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A NOVEL METHOD FOR THE QUANTITATIVE EVALUATION OF FIBRINOGEN COAGULATION

by

YIDAN LIU

Under the Direction of Binghe Wang

ABSTRACT

Fibrinogen aggregation is the last step in blood coagulation. Inhibition of fibrinogen aggregation could lead to anticoagulation effects. However, there is no good method for the ready evaluation of fibrinogen coagulation. A commonly used path method is slow and requires an expensive instrument. In this project, we have developed a microplate reader and In evaluating inhibitors of fibrinogen coagulations there is no good method. As an important process in hemostasis, fibrinogen coagulation is often detected by micro-plate reader. In our test of fibrinogen coagulation, we improved the observing and analyzing method by using photograph to see concentration-depended effect of thrombin inhibitors on the coagulation. Three known thrombin inhibitors, AEBSF, APMSF and PMSF, were applied to develop the method for detecting the fibrinogen coagulation. The results showed our method is of accuracy in determination of the amount of fibrin when compared with other types of methods.

INDEX WORDS: Fibrinogen, Coagulation, Platelet, Thrombin, Aptamer, SELEX, Thrombin inhibitor, AEBSF, APMSF, PMSF, Photograph

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YIDAN LIU

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Georgia State University

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Yidan Liu
2009

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TABLE OF CONTENTS

ACKNOWLEDGEMENTS iv

LIST OF TABLES vii

LIST OF FIGURES viii

LIST OF ABBREVIATIONS ix

CHAPTER

1. THE COAGULATION PROCESS—AN INTRODUCTION 1

2. FIBRINOGEN COAGULATION EVALUATION 11

2.1 Fibrinogen, Its Structure and Roles in Blood Coagulation 11

2.2 Importance of Inhibitor Development 13

2.2.1 General Overview 13

2.2.2 Literature Inhibitors 15

2.2.3 Aptamer as Inhibitors 17

2.2.4 Potential Application of Aptamers as Anticoagulant 19

2.3 Literature Methods in Evaluating Fibrinogen Coagulation 20

2.4 Method under Development 21

2.4.1 Introduction 21

2.4.2 Special Approaches 22

2.4.3 Methods and Materials 24

2.4.3.1 Sample preparation 24

2.4.3.2 Camera photographing 25

2.4.4 Results 25

2.4.5 Potential Problems 34

2.4.6 Conclusion and Discussion 35

REFERENCES 37

LIST OF TABLES

Table 1. Comparison of Structure, Inhibition Time and IC50 of AEBSF, p-APMSF and PMSF 34

LIST OF FIGURES

Figure 1. 3D Structure of Thrombin 6

Figure 2. Coagulation Cascade 10

Figure 3. Crystal Structure of the Complex Between Thrombin and the Central ‘‘E’’ Region of Fibrin 12

Figure 4. SELEX Process 18

Figure 5. Observation at Start 27

Figure 6. Observation after 3 minutes 27

Figure 7. Observation after 7 minutes 28

Figure 8. Observation after 10 minutes 28

Figure 9. Observation after 20 minutes 29

Figure 10. Observation after 30 minutes 29

Figure 11. Inhibition Curve of AEBSF 30

Figure 12. Inhibition Curve of p-APMSF 31

Figure 13. Inhibition Curve of PMSF 31

Figure 14. IC50 of AEBSF 32

Figure 15. IC50 of p-APMSF 32

Figure 16. IC50 of PMSF 33

LIST OF ABBREVIATIONS

ADP	Adenosin Diphosphate
TFPI	Tissue Factor Pathway Inhibitor
vWF	von Willebrand factor
TF	Tissue Factor

CHAPTER 1

THE COAGULATION PROCESS—AN INTRODUCTION

The coagulation cascade, as a critical part of the hemostasis, is composed of several steps, which can occur when a blood vessel is damaged. It includes reactions from blood platelets and some plasma proteins, which accurately act on the whole system. The end of coagulation cascade is the clotting of the blood and formation of the protective hemostatic plug, which can stop the flow of blood at the site of vascular injury.

