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Synthesis of recombinant blood coagulation factor VIII (FVIII) heavy and light chains and reconstitution of active form of FVIII

Sang-Hwan Oh[1,2], Mi-Young Lee[1] and Dong Weon Song[1]

1 Department of Biochemistry and Molecular Biology, The Institute of Genetic Science, College of Medicine, Yonsei University, Seoul 120-752, Korea

2 Corresponding author: Tel, +82-2-361-5182; Fax, +82-2-312-5041; E-mail, shoh@yumc.yonsei.ac.kr

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Abbreviations: rFVIII, recombinant blood coagulation factor VIII; FX, factor X; FIX, factor IX; rFVIII^{m}, recombinant mutant factor VIII; rFVIII-H^{m}, recombinant mutant factor VIII heavy chain; rFVIII-L, recombinant factor VIII light chain; FVIII-B, B domain deleted factor VIII; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; ECL, enhanced chemiluninescent; SDS, sodium dodecylsulfate

Abstract

FVIII is synthesized as a single chain precursor of approximately 280 kD with the domain structure of A1-A2-B-A3-C1-C2 and it circulates as a series of metal ion-linked heterodimers that result from cleavages at B-A3 junction as well as additional cleavages within B domain. Factor VIII is converted to its active form, factor VIIIa, upon proteolytic cleavages by thrombin and is a heterotrimer composed of the A1, A2, and A3-C1-C2 subunits. A1 subunits of factor VIIIa terminates with 36 residue segment (Met^{337}-Arg^{372}) rich in acidic residues. This segment is removed after cleavages at Arg^{336}by activated protein C, which results in inactivation of the cofactor. In the present study, site-directed mutagenesis of FVIII at Arg^{336} to Gln^{336} was performed in order to produce an inactivation resistant mutant rFVIII (rFVIII^{m}) with an extended physiological stability. A recombinant mutant heavy chain of FVIII (rFVIII-H^{m}; Arg^{336} to Gln^{336}) and wild-type light chain of FVIII (rFVIII-L) were expressed in Baculovirus-insect cell (Sf9) system, and a biologically active recombinant mutant FVIII (rFVIII^{m}) was reconstituted from rFVIII-H^{m} and rFVIII-L in the FVIII-depleted human plasma containing 40 mM $CaCl_2$. The rFVIII^{m} exhibited cofactor activity of FVIIIa (2.85 $\times 10^{-2}$ units/mg protein) that sustained the high level activity during *in vitro* incubation at 37˚C for 24 h, while the cofactor activity of normal plasma was declined steadily for the period. These results indicate that rFVIII^{m} (Arg^{336} to Gln^{336}) expressed in Baculovirus-insect cell system is inactivation resistant in the plasma coagulation milieu and may be useful for the treatment of hemophilia A.

Key words: recombinant FVIII, baculovirus, expression, mutation.

