Chapter 4. Batch-mode DEAE Adsorption

Introduction

Paleyanda et al[i] were able to express active hFVIII in the milk of transgenic swine. However, the activity of the rhFVIII that they were able to purify was extremely low relative to the total amount of FVIII antigen detected. The average amount of rhFVIII as measured by ELISA was 1.60 μg/mL. This rhFVIII had an average activity of 0.53 U/mL. One U is defined to be the FVIII activity normally present in 1 mL of plasma with plasma having a FVIII concentration of approximately 200 ng/mL. Thus, the rhFVIII purified from the swine milk has a specific activity 15 fold less than that obtained from plasma. This diminished activity could be the result of two factors. First, the transgenic swine may be unable to efficiently produce active FVIII. Secondly, the purification method used by Paleyanda et al could be partially destructive. The second factor was the basis for these experiments. It was hypothesized that an alternative purification method could be developed and higher activities could be obtained.

Paleyanda et al used a batch immunoaffinity purification technique. Antihuman FVIII Mab 9041 was immobilized on Sepharose-CL2B beads. These beads were then incubated overnight in transgenic swine milk that had been defatted using centrifugation. Following an overnight incubation step, the FVIII was eluted using a 40% ethylene glycol solution and activity was analyzed using an APTT assay.

DEAE is widely used as a process step in the purification of FVIII.[ii iii iv] In this work, DEAE was explored as a possible purification method to purify FVIII from transgenic swine milk. It was examined with both rhFVIII contained in transgenic swine milk and hFVIII contained in Antihemophilic Factor (Human) Method M Purified (from American Red Cross). Although a successful purification scheme resulting in active rhFVIII was not achieved, substantial progress was made in understanding the kinetic and thermodynamic limitations encountered in purifying rhFVIII from milk. The purification also allowed a clear visualization of the FVIII chains present in the milk. This visualization provided evidence that the Factor VIII chains are largely intact although not in an associated and thus active form.

Methods

Column-mode DEAE Binding of ARC Antihemophilic Factor Method M Purified

Load 1.5 mL of Antihemophilic Factor (Human) Method M Purified (from ARC) on 1.5 mL of DEAE Sepharose Fast Flow. AHF is in 55 mM histidine / 30 mM glycine. Save a sample of the Starting Material. Wash the column with 3 CV (column volumes) of Loading Buffer (25 mM Tris / pH 7.5). Collect three fractions by volume (1.5 mL each). Wash the column with 3 CV of Wash buffer (25 mM Tris / 250 mM NaCl / pH 7.5). Collect three fractions by volume (1.5 mL each). Elute with 3 CV of elution buffer (25 mM Tris / 500 mM NaCl / pH 7.5). Collect three fractions by volume (1.5 mL each). Wash the DEAE packing with 3 CV of 1 M NaCl and save the fractions (3 fractions by volume, 1.5 mL each) followed by 3 CV of 0.5 M NaOH and finally put the column back in loading buffer.

Column-mode DEAE Binding of transgenic / non-transgenic pig milk.

Load 1.5 mL of defatted pig milk on 3.0 mL of DEAE Sepharose Fast Flow. Save a sample of the Starting Material. Wash the column with 3 CV (column volumes) of Loading Buffer (25 mM Tris / pH 7.5). Monitor OD using flow spectrophotometer and collect fractions. Wash the column with 3 CV of Wash buffer (25 mM Tris / 250 mM NaCl / pH 7.5). Monitor OD by flow spec and collect fraction. Elute with 3 CV of elution buffer (25 mM Tris / 500 mM NaCl / pH 7.5). Monitor OD by flow spec and collect fraction. Wash the DEAE packing with 3 CV of 1 M NaCl and collect any fraction by OD. Wash packing with 3 CV of 0.5 M NaOH and put the column back in loading buffer.

Batch DEAE Column (small scale)

Equilibrate DEAE sepharose fast flow packing material in 100 mM phosphate / pH 7.2 (loading buffer). Put 4 mL of DEAE packing material in a test tube with 1 mL of defatted milk diluted 1:1 with 200 mM phosphate / pH 7.2 (6 mL total volume). Allow column to equilibrate on a rotator overnight in the cold room. Repack DEAE column in a

column. Wash with loading buffer and collect any peaks. Wash with 100 mM phosphate / 250 mM NaCl / pH 7.2 and collect any peaks. Elute with 100 mM phosphate / 500 mM NaCl / pH 7.2 and collect any peaks. Wash packing with 1 M NaCl followed by 0.5 M NaOH and put back in 20% EtOH for storage.

