A Novel Vector for Expression/Secretion of Properly Folded Eukaryotic Proteins: a Comparative Study on Cytoplasmic and Periplasmic Expression of Human Epidermal Growth Factor in *E. coli*

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ABSTRACT

Expression of eukaryotic proteins in *E. coli* often results in their aggregation. Proper folding and solubility of therapeutical proteins are the pre-requisite for their bioactivity. This is not achieved in cytoplasmic expression in *E. coli* because of the absence of disulfide bonds formation. A novel expression/secretion vector was constructed which exploited β-lactamase signal sequence to translocate processed and soluble proteins into the periplasm of cells. Secretion of model proteins, β-lactamase and human Epidermal Growth Factor (hEGF) in M15/pSB and M15/pSE systems respectively, was confirmed by SDS-PAGE analysis and bioactivity assay. Secreted hEGF was found to be identical to authentic protein, in size, N-terminal amino acid sequence, biological activity and Western-blotting. The radioimmunoassay revealed 10-fold-higher level of hEGF expression in M15/pSE secretion system compared to that of cytoplasmic expression of the protein. The properly processed and *in vivo* folded hEGF demonstrated high solubility and bioactivity. The data obtained, evidenced in favor of M15/pSE expression system, which might be suitable to produce the small eukaryotic disulfide-bonded proteins for therapeutical applications or structural studies. *Iran Biomed. J. 8 (2): 51-61, 2004*

INTRODUCTION

Many medically and industrially important proteins experience difficulties in proper folding and acquiring a biologically active form when expressed at high concentrations in a bacterial host [1, 2]. The most frequent problem in proper folding of newly synthesized proteins is the formation of disulphide bonds [2]. Human interferon-α2c [3] and epidermal growth factor family [4] are some of the most therapeutically important proteins, which contain disulfide bonds. These covalent links are essential to the folding pathway and consequently to the biological activity of recombinant proteins. They may also enhance the stability of the protein [2, 5].

Among expression systems for recombinant protein production, *E. coli* remains one of the most widely used although there are some limitations in cytoplasmic expression of the recombinant disulfide-bonded proteins. Previous studies have shown that the disulfide bond formation is a process mainly restricted to proteins outside the cytoplasmic compartment, such as those secreted into the lumen of the endoplasmic reticulum of eukaryotic cells or into the periplasm of Gram-negative bacteria [5]. The presence of thioredoxin and glutathione/ glutaredoxin pathways in *E. coli* renders its cytoplasm to function in a reduced state so that disulfide bonds do not normally occur [5, 6]. In the case of disulfide-bonded proteins, inclusion body formation can be anticipated if the protein is produced in the bacterial cytosol and the consequence is improper folding which results in aggregation [7]. This makes the purification of such proteins into therapeutic grade, tricky and their

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structural study impossible [2]. So, the important strategy in expression of recombinant disulfide-containing proteins is their secretion into the periplasmic space of *E. coli*, as the periplasm is the location of the disulfide-forming machinery [8]. Furthermore, periplasmic expression of recombinant proteins has several advantages over cytoplasmic production. The absence of N-terminal methionine, higher stability of proteins due to the lower amount of proteinase in periplasm and facilitated purification of target protein because of the fewer amounts of proteins (4% of total cell protein) make the secretion system the preferred one in many cases [9].

Prokaryotic β-lactamase is naturally a secretory protein, which confers the cells resistance to ampicillin. As the level of this protein in periplasm of *E. coli* cells is directly related to the rate of membrane translocation of the precursor protein, so it could serve as a model for the development of novel periplasmic expression system [10].

Periplasmic expression of β-lactamase has been reported by the previous studies [11]. Many researchers have also used it as a reporter gene or as a model protein to evaluate the potential of secretion systems by different studies [12, 13]. Some other studies have shown that β-lactamase with out-processing and proper folding cannot confer the resistance against ampicillin to the cells [13]. Human Epidermal Growth Factor (hEGF), (53 amino acids) is medically important eukaryotic protein [4, 14]. Previous studies have reported the therapeutical and diagnostic applications of recombinant hEGF [14]. Many studies have been focused on designing the expression/ secretion systems to secrete the eukaryotic proteins, some of which have directed the expression of hEGF [1, 3, 9, 15, 16]. Some reports have demonstrated that the cytoplasmic expression of small polypeptides, directly in *E. coli* is extremely inefficient [15, 16]. The striking feature of proteins from EGF family is the conservation of six cysteines, which form three disulfide bonds [4]. In this study, β-lactamase and hEGF were selected as model proteins in order to investigate the potential of novel system in secretion of prokaryotic and small eukaryotic recombinant proteins with disulfide bonds. We analysed the formation of proper disulfide bonds and correct folding in hEGF and compared the periplasmic expression of hEGF with cytoplasmic form based on the level of expression and bioactivity.

