

Research Article

Expression of Recombinant Human Coagulation Factor VII by the Lizard *Leishmania* Expression System

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The variety of recombinant protein expression systems have been developed as a resource of FVII gene expression. In the current study, the authors used a novel protein expression system based on the Iranian Lizard *Leishmania*, a trypanosomatid protozoan as a host for expression of FVII. Plasmid containing cDNA encoding full-length human FVII was introduced into Lizard *Leishmania* and positive transfectants were analyzed by SDS-PAGE and Western blot analysis. Furthermore, biological activity of purified protein was detected by PT assay. The recombinant strain harboring a construct was analyzed for expression of FVII at the mRNA and protein level. Purified rFVII was obtained and in order to confirm the purified compound was in fact rFVII. Western blot analysis was carried out. Clotting time in PT assay was reduced about 30 seconds with the purified rFVII. In Conclusion, this study has demonstrated, for the first time, that *Leishmania* cells can be used as an expression system for producing recombinant FVII.

1. Introduction

FVII is a vitamin-K-dependent serine protease synthesized in the liver and plays an important role in blood coagulation [1]. The gene for human FVII (NG_009262.1) is located on chromosome 13 (13q34); it has approximately 14 kb and consists of nine exons and eight introns. Alternative splicing patterns of the gene mRNA transcripts result in 2 coagulation factor VII isoforms, “a” and “b” precursors. Isoform “b” (NM_019616.2) is the prevailing form in normal liver; it does not include exon 1b and thus encodes a shorter signal peptide than isoform “a” (NM_000131.3). The mature protein encoded by the two transcript variants is identical with a single-chain peptide with 406 amino acid residues and molecular mass of approximately 50 kDa [2].

Upon activation, FVII is converted to the protease FVIIa, and a peptide bond between Arg152 and Ile 153 is separated.

So, the light chain, consisting of a γ -carboxyglutamic-acid containing (Gla) domain and two epidermal growth-factor-like (EGF) domains, is covalently connected to the heavy chain (the serine protease domain) through a single disulfide bond [3] (Figure 1).

Based on the understanding of the role of activated FVII in the treatment of the coagulation disorders, synthesis of recombinant activated FVII (rFVIIa) was considered. A review of application of rFVIIa (Novo Seven) over the past two decades indicates that initially this compound was approved for use in patients with congenital or acquired hemophilia who produce inhibiting antibodies toward factor VIII or IX. By this approach, high concentrations of FVIIa, which are achieved by pharmacological doses of rFVIIa, bind to preactivated platelets. Through this approach, it can bypass the need for VIIIa and IXa as a result of activation of FX to FXa. This would result in an increased generation

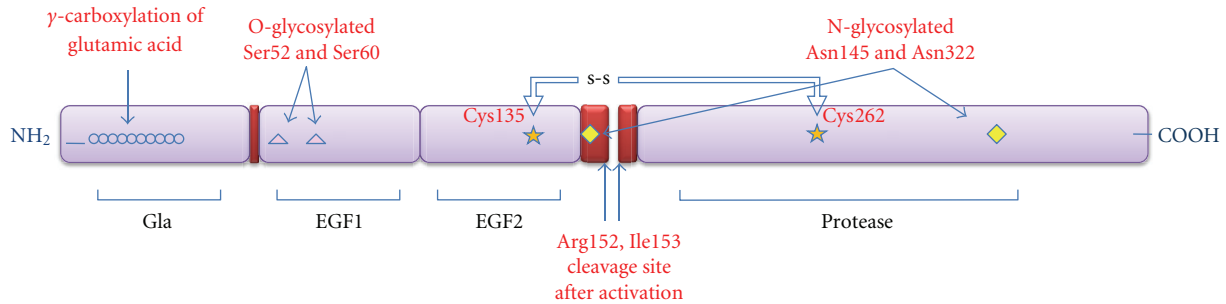


FIGURE 1: Human coagulation factor VII. FVII is a serine protease of 406 residues. Different domains of FVII are shown. Posttranslational modifications of the FVII molecule include (1) γ -carboxylation of 10 glutamic acid residues in the N-terminal part of the molecule, (2) N-glycosylation of asparagine residues in positions 145 and 322, and (3) O-glycosylation of serine residues 52 and 60. \circ , gamma-carboxyglutamic acid; \triangle , O-glycosylation sites; \diamond , N-glycosylation sites; Arg152 and Ile153, cleavage site generated after activation; \star , joining of two chains of FVIIa by a single disulfide bond between cys135 and cys262.

of thrombin which produces a tight, well-structured fibrin haemostatic plug, which is resistant to fibrinolysis [4–6].

