Blood Coagulation Factor IX: Purification, Activation, Crystallization

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Blood Coagulation Factor IX

Purification, Activation, Crystallization

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Honors Capstone Project

December 2023
Abstract

This paper presents readers with an optimized procedure for the purification, activation, and crystallization of selected blood coagulation Factor IX double mutant (FIX_2). Through the completion of this work, we aim to enhance future biochemical and structural studies by providing an easier means for the FIX_2 production, in order to increase understanding of the protein’s function within the blood coagulation cascade. The initiation of the blood coagulation cascade is brought on by activation of inactive Factor VIII (FVIII) protein though contact with tissue factor, the FVIII protein then binds to an activated platelet surface where it must wait for its serine protease cofactor, Factor IX (FIX)_1. Just as many other coagulation factors, FIX is produced and circulating in its inactive, catalytically silent form and must be activated by another protein Factor XIa, through cleavage of the amino acid sequence known as the activation peptide _2,^3_ Once both proteins have become activated, the platelet bound active FVIII (FVIIIa) coordinates with active FIX (FIXa) to form the potent Xase complex _1,^2_. This Xase complex formation results in structural rearrangement of the FIX’s 99-loop and 60-loop to permit the proteolytic cleavage action of FIXa on Factor X (FX) converting it into active FX (FXa)_4_. This FXa protein is subsequently used for the activation of thrombin which heavily induces clot formation _3_. However, structural understanding of the assembly and activity of the Xase complex is still relatively unknown therefore the study of the Xase complex and its operating co-factors, FVIII and FIX, remains a highly active field of research.
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**Introduction**

Blood coagulation is a process utilized within human bodies to react to and prevent blood loss due to vessel injury. Through the work of coagulation, blood transitions from a liquid to a semi-gel form, known as a blood clot, thus preventing any further bleeding. Blood clots are made up of red blood cells (RBC) netted together by platelets and fibrin protein. Platelets are a type of blood cell used to produce an initial loose clot by attaching themselves to the surface of multiple RBCs. This weaker platelet held structure, is then reenforced with the formation of fibrin protein that tightly nets the whole clot together through binding to platelets. However, coagulation of blood must be specific to the injured blood vessel to avoid random clotting (thrombosis) throughout the body, which is achieved by the collaborative action of tiered blood coagulation factors. Initiation of this coagulation cascade is maintained by two separate pathways, the intrinsic and extrinsic pathways which converge to produce active factor X (FXa) which in turn stimulates fibrin formation. This cascade begins with the extrinsic pathway initiated by trauma to blood vessels, which introduces tissue factor (TF) into the blood stream thus activating factor VIII (FVIII) into active FVIII (FVIIIa) which will eventually bind to activated platelet surfaces, this is known as the extrinsic Xase.

*Figure 1. Color coded schematic of the human blood coagulation cascade with labeled blood plasma factors.*
complex. However, this initial means of coagulation cannot support large scale coagulation, as more blood is lost, the intrinsic pathway is enabled leading to vastly increased clotting activity and stability. The intrinsic pathway is activated by contact activators that subsequently activate factor XII (FXII) resulting in the initiation of this pathway.

Siting at the intersection of these two pathways lies coagulation FIX and its cofactor FVIII that together form the highly active intrinsic Xase complex. The presence of these proteins within the center of the cascade highlights just how central of a role they play within blood coagulation. The intrinsic Xase complex is made up of a FVIIIa protein bound to either an endothelial cell or activated platelet, coordinated with the FIXa protein, however the exact mechanism of this coordination is still yet to be discovered.

