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Potential Expression of cDNA of BHK21 cells -Derived Recombinant Human Erythropoietin in *E.coli* cells

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Abstract: The erythropoietin is one of the important glycoprotein hormone plays a major role in regulating erythropoiesis and its deficiencies leads to anemia human system. Various attempts made to replacement therapy have been difficult due to the scarcity of purified material human erythropoietin and also required large quantities made to clinical demand for hormone. We used a cloned human erythropoietin gene to develop stably transformed *E.coli* cells that secrete large amounts of the hormone with potent biological activity. The full length cDNA of rHuEpo isolated from BHK 21cells by BamHI and HindIII digestion. The restricted fragment is inserted into pRSET vector and transformed into *E.coli* GJIJK58 strain. Erythropoietin gene fused to His tag was expressed at both 0.lM and 0.3M NaCl concentrations of which potential expression identified at 0.1M NaCl. Analysis of expression at different time points post induction with 0.1M NaCl showed maximum induction at 4 and 5h, afterwards the induction shows decrease.
Key words: cDNA, inducer, EPO gene, competent cells,

Introduction

Erythropoietin (Epo), a 30-38 *kDa* glycolprotein hormone is produced mainly by kidney as a 193 aminoacids pre-mature protein[1]. Erythropoietin is a member of hematopoietic growthfactors which stimulates the growth of red blood cells and as a consequence increases haemoglobin levels and also involved in regulating red blood cell circulation[2]. EPO hormone triggers the proliferation, differentiation andmaturation of bone marrow erythroid precursors cells into functionalerythrocytes when blood oxygen accessibility is decreased, such as during hypoxia[3]. EPO controls proliferation and differentiation of erythroid precursor cells both *in vitro* and *in vivo*[4-6].

The treatment of anemic patients with EPO significantly reduces their dependenceon blood transfusions and minimizes potentialside effects such as iron overload, infections and adversereactions to leukocyte antigens. In addition to its role in hematopoeisis, EPO is neuro protective in the nervous systemand can also protect other organs[3]. Administration of EPO protein as a therapeutic agent increases the quality of life in patients with cancer, renal and heart failure anemia[7]. The global EPO drugsmarket is thus driven by increasing number of patients suffering from anemic condition induced due to cancer, HIV and ESRD treatment; favorable reimbursements and increasing commercialization of EPO bio-similars. The global EPO drugs market would reach $11.9 billion by 2020, registering CAGR of 9.7%during 2014-2020[8].

Realizing the need to produce EPO in high quantities hadprompted the pioneering work of isolating 10 mg EPO from 2550:l of human urine [9-10]. The preparation allowed the identification of theamino acid sequence and synthesized human EPO DNAprobes for the isolation and cloning of the human EPO genefrom mRNA in kidney and liver which are the site for EPOproduction[9, 11, 12].

Recombinant human erythropoietin (rhuEPO) has been produced in various cell lines; in particular Chinesehamster ovary (CHO) and baby hamster kidney (BHK)cells [11, 13, 14].Currently, rhuEPO produced in CHO cell line isextensively used in the therapy to cure severe anemia [15].

The epo gene was first cloned in the year of 1985 and it is sialoglycoprotein hormone consists of 165 amino acids that form a single polypeptide chaincontaining two intra chain disulfide bonds (Cys7-161 and Cys 29-33) and four potential glycosylation sites [16, 17]. It has three N-linked (Asn24, Asn38 and Asn83) and one O-linked (Ser126) glycosylation sites [15, 17]. The molecular mass of EPO is 30-34 kDa and about 40% of its molecularweight is due to carbohydrate moieties and glycosylation may affect protein stability [18]. Recombinant EPOhu had been expressed in mammalian cells, such as CHO, BHK and COS cells in successfully [16, 17, 19]. It had also been expressed invarious hosts, such as Tobacco plant cells, *Spodoptera frugiperda* insect cells [20] and in yeasts such as *Saccharomyces cerevisiae* and *Pichiapastoris* [21].

