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Research Article

Molecular Cloning and Expression of Human Epidermal Growth Factor (hEGF) Gene in Yeast *Kluyveromyces lactis*

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ABSTRACT

Several attempts have been made to produce the biologically active recombinant hEGF in enhanced quantities in prokaryotic system. However, until recently they have been unsuccessful due to the absence of disulfide bond formation and improper folding of proteins in the prokaryotic system. Hence, in the present study we have attempted to express the EGF gene in an eukaryotic expression system using a suitable expression vector containing a strong promoter. To achieve this issue, the gene of interest was obtained by the chemical synthesis and subjected to PCR amplification using gene specific primers. The amplified gene was cloned into *E.coli* cells using pKLAC2 vector and the cloned gene was expressed in a yeast expression system *(Kluyveromyces lactis*). The expression of recombinant hEGF was confirmed by ELISA using anti-hEGF antibody. To the best of our knowledge, this is the first attempt of characterization and expression of hEGF in *Kluyveromyces lactis.* A comparison study of expressed recombinant hEGF in different expression systems showed that *K. lactis* is one of the best suitable expression systems for expressing hEGF as it had been already proved that *K. lactis* cells are effective for producing the highest yields of proteins that are normally secreted from cells. In addition to that, expression of hEGF gene in *K. lactis* cells result in the secretion of proteins from the cells that are significantly pure and do not require the lysis of yeast cells to isolate them. The ultimate objective of the present study was to optimize the production of the biologically active recombinant hEGF in yeast *Kluyveromyces lactis* in enhanced quantities.

Key Words: Molecular Cloning, Human Epidermal Growth Factor, Gene Expression, Yeast, *Kluyveromyces lactis.*

INTRODUCTION

The discovery of EGF dates back to 1986 where by Dr. Stanley Cohen of Vanderbilt University won a Nobel Prize in Physiology and Medicine (Hall, 1986). The discovery of EGF has opened new fields of widespread importance to basic science. Human epidermal growth factor (hEGF) or β-urogastron is a monomeric protein consisting of 53 amino acid residues with molecular weight of approximately 6,200 Daltons. It consists of intra molecular disulfide bonds needed for the biological activity. It has been classified as a member of EGF super family, an expanding group of growth factors containing several members including EGF, TGFα, Epiregulin, HB-EGF, AR, BTC and the neuregulins where all members have six-conserved cysteine residues in the active peptide, resulting in having a similar tertiary structure.

It has been already proven that hEGF has

many beneficial effects such as promoting proliferation of numerous cells in the body, inhibition of gastric acid, management of gastric ulcer healing, elimination of skin scars, hastening the healing process of epidermis damage on skin and so on. In patients with multiple co-morbidities and in high risk surgical cases, selective use of this molecule for healing ulcers remains as an attractive alternative.

Since isolation of EGF from natural sources like plasma or urine is technically laborious, expensive and time consuming, several studies have focused on the development of biologically active recombinant human epidermal growth factor (rec-hEGF) in large quantities by other alternatives. Till now many attempts have been made to produce biologically active recombinant human epidermal growth factor in *E.coli* (Kishimoto *et al.*,1986; Smith *et al.*, 1988; Coppella and Dhurjati, 1989; Shimizu et al., 1991), however due to the absence of disulfide bond formation and improper folding of protein, the recombinant protein expressed in the cytoplasm of *E.coli* is either degraded rapidly by cellular proteases or get accumulated to form aggregates or inclusion bodies (Shimizu et al., 1991). Furthermore production of hEGF in some eukaryotic hosts such as *S. cerevisiae* has its own difficulties. Expression in yeast *S. cerevisiae* may result in hyperglycosylation. Yeast *S. cerevisiae* has a majority of N-linked glycosylation of the high-mannose type; however, the length of the oligosaccharide chains added post translationally to proteins in other yeast expression system may be much shorter than those in *S. Cerevisiae.* (Grinna and Tschopp, 1989; Tschopp *et al.*,1987).

