Chapter 3. Isoelectric Focusing

Introduction

Isoelectric focusing is a technique whereby proteins are concentrated based upon their isoelectric point (pI). The isoelectric point for a protein corresponds to the pH at which the protein has no net charge. This isoelectric point is a function of the protein's primary structure as well as the spatial orientation of the protein side groups. Glycosylation also shifts the pI as a result of the charged carbohydrate groups added to the molecule during post-translational modification.

Primary Structure will dictate the pI of proteins in the absence of side chain charge interactions, glycosylation, or internal sequestering of charges.[i] The Henderson-Hasselbach equation can be used to approximate the pI of the protein based upon the pK_as of the ionizable side groups.

$$pH = pK_A + \log\frac{[A^-]}{[HA]}$$

For acidic side chains, the species in the numerator will have a negative charge. For basic side chains, the species in the denominator will have a positive charge. Rearranging and solving for the magnitude of the fractional charge for acidic sidechains yields:

$$[A^-] = \frac{10^{pH-pK_A}}{10^{pH-pK_A}+1}$$

For basic sidechains:

$$[HA^+] = \frac{1}{10^{pH-pK_A}+1}$$

These equations provide a way to predict the fraction of an amino acid species existing in the charged form at a given pH. By multiplying that fractional charge by the total ionizable side groups present for each species, it is possible to predict the net charge for the entire protein at a given pH. The amino acids with ionizable side groups are lysine, arginine, histidine, aspartic acid, and glutamic acid. A simple iterative procedure then allows the isoelectric point to be calculated. However, it is likely that this pI estimate will be best used as an indicator of the relative pI of one protein to another due to alteration of the local ionization potentials introduced as the result of tertiary structure.

Based solely upon the intact primary structure of FVIII, the pI is 7.04. Because of FVIII's tendency to degrade, it is also useful to predict the pI of those fragments that are likely to exist after degradation. The domain structure of FVIII is typically characterized as A1:A2:B:A3:C1:C2. In plasma, FVIII circulates as a heterodimer consisting of a light chain (A3:C1:C2) and a heavy chain (A1:A2:B). Under those conditions encountered in plasma, the heavy chain and the light chain are linked via a weak calcium dependent ionic interaction. Upon activation, the FVIII molecule is cleaved into FVIIIa such that A1, A2, B, and A3:C1:C2 are predominantly separate species. A1 has a pI of 5.69. A2 has a pI of 5.87. B has a pI of 9.36. A1A2B has a pI of 6.53. The A3C1C2 has a pI of 9.82. Branovic et al reported the pI of a highly purified plasma-derived FVIII to be 5.9. Since this is significantly lower than that predicted from primary structure alone, other factors such as glycosylation and the existence of proteolytic subspecies are clearly important.

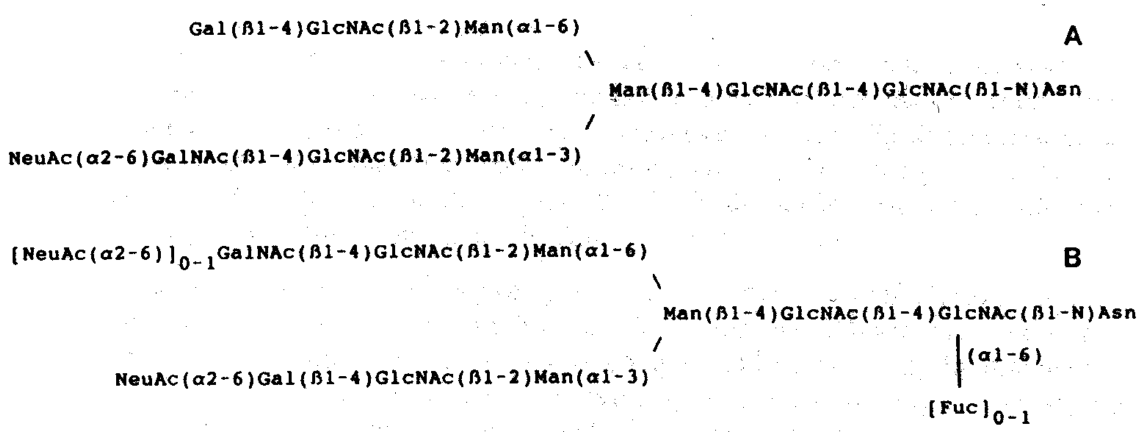


Figure 1. Primary Structure of a biantennary glycan from porcine lactoferrin [iii]

Factor VIII is a complex glycoprotein that will inherently exist as a broad heterogeneous population of molecules. Glycosylation affects the isoelectric point of proteins by adding charged groups to the protein. Factor VIII has 25 potential asparagine (N)-linked glycosylation sites.[ii] Eighteen of these are located in the B domain with 2 in the A1 domain, 1 in the A2 domain, 2 in the A3 domain, and 1 in the C1 domain. Each of these glycosylation sites can have one, two, or no sialic acid attached to them.(Figure 1) [iii] The number present depends upon the extent to which glycosylation proceeds with complete glycosylation being very rare. When complete glycosylation is assumed, the predicted pI for Factor VIII is 5.7 with A1 having a predicted pI of 5.3, A2 having a predicted pI of 5.6, B having a predicted pI of 5.1, A1A2B having a predicted pI of 5.25, and A3C1C2 having a predicted pI of 8.2. This would be particularly important for recombinant FVIII since rate limitations in post-translational processing would potentially generate larger diversity of pIs for each rFVIII chain. Isoelectric focusing would reveal the existence of these diverse subspecies.

