Indian Journal of Experimental Biology
Vol. 53, April 2015, pp. 195-201

Expression of GPI anchored human recombinant erythropoietin in CHO cells is devoid of glycosylation heterogeneity

Pankaj Kumar Singh[1], Mercy Devasahayam[1]* & Sobita Devi[2]

[1]Centre for Transgenic Studies, Directorate of Research building; [2]Department of Plant Protection, Allahabad School of Agriculture; Sam Higginbottom Institute of Agriculture, Technology and Sciences, Naini, Allahabad 211 007, Uttar Pradesh, India

Received 17 February 2014; revised 22 April 2014

Erythropoietin is a glycohormone involved in the regulation of the blood cell levels. It is a 166 amino acid protein having 3 N-glycosylation and one O-linked glycosylation sites, and is used to treat anaemia related illness. Though human recombinant erythropoietin (rEPO) is produced in CHO cells, the loss in quality control is 80% due to incomplete glycosylation of the rEPO with low levels of fully glycosylated active rEPO. Here, we describe the expression from CHO cells of fully glycosylated human rEPO when expressed as a GPI anchored molecule (rEPO-g). The results demonstrated the production of a homogenous completely glycosylated human rEPO-g as a 42 kD band without any low molecular weight glycoform variants as shown by affinity chromatography followed by SDS-PAGE and anti-human EPO specific western blot. The western blot using specific monoclonal antibody is the available biochemical technique to prove the presence of homogeneity in the expressed recombinant protein. The GPI anchor can be removed during the purification process to yield a therapeutically relevant recombinant erythropoietin molecule cells with a higher *in vivo* biological activity due to its high molecular weight of 40 kD. This is possibly the first report on the production of a homogenous and completely glycosylated human rEPO from CHO cells for efficient therapy.

Keywords: Anaemia, Decay acceleration factor, Glycophosphatidylinositol, Glycoprotein purification, rEPO.

Erythropoietin (EPO) is a glycoprotein hormone required for production of red blood cells (RBCs) in the human body[1]. It has 166 amino acids with O-linked glycosylation at position 126 and 3 N-linked glycosylation sites at Asn 24, 38 and 83[1,2]. Naturally occurring human erythropoietin and recombinant human erythropoietin from mammalian expression systems are heavily glycosylated accounting for 40% of its molecular weight[2-4]. Glycosylation of erythropoietin is essential for its secretion, stability, protein conformation and biological activity[2]. It has been shown that non-glycosylated erythropoietin is prone to aggregation[5]. The glycosylation pattern of glycoprotein is affected by several parameters including the protein primary and secondary structure[6-8], host system used to produce glycoprotein[9-12] and culture conditions[11] for large scale production[11-12]. The post-translational modification N-glycosylation of recombinant glycoproteins are expression system dependent[6,13-15]. Insect cells and various other cell lines attach oligosaccharides to the protein which are deleterious oligosaccharide linkages or have incomplete oligosaccharide modification resulting in an immunological reaction in humans[16-18].

Recombinant erythropoietin is used extensively to treat anaemia associated with chronic renal failure, HIV infection, rheumatoid arthritis, premature birth, cancer, ischemic stroke, chronic diseases and in blood transfusions[1]. However, therapeutic use of incompletely glycosylated glycoproteins, such as from CHO mammalian cells cause undesirable responses that can affect the efficacy of the treatment[19-20]. Though recombinant erythropoietin is produced in CHO cells[21], the loss in quality control is 80% due to the incomplete glycosylation[6-8,22].

The heterogeneity in glycosylation of human protein therapeutics is a major concern in the bio-industry[23]. Varying culture conditions for complete sequon occupancy to decrease glycosylation heterogeneity is not uncommon. Human tissue plasminogen activator (t-PA) is a 70 kD serine protease that contains 17 disulphide bonds which when expressed in CHO cells show variable sequon occupancy[24]. Conditions that

*Correspondence:
Phone: +91 9565824739
E-mail: mercy@shiats.edu.in

prevent disulphide bond formation have led to complete glycosylation of the sequon which otherwise showed variable glycosylation in untreated cells[24]. Similarly, recombinant human interferon-γ expressed in CHO-K1 cells exhibited a decrease in fully glycosylated glycoprotein[25]. This decrease could be prevented by batch feeding of the culture with glucose and glutamine[25]. The recombinant human erythropoietin (rEPO) expressed in CHO cells have demonstrated glycosylation heterogeneity as a mixture between 34-38 kD and 34-45 kD by western blot[26,27]. In this study, we have attempted to eliminate glycosylation heterogeneity by expressing human rEPO as a cell surface GPI anchored molecule (rEPO-g) by attaching a GPI anchor signal sequence from the human decay accelerating factor (DAF) protein (Fig. 1A).