The absence or disfunction of even one blood coagulation factor produces hemostasis that is severely compromised resulting in the bleeding disorder known as hemophilia. There exist multiple types of hemophilia that are categorized according to which protein within the cascade is defective, an ineffective FVIII protein will result in hemophilia type A whereas, ineffective FIX results in hemophilia type B. Hemophilia A has been found to be more common, with an incidence of 1 in 5000 male births, whereas hemophilia B is far less common with incidences of 1 in 30,000 male births. Current treatments for hemophilia B, include recombinant FIX protein replacement as well as a new adeno-associated viral (AAV)-FIX gene replacement therapy that comes with a $3.5 million dollar price tag inside the United States. However, there are major obstacles for patients with both treatments; the replacement therapy can be extremely disruptive.
to a patient’s normal life, with the possibly of having to receive multiple treatments every week, and due to the cost of the gene therapy it is inaccessible to most patients.

FIX is a mid-sized human protein at approximately 57,000-Da in size and possess five domains: consisting of a N-terminal Gla domain, followed by epidermal growth factor domains 1 and 2 (EGF 1 and 2), an activation peptide and finally a C-terminal protease domain \(^2\) (Figure 2a). Due to the powerful enzymatic activity of these coagulation proteins, they are initially produced in the body in their inactive form, known as a zymogen, but are converted into their active protease forms when blood vessel injury or damage occurs \(^2\) (Figure 2B).

Activation of FIX occurs though double proteolytic cleavage of the activation peptide spanning between Arg\(^{145}\) and Arg\(^{180}\). This cleavage of the activation peptide produces an approximately 14,000-Da light chain consisting of the calcium binding Gla, EGF1 and EGF2 domains, and an approximately 26,000-Da heavy chain containing the protease domain \(^3\). Additionally, these two domains are connected though a

Figure 2. (A) Amino acid sequence schematic of inactive factor IX, with labeled domains and disulfide bonds, from Kristensen et al., 2016. (B) Crude protein representation of a zymogen type protein requiring activation to become functional.
single disulfide-bond 2. The FIX protein, even when activated by FXIa, does not possess any inherently activity without the coordination of its cofactor FVIII, resulting in FIXa being unable in initiating the coagulation cascade alone 9. However, when formed, the Xase complex produces a 200,000-fold increase in FX activation to FXa by FIXa 1,2.

Figure 3. Protein schematic of activated and inhibited factor IX_2 from PDB: 2WPI. Heavy chain is denoted by red, light chain by tan, the green ion is Ca^{2+} and the PPACK inhibitor is designated by the light blue sticks.

Figure 4. Proposed protein schematic of activated factor IX coordinating with factor VIII to form the Xase complex. Factor VIII is denoted by cyan and purple colors and factor IX by tan.

Utilizing recent work by collaborators, this paper will attempt to provide an optimized procedure for purification, activation and crystallization of the FIX double mutant known as FIXa_2. FIX_2 is a mutant of human FIX protein (hFIX), produced at the University of Salzburg, containing two mutations, K98T and Y177T, that increase activity of the protein by ~150,000-fold when compared to active hFIX (hFIXa) 7. The structural and functional characteristics of this mutation could prove influential in gaining understanding of the assembly and activity of FIX within the Xase complex. Characterizations of FIX_2 could also be used to inform the possible
development of new, improved, cost effective therapeutics for those living with hemophilia B. Additionally, optimization of these procedures would allow the Spiegel lab to produce and characterize novel FIX mutants more reliably.

