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CLONING AND EXPRESSION OF RECOMBINANT HUMAN EPIDERMAL GROWTH FACTOR IN *Escherichia coli*

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CLONING AND EXPRESSION OF RECOMBINANT HUMAN EPIDERMAL GROWTH FACTOR IN *Escherichia coli*

By

AHMAD FAIZAL ABDULL RAZIS

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

September 2007

With the name of ALLAH,

To my parents ABDULL RAZIS and SITI RUKIAH and my siblings for their encouragement and support…….

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

CLONING AND EXPRESSION OF RECOMBINANT HUMAN EPIDERMAL GROWTH FACTOR IN *Escherichia coli*

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AHMAD FAIZAL ABDULL RAZIS

September 2007

Chairman: Associate Professor Zarida Hambali, PhD

Institute: Institute of Bioscience

The expression of recombinant hEGF (human epidermal growth factor) in *Escherichia coli* was conducted to produce hEGF with free of inclusion bodies and biologically active. The recombinant hEGF was constructed using sticky ends ligation and resulted in successful insertion of the hEGF gene into the multiple cloning sites of the pFLAG-ATS. This insertion was confirmed by restriction enzyme analysis, PCR (polymerase chain reaction) and DNA (deoxyribonucleic acid) sequencing with 100% homology. The recombinant hEGF was expressed in *E. coli* at 2, 4 and 6 hours with induction of 0.5 mM and 1.0 mM IPTG (isopropylthiogalactopyranoside).This recombinant was found to be expressed as periplasmic fraction and whole cell insoluble fraction as confirmed with SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) and western blotting analyses. These analyses showed the molecular weight of hEGF was approximately 6.8 kDa. There was no significant difference in the production of hEGF when the expression was induced with different IPTG concentration; however, for each IPTG concentration, there was significant difference between 0 hour and all the post-induction hours. In addition, hEGF was found to be significantly higher in

periplasmic fraction as compared to the whole cell insoluble fraction (196.5 ng/ml as compared with 167 ng/ml at 2 hour, 175.7 ng/ml as compared with 115.3 ng/ml at 4 hour and 168.3 ng/ml as compared with 140 ng/ml at 6 hour). Growth-stimulating activity of periplasmic hEGF was studied using BrdU (bromodeoxyuridine) cell proliferation assay and MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) cell proliferation assay. The periplasmic hEGF as compared to the standard hEGF was found to be biologically active and showed similar activity. There were significant findings of periplasmic hEGF in stimulating the growth of HEK (human epidermal keratinocytes) at 24, 48 and 72 hours of incubation. Even at the highest concentration of periplasmic hEGF (10 ng/ml), the growth-stimulating activity still occurred and incubation at 48 hour resulted with the highest stimulation (85.4% at 10 ng/ml, 77.7% at 1 ng/ml, 70.7% at 0.1 ng/ml, 55.9% at 0.01 ng/ml and 37.6% at 0.001 ng/ml). Besides, MTT cell proliferation assay of periplasmic hEGF on HDF (human dermal fibroblasts) showed significant increased in the growth-stimulating activity when the duration of incubation increased. Highest percentage of HDF growth was found at 72 hour incubation as compared with 24 and 48 hours incubation. In conclusion, the findings showed recombinant hEGF was successfully expressed in *E. coli* and the growth-stimulating activity of hEGF was determined.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PENGKLONAN DAN PENGEKSPRESAN REKOMBINAN FAKTOR PERTUMBUHAN MANUSIA DALAM *Escherichia coli*

Oleh

AHMAD FAIZAL ABDULL RAZIS

September 2007

Pengerusi: **Profesor Madya Zarida Hambali, PhD**

Institut: **Institut Biosains**

Pengekspresan rekombinan faktor pertumbuhan manusia dalam *Escherichia coli* telah dijalankan untuk menghasilkan hEGF (human epidermal growth factor) bebas daripada badan inklusi dan aktif biologi. hEGF rekombinan telah dibentuk menggunakan kaedah percantuman hujung lekit dan hasilnya menunjukkan kemasukan gene hEGF ke pelbagai tapak klon pFLAG-ATS. Kemasukan gene ini telah dibuktikan melalui analisa enzim pembatas, PCR (polymerase chain reaction) dan penjujukan DNA (deoxyribonucleic acid) menunjukkan homologi 100%. hEGF rekombinan telah diekspres dalam *E. coli* pada jam 2, 4 dan 6 dengan diinduksi oleh 0.5 mM dan 1.0 mM IPTG (isopropylthiogalactopyranoside). Rekombinan ini didapati telah diekspres sebagai fraksi periplasmik dan fraksi keseluruhan sel tak larut yang telah dibuktikan dengan analisa SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) dan kaedah 'western blotting'. Keputusan ini menunjukkan berat molekul hEGF lebih kurang 6.8 kDa. Tiada perbezaan penghasilan hEGF apabila diinduksi dengan berbeza kepekatan IPTG. Walau bagaimanapun, untuk setiap kepekatan IPTG terdapat perbezaan ketara penghasilan hEGF diantara induksi pada jam 0 dan semua jam pasca induksi. Tambahan lagi,