In addition, *S. cerevisiae* core oligosaccharides have terminal α1, 3 glycan linkages that may result in glycosylated proteins produced from *S.cerevisiae* to be primarily responsible for the hyper-antigenic nature of these proteins making them particularly unsuitable for therapeutic use. In the present study, expression of hEGF gene was done using *K. lactis* Protein Expression system, as it provides a method for cloning and expressing genes of interest in the yeast *Kluyveromyces lactis* in which the expressed protein could be secreted from the cell; therefore it made access to expressed proteins and evaluation of them more convenient. Although not yet proven, this is predicted to be less of a problem for glycoproteins generated in yeast *Kluyveromyces lactis* because it may resemble the glycoprotein structure of higher eukaryotes. The aim of the present study was to evaluate biological activity of expressed hEGF in yeast *Kluyveromyces lactis* and to produce recombinant human epidermal growth factor in enhanced quantities in *K. lactis.*

MATERIALS AND METHODS

DNA Template, Strains and Plasmid

The DNA template (hEGF gene) was chemically synthesized by Sigma-Aldrich chemicals Pvt. Ltd (Bangalore, India), based on the published hEGF gene sequence, available in the NCBI GenBank (Accession No: AY548762). *E.coli* strain JM 109 (endA1, recA1, gyrA96, thi, hsdR17 (r_k^-, m_k^+), relA1, supE44, Δ(lac-proAB), [F´ traD36, proAB, $laqI^qZ\Delta M15$]) cells as a host strain for cloning were obtained from Promega Corporation. Yeast *Kluyveromyces lactis* (*K. lactis*) strain GG799 (No auxotrophies or genetic markers) competent cells and pKLAC2 expression vector were procured from New England Biolabs (NEB),Inc.

PCR Amplification of hEGF

The synthesis hEGF was subjected to amplification by PCR using gene specific primers. The primers were designed based on the hEGF gene sequence obtained from NCBI GenBank and

K. lactis Protein expression kit manufacturer's instructions. The forward primer contained an XhoI restriction site and a Kex protease cleavage site immediately followed by the first codon of the gene's ORF and the reverse primer included a NotI restriction site followed by stop codon (5´-3´). Sequences of primers are as follows:

Forward ($^{5´}$CTC GAG AAA AGA AAT AGT GAC$^{3´}$)
XhoI Kex site
Reverse ($^{5´}$GC GGC CGC TTA GCG CAG TTC CCA CCA CTT C$^{3´}$)
NotI Stop

The amplification reaction was performed in 50 µl volume consisted of 5 µl of reaction buffer containing $MgCl_2$ (15 mM), 2 µl of DNA template (20 ng/µl), 1.3 µl of dNTPs (10 mM), 1.1 µl of each primer (0.2 mM), 1 µl of Taq DNA polymerase (5 u/µl) and 38.5 µl of distilled water. PCR was done for 30 cycles consisting of denaturation at 94ºC for 1 minute, annealing at 55ºC for 2 minutes and extension at 72ºC for 1.5 minutes. An initial denaturation of 94ºC for 1 minute and a final extension of 10 minutes were also included in the PCR reaction.

Construction of Recombinant pKLAC2

Amplified hEGF and pKLAC2 were digested with NotI and XhoI restriction enzymes and the purified digestion mixtures were cloned into linearized pKLAC2 as follows: 3 µl of vector, 10 µl of PCR fragment (insert DNA), 1 µl (~ 400 units) of T4 ligase, 2 µl of 10X ligase buffer and 9 µl of nuclease-free water were mixed by gentle pipetting for a few times and then, ligation mixture incubated at 16ºC overnight.

Gene Cloning and Bacterial Transformation

Competent *E.coli* cells strain JM 109 were used for cloning the gene of interest and transformation. The transformation mixture, containing the recombinant plasmids on transformation of *E.coli* strain JM 109 was plated on an agar plate supplemented with Ampicillin (100 µg/ml) and incubated overnight at 37ºC, whereby the transformants appeared as creamy-white colonies. Recombinant transformants were differentiated from non-recombinant transformants by PCR using specific primers for inserted genes. Plasmid DNA was prepared from PCR positive colonies by alkaline lysis method (Sambrook and Russel, 2001) and the existence of insert DNA was further appraised by restriction digestion analysis of the isolated plasmids. The integrity of DNA sequence of cloned gene was confirmed by sequence analysis.