Materials and Methods

Isolation of genomic DNA

BHK 21 cells along with cDNA of erythropoietin were used for production of human erythropoietin was obtained from ATTC. Genomic DNA was isolated from cells grown to a confluent state by isopropanol precipitation method (Kohli & Vyas 2000). The cDNA contained BHK 21 cells were isolated from the culture medium by centrifuged at 4000 rpm for 10 mins. The cell culture medium was discarded and the BHK 21cells were suspended in 500 µL of lysis buffer. The suspension was then incubated at 37°C for 2 hours with agitation in a shaker. Equal volume of ice cold isopropanol was then added and mixed slowly. The DNA was seen precipitating out. The solution was mixed well till there was no viscosity. The DNA was then spooled out into a fresh centrifuge tube and washed with 70% ethanol at 8000 rpm for 15 min. The cell culture pellet was collected and allow to air- dried and supernatant was decanted. The cell culture pellet was then dispersed in 40µL of TE buffer (pH 8.0). 2 µL of the genomic DNA sample was used for analysis on 0.8% Agarose gel.

Amplification of Genomic DNA

Add the following components of reaction mixture for PCR to a 0.2 ml micro centrifuge tube. Sterile water 12.8µL, 10 X amplification buffer 2.0 µL, 2mM dNTP 2.0 µL, Reverse primer (CAGGGGATTTGGAGGTTCCC) - 1.0 µL, Forwardprimer (GCTGCATGTGGATAAAGCCG) - 1.0 µL (Template (1/20 dil) 1.0 µL, Taq polymerase 0.2µL. Placed the tube in the thermal cycler and program for the following temperatures Initial denaturation at 95° C for 5 minutes, Final denaturation at 95 ° C for 1minutes , Annealing at 55° C for 1 min, extension reaction at 72° C for 1 min, Run the PCR for 35 cycles, final extension at 72° C for 10 minutes. Then the DNA sample of PCR product running in Agarose gel (1%), and analyzed with UV transilluminator.

Purification of PCR product

The PCR product was purified by using Eppendorf Gel Prep Clean Up kit. Erythropoietin specific primers had been designed with *Bam HI* site in the forward primer and *Hind* III site in the reverse primer. The DNA band was excised from the gel slice and added three volumes of binding buffer and heated at 50°C for 10 minutes. Then add one volume (of the gel volume) of isopropanol and mix by inversion or pipetting.

About 800 ul of sample was transferred to spin column in collection tube. It was then centrifuged at 6000 - 10000 g for 1 minute. The filtrate was discarded and the column was replaced in collection tube and washed with 750 ~l DWB. **I**t was again centrifuged at 6000-10000 g for one minute. The filtrate was discarded and the column was replaced in collection tube and centrifuged at 6000 g for one minute. The column was now placed in a new 2 ml centrifuge tube and 30 µL of elution buffer or core water was added and centrifuged at 6000-10000 g for 1 minute.

Restriction digestion and ligation of purified PCR product and vector pRSET

The purified PCR product was then restrict digested using *BamHl* and *Hind Ill.* The Restrict

digested PCR product was then purified directly using Eppendorf Gel Prep Clean up kit. pRSET vector is isolated and restrict digested with *BamHl* and *Hind 111.* The restriction mixture was incubated at 37°C for 4 hours then loaded onto a 1% agarose gel. The restricted vector was cut out, eluted and purified using the eppendorf gel Prep Clean up kit. The ligation treaction was performed with the BamHI & Hind III restricted vector and restrict digested EPO PCR product was carried out using T4 Ligase.

Amplification of EPO gene with pRSET

The completion of ligation reaction verified by the ligation mixture was used as template for PCR. The primers used were EPO specific forwardprimer(GCTGCATGTGGATAAAGCCG) andvector specific T7 reverse primer(TAATACGACTCACTATAGGG). Three reactions were setup to verify ligation. In the negative control, no template was added.

Preparation of Competent cells

Inoculate a single colony of the *E.coli* strain in 10 ml of LB medium, and then incubate overnight at 37°C, 180 rpm. One ml of the culture inoculated into 100 ml LB medium. Then regularly check OD of the culture at 0.6 OD and after reached the OD, the culture was kept it in ice for half an hour. Then the culture was centrifuged at 4000 rpm for 10 min. Discard the supernatant and suspended cells in 100mM. Keep it in ice for 15min. Spin at 4000 rpm for 10 minutes and discard the supernatant. Repeat the steps in two times and suspend the cells in 100mM sterile $CaCl_2$.

Bacterial cell transformation

The ligation product were mixed gently with competent cells and incubated on ice for 30 min. The cells were then subjected to heat shock at 42°C for 90 seconds in a water bath and then immediately chilled on ice for 10 min. The cells were mixed with 800 µL of LB medium without NaCl was added and incubated at 37°C with shaking at 250 rpm for 1 hr. The cells were then centrifuged at 7,500 rpm for 5minutes and plated on LB without NaCl, agar containing ampicillin and incubated at 37°C in the incubation for 12- 16 hours. After 12 hours, the plates were checked for positive transformant. The Epo gene with pRSET vector was transformed into *E.coli* by check the lysate with PCR amplication by using EPO specific primer.