When the small blood vessels and capillaries are damaged, they start to contract to diminish the loss of the blood and this is called vasoconstriction. As a result, a series of blood coagulation action are carried out in vivo. Generally, these processes are initiated by the damage of endothelial cells of the vessel wall, go through the “intrinsic” or “extrinsic” pathway, and end with the “common pathway”. Initially, platelets are activated and adhere to the site of injury. This is the result of both “intrinsic” and “extrinsic” pathway. These two pathways play important roles in different aspects in the coagulation cascade. Intrinsic pathway often helps in the growth and maintenance of fibrin formation in the coagulation cascade primarily while the second overlapping mechanism, extrinsic pathway, is critical in the initiation of fibrin formation. After activation of platelet, the aggregation of platelet happens and this lead to the formation of platelet plug that reduces or temporarily stops the loss of blood. Numerous proteins and small molecules are also released in activation of platelet to accelerate and increase platelet plug formation and begin the process tissue repair. The platelet aggregation and platelet plug formation is triggered by the bridges formed between activated platelets and fibrinogen and is stabilized by the insoluble fibrin clot.

Taking a look at the history on studying blood coagulation, it started from the discovery of fibrin which is the substance of a thrombus. Then the substance of product fibrin, fibrinogen was described and isolated. After that, scientists discovered and suggested that the process that fibrinogen was converted to fibrin was catalyzed by an enzyme and there was a precursor of thrombin called prothrombin. This progress was followed with the observation of the important role of calcium and platelets in the enzymatic reaction. Then it was found that thrombin was activated by a series of activation reactions involving several factors. The final activator is factor III which can activate thrombin with the help of calcium. In the 20th century, most factors involved in the whole pathway were discovered and studied. In the 1940s, Orwen found that bleeding diathesis in a young woman could not be explained by the "4 coagulation factor" concept which was suggested by Paul Morawitz in 1905 (Riddel, Aouizerat et al. 2007). He pointed out that the patient lacked a fifth coagulation factor which is we called now as factor V (Owren 1948). As the research on blood coagulation developed, the von Willebrand factor (VWF) was discovered at 1931, with factor VII discovered at 1951(Alexander, Goldstein et al. 1951), factor VIII discovered at 1936 (Patek and Stetson 1936), factor IX discovered at 1952 (Aggeler, White et al. 1952), factor XI discovered at 1953 (Rosenthal, Dreskin et al. 1953), and FX discovered at 1956 (Hougie, Barrow et al. 1957). In the 1960s, two models of explaining the coagulation pathway came out (Macfarlane 1964). The central idea was that blood coagulation was composed of a series of steps in which activation of one factor can stimulate the activation of another. In every step, clotting factors can be activated to active enzymes from inactive proenzyme. The whole process is composed of two pathways, which can be explained by "extrinsic pathway" and "intrinsic pathway". Either pathway would get into the common pathway after the activation of factor FX (Hougie, Barrow et al. 1957).

It was proposed that the formation of fibrin is caused by a series of stepwise reactions involving only proteins exiting in blood in a precursor or inactive form. Each of the plasma proteins was activated by minor proteolysis and many were converted to serine protease. The extrinsic pathway starts from the activation of factor VII by tissue factor(Engelmann 2006), an integral membrane glycoprotein tightly associated with phospholipids, which can be expressed by endothelial cells. This protein, which has a high affinity for FVII, forms a one-to-one complex with FVII in the presence of calcium ions. As a result, FVII is converted to a serine protease by minor proteolysis which happens on the Arg_{152}-Ile peptide bond in the precursor protein. With the participation of calcium ions and membrane phospholipids, the complex formed by TF and FVIIa lead to the activation of FX into FXa, which marks the first step of initiation of the common pathway by the cleavage of a single Arg_{52}-Ile peptide bond in the amino-terminal end of the heavy chain. But the activation of FXa can be immediately inhibited by tissue factor pathway inhibitor (TFPI) which can reversibly inhibit FXa. In addition, the factor IX can also be activated by the factor VIIa-tissue factor complex. This activation is also important in the initiation of the extrinsic pathway. Because the newly formed FIXa can interact with factor VIIIa in the presence of calcium ions and phospholipids, this complex will also activates FX to FXa by cleaving the same bonds that are hydrolyzed by the complex of FVIIa and tissue factor.

FXa remains bound to FV and to the membrane surfaces (phospholipids) which form the prothrombinase complex in the presence of the calcium ions. This leads to the activation from prothrombin to thrombin which is due to the hydrolysis of two internal peptide bonds that reduces the molecular weight of the precursor. In this catalytic reaction, FVa participates as a cofactor in prothrombin activation by increasing the V_{max} of the reaction, whereas the phospholipids provided by the activated platelets reduce the K_m for prothrombin. Then thrombin

activates several cofactors including FV, FVIII and FXI. FVIII is in the form of bound to von Willebrand factor (vWF) which is a large glycoprotein and plays an important role in generating the initial platelet plug (Andrews and Berndt 2004). After that, the vWF releases FVIII which form the tenase complex together with the FIXa. This complex can activates FX and so the pathway keeps on cycling.