Materials and Methods

Materials/reagents/services—Leibovitz L-15 medium with L-glutamine, trypsin and Cryo preserved Chinese Hamster Ovary (CHO) cells were obtained from Hi-media (Mumbai); DNA sequencing from Chromus Biotech Pvt Ltd (Bangalore); Platinum Taq DNA polymerase High fidelity, Geneticin and pcDNA3.3 Topo TA cloning kit from Invitrogen (US); Exact polymerase from 5 Prime (Germany), Ni-NTA His Bind resin and GeneJuice Transfection reagent from Novagen (US); Triton X-100, Triton X-114 and Protease Inhibitors cocktail set III, EDTA-free from Calbiochem (US); Human erythropoietin antibody Monoclonal mouse IgG 2A Clone #AE7A5 from R&D systems (US); DMEM and antibiotic-antimycotic solution from Seralab (UK); and DNA primers, Rabbit anti mouse IgG-ALP, Immobilon PVDF membrane, BCIP/NBT substrate, GeneiPure Gel Extraction kit and total blood cDNA from Genei-Merck (Bangalore). Human recombinant EPO cDNA pENTR233.epo was obtained from ASU Biodesign Institute (HsCD00514952), Arizona State University (Arizona, US).

Constructs—cDNA for GPI anchored human recombinant erythropoietin was constructed by overlapping PCR after amplifying the rEPO gene and GPI signal sequence from DAF independently. The rEPO gene was amplified using the following primers, Epo forward primer EF: 5′-GAGATGG GGGTGCACGAATG-3′; Epo reverse primer ER: 5′-CCTACCCTCGATGTGGTGATGGTGATGATG ACCCCTACCTCTGTCCCCTGTCC-3′. The Epo reverse primer contained the thrombin cleavage site, His6Tag for protein purification and the Factor Xa site. The GPI anchored protein decay accelerating factor (DAF) GPI anchor attachment signal sequence was obtained from the total human cDNA using the primers DAF forward primer DF: 5′-CACCACATC GAGGGTAGGCAAATAAAGGAAGTGG-3′ and DAF reverse primer DR: 5′-TCAAGTCAGCAAGC CCATGGT-3′. The DAF reverse primer contains the stop codon TGA 3′ of the GPI signal sequence (Fig. 1a). To construct the GPI anchored human rEPO, the human rEPO and human DAF GPI anchor attachment sequence were first amplified with exact polymerase using primers EF-ER and DF-DR from plasmid pENTR233.Epo and total blood cDNA, respectively. The fragments amplified for EPO and DAF signal sequence were 621 bp and 132 bp respectively both of which were agarose gel purified using the GeneiPure gel extraction kit. The purified fragments were combined[28] stoichiometrically approximately 50 ng of 606 bp fragment with 250 ng of 132 bp fragment. This was used in an overlap PCR[28] to amplify the human EPO fragment with the GPI anchor signal sequence 3′ of the EPO fragment (783 bp fragment) with 1 µM each primers EF & DR using Platinum Taq DNA polymerase, 200 µM dNTPs to generate A overhangs for ligation into the pcDNA3.3 Topo TA vector as specified by the kit manual. The EF & DR specific 783 bp band was excised and gel purified as mentioned above prior to cloning into pcDNA3.3. The pcDNA3.3 vector containing the human rEPO with DAF GPI anchor signal sequence in the right orientation in the vector was confirmed from Chromus Biotech Pvt Ltd sequencing facility.

The pCDNA3.3-epo-daf vector was re-suspended at a final volume of 0.3 µg/µl in $T_{10}E_1$ and used for transfection with the GeneJuice Transfection reagent. CHO cells were transfected as per the GeneJuice transfection reagent protocol. Briefly, CHO cells in 5% FBS DMEM were plated at 1×10^5 cells/ml in 12-well plate and transfected with 1 µg of DNA in DMEM at 37 °C with 5% CO_2. The cells were incubated in LM-15/DMEM complete media for 48 h. For stable cell line selection, the cells were detached using trypsin and sub-cultured (1:5 dilution) in complete growth medium LM-15/ DMEM containing 400 µg/ml geneticin in 12-well plate for the growth of stable cell lines at 37 °C (when using DMEM with 5% CO_2). Clones appeared after 2 weeks and were further grown for rEPO-g expression analysis.