MATERIALS AND METHODS

Bacterial stains. *E. coli* DH5α (for cloning), M15 *E. coli* cell (Qiagen/Germany) and BL21 plysisS (for expression), plasmids: pBR322, pUC18, pUC19, (New England Biolabs/USA), pUC118 (Panvera/ USA), pKK223-3 (Pharmacia/ Sweden), pBluescript II SK (Stratagene/USA). Plasmid pET23a (Novagen/ USA). All of the restriction enzymes, T4 DNA Ligase, T4 poly nucleotide kinase, Klenow fragment, mung bean nuclease, dNTP, Taq polymerase, and 1 Kb DNA ladder (Gibco BRL/USA), PfuTurbo™ (Stratagene/ USA), purification kits for PCR product and DNA fragment (Qiagen/Germany), EGF Radioimmuno-assay Assay kit, Vent polymerase and ECL western blotting detection reagent (Amersham Pharmacia Biotech/Sweden), Monoclonal anti-hEGF and hEGF (R and D company/USA). Nitrocefin (SR112C) (Oxoid/England), Methylthiazolyl-diphenyl-tetrazolium bromide (MTT) (Sigma/USA), Polyvinylidene difluoride (PVDF) membrane and protein molecular weight (Bio-Rad/USA) were used in this study.

PCR amplification of β-lactamase gene. The plasmid pBR322 was used as a template to amplify a fragment consisting of signal sequence and functional part of β-lactamase gene [17]. Following primers were used in the amplification protocol. The forward primer had an *Nde*I site and a methionine codon for translation initiation. The reverse primer was designed to include *Hind*III site after stop codon by changing one adenine nucleotide to guanine.

<div align="center">

***Nde*I**
</div>

Forward : 5' GAT AAA <u>CAT ATG</u> AGT ATT CAA CAT TTC CG 3'

<div align="center">

***Hind*III**
</div>

Reverse : 5' TGA GT<u>A AGC T</u>TG GTC TGA CAG TTA C 3'

Construction of expression vector. The strategy for construction of expression vector is illustrated in (Figs. 1 and 2). All the constructs were confirmed by restriction analysis. Plasmid pBR322 was digested with *Hind*III, end filled and self ligated to yield the plasmid pBH that is inactivated for *Hind*III. Plasmid pAM.ST (amp^r, tet^r) was constructed by sub-cloning of *EcoR*I-*Pvu*II fragment of pBH, consisting of tetracycline
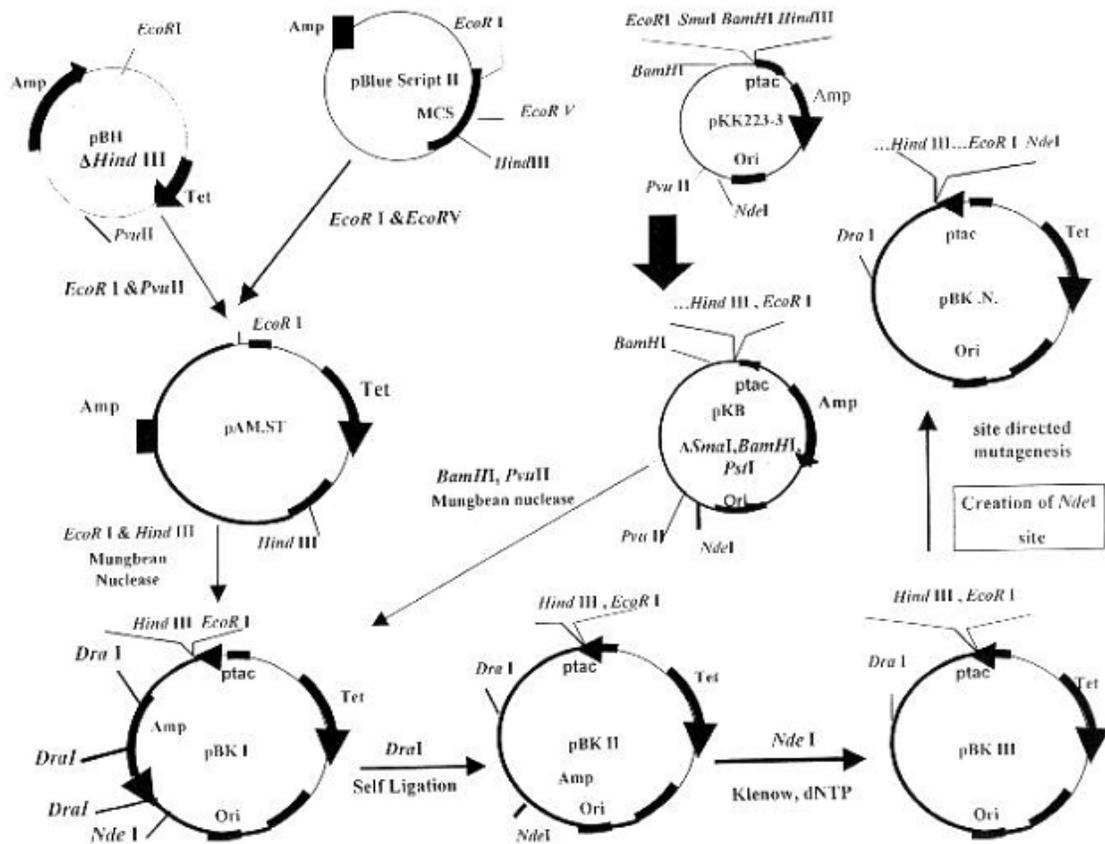
Fig. 1. The strategy for construction of pBK.N. plasmid Amp, β-lactamase resistance gene; Tet, tetracycline resistance gene; Δ, deletion of a gene or a site; Ori, origin of replication; ptac, tac promoter.

resistant gene, into pBluescript II SK. The removal of *Bam*HI site in Multiple Cloning Site (MCS) of pKK223-3 was performed by double digestion with *Sma*I and *Pst*I and the terminal flanking ends were flushed with mungbean nuclease. Self-ligation of this DNA fragment resulted into plasmid pKB (amp^r). Construction of pBK I (amp^r tet^r) was carried out by ligation of *EcoR*I-*Hind*III fragment of pAM.ST, treated with mung bean nuclease to the identically treated *Bam*HI-*Pvu*II fragment of pKB. Inactivation of ampicillin resistance gene by digestion of pBK I with *Dra*I and self-ligation of the fragment generated pBK II (amp^s tet^r). Plasmid pBK III was constructed by inactivation of *Nde*I site in pBK II by enzymatic digestion and end filling, using dNTP and Klenow.

