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(71) Applicant(s)
SK Chemicals Co., Ltd.

(72) Inventor(s)
Song, In-Young;Kim, Hun-Taek;Lee, Bong-Yong;Park, Mahn-Hoon;Lee, Ho-Soon;Lim, Yun Jung;Lee, Ji-Hye;Son, Seo Yeon;Kim, Min-Sun

(74) Agent / Attorney
Cullens Pty Ltd, Level 32 239 George Street, Brisbane, QLD, 4000

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(71) Applicant (*for all designated States except US*): **SK CHEMICALS CO., LTD.** [KR/KR]; 686, Sampyeong-dong, Bundang-gu, Seongnam-si, Gyeonggi-do 463-400 (KR).

(72) Inventors; and

(75) Inventors/Applicants (*for US only*): **SONG, In-Young** [KR/KR]; Woosung Apt. 304-1313, Sadang-2-dong, Dongjak-gu, Seoul 156-773 (KR). **KIM, Hun-Taek** [KR/KR]; Hanshin 22-cha Apt. 338-1111, Jamwon-dong, Seocho-gu, Seoul 137-030 (KR). **LEE, Bong-Yong** [KR/KR]; Bangbae Raemian Tower 201-2103, #485-3, Bangbae-dong, Seocho-gu, Seoul 137-060 (KR). **PARK, Mahn-Hoon** [KR/KR]; Daejoo Piorae Apt. B-danji 205-1703, Gongse-dong, Giheung-gu, Yongin-si, Gyeonggi-do 446-902 (KR). **LEE, Ho-Soon** [KR/KR]; Taeyoung Desian Apt. 1306-2304, Unjoong-dong, Bundang-gu, Seongnam-si, Gyeonggi-do 463-440 (KR). **LIM, Yun Jung** [KR/KR]; Youngpoong Madrevill Apt. 102-1504, Oryu-2-dong, Guro-gu, Seoul 152-100 (KR). **LEE, Ji-Hye** [KR/KR]; 101-ho, #495-16, Sinlim-4-dong, Gwanak-gu, Seoul 151-015 (KR). **SON, Seo Yeon** [KR/KR]; Mokryeon Apt. 1202-1402, #1088, Sanbon-dong, Gunpo-si, Gyeonggi-do 435-040 (KR). **KIM, Min-Sun** [KR/KR]; 102-2702, Heendolmaeul, #1194, Baek-seok-dong, Ilsandong-gu, Goyang-si, Gyeonggi-do 410-360 (KR).

(74) Agent: **FIRSTLAW LEE & KO**; Trust Tower, 275-7, Yangjae-Dong, Seocho-Ku, Seoul 137-739 (KR).

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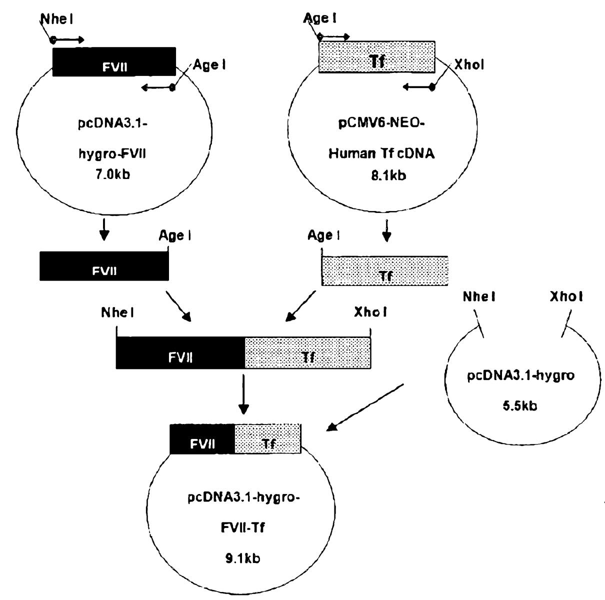
(54) Title: FUSION PROTEIN HAVING FACTOR VII ACTIVITY

[Fig.1]



(57) **Abstract**: A fusion protein comprising factor VII (FVII) and transferrin according to the present invention has an improved specific activity of FVII compared to existing FVII fusion proteins comprising other fusion partners than transferrin, and thus can be effectively used in a therapy using FVII.

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Description

Title of Invention: FUSION PROTEIN HAVING FACTOR VII ACTIVITY

Technical Field

[1] The present invention relates to a fusion protein having factor VII (FVII) activity; and, more particularly, to a fusion protein comprising FVII and transferrin and having 0.7 or more of FVII specific activity compared to the unfused natural type FVII, a DNA coding therefor, a recombinant vector comprising the DNA, and a host cell comprising the recombinant vector.

[2]

Background Art

[3] A variety of hemorrhagic disorders are caused by the lack of blood coagulation factors. The most common disorders are hemophilia A and B caused by deficiencies or abnormality of blood coagulation factors VIII and IX, respectively.

[4] Hemophilia A is a genetic bleeding disorder caused by an X-linked recessive trait of defective factor VIII gene. A concentrate of a plasma-derived or recombinant factor VIII has been used for the treatment of hemophilia A. Hemophilia B is caused by a deficiency or dysfunction of factor IX, which is treated by using a concentrate of plasma-derived or recombinant factor IX. However, the emergence of alloantibodies against the replacement factors remains as a serious medical problem in the treatment of hemophilia A and B. Antibodies against factor VIII are generated in up to 30% of patients with hemophilia A. Although antibodies against factor IX are less produced, they are less sensitive to an immune tolerance induction therapy, leading to more serious results.

[5] Blood coagulation is initiated by the formation of a complex between tissue factor exposed to circulating blood after a vessel wall damage and an activated form of factor VII (FVIIa). Such complex activates factor IX and factor X and the resultant factor Xa produces the limited amount of thrombin. In a positive feedback loop, thrombin activates a variety of factors (such as factor VIII, factor V, factor XI, etc.) of blood coagulation cascade, and the activated factors constitute a factor Xase complex or a prothrombinase complex. These complexes further amplify their own generation and the production of thrombin. This sufficient amount of thrombin called 'thrombin burst' converts fibrinogen at bleeding sites to fibrin, thereby achieving complete hemostasis. However, in case of hemophilia patients having a high concentration of neutralizing antibodies against factor VIII or factor IX, no sufficient hemostasis is attained since the factor Xase complexes mentioned above can't be produced. FVIIa has been used as a

major therapeutics for the patients who have neutralizing antibodies against factor VIII or factor IX, because it can activate factor X, even in the absence of factor VIII and factor IX, thereby ultimately producing a sufficient amount of thrombin to achieve the desired therapeutic effects.

[6] FVII is a single-chain glycoprotein consisting of 406 amino acids, has a molecular weight of 50kDa, and is secreted into blood stream as a zymogen. FVII consists of four distinct domains, i.e., an amino terminal-carboxyglutamic acid (Gla) domain, two epidermal growth factor (EGF)-like domains and a serine protease domain (Hagen FS et al., Proc. Natl. Acad. Sci. USA, 83(8):2412-2416, 1986). FVII is converted to its activated form, FVIIa, by forming two polypeptide chains linked by a disulfide bond, i.e., N-terminal light chain (24 kDa) and C-terminal heavy chain (28 kDa) through the proteolysis of a single peptide bond located at Arg152-Ile153. FVII is present at a concentration of 500 ng/mL in plasma, and 1% (i.e., 5 ng/mL) of FVII is present as FVIIa.

[7] Meanwhile, it has been reported that the half-life of FVII in plasma is approximately 4 hours (3~6 hours), while that of FVIIa is about 2.5 hours. Due to the short half-life, FVIIa is required to be administered via multiple intravenous injections or continuous injection. However, this would limit the therapeutic uses of FVIIa in terms of high treatment expenses and making the patient's discomfort.

[8] To overcome these problems, methods have been provided for preparing fusion proteins comprising FVII and a fusion partner linked thereto, but the resulting proteins had the problem of losing their biological activities, even though the short *in-vivo* half-life was somewhat improved compared to the unfused protein.

[9] Accordingly, there are needs for providing and securing a FVII fusion protein which has an improved *in-vivo* half-life while retaining the biological activity of the natural type FVII.

[10]

Disclosure of Invention

Technical Problem

[11] It is an object of the present invention to provide a fusion protein having the biological activity of natural type FVII.

[12] It is other object of the present invention to provide a gene coding for the fusion protein.

[13] It is a further object of the present invention to provide a recombinant vector comprising the gene.

[14] It is a still further object of the present invention to provide a host cell comprising the recombinant vector.

[15]

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Solution to Problem

[16] In accordance with one aspect of the present invention, there is provided a fusion protein comprising factor VII (FVII) and transferrin, wherein the transferrin is linked to the C-terminus of the FVII.

[17] In accordance with other aspect of the present invention, there is provided a DNA coding for the fusion protein.

[18] In accordance with a further aspect of the present invention, there is provided a recombinant vector comprising the DNA.

[19] In accordance with a still further aspect of the present invention, there is provided a host cell comprising the recombinant vector.

[19a] Definitions of the specific embodiments of the invention as claimed herein follow. According to a first embodiment of the invention, there is provided a fusion protein comprising factor VII (FVII), transferrin and a linker between the FVII and the transferrin, wherein said transferrin is linked to the C-terminus of said FVII.

[19b] According to a second embodiment of the invention, there is provided a DNA coding for the fusion protein of the first embodiment.

