Bacterial expression and purification of C1C2 domain of human factor VIII

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Abstract

With the aim of the production of human factor VIII antigen and its corresponding antibody an epitope coding fragment of the light-chain of hFVIII, fused to a His6-tag, was isolated and over-expressed in Escherichia coli. The over-expressed hFVIII-epitope containing peptide was confirmed by its reaction with a rabbit serum directed against native hFVIII as well as antiHis_6-tag antibody. An expression level of 6.5 mg/l (of culture) of the C1C2-related peptide was estimated. The purified product was used to develop antibody in rabbit. The immunoblotting experiment confirmed that the rabbit polyclonal antibodies developed against the purified bacterially expressed hFVIII sub-fragment, recognizes human plasma derived FVIII. Both the produced hFVIII-related antigen and its corresponding antibody are useful in experiments using for detection and purification of hFVIII as well as the clinical diagnosis of hFVIII related disorders.

Keywords: *Human factor VIII light chain, C1C2 domain, E. coli expression system.*

INTRODUCTION

Human coagulation factor VIII (hFVIII), a 300 kD glycoprotein with 2332 amino acids is an essential cofactor in the intrinsic pathway of blood coagulation (Gitschier *et al*., 1984; Toole *et al*., 1984). In its activated form, hFVIII serves as a cofactor and enhances the activation of factor X by factor IXa in the presence of Ca^{2+} ions and phospholipids. Human FVIII circulates as a heterodimer composed of A1-A2-B-A3-C1-C2 domains. This large protein is stabilized by the formation of a complex with von Willebrand factor (vWF), from which it is released after proteolytic activation by thrombin (Scandella, *et al.,* 1988, 1995; Toole *et al.,* 1984). Upon secretion the precursor protein is proteolytically processed to generate a heavy chain (A1-A2-B) of 90-200 kDa and a light chain (A3-C1-C2) of 80 kDa that are held together by metal ions. The absence or malfunction of hFVIII is associated with the X-chromosome-linked recessive bleeding disorder, known as hemophilia A (Scopes, 1982). In patients with hemophilia A, the bleeding tendency can be corrected by the administration of FVIII concentrates. Considering its key role in coagulation and its corresponding medical applications, most researches have been focused on the molecular aspects of hFVIII (Miao *et al*., 2004). At present a number of preparations of rhFVIII are commercially available and numerous forms of rhFVIII including several B-domain-deleted hFVIIIs (BDDhFVIII) have been expressed through eukaryotic expression systems (Meulien *et al*., 1988; Pittman *et al*., 1993; Amirizadeh *et al*., 2005).

After multiple infusions of hFVIII, some patients with hemophilia A develop antibodies that neutralize the procoagulant activity of FVIII (Toole *et al*., 1984). The roles of common epitopes in the A2 and C2 domains of hFVIII for the development of antibodies in hemophilia A patient after replacement therapy have been doccumented (Scandella *et al*., 1995; Van den Brink *et al*., 2000). It has been shown that the bacterially produced FVIII fragments containing the major epitopes are capable of neutralizing antibodies that inhibit hFVIII activity (Scandella, *et al*., 1988). Indeed, both hFVIII antigens and their corresponding antibodies have various applications in hFVIII-related studies. Therefore, to develop specific anti-hFVIII

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antibody, expression of some epitope-containing fragments of hFVIII in *E. coli* was considered.

In this report the over-expression of the hFVIII-C1C2 in a T7/lac regulated *E. coli* expression system is presented. The bacterially derived hFVIII sub-fragment was then used to develop polyclonal anti-hFVIII-C1C2 antibody in rabbit serum. The specificity of the generated antigen and its related antibody were examined and their further applications are discussed.