Also, it is reported in the literature that the recombinant factor VIIa has been used effectively in patients with other categories of coagulation defects or patients with normal coagulation system who, for instance, experience excessive bleeding, as a result of trauma or surgery. However, it should be mentioned that adverse events such as arterial thromboembolism have been reported among patients who received rFVIIa, particularly among those who were 65 years of age or older [7–9].

Initially, BHK cells and subsequently the variety of recombinant protein expression systems such as insect and mammalian cells have been developed as a resource of FVII gene expression [4, 10, 11].

Using mammalian systems has a number of drawbacks: (a) high price of cell-culture media, (b) possibility of contamination of products with viruses and prions depending on the components of animal origin, and (c) high purification cost [12, 13].

Recently, a novel protein expression system was introduced which was based on the nonpathogenic trypanosomatid flagellate *Leishmania tarentolae*, a protozoan parasite of lizards [14].

The main advantages of *Leishmania* expression system are as follows:

- (1) easy to handle like *E. coli* and yeast expression systems,
- (2) full eukaryotic protein folding,
- (3) The mammalian-type posttranslational modification of target proteins and cultivation in low-cost media,
- (4) high specific growth rate in comparison to mammalian cells [15, 16].

In the present study, attempt is made to express heterologous recombinant human FVII (rFVII) by the Iranian Lizard *Leishmania* (I.L.L.) Expression System [17].

2. Materials and Methods

2.1. Cell Line, Hosts, and Plasmid. Human hepatoma HepG2 cell line [18] was obtained from the National Cell Bank of Iran (ncbi code: c158). It was grown in RPMI₁₆₄₀ (Gibco, 52400; U.K.) medium at 37°C in 5% CO₂ supplemented with (a) 10% fetal bovine serum, (b) 1 μ g/mL vitamin K1, (c) 100 u/mL penicillin and (d) 100 μ g/mL streptomycin (GIBCO, Pen-Strep,15140). The *E. coli* Top10 strain, the bacterial host, was cultured in LB broth medium. pLEXY-hyg2 plasmid was prepared from Jena Bioscience company of Germany.

2.2. Preparation of FVII cDNA. Total RNA was extracted using a commercial kit (QIAGEN RNeasy Mini Kit, Germany) from the hepG2 cell line. The synthesis of the FVII encoding cDNA was performed by using SuperScript III reverse transcriptase (Invitrogen, USA). RT reaction was carried out at 25°C for 10 min, 55°C for 50 mins and 85°C for 5 min using random hexamer.

The PCR program consisted of an initial denaturation at 94°C for 5 minutes. A total of 35 cycles was carried out comprising of the following steps: 94°C for 30 s, 60°C for 60 s, 72°C for 60 s, followed by 72°C for 5 min as a final extension using the forward primer 5'CTCG-AGATGGTCTCCCAGGCCCTCAG3' and reverse primer 5'GCGGCCGCCTAGGGAAATGGGGCTCGCAG3'.

2.3. Plasmid Construction and Confirmation of the Prepared Construct. The purified PCR product of transcript isoform “a” was ligated to pTZ57R/T vector [19] and transformed in *E. coli* TOP10 strain competent cell as described previously [20]. The presence and accuracy of the insert was confirmed by restriction analysis and heminested PCR using two pairs of internal primers, forward primer 5'ATGGTCTCCCAGGCCCTCAG3' and reverse primer 5'AGATGGTGTGATCAGGGTCC3' flanking a 700 bp fragment inside the gene, and final verification was carried out using DNA sequencing.