Introduction

Factor VIII (FVIII) is a plasma glycoprotein cofactor that accelerates the activation of factor X by FIXa in the core reaction of blood coagulation cascade. Hemophilia A is an X-linked, recessive bleeding disorder (Anatonarakis *et al*., 1985), which affects 1 in 10,000 males, resulting in defective or deficient human clotting factor VIII molecules, necessitating exogenous replacement by either plasma- or recombinant-derived FVIII preparations. In an effort to avoid any further risk of unknown viral or hazardous element contamination from the human donor blood (Chan and Lembach, 1991), there have been concerted efforts to develop a safe and biologically active recombi-nant rFVIII. FVIII is synthesized as a 2,351-aa single-chain glycoprotein of 280 kDa with the domain structure of A1-A2-B-A3-C1-C2 and it circulates as a series of metal ion-linked heterodimers that result from cleavages at B-A3 junction as well as additional cleavages within B domain. FVIII is converted to its active form, factor VIIIa, upon proteolytic cleavages by thrombin and is a heterotrimer composed of the A1, A2, and A3-C1-C2 subunits. A1 subunits of factor VIIIa terminates with 36 residue segment (Met^{337}-Arg^{372}) rich in acidic residues. This segment is removed after cleavages at Arg^{336} by activated protein C (APC). Inactivation of FVIII occurs by proteolysis of FVIII subunits by APC which hydrolizes subunit protein at Arg^{336} in the heavy chain and Arg^{1721} in the light chain. However, recent observation suggests that cleavage at Arg^{1719} or Arg^{1721} in the light chain may be unrelated to FVIII inactivation (Fay, 1993). Numerous forms of rFVIII has been expressed as a biologically active rFVIII through eukaryotic expression system; i.e. the B-domain-deleted FVIII variants are undergoing clinical or preclinical trials and other factor VIII variants have been designed for treatment of particular clinical manifestation of hemophilia A patients. In the present study, site-directed mutagenesis of FVIII at Arg^{336} to Gln^{336} was performed in order to produce an inactivation resistant mutant rFVIII (rFVIII^{m}) to allow an extended stability in the physiological system. A mutant

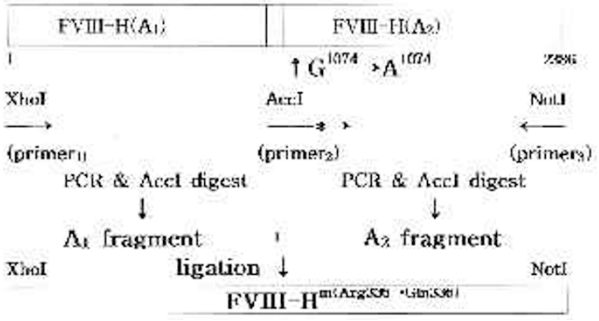

Figure1. Strategy for a site-directed mutagenesis of FVIII-H.

heavy chain of FVIII (FVIII-H^m; Arg^{336} to Gln^{336}) and wild-type light chain FVIII (FVIII-L) were expressed in Baculovirus-insect cell (Sf9) system and reconstitution of a biologically active rFVIII^m from these subunits was carried out.

Materials and Methods

Site-directed mutagenesis of FVIII-H

In order to extend a physiological half-life of rFVIII, substitution of G to A at Arg^{336} codon of FVIII cDNA was performed by PCR-associated site-specific mutagenesis (Figure 1). FVIII heavy chain (FVIII-H) cDNA corresponding to nucleotide No.1 through No. 2293 was PCR amplified from a cloned B-domain deleted FVIII cDNA plasmid (pSP64FVIII-B) using oligonucleotide primers containing specific restriction enzyme recognizing sites (sense primer: ACCGCTCGAGACCATGCAAATAGA-3', antisense primer: GAATCCGCGGCCGCTCATCTTGGTTCAATGGC-3'). For the construction of a mutant FVIII-H (Arg^{336} to Gln^{336}), PCR amplification of A2 domain of FVIII-H (nucleotide No.1040-2293) was performed using a mutant sense primer containing *Acc*I restriction site (CAAAGTAGACAGCT GTCCAGAGGAACCCCAACTACAAATGAAA-3') and substituted G by A at nucleotide No.1074 of FVIII-B cDNA. The mutant FVIII-H cDNA was made by combining A1 domain cleaved from the PCR amplified normal FVIII-H cDNA by *Acc*I restriction enzyme and A2 domain of mutant FVIII-H cDNA. For the expression of normal FVIII-H and FVIII-L, cloned wild type FVIII-H cDNA (nucleotide No.1 ~ 2293) and FVIII-L cDNA(nucleotide No. 2782 ~ 4768) were amplified by PCR. Both normal and mutant FVIII-H cDNAs were inserted into pAcSG2 plasmid (a Baculovirus transfer vector) and subcloned (Figure 2a). FVIII-L cDNA was inserted into pBacPAK9 (a Baculovirus transfer vector) and subcloned to get FVIII-L cDNA inserted recombinant transfer vector (Figure 2b). Subcloning was carried out by the method