MATERIALS AND METHODS

Bacterial strains, plasmids and primers: DH5α and BL21(DE3) strains of *E. coli,* were used as hosts for sub-cloning and expression steps, respectively. The PCR product cloning Kit (InsT/Aclone Fermentas, Canada) was used for the cloning of the PCR product containing hFVIII-C1C2. Plasmid pET26b+ (Novagen) was used for the construction of hFVIII-C1C2 expressing plasmid. A plasmid containing B-domain-deleted hFVIII cDNA was kindly provided by Dr. John H. MacVey (MRC- Clinical Science Center at the Imperial College School of Medicine, London, UK) (David *et al*., 2001) and used as template for the PCR amplification of the hFVIII-C1C2 cDNA. The following primers (synthesized by MWG-Germany) were designed for amplification of the coding region of hFVIII-C1C2. Primer C1C2-VIIIF (5´-CATGCCATGGATAAGTGTCAGACTCCCCTG-3´) and with *Nco*I restriction site (underlined) and primer C1C2-VIIIR (5´-CCGCTCGAGGTAGAGGTCCTGTGCCTCGC-3´) with *Xho*I restriction site (underlined) were designed respectively in forward and reverse directions from the C1-C2 domains of hFVIII cDNA.

Media, enzymes, antibodies and chemicals: Luria-Bertani (LB), containing 10 g/l Bacto-tryptone (Biotech. lab.), 5 g/l Bacto yeast extract (Merk-*Germany*), and 10 g/l NaCl adjusted to pH 7.0 with NaOH, was used as culture medium. Where necessary, 100 µg/ml ampicillin (during PCR product cloning) or 60 µg/ml Kanamycin for providing selective pressure and 1 mM Isopropyl-β-D-1-thyiogalactopyranoside (IPTG) for expression induction were added to the culture medium. Enzymes including *Nco*I, *Xho*I, *Taq* DNA polymerase and T4 DNA ligase were purchased from Roche-Germany. Polyclonal rabbit antiserum against the plasma derived hFVIII was prepared in NIGEB-Iran. Immuno-reactive material was detected using peroxidase conjugated antibody (Tebsan-Iran). Commercially prepared columns (Roche-Germany) were applied for the purification of plasmids and DNA fragments from agarose gel as well as PCR products. Plasma derived hFVIII samples (Biovitru,Sweden) were kindly donated by the Comprehensive Hemophilia Care Center-Iran.

DNA manipulation: Plasmid DNA isolation, DNA digestion and sub-cloning steps were performed according to standard methods (Sambrook and Russell 2001). PCR reaction for the amplification of the complete sequence coding for hFVIII-C1C2 domains was performed according to standard protocols. In addition to restriction analysis, PCR was employed for confirmation of recombinant plasmids using the specific primers, followed by nucleotide sequence analysis *(*MWG, Germany*)*. The comparison of the obtained sequences against those in the Gene-Bank was performed using the Blast program.

Growth and inducing conditions: Isolated colonies were used to inoculate shake flasks containing 5 ml of LB medium containing kanamicin. Cells were grown at 37°C until OD_{600}=0.5-0.7 was reached. Then the recombinant bacteria were induced by adding IPTG to a final concentration of 1mM. Incubation continued at 37°C for various post-induction times. The cells were harvested and the total protein content of samples were prepared for further analysis.

Periplasmic fluid preparation: Periplasmic-osmotic shock fluid from hFVIII-producing *E. coli* strains was obtained by a modified method described by Libby and co-workers (1987). Briefly, after reaching the OD_{600}=1.0, 50 ml of fermentation medium was centrifuged at 1500 ×g for 5 min and the pellet was placed on ice. All the subsequent steps were carried out at 4°C. The pellet was resuspended in 15 µl of ice cold TES buffer (Tris-HCl 0.2 M, EDTA 0.5 M, Sucrose 0.5 mM) pH 8.0. A volume of 22.5 µl of ice-cold double distilled H_2O was added and the incubation was continued for 30 min. The cells were centrifuged at 15800 ×g for 20 min. Tri-chloro acetic acid was added to the supernatant up to 12% of the final volume. The mixture was centrifuged at 15800 ×g for 20 min. The pellet was dissolved in sample buffer and saved as the periplasmic fraction for further protein analysis.

