Journal of Chromatography B, 898 (2012) 78–89

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Pilot production of recombinant human clotting factor IX from transgenic sow milk

Yu-ling Sun, Yuo-sheng Chang, Yin-shen Lin, Chon-ho Yen*

Division of Biotechnology, Animal Technology Institute Taiwan, P.O. Box 23, Chunan 350, Miaoli, Taiwan, ROC

ARTICLE INFO

Article history:
Received 30 January 2012
Accepted 17 April 2012
Available online 26 April 2012

Keywords:
Factor IX
Transgenic sow
Milk
Purification

ABSTRACT

Valuable pharmaceutical proteins produced from the mammary glands of transgenic livestock have potential use in the biomedical industry. In this study, recombinant human clotting factor IX (rhFIX) produced from transgenic sow milk for preclinical animal studies have been established. The transgenic sow milk was skimmed and treated with sodium phosphate buffer to remove abundant casein protein. Then, the γ-carboxylated rhFIX fraction was segregated through the Q Sepharose chromatography from uncarboxylated one. For safety issue, the process included virus inactivation by solvent/detergent (S/D) treatment. Subsequently, the S/D treated sample was loaded into the Heparin Sepharose column to recover the rhFIX fraction, which was then reapplied to the Heparin Sepharose column to enhance rhFIX purity and lower the ratio of activated form rhFIX (rhFIXa) easily. This was possible due to the higher affinity of the Heparin affinity sorbent for rhFIXa than for the rhFIX zymogen. Furthermore, an IgA removal column was used to eliminate porcine IgA in purified rhFIX. Finally, nanofiltration was performed for viral clearance. Consequently, a high-quality rhFIX product was produced (approximately 700 mg per batch). Other values for final rhFIX preparation were as follows: purity, >99%; average specific activity, 415.6 ± 57.7 IU/mL and total milk impurity, <0.5 ng/mg. This is the first report that described the whole process and stable production of bioactive rhFIX from transgenic sow milk. The overall manufacturing process presented here has the potential for industrial production of rhFIX for treatment of hemophilia B patients.

1. Introduction

Human clotting factor IX (hFIX) is a vitamin K-dependent plasma glycoprotein of approximately 57 kDa and is synthesized in the liver. This protein is a zymogen that acts as a serine protease to play a key role in the intrinsic coagulation pathway. Hemophilia B, a hereditary recessive X-linked bleeding disorder, is caused by a deficiency or absence of hFIX. At present, hemophilia B patients are treated using hFIX concentrates purified from the pooled plasma of many blood donors, such as MonoFIX (CSL Limited, Australia) and Immunine (Baxter AG, Vienna, Austria). The use of human plasma derivatives involves the risk of infections caused by viruses, such as human immunodeficiency virus type (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV). Application of recombinant DNA technology to manufacture recombinant hFIX (rhFIX) is an alternative method to overcome the limitations and dangers associated with plasma-derived FIX (pd-FIX).

Production of rhFIX in mammalian cells, such as rat hepatoma, baby hamster kidney cells, human hepatoma, mouse fibroblast, mouse hepatoma, and Chinese hamster ovary (CHO) cells, has been investigated [1–6]. BeneFIX (Wyeth Pharmaceuticals Inc., PA, USA), an rhFIX produced by a genetically engineered CHO cell line, was the first recombinant prescription drug used in hemophilia B treatment and was shown to be stable and free of infectious agents [7,8]. However, the production of rhFIX using mammalian host cells continues to have some limitations, such as low cell density and cost-effectiveness.

Transgenic livestock, including mice, cows, sheep, goats, and pigs, are potential hosts for the production of recombinant therapeutic proteins. Pigs are particularly promising for pharmaceutical application because of their fecundity and physiological similarity to humans [9]. Transgenic sow milk may be an attractive and cost-effective vehicle for large-scale production of biopharmaceuticals with the natural secretory properties of the mammary glands and appropriate post-translational modifications of recombinant proteins may also be possible [10]. Extensive studies have shown that pigs are appropriate bioreactors for the production of various recombinant proteins, including factor VIII (FVIII) [11], von Willebrand factor [12], protein C [13] and FIX [14,15].

Although milk from transgenic animals may express and produce considerable quantities of target proteins, the high cost of downstream processing and low product yields are significant

* Corresponding author. Tel.: +886 37 585785; fax: +886 37 585829.
E-mail address: chy01@mail.atit.org.tw (C.-h. Yen).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2012.04.022

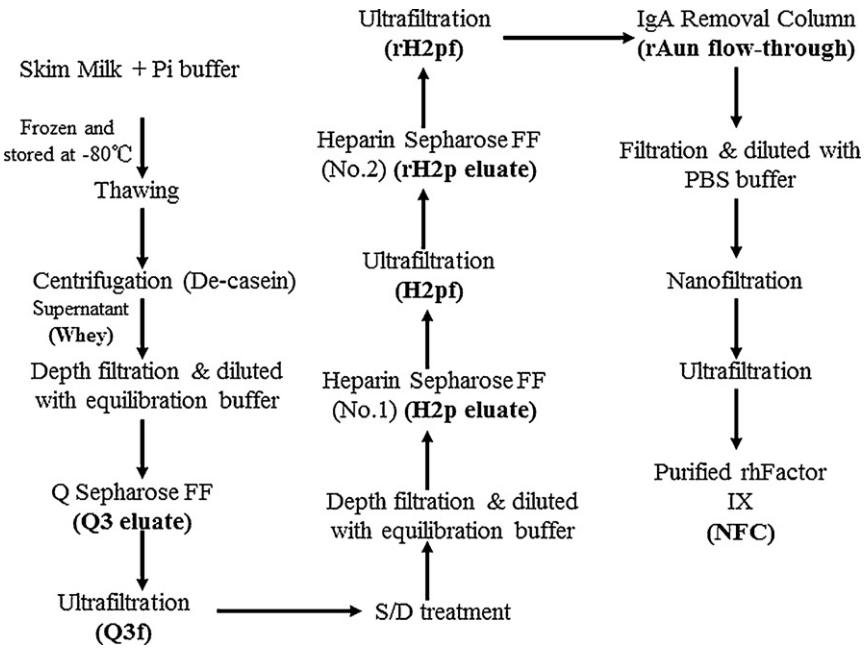

Fig. 1. Flowchart of the recombinant human factor IX production.

obstacles to pharmaceutical application of this technique [16]. Therefore, methods to reduce costs of manufacturing therapeutic proteins must be sought. Efforts have been made to purify rhFIX from transgenic sow milk [15,17]. In this study, we used the double transgenic pigs, which carry genes of both hFIX and exogenous porcine lactoferrin and were established by Wu et al. [18]. Both proteins are stably expressed and continuously secreted into milk during lactation.

Milk is a medium containing calcium and phospholipids and there have been measured an elevated level of FVIIa and FXIa in mastic sow milk, that is, the damage of mammary tissue will switch on the activation of rhFIX zymogen to rhFIXa in milk. If high content of rhFIXa still persists in the rhFIX product, it is fatal to patients with infusion of rhFIX. A similar problem was circumvented when producing rhFIX from CHO cells [19]. Therefore, it is important to minimize rhFIX activation when recovering the unactivated rhFIX produced in transgenic sow milk. Minimization processes include chelation of calcium, phospholipid removal, pH adjustment, storage in ultra-low freezers, controlled thawing procedures, addition of protease inhibitors, and purification procedures that maintain minimal activation of FIX [17]. In this work, we described a chromatographic method by Heparin Sepharose FF column that can be used to purify rhFIX from rhFIXa to overcome the forementioned problem.

In the present study, we describe an effective, alternative, and complete pilot production of rhFIX from the milk of previously established lineages of transgenic sows. The manufacturing process consists of pretreatment and removal of milk protein (especially casein), isolation of γ-carboxylated rhFIX, separation of the rhFIX zymogen from activated rhFIX, removal of impurities, and viral clearance. The biochemical and biological properties of rhFIX purified from different batches were also characterized.

2. Experimental

2.1. Reagents

The chromatographic gels of Q Sepharose Fast Flow (FF) and Heparin Sepharose 6 FF were purchased from GE Healthcare (Uppsala, Sweden) and the CaptureSelect Human IgA affinity matrix was obtained from BAC BV (Naarden, Netherlands). All buffer components were purchased from J. T. Baker (Phillipsburg, NJ, USA) or Sigma (St. Louis, MO, USA). The following buffers were used:

[1]Pi buffer: 1 M phosphate buffer, pH 5.8.
[2]B.eq buffer: 50 mM Tris, pH 7.2.
[3]B.2 buffer: 50 mM Tris, 0.2 M NaCl, pH 7.2.
[4]B.3 buffer: 50 mM Tris, 0.3 M NaCl, pH 7.2.
[5]B.6 buffer: 50 mM Tris, 0.6 M NaCl, pH 7.2.
[6]B.10 buffer: 50 mM Tris, 1 M NaCl, pH 7.2.
[7]B.CP buffer: 10 mM citric acid, 30 mM $Na_2HPO_4{\cdot}7H_2O$.
[8]PBS buffer: 8.1 mM $Na_2HPO_4{\cdot}7H_2O$, 1.47 mM KH_2PO_4, 137.93 mM NaCl, 2.67 mM KCl, pH 7.4.
[9]B.E buffer: 100 mM glycine, pH 3.0.
[10]B.F buffer (formulation buffer): 260 mM glycine, 1% sucrose, 10 mM L-histidine, pH 6.8.

