J Microbiol Immunol Infect
2006;39:366-371

Original Article

Secretion of biologically active human epidermal growth factor from *Escherichia coli* using *Yersinia pestis* Caf1 signal peptide

Yi-Lin Liu[1], Liang-Min Huang[2], Wen-Po Lin[2], Chung-Chin Tsai[2], Tsung-Shun Lin[2], Yu-Hua Hu[2], Hquan-Shin Chen[2], Jun-Ming Han[2], Hsian-Jenn Wang[2], Yu-Tien Liu[2]

[1]Department of Dermatology, Taipei Veterans General Hospital, Taipei; and [2]Institute of Microbiology and Immunology, National Defense Medical Center, Taipei, Taiwan

Received: August 9, 2005 Revised: January 14, 2006 Accepted: February 6, 2006

Background and Purpose: The Caf1 secretion pathway of *Yersinia pestis* is one of the most well-characterized export machineries. To facilitate the secretion of human epidermal growth factor (hEGF) in *Escherichia coli*, a DNA fragment containing the synthetic gene for hEGF was joined to a sequence encoding the signal peptide of *Yersinia pestis* Caf1 protein.

Methods: The gene for hEGF was synthesized by overlapping polymerase chain reaction technique and was placed under the control of the *caf1* gene promoter in the recombinant plasmid pHL401 which was used to transfect *E. coli* BL-21 for production of hEGF. The biological function of recombinant hEGF was measured by estimating its ability to stimulate the proliferation of human embryonic kidney-293 cells.

Results: The results indicated that the expressed hybrid protein was processed during the secretion process. The majority of the mature hEGF was recovered from the periplasm and medium fractions, with a small amount of the expressed hEGF deposited in the cytoplasm. Furthermore, it was found that the cell proliferation was enhanced by the recombinant hEGF.

Conclusion: These results suggested that the recombinant hEGF was successfully secreted through the inner membrane of cells into the periplasm and then through the outer membrane into the medium via the action of the signal peptide of *Y. pestis* Caf1 in *E. coli*. The mitogenic activity of hEGF in cells was demonstrated.

Key words: Bacterial proteins, Caf1, protein sorting signals, recombinant fusion proteins, *Yersinia pestis*

Introduction

Human epidermal growth factor (hEGF) is a single-chain polypeptide consisting of 53 amino acids present in many mammalian species [1]. It is highly mitogenic in a number of cell types, including epithelial, fibroblast and endothelial cells and is capable of preventing gastric acid secretion in human [2,3]. These properties of hEGF have led to its use in wound healing, corneal transplants and the treatment of gastric ulcers.

In an *Escherichia coli* expression system, a signal peptide is generally required for the secretion of target protein through the inner membrane of cells into the periplasm and then the outer membrane into the medium; however, the presence of a signal peptide does not ensure efficient protein secretion [4]. The Caf1 secretion pathway of *Yersinia pestis* is one of the most well-characterized export machineries [5]. The precursor of *caf1* gene product consists of a structural Caf1 protein and a signal peptide which leads to secretion of Caf1 protein from *Y. pestis* [6]. The potential Caf1 signal peptide sequence has been identified with a typical cleavage site (/): Ala-Thr-Ala-Asn-Ala/Ala-Asp which resembled the prokaryotic consensus signal sequence: Ala-Ser-Ala-Phe-Ala/Ala-Asp, and yields a leader peptide of 21 residues and a secreted Caf1 of 149 residues [7]. Recent studies indicated that the Caf1 secretion pathway was capable of facilitating the secretion of

Corresponding author: Yu-Tien Liu, Ph.D., Professor of Microbiology, Institute of Microbiology and Immunology, National Defense Medical Center, P.O. Box 90048-505, Neihu, Taipei, Taiwan.
E-mail: ytliu@mail.ndmctsgh.edu.tw

full-length heterologous proteins fused to the C-terminus of Caf1 signal peptide and the N-terminus of structure Caf1 protein, for example, human interleukin-1beta [8].

