A novel factor Xa inhibitor (PifXa) is an effective, reversible inhibitor of blood coagulation

Andersen, Mette Sondrup

Publication date:
2011

Document Version
Early version, also known as pre-print

PhD Thesis

A novel factor Xa inhibitor (PifXa) is an effective, reversible inhibitor of blood coagulation

Mette Sondrup Andersen

2011

Preface

This thesis is submitted to the Doctoral School of Biotechnology at Aalborg University. The PhD study was carried out from December 1st 2007 to July 31st 2011, at the Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Denmark, under the supervision of Associated Professor Kåre Lehmann Nielsen. The PhD project was cofounded by IfXa A/S Novi Innovation and Aalborg University.

In this thesis, chapters 1, 2 and 3 gives an introduction to the coagulation system, factor Xa, the Kunitz protease inhibitor from potato tubers, respectively. Chapter 4 describes the specific aims of the PhD thesis. Chapter 5 describes the materials and methods used, and chapter 6 presents the results obtained during the study. The thesis finishes of with a discussion and conclusion, and further perspectives in chapter 7 and 8, respectively. Appendix 1 and 2 contains an illustration of the vectors used in the experiments. Appendix 3 contains an alignment of the sequences used to create the phylogenetic three seen in the results. Appendix 4 describes the used *E. coli* expression strain, and finally appendix 5 contains the article written on the basis of these results and the first page of the patent. A CD is enclosed containing the full patent.

First and foremost I would like to give thanks to my supervisor Associated Professor Kåre Lehmann Nielsen for his skilled and excellent guidance throughout this PhD study, for always finding time when a discussion was needed, and for his positive outlook on things. I also thank chief physician Søren Risom Kristensen for helping me with the experiments performed at Aalborg Hospital, Doctor of veterinarian medicine Aage Kristian Olsen Alstrup for his work with the *in vivo* experiments, Associated Professor Allan Steensballe for his help with the mass spectroscopy part of the experiment, and Associated Professor Michael Toft Overgaard for helping me with the binding kinetics experiments and data analysis. I would also like to thank the rest of the functional genomics group for creating a good, fun, and inspiring working environment.

Last but not least I would like to give my appreciation to my boyfriend René and to my family. *"You never pushed my too hard just enough to make me fly"*

Mette Sondrup Andersen

Aalborg, July 2011

Contents

Summary **5**
Resumé **7**
Abbreviations **9**
1 The Coagulation System **12**
1.1 Cell-based model of coagulation 15
1.2 Platelets 18
1.3 Vitamin K 20
1.4 Naturally occurring anticoagulants 21
1.4.1 Antithrombin 22
1.4.2 Tissue Factor Pathway Inhibitor (TFPI) 24
1.4.3 Protein C 26
1.5 Thrombotic disorders 26
1.5.1 Arterial Thrombosis 26
1.5.2 Venous Thrombosis 27
1.6 Anticoagulant treatment 28
1.6.1 Antiplatelet agents 28
1.6.2 Anticoagulant Drugs 31
1.7 Cardio Pulmonary Bypass 37
2 Factor Xa – Structure and Mechanism **39**
3 Kunitz Protease Inhibitor A-k1 from Potato tubers **42**
3.1 Preliminary work 42
3.2 Primary structures of KPI A-k1 42
4 Aim **46**
5 Materials and Methods **47**
5.1 Materials 47
5.1.1 Chemicals 47
5.1.2 Equipment's 48
5.2 Methods 48
5.2.1 Purification of PifXa 48
5.2.2 In vitro Characterization of PifXa 50
5.2.3 *In vivo* Characterization of PifXa 54

5.2.4 Attempt to determine the half-life of PifXa *in vivo* 55
5.2.5 Directed Evolution and Recombinant Protein Expression....58
6 Results 63
6.1 Bioinformatics study of PifXa....63
6.2 PifXa can be purified from potato juice 63
6.2.1 Purification by ion exchange chromatography 65
6.2.2 Final purification by gel filtration....69
6.2.3 Validation of purity by MALDI TOF MS 71
6.3 *In vitro* characterization of PifXa....72
6.3.1 Determination of the kinetic constants of PifXa....72
6.3.2 PifXa Inhibition of selected coagulation proteins....75
6.3.3 The effect of PifXa can be reversed by a polyclonal antibody 77
6.3.4 PifXa does not inhibit fX activation 78
6.3.5 PifXa prolongs whole blood coagulation time....79
6.3.6 PifXa does not affect platelet aggregation *in vitro* 80
6.3.7 PifXa effect standard coagulation tests....81
6.4 PifXa is a potent specific anticoagulant *in vivo* 82
6.4.1 PifXa prolongs bleeding time but not bleeding amount 82
6.4.2 The effect of PifXa is additive to the effect of Aspirin....83
6.5 Attempts to determine the half-life of PifXa *in vivo*....84
6.5.1 Western Blot 85
6.5.2 iELISA 85
6.5.3 Iodine 131 labeled PifXa....86
6.6 Expression and purification of mutant variants of PifXa in *E. coli* 87
6.6.1 Insertion of fXa's recognition site in PifXa....89
6.6.2 Mutation in the putative reactive center loop....91
6.6.3 Random mutagenesis for phanning experiments 97
7 Discussion....99
7.1 *In vitro* characterization of PifXa....99
7.2 PifXa prolongs bleeding time in rats....101
7.3 Attempt to determine the half-life of PifXa *in vivo*....103
7.4 Directed Evolution 105
8 Conclusions and further perspectives 109

9 References..... 123

Summary

The blood coagulation cascade is primarily regulated by a series of specific proteases that activates each other in an amplification cascade that finally leads to the cleavage of fibrinogen converting it to fibrin which polymerizes and forms a stable blood clot. The ability of the coagulation to stop bleeding from wounds is obvious important. However, equally important are the naturally occurring protease inhibitors, like antithrombin and tissue factor pathway inhibitor, which limit the coagulation to the needed location and preventing the activated coagulation factors to spread to the surrounding areas. The formation of untimely blood clots in the circulatory system is one of the most common causes of death in the developed world today. The risk of untimely clot formation is increased in certain diseases, at increasing age, and at major surgical procedures. As a consequence high dose of anticoagulants are administered during surgeries. Heparin has been the drug of choice in a number of years despite the fact that it has multiple targets not only in the coagulation system, and that not all patients are treatable with heparin. However, it is still the only anticoagulant to which there is an antidote which is a major advantage in many surgeries.

Previous experiments have shown that the juice from the potato tube has the ability to prolong plasma coagulation *in vitro*. Later it was discovered that the effect came from a Kunitz protease inhibitor of the variant KPI A-k1. In this thesis the specificity of the inhibitor, named PifXa, was determined using different coagulation enzymes, and it was shown that PifXa was capable of inhibiting fXa, plasmin, and aPC, while it had no effect on either thrombin or t-PA. The inhibitor constant for the inhibition of fXa was determine to be ~6 nM and the binding constant was found to be ~170 nM. Despite the fact that PifXa both inhibited coagulation and anti-coagulation enzymes, the net effect of the inhibition was indeed anticoagulant. This was clearly seen in the *in vivo* results where PifXa was capable of significantly prolonging the tail bleeding time in rats. PifXa was shown to be highly specific to the coagulation cascade, and no interference with platelet plug formation could be detected *in vitro*. In fact, the effect of PifXa was additive to that of the anti-platelet drug acetylsalicylic acid and combined exceeded the *in vivo* effect of both heparin and the fXa inhibitor fondaparinux.

In contrast to existing fXa inhibitors, the effect of PifXa can be fully reversed by addition of a specific antibody as demonstrated *in vitro*. The specificity of the inhibitor to inhibit coagulation combined with the possibility to reverse the effect makes PifXa a candidate drug during cardio-pulmonary bypass where a large dose of anticoagulant is necessary, and reversal of the effect by administration of an antidote at the end of the surgical procedure is desired.

Although PifXa was effective in prolonging the bleeding time in rats, it is possible that an even more effective variant can be produced. Five different mutant variants, both intended to increase the affinity towards fXa but also pinpoint the RCL, was successfully expressed in Rosetta Gami B (DE3) *E. coli*, and the inhibitory effect towards fXa have been tested and compared both to the potato wild type and to a bacterial recombinant form of the wild type. None of the mutants were more effective than the wild type in inhibiting fXa. However, the results indicated that the amino acids in the putative RCL had to be without any charge in order for the interaction between fXa and PifXa to be most favorable.

Resumé

Blodkoaguleringskaskaden bliver primært regulerer af en række specifikke proteaser, der aktiverer hinanden i en amplifikationskaskade, der i sidste ende leder frem til kløvingen af fibrinogen, hvilket omdanner den til fibrin. Fibrin polymeriserer spontant og danne en stabil blodprop. Evnen til at standse blødningen er af åbenlyse årsager meget vigtig, men lige så vigtige er de naturlige forekommende antikoagulanter som f.eks. antithrombin og tissue factor pathway inhibitor, der begrænser koaguleringen til det beskadige område og forhindre aktiverede koagulationsfaktorer i at sprede sig til de omkringliggende væv. Dannelsen af uhensigtsmæssige blodpropper i blodbanen er en af de mest almindelige dødsårsager i den vestlige verden, og risikoen for dette øges f.eks. med alder, ved bestemte sygdommer og under store operationer. Som en konsekvens heraf gives der store doser af antikoagulerende medicin under operationer. Heparin har i en lang årrække været den fortrukne medicin, på trods af at den påvirker en række af forskellige proteiner, ikke kun i koagulationssystemet. Derudover er det ikke alle patienter, der kan behandles med heparin. Grunden til at heparin stadig er at fortrække, er at den er den eneste antikoagulant med en modgift, hvilket er en kæmpe fordel i en lang række situationer.

Tidligere forsøg har vist, at juice fra kartoffelknolde er i stand til at forlænge koaguleringen af plasma *in vitro*. Senere blev det opdaget, at effekten kom fra en Kunitz protease inhibitor af varianten KPI A-k1. I denne afhandling blev specificiteten af inhibitoren, navngivet PifXa, undersøgt. Inhibitor aktiviteten mod en række forskellige koagulationsenzymer blev testet, og det blev vist at PifXa var i stand til at inhiberer aktiviteten af fXa, plasmin og aPC, men at den ikke havde nogen effekt på hverken thrombin eller t-PA. Inhiberingskonstanten for inhiberingen af fXa blev bestemt til at være ~6 nM og bindingskonstanten blev fundet til at være 170 nM. Selvom PifXa både inhiberede koagulerings- og antikoaguleringsenzymer var det samlede effekt antikoagulant. Dette blev tydligt set i *in vivo* forsøgene, hvor PifXa var i stand til at øge haleblødningstiden i rotter signifikant. PifXa havde kun effekt på plasma koaguleringen, mens ingen indvirkning på blodpladeaggregeringen kunne detekteres. Faktisk var effekten af PifXa additiv med effekten af anti-blodplade medicinet acetylsalisylsyre, og en kombinationen af disse to *in vivo* gave en effekt, der var sammenlignelig med effekten af både heparin og fXa inhibitoren fondaparinux.

I forhold til eksisterende fXa inhibitorer kan effekten af PifXa i systemet ophæves fuldstændigt ved at tilsætte et antistof specifikt for PifXa, dette blev vist *in vitro*. Kombinationen af specificiteten af inhibitoren samt det faktum at effekten kan ophæves, gør at PifXa som medicin kan blive en kandidat til at blive benyttet i store hjerteoperationer, hvor der benyttes bypass. I disse operationer er store doser af antikoagulerende medicin nødvendt, og muligheden for at ophæve effekten tilslut i operationen ønskeligt.

Selvom PifXa effektivt forlængede blødningstiden i rotterne, er det sandsynligt, at det er muligt, at fremstille en endnu mere specifik variant. Fem forskellige mutantvarianter blev succesfult udtrykt i Rosetta Gami B (DE3) *E. coli*. Disse varianter havde til formål enten at øge affiniteten mod fXa eller at forsøge at lokaliserer det formodede RCL. Den inhiberende effekt mod fXa blev testet og sammenlignet med effekten for både vildtypen oprenset fra kartoffelknoldende og en vildtype udtrykt i *E. coli*. Ingen af mutanterne var mere effektive i inhiberingen af fXa end vildtyperne, men resultaterne indikerede, at de aminosyrer der udgør RCL, skal være uden ladning for at interaktionen mellem fXa og PifXa skal være optimal.

Abbreviations

ADAMTS	A Disintegrin And Metalloproteinase with Thrombospondin Motifs
ADP	Adenosine diphosphate
AMS	Ammonium sulphate
aPC	Activated protein C
aPTT	Activated partial thromboplastin time
ASA	Acetylsalicylic acid
AT	Antithrombin
BPTI	Bovine pancreatic trypsin inhibitor
COX-1	Cyclooxygenase 1
CPB	Cardio pulmonary bypass
CPI	Carboxypeptidase
CV	Column volume
CYP	Cytochrome P450
Da	Dalton
dNTP	Deoxyribonucleotide triphosphate
DVT	Deep vein thrombosis
ER	Endoplasmic reticulum
EtOH	Ethanol
FDA	Food and Drug administration
FT	Flow through
fXa	Factor Xa
Gla	γ-carboxyglutamic acid
Glu	Glutamic acid
GP-Ib/V/IX	Glycoprotein Ib/V/IX
HIT	Heparin induced thrombocytopenia
HMWK	High molecular weight kiniogen
iELISA	Indirect enzyme-linked immunosorbent assay
IgG	Immunoglobulin G
IMAC	Ion metal affinity purification chromatography
INR	International normalized ratio

ISI	International sensitivity index
IV	Intravenously
KPI	Kunitz protease inhibitor
LMWH	Low molecular weight heparin
MALDI	Matrix assisted laser desorption/ionization
MeOH	Methanol
MS	Mass spectrometry
MWCO	Molecular weight cut off
nt	Nucleotides
OEPCR	Overlap extension polymerase chain reaction
PAR-1	Protease-activated receptor
PBS	Phosphate buffered saline
PDI	Protein disulfide isomerase
PE	Pulmonary embolism
PF4	Platelet factor 4
P-gp	P-glycoprotein
PI	Protease inhibitor
PifXa	Protein Inhibitor of Factor Xa
PK	Pre-kallikrein
PL	Phospholipid
PPP	Platelet poor plasma
PRP	Platelet rich plasma
PT	Prothrombin time
PVDF	Polyvinylidene Fluoride
RCL	Reactive center loop
RFLP	Restriction Fragment Length Polymorphism
RT	Room temperature
RVV	Russell Viper Venom
SA	Sinapinic acid
SD	Sprague Dawley
SEC	Serpin-enzyme complex
STI	Soybean trypsin inhibitor

TF	Tissue factor
TFA	Triflouroacetic acid
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
TOF	Time of flight
TxA_2	Thromboxane A_2
UFH	Unfractionated heparin
VKA	Vitamin K antagonists
VKOR	Vitamin K epoxide reductase
VTE	Venous thromboembolism
vWF	Von willebrand factor

1 The Coagulation System

Blood is the fluid component of the cardiovascular system which also includes the heart, that pumps the blood though the entire body. The main function of blood is to transport oxygen from the lungs to the different tissues and remove carbon dioxide from these tissues and transport it back to the lungs. Blood also carries nutrients and hormones to target cells, and carries waste from the tissues to the kidneys for excretion. The fluent state of the blood is important for easy transport of cells, nutrients, and hormones. However, it is also important to stop the flow if there is a leak in the system. Haemostasis, cessation of bleeding, is a complex process that must be quick, local and carefully controlled in order to be effective in maintaining blood homeostasis. Three different mechanisms are used to reduce blood loss i) vascular spasm, ii) platelet plug formation, and iii) blood coagulation (Martini, Garrison et al. 2004).

Initially it was thought that exposure of blood to air was the initiator of blood coagulation, and it was not until the mid-19th century that it was discovered that the clotting ability of blood involved several different components. The early models described the conversion of fibrinogen to fibrin by thrombin, but other factors of the blood coagulation were not added to the models before the 1940s and 1950s. Newly identified coagulation factors were either named after their discoverer or after the first patient described with the deficiency. However, this gave rise to a lot of confusion and an international committee was established in 1954. This committee gave the different factors the roman numerals that are known today (Giangrande 2003).

In 1964 two independent groups proposed the cascade model that defined a series of enzymatic steps leading to the generation of a fibrin clot (Figure 1.1) (Davie, Ratnoff 1964), (Macfarlane 1964).

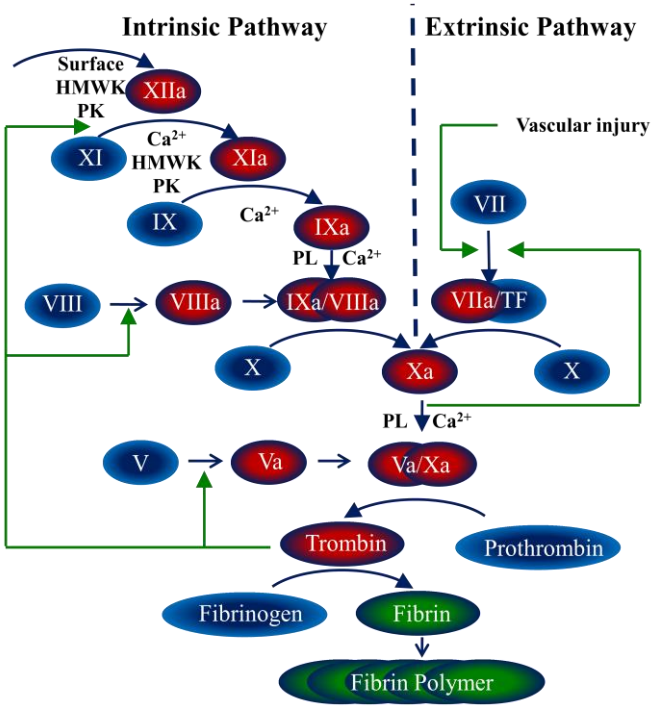


Figure 1.1: Blood coagulation cascade. The activated proteases are shown in red, zymogens in blue, and fibrin and fibrin polymer in green. PL denotes phospholipid, PK is pre-kallikrein, and HMWK is high molecular weight kiniogen. See text for further explanation.

The cascade model branches into two pathways, meaning that coagulation could either be initiated by the intrinsic or the extrinsic pathway. The initiation of either pathway, lead to the activation of factor Xa (fXa) in the final common pathway.

The intrinsic pathway was so named because all the components used are present in the blood. The pathway was thought to be initiated when the zymogen fXII comes into contact with a negatively charged surface such as glass, or the membrane of an activated platelet, which then activates fXII. High molecular weight kiniogen (HMWK) helps to anchor fXIIa to the charged surface, and serves as a cofactor of fXII. Once a small amount of fXIIa is formed it converts pre-kallikrein to kallikrein, which in turn accelerates the conversion of the zymogen fXII to fXIIa in a positive feedback. FXIIa cleaves fXI forming fXIa, which in return activates fIX. FIXa together with Ca^{2+} and fVIIIa on negatively charged phospholipids forms the tenase complex. The tenase complex activates fX, which is the first molecule of the common pathway (Riddel, Aouizerat et al. 2007). During clinical analysis the intrinsic and common pathway is evaluated using the activated partial thromboplastin time (aPTT). The aPTT is performed by adding a surface activator (e.g. kaolin or celite) and phospholipids to citrated plas-

ma. The reagent is called partial thromboplastin because tissue factor (TF) is absent. The plasma mixture is allowed to incubate in order to insure activation of the proteins in the pathway. Coagulation is initiated by addition of calcium and the clotting time is measured. The aPTT for a normal patient is typically in the range between 22-40 s (Bates, Weitz 2005).

In the extrinsic pathway the subendothelial cell membrane protein TF is needed to initiate coagulation. When the endothelial is damage fVIIa comes into contact with TF, and the two forms a complex which in turn activates fX (Hoffman 2003). The extrinsic pathway is monitored clinically using the prothrombin time (PT). Two variants of PT exist, Quick and Qwren. The Quick test depends entirely on the proteins present in the plasma, whereas the Owren test adds components of bovine plasma to compensate for variability in the content of fibrinogen and fV (Jackson, Esnouf 2005). Generally, a thromboplastin reagent containing TF and calcium is added to the citrated plasma and the coagulation time is measured. The PT can vary with reagents and among laboratories but is normally within 10-14 s. The variation is overcome by the international normalized ratio (INR). This ratio uses the international sensitivity index (ISI) that is supplied by the manufacturer of the reagent, the patients PT, and a mean normal PT calculated from 20 healthy subjects in the laboratory. PT can be calculated to INR with the following formula: INR = (patient PT/mean normal PT)^{ISI} (Bates, Weitz 2005, Kamal, Tefferi et al. 2007).

The common pathway begins with fXa, which have been activated in the intrinsic, extrinsic, or both pathways. FXa forms the prothrombinase complex together with fVa, Ca^{2+}, and phospholipids which converts prothrombin into its active form thrombin. The main action of thrombin is to convert fibrinogen to fibrin monomers. These monomers spontaneously polymerize to form a gel of fibrin polymers that trap the blood cells (Hoffman 2003).

Even though the cascade model has been useful in explaining the *in vitro* plasma coagulation it has become obvious that it could not adequately explain what is seen *in vivo*. For example, patients with a deficiency in the initial components, fXII, HMWK or pre-kallikrein (PK), in the intrinsic pathway have prolonged aPTT but no clinical bleeding tendency. However, the components of the intrinsic pathway had to play an important role in haemostasis since patients deficient in fVIII or fIX have severe bleeding tendency, although the extrinsic pathway is intact. Similarly, patients with a deficiency in fVII also have seriously bleeding tendency

although the intrinsic pathway is intact. It is therefore obvious that the two pathways does not operate as independently as first thought (Hoffman 2003).

1.1 Cell-based model of coagulation

The new understanding of the blood coagulation incorporates the role of cells, is believed to occur in three overlapping phases; initiation, amplification, and propagation, and takes place on two different cell types, namely TF-bearing cells and platelets (Figure 1.2).

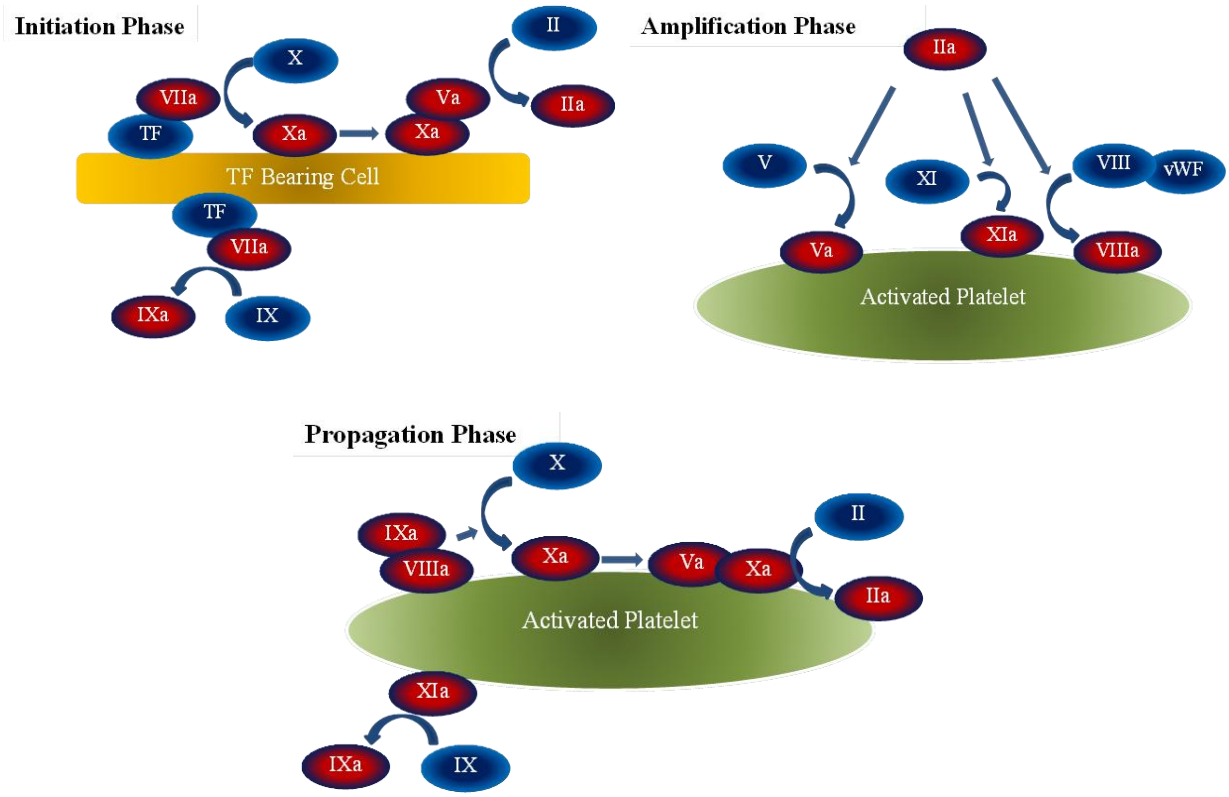


Figure 1.2: Cell –based model of coagulation. Coagulation occurs in three overlapping phases on two different cell types, TF bearing cells and activated platelets. The first phase is the initiation, in which small amounts of thrombin (fIIa) is being formed. In the amplification phase platelets are activated along with fV, fXI and fVIII. In the propagation phase the tenase and prothrombinase complexes are being formed resulting in generation of large amounts of thrombin.

The initiation phase takes place on cells that express TF. These cells can be fibroblasts or smooth muscle cells, and are normally located outside the vasculature. This prevents initiation of coagulation under normal circumstances. There is increasing evidence that TF also is present on micro particles located in the blood stream. However, this TF is present in a latent form that lacks coagulant activity, and may be activated by protein disulfide isomerase (PDI)

released by activated platelets (reviewed by (Furie, Furie 2008)). When the TF-bearing cells come in contact with the blood stream, fVIIa rapidly binds to TF and forms a complex that activates small amounts of fX and fIX. FXa then associates with fVa; this complex activates small amounts of thrombin (fIIa) from its precursor prothrombin (fII). The fVa in the complex can come from different sources: i) it can come from the granules of partially activated platelets, ii) fV can be activated by fXa (Monkovic, Tracy 1990), or iii) it can be activated by other non-coagulation proteases (Tracy 1995). FXa located on the cell surface is relatively protected from inactivation by antithrombin (AT) or tissue factor pathway inhibitor (TFPI) due to the size of the inhibitor (Toschi, Lettino 2011). However, if fXa dissociates from the cell it is rapidly inhibited. FIXa, in contrast to fXa, can move from the cell surface and onto a nearby platelet since it is not inhibited by TFPI and only slowly by AT (Monroe, Hoffman 2006). Importantly, since TF is always present in the perivasculature, any fVIIa that that escapes through minor breaks in the endothelial wall will bind to TF and potential initiate coagulation. However, the coagulation proceed to the amplification phase, only when damage to the vasculature allows the larger compounds of the coagulation process (platelets and fVIII/von willebrand factor (vWF)) to come in contact with the TF-bearing cells (Smith 2009), (Hoffman 2003).

The small amount of thrombin generated on the TF bearing cells in the initiation phase is insufficient to produce a hemostatic plug by converting fibrinogen to fibrin but it has several other different functions. One major function is the activation of platelets. Even though platelets have already adhered to the injured area, they are only partially active. The activation by thrombin results in a change in the shape of the platelet, and as a result the platelet will expose negatively charged phospholipids on the surface, which forms binding sites for activated coagulation factors (Veldman, Hoffman et al. 2003). The activated platelets release partially activated fV from their α-granules which are fully activated by thrombin or fXa. Another function of the thrombin generated in the initiation phase is to activate factors V and XI on the platelet surface, and to cleave vWF from fVIII, which circulate bound together, releasing it to mediate platelet adhesion and aggregation (Monroe, Hoffman 2006).

The propagation phase occurs on the large number of activated platelets there are at the site of injury. First, fIXa activated during the initiation phase forms the tenase complex with fVIIIa bound to the surface, additional fIX can be activated by fXIa also bound to the surface of the

platelet. Second, since fXa cannot move from the TF bearing cell it has to be activated directly on the platelet surface, this is facilitated by the tenase complex. The newly activated fX quickly associates with fVa forming the prothrombinase complex. This complex activates large amounts of thrombin, which then cleaves fibrinogen to fibrin, which spontaneously polymerizes and forms a fibrin mech. Thrombin also activates fXIII that forms crosslinks between the fibrin strands and improves the strength of the clot (Vine 2009), (Veldman, Hoffman et al. 2003). The high levels of thrombin generated during the propagation phase binds to fibrin, where it is protected from inhibition by antithrombin (AT). Untimely disruption of the clot brings fibrinogen into contact with the bound thrombin, and fibrin formation can be initiated immediately without the need of thrombin generation (Toschi, Lettino 2011).

Table 1.1 gives an overview of the different factors in the blood coagulation systems, their molecular weight, the plasma concentration, and the concentrations required for normal haemostasis (Tollefsen 2010).

Table 1.1: Concentrations of coagulation factors need for normal haemostasis (Tollefsen 2010)**.**

Factor	Molecular Weight [Da]	Plasma Concentration (µg/mL)	Required for Haemostasis (% of normal concentration)
Fibrinogen	330,000	300	30
Prothrombin	72,00	100	40
Factor V	300,000	10	10-15
Factor VII	50,000	0.5	5-10
Factor VIII	300,000	0.1	10-40
Factor IX	56,000	5	10-40
Factor X	56,000	10	10-15
Factor XI	160,000	5	20-30
Factor XIII	320,000	30	1-5

1.2 Platelets

Platelets are small subcellular disc shaped fragments that are formed from the cytoplasm of megakaryocytes. Once released, platelets circulated the blood stream for about 7 days (Hartwig, Italiano 2003). When an injury occurs in the vessel wall the circulating platelets are recruited to the site of injury, where they become a major part of the developing thrombus (Figure 1.3).

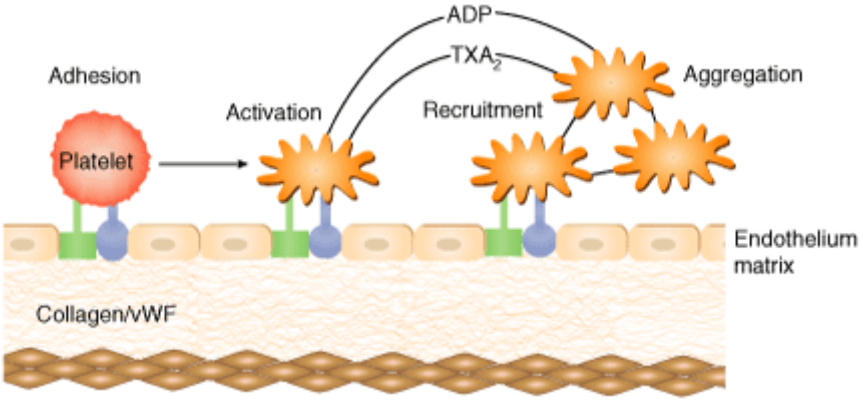


Figure 1.3: Platelet adhesion, activation, and aggregation. Platelets adhere to exposed collagen or vWF at the site of injury. After they adhere they become activated, change their shape and release adenosine diphosphate (ADP) and thromboxane A_2 (TxA_2), which in turn recruit and activate other platelets. Integrin $\alpha_{IIb}\beta_3$, which is exposed during activation, has high affinity towards fibrinogen which bridges platelets together (Gross, Weitz 2009).

When the vessel wall or endothelium is disrupted, collagen and TF comes into contact with the flowing blood. Collagen triggers the accumulation and activation of platelets, whereas TF, as mentioned in the previous section, initiates thrombin generation which both converts fibrin into fibrinogen and activates platelets. Collagen and thrombin acts as two independent pathways for platelet activation, and dependent on the injury one of these pathways may be dominating, but the net result is the same (Furie, Furie 2008). Adhesion of the platelets to the site of injury is a result of direct interactions between the collagen of the exposed vessel wall and the receptors; glycoprotein VI (GP-VI) and integrin $\alpha_2\beta_1$, or indirect interactions between collagen bound vWF and the receptors glycoprotein Ib/V/IX (GP-Ib/V/IX) and integrin $\alpha_{IIb}\beta_3$. Adhesion of the platelet is followed by morphological changes with rearrangement of the membrane and exposure of negatively charged phospholipids. The activation of platelets by thrombin is independent of disruption of vessel wall, vWF and GP-IV (reviewed by (Brass

2003) and (Furie, Furie 2008)). The thrombin generated by the presence of TF binds to and cleaves protease-activated receptor 1 (PAR-1) on the platelet surface resulting in activation of the platelet. Thrombin also binds to the GP-Ib/V/IX complex, where the binding site has been located to be on GP-Ib, which also has a vWF binding site (De Candia, De Cristofaro et al. 1999). Activation of the platelet causes it to release adenosine diphosphate (ADP) and thromboxane A_2 (TxA_2) form the platelet granules. ADP binds to the $P2Y_{12}$ receptor on the platelet surface, which causes a change in shape, mobilization of calcium, and initiate aggregation. The antagonists also recruits and activates other platelets in the area, and affect the contraction of the smooth muscle cells in the vessel wall. Further development of the growing platelet thrombus require activation of integrin $\alpha_{IIb}\beta_3$ by protein disulfide isomerase (PDI), which in turns promote formation of stable platelet-platelet contacts by increasing the affinity of the integrin for its ligands fibrinogen and vWF (reviewed by (Brass 2003) and (Furie, Furie 2008)).

Platelets have a unique ability to form stable adhesions in all types of blood flow, ranging from low flow in the large veins to the high flow conditions found in the arteries. Not only do they have to be able to form adhesive bonds during rapid flow, but they must also sustain them in order to prevent the thrombus from detachment (embolization) from the site of injury. Dependent on the blood flow there are three mechanisms of platelet aggregation. In low shear rates (< 1000 s^{-1}) aggregation is mediated by fibrinogen and integrin $\alpha_{IIb}\beta_3$. At medium shear rates (1000 - 10,000 s^{-1}) the platelet –platelet interaction becomes more reliable on vWF but also GP-Ib and integrin $\alpha_{IIb}\beta_3$ has an important role. In high shear rates (> 10,000 s^{-1}) the aggregation mechanism is exclusively mediated by vWF-GPIb bonds (reviewed by (Jackson 2007).

VWF plays a crucial role in the formation of a platelet thrombus, and its main functions are, as already mentioned, to promote platelet-platelet and platelet-collagen interactions. VWF is a multimeric protein which consists of identical subunits. Disulfide-bonds binds the subunits together to form dimers of approximately 500 kDa, the dimers are linked together into multimers of various sizes that may exceed 10,000 kDa. VWF is synthesized by endothelial cells and is stored here until use. Another storage site for vWF is in the granules within the platelets, which insures that the protein is present where it is needed. VWF is stored as ultra-large vWF which is cleaved to the desired length by the metalloproteinase ADAMTS-13 (A Disintegrin and Metalloproteinase with Thrombospondin Motifs) (reviewed by (Reninger 2008)).

1.3 Vitamin K

In 1943 the Nobel Prize in Physiology or Medicine went to the Dane Henrik Dam and the American Edward Doisy for their characterization of vitamin K. The vitamin was named K because a lack of it caused a defect in the blood *koagulation* (Nordic spelling) (Sadler 2004). Today it is known that some coagulation proteins (fVII, fIX, fX, and prothrombin) and regulatory proteins (protein Z, protein S, and Protein C) require a conversion of some of their glutamic acid (Glu) residues to γ-carboxyglutamic acid (Gla) to function properly. This modification is carried out by the vitamin K dependent enzyme γ-glutamyl-carboxylase. γ-glutamyl-carboxylase requires three cofactors to function; reduced vitamin K, CO_2, and O_2. In its naturally occurring state vitamin K is in its quinone oxidation state, and must be reduced to the hydroquinone state to function as a cofactor. The enzyme responsible for this conversion is vitamin K epoxide reductase (VKOR). During the carboxylation of the Glu residue the vitamin K hydroquinone state is converted to the vitamin K epoxide state, and before it can be reused it has to be converted back to the reduced form by VKOR (Figure 1.4). For each Glu residues carboxylated a molecule of vitamin K epoxide is formed (Furie, Bouchard et al. 1999),(Stafford 2005). Another enzyme DT-diaphrase can reduce the quinone form of vitamin K, but not vitamin K epoxide. Furthermore, this enzyme requires high concentrations of vitamin K, and it is not believed to play a role in vitamin K recycling at physiological concentrations (Furie, Bouchard et al. 1999).

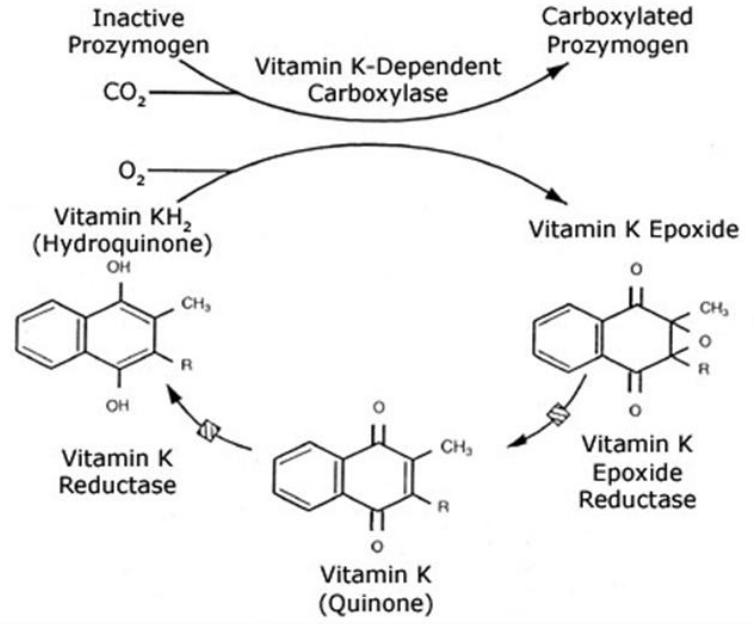


Figure 1.4: The vitamin K cycle. Vitamin K-dependent carboxylase uses the hydroquinone form of vitamin K together with CO_2 and O_2 to convert glutamic acid (Glu) residues into γ-carboxylglutamic acid (Gla). This reaction results in the formation of vitamin K epoxide that is converted back into the hydroquinone form by the enzyme vitamin K epoxide reductase (VKOR) (modified from (Sadler 2004)).

All vitamin K dependent coagulation enzymes contain 10-12 Gla-residues located within the first 40 amino acids in the N-terminal of the mature protein. When the proteins come into contact with calcium ions they undergo structural changes leading to exposure of a phospholipid binding site located in the N-terminal (Furie, Bouchard et al. 1999).

1.4 Naturally occurring anticoagulants

As important as the coagulation of the blood is, just as important is the control of the coagulation. The intact endothelium has many anticoagulant functions to keep the blood fluent. Many of the coagulation inhibitors like TFPI and AT are produced by, and expressed on, the surface of endothelial cells. These cells also secret the polysaccharide heparan sulfate which catalyzes the activity of AT, and express thrombomodulin (TM) which binds to thrombin (Tanaka, Key et al. 2009).

1.4.1 Antithrombin

The serine protease inhibitor antithrombin (AT) is a 58 kDa glycoprotein belonging to the serpin (serine proteinase inhibitor) family, which inhibits a number of proteases in the coagulation system including thrombin, fXa, fIXa, fXIa, and fXIIa. However, thrombin and fXa is regarded as the main targets. AT circulates in the blood at a concentration of 125 µg/mL (2.3 µM) (Pike, Buckle et al. 2005).The inhibitor consists of three β-sheets (A-C) and nine α-helices (A-I). The reactive Arg393 and Ser394 bond is located on an exposed loop at the surface of the molecule known as the reactive center loop (RCL) (Rezaie 2005). The inactivation of the target enzyme by AT is initiated by the enzyme recognizing the reactive bond of the inhibitor. The P1 residue (Arg-393) is essential for this recognition. Once the proteinase is bound, its catalytic serine attacks the P1 Arg bond in the RCL in AT, resulting in the formation of an acyl-intermediate complex, in which the RCL has been cleaved but remains covalently bound to the reactive serine through the P1 residue. The cleavage triggers a conformational change in AT, in which the cleaved RCL inserts into the center of the 5-stranded β-sheet (the A sheet), causing the bound proteinase to be relocated to the opposite end of the inhibitor and inactivated through conformational changes. The serpin-enzyme complex (SEC) is removed from circulation by a receptor known as the SEC receptor, which is present in the liver, hence AT acts as a suicide inhibitor. The SEC receptor is a member of the low-density lipoprotein (LDL) receptor family, all of which are responsible of binding a range of ligands and many are associated with proteases (Gettins 2002, Strickland 1997).