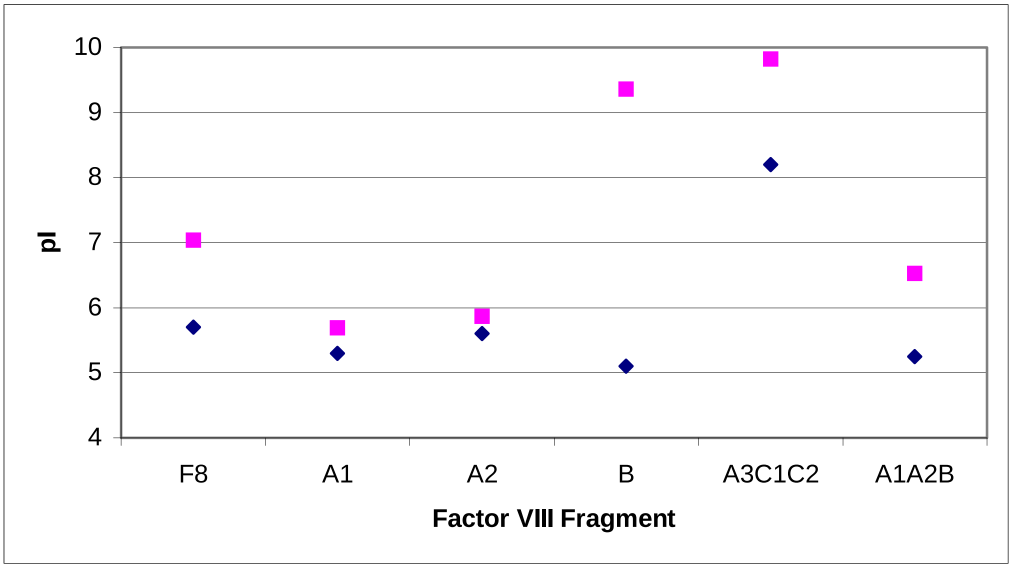
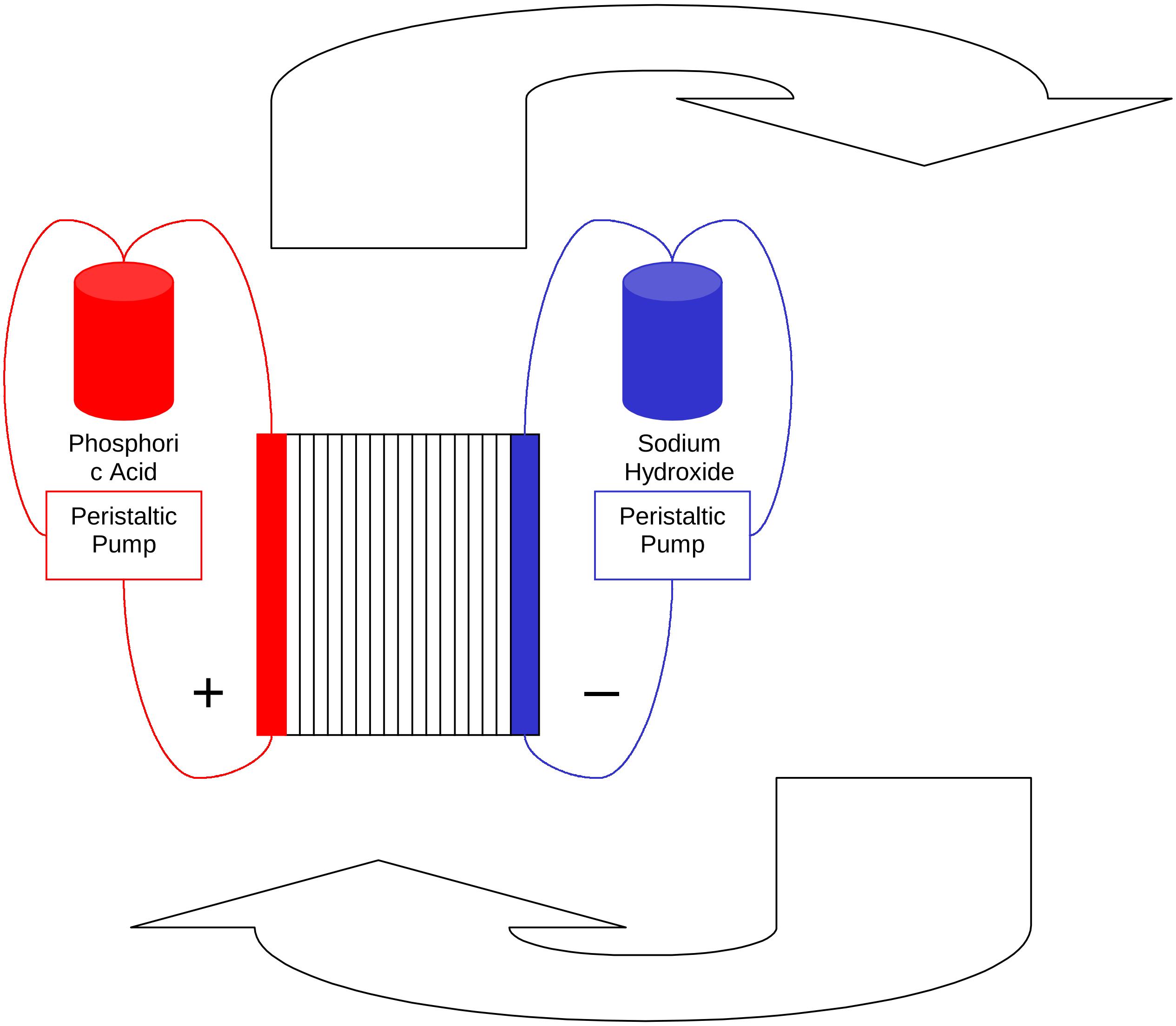


Figure 2. Expected pI range for Factor VIII Fragments Based upon Primary Structure and Glycosylation

Our goal is to use preparative isoelectric focusing to fractionate recombinant rFVIII from transgenic pig whey. The preparative isoelectric focusing apparatus (Figure 3) consists of a rectangular chamber subdivided into 50 channels. On each horizontal end of this chamber is an electrode buffer chamber. The left side contains phosphoric acid and is connected to the positive electrode. The right side contains sodium hydroxide and is connected to the negative electrode. During focusing, a current is applied across the chamber and the contents of both buffer wells are recirculated continuously. The bottom and top extremes of the lanes in the chamber are connected via individual tubes which pass through a peristaltic pump and direct the flow pattern upwards through the chamber to minimize the disruptive potential of buoyancy effects caused by density gradients. Upon completion of the focusing process, the chamber is drained leaving the contents of the tubing undisturbed. Finally, the liquid in each tube is collected individually.

Figure 3. Isoelectric Focusing Unit



In this work, preparative IEF was studied with three types of systems. First, a 2 dimensional western was performed to verify the actual pI distribution of FVIII antigen and to compare it to plasma-derived FVIII. Next, an azocasein / hemoglobin binary protein mixture was loaded onto the IEF unit. This was done to demonstrate the effectiveness of the IEF unit to resolve proteins in solution based upon their pI and to characterize typical pI distributions that occur with these protein mixtures. Finally, delipidated transgenic swine milk was loaded onto the unit with and without DEAE preprocessing. This was done to determine if preparative IEF could be used as a purification step to recover intact, active rhFVIII from swine milk.

Factor VIII is an inherently unstable molecule with diverse subpopulations having isoelectric points ranging (pI) from 5 to 6 (Branovic[iv] et al, 1998). Wolf [v] (1959) reported that FVIII is optimally stable between a pH of 6.9 and 7.2 with large decreases in stability below pH 6 and above pH 8. This inherent instability could greatly complicate any purification process based upon isoelectric focusing (IEF) because the pI of FVIII lies between pH 5 and pH 6. Additionally, calcium ions are necessary for stability. Mikaelsson [vi](1983) determined the optimal calcium concentration to lie between 0.3 and 1.0 mM. Any successful purification scheme based upon isoelectric focusing must take both of these stability issues into account. The preparative scale IEF experiments detailed below were used to determine if IEF could recover recombinant human Factor VIII (rhFVIII) in spite of the known instability of plasma derived FVIII at pH 5 to 6.

Methods

Milk Sample Treatment: 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. Elute with 100 mM phosphate / 500 mM NaCl / pH 7.2 and collect any peaks. Dialyze elute against DI water and concentrate using lyophilization.