In previous research, we constructed a recombinant plasmid pLT105 for producing the non-structural dengue virus type 2 NS1 protein in *E. coli* and *Salmonella enterica* serovar Typhimurium (unpublished data). The NS1 protein was fused to the C-terminus of the signal peptides and to the N-terminus of the structural protein of Caf1 such that the fusion protein could be delivered to the cell surface. In this study, the recombinant plasmid pHL401 was constructed from pLT105 by replacing the NS1 gene with hEGF gene which was fused with the DNA sequence encoding signal peptide but not fused with the sequence encoding structural Caf1, by using an additional stop codon at the 3'-end of hEGF gene. Although construction and cloning in bacteria and yeast cells of recombinant DNA containing a synthetic gene for hEGF have been reported elsewhere, this article is the first to describe the ability of *Y. pestis* Caf1 signal peptide to facilitate the secretion of hEGF from *E. coli* [9-13].

Methods

Synthesis and cloning of hEGF gene

The strategy for the synthesis and cloning of hEGF gene (GenBank accession number, AF274587) is illustrated in Fig. 1. Initially, the DNA fragment containing the

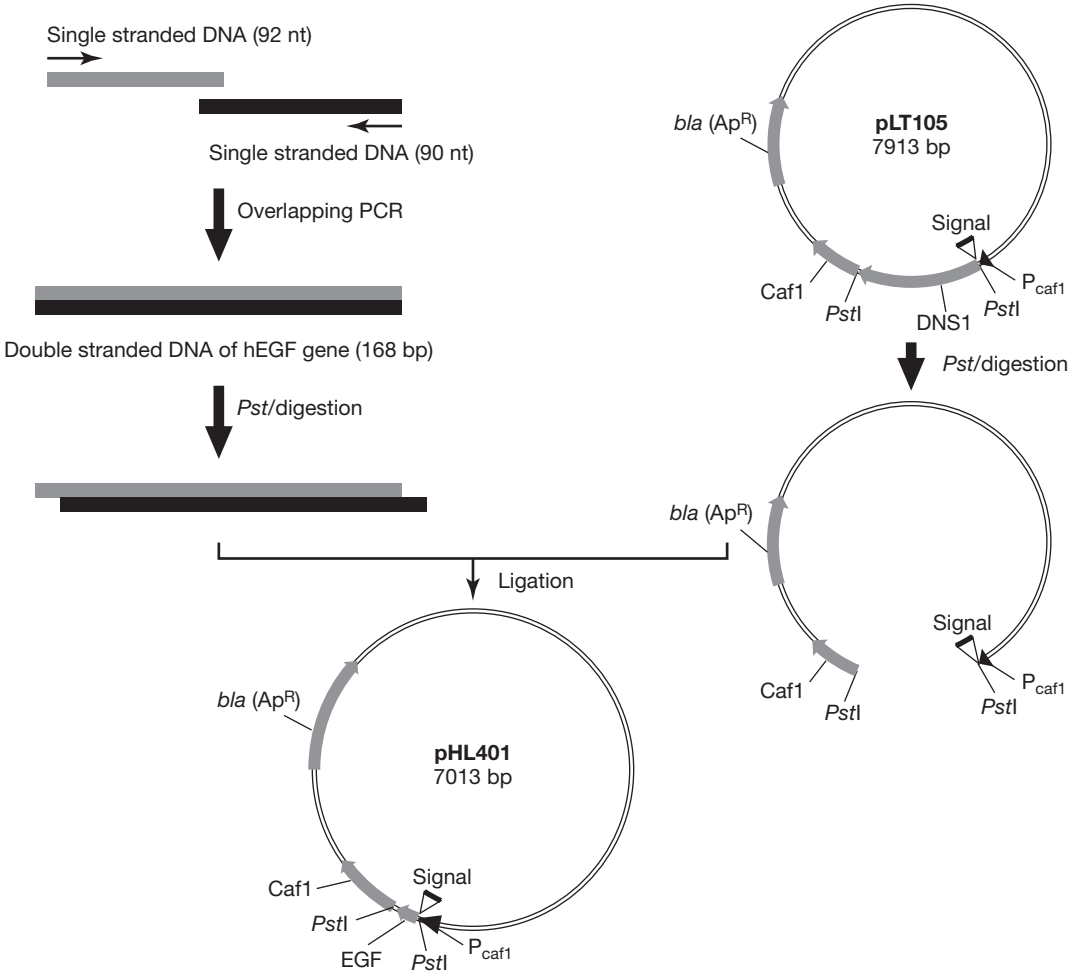

Fig. 1. Synthesis of human epidermal growth factor (hEGF) gene and construction of the recombinant plasmid, pHL401. Gene fragments and positions of restriction endonuclease cleavage sites are not drawn in their actual proportions. hEGF gene was synthesized by overlapping polymerase chain reaction (PCR) using two single stranded oligonucleotides with 20 overlapping complementary nucleotides of each 3'-terminal sequence and two primers indicated by arrows. "Signal" represents the DNA sequence encoding signal peptide of Caf1. Only the essential genes and restriction enzyme cutting sites on the plasmid pHL401 are indicated. *bla* = beta-lactamase gene; Ap^R = ampicillin resistance gene marker; nt = nucleotide; Pcaf = *caf1* gene promoter; signal = sequence encoding signal peptide of *Yersinia pestis* Caf1 protein.

gene for hEGF was synthesized by using two single-stranded oligonucleotide sequences including a 92-mer forward oligonucleotide and a 90-mer reverse oligonucleotide sequences with a 20-nucleotide complementary sequence at the 3' end, which were designed according to the sequence for hEGF (GenBank accession number, AF274587). Then, the hEGF gene was synthesized and amplified by overlapping polymerase chain reaction (PCR) using the forward primer: 5'-AAAACTGCAGAATTCTGATTCTGAA-3' and the reverse primer: 