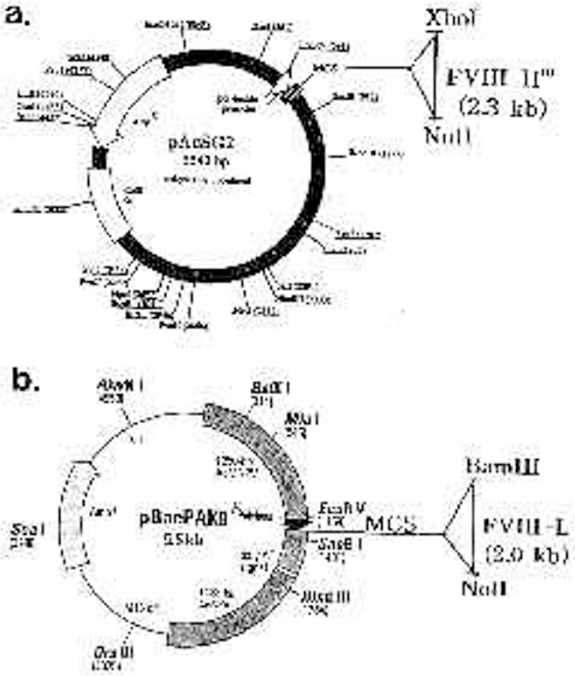

Figure 2. Construction of baculovirus transfer vector inserting FVIII-H^m and FVIII-L. a: FVIII-H^m inserted transfer vector. b.FVIII-L transfer vector.

described by Sambrook *et al*.(1989). Site specific mutation of FVIII-H cDNA (G^{1074} to A^{1074}) was confirmed by DNA sequencing performed by the method of Sanger and Coulson(1978).

Cotransfection of recombinant transfer vectors with viral DNA and expression of rFVIII subunits in insect cells

For the expression of rFVIII-H^m and FVIII-L in the Baculovirus expression system, insect cells (Sf9) were seeded in 35-mm tissue culture dishes(1×10^6) and incubated at 27˚C for 4 h in 2 ml BacPAK Grace's medium. Old medium was removed from cells and 2 ml BacPAK Grace's basic medium was added to the dishes and incubated for another 30 min. pAcSG2-FVIII-H^m (G^{1074} to A^{1074}) and pBacPAK9-FVIII-L recombinant transfer vectors (500 ng each) were cotransfected with 5 ml of pBacPAK6 DNA (*Bsu*I digest) into Sf9 insect cells according to the method described in the manual of Clontech Lab. Inc. The cells cotransfected with these vectors and viral DNA were cultured for 5 h at 27˚C and 1.5 ml of Grace's medium supplemented with 10% fetal bovine serum and antibiotics (Gentamycin) was added and cultured for 72 h at 27˚C. Recombinant Baculoviruses released from the cultured cells were harvested after 3 days and saved for plaque assay.

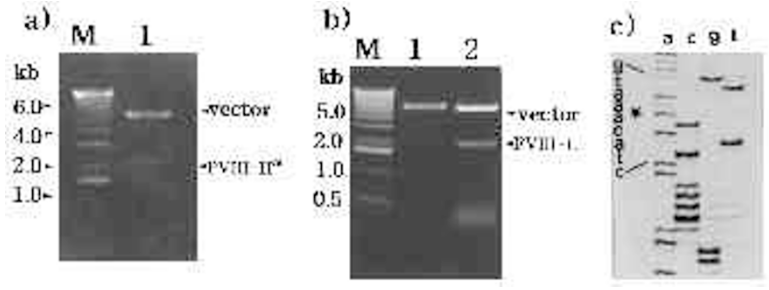

Figure 3. Confirmation of insertion of FVIII-H^m and FVIII-L into Baculovirus transfer vector. Agarose gel electrophoresis of *Xho*I and *Not*I digested pAcSG2-FVIII-H^m(A) and *Bam*HI and *Not*I digested pBacPAK9-FVIII-L(B). DNA sequencing of FVIII-H^m (C). Asterisk indicates the mutation (G to A).