SDS-PAGE and Western blotting: SDS-PAGE was performed by a method described by Laemmli (1970), with slight modifications and gels were stained with coomassie brilliant blue. The specificity of the expressed recombinant hFVIII-C1C2 was examined by Western blotting. Electroblotting of proteins onto nitrocellulose PVDF membrane (Roche, Germany)

was performed using the wet-blotting procedure. For the Western blotting experiment, electerophoresed proteins were transferred to a nitrocellulose membrane in a transfer buffer (Tris 25 mM, glycine 192 mM, methanol 20%) at 86 mA for 16h, and then probed with either polyclonal antiserum prepared against hFVIII-C1C2 or monoclonal anti-His_6. The C1C2-antibody complex was then treated with horseradish peroxidase-conjugated anti-rabbit (in the case of polyclonal anti-hFVIII) or anti-mouse immunoglobulin (in the case of anti-His_6) using a solution of 4-chloronaphtol with hydrogen peroxidase as the substrate.

Estimation of the expressed rhFVIII-C1C2: Total protein patterns of the recombinant bacteria, visualized on comassie brilliant blue stained gels, were scanned by a Beckmann model R-112 densitometric gel scanner for estimation (%) of the expressed rhFVIII-C1C2 among total bacterial proteins.

Protein purification and solubilization: After sonication, the lysed cells were centrifuged at 1500 ×g for 15 min and the resulting supernatant and pellet were taken as soluble and non-soluble fractions respectively, for further analysis. For the solubilization of non-soluble proteins, lysed cells were incubated with either 8 M urea or a solution containing; guanidine hydrochloride 6 M, Phenyl Methyl Sulfonyl Fluoride (PMSF) 1 mM, EDTA 5mM, Triton X100 0.1-1% (named as GPET), for 2-3 h at room temperature with shaking, followed by a centrifugation at 15800 ×g for 20-30 min. The obtained supernatant was taken as the solubilized protein. The dissolved proteins from the previous step were subjected to purification of the His_6-tagged C1C2 protein via either Ni-NTA resin (Qiagen, USA), or a two step ultra-filtration method using commercially produced filters (Millipore, USA), as follows; a 50 KD-cut off filter (XM-50) was used in the first-step. The permeated fraction, obtained at this step was passed through a 30 KD filter (PM30) and the concentrated fraction of this step was dialyzed and used for protein analysis by SDS-PAGE. The purified protein was quantified by the Lowry assay (Scopes 1982) and used for immunization of rabbit.

Rabbit Immunization: For antibody production, a mature female rabbit was immunized with a solution composed of 1 ml of antigen (the purified bacterially derived hFVIII C1C2-related peptide), mixed vigorously with 1 ml of complete Freud's adjuvant, and injected subcutaneously to rabbit at four sites (in shoulder and lumber regions). Before injection 5 ml of test blood was collected. First booster injection was performed similarly, using incomplete Freud's adjuvant in the 4th week. Finally the 2nd booster was took place on the 6th week with crude antigen (half dose, used in previous times). Blood samples were collected for antibody titration, before the 3rd immunization. After 2 months the animal was bled and serum was collected for further applications. In order to determine the specificity of the produced antibody, an immunoblotting experiment was carried out to demonstrate its recognition of the native hFVIII.

RESULTS

Construction of the hFVII-C1C2 expressing plasmid: After the first round of cloning in a T-vector, the 942 bp DNA fragment coding for the C1C2 domain was sub-cloned between the *Nco*I and *Xho*I sites of a T7-based periplasmic expression vector, next to a pelB signal sequence and in-frame with a His_6-tag coding sequence, on its 3´ end (Fig. 1). Therefore periplasmic

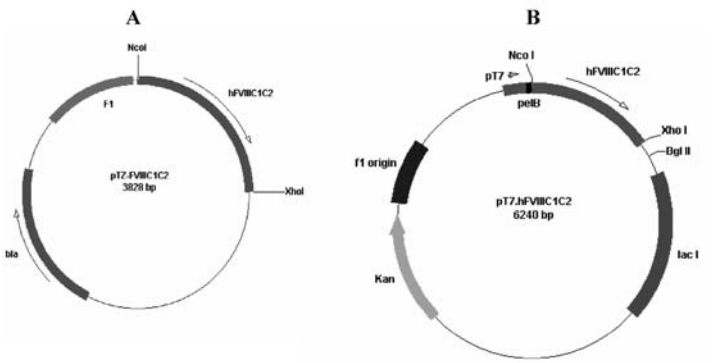

Figure 1. Physical maps of the constructed recombinant plasmids. (A) The recombinant T-vector (pTZ-FVIII-C1C2) was constructed by the insertion of a PCR product coverring a 942 bp subfragment of hFVIII lightchain sequence in a pTZ57R plasmid. (B) The insert was then isolated by *Nco*I/*Xho*I digestion and subcloned in a T7-based expression plasmid.