**PART I: Purification, Activation and Crystallization**

*Expression and Purification of FIX_2*

*Methods*

Prior to experimentation, the FIX_2 protein amino acid sequence was loaded into the software ProtParam to calculate theoretical protein parameters. Initial work up of the FIX_2 protein begins with a cell stock of BL21 *Escherichia coli* cells containing the pET22b+ FIX_2 mutant (K98T-Y177T) plasmid obtained by Dr. Kenneth Childers, PhD from researchers at the University of Salzburg. This frozen cell stock produced by Dr. Childers is utilized in 2XYT broth overnight (O/N) cultures containing ampicillin. The 50-milliliter (mL) O/N cultures are then used to inoculate a 1-liter large-scale bacterial growth using 2XYT broth and ampicillin. Cells are then grown at 37 degrees Celsius (°C) with 210 rotations-per-minute shaking until the cultures reached an optical density of 0.8, when protein expression is induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG). Following induction with IPTG, the culture temperature is decreased to 15°C and left shaking to produce protein O/N. Following expression, an approximate 9-gram cell pellet is harvested though centrifugation at 4°C, 4,000 x g for 15 minutes. The solubilized inclusion bodies (IBs), containing the desired protein, are then harvested from the whole cell lysate using lysis buffer [100 mM tris (pH 7.2), 20 mM EDTA, 500 mM NaCl, 2% Triton X-100, 1 mg/mL lysozyme, 1 mM PMSF], six 30-second pulses of sonication with a Branson 450 analog sonifier, and centrifugation at 4°C, 40,000 x g for 45
minutes. Afterward, the supernatant is wasted, the pellet is collected, and subsequently washed. The IBs pellet is washed with wash buffer [100 mM tris (pH 7.2), 20 mM EDTA, 500 mM NaCl, 2% Triton X-100] and spun down at 4°C, 20,000 x g for 20 minutes three times. Purification of the inclusion bodies is the next step to test by two methods.

The IBs are initially purified with the use of protein re-solubilization and precipitation (R/P) as detailed in the Zögg paper. This method uses the characteristics of FIX_2 protein to separate other contaminant proteins with the use of differing solutions. Initially, the IBs are left to solubilize in a solution of 6 M guanidine HCl, 100 mM tris (pH 8.5), 20 mM EDTA, 100 mM β-ME, at room temperature (RT) O/N. When fully solubilized, the IB solution is centrifuged at 4°C, 40,000 x g for 20 minutes to remove any non-soluble contaminates. The pure solubilized solution is then transferred into a 100x dialysis solution [20 mM EDTA (pH 3.5), 100 mM NaCl] to create precipitation of the FIX_2 protein from the solution O/N at 4°C. The precipitate is collected through centrifugation pelleting at 4°C, 40,000 x g for 30 minutes. This protein pellet is then re-solubilized using the earlier re-solubilization solution without β-ME [6 M guanidine HCl, 100 mM tris (pH 8.5), 20 mM EDTA]. These steps are repeated for a total of three rounds of R/P. Protein identity is then analyzed and confirmed using a 12.5% acrylamide, discontinuous SDS-PAGE analysis.

Additionally, we recalled a 6x His-Tag in the sequence of FIX_2’s and attempted an immobilized metal affinity chromatography (IMAC) purification with batch bound HisPur™ Ni-NTA resin. The 6-histidine residue stretch theoretically possesses a negative charge, allowing the FIX_2 protein to bind to the immobilized positively charged Ni^{2+} ions in the resin. To test the ability of Ni^{2+} resin to purify FIX_2 from IBs, two denaturant solutions, guanidine HCl (GHCl) and urea, are utilized to keep FIX_2 in solution as it is run through the column. For this
procedure, we start with frozen pre-prepared IB, which can be thawed with warm water because the proteins are already denatured. The IB pellets are solubilized in their respective denaturant solution of GHCl [6 M guanidine HCl, 20 mM tris (pH 8.0), 300 mM NaCl] and urea [8 M urea, 20 mM tris (pH 8.0), 300 mM NaCl]. These filtered samples are then batch bound with Ni$^{2+}$ resin at RT for 30 minutes, transferred to their respective gravity column, washed [6 M guanidine HCl/8 M urea, 20 mM tris (pH 8.0), 300 mM NaCl, 15 mM imidazole] and gradient eluted [6 M guanidine HCl/8 M urea, 20 mM tris (pH 8.0), 300 mM NaCl, 500 mM imidazole] with their respective buffers. Purification fractions are then analyzed by a 12.5% acrylamide, discontinuous SDS-PAGE to determine purity. Following gel analysis, the samples are flash frozen with liquid N$_2$ and placed in the -80°C freezer for later analysis.