rFVIII-H^m and rFVIII-L proteins express-ed in the cultured cells (Sf9) were extracted from the cells by homogenization with FVIII buffer (20 mM imidazol, pH 6.8, 0.15 M NaCl, 0.1 M L-lysine and 0.02% NaN_3) by glass homogenizer followed by centrifugation at 12,000 rpm for 10 min in a microcentrifuge.

Reconstitution of rFVIII

rFVIII-H^m and rFVIII-L were purified from insect cell extracts by anti-FVIII antibody conjugated Sepharose-4B affinity gel column chromatography as described previously (Oh *et al*., 1997). Reconstitution of biologically active rFVIII^m was carried out by adding an equimolar amount (200 nM) of the partially purified rFVIII-H^m and rFVIII-L in the 20 mM Hepes buffer, pH 7.2, containing 400 mM NaCl, 40 mM $CaCl_2$, 1.0 mM 2-mercaptoethanol, and 10% FVIII-free plasma, and allowed to incubate for 24 h at 4˚C. The association of rFVIII-H^m with rFVIII-L was monitored by FVIII-heterodimer ELISA assay (Oh *et al*.,1997) and emergence of cofactor activity was monitored by using Coamatic FVIII assay kit [Carlebjok *et al*.(1987)]. The reassociation constant for rFVIII-H^m with rFVIII-L was determined according to the method of Lenting *et al.*(1994). Total protein content was determined by Bradford method (Bradford,1976) and quantitative analysis of rFVIII-H^m and FVIII-L was performed by sandwitch type ELISA method (Johnston and Thorpe, 1987).

Western blot immunodetection of rFVIII subunits

Western blot analysis of rFVIII subunits was performed according to the method described by Burnette *et al*. (1981). Cell lysates including rFVIII-H^m and rFVIII-L expressed in insect cells were subjected to SDS PAGE (10%) according to the method of Laemmli (1970), and proteins in the gel were electrotransferred onto nitrocellulose membrane. Immuno detection of rFVIII subunits was carried out according to ECL Western blotting protocol (Amersham, Buckinghamshire, UK) using mouse monoclonal antibody to FVIIIc and polyclonal antibody to FVIII-H (specific for Asn^{169}~ Arg^{541}) as primary antibodies and peroxidase-conjugated antibody to mouse IgG as secondary antibody.

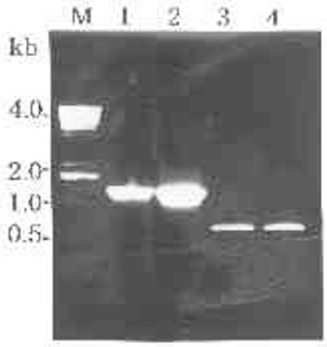

Figure 4. Identification of FVIII-H^m and FVIII-L in Sf.9 cells cotransfected with pAcSG2-FVIII-H^m or pBacPAK9-FVIII-L with pBacPAK6 DNA. 1,2: PCR products of FVIII-H^m (No.1040-2310). 3,4: PCR products of FVIII-L (No.4124-4770).

Results

Site specific mutation of FVIII-H

Mutation of Arg^{336} to Gln^{336} at N-terminal side of A2 domain in FVIII-H has been achieved by PCR amplification of A2 domain of FVIII-H using the mutant primer and ligation of it to A1 domain of FVIII-H amplified with PCR. Insertion of FVIII-H^m (G^{1074} to A^{1074}) and FVIII-L cDNA sequences into Baculovirus transfer vectors (pAcSG2 and pBacPAK9 plasmids) were confirmed by restriction analysis of plasmids prepared from *E. coli* cells transformed with the respective recombinant plasmids (Figure 3a, 3b). Substitution of nucleotide at Arg^{336} codon (CGA) to Gln^{336} (CAA) was verified by DNA sequencing (Figure 3c).

