

Chapter 3: Purification of Recombinant DNA-Derived Factor IX Produced in Transgenic Pig Milk and Fractionation of Active and Inactive Subpopulations

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3.1 Abstract

Transgenic animal bioreactors can be engineered to make gram per liter quantities of complex recombinant glycoproteins in milk. However, little is known about the limitations in post-translational processing that occurs for very complex proteins and how this impacts the task of purification. We report on the purification of recombinant factor IX (rFIX) from the milk of transgenic pigs having an expression level of 2-3 grams rFIX/liter/hour, an expression level that is about 20-fold higher than previously reported. This purification process efficiently recovers highly active rFIX and shows that even complex mixtures like pig milk, which contains 60 grams/liter total endogenous milk protein and multiple subpopulations of rFIX, can be processed using conventional, non-immunoaffinity chromatographic methods. Without prior removal of caseins, heparin-affinity chromatography was used to first purify the total population of rFIX at greater than 90% yield. After the total population was isolated, the biologically active and inactive subpopulations were fractionated by high-resolution anion-exchange chromatography using an ammonium acetate elution. Capillary isoelectric focusing of the active and inactive rFIX fractions demonstrated that the active subpopulations are the most acidic.

Keywords: transgenic animal, factor IX, heparin, chromatography, Hemophilia B

3.2 Introduction

Hemophilia B is a bleeding disorder caused by a congenital deficiency in circulating factor IX (FIX) activity. The ideal treatment of Hemophilia B is prophylactic treatment, which significantly reduces complications and improves the quality of life.¹ Unfortunately, this regimen is unavailable for many hemophiliacs because the only sources of replacement factor IX are plasma-derived factor IX (pd-FIX) and recombinant factor IX (rFIX) produced in Chinese hamster ovary (CHO) cells (BeneFIX[®]).² Pathogen safety concerns with pd-FIX, combined with the limited supply of pd-FIX and rFIX from CHO cells make prophylactic treatment of Hemophilia B expensive. Safer and more productive sources of rFIX are thus desired.

FIX is a complex protein with numerous post-translational modifications (PTMs). These PTMs are required for biological activity and pharmacokinetics, and include propeptide removal, vitamin K-dependent γ -carboxylation of Glu to γ -carboxyglutamate (Gla), Ser-phosphorylation, Tyr-sulfation, and glycosylation.³ In particular, γ -carboxylation and propeptide removal are required for the procoagulant activity of FIX (Figure 3-1). The complexity of these multiple PTMs also necessitates production of rFIX in mammalian cells. However, not all mammalian cells are equally proficient in performing all of these PTMs. For example, CHO cells were shown to have significant rate limitations in propeptide removal, γ -carboxylation, and Ser-phosphorylation.^{4,5,6} Additionally, transgenic sheep were not able to produce any significant amounts of biologically active rFIX in milk.⁷ We have previously published immunoaffinity methods for purification of biologically active rFIX from transgenic pig milk making lower expression levels of 100-200 μ g/ml containing 35-70 U/ml.⁸ The rFIX was fully γ -carboxylated at this level of production. Immunoaffinity chromatography was also used to purify biologically active rFIX from the milk of transgenic mice.⁹

In this work, we report on the purification of recombinant FIX from a new lineage of transgenic pigs having expression levels of 2-3 grams rFIX/liter milk containing about 70-140 U/ml FIX activity. With the ability to translate and secrete higher levels of recombinant polypeptide by transgenic methods comes the need for economical purification methods that can selectively isolate populations that have preferred post-translational structure. We demonstrate that non-immunoaffinity chromatographic methods are able to purify select rFIX populations to a high purity and a high specific activity. Importantly, we also demonstrate a rate limitation in γ -carboxylation occurs at rFIX expression levels of 0.2 to 0.3 g/liter.

3.3 Experimental

3.3.1 Reagents

All buffer components were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise noted. The following buffers were used:

- Heparin Loading Buffer - 20 mM Tris, 50 mM NaCl, pH 7.2
- Heparin Wash Buffer - 20 mM Tris, 200 mM NaCl, pH 7.2
- Heparin Elution Buffer - 20 mM Tris, 500 mM NaCl, pH 7.2
- Heparin Regeneration Buffer - 4 M NaCl
- Matrex Cellufine Sulfate Loading Buffer - 20 mM Tris, 125 mM NaCl, pH 7.4
- Matrex Cellufine Sulfate Wash Buffer - 20 mM Tris, 250 mM NaCl, pH 7.4
- Matrex Cellufine Sulfate Elution Buffer - 20 mM Tris, 500 mM NaCl, pH 7.4
- Q Loading Buffer - 20 mM Tris, pH 9.0

3.3.2 Transgenic Milk Collection and Storage

Transgenic pigs were generated as described previously,⁸ but using a 4.2 kb mouse whey acid protein promoter for the FIX cDNA construct.¹⁰ Milk was collected from lactating pigs in accordance with protocols approved by the Virginia Tech Animal Care Committee. Lactating sows were injected intramuscularly with 60 units of oxytocin (Vedco, St. Joseph, MO), and milked by hand. The milk was immediately aliquoted and frozen at -50°C . Milk used in this study had a FIX activity level of 100-140 U/ml milk, as measured by the aPTT assay.