Following purification through R/P, the soluble FIX_2 is folded through drop-wise dilution into refolding buffer [100 mM tris-HCl (pH 8.5), 100 mM NaCl, 20 mM CaCl$_2$, 3 mM cysteine, 0.3 mM cystine and 500 mM L-arginine] to produce a final dilution of 1:100 protein solution to buffer. At this point, the solution containing folded FIX_2 is concentrated to 5 mL for further purification through size exclusion chromatography (SEC) on an AKTA Prime Plus HPLC equipped with a Cytiva HiLoad Superdex 75 pg prepacked column. Next, buffer-exchange the purer folded FIX_2 protein to a storage buffer [20 mM tris-HCl, 100 mM NaCl, 10 mM CaCl$_2$] that is then eluted from the column as confirmed through SDS-PAGE analysis on a 12.5% acrylamide discontinuous gel. Collect each of the multiple peaks as different fractions for ease in sample identification. Following gel analysis, the folded FIX_2 fraction is concentrated, flash frozen with liquid N$_2$ then stored in a -80°C freezer for later experimentation.

Finally, the pure folded FIX_2 sample produced through R/P purification is characterized using mass spectroscopy. Samples are prepared by diluting concentrated sample to ~1 mg/mL in
a final volume of 50 µL with their storage buffer solution. The FIX_2 sample is then run with the Agilent 1290 UHPLC with AdvanceBio 6545XT Q-TOF instrument operated by Nathan Avery. In addition, mass spectroscopy was attempted, but non-conclusive as detailed below, with the FIX_2 sample from the IMAC purifications utilizing the same procedure as listed above.

Results

The purification through re-solubilization and precipitation results in the production of folded FIX_2 proteins (Figure 5A). Sample protein from the R/P procedure was determined to be ~40 kDa in molecular weight when analyzed by SDS-PAGE (Figure 5B) and this value agrees with the mass spectroscopy determined value of 43, 687 g/mol (Figure 5C) which is hardly a deviation from the ProtParam calculated mass of 43,688.91 g/mol (Table 1).
<table>
<thead>
<tr>
<th>FIX_2 Protein Segment</th>
<th>Molecular Weight (g/mol)</th>
<th>Extinction Coefficient (M$^{-1}$ cm$^{-1}$)</th>
<th>Theoretical pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactive Full-Length Protein</td>
<td>43,688.91</td>
<td>55110</td>
<td>5.81</td>
</tr>
<tr>
<td>Active Full-Length Protein w/ Inhibitor</td>
<td>40,041.09</td>
<td>53620</td>
<td>6.48</td>
</tr>
<tr>
<td>Heavy Chain</td>
<td>26,067.69</td>
<td>41285</td>
<td>6.81</td>
</tr>
<tr>
<td>Light Chain</td>
<td>13,688.06</td>
<td>12210</td>
<td>5.94</td>
</tr>
</tbody>
</table>

*Table 1. ProtParam calculated molecular weight, extinction coefficient, and theoretical pI values for FIX_2 protein based on amino acid sequence.*

However, the purification using Ni$^{2+}$ resin was unsuccessful in binding and isolating FIX_2 as seen by absence of migrating proteins at the correct MW, of approximately 43 kDa, in the SDS-PAGE analysis(Figures 6A/B). Gels for the Ni$^{2+}$ resin showed FIX_2 eluting off the column in the flow through solution (FT).