Batch DEAE Column (larger scale)

Equilibrate streamline DEAE packing material in 100 mM phosphate / pH 7.2 (loading buffer) Put 20 mL of DEAE packing material in each of two 50 mL test tube with 5 mL of defatted milk diluted 1:1 with 200 mM phosphate / pH 7.2 (30 mL total volume in each tube) Allow columns to equilibrate overnight in the cold room. Repack both batch DEAE columns into one column. Wash with loading buffer and collect any peaks. Wash with 100 mM phosphate / 250 mM NaCl / pH 7.2 and collect any peaks. Elute with 100 mM phosphate / 500 mM NaCl / pH 7.2 and collect any peaks. Wash packing with 1 M NaCl followed by 0.5 M NaOH and put back in 20% EtOH for storage.

Gels and Westerns

Gels and westerns were performed as per the instructions that came with the gels.

APTT Test

One cuvette per sample was placed in a test tube rack. 100 uL of FVIII deficient plasma and 100 uL of sample was added to each cuvette and mixed by vortexing. The samples were next placed into the warming spots on the coagulameter (Electra 750A) to bring them to 37 degrees C. 100 uL of the APTT reagent was added to one of the samples and 90 seconds were allowed to elapse before the cuvette was moved to the measuring spot and 100 uL of 25 mM $CaCl_2$ was added to the cuvette using the special pipette that starts the clock. The time required to form a clot was recorded. This was repeated with each standard and sample in duplicate. A standard curve was constructed using the standards and the samples were quantitated using this standard curve.

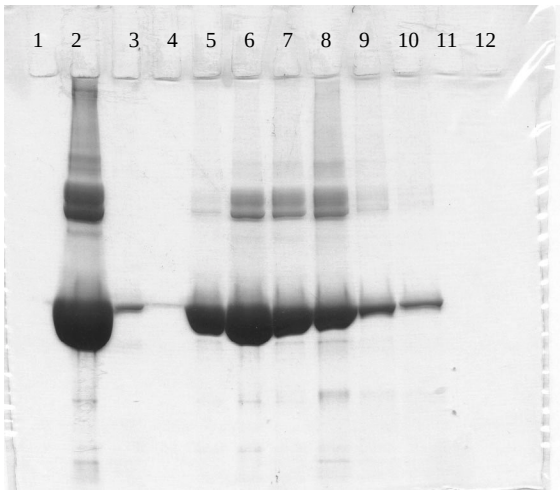
Selective Precipitation with Zinc Acetate

Samples are first dialyzed using Slide-A-Lyzers with a MWCO of 10,000 kD against a dilute buffer solution to remove high salt concentrations and to ensure that all samples are in similar buffer environments prior to the precipitation step.

After dialysis, samples are lyophilized completely to dryness and then resuspended in either DI H_2O (for the control) or varying concentrations of Zinc Acetate. Zinc Acetate is an ideal vehicle for Zn^{2+} precipitation because it combines the Zinc with a buffer that easily maintains the pH between 6 and 7. Other Zinc preparations (ie. $ZnCl_2$) perform similarly except in regards to the buffering capacity but are much more difficult to use because of the need to continually readjust the pH after addition of the Zinc.

After the Zinc Acetate solution is added to resuspend the samples to their original volume, they are vortexed to mix and then centrifuged to pellet any precipitated proteins in the bottom of the tube. The supernatant is then drawn off and analyzed.

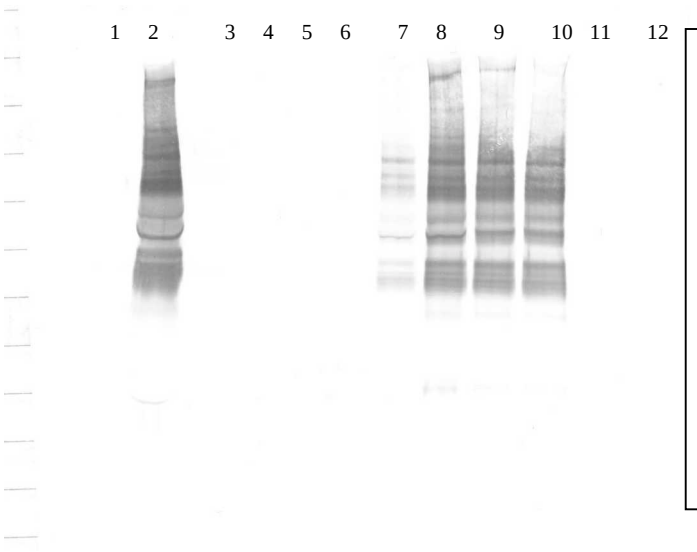
Results



Lane	Sample
1	Blank
2	Starting Material
3	Fallthrough 2
4	Fallthrough 3
5	Wash 1
6	Wash 2
7	Wash 3
8	Elute 1
9	Elute 2
10	Elute 3
11	Blank
12	Blank