Transformation of *K. lactis* GG799 Cells and Expression of Recombinant hEGF pKLAC2 containing gene of interest (hEGF) was digested with SacII restriction enzyme to generate an expression cassette. The DNA fragment was purified with GeNei™ gel extraction kit (Bangalore Genei, India) and transformation was done by introducing the linearized expression cassette into *K.lactis* cells as per *K. lactis* Protein expression kit manufacturer's instructions using the *K.lactis* GG799 competent cells and NEB yeast transformation reagent supplied with the kit. Transformants were selected by growing cells on yeast carbon base (YCB) agar medium plates containing 5 mM acetamide on 3rd day of incubation. Identification of properly integrated cells was done by colony PCR. A single colony of cells which were positive for integration of expression cassette were harvested by scraping with a sterile pipette tip and resuspended in 2 ml of YPGal medium in a sterile culture tube. The culture was incubated with shaking (180 rpm) at 30°C for 3-4 days to obtain a saturated culture (a culture density of > 30 OD600 units/ml). An aliquot (1 ml) of each culture was taken and centrifuged to pellet the cells. The culture supernatant was used for ELISA analysis.

ELISA Analysis

Different volumes of supernatant, 250 ng (5 µl), 500 ng (10 µl) and1 µg (20 µl) were coated onto 3 wells of microtiter well plates. The plate was incubated overnight at 4˚C. Wells were washed using 1XPBST to remove any unbound protein. Blocking buffer was added to the wells and incubated for 1hr at RT to avoid any nonspecific binding. Wells were washed again with 1XPBST and 5000 times diluted mouse primary antibody was added to each antigen coated well separately. It was incubated for 30 minutes at RT. Wells were washed again with 1XPBST. HRP conjugated secondary antibody specific against the primary antibody was then loaded to all the four wells. Again incubated for 30 minutes at RT. Wells were washed with 1XPBST and 1XTMB/H202 added and incubated for 10-15 minutes till the blue color developed. Sulfuric acid (1N) was added to stop the reaction which changes the color to yellow. Absorbance reading was taken at 450 nm.

SDS-PAGE Analysis

The gel was clamped and both buffer chambers

were filled with running buffer and 20 µl of sample with the bromophenol dye (5µl) along with protein molecular marker were loaded on to the lanes using micropipette. The gel was run at 15mA for 30 minutes. Typically once a SDS-PAGE gel was run, it was fixed in 25% acetic acid in water (containing 20% methanol) and the gel was stained with Coomasie blue dye R250. The gel was destained in same solution with no dye and dried (Laemmli 1970).

MTT Assay for Cell Proliferation of Recombinant Human EGF Protein

1. 500-10,000 cells were plated in 150 µl media per well in a 96 well plate.
2. The plate was then incubated (37 °C, 5% CO2) overnight to allow the cells to attach to the wells.
3. The supernatant was dissolved in serum free medium (as per concentration) to each well (1000µl).
4. It was incubated (37C, 5% CO2) for 2 days to allow the hEGF to take effect.
5. 2ml of MTT solution per 96 well plates was made at 5mg/ml in PBS.
6. 10µl MTT solution was added to each of the wells and placed on a shaking table at 150 rpm for some minutes, to thoroughly mix the MTT into the media.
7. The plate was incubated (37°C, 5% CO2) for 4 hours to allow the MTT to be metabolized.
8. After incubation, the formazan produced in the cell, appeared as a dark crystal in the bottom of the wells.
9. The media was removed and the plate was dried on paper towels to remove residue if necessary.
10. 100 µl of crystal dissolving solution (DMSO or ISOPROPANOL) was added to each well. This solution was supposed to dissolve the formazan crystals, producing a purple solution.
11. The absorbance to each concentration was measured at 570 nm using a microplate reader.

RESULT AND DISCUSSION

PCR Amplification of hEGF

The analyzed amplification product on 1.6% agarose gel was found to be a single intense band approximately of 182bp (Fig.1). The PCR products were ligated to pKLAC2 vector system.