Purification of human recombinant EPO by Ni-NTA resin affinity chromatography—Briefly, selected CHO cells expressing recombinant rEPO-g were incubated at 37 °C in complete media (LM-15 or DMEM with 5% CO_2). The cells were detached using 1 mM EDTA and washed in cold PBS. Thereafter, the cells were lysed on ice for 90 min in PBS pH 7.4 containing 1% Triton X-114[29], 1:200 dilution of protease inhibitor cocktail set III (Calbiochem), 10 mM imidazole, 500 mM Nacl. The lysate was centrifuged for 5 min at 3000 rpm at 4 °C to eliminate nuclei and the supernatant was collected carefully into a prechilled 15 ml sterile falcon tube[29]. To the supernatant, 5 volumes of chilled PBS pH7.4 containing 1:200 dilution of protease inhibitor cocktail set III (Calbiochem), 10mM imidazole, 500mM NaCl was added followed by pre-washed Ni-NTA resin beads, and the cells were incubated with rocking on ice for 45 min. The Ni-NTS beads were pelleted down at 750 rpm for 2 min and the supernatant carefully removed and stored for further analysis. The Ni-NTA beads were washed in 10 bed volumes of ice cold PBS pH 7.4 containing 300 mM NaCl and 10 mM imidazole containing protease Inhibitors cocktail set III. The His tagged GPI anchored recombinant EPO protein was eluted in 1 M imidazole containing protease inhibitors cocktail set III, and analysed on a discontinuous 12% SDS PAGE gel. The gel was stained with silver stain to visualize purified protein.

For purification by phase separation, cells were lysed using 1% Triton X-114 and nuclei and cell debris removed by centrifugation. The supernatant was incubated at 32 °C for 3-5 min and centrifuged at 300 g for 5 min at room temperature. The upper aqueous phase was stored for analysis, and 250 µl of 1X PBS/ml of initial lysis solution with 0.06% triton X-114 was added to the detergent phase at the bottom of the tube. The supernatant was again incubated at 32 °C for 3-5 min and centrifuged at 300 g for 5 min at room temperature. The upper aqueous phase was stored for analysis at −20 °C and 250 µl of 1X PBS containing protease inhibitors cocktail set III was added to the detergent phase at the bottom of the tube. This was followed by Ni-NTA affinity chromatography purification as mentioned above.

Detection of human recombinant EPO by western blotting—Recombinant human erythropoietin was detected on the cell surface of the transfected CHO cells by western blot using monoclonal mouse IgG 2A Clone #AE7A5 (R & D systems)[26] followed by rabbit anti-mouse IgG-ALP (Merck) and detected using BCIP/NBT substrate. Briefly, the purified human recombinant EPO protein was separated on 12% SDS-PAGE gel and transferred to immobilon membrane for 2 h at constant voltage of 32 V according to the immobilon manufacturers manual. The membrane was blocked at 4 °C in 2% BSA PBS pH 7.4 for 1 h and incubated in mouse anti-human EPO monoclonal antibody at 4 °C for 1 h at 1:3000 dilution. The membrane was washed 3 times for 10 min each in PBS containing 0.05% Triton X-100 and incubated in ALP conjugated anti-mouse antibody at 1:10000 dilution for 1 h at 4 °C. The membrane was washed three times for 10 min each in PBS containing 0.05% Triton X-100 and the BCIP/NBT substrate added for 10-20 min for colour development.

Results and Discussion

The GPI anchoring of secretory proteins such as growth hormone and rubella virus E1 glycoprotein using GPI attachment signals from DAF protein have been demonstrated in MDCK or COS cells[30,31]. Similarly, attachment of the GPI attachment signal from Thy-1 cell surface protein to normally transmembranous Herpes simplex virus 1 glycoprotein D (gD1) resulted in expression of gD1 in transfected Hela and COS cells[29]. More recently, CXCL10 containing a chemokine domain and the mucin stalk has been expressed in CHO cells as a fusion protein with the GPI anchor signal sequence for recruitment of natural killer cells when incorporated into endothelial cells[32]. However, these investigations have not addressed the issue of glycosylation heterogeneity of the GPI anchored fusion protein which is the objective of this manuscript. Here, we used western blot to confirm glycosylation homogeneity of the expressed human recombinant erythropoietin (rEPO) using anti-human erythropoietin monoclonal antibody IgG 2A Clone #AE7A5 (R&D systems) against the protein part of the rEPO. SDS-PAGE western blot is the only technique which can confirm whether the expressed protein is glycosylation heterogenous or homogenous by the presence of multiple bands or a single homogenous band respectively.