2.2. Collection, de-fated and storage of transgenic sow milk

Lactating sows were injected with oxytocin by intravenous injection and milked with patented machine. The collected transgenic sow milk was immediately centrifuged for 15 min at 12,000 rpm to separate the fat. The biological activity of rhFIX of sampled skim milk was determined by activated partial thromboplastin time (APTT) assay.

2.3. Batch production of rhFIX

The overall production steps of rhFIX were illustrated in Fig. 1. The detailed methods and all four chromatographic steps of operative parameters were described as follows and Table 1.

2.3.1. De-casein of skim milk

The Pi buffer was added to the skim milk and mixed well to a final concentration of 0.2 M and frozen to precipitate casein. The precipitated casein in skim milk/Pi buffer (SM/Pi) was centrifugation at 8000 rpm for 30 min. The resulting supernatant, named whey, was pre-filtrated by depth filter (0.65 μm/0.05 cm^2, Sartopure GF Plus,

Table 1
The operative parameters for all four chromatographic steps in the preparation of rhFIX.

Process parameter	Q Sepharose FF	Heparin Sepharose FF (No. 1)	Heparin Sepharose FF (No. 2)	IgA removal column
Column volume (CV)	5.6 L 20 cm (d) × 17.8 cm (h)	2.6 L 14 cm (d) × 17 cm (h)	2.6 L 14 cm (d) × 17 cm (h)	490 mL 5 cm (d) × 25.5 cm (h)
Operation temperature	4–8 °C	4–8 °C	4–8 °C	4–8 °C
Flow rate	850 mL/min	230 mL/min	230 mL/min	35 mL/min
Loading volume/name	78 L (QL)	18 L (HL)	4 L (H2pf)	200–500 mL (rH2pf)[a]
Equilibration buffer	B.eq (3 CV)	B.eq (3 CV)	B.eq (3 CV)	PBS (3 CV)
Wash buffer	B.eq (9 CV), followed by B.2 (2 CV)	B.eq (9 CV), followed by B.CP (5 CV) and B.2 (2 CV)	B.eq (9 CV), followed by B.CP (5 CV) and B.2 (2 CV)	PBS (2 CV)
Elution buffer	B.3	B.2	B.2	NA
Elution volume/name	20.2 L (Q3)	28 L (H2p)	4 L (rH2p)	(rAun)
Intermediate product after UF/DF	Q3f	H2pf	rH2pf	

[a] The loading volume was in accordance with the situation of rH2pf.

Sartorius, Goettingen, Germany) with the operation pressure less than 1.2 bar (the flow rate was usually <400 mL/min). Then, the filtrate was diluted 3 times with B.eq buffer to be the loading material, labeled QL. Forty kg SM/Pi was used as the starting material and applied to each batch.

2.3.2. Q Sepharose FF chromatography and ultrafiltration/diafiltration (UF/DF)

The loading material QL was loaded onto the Q Sepharose FF column and washed with B.eq buffer and B.2 buffer subsequently. The rhFIX-rich fraction was eluted with B.3 buffer and followed by B.10 buffer to regenerate the column. The rhFIX-rich eluate was concentrated 7 times by Pellicon 2 filter holder with cassette (0.5 m^2, Millipore, Billerica, MA, USA) and diafiltered against B.eq buffer to provide a consistent buffer for loading onto the Heparin Sepharose 6 FF column. The flow rate and transmembrane pressure (TMP) of UF/DF were maintained with 3–7.5 L/min/m^2 and 3–7 psi, respectively. The Q Sepharose FF column can be regenerated and reused according to the manufacture's instruction.

2.3.3. Solvent/detergent (S/D) treatment and depth filtration

The UF/DF rhFIX-containing fraction was added from a 10× concentrated stock solution to give a final concentration of 0.3% Tri(*n*-butyl) phosphate (TnBP; Fluka) and 1% Triton X-100 (Sigma) and incubated at 20–22 °C for 10–12 h to inactivate the enveloped viruses. Then, the S/D treated sample was filtered by depth filter (0.65 μm/0.05 cm^2, Sartopure GF Plus) and diluted 1.5 times with B.eq buffer to obtain HL.

2.3.4. Heparin Sepharose 6 FF chromatography (Heparin No. 1) and UF/DF

The HL was applied to the Heparin Sepharose 6 FF column. After washing with B.eq buffer and B.CP buffer, the column was then washed by B.2 buffer until the absorbance declined. Then, the column was washed with B.2 buffer continuously until the UV absorbance (output as electronic signal, mV) was raised again and collected the eluate. The collected sample was concentrated and dialyzed to B.eq buffer by Pellicon 2 filter holder with cassette (0.5 m^2, Millipore). The condition of UF/DF was described as Section 2.3.2. The heparin column was continuously washed and regenerated with B.6 and B.10 buffers, respectively.

2.3.5. Heparin Sepharose 6 FF chromatography (Heparin No. 2) and UF/DF

The ultrafiltered rhFIX fraction was loaded directly onto the Heparin Sepharose 6 FF column repeatedly. After similar washes as at step Heparin No. 1, the rhFIX was then eluted by B.2 buffer when the UV absorbance was raised. The rhFIX fractions were collected and analyzed for the ration rhFIXa/rhFIX. Then, the fractions of rhFIXa ratio ≤2.4% were taken to concentrate 7 times and dialyzed to PBS buffer by Pellicon mini filter holder with cassette (0.1 m^2, Millipore). The flow rate and TMP of UF/DF were 3–7.5 L/min/m^2 and 3–7 psi, respectively.

2.3.6. CaptureSelect human IgA affinity chromatography (IgA removal column)

The Heparin Sepharose rhFIX product was divided two parts equally. After equilibrating with PBS buffer, one part rhFIX-rich product was applied to the IgA removal column, to which the rhFIX was passed through and collected, and then the bounded IgA was washed out by B.E buffer. Another part of the rhFIX product was loaded onto the IgA removal column and collected the flow-through fraction, too. Combining the respective flow-through fractions and applied to the IgA removal column. After circulation, the pass-through rhFIX fraction was collected and diluted with PBS buffer at a protein concentration of 0.1 mg/mL.

2.3.7. Removal of viruses and final UF/DF

The method used for removing virus contamination is nanofiltration. The filter used for this purpose is Virosart CPV (Sartorius) with an area of 2000 cm^2 and a nominal pore-size of 20 nm. The filter was rinsed with PBS buffer prior to use. Then, the diluted rhFIX product was passed through the filter at a pressure of 2 bars. The filtrate volumes and collection time were monitored throughout the process and used to determine the flow-rate.

Finally, the rhFIX preparation was ultrafiltrated and concentrated in B.F buffer by Pellicon mini filter holder with cassette (0.1 m^2, Millipore) and stored at −80 °C for further analysis.

2.4. Analytical techniques

2.4.1. Protein quantitation

Protein concentration was quantified by UV_{280} with a full-wavelength spectrophotometer (Scnico, Seoul, Korea) using bovine serum albumin as a standard.

2.4.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

Collected samples were analyzed by SDS-PAGE using 4–12% NuPAGE Bis-Tris gel (Invitrogen, Carlsbad, CA, USA) followed by Coomassie Brilliant Blue R-250 (Sigma).

Protein sample was resolved by electrophoresis and electro-transferred onto PVDF membrane (Amersham Biosciences, USA). The membrane was blocked with 5% (w/v) non-fat milk in PBS and incubated for 2 h in 1:10,000 rabbit anti-human FIX (Sigma) diluted in PBS. Following washed with TBS (PBS containing 0.1% Tween 20) three times, the blot was reacted in a 1:10,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG in PBS for 1 h and washed with TBS. The protein bound enzymes on

the membrane were visualized with TMB liquid substrate system for membrane (Sigma).

2.4.3. Determination of factor IX coagulation activity

The clotting activity of FIX was performed by mixing FIX-deficient plasma with the diluted sample by the instructions of manufacture. Coagulation was triggered by adding $CaCl_2$ and the clotting time was measured by viscosity-based detection system of STA compact coagulometer (Diagnostica Stago, France). Coagulation deficient plasma, calibration plasma and other reagents were purchased from Diagnostica Stago, too.

2.4.4. Determination the bioactivity of active form factor IX (FIXa)

The bioactivity of active form FIX was assayed by using a chromogenic substrate method with Biophen FIX kit (Hyphen BioMed, France). In the presence of thrombin, phospholipids and calcium, the amount of FIXa in the assayed sample was measured by the factor Xa specific substrate SXa-11 cleaved to *p*-nitroaniline (pNA) which could be visualized at 405 nm.