expression of a $(His)_6$-tagged hFVIII-C1C2 was expected. The use of the His_6-tagged tail was considered for detection as well as purification of the expressed recombinant protein. After verification of the cloned fragment by restriction analysis (data not shown) and nucleotide sequencing, the 6240 base pair recombinant expression plasmid (pT7.hFVIIIC1C2) was used in the transformation of the BL21(DE3) strain of *E. coli*.

Expression analysis: The recombinant bacteria carrying the pT7.hFVIIIC1C2 plasmid were induced with IPTG for various post induction durations and their cytoplasmic as well as perplasmic proteins were subjected to protein analysis. Due to presence of the pelB signal peptide in the expression plasmid an over-expression of the recombinant protein in the periplasmic space was expected. However as the data indicates, an over-expression of a protein of approximately 37KD, compatible with the size of the hFVIII-C1C2 peptide, was observable among the cytoplasmic proteins (Fig. 2). Presence of the 37 KD protein among the cytoplasmic proteins could be explained by a possible formation of non-soluble particles (inclusion bodies) of the over expressed C1C2-related peptide (Fig. 2). The possibility of inclusion body formation was examined by the analysis of the soluble and non-soluble fractions of total bacterial proteins, obtained from the homogenized recombinant bacterial cells (Fig. 3). Indeed, the over-expressed protein was detectable in the non-soluble fraction of the bacterial proteins, and therefore the expressed protein was separated together with cytoplasmic proteins of the induced bacteria. The specificity of the over-expressed protein was further confirmed by the immunoblotting the cytoplasmic proteins of the induced bacteria after 2, 4 and 8 h of post-induction time, using rabbit serum directed against native hFVIII (Fig. 4). The results showed that a regulated expression of the hFVIII-C1C2 taking place, where a relatively higher level of expression occurs after 8 hrs of induction. Therefore, in the subsequent steps, total proteins obtained from the recombinant bacteria, induced with 1mM IPTG for 8 h, were subjected to purification of hFVIII-C1C2.

Purification of the hFVIII-C1C2::$(His)_6$: The recombinant hFVIII-C1C2 related protein was collected as non-soluble inclusion bodies trapped in cells. Therefore, to purify the recombinant protein, after homogenization of the induced recombinant cells, the non-soluble fraction was subjected to solublization using either 8 M urea or guanidine hydrochloride denaturants. As it is shown in Figure 5A, the non-soluble proteins were dissolved more efficiently in the solution containing guanidine hydrochloride. The solubilized proteins were then subjected to the purification of His_6-tagged C1C2-related protein, using a Ni-NTA-based resin. The specificity of the purified rhFVIII-C1C2 protein was confirmed by an immunoblotting experiment, using the anti-$(His)_6$ antibody (Fig. 5B and C). Appearance of two protein bands in the final products may indicate the presence of a residual fraction of the expressed protein which has been left unprocessed among the bacterial proteins. Considering the 22 amino acid legth of the signal peptide used for the periplasmic expression of the protein in *E. coli*, the size difference between the processed and unprocessed proteins is estimated around 2 KD.

A two-step ultra-filtration method was also used

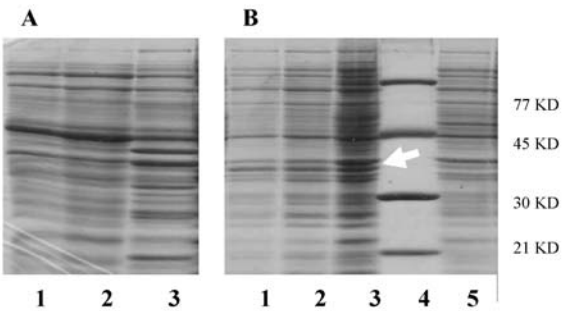

Figure 2. SDS-PAGE (10%) analysis of the periplasmic (A) and cytoplasmic (B) proteins of the recombinant hFVIII-C1C2 expressing *E. coli.* Panel A: lanes 1, 2 and 3: periplasmic proteins obtained after 2, 4 and 8 h of post induction, respectively. Panel B: lanes 1, 2 and 3: cytoplasmic proteins obtained after 2, 4 and 8 hrs of post-induction, respectively. Lane 4: protein size marker. Lane 5: negative control. The over- expressed protein band of approximately 37 KD is indicated with an arrow head.