5'-AAAACTGCAGTTAT CTCAATTCCCA-3'. Each primer contained a *Pst*I cutting site (underlined). PCR was performed according to a protocol previously described [14]. Finally, the PCR product was cloned into the vector plasmid pLT105 by ligating the *Pst*I-digested DNA fragment containing hEGF gene with the *Pst*I-digested pLT105 to create the recombinant plasmid pHL401 which was used to transfect *E coli* BL-21 (Yeastern Biotech Co., Ltd, Taiwan) for expression of hEGF. The DNA techniques were performed according to Sambrook et al [14].

Expression of hEGF

The LB broth (Difco, NJ, USA) supplemented with 100 μg/mL of ampicillin was used for culturing *E. coli* (pHL401). In cell growth, a 300 mL flask containing 50 mL of growth medium was inoculated with a single colony from a freshly spread plate and cultured in an incubator at 220 rpm for 7-9 h at 37°C. For the production of hEGF, a 300 mL flask containing 50 mL of LB medium supplemented with 100 μg/mL ampicillin was inoculated with 1 mL inoculum of the seed culture and incubated for 18 h at 37°C.

Preparation of crude extract

The hEGF-containing culture was clarified by centrifugation and the hEGF was recovered from the medium fraction by a salting out method using 35% saturation of ammonium sulfate at 4°C. After centrifugation at 10,000 rpm for 30 min at 4°C, the precipitate was re-suspended in 0.02 M phosphate-buffered saline and left for 4 h at 4°C. The suspension was clarified by centrifugation at 10,000 rpm for 30 min. The supernatant containing hEGF was then desalted and partially purified by means of using Microcon Centrifugal Filter Devices (Millipore, Billerica, MA, USA). The periplasm fraction of hEGF was prepared by osmotic shock method, with the lysate of the cells after osmotic shock, prepared by ultrasonification, representing the cytoplasm fraction [15]. The protein concentration of hEGF was determined by the BCA method following the manufacturer's recommendation (Pierce Biochemicals, Rockford, IL, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

hEGF protein was separated from other proteins by a 20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for the qualitative analysis of hEGF. Protein bands were visualized by Coomassie brilliant blue (Sigma, St. Louis, MO, USA) staining [16]. Mark 12 (Cat. No. LC5677) purchased from Invitrogen Co. (CA, USA) was used as protein markers in this study. The content of hEGF was quantified by One-Dscan™ software (Scanalytics, Inc. USA) for calculating the percentage of hEGF in the total protein of crude extract.

