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Original Article

Expression and purification of recombinant human coagulation Factor VII fused to His-Tag through Gateway technology

Raheleh Halabian[1], Mahdi Edalati Fathabad[1], Nasser Masroori[1], Amaneh Mohammadi Roushandeh[2], Sasan Saki[3], Nasser Amirizadeh[1], Ali Jahanian Najafabadi[4], Ahmad Gharehbaghian[1], Mehryar Habibi Roudkenar[1]

[1] Research Center, Iranian Blood Transfusion Organization, Tehran Iran
[2] Department of Anatomy, Faculty of Medicine, Medical university of Tabriz, Tabriz, Iran
[3] Islamic Azad University, Arak Branch, Arak, Iran
[4] Department of molecular biology, Pasteur institute of Iran, Tehran, Iran

Background. Factor VII is a plasma glycoprotein that participates in the coagulation process leading to generation of fibrin. Construction, expression and purification of recombinant FVII fused to poly histidin tag through gateway technology were aimed in this study.

Methods. To construct entry clone, blunt-end FVII cDNA and subsequent PCR product isolated from HepG2 cell line was TOPO cloned into pENTR TOPO vector. To construct expression clone, LR recombination reaction was carried out between entry clone and destination vector, pDEST26. CHO cells were transfected with 1μg of DNA of PDEST26–FVII using the FuGENE HD transfection reagent. Two cell lines that permanently expressed recombinant factor VII were established. The expression of recombinant FVII was confirmed by RT-PCR and ELISA. Culture medium containing his-FVII was added to the nickel-nitrilotriacetic acid resin column and bound protein was eluted. The purified protein was detected by SDS-PAGE and western blot analysis. Biological activity of the recombinant factor VII was determined by prothrombin time assay using factor FVII-depleted plasma.

Results. The results showed that human recombinant FVII successfully was cloned and accuracy of the nucleotide sequence of the gene and its frame in the vector were confirmed by DNA sequencing. Stable clones transfected with the construct expressed FVII mRNA and related protein but any expression was not detected in the CHO cells containing empty vector. A protein of about 52KDa was detected in SDS-PAGE and was further confirmed by western blot analysis. A three-fold decrease in clotting time was observed by using this rFVII.

Conclusion. As we are aware, this is the first report of expression of recombinant FVII fused with his-tag through gateway technology. The next steps including large scale expression, purification, activation and stabilization are underway.

Key words: Gateway technology, Hemophilia, Recombinant FVII, His-Tag, Purification

Introduction

Factor VII is a plasma glycoprotein that participates in the coagulation process leading to the generation of fibrin[1,2]. It is synthesized in the liver where vitamin K is required for the formation of approximately 10 γ-carboxyglutamic acid residues that are present in the amino-terminal region of the protein[2-4]. Factor VII is a single-chain glycoprotein that is secreted into the blood and circulates in a zymogen form[5-8]. It is converted to factor VIIa by factor Xa[7, 9], factor XIIa[7,10-12], and factor IXa[12] or thrombin[9] through minor proteolysis. Activated FVII (FVIIa) initiates the extrinsic coagulation pathway by binding to tissue factor on the surface of cells that have become exposed to circulating blood following injury[13,14]. Recombinant human FVIIa can counterbalance the factor VIII (FVIII) or factor IX (FIX) deficiencies and could be used for treatment of bleeding in hemophilia A or B patients that produce antibodies (inhibitors) against FVIII or FIX[13,15]. Nowadays two different types of FVII preparation exist: plasma-derived and recombinant.
Low yield and possible viral contamination are disadvantages of plasma-derived FVII. Commercially available FVIIa, NovoSeven, is produced in baby hamster kidney (BHK) cell line that secretes FVII into the culture medium in its single chain form. The product is purified with murine monoclonal anti-FVII antibodies and subsequent ion exchange chromatography further purifies the product. Because this type of purification is time-consuming and expensive, construction, expression and purification of recombinant FVII fused to poly histidin tag through gateway technology were aimed in this study.