AT is alone an inefficient inhibitor but the activity is, as mentioned, stimulated by the binding of heparin released from mast cells, or heparin-like molecules (heparan sulphate) present on the surface of endothelial cells (Dahlbäck 2000). Heparin activates AT due to an overall conformational change induced by the binding of AT to the unique core pentasaccharide present in heparin. The heparin induced change alters the conformation of the RCL of AT and gives a 300 fold increase in inhibitory activity against fXa (Jin, Abrahams et al. 1997). The full length heparins may additionally help the inhibition by AT by serving as bridging cofactors by binding to both AT and the enzyme, and promoting their interaction (Figure 1.5). Allosteric activation by the heparin pentasaccharide is enough to inhibit fXa. However, in order to acceleration the rate of inhibition of thrombin a heparin length of more than 18 saccharides is required (Weitz, Weitz 2009).

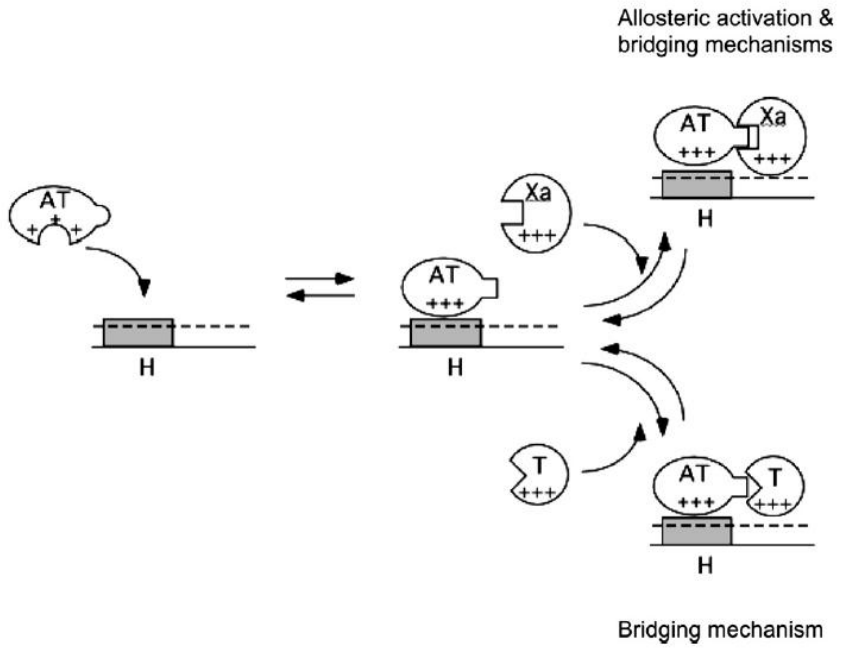


Figure 1.5: Mechanism of heparin activation of antithrombin. The scheme shows the inhibition of fXa and thrombin by AT in two different ways. In both mechanisms a positively changed site on AT binds to a specific negatively charged pentasaccharide (shaded) on the heparin molecule (H). This binding induces a conformational change in the heparin binding site that is allosterically transmitted to the RCL making binding to fXa more favorable. The rate of thrombin (T) inactivation is primarily enhanced by the binding of both thrombin and AT to the same heparin chain so that the interaction between the two is facilitated through a complex bridging (Olson, Richard et al. 2010).

The heparin binding site on AT is comprised of three distinct regions. The N-terminal region, the N-terminal end of helix A, and all of helix D together with its N-terminal loop. Comparison between free and heparin bound AT reveals conformational changes both in the heparin binding site, and the region surrounding the RCL of the inhibitor induced by the binding of heparin (Figure 1.6). In the heparin binding site, the N-terminal of helix A undergoes reorientations to widen the binding site and position the basic residues for binding with the heparin pentasaccharide (cyan stick representation). Additionally, the C-terminal of the helix D extends and a new P helix is formed in the loop preceding this helix (blue). In the RCL region, the RCL, initially buried through is N-terminal hinge in the A sheet, is expelled from the sheet and extended away from the inhibitor (yellow). The expulsion is driven by a closing of a gap in sheet A that in turn is linked together with the extension of helix D (reviewed by (Olson, Richard et al. 2010)).

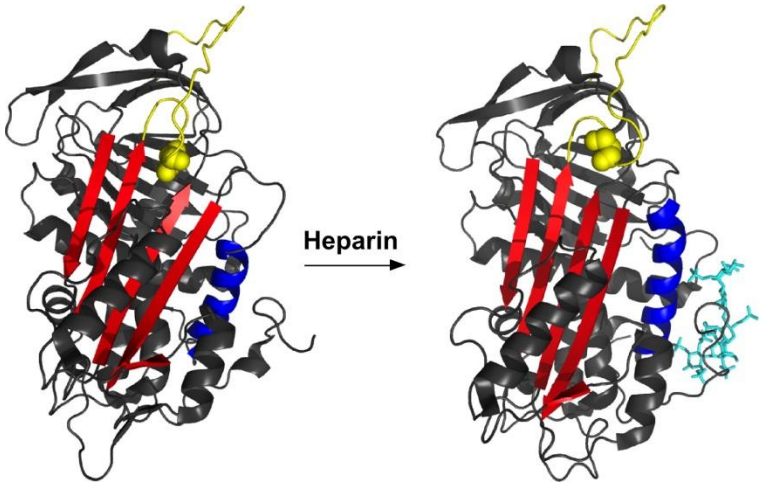


Figure 1.6: Conformational changes of antithrombin upon binding to heparin. A ribbon representation of free (left) and heparin (cyan stick) bound (right) AT. Helix D is extended and a new P helix (blue) is formed in the heparin binding region. The RCL (yellow) is expelled from the inhibitor driven by a closing of a gap in the A sheet (red) (Olson, Richard et al. 2010).

Both clot bound thrombin and fXa are protected from inhibition by AT, hence the role of AT is preventing the active coagulation enzymes to spread to other areas, rather than shutting the clotting down (Pike, Buckle et al. 2005).

1.4.2 Tissue Factor Pathway Inhibitor

Tissue factor pathway inhibitor (TFPI) is a mammalian Kunitz-type inhibitor with three Kunitz-type domains, and it is capable of rapidly inhibiting fXa independent of a cofactor. Besides the three Kunitz-domains TFPI has an acidic N-terminal and a basic C-terminal, the latter is required for the binding of TFPI to cell surfaces (Figure 1.7). TFPI is produced by microvascular endothelial cells and is found three places in the system. The majority of TFPI is bound to the vascular endothelium, approximately 10 % circulates as a complex with lipoproteins in the plasma, and a smaller portion is present in the platelets. Stored TPFI is released from the endothelial cells by the action of heparin and by platelet activation (Price, Thompson et al. 2004), (Lwaleed, Bass 2006).

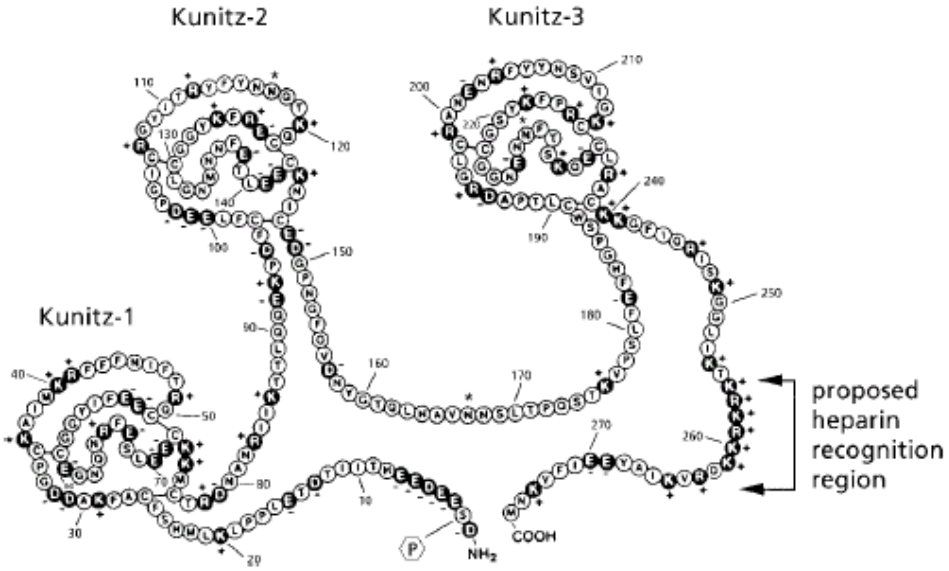


Figure 1.7: The primary amino acid sequence of TFPI. The Kunitz-type domains, the positively and negatively charged amino acid residues are shown. The arrows points to the heparin binding site found near the C-terminal (Lwaleed, Bass 2006)**.**

TFPI inhibits TF-initiated coagulation by binding to the fVIIa/TF complex in an fXa dependent manner. In the first step of inhibition the second Kunitz domain (K2) binds to the active groove of fXa. The second stage in the inhibition by TFPI involves binding of the fXa/TFPI complex to the fVIIa/TF-complex. This step requires binding of the P1 residue (Lys36) of the first Kunitz domain (K1) to the active site of fVIIa. This step is, unlike the first step, Ca^{2+} dependent and prevents additional activation of fX (Crawley, Lane 2008). The inhibition by TFPI results in the formation of the TFPI/fXa/TF/fVIIa complex on the plasma membrane, which has no catalytic activity. The inactivation of the fVIIa/TF complex by TPFI does not occur in the absence of fXa, indicating that coagulation must be initiated before TFPI can function (Price, Thompson et al. 2004). Hackeng and co-works showed in 2006, that protein S, classically known as a cofactor for activated protein C (aPC), also acted as a cofactor for TFPI increasing the affinity towards fXa. Their data suggested that the interaction between protein S and TFPI were mediated through the third Kunitz domain (K3) and the C-terminal (Hackeng, Seré et al. 2006). Similar to AT, fXa bound in the prothrombinase complex is protected from inhibition by TFPI.

1.4.3 Protein C

The protein C system regulates coagulation by changing the activity of the two cofactors fVIIIa and fVa. When thrombin binds to the membrane protein thrombomodulin (TM), which is bound to non-activated resting endothelial cells, it forms a complex that convert thrombin from a procoagulant to an anticoagulant protein. The thrombin-TM complex in turn activates the protein C (aPC). The anticoagulant effect of aPC is supported by the cofactor protein S. Protein S and aPC forms a membrane-bound complex, which can cleave fVIIIa and fVa, when they are bound to the phospholipid membrane, even when they are a part of the tenase or prothrombinase complex, respectively. APC does not cleave fVIII since it is bound together with vWF and cannot interact with the membrane. However, since both fV and fVa binds phospholipids equally well, aPC is able to cleave both forms. The consequence of aPC mediated cleavage of fV is a formation of an anticoagulant fVi that functions in synergy with protein S as a cofactor for aPC in the degradation of fVIIIa (Dahlbäck 2000).

1.5 Thrombotic disorders

A disrupt in balance between the procoagulant and anticoagulant molecules can lead to thrombosis. Thrombosis may result from physiological changes (e.g. ageing), hereditary causes, acquired diseases (e.g. cancer), and drugs (e.g. oral contraceptive) (Tanaka, Key et al. 2009). Both venous and arterial thrombosis incidences increase with age, which in part can be explained by athermanous diseases, various surgical procedures, and decreased mobility.

1.5.1 Arterial Thrombosis

The primary trigger for arterial thrombosis is the rupture of an atherosclerotic plaque, which is caused by accumulation of lipid on the artery wall (Figure 1.8.A). When the rupture occur platelets are quickly recruited to the site, and a thrombi rich in platelets are formed. TF is also present in the plaque, and this initiate the plasma coagulation (Mackman 2008). Acute arterial thrombosis is the cause of most cases of myocardial infraction, commonly known as a heart attack, where the blood supply to the heart is interrupted, causing the heart cells to die, resulting in damage or death of the heart tissue (Tortora, Derrickson 2009). Myocardial infraction and stroke are the major causes of mortality globally causing about 13 million deaths (WHO

2008). Antiplatelet drugs (described in section 1.6.1) are used prophylactic to reduce the incidences of arterial thrombosis.

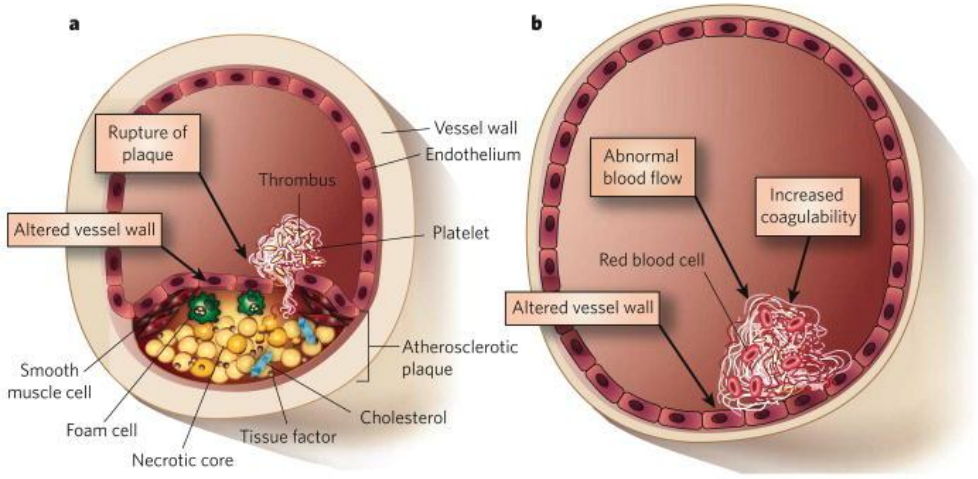


Figure 1.8: Arterial and venous thrombosis. (**A**) The primary trigger of arterial thrombosis is the rupture of an atherosclerotic plaque, which releases the plaque and TF into the blood stream. Arterial thrombi are rich in platelets. (**B**) Thrombi formed in the veins are rich in fibrin and can be caused, among other things, by abnormal blood flow (Mackman 2008).

1.5.2 Venous Thrombosis

Deep vein thrombosis (DVT) and pulmonary embolism (PE) are collectively referred to as venous thromboembolism (VTE). DVT normally occurs in the large veins of the legs. PE is a complication of DVT, where a part of the thrombus breaks away, travel to the lung, and gets trapped in a pulmonary artery, resulting in the disruption of the blood flow. The thrombi that form in the veins are rich in fibrin, and traps red blood cells (Figure 1.8.B) (Mackman 2008). The risk of DVT can be increased by both genetic and environmental factors. Acquired risk factors include cancer, obesity, and major surgery (Mackman 2008). Whereas an example on a genetic risk factor is a single nucleotide polymorphism in the factor V gene (commonly Arg506→Gln) named factor V Leiden. This mutation results in slower aPC-mediated inactivation of fVa than normal, since Arg506 is one of the three cleavage sites of aPC in fVa. The Leiden V mutation is common in northern Europeans (heterozygous 5-10 %), and increase the risk for venous thrombosis three to eight fold in heterozygotes, and up to 80-fold in homozygotes (Tanaka, Key et al. 2009). On the other hand women with fV Leiden have a reduced

bleeding tendency after given birth, which may account for the high prevalence of the mutation, due to higher survival rates among these women during evolution (Dahlbäck 2000).

1.6 Anticoagulant treatment

In order to prevent and treat thrombotic diseases, there are a large number of different anticoagulants available on the market. In broad they can be either antiplatelet drugs or anticoagulant drugs. In the last couple of year's new oral anticoagulant have been developed which, in the future, will have the possibility to replace some of the old less effective drugs. However to date, the most widely available and used drugs are unfractionated heparin (UFH), low molecular weight heparin (LMWH), Fondaparinux, and vitamin K antagonists like warfarin (Mannucci, Franchini 2011).

1.6.1 Antiplatelet agents

Antiplatelet drugs are used for both the prevention and the acute treatment of arterial thrombosis. There are a number of different drugs available that target the activation or aggregation of the platelets (Figure 1.9). The main targets are thromboxane, PAR-1, the ADP receptor, and the $\alpha_{IIb}\beta_3$ integrin.

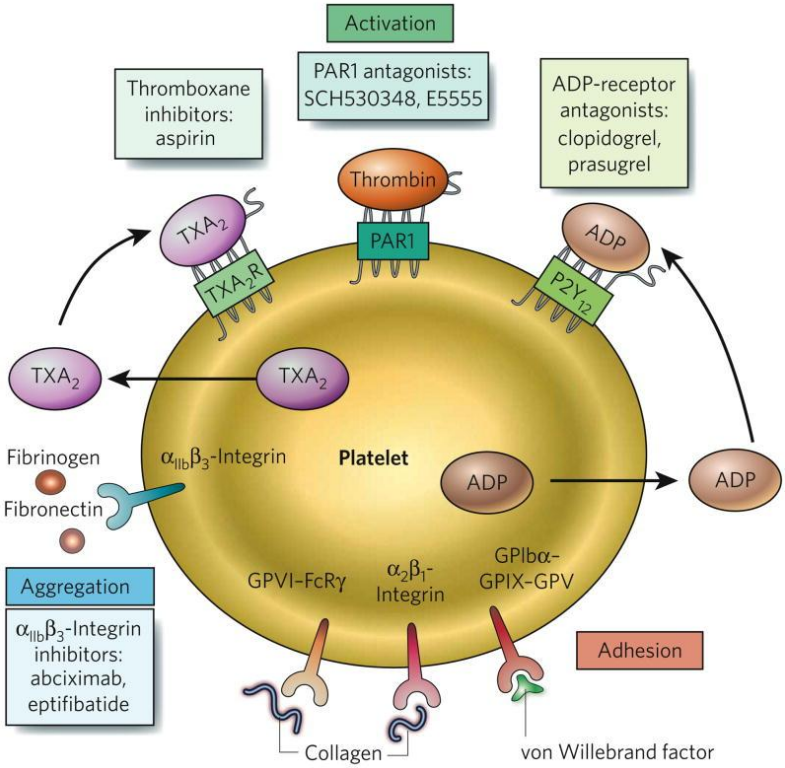


Figure 1.9: Targets of antiplatelet drugs. Platelets have a number of cell surface receptors that mediate their activation (green boxes), their adhesion to the vessel wall (red), and their aggregation with each other (blue). Antiplatelet drugs and their target are also shown (Mackman 2008)**.**

1.6.1.1 Thromboxane inhibitors - Aspirin

Acetylsalicylic acid (ASA) better known as aspirin is the most widely used drug in the world, and has been established as a first in line antithrombotic treatment for a wide range of cardio-vascular diseases. ASA irreversibly inhibits platelet prostaglandin G/H synthase 1 also known as cyclooxygenase 1 (COX-1), by selectively acetylating the hydroxyl groups of one serine residue (Ser530) located 70 amino acids from the C-terminal of the enzyme. This acetylation places a bulky substituent on the serine recidue, hereby inhibiting the binding of the substrate arachidonic acid (Vane, Botting 2003). TxA_2 is under normal conditions produced from arachidonic acid through enzymatic conversions by COX-1 and thromboxane synthase, hence the inactivation of COX-1 by aspirin results in decreased platelet activation and aggregation promoted by TxA_2 (Paikin, Eikelboom et al. 2010).

1.6.1.2 ADP-receptor antagonists

Thienopyridines are a family of drugs that irreversibly bind to the $P2Y_{12}$ receptor on the platelets, thereby preventing binding of ADP, and consequently inhibiting platelet activation and aggregation. The oral drug Clopidogrel (Plavix, Bristol-Meyers Squibb or Sanofi-Aventis) is one type of ADP-receptor antagonists, and is given as a prodrug. After ingestion, the majority (85 %) of the molecules are metabolized to form an inactive carboxylic-acid derivate. The remainder of the prodrug molecules is converted to the active form by a two-step process in the liver by the hepatic enzymes of the cytochrome P450 (CYP) system (predominantly CYP3A4). The active compound is an unstable, reactive, thiol derivate with a very short half-life. The active metabolite forms disulfide bridges between the cysteine residues on the $P2Y_{12}$ receptor, thereby irreversibly modifying it, preventing the binding of ADP. Since the $P2Y_{12}$ receptor inhibition is irreversible, the drug has a permanent effect on the platelet aggregation that lasts the life time of the platelet (5-10 days) (Paikin, Eikelboom et al. 2010, Raju, Eikelboom et al. 2008).

Another oral administered irreversible inhibitor of $P2Y_{12}$ is Prasugrel (Effient, Eli Lilly and company). Unlike clopidogrel, which, as mentioned, undergoes a two-step CYP450-dependent conversion to the active metabolite, prasugrel only requires a single-step activation. Prasugrel is a more potent inhibitor of ADP-induced platelet aggregation, and achieves a more consistent and faster inhibition (Paikin, Eikelboom et al. 2010, Raju, Eikelboom et al. 2008).

1.6.1.3 PAR-1 antagonists

PAR-1 inhibitors block the binding of thrombin to PAR-1, hereby inhibiting thrombin induced activation and aggregation. Two PAR-1 inhibitors, E-5555 and SCH530348 are currently being evaluated in clinical trials for the prevention of arterial thrombosis. The oral E-5555 is currently in phase two trials, and has demonstrated antiplatelet effects without increasing bleeding times. The safety and tolerability are under investigation. SCH530348 is an oral high affinity, low molecular weight, non-peptide, that competitive inhibits PAR-1. The molecule inhibits the thrombin induced activation and aggregation, without affecting the coagulation cascade and bleeding time (Angiolillo, Ueno et al. 2010).

1.6.1.4 $\alpha_{IIb}\beta_3$-Integrin inhibitors

The aggregation of platelets is a crucial step in growth of a thrombus. Inhibitors of platelet $\alpha_{IIb}\beta_3$-integrin are designed to reduce this aggregation by inhibiting the binding of fibrinogen to the activated platelets (Mackman 2008). There are currently three inhibitors available. Eptifibatide (Integrilin, Millennium Pharmaceuticals), which is a cyclic heptapeptide with a molecular weight of about 800 Da. It is highly specific for the $\alpha_{IIb}\beta_3$-integrin. Abciximab (ReoPro, Eli Lilly) is a monoclonal antibody Fab fragment with a tight receptor binding. Finally, Tirofiban (AGGRASTAT, Medicure Pharma), which is a small non-peptide with a rapid, selective, and reversible blockade of the integrin (Zeymer, Wienbergen 2007).

1.6.2 Anticoagulant Drugs

There are a number of different classes of anticoagulant drugs that reduce the activity of the proteases by either directly or indirectly inhibiting them, or by inhibiting their posttranslational modifications (Figure 1.10).

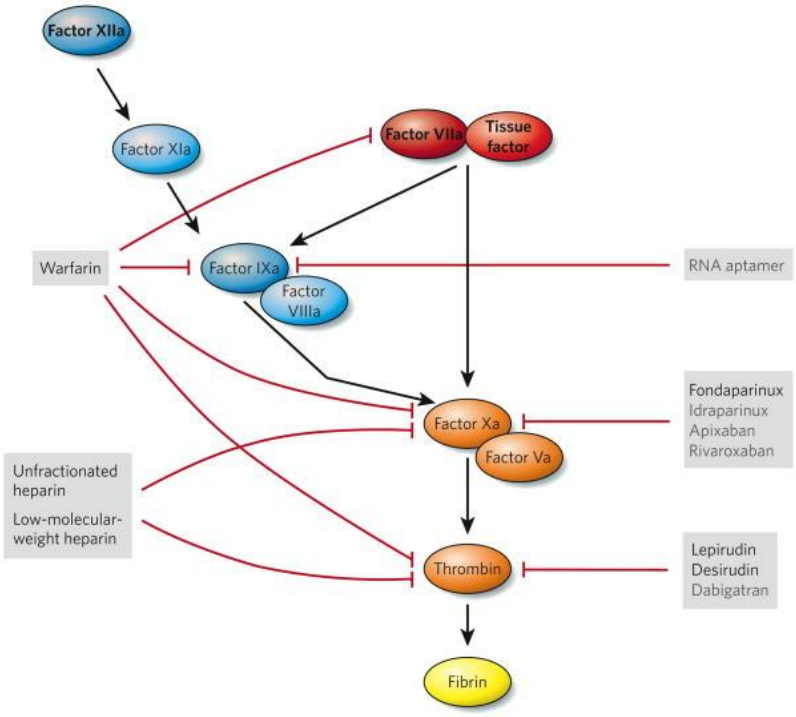


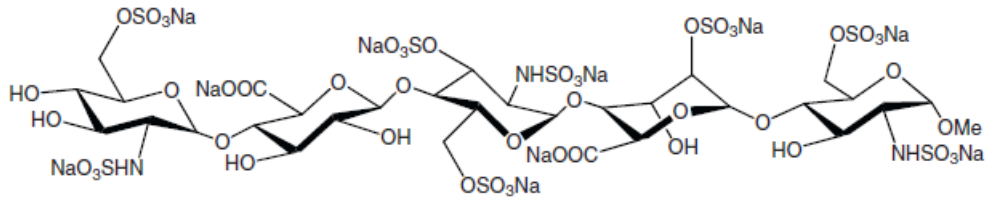
Figure 1.10: A simplified diagram of the coagulation cascade and various anticoagulant drugs. Components of the intrinsic pathway are shown in blue, extrinsic pathway in red, common pathway in orange, and the end product fibrin in yellow. The anticoagulants currently in use and in late development are listed, and their targets are indicated with a red arrow (Mackman 2008).

1.6.2.1 Heparins

Heparin is a heterogeneous mixture of unbranched negatively charged sulphated glycosaminoglycan. Heparin itself has no anticoagulant activity. However, as mentioned in section 1.4.1, it works by binding to AT and enhancing its capacity towards mainly fXa and thrombin. Today a variety of different heparin based drugs are available on the market. UFH and LMWH are both derived from animal tissue most often from porcine intestinal mucosa, whereas the pentasaccharide Fondaparinux (Arixtra, GlaxoSmithKline) is synthetically manufactured.

UFH catalyze the inhibition of fXa and thrombin to a similar extent. For the catalysis of fXa inhibition, heparin needs only to bind to AT via a unique pentasaccharide sequence which, as mentioned, alters the reactive center loop in AT. For the inhibition of thrombin, UFH binds to both thrombin and AT bridging the two together. This bridging can only be facilitated by UFH chains with 18 or more saccharide units, which corresponds to 5400 Da or higher. The mean molecular weight of UFH is 15000 Da, and consists of between 10-50 saccharide units, so UFH has an equal inhibitory activity against fXa and thrombin (Weitz, Weitz 2009). The mean molecular weight of LMWH is about 5000 Da, contains between 6-30 saccharides, and less than half the chains are of sufficient length to bridge thrombin and AT together. Thus, LMWH catalyze the inhibition of fXa to a greater extent than thrombin. Fondaparinux only consists of the unique pentasaccharide sequence that alters the reactive center loop in AT (Figure 1.11.A), and therefore only catalyze the inhibition of fXa (Weitz, Weitz 2009). Idraparinux is a synthetic pentasaccharide analogue to Fondaparinux, in which the hydroxyl groups are methylated and the *N*-sulphate groups are replaces by *O*-sulphate (Figure 1.11.B). Idraparinux has a long half-life (80-120) and administration is only necessary once a week. Is has been evaluated for the treatment of and prevention of VTE and is now in the late clinical development pipeline of Sanofi-Aventis (Gandhi, Mancera 2010). In the clinical trials hemorrhage complications were observed, why Idrabiotaparinux was developed. Idrabiotaparinux differs from idraparinux in that it contains a biotin moiety that enables reversal of the anticoagulant effect by the protein avidin. The drug is currently being tested in phase III trials (Toschi, Lettino 2011).

A



B

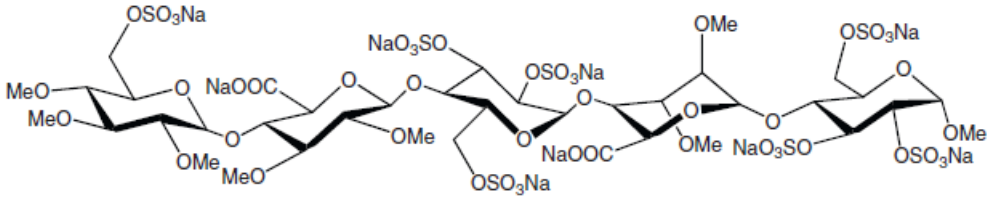


Figure 1.11: Chemical structures of (A) Fondaparinux and (B) Idraparinux (Gandhi, Mancera 2010)**.**

Heparin is cleared from the system in a biphasic fashion. First, in an initial rapid clearing phase heparin binds to endothelial cells and macrophages. This binding is chain length dependent with longer chains having higher affinity. Consequently, LMWH and Fondaparinux exhibit little or no binding. Once the binding sites on the endothelial cells and macrophages are saturated, the slower non-saturable clearing phase through the kidneys occurs. Heparin bound to macrophages is depolymerized, and smaller fragments reenter the system, and are being cleared through the kidneys. The half-life of heparin is hence dose dependent. The half-life of a small dose of heparin is short because of the rapid binding to the cells, while the half-life of a larger dose is longer; hence regular monitoring of patients is needed. The clearance of both LMWH and Fondaparinux is also dose-dependent, and their half-life is longer, hence their anticoagulant response is more predictable and regular monitoring is not necessary (Weitz, Weitz 2009).

Besides the need for monitoring patients receiving heparin, the drug has another side effect. Heparin can induce thrombocytopenia via immune or non-immune mechanisms. Non-immune heparin induced thrombocytopenia (HIT) type I is caused by direct platelet activation by heparin, involving the negatively charged side chains of heparin binding to the platelet. HIT type I is typically characterized with a mild drop in platelet count, within two days after heparin administration, and does not need any treatment. The more server HIT type II is an immune mediate disorder that occurs when antibodies (normally IgG) are produced against platelet factor 4 (PF4)-heparin complexes. The heparin-PF4-antibody complex binds to the surface of

platelets via the Fc portion of the immunoglobulin, which leads to platelet aggregation and further release of PF4 hereby, creating a positive feedback loop. The platelets are removed from circulation leading to thrombocytopenia and sometimes thrombosis (Day, Landis et al. 2004). HIT is not a rare complication after cardiac surgery, and approximately 25-50 % of all patients receiving heparin for cardiac surgeries will form HIT antibodies, and of them, 2-2.4 % will develop clinical HIT (Murphy, Marymont 2007). Patients receiving Fondaparinux have been reported to produce antibodies. However, since the drug does not bind to the platelets it should not cause HIT (Toschi, Lettino 2011).

Heparin not only has inhibitory effect towards thrombin and fXa, but is also known to affect platelet aggregation. One of the major glycoproteins expressed on the platelet surface is GP-Ib, which binds to thrombin and plays an important role in thrombin induced platelet activation (Day, Landis et al. 2004). Studies have shown that heparin by binding to thrombin interfere with thrombin's ability to bind to GP-Ib, thus inhibiting the platelet activation. The effect on the platelets was found to be more profound for UFH than LMWH (De Candia, De Cristofaro et al. 1999). Heparin has a similar effect on vWF-GP-Ib interactions. The heparin binding domain on vWF overlaps with the GP-Ib binding domain, hence heparin binding to vWF interferes with vWF induced platelet aggregation (Sobel, McNeill et al. 1991).

Despite the obvious disadvantages with heparin, it still has a major advantage over all other anticoagulants on the market, since it is the only one with an antidote. Protamine sulphate, a highly cationic peptide derived from salmon sperm, binds to heparin with high affinity, and the protamine-heparin complex is rapidly cleared by the kidneys. Binding of heparin to protamine is chain length dependent, with longer chains of heparin binding with higher affinity than shorter chains. This means that protamine is only capable of partially reversing the anticoagulant effect of LMWH. The antidote completely revers the inhibitory effect towards thrombin, but only 60 % of the anti-factor Xa activity of LMWH is affected. In contrast protamine sulphate has no effect on Fondaparinux (Weitz, Weitz 2009).

1.6.2.2 Vitamin K antagonist - Warfarin

As mentioned, some proteases in the coagulation system require γ-carboxylation of their Glu residues to function properly (section 1.3). Vitamin K antagonists like warfarin inhibits these proteins indirectly by inhibiting the enzyme VKOR, hereby preventing vitamin K to return to the reduced form (Jackson, Esnouf 2005). Both pro- and anticoagulant proteins are affected

by vitamin K antagonists (VKA). However, the net effect is anticoagulant, since the thrombin generation is suppressed by the nonfunctional prothrombin and fXa (Furie, Furie 1990). Until recently the VKAs were the only orally available anticoagulants on the market, as well as the most frequently prescribed. But although effective, warfarin has a number of unwanted limitations. The dose of warfarin differs among patients because of commonly occurring genetic polymorphisms affecting the metabolism, thereby making the patient either more or less sensitive towards the drug. Also the dietary intake of vitamin K and drug-drug interactions affect the anticoagulant response of the drug. Consequently, patients receiving warfarin needs regular monitoring to ensure that the therapeutic dose is meet (Gross, Weitz 2009).

1.6.2.3 Direct thrombin inhibitors

Hirudin is an anticoagulant peptide found in the saliva of leeches, and it is the most potent natural occurring thrombin inhibitor. Lepirudin and Desirudin are recombinant forms of hirudin. They differ from hirudin in the lack of a sulphate at position 63 on their molecules. Lepirudin differs from Desirudin in a substitution of a leucine for an isoleucine. Both have 65 amino acids in their polypeptide chain. Both drugs are bivalent direct thrombin inhibitors. They bind both to the active site and to exosite 1 on the protease, and once bound all of the pro- and anticoagulant functions of thrombin are inhibited. Moreover, they also inactivate clot-bound thrombin. Since they are derived from a non-human protein, anti-hirudin antibodies have been seen to form against both drugs. The clinical implication of the antibody release is a reduction in drug clearance, and some patients may experience skin irritation. Anaphylactic reactions have been reported very rarely (reviewed by (White 2005)).

Lepirudin is licensed for the treatment of thrombosis in patients with HIT, whereas Desirudin is approved for postoperative prevention of VTE in patients undergoing elective hip replacement surgery. Desirudin is also under current investigation as a potential anticoagulant for patients with HIT. Like with many other anticoagulants there is no antidote for either of the two drugs (Lee, Ansell 2011).

1.6.2.4 Direct oral anticoagulants

In 2008 Rivaroxaban (Xarelto, Bayer HealthCare) was the first small-molecule direct oral inhibitor that was approved for clinical use. It was approved by the European authorities for prevention of DVTE and PE after elective hip or knee replacement (Perzborn, Roehrig et al. 2011). Rivaroxaban has a molecular weight of 463 g/mol, and is a direct reversible fXa inhibi-

tor. It inhibits fXa in a concentration dependent manner (K_i = 0.4 nM) and is capable of both inhibiting prothrombinase-bound and clot-associated fXa. The bioavailability is high (80-100 %), the inhibitor has a half-life of 7-11 hours, and it does not interact with food and other drugs (Perzborn, Roehrig et al. 2010). A part of the drug is cleared unchanged by the kidney and a part is metabolized by the CYP3A4 pathway in the liver. The drug is substrate for the P-glycoprotein (P-gp) transporter, which is found in the intestine and the kidneys, and co-administration of P-gp inhibitors can increase the plasma level of Rivaroxaban by reducing the clearance (Toschi, Lettino 2011). The drug prolongs both the PT and aPTT time in patients. There is no known antidote for Rivaroxaban.

Apixaban is also an oral direct fXa inhibitor, which is currently in phase III trials for preventing VTE in patients undergoing total knee replacement. The drug has a half-life of 8-14 h, and is cleared through multiple pathways including renal and intestinal routes. The drugs metabolism is catalyzed by enzymes from CYP450 system (reviewed by (Lassen 2009) and (Paikin, Eikelboom et al. 2010)). Apixaban is capable of both inhibiting free, and prothrombinase- and clot-bound fXa, and although the inhibitor has no direct effects on platelet aggregation, it indirectly inhibits the process by reducing thrombin generation (Wong, Pinto et al. 2011).

In 2010 the US Food and Drug administration (FDA) approved the oral direct competitive thrombin inhibitor dabigatran etexilate (Pradaxa, Boehringer Ingelheim) for stroke prevention in atrial fibrillation (Hughes 2010). It is an orally active pro-drug, which is rapidly converted by esterases to dabigatran. Dabigatran has a half-life of 14-17 h, which permits for a once or twice daily administration. Dabigatran inhibits both clot bound and free thrombin, and also affect thrombin-induced platelet aggregation (Lassen 2009). The elimination occurs 80 % through the kidneys, and as Rivaroxaban it is a substrate of the P-gp transporter, which is found in the kidneys. Drugs that inhibit this transporter can increase the plasma level of Dabigatran by reducing the clearance. The drug has predictable pharmacokinetics, has no clinically relevant interactions with other drugs, and the effect of the drug can, at lower doses, be monitored by aPTT (reviewed by (Paikin, Eikelboom et al. 2010) and (Weitz 2010)).

1.6.2.5 RNA aptamers

Recently, an entirely new approach to anticoagulation drug design has been taken. Certain RNA and DNA molecules can adopt highly ordered three-dimensional structures, that allow them to bind to target proteins with high affinity and specificity, hence function as direct pro-

tein inhibitors (Gopinath 2008). Aptamers are allegedly non-immunogenic. Their small size and similarity to endogenous molecules theoretically makes them poor antigens. Interestingly, the effect of aptamers can be reversed using an anti-sense aptamer antidote. An antidote against a fIXa-aptamer has been developed. This antidote is capable of reversing > 95 % of the aptamers activity in 10 min when added in a slight molar excess (Potti, Rusconi et al. 2004). Currently aptamers have been developed that inhibits fIXa, thrombin, vWF, aPC and fVIIa. The aptamer targeting fIXa is currently in phase 2 trails, and has been used to replace UFH in a mini swine model of cardio pulmonary bypass (CPB) (Gopinath 2008, Becker, Povsic et al. 2010).

1.7 Cardio Pulmonary Bypass

The new oral anticoagulants, have a predictable pharmacokinetic, does not interact significantly with food and other drugs and, together with the fact that regular monitoring is not necessary, enhances the likelihood that, they can become an alternative to vitamin K antagonists. But, even though new promising oral anticoagulant is fast emerging in the medical world, there is still a field where UFH is the major drug of choice. In surgeries where CPB is required, UFH is still the preferred drug of choice.

Postoperative bleeding is one of the most common complications after cardiac surgery. Approximately 20 % of all patients bleed significantly after the surgery, and 5 % needs re-exploration (Wolfe, Bolsin et al. 2007). During CPB both fibrinogen and fibrin are deposited onto the CPB circuit, making an environment to where thrombin can adhere. When thrombin binds to fibrinogen/fibrin it becomes inaccessible for inhibition by AT. Thus, it remains active and can generate more thrombin. Thrombin also, as mentioned previously, activates platelets that binds to the fibrinogen/fibrin, and in addition provides a phospholipid membrane where, among other things, the prothrombinase complex can be assembled (Paparella, Brister et al. 2004). As a result, high doses of anticoagulant drugs are required during CPB, in order to prevent activation of platelets and the coagulation system. A number of different anticoagulant drugs can be used during CPB; UFH, LMWH, and Lepirudin are the most commonly used. The dose of UFH that are typically needed to maintain CPB results in a 20-30 % reduction in platelet count, and the probability for the patient to develop HIT antibodies is 25-50 % (Yavari, Becker 2008). Protamine is used to reverse the effect of UFH after the surgery. How-

ever, protamine administration may itself contribute to some unwanted side-effects including arterial hypotension, decreased cardiac output, and anaphylaxis. Besides that, protamine given in excess of heparin is known to alter the interactions between GP-Ib and vWF, thus affecting the platelet aggregation (Paparella, Brister et al. 2004). In some cases LMWH is used in CPB but, as with UFH, LMWH is not capable of inhibiting complex/clot bound fXa and thrombin. LMWH is less likely to trigger the formation of HIT antibodies, however LMWH is not recommended in patients that already have a history of HIT. Protamine can completely reverse the anti-thrombin effect of LMWH but only partially neutralizes the anti-fXa effect, limiting the overall usefulness of LMWH in CPB. Lepirudin is not associated with a risk of cross-reactivity to HIT antibodies, and clinical data have demonstrated that Lepirudin is a suitable alternative anticoagulant during CPS in patients with HIT. However, care must be taken in patients with abnormal kidney function, because of the possibility for the inhibitor to accumulate, leading to postoperative bleeding (Riess 2005). A drawback in the use of Lepirudin is that there are no approved reversal agents for the inhibitor, and that 44-75 % of the patients will develop antibodies against it (Yavari, Becker 2008).