Immunoblot analysis of hEGF

For immunoblotting, proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked by skim milk (5%) in phosphate-buffered saline-Tween and incubated with mouse monoclonal antibody (R&D Systems Inc., Minnespolis, MN, USA) specific to hEGF (R&D Systems Inc.). Binding of primary antibodies was visualized by peroxidase-conjugated goat anti-mouse antibodies (Bio-Rad, CA, USA) using ECL kit (Amersham Biosciences, Stockholm, Sweden).

Biological activity assay

The ability of hEGF to stimulate the proliferation of HEK-293 (human embryonic kidney cell, American Type Culture Collection CRL-1573) cells was measured by hemocytometer. The HEK-293 cells were directly adapted to grow readily in the serum-free medium, HYQSFM4HEK293 (HyClone, Logan, UT, USA), supplemented with 100 μg/mL of streptomycin, 100 units/mL of penicillin G, and 0.25 μg/mL of amphotericin B by a serum-free adaptation process in which the serum concentration of the culture medium was reduced by one-half (from 10% to 5%, and so forth) upon subsequent passage. Initially, the cells were seeded at 4×10^4 cells per well in a volume of 1 mL into 6-well culture plates and incubated for 12 h. The culture medium containing 10 ng/mL of the test hEGF was then added (1 mL per well). After one and two days of further incubation at 37°C in 5% carbon dioxide, the cells were trypsinized and the number of viable cells was determined using the trypan blue exclusion method: 2.5% trypan blue was added to the cell suspension

before counting to differentiate between viable (dye-excluding) and non-viable (blue staining) cells. Triplicate experiments were performed in this study and the data were shown as mean ± standard deviation.

Results

A DNA fragment coding the 53 amino acids of hEGF was successfully synthesized by overlapping PCR and cloned into a secretion vector (pLT105) for hEGF expression. The recombinant plasmid (pHL401) was transferred to *E. coli* BL-21. A fusion protein consisting of Caf1 signal peptide and hEGF is expressed from pHL401 under the control of the *caf1* promoter (Fig. 1).

The immunoblotting analysis (Fig. 2A) showed that a protein band from the culture medium of *E. coli* BL-21 (pHL401), with a molecular mass close to that of hEGF, was recognized by mouse anti-hEGF monoclonal antibody (R & D Systems Inc.). This result demonstrated that the expressed hEGF was successfully secreted from *E. coli* into the culture medium via the action of Caf1 signal peptide.

The cellular location of the expressed hEGF in *E. coli* BL-21(pHL401) was analyzed by SDS-PAGE. The result demonstrated that the majority of the expressed hEGF was found in the medium and periplasm fractions, with a minority of the expressed hEGF deposited in the cytoplasm (Fig. 2B and 2C). The content of hEGF in