[19c] According to a third embodiment of the invention, there is provided a recombinant vector comprising the DNA of the second embodiment.

[19d] According to a fourth embodiment of the invention, there is provided a host cell comprising the recombinant vector of the third embodiment.

[20]

Advantageous Effects of Invention

[21] The fusion protein according to the present invention has an improved *in-vivo* half-life compared to the natural type FVII while retaining a high biological activity of FVII, and thus can be effectively employed in the therapy using FVII.

[22]

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Brief Description of Drawings

[23] The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings, which respectively show:

[24]

[25] Fig. 1 is a schematic diagram showing a cloning procedure for constructing FVII-Tf expression vector from a vector comprising a cDNA coding for FVII sequence and a vector comprising a cDNA coding for transferrin (Tf) sequence;

[26] Fig. 2 is a schematic diagram showing a procedure for constructing FVII-GS1 (linker)-Tf expression vector by overlapping PCR;

[27] Fig. 3 is a schematic diagram showing a construction procedure of FVII-GS linker-Tf expression vectors comprising GS3, GS5, GS7, GS9, GS11, GS13, GS15 or GS-1-T as a linker;

[28] Fig. 4 displays Western blot results of VII-Tf, FVII-GS1-Tf, FVII-GS3-Tf, FVII-GS5-Tf, FVII-GS7-Tf, FVII-GS9-Tf, FVII-GS11-Tf, FVII-GS13-Tf, FVII-GS15-Tf, FVII-GS1-T-Tf and FVII-Helix-Tf fusion proteins of the present invention, FVII-albumin fusion protein (FVII-Alb) and FVII (NovoSeven^{TM});

[29] Fig. 5 is a graph showing specific activities of FVII-Tf, FVII-GS1-Tf, FVII-GS3-Tf, FVII-GS5-Tf, FVII-GS7-Tf, FVII-GS9-Tf, FVII-GS11-Tf, FVII-GS13-Tf, FVII-GS15-Tf, FVII-GS1-T-Tf and FVII-Helix-Tf fusion proteins of the present invention, and FVII-albumin fusion protein (FVII-Alb);

[Text continues on page 4.]

[30] Fig. 6 presents the structure of a linker and restriction recognition sequences at both termini in FVII-GS1-T-Tf fusion protein; and

[31] Fig. 7 shows western blot results of purified FVII-Tf, FVII-GS1-Tf, FVII-GS1-T-Tf, FVII-GS3-Tf, and FVII-GS15-Tf fusion proteins of the present invention, NovoSeven ™ and FVII.

[32]

Best Mode for Carrying out the Invention

[33] The present invention provides a fusion protein comprising factor VII (FVII) and transferrin.

[34] The FVII and transferrin of the fusion protein of the present invention may be derived from any mammal, preferably a human. More preferably, FVII and transferrin used in the present invention may have not less than 95% of sequence homologies with those of natural type of the proteins found in human blood, respectively. Most preferably, FVII has the amino acid sequence of SEQ ID NO: 1 and transferrin has the amino aicd sequence of SEQ ID NO: 2.

[35] In addition, FVII or transferrin used in the fusion protein of the present invention may be a functional equivalent or a functional derivative of natural type thereof which has a substantially equivalent functional activity. Exemplary functional equivalents include mutants induced by deletion, insertion or non-conservative or conservative substitution of any amino acid residues, or a combination thereof in amino acid sequences represented by SEQ ID NOs: 1 and 2, respectively, in which such changes do not substantially alter active sites or domains offering biological activities to FVII.

[36] In some cases, the fusion protein of the present invention may be modified, e.g., via phosphorylation, sulfation, acrylation, glycosylation, methylation, farnesylation, acetylation, amidation, and others, for the improvement or reduction of its physical or chemical properties, and such functional derivatives also fall within the scope of the present invention so long as the biological activity of FVII is substantially retained.

[37]

[38] In the fusion protein of the present invention, transferrin is preferably linked to the C-terminus of FVII. The fusion protein in the order of FVII-transferrin is superior to a fusion protein in the order of tranferrin-FVII, probably due to the exposure of the N-terminus of the FVII (see Table 3).

[39] The fusion protein of the present invention may further comprise recognition sequence(s) for a restriction enzyme between FVII and transferrin in order to facilitate the insertion of a linker as described below. The restriction recognition sequence may be any restriction recognition sequence known to one of ordinary skill in the art, and *Age*I recognition sequence (A/CCGGT) may be preferably used. In other words, fusion

proteins, in which the restriction recognition sequence is linked to the C-terminus of FVII and transferrin is linked to the restriction recognition sequence, are included within the scope of the present invention.

[40]

[41] The present invention provides a fusion protein comprising a linker between FVII and transferrin.

[42] The linker may have 1 to 100 amino acids, preferably 1 to 75 amino acids, more preferably 5 to 25 amino acids, and it may be any peptides which can functionally separate FVII and transferrin. The linker may have a stable secondary structure such as a helix or be originated from IgG hinge region. Preferably, the linker may rotate freely in aqueous solution and does not have a fixed structure, and, therefore, it would be non-immunogenic and would increase FVII activities of fusion proteins by minimizing the potential interference between two fusion partners. As an example, such linker may be a helix linker represented by the amino acid sequence of SEQ ID NO: 11. Further, such flexible linker may contain glycine (G) and serine (S) in a repeated or random pattern. For example, the linker comprises $(GGGGS)_N$ (wherein N is an integer ranging from 1 to 20), and preferably has any one selected from the group consisting of the amino acid sequences of SEQ ID NOs: 3 to 11 (see Table 1). In addition, any amino acid sequences having not less than 80% of homologies with the linker, preferably having not less than 85% of homologies may be also used in the fusion protein of the present invention.

[43]

[44] Furthermore, the linker may also include protease cleavage site(s) which is recognized by protease(s) abundant in an injured tissue. The cleavage site may be cleaved by a protease selected from the group consisting of thrombin, factor Xa, factor IXa, and factor VIIa. The fusion protein having such protease cleavage site is cleaved at the working site to produce each protein, i.e., FVII and transferrin, and the resulting proteins function as individual proteins. Preferably, the linker has the amino acid sequence of SEQ ID NO: 12 (see Table 1).

[45]

[46] The linker may be inserted into a fusion protein more easily via the restriction enzyme recognition sequence which is located between FVII and transferrin. Accordingly, the restriction enzyme recognition sequence may be present at any one end or both ends of the linker, and be in turn translated into amino acids encoded by the sequence. For example, when *Age*I restriction enzyme recognition sequence is used, Thr may be present at the N-terminus of the linker and Thr-Gly may be present at the C-terminus of the linker. That is, when a linker $(GGGGS)_3$ is used, The recognition sequence and linker may be present in a form of --T$(GGGGS)_3$TG--. The amino acids

translated at the N- and C-termini of the linker may vary depending on the restriction enzyme recognition sequence employed, but the presence thereof does not influence the activities of the fusion proteins (see Table 5).

[47]

[48] The fusion protein of the present invention exhibits not less than 0.7 of FVII specific activity compared to the unfused natural type FVII.

[49] In an aspect of the present invention, the fusion protein, which comprises FVII represented by the amino acid sequence of SEQ ID NO: 1 and transferrin represented by the amino acid sequence of SEQ ID NO: 2, has about 0.82 to about 0.92 of FVII specific activity compared to the unfused natural type FVII (see Tables 2 and 3).

[50] In addition, the fusion protein, which comprises FVII represented by the amino acid sequence of SEQ ID NO: 1, the linker represented by the amino acid sequence of SEQ ID NO: 3 and transferrin represented by the amino acid sequence of SEQ ID NO: 2, has about 0.97 of FVII specific activity compared to the unfused natural type FVII (see Table 2).

[51] The fusion proteins according to the present invention, in which other linkers are inserted between FVII and transferrin, have also about 0.74 to about 1 of FVII specific activities compared to the unfused natural type FVII (see Table 2).

[52] Furthermore, the fusion protein of the present invention has a half-life 3~4 times longer than that of FVII with no transferrin linked thereto (see Table 6).

[53]

[54] The present invention also provides a DNA coding for the fusion protein.

[55] The DNA coding for the fusion protein may be subjected to various changes and modifications due to the codon's degeneracy or considering codons preferred in the organism to express the fusion protein, unless the amino acid sequence of the fusion protein is substantially altered, and the modified DNAs are also included in the scope of the present invention. In the present invention, the DNA coding for the fusion protein may be preferably represented by any one of the nucleotide sequences of SEQ ID NOs: 13 to 24. The DNA coding for the fusion protein of the present invention may be provided by a vector for expressing the DNA.

[56]

[57] The present invention provides a recombinant vector comprising the DNA coding for the fusion protein.

[58] The term "vector" used herein refers to a means for introducing a DNA coding for said fusion protein into a host cell and expressing the fusion protein therein. The vector may include all conventional vectors such as plasmid vectors, cosmid vectors, bacteriophage vectors, virus vectors, and others, preferably a plasmid vector.

[59] A suitable expression vector contains expression regulatory elements such as

promoters, initiation codons, termination codons, polyadenylation signals and enhancers, as well as signal sequences or leader sequences for membrane targeting or secretion, and it may be diversely prepared according to the purposes. The initiation codon and termination codon should be sure to work in an organism where the gene construct is administered, and be in-frame with the coding sequence. Further, the expression vector contains a selective marker for selecting a host cell containing the vector, and an origin of replication if the expression vector is reproducible. The vector may self-replicate or be integrated into the DNA of a host cell.