3.3.3 Purification of rFIX

Heparin Chromatography

Heparin-Sepharose FF (Amersham Biosciences, Piscataway, NJ) was packed in Bio-Rad Econo-columns (Hercules, CA) and the columns were run on a Bio-Rad BioLogic DuoFlow chromatography system with 280 nm detection. Aliquots of milk were thawed and mixed 1:1 (v:v) with 200 mM EDTA pH 7.4 (to dissolve the casein micelles) and centrifuged for 15 minutes at 3000 x g at 4°C to separate the fat. The skim milk/EDTA was diluted five-fold with Heparin Loading

Buffer, and loaded onto the column (L = 9 cm, ID = 2.5 cm). All columns in this work were run at room temperature. In order to minimize rFIX losses in the flow-through and wash steps, the column was loaded to only 1 mg rFIX/ml matrix at a linear velocity of 1 cm/min. The column was subsequently washed with Heparin Loading Buffer at 2 ml/minute until the baseline absorbance was reached, and then washed with Heparin Wash Buffer at 2 ml/minute to remove adsorbed caseins. Recombinant factor IX was eluted with Heparin Elution Buffer at 3 ml/minute. The heparin product was either immediately diluted and processed by anion-exchange chromatography, or else immediately frozen at -70°C . The heparin-Sepharose column was regenerated with 4 M NaCl, and re-equilibrated.

A synthetic heparin analogue matrix, Matrex Cellufine Sulfate (Millipore, Billerica, MA), was also tested. The column was packed in a Bio-Rad Econo-Column (L = 8 cm, ID = 1 cm). The Cellufine Sulfate column was first equilibrated with Sulfate Loading Buffer and subsequently loaded at 1.5 ml/min with skim milk diluted identically as with the heparin-Sepharose columns. The column was then washed with Cellufine Sulfate Loading Buffer until baseline was reached, and then washed using Cellufine Sulfate Wash Buffer. FIX was eluted using Cellufine Sulfate Elution Buffer. All steps were performed at 1.5 ml/min. As with heparin Sepharose, the Cellufine Sulfate column was regenerated with 4 M NaCl and re-equilibrated.

The purity of the heparin-Sepharose product was analyzed by reverse phase HPLC on a Vydac 218TP5415 column (C18 stationary phase, L = 15 cm, ID = 4.6 mm, 5 μm particles, 300 Å pores, Hesperia, CA) with a Supelco Supelguard LC318 guard column (C18, 5 μm particles, 300 Å pores, St. Louis, MO). The solvents used were A: 0.1% (w/v) TFA in water, and B: 0.1% (w/v) TFA in acetonitrile. The heparin-Sepharose product and immunoaffinity purified pd-FIX standard (Mononine[®], Centeon LLC, Kankakee, IL) were diluted in deionized water and 200 μl were injected. The solvent program was as follows: 5 minutes 5% B, followed by 5-95% B over 45 minutes (2%/minute), followed by 5 minutes of 95% B. The flow rate was constant at 1 ml/min. Data were collected on a Beckman System Gold HPLC system with a Model 126 solvent delivery system and a Model 168 diode array detector. An Isco Foxy programmable fraction collector was used to collect fractions. Peak areas at 220 nm were integrated by the System Gold software.

Anion-Exchange Chromatography

A Mini Q 4.6/5.0 column (L = 5 cm, ID = 4.6 mm, nonporous 3 μm particles, Amersham Biosciences) was used to fractionate active from inactive rFIX subpopulations. The heparin-

Sepharose product was diluted with two parts Q-Loading Buffer, and loaded at a flow rate of 0.4 ml/minute. The column was washed with 90%:10% Q-Loading Buffer:Q-Elution Buffer for 10 CV at 0.8 ml/minute. Adsorbed rFIX was eluted by either a linear gradient or by a combination of gradient and isocratic elutions. Data were collected on the Beckman HPLC system. The specific conditions are given in the figure captions for the chromatograms shown.

3.3.4 SDS PAGE

Column fractions were analyzed by SDS PAGE (silver-stained) using Invitrogen (Carlsbad, CA) gels and the Invitrogen Minicell apparatus. All gels were NuPage 10% Bis-Tris run with MES Running Buffer (Invitrogen). Briefly, samples were mixed with 4x LDS Sample Buffer (Invitrogen) and deionized water followed by heating at 95°C for 10 minutes. For reduced gels, samples were adjusted to 0.05 M DTT prior to heating.

3.3.4 FIX Activity - aPTT Assay

The biological activity of the rFIX fractions was determined by the activated partial thromboplastin time (aPTT) assay.¹¹ Briefly, 100 µl each of Alexin (cephalin from rabbit brain, Sigma), FIX deficient plasma (Biomerieux, Durham, NC), and sample of interest were added to a cuvette and incubated at 37°C for 3 minutes. 100 µl of 20 mM CaCl₂ (Sigma) was then added and the clotting time was measured using a MLA Electra Coag-a-Mate coagulation timer. Units of FIX activity were calculated by comparison of the unknown sample to a standard curve created from dilutions of normal reference plasma (Biomerieux, 1 U/ml plasma) made using the same protocol. The concentration of purified rFIX was determined by measuring the optical density at 280 nm in a 1 cm quartz cuvette and using an extinction coefficient of 1.33 for a 1 mg/ml FIX solution.

3.3.5 Total Gla Analysis

Total Gla content of the purified rFIX fractions was determined by amino acid analysis from basic hydrolysis. Column fractions were dialyzed overnight against deionized water in a cold room (4°C) and then lyophilized to dryness in a Savant Speed-Vac system. The rFIX was then

reconstituted in deionized water, frozen, and shipped to Commonwealth Biotechnologies (Richmond, VA) for analysis. Briefly, 83 μl of 4N NaOH and 117 μl of water were added to 100 μl of sample. The tubes were sealed under vacuum and hydrolyzed for 20 hours at 100°C. The samples were then neutralized with 4 N acetic acid, diluted with a borate buffer, and then subjected to analysis. The molar ratio $R = \text{Glu}/\text{Gla}$ for each sample was obtained from the respective peak areas of the chromatogram. FIX has 40 total Glx residues (28 Glu + 12 potential Gla). The (moles Gla)/(moles FIX) was obtained by solving the simple set of equations

$$\text{Glu} + \text{Gla} = 40$$

$$\frac{\text{Glu}}{\text{Gla}} = R$$

where R is the experimentally determined ratio of (moles Glu)/(moles Gla) for the sample.