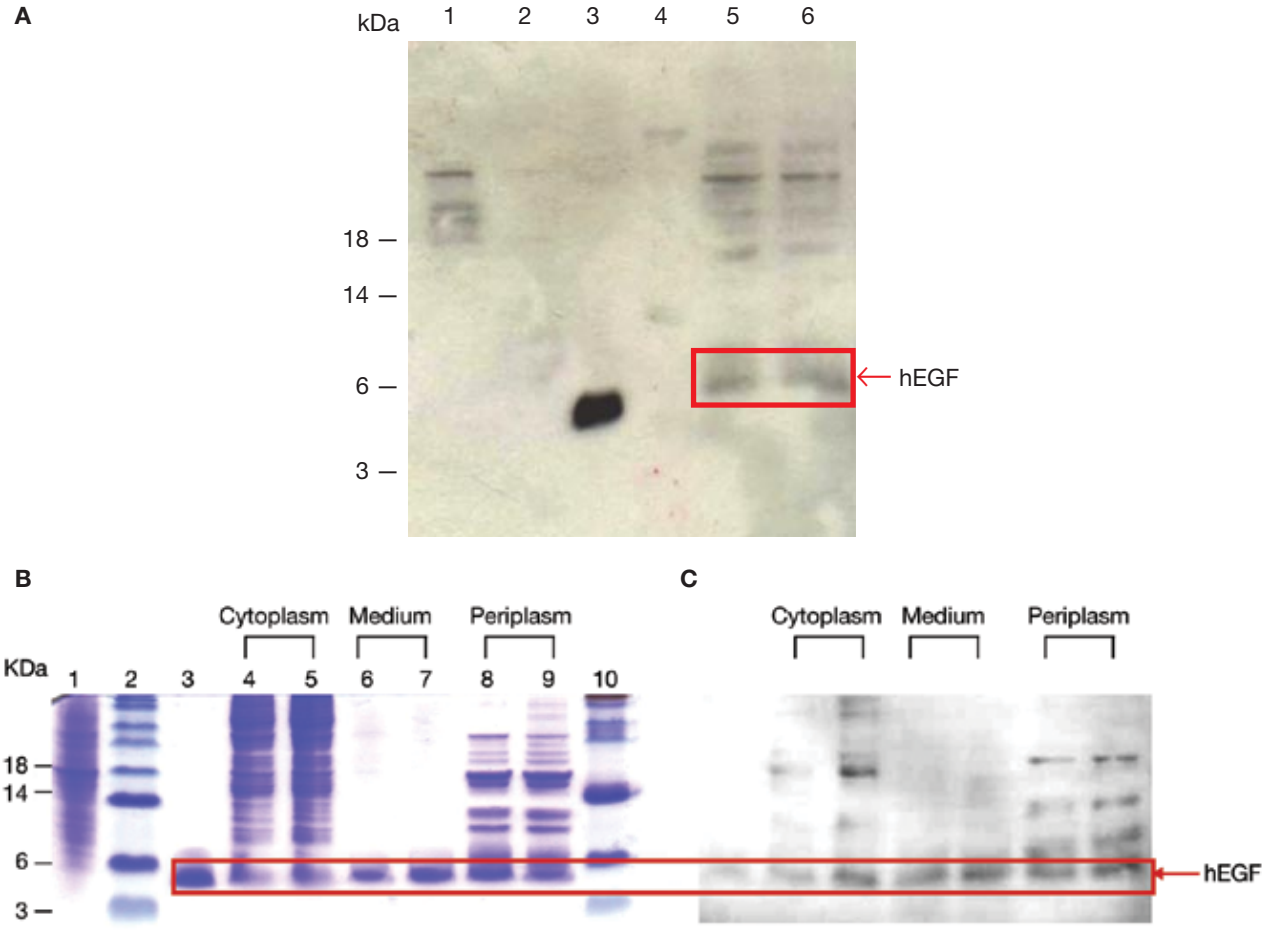

Fig. 2. Immunoblotting and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of human epidermal growth factor (hEGF). A) Immunoblotting analysis of hEGF; lane 1, culture medium of BL-21 as negative control; 2, whole cell lysate of BL-21 as negative control; 3, authenic hEGF as positive control; 4, protein markers; 5 and 6, crude extract of BL-21 (pHL401) culture medium. An aliquot of each sample, containing approximately 40 μg of protein, was used for this experiment. B) SDS-PAGE analysis of hEGF: lane 1, BL-21 culture medium as negative control; 2 and 10, protein marker; 3, authentic hEGF as positive control; 4 and 5, cytoplasm fraction of BL-21(pHL401); 6 and 7, medium fraction of BL-21(pHL 401); 8 and 9, periplasm fraction of BL-21(pHL 401). C) Immunoblotting analysis of hEGF: the samples of each preparation used in this experiment are the same as those used in panel B.

Table 1. Expression and secretion of human epidermal growth factor (hEGF) in *Escherichia coli*

Bacteria	Fraction	hEGF (μg/L of culture)[a]
BL-21(pHL401)	Medium	815[b]
	Periplasm	752
	Cytoplasm	216
BL-21(pUC18)	Medium	ND[c]

Abbreviation: ND = not detectable

[a]The content of hEGF was calculated from the total protein of each fraction prepared from *E. coli* BL-21(pHL401) culture times the percentage of hEGF in each fraction, determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of hEGF.

