Production of human epidermal growth factor

Ureporn Kedjarune-Leggat, Wilaiwan Chotigeat
and Supreya Pongprerachok-Wanichpakorn

Abstract

The human epidermal growth factor (hEGF) gene was synthesized and cloned in *Escherichia coli* with phagemid pBK-CMV as a vector that had both prokaryotic and eukaryotic expression systems. The expression protein was extracted using osmotic shock technique and was estimated about 788 nanogram per liter of the cultured bacteria by ELISA method. The recombinant hEGF product had biological property for promoting keratinocyte proliferation. The hEGF product required further process of purification to exclude some toxic substances. However the plasmid of hEGF gene in this study could be used to transfect mammalian cells in order to release hEGF in the affected area directly for tissue engineering purpose.

Introduction

Epidermal growth factor (EGF) was one of the first discovered polypeptide growth factors. In 1920s, Cohen described a protein isolated from submaxillary glands of male mice that caused premature eyelid opening and tooth eruption in neonatal mice (Cohen, 1962). The EGF gene in human is located on chromosome 4 and consists of a large complex of 24 exons separated by large non-coding regions. The initial transcript is approximately 110 kb after splicing and export from the nucleus, a 4.8 kb mRNA codes for a 1207 amino acid precursor, which is processed to be an active 6-kDa EGF in the submaxillary, pancreatic, small intestinal and mammary glands (Bell *et al.*, 1986). Human epidermal growth factor (hEGF) comprises of 53 amino acid residues containing three intramolecular disulfide bonds and is identical to β-urogastrone, a polypeptide which was recognized and isolated on the basis of its ability to inhibit gastric acid secretion (Gregory,1975) . This growth factor is involved in the regulation of cell proliferation and can promote wound healing, which has been reviewed elsewhere (Boonstra *et al,* 1995). There have been some studies cloning hEGF gene in order to obtain the recombinant hEGF product using many different types of microorganisms and vectors (Oka *et al.*, 1985; Yamagata *et al.*, 1989; Ebisu *et al.*, 1996). In this study the synthesized hEGF gene was cloned in *E. coli* with plasmid vector that have both prokaryotic and eukaryotic expression systems which can use either getting hEGF product or can be used further to transfect into mammalian cells to produce hEGF in the affected area directly.

Materials and Methods

Materials

Plasmid p092k2 and *Eschelichia coli* strain K12DH5α were obtained from Interactiva (Baden-Württemberg, Germany). Phagemid pBK-CMV and *Eschelichia coli* strain XLOLR were obtained from Strategene Inc. (WI., USA). All other biochemicals were of high quality and commercial grade.

Methods

Preparation of hEGF gene and cloning in E. coli

Human epidermal growth factor gene (EGF) was synthesized by nucleotide synthesizer according to Yamagata *et al.* (1989). The synthesized EGF nucleotide was cloned into pO9K2 at *E. coR* I site. Since the EGF was synthesized to contain *E. coR*I and *Xho* I at 5′end and 3 ′end respectively, therefore, the EGF was digested from the pO9K2 at *E. coR*I and *Xho* I site, subcloned to express in pBK-CMV and then transformed into *E. coli* (XLOR) as the standard molecular cloning method (Sambrook *et al.*, 1989). The EGF nucleotides were analyzed on 1.5% agarose gel electrophoresis after subcloned into pBK-CMV. The expected EGF band was about 200 bp.

Expression of the recombinant EGF

The recombinant protein EGF was extracted from periplasmic of recombinant *E. coli* cell by cold osmotic shock according to the method of Oka *et. al* (1985). Briefly, *E. coli* XLOLR carrying pBK-CMV with hEGF gene was cultivated in 1000ml of LB broth medium with kanamycin (50 μg /ml) at 37°C overnight and the recombinant cell suspension was spun at 8,000g for 10 min and resuspended in 250 ml of ice cold 20% (W/V) sucrose buffer containing 30 mM Tris-HCl, pH 8.0, 1 mM EDTA, incubated on ice for 10 min, then centrifuged as above. The pellet was resuspended in 100 ml of 5 mM cold $MgCl_2$

And shaken in ice bath for 10 min and spun at 8,000 g for 15 min. The rhEGF in the supernatant was filter sterilized and analyzed for quantity and biological activity.