Site directed mutagenesis in pBK III. A single *Nde*I site was introduced into pBK III by PCR based site directed mutagenesis [18], using the following primers that were exactly complementary to each other. The primers were mutated in 4 bases.

The resulted plasmid was called pBK.N (Fig. 1).

Mutated Primer I: 5'- ACA   GGA   AAC   *Nde***I**
*A C*A T*A*T G**G   A TAT CAA GCT T-3'

Mutated Primer II: 5'-AAG CTT GAT ATC CAT ATG TGT TTC CTG T-3'

Original Sequence: 5'-ACA GGA AACAG AATT CGA TATCAA GCT T-3'

PCR mediated cloning of *Nde*I-*Hind*III fragment consisting of □-lactamase signal sequence and the functional part of the gene was carried out in pUC118 at *Sma*I site and the recombinant clones were picked up from selective media containing ampicillin, Xgal and IPTG. The Plasmid pSB, (tet^r Amp^r) expression vector was produced by sub-cloning of PCR product (*Nde*I-*Hind*III) from pUC 118 into pBK.N.
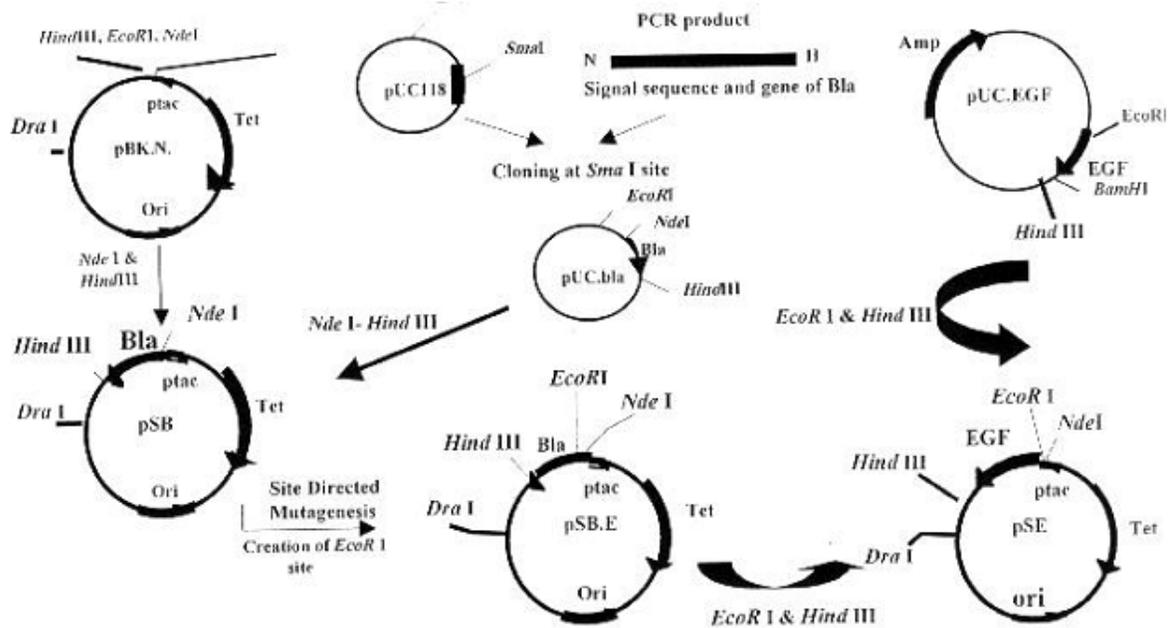
Fig. 2. The strategy for construction of expression/secretion vectors: pSB and pSE Bla, β-lactamase gene consisting of the signal sequence and the functional part of the gene; Tet, tetracycline resistance gene; Ori, origin of replication; ptac, tac promoter; EGF, the sequence coding for hEGF.

Computer analysis for signal sequence. By application of the procedures developed by Nielsen *et al.* [19], signal peptide cleavage sites were assigned. This prediction was carried out based on the primary sequences of β-lactamase and hEGF genes to estimate the optimal cleavage site [19].

Site directed mutagenesis in pSB. Based on computational analysis for cleavage site prediction, an *EcoR*I site was introduced into the junction between signal sequence and β-lactamase gene. The following primers, which were completely complementary to each other, were used in this amplification.