[b]The average of the results from duplicate experiments.

[c]All cultures were incubated at 37°C for 18 h.

each fraction was calculated by measuring the percentage of hEGF in the total protein of each fraction, using one-Dscan software. When *E. coli* BL-21 harboring the recombinant plasmid pHL401 was cultivated at 37°C for 18 h, almost equal amounts of hEGF were recovered from the medium and the periplasm fraction, but only a small amount of hEGF was found in the cytoplasm (Table 1). The expressed hEGF was efficiently secreted through not only the inner membrane into the periplasmic space but also the outer membrane into the medium. In this study, an hEGF crude extract, in which 85% of the protein was identified to be hEGF, prepared from the medium fraction by salting out method and partial purification using Microcon Centrifugal Filter Devices filtration (Millipore), was obtained and its biological activity determined.

Biological activity of the crude hEGF was measured by hemocytometer to assess its effectiveness in promoting HEK-293 cell proliferation. Since fetal bovine serum may contain EGF, serum-free medium was used in the determination of biological function of hEGF. The partially purified hEGF enhanced the growth of HEK-293 cells while the denatured hEGF and the crudely extracted protein of *E. coli* BL-21 did not show any activity on the proliferation of HEK cells (Fig. 3). This result indicated that the test hEGF was an active protein with mitogenic activity in HEK cells, showing activity comparable to that of the authentic hEGF.

Discussion

In this study, a cDNA encoding mature epidermal growth factor was synthesized and cloned into a pLT105 vector in which hEGF, with the Caf1 signal peptide at its amino-terminal end, was expressed and secreted through inner

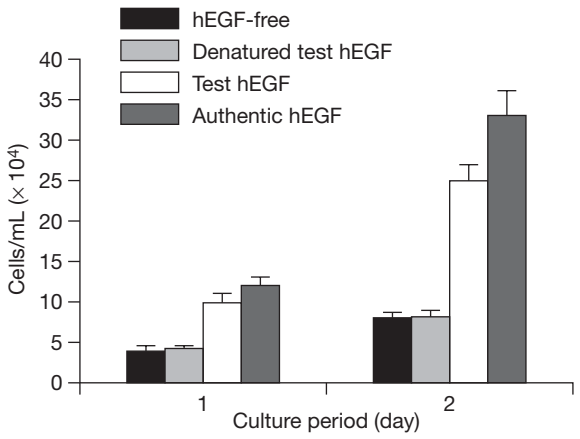

Fig. 3. Biological function of human epidermal growth factor (hEGF) produced by *Escherichia coli* BL-21(pHL401). Denatured test hEGF, treated by heating at 100°C for 15 min, was used as a negative control. Authentic hEGF was used as a positive control. Ten ng/mL each of the test hEGF, denatured test hEGF and authentic hEGF were added to HEK-293 cells (human embryonic kidney cell, American Type Culture Collection CRL-1573). In the hEGF-free group, 10 ng/mL of a crudely extracted protein of *E. coli* BL-21 was added to HEK-293 cells. After one and two days of further incubation at 37°C, the cells were trypsinized and the number of viable cells determined using the trypan blue exclusion method.

membrane into the periplasmic space and then outer membrane into the medium by *E. coli* BL-21. Since foreign peptides, especially rather short peptides, expressed in *E. coli* are degraded rapidly [17], it is possible that the small amount of hEGF observed in the cytoplasm fraction was due to protease activity in the cytoplasm (Table 1).

The recombinant hEGF produced by *E. coli* BL-21 (pHL401) stimulated the proliferation of HEK-293 cells as compared with control groups (Fig. 3). Its biological activity was comparable to, but somewhat less than, that of the commercially produced hEGF, suggesting that the hEGF preparation may contain a small fraction of denatured non-native hEGF in which some disulfide bond may be possibly destroyed by reduction during the extraction process [18].