Semi-quantitation of rhEGF

The rhEGF in the supernatant was semi-quantitated by using goat polyclonal anti-human EGF (Sata Cruz, Biotechnology, USA) and donkey antigoat- IgG, AP (Promega, USA) as the primary and secondary antibody respectively. The method was modified from the method of ELISA described by Johnstone and Thorpe (1996). Briefly, the supernatant of the rhEGF of 100µl was serial diluted with phosphate buffer saline pH 7.4 containing 0.02 sodium azide (PBSN), hEGF (Promega, USA) at the concentrations between 5-10 µg/ml were used as a standard and the supernatant, extracted from *E. coli* harbouring the expression plasmid without the hEGF gene, was used as negative control. All the samples were done in triplicate. The supernatant of the rhEGF, the standard solutions and negative control solution were coated in 96-well ELISA plate, incubated overnight at 4°C, and washed three times with washing buffer (0.05% Tween-20 in PBSN) by Titertek microplate washer (Flow Laboratories, UK). Then, 50 µl of blocking solution (0.05% bovine serum albumin in 0.1 M Sodium carbonate pH9.6) was added into each well and left for one hour before washing 3 times with washing buffer. After that, the goat polyclonal anti-human EGF (Sata Cruz, Biotechnology, USA) as primary antibody was added in each well and left for 45 minutes at room temperature then washed as previously, before 50 µl of donkey anti-goat IgG conjugated with alkaline phosphatase (Promega, USA) as secondary antibody was added and then left at room temperature for 45 minutes. The plate was washed as previously and substrate (10 mM of p-nitrophenylphosphate disodium salt (pNPP) in 0.1 diethanolamine, pH10.3 containing 0.5 mM $MgCl_2$) of 50 µl was added in each well and incubated for 30 minutes at room temperature before reading the absorbance at 405 nm using Titertek Multiscan Plus (Labsystems, Finland).

Biological activity of rhEGF

The rhEGF protein in the extracted supernatant fluid was dialysed with dialysis membrane against phosphate buffer saline to remove some substances especially some ions, such as calcium, which can differentiate keratinocytes before being concentrated about 10 times and determined biological property by the increase in cell numbers of keratinocyte cells with various dilutions of the tested rhEGF in the culture media (Carpenter and Zendegui, 1985). Primary gingival keratinocytes were isolated using direct explant technique (Kedjarune *et al.*, 2001). Keratinocytes at second passage were used in this study. Keratinocytes at 1×10^4 cells in 1 ml of keratinocyte - SFM media (Life Technology,USA) supplemented with mouse EGF as provided by the company and 50 μg/ml of gentamicin (Life Technology, USA) were seeded in each well of 24-well culture dishes (Costar, USA). After 24 hours, the media was changed and replaced with keratinocyte-SFM media without EGF supplemented mixed with tested materials These were hEGF (Promega, USA) at concentrations 0.1, 0.2, 1 and 2 ng/ml, which acted as standard controls, while the tested samples were the supernatant of extracted *E. coli* with hEGF plasmid (rhEGF) at 50, 100, and 200 times dilution with culture media and the supernatant of extracted *E. coli* with plasmid that did not have hEGF gene at dilution 50 and 100 times and also keratinocyte media without EGF supplement as negative controls and media with EGF supplemented with mouse EGF as positive control. The experiment in each tested material was done in triplicate. After three days, each well was tripsinized by 500 μl of 0.025% trypsin in EDTA (Life technology, USA) and the numbers of cells were counted by Coulter counter (Coulter Corporation, USA).

Results

The DNA duplex coding for the 53 amino acids of hEGF was synthesized and cloned into plasmid vector p09K2. Its sequences and the plasmid's restriction map are shown in Figures 1 and 2, respectively. The plasmid was cut with restriction enzymes EcoRI and XhoI and the hEGF gene given sized about 178 base pairs, which can be

shown in gel electrophoresis in Figure 3. This gene was subcloned into pBK-CMV expression vector (see Figure 4) using *lac* promoter and also had *lacZ* gene as well as Neo'-Kan' gene, which indicated that the positive clone (with hEGF insertion) can be selected by kanamycin.

