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Original Research Article

Amidolytic Activity of Factor VII Expressed in Iranian Lizard *Leishmania*

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HIGHLIGHTS

- Factor VII has important role in the treatment of coagulation and hemophilic disease.
- Recombinant FVII was expressed in Iranian Lizard *Leishmania*.
- The amidolytic activity of rhFVII was about 0.0125 IU/ml in cell pellet.
- No amildolytic activity was observed in the supernatant.

ABSTRACT

Over many years, variable gene expression systems have been used obtaining of Factor VII protein, but each one has certain limitations. Therefore, the main goal of this study was to assess the biological activity of purified and recombinant factor VII expressed in Iranian lizard *Leishmania*. After transferring recombinant construct containing FVII gene to *Leishmania*, first, the expression of 55 kDa FVII protein in transfected cells was confirmed by analyzing cell lysate using SDS-PAGE and Western Blotting techniques. Then, FVII was purified by NI-NTA-His Tag- resin through an affinity chromatography. The chromogenic activity of human recombinant Factor VII (amidolytic activity) in supernatant and pellet fractions of *Leishmania* promastigotes was done using ELISA method. The amidolytic activity of rhFVII was about 0.0125 IU/ml in the concentrated cell sediment and 0 IU/ml in the supernatant in the first 60 minutes and after that time, none of the samples showed acceptable activity.

Introduction

Factor VII is a plasma glycoprotein that plays an important role in the production of blood clotting fibrin (Davie et al., 1979). Factor VII in active form and the presence of calcium and TF, activates factor X and IX (Persson et al., 1997; Hoffman et al., 1998). Conversion of factor VII was activated by digestion between amino acid arginine 152 and isoleucine 153 (Chao-Hung et al., 2006). Factor VII, due to its important role in the treatment of coagulation and hemophilic disease, is of considerable interest (Hedner and Kisiel, 1983). So far, FVII gene was expressed in eukaryotic cells like BHK, insects and mammalian cells (Hagen et al., 1988; Masroori et al., 2010). The *Leishmania tarantulae* expression system has been considered according to following advantages; a) easy to work with organisms like yeast and *E. coli*, b) have the same folding as protein synthesis by eukaryotic systems, c) post-translational modifications similar to changes that occur in the mammalian cells, d) cells

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proliferation in cultured media are cheap, e) in contrast to the mammalian cell, the cell has a higher specific growth rate (Fritsche et al., 2007; Fritsche et al., 2008). These benefits led to the production of recombinant FVII in Iranian Lizard *Leishmania* (Mirzaahmadi et al., 2011). In this study, a similar production system was run and the chromogenic activity of human factor VII produced in *Leishmania tarantulae* was examined.

Materials and Methods

Production of protein (rFVII)

Human factor VII recombinant Plasmid pLEXY-hyg2 was gifted from Mirzaahmadi (Mirzaahmadi et al., 2011). Recombinant plasmid pLEXsY-hyg2 was digested by SwaI enzyme (Bioneer Germany). the transfection into the promastigotes of Iranian Lizard *Leishmania* was performed by electroporation (Eppendorf's GEN PLUSES) (Robinson and Beverley, 2003). Transfected promastigotes were cultured in RPMI1640 and BHI medium supplemented with FBS 10%, 100 mg/ml streptomycin (GIBCO, Pen-strep 15140) at 37°C. Transfected *Leishmania* was confirmed by PCR reaction using the primers: forward primer (F3001): 5'-GATCTGGTTGATTCTGCCAGTAG-3' (rDNA of *Leishmania* genome) and reverse primer (B2): 5'-AGATGGTGTTGATCAGGGTCC-3' (from 764 t0 784 bp of FVII gene). Expressed rhFVII in Iranian Lizard *Leishmania* was electrophoresed on SDS-PAGE and stained by coomassie brilliant blue R250. Gel transferred onto nitrocellulose membrane and FVII was confirmed by western blot analysis using a polyclonal antibody against FVII.

Protein (rFVII) purification

Transgenic *Leishmania* promastigotes were cultured in 10 ml of RPMI1640 and centrifuged at 500 rpm for 5 minutes and the supernatant was separated from cell pellet. Precipitate dissolved in lysis buffer containing 1mM PMSF. The Suspension was obtained by sonication (3 times with 20 seconds bursts), then, recombinant factor VII was purified by affinity chromatography using Ni-NTA His Tag Resin (Novagen).

The chromogenic activity of rFVII

Recombinant FVII activity was assayed by human FVII chromogenic activity assay kit (based on the indirect amidolytic activity). The specific hrFVII polyclonal antibody that was coated at the bottom of a microplate, connect to FVII. In this experiment, the amount of lipoprotein TF/FVIIa activating the FX was measured using an active substrate, which could produce a yellow color (from Para Nitro Alanine (PNA)) in the presence of enzyme. PNA changes in 405 nm shows enzymatic activity, directly.