Materials and methods

Plasmids and bacteria

Plasmids pENTR TOPO/D and *p*DEST26 (Invitrogen, USA) were used in different stages of cloning and expression procedure. pENTR TOPO/D was used for initial cloning and sequencing. Bacterial strain *E. coli DH5α* (Invitrogen, Carlsbad, CA) was used as a host for cloning of the constructs. pDEST26 expression vector provides the opportunity to clone the desired insert as a fusion protein with N-terminal polyhistidine 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.

Cell culture

HepG2 (human hepatoma cell line) and CHO (chinese hamster ovary cell line) were obtained from National Cell Bank of Iran (NCBI). These cell lines were grown in RPMI-1640 medium (Gibco-BRL, Eggenstein, Germany) containing 10% fetal bovine serum, 1µg/mL vitamin K1, 100 U/mL penicillin, and 100 µg/mL streptomycin. (Gibco-BRL, Eggenstein,Germany).

Isolation of FVII cDNA and plasmid construction

HepG2 cell line was used as a source of human FVII gene. Total RNA was extracted by Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. The quality of RNA was determined by electrophoresis. Reverse transcription was performed by SuperScript III reverse transcriptase (Invitrogen, USA) with 2µg of total RNA followed by DNaseI (Invitrogen, USA) treatment and heat inactivation. Full length human FVII cDNA was isolated using specific primers by RT-PCR. PCR was performed using Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA) in a GeneAmp PCR system 9600 (PerkinElmer Life And Analytical Sciences, Inc., Wellesley, MA, USA). After initial denaturation (5 min. at 94ºC), cDNA was subjected to 30 cycles of PCR. Primer set for amplification of full length human *FVII* gene containing Kozak sequence site included forward: 5'-ACCATGGTG GTC TCC CAG GCC CTC AGG CTC -3' and reverse: 5'-T AGG GAA ATG GGG CTC GCA G -3'. PCR annealing temperature was 60º C for human *FVII* gene. PCR products were separated in 2 % agarose gel.
Then the blunt-end PCR products were TOPO cloned into pENTR TOPO/D vector according to the manufacturer's protocol (Invitrogen, USA). The reaction mixture was incubated for 5 minutes at room temperature.

Then the reaction was placed on ice and pENTR TOPO/D-FVII construct was transformed to competent *E.coli* according to the manufacturer's protocol (Invitrogen, USA). Positive clones were selected on LB medium containing 100 µg/mL kanamycin. Plasmid DNA was isolated using high pure plasmid extraction kit (Roche, Germany). Presence of the insert was confirmed by PCR and finally to confirm the fidelity of the sequence, DNA sequencing was performed. This construct is called entry clone. Then, LR recombination reaction was carried out between entry clone and destination vector, pDEST26, according to manufacturer's instruction (Invitrogen, Carlsbad, CA). The products of LR recombination were transformed to competent *E.coli* according to the manufacturer's protocols. Positive clones were analyzed by culturing them on LB medium containing 100 µg/mL ampicillin and 30µg/mL chloromphenicol.
Afterwards, the plasmid DNA was isolated using a commercially available plasmid extraction kit and was further analyzed by restriction enzyme digestion and PCR. Finally this expression vector was transfected into CHO cell line.

Transfection and generation of stable FVII-expressing cells
CHO cells (5×10^5) were seeded and upon reaching 70% confluence were transfected with 1μg of pDEST26–FVII DNA using the FuGENE HD transfection reagent (Roche, Mannheim, Germany) according to the manufacturer's protocol. pDEST26 DNA was used as a control. CHO cells containing pDEST26-FVII construct were selected in a medium containing 600 μg/mL geneticin (Roche, Mannheim, Germany) for at least 14 days. Several stable clones were generated by dilution of the cells followed by their cultivation in 96 well culture plates. The expression of FVII was proved by RT-PCR and ELISA (Diagnostica Stago, France) according to the manufacturer's protocol.