During CPB the primary objective of anticoagulant therapy is to prevent clotting of the blood, and maintain the number of platelet. The anticoagulants available for CPB today has a great number of unwanted side effects, hence new drugs with a predicable pharmacokinetics, and the possibility to rapidly reverse the effect is wanted.

2 Factor Xa – Structure and Mechanism

In anticoagulation therapy, fXa has been identified as a particular interesting target because of its central place in the coagulation cascade (Alexander, Kanwar 2005). Additionally, because the blood coagulation cascade is an amplification cascade, circulating plasma concentrations of fXa is much lower than that of thrombin, in fact one molecule of fXa can generate more than 1000 molecules of thrombin (Toschi, Lettino 2011). Therefore, efficient anticoagulation treatment with lower doses of active drugs can be expected when using fXa as target.

FXa is synthesized in the liver as a single chain 73 kDa glycoprotein consistent of a pre-pro-leader sequence, a light chain, a connective peptide, and a heavy chain (Figure 2.1) (Krupiczojc, Scotton et al. 2008).

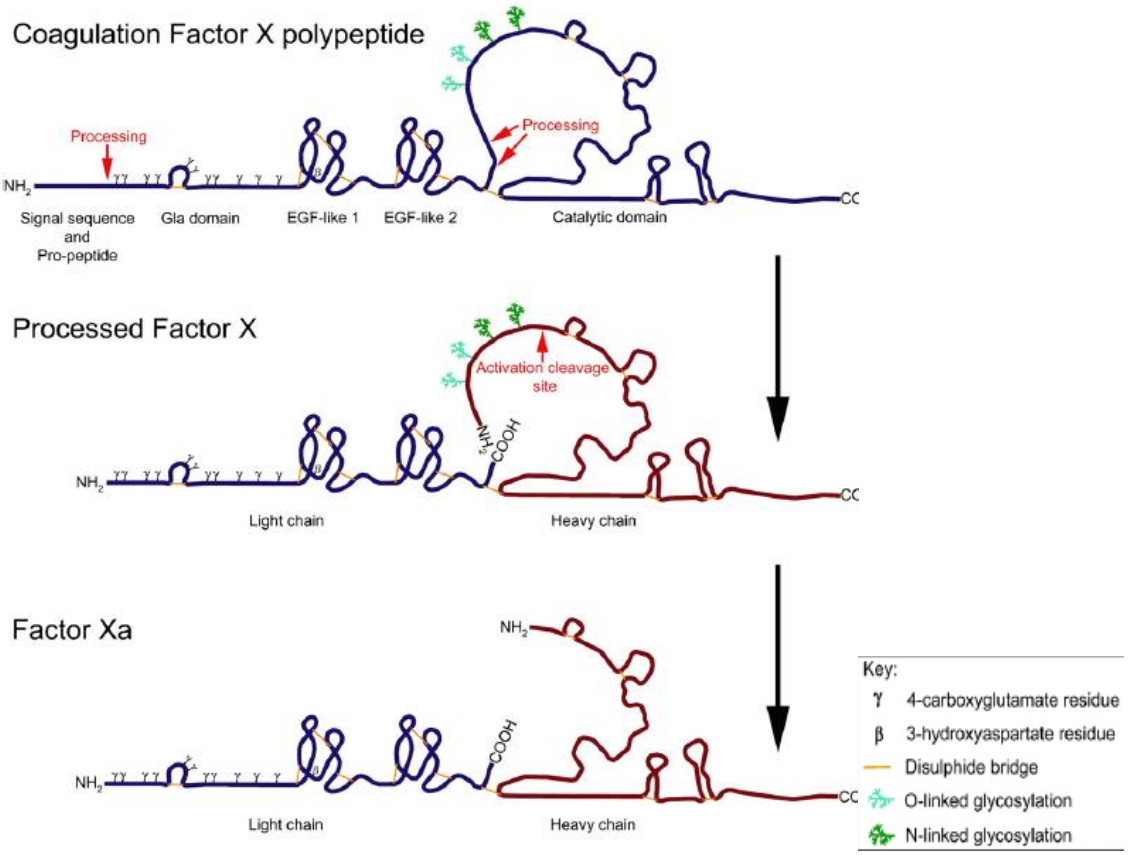


Figure 2.1: Post-translational modifications of factor X. In the endoplasmic reticulum the signal sequence is cleaved off and the protein undergoes some post-translational modifications (see text for further explanation). Before it is released into circulation it is cleaved into a heavy and a light chain held together by a disulfide bond. Factor X is activated by the hydrolysis of the bond between Arg194 and Ile195 (Krupiczojc, Scotton et al. 2008).

The single chain protein undergoes a number of post-translational modifications before it is released into circulation. First, the precursor protein is translocated to the endoplasmic reticulum (ER) where the signal sequence is cleaved off. The remaining pre-peptide directs the vitamin K dependent γ-carboxylation of 10-12 glutamic acid residues in the N-terminal of the protein as mentioned in section 1.3. Moreover the protein undergoes β-hydroxylation of Asp63 in the first EGF-like domain, and a high degree of glycosylation near the activation cleavage site. The final processing step is a cleavage of a peptide bond splitting the protein in a heavy and light chain held together by a disulfide bond. This cleavage results in the release of a basic tripeptide (Arg140-Lys141-Arg142) from the center of the molecule. The heavy chain (42 kDa) contains the activation peptide, and the light chain (16 kDa) contains the Gla- and EGF-like domains. These two chains make up the mature form of fX. FX is activated (to α-fXa) when the bond between Arg294 and Ile295 at the N-terminal of the heavy chain is cleaved, releasing a small 52 amino acid activation peptide (Krupiczojc, Scotton et al. 2008). FXa is a serine protease with an active site catalytic triad consisting of Ser195, His57, and Asp102. It belongs to the family of trypsin-like serine proteases characterized by the presence of a negatively charged Asp189 at the bottom of the S1 pocket. This pocket is formed by the 214-220 and 189-195 loops tied together by Cys220-Cys191 disulfide bond (Rai, Sprengeler et al. 2001).

As described in section 1.1 the function of fXa is the proteolytic activation of prothrombin to thrombin, which is obtained by the hydrolysis of the peptide bonds following Arg271 and Arg320. FXa assembles together with the cofactor fVa on membranes and forms the prothrombinase complex. This complex activates prothrombin by an initial cleavage at Arg320, which activates the catalytic site, and results in the formation of meizothrombin. This is followed by the cleavage of Arg271 yielding thrombin and the activation peptide fragment 1.2 (reaction 1 and 2 in Figure 2.2). In the absence of fVa, cleavage occurs in the alternative order with a slow initial cleavage of Arg271 resulting in the inactive prethrombin 2-Fragment 1.2 non-covalent complex, followed by cleavage of Arg320 to form active thrombin (reaction 3 and 4 in Figure 2.2) (reviewed by (Bock, Panizzi et al. 2007) and (Krishnaswamy 2005)).

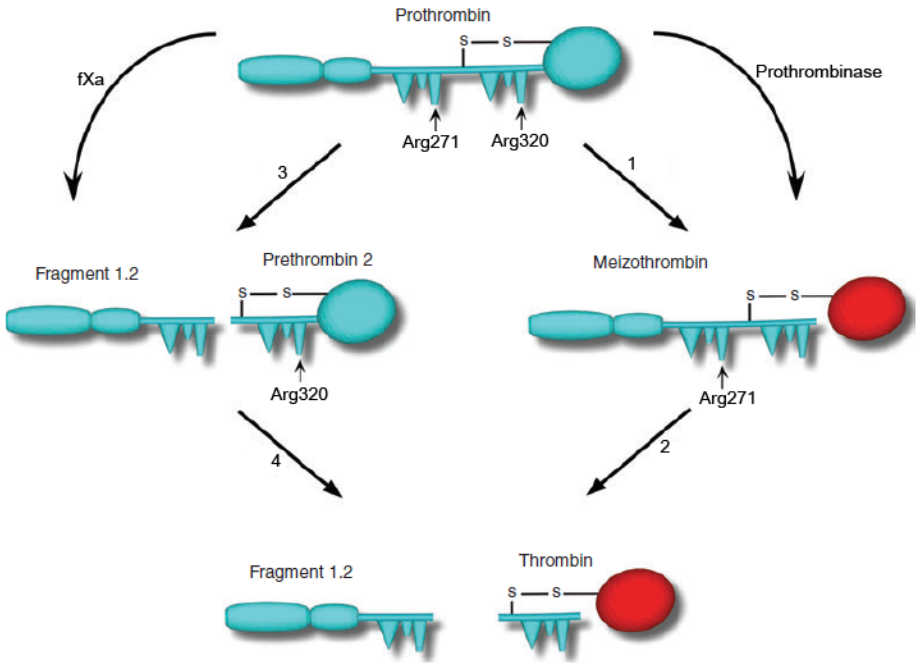


Figure 2.2: Pathways of thrombin activation by fXa and the prothrombinase complex. Cleavage of the peptide bonds following Arg271 and Arg320 in prothrombin results in the formation of thrombin and the activation peptide fragment 1.2. The activation can be catalyzed either by the prothrombinase complex or, in absent of fVa, by fXa alone (Modified from (Krishnaswamy 2005)).

FXa is, as before mentioned, inhibited naturally in the system primarily by AT and TFPI and medical inhibitors both indirect like Fondaparinux and direct like Rivaroxaban are available. However, none of these has an antidote. It is therefore not possible to reverse the inhibition in case this is needed.

3 Kunitz Protease Inhibitor A-k1 from Potato tubers

3.1 Preliminary work

The potato is the world's third most important food crop. Depending on the cultivar, the tuber contains 18 % starch and 2 % proteins. The major storage proteins in the potato tubers are patatin and protease inhibitors, where the latter include 28 different variants of Kunitz type protease inhibitors (KPI). Most of the tuber proteins are stored in the vacuole and is believed to be involved in the protection of the potato by inhibiting proteases of invading organisms ((Valueva, Revina et al. 2000) and (Jørgensen, Stensballe et al. Submitted)).

Since 2005 it has been known that potato juice has the ability to inhibit plasma coagulation (Kåre Lehmann Nielsen, personal communication). In my master thesis "A Potent Specific Kunitz Inhibitor of Coagulation Factor Xa from Potato Tuber" the proteins of the potato juice were separated, and it was found that the only one of them that was responsible for inhibiting plasma coagulation was a Kunitz protease inhibitor of the type A-k1. The proteins ability to inhibit fXa, thrombin and fVIIa was also tested, and it was found that the inhibitor only showed inhibitory effect towards fXa, why the inhibitor was named PifXa (Protein inhibitor of factor Xa) (Andersen 2007). In another master thesis "Characterization of the Interaction between Kunitz Protease Inhibitor, KPI A-k1 and Factor Xa for Improved Acute Anticoagulant Therapy" by (Andersen 2008) the purification of the inhibitor was improved, and recombinant KPI A-k1 was successfully expressed in the leafs of *Nicotiana benthamiana*. Later another recombinant line was developed. In 2010 different N-terminal variants of KPI A-k1 was expressed in the *E. coli* strain Rosetta Gami B, and all showed the same inhibitor profile as the wild type (Brøndum 2010). Crystallization of the inhibitor has been attempted, but today the 3D structure of KPI A-k1 is still unknown.

3.2 Primary structures of KPI A-k1

KPI A-k1 has been classified as member of the leguminous Kunitz-type protease inhibitor family, where soybean trypsin inhibitor (STI) is the archetypal member of the family. The family is recognized by the signature sequence [LIVM]-x-D-{EK}-[EDHNTY]-[DG]-[RKHDENQ]-x-[LIVM]-x-{E}-{Q}-x(2)-Y-x-[LIVM], where [] indicates residues allowed, x is any residue, {} indicates residues not allowed, and () indicates number of residues defined

by the preceding symbol (Prosite 2006). The leguminous Kunitz-type protease inhibitor normally consists of 170-200 amino acids with a molecular weight of around 21 kDa. The inhibitor normally has four cysteine residues that form two S-S bridges. KPIs interact with the active site of the proteases in a canonical substrate-like manner via an exposed reactive loop (Oliva, Sampaio 2008). In the tuber of the cultivar Kuras all of the identified KPIs were without N-glycosylation (Jørgensen, Stensballe et al. Submitted). The leguminous Kunitz-type protease inhibitor is not related to TFPI, a member of the mammalian Kunitz protease inhibitor family of which bovine pancreatic trypsin inhibitor (BPTI) is the archetypical. This is evident by the folding of the two protein families. While the leguminous Kunitz-type protease inhibitor family has a β-trefoil fold, the mammalian Kunitz-type protease inhibitor family has a BPTI-like fold (Rawlings, Tolle et al. 2004),(Murzin, Chandonia et al. 2009).

KPI A-k1 is expressed as a 220 amino acid polypeptide, where the 19 most N-terminal residues are an ER sorting signal. The ER sorting signal is follow by a propeptide of 11 residues containing the NIPR-like sequence specific vacuolar sorting determinant. Several variants of KPI A-k1 has been found in the tuber with a large number of different N- and C-terminals, the function of these truncated variants if any remain unclear (bracket followed by the number of times the variant were observed in Figure 3.1) (Jørgensen, Stensballe et al. Submitted).

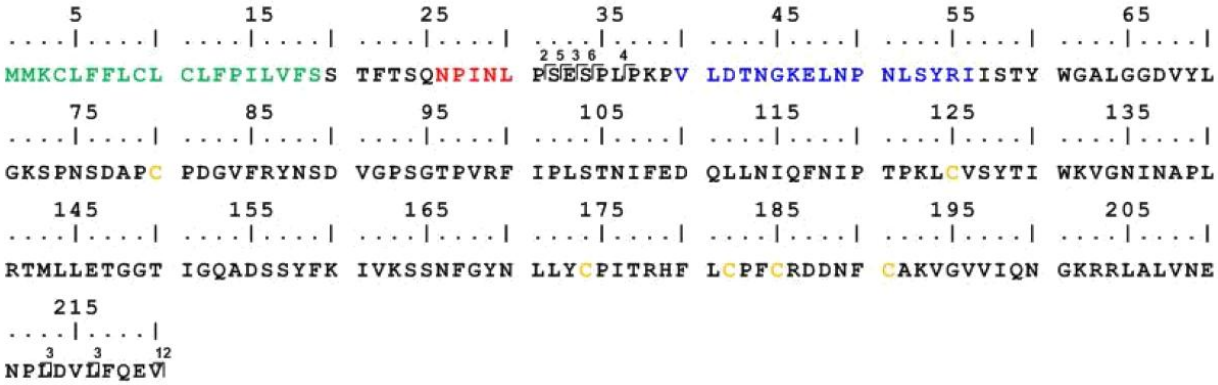


Figure 3.1: Amino acid sequence for KPI A-k1 (DQ168311). The green sequence indicate the ER sorting signal, red a vacuolar sorting signal, blue is the 17 residue Kunitz motif and the cysteines are colored in orange. The superscript number together with a bracket indicate the number of times the different N- and C- terminals were observed by LC MS/MS of potato vacuolar protein extracts **(Jørgensen, Stensballe et al. Submitted)**.

The closest relative to KPI with a known 3D structure is STI. The 3D structure of STI has been solved both for the protein alone and in complex with the substrate trypsin (Song, Suh 1998). The inhibitor consists of twelve antiparallel β-strands with long loops connecting these

strands. Six of the stands form a short antiparallel β-barrel with one side of the barrel being closed by a "lid" consisting of the other six strands. The fold is commonly known as the β-trefoil fold. The reactive site of STI (Arg63-Ile64) is located at one end of a loop. However, twelve amino acid residues have contact with the protease during inhibition. They are; Asp1, Phe2, Asn3, Pro61 (P3), Tyr62 (P2), Arg63 (P1), Ile64 (P1´), Arg65 (P2´), His 71, Pro72, Trp117, and Arg119. Most of the contact between the inhibitor and the protease involves the five residues in the reactive site loop (P3-P2´) (Song, Suh 1998, Oliva, Sampaio 2008). An alignment between KPI A-k1 and STI can be seen in Figure 3.2.

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50         60
KPI A-k1        -PVLDTNGKE LNPNLSYRII STYWGALGGD VYLGKSPNSD APCPDGVFRY NSDVGP-SGT
STI             DF...NE.NP .ENGGT.Y.L .DIT-.F..- --IRAA.TGN ER..LT.VQS RNELDKGI..

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         70         80         90        100        110        120
KPI A-k1        PVRFIPLSTN IFEDQLLNIQ FN-IPTPKLC VSY-TIWKVG NINAPLRTML LETGGTIGQA
STI             IISSPYRIRF .A.GHP.SLK .DSFAVIM.. .GIP.E.S.- -VEDLPEGPA VKI.ENKDAM

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        130        140        150        160        170        180
KPI A-k1        DSSYFKIVKS SN--FG-YNL LYCPITRHFL RDDNFCAKVG VVIQ--NGKR RLALVNENPL
STI             .G-W.RLERV .DDE.NN.K. VF..QQ---- AE.DK.GDI. IS.DHDD.T. ..VVSKNK..