Mutated primer I: 5'-T   CCT   GTT   TTT
 ***EcoR*I**
GC*G*A*AT* T* CA GAA ACG CTG GT- 3'

Mutated primer II : 5'-A CCA GCG TTT CTG AAT TCG CAA AAA CAG GA-3'

Original Sequence: 5'-T CCT GTT TTT GCT CAC CCA GAA ACG CTG GT-3'

The presence of *EcoR*I site was confirmed by

restriction mapping of recombinant clones versus the original plasmid. *EcoR*I site was later used to fuse EGF chimeric gene (*EcoR*I-*Hind*III) to β-lactamase leader sequence, which generated plasmid pSE (Fig. 2).

Construction of expression vector for cytoplasmic expression of EGF. The plasmid pBR.EGF was used as a template to amplify EGF gene. Two following primers were designed to produce *Nde*I and *Hind*III sites at N and C terminal of EGF gene, respectively for cloning in plasmid pET 23a (Fig. 3).

         ***Nde*I**
Forward primer: 5'-GACTTGAC**CATATG**AATT CC GATAGCGAGTGT- 3'

         ***Hind*III**
Reverse primer: 5'-GCATTA**AAGCT**TTTATCTA A GTTCCCACCA – 3'

PCR mediated cloning of EGF was performed in pUC118 at *Sma*I site and it was subsequently sub-cloned in pET23a (*Nde*I-*Hind*III) resulting in pET.EGF, which was further transformed into BL21 cells for expression purpose (Fig. 3).
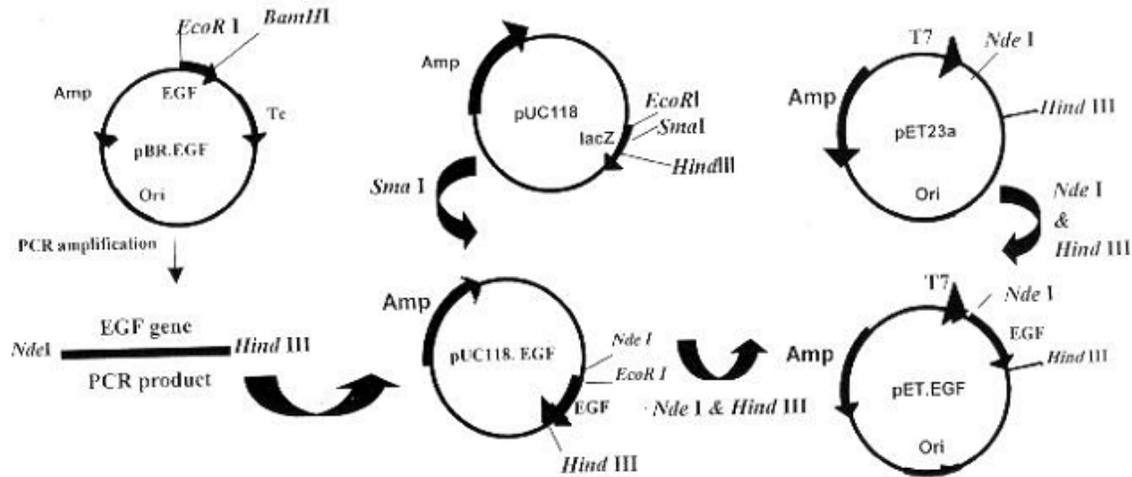
Fig. 3. A schematic representation of plasmid construction (pET.EGF) for cytoplasmic expression of hEGF. Amp, Ampicillin resistance gene; Tc, tetracycline resistance gene; ori, origin of replication; EGF, coding sequence for hEGF.

Protein expression. E. coli M15 cells were transformed with either pSB directing synthesis of β-lactamase or pSE expressing hEGF. The resulting cells were grown in LB medium containing tetracycline (12.5 μg/ml) and kanamycin (20 μg/ml) at 37°C. For BL21/pET.EGF, LB broth containing 100 μg ampicillin was inoculated by overnight cell cultures containing ampicillin and chloramphenicol. Cell growth of the cells was monitored by measuring optical density at A600 = 0.5. For M15 /pSB two final concentrations of IPTG: 1mM and 0.5 mM were used to induce the expression of β-lactamase. Temperature and incubation time after induction, including 22°C/over night and 37°C/3 h, were also tested for M15/pSB and M15/pSE. For BL21/pET.EGF, the addition of IPTG to a final concentration of 0.5 mM and the incubation for additional 3 and 9 hours after induction were done. Cells were harvested after incubation and the periplasmic proteins were obtained by osmotic shock using the standard protocol [20]. Soluble cytoplasmic EGF was prepared by resuspending the bacterial pellet in 10 mM Tris-HCl (pH 7.5), 10% sucrose, 0.2 M NaCl, 10 mM MgCl$_2$, followed by brief sonication and centrifugation. The supernatant contained soluble protein. The insoluble proteins from both systems were obtained by following protocol: bacterial precipitates were frozen (-80°C) and thawed 3 times and then were suspended and washed in a solution containing 0.5% Triton X-100, 0.2 M NaCl, 1mM EDTA followed by sonication. After three washings, inclusion bodies enriched in recombinant protein were pelleted.

Gel electrophoresis. A sample of recombinant β-lactamase was electrophoresed on 15% or 20% and recombinant hEGF was loaded on 25% SDS polyacrylamide gel. For better resolution, a modified procedure, (Anderson gel) [21] was also used (data not shown).