3.3.6 Isoelectric Focusing

The pI of rFIX subpopulations was determined by a two-step capillary isoelectric focusing and mobilization based on the method reported by Huang *et al.*¹² A two-step method was necessary because FIX is a very acidic protein. Polyacrylamide-coated capillaries (eCAP Neutral, Beckman Coulter, Fullerton, CA) of 50 μm ID and 27 cm total length were used with a Beckman PACE/5000 instrument with UV absorbance detection. The anolyte used was 60 mM phosphoric acid in 0.4% (w/v) hydroxypropylmethyl cellulose (HPMC, Sigma). The catholyte was 20 mM NaOH. All solutions were filtered with a 0.2 μm syringe filter before use. Ampholytes (pI 3-10) were purchased from Fluka (St. Louis, MO). Two standards, carbonic anhydrase I (Sigma, pI = 6.6) and amyloglucosidase (Sigma, pI = 3.6) were included in all focusing experiments. The sample solution was prepared by combining the FIX sample, the two protein standards (2 μl of 4 mg/ml carbonic anhydrase I and 1 μl of 4 mg/ml amyloglucosidase), ampholytes (1 μl), and 1% HPMC (80 μl , for final 0.4% HPMC) to a total volume of 200 μl . The capillary was first rinsed with deionized water for 2 minutes and then filled with the sample solution by rinse mode (20 psi) for 1 minute. Normal polarity (cathode at detector end) was used for focusing and mobilization. Focusing was performed by applying 15 kV for 6 minutes. After the focusing step, a low pressure rinse of anolyte (0.5 psi) while maintaining 15 kV was applied for 50 minutes to mobilize the focused proteins past the detector window. The separations were carried out at 20°C with detection at 280 nm. The capillary was rinsed with deionized water for 5 minutes after each run.

3.4 Results and Discussion

Efforts to purify FIX from donor plasma are decades old, and production of FIX concentrates by crude methods such as cryoprecipitation or Cohn fractionation has been replaced with high purity products using immunoaffinity and heparin-affinity chromatography.^{13,14,15} Milk and blood are equally challenging feedstocks for protein purification as both are multi-phase mixtures. Milk has a solids phase that includes cell debris and the casein micelles, an aqueous phase that contains whey proteins, carbohydrates, minerals and nutrients, and the milkfat phase that contains triglycerides, phospholipids, and cholesterol.¹⁶ The caseins are the most prevalent endogenous milk proteins at about 30 g/liter. Other major milk proteins include whey proteins (lactoferrin, alpha-lactalbumin, beta-lactoglobulin) and serum-passover proteins (serum albumin, immunoglobulins, transferrin). Even though rFIX is being produced at a very high level of 2-3 grams/liter in the milk, it is still less than 10% of the total protein content. In addition, the numerous variations in the PTMs that are made to this complex protein add another layer of complexity to the purification problem – how does one design a purification process that also selects for only the biologically active subpopulations?

The initial objective was to purify the entire population of rFIX from the milk so as to understand the nature of limits in post-translational processing of rFIX that occurs at these very high expression levels. The use of classical casein precipitation techniques was problematic, with as much as 20% of the rFIX partitioning into casein precipitates (data not shown). Thus, to circumvent this problem, the casein micelles were solubilized with excess EDTA. The heparin binding domain of FIX is located in the C-terminal end of the molecule, a region devoid of PTMs.¹⁷ We therefore used heparin-affinity chromatography as a capture step so that the entire population could be isolated. The rFIX was found to strongly bind to the heparin matrix. The column was underloaded (with respect to FIX capacity) to result in complete rFIX capture. Caseins as well as a number of other milk proteins also adsorbed to the column, but were washed off with 200 mM NaCl (Figure 3-2). Highly pure rFIX was eluted with 500 mM NaCl. The purity of the rFIX product was analyzed by reverse phase HPLC. Using TFA as an ion pairing agent and a 2%/minute acetonitrile gradient, the isoforms of both plasma-derived FIX and rFIX were not resolved, but eluted as a single peak (Figure 3-3). The purity from this single chromatographic step (98% peak area at 220 nm) was similar to the purity of pd-FIX purified by immunoaffinity chromatography. Western blots and activity assays of the column flow-through and 200 mM NaCl fractions had undetectable rFIX antigen and activity.

A synthetic heparin analogue, Matrex Cellufine Sulfate, has also been used in the purification of BeneFIX[®], a recombinant FIX produced in CHO cells.¹⁸ We were unable to obtain a pure rFIX fraction with this heparin analogue when clarified skim milk was used as the column feed (Figure 3-4). Washing the column with a 250 mM NaCl buffer resulted in the loss of rFIX, and a significant amount of the caseins co-eluted with rFIX in a 500 mM NaCl buffer. In fact, caseins remained adsorbed to the column and had to be removed during the regeneration step with 4 M NaCl. The data show that the heparin analogue did not have the specificity to discriminate between rFIX and the caseins, and non-specific ion-exchange interactions with the sulfated cellulose matrix were likely dominating for the conditions used. Thus, unless the caseins are removed, this heparin analogue is not useful for purification of heparin-binding proteins from porcine milk.