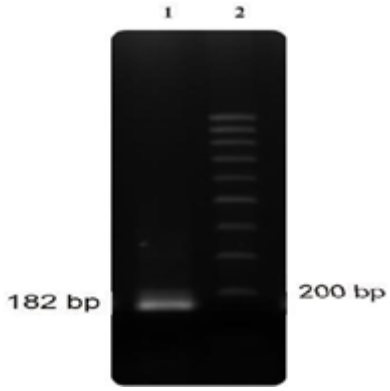

Fig .1 Agarose Gel Electrophoresis.
Lane-1, The PCR Amplified hEGF Gene;Lane-2, 100 bp DNA Ladder (CHROMOUS BIOTECH).

The transformation mixture, containing the recombinant plasmids on transformation of *E.coli* strain JM 109 when plated on an agar plate supplemented with Ampicillin (100 µg/ml) after overnight incubation at 37ºC, appeared as creamy-white colonies forming the transformants while non-transformants could not grow due to the absence of Ampicillin resistance gene (Fig .2). Recombinant transformants were differentiated from non-recombinant transformants by PCR using specific primers for inserted genes. Colony PCR for recombinant transformants showed amplification of DNA fragment approximately of 182bp while there was no amplification for non-recombinant transformants. PCR positive colonies were used for plasmid DNA preparation.

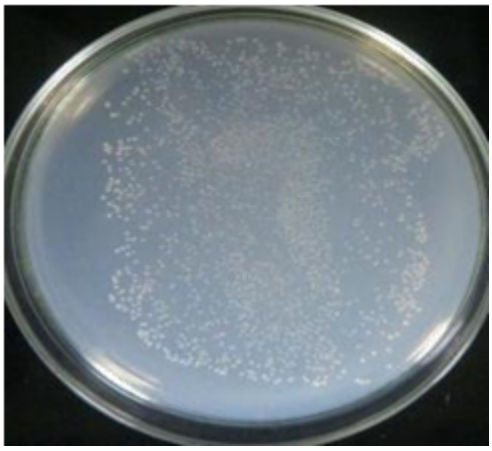

Fig .2 Transformed *E. coli* Colonies Grown on LB Agar
Plate Supplemented with Ampicillin (100 µg/ml).Transformants Appear as White Colonies.

Isolation and Restriction Digestion of Plasmids

Extracted plasmids from PCR positive colonies after running on 1% agarose gel showed the presence of three forms of plasmids (Fig .3). The presence of the DNA insert was confirmed by colony PCR; single intense band of expected size was observed (Fig.4). In addition, restriction digestion analysis of the recombinant plasmids using NotI and XhoI showed two bands-insert and the plasmid, of expected size which further confirmed that plasmids were recombinants and contained the correct type of DNA insert.

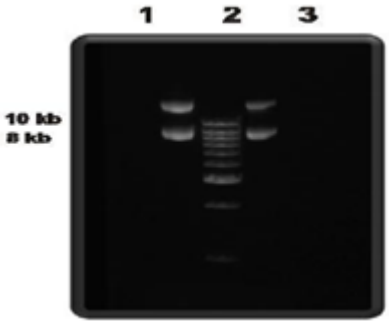

Fig.3 Agarose Gel Electrophoresis.
Lane- 1, 3, Isolated Recombinant Plasmid Containing hEGF. Gene From Transformed Cells; Lane-2, 1 kb DNA Ladder.

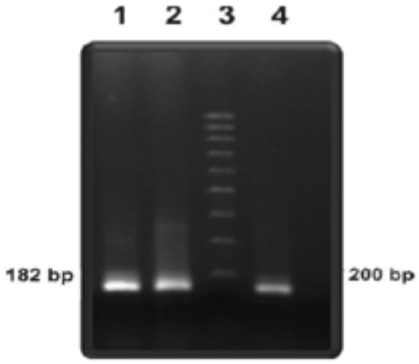

Fig.4 Agarose Gel Electrophoresis.
Lane- 1, 2, 4, PCR Amplified Recombinant. Transformants; Lane- 3, 100 bp DNA Ladder.