Immuno blotting. Western-blotting was carried out for recombinant hEGF. Monoclonal anti-hEGF (1.5 μg/ml) was used as first antibody and anti-mouse IgG labled by horseradish peroxidase (1:10000 dilution) as second antibody. Blocking step was achieved by using 5% skim milk in PBS containing Tween 20. Binding of the second antibody was revealed using chemiluminescence detection kit (ECL). EGF recombinant standard protein was also used in Western-blotting.

Minimum inhibitory concentration (MIC) of ampicillin. An overnight colony was suspended in 1 ml of 50 mM phosphate buffer (pH 7), 10 μl was spread on LB plates containing different concentrations of ampicillin (100 μg/ml-11 mg/ml). MIC was defined as the lowest concentration of ampicillin that inhibited growth [12]. The measurement was carried out for the cells containing pBR322 and the cells harboring pSB in triplicates.

Enzyme assay of β-lactamase. β-lactamase activity was determined spectrophotometrically using nitrocefin as substrate. The bioactivity of enzyme was detected on soluble material obtained

by osmotic shock from the cells carrying either pBR322 or pBK.N., pSB. Assay mixtures containing substrate solution and appropriate amounts of recombinant β-lactamase were incubated at room temperature for 10 minutes. All determinations were carried out in duplicates. One unit of β-lactamase was defined as the amount of enzyme that increased the absorbance at 486 nm by 0.001 in 1 min at room temperature [13]. β-lactamase from M15/pBR322 was measured to show the level of enzyme secretion under its own promoter and ribosome-binding site. It was also considered as positive control for assay. The periplasmic material from the cells carrying pBK.N., the construct without β-lactamase gene was used as a negative control. The samples were diluted before application for analysis [13].

Radioimmunoassay (RIA). Assay of recombinant EGF including cytoplasmic and periplasmic, soluble and insoluble forms was carried out by using RIA kit (Amersham-Pharmacia-Biotech) according to recommendation. Serial dilutions of authentic hEGF in the range of 0.08-10 ng/tube were prepared and the amount of recombinant EGF in the samples was measured automatically based on the standard curve using automatic gamma counter 1470 Wizard (Wallac, Finland). All measurements were performed in duplicate (Kit recommendation).

Bioactivity assay. A non-radioactive, colorimetric assay was used to detect the mitogenic effects of hEGF [22]. The assay was performed using BALB/c 3T3 cells (A31-714-C4, mouse fibroblast cell line). Seed preparation followed by necessary incubation periods at 37°C, 5% CO_2 in 90% DMEM with 10% calf serum and several sub-culturing. Sub-culturing was carried out using 0.0125% Trypsin and 0.02% EDTA in Ca^{++}, Mg^{++} free PBS. Cells (1.5×10^3) 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 serially diluted (10-fold steps) (from 10 ng/ml to 1 pg/ml), with the basal medium and were added to the wells (50 μl). Incubation at 37°C and 5% CO_2 for 48 h was followed by addition of methylthiazolyldiphenyl-tetrazolium bromide (MTT) solution in PBS. The plates were incubated for additional 4 h. Subsequently, 100 μl of 10% SDS and 0.01 N HCl were added to each well and the plates were incubated at 37°C and 5% CO_2 for 1 day. After the incubation, absorbance was measured at 570 nm. The bioactivity curve was prepared by plot corrected absorbance values 570nm (Y-axis) versus concentration of growth factor (X-axis, log scale). ED50 (one unit of bioactivity or the concentration of growth factor necessary to give half the maximal response) was measured by locating the X-axis value corresponding to one-half the maximum (plateau) absorbance value. The mitogenic activity was measured based on the bioactivity of standard EGF [22]. The well containing just media or the periplasmic material from a construct without EGF gene was both considered as negative controls. The samples for bioactivity assay consisted of the periplasmic material from M15/pSE (soluble protein), after osmotic shock and the soluble cytoplasmic EGF from BL21/pET.

N-terminal sequencing of EGF. After SDS-PAGE, the gel was stained and subsequently electroblotted onto a polyvinylidene difluouride (PVDF) membrane. The EGF band on the PVDF membrane was excised and sequenced using a protein sequencer (Model 491, Applied Biosystems, USA).

For sufficient amount of protein (40-100 pmol), running three to five lanes was helpful and multiple bands were cut out [23].

DNA sequencing of target genes in the constructs. The nucleotide sequences of DNA in pSB, pSE and pET.EGF were confirmed by dideoxy chain termination method.

RESULTS

Construction of expression vectors. Plasmid pBK.N. (tet^r) contained the tac promoter, ribosome binding site, transcription termination and origin of replication all from pKK223-3. Tetracycline selection marker was derived from pBR322. In order to study the expression of β-lactamase, ampicillin resistance gene was inactivated. Plasmid pBK.N. carried a single *Nde*I site down stream of the promoter as a result of site directed mutagenesis in pBK III by which 4 nucleotides were changed. Asterisks (in materials and methods) show mutated nucleotides. Plasmid pSB was constructed by PCR mediated cloning of the fragment (*Nde*I-*Hind*III) consisting of β-lactamase signal sequence and the functional part of the gene in pBK.N. (Fig. 1). A single *EcoR*I site was introduced into the adjacent site of signal sequence and the coding sequence for β-lactamase,

Table 1. The amounts of recombinant and native β-lactamase in the periplasmic material of M15/pSB and M15/pBR322, determined by enzyme assay. The assay was performed under the different conditions of expression for M15/pSB (IPTG concentration, incubation temperature and time course). One unit of β- lactamase was defined as the amount of enzyme that increased the absorbance at 486 nm by 0.001 in one minute at room temperature. The time course of enzymatic reaction was considered as 10 minutes. M15/pBK.N. (construct with out β-lactamase gene) was considered as negative control for the experiment.

