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Large-scale production and properties of human plasma-derived activated Factor VII concentrate

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Vox Sanguinis

Background and Objectives An activated Factor VII (FVIIa) concentrate, prepared from human plasma on a large scale, has to date not been available for clinical use for haemophiliacs with antibodies against FVIII and FIX. In the present study, we attempted to establish a large-scale manufacturing process to obtain plasma-derived FVIIa concentrate with high recovery and safety, and to characterize its biochemical and biological properties.

Materials and Methods FVII was purified from human cryoprecipitate-poor plasma, by a combination of anion exchange and immunoaffinity chromatography, using Ca^{2+}-dependent anti-FVII monoclonal antibody. To activate FVII, a FVII preparation that was nanofiltered using a Bemberg Microporous Membrane-15 nm was partially converted to FVIIa by autoactivation on an anion-exchange resin. The residual FVII in the FVII and FVIIa mixture was completely activated by further incubating the mixture in the presence of Ca^{2+} for 18 h at 10 °C, without any additional activators. For preparation of the FVIIa concentrate, after dialysis of FVIIa against 20 mM citrate, pH 6·9, containing 13 mM glycine and 240 mM NaCl, the FVIIa preparation was supplemented with 2·5% human albumin (which was first pasteurized at 60 °C for 10 h) and lyophilized in vials. To inactivate viruses contaminating the FVIIa concentrate, the lyophilized product was further heated at 65 °C for 96 h in a water bath.

Results Total recovery of FVII from 15 000 l of plasma was ≈ 40%, and the FVII preparation was fully converted to FVIIa with trace amounts of degraded products (FVIIaβ and FVIIaγ). The specific activity of the FVIIa was ≈ 40 U/μg. Furthermore, virus-spiking tests demonstrated that immunoaffinity chromatography, nanofiltration and dry-heating effectively removed and inactivated the spiked viruses in the FVIIa. These results indicated that the FVIIa concentrate had both high specific activity and safety.

Conclusions We established a large-scale manufacturing process of human plasma-derived FVIIa concentrate with a high yield, making it possible to provide sufficient FVIIa concentrate for use in haemophiliacs with inhibitory antibodies.

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Introduction

Haemophilia A and B are congenital diseases in which patients lack Factor VIII (FVIII) and Factor IX (FIX), respectively. Replacement therapies of FVIII and FIX have been adapted to haemophiliacs using FVIII and FIX concentrates. Antibodies (inhibitors) against FVIII and FIX develop in haemophilia patients, however, and those inhibitors make replacement therapies difficult. Activated prothrombin complex concentrates (APCCs) from pooled plasma were developed in the 1980s and have been given to haemophilia patients, ensuring 'FVIII bypassing activity' for haemophiliacs with inhibitors [1,2]. The use of such concentrates containing activated coagulation factors has met with limited success, however, because there have been some cases of disseminated intravascular coagulation (DIC) and thromboembolic complications [3,4].

Human Factor VII (FVII) is a 50 000 molecular-weight (MW) glycoprotein that is synthesized by the liver as a single-chain precursor and circulates in plasma at a concentration of ≈ 0·5 μg/ml. Activated FVII (FVIIa) is a serine protease and is generated by limited proteolysis of zymogen FVII with activated Factors XIIa, Xa, IXa, VIIa itself, and thrombin. Upon binding to its receptor, cofactor tissue factor (TF), FVIIa gains full catalytic activity and activates its substrates, Factor X (FX) and Factor IX (FIX), thereby initiating the coagulation pathway. High doses of recombinant FVIIa concentrate (rFVIIa) are used to control bleeding episodes in patients with haemophilia A or B who have inhibitory antibodies against FVIII or FIX [5,6]. It is now believed that high doses of rFVIIa bind to activated platelets, exposing negatively charged phospholipids and leading to the formation of a haemostatic plug at the site of injury [7].

Previous studies, although limited and preliminary, have suggested that FVIIa derived from plasma (i.e. prepared from human plasma on a small scale) might be useful in the treatment of haemophilia patients with inhibitory antibodies [8]. We previously established a method of preparation of FVIIa on a relatively large scale (using 500–1000 l of plasma) [9] and evaluated the usefulness of FVIIa as a haemostatic agent using a primate model of haemophilia B with inhibitory antibodies [10]. The recovery of FVIIa in our process was insufficient for use in haemophiliacs with inhibitors, however, because the process could not be sufficiently optimized. Thus, an FVIIa concentrate, prepared on an industrial scale from human plasma with both high recovery and safety was, prior to the present study, not available for clinical use in haemophiliacs with inhibitors. Therefore, in the present study, to provide a sufficient quantity and quality of the FVIIa concentrate, we attempted to establish a large-scale manufacturing process with a high yield of FVIIa and to characterize its biochemical and biological properties.

The conversion of FVII to FVIIa is a key process in the production of FVIIa concentrate. In 1986, Bjoern *et al.* reported that rFVII was autocatalytically activated to rFVIIa on an anion-exchange column [11]. Pedersen *et al.* also reported that rFVII from serum-free cell culture is autoactivated in a solution containing poly-D-lysine and Ca^{2+}, and explained the activation on the basis of the autoactivation theory [12]. On an industrial scale, the activation of FVII on an anion-exchange resin is a very useful technique, because this reaction does not require any other proteases, such as Factor Xa, Factor IXa, or Factor XIIa, all of which activate FVII to FVIIa. Therefore, we established a production-scale method for the activation of FVII using autoactivation with the following two steps:

(1) activation on the anion-exchange resin; and

(2) further activation in the solution after eluting from the resin.

This method is suitable for the large-scale production of FVIIa with a high yield. Furthermore, virus-spiking tests demonstrated that immunoaffinity chromatography, nanofiltration and dry-heating of FVIIa effectively removed and inactivated the spiked virus in FVIIa, indicating that the FVIIa concentrate is safe.