Induction of gene expression

A single colony of *E. coli GJl J*58 containing pRSET vector and pRSET with EPO insert were inoculated into3 ml of LB medium without NaCl to which 3 µL of ampicillin (100mglml stock) had been added. The tubes were kept for incubation at 37°C under static condition for the period of 12 hrs. After incubation period, an OD of 0.6 was reached the culture was transferred to eppendorf tubes. The bacterial cells were collected by centrifugation at 4000 rpm for 15 min.

The pellet was resuspended in 1.5 ml of fresh LB medium without NaCl. The cells were then induced for protein expression by the adding 0.3M NaCl into medium and incubated at 37^{O}C under shaker at 200 rpm. Ampicillin was added intermittently one hour after induction. The gene induction bacterial samples were taken after two hours and then collected through centrifugation at 6000 rpm for 15 min. The collected bacterial cells were suspended in 75 ~l of IX PBS. 25 ~l of 4X SSB was added and boiled for 20 min. The samples were then loaded on a 12% SDS-PAGE to check for protein expression.

Result and Discussion

The human epo cDNA was isolated from BHK 21 human cell line and sub cloned into *E.coli* through the bacterial expression vector pRSET successfully (Fig 1). Recombinant HuEPO protein was expressed and purified from the bacteria *E.coli*. The genomic DNA was isolated using isopropanol precipitation method and one µml of genomic DNA used as template for PCR using EPO specific primers provided preeminent result. pRSET vector was digested with BamHI and Hind III and digested fragments were run in gel (Fig 2). Ligation PCR using EPO specific forward primer and T7 reverse primer gave a band approximately 572bp (Fig 3). This is in concurrence with the expected product size (500bp + 72bp between Hind III site of vector and

T7 reverse priming site). Transformation of competent cells of *E. coli* GJl158 with the ligation mixture yielded two transformant. Both the transformant were found to be positive confirmed by lysate PCR (Fig 4).

Fig 1. Isolation of genomic DNA from BHK 21 cell line
Ll: l00bp Ladder
L2 and 3: EPO PCR Product