2.4.5. The purity and aggregation analysis of factor IX

The purity of the recombinant FIX product was analyzed by reversed-phase HPLC (RP-HPLC) on a Discovery BIO Wide Pore C18 column (25 cm × 4.6 mm i.d., 5 μm particles, Supelco, St. Louis, MO, USA) with a C18 guard column. The mobile phase components were: A: 0.08% (v/v) trifluoroacetic acid (TFA, Sigma) in water and B: 0.08% TFA in water:acetonitrile 2:8 (v/v). The gradient condition was as follows: 10, 80, 80 and 10% B at 0, 25, 35, and 36 min with a flow rate of 1 mL/min.

The aggregation of rhFIX product was determined by size-exclusion HPLC (SEC-HPLC) using Superdex 200 10/300 GL column (GE Healthcare) with a flow rate of 0.4 mL/min of 0.2 M ammonium acetate (Sigma).

2.4.6. Analysis of impurities, host cell proteins (HCP) and residual DNA

The analysis of impurities including IgA, IgM, IgG and albumin was performed with Pig IgA/IgM/IgG/Albumin ELISA Quantitation Kit (Bethyl Laboratories, Inc., Montgomery, USA), respectively. The content of β-lactoglobulin was detected by bovine β-lactoglobulin ELISA Quantitation Kit (Bethyl Laboratories, Inc.).

The detection of host cell proteins (HCP) was performed as follows. The 5000×-diluted rabbit anti-HCP polyclonal antibody (LTK BioLaboratories, Taoyan, TW) was coated on ELISA plate and incubated overnight. After washing and blocking, the sample/standard was added to the antibody-coated well. Then, the biotinylated rabbit anti-HCP polyclonal antibody was added and reacted with the bound sample/standard. After the ExtrAvidin-peroxidase (Sigma) was added and used in conjunction with biotin, the HCP bound enzymes were detected by TMB liquid substrate system for ELISA. The signal was analyzed by TECAN/Sunrise ELISA reader (Advance Biotechnology, Taipei, TW).

The residual DNA analysis of final product of rhFIX was performed by Real-time quantitative PCR (RT Q-PCR). The analysis was carried out by Biosafety Test Laboratory (BTL) of ATIT, which has received certification of its compliance with the principals on OECD/GLP during 2010.

3. Results and discussion

3.1. Removal of casein from milk

Transgenic animals are used for the production of valuable therapeutic proteins. However, the main protein casein comprises >50% of the total amount of milk protein, and therefore can be a source of impurity in the process of extracting recombinant proteins from milk. Caseins in milk exist as casein micelles [16] will cause difficulty in the purification process.

Existing methods of removing casein micelles include acid precipitation, PEG precipitation and EDTA/citrate dissolving followed ultrafiltration. These methods may disrupt the protein structure due to pH change, the recombinant target protein is co-precipitated and casein micelles will block the filter during ultrafiltration to result in low yields [20]. However, some efforts were still made to remove caseins from milk [16,21,22].

A method of precipitating caseins from transgenic sow milk under neutral or weakly acidic conditions was described in Yen et al. [23] and modified in this report. A sodium phosphate solution is added to the porcine skim milk and mixed well. The resulting mixture with a phosphate concentration of ≥40 mM is then frozen. The destructed casein micelle was aggregated like whitish-gel after the frozen samples were thawed at 4 °C (Fig. 2(a)). After centrifugation, the rhFIX-containing supernatant, i.e. whey, was separated from the casein-precipitate fraction as shown in Fig. 2(b)–(d). The whey protein fraction that contains >90% rhFIX is available for further purification (Fig. 2(e)). This separation might have been caused by the destruction of the structure of casein micelles because of the sequential procedures of addition of sodium phosphate buffer, freezing and thawing.

3.2. Purification of rhFIX

3.2.1. Q Sepharose fast flow chromatography

The first step in chromatography was the key to rhFIX production from transgenic milk. It involved finding a medium to separate rhFIX from other milk proteins.

Q Sepharose FF is a strong anion-exchange resin composed of a cross-linked agarose matrix that is covalently derivatized with a quaternary amine group through a short linker. Acidic proteins such as rhFIX and other polyionic substances with a net negative charge bind to Q Sepharose FF via charge interactions. Buffers with increased conductivity disrupt the charge interactions, and thus elute the bound target proteins from the column.

Recombinant hFIX present in the QL loading pool was isolated and purified on an anion-exchange resin, Q Sepharose FF. The objective of this step was to remove the contaminant proteins and isolate rhFIX. Chromatography and SDS-PAGE of the collected samples are shown in Fig. 3. As shown by the results, the Q Sepharose FF column adsorbed the rhFIX protein, and loosely bound contaminants, including uncharged and basic proteins, were removed in the unbound fraction (flow-through) during column loading (Fig. 3(a)). Some inactive (uncarboxylated) forms of rhFIX and other impurities containing antibodies and some milk proteins were washed out by adjusting the conductivity of the buffers (Fig. 3(b)). Using B.3 buffer, rhFIX was eluted from the column as a single peak (labeled Q3 in Fig. 3(a)). The eluted product was further processed.

The biological activity of rhFIX is highly dependent on post-translational modifications such as γ-carboxylation which is the rate-limiting step during rhFIX production [24,25]. Therefore, it is critical to properly produce carboxylated rhFIX during the production of functional rhFIX.

Several methods have been used to separate γ-carboxylated and uncarboxylated rhFIX. Gillis et al. [26] used NaCl gradient elution from an anion-exchange column (Mono Q column) to resolve in part the low specific activity of rhFIX in CHO cells. Wojcik et al. [27] raised a metal-dependent conformation-specific monoclonal antibody against the Gla domain to identify the carboxylation site of rhFIX. Anion-exchange chromatography using ammonium acetate elution can separate the biologically active and inactive subpopulations of rhFIX from transgenic sow milk [15]. Wajih et al. [28] used immunoaffinity chromatography was able to purify γ-carboxylated

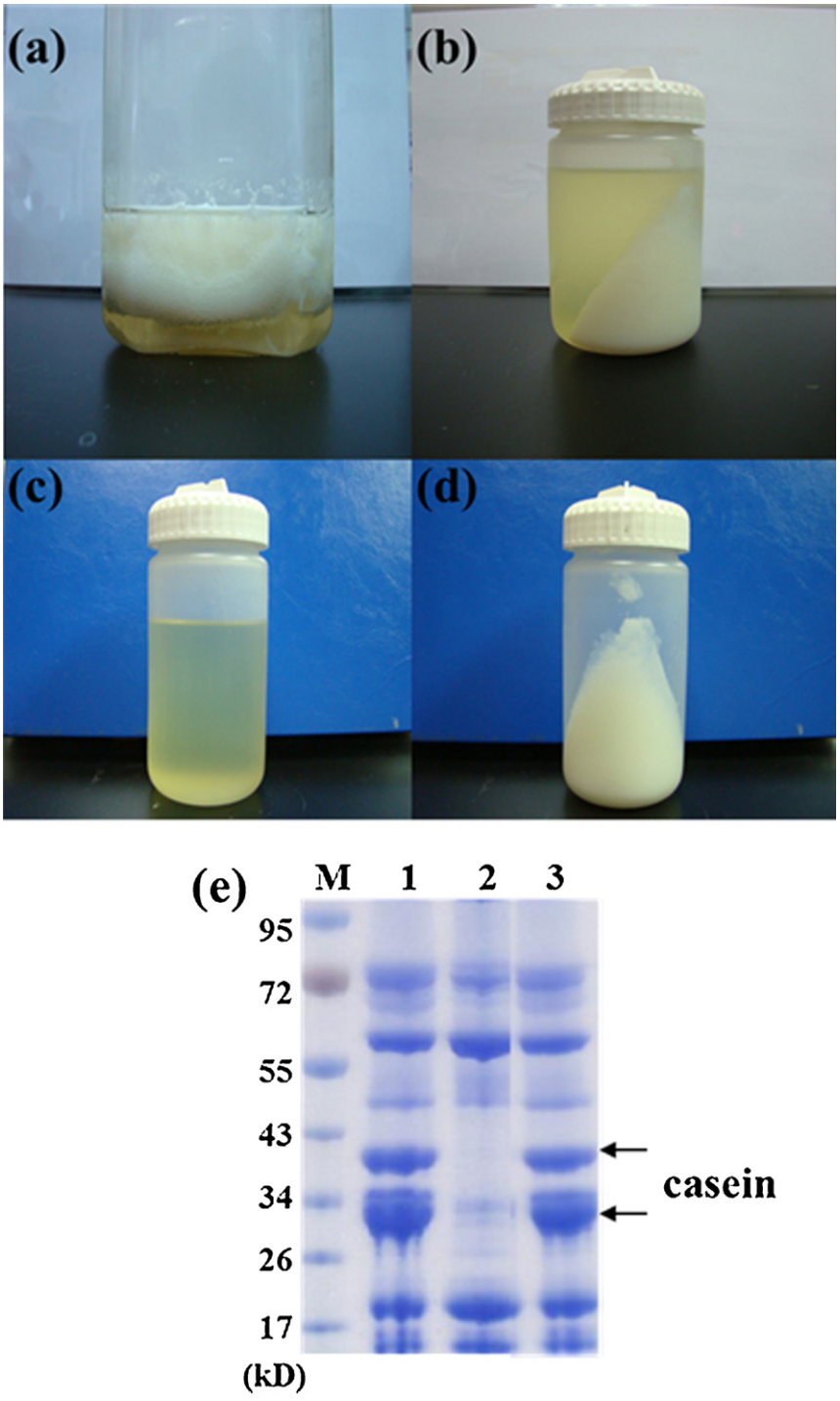

Fig. 2. Separation of casein from milk by phosphate precipitation. (a) Whitish gel-like aggregate appeared upon thawing after the skim milk mixed with Pi buffer and frozen; (b) the aggregate containing casein was precipitated by centrifugation; (c) the supernatant (whey) containing abundant rhFIX; (d) a large amount of casein-contained precipitate; (e) samples of casein removal were analyzed by using SDS-PAGE. Lane 1: skim milk; lane 2: rhFIX-contained supernatant; lane 3: casein precipitate, as arrow indicated and lane M indicated pre-stained protein marker.

rhFIX on a column with Ca^{2+}-induced conformation-specific antibodies, which were used as immobilized ligands. To quantify the efficiency of γ-carboxylated rhFIX from HEK 293 cells, hydroxyapatite chromatography was performed and carboxylated and uncarboxylated rhFIX were separated, as described by Blostein et al. [29].