Materials and methods

Materials

All chemicals used were of the highest analytical grade commercially available. FVII-depleted plasma, prothrombin-depleted plasma, human standard plasma and prothrombin time (PT) reagent were obtained from Baxter Diagnostics (Miami, FL); DEAE-Sepharose Fast Flow (DEAE-FF), Q-Sepharose Fast Flow (Q-FF), and protein A–Sepharose 4 Fast Flow were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). The TSK gel G3000SW_{XL} column was obtained from Tosoh (Tokyo, Japan). Recombinant neuraminidase was purchased from New England Biolabs (Beverly, MA).

Assay methods of coagulation factors

FVII activity was measured using the PT method with a CA-5000 automatic coagulation analyser (Sysmex, Kobe, Japan), FVII-depleted plasma and pooled normal human plasma as a reference (1 U/ml). FVIIa activity was measured using soluble tissue factor [13]. Prothrombin activity was also determined with a one-stage clotting assay using prothrombin-depleted plasma. FVII and FIX antigens were measured using enzyme-linked immunosorbent assay (ELISA) kits (Asserachrom®; Diagnostica Stago, Asnieres-Sur-Seine, France). FX antigens were also measured by sandwich ELISA using Ca^{2+}-dependent anti-FX monoclonal antibody (mAb) and peroxidase-conjugated rabbit anti-FX polyclonal antibodies (DAKO, Glostrup, Denmark). Anti-human FX mAb was developed by immunizing BALB/c mice with plasma-derived FX, using the method described by Sugo *et al.* [14].

Preparation of Ca^{2+}-dependent anti-FVII mAb-immobilized gel

Anti-FVII mAb [HFVII-1-20-25-133-19, immunoglobulin G1 (IgG1), κ subtype] was developed by immunizing BALB/c mice with plasma-derived FVII, and the resulting hybridoma cells were grown in bioreactors in serum-free medium. After the cultured cells were removed by filtration, the mAb was purified from the filtrate using a Q-FF column, followed by a protein A–Sepharose 4 Fast Flow column. The eluted mAb fraction was nanofiltrated using a Bemberg Microporous Membrane-15 nm (BMM-15; Asahi Chemical, Tokyo, Japan). For preparation of mAb-immobilized gel, Sepharose Fast Flow (Amersham Pharmacia Biotech) was activated with cyanogen bromide (Nakarai Tesque, Kyoto, Japan) and the mAb was coupled to the activated gel at a mean density of 5 mg of mAb/ml of gel [15]. Anti-FVII mAb was quantified by sandwich ELISA for murine IgG, using a modification of a previously described procedure [16]. The limit of detection for anti-FVII mAb was 0·25 ng/ml.

Purity of FVIIa and FVII

To examine the purity of FVIIa and FVII, we performed the analyses described below.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE analysis (acrylamide concentration of 12·5%) was performed using Laemmli's buffer system [17]. Following electrophoresis, proteins were visualized after staining with Coomassie Brilliant Blue R250. The intensities of the visualized bands were measured using a densitometer (CS-9000; Shimadzu, Kyoto, Japan). The amount of FVII and FVIIa was calculated as the intensities of the 50 000-MW band and the sum of the intensities of the light and heavy chains of FVIIa (molecular masses, 25 kDa and 35 kDa, respectively) in SDS–PAGE, under reducing conditions.

High-pressure gel-permeation chromatography

High-pressure gel-permeation chromatography was performed using an LC-10AS system (Shimadzu, Kyoto, Japan) and a TSK gel G3000SW_{XL} column (ø 7·8 mm × 30 cm) in 50 mM phosphate, pH 5·5, including 300 mM NaCl, at a flow rate of 0·5 ml/min. The effluent was monitored by measuring the absorbance at 280 nm (A_{280}).

Amino acid composition and N-terminal amino acid sequencing

The amino acid composition and the N-terminal amino acid sequence were analysed using an amino acid analyser (Accq·Taq; Waters, Milford, MA) and a protein sequencer (PROCISE™ protein sequencer model 492; PE Biosystems, Foster, CA), respectively.

Preparation of human plasma-derived FVIIa concentrate on a large scale

Human plasma-derived FVIIa concentrate was prepared using the following procedures in which the temperature was kept at approximately 4 °C unless stated otherwise.

Preparation of vitamin K-dependent protein-rich fraction

The ion-exchange chromatography method on Q-FF media was used to prepare a vitamin K-dependent protein-rich fraction from human cryoprecipitate-poor plasma. In brief, 3000 l of human plasma was applied to a Q-FF column (gel volume, 200 l) equilibrated with 20 mM citrate (pH 8·0) containing 0·1 M NaCl, and the bound fraction was eluted with the same buffer, containing 0·5 M NaCl (Fig. 1). For preparation of this fraction from 15 000 l of plasma, the chromatographic procedure was performed five times.

Purification of FVII

The eluted fraction was applied to an anti-FVII mAb-immobilized gel column (ø 25 cm × 2 cm) equilibrated with 50 mM Tris–HCl (pH 8·0) containing 50 mM NaCl and 5 mM $CaCl_2$. The column was then washed extensively with the same buffer, and the bound FVII fraction was eluted with 50 mM Tris–HCl, pH 7·5, containing 10 mM EDTA. The anti-FVII

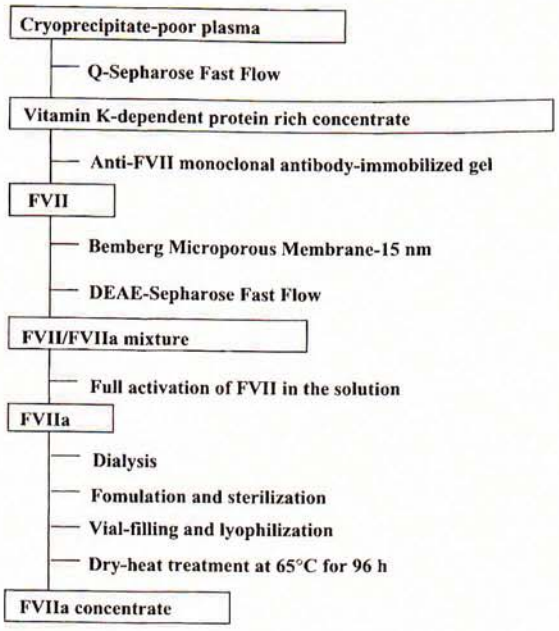

Fig. 1 Flow diagram of the purification process of activated Factor VIIa (FVIIa). See the text for details.

mAb-immobilized gel was regenerated by successive washing with the following solutions: 20 mM Tris–HCl, pH 7·25, containing 2 M NaCl; 6 M urea, pH 7·2, containing 1 M NaCl and 1% Tween-80.