Purification of polyhistidine-tagged FVII fusion protein and its characterization
The FVII 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 FVII was added to the column, and bound protein was eluted according to the manufacturer's instruction (Invitrogen, Carlsbad, CA).

Detection of the purified protein
The protein concentration was quantified by Bio-Rad protein assay kit according to the instructions of the supplier (Bio-Rad, USA). Purified protein was detected by running the samples heated in 1x SDS-PAGE sample buffer at 95 ^{o} C for 5 min on 12% gels followed by Coomassie blue staining. The proteins were also blotted onto PVDF paper (Hi-bond Amersham Biosciences, USA) and blocked with a solution containing 5 % skimmed milk and 0.1% Tween 20. The blocked membranes were washed with PBS containing 0.05% Tween 20 and incubated with horseradish peroxidase-conjugated His antibody according to the instructions of the supplier (Roche, Mannheim, Germany) at room temperature for 1 h. Afterwards, the membranes were washed 4 times with PBS containing 0.1 % Tween 20, and finally the membranes were developed with DAB solution (Sigma, Dusseldorf, Germany). Purified protein was also detected using goat anti-human coagulation factor VII antibody (R & D Systems, Minneapolis, MN) with 0.75 µg/mL peroxidase conjugated anti-goat IgG (Dako, Denmark) as the secondary antibody. The coagulant activity of purified rFVII was measured using FVII-depleted human plasma (Diagnostica Stago, France). Thromboplastin preparations utilized in the assay were from human (Hoechst Canada Inc., Behring Diagnostics, Montreal, Quebec, Canada). PT assays were performed using fibrin timer cups and a mechanical fibrometer with a built-in automatic timer device (BBL, Division of Bioquest, Cockeysville, MD, USA).

Results

Isolation and construction of the recombinant vectors

Summary of the experiment is depicted in Figure 1. Specific primers were designed to amplify full length of human factor VII gene. The expected size of the PCR product was about 1400 bp. The blunt-end FVII PCR product was directly TOPO cloned into pENTR TOPO/D vector and the accuracy of the nucleotide sequence of the gene and its frame in the vector were confirmed by DNA sequencing (the gene is accessible in the gene bank with accession number EU557239). Next, to construct expression clone LR recombinant reaction was carried out between pENTR TOPO/D-FVII and pDEST26 vector.