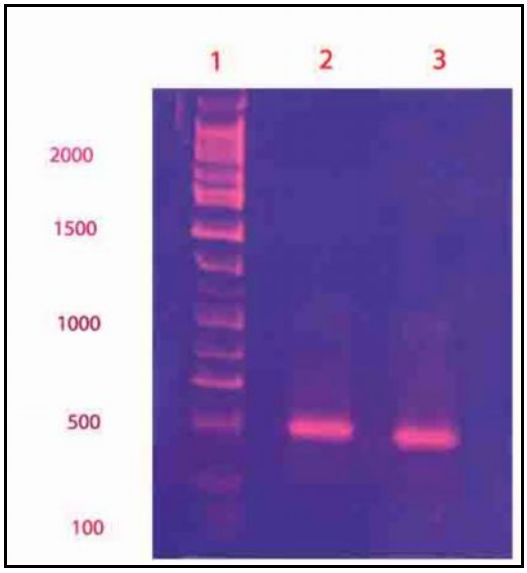

Fig 2 . Amplification of EPO cDNA (500bp) from genomic DNA from BHK 21 cell line

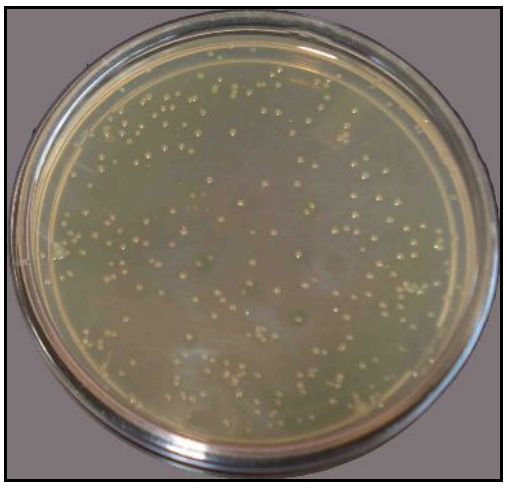

Fig 3. Transformation of EPO gene into *E. coli* GJ1158 by $CaCl_2$ method

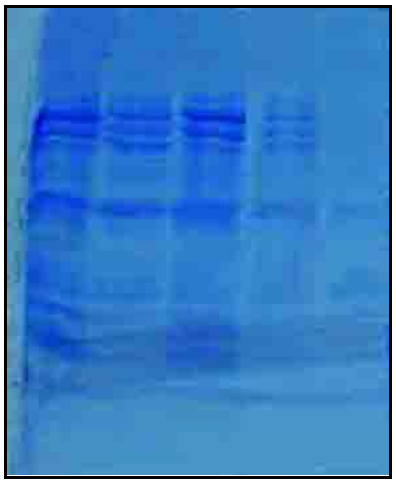

Fig 4. Induction of expression of EPO protein with *His* tag in *E. coli* GJ1158 IN LBON medium with 0.1M NaCl by SDS PAGE electrophoresis
L1: Induced clone, 2 hrs
L2: Induced clone, 3hrs
L3: Induced clone, 4hrs
L4: Induced clone, 5hrs
L5: Uninduced clone

Pure, soluble and functional proteins are of high demand in modem biotechnology. Natural protein sources rarely meet the requirements for quantity, ease of isolation or price and hence recombinant technology is often the method of choice. Recombinant cell factories are constantly employed for the production of protein preparations bound for downstream purification and processing. *Escherichia coli* is a frequently used host [19], since it facilitates protein expression by its relative simplicity, its inexpensive and fast high density cultivation, the well known genetics and the large number of compatible molecular tools available. In spite of all these qualities, expression of recombinant proteins with *E. coli* as the host often results in insoluble and/or nonfunctional proteins[22]. Although inclusion body formation can greatly simplify protein purification, there is no guarantee that the *in vitro* refolding will yield large amounts of biologically active product [24]

The expression of erythropoietin gene fused to histidine tag, vector (pRSET-EPO I) was carried out in expression strain of *E. coli* GJl158. Recombinant protein expression is induced by the addition of the sodium chloride in *E. coli* GJl158. The EPO expression in *E. coli* indicates the presence of EPO in both inclusion bodies and soluble forms, with majority in inclusion bodies[24] or in the membrane bound fraction[16]. The significant amount of EPO is produced in soluble form is as a thioredoxin fusion protein using the 'cell free' expression systems- 'Roche'sRTS systems' [23].

Poonam Bhandari and Gowrishankar [18] demonstrated that the propensity for inclusion body formation may be significantly reduced with the NaCl induction protocol. In *Ecoli* 011158, T7RNA polymerase has been placed under control of the osmotically inducible *proU* promoter of *E. coli.* IPTG being costly, NaCl induction poses an attractive alternative for large scale produce of therapeutic proteins [20].

The expression of erythropoietin gene in *E. coli* GJl158 by a salt inducible system was successfully. The expression kinetics of erythropoietin gene with or without histidine fusion tag in *E. coli* GJl158 cells under different inducer concentrations and at different time points. Erythropoietin gene fused to His tag was expressed at both 0.lM and 0.3M NaCl concentrations with better expression at 0.1M NaCl inducer concentration. Analysis of expression at different time points post induction with 0.1M NaCl showed maximum induction at 4 and 5h , afterwards the induction shows decrease.

Recombinant mammalian derived EPO is a stable, highly soluble protein, owing to its extensive glycosylation. However, the *E. coli derived* EPO and mammalian derived EPO treated with glycanases are both prone to aggregation[10]. This is mainly because of the exposure of basic residues such as asparagines that are not glycosylated in *E.coli* and make the protein prone to aggregation, and

therefore inclusion body formation [9].

Expression in a strain with a decreased tendency to form inclusion bodies therefore does not treat the problem completely as EPO in this study, was found to form inclusion bodies although a significant portion was also present in the soluble form. RNase H of *Saccharomyces cerevisiae* also has been shown to form inclusion bodies in *E. coli* G11158[22]. Erythropoietin gene was also expressed without His tag from pRSET vector in *E. coli* G11158.

Addition of 0.1M NaCI at OD of 0.6 induced expression of erythropoietin and secreted protein size comes around 18.6kDa. Expression was also obtained at 0.3M NaCl concentration but growth of bacteria was found to be inhibited. Allowing the culture to grow uninduced after 0.6 OD was reached did not lead to leaky expression of erythropoietin. However compared to expression of Epo fused to Histidine tag was significantly higher than that without tag indicating the positive influence of fusion tag on protein over expression. This is in agreement with the observation that fusion proteins appear to permit synthesis of otherwise poorly translated peptides[22] .

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