Production of Baculoviruses expressing rFVIII subunits

Insect cells were cotransfected with recombinant transfer vectors carrying the FVIII-H^m and FVIII-L cDNAs (pAcSG2-FVIII-H^m and pBacPAK9-FVIII-L) with *Bsu*I digested linearized Baculovirus DNA (pBacPAK6) and were cultured for 72 h. Viruses released into the culture media were harvested and saved for the plaque assay. The plaque

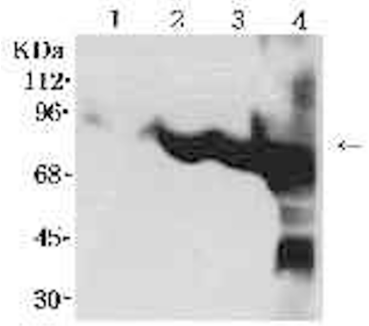

Figure 5. Western blot immunostaining of FVIII expressed in Sf.9 cells. After 72 h culture, cells(7×10^6) were harvested and lyzed by adding 500 ml of SDS-PAGE sample buffer, following the sonication 5 times for 20 sec. A portions of protein (20 mg) were subjected to SDS-PAGE and ECL-western blot immunodetection. 1, Insect cells cotransfected with pAcSG2 vector and pBacPAK6 DNA; 2, insect cellls cotransfected with pAcSG2-FVIII-H^m and pBacPAK6 DNA; 3, insect cells cotransfected with pBacPAK9-FVIII-L and pBacPAK6 DNA; 4, normal human plasma. Arrow indicates the rFVIII-H^m or rFVIII-L.

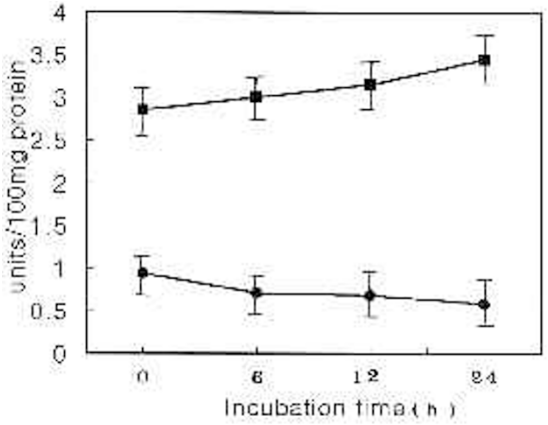

Figure 6. Changes of cofactor activities of reconstituted $rFVIII^m$ and normal plasma under physiological condition. $rFVIII^m$ was reconstituted from equimolar $rFVIII^m$ and FVIII-L in the 20 mM Hepes buffer, pH 7.2, containing 400 mM NaCl, l0% FVIII-free plasma,, 40 mM $CaCl_2$ and 1.0 mM 2-mercaptoethanol, followed by the incubation for 24 h at 4°C. -( )-: normal plasma; -(■): $rFVIII^m$.

assay was performed after serial dilution of viral stock solution and viral titer of 2.5×10^7 pfu/ml was determined. The presence of FVIII-H^m and FVIII-L sequences in recombinant viruses was confirmed by PCR amplification of the sequences corresponding to each subunits (Figure 4).

Identification and quantitative determination of rFVIII subunits expressed in insect cells

rFVIII-H^m and FVIII-L proteins expressed in insect cells (Sf9) were extracted by homogenizing with the FVIII buffer and identified by western blot immunodetection system (Figure 5). The amounts of rFVIII-H^m protein present in the culture media and in the insect cell lysates were 8.0 ng/ml and 526.3 ng/10^6 cells, respectively, and the amount of rFVIII-L protein presented in the cultured media and cell lysates were 83.2 ng/ml and 457.7 ng/10^6 cells, respectively (Table 1).