The cloned FVII gene was digested by NotI and SmaI restriction enzymes (Fermentas, Lithuania) and cloned into pLEXY-hyg2 plasmid (EGE-232, Jena Bioscience, Jena,

Germany). The presence and correct orientation of the insert was analyzed by PCR and NotI and SmaI restriction enzymes.

2.4. Transfection and Cultivation Condition of Lizard *Leishmania*. I.L.L. [17] was cultivated in RPMI1640 (Gibco-BRL, Eggenstein, Germany) medium supplemented with 10% fetal bovine serum, 100 u/mL penicillin and 100 µg/mL streptomycin (GIBCO, Pen-Strep, 15140), at 25°C, 15 µg/mL haemin (Sigma, St. Louis, Mo, USA). Plasmid containing cDNA encoding human FVII was linearized with *Sma*I (BIORON, Germany) [14] and used for transfection of the I.L.L. by electroporation in 4 mm cuvettes using an electroporator (Bio-Rad Gene Pulser) [21]. After two pulses, stable transfectants were selected on solidified RPMI media containing 25 µg/mL hygromycin B (Sigma, St. Louis, Mo, USA) [22]. For transcription and expression assays, recombinant I.L.L were cultured in RPMI-based suspension supplemented with haemin (5 µg/mL), penicillin (50 U/mL), and streptomycin (50 µg/mL) at 26°C in the absence of light and under shaking condition (140 rpm) [22, 23].

2.5. Expression and Purification of Recombinant FVII. The expression of rFVII was assayed by RT-PCR reaction on extracted total RNA of approximately 10⁸ recombinant *Leishmania* cells. Amplifications of 1400 bp of the FVII fragment were carried out by specific primers. Furthermore, the expression of rFVII protein in *Leishmania* was confirmed by SDS-PAGE and western blotting procedure.

To select positive transfectants of I.L.L., cells were harvested by centrifugation at 3000 rpm for 15 min and washed in PBS. The pellets were immediately lysed in 2x SDS-PAGE sample buffer (100 mM Tris-HCl pH 8, 20% glycerol, 4% SDS, 2% beta mercaptoethanol, 0.2% bromophenol blue) then boiled for 5 minutes. Samples were loaded on a 12% SDS-PAGE. The gel was stained by Coomassie brilliant blue R250, and gene expression was analyzed. The same procedures were carried out on the control sample in parallel for comparison.

For Western blot analysis, cell lysate was separated on 12% SDS-PAGE and electrophoretically transferred onto pvdf membrane. After UV crosslinking for protein fixation, the membrane was blocked with 3% BSA at room temperature. The specific factor VII polyclonal antibody, HRP-conjugated (US Biological, F0015-20B) was used in 1 : 500 dilutions. The protein band was detected by DAB (Diaminobenzoic Acid) and H₂O₂.

After selecting the appropriate clone and cell growth, cell pellet from 10 mL shake flask culture was obtained with centrifuge in 3000 rpm for 10 min and then resuspended in 5 mM Tris-HCl, pH 8.2. The suspension was immediately sonicated twice for 20 s. Then, cell lysate was applied to an affinity column containing specific factor VII polyclonal antibody coupled to CNBr-activated sepharose 4B resin (Amersham Biosciences, Sweden). According to the manufacturer's instructions, rFVII was eluted with the changes in pH and by using 0.1 M glycine, with the final pH of 2.8. Then, acetone precipitation of adsorbed rFVII was performed. The precipitated product was resuspended in 1x TBS, and dot blot analysis was carried out by spotting the suspension

