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RESEARCH ARTICLE

EXPRESSION OF HUMAN ERYTHROPOIETIN PROTEIN USING A BACULOVIRUS

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ABSTRACT

Bombyx mori cell (Bm5) was transfected with circular viral DNA from HyNPV and the baculvirus transfer vector with the cDNA of human Erythropeitin. The recombinant virus obtained was plaque purified and inoculated to early fifth instar larvae of the silkworm. At five days post infection, hemolymph was collected and the activity was measured .The rEPO was expressed successfully and was secreted into hemolymph by signal sequence derived from human EPo gene. The molecular weight of the rEPO in this study was approximately 33 KDa which is in confirmation of wide range of molecular weights existing recombinant erythropoietin.

INTRODUCTION

Recombinant Erythopoietin (rEpo) is one of the largest selling recombinant proteins in the market today. The demand for rEpo has increased rapidly in the recent years due to the wide range of clinical applications and its use. Recombinant human Epo (rHuEpo) is currently being used to treat patients with anemia's associated with chronic renal failure(Eschbach *et al*.,1989), AIDS patients with anemia due to treatment with zidovudine, nonmyeloid malignancies in patients treated with chemotherapeutic agents, perioperative surgical patients and autologous blood donation (Abels and Rudnick 1991; Koury and Bondurant 1992). The these clinical applications, it is clear that there is rEpo has commercial potential, but still the biotech industry is not able to support the demands due to time consuming cell culture techniques. Therefore, there is an urgent need to improvise or scale up the production of the rEpo using insects as bioreactors. Several rEpo producing companies are already marketing their product by the brand names EPOGEN, PROCRIT, EPREX and ERYPRO (Browne *et al* 2). An attempt is being made to use the silkworm as bioreactor to make rEpo in silkworm by using Baculovirus expression system (BEVS).

Silkworm Bombyx mori was proposed as an ideal laboratory tool by Tazima Y 1978; Silkworm has been explored for various molecular, physiological and pharmacokinetic studies.
A Novel Bombyx mori Nucleopolyhedrosis Virus (BmNpV) based Bac to Bac system was developed by Deng X *et al*.,2000 4. The Bac to Bac system simplified the usage of dual vectors which is a prerequisite for eukaryotic expression System as well this system facilitates speciesspecific infection of BmN cells (Luckow *et al*.,1991). The Bacmid system is a convenient, rapid and highly efficient method for the construction of recombinant BmNpV Bacmid vector which facilitates low cost production (Maeda 1989). Current production of recombinant erythropoietin is expensive and unaffordable to all classes of the society, due to conventional methods of animal cell culture and purification and cost of production strategies employed during recombinant Epo production. Here we explored and propose silkworm as a bioreactor for production of therapeutic proteins like erythropoietin which decreases the cost of production of recombinant Epo due to well understood and established methods available for exploring silkworms and its insect cell lines in this direction.

MATERIALS AND METHODS

Fetal calf serum (FCS) and culture media, TC-100 and Bm5, were purchased from Gibco BRL. The wild type BmNPV was collected and purified from diseased insect cadavers. A hybrid strain of silkworm CSR2xCSR4 were used in this study, the larvae were reared on mulberry leaves and reared in an environmental chamber with 25C temperature and 75% RH .

Cell Line

Bm5 insect cells were maintained as monolayer cultures at 27 °C in TC-100 insect medium (Gibco BRL) supplemented with 10% fetal calf serum.

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Construction of Recombinant Virus

The recombinant virus PBmEpo was constructed using the recombinant transfer vector The sequence of the EPo cDNA with signal sequence of 165amino acid residues was inserted into the transfer vector PBmKSK3. Recombinat Erythropoeitin was expressed by transfecting the recombinant bacmid into insect cell lines and recombinantion was confirmed by performing plaque assay Bm5 cells were used for transfection.