Figure 1. Non-reduced 4-12% Bis-Tris Gel Analysis of DEAE Purification of hFVIII

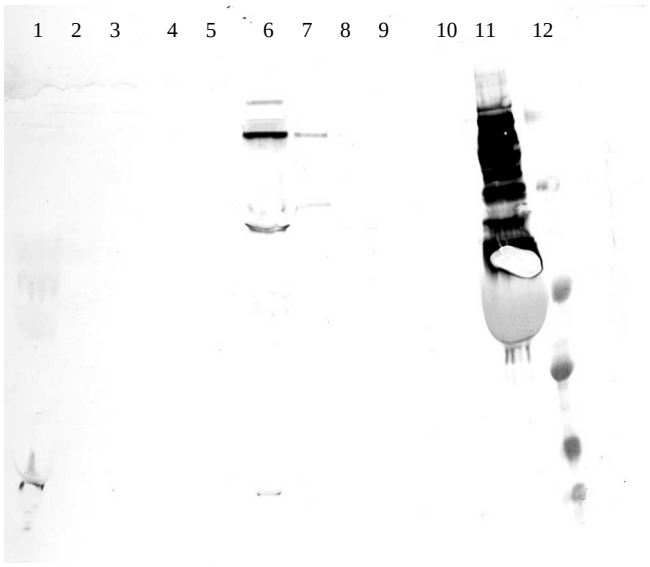
Figure 1 a non-reduced gel performed on samples from a DEAE purification of hFVIII obtained from American Red Cross. Column mode DEAE purification was done to demonstrate the ability of DEAE to bind hFVIII. Lanes 1, 11, and 12 are blank lanes. Lane 2 contains the starting material. Lane 3 contains the second fallthrough fraction. Lane 4 contains the third fallthrough fraction. Lane 5 contains the first wash fraction. Lane 6 contains the second wash fraction. Lane 7 contains the third wash fraction. Lane 8 contains the first elute fraction. Lane 9 contains the second elute fraction. Lane 10 contains the third elute fraction. All samples were prepared as 30 uL of sample + 10 uL of sample buffer with 15 uL total loaded on the gel. The major protein present in the starting material is the albumin. This protein is added to purified hFVIII to help stabilize it and to coat any surfaces to help ensure that the maximum amount of FVIII is delivered. The albumin is seen as the lowest smudgy band in the starting material. Some of it is visible in the fallthrough fractions but most of it binds to the column and is eluted with 250 mM NaCl. The FVIII itself isn't visible on the gel because of its low concentration.



Lane	Sample
1	Blank
2	Starting Material
3	Fallthrough 2
4	Fallthrough 3
5	Wash 1
6	Wash 2
7	Wash 3
8	Elute 1
9	Elute 2
10	Elute 3
11	Blank
12	Blank

Figure 2. Non-reduced 4-12% Bis-Tris Western Analysis of DEAE Purification of hFVIII

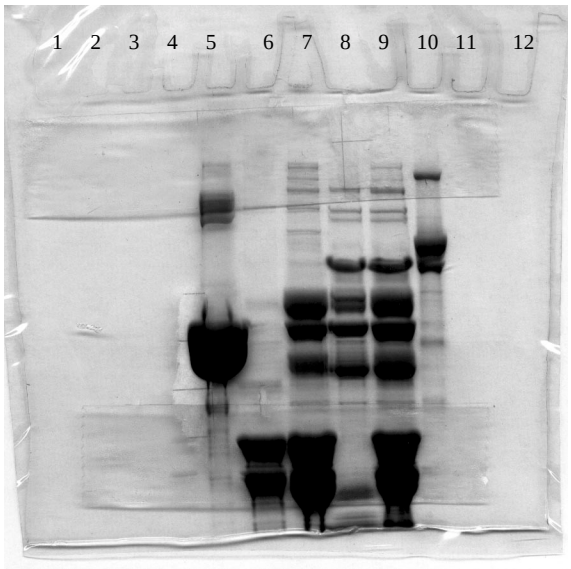
Figure 2 a non-reduced western performed on samples from a DEAE purification of hFVIII obtained from American Red Cross. Column mode DEAE purification was done to demonstrate the ability of DEAE to bind hFVIII. Lanes 1, 11, and 12 are blank lanes. Lane 2 contains the starting material. Lane 3 contains the second fallthrough fraction. Lane 4 contains the third fallthrough fraction. Lane 5 contains the first wash fraction. Lane 6 contains the second wash fraction. Lane 7 contains the third wash fraction. Lane 8 contains the first elute fraction. Lane 9 contains the second elute fraction. Lane 10 contains the third elute fraction. All samples were prepared as 30 uL of sample + 10 uL of sample buffer with 15 uL total loaded on the gel. The FVIII antigen is clearly visible in the starting material. All of it binds to the column as evidenced by the lack of FVIII in any of the fallthrough samples. A faint signal is present in the third wash fraction but most of the FVIII is eluted in the 500 mM NaCl fractions. By comparing the gel with the western, it is seen that the FVIII in the elution fractions is relatively more pure than the starting material. The second and third elution fractions have very strong FVIII signals as visualized on the western. The gel, however, shows that the total amount of protein is significantly decreased. This confirms the effectiveness of DEAE as a purification step for FVIII.



