
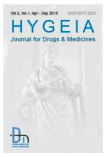

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Stable expression of modified Gene encoding functional Human coagulation Factor viii

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Abstract

Hemophilia A or factor VIII deficiency is a common X-linked genetic bleeding disorder in humans. FVIII contains a domain sequence organization designated A1-A2–B-A3-C1-C2 which B domain is not necessary in coagulation activity. We constructed a new B-domain deleted FVIII cDNA and cloned into N-terminal His tagged expression vector via Gateway technology. This vector transfected into three cell lines: NIH3T3 CHO and HepG2. rFVIII extracted purified and detected with SDS PAGE and western blot using anti His tag and anti FVIII antibodies and rFVIII activity was measured using ST4 kit. The results showed high expression and activity in NIH3T3 and CHO cell lines. B-domain containing glycosylation sites was removed in this construct but some elements were added to enhance expression level of this recombinant rFVIII. As a result of this Diminished glycosylation heterogeneity, B-domain-Truncated and modified variants of FVIII may also be a more suitable precursor for making well-characterized long acting FVIII variants.

Keywords: Blood Coagulation Factors, Factor VIII, Cloning, Expression, Gateway Technology

1. Introduction

Hemophilia A or factor VIII deficiency is a common X-linked genetic bleeding disorder in humans, occurring in about 10-20 males per million [1]. For those afflicted it is potentially life threatening and crippling. Human coagulation factor VIII is a large glycoprotein that participates in the intrinsic pathway of the coagulation cascade as a cofactor of factor IX in the proteolytic activating of factor X and contains a domain sequence organization designated as A1-A2-B-A3-C1-C2 [2].

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Currently recombinant factor VIII has become an important alternative for hemophilia -A therapy with equivalent efficacy to older therapies. This replacement therapy has a number of serious drawbacks as the development of inhibitory antibodies against the infused FVIII protein is a serious problem which occurs in over 20% of severe hemophiliacs [3, 4].In addition, the concern over the possible transmission of blood-born pathogens such as hepatitis viruses, HIV, and parvovirus B19 in addition to prions in the plasma derived products, has persuaded many researchers to pay special attention over the production of recombinant hFVIII [5].

Knowledge of the structure and function of FVIII ,as well as secretion pathways has led to certain advances in overcoming r FVIII production limitations such as mRNA instability interactions with chaperones in the endoplasmic reticulum misfolding and low transport efficiency from the ER to Golgi [6,7].

To produce a biologically active FVIII, a mammalian expression system is required to provide proper post-translation modification. Removal of the central B-Domain improved rFVIII production by increasing mRNA levels due to the removal of ER retention elements [6, 8].

In this study we described, the expression purification and characterization of a new recombinant FVIII in which the 908 amino acid residue B-domain was reduced to 18 amino acid linker sequence. This linker represents 10 amino residues from the N-terminal of the B domain linked to 8 amino acid residue from the C-terminal of B-domain. As this linker has the same C-terminal sequence as the naturally occurring β-domain, processing by the cell membrane bound subtilisin-like protease (furin) with specificity towards sequences of RXXR [9] at the C-terminal end of the linker would Be expected .Thus by inserting a coding sequence of Signal-A1-A2-Linker-C1-C2 into a mammalian expression vector a two chain of heavy and light recombinant FVIII is ensured.

2. Materials and Methods

The plasmids pENTR TOPO/D and pDEST26 (Invitrogen, Carslbad, CA, USA) were used in different stages of the cloning and expression procedure. pENTR TOPO/D was used for initial cloning and sequencing. The E. coli XL1 blue and DH5α bacterial strains (Invitrogen, USA) were used as a host for cloning of the constructs. The pDEST26 expression vector provides the opportunity to clone the desired insert as a fusion protein with an N-terminal poly-his tag. This tag facilitates detection of the expressed protein with anti- his antibody and also purification of the protein using the metal-binding site for affinity purification of the recombinant protein. Commercially available recombinant FVIII product, ReFacto (Wyeth; Europe Ltd. ,Berkshire ,UK)were purchased at the local pharmacy. The cell lines were purchased from ATCC (American Type Culture Collection) HepG2, (ATCC Number: HB-806) and CHO-K1 (ATCC Number CCL-61) and) NIH3T3, (ATCC Number: CRL-2795) .