Two-dimensional Western using IPGphor IEF System: The first dimension was processed using a 7cm IPG strip with a pH 3-10 linear gradient as per procedure described in Amersham Pharmacia manual[vii] Sample was loaded with the rehydration solution and allowed to equilibrate overnight before focusing. After focusing, the strip was frozen a -70°C prior to the second dimension being run. The first dimension was loaded onto a precast 4-12% Bis-Tris second dimensional gel obtained from Invitrogen. The gel was run until the tracking dye reached the bottom of the gel and then the electrical current was stopped. Upon completion of the electrophoresis, the gel was transferred to a PVDF membrane and probed as per standard western blot procedure.

Azocasein / Hemoglobin System: A mixture of azocasein and hemoglobin was loaded onto the preparative IEF unit (2 mL of 30 mg/mL azocasein / 30 mg/mL bovine hemoglobin / 2% Triton X-100) and allowed to focus overnight. The chamber was monitored and any precipitation was noted. The azocasein and the bovine hemoglobin were yellow and red respectively and could be monitored visually. Once the run was finished, the IEF unit was drained and the pH and OD_{280nm} of each lane was measured and recorded.

IEF Processing of Transgenic Swine Milk With DEAE Preprocessing: Transgenic swine milk obtained from pig 22-5 on 3/8/99 was first processed with DEAE Sepharose. Ten mL of the swine milk was mixed with 10 mL 100 mM Tris / 200 mM NaCl / pH 8. The mixture was centrifuged at 3800 RPM / 4 °C for 30 minutes to defat.

After spinning, the bottom layer was pulled off leaving the top fat layer intact and undisturbed. The resulting defatted milk was diluted 1:6 with 50 mM Tris / 100 mM NaCl resulting in a final optical density of 13.8. This milk (57 mL) was then loaded onto a DEAE Sepharose column with a volume of approximately 88 mL. The fallthrough fraction was collected as well as a wash (50 mM Tris / 100 mM NaCl). Next, 50 mM Tris / 250 mM NaCl was flowed over the column. An elution fraction and a wash fraction were collected. Then, 50 mM Tris / 500 mM NaCl was passed over the column and another elution fraction and another wash fraction were collected. Samples were analyzed by slot blot and by measuring the optical density. Based upon the ratio of slot blot darkness to optical density, optimal samples to load onto the IEF unit were determined. These optimal samples were then fed onto the IEF unit in an ampholyte carrier mixture containing 2% w/v Bio-Rad Biolyte 3-10 ampholytes + 0.5 mM $CaCl_2$. The unit was allowed to focus overnight and samples were collected. These samples were then analyzed by reduced western.

Direct IEF Processing of Transgenic Swine Milk: 50 mL of milk collected from pig 177-2 on 11/7/96 was spun at 4 degrees C to defat. The milk partitioned itself into three layers: a top fat layer, a middle layer which was drawn off (volume of 30 mL), and a smaller bottom layer containing debris. The middle layer was dialyzed against 1 mM $CaCl_2$. This 30 mL of dialyzed, de-fatted milk was then fed onto the isoelectric focusing unit. The ampholyte carrier mixture was 2% w/v of Biorad Biolyte 3/10 ampholytes + 0.5 mM $CaCl_2$. The IEF unit was run overnight and samples were collected. Any samples with heavy precipitate were centrifuged to pellet and the supernatant and the pellets were analyzed separately. The pellets were resuspended in 50 mM Tris / 10 mM $CaCl_2$ / pH 7.3. The pH gradient of the supernatant and the protein concentration (by optical density at 280 nm) of both the supernatant and the reconstituted pellets were measured.

Two-dimensional Western Analysis

Factor VIII partially purified from transgenic swine milk was analyzed using two-dimensional western blots. This was done to better understand the distribution of FVIII species in terms of their molecular weights and isoelectric points. These western blots were compared to those molecular weight and isoelectric points encountered with plasma-derived FVIII.

Figure 4 shows a two-dimensional western of human Factor VIII obtained from American Red Cross Anti-hemophilic Factor. It is wild-type and plasma derived. A pI scale is superimposed over the top of the western. At the top of the western, clear bands are observed in the pI range from 4.5 to 9.5. This corresponds to the heavy chain of FVIII. Approximations based upon primary structure and neglecting complex side-chain interactions predict the heavy chain to have a pI varying from 5 to 7 depending upon the extent of glycosylation. However, the greatest heterogeneity due to glycosylation is introduced in the B chain and it has a predicted pI from 5 to 9.5. The multiplicity of bands observed near the top of the western probably arises from small degradations that result in a lower molecular weight without affected the pI of the protein chain. Below the heavy chain bands, there are smaller bands. These bands correspond to the light chain. Approximations based upon primarily structure and neglecting complex side-chain interactions predict the light chain to have a pI varying from 8 to 10 depending upon the extent of glycosylation.

Figure 5 shows a two-dimensional western of human FVIII produced in the milk of transgenic swine. A pI scale is superimposed over the top of the western. This western is characterized by heavy precipitation towards the top of the gel in the pI range from 7 to 10. This is probably the heavy chain although if degradation is present, exact prediction is impossible as degradation affects both the primary structure and the concomitant tertiary structure making theoretical predictions of pI problematic. Below the heavy precipitate smear are numerous bands at various pI and molecular weight. These correspond to degradation products with exact characterization being impossible.

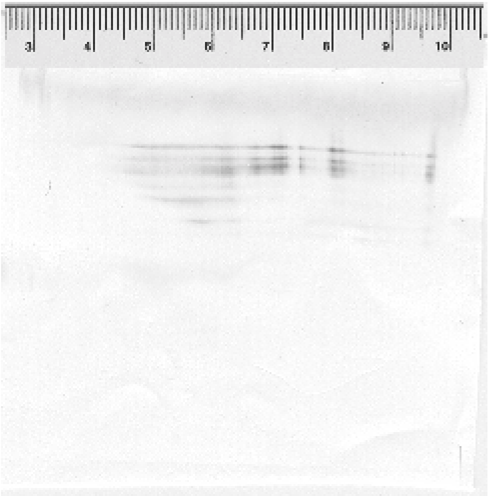


Figure 4. Two-dimensional Western of hFVIII obtained from American Red Cross Anti-hemophilic Factor