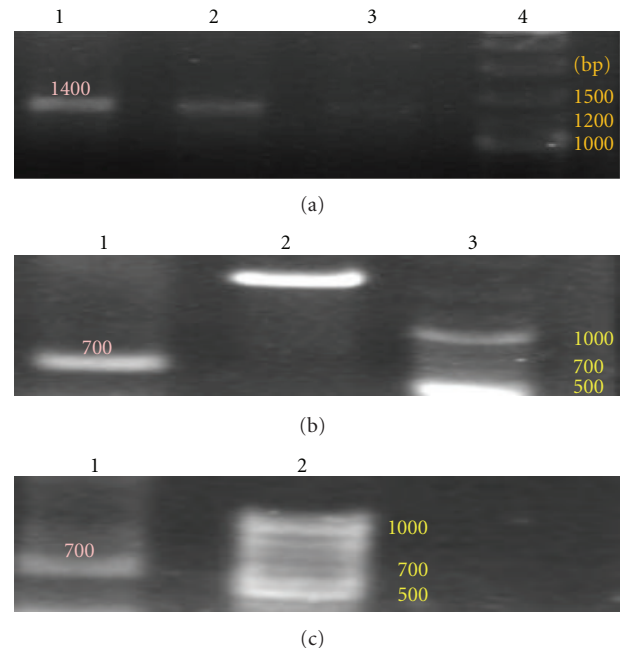


FIGURE 2: (a) FVII-PCR product, Lanes (1, 2, 3), 100 bp DNA ladder (4) (b) Restriction analysis of PCR product. Lane 1: digestion with *Hph*I that revealed a 700 bp fragment, Lane 2: PCR product without enzyme effect. (c) Lane 1: 700-bp PCR product obtained by heminested PCR.

onto a pvdf membrane. The specific factor VII polyclonal antibody was allowed to bind, and positive-colored spot was detected.

Subsequently, the glycine buffer composition of eluted rFVII was exchanged by 0.1 M imidazole, NaCl 0.2 M, pH 7.3 buffer through the dialysis.

After Dialysis, soluble protein was further characterized by SDS-PAGE and western blot analysis, which was performed as mentioned above. Also, purified rFVII concentration was determined spectrophotometrically by Biophotometer (Eppendorf, Germany) at 280 nm.

2.6. Analysis of Purified rFVII. For determination of coagulant activity of purified rFVII, clotting times of the dialyzed rFVII were obtained by a one stage clotting assay (PT-based assay for FVII). Hence, PT assay was performed based on human plasma immunodepleted of factor VII (Diagnostica Stago, France) in combination of purified rFVII and human thromboplastin-D (Thermo Scientific, USA). The coagulation was initiated by addition of PT reagent, and the coagulation time was recorded and compared with that of FVII (FVIIa, US Biological), as a positive control and purified sample obtained from untransfected I.L.L. (negative control), using fibrin timer cups and a semiautomated coagulation analyzer (Option 4 plus Biomerieux, Germany).

A standard curve was made, by using dilutions of (1/1), (1/2), (1/5), (1/10), (1/20), (1/40) of Unicalibrator (Stago, Ref. 00625) that related to defined 100%, 50%, 20%, 10%, 5%, and 2.5% of FVIIa levels, respectively. Also, several dilutions of dialyzed protein were made. An aliquot (100 µL)