                ....|....| ..
                         190
KPI A-k1        DVLFQEV--- --
STI             V.Q..KLDKE SL
```

Figure 3.2: Alignment between KPI A-k1 and STI. The blue sequence indicates the β-sheets secondary structure. The cysteines are marked in orange, and the reactive site loop in STI is marked in red. Identical residues are marked with a **.** while gaps are denoted with a **-**. A part of the sequence (Cys151, Pro152, Phe153, and Cys154) has been removed in order to improve the alignment.

The sequence identity between KPI A-k1 and STI is only 24 %, and STI is therefore not the best model. However, since the structure for KPI A-k1 is not known, STI can help to give an idea about where the reactive site loop of KPI A-k1 is located. Based on the alignment between KPI A-k1 and STI the putative reactive site loop of KPI A-k1 could be FIPL (P4-P1).

4 Aim

The aim of this PhD thesis is divided into three parts:

I) To *characterize the inhibitor in vitro,* by among other things determining the kinetic constants for the inhibition of and binding to fXa and test the inhibitory profile against a number of coagulation proteins.

II) To give *proof of technology* that the inhibitor can prolong the coagulation time *in vivo* and thereby have the possibility to in the future be used as a medicament. This was tested by measuring the tail bleeding using rats as the animal model.

III) To establish a *bacterial recombinant expression system* and generate different mutant variants of the inhibitor in order to pin point the binding area between fXa and PifXa.

5 Materials and Methods

5.1 Materials

5.1.1 Chemicals

Ammonium sulphate (AMS), glycine, hepes, IPTG, KCl, methanol (MeOH), sodium acetate (NaOAC), Tris (Applichem GmbH, Darmstadt, Germany), ADP, arachidonic acid (Bio/Data Corporation, Pennsylvania, USA), Proteinase Inhibitor Cocktail III (Calbiochem, San Diego, USA), Iodoacetic acid, Skim milk powder, trifluroacetic acid, tween 20 (Fluka Chemie GmbH, Buchs, Switzerland), K_3PO_4, sodium bisulphite, sodium chloride (JT Baker, Deventer, the Netherlands), Ethanol 99.9 % (Kemetyl A/S, Køge, Denmark), Prestained protein marker SM1811, protein molecular weight marker SM0431, Dream Taq, 10 X dream taq PCR buffer, dNTP mix, Not I, SalI, *pfu* polymerase (Fermentas, Helsingborg, Sweden), Acetic acid, Acetonitrile, 2.5-dihydroxybenzoic acid, Benzonase, Carbonic anhydrase, Cytochrome C, Formic acid, Heparin sodium salt from Bovine Intestinal Mucosa, Myoglobin, Nickel(II) sulfate heptahydrate (Sigma-Aldrich, Brøndby, Denmark), Chromozym t-PA (N-methylsulfonyl-$_D$-Phe-Gly-Arg-4-nitranilide acetate) (Roche Diagnostics, Indianapolis, USA), Antibody serum (Anti PifXa) (Genosphere Biotechnologies, Paris, France), ANS (1-anilino-8-naphthalene sulfonate), pENTR Directional TOPO Cloning Kit, PET-DEST 42 Gateway Vector, Secondary antibody Alexa Fluro 647 goat anti-rabbit (Invitrogen, Taastrup, Denmark), Factor X, Russell Viper Venom (RVV) (Haematologic Technologies Inc., Essex Junction, USA), Factor Xa (Molecular biological institute, Århus University, Denmark), Lysozyme, Na_2HPO_4 (Merck, Darmstadt, Germany), $Na^{131}I$ (Perkin Elmer, Massachusetts, USA), Rosetta Gami B (DE3) chemically competent *E. coli* cells (Novagen), Goat anti rabbit IgG horseradish peroxidase (HRP) conjugated, 1-step^{TM} Ultra tetramethylbenzidine (TMB) (Pierce, Rockford, USA), S-2366, S-2288, S-2222, S-2251, S-2238, Factor Xa (Chromogenix, Lexinton, MA USA), Human plasmin, activated protein C, bovine thrombin (Enzyme Research, South bend, IN USA), t-PA (Technoclone GmbH, Vienna, Austria), Pathromtin® SL reagent (Siemens Healthcare Diagnostics Products GMBH, Germany), UFH (Heparin Leo 5000 IE/mL, Leo Pharma, Denmark), Fondaparinux (Arixtra 2.5 mg/0.5 mL, GlaxoSmithKline, UK), Nycotest PT Reagent, Bovine thromboplastin (Medinor A/S, Brøndby, Denmark), Human thrombin (Biofac A/S, Esbjerg, Denmark), Primers (TAG Copenhagen, Copenhagen, Denmark), Human plasma

(Clinical Immunological Department, Aalborg Hospital, Denmark), Restriction enzymes NotI, SalI, (New England Biolabs, Ipswits, UK).

5.1.2 Equipment's

pENTR Directional TOPO Cloning kit, pENTR/SD/D TOPO vector, pET-DEST 42 Gateway vector (Invitrogen, Taastrup, Denmark), Fluoromax- 4 Spectroflurometer (Horiba Jobin Yvon, New Jersey, USA), Four channel light transmission aggregometer (Model PAP-4, Bio/data Corporation, Horsharm, USA), Juice extractor (Moulinex, Denmark), Heto master jet (Heto-Holten A/S, Allerød, Denmark), Filtering paper 0.45 µm mixed cellulose ester and 1.6 µm glass fiber (Advantec, Tokyo, Japan), Dialysis tube with MWCO 12,000-14,000 Da (Medicell International Ltd., London, United Kingdom), Nanodrop ND-1000 spectrophotometer (Saveen Werner AB, Limhamn, Sweden), Äkta purifier FPLC system, Ni-NTA super flow column, Q Sepharose HP Hiload 26/10 column, S sepharose Hilaod 26/10 column, Sephacryl 200 gel filtration column, (GE Healthcare, Buckinghamshire, United Kingdom), Sorval evolution RC centrifuge, Sorval SLA 3000 super-lite rotor (Kendro laboratory products, Frankfurt, Germany), Immbilon transfer PVC membrane pore size 0.45 µm (Millipore Corporation, USA), Typhoon 8600 (Amersham Pharmacia Biotech, USA), ELISA reader Labsystem multiscan RC (Bie & Berntsen A/S, Rødovre Denmark), Gel loadings tips (Eppendorf, Hamburg, Germany), Mass spectrometer (Bruker Daltronics, UK), Behring Coagulation timer (Siemens Healthcare, Germany), High binding ELISA plates (Greiner Bio One, Frickenhausen, Germany), Pierce Iodination beads (Piece Biotechnology, Rockford, IL, USA), Sprague Dawley rats (Taconic M&B A/S, Ry, Denmark), Mini Trans-Blot (BioRad, USA), NucleoSpin Extract II, Nucleospin Plasmid miniprep (Macherey-Nagel, Düren, Germany).

5.2 Methods

5.2.1 Purification of PifXa

5.2.1.1 Preparation of potato juice

5 kg of potato tubers (cv. Kuras) collected in Northern Jutland in 2007 was washed and cut into pieces of 3-4 cm. Potato juice were produced with a domestic juice extractor (Moulinex) directly into at beaker containing 20 g of bisulphite placed on ice. The juice was gently stirred

and left for s15 min in order to let the starch settle. The supernatant was hereafter centrifuged for 20 min at 10000 x g and 4°C. The supernatant was filtered through a 0.45 μm filter, and the pH was adjusted to 7-8 with NaOH.

The potato proteins were precipitated using a saturation concentration of 70 % ammonium sulphate (AMS). 472.28 g AMS pr. liter potato juice was slowly added under mild stirring on ice (Kielberg, Rasmussen 2000). Mild stirring was continued for 30 min. Afterwards the solution was centrifuged for 30 min at 10000 x g and 4 °C. The pellet was resuspended in 412 mL (½ the vol. of potato juice after filtration) 10 mM tris buffer pH 8 by gentle mixing.

Ethanol (EtOH) precipitation was used to separate the patatins and larger proteins from the other potato proteins. 40 % (v/v), corresponding to 666 mL/L, cold (-20 °C) EtOH was added to the potato protein solution slowly and under mild stirring (Scopes 1988, Idel 2006). The solution was incubated at 4 °C for an hour and centrifuged for 10 min at 5000 x g at 4 °C. The supernatant was dialyzed (molecular weight cut off (MWCO) 12-14 kDa) against 10 mM tris buffer pH 8.

5.2.1.2 Ion Exchange Chromatography

The protein solution was centrifuged at 10000 x g for 20 min, and the supernatant was filtered through a glass fiber filter (1.6 μm) and a 0.45 μm filter. The protein concentration was estimated by A_{280}. The solution was applied to a Q sepharose Hiload column, which had been equilibrated with 10 mM tris, pH 8. The proteins were eluted with a linear gradient of 0-1 M NaCl in 10 mM tris, pH 8 with a flow of 4 mL/min. The flow through was dialyzed (MWCO 12-14 kDa) against 20 mM NaOAC, pH 4, and the protein solution was filtered through glass fiber and 0.45 μm filters before it was applied to a S sepharose Hiload column equilibrated with 20 mM NaOAC, pH 4. The proteins were eluted as with the Q sepharose column. Selected fractions and the flow through from both the anion and cation exchange were analyzed on a 12 % SDS-gel, and the ability to inhibit fXa was tested using the amidolytic activity assay. The samples that showed ability to inhibit fXa were pooled, dialyzed against 10 mM tris, pH 8 and applied to the S sepharose column, equilibrated with 10 M tris, pH 8. The proteins were eluted with a linear gradient of 0-1 M NaCl in 10 mM tris, pH 8. The fXa inhibitor activity was tested again. The samples with inhibitor ability were pooled and applied to a sephacryl 200 gel filtration column equilibrated with 25 mM hepes with 100 mM NaCl, pH 7.2. The

purity of the final protein solution was analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI TOF MS).

5.2.1.3 MALDI TOF MS

The protein samples from the gel filtration were analyzed by MALDI-TOF MS using sinapinic acid (SA) as matrix. The SA was dissolved in 0.1 % triflouroacetic acid (TFA) in acetonitrile (2:1, v/v). The proteins were applied to GelLoader pipet tips packed with an Empore^{TM} C8 porous teflon, which had been rinsed with 10 µL 0.1 % TFA in acetonitrile (1:2, v/v) and 10 µL 5 % formic acid. The sample (5 µL) were slowly loaded by air pressure from a syringe, and washed with 20 µL 5 % formic acid. The proteins were eluted with 1 µL SA matrix solution and deposited directly onto a MALDI target plate. The drops were allowed to air dried. The instrument was internally calibrated with Cytochrome C (12360 Da), Myoglobin (16952 Da), and Carbonic anhydrase (29025 Da). The obtained mass spectra were analyzed using the freeware m/z software (Genomic solution, USA).

5.2.2 In vitro Characterization of PifXa

5.2.2.1 Amidolytic activity assay

fXa inhibition

The different samples from the purification of potato juice were tested for their ability to inhibit fXa using an amidolytic activity assay. Buffer (50 mM tris, 100 mM NaCl, 10 mM $CaCl_2$, 0.1 % PEG 6000, pH 8), enzyme (25 nM fXa) and inhibitor (various concentrations) were added to a 96 well plate and incubated for 10 min at room temperature (RT). The reactions were started by addition of chromozym t-PA to a final concentration of 0.5 mM, and the change in absorption was measured at 405 nm every 30 sec in an ELISA reader. Absorbance was converted into *p*-nitroaniline formation (mM) using the following equation (Equation 5.1):

$$A = \varepsilon * l * C \qquad \textbf{Equation 5.1}$$

Where the molar absorption coefficient (ε) was 9900 M^{-1} cm^{-1} (Tans, Janssen-Classen et al. 1987) and the light path length (l) was 0.285 cm. The light path length was calculated from

the volume of sample (100 µL), the total volume of the well (382 µL) and the height of the well (10.9 mm) (Greiner Bio One).

Determination of the inhibitory constant

The kinetic values for inhibition of fXa by PifXa were determined by varying the concentration of chromozym t-PA. PifXa was mixed with fXa (50 nM) in buffer and incubated for 10 min at RT before various concentrations of chromozym t-PA (0-1.25 mM) was added. The absorbance was measured at 405 nm every 30 s. for 20 min. Different concentrations of PifXa was used (0- 1.8 µM). Every concentration was carried out in triplicates. K_M and V_{max} were determined by fitting the data to Michaelis Menten kinetics, and the inhibition constant K_i was determined using a competitive, non-competitive, and mixed mode of inhibition model. The data were fitted using the program GraphPad Prims version 5 (GraphPad Software Inc., USA).

Reversible Inhibition of fXa

In order to see if it was possible to reverse the inhibition of PifXa, 100 µg/mL PifXa and 25 nM fXa was mixed with amidolytic activity buffer and incubated for 10 min where after 0.5 mM chromozym t-PA was added and the absorbance was measured every 30 sec. at 405 nm. After 5 min antibody serum specific for PifXa was added to a final concentration of 20, 40 and 60 µg/mL, and assay was continued for another 25 min.

Inhibition of other coagulation proteins

PifXas ability to inhibit thrombin, activated protein C (aPC), plasmin, fXa, t-PA, and trypsin was tested using the enzyme specific substrate S-2238, S-2366, S-2251, S-2222, and S-2288, respectively. S-2222 was also used as substrate for trypsin. 20 nM of thrombin, aPC, t-PA, and trypsin, 40 nM plasmin or 1 µM fXa were mixed with 0, 25 (1.2 µM), 50 (2.4 µM), or 100 (4.8 µM) mg/mL PifXa in buffer (50 mM tris, 100 mM NaCl, 10 mM $CaCl_2$, 0.1 % PEG 6000, pH 8) and incubated for 10 min at RT. The concentration of substrate corresponding to $2xK_m$, which according to the manufacture were 0.2, 0.4, 0.8, 0.8, and 2 mM for S-2238, S-2366, S-2251, S-2222, and S-2288, respectively, were added to the mixture and the absorbance at 405 nm were measured ever 30 s. for 20 min.

5.2.2.2 Measurement of the binding constant using fluorescence

The binding constant between fXa and PifXa were determined using fluorescence. FXa (0.5 µM) was mixed various concentrations of PifXa (0-5 µM) in buffer (25 mM hepes with 100 mM NaCl, pH 7.2) containing 60 µM 1-anilino-8-naphthalene sulfonate (ANS). The solution was incubated at RT for 5 min before a fluorescence emission scan was made between 400-600 nm with an excitation at 280 nm. The excitation and emission bandwidth were set at 3 and 5 nm, respectively. The signal intensity at 476 nm was plotted against the concentration of PifXa and the curved were normalized between 0-1, where 1 was considered full saturation of the binding sites. The binding constant was determined by fitting the data to the following equation (Equation 5.2).

$$\theta = \frac{K_D+[M_{tot}]+[L_{tot}]}{2[M_{tot}]} - \sqrt{\left(\frac{K_D+[M_{tot}]+[L_{tot}]}{2\,[M_{tot}]}\right)^2 - \frac{[L_{tot}]}{[M_{tot}]}}$$ **Equation 5.2**

θ is defined as the fractional saturation of the macromolecule with the ligand, K_D is the binding constant, M_{tot} is the total concentration of macro molecule, in this case fXa, and L_{tot} is the total concentration of ligand, here PifXa (Jensen 2010). The data was fitted to the equation using GraphPad Prism. The experiment was carried out in triplicates.

5.2.2.3 fX Activation assay

0.8 µM fX was incubated for 10 min. at room temperature with 2.4 µM PifXa in assay buffer (10 mM Hepes, 100 mM NaCl, 5 mM $CaCl_2$, 0.1 % PEG 6000, pH 7.5) where after 0.8 µM Russell Viper venom (RVV) was added and incubated for 45 min at room temperature. The solution was mixed with SDS PAGE loading buffer, heated for 5 min at 95 ^oC, and run on a 12 % SDS-gel under both reducing and non-reducing conditions. As controls a solution of fX and RVV, fX and PifXa, and RVV and PifXa was used.

5.2.2.4 Thrombin Inhibition

Thrombin (0.063 U/mL) was incubated with various concentrations of PifXa (0-4.8 µM) and buffer (50 mM tris, 150 mM NaCl, 30 mM $CaCl_2$ and 0.1 % PEG 6000, pH 7.5) for 15 min at 37^oC. 3 mg/mL fibrinogen was added, and the absorbance was measured every 30 s at 600 nm for 90 min.

5.2.2.5 Coagulation assays

All coagulation assays were carried out in human pooled citrated plasma with various concentrations of either PifXa (0-200 µg/mL), UFH, LMWH, or Fondaparinux (0-2 IU/mL). All assays were performed in a Behring Coagulation Timer from Siemens in trips.

Activated Partial Thromboplastin time (aPTT) assay

100 µL Pathromtin® SL reagent containing vegetable phospholipids and silicon dioxide activator (Siemens Healthcare Diagnostics Products GMBH, Germany) was mixed with 100 µL plasma containing various concentration of inhibitor (PifXa, UFH, LMWH, or Fondaparinux) and incubated at 37 ^{o}C for 5 min. Coagulation was initiated by adding 100 µL 20 mM $CaCl_2$ and coagulation time was measured for up till 600 s.

Prothrombin Time (PT)

Qwren PT: 100 µL Nycotest PT reagent (Medinor A/S, Brøndby, Denmark), containing thromboplastin from rabbit brain, fV and fibrinogen from bovine plasma, was mixed with 50 µL plasma containing various concentrations of inhibitor (PifXa, UFH, LMWH, or Fondaparinux). $CaCl_2$ was added to initiate coagulation. Coagulation was measured for 180 s.
Quick PT was performed like the aPTT assay except that 100 µL of Bovine thromboplastin (Medinor A/S, Brøndby, Denmark) was added instead of pathromtin reagent.

Thrombin time

50 µL human thrombin (Biofac A/S, Esbjerg, Denmark) was mixed with 100 µL of plasma containing various concentrations of inhibitor (PifXa, UFH, LMWH, or Fondaparinux). $CaCl_2$ was added to initiate coagulation. The coagulation vas measured for 180 s.

5.2.2.6 Whole Blood Coagulation Assay

A glass Pasteur pipette was glued to a 2 mL syringe and a 30 G needle was attached. 2 mL fresh citrated whole blood was mixed with varying concentrations of PifXa (4-32 µg/mL) and 8 mM $CaCl_2$. Heparin (2 U/mL) was used as control. The blood was kept at 37 ^{o}C until it was used. The needle was placed in 25 mL water and 2 mL blood solution was added to the Pasteur pipette. The time until the blood flow ended was measured. 1 % (w/v) SDS was added to

the water and the absorbance was measured at 405 nm. Blood from four different donors was used.

5.2.2.7 Platelet Aggregation Studies

Venous blood was collected in 3.8 % sodium citrate for a final ratio of 1:9. Platelet aggregation was measured in platelet rich plasma (PRP) in a four channel light transmission aggregometer (Model PAP-4, Bio/data Corporation, Horsharm, USA) at 37 ^{o}C and 626 nm. The PRP was obtained by centrifugation of whole blood at 1500 rpm for 6 min, and platelet poor plasma (PPP) was prepared by centrifugation of the remaining blood for an additional 10 min at 4000 x g. PPP was used to set the light transmission at 100 %. Platelet aggregation was initiated by adding ADP or arachidonic acid. To test the effect of heparin and PifXa on platelet aggregation, PRP was mixed with 20 U/mL heparin or 8 µg/mL PifXa and various concentrations of ADP (0.2 – 3.2 µM).
To test PifXas effect on the inhibition of aggregation by aspirin, PRP was mixed with 5, 8, and 30 µg/mL inhibitor, respectively, and/or 1 mg/mL acetylsalicylic acid (ASA). 1.5 mM arachidonic acid was used as activator.

5.2.3 *In vivo* Characterization of PifXa

In all *in vivo* experiments Sprague Dawley (SD) rats obtained from Taconic M&B A/S (Ry, Denmark) were used. They were allowed to acclimatize for minimum three days prior to the experiment with unlimited access to standard feed and water. The rats were sedated with 5 % isofluran in an induction chamber, placed on a heating blanket and injected intravenously (IV) with inhibitor solution or placebo, through a Neoflon catheter placed in the tail vein. After 10 min the outer 2 mm of the tail was cut off and the tail was placed in 37 ^{o}C warm saline (0.9 % NaCl). The bleeding time was measured for 30 min or until the bleeding stopped. 1 % (w/v) SDS was added to the solution and the absorbance was measured at 410 nm as an estimation of the bleeding amount. The rats were killed by an overdose of the anesthetic after the experiment.

5.2.3.1 Preliminary *in vivo* experiments

28 rats weighing about 250 g were used in the preliminary experiment. They were after sedation blindly injected with various concentrations of inhibitor (0, 0.2, 0.75, or 1.3 mg). In order to insure that all groups were injected with the same amount of protein (1.3 mg), bovine serum albumin was added to the samples. As controls one group of rats was injected with heparin (50 IU) and one group was not given anything. Each group contained 3 rats.

5.2.3.2 2^{end} round of *in vivo* experiments

In the second round of *in vivo* experiments 49 rats were used. The rats where treated as before and given either 0, 0.063, 0.125, 0.25, or 0.5 mg of the inhibitor or heparin (50 IU), again bovine serum albumin was added to the samples to insure that all rats received the same amount of protein. One group was not given anything.

5.2.3.3 3th round of *in vivo* experiments

40 rats were used in this experiment. The rats were treated with either 0 or 0.8 mg of the inhibitor, a combination of 4 mg ASA and 0.8 mg inhibitor, or a combination of 4 mg ASA and 0.8 mg bovine serum albumin. Heparin (50 IU) and Fondaparinux (0.025 mg) was given as controls. One group was not given anything. The bleeding time was measured for 60 min.

5.2.3.4 *In vivo* clearings rate

6 rats were given 0.2 mg PifXa. At the time points 0, 5, 10, 15, 20, 25, 30, 40, 50, 60, 80, 100, 120, 150, and 180 min a blood sample of 150 µL was drawn. The blood was immediately mixed with EDTA to a final concentration of 5 mM. The samples were centrifuged for 10 min at 2500 x g to separate the plasma from the cells. The plasma was kept at -20 ^{o}C until use.

5.2.4 Attempt to determine the half-life of PifXa *in vivo*

5.2.4.1 Western Blot

Samples with various concentrations of PifXa (0.8 ng-4 µg) diluted in either plasma or buffer (25 mM hepes with 100 mM NaCl, pH 7.2), and a control containing only human plasma were run on a non-reducing 12 % SDS PAGE gel. The marker used was prestained SM1811 (Fermentas, Helsingborg, Sweden). The gel was run as normal and soaked in blotting buffer

(20 % methanol (MeOH) (v/v), 200 mM glycine, 20 mM tris, 0,01 % (w/v) SDS) for 10 min together with fiber pads and filter paper. A polyvinylidene fluoride (PVDF) membrane were wetted in MeOH for 30 s and equilibrated in blotting buffer for 10 min. The gel, membrane, pads and filter paper were assembled according to the manufacturer (Biorad). The gel was blotted at 100 V for 1 h. for 0.75 mm SDS-gels and 2 h. for 1.5 mm SDS-gels. The membrane was placed in a 50 mL tube and blocked for an hour with blocking buffer (5 % (w/v) skim milk powder in basic buffer (20 mM Tris, 150 mM NaCl, 0.05 % (v/v) Tween 20, pH 7.5)) at 4^{o}C. The blocking buffer was replaced with binding buffer (0.04 % (v/v) NaN_3, 5 % (w/v) skim milk powder in basic buffer) containing 1:2000 dilution of anti-PifXa primary antibody serum and the membrane was incubated at 4^{o}C overnight. Afterwards it was washed for 5, 10, and 10 min with washing buffer (0.1 % (v/v) triton X 100 in basic buffer) and incubated for 2 h. in the dark at 4 ^{o}C with binding buffer containing 1:5000 dilution of secondary antibody (Alexa Fluro 647 goat anti rabbit IgG, Invitrogen, Taastrup, Denmark). The membrane was washed and scanned using a typhoon 8600.

5.2.4.2 Indirect enzyme-linked immunosorbent assay (iELISA)

Prior to the experiment the optimal concentration of primary and secondary antibody was determined using a checkerboard titration experiment. The optimal dilution was found to be 1:1600 for the anti PifXa serum and 1:20000 for the goat anti rabbit HRP-conjugated antibody. The iELISA was carried out in 96 well microtiter plates. The wells were coated with 75 µL PifXa diluted to different concentrations in human plasma or PBS buffer (0-64 µg/mL) and incubated overnight at 4^{o}C. The wells were washed three times with PBS buffer (16.3 mM Na_2PO_4, 2.7 mM KCl, 0.9 mM K_3PO_4 and 136.9 mM NaCl pH 7.4) containing 0.05 % (v/v) tween-20 and remaining protein binding sites were blocked with 200 µL per well of 5 % (w/v) skim milk powder in PBS (blocking buffer) for 2 hours at room temperature. After blocking the wells were washed twice with PBS/tween. 100 µL primary antibody diluted 1:1600 in blocking buffer were added to each well and the plate was incubated for 2 hours at room temperature. The plate was washed again four times in PBS/tween and 100 µL of secondary antibody diluted 1:20000 in blocking buffer was added to each well and incubated for 2 hours at room temperature. After another four washes 100 µL 1-step^{TM} Ultra TMB was added to each plate. The color was allowed to develop for 30 min before 100 µL 2 M sulfuric

acid were added and the absorbance were measured at 450 nm. The experiment was carried out in both normal and high binding ELISA plates.

5.2.4.3 Iodine 131 labeling of PifXa

The labeling of PifXa was done using Pierce Iodination beads (Piece Biotechnology, Rockford, IL, USA). In brief, beads were washed in buffer (25 mM hepes, 100 mM NaCl, pH 7.2) and two beads were mixed with 5 mCi $Na^{131}I$ (Perkin Elmer, Massachusetts, USA) in buffer and incubated for 5 min at RT. 0.5 mg PifXa was added and the solution were incubated for another 15 min. The reaction was stopped by removing the beads from the solution. To separate non bound ^{131}I from $PifXa^{131}I$ the solution were applied to a G-50 gel filtration column. Samples were collected in drops of five. The fractions were run on a 12 % non-reducing SDS-PAGE gel, and those containing protein were pooled.

5.2.4.4 *In vivo* experiments with $PifXa^{131}I$

This *in vivo* experiment was carried out like the previous. 8 female SD rats weighing about 300 g were used. They were divided in two groups, with and without tail cut. 0.5 mg $PifXa^{131}I$ were injected through the tail vein and after 10 min the tip of the tail were cut off in one of the group. The bleeding tail was placed on gaze. After 40 min the rats were killed. The blood from the gaze, the cut off piece of tail, the next two and four mm of tail, a piece of the liver, kidney, heart, muscle from the right thigh, lung, spleen, and a blood sample, were taken after the rat was killed and the radioactivity was measured over five min. The radioactive count was adjusted to decay/min/mg tissue. In three of the rats an additional blood sample was drawn 10 min into the experiment. Extra blood and urine from four rats were extracted after they were killed. These samples were filtered through 0.45 µm and the proteins were separated on a Mono S column, which had been calibrated with 10 mM tris pH 8. The protein were eluted from the column with a gradient of 0-15 % 10 mM tris, 1 M NaCl, pH 8 over 2 CV, 15 -60 % over 7 CV and 60-100 % over 0.5 CV. The radioactivity from selected fractions from the separation, the non-bound proteins, and the initial protein sample were measured.

5.2.5 Directed Evolution and Recombinant Protein Expression

5.2.5.1 Design of different mutant PifXa for recombinant protein expression

The sequence for PifXa (KPI A-k1) was synthesized and cloned into pUC57 by GenScript (Piscataway, New Jersey). This was used as template for the PCR. Five different mutant PifXa construct were produced. Two of the mutants (IEGR 1 and 2) contained the recognition sequence for fXa (IEGR), which were incorporated into two different places of the putative RCL of PifXa by overlap extension PCR (OEPCR) using the following primers KPI A-k1sense 5'-caccatgtcaactttcacttcccaaaatc-3', KPI A-k1antisense 5'-tcagtcgactcaatggtgatg-gtgatgatggtcgacttcctggaataagacatc-3', IGER1 sense 5'-gcggtacacccgttagaatcgagggccggtct-acaaatatctttgaag-3', IGER1 antisense 5'-cttcaaagatat-tgtagaccggccctcgattctaacgggtgtaccgc-3', IGER2 sense 5'-ggtacacccgttagattcatcgagggccggaca-aatatctttgaagatc-3', and IGER2 antisense 5'-gatcttcaaagatatttgtccggccctcgatgaatctaacgggtgtacc-3'. The KPI A-k1 antisense primer contains a recognitions site for SalI and the codes for six His, while the KPI A-k1 sense contains a TOPO cloning overhang.

In the first reaction the primers IEGR-sense and KPI A-k1antisense was used and in the other KPI A-k1sense and IEGR-antisense was used. 0.2 ng of the synthesized DNA was used as template and mixed with 10 x PCR buffer, 0.5 mM dNTP, 0.5 µM of each primer, 2 mM $MgCl_2$, and 0.05 U Dream taq. DNase free water was added to 50 µL. Following program was used for the reactions: One cycle of 94 ^{o}C for 2 min, 17 cycles of 94 ^{o}C for 30 s, 54 ^{o}C for 30 s and 72 ^{o}C for 1 min 45 s and finally one cycle of 72 ^{o}C for 5 min. The products were loaded on a 1 % agarose gel and the product was purified from the gel using NucleoSpin extract II (Macherey- Nagel). 5 µL of each of the two purified products were mixed and used as template for a new PCR reaction where the primers KPI A-k1 sense and antisense where used.

Two loop swap constructs were also produced. The first construct (*KPI A-k1) contained the N- and C terminal part from the KPI A-k1 variant and the putative RCL part from the KPI B-k1 variant (A_1, A_3, and B_2), whereas the second construct (*KPI B-k1) had the N- and C terminal part from the KPI B-k1 variant and the putative RCL part from the KPI A-k1 variant (B_1, B_3, and A_2).

First all six pieces were produced by PCR using the following primers KPI A-k1 sense 5'-caccatgtcaactttcacttcccaaaatc-3', KPI A-k1 antisense 5'-tcagtcgactcaatggtgatggtgatgatggt-cgacttcctggaataagacatc-3', KPI B-k1 sense 5'-caccatgaagtgtttatttttgtta-3', KPI B-k1 antisense

5’-gcatgtcgactcagtgatggtgatggtgatggtcgacctggacttgcttgaaggag-3’, A_1andB_1 antisense 5’-ggacctagcggtacacccgttagattcatt-3’, A_2andB_2 sense 5’-aatgaatctaacgggtgtaccgctaggtcc-3’, A_2 antisense 5’-tgtgttagttatacaatttggaaagtcgg-3’, A_3 sense 5’-tgtgttagttatacaatttggaaagtcgg-3’, B_2 antisense 5’-cccactttccaaattgtataactaacaca-3’, B_3 sense 5’-tgtgttagttgtacaatttggaaagtggg-3’. A_1, A_2, and A_3 were produced using the sequence for KPI A-k1 as template and the primer pairs KPI A-k1 sense and A_1 antisense, A_2 sense and antisense, and A_3 sense and KPI A-k1 antisense, respectively. The three B pieces were produced using the sequence for KPI B-k1 as template, the sequence was synthesized by GenScript USA Inc., Piscaway, USA. The following primer pairs were used; KPI B-k1 sense and B_1 antisense, B_2 sense and antisense, and B_3 sense and KPI B-k1 antisense. The conditions for the PCR reactions were the same as above. The products were loaded on a 3 % agarose gel and the product was purified from the gel using NucleoSpin extract II (Macherey- Nagel). 15 ng of each of the products A_1, B_2, and A_3 or B_1, A_2, and B_3 were mixed and used as a template in two new PCR reactions to generate *KPI A-k1 and *KPI B-k1, respectively, using either the primer pairs KPI A-k1 sense and antisense or KPI B-k1 sense and antisense. The final products were loaded on a 1% gel and purified.

A final mutant was synthetically synthesized by GenScript USA Inc., Piscaway, USA. Here two amino acids in the putative reactive site loop (P102 and L103) were exchange with two alanine residues.

5.2.5.2 Cloning and Recombination

The generated PCR products were cloned into pENTR/SD/TOPO vector (see Appendix 1) (pENTR*PifXa*), as described in (Invitrogen 2006a), using the pENTR Directional TOPO Cloning Kit. A recombinant wild type PifXa and the KPI B-k1 variant were also produced. Prior to the cloning step the PCR products were polished to remove 3´adenine overhang generated by the Taq polymerase by incubating with ~1 U pfu polymerase per µg DNA and 2 mM Mg^{2+} at 72 ^{o}C for 30 min. 1 µL polished PCR product was added to the TOPO cloning reaction, and 2 µL of this was transformed into One Shot Top 10 electro competent *E. Coli* cells for amplification. Selected PifXa mutation Entry Clones were extracted using NucleoSpin Plasmid kit and analyzed using Restriction Fragment Length Polymorphism (RFLP). ~ 0.2 µg purified plasmid DNA was incubated with 5 U/µg DNA SalI and NotI at 37 ^{o}C for 2 h. The reaction was analyzed by gel electrophoresis on a 1 % agarose gel. Clones tested positive by RFLP were sequenced (Eurofines MGW Operon, custom DNA sequencing).

The correct Entry Clones were recombined with the pET-DEST 42 Gateway vector (see Appendix 2), as described by (Invitrogen 2010), to produce PifXa IEGR1, IEGR2, *KPI A-k1, *KPI B-k1 or PL-AA Expression Vectors (pEXP*PifXa*). 1 µL of each recombination reaction was transformed into One Shot Top 10 electro competent *E. Coli* cells, the plasmids were purified using NucleoSpin Plasmid kit and were analyzed using RFLP. Positive plasmids were transformed into *E. Coli* Rosetta Gami B.

5.2.5.3 Protein expression

One colony of positively transformed cells was transferred to 50 mL of LB media containing 100 mg/mL ampicillin and 34 mg/mL chloramphenicol. Cells were grown overnight at 37 ^{o}C and 200 rpm. 4x500 mL selective LB media with 0.2 % glucose (w/v) in 2000 mL baffled flasks were induced with 10 mL of the inoculum. At OD_{600} = 0.6-0.8 cells were induced by adding IPTG to at final concentration of 1 mM and incubated for 16 h. at 25 ^{o}C. The cells were harvested by centrifugation (5000xg at 4 ^{o}C for 30 min). The pellet were resuspended in fresh lysis buffer (5 mL/g wet pellet) (20 mM Tris pH 7.5, 50 mM NaCl, 5 mM imidazole, 0.5 % v/v proteinase inhibitor, 2 mM $MgSO_4$, 1 mg/mL lysozyme, 2.5 U/mL benzonase), incubated on ice for 30 min, and exposed for five freeze/thaw cycles. The cell lysate were centrifuged (16,000 x g for 30 min), the supernatant were filtered through a 0.2 µm filter, and stored at 4 ^{o}C.

5.2.5.4 Protein purification

The recombinant PifXa variants were purified from the cleared cell lysate by Ion Metal Affinity purification Chromatography (IMAC) on a 5 mL Ni-NTA column. The column was calibrated with 20 mM tris, 300 mM NaCl, 5 mM Imidazole, pH 7.5 before the sample was loaded. The protein were eluted over a 10 CV gradient of 0-5 % 20 mM tris, 300 mM NaCl, 500 mM Imidazole, pH 7.5, following a 20 CV gradient from 5-50 % 20 mM tris, 300 mM NaCl, 500 mM Imidazole, pH 7.5. Selected fractions were analyzed on a 15 % non-reducing SDS PAGE gel, and the inhibition effect towards fXa was tested using the amidolytic activity assay, as previously described. 40 µg/mL of the inhibitor was used in the assay.

5.2.5.5 Identification of purified protein by MS/MS

The purified recombinant proteins were analyzed by MS/MS sequencing of tryptic protein digests. Protein bands were excised from the SDS PAGE gel, sliced into 1 mm^3 pieces and

washed in cycles of 15 min in 100 µL 100 mM NH_4CO_3 and 15 min in 100 µL 50 mM NH_4CO_3, 50 % v/v acetonitrile until no traces of Coomassie Brilliant Blue from staining of SDS-PAGE gel remained. The gel pieces were then incubated in 20 µL acetonitrile for ~20 min. The acetonitrile was removed and the proteins were reduced by adding 20 µL 10 mM DTT in 0.1 M NH_4CO_3 and incubating at 56° C for 45 min. Following incubation the suspension was cooled to RT and leftover liquid removed. Proteins were then alkylated by adding 20 µL 10 mg/mL iodoacetic acid and incubating at RT in the dark for 30 min. After the incubation leftover liquid was removed and gel pieces were washed twice for 15 min in 100 µL 100 mM NH_4CO_3 and in 100 µL 50 mM NH_4CO_3, 50 % v/v acetonitrile, respectively. The gel pieces were then incubated in 20 µL acetonitrile for ~20 min. Acetonitrile was removed and gel pieces were re-hydrated in 25 µL pre-chilled 12 ng/µL porcine trypsin in 50 mM NH_4CO_3, pH 8 and incubated on ice for 45 min. Leftover liquid was discarded, the gel pieces were covered with 20 µL 50 mM NH_4CO_3, and proteins were left for digestion overnight at 37° C. Following the overnight digestion the peptide solution were transferred to 0.5 mL tube and the remaining peptides were extracted from the gel pieces by two successive cycles of adding 10 µL 5 % v/v formic acid, incubating at RT for 15 min, adding 10 µL acetonitrile, incubating at RT for 15 min and then transferring the peptide extract to 0.5 tube. The peptide solution was evaporated to ~1-2 µL using a Labconco Centrivap concentrator at RT and 1 atm and the peptides were re-dissolved in 20 µL 5 % v/v formic acid. Mass spectra were measured using µLC coupled to an ES-QTOF-MS/MS instrument externally calibrated using Tunemix. Peptide masses and sequences were automatically calculated by Bruker Daltonics microTOF DataAnalysis3.4. Finally, the resulting files were searched against the NCBI database and local database STGI12-kuras-DQ using Mascot MS/MS Ion Search.

5.2.5.6 Random mutagenesis of a part of the sequence of PifXa

A selected part of the sequence of PifXa was mutagenized randomly. The sequence was divided in three parts; an N-terminal part, a C-terminal part, and at middle mutagenized part. The N- and C-terminal part was generated by PCR using the primers; For the N-terminal: WTf(EcoRV) 5´-ggtttgatatcgaatctcctctaccta-3´ and N-termR 5´-aatgaatctaacgggtg-3´ and for the C-terminal: C-termF 5´-ctacttaacatacaa-3´ and WTr(NotI) 5´-ggtttgcggccgctcaatggtgatggtgatgatggacttcctggaataagac-3´. The primers contains a recognition site for EcoRV or NotI, a stop codon and a hexa his tag. 50 ng KPI A-k1 was used as template and mixed with 10 X

PCR buffer, 0.5 mM dNTP mix, 0.5 µM of each primer pair, 0.05 U/µL Dream taq and 2 mM $MgCl_2$. DNase free water was added to 50 µL. The solution was run under the following conditions: One cycle of 94 ^oC for 2 min, 18 cycles of 94 ^oC for 30 s, 54 ^oC for 30 s and 72 ^oC for 1 min 45 s and finally one cycle of 72 ^oC for 5 min. The PCR products were separated on a 3 % agarose gel and the bands were purified using NucleoSpin extract II (Macherey-Nagel). The mutagenized part was ordered as a single strand DNA with the following sequence 5´-cacccgttagattcattNNNNNNNNNNNNNNNNNNNNNNNNNNNNNctacttaacatacaa-´3,
where N is any of the four bases (TAG Copenhagen, Copenhagen, Denmark).
OEPCR was used to combine the N-terminal, the middle mutagenize part and the C-terminal. 25 ng of each of the three sequence-parts were mixed with 10xPCR buffer, 0.5 mM dNTP mix, 0.5 µM of each primer (WTf(EcoRV) and WTr(NotI)), 0.05 U/µL Dream taq and 2 mM $MgCl_2$. DNase free water was added to 50 µL. The following program was run: One cycle of 94 ^oC for 2 min, 12 cycles of 94 ^oC for 30 s, 54 ^oC for 30 s and 72 ^oC for 1 min 45 s and finally one cycle of 72 ^oC for 5 min. The PCR-product was analyzed on a 1 % agarose gel and the band was purified.
In order to test the mutagenesis frequency the PCR product was cloned into a TOPO vector using the TOPO TA Cloning kit from Invitrogen (Invitrogen 2006b). The vector was transformed into TOP10 *E. coli*. The plasmids from 15 random cells were purified using the NucleoSpin Plasmid Kit (Macherey Nagel) and sequenced (Eurofins mwg operon, Ebersberg, Germany). The PCR product was cloned into a T7Select 10-3b phage vector from Novagen by the company Rx Biosciences, Maryland, USA.