The most effective production of pharmaceutically active glycoproteins is using Chinese hamster ovary (CHO) cells mammalian expression systems[33]. With the exception of minor variations in sialylation and fucosylation, the oligosaccharide profile for a recombinant glycoprotein produced in CHO-K1 is

constant and reproducible, and is used for large scale production of pharmaceutically relevant recombinant glycoproteins[13,16,34-35]. It has been shown that the expression of secreted immunoglobulin super family molecule Thy-1 resulted in heterogeneity in glycosylation while recombinant GPI anchored Thy-1 was expressed as a homogenous glycoprotein band at 28 kD from CHO-K1 cells[7]. It was, therefore, decided to express human recombinant erythropoietin as a GPI anchored cell surface protein in CHO cells.

Human rEPO was found to show variable site occupancy when expressed in dihydrofolate reductase-deficient (DHFR-) CHO cells with a molecular weight range of 32-38 kD by silver staining and western blot[27]. Similarly, human rEPO when expressed in CHO cells at low CO_2 levels was in the molecular weight range of 33-48 kD as demonstrated by the western blot using the anti-human erythropoietin mab Clone #AE7A5 (R & D systems)[26]. In the present study, to eliminate N-glycosylation sequon variable site occupancy and glycosylation heterogeneity at each glycosylation site, the C terminus of the human EPO gene was attached to the GPI anchor signal sequence of the decay accelerating factor (DAF) as described earlier using overlapping primers as shown (Fig. 1B). The clone rEPO-g contained the sequence as designed with the entire sequence of the DAF GPI attachment site, primers DF DR and ER with a portion of the EPO gene shown in the 5′ to 3′ direction of the anti-parallel strand (Fig. 2). This is the first description of a therapeutically relevant glycoprotein such as EPO constructed to be expressed as a GPI anchored cell surface molecule. This was designed to eliminate glycosylation heterogeneity obtained during expression of secreted recombinant glycoproteins by CHO cells[7,8,26-27].

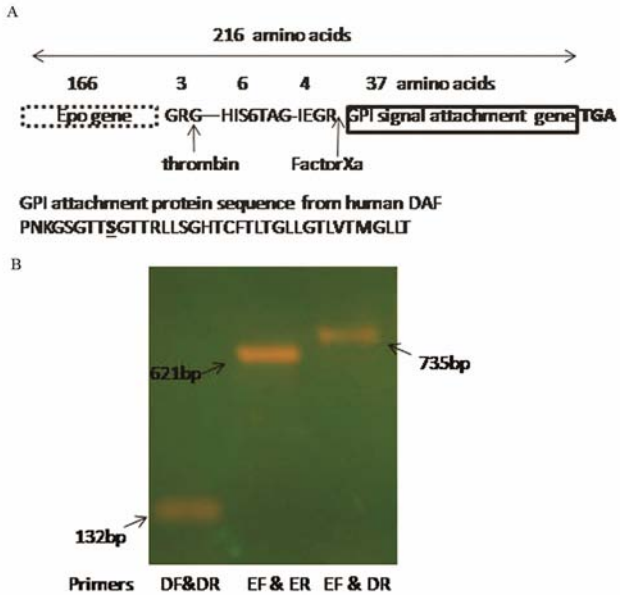

Fig. 1—(A) Schematic representation of the construct expressing human recombinant EPO. with a His6Tag and a Thrombin site and Factor Xa clevage site 5′ and 3′ of the His6Tag, respectively and DAF sequence with the TGA stop codon. The DAF GPI attachment protein sequence is shown with the GPI anchor attachment site in bold underlined; (B) Agarose gel electrophoresis showing PCR amplification of 132 bp DAF attachment site using primers DF and DR; amplification of 606 bp human EPO gene using primers EF and ER and; overlap amplification of the entire 735 bp human EPO gene with the DAF GPI anchor attachment site using primers EF and DR.

Stable CHO cell line expressing rEPO-g were obtained in complete DMEM/LM-15 media containing 400 µg/ml geneticin. After lysis and Ni-NTA affinity purification of rEPO-g, the His rEPO-g was eluted using 1 M imidazole and analysed by silver staining (Fig. 3). The His rEPO-g at 42 kD was obtained along with high molecular weight contaminant in the supernatant fraction (Fig. 3; lane 1 and 3). The 42 kD band was not obtained by silver staining in untransfected CHO cells (data not shown).