Reconstitution of biologically active $rFVIII^m$

The reconstitution of a biologically active $rFVIII^m$ molecule was carried out by combining an equimolar concentration (200 nM) of rFVIII-H^m and rFVIII-L in 10% FVIII-free plasma containing 40 mM $CaCl_2$ and allowed to incubate for 24 h at 4˚C. Cofactor activity of the reconstituted $rFVIII^m$ was 2.85 x 10^{-2} units/mg of reconstituted plasma protein (table 2). The stability of $rFVIII^m$ (inactivation resistance) were assessed by measuring the cofactor activity in comparison with normal plasma by the *in vitro* incubation at 37˚C in the coagulation milieu for the time periods of 6, 12, and 24 h. Cofactor activity of $rFVIII^m$ was slightly increased while that activity of the normal human plasma was decreased steadily for the incubation period of 24 h at 37˚C. The reassociation constant (k_a) for the rFVIII-H^m and rFVIII-L was determined as 35 $M^{-1}.sec^{-1}$.

Discussion

Table 1. Expression of FVIII-H^m and FVIII-L in Sf.9 cells transduced by recombinant baculoviruses.

Group	FVIII subunits transduced	FVIII content	
		media (ng/ml)	cell lysate (ng/10^6cells)*
Control	-	8.0	6.5
rFVIII-H^m	FVIII-H^m	174.4	526.3
rFVIII-L	FVIII-L	83.2	457.7
Reference plasma		120.0	-

Sf.9 cells (~7 x10^6) were cotransfected with pAcSG2-FVIII-Hm or pBacPAK9-FVIII-L with pBacPAK6 DNA (BSU36I digested). Cells were cultured for 4 h and then media were changed with the fresh one (4 ml) and cultured another 72 h. FVIII content was measured by ELISA and reference plasma was used a standard. All values are means of duplicate.

Table 2. Reconstitution of FVIII subunits and regeneration of FVIII activity.

Source	Subunits* reassociated	FVIII activity (x 10^{-2})
Cultured media	FVIII-H^m + FVIII-L	1.5 ± 0.09 units/ml
Sf.9 cell lysate	FVIII-H^m + FVIII-L	2.85 ± 0.45 units/mg protein
Reference	plasma (Sigma)	0.94 ± 0.07 units/mg protein

* Subunits in equimolar concentration were mixed in a 20 mM Hepes buffer, pH 7.2, containing 400 mM NaCl, 10% FVIII-free plasma, 40 mM $CaCl_2$ and 1.0 mM 2-mercaptoethanol and subjected to incubation for 24 h at 4˚C. All values are means of triplicate determinations.