The purified plasmids when sequenced bidirectionally using the insert specific primers, the sequence of hEGF was found to be exactly matching with the sequence data obtained from NCBI.

Transformation of *K. lactis* GG799 Cells

Transformants were selected by growing cells on yeast carbon base (YCB) agar medium plates containing 5 mM acetamide. When transformation mixture was spread on YCB agar medium plates containing acetamide and incubated at 30ºC on 3rd day , the transformed cells appeared on plates. Due to the presence of *amdS* gene in pKLAC2, only transformed cells can grow on YCB medium (Fig .5). Since YCB is a nitrogen free medium, the product of the *amdS* gene, acetamidase can utilize acetamide as a source of nitrogen. Plates were stored at 4ºC for further experiments.

Identification of the properly integrated cells were done by colony PCR after monitoring the PCR products of 5 different single colonies on 1.6% agarose gel, had shown that out of the total 5 samples, 4 samples were positive for the presence of insert (Recombinant) by producing the expected amplicons of approximately 2.4 kb size (Fig .6).

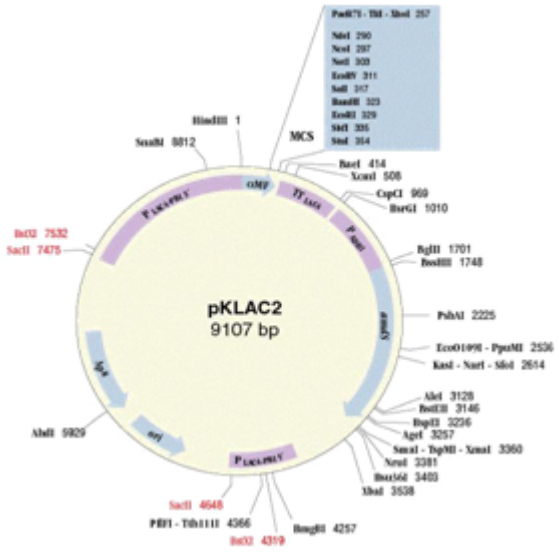

Fig .5- Map of pKLAC2 expression vector.

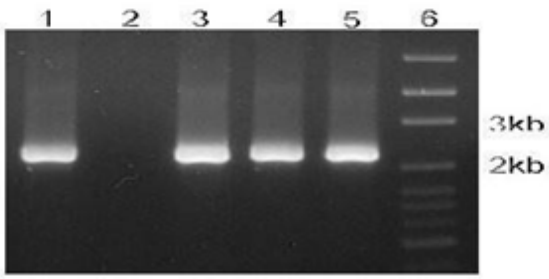

Fig.6- Gel electrophoresis pattern of colony PCR.
Lane-1, 3, 4, 5, PCR positive colonies by producing the expected amplicons of approximately 2.4 kb size; Lane-3, PCR negative colony; Lane-6, 1 kb DNA ladder.

ELISA Analysis

ELISA Assay for detection of recombinant protein showed positive result as shown in the following chart by absorbance of each well at 450 nm (Table .1).

Well no.	Antigen	Primary Ab	HRP conjugated Secondary Ab (1:1000 dil)	Absorbance (450nm)
1	250 ng (5 µl)	200 ng	200	0.785
2	500 ng (10 µl)	200 ng	200	1.635
3	1 ng (20 µl)	200 ng	200	2.127
+ve	Control			1.974
-ve	Control			0.102

Table .1 Showing ELISA Assay of rhEGF Using 5, 10 and 20 µl. It had Shown to have Positive Results when Compared with the Controls.

SDS-PAGE analysis of hEGF recombinant protein expressed in *K. lactis*, obtained a band approximately 6600 Da, compared with protein marker (fig .7).

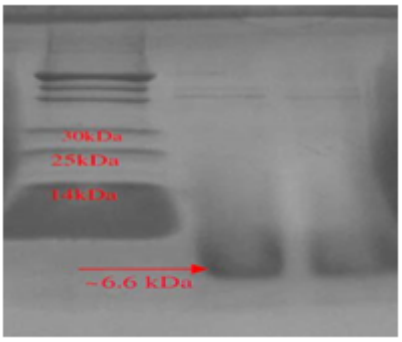

Fig.7 SDS-PAGE Analysis of Recombinant hEGF Protein. Coomassie Blue-stained SDS-PAGE gel of *K. lactis* was Shown over Expression.
Lane-1, protein marker; Lane-2,3 Recombinant hEGF approximaltely 6.6 kDa.