The cell lysate and the periplasmic fractions prepared by the osmotic-shock were assayed for hEGF by ELISA and the result can be seen in Table 1. The concentration of the hEGF was calculated from a standard calibration curve, as shown in Fig. 5. The supernatant extracted from *E. coli* with plasmid that have hEGF gene gave the mean (± SD) of absorbance at 0.013 (± 0.01), which may contained the hEGF about 7.88 ng/ml.

The biological property of rhEGF tested by growth assay of oral keratinocytes that have the tested products in culture media was shown in Table 2, which showed that the supernatant of *E. coli* extracted from positive clone that should have rhEGF mixed with keratinocyte SFM media had higher final cell numbers compared with the culture media that had the supernatant extracted of negative clone at the same dilution, but lower than the final cell number in culture media without EGF except in the 1/200 dilution of rhEGF. This meant that there may be some toxic substance(s) in the extracted supernatant fluid that interfered with the action of hEGF. It was noted that the cell numbers of standard hEGF concentrated at 0.1, 0.2, 1.0 and 2.0 ng/ml was not directly correlated.

Discussion

There have been a number of studies that have been concerned with the synthesis and secretion of hEGF using different types of microorganisms and vectors (Oka *et al.*, 1985; Yamagata *et al.*, 1989; Ebisu *et al.*,1996). In this study, *Eschelichia coli* was used as a host bacteria and the expression vector used in this study was pBK-CMV phagemid vector that has both prokaryotic and eukaryotic expression systems, which means that this bacterial clone can produce hEGF by itself. Moreover, this plasmid can be extracted and transfected in mammalian cells in order to secrete this growth factor, which can be further use in tissue engineering, another potential technique that is of interest in the current medical research involved with the promotion of wound healing and regeneration of new tissue.

The technique of ELISA used in this study is a semi-quantitative procedure detected the positive clone bacteria that could produce hEGF. It could be roughly estimated that the amount of the product's concentration was about 7.88 ng/ml of the 100 ml of the supernatant prepared from 1000 ml of bacteria culture overnight or the total product was about 788 ng per one liter of the cultured bacteria. However, protein released by osmotic shock that was used in this study was generally relatively low, being in the order of 4 to 7% of the total soluble protein (Bucke, 1983).

Acknowledgements

This study was supported by a Prince of Songkla University research grant 2001.

References

Bell, G.I., Fong, N.M., Stempien, M.M., Wormsted, M.A., Caput, D., Ku, L., Urdea, M.S., Rall, L.B., Sanchez-Pescador, R. (1986). Human epidermal growth factor precursor: cDNA sequence, expression *in vitro* and gene organization. Nucl. Acid Res. 14: 8427-8446.

Boonstra, J., Rijken, P., Humbel, B., Cremers, F., Verkleij, A., Henegouwen, P.B. (1995). The epidermal growth factor. Cell Biol. Int. 19: 413-430.

Bucke, C. (1983). Principles of Biotechnology, A. Wiseman (ed.). Surrey University Press, Glasgow, Scotland, pp. 151-171.

Carpenter, G., Zendegui, J. (1985). A Biological assay for epidermal growth factor/Urogastrone and related polypeptides. Anal. Biochem 153: 279-282.

Cohen, S. (1962). Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the new born animal. J. Biol. Chem. 237: 15 – 1562.

Ebisu, S., Takagi, H., Kadowaki, K., Yamagata, H., Udaka, S. (1996). The efficient production of human epidermal growth factor by *Bacillus brevis*. Anal. N. Y. Acad. Sc; 782: 115-122.

Gregory, H. (1975). Isolation and structure of urogastrone and its relationship to epidermal growth factor. Nature (London) 257: 325-327.

Johnstone, A., Thorpe R. (1996). Immunoassays. In. Immunochemistry in Practice. Third edition. Blackwell Science,Victoria, Australia, pp.290-297.

Kedjarune, U., Pongprerachok, S., Arpornmaeklong, P., Ungkusonmongkhon, K. (2001). Culturing primary human gingival epithelial cells: comparison of two isolation techniques. J. Cranio-maxillofacial Surg. 29: 224-231.