Nanofiltration of FVII

The eluted FVII fraction was nanofiltered for virus elimination using BMM-15.

Partial conversion of FVII to FVIIa

Twenty-two litres of nanofiltered FVII fraction (A_{280} = 0·280–0·290), yielding ≈ 4·5 g of FVII from 15 000 l of plasma, was applied to a DEAE-FF column (ø 9 cm × 5 cm) equilibrated with 50 mM Tris–HCl, pH 7·8, containing 30 mM NaCl, at 10 °C. After washing with the same buffer, the partially activated FVII was eluted with 50 mM Tris–HCl, pH 7·8, containing 30 mM NaCl and 1·75 mM $CaCl_2$, at a linear flow rate of 4·8 cm/min.

Full activation of FVII and preparation of FVIIa

For full activation of FVII in the mixture of FVII and FVIIa, the eluate was incubated for 18 h at 10 °C. After incubation, the FVIIa preparation was dialysed against 20 mM citrate, pH 6·9, containing 240 mM NaCl and 13 mM glycine, and the dialysed FVIIa was frozen and stored at −60 °C.

Preparation of FVIIa concentrate

The frozen FVIIa preparation was thawed in a water bath at 37 °C and human albumin was added to the solution to give a final concentration of 2·5%. The albumin was pasteurized in advance at 60 °C for 10 h. Around 12 000 U/ml of FVIIa solution was sterilized by using a filter (0·22 µm), and 2·5 ml of FVIIa was placed into glass vials. Lyophilization was performed under the following conditions: freezing temperature of the vials, < −35 °C; vacuum level, 30 mTorr; shelf temperatures for primary and secondary drying, 5 °C and 30 °C; respectively. After lyophilization, the vials were stoppered under vacuum with rubber stoppers and sealed with flip caps, and they were further heated in a water bath at 65 °C for 96 h.

Virus-spiking test

To test the efficacy of removing viruses by immunoaffinity chromatography, a virus solution containing sindbis virus or poliovirus of sabin type I was added to the vitamin K-dependent protein-rich fraction at a volume ratio of 1 : 9, and the mixture was then applied to an anti-FVII mAb-immobilized gel column (ø 2·5 cm × 2·0 cm). The validity of the scale-down of immunoaffinity chromatography for the spiking test was shown by determining the recovery and purity of FVII in the eluate using SDS–PAGE analysis and high-pressure gel-permeation chromatography with TSK gel G3000 SW_{XL}. The recovery and purity of FVII were comparable to those of FVII in the manufacturing process (data not shown). Virus titre in an eluted fraction was determined by a titration method using tissue culture. In the nanofiltration process of FVII, porcine parvovirus (PPV), poliovirus, Japanese encephalitis virus (JEV), cytomegalovirus (CMV), or bovine viral diarrhoea virus (BVDV) was added to the FVII fraction at a volume ratio of 1 : 9, which was eluted from anti-FVII mAb-immobilized gel, and the mixture was then applied to BMM-15 (30 cm^2). To investigate the effect of dry-heating of FVIIa on the inactivation of possible contaminant viruses, human immunodeficiency virus (HIV), poliovirus, CMV, or BVDV were added to the FVIIa preparation at a volume ratio of 1 : 9. Furthermore, the mixture was lyophilized by methods similar to those employed in the large-scale process (e.g. glass vial, rubber stoppers, flip caps, filled volume, temperature for freezing, vacuum level, shelf temperatures for primary and secondary drying) and the lyophilized samples were heated in a water bath at 65 °C for 96 h. Titration of the viruses was performed by measuring the median (50%) tissue culture infective dose ($TCID_{50}$/ml) or plaque-forming units (PFU/ml). HIV was titrated using the cytopathic effect to the SKT-1B cell line, as previously described [18]. The removal efficacy of the spiked viruses was estimated by comparing virus titres or total virus titres ($TCID_{50}$ or PFU/ml × volume) in the samples between pre- and postprocedures, and the logarithm reduction value (LRV) was calculated. In this study, prior to the virus elimination test, we analysed the effect of the vitamin K-dependent protein-rich fraction and FVII solutions on the titre of the spiked viruses. The results showed that all of the spiked viruses were detected in each solution without significant loss of the virus, indicating that these solutions did not contain neutralizing antibodies against the spiked viruses (data not shown). Virus-spiked solution was subjected to the processes after filtration through a sterile filter (0·45 µm).

Preparation of asialo FVIIa

To prepare asialo FVIIa lacking sialic acid, neuraminidase (6 U) was added to the purified FVIIa (7·5 mg), and the mixture was incubated at 37 °C for 10 h, as previously described [19]. The digested FVIIa was then applied to an anti-FVII mAb-immobilized Sepharose Fast Flow column (ø 1·5 cm × 6·0 cm) to purify the asialo FVIIa. The sialic acid content of FVIIa and asialo FVIIa was quantified using the thiobarbituric acid method [20].

Results

Preparation of human plasma-derived FVII

Human plasma-derived FVII was highly purified by immunoaffinity chromatography from cryoprecipitate-poor plasma

using anti-FVII mAb-immobilized gel (Fig. 1). The purified FVII gave a single band under non-reducing conditions and had > 90% purity, as judged from the SDS–PAGE analysis (see Fig. 4a, lane 1). The specific activity of purified FVII was 1·8 U/μg and the activated form (FVIIa) in the FVII preparation measured using soluble tissue factor was less than 1·5%. FX and FIX antigens were not detected in the FVII solution by ELISA (< 0·1 ng/ml), and prothrombin was also undetectable in a one-stage clotting assay using prothrombin-depleted plasma (data not shown).