*Conclusions*

Purification of FIX_2 is most successful in producing protein when the re-solubilization and precipitation procedure is utilized compared to the IMAC purification. Folded FIX_2 can be reasonably purified by R/P followed by SEC polishing, yet the IMAC purification of the same protein is completely ineffective at producing any pure FIX_2 protein. The lack of FIX_2 His-Tag binding to the Ni$^{2+}$ resin could possibly be explained by native proteins in the IB binding Ni$^{2+}$ preferentially. This would explain why FIX_2 was eluted in the flow through for each of the IMAC attempts, however, more experimentation would be required to determine the cause of little to no binding.
Activation of FIX_2 Protein with FXIa

Methods

Following the folding and concentration of FIX_2 protein, the protein must be activated through proteolytic cleavage of the activation peptide by FXIa. The protein is activated at 25°C while shaking at 150 RMP for 4 hours in the presence of FXIa, at a ratio of 20 µg of FIX_2 to 40 ng of FXIa. Two separate activation reactions are carried out, one utilized a volume of 10 mL with a FIX_2 concentration of 1 mg/mL (HVLC-A) and the other reaction contained 5 mL at a concentration of 6 mg/mL (LVHC-A). Immediately after the activation of each FIX_2 reaction the samples are flash frozen with liquid N₂ and stored in the -80°C freezer for future inhibition. Finally, prior to use, the active FIX_2 protein (FIXa_2) was thawed on ice and the serine protease activity is inhibited O/N at 37°C, 1000 RPM, using 5 mM of the small molecule known as PPACK, to avoid sample loss due to self-cleavage.
Results

Based on the SDS-PAGE analyses of each activation reaction both conditions were able to produce the desired FIXa_2 protein displaying a heavy chain domain at approximately 26 kDa and a light chain domain at approximately 14 kDa, which approximately agrees with the calculated mass based on amino acid sequences (Figure 7B/C). The intensity of protein bands within the LVHC-A sample is increased compared to the protein bands of the HVLC-A sample (Table 1).

Figure 7. (A) Chromatogram of purification for activated and inhibited FIX_2 with a HiLoad Superdex 75 SEC column, unfolded protein elutes at ~12 mL and activated FIXa_2 protein elutes at ~14 mL. (B) 17.5% acrylamide, discontinuous SDS-PAGE analysis of high volume-low concentration activation’s (HVLC-A) SEC purification of FIXa_2; protein mass maintains at ~40 kDa insinuating product produced was folded FIX_2 rather than FIXa_2. (C) 17.5% acrylamide, discontinuous SDS-PAGE analysis of low volume-high concentration activation’s (LVHC-A) SEC purification of FIXa_2; protein mass separates into ~26 and ~14 kDa fragments representing the known FIXa heavy and light chain respectively.
Conclusions

When attempting to produce increased levels of FIXa_2 protein, it appears most advantageous to use a highly concentrated, small volume sample. The highly concentrated sample showed greatly increased levels of active protein compared to its low concentration counterpart, suggesting this procedure is more effective and accurate in active protein production.

Crystallization of FIXa_2 Protein

Methods

Once pure, activated FIX_2 protein was produced and concentrated to 3.990 mg/mL it was utilized for protein crystallization. We began by screening conditions around those noted in the Zögg paper for crystallization of FIX_2, 100 mM MES (pH 7.1) and 24% of PEG 6,000. Following crystallization of protein within the drops, the crystals are removed using a microscopic nylon loop and immediately flash frozen with liquid nitrogen but no cryo-protecting solution. These frozen crystal samples are then shipped to a synchrotron in the Lawrence Berkley National Laboratory at the University of California, Berkley, which utilizes a partial accelerator to produce high energy X-ray beams. Each crystal sample is placed on a rotating mount, known as a goniometer, to allow for analysis at many angles. Crystal samples were shot with a high energy X-ray beam and diffraction data was collected by Dr. Childers, operating the instrumentation remotely.
Results
Successful crystallization resulted after 6 days on a plate with 1 µL of reservoir, 100 mM MES (pH 6.5-8.0) and 20-30% PEG 6,000, in 2 µL of concentrated FIX_2 protein solution plated and left to sit at RT. After five days small crystals appeared and they were left to keep crystalizing, but after seven days of growth seven larger crystals were recovered, looped, and shipped to UC Berkeley. During testing of the samples, the protein loops started to form ice crystals, partially obscuring some protein crystals. Despite the large amount of crystal samples tested, not one produced a diffraction pattern.