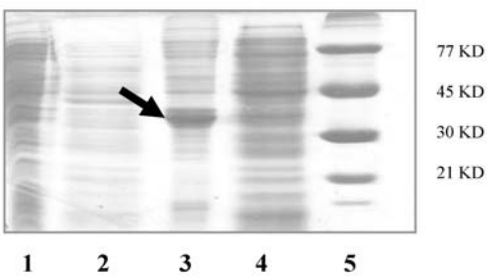

Figure 3. Analysis of soluble and non-soluble fractions of the total proteins of the rhFVIII-C1C2 expressing bacteria by SDS-PAGE (10%). Lane 1: non-soluble proteins of the negative control. Lane 2: soluble proteins of the negative control. Lane 3: non-soluble proteins of the induced recombinant bacteria. Lane 4: soluble proteins of the induced recombinant bacteria. Lane 5: protein size marker. The C1C2-related protein-band is indicated by arrow-head.

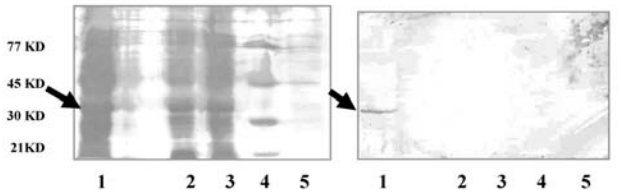

Figure 4. Analysis of the cytoplasmic proteins of the rhFVIII-C1C2 expressing bacteria by SDS-PAGE (10%) (Panel A) and Western blotting using polyclonal anti-hFVIII antibody (Panel B). The hFVIII-C1C2 corresponding band is indicated by arrow-heads. Lanes 1, 2 and 3 : 8, 4 and 2 h of post induction with IPTG, respectively. Lane 4: protein size marker. Lane 5: negative control.

for the purification of the expressed hFVIII-C1C2 and appeared to be more efficient than the previous method (Fig. 6). Based on the quantitative measurement of hFVIII-C1C2 obtained from the two purification procedures, an expression rate of approximately 6.5 mg of rhFVIII-C1C2 per liter of the culture was estimated.

Development of polyclonal anti-hFVIII-C1C2: With the aim of the production of a specific polyclonal anti-hfVIII antibody, the bacterially derived hFVIII-C1C2 (brhFVIII-C1C2) was used to direct rabbit serum against a major epitopic region of hFVIII (C1C2 domains). The serum obtained from the immunized rabbit was used for a set of immunoblotting experiments to confirm its specificity (Fig. 7). As the results indicate, the rabbit anti- bacterially-derived hFVIII-C1C2 (brhFVIII-C1C2) polyclonal antibody recognizes the human plasma-derived hFVIII. Appearance of multiple bands in the Western blotting experiment of the native hFVIII, shown in Figure 7B, suggests the presence of various hFVIII-derived fragments of different sizes in human plasma.

DISCUSSION

Two major epitopes of hFVIII are localized at amino acid sequences Arg484-Ile508 and Val2248-Ser2312, within the A2 and C2 domains respectively (Scandella *et al*., 1995). In the present work, a 314-amino acid length peptide derived from the light-chain of hFVIII containing the entire C1C2 domains of hFVIII coagulation factor, carrying a His-tag on its C-terminal was expressed in *E. coli*. The cytoplasmic as well as periplasmic protein patterns of the isolated clone taken after induction with IPTG showed an over-expression of a protein, comparable to the expected size of the hFVIII-C1C2 peptide. No protein of expected size appeared in the osmotic shock-fluid obtained from periplasmic fractions that can be explained by a possible formation of non-soluble particles of the over expressed C1C2-fragment. The formation of inclusion bodies is most likely, the major cause of the processing inhibition of the protein precursor (kiany *et al*., 2003; Ghasemi *et al*., 2004; Lim *et al*., 2000). The partial processing of the C1C2 preprotein in this work may also be caused by the formation of inclusion bodies. It is thought that the optimization of inducer concentra-