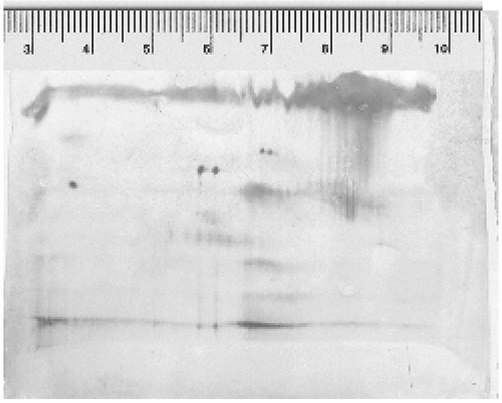


Figure 5. Two-dimensional Western of rhFVIII obtained from Transgenic Swine Milk

Azocasein / Hemoglobin Results

A binary mixture of azocasein and hemoglobin was loaded on the IEF unit to evaluate the performance of the preparative IEF unit. Figure 6 shows a typical set of results for these azocasein / hemoglobin experiments. Figure 6 shows a graph of the pH measured in each lane as well as the protein concentration as measured by OD_{280}. The pH gradient appears relatively smooth and linear from lanes 13 to 40 with relatively constant pH ranges below 13 and above 40. A sharp peak in the protein concentration is observed around pH 5 with a smaller, less well defined peak from pH 7 to pH 8. Casein has a pI of approximately 5. This peak is clearly visible between lanes 11 and 16. Additionally a yellow precipitate was clearly visible between lanes 15 and 25 both in the tubes and adsorbed to the chamber walls. Hemoglobin has a pI of approximately 7. A small peak is visible for the hemoglobin between lanes 26 and 40. Heavy red precipitate was visible between lanes 39 and 48 both in the tubes and adsorbed to the chamber walls. Of the 60 mg of azocasein loaded, about 18 mg was recoverable in the sample tubes (3.5 mL per sample tube). There is also approximately 2.0 mL of lane volume per lane that is contained in the chamber and can not be recovered as separate fractions. Assuming uniform concentration throughout the sample tube and chamber flow path, approximately 28 mg of protein can be accounted for or approximately 50%. The remainder is believed to have been precipitated and adsorbed to the walls of the IEF unit. Of the 60 mg of hemoglobin loaded, about 5 mg was recoverable in the sample tubes (3.5 mL per sample tube). If the unrecoverable 2.0 mL of chamber volume per channel is included, 8 mg of protein can be accounted for or approximately 13%. This much lower recovery relative to the azocasein is believed to be due to the hemoglobin precipitating more readily and sticking to the walls of the IEF unit.

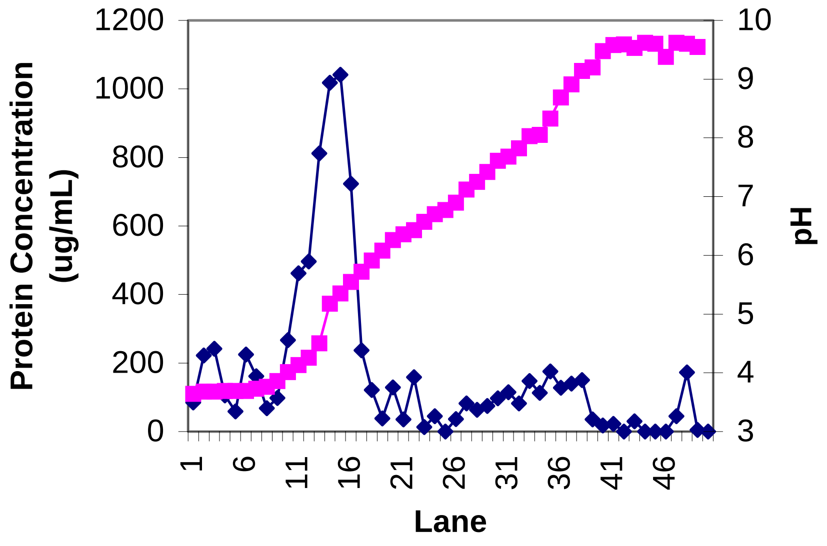


Figure 6. Protein Concentration and pH gradient for Azocasein / Hemoglobin IEF Run

IEF Processing of Transgenic Swine Milk With DEAE Preprocessing Results

Based upon the results from the azocasein / hemoglobin experiments, IEF processing of swine milk was viewed as a viable procedure. The defatted swine milk was first processed using a DEAE column in an attempt to reduce the total protein load. Figure 7 shows a slot blot performed on the DEAE column fractions. Slot blots allow greater sensitivity and faster results than westerns but suffer from some non-specificity because of the non-reduced nature of the assay. The column A and column B samples were diluted 1:1 with slot blot loading buffer and loaded onto the apparatus (400 uL total volume). The column B samples were diluted with slot blot loading buffer to render the final amount of hFVIII loaded at the desired level (200 uL total volume). In column A, a strong signal is observed in the starting material and the 250 mM Elute. Smaller amounts are observed in the fallthrough, the first wash, the second wash, and the 500 mM Elute. In column B, a clean, linear standard curve is observed with the assay still sensitive and significantly different from the blank at levels of 3.125 ng of total hFVIII.

Table 1 shows the sample volumes collected from the DEAE column as well as the OD_{280} of these samples. It can be seen that most of the protein came off the column in the fallthrough fraction with a large amount of protein also coming off in the 250 mM Elute. The total amount of protein eluted from the column was 404 mg or a little more than 50% of the amount loaded.

By visual inspection of the slot blot and spectrophotometric analysis of the samples, an approximation can be made to determine which samples have the highest concentration of rhFVIII relative to total protein. Based upon the slot blot, the second wash fraction appears promising in terms of FVIII purity because the signal on the slot blot is higher than the blank while the total protein concentration in that fraction is very low. It also appears that most the rhFVIII came off in the 250 mM Elute. The signal on the slot blot is very strong for this fraction. The total protein in this fraction is also high. Based upon these results, the second wash fraction and the 250 mM elution fractions were processed further using preparative isoelectric focusing.

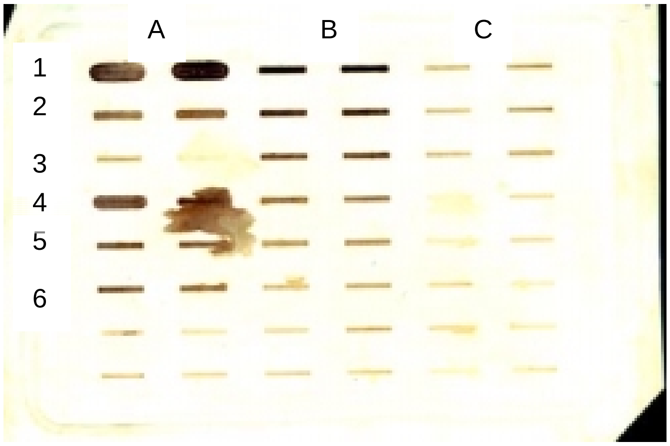
Figure 9 shows the pH profile that was obtained when 60 mL of the second wash fraction was loaded onto the IEF unit. The pH curve is relatively linear from pH 4 to pH 8. The extreme acid and base side of the chamber have pHs that exceed the standard range of the ampholytes which should be between 3.5 and 10. This could be the result of the higher salt concentration that was loaded onto the unit.

Figure 10 shows a reduced western analysis of the IEF samples obtained from loading the second wash from the DEAE column. Cross-reactivity is observed in the swine IgG sample although it clearly has a different appearance than the hFVIII obtained from ARC. This Swine IgG is possibly the source of the cross-reactivity in the slot blot. However, in those lanes where there should be Factor FVIII antigen, none is observed.

Figure 11 shows the pH gradient that resulted from loading 70 mL of the 250 mM elute dialyzed against DI H_2O and lyophilized down to a volume of 15 mL prior to loading. The pH gradient is relatively flat from lanes 1 to 8 with a pH of approximately 3.1. Lanes 42 to 50 are also relatively flat with a pH of approximately 9. Between these two flat regions, the pH gradient is relatively linear.