The specific activity of the heparin product was low (typically 30-35 U/mg protein) compared to the theoretical pd-FIX specific activity of 200-250 U/mg. This indicated that only a fraction of the total rFIX population was biologically active. The biological activity of FIX is highly dependent on the nature and extent of PTMs. Of primary importance are the PTMs that are made to the N-terminal region of FIX – the Gla domain. For FIX to be active, the propeptide must be removed, and the Glu residues in the Gla domain must be γ -carboxylated.³ The conformation of a fully γ -carboxylated Gla domain undergoes a dramatic change in the presence of Ca^{2+} .^{19,20} This Ca^{2+} -dependent conformational change enables localization of FIX on the endothelial or platelet cell surface, where it associates with factor VIIIa to activate factor X. Inadequate γ -carboxylation or the presence of propeptide severely diminishes this metal-dependent conformational change and renders the FIX inactive.^{21,22}

Several different methods have been used to fractionate highly γ -carboxylated, active rFIX from inactive rFIX, and they are all based on the metal-dependent conformational changes of the Gla domain. Barium citrate adsorption was one of the earliest methods used,²³ but this has the disadvantage of long processing times and is not easily scaled up. More recently, metal-dependent monoclonal antibodies have been raised against the Gla domain.²⁴ Other pseudo-affinity chromatographic methods have also been developed: Yan *et al.*²⁵ used a calcium elution off an anion-exchange resin to fractionate fully γ -carboxylated recombinant Protein C from uncarboxylated subpopulations. This method, as well as hydroxyapatite chromatography, has also been adapted for BeneFIX[®] production.¹⁸ For the rFIX heparin chromatography product from transgenic pigs, hydroxyapatite chromatography and the pseudo-affinity calcium elution off an anion-exchange column did not cleanly fractionate active from inactive subpopulations. Barium

citrate adsorption was moderately successful in fractionating active from inactive subpopulations, but the specific activity of the pellet fraction was low (~50 U/mg) due to the fact that propeptide-containing rFIX also partitions into the pellet fraction,^{26,27} which was confirmed by N-terminal amino acid sequencing (data not shown).

Gamma-carboxylated proteins are highly acidic and this favors the use of anion-exchange to obtain the most highly biologically active rFIX species. We adapted the method of Gillis *et al.*,²⁸ which used a NaCl gradient elution from a high-resolution anion-exchange column to partially resolve rFIX made in CHO cells having 10, 11, or 12 Gla residues. The chromatogram from a NaCl gradient elution of rFIX on a Mini Q column is given in Figure 3-5A. The FIX begins eluting at about 0.2 M NaCl, and multiple subpopulations elute as the salt concentration is increased at a rate of 5 mM NaCl/CV. Analysis of the fractions showed that the biologically active subpopulations eluted in the tail end of the peak that corresponded to about 10% of the peak area. However, even with this high-resolution column matrix there is poor resolution between the subpopulations in this region, and low specific activities of collected fractions indicated that subpopulations with inadequate Gla content and/or proFIX were not well resolved. Resolution was not significantly improved by using a gradient slope of 2.5 mM NaCl/CV, and resolution was even poorer at higher rFIX loading.

In an effort to obtain better resolution between the active and inactive subpopulations, a different displacing salt was tried. Kopaciewicz *et al.*²⁹ showed that the resolution between soybean trypsin inhibitor and ovalbumin on a strong anion-exchange column was dependent on the counterion. We therefore used an elution buffer with ammonium acetate for gradient elution of rFIX. The results of an ammonium acetate gradient on the same Mini Q column are shown in Figure 3-5B. Again, the biologically active rFIX eluted in the last 10% of the peak area (beginning at 61 minutes). However, the use of ammonium acetate resulted in better resolution between subpopulations, especially in the later eluting subpopulations where biologically active rFIX elutes. There are several factors that are likely contributing to this result. The acetate counterion is known to have a weaker elution strength than chloride, and the ammonium ion is more chaotropic than the sodium ion. Additionally, in a detailed study of the effect of salt counterion on protein retention in anion-exchange chromatography, Malmquist and Lundell³⁰ concluded that non-specific effects could play an important role also. A more detailed mechanistic explanation of the cause of this behavior is beyond the current scope of this work, but the data show that the salt species can play a significant role in improving the resolution between closely related subpopulations of the same protein.

The elution program was then modified to obtain a more concentrated product (Figure 3-5C). The active rFIX subpopulations were eluted in the triplet peak obtained after the sharp increase in ammonium acetate from 0.6 M to 1.0 M, and corresponded to 10% of the total elution peak area. The overall Gla content of a pool of fractions of 63-67 minutes from six replicate runs was measured by amino acid analysis after basic hydrolysis, and was determined to be 6.0 moles Gla/mole rFIX. Parallel analysis of Mononine[®] gave a result of 10.5 moles Gla/mole FIX (theoretical value is 12 moles Gla/mole FIX). Our results are similar to rFIX that was produced in CHO cells, where the average Gla content was 6.5 moles Gla/mole rFIX,⁵ and again demonstrate that γ -carboxylation of *all* 12 Glu residues in the Gla domain is not a necessity for biological activity.

The results from the Gla analysis also revealed why previously published FIX purification techniques were unsuccessful in fractionating active from inactive subpopulations. It is known that *human* γ -glutamyl carboxylase modifies human FIX by a tethered-processive mechanism.^{31,32} That is, the carboxylase binds to the FIX propeptide and sequentially modifies all Glu residues in the Gla domain during that single binding event. The unmodified Glu residues in rFIX produced in the pig are most likely at the C-terminal end of the Gla domain. Zhang *et al.*^{33,34} found that conserved mutations of Glu to Asp in the Gla domain of Protein C (a protein with a homologous Gla domain) did not necessarily abolish biological activity, but did affect the Ca²⁺-dependent conformational changes. Therefore, subtle differences in the Ca²⁺-dependent conformation of rFIX caused by *partial* γ -carboxylation are likely the reason why hydroxyapatite and Ca²⁺-specific pseudoaffinity fractionation methods were not successful for rFIX produced at these very high expression levels.

