Chapter 5. Conclusions and Future Work

Factor VIII is a protein which has therapeutic importance for the treatment of hemophilia A. Its deficiency, either qualitative or quantitative, results in Hemophilia A, a disorder affecting approximately 1 in 10,000 males. Currently, FVIII replacement therapy uses FVIII derived from plasma or cell culture. However, both of these sources have limitations that make the generation of alternative sources attractive.

The use of plasma poses a risk of transmitting a blood-borne pathogen to the patient such as AIDS, Hepatitis, or one of the emerging prion diseases. Cell culture is pathogen free but is prohibitively expensive for most people with treatments costing $150,000 / patient / year. If transgenic animals could be made to produce FVIII, a relatively inexpensive and plentiful pathogen-free source for FVIII would be available. This would open up the possibility of safe treatment for all hemophiliacs, including those living in less affluent countries. These salient features provide the motivation for the study of FVIII.

Paleyanda et al have expressed rhFVIII in the milk of transgenic swine. However, their recovered activity is extremely low precluding its current use as a replacement source for FVIII. The specific goals of this research were to characterize those factors hindering the production and recovery of active rhFVIII from the milk of transgenic swine. It was hoped that this would facilitate future work in the generation of transgenic animals capable of producing active and recoverable FVIII activity. The specific approaches taken included attempts at assay development and novel purification strategies.

Immunoprecipitation-based assays were explored as a possible means to assay FVIII. None of the assay methods produced an acceptable assay. Instead of clearly resolved antibody-mediated precipitation patterns, an unresolvable smeary precipitate was observed. Various techniques were attempted with all yielding similar results for all assay conditions. It appears that the extreme tendency for FVIII to precipitate even in the absence of antibody will confound any assay based upon precipitation. The high degree of molecular weight as well as charge distribution heterogeneity also complicates immunoprecipitation assays preventing discrete easily quantified precipitate bands.
Analytical isoelectric focusing was explored as a characterization tool. Two-dimensional westerns were used to characterize FVIII obtained from human plasma as well as rhFVIII obtained from transgenic swine milk. The FVIII produced in transgenic swine is much more heterogeneous and degraded than that obtained from plasma. This is believed to be due to incomplete processing that results in the secretion of separate chains. The endogenous proteins present in milk are also believed to be responsible for additional degradation products.

Preparative isoelectric focusing failed to recover any detectable FVIII. It appears that the degradation occurring in the milk is exacerbated further by the isoelectric focusing unit. As the protein degrades in the isoelectric focusing unit, the degradation products are pulled away towards the isoelectric point of the new fragments. In this way, the equilibrium is continually shifted towards the degradation products.

Batch-mode DEAE was explored as a purification technique after failures to bind FVIII with column-mode DEAE. Batch-mode binding resulted in nearly complete binding and supported the hypothesis that FVIII is being sequestered or physically adsorbed to casein micelles. The success of the batch-mode DEAE demonstrated that the adsorption kinetics are limiting. Analysis of the purified FVIII showed proteolytic processing very similar to that seen in FVIII obtained from human plasma. The chains were also present in the proper stoichiometric amounts. Despite the native appearance of the FVIII chains on a western blot, no activity was detected.

All the limitations in assay development and purification essentially reduce to two fundamental problems. Wild-type FVIII is highly labile and highly heterogeneous. Any future effort to produce functional rhFVIII in the milk of transgenic livestock should strive to minimize these two problems. One possible solution appears to be the use of a construct coding for a modified molecule with FVIII activities.

One such construct likely to yield excellent results is IR8 as developed by Pipe et al. IR8 is an inactivation resistant coagulation factor VIII created by judicious gene splicing. The most notable features of this modified FVIII are the replacement of the B domain by a short linker sequence composed of a short section of the wild-type B domain. This linker sequence causes the A2 and A3 domains to be covalently linked instead of being linked by the much weaker ionic interaction existing in the wild-type.
These covalently linked A2 and A3 domains also eliminate some of the vWF stabilization requirements because the molecule exists as a single chain. As a result of these modifications, the rFVIII retains 38% of its activity after 4 hours whereas wild-type FVIII becomes inactive after only 10 minutes. Additionally, a 5-fold increase in specific activity is observed compared to wild-type FVIII.

In addition to being more stable, IR8 is also far more homogeneous. The majority of the glycosylation-based heterogeneity is due to the B domain. With IR8, most of this domain has been eliminated. Figure 1 shows the expected isoelectric ranges for the IR8 construct. With the removal of the B domain, the ranges are far more narrow. This would potentially facilitate the purification of IR8 using an isoelectric focusing strategy since most of the active population would be confined to a very narrow range.

![Figure 1. Theoretical pI of IR8 Fragments](image)

The IR8 construct would also have far less heterogeneity in the size of its chains. The zymogen form of wild-type FVIII consists of an 80 kDa light chain and a heavy chain that varies in size from 90 kDa to 200 kDa. There is the possibility for activity over this entire range of variability. In contrast, the zymogen form of IR8 is composed of a single chain instead of two chains linked by an ionic interaction. This single chain when intact and functional will always have a mass of 180 kDa. While nonspecific proteolysis may exist, the products of these degradations will not have activity and thus not require purification.
All of the modifications present in IR8 make it much more suitable than wild-type hFVIII as a construct used to produce Factor VIII. Specific approaches that should be explored in the future are the same approaches that were explored with the wild-type FVIII expressed using a cDNA construct.

The more homogenous nature of IR8 would facilitate assays based upon immunoprecipitation. Non-specific precipitation may still pose problems but the approach has more promise than ELISAs because of the necessity of either developing an antibody specific to IR8 or using existing FVIII antibodies that will intrinsically have lower binding affinities. These lower binding affinities would require larger amounts of antibody and be even more costly than ELISAs for wild-type FVIII.

Isoelectric focusing and batch DEAE based purification also are likely to yield promising results. The increased stability of IR8 make it more likely to survive the preparative isoelectric focusing process. Additionally, it’s homogeneous nature make the purification much more efficient in terms of the pI ranges that must be collected. Batch DEAE is likely to yield similar results as were obtained with FVIII. The casein micelles are likely to present similar hindrances to purification. However, as with wild-type FVIII, the batch-mode binding should be nearly complete and because or IR8’s increased stability, the activity should be recoverable.

There will clearly be new problems encountered with using the IR8 construct but it appears to solve most of the fundamental problems encountered in my research. I postulate that IR8 could make the goal of creating a large, inexpensive source of active transgenically produced FVIII a reality.

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ii http://www.nmia.com/~mdibble/prion.html

iii Pipe, Steven W., Randal J. Kaufman. Proceedings of the National Academy of Sciences, 1997; 94:11851-11856