Initiation of the common pathway is the activation of FX from either the extrinsic pathway or intrinsic pathway or both. The first protease FXa, with the aid of FV, calcium ions and phospholipids, activates prothrombin to thrombin. Prothrombin is produced in the liver and is modified by vitamin K. Sufficient vitamin K can generate the Gla domain which can localize into the phospholipids bilayers. Thrombin also binds to throbomodulin in order to activate protein C which is a vitamin K-dependent serine protease enzyme to activated protein C. Activated protein C can act with protein S to inhibit FVIIIa and FVa. This process is called protein C pathway which will not be discussed further.

Thrombin is generated by the enzymatic cleavage of two sites on prothrombin by FXa. Binding to FVa can enhance the activity of FXa greatly. Actually, in the initiation process, FXa stuck to the tissue factor on cell surface can combine with FVa to generate a small amount of thrombin (Monroe, Hoffman et al. 2002). . Although this small amount of thrombin cannot catalyze the fibrinogen clotting, the thrombin can be generated continuously. Then in the second process called priming, the thrombin binds to the platelet by binding to von Willebrand factor the matrix proteins can activate platelets such as FXI, FV and FVIII. They can be localized near the site of TF. The TF/VIIa complex can be inhibited by TF pathway inhibitor in the presence of FXa. In the propagation process, when the FIXa arrives on the surface of the platelet, the factor IXa/VIIa complex forms. This action can activate FX into FXa which can move into a protected

complex with FVa. This factor complex can generate a lot of thrombin which is enough to form the fibrin clot.

When thrombin is formed, it converts fibrinogen to fibrin by limited proteolysis. The formation of fibrin is due to the formation of cleavage of a peptide bond in each of the two α chains and in each of the two β chains. As a result, four fibrinopeptides and fibrin monomers with new amino-terminal sequences of Gly-Pro-Arg in the α chains and Gly-His-Arg in the β chains. The newly generated Gly-Pro-Arg residues in the α chain of one monomer then bind to the D domain in the adjacent fibrin monomer, leading to the linear polymerization of fibrin. The newly formed Gly-His-Arg from the β chain binds to the D domain of an adjacent fibrin monomer, leading to the side-by-side polymerization of fibrin. These polymerization reactions generate the insoluble fibrin clot. Factor XIII is also an important factor participating in the fibrin formation. It can be activated by thrombin in the presence of calcium ions. FXIIIa can crosslink to fibrin in covalent form which makes this linkage very strong. FXIIIa can also crosslink other plasma proteins such as fibronectin and α_2-antiplasmin to the α chains of fibrin, resulting in their incorporation into the fibrin clot.

In this process, thrombin is the key enzyme in catalyzing the fibrinogen to fibrin. Thrombin, a coagulation protein with 36 kD molecular weight that plays an important role in the coagulation cascade, is an allosteric serine protease of the chymotrypsin family. When prothrombin is activated, the active domain goes out of it. The function of thrombin is, firstly, the conversion of fibrinogen to fibrin that can allow platelets to adhere to it and protect the bleeding site to repair. FXIIIa activated by thrombin can stabilize the formation of the fibrin gel. Secondly, thrombin is the activator of protein C (Di Cera 2003). In the protein C pathway, thrombin, thrombomodulin, the endothelial cell protein C receptor (EPCR), protein C and protein

S play the key role in the process. Although thrombin binding to thrombomodulin will decrease the ability of thrombin to degrade fibrinogen and protein-activated receptor, the specificity to protein C increases apparently. Protein C activation reaction is enhanced when it is bound to the endothelial cell protein C receptor (EPCR). Due to the efficient activation of thrombin acting on protein C, when thrombin stops generating, the activated-protein C stops generating. The APC can bind protein S and FVa, FV and FVIIIa and then inactivate FVa and FVIIIa which participate in the activation of FX and FIX that are required for thrombin generation. What's more, thrombin can be inhibited to bind to fibrinogen by antithrombin and the assistance of heparin and heparin cofactor II. Besides, thrombin also has effect on PAR1 (Vu, Wheaton et al. 1991), PAR3 (Ishihara, Connolly et al. 1997) and PAR4 (Kahn, Zheng et al. 1998), Protease Activated Receptors, which belongs to protease-activated receptors. These mechanisms will not be mentioned in this article.