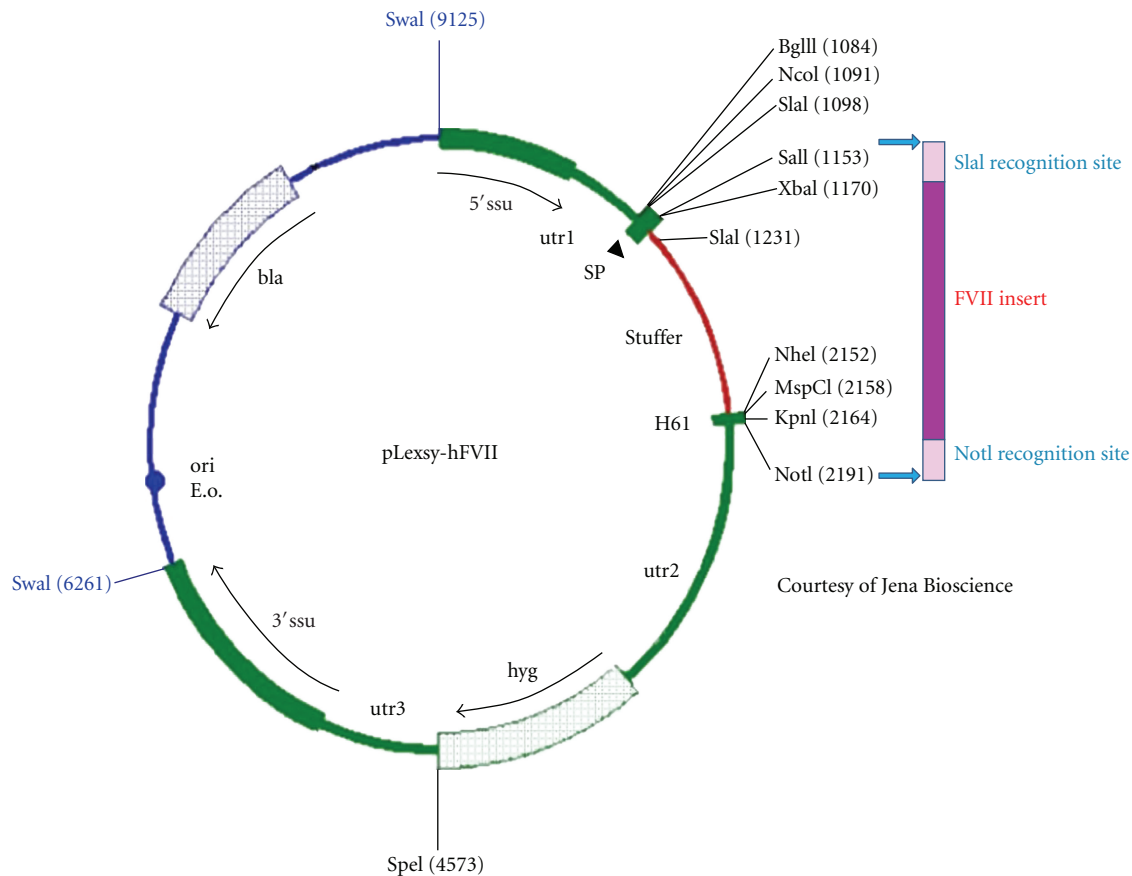


FIGURE 3: Construction of expression plasmid containing human FVII (pLexsy-hFVII). Map of pLEXSY-2 vector and the strategy used for plasmid construction are shown. The cDNA sequence encoding human FVII was inserted in corresponding restriction sites SmaI and NotI. More information about the vector itself can be found on <http://www.jenabioscience.com/>, pLEXSY-hyg2 (EGE-232).

of each diluted sample was added to 100 μ L of human plasma immunodepleted of factor VII; 200 μ L of thromboplastin was added and the clotting time was recorded immediately [24]. All determinations were carried out in duplicate and were compared to the standard curve. Hence, specific activity of the purified rFVII was determined.

3. Results

3.1. FVII Fragments Preparation and Its Confirmation. Specific primers were used for the isolation of full-length rFVII, and the amplicon with expected size (about 1400-bp) was produced (Figure 2(a)). In order to confirm the correctness of the amplicon, purified PCR product was digested with *HphI* which resulted in two fragments of 700-bp (Figure 2(b)).

3.2. Construction of the Recombinant pLEXSY-hyg2-FVII Plasmid. To generate pTZ57r-FVII, the PCR product was cloned into pTZ57r vector. The accuracy of the gene was determined by heminested PCR approach which yielded the specific 700-bp amplified product (Figure 2(c)), and by *KpnI* enzyme digestion which revealed a 1200-bp fragment. Additional confirmation was obtained by DNA sequencing.

Subsequently, the resultant pTZ57r-FVII was digested with SmaI and NotI restriction enzymes. In order to generate pLexsy-hFVII construct, the correct fragment was inserted into the corresponding restriction sites of pLEXSY-hyg2 expression vector (Figure 3).

3.3. Expression and Purification of Recombinant Human FVII in I.L.L. Cells. Approximately 5 μ g pLEXSY-hyg2-FVII plasmid was linearized by SwaI restriction enzyme (Figure 4(a)). It was transfected into I.L.L. cells by electroporation, and stable transfectants were selected by culturing cells on solid medium with hygromycin. Subsequently, two to three passages in liquid medium containing hygromycin were performed, and the recombinant promastigotes harboring a construct was analyzed for expression of rFVII.