Human plasma as a positive control and PBS as a negative control were considered. 100 µl of standards were added into the separated wells. Then, 100 µl of concentrated culture supernatant, concentrated cell sediments, and cell sediment were added to the other separated wells and incubated for 2 h at room temperature. Then, the microplate was washed with Wash Buffer for 5 times. 80 µl of Assay Mix was added to each well and incubated for 30 minutes at 37°C and after that, 20 µl of FXa substrate was added to each well and the absorbance was read immediately at 405 nm. It was also studied after incubated at 37°C again for, 10 seconds, 25, 60 minutes.

Results and Discussion

Accuracy of recombinant pLEXSY-hyg2-FVII

To confirm the accuracy of the recombinant plasmid containing factor VII gene, PCR reaction was done using these primers: forward primer (F3001): 5'-GATCTGGTTGATTCTGCCAGTAG-3' and reverse primer (B2): 5'-AGATGGTGTTGATCAGGGTCC-3' and 980 bp PCR product of FVII gene and *Leishmania* rDNA gene was confirmed by agarose gel electrophoresis (Fig. 1).

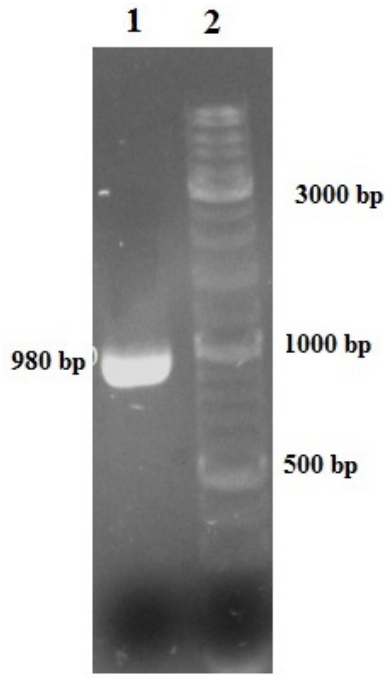

Figure 1. Agarose gel electrophoresis of pLEXSY-hyg2-FVII: lane 1: 980 bp as PCR product. Lane 2: the 100 bp DNA ladder marker.

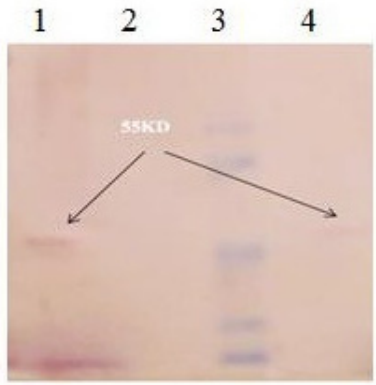

Figure 2. Western blot analysis. Lane 1 and 4: produced rhFVII protein, lane 2: empty. Lane 3: protein weight marker.

Expression of rhFVII by Iranian lizard Leishmania

Because N-glycosylation pattern and post-translational modification of *Leishmania* are similar to mammals (Niimi, 2012; Khan et al., 2017), we use this system for FVII expression.

Expression of rhFVII by *Leishmania* was confirmed by SDS-PAGE and western blot. *Leishmania* promastigote was lysed and loaded on 10% SDS-PAGE. The gel was transferred onto the nitrocellulose membrane and rhFVII was detected by polyclonal antibody against factor VII (Fig. 2).

Verification of the purified product was also performed. *Leishmania* lysate passed through a column chromatography of Ni-NTA-His Tag resin. The resin was loaded on the SDS-PAGE gel and after the electrophoresis, the gel was transferred to the nitrocellulose membrane for western blot analysis using a polyclonal antibody against FVII (Fig.3).

Measurement of rhFVII activity by chromogenic activity

Factor VII Human Chromogenic Activity Assay Kit (ab108830) has been developed to determine human FVII activity in plasma, serum and cell culture supernatants. The assay couples immuno-functional and indirect amidolytic assay (Abcam catalog for Factor VII Human Chromogenic Activity Assay Kit, ab108830). Osterud in 1983 was studied how to measure FVII and FVIIa activities by using a direct method for FVIIa activity measurement. For FVII coagulant activity (FVIIc) coincides three methods considering, 1) quantification of the FVII antigen (FVII Ag), 2) assaying amidolytic activity (FVII am), 3) coagulation tests using bovine tissue thromboplastin (VII bt) (Østerud, 1983).

In the present study, the rhFVII was expressed in Iranian Lizard *Leishmania* and was auto activated during purification (Pedersen et al., 1989). FVII amidolytic activity was measured by kit (ab108830).