Cell/construct	IPTG concentration	Time course	Temperature	β-lactamase unit/µl
M15/pSB	0.5 mM	3 h	37°C	42.94
M15/pSB	1.0 mM	3 h	37°C	81.60
M15/pSB	1.0 mM	overnight	22°C	2040.0
M15/pBK.N	-	3 h	37°C	0.00
M15/pBR322	-	3 h	37°C	0.019

which led to 4, mismatch nucleotides (shown by asterisks). *EcoR*I site was later used to fuse EGF synthetic gene (*EcoR*I-*Hind*III) to β-lactamase leader sequence, which generated plasmid pSE (Fig. 2). This strategy led to EGF gene substitution for β-lactamase. In order to express cytoplasmic hEGF, PCR mediated cloning of hEGF (*Nde*I-*Hind*III) was carried out in pET 23a (Fig. 3).

Recombinant β-lactamase bioactivity. The MIC of ampicillin for pSB transformants (11 mg/ml) was approximately 2.75 times more than this value for the cells carrying pBR322 (4 mg/ml). Periplasmic extracts from cells bearing pBK.N. without β-lactamase gene gave no measurable activity! in enzyme assay, indicating that the activity was due to β-lactamase alone. The results of enzyme assay for β-lactamase from M15/pBR322 and M15/pSB under different conditions are presented in Table 1.

β-lactamase in M15/pSB cells had two times more activity (81.6 unit/ µl versus 42.94 unit/ µl) when the expression induction was carried out using 1 mM instead of 0.5 mM IPTG. Overnight incubation of M15/pSB cells at 22°C after induction showed a higher activity of β-lactamase in compare with 3 h/37°C (2040 unit/ µl versus 81.6 unit/ µl) (Table 1).

Recombinant protein analyses. Both the soluble and insoluble material from M15/pSB were loaded on 15-20% SDS-PAGE. Although there was a predominant band with the size about 28 kDa on SDS-PAGE gel (Fig. 4), no equal band was found for insoluble material after sonication. The cell lysate from BL21/pET.EGF and the periplasmic fractions from M15/pSE were loaded on Anderson gel [20] and gave a single predominant band which corresponded to the mature and native form of protein with approximately 6 kDa size according to the protein size marker (data not shown). Expression of β-lactamase using M15/pSB showed

that the expression was controlled and there was no significant background in the absence of IPTG (Fig. 4). Immunoblotting of recombinant EGF, both cytoplasmic and periplasmic, revealed a band with the proper size, as compared with standard hEGF using a chemiluminescence detection kit (Fig. 5).
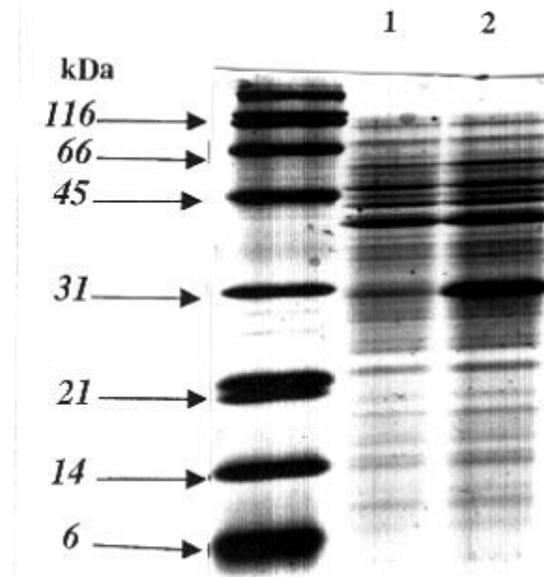

Fig. 4. SDS-PAGE analysis for recombinant β-lactamase from periplasmic material of M15/pSB after induction using 1 mM IPTG. 1, uninduced periplasmic extract and 2, induced periplasmic extract.

EGF RIA. The expression rate of hEGF in M15/pSE strains cultivated at 37°C for 3 h after induction was 120 µg/l as measured by RIA kit in the cold-water wash after the osmotic shock procedure. The amount was increased to 200 µg/l by incubation at 22°C overnight. However, only 20 µg/l of hEGF was detected in the cell lysate from BL21/pET.EGF cultured at 37°C/3 h after induction and the longer incubation time (9 h) even led to decreased amount of soluble EGF (7 µg/l).
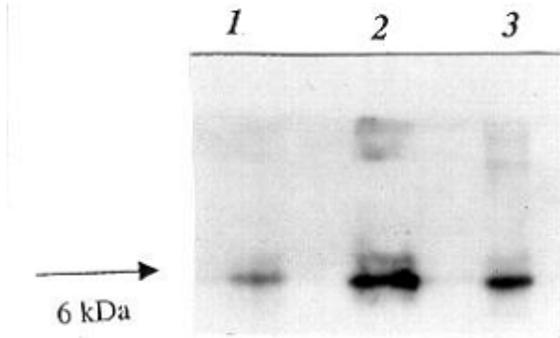
Fig. 5. Western-blot analysis of cytoplasmic and periplasmic recombinant hEGF. 1, Cell lysate from BL21/pET.EGF, the soluble cytoplasmic fraction after induction with 0.5 mM IPTG; 2, Periplasmic soluble fraction from M15/pSE, obtained by osmotic shock procedure after induction with 1 mM IPTG and 3, Standard hEGF.