kepekatan hEGF didapati lebih tinggi dalam fraksi periplasmik berbanding dengan fraksi keseluruhan sel tak larut (196.5 ng/ml berbanding 167 ng/ml pada jam 2, 175.7 ng/ml berbanding 115.3 ng/ml pada jam 4 dan 168.3 ng/ml berbanding 140 ng/ml pada jam 6. Aktiviti rangsangan pertumbuhan oleh hEGF periplasmik telah dikaji menggunakan kaedah proliferasi sel BrdU (bromodeoxyuridine) dan MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide). hEGF periplasmik didapati aktif secara biologi dan mempuyai aktiviti yang sama apabila dibandingkan dengan Standard hEGF. Terdapat keputusan ketara menunjukkan hEGF periplasmik merangsang pertumbuhan HEK (human epidermal keratinocytes) pada jam 24, 48 dan 72 inkubasi. Aktiviti rangsangan pertumbuhan masih berlaku meskipun pada kepekatan tertinggi hEGF periplasmik digunakan (10 ng/ml) dan inkubasi pada jam 48 menunjukkan rangsangan yang tertinggi (85.4% pada kepekatan 10 ng/ml, 77.7% pada kepekatan 1 ng/ml, 70.7% pada kepekatan 0.1 ng/ml, 55.9% pada kepekatan 0.01 ng/ml dan 37.6% pada kepekatan 0.001 ng/ml). Disamping itu, kaedah prolifersi sel MTT pada hEGF ke atas HDF (human dermal fibroblasts) menunjukkan pertambahan ketara dalam aktiviti rangsangan pertumbuhan dengan pertambahan tempoh inkubasi. Peratusan pertumbuhan HDF adalah tertinggi pada jam 72 inkubasi dalam perbandingan dengan inkubasi pada jam 24 dan jam 48. Kesimpulannya, keputusan menunjukkan bahawa hEGF rekombinan telah berjaya diekspres dalam *E. coli* dan aktiviti rangsangan pertumbuhannya telah ditentukan.

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I certify that an Examination Committee has met on 10th September 2007 to conduct the final examination of Ahmad Faizal Abdull Razis on his Master of Science thesis entitled "Cloning and expression of recombinant human epidermal growth factor in *Escherichia coli*" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Master of Science.

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Date: 22 November 2007

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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Date: 13 December 2007

DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

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AHMAD FAIZAL ABDULL RAZIS

Date: 6 November 2007

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LIST OF ABBREVIATIONS

^{51}Cr-labeled	^{51}Chromium-labeled
Amp	Ampicillin
ARF	Acute Renal Failure
ATCC	American Type Culture Collection
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin
BUN	Blood Urea Nitrogen
C-terminal	Carboxyl-terminal
DAB	3, 3'-diaminobenzidine
E. coli	*Escherichia coli*
EBA	Expanded Bed Adsorption
EGF	Epidermal Growth Factor
EGF-R	Epidermal Growth Factor Receptor
fMet	*N*-formyl methionine
FTNF	Feline Tumor Necrosis Factor
HB-EGF	Heparin binding EGF-like growth factor
HDF	Human Dermal Fibroblasts
hEGF	Human Epidermal Growth Factor
HEK	Human Epidermal Keratinocytes
hOB	Human obese
K1 (SK)	Kringle 1 (Streptokinase)
MCS	Multiple cloning site
mEGF	Mouse Epidermal Growth Factor
MRC-5	Normal Human Fetal Lung Fibroblast

NHEK	Normal Human Epidermal Keratinocytes
NMWL	Nominal Molecular Weight Limit
N-terminal	Amino-terminal
OmpA	Outer membrane protein A
ORF	Open Reading Frame
Prepro	Protein precursor
rEGF	Rat Epidermal Growth Factor
T3	Triiodothyronine
TGFα	Transforming Growth Factor alpha
TNF	Tumor Necrosis Factor

CHAPTER 1

INTRODUCTION

Human epidermal growth factor (hEGF), consisting of 53 amino acid residues, is a single chain polypeptide with a molecular weight of about 6,200 Da. hEGF is considered to be identical with human urogastrone (Gregory, 1975), which is produced by the human duodenum and salivary glands (Cohen and Carpenter, 1975). hEGF is widely used in clinical and cosmetic fields because it stimulates the growth of a variety of cell types in cultures as well as the growth and differentiation of certain tissues *in vivo* (Carpenter and Cohen, 1979). It also promotes protein synthesis, RNA synthesis and metabolic uptake in epidermal cells (Kim and Muller, 1999), repairs corneal wounds and gastric ulcers and inhibits the proliferation of human gastric cancer cells (Jiang *et al*., 1999; Reim *et al*., 1988). Besides, hEGF has been observed to have many biological actions both *in vitro* and *in vivo* including proliferative effects on fibroblasts, keratinocytes and epithelial cells.