Viral Plaque assay

To determine the titer of a recombinant baculoviral stock, viral plaque assay was carried out. Bm5 cells were plated in 35mm culture plates at 1.5 x 10^1 to 10^4 cells in 2ml medium and incubated for 2 hours. 5ml of low melting agarose overlay was premixed with medium and heated to 45ºC. After incubation for 7 days in the incubator, 12 plaques were picked randomly from 1.5 X 10^4 cells concentration plates and infected to the fresh Bm-5 cells plates. Viral DNA 5 days post infection was used for PCR analysis using the following primers, EPO gene specific primers (Forward-5' TCA CTG TCC CAG ACA CCAAA 3' and Reverse-5' CAC TGA CGG CTT TAT CCA CA 3'). The PCR product was analyzed on 0.8% Agarose gel.

Expression of Recombinant EPo

In Insect cell lines

The Bm5 insect cell lines grown in standard cell culture methods were infected with the recombinant bacmid with Epo gene and also a control batch was maintained and observed under inverted microscope to check the progress of infection.

In Insects

Silkworm larvae were fed with stock solution of wild type BmNPV and recombinant virus with Epo gene. The larvae were reared at 27°C for an appropriate period on mulberry leaves and hemolymph was collected by cutting proleg, the difference in hemolymph turbidity was checked.

SDS PAGE Analysis of Recombinant Protein Expression

Protein expression from the recombinant baculovirus with EPo gene was analyzed by using SDS PAGE. The cells were grown in 24 well plates for expression analysis and were harvested at different intervals from 24 to 96 hours post infection. The supernatant media was removed and fresh media was replaced. The Baculoviral stock with EPo gene was added to each well also by maintaining appropriate controls (uninfected cells) and incubated in humidified incubator at 28ºC. The cells were harvested by removing media and rinsing cells once with serum-free medium and cells lysed with 400 µl of 1X SDS-PAGE Buffer. The hemolymph from the recombinant virus infected larva was also processed by similar method.

RESULTS

Construction of Recombinant Virus

The synthetic Epo gene from PUC vector was released by using BamH1 and EcoRI enzymes, to release the 540 bp Erythropoetin gene (fig.1). Further the Epo gene was ligated into the transfer vector PBmksk3 which was linearized priorly using BamH1 and EcoRI restriction enzymes (Fig.2).

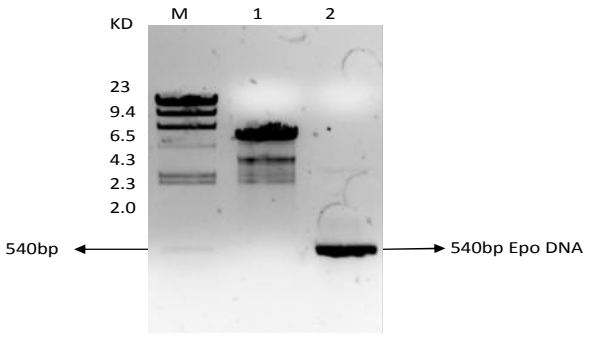

Fig.1 Agarose gel electrophoresis of restriction digestion of pBmKsK3 vector
M: marker 1:pBmKsK3 DNA with Epo (5.9Kb) 2: Epo DNA (540bp)

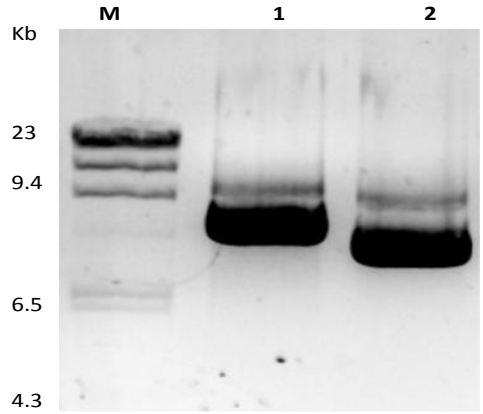

Fig.2 Agarose gel electrophoresis of Plasmid DNA analysis M: marker Lamda
DNA/Hind III marker 1:PUCEPo DNA with Epo (3.25Kb) 2: PBmKSK3 DNA (5.36 Kb).

