Middle-East Journal of Scientific Research 7 (4): 515-520, 2011
ISSN 1990-9233

Enhanced Recombinant Protein Expression in the BTI-TN-5B1-4 Insect Cells

[1]Azizi Saraji Alireza, [1,3]Nasser Masroori, [2]Ahmad Reza Kamyab,
[4]Bayat Mansour and [5]Entezari Maliheh

[1]Scientific Society of Veterinary Science, Department of Veterinary Laboratory of Sciences,
Faculty of Specialized Veterinary Sciences, Science and Research Branch,
Islamic Azad University, Tehran, Iran
[2]Islamic Azad University, Science and Research Branch, Tehran, Iran
[3]Research Centre, Iranian Blood Transfusion Organization, Tehran, Iran
[4]Department of medical and Veterinary Mycology, Faculty of Specialized Veterinary Sciences,
Science and Research Branch, Islamic Azad University, Tehran, Iran
[5]Department of Genetics, Islamic Azad University, Tehran Medical Branch, Tehran, Iran

Abstract: Hemophilia is a bleeding disorder that patient suffers from poor blood clotting and continues bleeding. Factor VIII and IX deficiencies are the major causes that inherited through mutations in genes. FVII is one of the important factors in the coagulation cascade can initiates the clotting process through extrinsic pathway instead, therefore the possibility of producing FVII in high level by baculovirus and insect cells may be useful for therapeutic applications. In the present study, the full-length cDNA of human coagulation FVII was isolated from total cDNA of human liver cells and cloned into pENTR/D-TOPO vector using TOPO cloning strategy and then recombinant baculoviruses were generated using LR recombination reaction, Gateway technology. To express recombinant protein, the BTI-TN-5B1-4 cells (High Five) transfected with viruses and the transfected medium was separated in different time intervals. The ELISA analysis was performed for all protein fractions and the result shown the highest expression level achieved in 72 hrs post-infection (100µg/ml). To further asses, expression of human FVII was analyzed by RT-PCR, SDS-PAGE and Western blot. In conclusion, the expression level showed an upward trend over the time but decreased significantly 96 hrs after infection. Most surprisingly, the BTI-TN-5B1-4 cells prepared large scale protein expression level which could be more considerable if optimized as a powerful foreign gene expression vehicle.

Key words: High Five · FVII · Baculovirus · Expression

INTRODUCTION

Haemostatic challenges in patients with hemophilia A and B are treatable with highly purified plasma derived and recombinant DNA- derived factor VIII and factor IX concentrates. However, a well recognized and potentially life-threatening complication of hemophilia is the development of neutralizing antibodies against the missing factor. Up to 25 % of patients develop an inhibitor to factor VIII and 3-5% to factor IX. This makes the management of bleeding episodes difficult and poses a challenge for elective or emergency surgical procedures [1].

To date, therapeutic strategies in these situations have based on overcoming the inhibitors with large doses of factor VIII, but this is only feasible when the inhibitor titer is relatively low [2,3]. Other approaches such as the use of activated and non-activated prothrombin complex concentrates and porcine FVIII [4,5] and plasmapharesis with or without adsorption of antibody can have significant drawbacks including high cost, unpredictability of response, transmission of blood-derived infections, thromboembolic complications and in the case of porcine FVIII, development of anti-porcine antibodies. All these existing therapeutic caveats led to the development of recombinant factor VII (rFVII) as a

Corresponding Author: A.R. Azizi Saraji Scientific Society of Veterinary Science, Science and Research Branch, Department of Veterinary Laboratory of Sciences, Faculty of Specialized Veterinary Sciences, Science and Research Branch, Islamic Azad University, Tehran, Iran.

potential solution for treating hemophilia patents with inhibitors. Also data from several case reports and studies indicate that rFVII could be a valuable general haemostatic agent for non-hemophilic bleeding episodes.

Factor VII is central component in the initiation of coagulation [6] and wild-type recombinant factor VIIa can effectively treat patients with factor VIII, IX and VII deficiencies [7-9]. With concerning to the role in coagulation cascade, it can be used as replacement of the missing clotting factor (VIII or IX). Expression of FVII has been reported in mammalian cell lines, however low expression level is a major concern [10]. Recently, Insect cells have been used as higher eukaryotic expression systems not associated with some of the deficiencies common to prokaryotic systems (such as lack of post-translational modification) and mammalian cell systems (such as low expression level).