Since the finding of hEGF properties as strong anti-ulcer (Gregory, 1975) and growth-stimulating factors (Hashimoto *et al*., 1994), the sufficient production for medical application has been in great demand. Conventional method such as recovery of hEGF from human urine via complicated steps is established (Starkey *et al*., 1975). However, it failed to provide sufficient amount of homogenous hEGF with well-defined quality for clinical application and inappropriate for human consumption.

Many studies have reported using recombinant DNA technique to produce high hEGF productivity. However, the cytoplasmic expression of heterologous protein in *E. coli,* particularly small peptides, has encountered many obstacles. It is commonly found that proteins expressed in this form are rapidly degraded by proteases within the cell (Skipper *et al*., 1985). It was reported that the hEGF expressed in high density precipitated intracellularly form aggregates called inclusion bodies (Shimizu *et al*., 1991). This causes the expressed proteins to be severely misfolded. Although there are many protocols available for refolding this protein, the production is inefficient and the procedure is costly.

Thus, to obtain hEGF in sufficient quantities for this study, the recombinant hEGF is developed. It is desirable that recombinant hEGF be secreted into the cell growth medium. Purification of this protein would then be simpler for an intracellular protein as the product would not be contaminated with cytoplasmic components. In addition, the formation of inclusion bodies would be avoided and possible toxic effects of the hEGF polypeptide product on the host cell would be reduced.

In this study, a secretory plasmid is used, which contains strong regulatory elements such as *tac* promoter, the consensus ribosome-binding site, and the ompA leader sequence for efficient expression and secretion of hEGF in *E. coli.* This plasmid also encodes a *lac* repressor and an ampicillin (Amp) resistance gene. It was shown that secretory recombinant *E. coli* system was able to express and accumulate authentic hEGF in the culture medium (Wong and Sutherland, 1993). Normally, the secretion of high-level expressed protein in *E. coli* will eventually elicit a stress response in the host such as energy drain, membrane perturbation and lethal effect which leads to the

phenomenon known as “overproduction lethality”. Andersson *et al*. (1996) suggested that overexpression of foreign protein was due to the appearance of non-culturable cells which do not lose all metabolic activities and even continued to maintain glucose uptake and respiratory ability. However, only few studies reported the optimization of conditions for the process control in the expression and secretion of hEGF in *E. coli*.

In the present study, recombinant hEGF is expressed as a fusion protein using pFLAG expression system (Sigma, U.S.A.). This system allows recombinant protein to be expressed as a fusion protein containing the FLAG peptide sequence. Monoclonal antibodies recognizing the FLAG peptide can then be used to purify the fusion protein using affinity chromatography. The purification of hEGF protein has been made more convenient and efficient by using this system.

The aim of this study is to express the recombinant hEGF in *E. coli* and study the growth-stimulating activity of hEGF on human epidermal keratinocytes and human dermal fibroblasts cultures. The objectives involved in this study were:-

1. cloning and sequencing of hEGF gene.
2. expression, isolation and purification of hEGF.
3. determination of the growth-stimulating activity of hEGF on human epidermal keratinocytes and human dermal fibroblasts cultures.

CHAPTER 2

LITERATURE REVIEW

2.1 Discovery and Characterization of Epidermal Growth Factor

Epidermal growth factor (EGF) was discovered as a contaminant of nerve growth factor in 1960 by Stanley Cohen (Cohen, 1962). EGF was recognized by its ability to accelerate the eruption of mouse teeth and the opening of eyelids of newborn mice. The abundance of EGF in mouse salivary glands facilitated its purification and in 1972 the full amino acid sequence of mouse EGF was determined (Savage *et al*., 1972). Although no human equivalent of EGF had been purified during that time, it was known that a similar protein was present in concentrates of human urine (Starkey *et al*., 1975). The human urinary protein responsible for the inhibition of gastric acid secretion (β-urogastrone) was finally purified and analyzed. Harry Gregory (1975) recognized that the amino acid composition of murine EGF, human urinary EGF and β-urogastrone were closely related. The biology of urogastrone indicated that it might also promote the proliferation and epithelialization of gastric mucosa, so he concluded that urogastrone and human epidermal growth factor are one and the same (Gregory, 1975). The amino acid sequence of EGF is as displayed in Table 1.

Table 1. Amino acid sequence of Epidermal Growth Factor

EGF	AMINO ACID SEQUENCE
rat EGF	N S N T G C P P S Y D G Y C L N G G V C M YV E S V D RY V C N C V I G Y I G E R C Q H R D L R
mouse EGF	N S Y P G C P S S Y D G Y C L N G G V C M H I E S L D S Y T C N C V I G Y S G D R C Q T R D L R W W E L R
guinea pig EGF	Q D A P G C P P S H D G Y C L H G G V C M H I E S L N T Y A C N C V I G Y VG E R C E H Q D L D D W E
human EGF	N S D S E C P L S H D G Y C L H D G V C M Y I E AL D K Y A C N C V VGY I G E R C Q Y R D L K W W E L R

(Source: Burgess, 1989)