Figure 12 shows a reduced western analysis done on the IEF fractions obtained from loading the dialyzed, lyophilized, 250 mM Elute from the DEAE column onto the IEF unit. Based upon slot blot analysis, enough FVIII antigen was loaded that a signal should be visible in at least some of the lanes analyzed. Nothing is observed however. It appears that any Factor FVIII loaded was sufficiently denatured that it can no longer be recognized by the detecting antibodies.

Because of the lack of Factor VIII signals seen in the two reduced westerns performed on the IEF samples, a reduced western was performed on the column fractions to better determine where the FVIII came off the column. Figure 8 shows a reduced western blot performed on column fractions from a DEAE column loaded with transgenic milk for pig 22-5. Clear FVIII antigen signals are only observed in the ARC standard, starting material, and the fallthrough lanes. There are very faint bands present in other lanes on the original blot but they aren’t visible after being scanned in. This experiment highlights two important phenomena. First, while slot blots are more sensitive than westerns, they have unacceptable cross-reactivity. Second, DEAE preprocessing is not a viable pre-processing step to use before preparative IEF. The FVIII falls through the DEAE column even when the column media is in excess to the total amount of protein loaded. In fact, the majority of protein falls through the column. Most of the fallthrough is presumably the casein micelles that exist in a state that makes them unable to bind to the column packing. Because of tight association of rhFVIII with the casein micelles, they also come out in the fallthrough. Better results should be obtainable by directly loading the swine milk onto the IEF unit. In this way, the rhFVIII should be separable from the micelles as a result of the driving force provided by the electrical field.

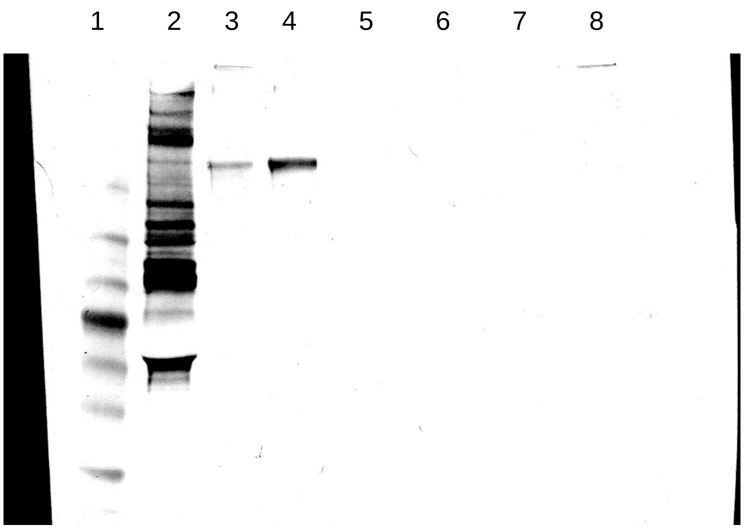


A1	Starting Material
A2	Fallthrough
A3	Wash 1
A4	250 mM Elute
A5	Wash 2
A6	500 mM Elute
B1	50 ng hFVIII Standard
B2	25 ng hFVIII Standard
B3	12.5 ng hFVIII Standard
B4	6.25 ng hFVIII Standard
B5	3.125 ng hFVIII Standard
B6	1.5625 ng hFVIII Standard
C1	Blank
C2	250 mM Blank
C3	500 mM Blank

Figure 7. Slot Blot Performed on Fractions From DEAE Column Performed on Milk from Pig 22-5

Table 1. Sample volumes and OD_{280} Absorbance of DEAE Column Performed on Milk from Pig 22-5

Fraction	Volume (mL)	OD_{280}	Total Protein by OD_{280} (mg)
Starting Material	57	13.8	786.6
Fallthrough	140	1.5	210.0
Wash1	55	0.13	7.2
250 mM Elute	73	2.24	163.5
Wash2	65	0.11	7.2
500 mM Elute	55	0.29	16.0



1	Molecular Weight
2	FVIII Standard
3	Starting Material
4	Fallthrough
5	Wash 1
6	250 mM Elute
7	Wash 2
8	500 mM Elute

Figure 8. 4-12% Bio-Rad Tris-Glycine Gel: Reduced Anti-FVIII Western on DEAE Column Fractions. Lane 1 is prestained molecular weight markers obtained from Biorad. Lane 2 is 130 ng of hFVIII standard (plus albumin) obtained from American Red Cross. Lane 3 is the starting material (1.2 uL of sample + 8.8 uL of buffer + 10 uL of reducing cocktail). Lane 4 is the fallthrough fraction (12.5 uL of sample + 12.5 uL of reducing cocktail). Lane 5 is the first wash fraction (12.5 uL of sample + 12.5 uL of reducing cocktail). Lane 6 is the 250 mM Elute fraction (1.2 uL of sample + 8.8 uL of buffer + 10 uL of reducing cocktail). Lane 7 is the second wash fraction (15 uL of sample + 15 uL of reducing cocktail). Lane 8 is the 500 mM Elute fraction (15 uL of sample + 15 uL of reducing cocktail).

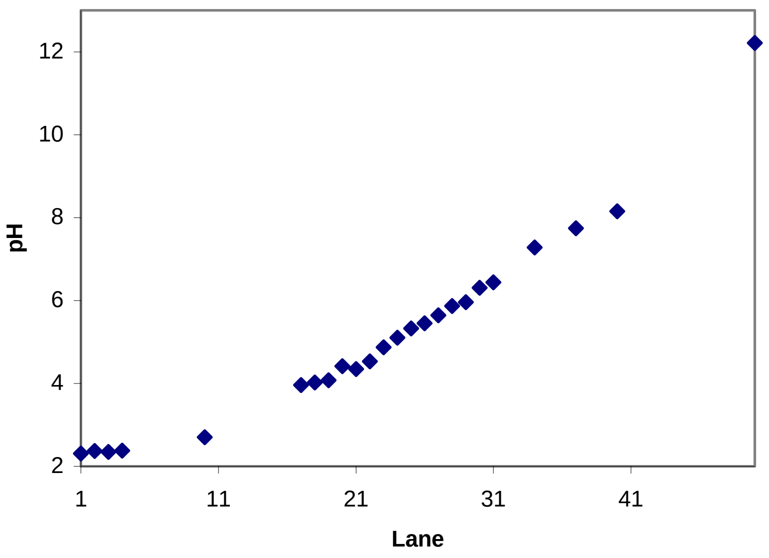
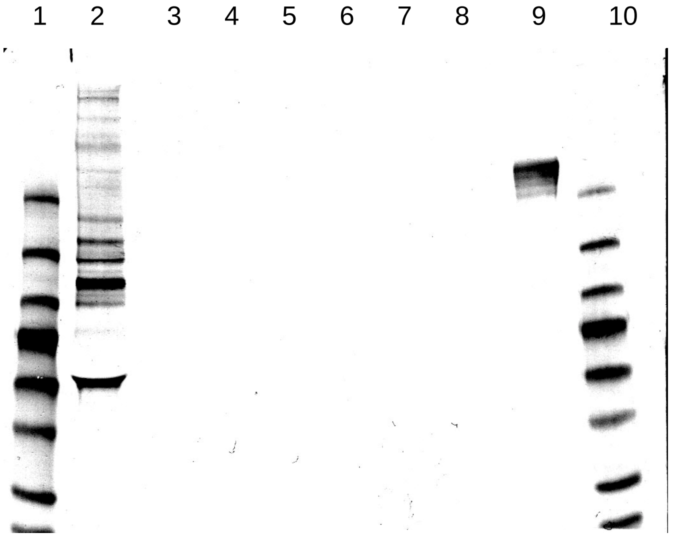