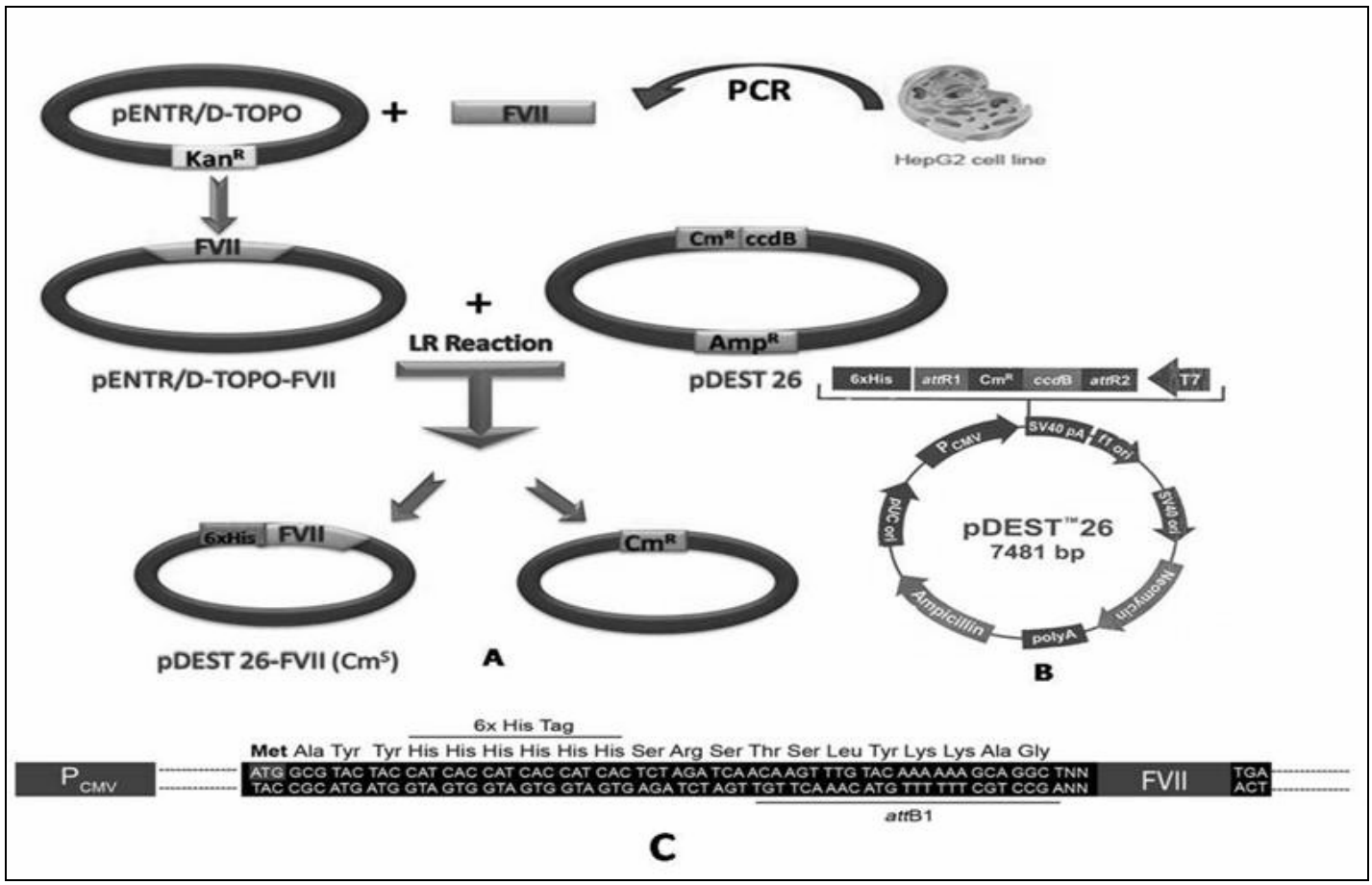

Figure 1 - A; Summary of construction of **the** entry and expression clones. HepG2 cell line was used as a source of FVII cDNA. Blunt-end PCR product was TOPO cloned into pENTR TOPO/D vector. The construct was **used to** transform competent *E.coli* and positive clones were selected on LB medium containing a proper antibiotic. This construct is called entry clone. Next, LR recombinant reaction was carried out between pENTR TOPO/D-FVII and pDEST26 vector to construct expression clone. The resulted pDEST26-FVII which is called expression clone was transfected into CHO cells. Stable clones expressing recombinant FVII were established in the presence of geneticin. B; pDEST26 vector. C; N-terminal sequence of the fusion protein.

To check the putative expression clones, they were grown in the presence of chloramphenicol and the desired clones which were ampicillin-resistant and chloramphenicol-sensitive were chosen for further analysis using PCR, which proved the existence of the insert. Finally, to confirm that the FVII gene is in frame with appropriate tag, DNA sequencing was performed. Figure 1 – letter C represents the N-terminal sequence of the construct and its frame in detail.

Expression of FVII by CHO cell line

To investigate whether pDEST26-FVII transfected cells express human FVII, RT-PCR was performed. Stable clones transfected with the construct expressed FVII mRNA but there was not any expression in the CHO cells transfected with pDEST26 vector (Figure 2). The cell culture medium of stably pDEST26-FVII transfected cells was used for detection of FVII protein by ELISA. The cells transfected with pDEST26-FVII expressed FVII protein while cells transfected with pDEST26 vector not (Figure 3). About 40% of His-FVII was found to be intracellular and not secreted to the medium. In another study, in which the FVII gene was cloned into pcDNA3.1 expression vector, which lacks a his-tag, the intracellular part of the FVII was 20%, which indicates the fusion of FVII to his-tag may impair its secretion.