In the present study, we used NaCl gradient elution and an anion-exchange Q-Sepharose FF column to fractionate carboxylated rhFIX from uncarboxylated subpopulations. As illustrated in Fig. 3, rhFIX was eluted in B.2 buffer (Q2 eluate) and B.3 buffer (Q3 eluate). Both eluates were analyzed for coagulation activity and Gla content. The Q3 eluate exhibited clotting activity

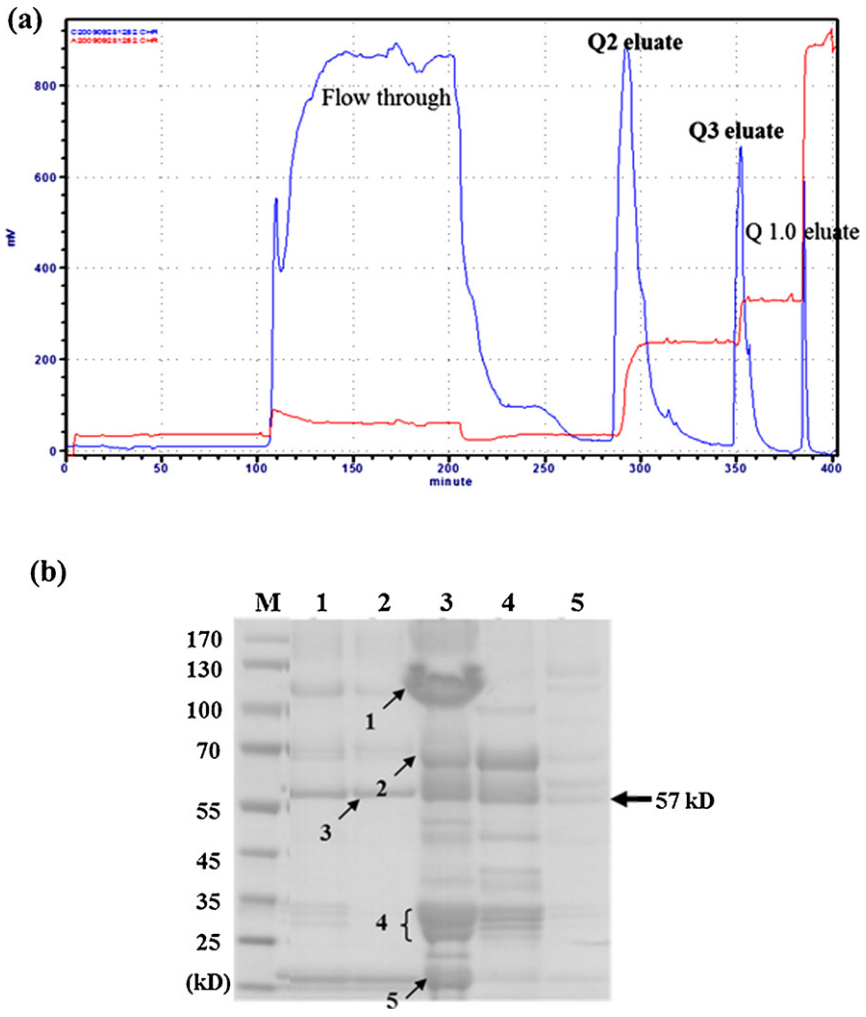

Fig. 3. Elution profile in production of recombinant rhFIX by Q Sepharose FF chromatography (Batch 6). (a) The QL loading pool was passed through the column. Uncarboxylated rhFIX was eluted as Q2 and the γ-carboxylated rhFIX was collected in the Q3. (b) Samples of chromatographic fractions were analyzed by SDS-PAGE under non-reducing condition. Lane 1: QL loading pool; lane 2: unbound flow-through; lane 3: Q2 eluate; lane 4: Q3 eluate; lane 5: eluate Q1.0 eluted with B.10 buffer and lane M indicated pre-stained protein marker. The size and relative position of rhFIX was indicated. The contaminant proteins were identified by MALDI-TOF and pointed out as arrows. 1: antibodies; 2: lactoferrin; 3: albumin; 4: casein and 5: β-lactoglobulin.

and produced a result of 11.89 ± 0.17 mol Gla mol/rhFIX, which was almost equal to the theoretical value of completely carboxylated hFIX (12 mol Gla mol/hFIX) [4] and not of rhFIX produced in CHO cells (6.5 mol Gla mol/rhFIX) [15] and transgenic sow milk (6.5 mol Gla mol/rhFIX). No clotting activity was detected in the Q2 eluate and the average number of Gla residues was 3.32 ± 0.68 (unpublished data). This indicated that carboxylated and uncarboxylated rhFIX can be easily separated by anion-exchange chromatography.

The Q3 eluate containing γ-carboxylated rhFIX was then subjected to UF/DF to obtain Q3f. As shown in Table 2 of Batch 3 (for example), the specific activity of Q3f was 64.69 IU/mg protein, and the activity yield reached approximately 92.33%.

3.2.2. S/D treatment-virus inactivation

S/D treatment is widely used for ensuring virus-free plasma products such as coagulation factors, immunoglobulin (Ig), and albumin. Many studies have shown this procedure to be effective for inactivating enveloped viruses under manufacturing conditions. Johnston et al. [30] performed S/D treatment by adding 1% (w/v) Tween and 0.3% (w/v) TnBP to validate the effectiveness of HIV-1, HAV, HBV, and HCV inactivation during the manufacture of

Table 2
Analytical results of the intermediated products of one batch of rhFIX (Batch 3).

Sample	Protein (mg/mL)	rhFIX activity (IU/mL)	Activity Yield (%)	rhFIX specific activity (IU/mg protein)	FIXa/FIX (%)	IgA (μg/mL)	IgM (μg/mL)	IgG (μg/mL)	Albumin (μg/mL)	β-LG (μg/mL)
Whey	25.02	34	100	1.36	–	3581	1027	474	1629	15.8
Q3f	3.03	196	92.33	64.69	11.48	271	81.5	1.65	1.27	1.32
H2pf	0.92	307	77.3	333.7	3.7	3.36	1.88	0.02	0.11	0.02
rH2pf	944.8	372	34.53	383.51	0.36	0.51	0.21	0.01	0.08	0.02
rAun	0.33	119	33.52	360.61	1.16	0.03	0.03	<0.01	0.03	0.01
NFC	1.05	414	35.9	394.29	1.07	<0.02	<0.02	<0.01	0.06	<0.01