Cells were grown at 37° C with 70% humidity and 5% CO_2 atmosphere on RPMI media (GIBCO) and HAM F12 media (HYclone) respectively, supplemented with 10% of fetal bovine serum (FBS).

FVIII heavy and light chains were amplified separately by PCR from a human liver cDNA library purchased from *Clontech* and further fused to generate a shortened B-linked single chain rFVIII , which will transfect to a mammalian cell expression vector for transfection experiments The primer set for amplification of the heavy chain of human FVII gene containing Kozak sequence site included the forward primer: 5'- CACCATGGTC TCC CAG GCCCTC AGG CTCC -3' and reverse primer: 5'-T AGG GAA ATG GGGCTC GCA G -3' And the primer set for amplification of light chain is 5'-GTACCATGCTAGGCTAACTGTCA ,CAATTCC-3' and reverse primer: 5'-CTAGGT TCGGTCCAATCGCGTAACTG -3' . The blunt-end PCR products were then TOPO- cloned into pENTR TOPO/D vector according to the manufacturer's protocol (Invitrogen, USA). The reaction mixture was incubated for 5 min at room temperature. The reaction was then placed on ice and the pENTR TOPO/D-FVIII (Entry clone) construct was transformed to competent E. coli according to the manufacturer's protocol (Invitrogen, USA) .

The LR recombination reaction was then carried out between the entry clone and destination vector, pDEST26, according to the manufacturer's instructions (Invitrogen, USA). The Plasmid obtained were cloned in escherchia coli (XL1 Blue and DH5 α) .The cell lines were transfected with cDNA contained expression vector using the Lipofectamine 2000 (Invitrogen) according the manufacturer's protocol. pDEST26 DNA was used as a control. NIH3T3, HepG2 and CHO cells containing pDEST26-FVIII construct were selected in a medium containing 600 g/mL geneticin (Roche, Germany) for at least 14 days.

Several stable clones were generated by dilution of the cells and their culture in 96-well culture plates. The FVIII encoded by pDEST26 carries six histidine residues at its N-terminus. Polyhistidine has a high affinity for a nickel-nitrilotriacetic acid resin permitting single-step purification of the fusion protein. The nickel-nitrilotriacetic acid resin was washed and culture medium containing FVIII was added to the column; the bound protein was eluted according to the manufacturer's instruction (Invitrogen, USA). Samples were denatured and reduced at 70° C for 10 min in LDS sample buffer (Invitrogen Corpora-Tion ,Carlsbad ,CA ,USA) containing 50 mm DTT. Electrophoresis was performed using 7% Tris-acetate (TA)Pre-Cast Novexgels (Invitrogen)and TA Buffer (Invitrogen).Silver staining was carried out Using Silver Quest (Invitrogen) and Coomassie stain- Ing using Gel Code(Pierce ,

Thermo Fisher Scientific Inc .,Rockford ,IL,USA) according to the manufacturer's description and blocked with a solution containing 5% skimmed milk and 0.1% Tween 20. The blocked membranes were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and incubated with horseradish peroxidase- conjugated histidine antibody according to the supplier's instructions (Roche, Germany) at room temperature for 1 h.

Afterwards, the membranes were washed four times with PBS containing 0.1 % Tween 20, and finally the membranes were developed with 3,3 diaminobenzidine (DAB) solution (Sigma, Dusseldorf, Germany) Purified protein was also detected using goat anti-human FVIII antibody (R&D Systems, Minneapolis, MN, USA) with 0.75 µg/mL peroxidise-conjugated anti-goat IgG (Dako, Denmark) as the secondary antibody .

Coagulation activity of factor VIII was assayed with a one-stage method and performed on the ST4 Blood Coagulation System (Diagnostic stago, France). FVIII antigen was quantized by an anti-FVIII light chain sandwich enzyme-linked immune sorbent assay(ELISA) method using both the Asserachrom VIII:C Ag and FVIII:C-EIA commercial kits according to the manufacturers' instructions.