The gradient elution depicted in Figure 3-5C was further modified to obtain a shorter run time and be more amenable for future scale-up. As shown in Figure 3-6A, the majority of the inactive rFIX subpopulations were eluted by a sharp increase to 0.5 M ammonium acetate. The specific activity in the pooled fraction from 27-44 minutes was 0.3 U/mg. The expanded view of the elution peak is given in Figure 3-6B. As the ammonium acetate was slowly increased up to 0.6 M, rFIX subpopulations with low specific activity began to elute. rFIX with high activity was then eluted by a sharp increase to 1 M ammonium acetate. Again, a characteristic triplet peak was obtained, and the area percentage of this elution peak (46-50 minutes) was approximately 10% of the total peak area of the chromatogram for all replicate runs. The average specific activity of the fraction from 46-50 minutes from a representative isolation was 197 U/mg. This translates to a net amount of about 0.3 g/liter rFIX that is biologically active. Thus, this level of active rFIX

production is similar to the previously reported lineage of transgenic pigs producing 100% biologically active rFIX at a total expression level of 0.2 g/liter, and it indicates that a rate limitation in γ -carboxylation occurs in the range of 0.2-0.3 g/liter.

The anion-exchange column with ammonium acetate elution method is highly reproducible, and results in a cleaner fractionation of active subpopulations compared to a NaCl elution. Isoelectric focusing of the products confirmed that the most active rFIX subpopulations that are fractionated by anion-exchange chromatography are also the most acidic (Figure 3-7). However, the biologically active rFIX produced by the transgenic pig is less acidic than plasma-derived FIX. This is at least partially due to the fact that overall Gla content of the entire 46-50 minute elution fraction was lower than pd-FIX. This lower acidity may also be due to differences in sialic acid content, although Bharadwaj *et al.*³⁵ demonstrated that removal of sialic acid had no effect on FIX activity. Further characterization of glycosylation, phosphorylation, and sulfation made to rFIX subpopulations produced in the transgenic pig is in progress.

3.5 Conclusions

The purification of complex recombinant proteins is complicated by the fact that multiple subpopulations corresponding to differences in post-translational processing are usually present. In many cases, the PTMs have a significant impact on the biological activity and pharmacokinetics, and so the purification process will need to select for the best subpopulations. Heparin-affinity chromatography was found to be an ideal method for purifying the total rFIX population in a single chromatographic step. However, a synthetic heparin analogue, Matrex Cellufine Sulfate, did not have the specificity required for purification of a heparin-binding protein from milk. Because the rFIX produced at expression levels of 2-3 grams/liter was partially γ -carboxylated, conventional chromatographic techniques that have been used in the past for fractionation of active from inactive FIX subpopulations were not efficient. The heparin-chromatography product was fractionated into active and inactive subpopulations by high-resolution anion-exchange chromatography. Changing the salt displacer from NaCl to ammonium acetate enabled us to more cleanly resolve the biologically active and inactive rFIX subpopulations. *In vitro* coagulation activity assays and capillary IEF confirmed that the most acidic subpopulations that eluted at higher acetate concentrations were the most active, and accounted for about 10% of the rFIX that is being produced in the milk at an overall expression level of 2-3 grams/liter. Thus, a highly pure and biologically active rFIX product was obtained from transgenic animal milk without the use of

immuno-affinity chromatography.

Acknowledgements

The technical support of T. O'Sickey and S. Butler is acknowledged. While this work was being developed, a key scientist in the history of this project, Dr. Christopher G. Russell, passed away. The authors gratefully acknowledge his contributions to the design and cloning of the FIX transgene construct.

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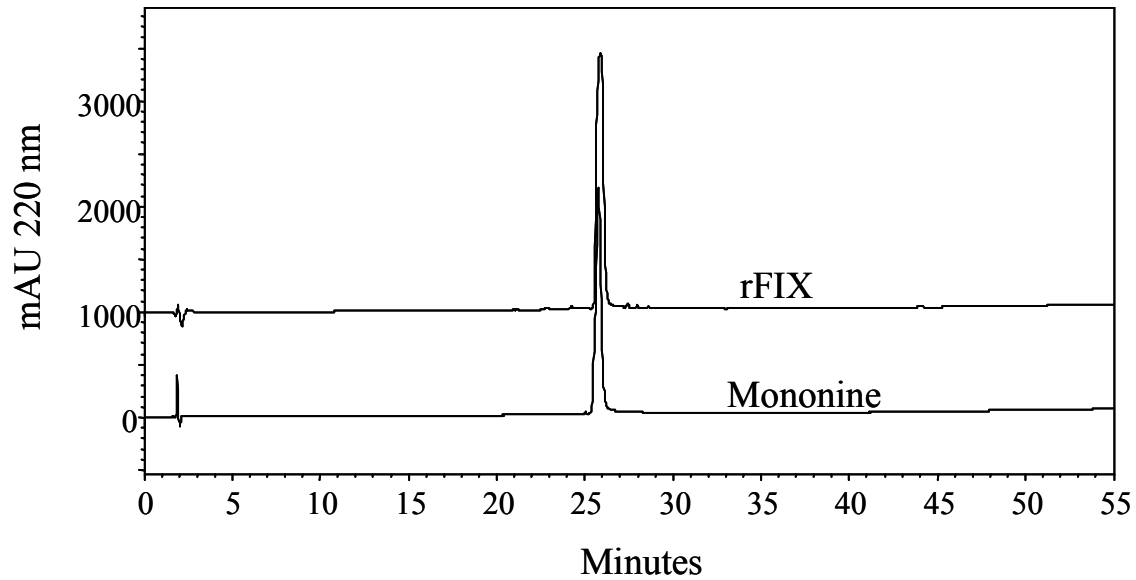