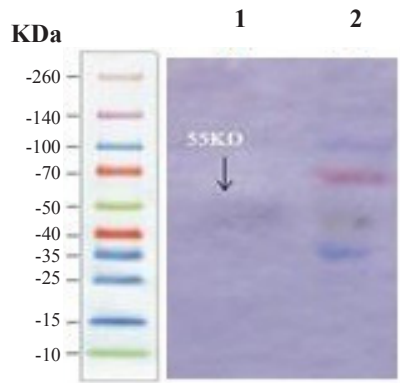

Figure 3. Western blot analysis of purified protein; Lane 1: western blot of purified rhFVII. Lane 2: proteins size marker.

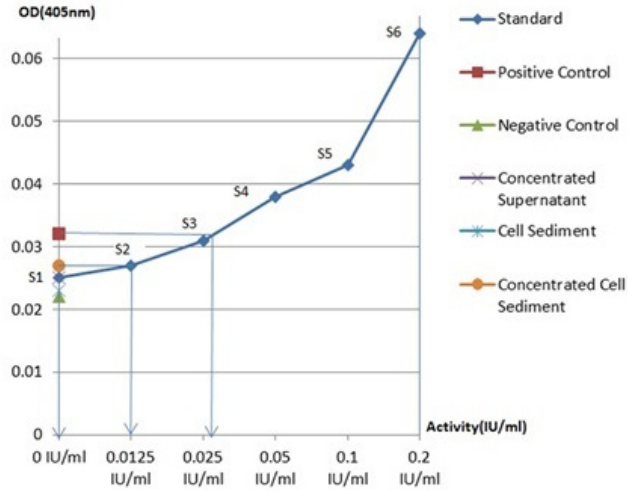

Figure 4. The rhFVII chromogenic activity measured at 405 nm.

The chromogenic activity of rhFVII was 0.0125 IU/ml in concentrated cell sediment and 0 IU/ml in the concentrated supernatant in the first 60 minutes and after 60 minutes any of the samples had not acceptable activity (Fig. 4). Based on the kit manual (ab108830), the standard dilutions (1/8), (1/16), (1/32) (1/64), (1/128), (1/256) of stock solution shows the 0.2, 0.1, 0.05, 0.025, 0.0125, 0.000 IU/ml activity of FVII.

Conclusion

Upon vascular injury and tissue damage, the tissue factor (TF) and cell surface receptor binds together and activates FVII to FVIIa. The TF/FVIIa complex catalyzes the conversion of both factors IX to factor IXa and factor X to factor Xa to initiate coagulation via the extrinsic pathway (Seligsohn et al., 1978; van Dieijen-Visser et al., 1982). Very low levels of FVII are associated with severe coagulation disorders and elevation of factor VII in middle-aged subjected to an independent risk factor for subsequent ischemic heart disease (Hoffman et al., 1989; Suzuki et al., 1991).

Basile *et al.* proposed the production of recombinant proteins in *Leishmania tarantulae* in 2009. The *Leishmania* can successfully produce glycoproteins with 10% fructose and galactose as oligosaccharides, which are similar to mammals. The *Leishmania tarantulae* expression system was proposed as a eukaryotic expression for heterologous proteins (Basile and Peticca, 2009). Sodoyer in 2004 used BHK, HEK293, and CHO for expression of rhFVII. The rhFVII was also expressed in insect cells, SF9 (Spodoptera Frugipedra) and also, *Baculovirus*. Mammalian cell expression systems has several limitations, such as high-cost medium, the possibility of contamination with viruses or prions, and the high cost of purification. In insect cells as a model, the addition of complexes oligosaccharides in post-translational modifications are defective, in comparison with mammalian cells, and as a result, recombinant protein may exhibited altered bioactivity (Sodoyer, 2004).

Fritsche *et al.* in 2007 were studied characterizations of *Leishmania tarentolae* as an expression system for recombinant protein expression (Fritsche and Sitz, 2007). Coagulation FVIIa expressed in *Leishmania Tarentolae*, due to many advantages of this expression system, such as easy to work with this organism like *E. coli* and yeast, same folding and post-translational modifications of proteins, similar to eukaryotic systems, especially mammalian cells, reproducibility of Iranian lizard *Leishmania* cells (Kazemi et al., 2004), low cost culture media with high specific growth rate (Fritsche et al., 2008) than any other expression systems such as BHK, HEK293, CHO, SF9 (*Spodoptera frugiopedra*) and mammalian cell expression system (Berkner et al., 1988; Halabian et al., 2009) and the activity of expressed FVII was examined. Mirzaahmadi *et al.* were also expressed rhFVII in Iranian Lizard *Leishmania* expression system and could reduce clotting time of the sample to 30 seconds (Mirzaahmadi et al., 2011). In this study, rhFVII was successfully expressed by Iranian Lizard *Leishmania*, in the cell pellet, and exhibited acceptable amidolytic activity. Therefore, Iranian lizard *Leishmania* can be a suitable expression system for production of active rFVII.

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Competing Interests

The authors declare that there are no conflicts of interest.

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