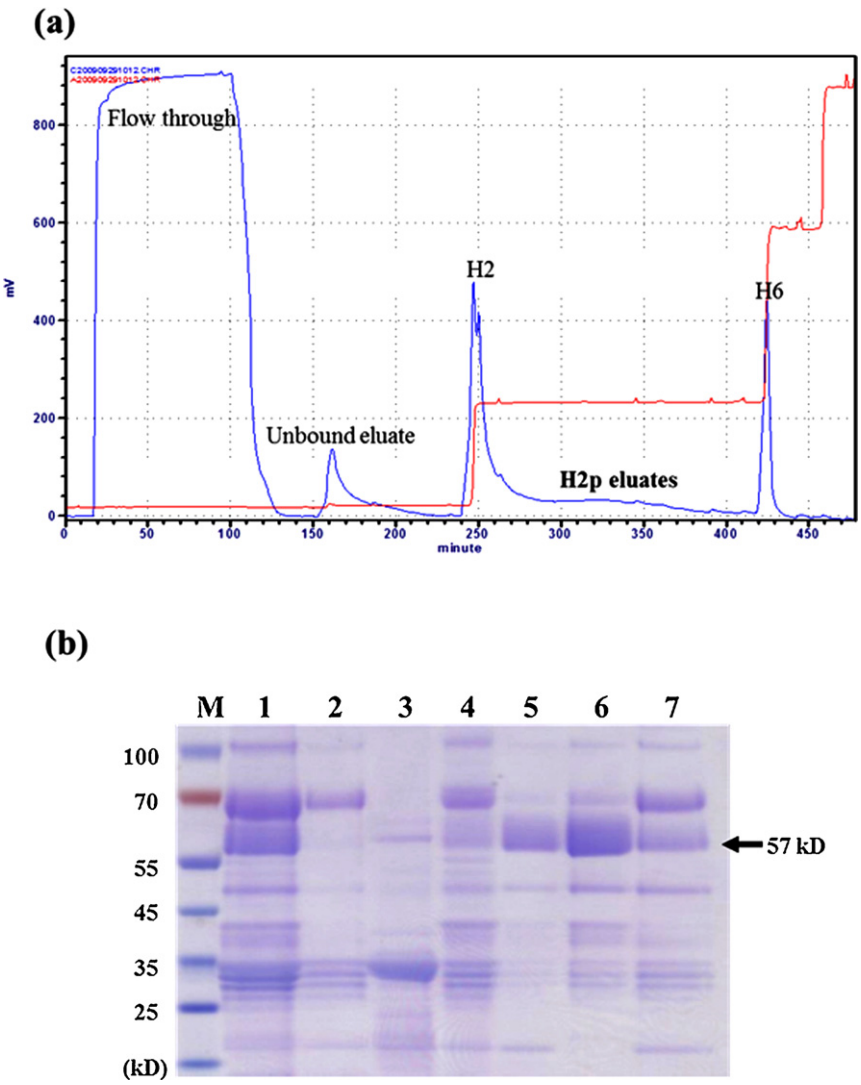

Fig. 4. Chromatogram for rhFIX purification on Heparin Sepharose FF chromatography (Heparin No. 1). (a) The solvent/detergent and other contaminant proteins were removed in flow through, unbound eluate and H2. The fraction of rhFIX zymogen was collected by B.2 buffer to be the H2p eluates. The most active rhFIX was eluted with B.6 buffer (H6). (b) SDS-PAGE analysis of rhFIX purification on Heparin No. 1. Lane M was pre-stained protein marker and the size and relative position of rhFIX was indicated. Lane 1: Q3f; lane 2: flow through; lane 3: unbound eluate; lane 4: H2; lane 5: H2p; lane 6: H2pf; lane 7: H6.

MonoFIX®-VF. Robert and Dunkerley [31] investigated the effects of different manufacturing process parameters on virus inactivation by treatment with polysorbate 80 and tri-*n*-butyl phosphate during Replenine®-VF production.

TnBP and non-ionic detergents such as polysorbate 80, Tween 20, and Tween 80 are most often used for S/D treatment of plasma products. Recently, 0.25–1% Triton X-100 has been confirmed to be effective for inactivating the pseudorabies virus during rhFIX production from transgenic sow milk [32].

The effects of different manufacturing process parameters on virus inactivation by S/D treatment of plasma products, including S/D concentration, temperature, treatment time, pH concentration, and protein concentration, have been investigated during pd-FIX production [31]. In this study, Q3f was treated with 0.3% TnBP and 1% Triton X-100 to inactivate the enveloped viruses for 10–12 h at 20–22 °C. The average protein concentration of Q3f in various batches was approximately 4 mg/mL, and the standard pH for S/D treatment was 7.2. In our validation of virus inactivation using a scale-down process, the viral reduction factor was $\geq 3.2 \pm 0.519\ \mathrm{Log}_{10}$ for the xenotropic murine leukemia virus (X-MuLV) (unpublished data).

3.2.3. *Heparin Sepharose 6 FF chromatography (Heparin No. 1 and No. 2)*

The protease domain of hFIX contains patches of positively charged amino acids to mediate the interaction with negatively charged glycosaminoglycans such as heparin. This characteristic of hFIX allows chromatographic separation using immobilized heparin [33]. The activation of FIX to FIXa not only removes the activation peptide, but also rearranges of the protease domain [34]. In the pilot production process presented here, purification using the heparin column was selected for the separation of rhFIX and hFIXa.

The Heparin Sepharose 6 FF column is composed of heparin, which is a naturally occurring glycosaminoglycan linked to the Sepharose matrix by reductive amination. Therefore, heparin can be used as an effective affinity-binding and ion exchange ligand for a wide range of biomolecules including coagulation factors (such as rhFIX) and other plasma proteins.

In the second chromatography step, the S/D-treated sample was directly loaded onto the Heparin Sepharose 6 FF column. The objective of this chromatography step was to isolate the recombinant target protein rhFIX.

As shown in the results of the chromatographic profile (Fig. 4(a)) and SDS-PAGE (Fig. 4(b)), the solvent/detergent and unbound proteins were removed in the flow-through, and the remaining casein and loosely bound proteins were washed out in B.CP buffer (labeled unbound eluate in Fig. 4(a)). Then, the more tightly bound contaminants (labeled H2) were removed by 3 CV wash with B.2 buffer. The Heparin Sepharose 6 FF column was then continuously flushed with B.2 buffer for further 3 CV. Then, the UV absorbance was raised again and the bound rhFIX was collected as a single fraction, labeled H2p. The H2p eluate was further concentrated and dialyzed with B.eq buffer to obtain H2pf. The specific activity of H2pf was 333.7 IU/mg protein, and the activity yield was 77.3% (Table 2 of Batch 3).

H2pf was directly loaded onto the Heparin Sepharose 6 FF column (Heparin No. 2). Fig. 5 shows the protein absorbance elution profile and SDS-PAGE for the Heparin No. 2 column. No obvious eluted peak was observed in the flow-through. The weakly bound impurities and minor rhFIX were co-eluted from the column, as demonstrated by SDS-PAGE. Then, the majority of rhFIX began to elute and collect when the absorbance peak was raised by B.2 buffer. The FIXa/FIX ratio was analyzed in the collected fractions for process control. Eluates with rhFIXa/rhFIX ratios ≤2.4% were selected for further UF/DF processing in PBS buffer to obtain rhFIX-rich rH2pf. To ensure that the rhFIX final product (rhFIXa/rhFIX ratio ≤1.7%) will not form a thrombus when infused into mice (data not shown), the threshold value of the rhFIXa/rhFIX ratio of rH2pf must be ≤2.4%. As indicated in Table 1, the specific activity of rH2pf was 383.51 IU/mg protein, and the activity yield was 34.53%.

FIX, a zymogen, belongs to the family of vitamin K-dependent serine protease precursors and can be proteolytically cleaved by physical activators (FVIIa-tissue factor complex or factor XIa) in the presence of calcium and phospholipids. It is then converted into its active enzymatic derivative, FIXa [35–37]. Activation is a 2-step process involving sequential cleavages at positions arginine (Arg)^{145} and Arg^{180} to convert to rhFIXa consisting of light and heavy chains held together by a single disulfide bond [34,38].

In Heparin chromatography, especially with Heparin No. 2, the rhFIX zymogen was gradually eluted by B.2 buffer. However, rhFIXa was released mostly using B.6 buffer of high NaCl concentration, as shown in Fig. 5(a) and Table 3. These results suggest that the interaction between rhFIXa and heparin was stronger than that of the rhFIX zymogen. Accordingly, the rhFIX zymogen was easily separated from rhFIXa. As Rohlena disclosed [38], heparin recognizes the binding region of the protease domain in both hFIX and in its inactive precursor, hFIX. Nevertheless, there was a lower response was observed for the hFIX zymogen compared to the hFIXa protease. This is in agreement with the fact that the rhFIX zymogen is more easily released than rhFIXa when separated by the heparin column in this report. Recombinant hFIXa may appear in milk because proteases from mammary tissue damaged during milking activated the rhFIX zymogen.

3.2.4. CaptureSelect Human IgA affinity chromatography (IgA removal column)

Compared to other impurities (e.g., albumin, β-lactoglobulin, IgG, and IgM), higher IgA levels were measured in casein-precipitated whey in this study. The previously described purification steps were adequate for most impurities. However, IgA could not be effectively discarded using these methods (Table 2). Therefore, IgA residues present in the rhFIX-rich concentrate rH2pf were removed by CaptureSelect Human IgA affinity chromatography.

The matrix of the IgA removal column contains a llama antibody fragment that recognizes IgA coupled to NHS-activated Sepharose FF. The two equal parts of rH2pf were loaded onto the IgA removal column. The flow-through containing rhFIX was collected, and the bound IgA removed in a washing step, as shown in Fig. 6(a). The

Table 3
The FIXa/FIX ratio from 8 batches of rhFIX.