Figure 3-3. HPLC analysis of the purity of the heparin-Sepharose product: 0.35 mg/ml; 100 μ l injected. For comparison, an immunoaffinity purified plasma-derived FIX (Mononine): 0.5 mg/ml, 50 μ l injected. Column: Vydac C18, 4.6 mm x 15 cm. Solvents: A: 0.1% (w/v) TFA in water, and B: 0.1% (w/v) TFA in acetonitrile. Flow rate: 1 ml/min. Solvent program: 5 minutes 5% B, followed by 5-95% B over 45 minutes, followed by 5 minutes of 95% B.

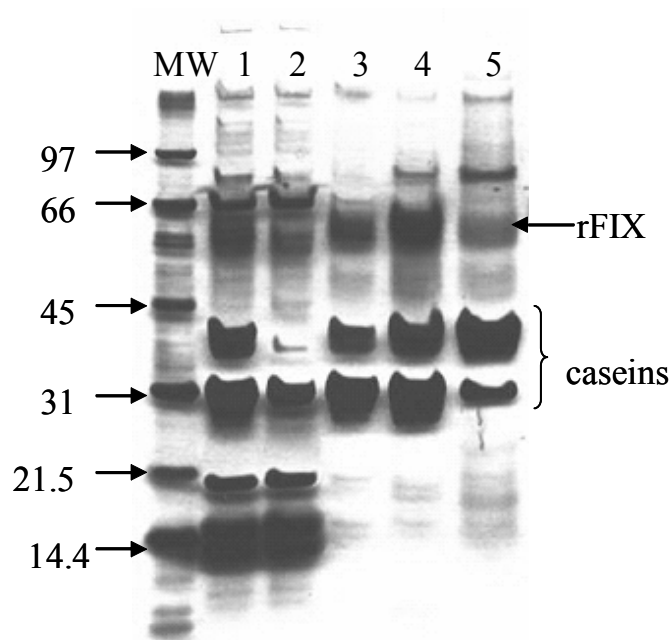


Figure 3-4. Silver-stained SDS PAGE of column fractions from Matrex Cellufine Sulfate, a synthetic heparin analogue. Lane 1: Column feed (skim milk/EDTA). Lane 2: Column flow-through and wash. Lane 3: 250 mM NaCl eluate. Lane 4: 500 mM NaCl eluate. Lane 5: 4 M NaCl eluate.