[60] Specifically, the recombinant expression vector according to the present invention may be prepared by inserting a DNA coding for the fusion protein sequence into pcDNA3.1-hygro vector.

[61]

[62] Further, the present invention provides a host cell which produces the fusion protein by transformation with said recombinant expression vector.

[63] Since the expression levels and modifications of the proteins vary depending on the type of host cells, it is preferred to choose a host cell most suitable for the purpose. Examples of host cells include mammal cells, e.g., Chinese hamster ovary (CHO) cells, human embryonic kidney cells (HEK293), hamster kidney cells (BHK 21), human liver cancer cells (Hep G2), and others, but not limited thereto.

[64] In order to transform a host cell with the recombinant expression vector according to the present invention, any method known to those of ordinary skill in the art may be employed, and the example of such method includes, but not limited to, electroporation, protoplast fusion, calcium phosphate ($CaPO_4$) precipitation, calcium chloride ($CaCl_2$) precipitation, and others.

[65]

Mode for the Invention

[66] The following Examples are given for the purpose of illustration only, and are not intended to limit the scope of the present invention.

[67]

[68] **Example 1: Preparation of factor VII (FVII) plasmid vector (pcDNA3.1-hygro-FVII)**

[69]

[70] Total RNA purified from Hep G2 cells (KCLB No. 88065) was used as a template for reverse transcription. Complementary DNA (cDNA) transcript was amplified by PCR using FVII gene specific primers, FVII-F and FVII-R (SEQ ID NOs: 25 and 26) to obtain open reading frame of human FVII gene. The PCR was performed by treating 50 μL of reaction solution (0.5 μL of cDNA, 0.4 μM (10 pmol/μL) of primers of SEQ

ID NOs: 25 and 26, 0.2 mM of dNTP, 5 unit of Taq DNA polymerase and water) under the following condition: 1 cycle of denaturation at 94°C for 5 min, 35 cycles of amplification at 94°C for 1 min, at 60°C for 1 min, and at 72°C for 2.5 min, and 1 cycle of final extension at 72°C for 5 min. The purified PCR product was cloned into pGEM-T easy vector (Promega, Cat #: A1360). Positive clones were selected by restriction digestion using *EcoR*I and *Nco*I. The Selected clones were further verified by DNA sequencing. To transfer ORF of FVII (FVII-ORF) to an expression vector, FVII-ORF cleaved with *Not*I was blunted by T4 DNA polymerase and ligated with pcDNA3.1-hygro vector (Invitrogen) digested with *Hind*III/*Xba*I and blunted. The ligated vector was confirmed by restriction digestion with *Apa*I, *Xba*I, *EcoR*I, *Nco*I, *Pst* I and DNA sequencing. This vector was designated 'pcDNA3.1-hygro-FVII'.

[71]

[72] **Example 2: Construction of FVII-Tf expression vector (pcDNA3.1-hygro-FVII-Tf)**

[73]

[74] FVII cDNA prepared in Example 1 was fused to human transferrin (Tf) cDNA in order to express as a single zymogen in an animal cell. Human tranferrin cDNA was purchased from Origene (Cat #: SC322130) and was verified whether it is equal to the sequence of GenBank accession #: NM_001063.2. Primers used in the fusion were designed to remove termination codon of FVII and signal peptide of transferrin. In order to facilitate the insertion of the varying sizes of linkers between FVII and Tf, *Age* I site (ACCGGT), which will be translated to threonine (Thr) and glycine (Gly), was added to the linking primers. The resulting fusion protein would have the following structure: (leader peptide)-(mature FVII)-(Thr-Gly)-(mature Tf) (in which the leader peptide consists of a signal peptide (prepeptide) not present in mature FVII and a propeptide to be cleaved by a processing enzyme, which is composed of 38 amino acids and corresponds to amino acids ranging from positions 1 to 38 in the amino acid sequence of SEQ ID NO:1). cDNAs of FVII and Tf were amplified by using primers FVII-S1, FVII-AS1, Tf-S1 and Tf-AS1 (SEQ ID NOs: 27 to 30) and the vector as described in Example 1 was used. The primers of SEQ ID NOs: 27 and 30 contains *Nhe*I and *Xho*I sites, respectively.

[75] A cloning strategy for linking of FVII cDNA and Tf cDNA is depicted in Fig. 1. First, FVII cDNA was amplified from pcDNA3.1-hygro-FVII vector by PCR. The PCR was performed by treating 50 μL of reaction solution (1 μL of vector template, 1 μL of a primer set, FVII-S1 and FVII-AS1(10 μM), 10 μL of 5x Phusion HF buffer, 200 μM of dNTP, 0.5 μL of Phusion DNA polymerase (FINNZYMES, #F-530S, 2 units/μL) and 35.5 μL of water) under the following condition: 1 cycle of denaturation at 98°C for 30 sec, 30 cycles of amplification at 98°C for 10 sec, at 60°C for 45 sec, and

at 72°C for 30 sec, and 1 cycle of final extension at 72°C for 7 min.

[76] Next, Tf was amplified using transferrin cDNA as a template. The above mentioned PCR procedure was repeated except for using a primer set (Tf-S1: 10 μM; Tf-AS1: 10 μM).

[77] The amplified FVII and Tf cDNA were joined by a series of restriction and ligation. Each DNA amplified by PCR was digested with *Age*I and *Xho*I, or with *Nhe*I. The digested DNAs were purified and ligated at 1:1 molar ratio. The ligated DNA was subcloned into pcDNA3.1-hygro vector (Invitrogen) digested with *Nhe*I/*Xho*I. The size and sequence of insert was further verified by DNA sequencing.

[78]

[79] **Example 3: Construction of FVII-GS linker-Tf expression vector**

[80]

[81] A peptide consisting of 5 amino acids comprising glycine and serine was used as a basic linker unit. The basic linker unit comprises four glycines and one serine with following sequence: 'GGGGS'. The basic linker unit (hereinafter, referred to "GS-X linker" in which X is the repeat number of the basic GS linker unit) was utilized to constitute the longer GS linkers. In this Example, the linkers ranging from GS-1 to GS-15 were constructed.

[82]

[83] 1) Construction of FVII-GS-1 linker-Tf expression vector

[84]

[85] A set of primers, GS-FV-AS1 and GS-Tf-S1 (SEQ ID NOs: 31 and 32), containing the basic GS linker unit was synthesized and inserted between FVII and Tf by overlapping PCR (see Fig. 2).

[86] The GS-1 linker was linked to FVII by PCR using a set of primers FVII-S1 and GS-FV-AS1 (SEQ ID NOs: 27 and 31) and Phusion DNA polymerase (FINNZYMES, #F-530S). The PCR was performed by treating 50 μL of reaction solution (1 μL of pcDNA3.1-hygro-FVII-Tf vector, 1 μL of FVII-S1 (10 pmole/1μL), 1 μL of GS-FV-AS1 (10 pmole/μL), 1 μL of 10 mM dNTP, 10 μL of 5x Phusion HF buffer, 35.5 μL of water and 0.5 μL of Phusion DNA polymerase (2 unit/μL)) under the following condition: 1 cycle of denaturation at 98°C for 30 sec, 35 cycles of amplification at 98°C for 10 sec, at 64°C for 30 sec, and at 72°C for 45 sec, and 1 cycle of final extension at 72°C for 7 min. Meanwhile, to connect the GS-1 linker to Tf, the above PCR procedure was repeated except for using a set of primers GS-Tf-S1 and Tf-AS1 (SEQ ID NOs: 32 and 30). The amplified PCR products were utilized as overlapping PCR templates. The overlapping PCR was performed by treating the reaction solution (1 μL of amplified PCR products, 1 μL of FVII-S1 (10 pmole/μL, SEQ ID NO: 27), 1 μL of antisense primer (Tf-AS1 10 pmole/μL, SEQ ID NO: 30), 10 μL of 5x Phusion HF buffer, 1 μL

of 10 mM dNTP, 34.5 μL of water, 0.5 μL of Phusion DNA polymerase (2 units/μL)) under the following condition: 1 cycle of denaturation at 98°C for 1 min, 45 cycles of amplification at 98°C for 10 sec, at 66/68°C for 30 sec, and at 72°C for 45 sec, and 1 cycle of final extension at 72°C for 7 min. The amplified overlapping PCR product was cloned into pcDNA3.1-hygro-lacZ digested with *Nhe*I and *Xho*I.

[87]

[88] 2) Construction of FVII-GS-3 linker-Tf expression vector

[89]

[90] A primer set, GS3-S and GS3-AS (SEQ ID NOs: 33 and 34), containing GS-3 and *Age*I site was synthesized. To make a GS-3 double stranded linker, the primers were annealed by heating a mixture (5 μL of GS3-S (100 pmole/μL), 5 μL of GS3-AS (100 pmole/μL), 2 μL of 10X annealing buffer (100 mM Tris-Cl [pH 8.0], 1 M NaCl, 10 mM EDTA) and 8 μL of water) at 98°C for 10 min and cooling at 25°C for 1 hour. The annealed linker was digested with *Age*I and pcDNA3.1-hygro-FVII-Tf vector prepared in Example 2 was also digested with *Age*I. The digested vector was treated with 1 μL of CIP (Calf intestinal phosphatase; NEB, #M0290S) at 37°C for 1 hour and subjected to gel extraction procedure (QIAGEN, #28704), followed by ligation at a molar ratio of 1:3 (vector : insert) using T4 DNA ligase (TAKARA, #2011A).