In contrast to a prokaryotic expression system like E.coli, insect cells are able to glycosylate the proteins although the glycosylation pattern is not identical to mammalian cells [11]. This unique tool usually yields high amounts of the produced protein, making the baculovirus expression system highly cost effective in comparison to other eukaryotic expression systems and recently has become one of the most widely used systems for heterologous protein expression in Lepidopteran insect cells [12-14], whereas this system is known to express exogenous proteins at levels ranging from 1 to 500 mg/lit [13]. Therefore the possibility of producing FVII in high level by baculovirus and insect cells may be useful for therapeutic applications.

According to previous studies, in compare with other Lepidopteran insect cells the relative potency of protein expression in the BTI-TN-5B1-4 (High Five) cells is 4-10 folds higher than other cells [15,16]. Therefore in this study, high level expression of recombinant human FVII (rhFVII) using Baculovirus expression vector system (BEVS) and Gateway technology was the main aim and we assumed that directly transfection of insect cells with recombinant baculovirus carrying human FVII would lead to produce hFVII in high scale.

MATERIALS AND METHODS

Isolation and Cloning of a Full-length hFVII cDNA: Two to three million of HepG2 cells in 1ml of Trizol reagent were ground in a mortar and total RNA was extracted according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). First strand normal human liver cDNA was synthesized with cDNA synthesis kit (Invitrogen) by random hexamers as primer. RT-PCR reaction conditions were: 25°C for 10 min, 50°C for 50 min and 85°C for 5 min.

The following forward primer (CACCATGGTCTCC CAGGCCCTCAGGCTCCTCTG) and reverse primer (GGGAAATGGGGCTCGCAGGAGGACTCCTG)wereused to isolate hFVII cDNA, a 1335bp fragment (GenBank accession no. NM_019616). The underlined nucleotides were added to the forward primer for performing TOPO Cloning reaction. The PCR was performed under the following conditions: predenaturing the template at 95°C for 5 min; 95°C for 30s, 59°C for 30s, 72°C for 45s for 32 cycles; followed by 72°C for 5 min as final extension. The PCR product was purified using PCR purification kit (Roche, Germany) and analyzed by Gel-Electrophoresis.

The pENTR Directional TOPO Cloning kit (Invitrogen) was used for directional cloning of FVII gene into pENTR/D-TOPO vector. The reaction was performed according to the manufacturer's instruction. Two µl of cDNA was mixed with 1µl TOPO vector and incubated for 5 min at room temperature. Full length FVII cDNA was subcloned into pENTR/D-TOPO vector. The recombinant plasmid pENTR-hFVII was analyzed for proper orientation of the insert by PCR and then transformed into chemically competent E. coli cells (cold CaCl2). Recombinant bacteria were screened with selective LB agar medium containing 50µg/ml kanamycin. The positive colonies were analyzed by PCR and further characterized by sequence analysis. Recombinant pENTR-hFVII was isolated using High Pure Plasmid Isolation Kit (Roche, Germany) from bacteria.

Production of Recombinant Baculoviruses Carrying hFVII: To construct recombinant baculovirus, we utilized Gateway technology therefore the LR recombinant reaction was performed to transfer hFVII gene into the Baculovirus linear DNA, according to the manufacturer's instructions (Invitrogen). Three hundred nanogram of linearized baculovirus DNA and 200ng of pENTR-hFVII were reacted using LR clonase II enzyme mix. The accuracy of reaction analyzed by PCR using polyhedrin forward primer (AAATGATAACCATCTCGC) and V5 reverse primer (Invitrogen).

Cell Culture: HepG2 (human hepatoma) cell line was obtained from National Cell Bank of Iran (NCBI). This cell line was grown in RPMI-1640 medium (Gibco-BRL, Eggenstien, Germany) containing 10% fetal bovine serum, 1µg/ml vitamin K1, 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco).

The BTI-TN-5B1-4, High Five, cell line (Invitrogen) was grown in complete TNM-FH [supplemented Grace's insect medium (Invitrogen) / 10% fetal bovine serum (Gibco) / Penicilin-Streptomycin (100µg/ml)] at 27°C without Co2 exchange (17).