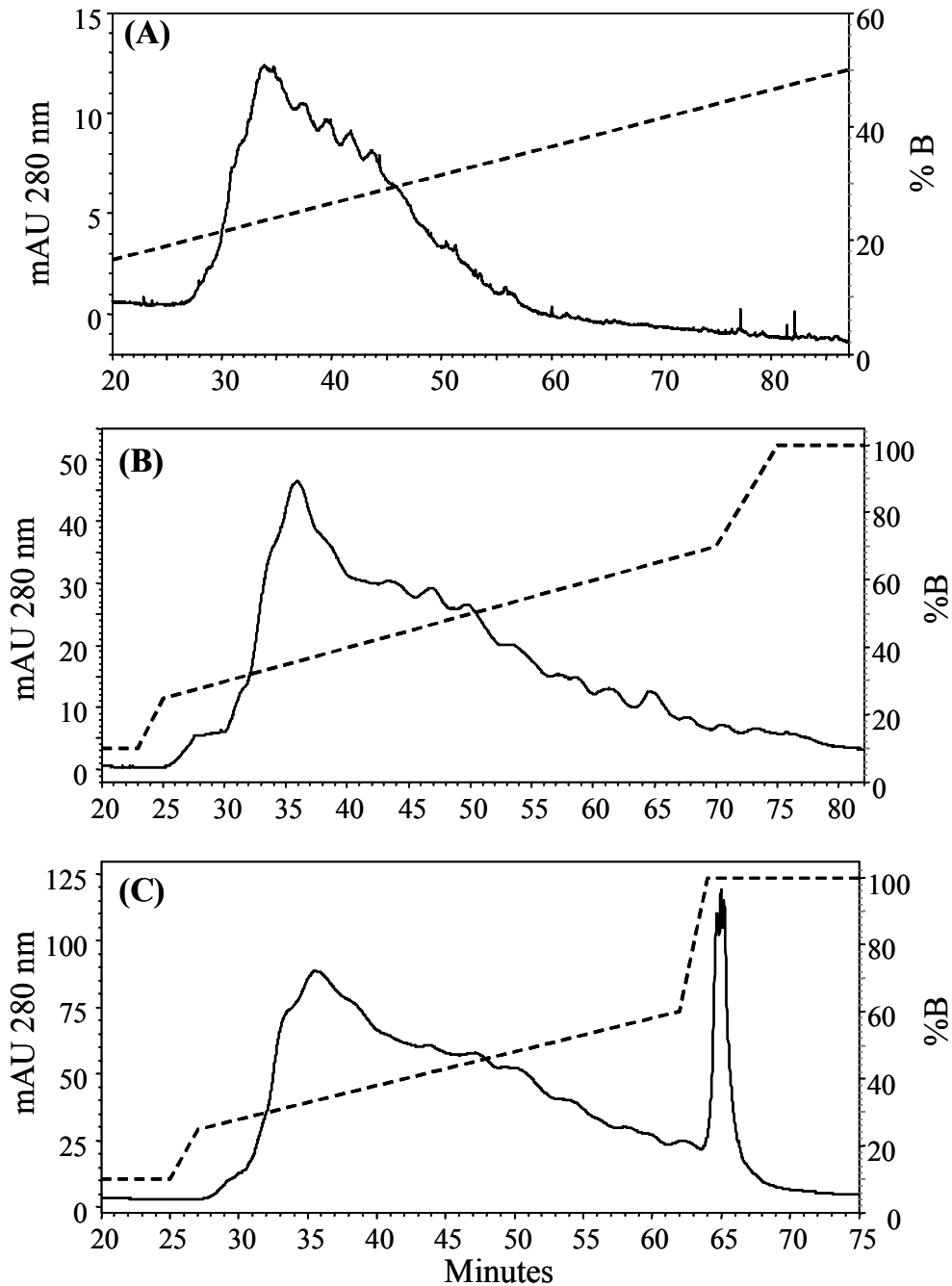


Figure 3-5. Anion-exchange chromatography of heparin-purified rFIX on a Mini Q column. **(A)** NaCl gradient elution. Buffer A: 20 mM Tris, pH 9.0. Buffer B: 20 mM Tris, 1 M NaCl, pH 9.0. Load rFIX at 0.4 ml/min at 10% B. Wash with 10% B at 0.8 ml/min. Elute with linear gradient of 10-50% B over 80 minutes (t = 7-87 min). Loaded 0.3 mg rFIX. **(B)** Ammonium acetate gradient elution. Buffer A: 20 mM Tris, pH 9.0. Buffer B: 20 mM Tris, 1 M ammonium acetate pH 9.0. Load rFIX at 0.4 ml/min at 10% B. Wash with 10% B at 0.8 ml/min. Elution program: 10-25% B over 2 minutes, 25-70% B over 45 minutes, 70-100% B over 5 minutes, 100% B for 5 minutes. Loaded 0.6 mg rFIX. **(C)** Modified ammonium acetate gradient elution. Elution program: 10-25% B over 2 minutes, 25-60% B over 35 minutes, 70-100% B over 2 minutes, 100% B for 10 minutes. Loaded 1.6 mg rFIX.

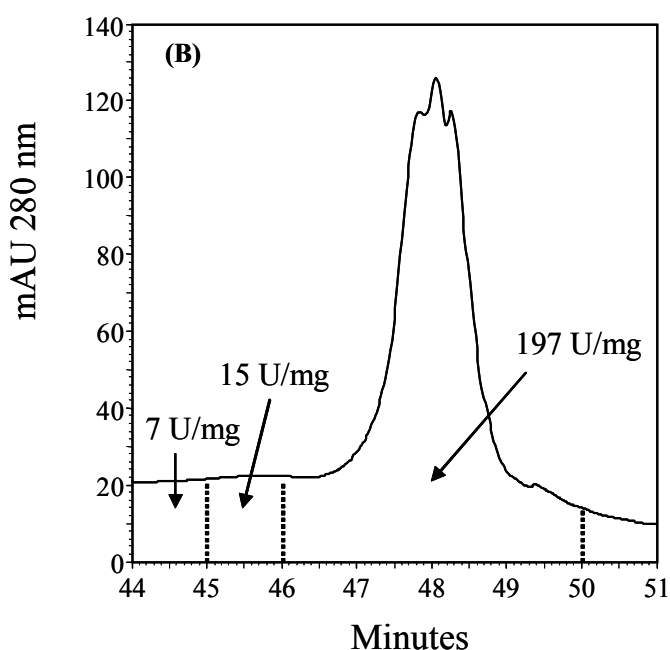
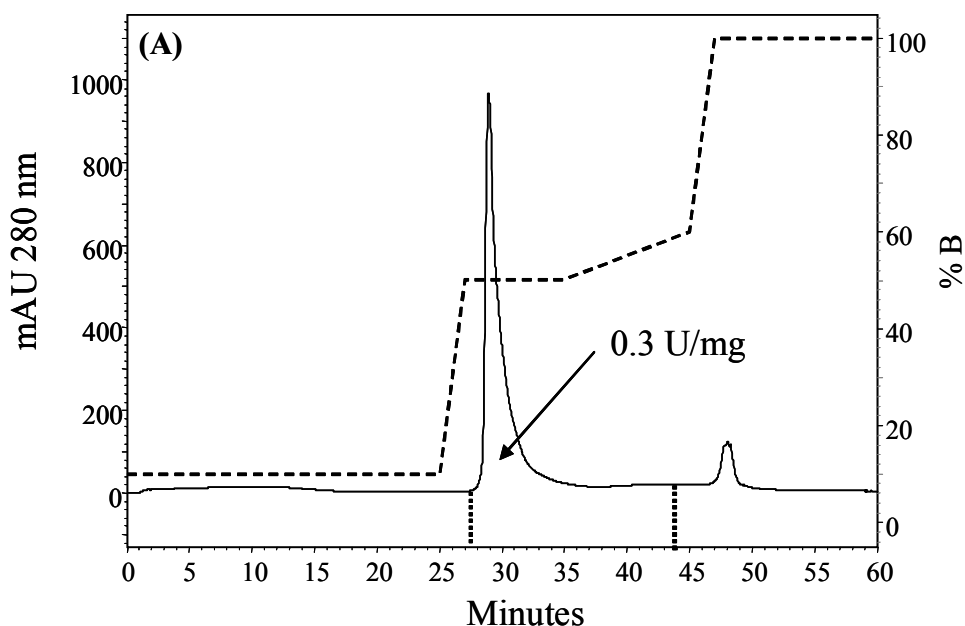


Figure 3-6. Anion-exchange fractionation of biologically active and inactive subpopulations of rFIX on a Mini Q column. Loaded 1.6 mg rFIX.