Batch	Test											
	FIX activity (IU/mL)				FIXa activity (IU/mL)				FIXa/FIX ratio (%)			
Sample	QL	rH2pf	rH6	NFC	QL	rH2pf	rH6	NFC	QL	rH2pf	rH6	NFC
1	17.73	439.2	14.73	452.3	2.97	5.06	5.03	5.56	16.75	1.15	34.15	1.23
2	12.9	127	11	382	1.34	1.35	2.32	4.15	10.39	1.06	21.09	1.09
3	13	372	13	414	1.69	3.43	2.82	4.41	13	0.92	21.69	1.07
4	13	255	3	346	2.18	4.63	1.42	5.26	16.77	0.92	47.33	1.52
5	10	451	5	349	0.8	3.86	0.76	2.9	8	0.86	15.2	0.83
6	18	455	15	462	3.17	5.21	6.54	6.15	17.61	1.15	43.6	1.33
7	23	534	25	466	2.42	5.1	7.8	4.25	10.51	0.95	31.44	0.91
8	25.8	550.6	23.3	489	3.86	8.51	8.37	7.51	14.96	1.54	35.92	1.54
Mean ± SD	16.7 ± 5.5	398 ± 143	13.8 ± 7.8	420 ± 56	2.3 ± 1.0	4.6 ± 2.0	4.4 ± 3.0	5.0 ± 1.4	13.5 ± 3.6	1.1 ± 0.2	31.3 ± 11	1.19 ± 0.3

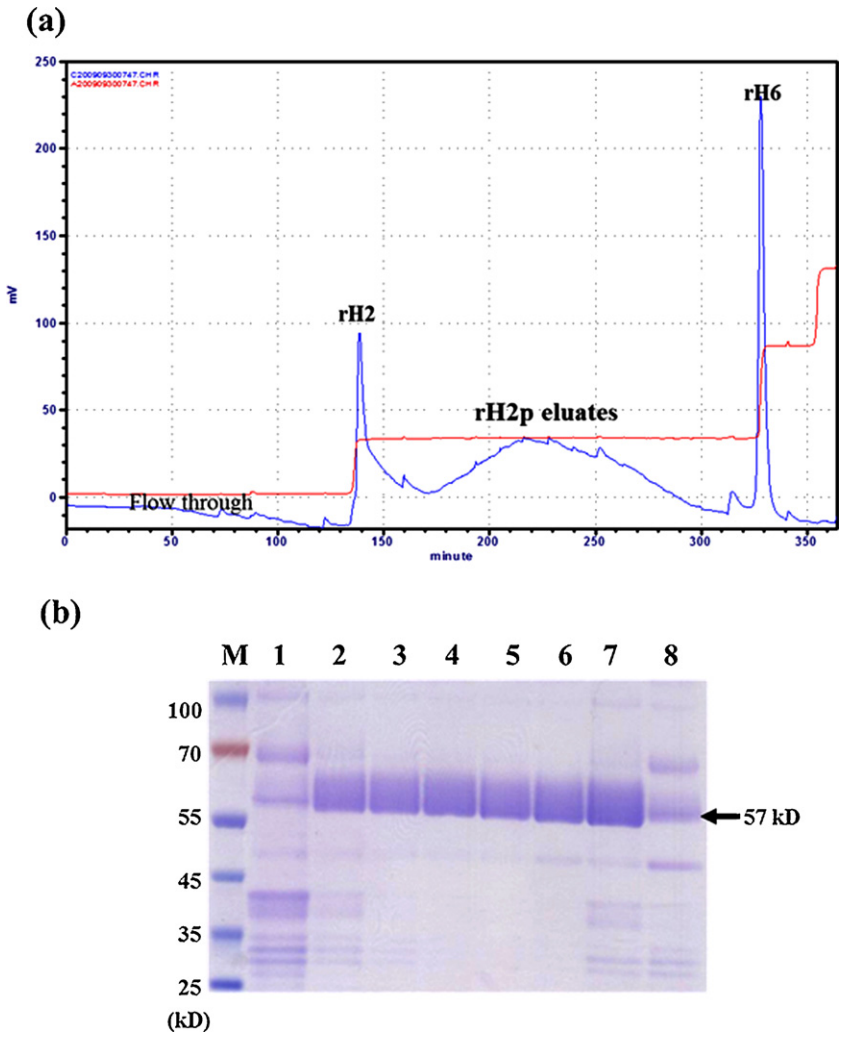

Fig. 5. Purification of rhFIX on Heparin Sepharose FF chromatography (Heparin No. 2). (a) Some contaminant proteins were shown in rH2. The high purity of rhFIX was eluted as rH2p eluates. The active rhFIX was presented in rH6 with 0.6 M NaCl concentration. (b) SDS-PAGE analysis of rhFIX purification on Heparin No. 2. Lane M was pre-stained protein marker and the size and relative position of rhFIX was indicated. Lane 1: rH2; lanes 2–6: rH2p-1 to rH2p-5; lane 7: rH2pf; lane 8: rH6.

pools of rhFIX-rich flow-through were applied to the IgA removal column and circulation. Fig. 6(b) shows that the unbound rhFIX fraction was collected and separated from the IgA contaminant. After processing with the IgA removal column, IgA concentration was reduced to 0.03 μg/mL. The activity yield of the resulting product, named rAun, was 33.52%, and the specific activity was 360.61 IU/mg protein (Table 2).

Van Cott et al. [39] observed an increase in IgA in transgenic sow milk, revealing that transgenic sows with average recombinant human protein C (rhPC) expression levels of approximately 1000 μg/mL demonstrated statistically significant increases in Igs and transferrin. IgA is the most abundant in transgenic sow milk, followed by IgG and IgM. The straightforward explanation for this phenomenon is that immunoglobulins are transported into milk by transcytosis, which is probably upregulated in sows with high rhPC expression levels. However, increased IgA concentrations may also be caused by production of antibodies to rhFIX. The direct cause of this phenomenon needs to be determined.

3.2.5. *Nanofiltration: virus removal*

Nanofiltration is one of the several methods of eliminating potential virus contamination and is especially effective in the removal of non-enveloped viruses resistant to other inactivation methods, such as S/D treatment. Therefore, this method is commonly used with plasma products such as coagulation factors and IgG to further enhance their safety. In this study, Virosart CPV was used for this purpose.

Virosart CPV efficiently removes both small non-enveloped viruses (20 nm) and large enveloped viruses (>50 nm) from the biopharmaceutical feed stream by size exclusion. It has the ability to retain large proteins and viral particles while allowing the rhFIX solution to pass through the nanofilter. In this manner, the high rhFIX filtrate was collected and labeled NF.

The efficacy of nanofiltration for virus removal was investigated using down-scale experiments performed with Virosart CPV Minisart on products spiked with two viruses: X-MuLV and porcine parvovirus (PPV). Our unpublished data indicated that no substantial changes were observed in the functional bioactivity of the filtered rhFIX in comparison with those before filtration. Furthermore, the results indicated that both the non-enveloped PPV and the enveloped X-MuLV were effectively removed during filtration. Log reduction factors were 5.749 ± 0.188 Log_{10} for PPV and $\geq 6.269 \pm 0.172$ Log_{10} for X-MuLV, respectively.

3.2.6. *The characteristics of purified rhFIX*

NF was processed in a final UF/DF step to exchange the PBS buffer to B.F buffer and the final product was labeled NFC. NFC was further identified by Western blotting and the result was shown in