Advances in recombinant DNA technology have led to the successful cloning of both cDNA and genomic sequences of FVIII, and the availability of full length FVIII cDNA has made it possible to construct a vector for the expression of FVIII in mammalian cells (Toole *et al*., 1984; Wood *et al*., l984; Kaufman *et al*., 1988; Klein, 1991; Pittman *et al*.,1993). FVIII has been known to be synthesized as a single polypeptide having a sequential domain of A1-A2-B-A3-C1-C2 of which B domain is dispensable for the cofactor activity (Vehar *et al*., 1984). The generation of active FVIII from the isolated subunits has been demonstrated (Nordfang and Ezben,1988), and an efficient production of rFVIII by coexpression of the heavy and light chains in cell culture system has been attained (Yonemura *et al*.,1993). In the present study, we have synthesized recombinant variant, rFVIII-H^m , of which Arg^{336}, a APC cleavage site, was substituted to Gln^{336} in order to prevent APC induced cleavage and inactivation of $rFVIII^m$. rFVIII-L was synthesized in the same system and assembled with rFVIII-H or rFVIII-H^m to functional rFVIII or $rFVIII^m$. Both reconstituted rFVIII and $rFVIII^m$ had similar cofactor activities in a molar base, but $rFVIII^m$ retained relatively higher cofactor activity than that of rFVIII when they were incubated at 37˚C for 24 h. The high level of $rFVIII^m$ cofactor activity during the incubation period was likely due to the loss of APC attacking site in rFVIII-H although inadequate amount of inactivating factors in the reconstituted $rFVIII^m$ pre-paration has not been ruled out. The lower clearance rate and the longer half-life of rFVIII than that of the highly purified plasma-based FVIII preparation in circulating blood have been reported (Morfini *et al*.,1992, 1993). In their report, the half-life of rFVIII in hemophilia patients was 14.6 ± 7.6 h while that of von Willebrand disease was 4.5 h, indicating that the half-life of rFVIII was much influenced by the presence of von Willebrand factor, a stabilizing molecules in blood. The relatively higher cofactor activity of $rFVIII^m$ in this study implicates that $rFVIII^m$ may be useful for the particular hemorrhagic diathesis in some hemophilia A patients. Activation process of FVIII is known to involve the limited proteolytic cleavage of single chain FVIII by specific proteases, thrombin and FXa (Harris *et al*., l986) and further pro-teolysis of heavy chain of FVIII by thrombin and APC inactivates FVIII (Eaton *et al*., 1986; Fass, 1991). Thus, it is expected that limitation of inactivating process by site-directed mutagenesis of its cDNA may improve the pharmacokinetic characteristics. The stability of both wild type FVIII and $rFVIII^m$ measured in our study was somewhat higher than the previous *in vivo* data of Morfini *et al*.(1992). The difference between these two results might be due to the different assay systems (*in vitro* system vs *in vivo* system) and the quality of products used. The protection of $rFVIII^m$ from APC-mediated inactivation may have a sparing effect of rFVIII infused into hemophilia A patients but the side effects associated with thrombophilia which is frequently observed in abnormal Factor V accumulation due to APC resistance should be considered. In fact, $rFVIII^m$ application may not be a serious risk factor for thrombophilia since FVIII resistance to APC did not cause thrombophilia (Ingerslev *et al*., 1996). The benefits of rFVIII for the treatment of previously untreated patients with hemophilia A have been shown to outweigh the risks associated with inhibitor antibodies (Lusher *et al*., 1993). It is known that glycoproteins produced in insect cells are smaller in size than those of mammalian cells due to the presence of high mannose-type, small oligosaccharides in the molecules (Luckow, 1991). $rFVIII^m$ produced in insect cells in the present study could reassemble functionally active molecules with similar cofactor activity produced in other eukaryotic cell expression system (Yonemura *et al*., 1993). These results indicate that size of oligo-saccharides in rFVIII may not be critical for the activity. The molecular weights of rFVIII-H^m and rFVIII-L produced in Sf9 cells in the present study were close to 81 kDa which is the size to be expected (Figure 4). The appear-ance of FVIII with heterogeneous size was thought to be due to the partial processing or degradation of the products in normal human plasma. It is likely that cofactor activity and stability of $rFVIII^m$, obtained from individual subunits (rFVIII-H^m, rFVIII-L) expressed in insect cell system might be influenced by the presence of stabilizers in cells as well as in media. Therefore, optimization of rFVIII

expression system in insect cell by coexpression of rFVIII subunits and vWF may lead to the yield of a biologically active rFVIII with better stability.

In summary, functionally active and relatively stable $rFVIII^m$ was produced by site-directed mutagenesis at G^{1074} of FVIII-H cDNA (Arg^{336} to Gln^{336}) and reconstitution of it from $rFVIII\text{-}H^m$ and rFVIII-L expressed in baculovirus insect cell system. Further study is required for the safety of the product in terms of thrombophilia due to APC resistance and biological half-life of the product *in vivo*.

Acknowledgement

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