Oka, T., Sakamoto, S., Miyoshi, K., Fuwa, T., Yoda, K., Yamasaki, M., Tamura, G., Miyake, T. (1985). Synthesis and secretion of human epidermal growth factor by *Escherichia coli* . Proc Natl. Acad. Sci. USA 82: 7212-7216.

Sambrook, J., Fritsch, E.F., Maniatis, T. (1989). Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Yamagata, H., Nakahama, K., Sazuki, Y., Kakinuma, A., Tsukagoshi, N.and Udaka, S. (1989). Biochemistry 86, 3589-3593.

Table 1. Means (SD) of absorbances as the results of ELISA.

Standards hEGF (ng/ml)				Supernatant of *E.coli* extraction		
2	20	200	2000	Negative clone	rhEGF	Concentrated rhEGF
0.008	0.017	0.034	0.038	0.004	0.013	0.031
(0.007)	(0.007)	(0.012)	(0.017)	(0.004)	(0.010)	(0.026)

n = 3 in each group

Table 2. Final cell numbers of keratinocytes in hEGF growth assay.

Standards hEGF (ng/ml)				Media without EGF	Supernatant of *E. coli* extraction				
0.1	0.2	1.0	2.0		1/50 rhEGF	1/100 rhEGF	1/200 rhEGF	1/50 Negative clone	1/100 Negative clone
$1.51x10^4$	$1.28x10^4$	$1.0x10^4$	$3.6x10^4$	$1.17 x10^4$	$8.3x10^3$	$1.11x10^4$	$1.3x10^4$	$4.67x10^3$	$8.5x10^3$

1 **EcoRI**

GAATTCTATGAACAGTGATTCAGAATGTCCTCTCTCACACGATGG

CTTAAGATACTTGTCACTAAGTCTTACAGGAGAGAGTGTGCTACC

46

ATACTGCCTCCATGACGGCGTGTGTATGTATATTGAAGCACTAGACAAAT

TATGACGGAGGTACTGCCGCAGACATACATATAACTTCGTGATCTGTTTA

96

ACGCATGCAACTGTGTAGTTGGCTATATTGGTGAACGATGCCAGTACCGA

TGCGTACGTTGACACATCAACCGATATAACCACTTGCTACGGTCATGGCT

146 178

GATCTGAAATGGTGGGAACTGCGATAGCTCGAG

CTAGACT TTACCACCC TTGACGCTATCG**AGCTC**

XhoI

Fig. 1. The synthesized DNA duplex encoding for hEGF.