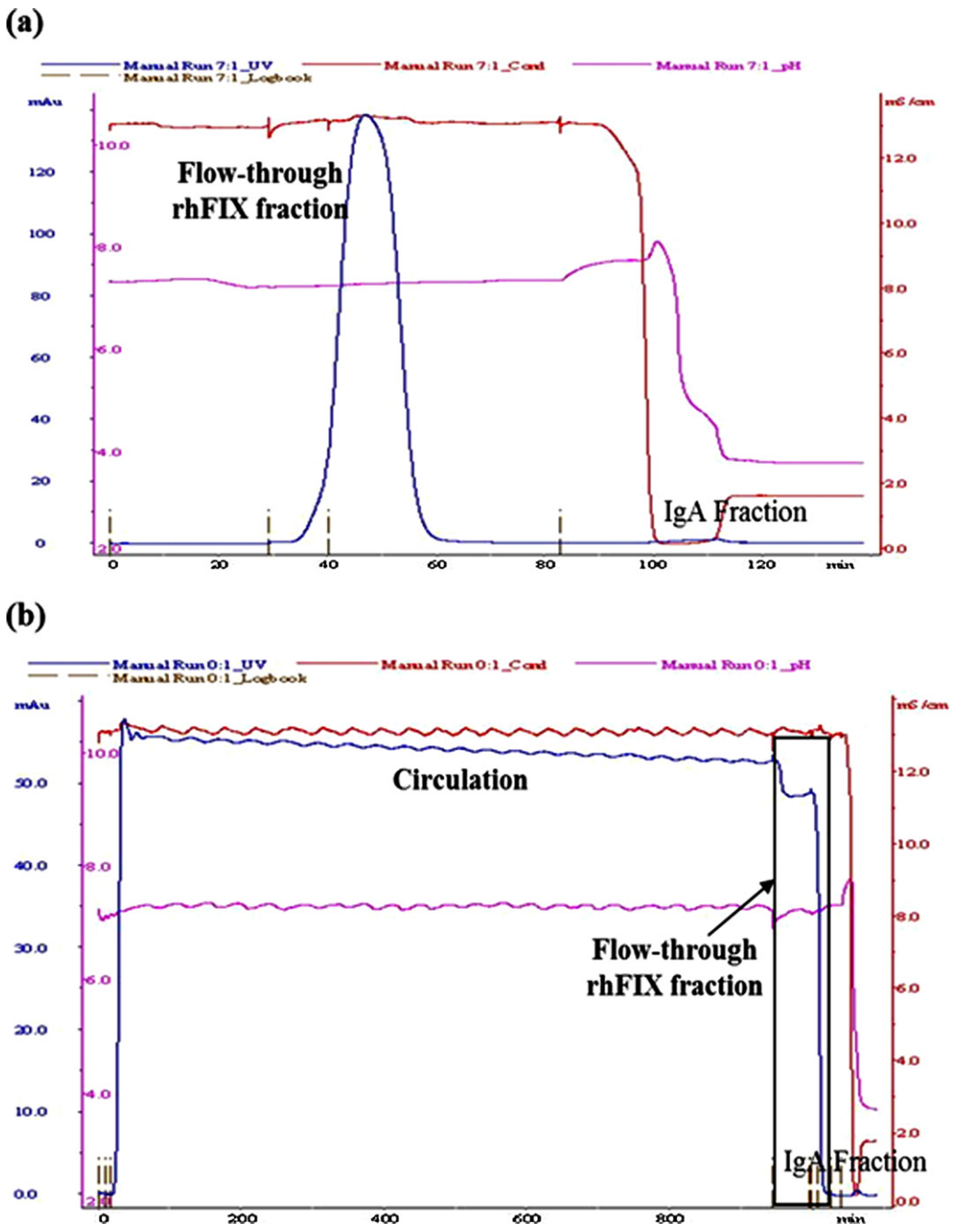

Fig. 6. Chromatographic step in removing IgA on CaptureSelect Human IgA affinity chromatography. (a) IgA was separated from rhFIX which was collected in flow-through. (b) Two parts of rhFIX-containing flow through were combined to load on IgA removal column and circulated. Then, the rhFIX-rich fraction (rAun) was still collected in unbound pass through.

Fig. 7. As presented in Fig. 7(a) and (b) (lanes 3–8, the solid arrows indicated) of Batch 3, the broad bands of purified rhFIX exhibited size heterogeneity apparently due to the difference of glycosylation. Additional bands ranging 40–55 kDa were also observed in Fig. 7(a) and (b) (lanes 3–8, the dotted arrows indicated). These were activated form of rhFIX (rhFIXa) which existed originally in transgenic sow milk [18]. rhFIXa could be separated from rhFIX and reduced gradually by the present manufacturing process as shown in Fig. 7(b).

The purity chromatogram of the final rhFIX by RP-HPLC was shown in Fig. 7(c) (Batch 3 as example) and the purity values of each batch were described in Table 4. The results indicated that rhFIX produced in this pilot production to be >99% of the total protein (mean ± SD: 99.64 ± 0.3). Furthermore, purified rhFIX aggregation was determined by SEC-HPLC analysis, and the profile showed a single peak to indicate that there was no aggregation of rhFIX but monomeric (Fig. 7(d)). These results indicated that a high purity of expressed rhFIX in the sow milk could be obtained.

The results of analyses of rhFIX produced from 8 batches are shown in Table 4. These results show that production was stable and reproductive across all batches; an average specific activity of 415.6 ± 57.7 IU/mg protein and an average lower rhFIXa/rhFIX ratio of 1.19 ± 0.3 (a threshold value of the final rhFIX product: 1.7). The activity yield ranged from 15.9% to 27% (mean ± SD: 23.95 ± 6.3). The variation in activity yield was related to the collected fractions during purification using Heparin No. 2, i.e., to that of the

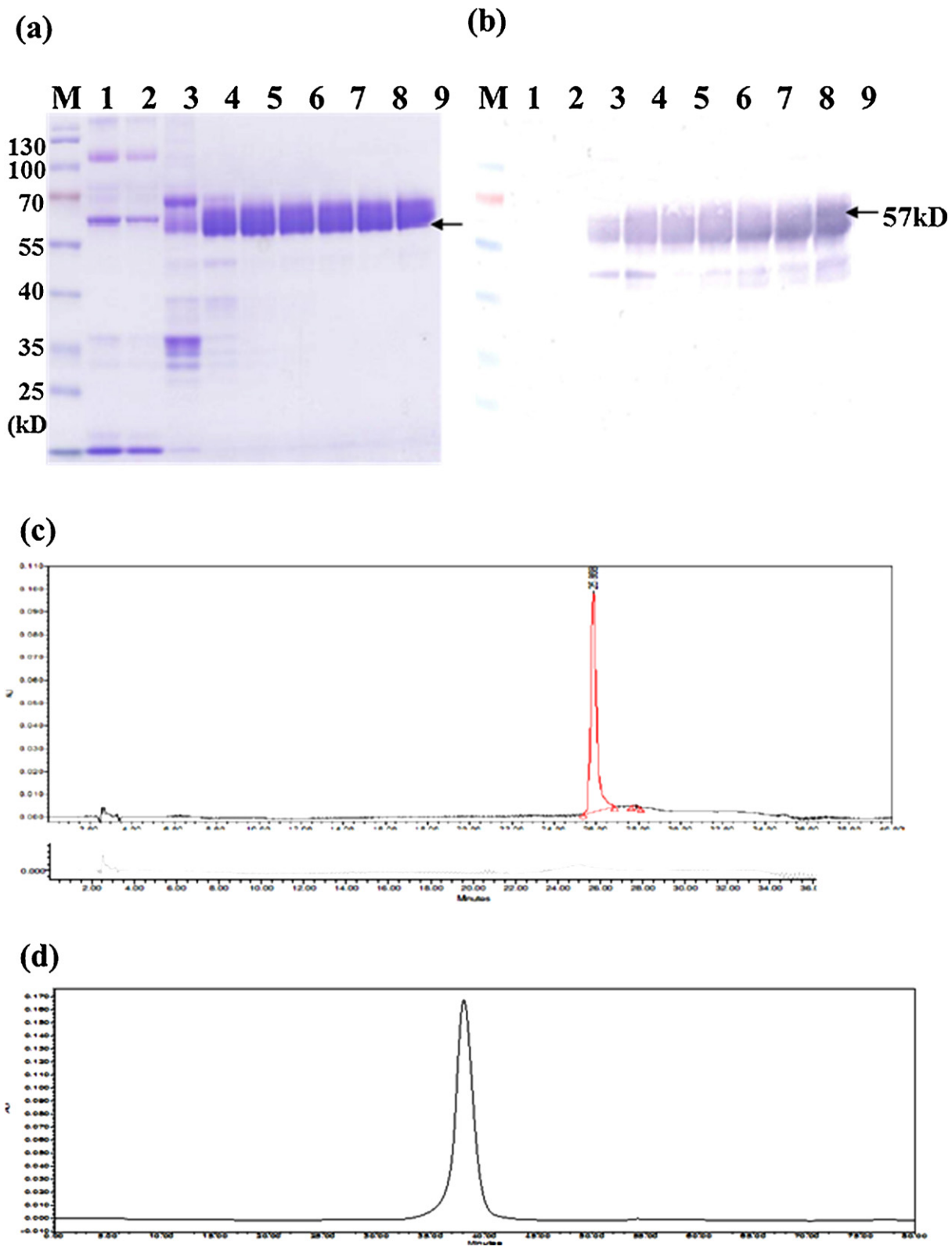

Fig. 7. Analysis of the presence of rhFIX in every established purification step (Batch 3). (a) 5 μg protein was loaded to each well and separated in non-reducing SDS-PAGE. Then, the gel was stained with Coomassie Blue. (b) Western blotting to identify the rhFIX. The proteins were transferred to PVDF membrane and exposed to rabbit anti-human FIX. Bound antibodies was detected by anti-rabbit IgG conjugated HRP. Lane M was pre-stained protein marker and the size and relative position of rhFIX was indicated. Lane 1: whey; lane 2: QL; lane 3: Q3f; lane 4: H2pf; lane 5: rH2pf; lane 6: rAun; lane 7: NFC; lane 8: NFC from Batch 3; lane 9: internal standard, the final product from Batch 1. The purity chromatogram of the final rhFIX was detected by RP-HPLC (c) and by SEC-HPLC (d).

Table 4
Summary characteristics for 8 batches of purified rhFIX.

Test item	Batch								Mean ± SD
	1	2	3	4	5	6	7	8	
Protein concentration (mg/mL)	0.9	1.1	1.05	1.01	0.9	1.07	1.05	1.03	1.01 ± 0.08
rhFIX activity (IU/mL)	452	382	414	346	349	462	466	489	420 ± 55.6
Specific activity (IU/mg)	502	347	394	343	388	432	444	475	415.6 ± 57.7
Ratio of FIXa/FIX (%)	1.23	1.09	1.07	1.52	0.83	1.33	0.91	1.54	1.19 ± 0.26
Aggregation by SEC-HPLC	0	0	0	0	0	0	0	0	0
Purity by RP-HPLC (%)	99.89	99.56	99.64	99.37	100	100	99.42	99.24	99.64 ± 0.3
Total impurities of HCP (ng/mg)	0.15	0.29	0.27	0.56	0.73	0.75	0.28	0.21	0.41 ± 0.2
Residual DNA (pg/dose)[a]	<250	<250	<250	ND[b]	ND	ND	ND	ND	
Activity Yield (%)	25	23.4	35.9	15.9	26.7	18.1	19.6	27	23.95 ± 6.3

[a] The detection limit was 250 pg/mL.
[b] ND means "not determined."

rhFIXa/rhFIX ratio. This phenomenon verified that the uniqueness and importance of the purification steps established in the present report. For further evaluation of bioactivity, Chang et al. [40] studied the distribution and pharmacokinetics of the purified-rhFIX *in vivo*. The rhFIX products also have exhibited potential use in hemophilic B prophylaxis with prepared long-acting rhFIX microspheres by Chang et al. [41].