Lane	Sample
1	NTG (Non-transgenic) starting material
2	NTG Fallthrough
3	NTG Wash
4	NTG Elute
5	NTG Salt Wash
6	TG (Transgenic) starting material
7	TG Fallthrough
8	TG Wash
9	TG Elute
10	TG Salt Wash
11	FVIII Standard
12	MW Markers

Figure 3. Western Analysis of Column-mode DEAE treatment of pig milk containing rhFVIII

Figure 3 shows a western analysis performed on samples from a DEAE column processing transgenic milk using a reduced 4-12% Bis-Tris Gel. Lane 1 contains non-transgenic defatted pig milk (from W-10 collected on 4/6/99) that was loaded onto the DEAE column. Lane 2 contains the non-transgenic fallthrough fraction from that column. Lane 3 contains the non-transgenic wash fraction. Lane 4 contains the non-transgenic elute fraction. Lane 5 contains the non-transgenic salt wash fraction. Lane 6 contains the transgenic defatted pig milk (from 3-4 collected on 4/6/99) that was loaded onto the DEAE column. Lane 7 contains the transgenic fallthrough fraction from that column. Lane 8 contains the transgenic wash fraction. Lane 9 contains the transgenic elute fraction. Lane 10 contains the transgenic salt wash fraction. Lane 11 contains the hFVIII standard obtained from American Red Cross. Lane 12 contains broad range prestained molecular weight markers (5 uL loaded on gel). All samples except the molecular weight markers loaded on the gel contain 5 uL of sample, 2.5 uL of sample buffer, 1 uL of reducing agent, and 1.5 uL of DI H_20. A relatively strong FVIII signal is present in the starting material but none of it binds to the column. Only the fallthrough fraction has any FVIII in it. This demonstrates the difficulty that arises when column mode DEAE is used with milk. Something in the milk prevents the FVIII from binding to the packing material.



Lane	Sample
1	Blank
2	Blank
3	Blank
4	Non-transgenic Whey
5	FVIII Standard
6	500 mM NaCl Elute
7	250 mM NaCl Elute
8	Fallthrough
9	Starting Material
10	MW Standards
11	Blank
12	Blank

Figure 4. Reduced 4-12% Bis-Tris Gel Analysis of Batch DEAE Processing of Transgenic Milk

Figure 4 shows a reducing 4-12% Bis-Tris gel analysis of a batch DEAE run on transgenic milk. Lanes 1, 2, 3, 11, and 12 are blank. Lane 4 contains non-transgenic whey (2.5 uL of sample + 10.5 uL of DI H_2O + 7 uL of sample buffer). Lane 5 contains hFVIII Standard (5 uL of sample + 8 uL of DI H_2O + 7 uL of sample buffer). Lane 6 contains 500 mM NaCl Elute (13 uL of sample + 7 uL of sample buffer). Lane 7 contains 250 mM NaCl Elute (13 uL of sample + 7 uL of sample buffer). Lane 8 contains the fallthrough fraction (13 uL of sample + 7 uL of sample buffer). Lane 9 contains the defatted transgenic pig milk (from pig 3-3 collected on 2/4/98) used as the starting material (5 uL of sample + 8 uL of DI H_2O + 7 uL of sample buffer). Lane 10 contains broad range molecular weight markers obtained from Bio-Rad (5 uL loaded).