In this study, we have demonstrated that the secretory apparatus of *E. coli* functions well in the secretion of a human polypeptide derived from a bacterially synthesized precursor with the *Y. pestis* Caf1 signal-peptide sequence. The *E. coli* expression and secretion system developed in this study is characterized by accumulation of the gene product in the periplasmic space and the culture medium. Thus, the system is useful in the production and recovery of foreign protein synthesized in *E. coli*.

Acknowledgments

This study was supported by grants DOD-93-1-01 and DOD-94-1-02 from the Ministry of National Defense, ROC.

References

1. Gregory H. Isolation and structure of urogastrone and its relationship to epidermal growth factor. Nature 1975;257:325-7.
2. Nakagawa S, Yoshida S, Mirao Y, Kasua S, Fuwa T. Biological effects of biosynthetic human EGF on the growth of mammalian cells in vitro. Differentiation 1985;29:294-8.
3. Clare JJ, Romanos MA, Rayment FB, Rowedder JE, Smith MA, Payne MM, et al. Production of mouse epidermal growth factor in yeast: high-level secretion using *Pichia pastoris* strains containing multiple gene copies. Gene 1991;105:205-12.
4. Jeong KJ, Lee SY. Secretory production of human leptin in *Escherichia coli*. Biotechnol Bioeng 2000;67:398-407.
5. Galyov EE, Smirnov OYu, Karlishev AV, Volkovoy KI, Denesyuk AI, Nazimov IV, et al. Nucleotide sequence of the *Yersinia pestis* gene encoding F1 antigen and the primary structure of the protein. Putative T and B cell epitopes. FEBS Lett 1990;277:230-2.
6. Galyov EE, Karlishev AV, Chernovskaya TV, Dolgikh DA, Smirnov OYu, Volkovoy KI, et al. Expression of the envelope antigen F1 of *Yersinia pestis* is mediated by the product of *caf1M* gene having homology with the chaperone protein PapD of *Escherichia coli*. FEBS Lett 1991;286:79-82.
7. von Heijne G. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res 1986;14:4683-90.
8. Zavialov AV, Batchikova NV, Korpela T, Petrovskaya LE, Korobko VG, Kersley J, et al. Secretion of recombinant protein via the chaperone/usher pathway in *Escherichia coli*. Appl Environ Microbiol 2001;67:1805-14.
9. Kim E, Fu J, Wilson DB, Shuler ML. Expression of human epidermal growth factor by *Escherichia coli* in continuous culture. Biotechnol Lett 1992;14:339-44.
10. Yadwad VB, Wilson S, Ward OP. Production of human epidermal growth factor by an ampicillin-resistant recombinant *Escherichia coli* strain. Biotechnol Lett 1994;16:885-90.
11. Clements JM, Catlin GH, Prince MJ, Edwards RM. Secretion of human epidermal growth factor from *Saccharomyces cerevisiae* using synthetic leader sequences. Gene 1991;106:267-71.
12. Ebisu S, Takagi H, Kadowaki K, Yamagata H, Udaka S. Production of human epidermal growth factor by *Bacillus brevis* increased with use of a stable plasmid from *B. brevis* 481. Biosci Biotechnol Biochem 1992;56:812-3.
13. Engler DA, Matsunami RK, Campion SR, Stringer CD, Stevens A, Niyogi SK. Cloning of authentic human epidermal growth factor as a bacterial secretory protein and its initial structure-function analysis by site-directed mutagenesis. J Biol Chem 1988;263:12384-90.
14. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
15. Neu HC, Heppel LA. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J Biol Chem 1965;240:3685-92.
16. Schagger H, von Jagow G. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal Chem 1987;166:368-79.
17. Derynck R, Roberts AB, Winkler ME, Chen EY, Goeddel DV. Human transforming growth factor-alpha: precursor structure and expression in *E. coli*. Cell 1984;38:287-97.
18. Savage CR Jr, Hash JH, Cohen S. Epidermal growth factor. Location of disulfide bonds. J Biol Chem 1973;248:7669-72.