6 Results

6.1 Bioinformatics study of PifXa

The relationship between PifXa (KPI A-k1) and the other proteins in the potato tuber was examined using the newly released potato genome database (PGSC 2011).

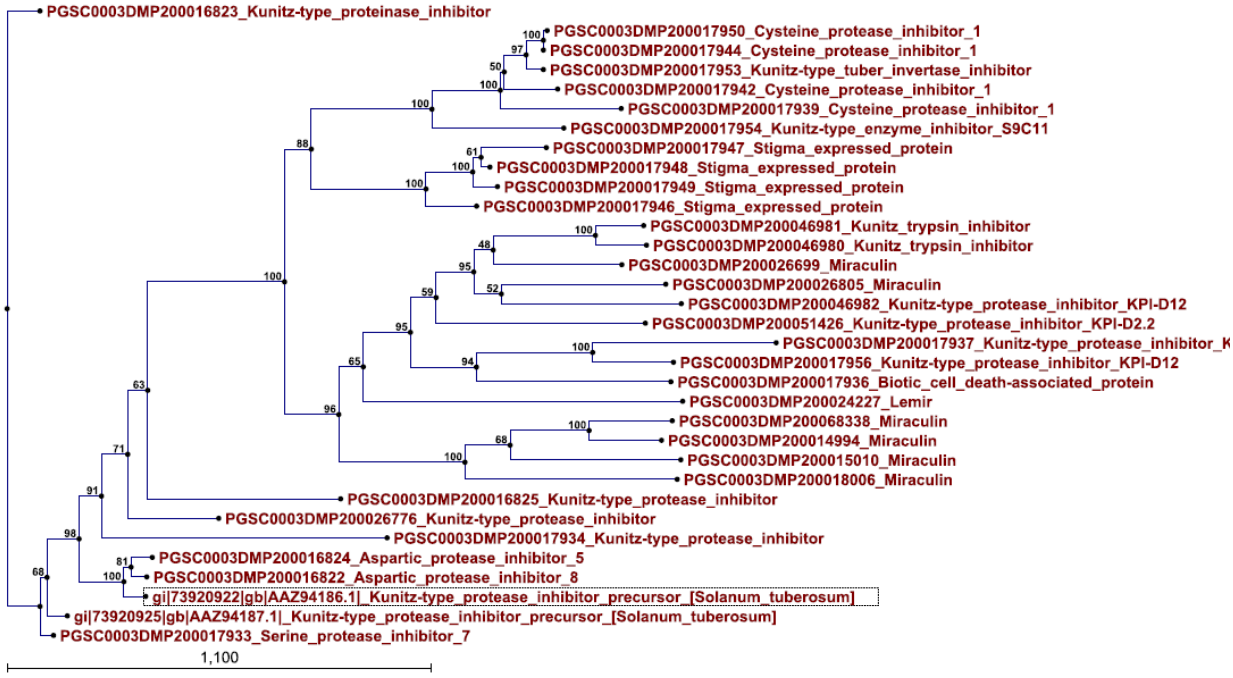


Figure 6.1: Phylogenetic tree based on a Blast search with the sequence of KPI A-k1 against the potato genome. The place of KPI A-k1 in the tree has been marked. The tree was constructed using CLC workbench.

Figure 6.1 shows a phylogenetic tree created based on a blast search with the sequence for KPI A-k1 against all protein sequences found in the potato genome. As a reference the sequence of KPI B-k1 was also included (gi | 73920925). The place of KPI A-k1 is marked in the tree and it appear only to have two fairly close relatives namely Aspartic protease inhibitor 5 and 8. The alignment of the sequences of the tree can be seen in Appendix 3.

6.2 PifXa can be purified from potato juice

PifXa was purified from raw potato juice via a series of precipitation and chromatographic steps as summarized in Figure 6.2.

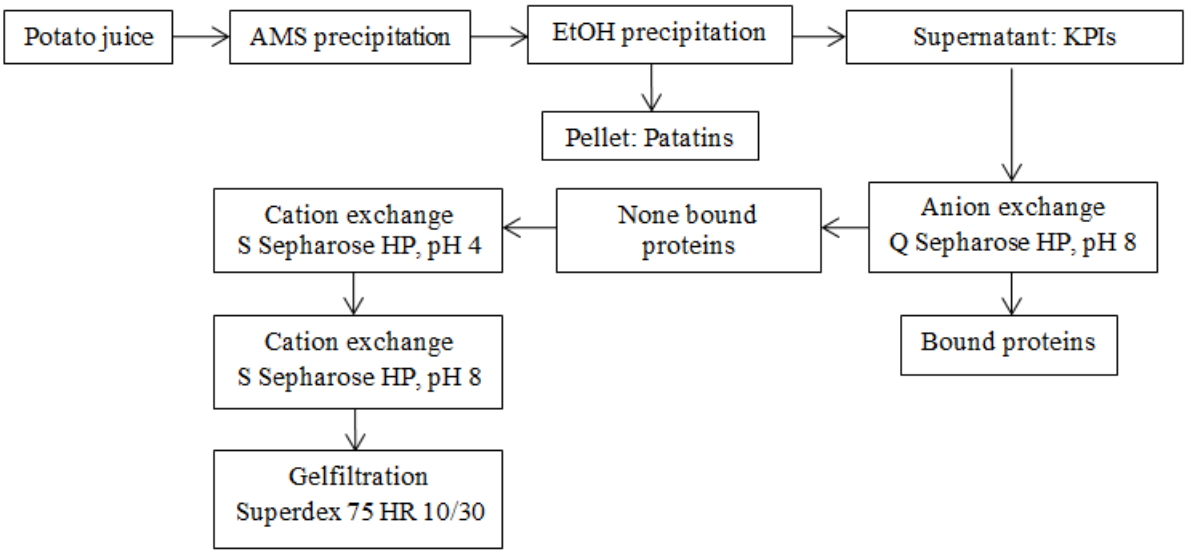


Figure 6.2: Schematic representation of the purification steps of PifXa from potato juice.

Potato juice was processed from field grown potatoes of the cultivar Kuras. In order to separate the KPIs from the additional proteins in the potato juice a crude separation was done. First an AMS precipitation with a saturation of 70 % (w/v) was performed to precipitate all proteins. An EtOH precipitation with 40 % (v/v) EtOH was used in order to separate the proteins in two groups dependent on their molecular weight (Idel 2006).

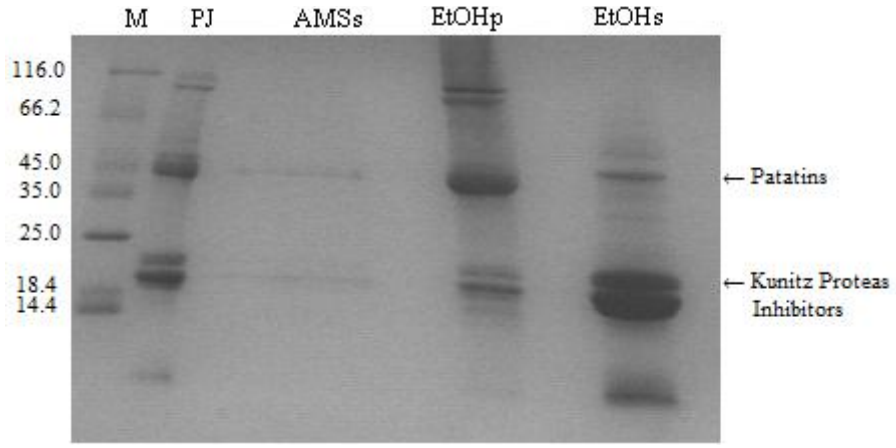


Figure 6.3: Initial fractionation of the potato juice (PJ). A non-reducing 12 % SDS PAGE gel with potato juice (PJ), the supernatant from the AMS precipitation (AMSs), and the pellet, and supernatant from the ethanol precipitation (EtOHp and EtOHs), respectively. The arrows indicate the band of the patatins and the KPIs. The marker (M) is sm0431 from Fermentas.

The SDS gel in Figure 6.3 shows the initial purification of the potato juice. After AMS precipitation, almost all proteins were, as expected, found in the pellet. The ethanol precipitation resulted in a crude separation of the large and small molecular weight proteins. Most of the

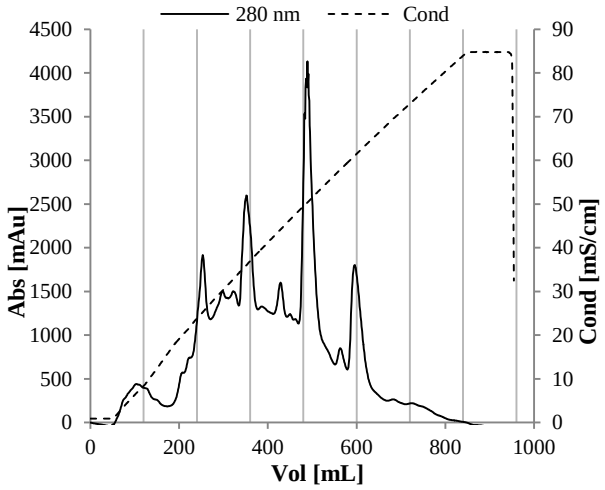
patatins (~ 45 kDa) and the other high molecular weight proteins like lipoxygenase (~100 kDa) were found in the pellet, while the majority of the KPIs (~18-20 kDa) were in the supernatant together with protease inhibitor I and II (Jorgensen, Bauw et al. 2006).

6.2.1 Purification by ion exchange chromatography

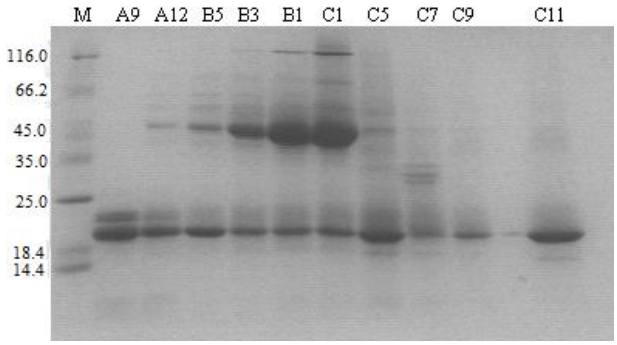
The proteins from the supernatant from the ethanol precipitation were further fractionated using a series of chromatic steps, starting with anion exchange at pH 8, and followed by cation exchange at both pH 4 and 8.

The fraction containing KPI's from the EtOH precipitation was filtered and applied to a Q Sepharose column, which had been equilibrated with 10 mM tris, pH 8. The proteins were eluted with a linear gradient of 0-1 M NaCl in 10 mM tris, pH 8 with a flow of 4 mL/min. Selected fractions were visualized on a 15 % non-reducing SDS PAGE gel (Figure 6.4).

A



B



C

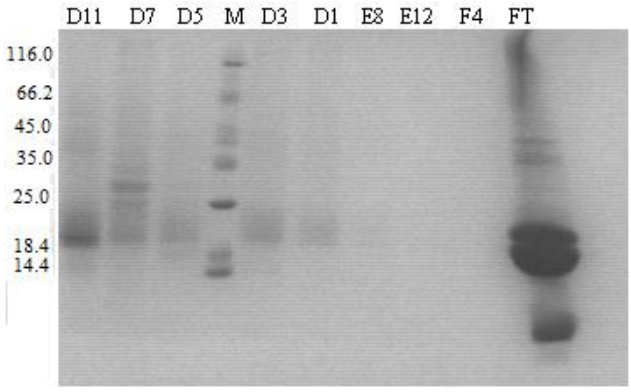
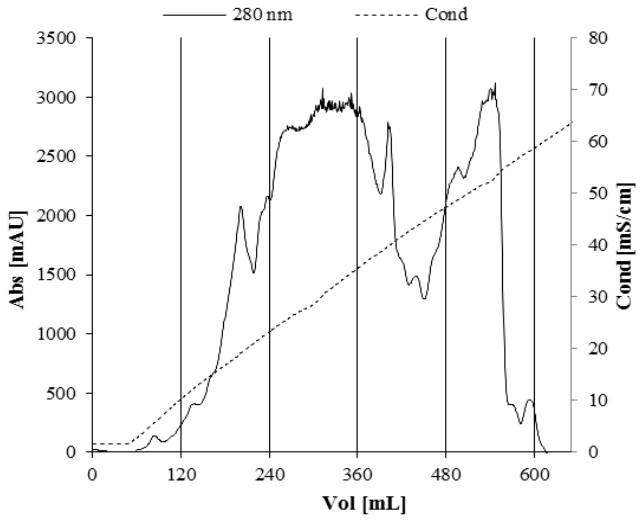


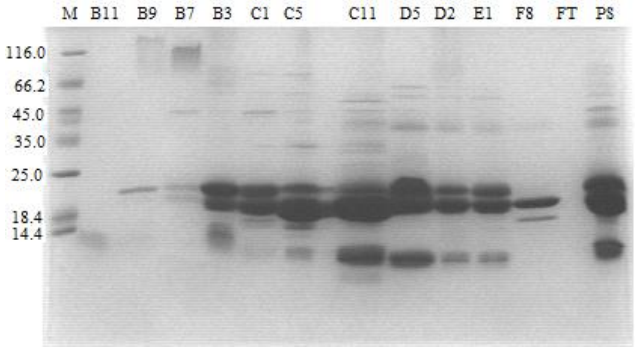
Figure 6.4: Anion exchange on the potato protein solution. (**A**) Anion exchange of the supernatant from the EtOH precipitation on a Q Sepharose column. (-) denotes the absorption at 280 nm and (¨) denotes the conductivity. The vertical lines divide the chromatograph in the different fractions. The first contains the fractions A1-A12, the second contains B12-B1 and so on. (**B** and **C**) selected fractions from the anion exchange and the flow-through (FT) on a 12 % non-reducing SDS-gel.

Even though both the chromatogram and the SDS PAGE gel shows a fractionation of the proteins bound to the column, previous studies have shown that PifXa does not bind to the anion exchange column, and is therefore found in the flow-through (Andersen 2007). The flow-through was thus applied to a cation exchange column equilibrated with 20 mM NaOAC, pH 4.

A



B



C

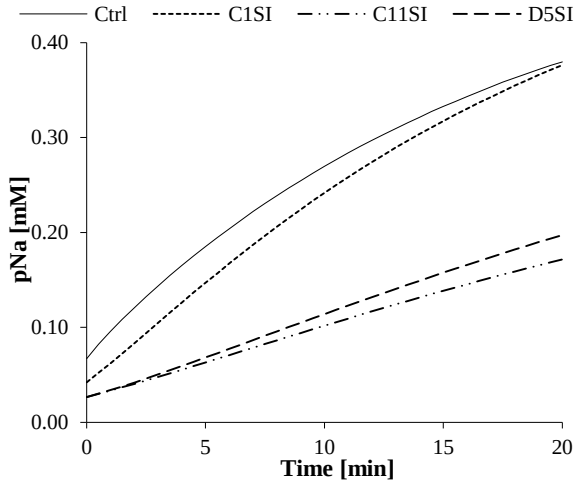
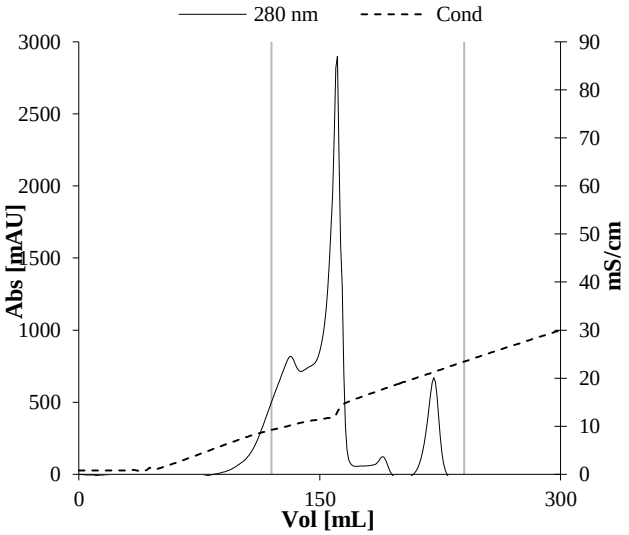
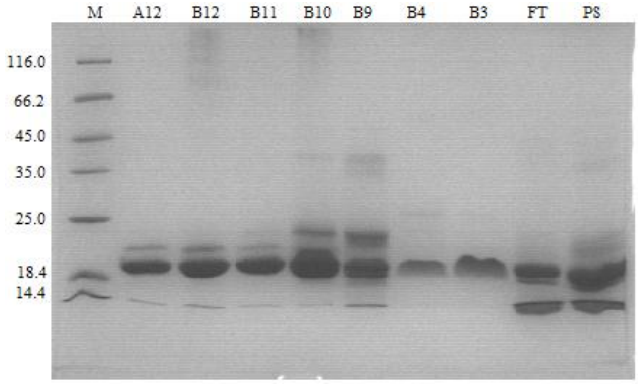


Figure 6.5: Cation exchange at pH 4 on the flowthrough from the anion exchange. (**A**) The chromatogram from the cation exchange on a S sepharose column. (-) denotes the absorption at 280 nm and (¨) denotes the conductivity. The vertical lines divide the chromatograph in the different samples. The first contains the fractions A1-A12; the second contains B12-B1, and so on. (**B**) Selected fractions from the cation exchange, flowthrough (FT), and protein sample (PS) on a non-reducing 12 % SDS PAGE gel. (**C**) Amidolytic assay on selected fractions from cation exchange, the control (Ctrl) is fXa without added inhibitor fraction. 100 µg/mL sample was mixed with 25 nM fXa and incubated 10 min before 0.5 mM substrate was added.

Figure 6.5 shows the results from the cation exchange purification at pH 4. The chromatogram shows several different peaks; hence fractionation of the proteins has occurred (Figure 6.5.A). This is also observed on the SDS PAGE gel, where KPI are present in all samples, however the pattern of the band are not the same throughout the samples. A number of samples were tested for their ability to inhibit fXa using the amidolytic activity assay (Figure 6.5.C). Based on their ability to inhibit fXa and the peak pattern in the chromatogram, samples spanning from C11 till D4 were pooled and applied to the same cation exchange column at pH 8.

A B


280 nm
Cond
Abs [mAU]
mS/cm
Vol [mL]
3000
2500
2000
1500
1000
500
0
90
80
70
60
50
40
30
20
10
0
0
150
300


M
A12
B12
B11
B10
B9
B4
B3
FT
P8
116.0
66.2
45.0
35.0
25.0
18.4
14.4

C

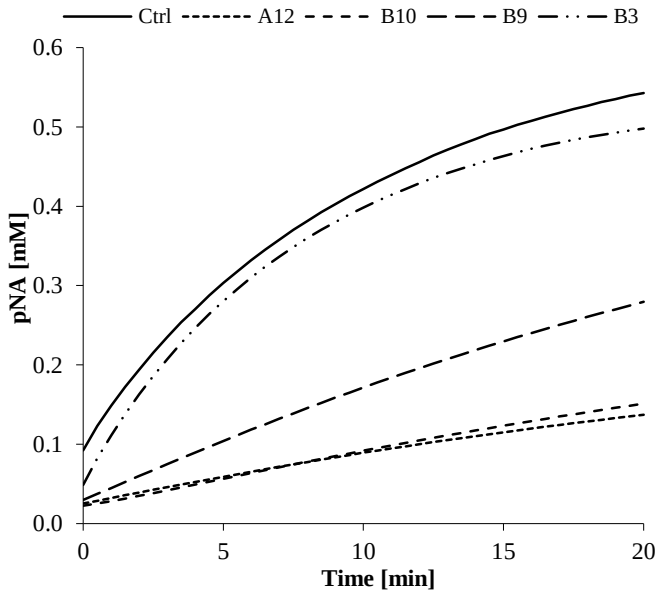


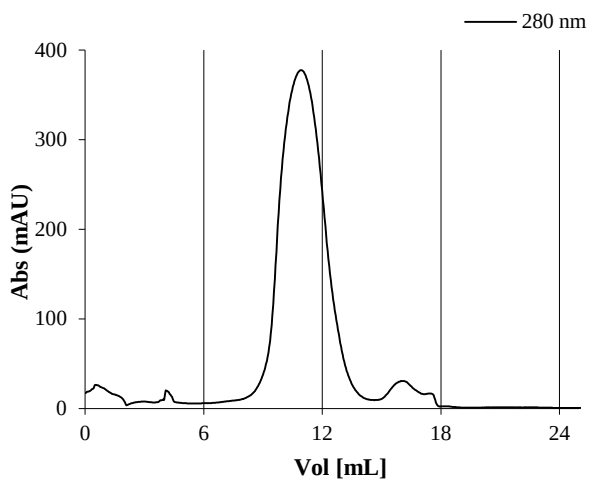
Figure 6.6: Cation exchange at pH 8 on pooled samples from the cation exchange at pH 4. (**A**) The chromatogram from the cation exchange at pH 8 on a S sepharose column. (-) denotes the absorption at 280 nm and (···) denotes the conductivity. The vertical lines divide the chromatograph in the different fractions. The first contains the fractions A1-A12; the second contains B12-B1, and so on. (**B**) Selected fractions on a non-reducing 12 % SDS-gel. (**C**) Amidolytic assay on selected fractions from cation exchange, the control (Ctrl) is fXa without added inhibitor fraction. 100 µg/mL sample was mixed with 25 nM fXa and incubated 10 min before 0.5 mM substrate was added.

The different peaks in the chromatogram indicates a separation of the different variants of the KPI in the sample, this is supported by the SDS PAGE gel, that in all lanes shows bands around 20 kDa but not in the same pattern in all samples (Figure 6.6.B). Selected samples were tested for their ability to inhibit fXa (Figure 6.6.C). Based on these results the samples A10-B10 were pooled and applied to a superdex 75 10/300 gel filtration column for at final purification step.

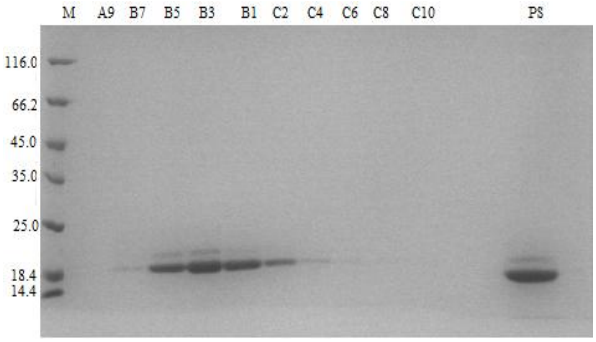
6.2.2 Final purification by gel filtration

The gel filtration chromatogram, the matching SDS PAGE gel, and the results from the amidolytic activity assay on selected samples can be seen in Figure 6.7.

A



B



C

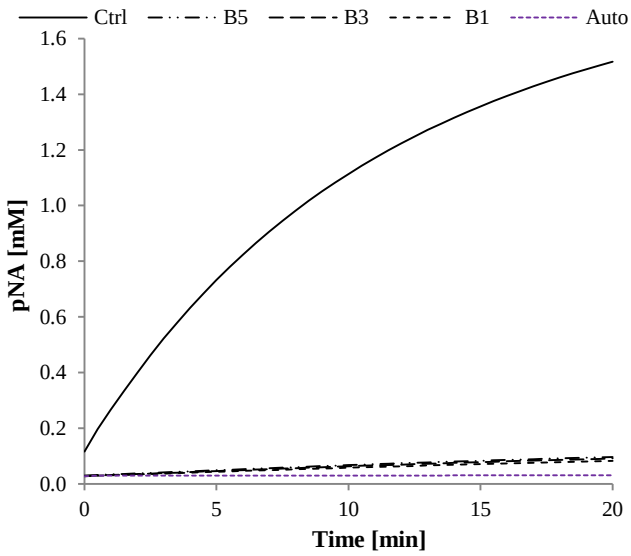


Figure 6.7: Gelfiltration on pooled inhibiting samples from the cation exchange at pH 8. (**A)** The chromatogram from the gel filtration on a superdex 75. (-) denotes the absorption at 280 nm. The vertical lines divide the chromatograph in the different fractions. The first contains the fraction A1-A12, the second contains B12-B1, and so on. (**B**) 12 % non-reducing SDS PAGE-gel with selected samples from the gelfiltration. (**C**) Amidolytic activity assay on selected fraction. 100 µg/mL sample was mixed with 25 nM fXa and incubated 10 min before 0.5 mM substrate was added. Auto represents the substrate auto-hydrolysis over the measured time.

The gelfiltration chromatogram showed a large peak followed by a smaller one. The SDS PAGE gel shows that the large peak contains a protein with the size corresponding to KPI. According to the gel two protein bands are present in some of the samples. However, previous studies have shown, that these band corresponds to different N-terminal variants of the same

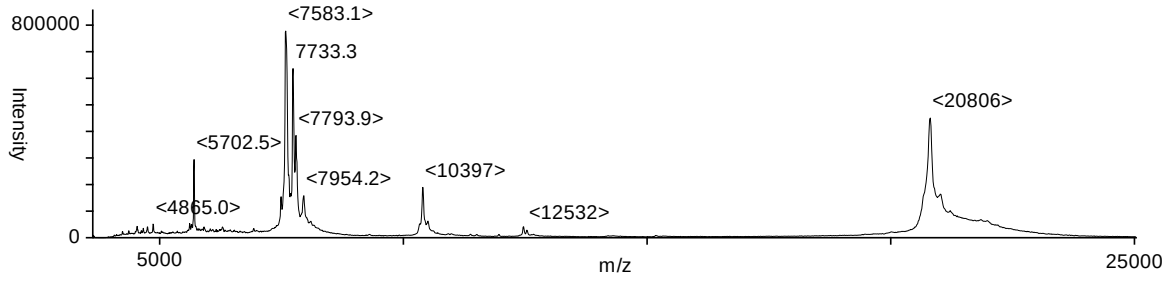
Kunitz type (KPI A-k1) (Figure 6.7B) (Andersen 2008). The proteins from the smaller peak were not visible on the gel, due to the small size and the range of the particular SDS PAGE gel.

Samples were tested for their ability to inhibit fXa and, as can be seen on Figure 6.7.C, an almost complete inhibition of fXa, was obtained with the tested samples.

6.2.3 Validation of purity by MALDI TOF MS

The samples that were able to inhibit fXa were pooled and the purity of the final sample was validated by MALDI TOF MS, as comparison the sample before gelfiltration was also tested. The spectra can be seen in Figure 6.8.

A



B

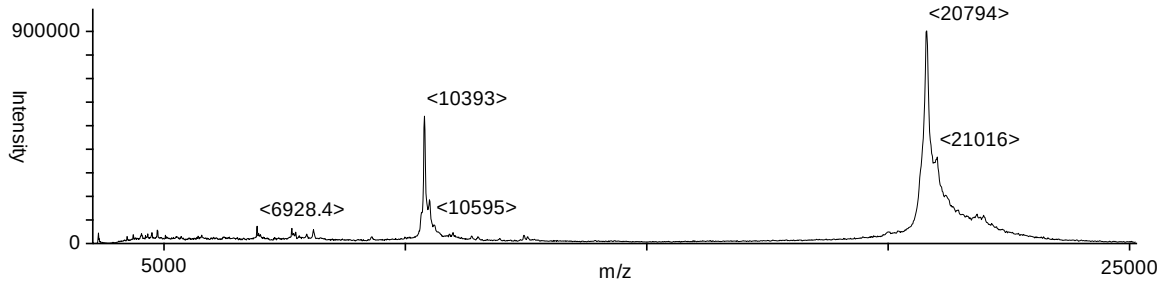


Figure 6.8: Purity determination by MALDI TOF MS. MALDI TOF MS spectra of **(A)** the protein solution before gel-filtration and **(B)** the protein solution after gel-filtration.

The most notable difference between the two spectra is the disappearance of a number of lower mass peaks. Figure 6.8.A shows the content of the protein sample before gel-filtration. The small peaks at 4865 and 5702, corresponds to carboxypeptidase inhibitor (CPI) and a type of definsin, respectively. The peaks at 7.7-7.9 kDa most likely corresponds to variants of protease inhibitor I (PI I), and the peak at 12532 corresponds to protease inhibitor II (PI II). The two peaks at 10397 and 20806 correspond to KPI A-k1 in two different ionization states. In

the second spectrum (Figure 6.8.B), which shows the protein content of the sample after gel-filtration, there are three peaks present which all represent the same protein (KPI A-k1, Genbank accession number DQ168318) in different ionization states. The peak around 20800 is very broad, indicating the presence of various N-terminal variants of KPI A-k1. The final yield of purified PifXa from the tubers was ~9 mg/kg tuber (wet weight).

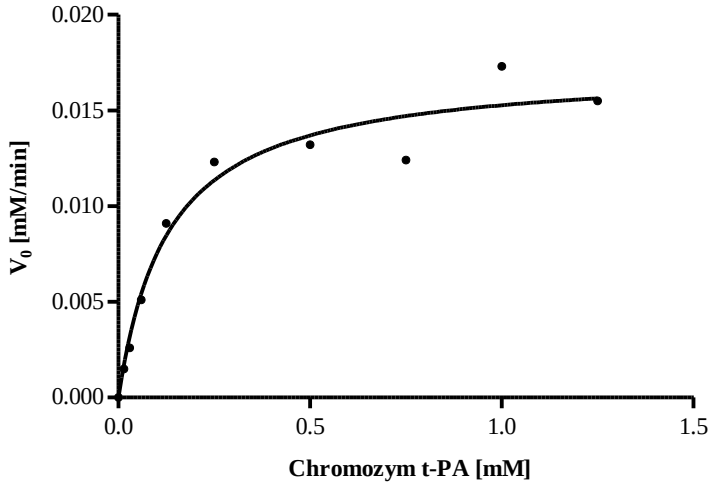
6.3 *In vitro* characterization of PifXa

The kinetic values for PifXa's inhibition of and binding to fXa were determined. The specificity of the inhibitor towards selected coagulation enzymes and the effect towards platelet aggregation were also examined. The inhibitor was tested in a whole blood coagulation assay, it was examined whether or not PifXa was capable binding to fX and hereby inhibiting the activation by Russell's viper venom, and finally is was tested if the effect of PifXa could be reversed by the addition of a inhibitor specific polyclonal antibody.

6.3.1 Determination of the kinetic constants of PifXa

The kinetic values of PifXa were measured using fXa and the substrate chromozym t-PA. First the kinetic constants for fXa was determined using various concentrations of substrate (0-1.25 mM)

A



B

	fXa
V_{max} [mM/min]	0.017±0.001
K_M [mM]	0.130±0.030

Figure 6.9: Michaelis-Menten curve for fXa. (**A**) Various concentrations of substrate (chromozym t-PA) were mixed with 50 nM of fXa. The reaction was followed for 20 min, and the reaction velocity was calculated, as the slope of the curve in the first 2 min of the reaction. The reaction velocity was plotted against the substrate con-

centration. (**B**) The curve was fitted to the Michaelis-Menten equation to obtain the constants K_M and V_{max} using the program GraphPad Prism.

By fitting the curve obtained by plotting the reaction velocity as a function of substrate concentration to the Michaelis-Menten equation, the Michaelis constant, K_M, and the maximum velocity, V_{max}, was found to be 0.13 mM and 0.017 mM/min, respectively (Figure 6.9.B). The inhibitor constant (K_i) was found by adding different concentrations of PifXa to fXa and then adding various concentrations of substrate (0-0.125 mM). The substrate concentration was kept under the value of K_M calculated for fXa.

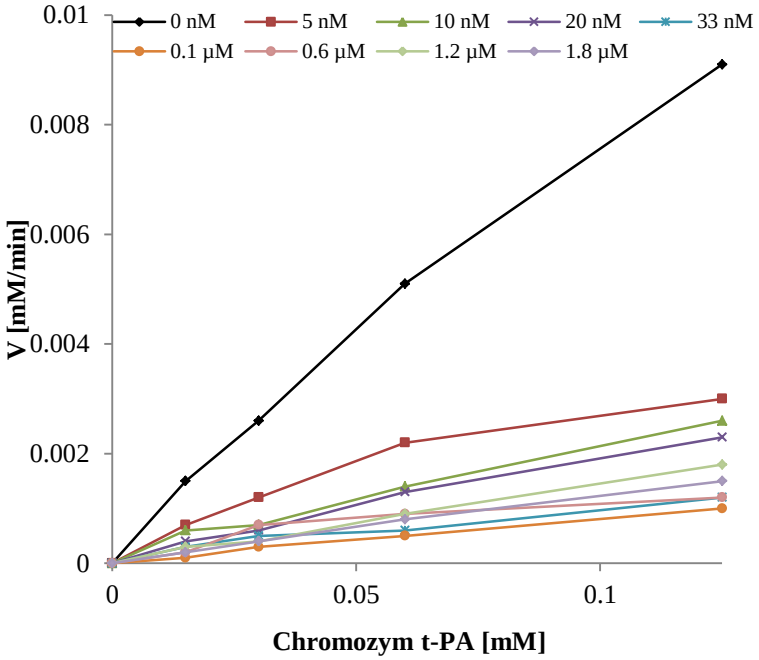


Figure 6.10: Inhibition curves for various concentrations of PifXa. The reaction rate versus the substrate concentration with various concentrations (0-1.8 µM) PifXa added. The reaction rate was calculated from the slope of the curves between 0-2 min.

In Figure 6.10 it can be seen that the reaction rate as expected decreases when various concentrations of the inhibitor is added. The curves were fitted to the Michaelis-Menten equation using the program GraphPad Prism and V_{max}, K_M, and K_i was determined. The results can be seen in Table 6.1.

Table 6.1: The kinetic constants for the inhibition of fXa by PifXa. V_{max} and K_M calculated for various concentrations (0-1.8 µM) PifXa using the program Graph Pad Prism. The inhibitor constant was calculated for a competitive, non-competitive and mixed mode of inhibition.

	0 nM	**5 nM**	**10 nM**	**20 nM**	**33 nM**	**0.1 µM**	**0.6 µM**	**1.2 µM**	**1.8 µM**
V_{max}	0.017	0.005	0.010	0.010	0.003	0.008	0.002	0.030	0.009

[mM/min]									
K_m [mM]	0.130	0.094	0.347	0.140	0.158	0.899	0.065	0.194	0.656
K_i mixed [nM]	5.944±3.925								
K_i comp. [nM]	2.993±0.698								
K_i non-comp. [nM]	4.033±0.640								

The different concentrations of PifXa gives rise to different values of K_M and V_{max}. Normally this would indicate that PifXa inhibits fXa in a mixed mode. However, the values of K_M and V_{max} seemed to be random. The inhibitory constant was therefore determined for both a mixed, competitive, and non-competitive mode of inhibition. This means that the inhibitory constant most likely is in the range from 3-6 nM.

The binding constant for PifXa to fXa was determined using fluorescence. Various concentrations of PifXa (0-5 µM) was added to 0.5 µM fXa in a buffer containing ANS. The solution was incubated for 5 min before the emission at 476 nm were measured and the data were plotted as a function of the inhibitor concentration.

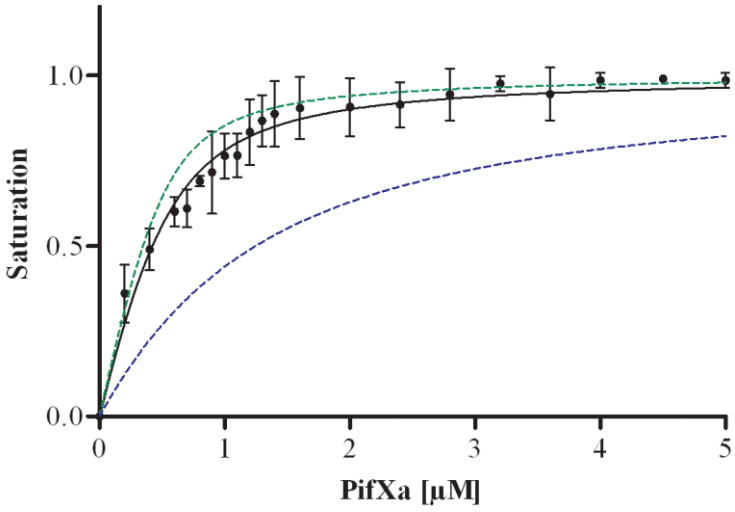


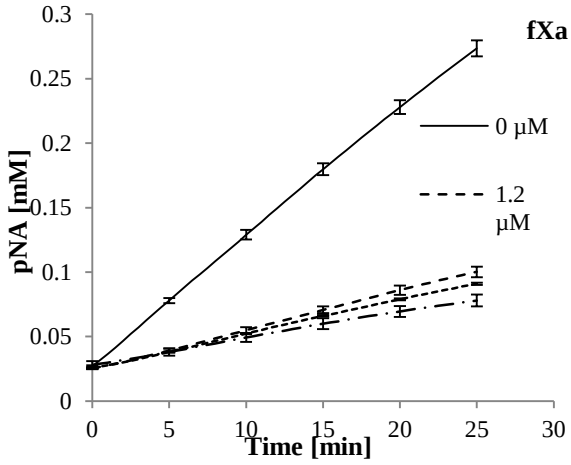
Figure 6.11 Binding curve for PifXa. The obtained data for emission at 476 nm were normalized between 0-1 and plotted as a function of the inhibitor concentration. The data points were fitted to Equation 5.2 (black line). The green and blue curves are the simulation of the data with a binding constant of 10^{-6} M or 10^{-7} M, respectively.

By fitting the measured data to Equation 5.2 the black curve seen in Figure 6.11 was obtained, and the K_D was calculated to be 0.173±0.014 µM. A simulation of the binding curves with a binding constant of either 10^{-6} M or 10^{-7} M was calculated for comparison (blue and green in the figure, respectively).

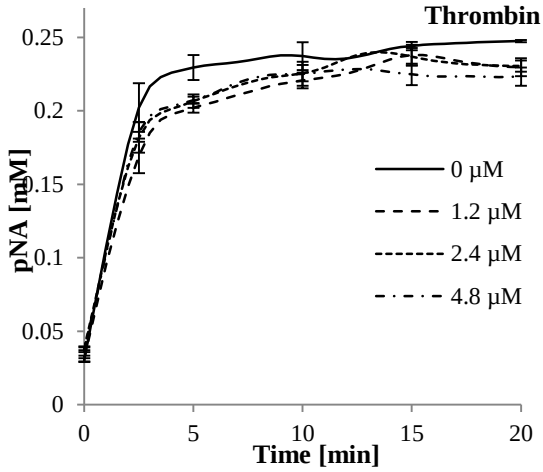
6.3.2 PifXa Inhibition of selected coagulation proteins

PifXa's specificity towards selected coagulation proteins (fXa, thrombin, aPC, t-Pa, and plasmin) was evaluated. The specificity towards trypsin was also examined (Figure 6.12). The affinity was tested using a chromogenic substrate specific towards the individual enzymes. Besides that the affinity towards thrombin was also tested in a fibrinogen cleavage assay (Figure 6.12.G).

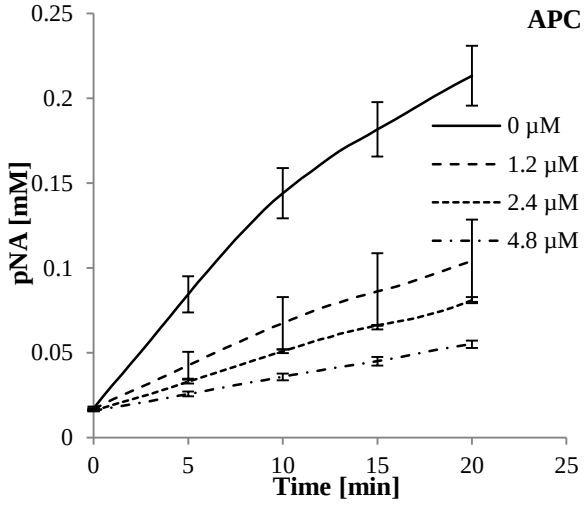
A



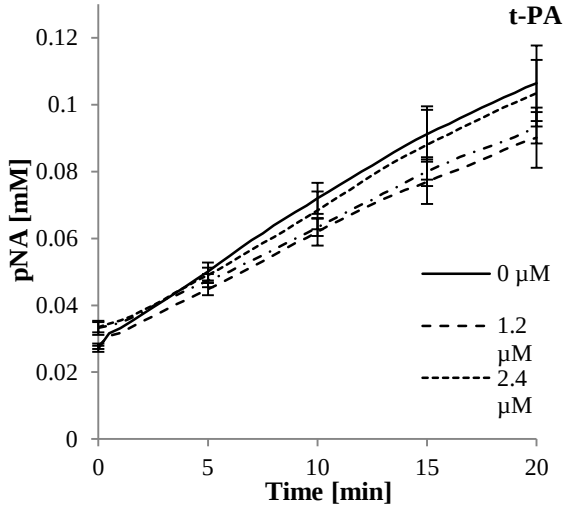
B



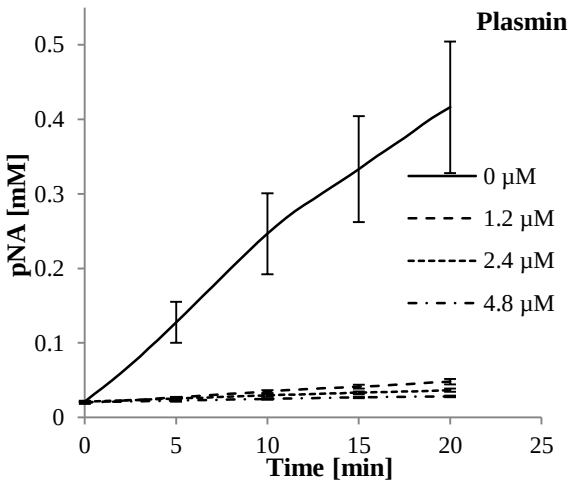
C



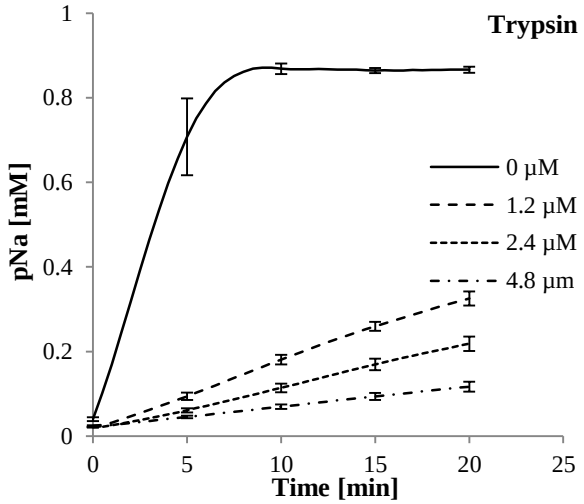
D



E



F



G

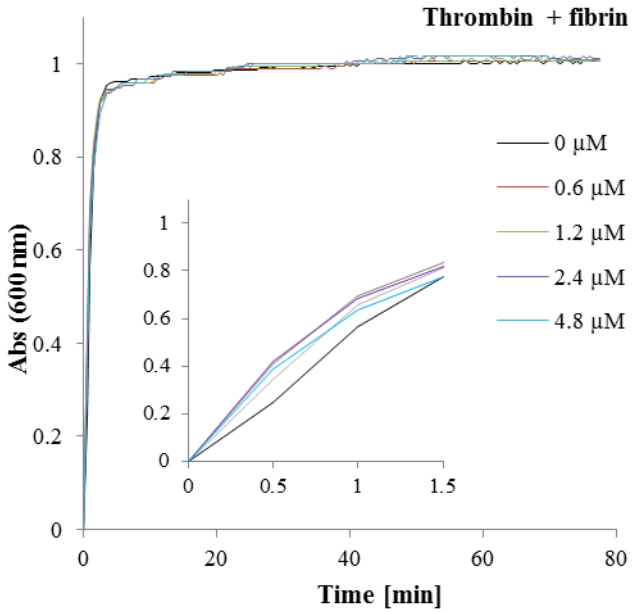


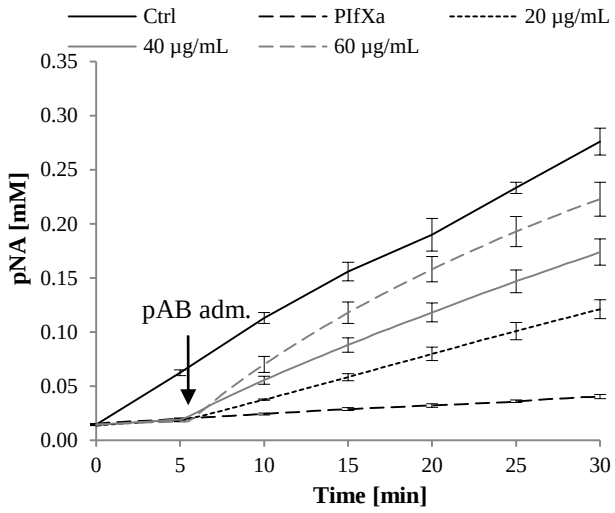
Figure 6.12: PifXa inhibition of fXa, thrombin, aPC, t-Pa, plasmin, and trypsin. Inhibition of (**A**) fXa (1 µM), (**B**) thrombin, (**C**) activated protein C, (**D**) t-PA, (**E**) plasmin, and (**F**) trypsin was evaluated with various concentrations of PifXa (0-4.8 µM). PifXa and the enzyme (20 nM) was mixed and incubated for 10 min at RT, where after enzyme specific substrate was added and absorbance were measured at 405 nm for 20 min. All data is presented as the mean ± the SD. For simplification the SD is only displayed for some of the data points. (**G**) Fibrinogen (3 mg/mL) cleavage assay with thrombin (0.063 µg/mL) and various concentrations (0-4.8 µM) of PifXa. PifXa and thrombin was mixed for 15 min before fibrinogen was added and turbidity was measured for 80 min. The smaller window shows the graphs from 0-1.5 min.

PifXa was according to the results capable of inhibiting fXa, aPC, plasmin, and trypsin (Figure 6.12.A, C, E, and F) in a concentration dependent manner. It was, however, not able to inhibit thrombin and t-PA. The inhibition of thrombin was tested both with a synthetic chromogenic substrate, and with the natural substrate fibrinogen. Both experiments strongly implied that PifXa does not inhibit thrombin (Figure 6.12.B and G).

6.3.3 The effect of PifXa can be reversed by a polyclonal antibody

A polyclonal antibody against PifXa was raised in rabbits. By using the amidolytic activity assay it was tested whether it was possible to reverse the inhibiting effect by the addition of the polyclonal antibody. PifXa was incubated with fXa, for 10 min where after the substrate was added and the color development was measured at 405 nm. After five min various concentrations of the antibody was added and the measurements were continued for 25 min.

A



B

	Reaction rate ± SD [µM/min]
Ctrl	9.43 ± 0.35
PifXa	0.80 ± 0.00
20 µg/mL	4.17 ± 0.40
40 µg/mL	6.13 ± 0.49
60 µg/mL	8.23 ± 0.47

Figure 6.13: Inhibition of fXa can be reversed by addition of antibody specific for PifXa. (**A**) FXa with or without PifXa (4.8 µM) was incubated for 10 min before the substrate was added. After another 5 min various concentrations (0-60 µg/ml) of polyclonal antibody serum specific for PifXa was added and the reaction was followed for another 25 min. The arrow indicates the polyclonal antibody (pAB) administration. All data is presented as the mean ± the SD. For simplification the SD is only displayed for some of the data points. (**B**) The reaction rate was calculated for all reactions after addition of the antibody.

After the addition of polyclonal antibody serum to the reaction mixture, the absorbance immediately began to rise (Figure 6.13.A) indication that the antibody binds to PifXa and prevents it from inhibiting fXa. Indeed, addition of 60 µg/mL antibody resulted in an almost

complete reversion of the inhibition with a reaction rate of 8.23 µM/min when the antibody was added compared to 9.43 µM/min for the control (Figure 6.13.B).

6.3.4 PifXa does not inhibit fX activation

To see if PifXa bound to fX could inhibit the activation of the enzyme, a factor X activation assay using Russell's viper venom (RVV) was designed. FX was incubated with PifXa before RVV was added. The sample was analyzed, together with a number of different controls, on a 15 % SDS PAGE gel under both reducing and non-reducing conditions.

A B

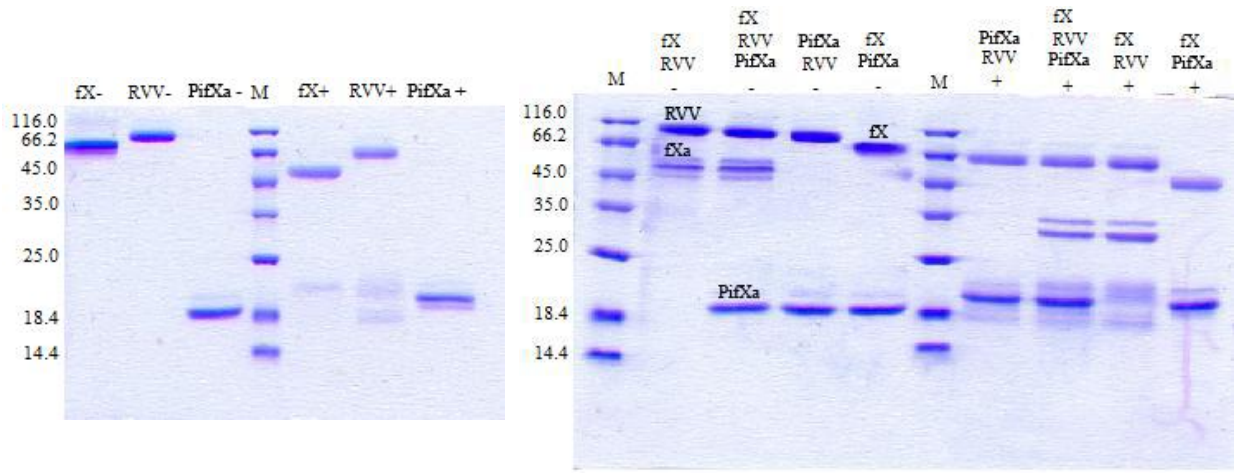


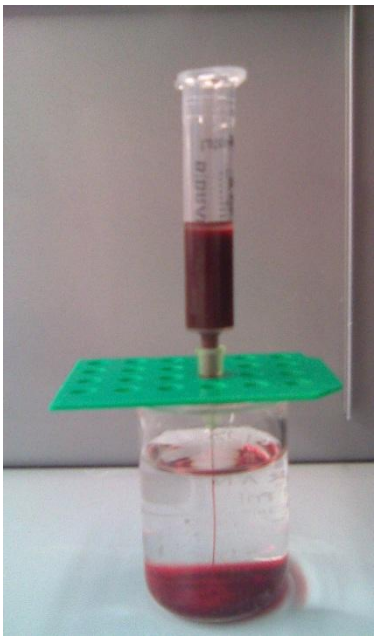
Figure 6.14: FX activation with RVV with and without added PifXa. (**A**) fX (1.6 µM), Russell's Viper Venom (RVV) (1.6 µM), and PifXa (4.8 µM) on a 12 % SDS-gel. (**B**) 12 % SDS-gel with mixtures of fX, RVV, and PifXa. fX and PifXa were incubated at RT for 10 min before RVV was added. The solution was incubated for 45 min at RT before it was mixed with loading buffer, heated for 5 min at 95 °C, and loaded on the gel. The additional samples were incubated fore 45 min at RT. – and + indicates that either non-reduced or reduced loading buffer was used.

Figure 6.14.A shows the protein band pattern of fX, RVV and PifXa under reducing and non-reducing conditions, respectively. When fX was incubated with RVV there was a shift in protein band size from ~60 kDa to ~50 kDa indicating an activation of factor X. This protein band shift was also observed in the sample containing fX, RVV, and PifXa, showing that PifXa does not affect the activation of fX. PifXa alone did not influence the protein band of either fX or RVV (Figure 6.14.B).

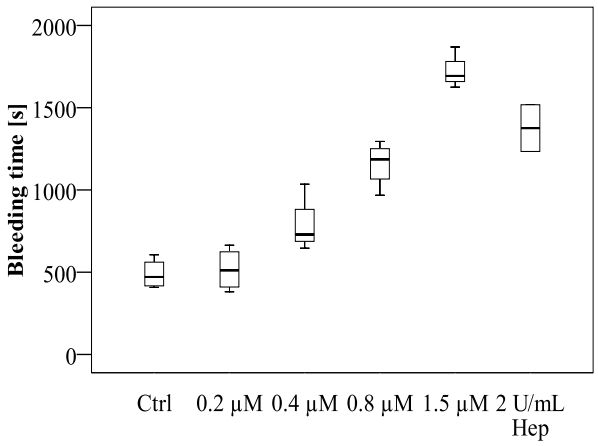
6.3.5 PifXa prolongs whole blood coagulation time

Various concentrations of PifXa (0-1.5 µM) was added to fresh human citrated whole blood and incubated for 10 min before $CaCl_2$ was added to initiate coagulation. The blood mixture was added to a glass Pasteur pipet placed inside syringe with a needle attached, and the needle was placed in water (Figure 6.15.A). The time until the bleeding stopped was measured together with the absorption at 405 nm of the water.

A



B



C

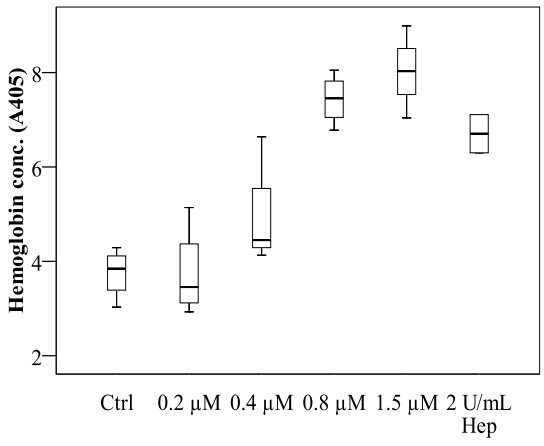


Figure 6.15: Inhibition of whole blood coagulation by PifXa. (**A**) The experimental set up. 0-1.5 µM of PifXa was added to fresh whole blood. 2 IU/ mL heparin was used as a positive control for the inhibition. (**B**) The coagulation time and (**C**) hemoglobin amount (A405) were measured. $CaCl_2$ was used to initiate coagula-

tion. Each concentration was done with four different donors. The data are presented as boxplots, where the top and bottom of the box represent the upper and lower quartile, respectively, the line in the middle of the box is the median, and the last lines are the highest and smallest observation.

Figure 6.15B and C shows a concentration dependent prolongation of both the bleeding time and amount of red blood cells flowing through the syringe. At a concentration of 1.5 µM of PifXa the bleeding time was about 30 min, which exceeded that of the control sample containing a therapeutic dose of heparin (2 U/mL).

6.3.6 PifXa does not affect platelet aggregation *in vitro*

The effect of PifXa on platelet aggregation was tested in platelet rich plasma. Figure 6.16.A shows platelet aggregation induced with various concentrations of ADP in the absence or presence of PifXa. At concentrations between 0-3.2 µM ADP, PifXa showed no effect of platelet aggregation compared to the control.

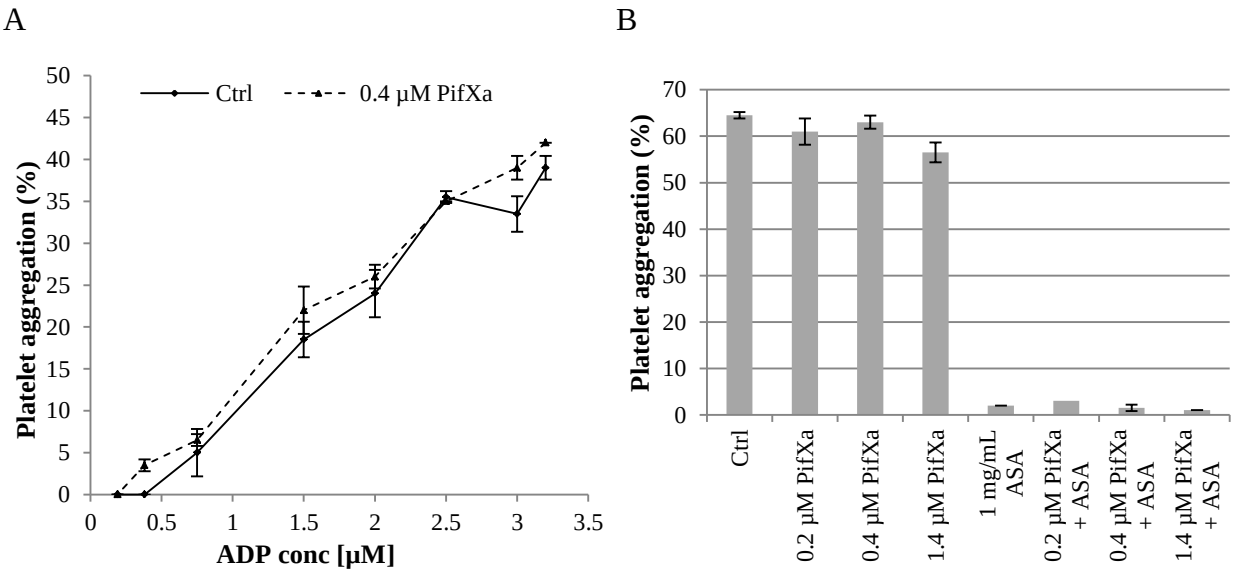


Figure 6.16: PifXa does not affect platelet aggregation. (**A**) Platelet rich plasma with and without 0.4 µM PifXa added was activated with various concentrations of ADP (0-3.2 µM). All measurements were carried out twice. (**B**) The effect of PifXa (0.2-1.4 µM), acetylsalicylic acid (1 mg/mL) or a combination of both was tested. The aggregation was activated using 1.5 mM arachidonic acid and the aggregation was measured at 37 ^{o}C. Platelet rich plasma was used as a control. The data is presented as mean ± SD.

In another aggregation experiment various concentrations of PifXa were tested both in the presence of aspirin and alone. As can be seen in Figure 6.16.B addition of 1 mg/mL aspirin

dramatically inhibited platelet aggregation, while none of the tested concentrations of PifXa had any influence on the aggregation or the inhibition by aspirin, respectively.

6.3.7 PifXa effect standard coagulation tests

Different standard coagulation tests were conducted with various concentrations of PifXa (0-200 µg/mL), UFH, LMWH, or Fondaparinux (0-2 IU/mL).

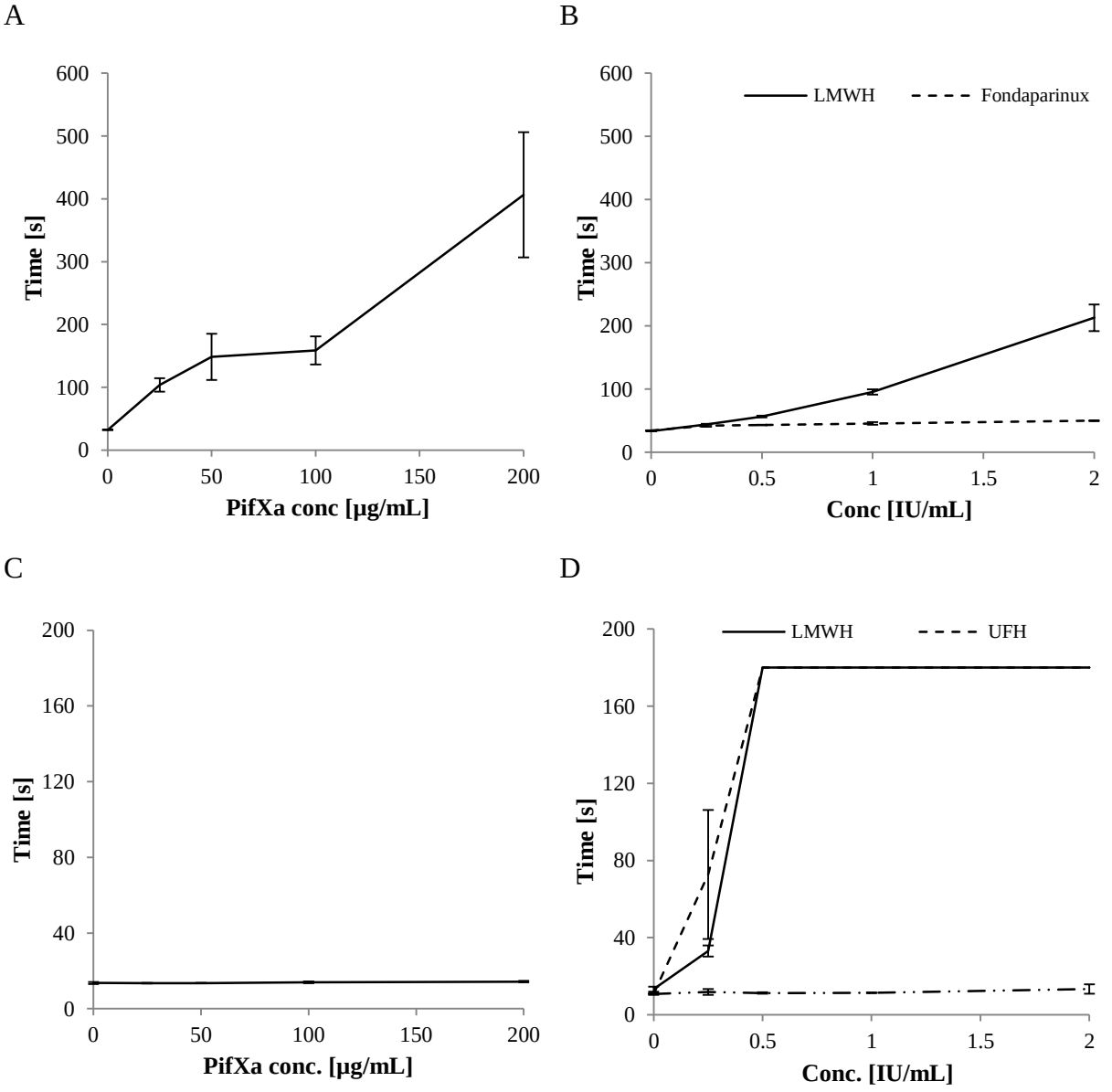


Figure 6.17: APTT and thrombin time assay with PifXa, LMWH, UFH, and Fondaparinux. Plasma with (**A**) various concentrations of PifXa, UFH, (**B**) LMWH, or Fondaparinux was mixed with aPTT reagent. Coagulation was initiated by adding $CaCl_2$, and was measured for 600 s. The thrombin time were measured for plasma containing (**C**) PifXa, (**D**) UFH, LMWH, or Fondaparinux.

PifXa showed a concentration dependent prolongation of the aPTT (Figure 6.17.A) to about 400 s at the highest tested concentration, in comparison the highest concentration of LMWH only prolonged aPTT to 200 s, while Fondaparinux had no effect in the assay (Figure 6.17.B). All tested concentrations of UFH extended the coagulation time beyond 600 s.
Neither PifXa nor Fondaparinux prolonged the coagulation time in the thrombin time assay, while both LMWH and UFH even a low concentrations extended coagulation beyond 180 s (Figure 6.17.C and D). None of the tested inhibitors were able to prolong the prothrombin time to above that of the normal range (20 s) in either the Owren or the Quick PT test (data not shown). The results from the standard coagulation tests are summarized in Table 6.2.

Table 6.2: PifXa is able to prolong aPTT, but does not affect PT and thrombin time. A summarization of the results from the standard coagulation assays with PifXa, LMWH, UFH and Fondaparinux.

	PifXa	**LMWH**	**UFH**	**Fondaparinux**
aPTT	Slight prolongation	Slight prolongation	Prolong	No effect
PT	No effect	No effect	No effect	No effect
Thrombin time	No effect	Prolong	Prolong	No effect

6.4 PifXa is a potent specific anticoagulant *in vivo*

The effect of PifXa was tested *in vivo* using rats as the model animal. The rats were given a dose of inhibitor through the vein tail under sedation. After 10 min the outer tip of the tail was cut off and the remainder was placed in warm saline. Both the bleeding time, and the amount of released red blood cells were measured.

6.4.1 PifXa prolongs bleeding time but not bleeding amount

To evaluate the efficiency of PifXa *in vivo* doses of up to 1.25 mg of inhibitor (5.2 mg/kg) were administered intravenously to anesthetized rats. The rats were divided into a low, middle and high dose group. The bleeding was followed for 30 min, and the bleeding time and amount of bleeding in to saline was recorded (Figure 6.18).

A B

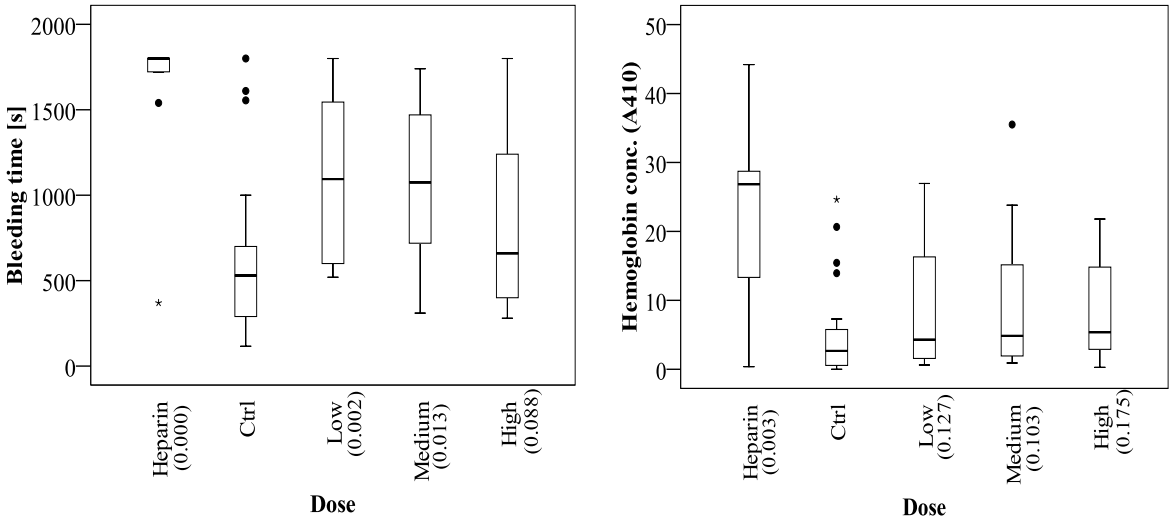


Figure 6.18: PifXa inhibits blood coagulation *in vivo*. Results from the dose dependent *in vivo* experiment with an observation time of 30 min. Both the bleeding time (**A**) and amount of red blood cells (**B**) were measured. Following doses was given to the rats: 200 IU/kg heparin (n=11), Control (Control or 0 mg/kg) (n=21), Low dose (0.25 or 0.5 mg/kg) (n=14), Medium dose (0.8 or 1.0 mg/kg) (n= 10) and High dose (2, 3 or 5.2 mg/kg) (n=13). All data is presented as boxplots, where the top and bottom of the box represent the upper and lower quartile, respectively, the line in the middle of the box is the median, and the last lines are the highest and smallest observation. ● and * indicates outliers and extreme outliers, respectively. The *P* values are given in parentheses below the label, and are a comparison between the control group and the given group.

The rats receiving heparin bleed in average the entire duration of the experiment. The rats receiving PifXa did not bleed as long as the rats given heparin, but still significantly longer than the control group (P=0.002 for the low dose group). Surprisingly full effect was obtained with the lowest dose of inhibitor. There was not a significant difference in bleeding time between the three different dose groups of PifXa.

The bleeding amount of the rats given heparin was significant different than the control group (P=0.003). This was, however, not the case with either of the groups that were given PifXa; here the bleeding amount was not significantly different from the control (P>0.1).

6.4.2 The effect of PifXa is additive to the effect of Aspirin

Prompted by the different effects of PifXa observed in the bleeding time and the amount of blood cells leakage the additive effect of the anti-platelet drug aspirin in the rats receiving PifXa, were tested. Besides that, groups of rats were given Fondaparinux or heparin to serve as controls (Figure 6.19).

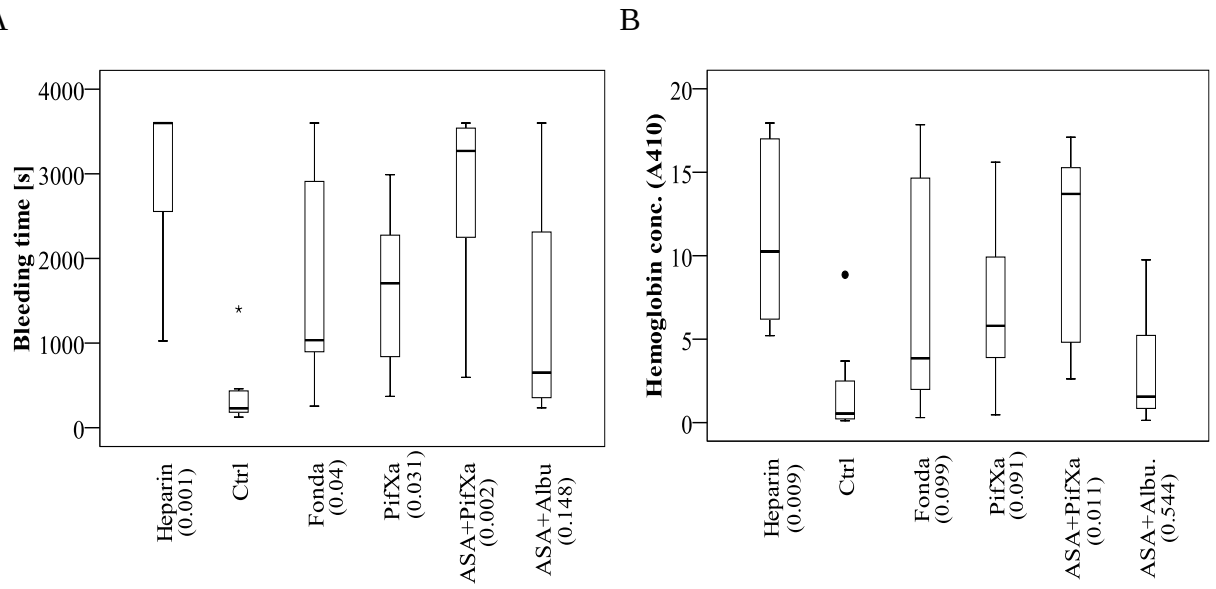


Figure 6.19: Aspirin has an additive effect on PifXa *in vivo*. The additive effect of aspirin was tested with an observation time of 60 min. Both the bleeding time (**A**) and released hemoglobin concentration (**B**) was measured. The following doses was given to the rats: Ctrl (control or 2 mg/kg albumin) (n=7), 200 IU/kg heparin (n=6), 0.1 mg/kg Fondaparinux (n=7), 2 mg/kg PifXa (n= 6), 2 mg/kg PifXa and 16 mg/kg acetylsalicylic acid (n=7), and 16 mg/kg acetylsalicylic acid and 2 mg/kg albumin (n=7). All data is presented as boxplots, where the top and bottom of the box represent the upper and lower quartile, respectively, the line in the middle of the box is the median, and the last lines are the highest and smallest observation. ● and * indicates outliers and extreme outliers, respectively. The *P* values are given in parentheses below the label, and are a comparison between the control group and the given group. A *P* value < 0.05 is considered significant. The *P* value of the comparison between the ASA and PifXa group and the ASA and albumin group are *P* = 0.102 for the bleeding time and *P* = 0.028 for the hemoglobin concentration.

Aspirin alone did not have a significant effect on either the bleeding time or amount. However, when given together with PifXa, both the bleeding time and amount was significantly different from the control. The effect of Fondaparinux in the assay was similar to that of PifXa given alone, both prolonged the bleeding time significantly, but did not affect the bleeding amount.

6.5 Attempts to determine the half-life of PifXa *in vivo*

In order to determine the rate of clearance of PifXa from the system *in vivo*, rats were given 0.2 mg IV, and blood samples were taken at 15 different time points (see section 5.2.3.4). Due to the very low concentration of PifXa in the plasma sample (~ 0.8 µg) already at the first time point, different detection methods were investigated.

6.5.1 Western Blot

The detection limit of the western blot was determined by diluting PifXa in either buffer (25 mM hepes with 100 mM NaCl, pH 7.2) or human plasma (Figure 6.20).

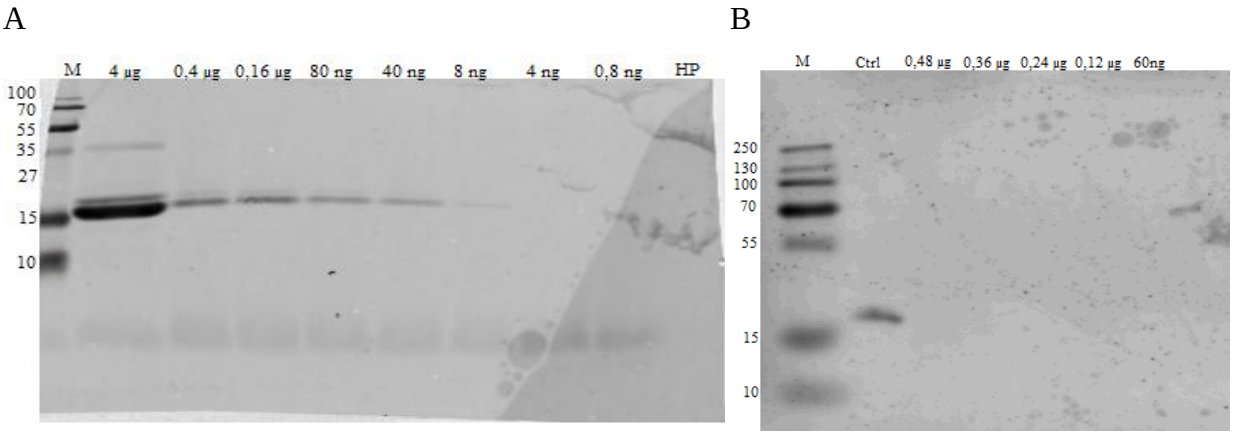


Figure 6.20: Western Blot on a PifXa dilution series. (A) PifXa was diluted in buffer (25 mM hepes, 100 mM NaCl pH 7.2) mixed with loading buffer, loaded on a 12 % SDS PAGE gel, run and blotted. A sample of human plasma (HP) was added as a negative control. **(B)** PifXa diluted in human plasma. The control sample is 4 µg PifXa diluted in buffer.

As can be seen on the figure (Figure 6.20.A) it was possible to see a band at ~20 kDa in all samples except the two lowest concentrations (4 and 0.8 ng) and in the negative control (human plasma). Another experiment where PifXa was diluted in plasma was also carried out. In this experiment 4 µg PifXa in buffer served as a positive control. Only the control sample was visible on the gel, while none of the tested concentrations of PifXa in plasma could be seen on the blot (Figure 6.20.B). The poor detection limit of the control samples with PifXa in plasma made western blotting unfit for use in detection of the inhibitor in the blood samples drawn from the rats.

6.5.2 iELISA

Since the western blot experiment was not sensitive enough to see the very low concentrations of PifXa in the clearings-rate samples. The sensitivity of iELISA was tested for both PifXa in plasma and buffer.

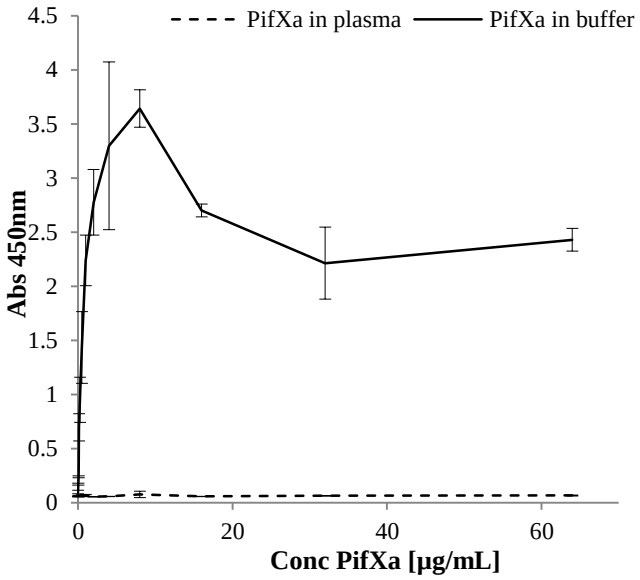


Figure 6.21: iELISA on various concentrations of PifXa diluted in buffer or human plasma. Various concentration of PifXa (0-64 µg/mL) in either buffer (–) or human plasma (--) was bound to microtitter plates. Antibody specific for PifXa was added and thereafter secondary antibody. Substrate was added and the color was allowed to develop for 30 min where after the absorbance was measured at 405 nm.

Various concentrations of PifXa (0-64 µg/mL) in either human plasma or PBS buffer was bound to the wells of the ELISA plate and tested. In the samples with PifXa diluted in buffer a concentration dependent absorbance signal was seen. However, there was no difference in absorbance in either of the samples PifXa diluted in plasma (Figure 6.21). Performing the experiment in high binding ELISA plate did not change the result. An attempt to purify the inhibitor from the plasma by a mono S ion exchange column followed by ELISA was also tried, but this did not show any improvement of the results. Hence, iELISA was also not sensitive enough to detect the inhibitor in the blood samples from the rats.

6.5.3 Iodine 131 labeled PifXa

Instead of detecting the inhibitor in the blood samples drawn from the rats at different time points, an entire new approach was tried out. PifXa was labeled with radioactive isotope iodine 131 and injected IV in tail of 8 rats. 4 had the tip of the tail cut off and 4 were left untouched. After 60 min selected organs were removed and the radioactivity was measured. The results are shown in the table below (Table 6.3).

Table 6.3: The radioactivity measured from different organs in rats with or without tail cut. The radioactive count from the different tissues was adjusted to decay/min/mg tissue.

Organ	Tail cut (n=3) [decay/min/mg tissue]	No tail cut (n=3) [decay/min/mg tissue]
Blood from gaze	68 ± 6	-
Blood sample after 40 min	30 ± 4	32 ± 6
Cut of tail tip	67 ± 3	60 ± 9
2 mm tail after cut	75 ± 10	65 ± 36
Next 2 mm tail	93 ± 2	102 ± 3
Heart	16 ± 2	16 ± 2
Lung	30 ± 5	41 ± 9
Liver	25 ± 3	27 ± 1
Kidney	1119 ± 85	1133 ± 102
Spleen	51 ± 3	58 ± 7
Muscle	7 ± 2	6 ± 1

Because of injection problems in two of the eight rats, the results in Table 6.3 are based on only six rats, three in each group. As it appears from the table there are no significant differences in the radioactive decay in the different organs in the two groups. The greatest level of activity was found in the kidneys, whereas little activity was seen in the other organs. There is no obvious difference in the radioactivity in any of the three pieces of tail. Blood samples drawn from three of the rats after 10 min showed a decay/min/mg at 1070 ± 714.

Unfortunately none of the used experiment proved to be effective to determine the half-life of PifXa *in vivo*. In the first two cases the detection limit was too high to measure the low concentration of the inhibitor. In the last case there were no significant different of radioactive signal in any of the measured tissues, except in the kidney where the signal was thought to stem from unbound iodine.

6.6 Expression and purification of mutant variants of PifXa in *E. coli*

A bacterial recombinant variant of PifXa was made in the Gateway system from Invitrogen and expressed in Rosetta Gami B (DE3) *E. coli* cells. The recombinant wildtype (rWT) was used as a control together with the potato derived protein (WT) when the inhibiting activity

toward fXa of different mutants was tested. Figure 6.22 shows the chromatogram from the IMAC purification of the recombinant variant of PifXa from cleared cell lysate from 2 L cell culture and an SDS gel with selected samples, the protein solution before purification, and the flowthrough.

A B

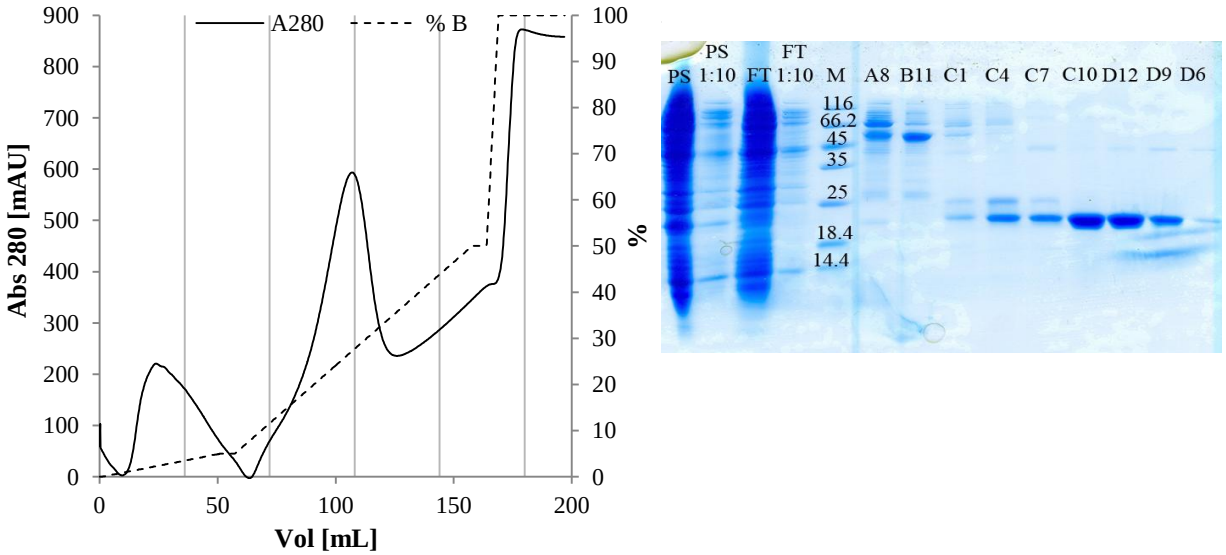


Figure 6.22: Purification of recombinant PifXa expressed in Rosetta Gami B (DE3) *E. coli* cells. (**A**) Chromatogram from the Ni-NTA purification of cleared lysate from 2 L cell culture. Fraction size during elution was 3 mL. The vertical lines divide the chromatogram into the different fractions. The first line contains fraction A1-12, the second contains B12-1, and so forth. (**B**) 15 % non-reducing SDS PAGE gel of selected fractions from the purification. PS indicate the protein sample before purification, while FT is the flow through.

The chromatogram displays two different peaks. The first peak from ~7-60 mL contains loosely bound protein with a size of around 60 kDa. The assumed recombinant PifXa was eluted between ~ 60 and 125 mL and had a molecular weight at around 21 kDa. When tested in the amidolytic activity assay the inhibitory activity against fXa of these fractions were comparable with the inhibitory activity of the wildtype PifXa. Moreover, using MS/MS, the protein in in these fractions was indeed confirmed to be the recombinant PifXa. The protein band at 60 kDa was identified to be protein alkyl hydroperoxide reductase, F52a subunit (ahpF) (gi|24111954) from *E. Coli*.

6.6.1 Insertion of fXa's recognition site in PifXa

The area around the putative RCL from PifXa (see section 3.2) was exchange with the recognition site for fXa found in prothrombin (IEGR) by overlap extension PCR (OEPCR). The putative loop was determined based on a prediction on the secondary structure and an alignment with STI. The secondary structure for PifXa was determined using the Discrimination of protein Secondary structure Class (DSC) algorithm in the program Genamics Expression (Figure 6.23).

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50         60
KPI A-k1        -PVLDTNGKE LNPNLSYRII STYWGALGGD VYLGKSPNSD APCPDGVFRY NSDVGP-SGT
STI             DF...NE.NP .ENGGT.Y.L .DIT-.F..- --IRAA.TGN ER..LT.VQS RNELDKGI..

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         70         80         90        100        110        120
                    IEGR2
                   IEGR1
KPI A-k1        PVRFIPLSTN IFEDQLLNIQ FN-IPTPKLC VSY-TIWKVG NINAPLRTML LETGGTIGQA
STI             IISSPYRIRF .A.GHP.SLK .DSFAVIM.. .GIP.E.S.- -VEDLPEGPA VKI.ENKDAM

                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        130        140        150        160        170        180
KPI A-K1        DSSYFKIVKS SN--FG-YNL LYCPITRHFL RDDNFCAKVG VVIQ--NGKR RLALVNENPL
STI             .G-W.RLERV .DDE.NN.K. VF..QQ---- AE.DK.GDI. IS.DHDD.T. ..VVSKNK..

                ....|....| ..
                        190
KPI A-k1        DVLFQEV--- --
STI             V.Q..KLDKE SL
```