In order to verify rFVII mRNA synthesis in the host, RT-PCR was performed on total RNA extracted from transformed cells using FVII primers. The desired band with the expected size of 1400 bp was obtained (Figure 4(b)). Furthermore, the expression of rFVII in Leishmania cells was determined by SDS-PAGE, and western blot analyses. Expressed protein was run onto 12% (V/V) SDS-PAGE and the protein band about 55 KD was detected which indicates protein expression of rFVII as shown in Figure 5(a).

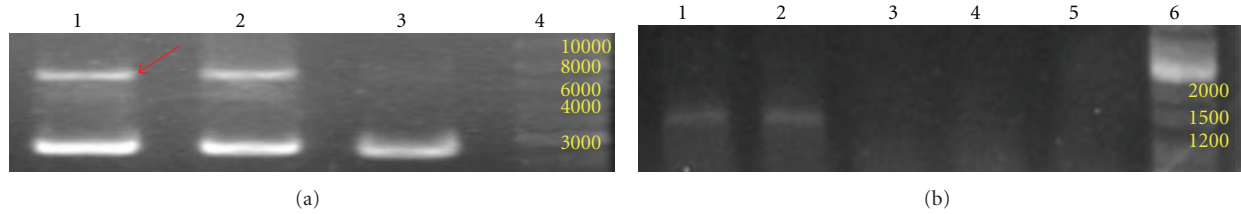


FIGURE 4: (a) Linearized pLEXSY-hyg2-FVII construct with *Swa*I (Lanes 1, 2), control plasmid (Lane 3). The arrow head shows a 6500-bp fragment containing cDNA sequence encoding human FVII, hygromycin B resistance gene, 5' and 3' portion of the small subunit of *L. tarentolae* RNA gene (5' and 3' ssu) and three UTRs: utr1, utr2, and utr3. (b) Confirmation of FVII expression by transfected *Leishmania* cells Lanes (1, 2), Control cells (Lanes 3, 4), Control plasmid (Lane 5).

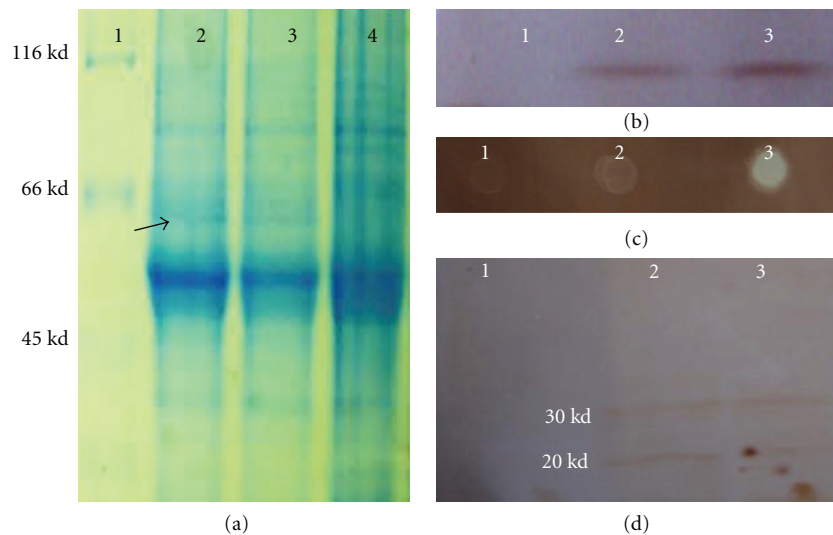


FIGURE 5: Analysis of expression of the human FVII by SDS-PAGE and western blotting and detection of purified rFVII by dot blot and western blot. (a) FVII protein band on 12% SDS-PAGE from transfected (Lanes 2, 3) and untransfected I.L.L. as a negative control (Lane 4). (b). expressed FVII protein band in transfected I.L.L. (Lane 2) and negative control untransfected cells (Lane 1), commercially available rFVIIa as a positive control (Lane 3). (c) Positive control sample rFVIIa (Lane 1), purified rFVII appears as a brown colored spot (Lane 2), purified product of negative control untransfected cells. (d) the purified rFVII from transfected I.L.L. (Lane 2, 3) was analyzed by western blot. Two chains of activated form of rFVII with corresponding length are shown; Lane 1, untransfected I.L.L.