In the present study, the His tagged rEPO-g was purified by Ni-NTA affinity chromatography and visualized both by silver staining (Fig. 3: lane 1 and 3) and coomasie staining (data not shown) at 42 kD. The purified protein was observed as a single homogenous band at 42 kD and not as a smear due to variable glycosylation seen by other groups which show secreted rEPO in a molecular weight range of 32-48 kD by the western blot[26,27]. To standardise the purification protocol, phase separation followed by Ni-NTA affinity chromatography showed the incomplete separation of the His Tagged rEPO-g from the first aqueous phase (Fig. 3; lane 1) to the detergent phase (Fig. 3; lane 3). However, the second aqueous phase did not show any 42 kD His-rEPO-g protein (Fig. 3; lane 2) with all the His tagged rEPO-g remaining in the detergent fraction (Fig. 3; lane 3) with a faint purification contaminant band at 25 kD (Fig. 3; lane 3). The faint 25 kD band is a contaminant since it was not observed by the rEPO specific western blot (Fig. 4).

In addition to His tagged verification of the protein, the band at 42 kD was further verified as specific for human EPO by western blot using human EPO specific monoclonal mouse IgG 2A Clone #AE7A5 (R & D systems) as done previously[26] followed by rabbit anti mouse IgG-ALP (Merck) and detected

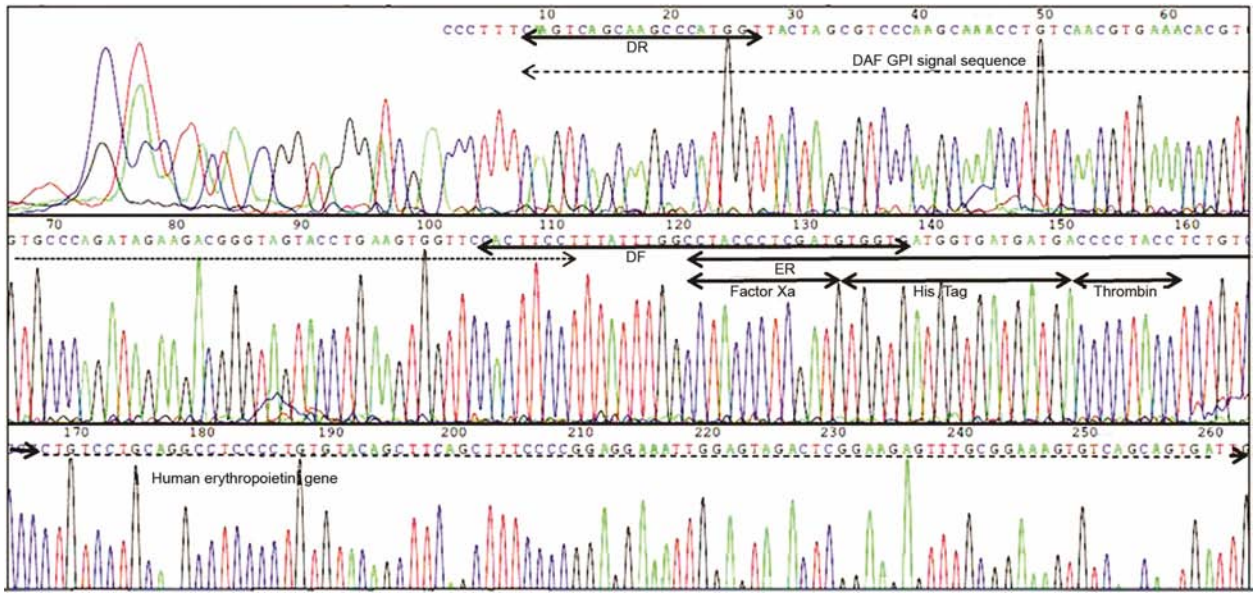

Fig. 2—Sequencing of the rEPO-g pCDNA3.3 construct: Fluorogram of the 3′ to 5′ human recombinant EPO DNA sequence in the 5′ to 3′ direction using the pCDNA3.3 kit reverse primer showing the DAF GPI anchor signal sequence (dashed arrow) followed by the ER primer containing the Factor Xa site, His6 tag and the thrombin cleavage site followed by a section of the human erythropoietin gene (dashed arrow). The primers DF, DR and ER are shown (solid double arrow lines).