We could detect only 0.9 μg/l of EGF in insoluble fraction of M15/ pSE, which is less than 1% of the total produced EGF in this system, while the insoluble EGF detected in BL21/pET.EGF was 4 μg/l which is 19% of expressed EGF in cytoplasmic expression system (Fig. 6).

N-terminal sequencing of secreted EGF. N-terminal sequencing of secreted EGF resulted in Asn-Ser-Asp-Ser that is the same sequence as authentic hEGF in the amino terminal region.
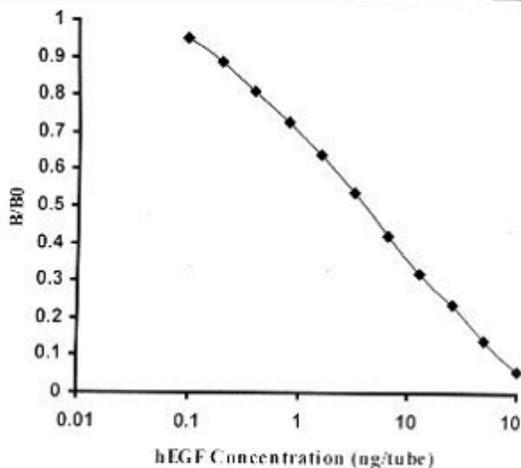

Fig. 6. Radioimmunoassay (RIA) standard curve for different concentrations of standard hEGF. B0 is binding of labled hEGF in the absence of unlabeled competitor (considered 100%) and B is binding of labled hEGF in the presence of unlabeled hEGF.

Biological activity of EGF. The bioactivity of secreted EGF in producing mitogenic effect on sensitive cells was 100% and it was completely

identical to authentic protein and standard hEGF. The bioactivity of soluble EGF in cytoplasmic expression was measured as 17% of the standard EGF biological activity (Fig. 7).
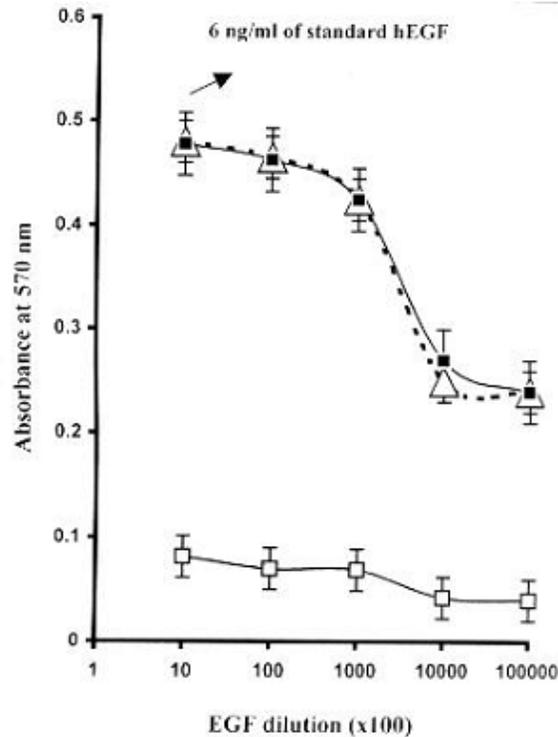

Fig. 7. Bioactivity assay of hEGF on BALB/c 3T3 fibroblast cells by using MTT colorimetric assay: (■) Standard hEGF (△) Periplasmic recombinant hEGF from M15/pSE by osmotic shock (□) cytoplasmic recombinant hEGF from BL21/pET.EGF. Standard (6 μg/ml) and samples were prepared in different dilutions (6 ng/ml-0.06 pg/ml). BALB/c 3T3 cells were cultured (10^3 cells/well). Assay is based on the cellular conversion of a tetrazolium salt into a formazan product. MTT formazan formed in the cells was solubilized with SDS solution. Absorbance at 570 nm was measured and the proliferation effect of hEGF on the cells was calculated and plotted against the cells treated with media alone. Each point and vertical line show the mean value and the standard deviation of triplicates.

DISCUSSION

This study was an effort to design a secretion system that is able to secrete the biologically active form of hEGF, a small protein, with 6 cysteines participating in folding. A novel and inducible protein expression/secretion system that facilitates periplasmic localization of mature β-lactamase is constructed. This system can also successfully translocate mature, correctly processed and folded hEGF that was able to induce mitogenic responses

in sensitive 3T3 cells. For exportation of both recombinant β-lactamase and hEGF into the periplasmic space, the secretion system employed the transcriptional control of tac promoter and the signal sequence of β-lactamase. β-lactamase was chosen as a prokaryotic model protein because *E. coli* cells secreting β-lactamase are resistant to ampicillin and, therefore can be positively selected.

Since cytoplasmic β-lactamase cannot protect a cell against ampicillin and catalytic activity of pre-β-lactamase is very low, the growth of ampicillin resistant transformants in *E. coli* would require both the correct translation and processing of pre-β-lactamase [13].