3. Results

Bioengineered, FVIII: heavy and light chains were amplified, sequenced and fused to generate a single chain rFVIII Connected by a linker. The final construct was 4.2 Kb in length and contained the organization LP-A1-A2-MB-A3-C1-C2, where LP was the signal sequence of FVIII and MB was the conserved minimal B domain linker that preserved protease processing sites needed for FVIII thrombin activity.

After transfection and selection, human FVIII was expressed in all three target cell lines (Table 1) . The highest FVIII expression was observed in transfected NIH3T3 cells 485 ng/10^6 cells $ml^{-1}24hrs^{-1}$ with a coagulant activity of 1.07 U/10^6 cells.24 hrs^{-1} .FVIII coagulant activities by transfected CHO cells were 0.18 U/10^6 cells.24 hrs^{-1} with expression level of 76. FVIII activity and the expression was relatively low in HepG2 cells (0.03 U/10^6 cells.24 hrs^{-1} and 7.8 ng /10^6 cells.24 hrs^{-1} respectively) .The commercially available FVIII ReFacto activity was 1.18 U.

No bioactivities or antigen of FVIII were found in any above cell lines before the transfection of the FVIII cDNA.. The recombinant fusion protein was purified using a nickel–sepharose column and the eluted protein was analyzed by SDS-PAGE and western blotting. A protein of approximately 184 KDa (single chain) and thrombin treated single chain resulted in HC and LC was detected by SDS-PAGE (Figure 1) and further confirmed by western blot analysis using both anti-his and anti-FVIII antibodies (Figure 2).

To test whether the purified protein is biologically active, 500 ng/mL of recombinant his-tagged FVIII protein was obtained from 10 mL culture medium and a prothrombin time test was carried out. A three-fold decrease in clotting time was observed when human FVIII-depleted plasma was used in combination with human thromboplastin and the recombinant FVIII produced in this study (Table 1).

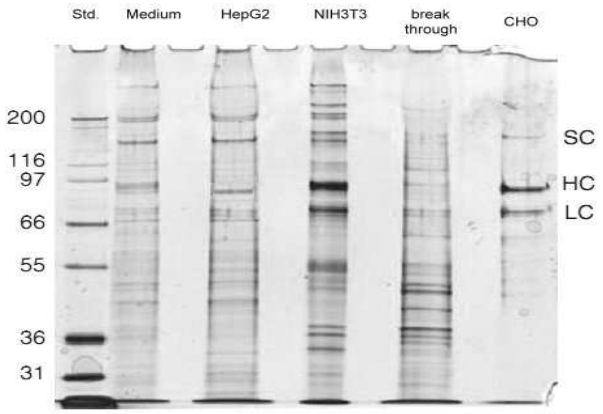

Fig1. SDS-PAGE of samples taken during purification .Molecular weights are given in kDa for the standard marker to the left side of the gels.

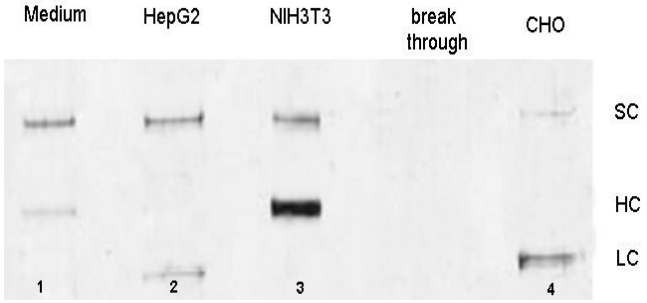

Fig 2. Western blotting of rFVIII using anti His antibody (lane 1,3) and anti FVIII antibody (lane2 ,4) (SC: Single Chain, HC: Heavy Chain, LC: Light Chain)