[91]

[92] 3) Construction of FVII-GS-5 linker-Tf to FVII-GS-15 linker-Tf expression vectors

[93]

[94] In order to construct a fusion protein expression vector containing GS-5 linker, a new strategy was implemented. FVII-Tf fusion vectors containing extended linkers were constructed by following two steps.

[95] First step is adding a synthesized double stranded (ds) GS2 linker to the previously obtained linker. After assuring the extension of linker, the linker was cut out and inserted into between FVII and Tf genes in pcDNA3.1-hygro-FVII-Tf vector. For example, to extend the GS-3 linker to GS-5 linker, the synthesized dsGS-2 linker unit of SEQ ID NO: 35 was digested with *Bgl*II and ligated with pcDNA3.1-hygro-FVII-GS3-Tf vector treated with *BamH*I and *Stu*I. Next, after confirming the extension of the linker by *BamH*1 and *Age*I digestion, the extended linker was cut out with *Age*I and subcloned into pcDNA3.1-hygro-FVII-Tf vector treated with *Age*I and CIP. FVII-Tf fusion expression vectors containing GS-7, GS-9, GS-11, GS-13 and GS-15 linkers were constructed by the same strategy (see Fig. 3).

[96]

[97] **Example 4: Construction of FVII-Tf expression vector (pcDNA3.1-hygro-FVII-GS1-T-Tf) containing a linker comprising thrombin cleavage site**

[98]

[99] A linker containing thrombin cleavage site was prepared by adjoining a GS-1 unit to both ends of thrombin recognition sequence (hereinafter, referred to "GS1-T linker"). dsGS1-T linker of SEQ ID NO: 36 (sense) was designed and synthesized to contain *Age*I sites at both ends. The dsGS1-T linker was digested with *Age*I and purified using PCR purification kit (Qiagen, cat #: 28104). The purified linker was ligated into pcDNA3.1-hygro-FVII-Tf vector treated with CIP/*Age*I.

[100]

[101] **Example 5: Construction of FVII-Tf expression vector (pcDNA3.1-hygro-FVII-Helix-Tf) containing a helix linker**

[102]

[103] A helix linker DNA was prepared by the method disclosed in U.S Laid-open Publication No. 2009/0170163. *Age*I site was added to the both ends of the prepared helix linker DNA by using primers, Helix linker S and Helix linker AS (SEQ ID NOs: 37 and 38). The primers Helix linker S and Helix linker AS were annealed and digested with *Age*I, followed by insertion into pcDNA3.1-hygro-FVII-Tf vector treated with *Age*I and CIP. The constructed vector was confirmed by DNA sequencing.

[104]

[105] **Comparative Example 1: Construction of FVII-albumin fusion expression vector (FVII-Alb)**

[106]

[107] The FVII-albumin fusion protein disclosed in EP Patent No. 1816201 was constructed. Human albumin cDNA was obtained by RT-PCR using human liver mRNA (Clontech) as a template and albumin gene-specific primers, Albumin-S and Albumin-AS (SEQ ID NOs: 39 and 40). RT-PCR was performed by using AccuScript High Fideleity RT-PCR system kit (Cat# 600180) according to the manufacturer's manual. First, 10 μL of reverse transcription reaction solution (1 μL of 10X Reverse Transcriptase buffer, 0.6 μL of oligo-dT primer, 1 μL of dNTP, 0.4 μL of water, 5 μL of human liver mRNA (10 ng/μL)) was kept at 65°C for 5 min and at a room temperature for 5 min, and was subjected to a reaction with 1 μL of 100 mM DTT and 1 μL of Reverse Transcriptase at 42°C for 1 hour. A human albumin sequence was obtained by PCR using the synthesized cDNA as a template and primers Albumin-S and Albumin-AS. PCR was performed by treating 50 μL of a reaction solution (1 μL of cDNA, 10 μL of 5x Phusion HF buffer, 1 μL of primers Albumin-S and Albumin-AS, respectively, 1 μL of 10 mM dNTP, 0.5 μL of Phusion DNA polymerase (FINNZYMES, #F-530S; 2 units/μL) and 35.5 μL of water) under the following condition: 1 cycle of denaturation at 98°C for 1 min, 30 cycles of amplification at 98°C for 10 sec, at 62°C for 30 sec, and at 72°C for 60 sec, and 1 cycle of final extension at 72°C for 7 min. The

synthesized oligonucleotides of SEQ ID NOs: 41 and 42 were annealed to the GS linker [SS(GGS)$_9$GS] (SEQ ID NO: 45) disclosed in EP Patent No. 1816201. To connect the FVII cDNA prepared in Example 1 to the above linker, FVII termination codon in pcDNA3.1-hygro-FVII vector was replaced with *Xho*I site by PCR-based mutagenesis using primers mut FVII(XhoI)-S and mut FVII(XhoI)-AS (SEQ ID NOs: 43 and 44). Using *Xho*I/*Apa*I sites, the GS linker [SS(GGS)$_9$GS] was fused to 3' end of FVII cDNA in pcDNA3.1-hygro-FVII vector. Finally, the human albumin cDNA digested with BamHI was inserted into pcDNA3.1-hygro-FVII-GS-linker vector. The prepared pcDNA3.1-hygro-FVII-GS-linker-albumin expression vector was verified by DNA sequencing.

[108]

[109] The characteristics of expression vectors constructed in Examples 2 to 5 and Comparative Example 1 were shown in Table 1.

[110] Table 1

[Table 1]

FVII fusion protein	C-terminus of FVII	Linker sequence (SEQ ID NO)	N-terminus of fusion partner	Number of amino acids in linker
FVII-Tf	APFP		VPDKTV	0
FVII-GS1-Tf	APFP	GGGGS (SEQ ID NO: 3)	VPDKTV	5
FVII-GS3-Tf	APFP	$(GGGGS)_3$ (SEQ ID NO: 4)	VPDKTV	15
FVII-GS5-Tf	APFP	$(GGGGS)_5$ (SEQ ID NO: 5)	VPDKTV	25
FVII-GS7-Tf	APFP	$(GGGGS)_7$ (SEQ ID NO: 6)	VPDKTV	35
FVII-GS9-Tf	APFP	$(GGGGS)_9$ (SEQ ID NO: 7)	VPDKTV	45
FVII-GS11-Tf	APFP	$(GGGGS)_{11}$ (SEQ ID NO: 8)	VPDKTV	55
FVII-GS13-Tf	APFP	$(GGGGS)_{13}$ (SEQ ID NO: 9)	VPDKTV	65
FVII-GS15-Tf	APFP	$(GGGGS)_{15}$ (SEQ ID NO: 10)	VPDKTV	75
FVII-GS1-T-Tf	APFP	GGGGSLVPRGSGGGS (SEQ ID NO: 12)	VPDKTV	15
FVII-Helix-Tf	APFP	$GA(EAAAK)_4A$ (SEQ ID NO: 11)	VPDKTV	23
FVII-Alb	APFP	$SS(GGS)_9GS$ (SEQ ID NO: 45)	DAHK	31
* For FVII-Tf, Thr-Gly derived from *Age*I is present.* For the linkers of SEQ ID NOs: 4 to 12, Thr derived from *Age*I is present at the N-terminus, and Thr-Gly derived from *Age*I is present at the C-terminus.				

[111]

[112] **Experimental Example 1: Measurement of specific activities of FVII-fusion proteins**

[113]

[114] The FVII-fusion proteins constructed in Example 2 to 5 and Comparative Example 1 were expressed in a CHO cell (CHO(VK2)) which stably expresses VKORC1 (vitamin K epoxide reductase complex subunit 1).

[115] The expression vectors constructed in Example 2 to 5 and Comparative Example 1 were purified by using Endo-free plasmid maxi kit (Qiagen, #27104). β-galactosidase was used as an internal control for transfection. CHO (VK2) cells were seeded at a density of 1.5×10^6 cells/well in 6-well plates. The cells were incubated in α-MEM (Lonza, #12-169F) supplemented with 10% FBS (Lonza, #14-501F), 1X HT

(Invitrogen,#11067-030), 4 mM L-glutamine (Lonza, #17-605E) and 200 μg/mL of hygromycin (Invitrogen, #10687-010) for 24 hours, and then transfected using lipofectamine 2000 (Invitrogen) according to the manufacturer's manual. Four hours after transfection, the medium was replaced with serum-free medium (OptiMEM), and 5 μg/mL of vitamin K was supplemented. After 48 hours of incubation, the culture medium was sampled and stored at -70°C.

[116] FVII-fusion proteins expressed were analyzed for their chromogenic activities and antigen amounts by using COATEST factor VII assay kit (Chrmogenix, #821900-63) and FVII ELISA kit (Cedarlene Lab, #CL20030K), respectively. The assays were performed according to the manufacturer's manual. Standard human plasma normalized against WHO standard was used as a control FVII in both assays. The expression of protein was assessed via western blot analysis. Equal amounts of FVII fusion proteins were loaded based on the ELISA results. The expressed FVII-fusion proteins were found to have the expected sizes without detectable fragmentation (see Fig. 4).