Figure 6.23: Secondary structure determination of PifXa. The secondary structure has been predicted for PifXa using the DSC algorithm in the program Genamics expression, while the structure for STI has been determined experimental ((Song, Suh 1998)). The sequence for β sheets is highlighted in blue, the cysteins are marked with orange, and the RCL in STI are marked in red. Identical residues are marked with a (.) while gaps are denoted with a (-). The place of the two mutations made by OEPCR can also be seen.

It should be noted that a part of the sequence for PifXa (Cys151, Pro152, Phe153, Cys154) has been removed in order to get a better alignment, since it is believed that this small sequence make up a little loop that does not contribute much to the secondary structure.

The gene sequences containing the two mutants were as mentioned produced by OEPCR and cloned into an expression vector using the Gateway System from Invitrogen. After the sequence had been validated the proteins were expressed in Rosetta Gami B (DE3) *E. coli* cells. The SDS gel with selected protein samples from the IMAC purification of the cleared cell lysate can be seen in Figure 6.24.

A B

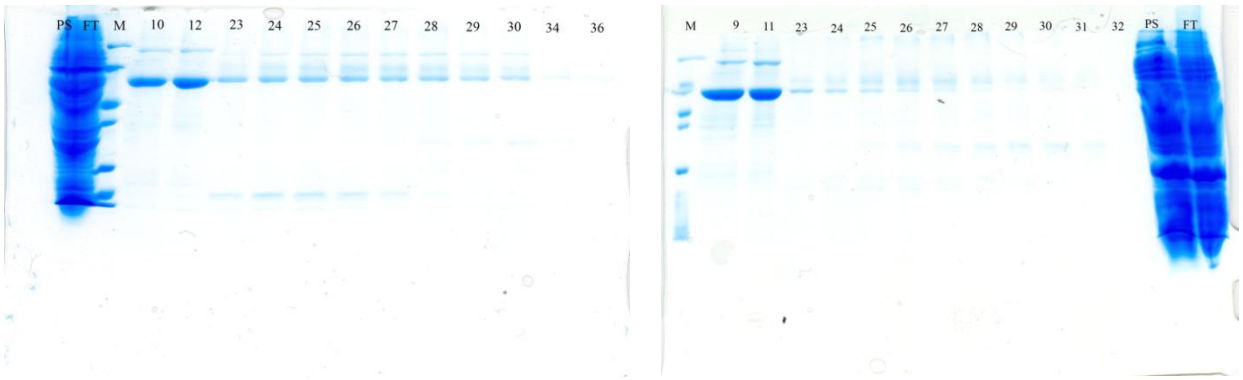


Figure 6.24: Purification of IEGR 1 and 2: 15 % non-reducing SDS PAGE gel with selected samples from the purification of (**A**) IGER 1 and (**B**) IGER 2, respectively. PS is the protein sample before purification and FT is the flowthrough.

The expression of the two IGER mutations was not as successful as the wild type, as can be seen by the very weak bands on the two SDS gels, and the samples containing the mutants was not entirely pure. However, the samples contain the mutant proteins were pooled and concentrated and the inhibitory activity towards fXa was tested in the amidolytic activity assay with rWT and WT as controls (Figure 6.25).

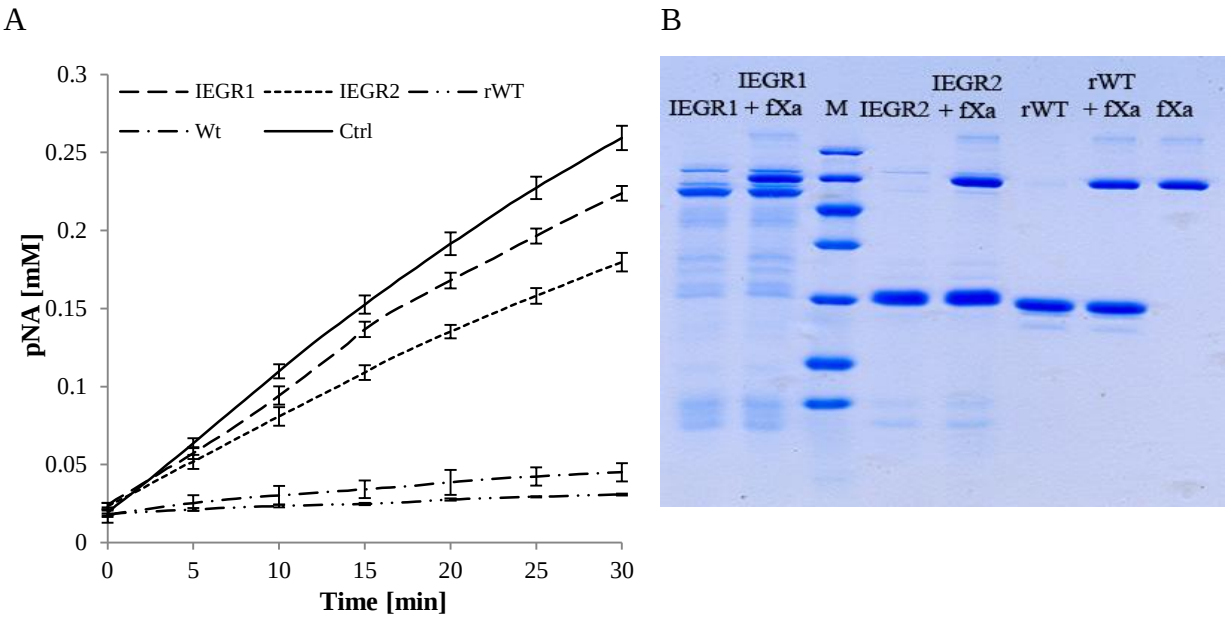


Figure 6.25: Amidolytic activity assay of wildtype, recombinant wildtype and the two IEGR mutant variants. (**A**) 100 µg/mL (1.8 µM) of each of the four variants of PifXa was mixed with 1 µM of fXa and incubated at RT. After 10 min 0.5 mM chromozym t-PA was added and the absorbance was measured for 30 min at 405 nm. (**B**) 12 % reducing SDS PAGE gel displaying pooled and concentrated samples of IEGR1, IEGR2 and rWT either alone or together with fXa. The protein solution was allowed to incubate for 30 min at RT before reducing loading buffer was added and the sample was heated.

None of the two variant types of PifXa were according to the assay capable of inhibiting fXa in the same degree as the two controls (Figure 6.25.A). The SDS PAGE gel shows that the content of the IEGR1 variant in the sample is very low, hence the expression of this variant is unsuccessful. This again explains the lack of inhibitory activity of the sample. The concentration of IEGR2 is, however, similar to that of the rWT variant meaning that the lack of inhibitory activity of this sample is not due to low concentration. It was speculated if the weak inhibition by the IGER2 mutation was caused by the inhibitor being cleaved by fXa. However, the gel does not show any change in pattern of the protein band for the inhibitor when fXa is added. Hence, it is not likely that the poor inhibition can be explained by a cleavage of the inhibitor.

6.6.2 Mutation in the putative reactive center loop

In order to pinpoint the area of interaction between PifXa and fXa, three different mutant variants of PifXa were produced. Two loop swap mutations were produced since previous experiments have shown that only the KPI A-k1 variant, but not the KPI B-k1 variant, is capable of

inhibiting fXa (Andersen 2007). DNA constructs were therefore made using OEPCR, where the putative RCL in KPI A-k1 was replaced with that from KPI B-k1 and vice versa. The strategy can be seen in Figure 6.26.

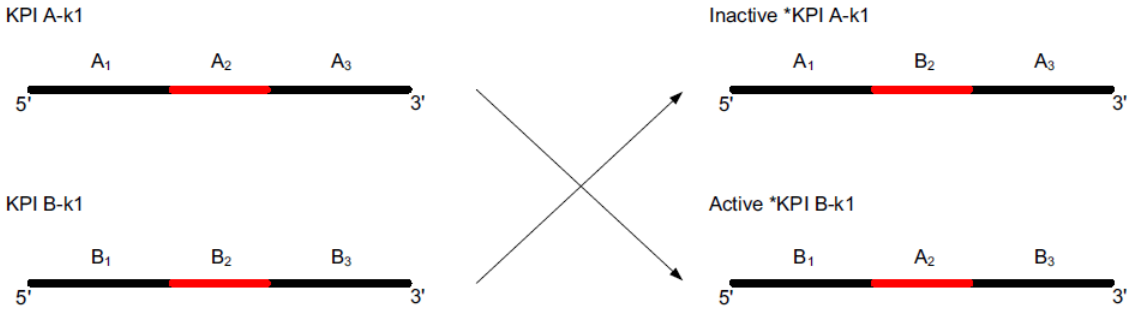


Figure 6.26: Strategy for producing *KPI A-k1 and *KPI B-k1. Both templates were divided into three parts; an N-terminal, C-terminal, and at middle part. All six parts were produced by PCR, purified, and reassembled using OEPCR.

In the other mutant (rKPI A-k1(PL-AA)) two amino acids in the putative RCL was replaced (P102A, L103A, numbering according to Figure 6.27). The sequence was synthetically produced by the company GeneScript and cloned into an expression vector using the Gateway system from Invitrogen. The sequences of all three mutants and the sequences for KPI A-k1 and KPI B-k1 can be seen in Figure 6.27.

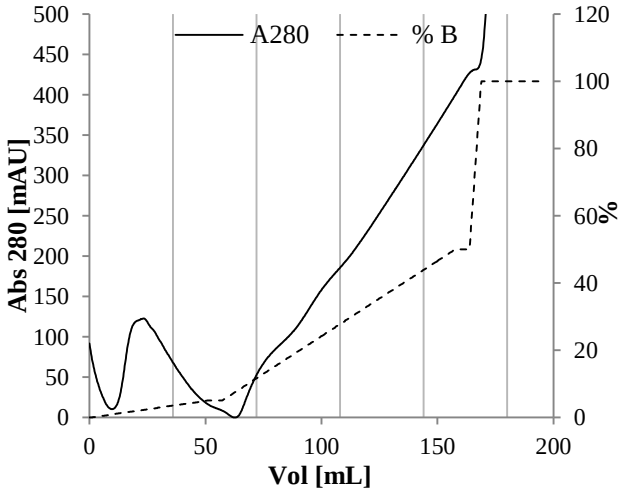
```
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     10         20         30         40         50         60
KPI A-k1    MMKCLFFLCL CLFPILVFSS TFTSQNPINL PSESPLPKPV LDTNGKELNP NLSYRIISTY
KPI B-k1    -.....L... ..V..V.... .......... ..DA---T.. ..VT....D. R........F
*KPI A-k    .......... .......... .......... .......... .......... ..........
*KPI B-k    -.....L... ..V..V.... .......... ..DA---T.. ..VT....D. R........F
PL-AA       .......... .......... .......... .......... .......... ..........

            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     70         80         90        100        110        120
KPI A-k1    WGALGGDVYL GKSPNSDAPC PDGVFRYNSD VGPSGTPVRF IPLS----TN IFEDQLLNIQ
KPI B-k1    .......... .......... AN........ .......... .GS.SHFGPH ...GE.....
*KPI A-k    .......... .......... .......... .......... .GS.SHFGP. ..........
*KPI B-k    .......... .......... AN........ .......... ....----.H ...GE.....
PL-AA       .......... .......... .......... .......... .AA.----.. ..........

            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                    130        140        150        160        170        180
KPI A-k1    FNIPTPKLCV SYTIWKVGNI NAPLRTMLLE TGGTIGQADS SYFKIVKSSN FGYNLLYCPI
KPI B-k1    .D.S.V.S.. ........DY D.S.G..... .......E.. .W.......Q L........A
*KPI A-k    .......... .......... .......... .......... .......... ..........
*KPI B-k    .D.S.V.S.. ........DY D.S.G..... .......E.. .W.......Q L........A
PL-AA       .......... .......... .......... .......... .......... ..........
```

```
            ....|....| ....|....| ....|....| ....|....| ....|
                    190        200        210        220
KPI A-k1    TRHFLCPFCR DDNFCAKVGV VIQNGKRRLA LVNENPLDVL FQEV-
KPI B-k1    .--MI....S ..E..L.... IH........ ..KD.....S .KQ.Q
*KPI A-k    .......... .......... .......... .......... ....-
*KPI B-k    .--MI....S ..E..L.... IH........ ..KD.....S .KQ.Q
PL-AA       .......... .......... .......... .......... ....-
```

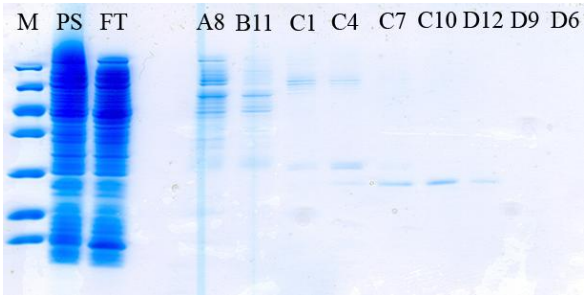
Figure 6.27: Protein sequence of KPI A-k1 (PifXa), KPI B-k1, the two loop swap mutants, and the PL-AA mutant. The protein sequence from KPI A-k1 (gi 73920922) and KPI B-k1 (gi 73920925) was aligned and the sequence in the putative RCL from KPI A-k1 was inserted into the KPI B-k1 sequence and vise visa creating the two sequences *KPI B-k1 and *KPI A-k1, respectively. The loops that were exchange and the PL-AA mutation are marked with red in the sequence.

The DNA constructs for the three mutants were validated by RFLP analysis and sequencing before they were transformed into a Rosetta Gami B (DE3) *E. coli* strain where the proteins were expressed. The proteins were purified using a Ni-NTA column and selected samples were analyzed using SDS PAGE (Figure 6.28).

A



B



C

D

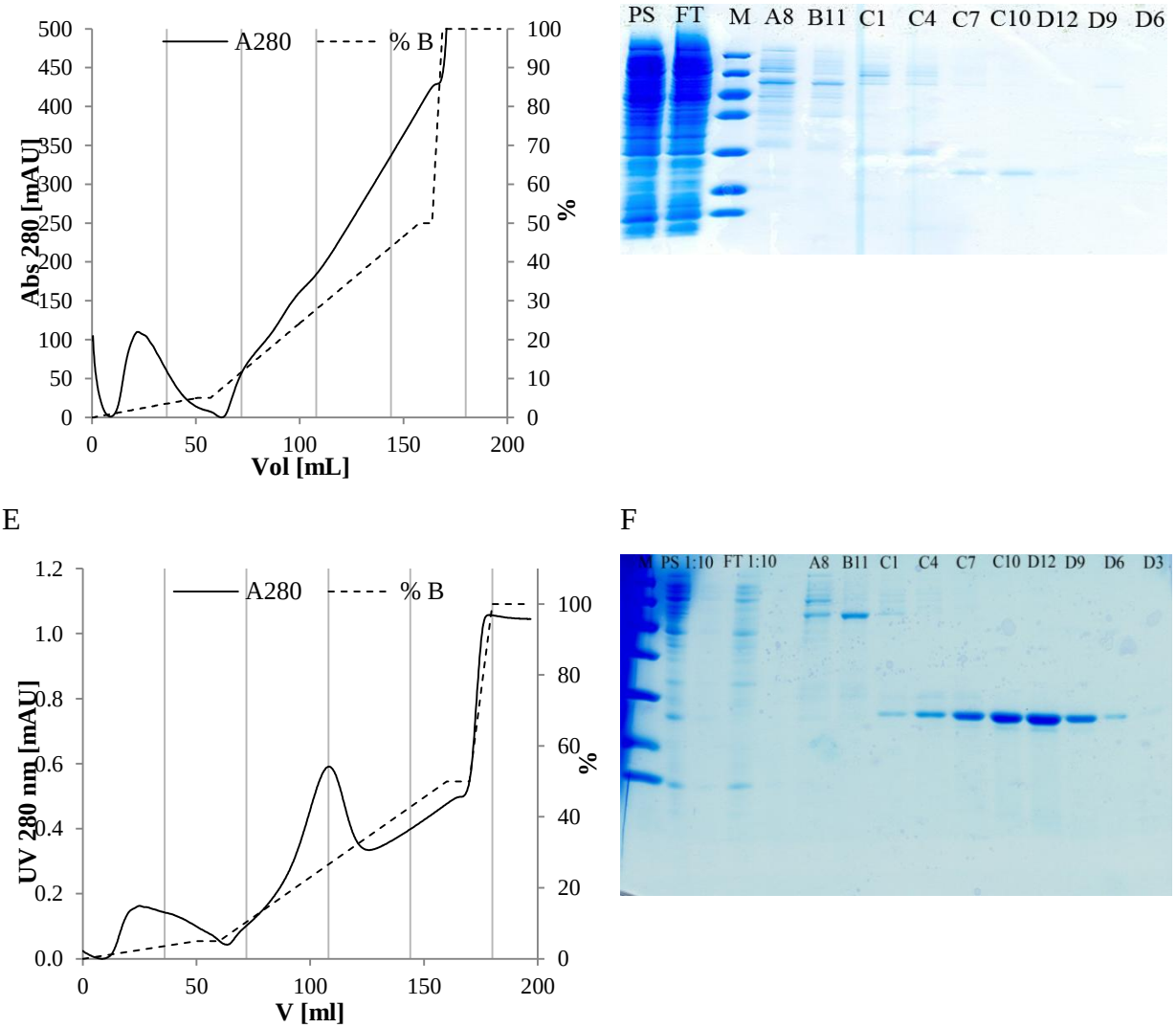
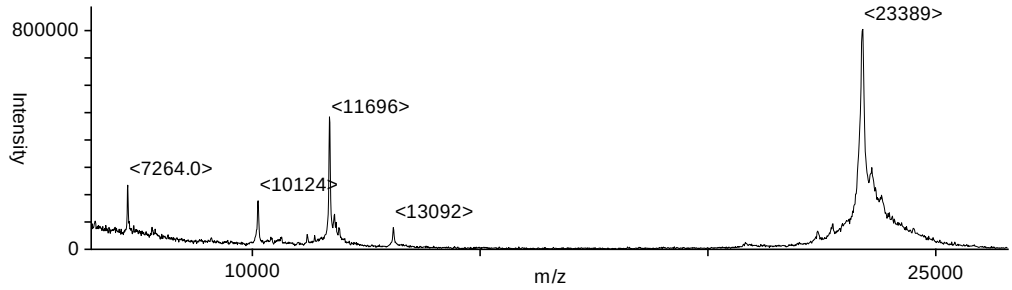


Figure 6.28: Purification of *KPI A-k1, *KPI B-k1, and rKPI A-k1(PL-AA) expressed in Rosetta Gami B (DE3) *E. coli* cells. (**A**, **C**, and **E**) The chromatograms from the Ni-NTA purification of cleared lysate from 2 L culture with *KPI A-k1, *KPI B-k1, and rKPI A-k1(PL-AA) respectively. Fraction size during elution was 3 mL. The vertical lines divide the chromatogram in the different fractions. The first contains the fractions A1-A12; the second contains B12-B1 and so on. (**B**, **D**, and **F**) Selected fraction from the purification analyzed on a 15 % non-reducing SDS PAGE gel. PS: protein sample before purification, FT: flowthrough.