MTT Assay Analysis

Biological activity of recombinant human EGF was evaluated on Human breast cancer (MCF-7), Cervical cancer (SIHA), Colon cancer (HCT-116), Ovarian cancer (OVCAR-8), Human embryonic kidney (HEK-293) and Amphophoenix cell lines.

Cell lines were observed under microscope (20X, NIKON Microscope) after addition of recombinant hEGF in different concentrations. Crystal formation of cell lines after adding the MTT reagent was clear. The plate was measured at 570 nm in each wells in a microplate reader. Absorbance values were compared with the blank which did not contain any protein. Higher absorbance rate indicates an increase in cell proliferation (Fig 8 & 9)

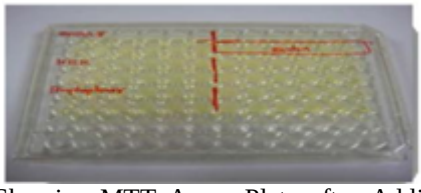

Fig.8 Showing MTT Assay Plate after Addition of the MTT Reagent.

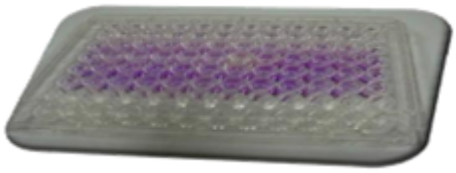

Fig .9 . Results of MTT Assay Showing Purple Color after Addition of the DMSO.

CONCLUSIONS

Production of recombinant hEGF is interesting as it could pave way to an improvised method of the expression of recombinant human epidermal growth factor as well as a cost effective production protocol for this protein which has various industrial and medical applications. Hence in the present study an attempt was made to clone, amplify and evaluate biological activity of expressed hEGF in yeast

Kluyveromyces lactis. The results obtained are summarized below: The chemically synthesized hEGF gene was amplified using specific primers. The amplified hEGF and pKLAC2 vector were digested with NotI and XhoI restriction enzymes. After ligation process the recombinant pKLAC2 was transferred into *E. coli* strain JM 109.

The transformation mixture, containing the recombinant plasmids on transformation of *E. coli* strain JM 109 was plated on an agar plate supplemented with Ampicillin. The transformants appeared as creamy-white colonies due to the presence of Ampicillin resistance after overnight incubation at 37ºC. Recombinant transformants were differentiated from non-recombinant transformants by colony PCR using specific primers for inserted gene (hEGF) and sequence analysis of recombinant plasmid with specific insert primers showed 97 percentage matching with the sequence data obtained from NCBI.

hEGF gene was expressed in yeast *Kluyveromyces lactis* eukaryotic expression system possibly one of the first attempts, so the pKLAC2 plasmid containing hEGF gene was linearized using SacII restriction enzyme and the expression cassette introduced into *K. lactis* cells strain GG799 by chemical treatment. Transformants were selected by growing cells on yeast carbon base (YCB) agar medium plates containing 5 mM acetamide. Identification of properly integrated cells by colony PCR showed the presence of insert (Recombinant) by producing the expected amplicons of approximately 2.4 kb size. SDS-PAGE analysis of recombinant hEGF expressed in *K. lactis* was done for the determination of expression pattern of the recombinant protein. Presence of recombinant hEGF was analyzed by an enzyme linked immunosorbent assay (ELISA) using monoclonal Anti-EGF antibody showing positive result.

EGF was evaluated on Human breast cancer (MCF-7), Cervical cancer (SIHA), Colon cancer (HCT-116), Ovarian cancer (OVCAR-8), Human embryonic kidney (HEK-293) and Amphophoenix cell lines. It was found that the recombinant hEGF had more activity on ovarian cancer (OVCAR-8) cell line but no activity on Human breast cancer (MCF-7) cell line.

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