[117] Meanwhile, the specific activities of FVII-transferrin fusion proteins were 0.74 to 1, which were higher compared to that of FVII-albumin fusion protein (0.52) (see Table 2). The FVII-transferrin fusion proteins containing linkers also retained not less than 70% of FVII activities. There was no relationship between the linker lengths and the specific activities, but FVII fusion proteins with shorter GS linkers showed somewhat higher specific activities than fusion proteins with longer ones. In particular, FVII-GS1-Tf and FVII-GS1-T-Tf fusion proteins showed comparable specific activities (see Fig. 5).

[118] Table 2

[Table 2]

FVII fusion protein	Antigen (%)	Activity (%)	Specific activity (activity/antigen)
FVII-Tf	53.2 ± 5.0	43.9 ± 0.3	0.82
FVII-GS1-Tf	53.4 ± 3.1	52.0 ± 0.5	0.97
FVII-GS3-Tf	61.9 ± 8.0	57.7 ± 0.2	0.93
FVII-GS5-Tf	69.3 ± 5.6	55.9 ± 1.4	0.81
FVII-GS7-Tf	70.9 ± 8.2	59.3 ± 1.1	0.84
FVII-GS9-Tf	64.2 ± 8.6	47.5 ± 0.7	0.74
FVII-GS11-Tf	59.1 ± 3.9	45.3 ± 0.9	0.77
FVII-GS13-Tf	59.7 ± 5.1	49.1 ± 0.8	0.82
FVII-GS15-Tf	59.2 ± 6.0	50.2 ± 0.5	0.85
FVII-GS1-T-Tf	70.8 ± 8.7	71.0 ± 2.6	1.00
FVII-Helix-Tf	89.0 ± 5.7	78.9 ± 2.2	0.89
FVII-Alb	106.6 ± 5.4	54.9 ± 3.3	0.52

[119]

[120] **Example 6: Characterization of FVII fusion proteins according to the direction of Tf fusion**

[121]

[122] In this Example, a fusion protein in which human transferrin (Tf) is linked to N-terminus of FVII was prepared and compared with a fusion protein in which transferrin is linked to the C-terminus of FVII, in order to examine the change of characteristics according to the direction of fusion in fusion proteins. Detailed procedure is as follows.

[123]

[124] <6-1> Construction of Tf-FVII and Tf-GS1-T-FVII expression vectors

[125]

[126] Two fusion proteins with Tf linked to N-terminus of FVII were designed as follows: (1) (leader peptide of Tf)-(mature Tf)-(Thr-Gly)-(mature FVII); and (2) (leader peptide of Tf)-(mature Tf)-(Thr)-(GS1-T; SEQ ID NO: 12)-(Thr-Gly)-(mature FVII).

[127] First, in order to obtain a Tf gene sequence containing a leader peptide, a forward primer (Nhe-Tf: SEQ ID NO: 46) was designed to contain *Nhe*I site for the purpose of cloning and a reverse primer (Tf-Age: SEQ ID NO: 47) was designed to contain *Age*I site for the purpose of removing the termination codon of transferrin and cloning. For cloning of mature FVII with leader peptide removed, a forward primer (Age-FVII:

SEQ ID NO: 48) was designed to contain *Age*I site and a reverse primer was designed to contain *Xho*I site.

[128] For Tf gene, cDNA purchased from Origene (Cat #: SC322130) as in Example 2 was used as a PCR template. The PCR was performed by treating 50 μL of a reaction solution (1 μL of vector template, 2 μL of primers *Nhe*-Tf and Tf-*Age*I (10 μM), 10 μL of 5× Phusion HF buffer, 1 μL of 10 mM dNTP, 0.5 μL of Phusion DNA polymerase (FINNZYMES, #F-530S, 2 units/μL) and 33.5 μL of water) under the following condition: 1 cycle of denaturation at 98℃ for 30 sec, 25 cycles of amplification at 98℃ for 10 sec, at 70℃ for 30 sec, and at 72℃ for 36 sec, and 1 cycle of final extension at 72℃ for 10 min. FVII was amplified by PCR using pcDNA3.1-hygro-FVII-GS1-T-Tf vector as a template as in Example 4. The PCR conditions were same with the above Tf PCR conditions, except for using primers Age-FVII (10 μM) and VII-Xho (10 μM).

[129] The amplified Tf gene was inserted into pcDNA3.1-hygro-FVII-GS1-T-Tf vector by using *Nhe*I/*Age*I to obtain pcDNA3.1-hygro-Tf-Tf vector. The pcDNA3.1-hygro-Tf-Tf vector and the FVII PCR product were digested with *Age*I/*Xho*I and ligated to construct an expression vector containing pcDNA3.1-hygro-Tf-FVII fusion protein. pcDNA3.1-hygro-Tf-GS1-T-FVII expression vector was constructed by inserting the dsGS1-T sequence synthesized in Example 4 via *Age*I. The constructed expression vectors were confirmed by restriction mapping and DNA sequencing.

[130]

[131] <6-2> Expression of fusion proteins and characterization

[132]

[133] In order to characterize fusion proteins with Tf linked to the N-terminus of FVII, the expression vectors thereof, i.e., pcDNA3.1-hygro-FVII-Tf, pcDNA3.1-hygro-FVII-GS1-Tf-VII, pcDNA3.1-hygro-Tf-FVII and pcDNA3.1-hygro-Tf-GS1-T-FVII, were transiently expressed in CHO cells.

[134] The constructed four plasmid DNAs were isolated using Endo-free maxi prep kit (Qiagen). On one day before transfection, CHO (DG44) cells cultured in T75 flasks were isolated by trypsin, and seeded at a density of 1.5×10^6 cells/well in 6-well plates. After 24 hours, the cells were transfected according to the manufacturer's manual. Four hours after transfection, the medium in each well was removed and replaced with 2 mL of a growth medium supplemented with 5 μg/mL of vitamin K. After transfection, the 6-well plate was incubated in a 37℃, 5% CO_2 incubator, and after 48 hours, the medium was harvested. The harvested supernatant was transferred to 1.5 mL tubes and stored -70℃ for chromogenic assay and ELISA of FVII. The plate with the medium removed was washed with 2 mL of HBSS per a well and lysed with 250 μL of a lysis solution (Tropix, #ABX210LM, 1 mM DTT addition), followed by being stored at -70℃ for β-galactosidase assay.

[135] The chromogenic assay and FVII ELISA were performed as in Experimental Example 1. Samples for analysis were prepared by thawing the frozen-stored media following the transfection just before the experiment and obtaining the supernatant via centrifugation. Standard human plasma (Dade Behring, # ORKL13, Lot#503216F) was used as a standard in the assay.

[136]

[137] The measurement results are shown in Table 3. For Tf-FVII and Tf-GS1-T-Tf with Tf linked to N-terminus of FVII, no activities were measured unlike FVII-Tf and FVII-GS1-T-Tf with Tf linked to the C-terminus of FVII. Further, low amounts of fusion proteins with Tf linked to N-terminus were detected in FVII ELISA. However, the fusion proteins showed similar detection sensitivities, irrespective of fusion directions, in western blot results using polyclonal antibodies of Tf. The results indicate that, when Tf is linked to N-terminus of FVII, fusion proteins with no FVII activities were generated, even though translations of amino acids were normally conducted.

[138] Table 3

[Table 3]

Fusion protein	FVII activity (%)	FVII antigen (%)	Specific activity (activity/antigen)
FVII-Tf	33.8 ± 0.73	37.0 ± 2.17	0.92
Tf-FVII	not detected	8.0 ± 1.51	-
FVII-GS-1-T-Tf	46.6 ±0.29	43.0 ± 4.75	1.08
Tf-GS-1-T-Tf	not detected	13.5 ± 1.01	-

[139]

[140] **Example 7: Characterization of fusion proteins according to the deletion of restriction enzyme recognition sequence used in the fusion**

[141]

[142] In Example 2, restriction enzyme (*Age*I) recognition sequences were used to facilitate the insertion of various linkers between FVII and Tf. As a consequence, some fusion proteins became to have Thr and Gly which are encoded by above restriction enzyme, at both ends of the linkers. In this Example, it was examined whether the properties of fusion proteins are altered or not by the presence of the restriction enzyme (*Age*I) recognition sequence.

[143] The restriction enzyme recognition sequences were deleted from FVII-GS1-T-Tf fusion protein containing GS1-T linker, by PCR-based site-directed mutagenesis using mutagenic primers. As shown in Fig. 6, this experiment was designed to delete "Thr" at the N-terminus and "Thr-Gly" at the C-terminus and primers used in the experiment

are listed in Table 4.