(A) Purified rFIX from the heparin-Sepharose column was diluted three-fold with Q Loading buffer and loaded at 0.4 ml/minute. After washing with 0.1 M ammonium acetate, the inactive rFIX was eluted by a sharp increase to 0.5 M ammonium acetate over a 2 minute period. After holding at 0.5 M ammonium acetate for 8 minutes, the ammonium acetate was increased to 0.6 M over a period of 10 minutes. The biologically active rFIX was then eluted by a sharp increase to 1.0 M ammonium acetate. The effluent from 27-44 minutes was pooled.

(B) An expanded view of the biologically active rFIX elution peak, along with the specific activity of the fractions as determined by the aPTT assay. Duplicates of two dilutions of each fraction were submitted to the aPTT assay.

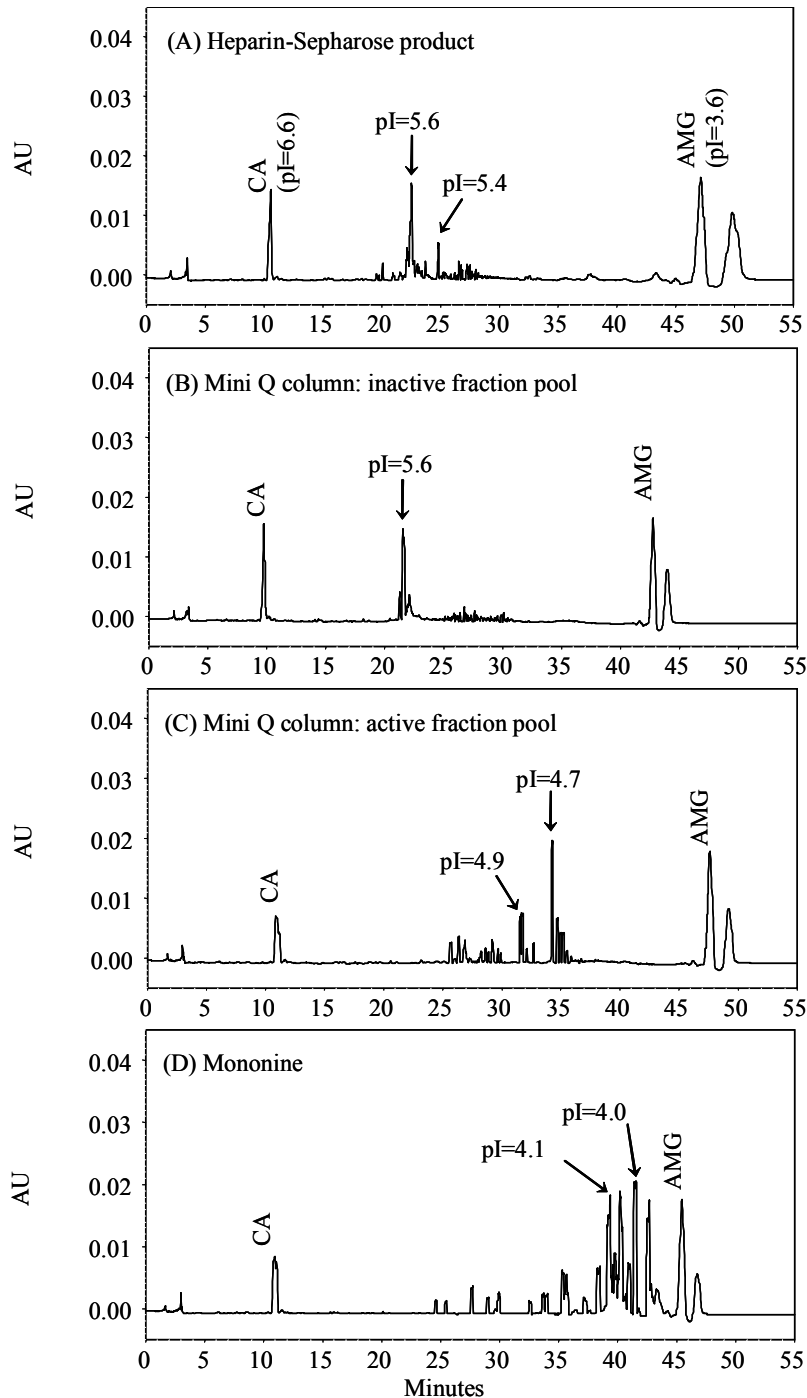


Figure 3-7. cIEF electropherograms of rFIX subpopulations and plasma-derived FIX. **(A)** 40 $\mu\text{g/ml}$ of rFIX purified by heparin-Shepharose, **(B)** 30 $\mu\text{g/ml}$ of rFIX - inactive fraction pool from Mini Q column, $t = 27\text{-}44$ minutes, **(C)** 50 $\mu\text{g/ml}$ of rFIX - active fraction pool from Mini Q column, $t = 46\text{-}50$ minutes, **(D)** 40 $\mu\text{g/ml}$ of Mononine – immunoaffinity purified plasma-derived FIX. Estimates of the pI of major rFIX and Mononine subpopulations are given using a linear interpolation of pI vs. time for the two standards: carbonic anhydrase I (CA, pI = 6.6) and amyloglucosidase (AMG, pI = 3.6).