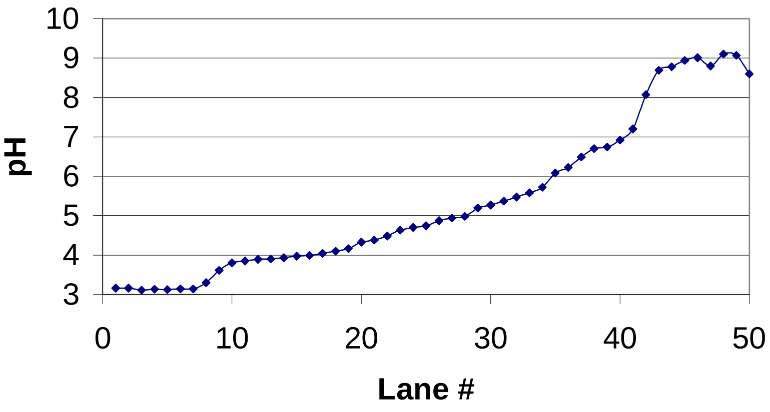
Figure 9. pH Profile of IEF run on Wash 2 from DEAE Column

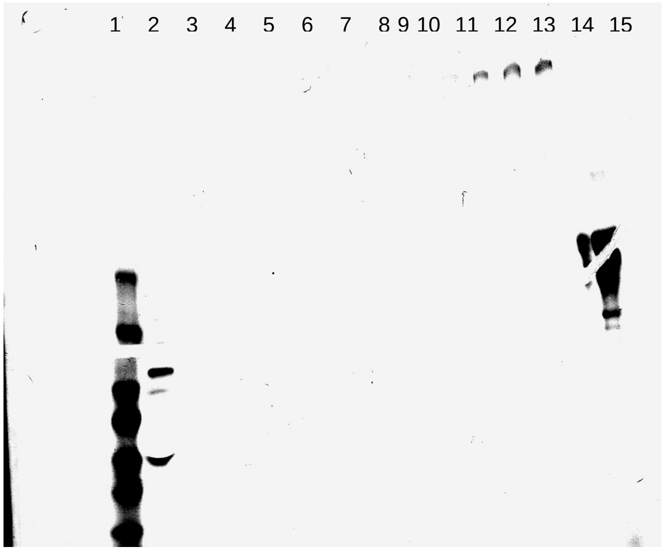


1	MW Markers
2	Factor VIII Standard
3	IEF channel 11
4	IEF channel 12
5	IEF channel 26
6	IEF channel 27
7	IEF channel 29
8	IEF channel 35
9	Swine IgG
10	MW Markers

Figure 10. 4-12% Bio-Rad Tris-Glycine Gel: Reduced Anti-FVIII Western on Wash1 IEF Fractions. Lanes 1 and 10 contain prestained molecular weight markers (10 uL loaded on gel) obtained from Bio-Rad. Lane 2 is 130 ng of hFVIII standard (plus albumin) obtained from American Red Cross. Lanes 3 through 8 contain samples of various channels from the IEF unit. Lane 3 contains IEF Channel 11 (5.4 uL of sample + 4.6 uL of buffer + 10 uL of reducing cocktail). Lane 4 contains IEF Channel 12 (11.75 uL of sample + 0.75 uL of buffer + 12.5 uL of reducing cocktail). Lane 5 contains IEF Channel 26 (15 uL of sample + 15 uL of reducing cocktail). Lane 6 contains IEF Channel 27 (15 uL of sample + 15 uL of reducing cocktail). Lane 7 contains IEF Channel 29 (15 uL of sample + 15 uL of reducing cocktail). Lane 8 contains IEF Channel 35 (15 uL of sample + 15 uL of reducing cocktail). No Factor VIII antigen is detected in any of these lanes. Lane 9 contains Swine IgG.

Figure 11. IEF Run on Elute 1 from DEAE Column





1	MW Markers
2	Factor VIII Standard
3	IEF channel 19
4	IEF channel 20
5	IEF channel 21
6	IEF channel 22
7	IEF channel 23
8	IEF channel 24
9	IEF channel 25
10	IEF channel 26
11	IEF channel 27
12	IEF channel 28
13	IEF channel 29
14	IEF channel 30
15	Swine IgG

Figure 12. 4-15% Novex Tris-Glycine Gel: Reduced Anti-FVIII Western on 250 mM Elute IEF Fractions. Lane 1 contains prestained broad range molecular weight markers (10 uL loaded on gel) obtained from Bio-Rad. Lane 2 contains 130 ng of hFVIII standard (plus albumin) obtained from American Red Cross. Lane 3 contains IEF Channel 19 (10 uL of sample + 10 uL of reducing cocktail). Lane 4 contains IEF Channel 20 (7.4 uL of sample + 2.6 uL of buffer + 10 uL of reducing cocktail). Lane 5 contains IEF Channel 21 (4 uL of sample + 6 uL of buffer + 10 uL of reducing cocktail). Lane 6 contains IEF Channel 22 (3.4 uL of sample + 6.6 uL of buffer + 10 uL of reducing cocktail). Lane 7 contains IEF Channel 23 (2.8 uL of sample + 7.2 uL of buffer + 10 uL of reducing cocktail). Lane 8 contains IEF Channel 24 (3.1 uL of sample + 6.9 uL of buffer + 10 uL of reducing cocktail). Lane 9 contains IEF Channel 25 (2.5 uL of sample + 7.5 uL of buffer + 10 uL of reducing cocktail). Lane 10 contains IEF Channel 26 (3.2 uL of sample + 6.8 uL of buffer + 10 uL of reducing cocktail). Lane 11 contains IEF Channel 27 (3.4 uL of sample + 6.6 uL of buffer + 10 uL of reducing cocktail). Lane 12 contains IEF Channel 28 (4.8 uL of sample + 5.2 uL of buffer + 10 uL of reducing cocktail). Lane 13 contains IEF Channel 29 (10 uL of sample + 10 uL of reducing cocktail). Lane 14 contains IEF Channel 30 (10 uL of sample + 10 uL of reducing cocktail). Lane 15 contains 6 ug of Swine IgG. No FVIII signals are observed in any of the IEF Channel lanes.

On the basis of the IEF results obtained when DEAE preprocessing was done, direct IEF processing of transgenic swine milk was explored. The IEF unit has 50 separate channels with 3.5 mL of volume that can be recovered from each one. For the runs that directly processed whey, many of the IEF channels contained heavy precipitate due to the insolubility of proteins near their pI when at high concentration. Each of the focused samples was centrifuged. The pellets and the supernatants were separated. The pellets were resuspended. The absorbance of the resolubilized pellets was between 100 and 125 (OD_{280nm}). The absorbance of the supernatants is shown in Figure 14. For the purpose of running gels, these pellet samples were diluted 4-fold in 100 mM EDTA to clarify and dilute them to the range of 25 to 30 mg / mL total protein.