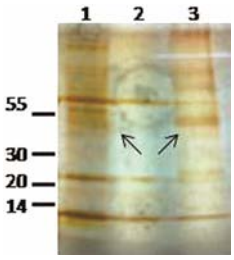

Fig. 3—Human rEPO-g identification by silver staining after Ni-NTA His tag purification: Silver staining of phase separation purified protein first aqueous layer after phase separation (lane 1); second aqueous phase after phase separation (lane 2); and Ni-NTA bead eluant of detergent phase (lane 3) with marker position indicated. The human rEPO-g is indicated by an arrow.

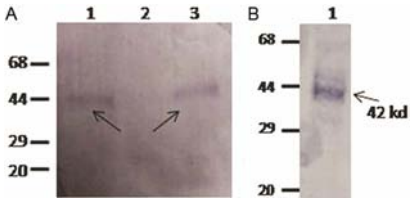

Fig. 4—Human rEPO-g identification by western blot using anti human mab Clone #AE7A5 (R & D systems) at 1:3000 dilution as described. (A) Western blot of phase separation purified rEPO-g from the first aqueous layer after phase separation (lane 1); second aqueous phase after phase separation (lane 2); and Ni-NTA bead eluant of rEPO-g from the detergent phase (lane 3); (B) Western blot of Ni-NTA purified rEPO-g to BCIP/NBT substrate for more than 24 h (lane 1). The human rEPO-g is indicated by an arrow.

using BCIP/NBT substrate. The Human rEPO-g was detected within 10 min of adding the substrate at 42 kD in both the first aqueous phase (Fig. 4A; lane1) and in the detergent phase after His tag purification and elution (Fig. 4A; lane 3) while none was observed in the second aqueous phase (Fig. 4A; lane 2). On over exposure, a homogenous band of His tagged rEPO-g at 42 kD was observed (Fig. 4B; lane 1). Glycosylation heterogeneity was not observed (Fig. 4B; lane 1) in the absence of low molecular weight glycosylation variants below 42 kD[26,27]. The 42 kD band was not observed in duplicate experiments with control CHO cells (data not shown). This is the first report of a protein primary structure engineered therapeutically relevant glycoprotein without any glycosylation heterogeneity or variable N-glycosylation site occupancy with its native primary structure intact containing all the three N-glycosylation sites and O-glycosylation site.

Thus, the His tagged rEPO-g was expressed without any glycosylation heterogeneity at individual sites and without variable N glycosylation site occupancy. This is possibly the first of its kind report on a therapeutic glycoprotein such as human rEPO expressed as a homogenous band at 42 kD. While the purified rEPO protein is quantitatively low, standardization of the large scale purification protocol will increase the recovered yield of the homogenous rEPO-g. The engineered rEPO with an additional Gly-Arg dipeptide at the C terminal can be further cleaved

from the His tag and GPI anchor using the protease thrombin for potential therapeutic applications (Fig. 1A).

The ability to produce completely glycosylated human recombinant erythropoietin is essential for biological activity since incompletely processed N-linked oligosaccharides is at least 500-fold less effective in stimulating erythropoiesis *in vivo*[19]. Further, EPO with incomplete N-linked oligosaccharides exhibits a 7-fold increased rate of clearance[19]. The appropriate complex N-linked oligosaccharides for the intrinsic biologic activity of EPO *in vivo* is an essential requirement for therapy[19]. The results have demonstrated, possibly for the first time, production of completely glycosylated human rEPO at 42 kD without any glycosylation heterogeneity from CHO cells.

Conclusion

The purified His tagged rEPO-g when expressed in CHO cells appeared as a single protein band at 42 kD by SDS-PAGE followed by western blot without any lower molecular weight glycoforms. The results clearly demonstrate glycoform homogeneity of recombinant glycoprotein expressed from CHO cells can be obtained by the addition of a GPI anchor signal sequence C terminal to the recombinant protein. This protocol can be adapted for large scale production of homogenous completely glycosylated therapeutic glycoproteins such as GMCSF, interleukins, Factor IX, etc., from CHO mammalian cells.

Acknowledgement

The authors acknowledge financial support from the Department of Science and Technology (DST), Govt of India (Grant number SR/SO/HS/053/2010); and PKS, for the award of Junior Research Fellowship. The authors also thank Prof. Pramila Gupta, Department of Plant Protection, Allahabad School of Agriculture, SHIATS, Allahabad for laboratory support.

References

1 Egrie JC, Dwyer E, Browne JK, Hitz A & Lykos MA, Darbepoetin alfa has a longer circulating half-life and greater *in vivo* potency than recombinant human erythropoietin. *Exp Hematol*, 31 (2003) 290.