Optimization of full activation of FVII on a small scale and characterization of FVIIa

To obtain a high yield of FVIIa on the production scale, it was essential to optimize the conversion process of zymogen FVII to the active form of FVIIa. Therefore, we first tried to optimize the activation condition of FVII on a small scale using anion-exchange chromatography, because Bjoern *et al.* [11] had reported previously that rFVII was autocatalytically activated to rFVIIa on an anion-exchange column. Seventy-five millilitres of the purified FVII solution (A_{280} = 0·260) was applied to a DEAE-FF column (ø 0·5 cm × 5 cm) and, after washing the column, the bound FVII was eluted with Tris buffer (containing $CaCl_2$) at different flow rates. The eluted FVII was immediately mixed with 500 mM acetic acid, pH 3·0, at a volume ratio of 9 : 1, in order to stop further activation. To determine the activation rate of FVII, the eluate was then subjected to SDS–PAGE. As shown in Fig. 2(a), 70% and 60% of FVII were activated to FVIIa, which consists of two chains (heavy and light chains), when FVII on a DEAE-FF column was eluted at a linear flow rate of 2 and 3 cm/min, respectively, and the activation rate of FVII decreased at a relatively high flow rate, indicating that the linear flow rate at elution determines the degree of FVII activation. In addition, a trace of Gla-domainless (GD-less) FVII/

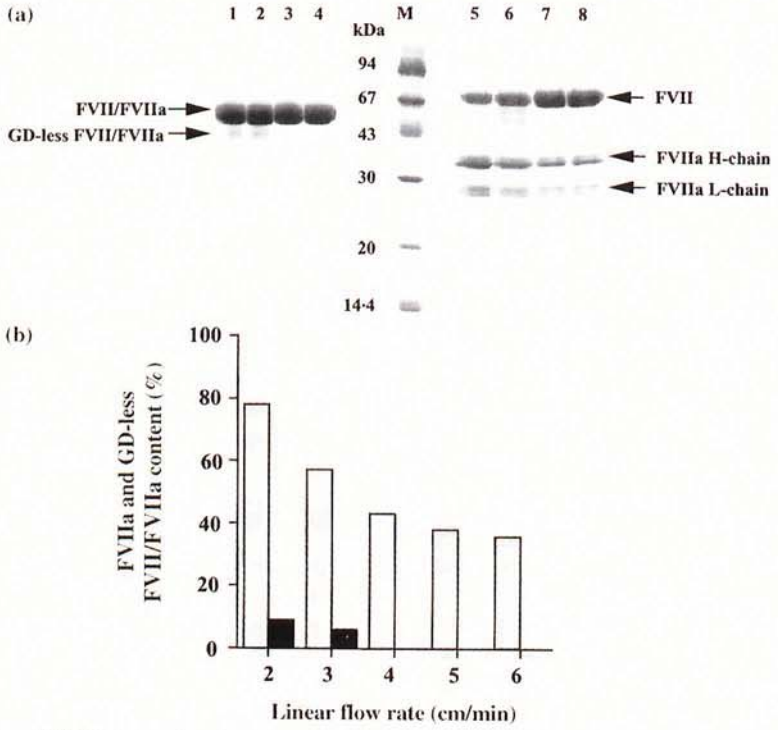

Fig. 2 Partial activation of Factor VIIa (FVIIa) on small-scale anion-exchange chromatography. (a) Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of FVIIa under non-reducing (lanes 1–4) and reducing (lanes 5–8) conditions. After nanofiltration using the Bemberg Microporous Membrane-15 nm, 75 ml of the filtrate (A_{280} = 0·260) was applied to a DEAE-Sepharose Fast Flow (ø 0·5 cm × 5 cm) column at 10 °C. After washing with 10 column volumes of equilibration buffer (50 mM Tris–HCl, pH 7·8, containing 30 mM NaCl) at a linear flow rate of 5 cm/min, proteins were eluted with the buffer, containing 1·75 mM $CaCl_2$, at different linear flow rates. Panel (a) shows the results of SDS–PAGE analyses (12·5% acrylamide): lanes 1 and 5, 2 cm/min; lanes 2 and 6, 3 cm/min; lanes 3 and 7, 4 cm/min; lanes 4 and 8, 5 cm/min; lane M, molecular mass markers. (b) Quantification of FVIIa and Gla-domainless (GD-less) FVIIa/FVII. The generation of FVIIa and GD-less FVIIa/FVII was quantified by densitometry of the light (25 kDa) and heavy (35 kDa) chains observed after SDS–PAGE under reducing conditions, and of GD-less FVIIa/FVII (47 kDa) under non-reducing conditions. The generation of FVIIa (grey column) is expressed as a percentage ratio of the sum of light and heavy chains, and GD-less FVIIa/FVII (black column) is expressed as a percentage ratio of the 47 kDa band. Note that the two experiments were performed independently.

FVIIa, which were cleaved in the light chain, also appeared during this activation procedure (see Fig. 2a). Generation of these degradation products, however, could be controlled by suppressing FVII activation to ≈ 50%, because GD-less FVII/FVIIa were generated when > 60% of FVII was activated (Fig. 2b).

Second, to optimize full activation of the residual FVII in the mixture of FVII and FVIIa, the mixture was further incubated in the presence of 1·75 mM $CaCl_2$ for 20 h at 10 °C without an anion exchanger or other activators. As a result, partially activated FVII solution (total protein concentration of FVIIa/FVII mixture, 1·5 mg/ml), in which 25% of FVII had been activated in advance, was converted to FVIIa by incubation in a time-dependent manner, and completely activated at 20 h without further degradation products (Fig. 3).

Finally, to characterize the biochemical properties of the FVIIa obtained, N-terminal amino acid sequence analysis of the heavy and light chains in FVIIa, and amino acid composition analysis of the FVIIa, were performed. As a result, two bands on SDS–PAGE that corresponded to the heavy chain in FVIIa had the sequence I^{153}VGGK, while two bands of the light chain had the sequence A^{1}NAFL. Furthermore, the amino acid composition of FVIIa was in good agreement with that of FVIIa deduced from the cDNA of FVII [21].