Insertion of the Epo gene was reconfirmed by using the same restriction digestion and two clear bands of 5.3Kb representing the molecular size of pBmKsK3 and smaller fragment of 540 bp Epo DNA could be visualized and confirmed (Fig.3), and designated as pBmEPo

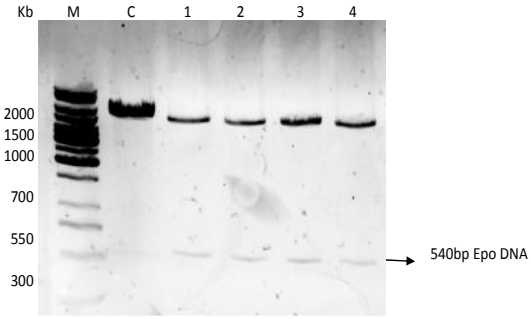

Fig 3 Confirmation of recombinant construct by restriction digestion
M= Marker, 1kb DNA ladder
C= control undigested PBmKSK3 DNA Lane,
1to4= pBmKSK3+Epo, larger band of 5.3Kb transfer vector and the lower Smaller band of 540 bp is Epo gene, thus confirming the presence of Gene.

The Bac to Bac system (pBmGoza) was used to generate the recombinant virus carrying the Epo gene. E.coli DH_{10} Beta maximum efficiency cells transferred by $pBmKSK_3$ and plated onto LB agar plates containing 50 µg/ml Ampicillin. The

recombinant bacmids were isolated from transformed bacterial cells and used to transfect Bm5 cells with lipopfectin reagent following manufacturer's instructions.

The Recombinant baculoviruses with Epo gene were harvested from cell culture medium 72 hrs post transfection and amplified to yield higher titer virus stocks.

Recombinant DNA technology is well established (Miller 1988).Further the expression in insect cells has accomplished by Miller 1993.During our study we could successfully transfer the human erythropoietin gene by using shuttle vector as followed in conventional eukaryotic expression systems.

Viral Plaque Assay

The viral plaque assay was performed by using Epo primers and randomly picking viral plaques for checking the recombination of human Erythropoietin gene was performed and all the 12 plaque showed the clear recombination of the gene (fig4). Viral plaque assay is a common procedure employed in realizing the insertion of gene of interest to ensure the recombination is successful in all the clones (Luckow 1991).

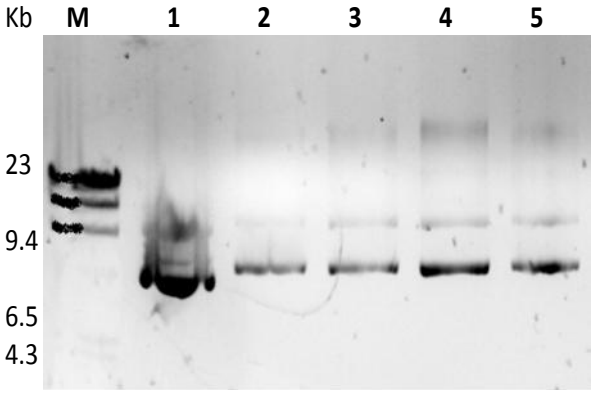

Fig 4 Ligation of EpoDNA into shuttle vector pBmKSK3 with Epo gene,

Expression of Recombinant Epo in insect cells and silkworms

Expression in cell lines

Five days post infection the cells in the control batch were intact as there was no virus infection, while the recombinant virus infected cells were ruptured and lysed to release the baculovirus (Fig 5), which is a common strategy during viral replication due to the late expression factors associated with the viral genome expression. Several proteins were earlier expressed in insect cell lines using similar strategy (Barrault *et al*.,2005;Barral *et al*.,2005)