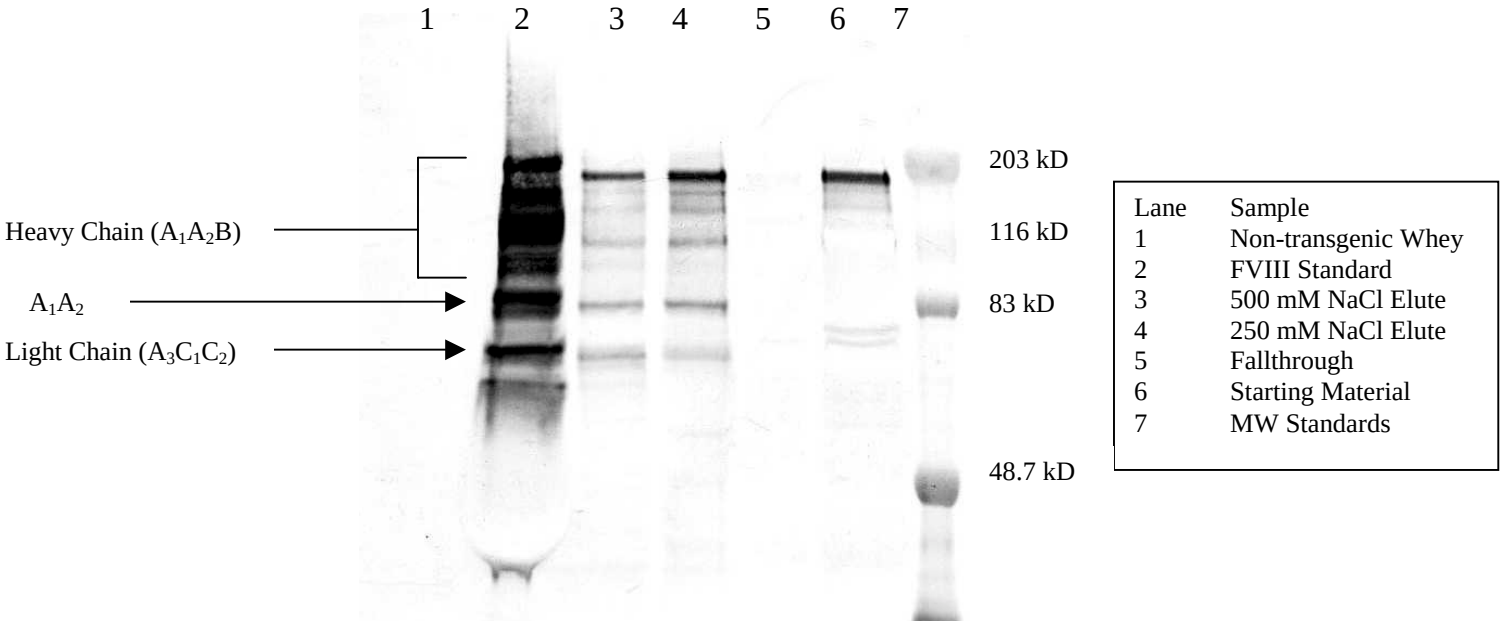
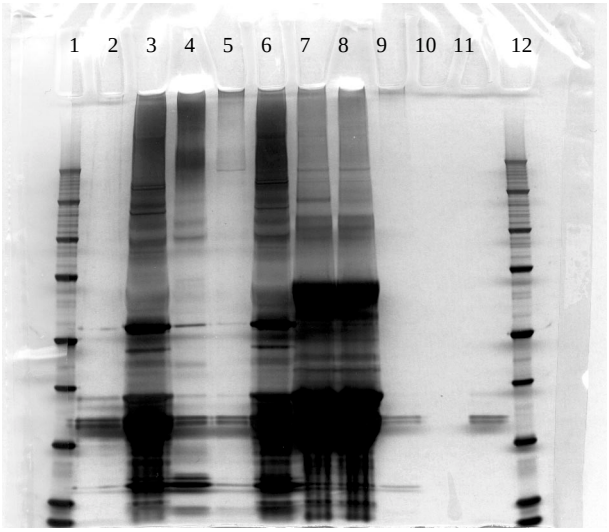


Figure 5. Reduced 4-12% Bis-Tris Western Analysis of Batch DEAE Processing of Transgenic Milk

Figure 5 shows a reducing 4-12% Bis-Tris western analysis of a batch DEAE run on transgenic milk. The locations of the heavy chain (A_1A_2B) and the light chain ($A_3C_1C_2$) are labeled. Lane 1 contains non-transgenic whey (2.5 uL of sample + 10.5 uL of DI H_2O + 7 uL of sample buffer). Lane 2 contains hFVIII Standard (5 uL of sample + 8 uL of DI H_2O + 7 uL of sample buffer). Lane 3 contains 500 mM NaCl Elute (13 uL of sample + 7 uL of sample buffer). Lane 4 contains 250 mM NaCl Elute (13 uL of sample + 7 uL of sample buffer). Lane 5 contains the fallthrough fraction (13 uL of sample + 7 uL of sample buffer). Lane 6 contains the defatted transgenic pig milk (from pig 3-3 collected on 2/4/98) used as the starting material (5 uL of sample + 8 uL of DI H_2O + 7 uL of sample buffer). Lane 7 contains broad range prestained molecular weight markers obtained from Bio-Rad (5 uL loaded). A strong heavy chain rhFVIII signal is present in the starting material. Portions of the heavy chain are clearly visible but the light chain is very faint. No rhFVIII is present in the fallthrough meaning that essentially all the rhFVIII bound to the DEAE packing.

Of particular interest is the appearance of native proteolytic processing in the elutes. The apparent molecular weights of the visible chains in the elutes are identical to

the FVIII chains derived from human plasma. This clearly demonstrates there is not significant proteolytic degradation of the individual chains in the milk although it is unclear whether those chains are still associated with each other as would be required for activity. The light chain of FVIII is clearly visible in both the 250 mM and the 500 mM fractions demonstrating the success of the purification step. The DEAE column has allowed the light chain to be sufficiently concentrated that it is clearly visible. By comparing the gels and the westerns, it is apparent that the total amount of protein in the 250 mM and 500 mM fractions is decreased while the strength of the FVIII signal is significantly increased. This clearly demonstrates the effectiveness of batch DEAE as a useful purification step for FVIII present in swine milk.