Figure 8. (A) Protein crystallization drop containing a long, skinny crystal from conditions, 100 mM MES (pH 7.4) and 24% of PEG 6,000. (B) Light microscope photograph of protein crystal within a nylon loop, ice crystallization is present as the darker areas. (C) X-ray light scattering data collected for a FIX_2 crystal with no diffraction present.
Conclusions

Initially utilizing the published crystallization conditions from Zögg allowed for decreased crystal production time since there was no need to screen a wide range of reservoir conditions. Additionally, the tested conditions must be very close to those optimal for crystallization due to the presence of crystal formation. However, more work is required to produce a protein crystal that will diffract. Although not ideal, receiving no diffraction data for the crystals suggests that the crystal is truly protein rather than salt, since salt crystals are known to produce high levels of diffraction regardless of their size. Unlike salt, for a protein crystal to diffract light it must be fairly large, with the longest portion being a minimum length of 0.1 mm, and highly ordered. Therefore, if the FIX_2 crystals are enhanced in size and latus structure diffraction could be achievable. The increasing of crystal size and ordering can be achieved through slowed, gradual formation. There are many different means in which a researcher can delay crystallization, and these will be presented in the next section describing future research directions.

Summary

When using BL21 E. coli cells, the FIX_2 protein will be expressed in IBs within the cells, that must be purified. Following expression, we found that these IBs are most effectively purified using 3 rounds of the R/P procedure. Furthermore, activation of the FIX_2 protein with FXIa should be carried out in high concentration, low volume samples to avoid precipitation of protein within solution and obtain the highest desired protein yield. Successive to activation, the flash freezing of samples resulted in lower levels of precipitate protein when the samples were eventually thawed and inhibited prior to use. Finally, these procedures were effective in
producing pure protein for crystallization. When crystallization of FIX_2 protein occurred, the resulting crystals were unable to produce diffraction data for structural characterization; however, the lack of any diffraction data suggests that the tested crystals were not salt. This informs researchers that current FIX_2 crystallization conditions need only to be optimized to produce diffracting crystals. Success with future crystallization of FIX_2 would prove useful not only in understanding of that protein but also in directing initial research and procedures for any novel FIX proteins.

**Part II: Future Research Directions**

Optimization of FIx_2 Crystallization Conditions

Continuing into the future, experiments will be focused towards producing larger and more ordered crystals that will diffract when analyzed with X-ray crystallography. To achieve this, we will work to slow the rate of crystal formation within the drop through manipulations in reservoir conditions (pH, polyethylene glycol (PEG) percentage), plate temperature, drop ratio, presence of oil covering the reservoir, and crystal seeding. When producing new reservoir solutions, we will perform a fine screen around one pH unit above the pI of 6.48 for FIX_2 protein. Within the reservoir, we can also examine old PEG solution compared to new PEG solutions to determine if there is an effect of PEG ageing on crystal formation. PEG has been shown to degrade overtime with access to light, warm temperatures, and oxygen, which could cause issues with reproducibility of protein crystals due to unintended condition deviations. This experiment will allow us to determine if there is a deviation between PEG solutions and increases our confidence in reproduceable results. Temperature during plating and crystal
formation can have great effects on enhancing or preventing crystallization of proteins too, therefore we will test at multiple temperature below and above our current standard of room temperature (25°C) to determine the optimal temperature\textsuperscript{14}. We will also work to edit drop ratios and increase drop volume which intern increases protein crystal size\textsuperscript{14}. Crystal formation can also be slowed by the presences of an oil layer on the top of the reservoir which partially inhibits its vapor diffusion with the drop. Finally, we could attempt to seed a new crystallization plate with small protein crystals from previously successful conditions to increase the probability that large, orderly crystals are produced\textsuperscript{14}. By placing pre-formed crystals into protein drops, more crystallization is initiated due to the old crystal acting as a scaffold for any new formations.