Expression of recombinant protein in Silkworm

The larvae fed with recombinant virus and wild type viruses showed milky white hemolymph in wild NPV infected larval hemolymph due to production of polyhedrin (Fig 6A). While the recombinant virus infected larval hemolymph was transparent (Fig 6B). In wild type NPV the polyhedrin gene is intact and hence the polyhedrin protein is expressed abundantly, while in the recombinant virus infected insects the polyhedrin gene is replaced by Erythropoietin gene which is expressed abundantly instead of the polyhedrin protein.Recombinant protein expression in larvae was reported by Liu *et al*., 2005

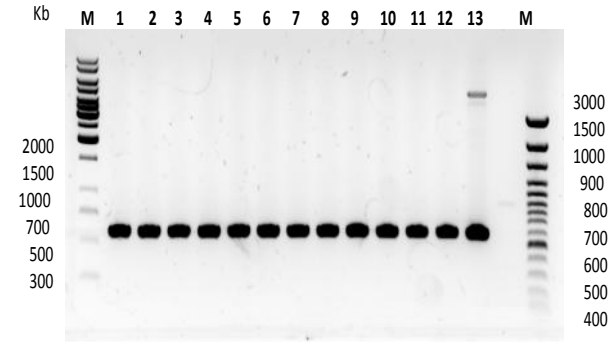

Fig5 Plaque assay PCR analysis of recombinant virus for confirmation of EPo gene,
M=marker (1Kb DNA ladder), C=Control (pBmKSK3) lane 1-12 :DNA from
12 colonies picked randomly, lane 13: Positive control PBm-Epo; lane 14:
Negative control Bacmid without EPo gene ; m:marker 100bp ladder (Primers :Kanamycin resistance).

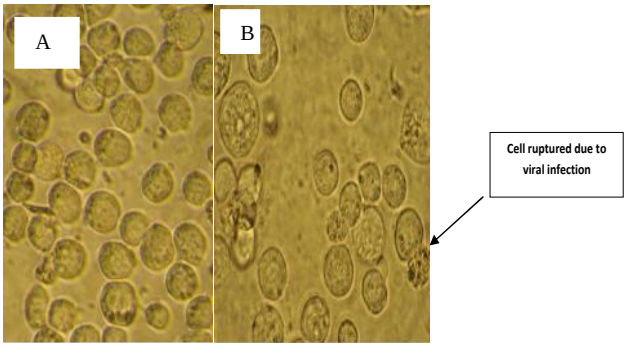

Fig 6 Bm5 *Bombyx mori* insect cell lines A: Heamolymph of control cell lines B: Hemolymp from infected cell lines.

Fig 7 Expression of recombinant protein in insects A: Silkworm infected with wildtype NPV showing milky white hemolymph B: recombinant virus with Epo infected silkworm without polyhedra in the hemolymph.

SDS PAGE of recombinant protein expressed In Bm5 cell lines

The analysis of cell lysate of Bm5 cell lines infected with recombinant virus with Epo gene expressed the predicted 35KDa recombinant Erythropoietin protein lane 1 to 3 (Fig7), and the control hemolymph uninfected with the virus didn't show the Epo protein while the insect cell infected with wild BmNPV showed the thick protein band representing the polyhedrin protein, while in the recombinant protein expressed

cells the polyhedrin gene is replaced by the Erythropoetin analysis of the cell lysate by elisa showed about 63,000U of Epo per milliliter of hemolymph. Analysis of hemolymph proteins by SDS PAGE elucidates the protein profile of the samples (Laemelli 1970).Elisa to mesure the level of erythropoietin expressed is a reliable method to check the level of recombinant protein expression (Noe *et al*.,1992).