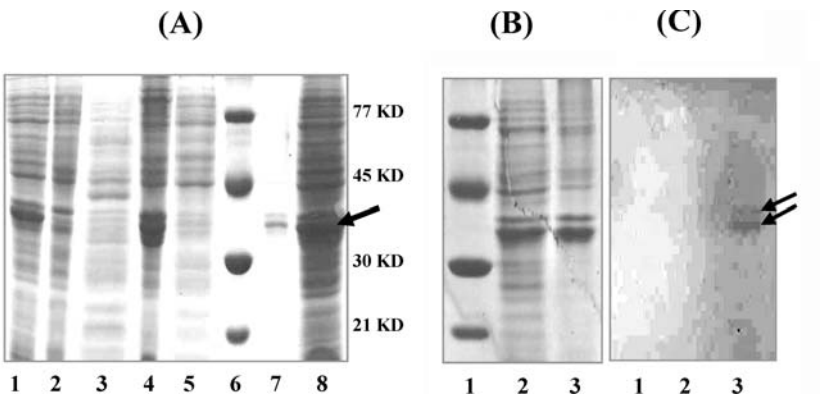

Figure 5. Solubilization and Purification of the bacterially expressed hFVIII-C1C2. Bands corresponding to the purified hFVIII-C1C2 are indicated by arrow-heads. Panel A: SDS-PAGE (10%) analysis of the bacterial proteins during solubilization of: Lane 1: total protein pattern of the recombinant bacteria. Lane 2 & 3: non-soluble and soluble fractions of normal bacterial proteins (negative control), after treatment with urea, respectively. Lanes 4 & 7: non-soluble fraction of the recombinant bacteria after treatment with either urea or a solution containing guanidine hydrochloride 6M, containing PMSF 1mM, EDTA 5 mM, Triton X100 0.1-1% (GPET), respectively. Lanes 5 & 8: soluble fractions of the recombinant bacteria after treatment with urea and GPET, respectively. Lane 6: protein size marker. Panels B & C: SDS-PAGE (10%) and western blot of the purified rhFVII-C1C2, respectively. Lanes 1: protein size marker. Lanes 2: total bacterial proteins dissolved in GPET. Lanes 3: eluted fraction.

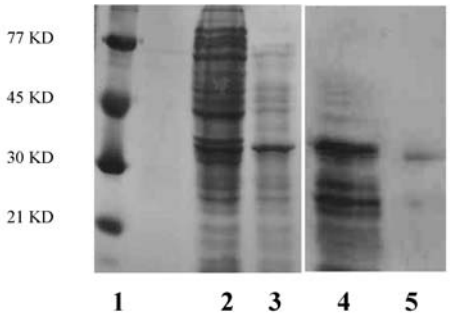

Figure 6. SDS-PAGE analysis of the fractions obtained from two-step purification of the GPET-solubilized rhFVIII-C1C2. Lane 1: protein size marker, Lane 2: concentrated fraction obtained from the XM-50 filter. Lanes 3 & 4: permeated fraction of XM-50. Lane 5: concentrated fraction obtained from the PM-30 amicon filter.