Many researchers have reported that inclusion body formation and misfolding are the consequences of high expression rates of mammalian proteins, specially those with several disulfide bonds, regardless of the system or protein used [3, 7]. Our results on enzyme assay and MIC determination indicated the over-expression of β-lactamase in proposed system (M15/pSB) compared with that under condition where β-lactamase was expressed under the control of its own promoter and ribosome binding site (M15/pBR322). Georgiou *et al.* [11] showed that over-expression of β-lactamase in *E. coli* RB791 cells under the transcriptional control of tac promoter and using phoA signal sequence led to the formation of inclusion bodies in periplasm. In spite of over-expression of β-lactamase in our system, there was no inclusion body formation of protein. The protein was completely soluble, as there was no band on SDS-PAGE for insoluble fraction of M15/pSB. It was also correctly folded and processed into biologically active form. This might be the result of the different systems and growth conditions used in our study and by the others.

In bacterial system, because of the higher speed of translation compared to eukaryotic cells, the folding of proteins is post-translational which leads to aggregation of eukaryotic recombinant proteins [24, 25]. The usage of tac promoter which is not as strong as T7 promoter, and the incubation at low temperature after induction!seems to be able to make the protein production slower and more sustained. Our results on the higher enzymatic activity of β-lactamase and the higher amount of secreted hEGF at longer incubation time with induction at lower temperature is compatible with the results presented by Chalmers *et al.* [26]. They reported that the lower temperature increased significantly the total amount of β-lactamase activity and the level of hEGF. This result might show that the slower growth could make enough

time for protein translocation and folding. Furthermore, the higher temperature might cause the higher rate of cell lysis. Taking the advantage of lower temperature might be also a useful way to decrease the proteolytic activity of the proteases present in periplasmic space.

Although some eukaryotic proteins have been successfully exported by bacteria, the information for export does not seem to reside exclusively in signal peptide and many attempts to promote the export by simply adding a signal peptide have failed [27, 28]. Our results show that the designing of precise site for in-frame fusion of β-lactamase signal sequence to EGF chimeric gene, conferred translocation of hEGF to the periplasm and *E. coli* could successfully recognize the attachment site of the signal sequence to EGF coding region and was able to cleave it off by specific signal peptidase. Results of protein sequencing showed that the N-terminal amino acid sequence of recombinant hEGF is identical to authentic hEGF, which has the advantage of avoiding the problem with the N-terminal formyl-methionine residue. This is an important point in the recombinant therapeutical proteins expression with active site at the N-terminal [3].

Several researchers have reported that small proteins are more stable in the periplasm than in the cytoplasm [15]. Furthermore, there is a body of proofs for the role of disulfide bonds in stabilizing the proteins and their pre-requisite for proper folding [6]. The results presented here, show that the cytoplasmic expression of EGF in pET.EGF/BL21 system (20 µg/l at 3 h/37°C, 7 µg/l at 9 h/37°C) was remarkably lower than its periplasmic expression (200 µg/l), while T7 is stronger than tac promoter and pET/BL21 is very powerful system in expression of heterologous proteins. This finding which is compatible with the results from other studies [15, 16], might be due to instability of cytoplasmic hEGF, which could be in turn either the result of the higher sensitivity of a very small protein (6 kDa) to proteases existing in the cytoplasm of bacteria or might be due to the absence of disulfide bonds in cytoplasmic EGF.

The disulfide bonds in EGF generate the loops presenting important residues for EGF receptor binding [28], so the mitogenic effects of EGF is the outcome of right folding and correct disulfide bridges. Our results showed in comparison to periplasmic hEGF, the mitogenic effect of cytoplasmic hEGF on 3T3 cells is much lower and it is consistent with the previous studies on protein folding [2, 30]. The bioactivity of secreted hEGF in our study was compatible with authentic protein

that indicated the native conformation of the protein with the three-disulfide bridges [6]. To our knowledge, there is no previous report outlining comparison of biological activity of cytoplasmic and periplasmic hEGF. Several previous studies have used different secretion systems by which 100-1000 μg/l of recombinant hEGF have been received in the periplasm [9, 15, 16 and 30]. The difference between the result of our study (200 μg/l) and the others might be due to the different factors involved, such as host cells, expression vectors, the type of signal sequence, translational signals consisting of start codon and ribosome binding site, growth conditions including time course and induction, the coding sequence for EGF gene including the chimeric gene and the recovery procedure. Although the production of hEGF in this study is moderate, the result of *in vivo* folding of this protein makes the introduced system an attractive one. Four μg/l (25%) of total EGF was detected in inclusion bodies in the cytoplasm of BL21/pET.EGF, while the insoluble and aggregated EGF in secretion system was measured less than 1 μg/L (0.5%) by RIA kit. This finding indicates the high solubility of hEGF in proposed secretion system. The high yield of biologically active hEGF (100%) in this study might be count for the advantages of the system especially for the therapeutical proteins with disulfide bonds, for which the *in vitro* folding is not usually recommended [2, 3].

Tetracycline resistance selection marker in our expression vector might also provide an advantage versus ampicillin selection marker because of some problems reported when large-scale expression or long-term fermentation of *E. coli* was needed [10].

Finally, our results demonstrated that the difficulties, which are observed in cytoplasmic expression of hEGF, could be overcome by using the introduced secretion system. The high yield of biologically active hEGF in our study suggests that such a secretion system might be efficient to secrete other small eukaryotic proteins with the structural homology to EGF family.

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