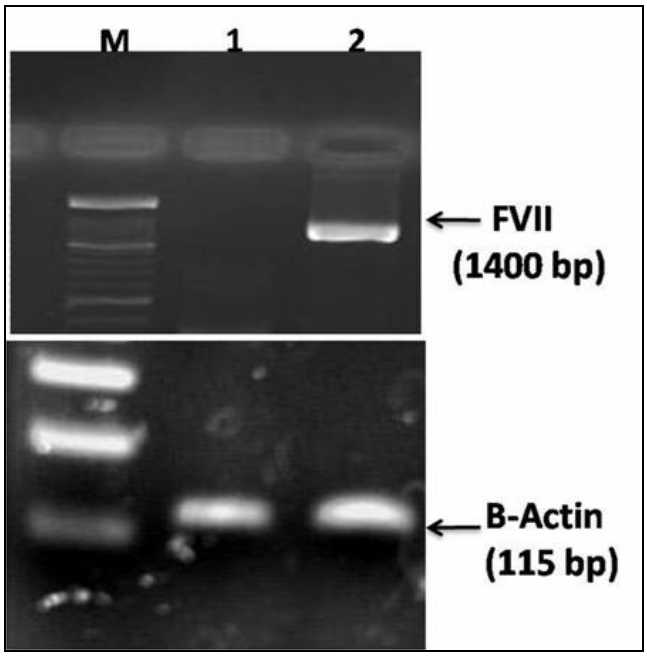

Figure 2 - Expression of the rFVII by stable clones of CHO. RNA was extracted from **the** CHO cells transfected with the pDEST26-FVII construct and **the** CHO cells transfected with the empty vector. cDNA was synthesized and RT-PCR was performed. CHO cells transfected with the construct expressed FVII mRNA, an approximately 1400 bp length fragment (Lane 2), while in CHO cells transfected with empty vector any expression was not observed (Lane 1). Lower figure shows the expression of β-actin in both stable clones of CHO, i.e. transfected with the pDEST26-FVII or empty vector. M: 100 bp ladder marker.