The protein identity was verified by western blot analysis. The assay revealed specifically recognition of rFVII coding gene by human FVII antibody (a sheep polyclonal antibody to human FVII, IgG HRP-conjugated). The results were compared with the positive control sample FVII (FVIIa, US Biological), and no reactivity was observed with negative control cells (Figure 5(b)).

Purification of rFVII was achieved using affinity column and by passing a cell lysate of *Leishmania* over a CNBr-activated sepharose 4B resin coupled to specific factor VII polyclonal antibody. The presence of purified rFVII was confirmed by visualizing a brown spot in a dot blot membrane against specific polyclonal antibody (Figure 5(c)).

Furthermore, western blot analysis of the purified rFVII revealed that, during purification, it was converted into the two-chain-activated form (Figure 5(d)).

The resulting two chains were light and heavy chains with a molecular weight of ~20 kDa and ~30 kDa, respectively. The purified rFVII concentration was estimated to be about 10 μ g per 10 mL of culture medium.

Dialyzed rFVII was added to human plasma immunodepleted of factor VII, and coagulation times were determined. The biological activity of dialyzed rFVII from I.L.L. in comparison with FVII (FVIIa, US Biological) was performed. In the initial experiments, a reduction in clotting time of about (33 ± 3) seconds with the purified rFVII was observed (Figure 6).

Also, comparison between different dilutions of dialyzed rFVII in a similar assay with standard curve indicated that dialyzed rFVII (rFVIIa) at dilution of (1/1) retained approximately 9% of the activity of Unicalibrator with 100% FVIIa level (Figure 7).

4. Discussion

In recent years, there has been increasing interest in the use of recombinant activated factor VII (rFVIIa) as an adjunct to the coagulation process. rFVIIa is a useful treatment option for patients with congenital or acquired hemophilia. The findings represent significant advancement

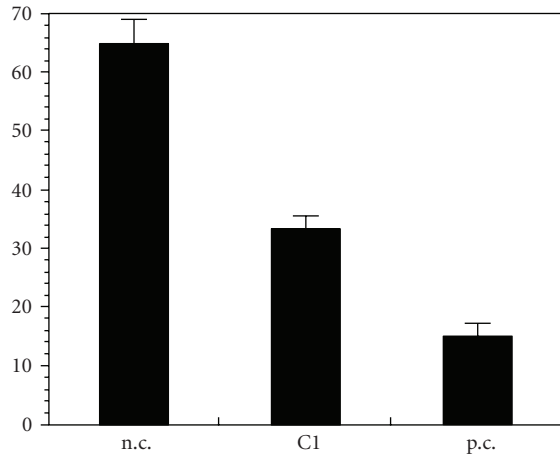


FIGURE 6: Prothrombin time assay. PT assay was performed for determination of coagulant activity of dialyzed rFVII. Coagulation time measured for dialyzed rFVII produced from transfected I.L.L. (Cl: clone.1), commercially available rFVIIa as a positive control (p.c.), purified sample obtained from untransfected I.L.L. as a negative control (n.c.). A reduction in clotting time of about (33 ± 3) seconds with the purified rFVII was observed. Data are shown as mean \pm SD.

in the management of patients who produce antibodies against FVIII or FIX [7].

Initially, BHK cells and subsequently the variety of recombinant protein expression systems such as insect and other mammalian cells have been developed as a resource of FVII gene expression [4, 10, 11]. However, in this study, leishmania expression system was used as a host cell.

I.L.L. cells were transfected with the vector pLexsy-hFVII, which encoded a full-length recombinant human FVII of 406 amino acids, and was integrated properly between 5' and 3' portion of the small subunit of rRNA gene and powerfully transcribed by RNA polymerase I of Leishmania cells. Interestingly, gene regulation, in Trypanosomatidae, occurs predominantly after transcription through intergenic untranslated regions (UTRs) [25]. Therefore, suitable UTRs should be chosen. The origins of UTRs of pLEXSY-hyg2 expression vector are as follows: UTR1, 5' nontranslated region of aprt gene of Leishmania with splice acceptor site for target gene; UTR2, 1.4 kb intergenic region from cam operon of Leishmania with polyA site for target gene and splice acceptor site for marker gene; UTR3, 5' UTR of dhfr-ts gene of Leishmania with polyA site for marker gene. Results of this study are also in agreement with earlier observations that Leishmania species are able to produce heterologous proteins.