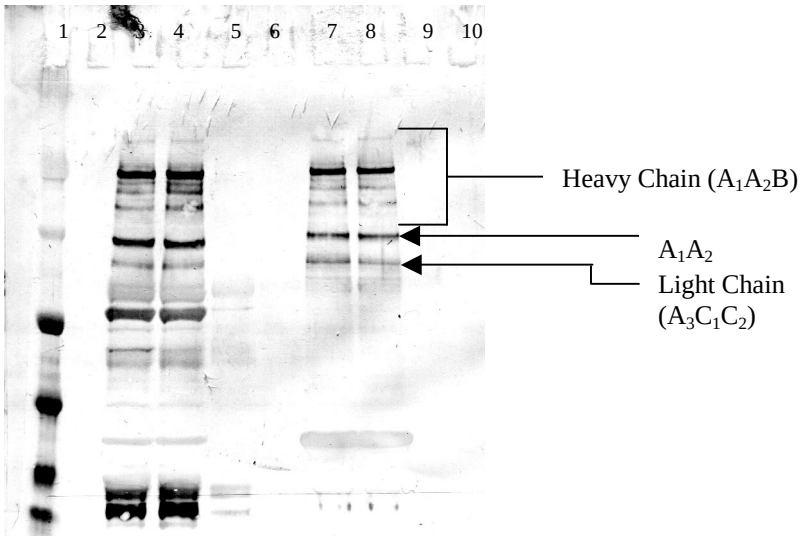


Lane	Sample
1	MW Markers
2	500 mM Elute + 30 mM Zn
3	Raw 250 mM Elute
4	250 mM Elute + 7.5 mM Zn
5	250 mM Elute + 15 mM Zn
6	250 mM Elute + 30 mM Zn
7	Raw 500 mM Elute
8	500 mM Elute + 7.5 mM Zn
9	500 mM Elute + 15 mM Zn
10	Blank
11	500 mM Elute + 30 mM Zn
12	MW Markers

Figure 6. Non-reduced 4-12% Bis-Tris Gel Analysis of Zinc Acetate Precipition of DEAE column fractions

Figure 6 shows a non-reduced 4-12% Bis-Tris Gel analysis of Zinc Acetate precipitation of DEAE column fractions. Lanes 1 and 12 contain broad range molecular weight markers obtained from Bio-Rad (5 uL loaded). Lanes 2 and 11 contain the 500 mM Elute from a larger scale batch DEAE processing run with 30 mM Zinc. Lane 3 contains the raw 250 mM Elute from a larger scale batch DEAE processing run. Lane 4 contains the 250 mM Elute + 7.5 mM Zinc. Lane 5 contains the 250 mM Elute + 15 mM Zinc. Lane 6 contains the 250 mM Elute + 30 mM Zinc. Lane 7 contains the raw 500 mM Elute. Lane 8 contains the 500 mM Elute + 7.5 mM Zinc. Lane 9 contains the 500

mM Elute + 15 mM Zinc. Lane 10 is a blank lane. All samples loaded on the gel contain 15 uL of sample + 5 uL of sample buffer.



Lane	Sample
1	MW Markers
2	Blank
3	Raw 250 mM Elute
4	250 mM Elute + 7.5 mM Zinc
5	250 mM Elute + 15 mM Zinc
6	250 mM Elute + 30 mM Zinc
7	Raw 500 mM Elute
8	500 mM Elute + 7.5 mM Zinc
9	500 mM Elute + 15 mM Zinc
10	500 mM Elute + 30 mM Zinc

Figure 7. Reduced 4-12% Bis-Tris Western Analysis of Zinc Acetate Precipitation of DEAE column fractions

Figure 7 shows a reduced 4-12% Bis-Tris Western Analysis of Zinc Acetate Precipitation of DEAE column fractions. Lane 1 contains prestained broad-range molecular weight markers obtained from Bio-Rad (5 uL loaded on gel). Lanes 2 is a blank lane. Lane 3 contains the raw 250 mM elute from a larger scale batch DEAE processing run. Lane 4 contains the 250 mM elute + 7.5 mM Zinc. Lane 5 contains the 250 mM Elute + 15 mM Zinc. Lane 6 contains the 250 mM Elute + 30 mM Zinc. Lane 7 contains the raw 500 mM elute from a larger scale batch DEAE processing run. Lane 8 contains the 500 mM elute + 7.5 mM Zinc. Lane 9 contains the 500 mM elute + 15 mM Zinc. Lane 10 contains the 500 mM elute + 30 mM Zinc.

