDa) and B (9.8 kDa) and standard hEGF.

DISCUSSION

Nowadays a lot of efforts were done for the production of recombinant proteins using simple and inexpensive methods as we can see them in literatures [12]. Human epidermal growth factor is a small protein which its advantages attracted many researchers [13]. In this study optimized synthetic gene for production of recombinant hEGF protein was used because synthetic genes for recombinant production of human proteins have some advantages [14]. Different methods for recombinant protein purification by affinity chromatography were used and among them FLAG tag was the best one for high degree of purification and His_6-tag was the common method [15]. In this study with regard to small size of hEGF (53 amino acids) and minimal alterations in hEGF's conformation, his_6-tag was preferred among other signal peptides for purification of this recombinant protein. By comparing the yields of purification method and biological activity assessment of recombinant hEGF protein with the molecular weight of 9.8 kDa with researches conducted by Ferrer Soler *et al* (fusion of thioredoxin gene to hEGF gene) [10] and Ebrahimi Rad *et al* study (fusion β-lactamase with hEGF gene) [8], results were benefited for our fused protein. Another recombinant hEGF protein in this study (6.4 kDa) was purified by the novel method in which purification time and biological activity assessment in comparison with other studies (purification by RP-HPLC) [6,7] was benefited for our recombinant hEGF protein. As noticed above, in some cases which affinity chromatography was used, the size of recombinant hEGF protein was changed because of its fusion peptide and in cases which RP-HPLC was used, the final costs and time expended were increased.

In comparison with purification method for recombinant hEGF protein (from pET21a(+)-EGF construct), our novel method for purification of recombinant hEGF protein(from pET28a(+)-EGF construct) did not have much changes in the size of protein and also purification was done very fast and inexpensive. The results of biological activity

assessment in this study shown better results than other relative studies. Sharma *et al* have reported same biological activity for standard hEGF and recombinant hEGF purified by using expanded-bed adsorption chromatography [16]. Ebrahimi Rad *et al* at the synchronizing cytoplasmic and periplasmic production of hEGF in *E. coli*, identical biological activity for pripelasmic hEGF and 17% biological activity for cytoplasmic hEGF have been reported [8]. Also Ferrer soler *et al* by producing recombinant hEGF protein in three expressing constructs and ultimately comparing theirs activities by Wesrern blot analysis were shown 94% activity for cytoplasmic hEGF and 42.5 and 67.5 % activity for soluble and insoluble hEGF fused with thioredoxin respectively [10].

CONCLUSION

In present study we produced two recombinant hEGF proteins and purifying them by two different methods. Comparing the biological activities of these recombinant hEGF proteins showed that sample without any fusion peptide is more active than recombinant hEGF protein containing fusion peptide and also both of them are more active rather than recombinant standard hEGF protein purified with RP-HPLC.

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