Next, to examine the effect of sialic acid residues on the broad bands of heavy and light chains of FVIIa in SDS–PAGE under reducing conditions (see Fig. 2a, lanes 5–8), FVIIa was treated with neuraminidase, and asialo FVIIa was purified by immunoaffinity chromatography. This neuraminidase treatment released an average of 6 moles of sialic acid per mole of FVIIa – comparable to that of a previous report [22]. In addition, two broad bands of the heavy and light chains of FVIIa in SDS–PAGE under reducing conditions shifted to bands with high mobility after neuraminidase treatment (data not shown). Also, asialo FVIIa repurified with mAb-immobilized gel had the same specific activity as that of

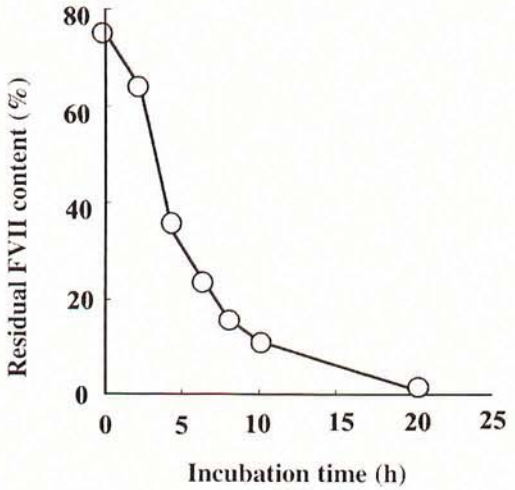

Fig. 3 Full activation of residual Factor VII (FVII) in the mixture of FVII and activated FVII (FVIIa). To optimize full activation of FVII in the mixture of FVII and FVIIa, the FVIIa solution was mixed with the FVII solution at a ratio of 25%, and the mixture, at a total protein concentration of 1·5 mg/ml, was then incubated at 10 °C for 20 h. The residual FVII content (% of the ratio of FVII and FVIIa) in the mixture was determined at incubation times by densitometry on SDS–PAGE, under reducing conditions, as described in the legend for Fig. 2.

Table 1 Recovery of activated Factor VIIa (FVIIa) from large-scale production

Step	Specific activity (U/mg)[a]	FVII antigen recovery (%)[b]
Cryoprecipitate-poor plasma	0·022 ± 0·002 (n = 50)	100
Q-FF	0·85 ± 0·05 (n = 50)	70 ± 3·2 (n = 50)
mAb-immobilized gel	1830 ± 130 (n = 50)	60 ± 2·5 (n = 50)
BMM-15	2330 ± 210 (n = 5)	58 ± 3·1 (n = 5)
DEAE-FF/full activation[c]	40300 ± 3500 (n = 5)	39 ± 2·1 (n = 5)
Dialysis	42000 ± 2140 (n = 5)	38 ± 1·2 (n = 5)
Post heat-treatment	470 ± 49[d] (n = 5)	36 ± 2·1 (n = 5)

Values are denoted as the mean value ± standard deviation (SD), with the number of samples (n) in parenthesis.

[a]FVII and FVIIa activities were measured using the prothrombin time (PT) method with FVII-depleted plasma, and protein content was calculated as the average extinction coefficient of 1·0 (mg/ml)-1 cm at 280 nm through the Q-Sepharose Fast Flow (Q-FF) fraction, while in subsequent steps, the extinction coefficient of 1·4 (mg/ml)-1 cm at 280 nm was used [22].

[b]Recovery of FVIIa or FVII was calculated as FVII antigen recovery, as measured by using enzyme-linked immunosorbent assay (ELISA).

[c]After partial activation of FVII using DEAE Sepharose Fast Flow (DEAE-FF), FVII/FVIIa mixture was fully activated in the solution.

[d]Heat treatment (65 °C for 96 h) was performed after lyophilization of FVIIa solution (≈ 12 000 U/ml) including 2·5% human serum albumin. FVII activity was determined after reconstitution by water (see the Materials and methods).

non-neuraminidase-treated FVIIa in the PT assay. This result suggests that the heterogeneities of the heavy and light chains, detected by SDS–PAGE analysis, were caused by differences in the sialic acid content of the FVIIa.

Large-scale preparation and characterization of FVIIa

To prepare FVIIa on a large scale, after the nanofiltrated FVII fraction was partially activated on a DEAE-FF column, the eluted FVII/FVIIa mixture was fully activated in the solution and the FVIIa obtained was finally dialysed against filling buffer. As shown in Table 1, the mean recovery of FVII antigen after activation of FVII, and the specific activity of the obtained FVIIa, were 39% and ≈ 40 000 U/mg, respectively. Leakage of anti-FVII mAb from the immunoaffinity gel was not detected in the FVIIa preparation (< 0·25 ng/ml). High-pressure gel-permeation chromatography of FVIIa demonstrated that FVIIa was 97% pure (data not shown), which was consistent with results obtained of the SDS–PAGE under non-reducing conditions (see Fig. 4a, lane 7). SDS–PAGE analysis of FVIIa under reducing conditions showed four bands derived from presumably degradation products, as well as intact heavy and light chains of FVIIa, although these bands were very minor (see Fig. 4a, lane 14). The N-terminal amino acid sequence analysis of these bands had the