Amplifying of Recombinant Baculoviruses: The High Five cells were transfected with the recombinant baculovirus carrying hFVII using Cellfectin as detailed by the manufacturer (Invitrogen). The recombinant viruses were screened by Ganciclovir (a nucleoside analog). The supernatant containing recombinant budded viruses from each cell culture, harvested after 96 hrs post-infection and centrifuged separately at 4000rpm for 5min to remove cell debris. The selected viruses were used three times to amplify the viral stock by infecting additional insect cells.

Expression of Recombinant hFVII: To express hFVII, approximately 8×105 High Five cells were transfected with the third stock of the recombinant virus at MOI of 100 and were grown in complete TNM-FH. Whereas hFVII was secreted into extracellular media, cellular and medium fractions of the transfected culture were separated in deferent time intervals (48, 72, 96, 120 hours post-infection). First, expression of FVII was investigated by RT-PCR on day 2 and 3 after infection, then a sandwich enzyme-linked immunosorbent assay (ELISA) method was performed to detect hFVII at three times for all fractions, as instructed by the manufacturer and utilized a specific rabbit anti-human FVII antibody coupled with peroxidase (Asserachrom VII:Ag, Diagnostica, France). The results were expressed as mean ± SD of three independent preparations.

Protein Assays: Immunoprecipitation of recombinant hFVII was carried out at 4°C in order to pruning of FBS proteins (abcam, USA). The protein sample was combined with goat anti human FVII, then incubated with protein-G conjugated beads for 4 hrs on ice and centrifuged three times at 13000rpm for 5 min. The partial purified protein was further characterized by SDS-PAGE and Western blot analysis.

To further assess, Sodium dodecyl sulfat-polyacrylamid gel electrophoresis (SDS-PAGE) and Western blot analysis were performed as previously described [18,19]. For Western blot analysis, the partial purified protein sample was separated by 12 % SDS-PAGE and subsequently transferred to a PVDF membrane (Hi-bond Amersham Biosciences, USA) (125 V) within 1.5 hr using the Mini Trans-Blot Electrophoresis Transfer Cell System from Bio-Rad (Hercules, CA, USA) in Tris/glycine buffer, pH 8.4, containing 20% (v/v) methanol. Then, the membrane was blocked with a solution containing 5 % skimmed milk and 0.1% Tween 20. The blocked membrane was washed with PBS containing 0.05% Tween 20. For detection of hFVII, the membrane was incubated with the following antibodies at room temperature for 1h, according to the suppliers' recommendations: polyclonal goat anti-human coagulation factor VII antibody (R and D, USA). Then, the membrane was washed 4 times with PBS containing 0.1 % Tween 20 and incubated with 100mU/ml horseradish peroxidase-coupled secondary antibody, polyclonal goat IgG-HRP (Abcam, USA), for 1 hr. Once, the membrane was washed 4 times with PBS containing 0.1 % Tween 20 and finally developed by ECL kit (Amersham, USA).

RESULTS

Isolation of hFVII cDNA and Generation of the Recombinant Baculovirus: Specific primers were designed to isolation of full-length hFVII. The expected size of 1.3kb in gel electrophoresis confirmed the correctitude. To demonstrate whether the hFVII cDNA was cloned correctly into pENTR/D-TOPO, we performed PCR to amplify a ~1300bp sequence from 5 bacterial colonies using the same primer pair which was used for isolation of FVII cDNA (Fig. 1). In addition, we performed PCR to amplify a ~1500bp sequence from colony 4 (randomly) using M13 universal primers. There was 200bp deference in length between two PCR products consequently, the result confirmed insertion of hFVII into pENTR. Finally, the accuracy of the nucleotides sequence and to be in frame was confirmed by DNA sequencing.

To determine that the hFVII cDNA was transferred into the baculovirus expression vector by LR recombination reaction, we performed PCR to amplify a 1575-bp sequence using polyhedrin forward primer and hFVII reverse primer which was used for isolation of hFVII cDNA.

Expression and Purification of Recombinant Human FVII: The presence of hFVII mRNA in High Five cells was confirmed by RT-PCR and the results showed an increase in expression of hFVII during the time intervals (Fig. 2).