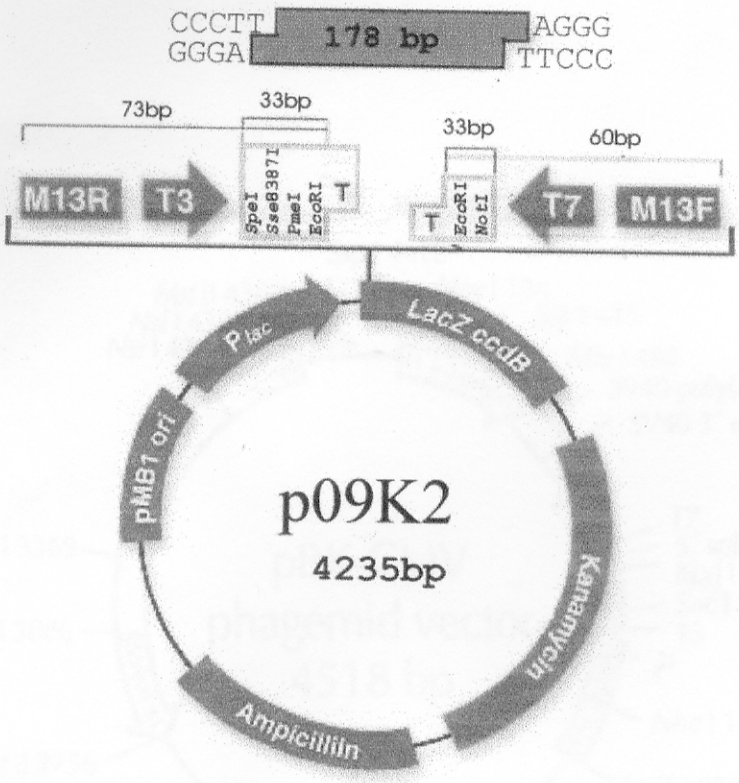


Fig. 2 Restriction map of plasmid P09K2 with synthesized hEGF gene

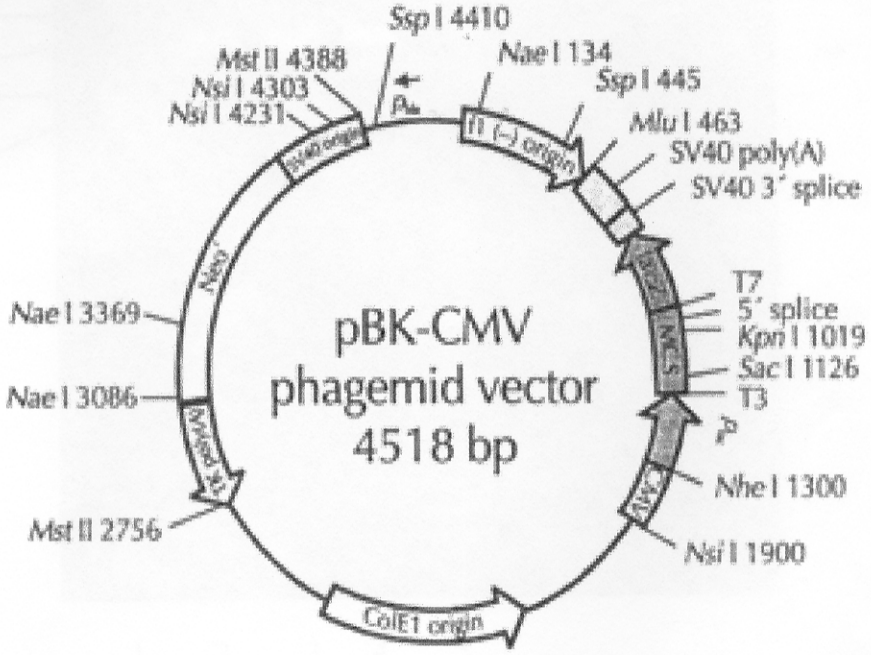


Fig. 3. Structure of pBK-CMV expression vector which can be cut with EcoRI and Xho I at multiple cloning site (MCS).