2 Narhi LO, Arakawa T, Aoki K, Wen J, Elliott S, Boone T & Cheetham J, Asn to Lys mutations at three sites which are N-glycosylated in the mammalian protein decrease the aggregation of *Escherichia coli*-derived erythropoietin. *Protein Eng*, 14 (2001) 135.

3 Davis JM, Arakawa T, Strickland TW & Yphantis DA, Characterization of recombinant human erythropoietin produced in Chinese hamster ovary cells. *Biochem*, 26 (1987) 2633.

4 Kolvenbach CG, Langley KE, Strickland TW, Kenney WC & Arakawa TJ, Densimetric determination of carbohydrate content in glycoproteins. *J Biochem Biophys Methods*, 23 (1991) 295.

5 Endo Y, Nagai H, Watanabe Y, Ochi K & Takagi TJ, Heat-induced aggregation of recombinant erythropoietin in the intact and deglycosylated states as monitored by gel permeation chromatography combined with a low-angle laser light scattering technique. *Biochem*, 112 (1992) 700.

6 Devasahayam M, N-Glyosylation Sequon Asn Residue Causes Variable Site Occupancy in Recombinant Soluble Thy-1. *Curr Sci* 92 (2007) 1087.

7 Devasahayam M, Catalino PD, Rudd P, Dwek RA & Barclay AN, The glycosylation of recombinant Thy-1 is markedly affected by the presence of a glycosylphosphatidylinositol anchor. *Glycobiol*, 9 (1999) 1381.

8 Devasahayam M, Factors Affecting the Expression of Recombinant Glycoproteins. A Review. *Indian J Med Res*, 126 (2007) 20.

9 Sheeley DM, Merrill BM & Taylor LC, Characterization of monoclonal antibody glycosylation: comparison of expression systems and identification of terminal alpha-linked galactose. *Anal Biochem*, 247 (1997) 102.

10 Devasahayam M, Characterization Of Glycosylation In COS-7 Cells Using Recombinant Soluble Thy-1. *Curr Sci*, 90 (2006) 549.

11 Schweikart F, Jones R, Jaton JC & Hughes GJ, Rapid structural characterisation of a murine monoclonal IgA alpha chain: heterogeneity in the oligosaccharide structures at a specific site in samples produced in different bioreactor systems. *J Biotechnol*, 69 (1996) 191.

12 Rudd PM, Wormald MR, Harvey DJ, Devasahayam M, McAlister MSB, Brown MH, Davis SJ, Barclay AN & Dwek RA, Oligosaccharide analysis and molecular modeling of soluble forms of glycoproteins belongong to the Ly-6 scavenger receptor and IgSF expressed in Chinese hamster ovary cells. *Glycobiol*, 9 (1999) 443.

13 Takeuchi M, Inoue N, Strickland TW, Kubota M, Wada M, Shimizu R, Hoshi S, Kozutsumi H, Takasaki S & Kobata A, Relationship between sugar chain structure and biological activity of recombinant human erythropoietin produced in Chinese hamster ovary cells. *Proc Natl Acad Sci USA*, 86 (1989) 7819.

14 Takeuchi M & Takasaki S, Role of Sugar Chains in the in vitro biological activity of human erythropoietin produced in recombinant Chinese hamster ovary cells. *J Biol.Chem*, 265 (1990) 12127.

15 Hamilton SR, Davidson RC, Sethuraman N, Nett JH, Jiang Y, Rios S, Bobrowicz P, Stadheim TA, Li H, Choi BK, Hopkins D, Wischnewski H, Roser J, Mitchell T, Strawbridge RR, Hoopes J, Wildt S & Gerngross TU, Humanization of yeast to produce complex terminally sialylated glycoproteins. *Science*, 313 (2006) 1441.

16 Kagawa Y, Takasaki S, Utsumi J, Hosoi K, Shimizu H, Kochibe N & Kobata A, Comparative study of the asparagine-linked sugarchains of natural human interferon-beta 1 and recombinant human interferon-beta 1 produced by three different mammalian cells. *J Biol Chem*, 263 (1988) 17508.

17 Galili U, Basbaum CB, Shohet SB, Buehler J & Macher BA, Identification of erythrocyte Gal alpha 1-3Gal glycol-sphingolipids with a mouse monoclonal antibody, Gal-13. *J Biol Chem*, 262 (1987) 4683.