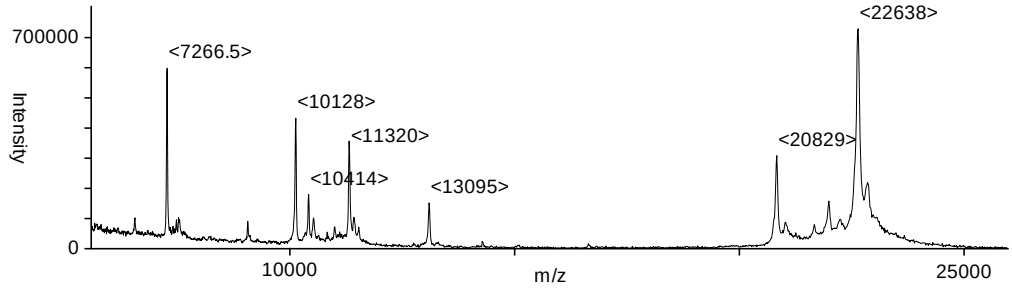
The chromatogram from the purification of both loop swap mutation indicates that the expression of the protein has been very low. On both chromatograms there are, however, a small peak in the area were the protein is expected to elute (60-125 mL). This is also observed on the SDS PAGE gel, where faint band with a size of around 21 kDa is seen (Figure 6.28.B and D). In contrast the expression of the rKPI A-k1(PL-AA) variant resulted in a large peak in the chromatogram which on the SDS PAGE gel revealed that proteins of the expected size had been expressed (Figure 6.28.E and F). MALDI TOF MS was used to verify that the two puri-

fied loop swap mutations were in fact the two mutations and not just wild type protein. The theoretical mass for *KPI A-k1, *KPI B-k1, and rWT is 25704.17 Da, 24685.11 Da, and 23157.33 Da, respectively. The theoretical masses have been calculated using the program GPMAW version 6. The results are seen in Figure 6.29.

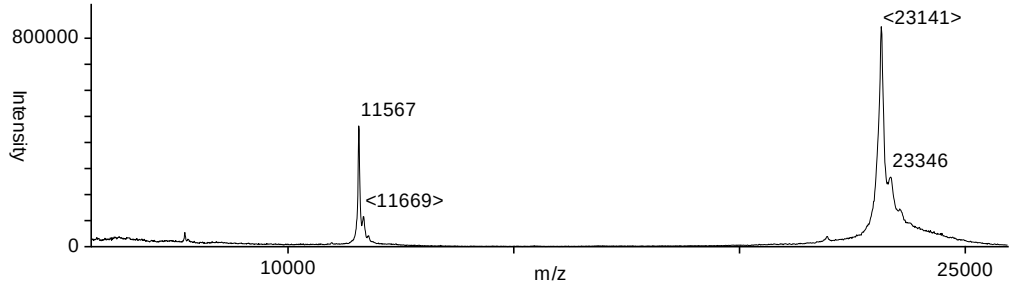
A



B



C



D

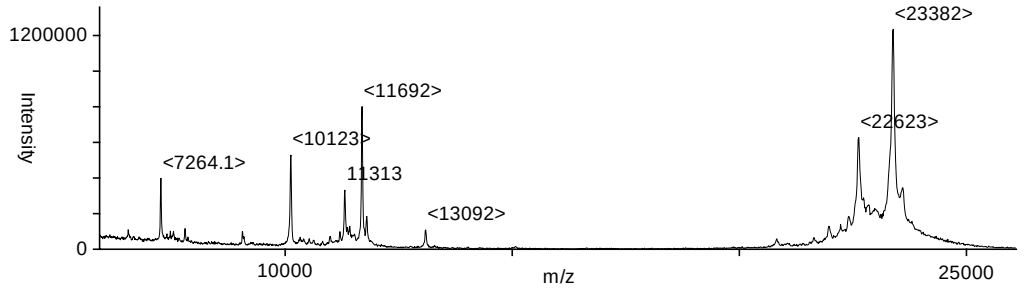


Figure 6.29: MALDI TOF MS analysis of the two loop swap mutations. 5 µL of either (**A**) *KPI A-k1, (**B**) *KPI B-k1, (**C**) rWT, or a (**D**) mixture of the two mutations was analyzed by MALDI TOF MS.

The MALDI TOF spectra of the two loop swap mutants validated that each sample contains proteins with a size that are different from the rWT, and that the two types of mutants as expected does not have the same weight. It can also be seen that the sample containing rWT does not contain any contaminants, whereas the spectra for both mutant shows a peak around 13000 Da. The sample with *KPI B-k1 also has a smaller peak at around 20800 Da. Moreover, none of the observed masses corresponds to the theoretical ones. In the spectra for *KPI A-k1 the peak is seen at 23389 Da, hence the mass is 2314 Da lower than expected. The same is observed for *KPI B-k1 where the measured mass is 2047 Da lower than expected. The difference between the theoretical and observed mass of rWT is only 16 Da.

The inhibitory activity of both loop swap mutations and the rKPI A-k1(PL-AA) mutation was tested using the amidolytic activity assay with rWT and WT as controls (Figure 6.30).

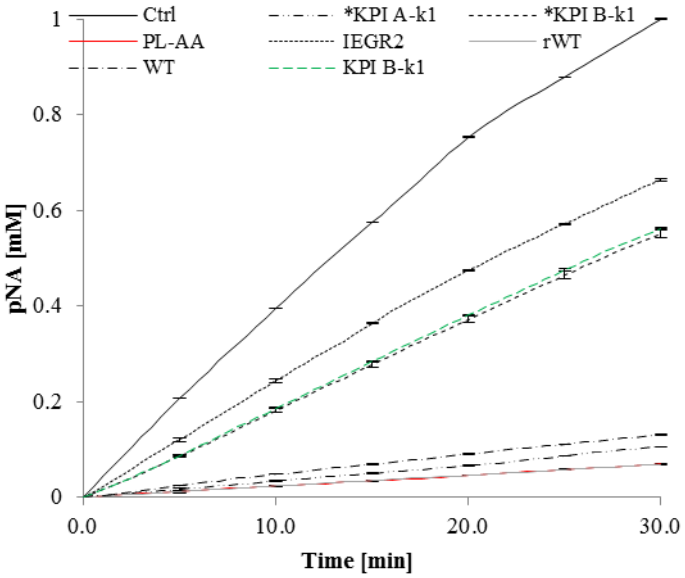


Figure 6.30: The mutant proteins *KPI A-k1 and KPI A-k1(PL-AA) inhibits fXa as good as the wt. 100 µg/mL of each of the protein variants were mixed with 1 µM fXa and incubated at RT for 10 min. 0.5 mM chromozym t-PA was added and the absorption at 405 nm were measured every 30 s. for 30 min. IEGR2 is added as comparison.

The amidolytic activity assay indicated that the two mutants *KPI A-k1 and rKPI A-k1(PL-AA) inhibits fXa to the same degree as both the recombinant wild type and the purified wild type (Figure 6.30). The *KPI B.k1 variant did not inhibit fXa to the same extend as the other variants, but it showed the same inhibitory activity against fXa as the KPI B-k1 variant. The experiment was also carried out using the fXa specific substrate S2222. However, this gave similar results (data not shown).

6.6.3 Random mutagenesis for phanning experiments

The area with the putative RCL was randomly mutagenized using a synthesized single stranded nucleotide. The N-terminal and C-terminal were generated from the sequence of PifXa by PCR, and the three DNA parts were assembled using OEPCR. In order to test the mutagenization frequency, the sequences were coned into an *E. coli* stain and 15 clones were selected for sequencing.

```
            ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  640        650        660        670        680        690        700
KPI A-k1    CTAGCGGTAC ACCCGTTAGA TTCATTCCTT TATCTACAAA TATCTTTGAA GATCAACTAC TTAACATACA
RDmut1_Wt   .......... .......... ......AAGG G.GTA.GGG. .GGTCCGT.T ..CAGG.... ..........
RDmut2_Wt   .......... .......... ......AT.C AT.AA.G.TT ATAAAAA.GG ...AGG.... ..........
RDmut3_Wt   .......... .......... ......AT.C A..G.NNNNT GC.GGAC..T .G.GG..... ..........
RDmut4_Wt   .......... .......... ......AGG. GGA.AC.CGG GGATAC.CT. TCATTT.... ..........
RDmut5_Wt   .......... .......... ......AT.G AG.G.TTNNN NNNA.AACTT CG.GT..... ..........
RDmut6_Wt   .......... .......... ......AAG. ATCTACGT.T CCATGAG.GT A.CAGT.... ..........
RDmut7_Wt   .......... .......... ......GAGG A.A.CG..GT .T..AGACG. ...AG..... ..........
RDmut8_Wt   .......... .......... ......ATG. ATG.GTTCG. AGAT.A.A.G TGAAT-.... ..........
RDmut9_Wt   .......... .......... .......... .......... .......... .......... ..........
RDmut10_Wt  .......... .......... .......... .......... .......... .......... ..........
RDmut11_Wt  .......... .......... ......AGA. GCG.A.T.GT GGA...A..G .GGTGG.... ..........
RDmut12_Wt  .......... .......... .......... .......... .......... .......... ..........
RDmut13_Wt  .......... .......... ......AG.A ATAGA.AC.G C.GGG.ATTT T..TGC.... ..........
RDmut14_Wt  .......... .......... ......AGC. GCAAATT.GG ..AT.GG..C CT.TT-.... ..........
RDmut15_Wt  .......... .......... .......... .......... .......... .......... ..........
```

Figure 6.31: The mutagenized area in the sequence for PifXa. 15 random cells were selected and sequenced. 11 out of the 15 showed to be randomly mutated. Part of two of the sequences was not fully sequenced, which is denoted by N.

Figure 6.31 show that 11 out of the 15 clones have a sequence that is different from the wild type in the desired area. Two of the sequences (nr 3 and 5) are not fully sequenced in the area, but the part that is, is different from the wild type. In three of the sequences (nr 2, 5, and 8) there is a nonsense mutation, and in one of the clones (nr 14) there is a deletion, meaning the amino acids following the RCL will be altered. Bases on the 15 sequenced clones there is reason to believe that when the randomly mutated sequences are cloned in a phage vector it will generate a library with a high diversity.

7 Discussion

PifXa (KPI A-k1) was isolated from potato tubers of the cultivar Kuras via a number of different precipitation and chromatographic steps. MALDI TOF MS analysis of the final sample showed peaks corresponding to the theoretical weight of the protein and peaks corresponding to the protein in other ionization states. The peak around 20800 Da is very broad which could indicate the presence of various N- and C-terminal variant of the protein. The different KPIs is in the potato known to be in many different truncated variants (Jorgensen, Bauw et al. 2006), and earlier results have identified the presence of at least four different variants of KPI A-k1 in the final purified sample (Andersen 2007). The yield of the purification of PifXa was ~9 mg/kg potato (wet weight).

From the phylogenetic tree generate from a blast search done with the sequence of PifXa against the potato genome it can be seen that PifXa have evolved early and that it only have two close related relative Aspartic protease inhibitor 5 and 8. According to the alignment the three proteins only differ en a few amino acids and have a lot of conserved regions. The phylogenetic tree also indicate that KPI B-k1 variant is an ancestor to the KPI A-k1 variant, and like with the two aspartic protease inhibitors the A and B variant only differs in a few areas. It is not known whether PifXa is specific for the cultivar Kuras or if it is found in other species of potato.

7.1 *In vitro* characterization of PifXa

Both small molecular chromogenic substrates and the natural substrate thrombin were used to determine the specificity of PifXa. The inhibitor effect towards five different coagulation enzymes and trypsin were tested with the chromogenic substrate specific for each enzyme. PifXa was able to increase but not completely abolish the activity towards fXa, likewise a decreased activity were seen in aPC, plasmin and trypsin. The latter is not surprising since the KPIs found in potato tubers in other studies have shown an affinity towards trypsin (Jorgensen, Bauw et al. 2006). In contrast, no inhibitory effect against thrombin was observed using either the small chromogenic substrate or the fibrin polymerization assay. Likewise, no inhibitory effect was seen against t-PA.

The physiological function of aPC and plasmin is to inhibit fVa and fVIIIa and degrade the fibrin clots, respectively (Dahlbäck 2000). Since PifXa is also able to inhibit both aPC and plasmin it is likely that the *in vivo* functions of these enzymes are lowered, which could have

a potential pro-coagulation effect. However, the dominant effect observed in the rat experiments is indeed the anti-coagulant seen by the prolonged bleeding time. Hence, *in vivo* the strongest effect of PifXa is the inhibition of fXa.

The inhibitory profile of PifXa raised the question to whether or not PifXa also was capable of inhibiting the activation of fX. However, the SDS PAGE gel based experiment showed that PifXa did not affect the activation of fX by Russell's viper venom. This result indicates that PifXa does not bind or overlap the region on fXa where the activation occurs.

The kinetics of inhibition of fXa was determined using the small chromogenic substrate chromozym t-PA. The calculated values of K_M and V_{max} seemed to be random why the inhibitory constant were determined for competitive, non-competitive and mixed mode of inhibition and the K_i was all determined to be in the area between 4-6 nM. The random values of the two Michaelis Menten constants together with the inability of PifXa to completely abolish the activity of fXa suggests that the binding interface between PifXa and fXa is only partially overlapping with the active site allowing the small substrate to slowly dissociate into and product to dissociate out of the active site. However, the physiological relevance of this phenomenon is likely to be marginal since that natural substrate for fXa, prothrombin, is much more bulky and cannot be expected to access the active site when fXa is in complex with PifXa. Indeed, the fact that even lowest tested dose of PifXa was sufficient to obtain full effect in the *in vivo* experiment supports that PifXa is a more effective inhibitor of fXa against large substrates. The binding constant (K_D) for the binding between fXa and PifXa were determined using fluorescence. By fitting the obtained data to Equation 5.2, the K_D was calculated to be 0.17 µM indicating a strong binding. It was expected that the K_i and the K_D would be of similar order. However the calculated K_D is 100 times larger than the estimated K_i. Hvorfor?

Three different standard clinical coagulation assays were performed with PifXa. UFH, LMWH, and Fondaparinux served as controls. None of the tested compounds were able to prolong the prothrombin time to above the normal values. This assay measures the effect of the extrinsic and final common pathway and it represents the time in seconds for the plasma to clot after the addition of TF and Ca^{2+}. Prothrombin time is known to be most sensitive to-

wards fVII and less sensitive toward the factor of the common pathway (factors V, X and thrombin), and is most often used to monitor the effect of vitamin K antagonists like warfarin (Kamal, Tefferi et al. 2007). In theory the effect of heparin and LMWH should also be seen using the prothrombin assay. However, the effect of these inhibitors is overcome with the addition of heparin neutralizers (e.g. Polybrene). When added to the PT reagent they can neutralized up to 2 U/mL of heparin (Kamal, Tefferi et al. 2007). Polybrene is a cationic polymer that binds to, thus neutralizes heparin and can in the same manner bind to proteins. The presence of the polymer can very well be the reason for the lack of sensitivity of the PT assay towards PifXa as well.

The effect of LMWH and Fondaparinux can according to literature not be measured using the aPTT assay. This was confirmed by the results in this thesis. LMWH showed as expected a slight prolongation of the aPTT, while Fondaparinux had no effect. It has been argued that the assay is only sensitive towards thrombin inhibitors rather than fXa inhibitors (Linkins, Julian et al. 2002). However, PifXa does not inhibit thrombin but the effect is still detectable in the aPTT assay. The effect that is seen could be caused by the fact that PifXa in contrast to antithrombin not only is able to bind and inhibit free fXa but also is capable of binding to and inhibiting fXa in the prothrombinase complex (Phospholipid:fXa:fVa:Ca^{2+}). The results indicate that the aPTT assay can potentially be used to monitor the anticoagulant effect of PifXa in patients.

Both heparin and LMWH was capable of prolonging the thrombin time to above the time frame set for the experiment, whereas neither PifXa nor Fondaparinux as expected had any effect in this assay. Hence, another strong indication that PifXa, as seen from the results in the chromogenic activity assay, has no inhibitory affinity towards thrombin.

7.2 PifXa prolongs bleeding time in rats

The anticoagulant characteristics of PifXa in whole blood *in vitro* showed a concentration dependent increase of both the bleeding time and the amount of red blood cells flowing through the syringed. At a concentration of 1.5 µM both the bleeding time and amount of red blood cells exceeded that of the control sample containing the therapeutic dose of heparin (2 U/mL). The same was not observed in the *in vivo* results. The bleeding time for the rats receiving PifXa was significantly different from the control group (P=0.002 for the low dose group), but somewhat lower than that of the rats given a full dose of therapeutic heparin (1100

s for the low dose group compared to 1800 s for the heparin group). Surprisingly, full effect as seen by the bleeding time was obtained with the lowest dose of PifXa, suggesting that maximal efficiency is reached even with lowest tested dose.

In contrast to the bleeding time, the amount of red blood cell leakage in the groups receiving PifXa were not significant different from the control, as it was the case in the heparin group. Hence, at first glance, the rat bleeding studies could suggest a relative poor effect of PifXa, especially considering that the bleeding amount is generally considered more reliable than the bleeding time (Johansen, Henriksen et al. 2008). However, a reason for the low amount of cell leakage could be explained by the unaltered platelet activity in the groups of rats giving PifXa, hence allowing a platelet plug to be formed in the tip of the tail not allowing the escape of red blood cells but allowing plasma leakage, which could be detected as prolonged bleeding time. This hypothesis prompted the investigation of the effect of PifXa on platelet aggregation *in vitro*. The experiment revealed that PifXa did not affect platelet aggregation, and that the co-administration of the antiplatelet drug acetylsalicylic acid (aspirin) had an additive effect. On the basis of these results rats were administered a fixed dose of PifXa (2 mg/ml) either alone or in combination with aspirin, and the bleeding time and amount were determined as previously. As expected the rats administered PifXa alone had a prolonged bleeding time, but minimal leakage of red blood cells. In contrast, the rats receiving both aspirin and PifXa leaked a much higher amount of red blood cells and bled longer. In fact, the effect of the combination was indistinguishable from the groups of rats receiving therapeutically doses of either heparin or the direct fXa inhibitor Fondaparinux. Therefore, in contrast to heparin which is known to bind and alter the activity of a lot of different targets, including vWF that mediates the adhesion and aggregation of platelets (Sobel, McNeill et al. 1991), PifXa seems to specifically inhibit plasma coagulation also *in vivo*. Interestingly to note, is that the effect of PifXa alone was compatible to that of the therapeutic dose of Fondaparinux, which also not effects platelet aggregation.

The leakage of blood cells from the rats in the heparin group was somewhat different in the two experiments, whereas the response from the control and PifXa group were similar. The difference could reflect the difficulty in determining the dose-response of heparin in different individuals. Moreover, the results among the rats in the same group varied, underlining the difficulties of working with animals as model systems and the necessity of having a high number of individuals in each group.

An important advantage in working with proteins as drugs is the possibility to produce an antibody based antidote. Treatment with such an antidote could result in fast clearance of the Ab:PifXa complex from the system, effectively reversing the effect of the drug. This was demonstrated *in vitro* using a polyclonal antibody raised against PifXa in rabbits. At a concentration of 60 mg/mL of antibody serum an almost fully reversal was observed. Moreover, the effect of the antibody began as soon as it was added to the solution. An antidote against an inhibitor is relevant since dose calculation is difficult (Hirsh, Warkentin et al. 2001) and because high doses of anticoagulant is needed under certain conditions (e.g. during cardio pulmonary bypass), but prolonged anticoagulation activity increase the risk of bleeding. Such a drug/antidote system is already in place in form of heparin and its antidote protamine sulfate. However, protamine sulfate has little effect on LMWH and no effect on Fondaparinux, for which there is no known antidote against. The lack of antidote limits the use of the anticoagulant in applications where an antidote is desired. Because of the rather large group of patients that develop HIT antibodies, the development of non-heparin based alternatives is important.
Even though none of the rats showed signs of an immediate allergic reaction, as with other protein therapeutics, especially non-human ones, there is a risk that this potato derived protein can cause an immunological response. Indeed, the fact that high affinity polyclonal antibodies could be raised against PifXa in rabbits seems to suggest that this is the case. However, protein based anticoagulant drugs like the two direct thrombin inhibitors Lepirudin and Desirudin are indeed used today demonstrating that this obstacle is not impossible to overcome (Gajra, Husain et al. 2008, Warkentin 2004).

7.3 Attempt to determine the half-life of PifXa *in vivo*

Various methods were tried in order to determine the half-life of the inhibitor in the rats. 0.2 mg PifXa was administered to the rats and blood samples were extracted at 15 different time points. The blood was separated and the plasma was analysed for the presence of PifXa at each time point. Two different methods were tried, western blotting and iELISA. However, in both cases the detection limit was too high to measure the very low amount of PifXa in the plasma sample. In the western blotting experiment the detection limit was first determined with PifXa in buffer, and it was possible to detect as low as 8 ng. However, when PifXa was

diluted in human plasma not even 0.48 µg was visible after blotting, wherefore the attempt to determine the half-life of PifXa in rats by western blotting was terminated.
In the iELISA experiment various concentrations of PifXa was tested both in buffer and in human plasma. In buffer even the lowest concentration of the inhibitor (0.03 µg/mL) could to be detected, but when PifXa was diluted in human plasma no detectable colour development was seen. The reason for that is explained by the high ratio of proteins in the plasma compared to PifXa, which means that the plasma proteins occupies most of the binding sites in the ELISA plate, hence resulting in very little binding of the antibodies to PifXa and thereby no colour development. Changing to a high-binding plate did not change the result. It was attempted to purify PifXa from the plasma by running the solution over a Mono S column before it was to be added to the ELISA plates, but this did not change the results.

Instead of investigating the blood samples drawn at different time points a totally different approach was attempted. PifXa was labelled with radioactive iodine 131 and injected IV into 8 rats. Four of the rats had the tip of their tail removed after 10 min, while the remaining four was left untouched. After 60 min the radioactivity of different tissues was measured. No significant difference in activity between the different tissues was observed in the two groups of rats. The highest level of radioactivity was found in the kidneys, where 1119 and 1133 decay/min/mg tissues were seen in the rats with and without tail cut, respectively. However, this may not reflect the degradation of I^{131}PifXa; instead it is believed to be caused by free iodine. Iodine is naturally mainly cleared from the system by the kidneys (Cavalieri 1997) and this can explain the high amount of radioactivity found in this particular organ. The tail of the rat was cut off in three different places, the outer 2 mm, the 2 mm after the cut, and the next 2 mm tail. No obvious difference in radioactivity was seen in either of the pieces of tail, and there were no statistically significant difference in the amount of radioactivity found in the rats with tail cut after 10 min and those with no tail cut. These results could indicate that PifXa does not accumulate in the place of injury. Summarizing the results from this experiment it is impossible to conclude where and how fast the degradation of PifXa occurs. A high number of drugs are metabolized by the cytochrome P450 system liver (Ogu, Maxa 2000). However, the radioactivity in the liver was not elevated compared to the other tissues. This could mean that either PifXa is not degraded in the liver, the half-life of PifXa is so high that

no significant degradation have begun in the time of the experiment, or that the radioactivity of the inhibitor is so low that it cannot be detected.
Unfortunately none of the three attempts to determine the rate of PifXa clearance from the system was successful. Furthermore, based on the results it is impossible to conclude where the degradation of PifXa occurs in the rat.

7.4 Directed Evolution

Potato tuber proteins and proteins in the blood coagulation system are highly unlikely to have a history of co-evolution. Therefore, the specific inhibitory effect seen by PifXa must be considered a coincidence. Consequently, it is reasonable to believe that a stronger binding variant of the inhibitor can be generated following directed evolution of PifXa, possibly also altering the inhibition profile to be even more specific for fXa. Based on these assumptions five different mutants were expressed in the *E. coli* strain Rosetta Gami B (DE3). The *E. coli* strain was selected due to its ability to translate none bacterial codons and an increased ability to form disulphide bridges (see Appendix 4). The purified rWT was as effective in inhibiting fXa in the amidolytic activity assay as the wild type purified from the potato tubers. Hence, despite the presence of three disulphide bridges in PifXa, the recombinant protein was folded correctly. The expression and purification of the rWT yielded in two proteins of different sizes as seen by the elution profile during the IMAC purification and the SDS PAGE gel analysis afterwards. The first protein to elute from the IMAC column was around 60 kDa, whereas the second protein was around the expected 21 kDa in size. The two protein were analysed using MS/MS and was identified to be the *E. coli* protein alkyl hydroperoxide reductase, F52a subunit (ahpF) and KPI A-k1, respectively. Previous experience with expression in Rosetta Gami B of other His-tagged proteins, likewise purified by IMAC, and under nearly identical conditions, showed no signs of co-expression or purification of ahpF (Brøndum personal communication). Thus, the sudden co-expression of ahpF must be induced by changes in the bacterial environment, caused by conditions specific to the KPI A-k1 expression platform. The function of ahpF in *E. coli* is the protection against oxidative stress (Wood, Poole et al. 2001). However, the two protein peaks during the IMAC elution was well separated and did not resulted in any contamination from the ahpF in the final protein sample.

In two of the expressed variants the recognition site of fXa on prothrombin, IEGR, was put in place of four amino acids in the putative RCL in two different locations. These mutants were prepared in the hope that the recognition site of fXa would make the protein bind tighter and more specific to the inhibitor, hence reducing the dose required to inhibit bleeding when used in patients. The protein expression of the IGER1 variant was unsuccessful, and the lack of inhibition seen in the amidolytic activity assay cannot be ascribed to the inhibitor itself but rises from the low concentration of inhibitor found in the sample. The protein expression of the other IEGR (IEGR2) variant was more successful and the SDS PAGE gel of the concentrated sample of the variant showed a concentration similar to that of the rWT. The amidolytic activity assay revealed that the IEGR2 mutant variant did not inhibit fXa to the same degree as either the rWT or the WT. We speculated whether the lack of inhibition could be because fXa instead of binding to the inhibitor cleaved it after the inserted sequence as it would do after the sequence in the natural substrate prothrombin. If this was the case it would have resulted in a fragmentation of the inhibitor, which would be visible on a SDS PAGE gel. FXa and the two IEGR mutants were allowed to incubate for 30 min before reducing SDS PAGE loading buffer was added and the sample was loaded onto the gel. However, no shift in protein size was observed for the inhibitor after the incubation with fXa. The absence of fragments from the mutant could suggest that fXa does not or at least only slowly cleave the inhibitor which means that the low inhibition is caused by interference in the binding area between the inhibitor and fXa caused by the altered amino acids.

The P102A,L103A mutant was designed to pinpoint the RCL more precisely. Since two amino acids were replaced with alanine, the inhibitory activity would cease if this area was the only one important for the binding of PifXa to fXa. The protein expression of this mutant was as successful as the expression of the rWT (8 mg/L culture for the mutant compared to 6 mg/L for the rWT). However, the inhibitory activity against fXa of the mutant was as good as both rWT and WT. This means that the two amino acids (P and L) in the putative RCL do not contribute significantly to the binding of the inhibitor to fXa.

In the last two mutants a variable area around the RCL was interchanged with the same area of another Kunitz protease inhibitor variant KPI B-k1. This variant is from earlier studies known not to have the same inhibitory effect towards fXa as the PifXa (KPI A-k1), although

most of the KPIs from potato tubers have a slight inhibiting effect ((Andersen 2007)). When the putative RCL and the area around it from PifXa is replaced by the same area from KPI B-k1 (*KPI A-k1) the expressed protein will in theory lose most of its inhibitory effect if this is the only area that is responsible for the inhibition of fXa. Likewise, when the loop from KPI B-k1 is replaced with the loop from PifXa (*KPI B-k1), one would get an inhibitor that have the same ability to inhibit fXa as the wild type. The expression of these two mutants was not as successful as the rWT (2 mg/L culture for the mutants compared to 6 mg/L culture for the rWT). However, this is often seen when trying to alter the sequence of a protein. Changing the sequence of the protein can interfere with its ability to fold properly, which will result in a lower final yield of correct protein.

The MALDI TOF MS analysis of the two mutants revealed that the expressed proteins did not have the expected size. The theoretical sizes is 25704 Da and 24685 Da for the *KPI A-k1 and *KPI B-k1 variant, respectively. However, the mass spectrum revealed that the masses of the protein were 23389 Da and 22638 Da for the two mutations, hence, 2314 and 2047 Da, respectively, less than expected. One of the reasons for this could be prompted by truncation of the N- and/or C-terminal part of the protein during processing in *E. coli*. In the potato tuber a number of different N- and C-terminal variants of both the A and B type of KPI have been observed (Jørgensen, Stensballe et al. Submitted). This could suggest that both ends of the proteins are exposed to the environment and hence vulnerable towards degradation. Indeed, calculations of the masses indicate that both variants have been cleaved in the same location namely after the ER sorting signal (green in Figure 3.1). Cleavage after the ER sorting signal would result in a 2335 and 2021 Da lower mass for the *KPI A-k1 and *KPI B-k1, respectively, which is much more consistent with the observed masses.

The mass spectrum for the rWT showed at peak around 23141 Da, which is consistent to that of the expected mass at 23157 Da. The N-terminal of rWT have been observed in the potato tuber, however, the N-terminal of this variant is still expected to protrude from the folded protein, thus be more vulnerable to proteolytic degradation.

Despite the low level of expression of the two loop swap mutations, enough protein was produced to test the inhibitory activity. The result of the amidolytic activity assay was not as expected. *KPI A-k1 had the same ability to inhibit fXa as rWT, while *KPI B-k1 was not as effective; hence the opposite effect of what was expected. These results could indicate, that the area with the putative RCL is not solely responsible for the interaction with fXa since the

effect seen by *KPI B-k1 originates from the effect seen by the KPI B-k1 variant. The KPI B-k1 variant does not inhibit fXa to the same extend as PifXa but it still has some effect towards it. Common for the two loops in the two mutations is the charge of the amino acids making up the loop. In both cases the loop consists of amino acids without any charge, this is also the case for the rKPI A-k1(PL-AA) mutation. Considering the IEGR2 mutation, which had a lower inhibitor effect, two of the four amino acids (Glu and Arg) have a negative and positive charge, respectively. These charges could very well interfere with the binding of fXa and cause the observed lowered inhibitory effect seen by the IEGR2 mutant. These observations points to the fact that the putative RCL loop has to be without any charge in order for the inhibitor to have a strong effect on fXa.

When the five mutants are compared the mutant with the least affinity towards fXa is the IEGR mutant. Beside from the charge of the loop, the IGER2 variant differs from the other mutants in another point. The IEGR2 variant is the only mutant were a serine residue near the putative RCL has been altered change in this case to an arginine. In all the other mutants this particular serine is unaltered. It is possible that the serine has an important role in the binding of PifXa to fXa. However, more studies have to be done to support this, and it is most likely that the inhibitory effect of PifXa is caused by a number of things and not just one single amino acid residue.

8 Conclusions and further perspectives

The wildtype PifXa purified from potato tubers proved to be capable of prolonging whole blood coagulation time *in vitro*, but did not have any effect of the activation or aggregation of platelets. Moreover, PifXa could *in vitro* inhibit the activity of fXa, plasmin, aPC, and trypsin but had no effect on either tPA nor thrombin. Even though PifXa inhibited plasmin and aPC which functions as anti-coagulant the effect seen *in vivo* was indeed anti-coagulant, hence favouring the inhibition of fXa. PifXa was capable of significantly prolonging the tail bleeding time in rats with full effect seen at the lowest tested dose (0.25 mg/kg). Moreover, given together with aspirin the effect of PifXa *in vivo* was comparable to that of the therapeutic dose of heparin.

The great advantage of PifXa as a protein based inhibitor is the possibility to produce a specific antidote in the form of an antibody, in order to fast and reliable remove the inhibitor from the system. This was shown *in vitro* where the inhibitory effect of PifXa was fully reversed by the addition of specific polyclonal antibody.

Different mutant variants of PifXa were produced in the *E. coli* strain Roseatta Gami B to see if it was impossible to localize the binding area and improve the inhibitory profile. The recombinant wildtype of PifXa was as effective as the inhibitor derived from potato tubers. None of the produced mutant variant showed improved inhibition. Two of the variants IGER2 and *KPI B-k1 were less effective, whereas the other were as effective as the wild type. Comparison between the five mutants and the wildtype lead to the hypothesis that the amino acids in putative RCL have to be without charge in order for the inhibitor to function properly. However, more experiments are needed to draw a final conclusion.

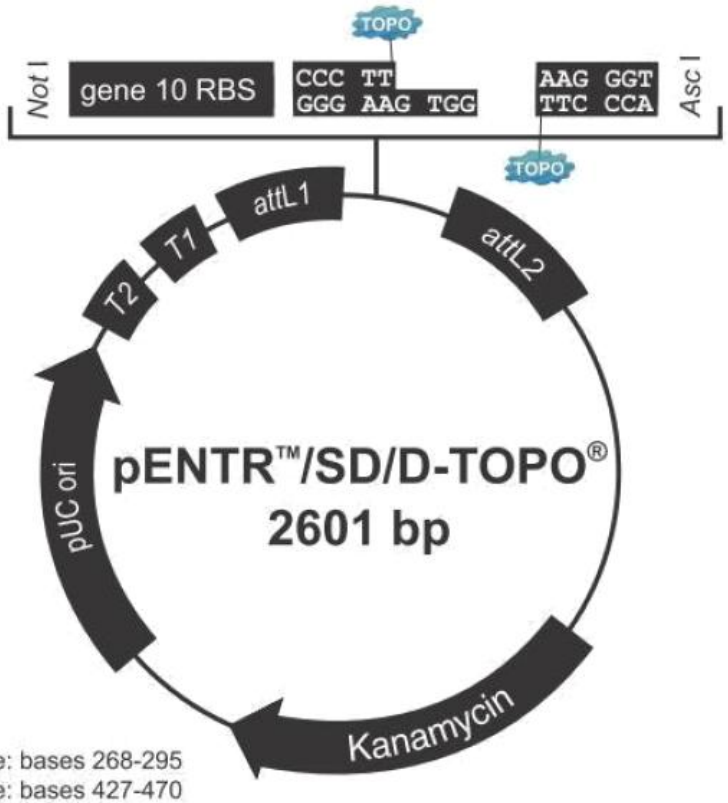
We believe that it is possible to design a variant of PifXa that has a more specific inhibition of fXa, which is why the area around the putative RCL have be randomly mutagenized and a phage display library have be produced. It is the hope that one of the many different variants in the library will show a stronger and even more specific inhibition of fXa, hence in the end reducing the concentration of the inhibitor given to the patient before surgery.

As mentioned there is a lot of new anticoagulant drug emerging on the marked. Most of them being oral small molecules that inhibits fXa directly, and while these new drugs have many advantages, there are also problems involved. One of them being that for most of them there are no antidote. This means that in surgeries where it is important to be able to reverse the

inhibitory effect, these new medicaments are not to be preferred. PifXa has the advantages over these drugs by providing an antidote in the form of a specific antibody, and it is the hope that one day PifXa can, at least in some cases, replace the use of heparin in CPB surgeries. Before that can happen there are a lot of obstacles to overcome one of them being the potential immunological problems with the inhibitor. However, today two protein-based inhibitors, Lepirudin and Desirudin, are approved as medical drugs, showing us that it is not an impossible problem to overcome. But there is still a long way to go.