Figure 13 shows a typical pH profile gradient that was established in the IEF unit in the presence of 900 mg of total loaded protein. In the range from channel 1 to channel 20, the pH is relatively constant at about pH 4.7. This relatively fixed pH section is caused by high protein concentrations (caseins) that act as ampholytes themselves. Channel 21 to channel 26 spans a steep gradient from pH 5 to 6 and thus represents a potential concentration effect of about 1 to 2 fold for protein which is focused entirely in these few channels. Greater than 90% of the FVIII antigen focused in these channels based upon slot blot analysis.

Figure 14 shows the absorbance at 280 nm for the IEF supernatant samples. From channels 1 to 23, the protein concentration remains relatively constant and is estimated to be about 2 mg/mL (by OD_{280nm}). The absorbance rises slightly from channels 24 to 27 followed by a sharp decline in OD_{280nm} to about 0.5 mg/mL.

Figure 13. pH Profile of IEF Run

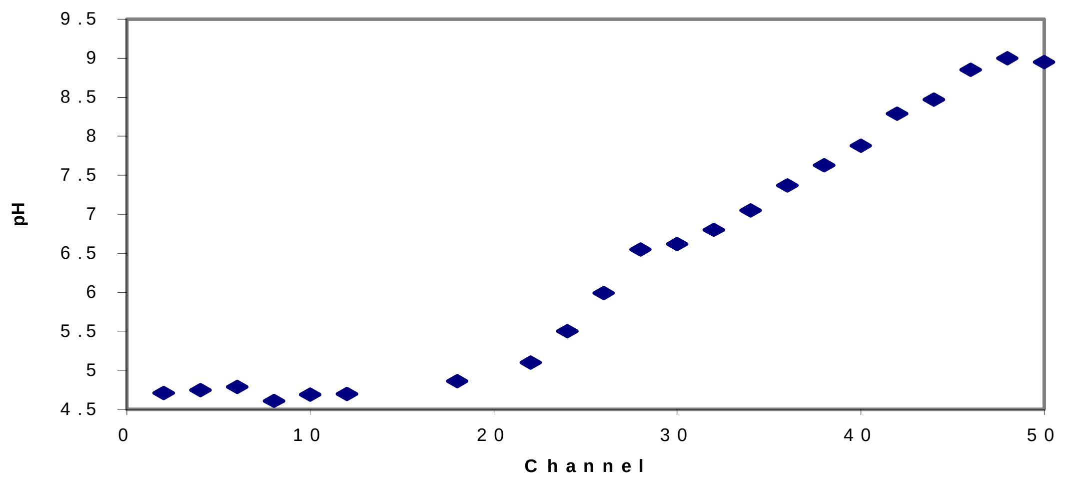
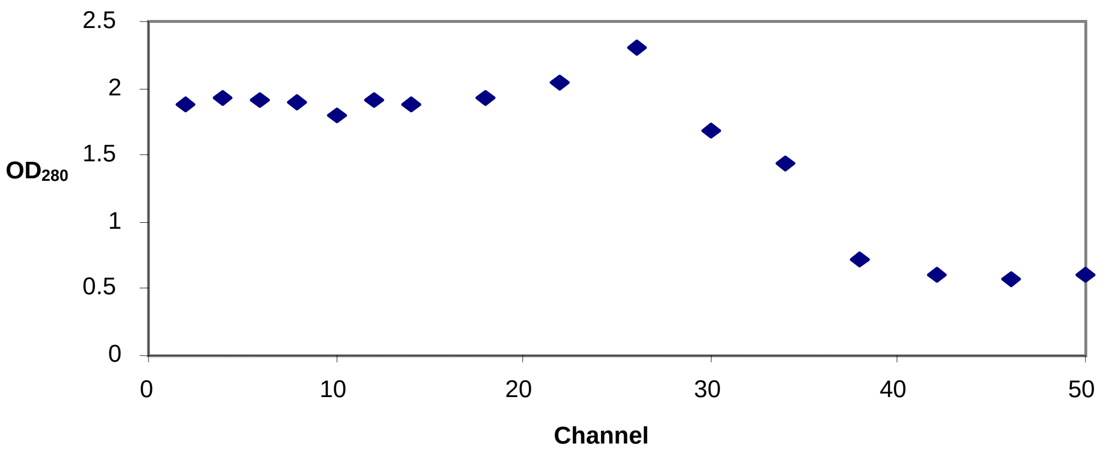
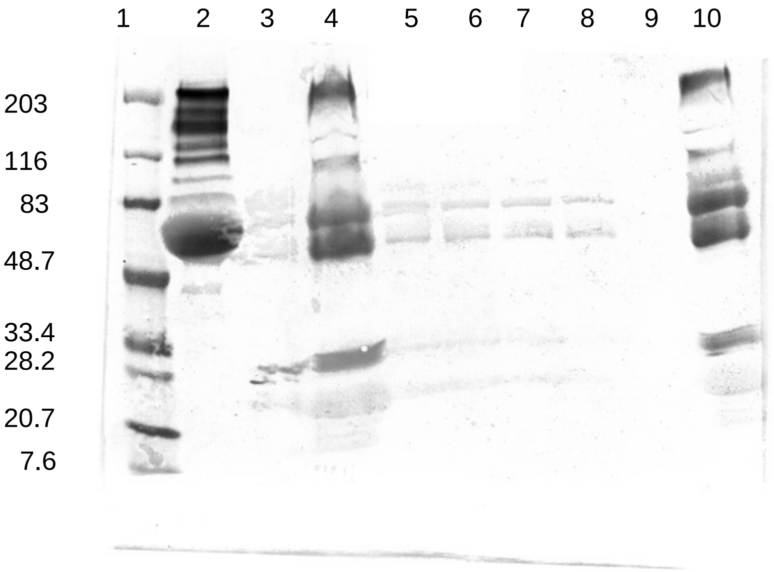


Figure 14. Protein Concentration of IEF Supernatant Samples



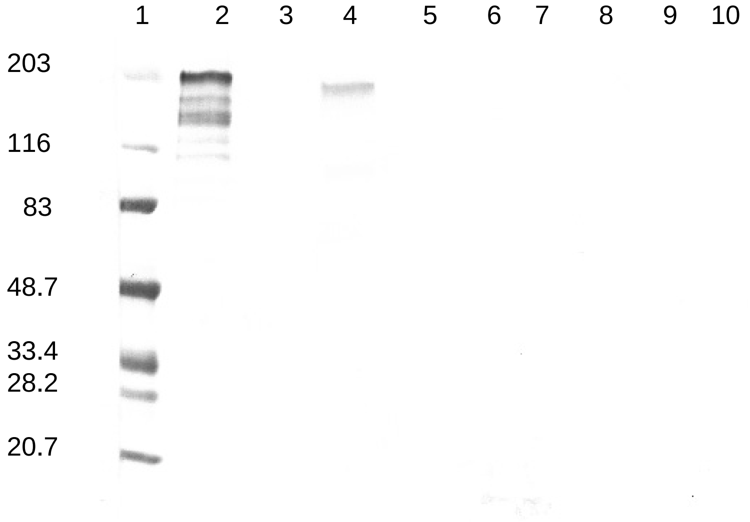


Lane	Sample
1	MW Markers
2	Factor VIII Standard
3	Nontransgenic Whey
4	Starting Material
5	IEF channel 23
6	IEF channel 24
7	IEF channel 25
8	IEF channel 26
9	Blank
10	Starting Material