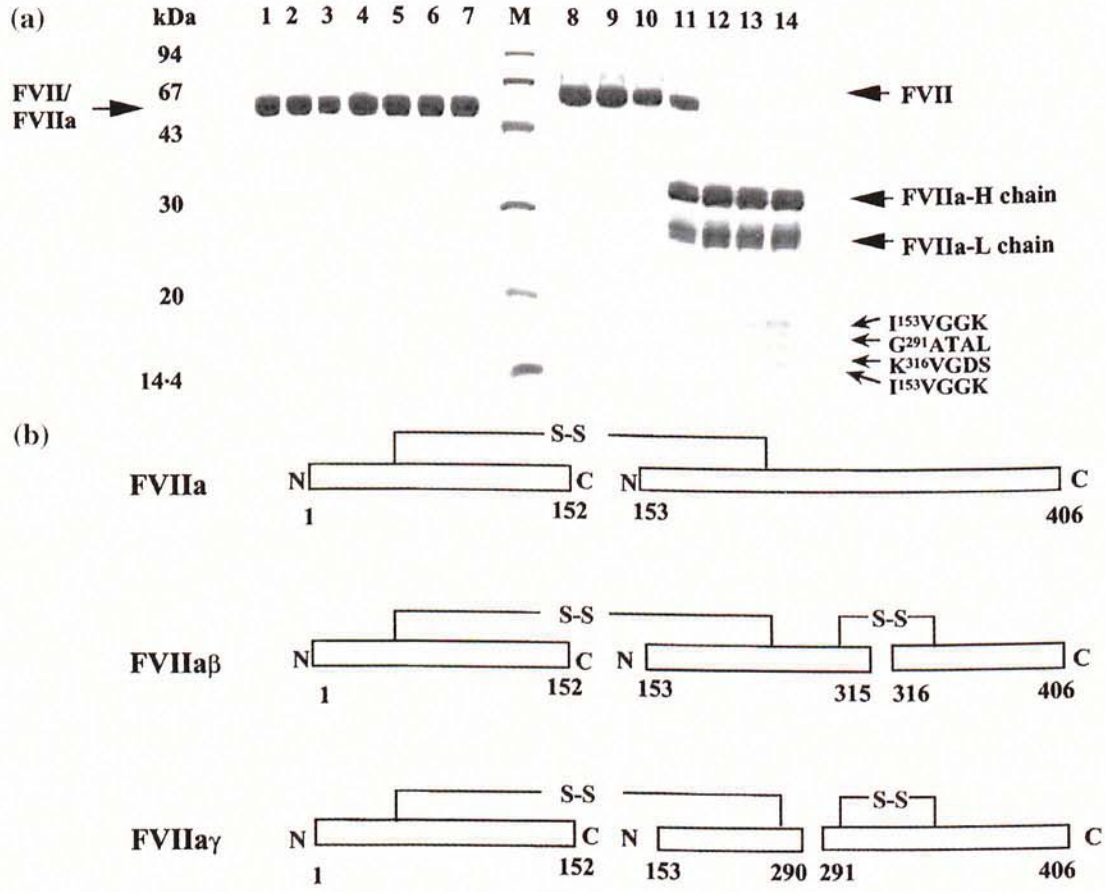

Fig. 4 Preparation of activated Factor VII (FVIIa) on a large scale. (a) Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of FVIIa. The FVIIa solution was prepared on a large scale, as described in the Materials and methods. After the column was washed with a buffer (50 mM Tris-HCl, pH7·8, containing 30 mM NaCl), protein was eluted with the same buffer containing 1·75 mM $CaCl_2$. The protein concentration of the eluted fraction was adjusted to 1·5 mg/ml, and the protein was incubated in solution for 18 h. After full activation, FVIIa was dialysed against a filling buffer (20 mM citrate, pH 6·9, containing 13 mM glycine and 240 mM NaCl). The FVIIa thus obtained was subjected to SDS–PAGE (acrylamide concentration of 12·5%) under non-reducing (lanes 1–7) and reducing (lanes 8–14) conditions. Lanes 1 and 8, before filtration using Bemberg Microporous Membrane-15 nm (BMM-15); lanes 2 and 9, after filtration using BMM-15; lanes 3 and 10, before application onto a DEAE-Sepharose Fast Flow (DEAE-FF) column; lanes 4 and 11, fraction eluted from the DEAE-FF column; lanes 5 and 12, after incubation for 3 h; lanes 6 and 13, after incubation for 18 h; lanes 7 and 14, after dialysis; lane M, molecular mass markers. Four minor bands with molecular weights of 18 000, 17 000, 16 000 and 15 000 were observed in lanes 13 and 14, and the N-terminal sequence analysis indicated that these bands have the sequence: I^{153}VGGK, G^{291}ATAL, K^{316}VGDS, and I^{153}VGGK. (b) Schematic representation of intact FVIIa, FVIIaβ and FVIIaγ.

sequences of I^{153}VGGK (18 kDa), G^{291}ATAL (17 kDa), K^{316}VGDS (16 kDa) and I^{153}VGGK (15 kDa), indicating that traces of FVIIaβ and FVIIaγ are generated autocatalytically in the FVIIa preparation (see Fig. 4b).

Properties of FVIIa concentrate

For manufacturing the FVIIa concentrate, the lyophilized FVIIa was dry-heated in a water bath at 65 °C for 96 h. To check the biochemical and biological properties of the FVIIa concentrate, the FVIIa concentrate was dissolved in 5 ml of water. Even when the FVIIa concentrate was dry-heated, the residual FVIIa activity was retained at > 95%, indicating that this dry-heating treatment had no significant effect on FVIIa activity. The mean moisture content of the FVIIa concentrate in the large-scale process was 1·91 ± 0·17% (n = 5), and the contents of individual preparations ranged from 1·7–2·1%. The components and properties of the FVIIa concentrate are summarized in Table 2. Furthermore, stability tests were performed on the FVIIa concentrate at 10 °C for 2 years; it was found that the pH, solubility, moisture content and FVIIa activity in the FVIIa concentrate did not change during this time-period (data not shown). To further examine the stability of FVIIa activity in a solution, the solubilized sample was kept at 25 °C for 24 h. There was no loss of FVIIa activity.