Appendix 1

pENTR/SD/D TOPO Vector



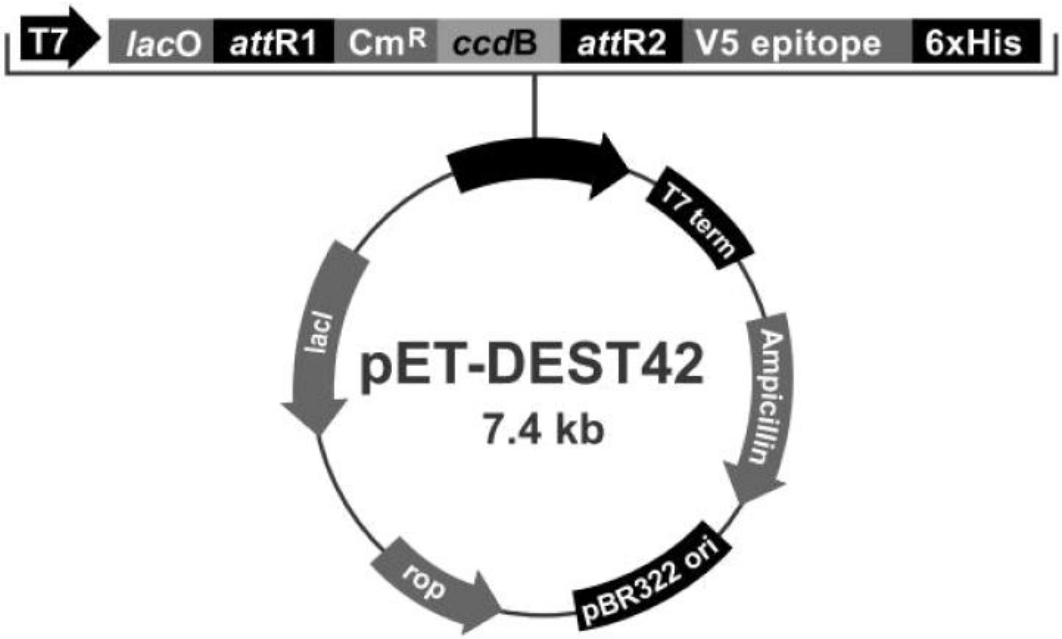
Comments for pENTR™/SD/D-TOPO®
2601 nucleotides

*rrn*B T2 transcription termination sequence: bases 268-295
*rrn*B T1 transcription termination sequence: bases 427-470
M13 forward (-20) priming site: bases 537-552
*att*L1: bases 569-668 (c)
T7 gene 10 translational enhancer: bases 684-692
Ribosome binding site: bases 694-700
TOPO® recognition site 1: bases 701-705
Overhang: bases 706-709
TOPO® recognition site 2: bases 710-714
*att*L2: bases 726-825
T7 Promoter/priming site: bases 842-861 (c)
M13 reverse priming site: bases 866-882
Kanamycin resistance gene: bases 995-1804
pUC origin: bases 1925-2598

(c) = complementary sequence

Appendix 2

pET DEST 42 Gateway Vector



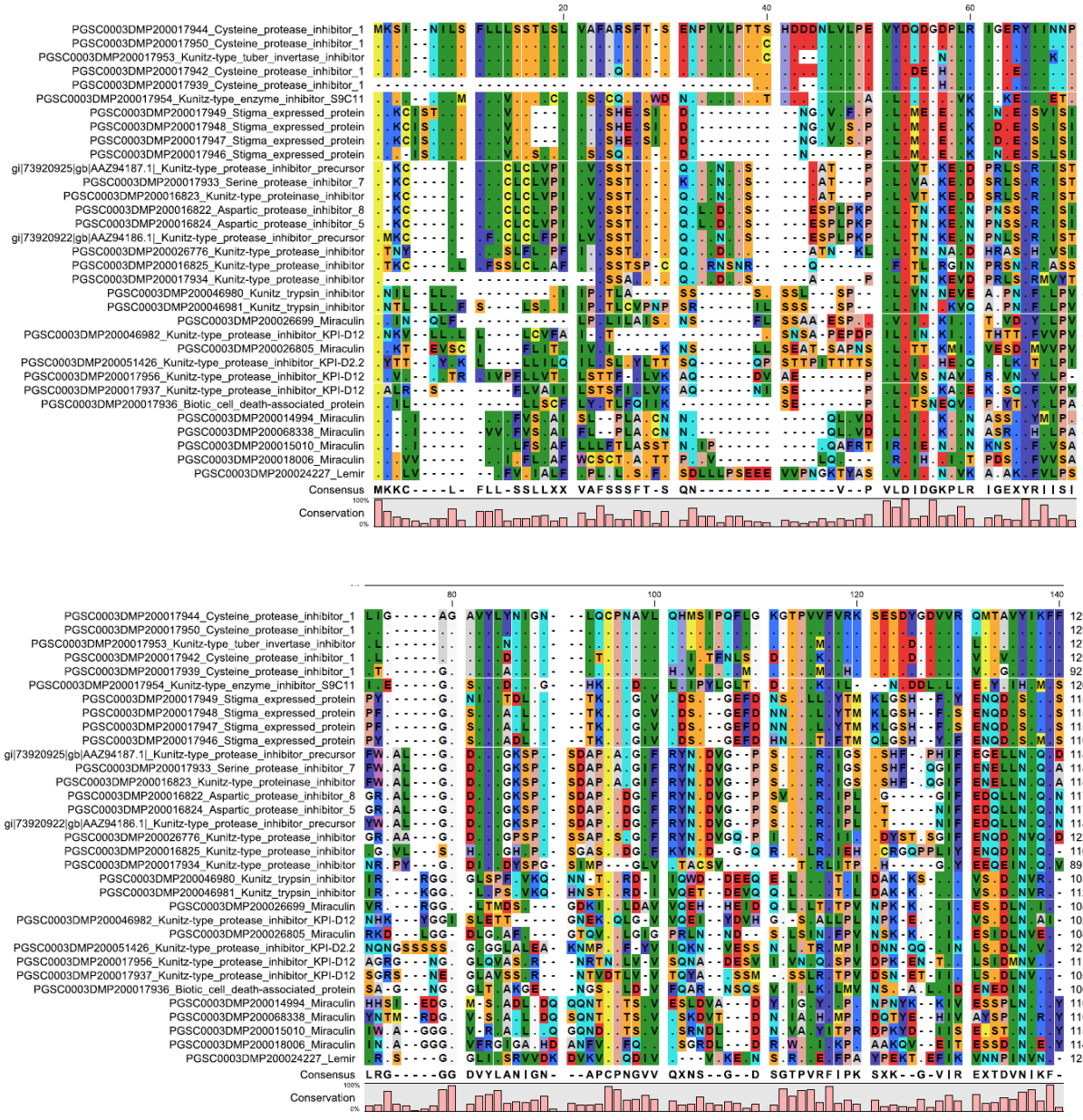
Comments for pET-DEST42
7440 nucleotides

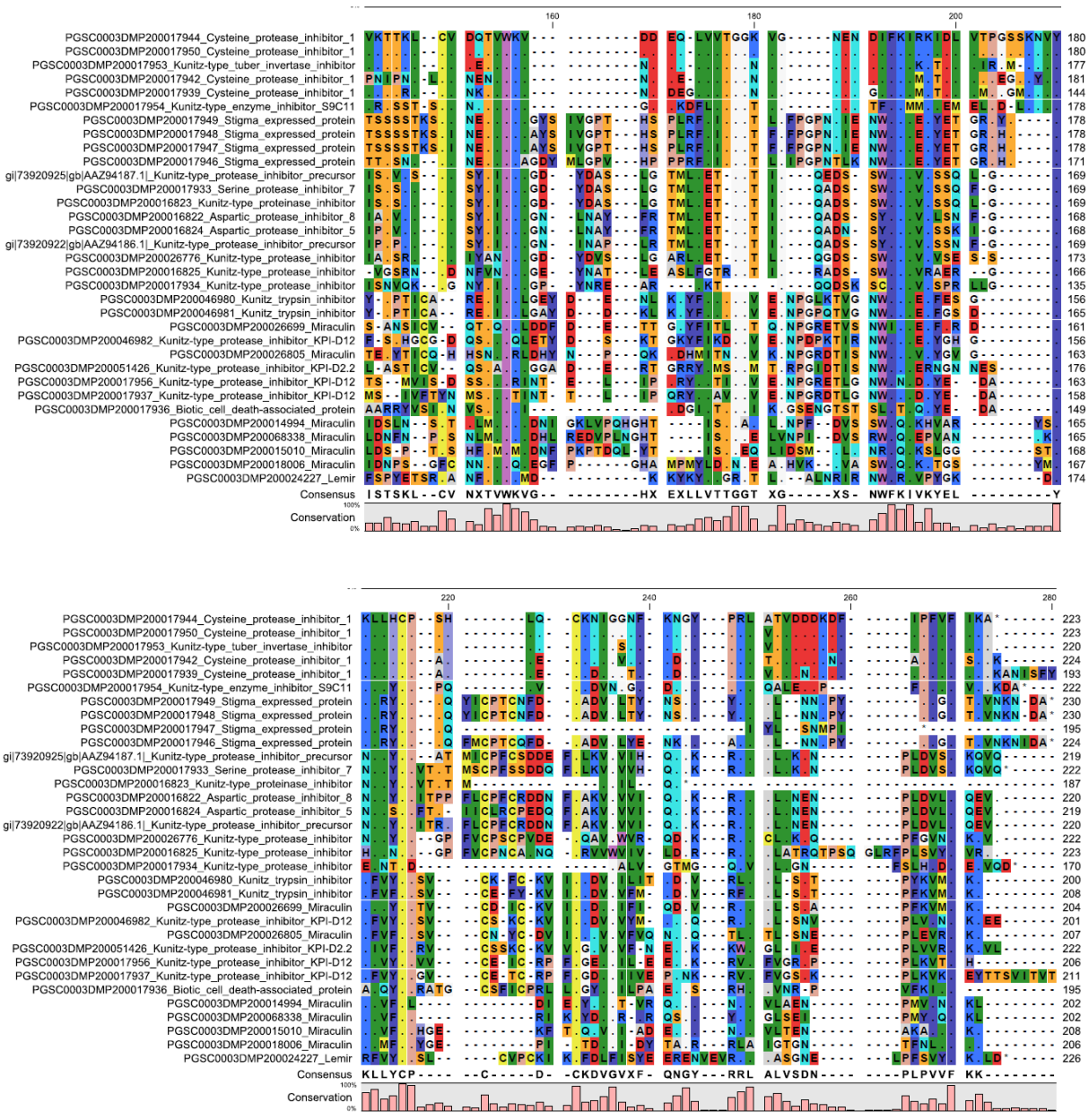
T7 promoter: bases 318-334
lac operator (*lac*O): bases 337-361
*att*R1 recombination site: bases 402-526
Chloramphenicol resistance gene: bases 635-1294
*ccd*B gene: bases 1636-1941
*att*R2 recombination site: bases 1982-2106
V5 epitope: bases 2126-2167
Polyhistidine (6xHis) region: bases 2177-2194
T7 transcription termination region: bases 2209-2337
bla promoter: bases 2638-2736
Ampicillin (*bla*) resistance gene (ORF): bases 2737-3597
pBR322 origin: bases 3742-4415
ROP ORF: bases 4786-4977 (complementary strand)
*lac*I ORF: bases 6289-7380 (complementary strand)

Appendix 3

Alignment of sequences from the phylogenetic tree

All the sequences from the phylogenetic tree were aligned. Both the phylogenetic three and the alignment were created using CLC workbench.




PGSC0003DMP200017944_Cysteine_protease_inhibitor_1
PGSC0003DMP200017950_Cysteine_protease_inhibitor_1
PGSC0003DMP200017953_Kunitz-type_tuber_invertase_inhibitor
PGSC0003DMP200017942_Cysteine_protease_inhibitor_1
PGSC0003DMP200017939_Cysteine_protease_inhibitor_1
PGSC0003DMP200017954_Kunitz-type_enzyme_inhibitor_S9C11
PGSC0003DMP200017949_Stigma_expressed_protein
PGSC0003DMP200017948_Stigma_expressed_protein
PGSC0003DMP200017947_Stigma_expressed_protein
PGSC0003DMP200017946_Stigma_expressed_protein
gi|73920925|gb|AAZ94187.1|_Kunitz-type_protease_inhibitor_precursor
PGSC0003DMP200017933_Serine_protease_inhibitor_7
PGSC0003DMP200016823_Kunitz-type_proteinase_inhibitor
PGSC0003DMP200016822_Aspartic_protease_inhibitor_8
PGSC0003DMP200016824_Aspartic_protease_inhibitor_5
gi|73920922|gb|AAZ94186.1|_Kunitz-type_protease_inhibitor_precursor
PGSC0003DMP200026776_Kunitz-type_protease_inhibitor
PGSC0003DMP200016825_Kunitz-type_protease_inhibitor
PGSC0003DMP200017934_Kunitz-type_protease_inhibitor
PGSC0003DMP200046980_Kunitz_trypsin_inhibitor
PGSC0003DMP200046981_Kunitz_trypsin_inhibitor
PGSC0003DMP200026699_Miraculin
PGSC0003DMP200046982_Kunitz-type_protease_inhibitor_KPI-D12
PGSC0003DMP200026805_Miraculin
PGSC0003DMP200051426_Kunitz-type_protease_inhibitor_KPI-D2.2
PGSC0003DMP200017956_Kunitz-type_protease_inhibitor_KPI-D12
PGSC0003DMP200017937_Kunitz-type_protease_inhibitor_KPI-D12
PGSC0003DMP200017936_Biotic_cell_death-associated_protein
PGSC0003DMP200014994_Miraculin
PGSC0003DMP200068338_Miraculin
PGSC0003DMP200015010_Miraculin
PGSC0003DMP200018006_Miraculin
PGSC0003DMP200024227_Lemir
Consensus
Conservation
160
180
200
220
240
260
280

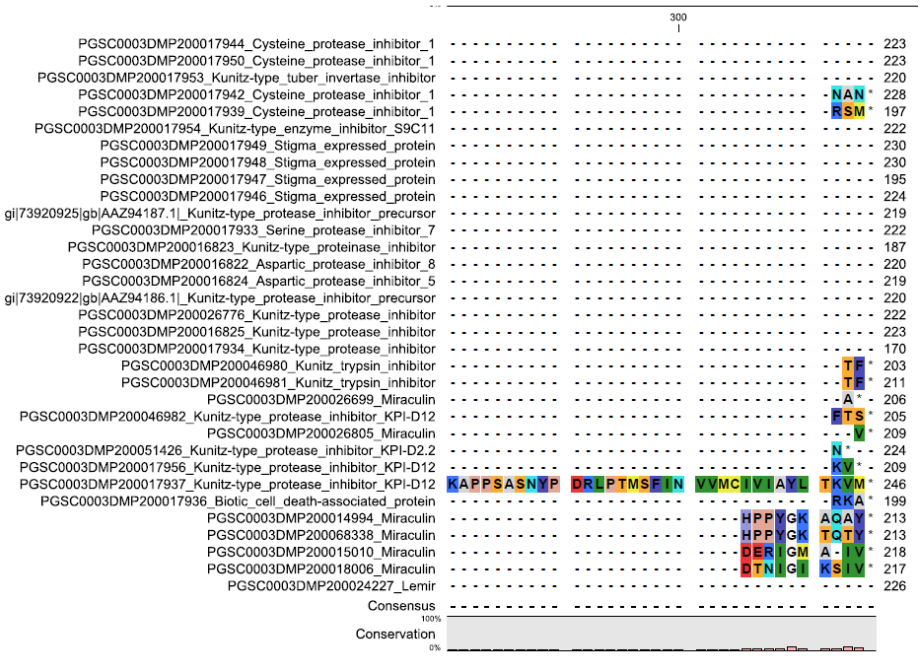


Figure A.1: Alignment of the sequences used to generate the phylogenetic tree. A (.) represents bases identical to the first sequence, while a (-) denotes a gap. A conservation bar is seen under the alignment. The alignment was created using CLC workbench.

Appendix 4

Expression Strain

Rosetta Gami B (DE3):

The Rosetta Gami B (DE3) strain is a BL21 (DE) derivative and it combines the key features of the Tuner, Origami, and Rosetta strains. The Tuner strains are *lacZY* deletion mutants of BL21, thus lacking lac permease activity. This allows the induction with IPTG to occur in a concentration dependent manner, which enables adjustable levels of protein expression. The Origami strain has mutations both in the thioredoxin reductase (*trxB*) and the glutathione reductase (*gor*) genes, which greatly enhance disulfide bond formation in the cytoplasma. The Rosetta stain enhances the expression of eukaryotic proteins by supplying tRNA for the codons rarely used in *E. coli* (AUA, AGG, AGA, CUA, CCC, and GGA) (Novagen 2004).

Genotype: F^- ompT hsdS_B (r_B^- m_B^-) gal dcm lacY1 ahpC (DE3) gor522::Tn10 trxB pRARE (Cam^R, Kan^R, Tet^R).

Table A.1: Genetic marker description (Novagen 2003)**.**

Marker	Description	Marker	Description
ahpC	Mutation in alkyl hydroperoxide reductase conferring disulfide reductase activity. Al-lows normal growth rates for *trxB*/*gor* double mutant in the absence of supplemental reducing agent.	*hsdS*	Abolishes both restriction and methylation of DNA at certain sites (r- m-).
dcm	No methylation of cytosines in the sequence CCWGG	*lacY*	Deficient in lactose transport. Abolishes lac permease (lacY1) and allows for uniform entry of IPTG into all cells.
DE3	Contains a lambda prophage in which the gene for T7 RNA polymerase is under control of the lacUV5 promoter.	*ompT*	Lacks an outer membrane protease; improves recovery of intact recombinant proteins.
F´	Strain does not contain the F episome, which encodes lacZΔ M15 and lacIq mutations; not appropriate for blue/white screening or expression from lac operated promotor in the absence of an additional source of lac repressor.	*pRARE*	Contains a Cam^R plasmid (pACYC184), which carries the tRNA genes for the six codons AGG, AGA, AUA, CUA, CCC, and GGA, rarely used in *E. coli*.
gal	Unable to utilize galactose.	*Tn10*	Contains the Tet^R transposable element, Tn10.
gor	Mutation in glutathione reductase. Allows formation of disulfide bonds in *E. coli* cytoplasm.	*trxB*	Abolishes thioredoxin reductase. Allows formation of disulfide bonds in *E. coli* cytoplasm.

Appendix 5

Supporting Material

Article: A Novel Factor Xa inhibitor (PifXa) is an effective, reversible inhibitor of blood coagulation

Co-Author Declaration

Title: A Novel Factor Xa inhibitor (PifXa) is an effective, reversible inhibitor of blood coagulation.

Authors: Andersen, Mette Sondrup; Alstrup, Aage Kristian Olsen; Andersen, Julie Kirstine; Kristensen, Søren Risom, and Nielsen, Kåre Lehmann.

Journal: To be submitted

Mette Sondrup Andersen purified the inhibitor from potato juice, performed the experiments and wrote the article.

Aage Kristian Olsen Alstrup performed the *in vivo* experiments.

Julie Kirstine Andersen improved the purification protocol.

Søren Risom Kristensen supervised the coagulation experiments.

Kåre Lehmann Nielsen supervised the project and wrote the article.

All authors read and approved the final manuscript.

We hereby declare that we are aware that the article in question will form a part of the PhD dissertation by Mette Sondrup Andersen and that the statements listed above are correct.

Co-authors signatures:

11/7-2011 — Date

Doctor of veterinary medicine, PhD. Aage Kristian Olsen Alstrup

7/7 - 2011 — Date

Cand. Polyt. Julie Kirstine Andersen

12.07.11 — Date

Professor, MD, Søren Risom Kristensen

Date

Associated Professor, PhD. Kåre Lehmann Nielsen

Patent: Protease Inhibitor

Co-Author Declaration

Title: Protease Inhibitor

Patent number: WO 2009/080054

Inventors: Kåre Lehmann Nielsen and Mette Sondrup Andersen

Agent: Højberg A/S

The full patent can be found on the enclosed CD rom.

I hereby declare that we are aware that the article in question will form a part of the PhD dissertation by Mette Sondrup Andersen and that the statements listed above are correct.

Co-authors signatures:

Date Associated Professor, PhD. Kåre Lehmann Nielsen

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
2 July 2009 (02.07.2009)

PCT

(10) International Publication Number
WO 2009/080054 A1

(51) International Patent Classification:
C07K 14/81 (2006.01) *A61K 38/55* (2006.01)

(21) International Application Number:
PCT/DK2008/050334

(22) International Filing Date:
19 December 2008 (19.12.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2007 01851 21 December 2007 (21.12.2007) DK
61/009,007 21 December 2007 (21.12.2007) US

(71) Applicant *(for all designated States except US)*: **IFXA A/S** [DK/DK]; Niels Jernes Vej 10, DK-9220 Aalborg Øst (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: **NIELSEN, Lehmann Kåre** [DK/DK]; Landmandsgade 25, DK-9293 Kongerslev (DK). **ANDERSEN, Sondrup Mette** [DK/DK]; Lyngholmsvej 11, st. th., DK-9200 Aalborg (DK).

(74) Agent: **HØIBERG A/S**; St. Kongensgade 59A, DK-1264 Copenhagen K (DK).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

WO 2009/080054 A1

(54) Title: PROTEASE INHIBITOR

(57) Abstract: The present invention relates to a polypeptide exhibiting a protease inhibitory activity and uses of said polypeptide in methods for inhibiting, directly or indirectly, one or more proteases of the blood clotting cascade. The invention also relates to use of said polypeptide as a pharmaceutical e.g. for prophylactic or ameliorating treatment of blood clots. In addition the invention comprises methods for production of said polypeptide.

9 References

ALEXANDER, J.H. and KANWAR, P.S., 2005. Inhibition of Factor Xa - A Potential Target for the Development of New Anticoagulants. *Am J Cardiovasc Drugs,* **5**(5), pp. 279-290.

ANDERSEN, J.K., 2008. *Characterization of the Interaction Between Kunitz Protease Inhibitor KPI A-k1 and Factor Xa for Improved Acute Anticoagulant Therapy.* MSc thesis edn. Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University.

ANDERSEN, M.S., 2007. *A Potent Specific Kunitz Inhibitor of Coagulation Factor Xa from Potato tuber.* MSc thesis edn. Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University.

ANGIOLILLO, D.J., UENO, M. and GOTO, S., 2010. Basic Principles of Platelet Biology and Clinical Implications. **74**(April),.

BATES, S.M. and WEITZ, J.I., 2005. Coagulation Assays. *Circulation,* **112**(4), pp. e53-60.

BECKER, R., POVSIC, T., COHEN, M., RUSCONI, C. and SULLENGER, B., 2010. Nucleic acid aptamers as antithrombotic agents: Opportunities in extracellular therapeutics. *Thrombosis and haemostasis,* **103**(3), pp. 586-95.

BOCK, P.E., PANIZZI, P. and VERHAMME, I.M.A., 2007. Exosites in the substrate specificity of blood coagulation reactions. *Journal of Thrombosis and Haemostasis,* **5**, pp. 81-94.

BRASS, L.F., 2003. Thrombin and Platelet Activation*. *Chest,* **124**(3), pp. 18S-25S.

BRØNDUM, M., 2010. *Expression, Purification and Preliminary Crystallisation of a Kunitz Inhibitor of Factor Xa.* MSc thesis edn. Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University.

CAVALIERI, R.R., 1997. Iodine Metabolism and Thyroid Physiology: Current Concepts. *Thyroid,* **7**(2), pp. 177-181.

CRAWLEY, J.T.B. and LANE, D., 2008. The haemostatic role of tissue factor pathway inhibitor. *Arteriosclerosis, Thrombosis, and Vascular Biology,* **28**(2), pp. 233-42.

DAHLBÄCK, B., 2000. Blood coagulation. *The Lancet,* **355**(9215), pp. 1627-1632.

DAVIE, E.W. and RATNOFF, O.D., 1964. Waterfall Sequence for Intrinsic Blood Clotting. *Science,* **145**(3638), pp. pp. 1310-1312.

DAY, J.R.S., LANDIS, R.C. and TAYLOR, K.M., 2004. Heparin is much more than just an anticoagulant. *Journal of cardiothoracic and vascular anesthesia,* **18**(1), pp. 93-100.

DE CANDIA, E., DE CRISTOFARO, R. and LANDOLFI, R., 1999. Thrombin-Induced Platelet Activation Is Inhibited by High- and Low-Molecular-Weight Heparin. *Circulation,* **99**(25), pp. 3308-3314.

FURIE, B. and FURIE, B., 1990. Molecular basis of vitamin K-dependent gamma-carboxylation. *Blood,* **75**(9), pp. 1753-1762.

FURIE, B., BOUCHARD, B.A. and FURIE, B.C., 1999. Vitamin K-Dependent Biosynthesis of gamma -Carboxyglutamic Acid. *Blood,* **93**(6), pp. 1798-1808.

FURIE, B. and FURIE, B.C., 2008. Mechanisms of Thrombus Formation. *The New England journal of medicine,* **359**(9), pp. 938-949.

GAJRA, A., HUSAIN, J. and SMITH, A., 2008. Lepirudin in the management of heparin-induced thrombocytopenia. *Expert Opinion on Drug Metabolism & Toxicology,* **4**(8), pp. 1131-1141.

GANDHI, N.S. and MANCERA, R.L., 2010. Heparin/heparan sulphate-based drugs. *Drug discovery today,* **15**(23-24), pp. 1058-1069.

GETTINS, P.G.W., 2002. Serpin Structure, Mechanism, and Function *Chemical reviews,* **102**(12), pp. 4751-4804.

GIANGRANDE, P.L.F., 2003. Six Characters in Search of An Author: The History of the Nomenclature of Coagulation Factors. *British journal of haematology,* **121**(5), pp. 703-712.

GOPINATH, S.C.B., 2008. Anti-coagulant aptamers. *Thrombosis research,* **122**(6), pp. 838-847.

GREINER BIO ONE, , 96 Well Polystyrene Microplate, clear. Available: http://www.greinerbioone.com/en/row/articles/catalogue/article/73_11/13243/ [March, 2010].

GROSS, P.L. and WEITZ, J.I., 2009. New Antithrombotic Drugs. *Clin Pharmacol Ther,* **86**(2), pp. 139-146.

HACKENG, T.M., SERÉ, K.M., TANS, G. and ROSING, J., 2006. Protein S stimulates inhibition of the tissue factor pathway by tissue factor pathway inhibitor. *Proceedings of the National Academy of Sciences of the United States of America,* **103**(9), pp. 3106-3111.

HARTWIG, J. and ITALIANO, J., 2003. The birth of the platelet. *Journal of Thrombosis & Haemostasis,* **1**(7), pp. 1580-1586.

HIRSH, J., WARKENTIN, T.E., SHAUGHNESSY, S.G., ANAND, S.S., HALPERIN, J.L., RASCHKE, R., GRANGER, C., OHMAN, E.M. and DALEN, J.E., 2001. Heparin and Low-Molecular-Weight Heparin Mechanisms of Action, Pharmacokinetics, Dosing, Monitoring, Efficacy, and Safety. *American College of Chest Physicians,* **119**(1), pp. 64-94.

HOFFMAN, M., 2003. Remodeling the Blood Coagulation Cascade. *Journal of thrombosis and thrombolysis*, **16**(1),.

HUGHES, B., 2010. First oral warfarin alternative approved in the US. *Nature reviews.Drug discovery*, **9**(12), pp. 903.

IDEL, I., 2006. *Structural and functional characterization of protease inhibitors from potato tuber.* MSc thesis edn. Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University.

INVITROGEN, November 7. 2010-last update, Gateway® Technology with Clonase™ II. Available: http://tools.invitrogen.com/content/sfs/manuals/gateway_clonaseii_man.pdf [November, 2010].

INVITROGEN, April 6. 2006a-last update, pENTR™ Directional TOPO® Cloning Kits. Available: http://tools.invitrogen.com/content/sfs/manuals/pentr_dtopo_man.pdf [November, 2009].

INVITROGEN, 10 April, 2006b-last update, TOPO TA Cloning Kit for Sequencing. Available: http://tools.invitrogen.com/content/sfs/manuals/topotaseq_man.pdf [November, 2009].

JACKSON, C.M. and ESNOUF, M.P., 2005. Has the Time Arrived to Replace the Quick Prothrombin Time Test for Monitoring Oral Anticoagulant Therapy? *Clinical chemistry*, **51**(3), pp. 483-485.

JACKSON, S.P., 2007. The growing complexity of platelet aggregation. *Blood*, **109**(12), pp. 5087-5095.

JENSEN, L.S., 2010. Lecture Notes to Physical Biochemistry 1. Part B: Mass Spectrometry, Macromolecule-Ligand Interactions and Kinetics.

JIN, L., ABRAHAMS, J.P., SKINNER, R., PETITOU, M., PIKE, R.N. and CARRELL, R.W., 1997. The anticoagulant activation of antithrombin by heparin. *Proceedings of the National Academy of Sciences of the United States of America*, **94**(26), pp. 14683-14688.

JOHANSEN, P.B., HENRIKSEN, L., ANDRESEN, P.R., LAURITZEN, B., JENSEN, K.L., JUHL, T.N. and TRANHOLM, M.T., 2008. Automated registration of tail bleeding in rats. *Thromb Haemost*, **99**, pp. 956-962.

JØRGENSEN, M., STENSBALLE, A. and WELINDER, K.G., Submitted. *Extensive post-translational processing of potato tuber storage proteins and vacuolar targeting.*

JORGENSEN, M., BAUW, G. and WELINDER, K.G., 2006. Molecular Properties and Activities of Tuber Proteins from Starch Potato Cv. Kuras. *Journal of Agricultural and Food Chemistry*, **54**(25), pp. 9389-9397.

KAMAL, A.H., TEFFERI, A. and PRUTHI, R.K., 2007. How to Interpret and Pursue an Abnormal Prothrombin Time, Activated Partial Thromboplastin Time, and Bleeding Time in Adults. *Mayo Clinic proceedings,* **82**(7), pp. 864-873.

KIELBERG, V. and RASMUSSEN, L., 2000. Dannelse af celleekstrakter og grovoprensning af proteiner. *Proteiner - oprensning og karakterisering.* 1 edn. København: Gads forlag, pp. 31.

KRISHNASWAMY, S., 2005. Exosite-driven substrate specificity and function in coagulation. *Journal of thrombosis and haemostasis,* **3**(1), pp. 54-67.

KRUPICZOJC, M.A., SCOTTON, C.J. and CHAMBERS, R.C., 2008. Coagulation signalling following tissue injury: Focus on the role of factor Xa. *The international journal of biochemistry & cell biology,* **40**(6-7), pp. 1228-1237.

LASSEN, M.R., 2009. Recent developments in the use of oral anticoagulants. *Expert opinion on pharmacotherapy,* **10**(11), pp. 1769-1781.

LEE, C.J. and ANSELL, J.E., 2011. Direct Thrombin Inhibitors. *British journal of clinical pharmacology,* .

LINKINS, L.-., JULIAN, J.A., RISCHKE, J., HIRSH, J. and WEITZ, J.I., 2002. In vitro comparison of the effect of heparin, enoxaparin and fondaparinux on tests of coagulation. *Thrombosis research,* **107**, pp. 241-244.

LWALEED, B. and BASS, P., 2006. Tissue factor pathway inhibitor: structure, biology and involvement in disease. *The Journal of pathology,* **208**(3), pp. 327-39.

MACFARLANE, R.G., 1964. An Enzyme Cascade in the Blood Clotting Mechanism, and its Function as a Biochemical Amplifier. *Nature,* **202**, pp. 498-499.

MACKMAN, N., 2008. Triggers, targets and treatments for thrombosis. *Nature,* **451**(7181), pp. 914-918.

MANNUCCI, P.M. and FRANCHINI, M., 2011. Old and new anticoagulant drugs: A minireview. *Ann Med,* **43**(2), pp. 116-123.

MARTINI, F.H., GARRISON, C.W., WELCH, K., HUTCHINGS, R.T. and IRELAND, K., 2004. Blood. *Fundamentals of Anatomy and Physiology.* 6 th. edn. USA: Benjamin Cummings Pearson Education International, pp. 651-681.

MONKOVIC, D.D. and TRACY, P.B., 1990. Activation of human factor V by factor Xa and thrombin. *Biochemistry,* **29**(5), pp. 1118-1128.

MONROE, D.M. and HOFFMAN, M., 2006. What does it take to make the perfect clot? *Arteriosclerosis, Thrombosis, and Vascular Biology,* **26**(1), pp. 41-48.

MURPHY, G.S. and MARYMONT, J.H., 2007. Alternative Anticoagulation Management Strategies for the Patient With Heparin-Induced Thrombocytopenia Undergoing Cardiac Surgery. *Journal of cardiothoracic and vascular anesthesia,* **21**(1), pp. 113-126.

MURZIN, A.G., CHANDONIA, J., ANDREEVA, A., HOWORTH, D., CONTE, L.L., AILEY, B.G., BRENNER, S.E., HUBBARD, T.J.P. and CHOTHIA, C., June, 2009-last update, SCOP: Structural Classification of Proteins. Available: http://scop.mrc-lmb.cam.ac.uk/scop/ [March, 2011].

OGU, C.C. and MAXA, J.L., 2000. Drug interactions due to cytochrome P450 *Proceedings (Baylor University.Medical Center),* **13**(4), pp. 421-423.

OLIVA, M.L.V. and SAMPAIO, U., 2008. Bauhinia Kunitz-type proteinase inhibitors: structural characteristics and biological properties. *Biological chemistry,* **389**(8), pp. 1007-13.

OLSON, S.T., RICHARD, B., IZAGUIRRE, G., SCHEDIN-WEISS, S. and GETTINS, P.G.W., 2010. Molecular mechanisms of antithrombin–heparin regulation of blood clotting proteinases. A paradigm for understanding proteinase regulation by serpin family protein proteinase inhibitors. *Biochimie,* **92**(11), pp. 1587-1596.

PAIKIN, J.S., EIKELBOOM, J.W., CAIRNS, J.A. and HIRSH, J., 2010. New antithrombotic agents—insights from clinical trials. *Nature reviews.Cardiology,* **7**(9), pp. 498.

PAPARELLA, D., BRISTER, S. and BUCHANAN, M., 2004. Coagulation disorders of cardiopulmonary bypass: a review. *Intensive care medicine,* **30**(10), pp. 1873-81.

PERZBORN, E., ROEHRIG, S., STRAUB, A., KUBTZA, D. and MISSELWITZ, F., 2011. The discovery and development of rivaroxaban, an oral, direct factor Xa inhibitor. *Nature reviews.Drug discovery,* **10**(1), pp. 61.

PERZBORN, E., ROEHRIG, S., STRAUB, A., KUBITZA, D., MUECK, W. and LAUX, V., 2010. Rivaroxaban: a new oral factor Xa inhibitor. *Arteriosclerosis, Thrombosis, and Vascular Biology,* **30**(3), pp. 376-81.

PGSC, 2011-last update, PGSC. Available: http://www.potatogenome.net/index.php/Main_Page [7/19/2011, 2011].

PIKE, R.N., BUCKLE, A.M., LE BONNIEC, B.F. and CHURCH, F.C., 2005. MINI-REVIEW: Control of the coagulation system by serpins. *FEBS Journal,* **272**(19), pp. 4842-4851.

POTTI, A., RUSCONI, C.P., SULLENGER, B.A. and ORTEL, T.L., 2004. Regulatable aptamers in medicine: focus on antithrombotic strategies. *Expert Opinion on Biological Therapy,* **4**(10), pp. 1641-1647.

PRICE, G.C., THOMPSON, S.A. and KAM, P.C.A., 2004. Tissue factor and tissue factor pathway inhibitor. *Anaesthesia,* **59**(5), pp. 483-492.

PROSITE, April, 2006-last update, Soybean trypsin inhibitor (Kunitz) protease inhibitors family signature [Homepage of Swiss Institute of Bioinformatics], [Online]. Available: http://expasy.org/prosite/PDOC00255 [February, 2011].

RAI, R., SPRENGELER, P.A., ELROD, K.C. and YOUNG, W.B., 2001. Perspectives on factor Xa inhibition. *Current medicinal chemistry,* **8**(2), pp. 101-19.

RAJU, N., EIKELBOOM, J. and HIRSH, J., 2008. Platelet ADP-receptor antagonists for cardiovascular disease: past, present and future. *Nature clinical practice cardiovascular medicine,* **5**(12), pp. 766-80.

RAWLINGS, N., TOLLE, D. and BARRETT, A., 2004. Evolutionary families of peptidase inhibitors. *Biochemical journal,* **378**(Pt 3), pp. 705-16.

RENINGER, A.J., 2008. Function of von Willebrand factor in haemostasis and thrombosis. *Haemophilia,* **14**, pp. 11-26.

REZAIE, A.R., 2005. Determinants of Specificity of Factor Xa Interaction with its Physiological Inhibitors. *Mini-Reviews in Medical Chemistry,* **6**(8), pp. 859-865.

RIDDEL, J.P.,JR., AOUIZERAT, B.E., MIASKOWSKI, C. and LILLICRAP, D.P., 2007. Theories of Blood Coagulation. *Journal of Pediatric Oncology Nursing,* **24**(3), pp. 123-131.

RIESS, F., 2005. Anticoagulation management and cardiac surgery in patients with heparin-induced thrombocytopenia *Seminars in thoracic and cardiovascular surgery,* **17**(1), pp. 85-96.

SADLER, J.E., 2004. Medicine: K is for koagulation. *Nature,* **427**(6974), pp. 493-4.

SCOPES, R.K., 1988. *Protein Purification - Principples and Practice.* 2. edn. New York: Springer-Verlag.

SMITH, S.A., 2009. The cell-based model of coagulation. *Journal of Veterinary Emergency & Critical Care,* **19**(1), pp. 3-10.

SOBEL, M., MCNEILL, P.M., CARISON, P.L., KERMODE, J.C., ADELMAN, B., CONROY, R. and MARQUES, D., 1991. Heparin Inhibition of von Willebrad Factor-dependent Platelet Function In Vitro and In Vivo. *The Journal of clinical investigation,* **87**, pp. 1787-1793.

SONG, H.K. and SUH, S.W., 1998. Kunitz-type soybean trypsin inhibitor revisited: refined structure of its complex with porcine trypsin reveals an insight into the interaction between a homologous inhibitor from Erythrina caffra and tissue-type plasminogen activator. *Journal of Molecular Biology,* **275**(2), pp. 347-363.

STAFFORD, D.W., 2005. The vitamin K cycle. *Journal of thrombosis and haemostasis,* **3**(8), pp. 1873-8.

STRICKLAND, D., 1997. Mechanisms of Cellular Uptake of Thrombin-Antithrombin II Complexes Role of the Low-Density Lipoprotein Receptor-Related Protein as a Serpin-Enzyme Complex Receptor *Trends in cardiovascular medicine,* **7**(1), pp. 9-16.

TANAKA, K.A.M., KEY, N.S.M.+. and LEVY, J.H.M., 2009. Blood Coagulation: Hemostasis and Thrombin Regulation. [Review]. *Anesthesia & Analgesia,* **108**(5), pp. 1433-1446.

TANS, G., JANSSEN-CLASSEN, T., ROSING, J. and GRIFFIN, J.H., 1987. Studies on the effect of serine protease inhibitors on activated contact factors. Application in amidolytic assays for factor XIIa, plasma kallikrein and factor XIa. *European Journal of Biochemistry,* **164**(3), pp. 637-642.

TOLLEFSEN, D.M., June, 2010-last update, Blood Coagulation [Homepage of Washington University in St. Louis], [Online]. Available: http://tollefsen.wustl.edu/coagulation/coagulation.html [March, 2011].

TORTORA, G.J. and DERRICKSON, B.H., 2009. The Cardiovascular system: The Heart. *Principles of Anatomy and Physiology.* Jonh Wiley & Sons, Inc, pp. 717.

TOSCHI, V. and LETTINO, M., 2011. Inhibitors of propagation of coagulation: factors V and X. *British journal of clinical pharmacology,* .

TRACY, P.B., 1995. Human Coagulation Factor V Is Activated to the Functional Cofactor by Elastase and Cathepsin G Expressed at the Monocyte Surface. *Journal of Biological Chemistry,* **270**(3), pp. 1408-1415.

VALUEVA, T.A., REVINA, T.A., MOSOLOV, V.V. and MENTELE, R., 2000. Primary structure of potato kunitz-type serine proteinase inhibitor. *Biological chemistry,* **381**(12), pp. 1215-21.

VANE, J.R. and BOTTING, R.M., 2003. The mechanism of action of aspirin. *Thrombosis research,* **110**(5-6), pp. 255-258.

VELDMAN, A., HOFFMAN, M. and EHRENFORTH, S., 2003. New Insights into the Coagulation System and Implications for New Therapeutic Options with Recombinant Factor VIIa. *Current medicinal chemistry,* **10**(10), pp. 797-811.

VINE, A.K., 2009. Recent advances in haemostasis and thrombosis *Retina (Philadelphia, Pa.),* **29**(1), pp. 1-7.

WARKENTIN, T.E., 2004. Bivalent direct thrombin inhibitors: hirudin and bivalirudin. *Best Practice & Research Clinical Haematology,* **17**(1), pp. 105-125.

WEITZ, D. and WEITZ, J., 2009. Update on heparin: what do we need to know? *Journal of thrombosis and thrombolysis,* .

WEITZ, J., 2010. New oral anticoagulants in development. *Thrombosis and haemostasis*, **103**(1), pp. 62-70.

WHITE, C.M., 2005. Thrombin-directed inhibitors: Pharmacology and clinical use. *American Heart Journal*, **149**(Supplement 1), pp. S54-S60.

WHO, October, 2008-last update, The Top 10 causes of death. Available: http://www.who.int/mediacentre/factsheets/fs310/en/index.html [February, 2011].

WOLFE, R., BOLSIN, S., COLSON, M. and STOW, P., 2007. Monitoring the rate of re-exploration for excessive bleeding after cardiac surgery in adults. *Quality and Safety in Health Care*, **16**(3), pp. 192-196.

WONG, P.C., PINTO, D.J. and ZHANG, D., 2011. Preclinical discovery of apixaban, a direct and orally bioavailable factor Xa inhibitor *Journal of thrombosis and thrombolysis*, .

WOOD, Z.A., POOLE, L.B. and KARPLUS, P.A., 2001. Structure of Intact AhpF Reveals a Mirrored Thioredoxin-like Active Site and Implies Large Domain Rotations during Catalysis *Biochemistry*, **40**(13), pp. 3900-3911.

YAVARI, M. and BECKER, R., 2008. Anticoagulant therapy during cardiopulmonary by-pass. *Journal of thrombosis and thrombolysis*, **26**(3), pp. 218-28.

ZEYMER, U. and WIENBERGEN, H., 2007. A Review of Clinical Trials with Eptifibatide in Cardiology. *Cardiovascular drug reviews*, **25**(4), pp. 301-315.