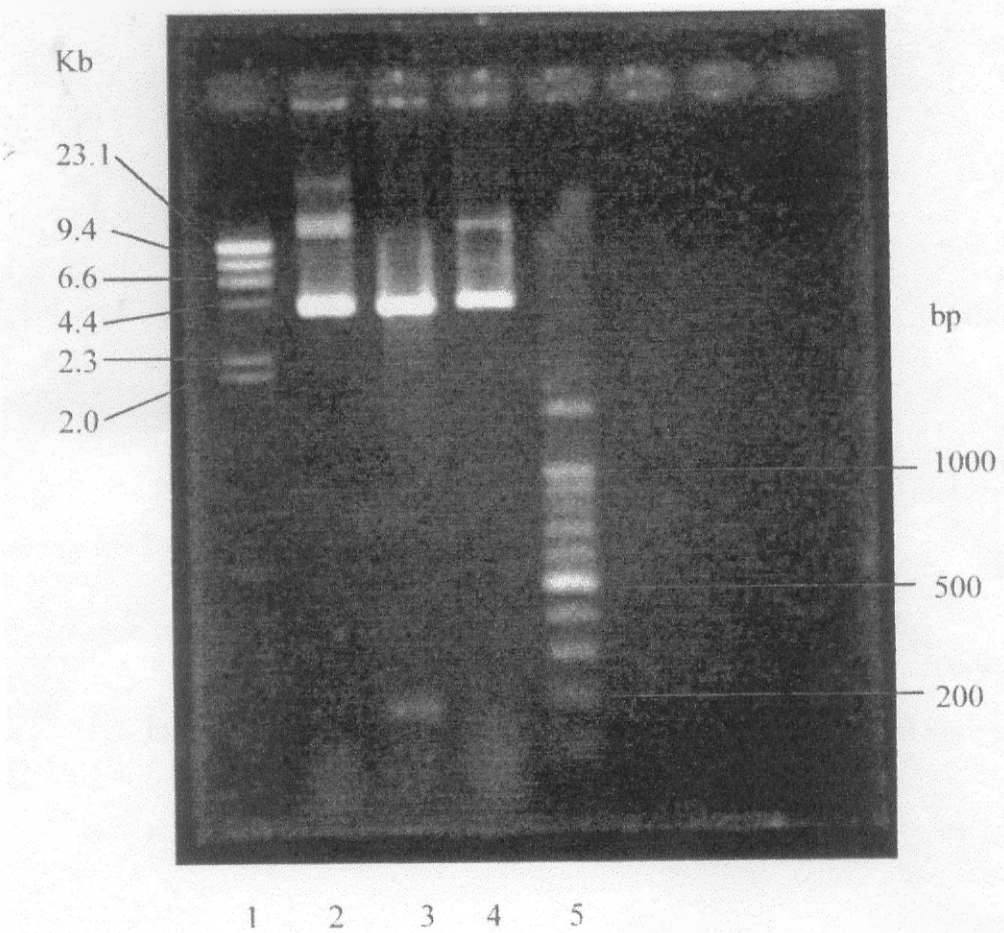


Fig. 4. Synthesized hEGF gene in plasmid p09K2 seperated on 1.5 % agarose gel Electrophoresis.

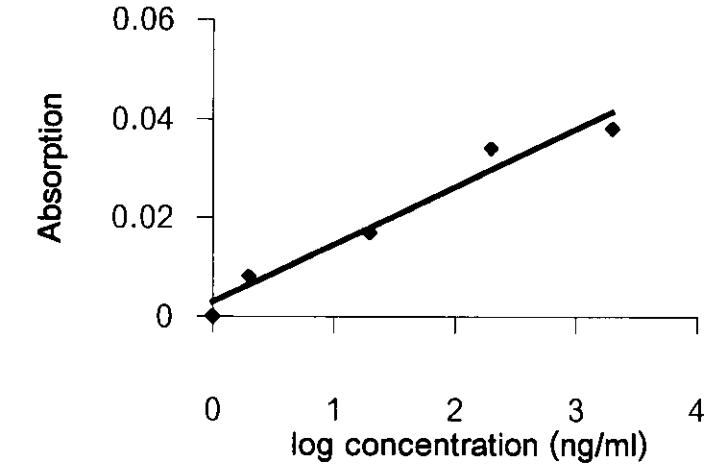
Lane1: HindIII standard molecular weight marker

Lane2: uncut plasmid hEGF-p09K2

Lane3: cut plasmid hEGF-p09K2 with XhoI / EcoRI

Lane4: cut plasmid hEGF-p09K2 with Bgl II

Lane5: 100 bp standard molecular weight marker



$y = 0.0116x + 0.0026$

$R^2 = 0.9613$

Fig. 5. Standard calibration curve between absorbances and log of the hEGF concentrations.

การผลิต Epidermal Growth Factor

PRODUCTION OF EPIDERMAL GROWTH FACTOR

วิไลวรรณ โชติเกียรติ[1*], อุรีพร เกตุจรูญ-เล็กกัต[2], มลธิรา ทองอนันต์[1] และ สุปรียา พงศ์พีรโชค-วานิชปกรณ์[1]
Wilaiwan Chotigeat[1*], Ureporn Kedjarune-Leggat[2], Montira Tonganun[1] and Supreya Pongprerachok-Wanichpakorn[1]
[1]Department of Biochemistry, Faculty of Science, Prince of Songkla University, Songkhla 90112, Thailand; [2]Department of Oral Biology and Occlusion, Faculty of Dentistry, Prince of Songkla University, Songkhla 90112, Thailand;
e-mail address: cwilaiwa@psu.ac.th