[144] Table 4

[Table 4]

Primer	Sequence (5'→3')	SEQ ID NO
TG del-S	CAG CGG AGG CGG TTC AGT CCC TGA TAA AAC TG	50
TG del-AS	CAG TTT TAT CAG GGA CTG AAC CGC CTC CGC TG	51
T del-S	CGA GCC CCA TTT CCC GGT GGA GGC GGA TC	52
T del-AS	GAT CCG CCT CCA CCG GGA AAT GGG GCT CG	53

[145]

[146] <7-1> Deletion of Thr-Gly

[147]

[148] PCR-based mutagenesis was conducted. The PCR was performed by treating a reaction solution (1 μL of pcDNA3.1-hygro-FVII-GS1-T-Tf vector, 0.2 μL of sense primer (TG del-S 10 μM), 0.2 μL of antisense primer (TG del-AS 10 μM), 1 μL of 10 mM dNTP, 4 μL of 5X PCR buffer, 14 μL of water, and 0.2 μL of Phusion DNA polymerase (FINNZYMES, #F-530S)) under the following condition: 1 cycle of denaturation at 98℃ for 30 sec, 18 cycles of amplification at 98℃ for 10 sec, at 58℃ for 30 sec, and at 72℃ for 3 min, and 1 cycle of final extension at 72℃ for 7 min. In order to remove the original template DNA, the amplified PCR product was treated with 1 μL of *Dpn*I (NEB, #R0176S) and incubated at 37℃ for 1 hour. 50 μL of HIT competent cell (DH5α, RH617) was transformed using 10 μL of the *Dpn*I-treated DNA and incubated in at an LB+amp (10 mg/mL) solid medium overnight. Four clones thus obtained were analyzed by DNA sequencing and two clones were verified as mutants.

[149]

[150] <7-2> Deletion of Thr

[151]

[152] In order to delete Thr, the similar method as in Example <7-1> was conducted by using different primers. Briefly, PCR-based mutagenesis was performed by using, as a template, 1 μL of plasmid DNA of the clones in which the mutation was confirmed in Example <7-1> and 1 μL of sense primer (T del-S; 10 pmole) and 1 μL of antisense primer (T del-AS; 10 pmole), under the same condition. By DNA sequencing of four clones which were selected, three clones were confirmed to have mutations. The secured expression vector was named "pcDNA3.1-hygro-FVII-GS1-T-Tf(M3)".

[153]

[154] <7-3> Characterization of fusion proteins with restriction enzyme recognition sequences deleted

[155]

[156] CHO cells were transfected with FVII-GS1-T-Tf and FVII-GS1-T-Tf(M3) expression vectors and FVII-Alb expression vector as a control, and the supernatants of the media were obtained. The obtained supernatants were subjected to FVII chromogenic assay (Chromogenix) and FVII ELISA (cedarlane) to verify the change in the ratio of activity/antigen. As shown in Table 5, the antigen amounts and activities of FVII-GS1-T-Tf and FVII-GS1-T-Tf(M3) fusion proteins were almost equal each other, and the ratios (specific activities) also did not vary. In addition, the specific activities were confirmed to be significantly higher than that of FVII-Alb fusion protein.

[157] Table 5

[Table 5]

	FVII antigen (%)	FVII activity (%)	Specific activity (activity/antigen)
FVII-GS1-T-Tf	34.1 ± 2.1	39.6 ±2.2	1.16
FVII-GS1-T-Tf(M3)	33.5 ±4.7	38.0 ±0.7	1.14
FVII-Alb	50.1 ±1.2	26.3 ±0.8	0.53

[158]

[159] **Example 8: Measurement of half-life of fusion proteins**

[160]

[161] In order to examine the increase of half-life in the fusion proteins according to the present invention, FVII-Tf, FVII-GS1-Tf, FVII-GS3-Tf, FVII-GS15-Tf and FVII-GS1-T-Tf were used as experimental groups, and a wild type FVII and commercially available FVIIa (NovoSeven®; Novo Nordisk) were used as control groups.

[162]

[163] <8-1> Sample preparation

[164]

[165] 1) Securing expression medium

[166]

[167] Wild type FVII protein and five Tf-fused FVII fusion proteins were expressed in FreeStyle™ CHO-S cell line (Invitrogen, Cat. no. R800-07). The CHO-S cells were cultured in suspension in a spinner flask with freestyle CHO expression medium supplemented with 8 mM L-glu (GIBCO, L-glutamine 200 mM (100X), Cat. No. 25030-081). The cultured cells were seeded at a density of 4×10^5 cells/mL before 24 hours to transfection, and were transfected when the density becomes 1×10^6 cells/mL.

The DNAs used in the transfection were prepared by using Endo-free maxi prep kit (QIAGEN, Cat. No.12362) or Endo-free plasmid mega prep kit (QIAGEN, 12381), and the transfection was conducted in reference to the transfection protocol of FreeStyle MAX Reagent (Invitrogen, Cat. No. 16447-100). 500 μg of DNA was added to 8 mL of OptiPRO SFM (Invitrogen, Cat. No. 12309-019) and mixed. To another tube, 8 mL of OptiPRO SFM (Invitrogen, Cat. No. 12309-019) and 500 μL of FreeStyle Max Reagent was added, and then the above two mixture was slowly mixed and stored at room temperature for 10 min. After 10 min, FreeStyle™ CHO-S cells were transfected with the mixture. The transfected cells were cultured in a 37℃, 5% CO_2 incubator for 3~5 days, and then the supernatant was obtained.

[168]

[169] 2) Purification of expression medium

[170]

[171] The medium obtained by a spinner flask culture was filtered through 0.22 μm of filter (Corning) to remove remaining cells and debris. The filtered medium was concentrated 10-fold by ultrafiltration using tangential-flow membrane (satorious, 30KDa). The concentrated medium was applied to an XK16/20 column (GE healthcare) charged with Ceramic Hydroxyapatite (BIO-RAD, 157-0040) resin. The Ceramic Hydroxyapatite column was equilibrated with more than 10 column volume of equilibration buffer (25 mM imidazole, 0.02% Tween 80 and 150 mM NaCl, pH 6.5). After the concentrated medium was loaded, the column was washed with the equilibration buffer and wash buffer-1 (25 mM imidazole, 0.02% Tween 80, 100 mM sodium phosphate, pH 6.3) and wash buffer-2 (25 mM imidazole, 0.02% Tween 80, 100 mM sodium phosphate, 1M NaCl, pH 6.3). After washing, the fusion protein captured to the column was eluted with an elution buffer (25 mM imidazole, 0.02% Tween 80, 500 mM sodium phosphate, pH 6.3). The eluted protein was analyzed by FVII-chromogenic assay, FVII ELISA assay and SDS-PAGE/western blot.

[172]

[173] <8-2> Western blot assay

[174]

[175] FVII and FVII/Tf fusion proteins partially purified via two-step columns were confirmed to have 45% or more of purities by SDS-PAGE/Coomassie Blue staining. The presence of FVII-derived fragments in the purified proteins was assessed by western blot, since the fragmented FVIIs in purified fusion proteins might have shorter half-life than intact FVII fusion protein and mislead the determination of half-life of each FVII fusion protein. NovoSeven®(Novo Nordisk, 1.2 mg/vial, 60 KIU) and the purified samples were prepared at 0.1 IU (FVII activity)/10 μL, and then SDS-PAGE was conducted by using NuPage 4-12% bis-Tri gel (Invitrogen). After the completion

of electrophoresis, the gel was transferred to a PVDF membrane and the membrane was blocked at room temperature for 1 hour by adding 10 mL of blocking buffer (25 mM Tris, 150 mM NaCl (pH 7.2), 5% skin milk and 0.1% Tween 80). The blocking solution was decanted, and 10 mL (5% skim milk in PBS-T) of anti-FVII antibody (Cat. No. F8146, Sigma) or mouse anti-transferrin antibody (sc52256, santa cruz) was added at a ratio of 1:5000 and 1:500, and incubated for 1 hour in a rocking shaker. The membrane was washed four times with a washing solution (25 mM Tris, 150 mM NaCl, pH 7.2) and incubated for 1 hour in 10 mL (5% skim milk in PBS-T) of solution in which goat anti-mouse IgG-HRP antibody (Cat. No. G21040, Invitrogen) as a secondary antibody has been added at a ratio of 1:50,000. After the membrane was washed four times with a wash solution (25 mM Tris, 150 mM NaCl, pH 7.2), 2 ml of Super-signal west Femto mix (Thermo) was added onto it for 5 min. After the completion of the reaction, the film was developed.

[176] The western blot results were shown in Fig. 7. As shown in Fig. 7, no FVII-derived fragments were detected in the purified proteins. No fragmented transferrins were detected on the blot probed by anti-transferrin antibody.

[177]

[178] <8-3> Measurement of half-life

[179]

[180] The half-lives of the fusion protein having no linker, the fusion proteins having four linkers (GS1, GS1-T, GS3 and GS15), and a wild type FVII expressed and purified under the same condition and a commercially available NovoSeven as controls were measured and compared each other in rats. The quantitative analysis of FVII amount in samples to be administered and samples collected from animal experiment was conducted by human FVII ELISA (Cedarlane, Paired Antibodies for ELISA factor VII, #CL20030K), according to the manufacturer's instruction. The concentrations of samples to be administered were determined by averaging the values from three different dilutions of a sample. Administration dilution solution (NaCl 3 mg/mL, $CaCl_2$ dihydrate 1.5 mg/mL, glycylglycine 1.3 mg/mL, polysorbate 80 0.1 mg/mL and mannitol 30 mg/mL, pH 5.5) was used as a diluent. After FVII ELISA quantification, each protein was diluted with the administration dilution solution, and the diluted sample was intravenously administered to rats (250~300 g of Sprague Dawley, three rats per group) via tail vein at 150 IU/kg based on the weights of rats measured on the day of experiment. The bloods were taken at total eleven time points, i.e., 0 min, 5 min, 15 min, 30 min, 60 min, 1.5 hour, 2 hour, 4 hour, 6 hour, 8 hour and 24 hour after administration of the drug. 225 μL of the blood and 25 μL of 3.2% sodium citrate were mixed and centrifuged at 4°C and 13,000 rpm for 1 min, followed by storing the supernatant at -70°C. Rat plasma was analyzed by dilution of 1/50 or 1/100 with a wash

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buffer used in FVII ELISA kit (cedarlane). Regression curve was obtained by plotting logarithm of human FVII antigen concentration versus the time points of sampling. The half-life of each FVII was determined by calculating from the formula 'half-life = ln2 / slope of regression curve'. As shown in Table 6, the fusion proteins of the present invention showed 3~4 folds of improved half-life compared to wild type FVII.