Figure 15. 4-15 % Tris-Glycine gel: reduced Anti-FVIII Western on IEF Supernatant Samples. Lane 1 is prestained broad range molecular weight markers (10 uL loaded on gel) obtained from Biorad. Lane 2 is 130 ng of hFVIII standard (plus albumin) obtained from American Red Cross. Lane 3 is non-transgenic pig whey (10 uL of sample + 10 uL of reducing cocktail with 60 mM EDTA (RC+EDTA) loaded on the gel). No cross-reactivity is observed in this control whey in the molecular weight range where the FVIII is located. Lanes 4 and 10 are the material (10 uL of sample + 10 uL RC+EDTA loaded) that was loaded onto the IEF unit.

Figure 15 shows a reduced western blot analysis done which is typical of the supernatant samples from IEF run of directly processed whey. The banding pattern observed in the starting material lanes is very similar to that hFVIII standard. The concentration of rhFVIII in this starting material is estimated to be about 5 ug/mL. Lanes 5, 6, 7, and 8 are supernatant samples from the IEF unit (10 uL of sample + 10 uL RC+EDTA loaded). No FVIII signal is seen. There are faint bands between 50 kD and 80 kD molecular weight but the control whey also has these bands suggesting cross-reactivity rather than a real signal.

Figure 16 shows a reduced western analysis done on the resuspended pellet samples. There is no cross reactivity with the control whey. There is a band that corresponds to a band in the hFVIII standard that can be seen in the starting material. The concentration of FVIII in the starting material is estimated to be about 5 ug/mL. No FVIII signal is seen in any of the pellet samples.



1	MW Markers
2	Factor VIII Standard
3	Nontransgenic Whey
4	Starting Material
5	IEF channel 22
6	IEF channel 23
7	IEF channel 24
8	IEF channel 25
9	IEF channel 26
10	IEF channel 27

Figure 16. 4-15 % Tris-Glycine gel: Reduced Anti-FVIII Western on IEF pellet samples. Lane 1 is prestained broad range molecular weight markers obtained from Biorad. Lane 2 is 130 ng of hFVIII standard (plus albumin) obtained from Red Cross. Lane 3 is non-transgenic pig whey (10 uL of sample + 10 uL of reducing cocktail with 60 mM EDTA (RC+EDTA) loaded on the gel). Lane 4 is the material (10 uL of sample + 10 uL RC+EDTA loaded) that was loaded onto the IEF unit. Lanes 5 through 10 are pellet samples from the IEF unit (10 uL of resuspended sample + 10 uL RC+EDTA loaded).

Two-dimensional Western Discussion

The two-dimensional western analysis was done to characterize the populations of FVIII existing in transgenic swine milk and to compare them to those present in plasma-derived hFVIII. Also of interest was a comparison between the actual pI observed and the pI predicted from primary protein structure. The theoretical predictions of pI based upon primary structure serve mainly to show the high variability in isoelectric points. The predictions obtained for the heavy chain (A1A2B and B) are reasonably accurate. However, those pIs predicted for the light chain are substantially different than what is observed experimentally. The light chain lies between 4.5 and 6.3 with the predicted range being from 8.1 and 9.9. These differences are probably due to side chain interactions and internal sequestering of charges. Of most interest are the substantial differences between plasma-derived hFVIII and hFVIII produced in transgenic swine milk. The FVIII from swine milk is extremely degraded and precipitated. Very few clean bands of intact FVIII species are observed. The FVIII is so heavily degraded even before purification steps are performed that recovery of purified active FVIII appears to be extremely difficult.

Azocasein / Hemoglobin Discussion

The azocasein / hemoglobin experiment was done as a calibration experiment in preparation for the transgenic pig processing. The azocasein / hemoglobin focusing run showed approximately a 50% recovery of the azocasein in a relatively narrow distribution. A distribution is expected because the electrodiffusive driving force decreases as proteins approach their pI and their charge decreases. At some point, the electrodiffusive driving force is balanced by the concentration driving force and equilibrium is established. This same phenomenon should be observed in the milk / rhFVIII systems and, in theory at least, should yield similar results. With Factor VIII, however, even a wider distribution should be observed because of the heterogeneity of FVIII. This heterogeneity is most manifested in the heavy chain because of the degradations that occur in the B domain resulting in its molecular weight varying from 90 kDa to 200 kDa.

Transgenic Swine Milk Discussion

Several IEF runs were done directly on whey or whey pre-purified with anion exchange chromatography. The average total rhFVIII input was 150 – 200 ug. None of the IEF products contained rhFVIII antigen at levels which would be predicted from 150 ug – 200 ug input.

No detectable rhFVIII antigen is present in either the IEF focusing run performed on the wash fraction from the DEAE column or on the elute fraction from the same column. This is indicative of sufficient degradation of the factor VIII that was initially loaded on the IEF unit to make immunochemical detection extremely difficult. The combination of degradation due to the pH and a lack of sequestered Ca^{2+} ions is believed to degrade FVIII so totally as to make it unrecognizable to the antibodies.

Based upon material balances of FVIII antigen input, the IEF unit was expected to concentrate native FVIII within the pH range from 5 to 6. An initial milk volume of 30 mL was loaded on the IEF unit. The concentration of FVIII in this 30 mL is estimated to be about 5 ug/mL (by reduced western analysis) for a total of 150 ug total loaded on the IEF unit. The FVIII in the milk should have focused between pH 5 and pH 6. Channels 21 to 26 span this range. The volume of each channel is approximately 3.5 mL. This represents a total volume of approximately 21 mL that should contain most of the FVIII. However, no Factor VIII is detected in either the supernatant or the pellet. It is believed that the Factor VIII was sufficiently degraded during the IEF process that the detecting antibody can no longer recognize the FVIII. Because we did not evaluate all 50 channels (only those from pH 5 to 6 range), it is possible that other channels contained FVIII antigen but EIA data as well as values published in the literature shows this to be unlikely. Also, because of the heterogeneous nature of FVIII, at least the tails of the distribution should fall within the pH 5 to 6 range even if the peak concentration is centered at pH ranges slight outside the analyzed range.

There are numerous factors that make IEF-based purification of rhFVIII a bad idea. Most importantly is the known instability of hFVIII at its pI. Also calcium ions are required for hFVIII stability especially once proteolytic cleavage has started to occur.

This presents a problem in an IEF environment because the calcium ions are drawn away from the rhFVIII by two factors. First, as a charged ion, the calcium is drawn away from the rhFVIII and towards the cathode. Secondly, carrier ampholytes act as weak chelators.[viii] All these factors combined together make purification of rhFVIII using simple free-flow isoelectric focusing a difficult if not impossible task.

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