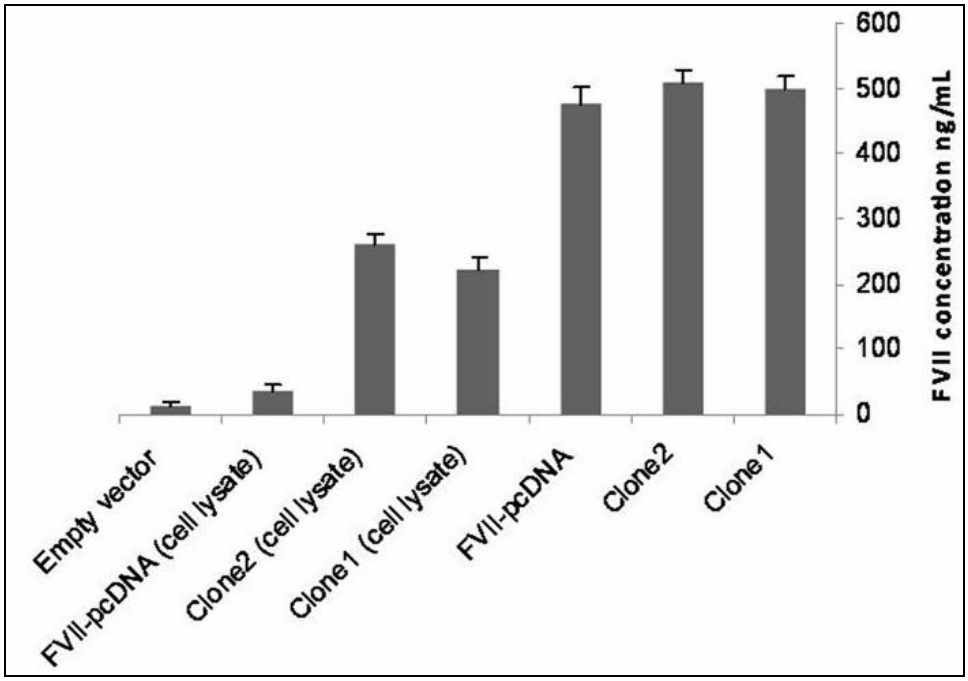

Figure 3 - Detection of rFVII by ELISA. Cell culture medium and lysate of **the** stable clones were evaluated. The expression of rFVII was confirmed by ELISA. His-FVII was detected both in cell culture medium and cell lysate. Two stable clones (Clone 1 and 2), CHO transfected with pcDNA 3.1-FVII and empty vector are shown in the figure. Any FVII expression was not detected in the medium of CHO cells transfected with empty vector (pcDEST26 3.1). Data are presented as mean ± SD.

Purification and characterization of the expressed rFVII

The recombinant fusion protein was purified using a nickel–sepharose column and eluted protein was analyzed by SDS-PAGE and western blotting. An approximately 52KDa protein was detected in SDS-PAGE (Figure 4, letter A) which was further confirmed by western blot analysis using both anti-his and anti-FVII antibodies (Figure 4 letter B and letter C). 500 ng/mL of recombinant fusion His-FVII protein was obtained from 10 mL culture medium. To test whether the purified protein is biologically active, prothrombin time test was carried out.