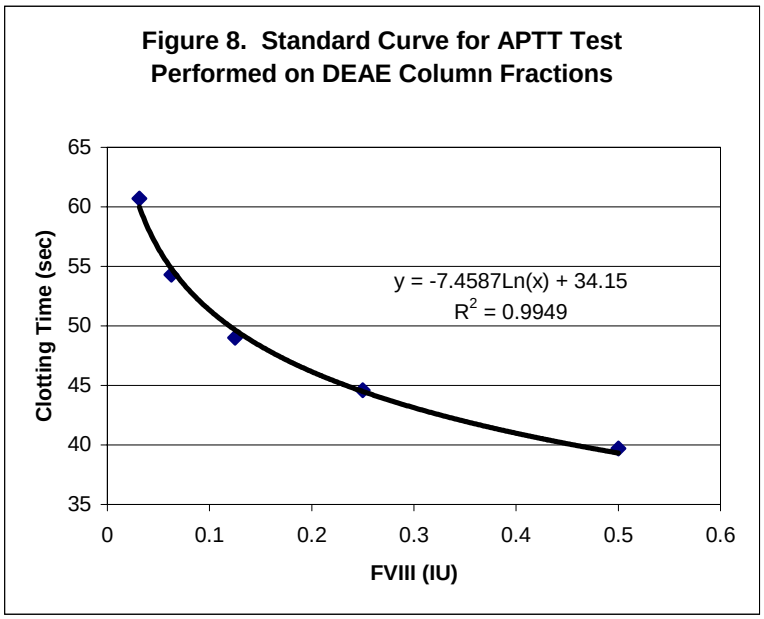


Figure 8 shows the standard curve obtained when the FVIII Standard obtained from American Red Cross was used. When the clotting assay was performed on the various column fractions, no activity was seen. Samples from the small scale batch experiment as well as the larger scale batch experiment was assayed. Prior to analysis, these samples were variously concentrated with centricons and by dialysis followed by lyophilization. Several dilutions were done of each sample and in all cases, the clotting time was not significantly different from the blank used in the standard curve.

Discussion

Work attempting to use column wise DEAE to purify rhFVIII from pig milk met with limited success because almost all the rhFVIII routinely fell through the column. Figure 3 shows typical results obtained for FVIII purified using column-wise DEAE adsorption. Almost all the rhFVIII falls through the column unabsorbed even though the column size is well in excess of what is commonly used with plasma-derived hFVIII. To verify the method we were using to attempt to purify the rhFVIII from milk, a test run was done on hFVIII obtained from American Red Cross. Figures 1 and 2 show the results typical of this type of purification. Only a small amount of the hFVIII is seen in the first fallthrough fraction. Additionally, the hFVIII appears to be very structurally similar, at least by its appearance on the western blot, to the starting material. These

experiments seem to indicate that the problem arises as the result of something in the milk environment in which the rhFVIII exists.

Milk is a complex mixture with many different types of proteins. Of particular interest to this work are the caseins contained in milk. These caseins typically exist as micelles in the milk solution. These micelles would present domains similar to the phospholipids on blood vessel walls with which FVIII normally associates. It was hypothesized that these FVIII micellular interactions were causing the purification to be kinetically rather than equilibrium limited. Based upon this hypothesis, a batch-wise DEAE adsorption study was performed to determine if the FVIII adsorption was kinetically limited.

All ionic exchange chromatography purification strategies, whether implemented in batch or column mode, are based on the same underlying phenomena. The chromatography packing material and any proteins in solution both present charged surfaces which can interact with each other. The strength of these interactions is a function of the pH of the buffer solution and the ionic character of the column packing and protein surface. The buffer system and the column packing used in a chromatography purification system is selected to promote attractive interactions between the proteins and the column packing. Every protein has a slightly different charged surface and thus binds to the column packing with varying avidity. It is this phenomena that is exploited to selectively purify proteins of interest away from their constituents.

In column-mode ionic exchange chromatography, the protein solution containing the protein of interest is pumped through a column containing packing material. The contact time between the protein in solution and the packing material is governed by the linear velocity of the protein solution and the length of the column. As the charged sites on the packing are occupied by protein, the packing will start to become saturated and unable to bind more protein. Longer contact times result in more complete saturation of the packing material and result in a higher binding capacity. Kinetic limitations can exist with column-mode chromatography. In cases where the adsorption time for a protein is approximately the same as the contact time, binding can be greatly reduced or nonexistent.

In batch-mode ionic exchange chromatography, the protein solution is incubated with the packing material. The contact time between the protein in solution and the packing material is simply the incubation time. With long incubation times, the charged regions of the packing material will be completely occupied and kinetic limitations will cease to be important. In this case, the binding is governed by equilibrium rather than kinetic considerations.

Figures 4 and 5 show the results typically obtained when Batch-wise DEAE adsorption was used. The DEAE packing is seen to bind rhFVIII with almost complete efficiency. No rhFVIII is observed in any of the fallthrough fractions. The longer time afforded by the batch processing has presumably allowed all the rhFVIII to disassociate from the casein micelles and reach an equilibrium state. This technique at least succeeded in yielding relatively pure rhFVIII as seen by comparing the gel and western analysis of that run.