The efficacy of removal and inactivation of spiked viruses

Virus-spiking tests were performed to determine the efficacy of removing spiked viruses by immunoaffinity chromatography and BMM-15. For immunoaffinity chromatography, sindbis virus (enveloped virus) and poliovirus (non-enveloped virus) were added to vitamin K-dependent protein-rich concentrate (see Fig. 1), and immunoaffinity chromatography was performed. After this chromatography, virus titres and antigens in each sample were quantified, and an LRV was determined. The results showed that most of the spiked viruses were removed into the pass-through and washing fractions, and the remaining small amount of viruses were eluted with FVII and washed with a solution for regeneration of the gel (2 M NaCl solution). LRVs for sindbis virus and poliovirus were 4·4 and 2·8, respectively, indicating that a high degree of reduction was obtained (Table 3). The efficacy of removing poliovirus, JEV, BVDV and CMV, which have different particle sizes, was tested in a nanofiltration process, resulting in a high-efficacy removal of these viruses of > 6·0, 3·3, 3·5 and 3·1 LRV, respectively (Table 4). In this

Table 2 Components and properties of activated Factor VIIa (FVIIa) concentrate from large-scale production

	Value
Component/vial	
Glycine	2·5 mg
Sodium chloride	35 mg
Citrate	30 mg
Human serum albumin	62·5 mg
Property	
Reconstitution volume	5 ml
Reconstitution time	< 1 min (n = 5)
pH	7·0 ± 0·1 (n = 5)
FVII activity	5900 ± 610 U/ml (n = 5)
Moisture content	1·91 ± 0·17% (n = 5)
Murine antibody[a]	Not detected (n = 5)
HCV antibody[b]	Not detected (n = 5)
HIV antibody[c]	Not detected (n = 5)
HBsAg[d]	Not detected (n = 5)
Safety	Sterile and non-pyrogenic (n = 5)

Values are denoted as the mean value ± standard deviation (SD), with the number of samples in parentheses.

[a]Murine antibody was measured by using an enzyme-linked immunosorbent assay (ELISA) (see the Materials and methods).

[b]Hepatitis C virus (HCV) antibody was measured by using a particle-agglutination test kit (Ortho HCV Ab PA test II).

[c]Human immunodeficiency virus (HIIV) antibody was measured by using an enzyme immunoassay (EIA) kit (AXSYM®; Dinabot).

[d]Hepatitis B surface antigen (HBsAg) was measured by using a radioimmunoassay kit (AUSRIA® II; Dinabot).

Table 3 Removal efficacy of spiked viruses by immunoaffinity chromatography

	Total virus titre[a]					
	Pre-application (A)	Pass-through and washing	Effluent (B)	Regeneration I	Regeneration II	LRV[b] log (A/B)
Sindbis virus	$10^{8.2}$	$10^{7.7}$	$10^{3.8}$	$10^{3.4}$	$< 10^{3.2}$	4·4
Poliovirus	$10^{6.4}$	$10^{5.9}$	$10^{3.6}$	$10^{3.4}$	$< 10^{3.2}$	2·8

At the first and second regeneration steps, two solutions were employed: 20 mM Tris–HCl, pH 7·25, containing 2 M NaCl; and 6 M urea, pH 7·2, containing 1 M NaCl and 1% Tween-80.

[a]Total virus titre is expressed as the 50% tissue culture infective dose ($TCID_{50}$)/ml × volume (ml).

[b]LRV, logarithm reduction value.

Table 4 Removal efficacy of spiked viruses by nanofiltration (using Bemberg Microporous Membrane-15 nm)

Virus (particle size)[b]	Total virus titre[a] Prefiltration (A)	Postfiltration (B)	LRV[c] log (A/B)
PPV (18–26)	$10^{7.1}$	$< 10^{2.5}$	> 4·6
Poliovirus (25–30)	$10^{8.5}$	$< 10^{2.5}$	> 6·0
JEV (40–50)	$10^{5.8}$	$< 10^{2.5}$	> 3·3
BVDV (46–57)	$10^{6.0}$	$< 10^{2.5}$	> 3·5
CMV (150–200)	$10^{5.2}$	$< 10^{2.1}$	> 3·1

[a]Total cytomegalovirus (CMV) titre is expressed as plaque-forming units (PFU)/ml × volume (ml), and other titres are expressed as 50% tissue culture infective dose ($TCID_{50}$)/ml × volume (ml).
[b]Particle sizes of the viruses are shown as the diameter (in nm).
[c]LRV, logarithm reduction value.
BVDV, bovine viral diarrhoea virus; JEV, Japanese encephalitis virus; PPV, porcine parvovirus.

Table 5 Inactivation of spiked viruses by dry-heat treatment at 65 °C

Virus	Total virus titre[a] (A) Pre-lyophilization	Post-lyophilization	Time course				(B) Post-heat-treatment	LRV[b] log (A/B)
CMV	$10^{4.7}$	$10^{1.6}$	$< 10^{0.7}$ (16 h)	$< 10^{0.7}$ (32 h)	$< 10^{0.7}$ (64 h)	$< 10^{0.7}$ (96 h)	$< 10^{0.7}$	> 4·0
HIV	$10^{7.0}$	$10^{6.0}$	$< 10^{0.5}$ (12 h)	$< 10^{0.5}$ (48 h)	$< 10^{0.5}$ (48 h)		$< 10^{0.5}$	> 6·5
BVDV	$10^{4.8}$	$10^{4.5}$	$< 10^{0.5}$ (12 h)	$< 10^{0.5}$ (24 h)	$< 10^{0.5}$ (96 h)		$< 10^{0.5}$	> 4·3
Poliovirus	$10^{6.2}$	$10^{1.0}$	$< 10^{0.5}$ (16 h)	$< 10^{0.5}$ (32 h)	$< 10^{0.5}$ (64 h)	$< 10^{0.5}$ (96 h)	$< 10^{0.5}$	> 5·7
PPV	$10^{5.5}$	$10^{5.2}$	$10^{4.8}$ (24 h)	$10^{3.8}$ (48 h)	$10^{3.7}$ (72 h)	$10^{3.3}$ (96 h)	$10^{3.3}$	2·2

[a]Cytomegalovirus (CMV) titre is expressed as plaque-forming units (PFU)/ml and the other titres are expressed as 50% tissue culture infective dose ($TCID_{50}$)/ml.
[b]LRV, logarithm reduction value.
BVDV, bovine viral diarrhoea virus; HIV, human immunodeficiency virus; PPV, porcine parvovirus.