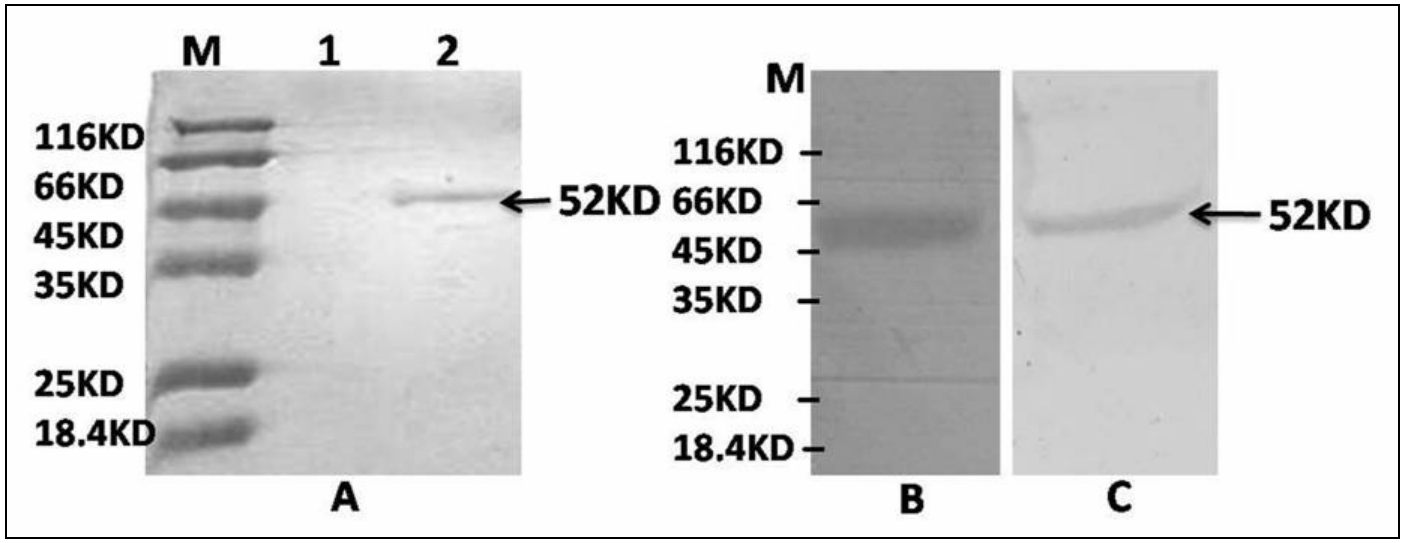

Figure 4 - SDS-PAGE and Western blot analysis of recombinant FVII fusion protein. A; SDS-PAGE analysis. The recombinant fusion protein was purified using a nickel–sepharose column. After purification, a single protein band was detected in 12% SDS-PAGE (Lane 2). The cell culture medium of CHO cells transfected with empty vector was also passed through nickel–sepharose column. However, any band was not observed (Lane 1). B; western blot analysis of eluted protein using anti-his-tag and anti-FVII antibodies (C). Eluted protein reacted with both antibodies, visualized by developing using DAB solution.

A three-fold decrease in clotting time was observed when human FVII-depleted plasma was used in combination with human thromboplastin and the rFVII produced in this study. Similar results were observed when prepared his-tag free recombinant FVII, expressed using pcDNA3.1 expression vector and also a commercially available FVII ,NovoSeven, were used (Figure 5).

Taken together, this confirms the biological activity of the expressed His-rFVII and the fact that its function is not affected following fusion.

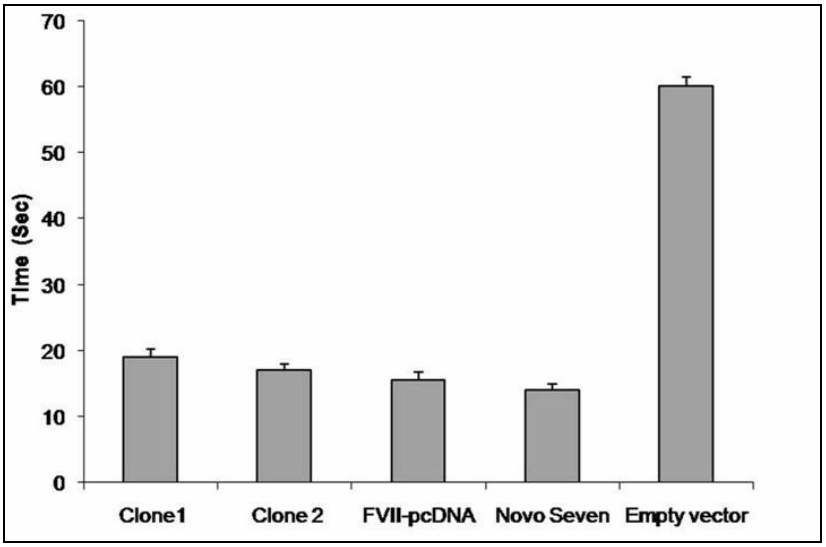

Fig 5 - Prothrombin time assay. Biological activity of the purified recombinant his-FVII was evaluated by prothrombin time test. A three-fold decrease in clotting time was observed when human FVII-depleted plasma was used in combination with human thromboplastin and the his-rFVII produced in this study. About four-fold decrease in clotting time was also observed when a commercially available preparation of rFVII, NovoSeven, was used. The experiment was performed in triplicate and the Data are presented as mean ± SD.

Discussion

In this study, for the first time we reported expression and purification of human recombinant FVII fused to his-tag. The gateway technology was employed for construction of the recombinant vectors. Gateway technology is a universal cloning method based on the site-specific recombination properties of lambda bacteriophage[16]. This technology provides a rapid and highly efficient way to clone DNA sequences into multiple vector systems for functional analysis and protein expression[17]. Briefly, in this technology there are two types of vectors. One is entry vector and other is destination or expression vector and accordingly there are two type of clones i.e. entry and expression. In the present study, pENTR TOPO/D and pDEST26 were entry and expression vectors, respectively. PCR product, FVII cDNA, was directly TOPO cloned into pENTR TOPO/D vector during 5 minutes with 100% efficiency, and with no ligase, post-PCR procedures, or restriction enzymes required. pDEST26 vector was used as expression vector. During LR recombination between pENTR TOPO/D-FVII and pDEST26, FVII gene was inserted into the destination vector resulted in construction of pDEST26-FVII which was in frame with appropriate his-tag as determined by DNA sequencing. CHO cell line was used as a host for expression of recombinant FVII protein. Kemball-Cook et al also expressed recombinant FVII in CHO cell line. However, they used different vector, pNeoIG502, compared to ours i.e. pDEST26.