3.2.7. Analyses of residual DNA and HCP

The residual DNA and HCP of purified rhFIX were also investigated and the results were shown in Table 4.

The World Health Organization has set a requirement for the residual host DNA in biopharmaceutical formulations of <10 ng/dose. In the present study, the analyses of residual DNA in the final purified rhFIX from 3 batches (Batches 1, 2 and 3) were all <300 pg/dose. This indicated that there was a low level of residual DNA in the final products and fit the recommended level of residual DNA by WHO.

There is no official criterion for the HCP level as residual DNA, but the HCP level must be reduced and controlled during purification. In this report, the level of residual host cell proteins in the purified rhFIX was 0.41 ± 0.2 ng/mg on average. This result implied that the HCP could be controlled and separated from the target protein by the production process described herein.

4. Conclusions

We have established a pilot production process for purification of high-quality, functional rhFIX from transgenic sow milk involving safe removal of casein, other impurities and viruses. The present process has the potential to purify rhFIX on a large scale in order to provide sufficient rhFIX for controlling bleeding in hemophilia B patients.

Acknowledgements

This work was supported by the grant of 96-EC-17-A-17-R7-0454 from the Ministry of Economic Affairs of Executive Yuan, Taiwan, R.O.C. The authors gratefully thank to Dr. Shih-Rong Wang for the technical operation of viral reduction factor at Biosafety Test Laboratory and persons who have participated in the execution of this production process of the Animal Technology Institute Taiwan.

References

[1] D.S. Anson, D.E. Austen, G.G. Brownlee, Nature 315 (1985) 683.
[2] S. Busby, A. Kumar, M. Joseph, L. Halfpap, M. Insley, K. Berkner, K. Kurachi, R. Woodbury, Nature 316 (1985) 217.
[3] H. de la Salle, W. Altenburger, R. Elkaim, K. Dott, A. Dieterlé, R. Drillien, J.P. Pazenave, P. Tolstoshev, J.P. Lecocq, Nature 316 (1985) 268.
[4] R.J. Kaufman, L.C. Wasley, B.C. Furie, C.B. Shoemaker, J. Biol. Chem. 261 (1986) 9622.
[5] S. Jallat, F. Perraud, W. Dalmans, A. Balland, A. Dieterle, T. Faure, P. Meulien, A. Pavirani, EMBO J. 9 (1990) 3295.
[6] S. Harrison, S. Adamson, D. Bonam, S. Brodeur, T. Charlebois, B. Clancy, R. Costigan, D. Drapeau, M. Hamilton, K. Hanley, B. Kelley, A. Knight, M. Leibard, M. McCarthy, P. Oakes, K. Sterl, M. Switzer, R. Walsh, W. Foster, Semin. Hematol. 35 (1998) 4.
[7] P. Chowdary, H. Dasani, J.A.H. Jones, C.M. Loran, A. Eldridge, S. Hughes, P.W. Collins, Haemophilia 7 (2001) 140.
[8] A.D. Shapiro, J. Di Paola, A. Cohen, K.J. Pasi, M.A. Heisel, V.S. Blanchette, T.C. Abshire, W.K. Hoots, J.M. Lusher, C. Negrier, C. Rothschild, D.A. Roth, Blood 105 (2005) 518.
[9] H.C. Hughes, Lab. Anim. Sci. 36 (1986) 348.
[10] A.J. Clark, J. Mammary Gland Biol. Neoplasia 3 (1998) 337.
[11] R.K. Paleyanda, W.H. Velander, T.K. Lee, D.H. Scandella, F.C. Gwazdauskas, J.W. Knight, L.W. Hoyer, W.N. Drohan, H. Lubon, Nat. Biotechnol. 15 (1997) 971.
[12] H.G. Lee, H.C. Lee, S.W. Kim, P. Lee, H.J. Chung, Y.K. Lee, J.H. Han, I.S. Hwang, J.I. Yoo, Y.K. Kim, H.T. Kim, H.T. Lee, W.K. Chang, J.K. Park, J. Reprod. Dev. 55 (2009) 484.
[13] W.H. Velander, J.L. Johnson, R.L. Page, C.G. Russell, A. Subramanian, T.D. Wilkins, F.C. Gwazdauskas, C. Pittius, W.N. Drohan, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 12003.
[14] K.E. Van Cott, S.P. Butler, C.G. Russell, A. Subramanian, H. Lubon, F.C. Gwazdauskas, J. Knight, W.N. Drohan, W.H. Velander, Genet. Anal. 15 (1999) 155.
[15] M. Lindsay, G-.C. Gil, A. Cadiz, W.H. Velander, C. Zhang, K.E. Van Cott, J. Chromatogr. A 1026 (2004) 149.
[16] T. Morçöl, Q. He, S.J. Bell, Biotechnol. Prog. 17 (2001) 577.
[17] W.H. Velander, W.N. Drohan, H. Lubon, J.L. Johnson, US Patent 6,344,596 (2002).
[18] S.C. Wu, T.K. Cheng, C.M. Chen, S.P. Lin, C.H. Yen, P.C. Yang, US Patent 7,087,808 (2006).
[19] W.H. Kim, J.S. Kim, Y. Yoon, G.M. Lee, J. Biotechnol. 142 (2009) 275.
[20] J. Denman, M. Hayes, C. O'Day, T. Edmunds, C. Bartlett, S. Hirani, K.M. Ebert, K. Gordon, J.M. McPherson, Biotechnology (NY) 9 (1991) 839.
[21] A.J. Clark, H. Bessos, J.O. Bishop, P. Brown, S. Harris, R. Lathe, M. McClenaghan, C. Prowse, J.P. Simons, C.B.A. Whitelaw, I. Wilmut, Nat. Biotechnol. 7 (1989) 487.
[22] C.H. Yen, M.L. Lin, U.S. Patent 7,214,319 (2007).
[23] C.H. Yen, S.L. Lee, M.Y. Chen, Y.S. Lin, X.H. Huang, C.H. Shih, H.F. Tai, U.S. Patent 7,943,739 (2011).
[24] B. Furie, B.C. Furie, Blood 75 (1990) 1753.
[25] B. Furie, B.A. Bouchard, B.C. Furie, Blood 93 (1999) 1798.
[26] S. Gillis, B.C. Furie, B. Furie, H. Patel, M.C. Huberty, M. Switzer, W.B. Foster, H.A. Scoble, M.D. Bond, Protein Sci. 6 (1997) 185.
[27] E.G. Wojcik, W.F. Cheung, M. van den Berg, I.K. van der Linden, D.W. Stafford, R.M. Bertina, Biochim. Biophys. Acta 1382 (1998) 91.
[28] N. Wajih, S.M. Hutson, J. Owen, R. Wallin, J. Biol. Chem. 280 (2005) 31603.
[29] M. Blostein, J. Cuerquis, S. Landry, J. Galipeau, Haemophilia 14 (2008) 1063.
[30] A. Johnston, A. MacGregor, S. Borovec, M. Hattarki, K. Stuckly, D. Anderson, N.H. Goss, A. Oates, E. Uren, Biologicals 28 (2000) 129.
[31] P.L. Robert, C. Dunkerley, Vox Sang. 84 (2003) 170.
[32] J.T. Chang, Y.C. Chou, M.S. Lin, S.R. Wang, Jpn. J. Vet. Res. 58 (2010) 179.
[33] L.O. Andersson, H. Borg, M. Miller-Andersson, Thromb. Res. 7 (1975) 451.
[34] D. Gailani, Trends Cardiovasc. Med. 10 (2000) 198.
[35] R.G. Di Scipio, K. Kurachi, E.W. Davie, J. Clin. Invest. 61 (1978) 1528.
[36] B. Furie, B.C. Furie, Cell 53 (1988) 505.
[37] L. Yang, C. Manithody, A.R. Rezaie, J. Biol. Chem. 277 (2002) 50756.
[38] J. Rohlena, Molecular interactions between coagulation factor IX and low density lipoprotein receptor-related protein, thesis, 2004 (Chapter IV).
[39] K.E. Van Cott, H. Lubon, F.C. Gwazdauskas, J. Knight, W.N. Drohan, W.H. Velander, Transgenic Res. 10 (2001) 43.
[40] C.H. Chang, T.K. Chou, C.Y. Yang, T.J. Chang, Y.H. Wu, T.W. Lee, In Vivo 22 (2008) 693.
[41] L.C. Chang, C.Y. Yang, A.C. Chua, Y.J. Lin, S.M. Lai, Mol. Pharmacol. 8 (2011) 1767.