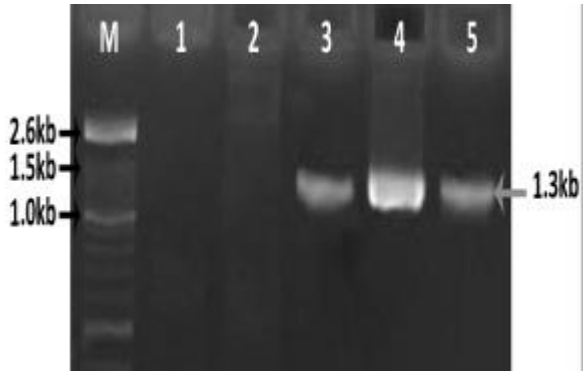

Fig. 1: Analyzing of five positive clones by PCR using hFVII primers. The colonies 3, 4 and 5 showed amplifying a 1.3kb sequence. The colony number 4 was selected for the next step. M; marker

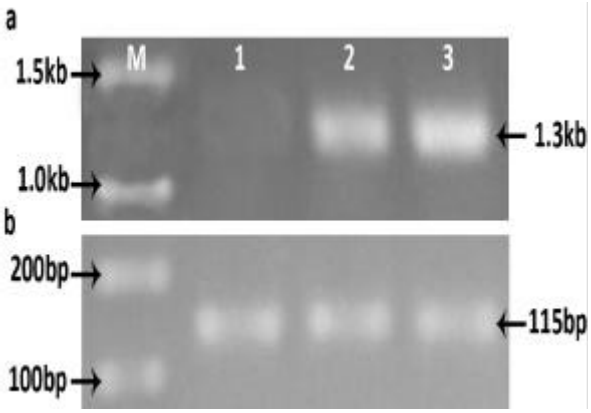

Fig. 2: (a) Analysis of expression of hFVII by RT-PCR at (1) 24hrs, (2) 48hrs and (3) 72hrs post-infection. Total RNA of High Five cells were extracted in deferent time intervals and analyzed for hFVII cDNA. The presence of hFVII mRNA in the cell lysis (lane 2, 3) demonstrated the expression of hFVII in the cell was increasing. (b) Analyzing of beta actin in transfected cells in order to confirm the accuracy of process. M; marker

Expression of hrFVII in the insect cell was also determined by ELISA, SDS-PAGE and Western blot analysis. The ELISA results were highly statistically significant and indicated that the expression level of recombinant protein reaches a peak 72 hrs post-infection (100µg/ml) (Table 1) (Fig. 3). The partial purified human rFVII derived from immunoprecipitation migrated as a prominent band with approximate molecular weight of 55 KDa in SDS-PAGE and Western blot analysis (Fig. 4a, 4b).

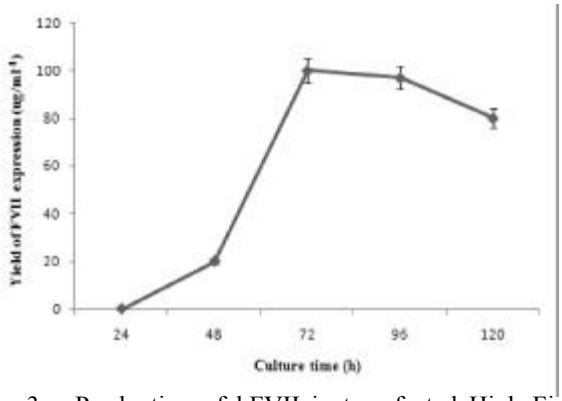

Fig. 3: Production of hFVII in transfected High Five cells. The High five cells, transfected with recombinant baculovirus, were cultured in TNM-FH media and infected medium analyzed by ELISA in appropriate time intervals. The results are shown in average (± SD) from three independent preparations (P<0.001) and as indicated by dotted line the concentration of hFVII increases over the time, however the optimal protein expression was obtained at 72 hrs post-infection.

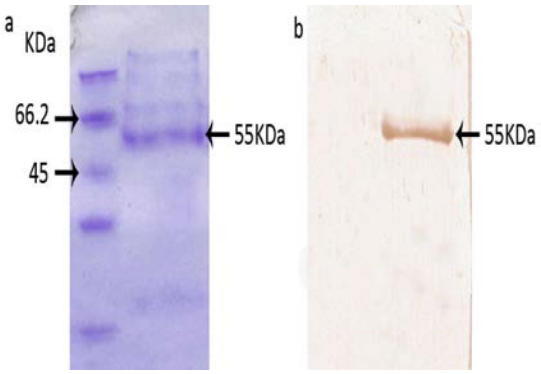

Fig. 4: (a) SDS-PAGE and subsequently (b) Western blot analysis were performed to identification of hFVII in infected medium at 72 hrs post-infection. A 55 KDa protein band was detected by goat anti-human FVII antibody.