[181] Table 6

[Table 6]

Type	Half-life (min)
FVII-GS1-Tf	254.2 ± 19.1
FVII-GS3-Tf	227.4 ± 23.5
FVII-GS1-T-Tf	235.4 ± 27.4
FVII-GS15-Tf	257.0 ± 23.9
FVII-Tf	277.0 ± 24.5
NovoSeven	80.3 ± 27.4
Natural type FVII	59.6 ± 2.9

[182] The term "comprise" and variants of the term such as "comprises" or "comprising" are used herein to denote the inclusion of a stated integer or stated integers but not to exclude any other integer or any other integers, unless in the context or usage an exclusive interpretation of the term is required.

[183] Any reference to publications cited in this specification is not an admission that the disclosures constitute common general knowledge in Australia.

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CLAIMS

1. A fusion protein comprising factor VII (FVII), transferrin and a linker between the FVII and the transferrin, wherein said transferrin is linked to the C-terminus of said FVII.

2. The fusion protein of claim 1, wherein said FVII is a polypeptide represented by the amino acid sequence of SEQ ID NO: 1.

3. The fusion protein of claim 1 or claim 2, wherein said transferrin is a polypeptide represented by the amino acid sequence of SEQ ID NO: 2.

4. The fusion protein of any one of claims 1 to 3, which further comprises a restriction enzyme recognition sequence between C-terminus of said FVII and said transferrin.

5. The fusion protein of claim 4, wherein the linker consists of 1 to 100 amino acids.

6. The fusion protein of claim 5, wherein the linker consists of 1 to 75 amino acids.

7. The fusion protein of claim 5, wherein the linker consists of 5 to 25 amino acids.

8. The fusion protein of any one of claims 5 to 7, wherein one or more amino acids translated from the restriction enzyme recognition site are present at one end or both ends of the linker.

9. The fusion protein of claim 5, wherein the linker is a peptide represented by any one of the amino acid sequences of SEQ ID NOs: 3 to 11.

10. The fusion protein of claim 5, wherein the linker comprises a protease cleavage site, which is capable of being cleaved by a protease selected from the group consisting of thrombin, factor Xa, factor IXa, and factor VIIa.

11. The fusion protein of claim 10, wherein the linker is a peptide represented by the amino acid sequence of SEQ ID NO: 12.

12. The fusion protein of any one of claims 1 to 11, wherein the fusion protein has not less than 0.7 of FVII specific activity compared to an unfused natural type FVII.

13. A DNA coding for the fusion protein of any one of claims 1 to 12.

14. The DNA of claim 13, wherein the DNA is represented by any one of the nucleotide

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sequences of SEQ ID NOs: 13 to 24.

15. A recombinant vector comprising the DNA of claim 13 or claim 14.

16. A host cell comprising the recombinant vector of claim 15.

17. The host cell of claim 16, which is selected from the group consisting of CHO cell, BHK21 cell, HEK293 cell and Hep G2 cell.

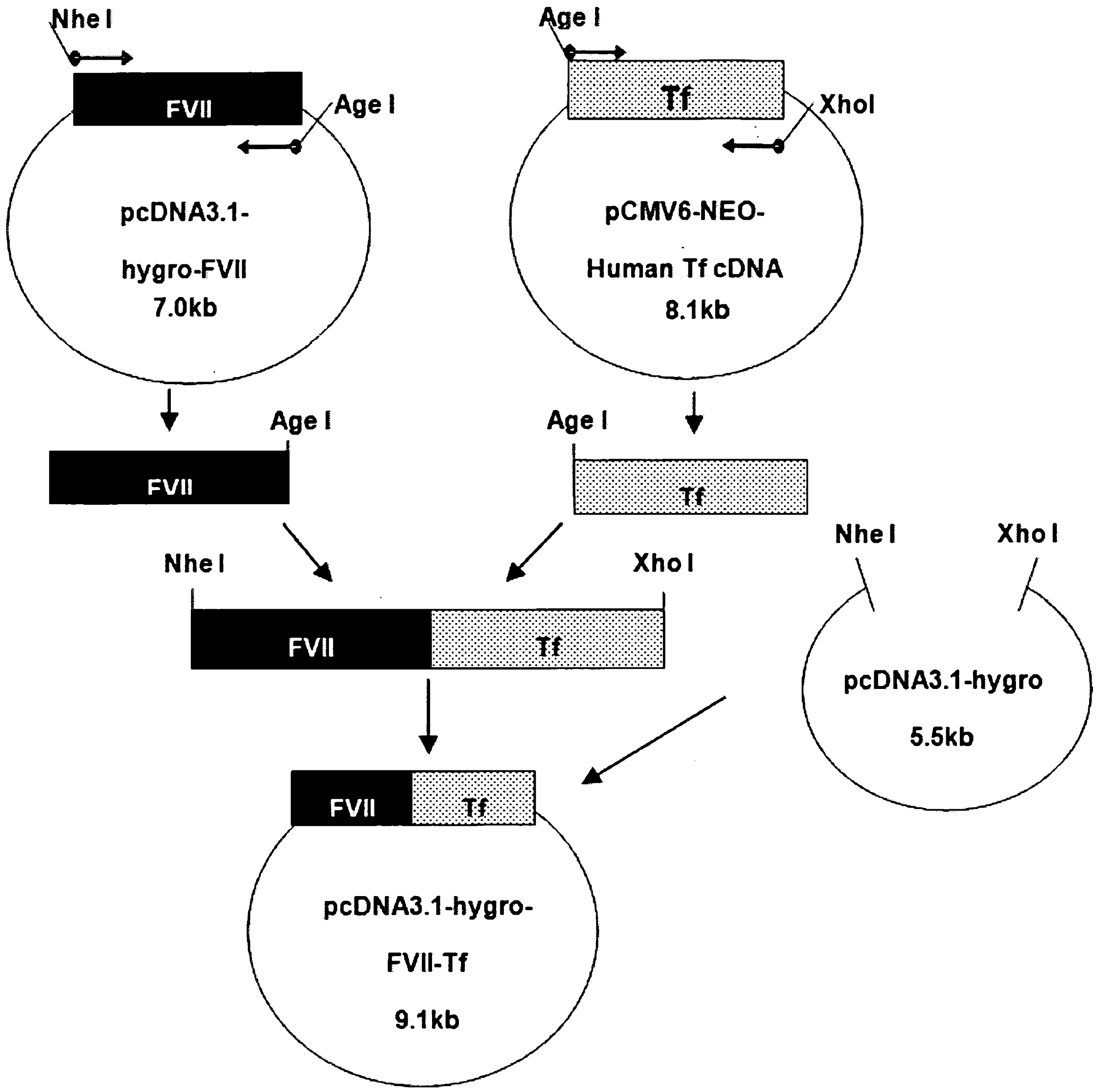
18. A fusion protein comprising factor VII (FVII), transferrin and a linker between the FVII and the transferrin, as defined in claim 1 and substantially as hereinbefore described with reference to the accompanying examples, excluding comparative examples.

19. A DNA coding for the fusion protein of claim 18.

20. A recombinant vector comprising the DNA of claim 19.

Dated: 6 November 2015

[Fig.1]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pcDNA3.1-
hygro-FVII
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Age I
Tf
XhoI
pCMV6-NEO-
Human Tf cDNA
8.1kb
Age I
FVII
Age I
Tf
Nhe I
Xho I
FVII
Tf
Nhe I
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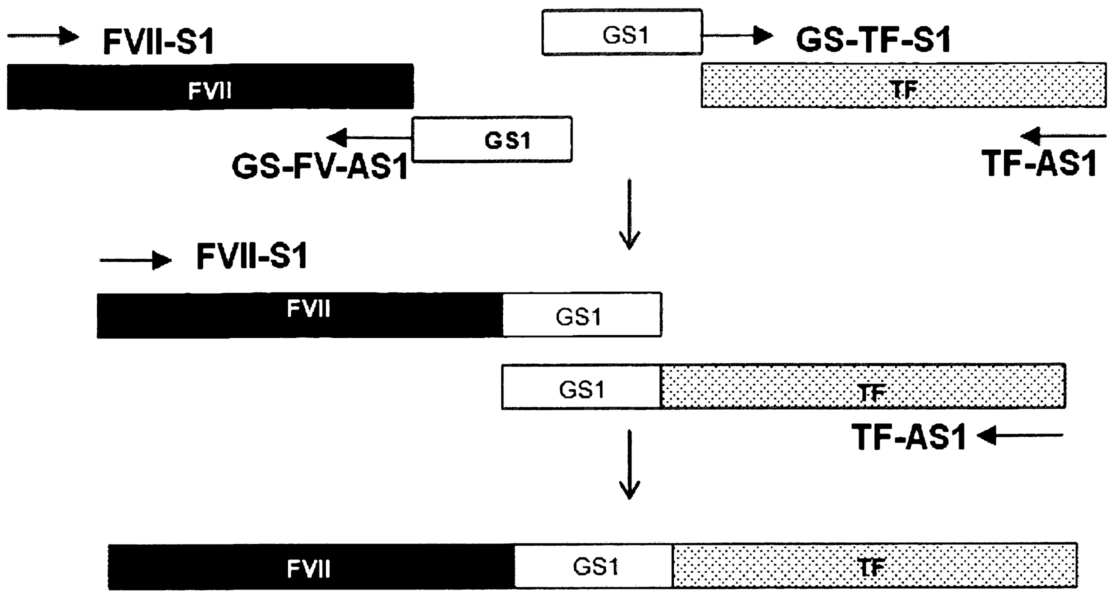
9.1kb