By using native human signal sequence, Breitling et al. [14] could obtain the clone with the maximum expression-produced recombinant erythropoietin at approximately 30 mg/L of suspension culture.

In this study, the final yield of the purified rFVII was estimated to be about $1 \mu\text{g}/\text{mL}$ of culture medium. This yield was comparable to that of rFVII-producing mammalian and insect cell lines [10, 26, 27]. Furthermore, it has been demonstrated that *Leishmania tarentolae* is able to produce

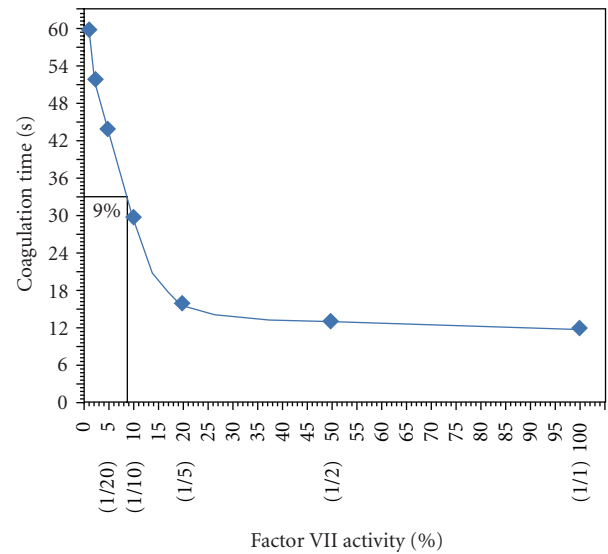


FIGURE 7: Estimation of the yield of functional rFVII (rFVIIa) by one-stage clotting assay (PT-based assay for FVII). Standard curve of different dilutions of unicalibrator was made. Also, clotting times of dilutions of (1/1), (1/2), (1/5), (1/10) of dialyzed rFVII were recorded. The clotting times of dilutions of dialyzed rFVII were 33, 42, 44, and 48 (s), respectively. The black line mark the (1/1) dilution of dialyzed rFVII activity at the point of intersection of the standard curve through this the yield of functional rFVII (rFVIIa) was estimated 9% of Unicalibrator with 100% FVIIa level. The clotting time of the negative control sample (without FVII) was 60 (s).

mammalian-like biantennary N-glycans [28]. So, according to the estimated molecular weight of rFVII produced by Leishmania cells and its comparison with carefully selected positive control of FVII (FVIIa, US Biological), it is thought that N-glycosylation profile and probably the protein folding of this product are similar to those in mammalian cells [29].

Before purification of rFVIIa, electrophoretic and western blot analysis indicated that it consisted of a single band with an estimated molecular weight of 55 kDa on SDS-PAGE. It indicates that rFVII has been synthesized as a single-chain form and then cleaved to the biologically active form during protein purification. Western blot analysis indicated that the resultant active form is composed of light and heavy chains with a molecular weight of ~ 20 kDa and ~ 30 kDa, respectively [30].

The results of PT assay showed a reduction in clotting time of about 30 seconds with the purified rFVII. As demonstrated by comparing clotting time of the dialyzed rFVII with standard curve, the yield of functional rFVII (rFVIIa) was estimated 9%. The specific findings with this product may be related to its posttranslational modification like γ -carboxylation. And it should be noted that considerably more investigations are required in order to determine posttranslational γ -carboxylation of glutamic acid residues in Gla domain of this product in Leishmania cells.

In conclusion, this study has demonstrated for the first time that Leishmania cells can be used as a powerful host for production of recombinant human FVII. The main

advantages of this expression system are its easy handling like *E. coli* and yeast expression systems, proper protein folding, posttranslation modification and cultivation in low-cost media such as LB.

Acknowledgments

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