For transfection of the construct into CHO cells they utilized electroporation while in the present study FuGENE HD transfection reagent was used[18]. Simple methodology, minimal optimization, low cytotoxicity and ability to provide high transfection efficiency even in the presence of serum are advantages of FuGENE HD transfection reagent. Expression of recombinant rabbit FVII has been reported by Ruiz et al. They utilized pCMV5 for cloning and expression of rabbit recombinant FVII in human 293 cell line[19]. Rabbit rFVII was purified using a variation of the barium citrate precipitation technique followed by DEAE sepharose FF and benzamidine agarose column chromatography [19,20]. Commercially available activated recombinant human coagulation factor VII (rFVIIa*)* or NovoSeven is produced in baby hamster kidney (BHK) cells and is purified by consecutive chromatography steps, including immunoaffinity chromatography using murine monoclonal antibodies.

In our study, the rFVII encoded by pDEST26 carries six histidine residues at its N- terminus. Polyhistidine has high affinity for a nickel-nitrilotriacetic acid resin permitting single-step and fast (in about 1.5 hr) purification of recombinant FVII protein. This recombinant FVII protein was detected as a single band in SDS-PAGE and western blot analysis indicating high purity of the protein, obviating additional purification steps.

Considering the fact that FVII is a secretion glycoprotein and its signal peptide recognition by SRP is essential for entering into the ER lumen, this question might raise that how his tag remains in fusion protein? In other words, is the signal peptide removed? Several hypothesis can be suggested to address this question including 1, The fusion protein unspecifically enters into ER which means without the involvement of SRP, 2, the protein is recognized by SRP and enters into ER but signal peptide is not removed by signal peptidase and 3, some part of protein enters into ER normally in which signal peptide is removed and others go through either the first or second mentioned pathways. However, further and complementary studies are required to clarify the precise mechanism in this regard. The results clearly confirm the existence of the his-FVII fusion protein in the cell culture medium as determined following elution using Ni^{++} column, SDS-PAGE and western blot analysis using both anti-his and anti-FVII antibodies. It is noteworthy that any rFVII protein was not detected in the culture medium of CHO cells producing his-tag free rFVII by expression of a pcDNA3.1 vector following passage of the medium through Ni^{++} column (data not shown), which proves the specific elution of His-FVII. However, Intracellular accumulation of His-FVII expressed by pDEST26 vector was much more higher compared to his tag free recombinant FVII expressed by pcDNA 3.1. This indicates that the fusion of FVII to his-tag might impair its secretion.

Finally, a three-fold decrease in clotting time induced by rFVII was observed, indicating that the rFVII produced in this study was biologically active and its function was not affected by fusion. However, because of the cultivation of CHO cells in serum containing medium, the contamination of the purified FVII protein with FBS is possible. Thus, in later clinical studies and uses the application of serum-free mediums should be considered to overcome this problem.

Conclusion

As we are aware, this is the first report of expression of a recombinant FVII fused with his-tag through gateway technology. Our results revealed that the recombinant hybrid protein was biologically active. Simple methodology, minimal optimization, high efficiency, low costs and fast procedure might be the advantages of our study. The next steps including large scale expression, purification, activation and stabilization are underway.

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Corresponding author: Mehryar Habibi Roudkenar
Research Center, Iranian Blood Transfusion Organization
Tehran, Iran
E-mail: Roudkenar@ibto.ir