18 Kim YK, Shin HS, Tomiya N, Lee YC, Betenbaugh MJ & Cha HJ, Production and N-glycan analysis of secreted human erythropoietin glycoprotein in stably transfected Drosophila S2 cells. *Biotechnol Bioeng*, 92 (2005) 452.

19 Wasley LC, Timony G, Murtha P, Stoudemire J, Dorner AJ, Caro J, Krieger M & Kaufman RJ, The importance of N- and O-linked oligosaccharides for the biosynthesis and in vitro and in vivo biologic activities of erythropoietin. *Blood,* 77 (1991) 2624.

20 Yuen CT, Storring PL, Tiplady RJ, Izquierdo M, Wait R, Gee CK, Gerson P, Lloyd P & Cremata JA, Relationships between the N-glycan structures and biological activities of recombinant human erythropoietins produced using different culture conditions and purification procedures. *Br J Haematol,* 121 (2003) 511.

21 Cointe D, Beliard R, Jorieux S, Leroy Y, Glacet A, Verbert A, Bourel D & Chirat F, Unusual N-glycosylation of a recombinant human erythropoietin expressed in a human lymphoblastoid cell line does not alter its biological properties. *Glycobiol,* 10 (2000) 511.

22 Zoph D & Vergis G, Glycosylation: a critical issue in protein development and manufacturing. *Pharm Visions*, Spring (2002) 10.

23 Jenkins N, Parekh RB & James DC, Getting the glycosylation right: implications for the biotechnology industry. *Nature Biotechnol,* 14 (1996) 975.

24 Allen S, Naim HY & Bulleid NJ, Intracellular folding of tissuetype plasminogen activator. Effects of disulfide bond formation on N-linked glycosylation and secretion. *J Biol Chem,* 270 (1995) 4797.

25 Castro PM, Ison AP, Hayter PM & Bull AT, The macroheterogeneity of recombinant human interferon-gamma produced by Chinese-hamster ovary cells is affected by the protein and lipid content of the culture medium. *Biotechnol Appl Biochem,* 21 (1995) 87.

26 Yoon SK, Ahn Y & Han K, Enhancement of recombinant erythropoietin production in CHO cells in an incubator without CO2 addition. *Cytotech,* 37 (2001) 119.

27 Surabattula R, Rao KRSS & Polavarapu R, An Optimized Process for Expression, Scale-Up and Purification of Recombinant Erythropoietin Produced in Chinese Hamster Ovary Cell Culture. *Research in Biotech,* 2 (2011) 58.

28 Teh SH, Fong MY & Mohamed Z, Expression and analysis of the glycosylation properties of recombinant,human erythropoietin expressed in *Pichia pastoris*. *Genet and Mol Biol*, 34 (2011) 464.

29 Beghdadi-Rais C, Schreyer M, Rousseaux M, Eisenberg RJ, Cohen GH, Bron C & Fasel N, Carboxyl terminus structural requirements for glycosylphospahtidylinositol anchor addition to cell surface proteins. *J of Cell Sci*. 1993, 105:831.

30 Lisanti MP, Carac IW & Rodrique-Boulan E, Fusion proteins containing a minimal GPI-attachment signal are apically expressed in transfected MDCK cells. *J Cell Sci,* 99 (1991) 637.

31 Bernasconi E, Fasel N & Wittek R, Cell surface expression of a functional rubella virus E1 glycoprotein by addition of a GPI anchor. *J of cell Science,* 109 (1996) 1195.

32 Muenchmeier N, Boecker S, Bankel L, Hinz L & Rieth N, A Novel CXCL10-Based GPI-Anchored Fusion Protein as Adjuvant in NK-Based Tumor Therapy. *PLoS ONE* 8 (2013): e72749. doi: 10.1371/journal.pone.0072749

33 Durocher Y & Butler M, Expression systems for therapeutic glycoprotein production. *Curr Opin Biotechnol*, 20 (2009) 700.

34 Smith DF, Larsen RD, Mattox S, Lowe JB & Cummings RD, Transfer and expression of a murine UDP-Gal:beta-D-Gal-alpha 1,3-galactosyltransferase gene in transfected Chinese hamster ovary cells. Competition reactions between the alpha 1,3-galactosyltransferase and the endogenous alpha 2,3-sialyltransferase. *J Biol Chem*, 265 (1990) 6225.

35 Davidson DJ & Castellino FJ, Oligosaccharide structures present on asparagine-289 of recombinant human plasminogen expressed in a Chinese hamster ovary cell line. *Biochem,* 30 (1991) 625.