Most importantly, the appearance of the FVIII on the westerns is nearly identical to the FVIII derived from human plasma. The heavy chain (A_1A_2B) and the light chain ($A_3C_1C_2$) are clearly visible in the correct stoichiometric amounts. An A_1A_2 fragment is also visible. The molecular weights of the fragments are very slightly lower those obtained from human plasma although this is likely due to differing glycosylation rather than proteolytic degradation. Based upon its appearance on the western, it was hypothesized that the FVIII might be active. An APTT test was performed. Figure 8 shows the standard curve obtained in that assay. In spite of the structural similarity on reduced SDS-PAGE westerns, no activity was measured in any fractions and therefore degradation may not be the chief cause of inactivity.

The rhFVIII produced in the swine mammary gland is very structurally similar to wild-type FVIII based upon western blot analysis. However, the activity is too low to be measurable. This is likely the result of incomplete processing and poor stabilization due the lack of vWF. In plasma, FVIII is closely associated with von Willebrand Factor (vWF). von Willebrand Factor serves two functions in its association with Factor VIII. It serves to stabilize the FVIII and also promotes the association of the light and heavy chains of factor VIII.[v] [vi] [vii] In the absence of vWF, FVIII tends to be secreted as separate chains rather than the native heterodimeric form. These separate chains would appear to

be identical to those present in native, active FVIII when visualized using western analysis. However, the separate unassociated chains would have no activity.

Proteases probably also play a role in FVIII's inactivity particularly with respect to the light chain. The light chain has a phospholipid binding domain which is likely to associate with the casein micelles. [v] When transgenic milk is analyzed with a western blot (Figure 5), a clear difference is observed in the relative ratios of the heavy chain and the light chain. The heavy chain is easily seen in the starting material. The light chain, however, is not visible until after further purification indicating that a lower concentration is present in the milk. This may be due to sequestering by other protein interactions.

Proteases have been reported to exist in the milk of humans, buffalo, and goat and it is highly probable that pigs also have proteases in their milk. One of the most widely studied milk proteases is Milk Alkaline Proteinase (MAP) also known as plasmin, the active form of plasminogen. This MAP is associated with the casein in milk (just as the light chain of rhFVIII is believed to be) and is also encountered in blood as the protease that solubilizes fibrin clots. Plasmin is capable of digesting a broad range of proteins and is a likely culprit in the partial destruction of the light chain of rhFVIII.[viii]

Zinc precipitation was explored. At 7.5 mM Zinc concentrations, the overall protein load decreases substantially while leaving the rhFVIII in solution. Figures 6 and 7 show results typical of the Zinc precipitation of the 250 and 500 mM NaCl elutions from batch DEAE processing runs. In the 250 mM NaCl fraction, the total protein left in solution is seen to decrease dramatically while not affecting the rhFVIII concentration. This appears to be a promising purification step in the possible purification of rhFVIII from transgenic swine milk. The zinc precipitation products also show native proteolytic processing. The zinc precipitation allows visualization of all the major FVIII fragments and they are clearly native. The improved purification relative to the batch DEAE allows additional lower molecular weight degradation products to be resolved but most of the rhFVIII is clearly native and intact.

This work resulted in two very important discoveries. First, while some proteolytic degradation is occurring, the majority of the rhFVIII chains are intact although not properly associated. This bodes well for future constructs that may be

expressed in transgenic animals as it suggests that proteolytic degradation is minimal. Secondly, the effectiveness of a batch DEAE strategy for purifying FVIII is demonstrated. Regardless of the precise interactions between the rhFVIII and the endogenous milk proteins, those interactions can be overcome via batch processing and a purer FVIII mixture can be obtained.

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[i] Paleyanda, Rekha K., William H. Velander, Timothy K. Lee, Dorethea H. Scandella, Francis C. Gwazdauskas, James W. Knight, Leon W. Hoyer, William N. Drohan, Henryk Lubon Nature Biotechnology, 1997; 15:971-975

[ii] Josic, Dj., H. Schwinn, M. Stadler, A. Strancar Journal of Chromatography B, 1994; 662:181-190

[iii] Booy, Marcel P. W. M. Te, Anita Faber, Egge de Jonge, Ernst P. Wolterink, Waander Riethorst, Tom Beugeling, Adriaan Bantjes, Jan Over, and Boudewijn W. Konig Journal of Chromatography, 1990; 503:103-114

[iv] Branovic, Karmen, Branka Gebauer, Anda Trescec, Bojan Benko Applied Biochemistry and Biotechnology, 1998; 69:99-111

[v] Kaufman, Randal. Annual Review of Medicine, 1992; 43:325-339

[vi] Wise, R. J., A. J. Dorner, M. Krane, D. D. Pittman, R. J. Kaufman. Journal of Biological Chemistry, 1991; 266:21945-21955

[vii] Fay, P.J. Archives of Biochemistry and Biophysics, 1988; 1988; 262: 525-531

[viii] Grufferty Mary B., Patrick F. Fox, Journal of Dairy Research, 1988; 55:609-630