Table 1. FVIII coagulation activity

Target cells	**FVIII activity ($U/10^6 cells ml^{-1}$ $24hrs^{-1}$)**	**FVIII Ag ($ng/10^6$ cells $ml^{-1}24hrs^{-1}$)**
CHO	0.18	76
HepG2	0.03	7.8
NIH3T3	1.07	485
ReFacto	1.18	-

The FVIII coagulation activity of of pooled plasma of health population is defined as $U/10^6$ cells ml^{-1} $24hrs^1$

4. Discussion

The recombinant DNA technology has enabled manufacturers to increase the production of FVIII concentrates to meet the needs of patients with hemophilia A. There has been a continued interest in the bioengineering of rFVIII with increased mRNA and protein synthesis, decreased proteolytic degradation in the final formulation with improved function to overcome some of the limitations in current treatment and the high costs of therapy.

In this study , we describe the construction of a new recombinant FVIII , in which the naturally occurring β-domain of 908 amino acid residues have been replaced by a 18 amino acid residue linker sequence (consists of 10 amino acid residues from the N-terminal of the β -domain linked to 8 amino acid residues from the C-terminal of the - β domain). Thus the new FVIII is devoid of the main part of the β -domain representing 890 amino acid residues.

While constructing the encoding sequence, the C- terminal part of the linker was kept identical to the C-terminal sequence of the - β domain. This ensures correct furin processing of the single chain FVIII molecule. The SDS page analysis of purified rFVIII (Fig.2), indicated the production of elevated levels of secreted molecules. This single chain form of rFVIII contains a truncated β domain of 18 amino acid residues and is similar to the single chain form of ReFacto containing a 14amino acid residue truncated β -domain [13, 14]. The reasons for making a - β domain truncated version of the FVIII molecule are several .The β -domain does not seem to have any function with respect to clotting activity [6],and also the expression of full-length FVIII in mammalian systems have been considerably more difficult than β -domain- truncated variants[9].

From a protein Chemistry point of view, the purification and characterization of full-length FVIII molecule containing 19 potential glycosylation sites in the β -domain would also be considerably more complex that a β -domain-truncated molecule. As a result of this diminished glycosylation heterogeneity, the β – domain truncated variants of FVIII may be a better precursor for making well-characterized long acting full length FVIII variants.

Toole etal (1996)also reported the reconstruction of the same FVIII-ΔBcDNA , as obtained in our experiment [15].The expression yield of this β- domain FVIII was 0.114U/mL/106cells/day ,compared with that of the full length FVIII, 0.015U /mL /10^6cells/.

In the study of the expression level of FVIII-DB in different cell lines by using the vector pDEST26,it was indicated that the yield was found highest in the NIH3T3 cell line. We observed that the addition of a short β-domain sequence with C-terminal sequence of the β-domain containing furin processing sequence resulted in high expression and increase in secretion compared with previous studies [13, 14,16].

Thus, FVIII with minimal β- domain content could provide more efficient expression in vitro to improve the efficiency of commercial production of BDDFVIII. The ability to produce recombinant FVIII variants may lead to higher yields with current manufacturing strategies. Further rigorous biochemical characterization is required to determine the bioequivalence of the bioengineered FVIII variants compared with currently approved BDD-FVIII and full –length FVIII recombinant products.

The FVIII amount in CHO and NIH3T3 cell supernatants could be directly measured HepG2 cells supernatant had to be diluted in culture media (1:5) in order to ensure linearity of the ST4 FVIII kit. The apparent disagreement with data reported by [10,11] for *Bacillus stearothermophyllus* alpha-amylase and human placenta secreted alkaline phosphatase , stable clones, is probably a result of FVIII protein complexity. These results confirm the biological activity of the expressed novel β-domain deleted, *his*-tagged recombinant FVIII.

The results clearly confirmed the existence of the *his*-tagged FVIII fusion protein in the cell culture medium, determined by the elution using a nickel column, SDS-PAGE and western blot analysis with both anti-his and anti-FVIII antibodies. In summary the present version of a new recombinant FVIII have some differences with respect to O-glycosylation and product weight with native FVIII and showed considerable activity in NIH3T3 and CHO cells.

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