[Fig. 2]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GS1
GS-FV-AS1
GS1
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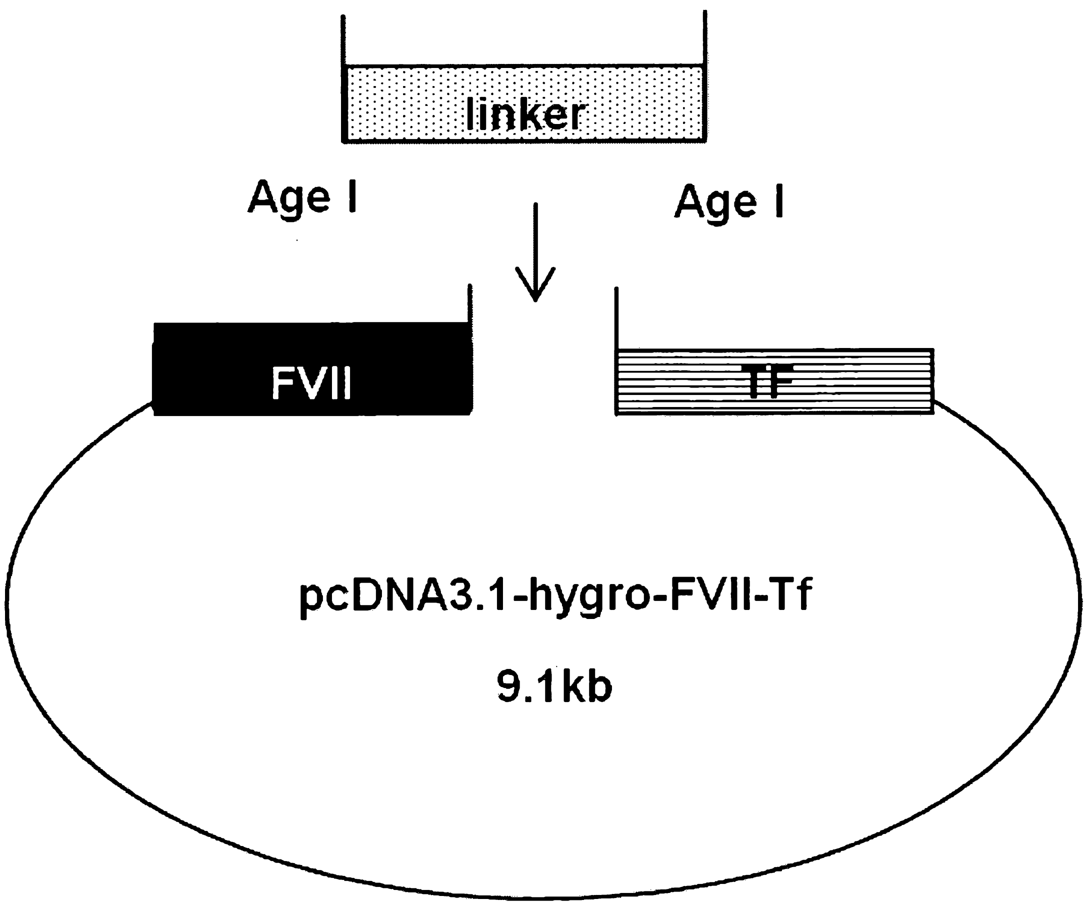
FVII
GS1
TF

[Fig.3]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FVII
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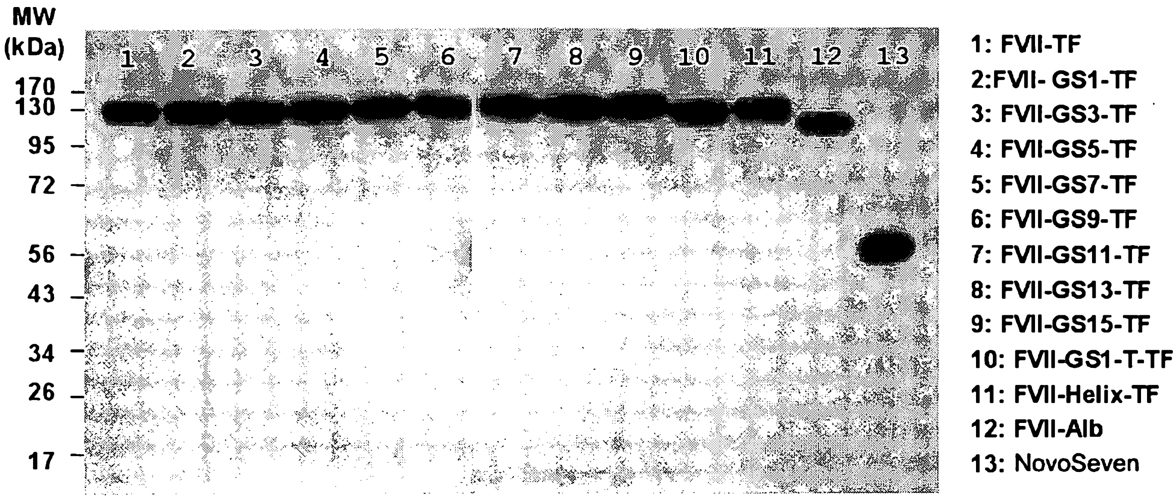
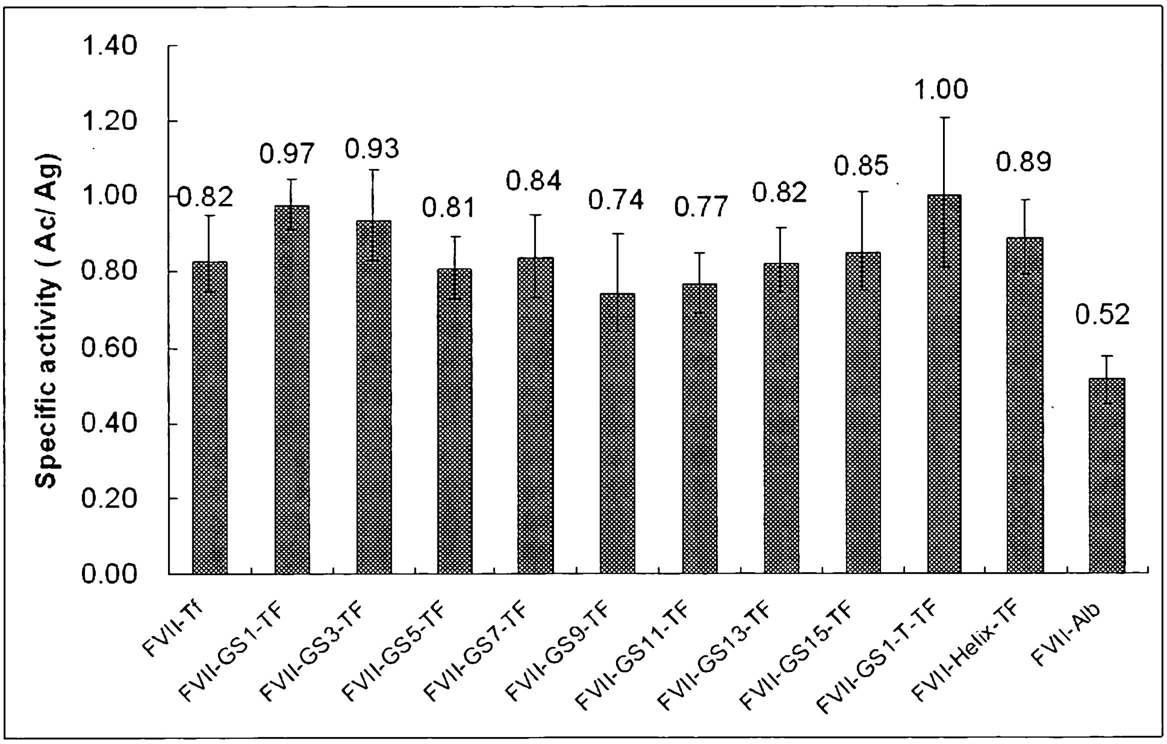
[Fig.4]



[Fig.5]



[Fig.6]

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[Fig. 7]

1: NovoSeven
2: FVII
3: FVII-Tf
4: FVII-GS1-Tf
5: FVII-GS1-T-Tf
6: FVII-GS3-Tf
7: FVII-GS15-Tf

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 65                  70                  75                  80

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Gly Ser Cys Lys Asp Gln Leu Gln Ser Tyr Ile Cys Phe Cys Leu Pro
            100                 105                 110

Ala Phe Glu Gly Arg Asn Cys Glu Thr His Lys Asp Asp Gln Leu Ile
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