\textit{Characterization of New FIX Mutants}

Though collaborative work with researchers at Emory University School of Medicine, we have identified two different FIX mutants, FIX-403 and ET9, that display increased activity when compared to the current human wild type FIX (hFIX)\textsuperscript{11}. One of the mutants was discovered though analysis of an ancestorial FIX construct known as FIX-An96, which contains 42 mutations when compared to hFIX. FIX-An96 is the reconstructed early mammalian FIX, believed to have arisen before the Cretaceous - Paleogene boundary, and is shown to have
activity increase 11-fold that of hFIX \(^{11}\). However, when further studied by our collaborators at Emory University, FIX-An96 was determined to only possess 5 mutations, out of the total 42, of consequence for the previously described increased activity of the protein \(^{11}\). These influential five non-human amino acids mutations consist of Val132Ala, Glu323Lys, Asp338Asn, Lys362Arg, and Leu367Ser (Figure 9).

Another commonly known FIX mutant with increased activity was found within a family of patients residing in Padua, Italy, this mutation in a single amino acid result in thrombophilia, increased blood clotting throughout the body \(^{11}\). A single mutation of Arg384Leu (Figure 9) within the protein results in an increase in activity of FIX-Padua by 7-8-fold compared to hFIX \(^{11}\). In addition, the Padua variation has also been found to produce increased immune tolerance in mouse and dog models \(^{16}\). However, the researchers at Emory University created a FIX mutant containing both the five active FIX-An96 mutations (FIX-403) and the one FIX-Padua mutation deemed An96-Padua (Figure 9) \(^{11,15}\). This An96-Padua FIX protein was found to have an almost 60-fold higher activity level, possibly earning it title of most active FIX mutant to date \(^{11}\).
Based on the findings from collaborators, the Spiegel lab has ordered plasmids to be produced containing these specific mutations, FIX-403 or An96-Padua, for the us to attempt further protein analysis. Following crystal diffraction and characterization of the FIX_2 mutant the Spiegel lab will attempt to work up these recently ordered FIX mutants for characterization using previously successful procedures outlined earlier in part one. The Spiegel lab will be focused on obtaining pure FIX-403 and An96-Padua protein to use in crystallization allowing for an X-ray crystallography analysis of protein structure. Though analysis of these protein structures we can work to further determine how these amino acid mutations interact to produce the vast increase in activity seen with both FIX-403 an An96-Pauda 11.
Acknowledgements

None of this work would have been possible without the aid, expertise and encouragement of Dr. P. Clint Spiegel and Dr. Kenneth Childers. The author wishes to thank Dr. Spiegel initially for the opportunity to research in his lab, which proved to be one of the best experiences in their entire undergraduate schooling. The author also wishes to thank Dr. Spiegel and Dr. Childers for their help in planning and execution of the research as well as providing access to the materials and lab spaces for this project. Furthermore, it should be recognized that Dr. Childers served as a co-advisor for the entirety of the project and was beyond influential in the author’s progression as a scientist. Due to all the help presented to the author by Dr. Childers, they would like to sincerely thank him for all he has done, including being a friend when lab got difficult.

The author would also like to extend their deepest gratitude toward Jordan Vaughan, the master’s student who initially trained them in the Spiegel lab. He proved instrumental in the author’s development as not only a scientific researcher but also as a critical thinker. Jordan also helped grow the author’s love for biochemistry despite the harsh reality of research setbacks and poor results. Additionally, the author would like to thank Nathan Avery for his lovely presence in the lab as well as his invaluable help with experimentation and the author’s understanding of lab results.
Resources


8 Freato, N.; Ebberink, E. H. T. M.; van Galen, J.; Fribourg, C.; Boon-Spijker, M.; van Alphen, F. P. J.; Meijer, A. B.; van den Biggelaar, M.; Mertens, K. Factor VIII-driven changes in