บทคัดย่อ: Epidermal growth factor (EGF) มีความสำคัญมากในการเร่งการเจริญเติบโตของเซลล์ผิวหนังซึ่งช่วยในการรักษาบาดแผล ในปัจจุบันสามารถใช้เป็นองค์ประกอบของเครื่องสำอางค์ เป็นสารต้านพิษ และใช้เชื่อมกับยอกซินในรูปของ immunotoxin เพื่อใช้ในการรักษาเซลล์มะเร็ง ดังนั้นจึงได้ทำการผลิต EGF โดยการสังเคราะห์ สายนิวคลีโอไทด์ของยีน EGF และ โคลนเข้าเวคเตอร์ pBK-CMV EGFที่ผลิตได้ทดสอบโดยวิธี indirected ELISA มีปริมาณ 60 นาโนกรัม/มลของน้ำเลี้ยงแบคทีเรีย และสามารถเร่งการเจริญเติบโตของเซลล์ keratinocyte ได้ดี การผลิตปริมาณมาก การทำให้บริสุทธิ์เพื่อนำไปประยุกต์ใช้ในทางการแพทย์ และการโคลนเข้าเซลล์ keratinocyte อยู่ในระหว่างการทดลอง

Abstract: Epidermal growth factor (EGF) plays very important role in wound healing. Currently, it has been used as cosmetics, antitoxin and immunotoxin, therefore, the production of EGF for use in several aspects has been tried. The EGF nuclotides containing 178 bp was synthesized, cloned into pO9K2, then subcloned to pBK-CMV expression vector. The recombinant human EGF (rhEGF) was determined about 60 ng/ml of the recombinant culture by in-directed ELISA. The produced EGF could stimulate growth of keratinocyte very well. The mass production, purification for medical uses in and cloning of EGF in keratinocyte cell are in the process.

Methodology: Human epidermal growth factor gene (EGF) was synthesized by nucleotide synthesizer according to Yamagata *et al.*(1). The EGF was cloned into pO9K2 at *E.coR* I site, subcloned into pBK-CMV at *E.coR*I and *Xho* I. and then transformed into *E.coli* as the standard molecular cloning method (2). The recombinant protein EGF was extracted from periplamic of recombinant *E.coli* cell by cold osmotic shock. Briefly, the recombinant cell suspension was spun at 8,000g for 10 min and resuspended in ice cold sucrose buffer, incubated on ice for 10 min, then centrifuge as above. The pellet was resuspended in 5 mM $MgCl_2$ stirred for 10 min on ice and centrifuge at 8,000 g for 15 min. The supernatant was semi-quantitated by using goat polyclonal anti EGF (Santa Cruz Biotechnology, USA) and monkey anti-goat IgG. AP (Promega, USA) as primary and secondary antibody respectively. The biological assay of the recombiant EGF was estimated by comparing with the commercial human EGF (promega, USA) in promoting growth of the keratinocyte cell. Keratinocyte was plated about 3,000 cell/200 µl in each well of 96 microtitre plate and the recombinant and commercial EGF was added in the keratinocyte culture after 24 hr. After incubation for 3 days , cells were fixed with 25% glutaraldehyde for 20 min and stained with 0.4% CV-methanol solution for 30 min. The plate was then washed in water and air dried. Absorbance of each well at 590 nm was measured by an automatic microplate reader (3).

Results, Discussion and Conclusion: The rhEGF was cloned in pBK-CMV at *E.coR*I and *Xho* I site, 178 bp of hEGF was digested from the recombinant clone by *E.coR*I and *Xho* I. The rhEGF from periplasmic space of the 100ml culture was extracted and detected by indirected ELISA. The extracted rhEGF was found about 60 ng/ml. Since the rhEGF was only extracted from the periplasmic space of the bacterial clone in order to achieve the well folding of the rhEGF therefore the content gain was lower than other reports (4) which extracting the rhEGF from the whole cell. The rhEGF from this study was able to promote the growth of keratinocyte as well as the commercial hEGF (Promega, USA). Since the rhEGF was active therefore, more experiments such as mass production of the rhEGF, the formulation as drug and engineering the keratinocyte cell to produce EGF are being investigated.

Acknowledgement: This work was supported by Prince of Songkla University research grant

References: (1) Yamagata, H., Nakahama, K., Sazuki, Y., Kakinuma, A., Tsukagoshi, N.and Udaka, S. (1989) *Biochemistry* **86**, 3589-3593.
(2) Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning :A laboratory manual, 2nd .ed. Cold Spring Harbor Laboratory Press, New York.
(3) Saoteme K, Morita H, Umeda M. (1989) *Toxic in Vitro* **3**, 317-321
(4) Yadwad, V.B., Wilson, S and Ward, O.P. (1994) *Biotechnology Leiters* **16**, 885-890.