Table 1: The ELISA results

samples	OD 492
Ctrl(+)*	2.105±0.113
48 hrs	1.591±0.076
72 hrs	1.960±0.076
96 hrs	1.951±0.186
120 hrs	1.785±0.085
Ctrl(-)**	0.050±0.010

*P<0.05, **P<0.01

The results indicated that hrFVII was expressed with an approximate molecular weight on increasing of 50 to 55 KDa that is due to the FVII encoded by the baculovirus carries six histidine residues at its C-terminus for purifying by Ni-resin column.

DISCUSSION

Nowadays, low density of plasma coagulation factors and transmission of blood-derived infections, production of recombinant coagulation factors is highly considered. This is first report of high level expression of recombinant hFVII by baculovirus in High Five insect cells. In the present study, insect cells were transfected with the baculovirus linear DNA containing human factor VII cDNA which encoded a full-length hFVII of 406 amino acids and then high level expression of functional hFVII has been achieved 72 hrs after transfection (100µg/ml/week). There was a significant drop in protein concentration after 96 hrs may have been due to increasing of proteolysis activity, the consequence of cell lysis.

Kemball-Cook *et al.,* expressed recombinant FVII in CHO cell line [20] and expression of recombinant rabbit FVII has been reported by Ruiz *et al.* [21]. They utilized pCMV5 for cloning and expression of rabbit recombinant FVII in human 293 cell line. Commercially available activated recombinant human coagulation factor VII (rFVIIa) or NovoSeven is produced in baby hamster kidney (BHK) cells and purified by consecutive chromotography steps, including immunoaffinity chromatography using murine monoclonal antibodies. However, in the mentioned studies the yield was lower than 1µg/ml/week except Ruiz *et al.* (5-10µg/ml/week). The Production of a functionally Enhanced recombinant FVII has been reported by Wajih *et al.* [22]. In their study, HEK 293 cells were transfected with vitamin K 2,3-epoxid reductase C1 (VKORC1) that gamma-carboxylase inhibitor calmenin is stably suppressed by shRNA transfection. The yield of rFVII produced in this study was 273ng/ml. Recently, we established a cell line expressing human FVIIa CHO cell line by co-transfection of hFVII and Hepsin, although the yield was lower than 500 ng/ml [10].

In our study, the rFVII encoded by baculovirus carries six histidine residues at its C- terminus. Polyhistidine has high affinity for a nickel-nitrilotriacetic acid resin providing single-step and fast purification (in about 1.5 hr) of recombinant FVII protein. This recombinant FVII protein was detected as a single band in SDS-PAGE and Western blot finally and about a four-fold rise observed in level of expression in compare with other insect cell [23]. There was no decrease in clotting time when the rFVII expressed by High Five cells which may be due to lack of gamma carboxylation in insect cells [24,25] but according to previous study[23], it would be possible to express large scale expression of functional hFVII in High Five cells using co-transfection of cells with FVII and human gamma carboxylase.

REFERENCES

1. Shapiro, A.D., G.S. Gilchrist, W.K. Hoots, H.A. Cooper and D.A. Gastineau, 1998. Prospective randomized trial of two doses of r-FVIIa in Hemophilia patients with inhibitors undergoing surgery. Thromb. Haemost., 80: 773-78.
2. Maick, B.G., 1993. Treatment of factor VII inhibitors: products and strategies. Semen Thromb Hemost., 19: 13-24.
3. Nillson, I.M., E. Berntop and C. Freiburghas, 1993. Treatment of patients with Factor VII and IX inhibitors. Thromb. Haemost., 70: 56-9.
4. Bray, G., E.D. Gomperts, S. Courte, R. Gruppo and E.M. Gordon, 1994. A muticenter study of recombinant factor VIII: safety, efficacy and inhibitor risk in previously untreated patients with hemophilia A. Blood, 83: 2428-35.
5. Hay, C.R., J.N. Lozier, C. Lee, M. Lafan, H. Tradati and E. Santagostino, 1994. Porcine factor VIII therapy in patients with congenital hemophilia and inhibitors: efficacy, patient selection and side effects. Semin Hematol. Apr., 31(2 Suppl 4): 20-5.
6. Furie, B. And B.C. Furie, 2005. Thrombus formation *in vivo.* J. Clin. Invest., 115(12): 3355-62.
7. Bauer, K.A., 1996. Treatment of factor VII deficiency with recombinant factor VIIa. Haemostasis. 26 Suppl, 1: 155-8.
8. Glazer, S., U. Hedner and J.F. Falch, 1995. Clinical update on the use of recombinant factor VII. Adv. Exp. Med. Biol., 386: 163-74.
9. Hedner, U., 1996. Dosing and monitoring NovoSeven treatment. Haemostasis. 26 Suppl, 1: 102-8.
10. Halabian, R., M.H. Roudkenar, N.S. Esmaeili, N. Masroori, A.M. Roushandeh and A.J. Najafabadi, 2009. Establishment of a cell line expressing recombinant factor VII and its subsequent conversion to active form FVIIa through hepsin by genetic engineering method. Vox Sang, 96(4): 309-15.