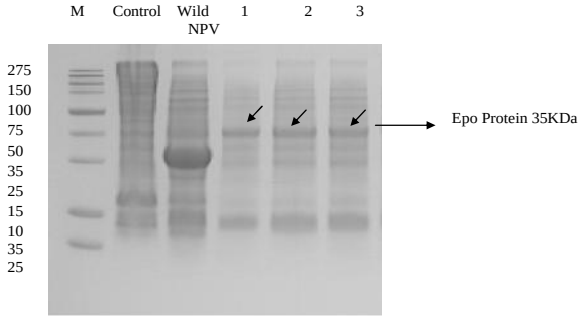

Fig 8 SDS PAGE analysis of insect cell extract for expression of Epo protein M : SDS PAGE marker; Wild NPV with polyhedrin protein Lane 1,2,3: Epo protein expressed (35Kda)

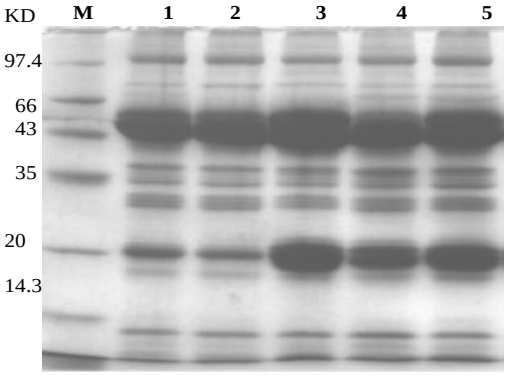

Fig 9 SDS PAGE analysis of insect hemolymph of Epo protein M : SDS PAGE marker;Wild NPV with polyhedrin protein Lane 1,2,3: Epo protein expressed (35Kda)

In Insect

The insect hemolymph analysis showed similar protein of 35 kDa and proves that there was successful expression of the recombinant erythropoietin both in silkworms and insect cell lines. ELISA assay showed that the Epo gene was expressed with high level in the silkworm. and larvae produced as high as 62,800U in 1 ml hemolymph on the 5th day (larva) after infection with the recombinant virus rBmNPVEpo, respectively. Inspite of several house keeping proteins in the insect system the expressed recombinant human erythropoietin was detected by ELISA (Noe *et al*.,1992).Several recombinant proteins were expressed in silkworms using baculovirus expression system. The pioneering work of Maeda *et al*.,1985; Liu *et al*., 2005; Lowin *et al*.,2005

DISCUSSION

Nuclear polyhedrosis viruses are members of the family baculoviridae and have a genome size of approximately 130kb of double stranded circular DNA (Aba *et al*.,2004; Miao 2005).After infection to insects, the virus particles are embedded within a protein capsule (polyhedron) nearly composed of a single protein known as polyhedrin. Polyhedrin constitutes the major % of the total protein of the infected cells but it is not essential for virus replication (Mori 2005;Motohashi 2005). The baculovirus expression vector system takes advantage in a large scale.expression of foreign gene products owing to the strong polyhedrin promoter(Taylor 2005). Pioneering work on expressing human genes in silkworm Bombyx mori paved a way for advanced recombinant. The system utilizing AcNPV and sf21 cells line is widely used for the production of many heterologous proteins, because of the ease of maintainging a large scale culture.Success of expressing recombinant proteins is well documented (Tomoko 2005., Wei Sheng 2005; Xu *et al*., 2005).The glycosylation of the expressed recombinant proteins varies in various expression systems during heterologous gene expression (Takenchi and Kobata 1991; Storring *et al*.,1998).

CONCLUSIONS

Human erythropoietin (Epo) gene has been efficiently expressed in insect cells and larvae or pupae of the silkworm using AcMNPV or BmNPV vector. The result may provide a new way for the production of rhEpo on large scale with low cost. The Epo has being highly expressed in our lab. The Epo gene was inserted into the transfer vector pBmksk3 to generate the recombinant transfer plasmid pBmEpo. Cotransfection of BmN cells with pBmEpo DNA and Wild BmNPV DNA generated the recombinant virus rBmNPVEpo carrying Epo gene driven by the strong promoter of AcNPV polyhedrin gene.) in vitro. Site specific transportation of an expression cassette, the bacmid contains the low copy number, mini F replicon, a Kanamycin resistance marker.

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