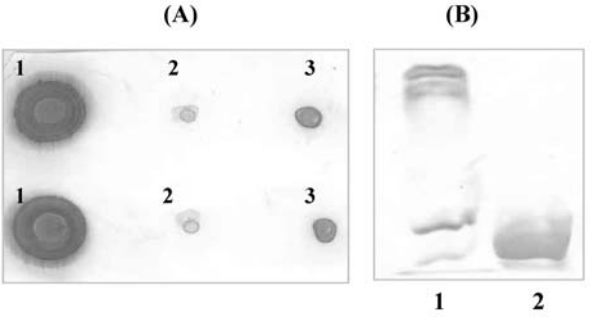

Figure 7. Immunoblotting the native plasma derived hFVIII and the rhFVIII-C1C2, using rabbit serum directed against the bacterially expressed hFVIII-C1C2. Panels A: use of 10^{-4} dilution of the immunized rabbit serum. All the samples were replicated to exclude any false signals. Sample 1: rhFVIIIC1C2 (dissolved proteins obtained from the recombinant bacteria). Sample 2: negative control (dissolved proteins obtained from normal bacteria). Sample 3: native hFVIII. Panel B: western blotting of the plasma derived hFVIII and the recombinant hFVIII-C1C2 protein, using polyclonal anti-hFVIII-C1C2. Lane 1: plasma derived native hFVIII. Lane 2: purified rbhFVIII-C1C2.

tion in combination with different parameters involved in expression efficiency may improve the processing efficiency of the preprotein. Many other factors are involved in the maturation process of recombinant proteins, including; host strain, growth and inducing conditions as well as expression level (Clave *et al*., 1996; Chang *et al*., 1987; Ghorpade and Garg, 1993; Goldstein, 1990). These factors are very much influenced by the nature of the signal sequence and its combination with corresponding protein (Berges *et al*., 1996). Therefore, the competence of the fusion protein being processed and translocated through the inner membrane depends on both the amino acid sequences in the signal peptide (Denefle *et al*., 1989) and bioprocess conditions (Chang *et al*., 1987; Cheah *et al*., 1994; Ghorpade and Garg, 1993; Goldstein, 1990; Hockney, 1994). PelB signal peptide is an example of well characterized signal peptides that is not efficiently processed, when used for the secretion of human growth hormone (Kiany *et al*., 2003) and G-CSF (Chung *et al*., 1998). In a previous work, we also showed that a suitable condition for efficient processing of pelB::hGM-CSF is achievable by using lower concentrations of lactose (under preparation). Therefore, in the cases of lac-operator regulated systems that the over-expression of proteins results in formation of inclusion bodies a slower induction rate by lactose can be considered as an alternative option. The over-expression of heterologous proteins may cause a drastic decrease in normal metabolic load known as metabolic burden (Mattanovich *et al*., 1998). In this regard, a number of molecular approaches have been suggested to decrease the extent of the metabolic load, such as the use of low copy number plasmid vector (Makrides, 1996). In addition, bioprocess strategies for optimization of the level of the target protein including manipulation of induction time (Neubauer *et al*., 1992), inducer availability (Gombert and Kiliian, 1998) and control of pre-induction specific growth rate (Curless *et al*., 1994) have been recommended.

The specific reaction of the plasma-derived hFVIII and the polyclonal antibody against the expressed hFVIII-C1C2 showed that, neither the carbohydrates nor the conformation of the native hFVIII, play critical roles in the epitopes of these domains. The data provided by Western blotting experiments demonstrate the potential of the generated antibody in experimental applications such as detection and purification of hFVIII. Based on these results and reports from other researchers (Scandella *et al*., 1988), it is assumed that the bacterially generated hFVII-C1C2 peptide might be able to recognize the inhibitory antibodies (inhibitors) generated against the infused hFVIII, in hemophilia A patients. This assumption is further supported by the fact that most inhibitors are directed against epitopes located in the A2 or C2 domains of hFVIII (Fulcher *et al*., 1985; Scandella *et al*., 1988; Toole *et al*., 1984). Using the peptide produced in this work and a number of plasma samples taken from hemophilia inhibitor patients in an ELISA, we have recently demonstrated a specific Ab::Ag reaction between the rbhFVIII-C1C2 and the patient's plasma (data not shown) that supports the idea of medical application of bacterially produced hFVIII-C1C2 as neutralizing factor in FVIII inhibitor patients (Scandella *et al*., 1995). The possible antigenic activity of the hFVIII sub-fragments that may cause further

immunological problem for the inhibitor patients should be considered in future investigations. Both the recombinant hFVIII-light chain sub fragment as well as its specific polyclonal antibody has provided valuable tools for further studies concerning the structure of rhFVIII and its related clinical and diagnostic applications.

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