11. Van Die, I., A. Van Tetering, H. Bakker, D.H. Van den Eijnden and D.H. Joziasse, 1996. Glycosylation in lepidopteran insect cells: identification of a beta 1-->4-N-acetylgalactosaminyltransferase involved in the synthesis of complex-type oligosaccharide chains. Glycobiology, 6(2): 157-64.
12. Cha, H.J., N.G. Dalal, V.N. Vakharia and W.E. Bentley, 1999. Expression and purification of human interleukin-2 simplified as a fusion with green fluorescent protein in suspended insect Sf-9 cells. J. Biotechnol., 69: 9-17.
13. O'Reilly, D.R., L.K. Miller, V. Luckow and A. Baculovirus, 1992. Expression Vectors: A Laboratory Manual. New York: W.H. Freeman.
14. Miller, M.K., 1988. Baculoviruses as gene expression vectors. Ann. Rev. Microbiol., 42: 177-99.
15. Davis, T.R., K.M. Trotter, R.R. Granados and H.A. Wood, 1992. Baculovirus expression of alkaline phosphatase as a reporter gene for evaluation of production, glycosylation and secretion. Biotechnology (N Y), 10(10): 1148-50.
16. Lee, D.F., C.C. Chen, T.A. Hsu and J.L. Juang, 2000. A baculovirus superinfection system: efficient vehicle for gene transfer into Drosophila S2 cells. J. Virol., 74(24): 11873-80.
17. Lynn Dwight, E., 2002. Methods for Maintaining Insect Cell Cultures. J. Insect Sci., 2(9): 1-6.
18. Kyoung, R.K., K.K. Yeon and J.C. Hyung, 2008. Recombinant baculovirus-based multiple protein expression platform for Drosophila S2 cell culture. J. Biotechnol., 133: 116-22.
19. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 15(227)(5259): 680-5.
20. Kemball-Cook, G., I. Garner, Y. Imanaka, T. Nishimura, D. Brien and G.E. Tuddenham, 1999. High-level production of human blood coagulation factors VII and XI using a new mammalian expression vector. Gene., 139: 275-9.
21. Ruiz, S.M., S. Sridhara, M.A. Blajchman and B.J. Clarke, 2000. Expression and purification of recombinant rabbit factor VII. Thromb. Res., 15; 98(2):203-11.
22. Wajih, N., J. Owen and R. Wallin, 2008. Enhanced functional recombinant factor VII production by HEK 293 cells stably transfected with VKORC1 where the gamma-carboxylase inhibitor calumenin is stably suppressed by shRNA transfection. Thromb Res., 122(3): 405-10.
23. Masroori, N., R. Halabian, M. Rouhbakhsh, A.M. Roushandeh, A.J. Najafabadi, M. Mohammadipour, 2010. High-Level Expression of Functional Recombinant Human Coagulation Factor VII in Insect Cells. Biotechnol Lett; in press.
24. Roth, D.A., A. Rehemtulla, R.J. Kaufman, C.T. Walsh, B. Furie and B.C. Furie, 1993. Expression of bovine vitamin K-dependent carboxylase activity in baculovirus-infected insect cells. Proc. Natl. Acad. Sci. U S A., 15;90(18): 8372-6.
25. Tao Li, Y. Chao-Tsung, J. Dayun and D.W. Stafford, 2000. Identification of a Drosophila Vitamin K-dependent gamma-Glutamyl Carboxylase. Biological Chemistry, 275(24): 18291-6.