virus-elimination system, the LRV of PPV, which has the smallest particle size (ø 18–26 nm), was also > 4·6 LRV (Table 4). Furthermore, the efficacy of inactivating spiked viruses by dry-heating at 65 °C for 96 h was examined using several viruses, including CMV, HIV, BVDV (a model virus of hepatitis C), poliovirus and PPV, which have different degrees of heat resistance. The moisture content of the lyophilized product for the spiking test was < 1·7%, which was slightly lower than the moisture content of the FVIIa concentrate in the large-scale process. Furthermore, the residual FVIIa activity after dry-heating was > 95%, and this recovery rate was the same as that of the production-scale process. These results demonstrate the validity of the scale-down for the virus-spiking test, and indicate that the efficacy of the inactivation of the virus by dry-heating would not be overestimated. The inactivation efficacy of these spiked viruses, except for PPV, was > 4·0 LRV (Table 5). In contrast, PPV was not sufficiently inactivated by dry-heating treatment. However, in view of the efficacy of removing PPV by nanofiltration, our FVIIa concentrate would be extremely safe, even though the dry-heating process was less effective for inactivation of PPV than for other viruses (Tables 4 and 5).

Discussion

rFVIIa is currently used to control bleeding episodes in patients with haemophilia A or B who have inhibitory antibodies against FVIII or FIX, and the usefulness of rFVIIa as a haemostatic agent has been recognized [6]. Plasma-derived FVIIa concentrate, however, which is prepared on an industrial scale from human plasma, is not yet available for clinical use for haemophiliacs with antibodies to FVIII and FIX. To safely provide such patients with FVIIa concentrate, the following critical issues had to be addressed:

(1) establishment of a large-scale manufacturing process of plasma-derived FVIIa concentrate, by which FVIIa can be obtained from human plasma with high yield and recovery; and

(2) ensure the safety of FVIIa concentrate by detailed characterization of its biochemical and biological properties, and use of a virus-spiking test.

Therefore, we first attempted to establish a large-scale process for manufacturing FVIIa concentrate using human cryoprecipitate-poor plasma as a starting material. As shown in Table 1, we obtained 4·5 g of purified and nanofiltered FVII preparation from 15 000 l of human plasma using this manufacturing process, which corresponded to 58% recovery. After activation of FVII and dialysis, FVIIa was finally prepared at 38% recovery (Table 1). In our procedure for FVIIa preparation, the specific activity of the purified FVIIa was 40 000 U/mg (Table 1), comparable to values reported previously [23,24]. FVIIa was lyophilized and dry-heated at 65 °C for 96 h. There was no significant loss of FVIIa activity during these processes.

The activation process of FVII is important for obtaining a high yield through the whole process. Full activation of FVII on the anion-exchange resin, however, promoted the generation of GD-less FVII/FVIIa (Fig. 2a, 2b). To avoid this, the activation process was divided into two steps. In the first, 50% of FVII was activated to FVIIa on a gel, and in the next, the remaining FVII was fully activated in solution. Throughout those steps, FVII was completely activated to FVIIa. Sakai *et al.* [25] previously reported that GD-less rFVIIa was spontaneously generated from rFVIIa in EDTA solution, presumably mediated via autodigestion of FVIIa between K^{38} and L^{39} in the light chain. In our experiment, GD-less FVIIa was also generated in solution, without phospholipid, in < 0·5 mM Ca^{2+} (data not shown). Therefore, to avoid generation of GD-less FVIIa/FVII, the mixture of FVII and FVIIa was incubated in solution containing $CaCl_2$ at > 1 mM. It was previously reported that excessive digestion of rFVIIa with activated FX or FIX yielded degradation products such as rFVIIaβ and rFVIIaγ, which were cleaved at the peptide bonds of R^{315}-K^{316} and R^{290}-G^{291}, respectively [26]. Our plasma-derived FVIIa also had minor bands with a molecular mass of 15–18 kDa, which was a lower mass than that of the light chain of FVIIa. N-terminal amino acid sequence analyses of these bands further demonstrated that they resulted from degraded FVIIa molecules (FVIIaβ and FVIIaγ) (see Fig. 4a, lane 14, and Fig. 4b).

Next, to ensure the safety of plasma-derived FVIIa, we first had to demonstrate no or only a low possibility of transmission of contaminating viruses from our FVIIa product. Therefore, we introduced three steps into our process to effectively eliminate and inactivate these viruses. As shown in Tables 3, 4 and 5, the virus-spiking tests clearly demonstrated that our process, which consists of immunoaffinity chromatography, nanofiltration (15 nm) and dry-heating, effectively removed and inactivated every test virus, except for PPV in dry-heating. In the present study, we used PPV as a test virus for human parvovirus B19 and evaluated the efficacy of our process for removing and inactivating this virus. Nanofiltration of FVII was very useful for removing PPV, although this virus has a high resistance to dry-heating (see Tables 4 and 5). In Japan, each unit of donated plasma (resource materials of FVIIa concentrate) is currently tested using the receptor-mediated haemagglutination (RHA) method to detect human parvovirus B19 antigen [27]. Thus, our manufacturing process for FVIIa concentrate has the potential to decrease the risk of parvovirus B19, as well as other viruses. Furthermore, we confirmed that the leakage of mouse IgG in the FVII preparation from the immunoaffinity resin can be removed by DEAE-FF chromatography to the pass-through fraction and the mouse IgG was not detected (by using ELISA) in the eluate of DEAE-FF or the FVIIa concentrate (see Table 2).

In summary, we have established a procedure for the large-scale production of highly purified FVIIa from cryoprecipitate-poor plasma, with both high recovery and safety. The present method could be useful for the large-scale preparation of plasma-derived FVIIa and may improve the efficient utilization of plasma. Thus, it appears that this process makes it possible to provide haemophilia patients with inhibitory antibodies against FVIII and FIX with sufficient FVIIa concentrate to control their bleeding episodes.

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