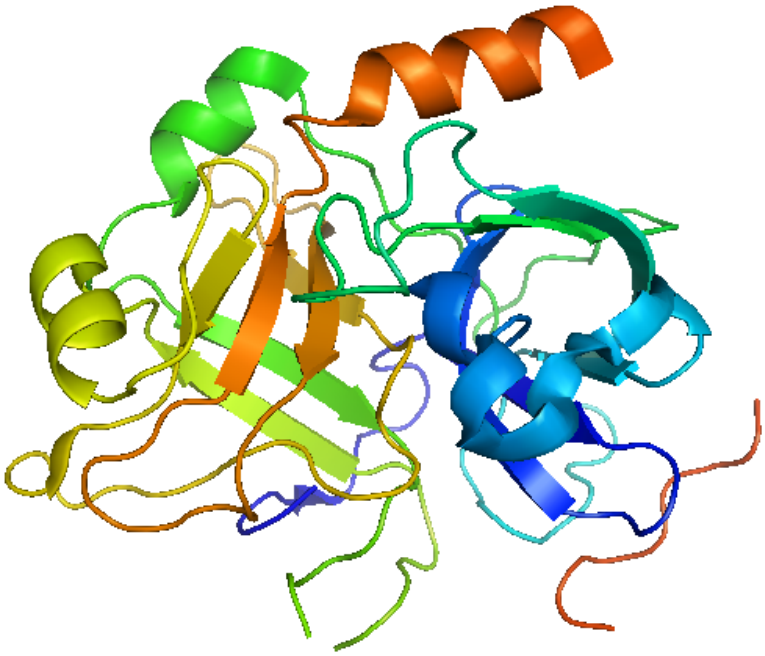


Figure 1 3D Structure of Thrombin （PDB ID: 2BVS (Neumann, Junker et al. 2005)）

The X-ray structure of thrombin was published in 1989 (Bode, Mayr et al. 1989). Generally, thrombin has two chains, A and B. The A chain is composed of 36 residues and is not important in the proteolytic activities. The B chain has 259 amino acids which come from the C-terminal sequence of the prothrombin. The active site of the B chain includes His57, Asp102, and Ser 195. These 2 chains are covalently linked to each other through the disulfide bond between residues C1 and C122 (Bode 2006). The whole structure of two chains is that, the A chain is put in the back of the enzyme and the B chain with all the active sites and functional domains are located in the front of the molecule. The special structure of the thrombin is that, like most of the chymotrypsin, the folding form of the protein is two Greek key β-barrels lie on top of and perpendicular to one other. All the catalytic residues lie in the gap between the barrels making it deeper and narrower than that of trypsin. There are two additional regions called exosite which locate out of the active site can induce the recognition of the substrate. The function of each barrel is catalytic and regulatory respectively. Thrombin has a "60-loop" which locates near the hydrophobic wall of the S2 site. This loop can restrict the P2 side chains to small and hydrophobic residues, such as Val or Pro. At the bottom of the S1site, the catalytic pocket is occupied with the Arg residue at P1 site. Residues of P3 leave away from the surface of thrombin whereas residues composed of aromatic and hydrophobic amino acids go to the surface of the enzyme. There is an autolysis loop circulate the active site of thrombin which guide the fibrinogen to it. This loop is the similar to the Ca^{2+} binding loop of trypsin and chymotrypsin. Exosite1 has a strong electrostatic field because of the containing of several positively charged residues. This site gives the guidance for fibrinogen, fibrin, thrombomodulin and PAR1 and PAR3. At the opposite side to exosite 1, the exosite 2, is the location for interaction with polyanionic ligands and glcosaminoglycans. For example, the heparin can locate into the exosite

2 in order to increase the inhibition of antithrombin. There is a special residue different from the serine protease is the replacement from glutamine to glutamate. This change can make thrombin more effective in activating the protein C in the absence of thrombomodulin.

Actually, Na^+ is important to the activity of thrombin because it can cause the conformational changes of thrombin. With the conformational changes, the enzyme shift from Na^+-free slow form with low activity to the Na^+-bound fast form with high activity (Di Cera, Dang et al. 1997). To explain from the structure, Na^+ binds 16-20Å away from residues of the catalytic triad and within 5Å from D189 in the primary specificity pocket. Nestled between the 220- and 186-loops, this site has very high affinity to the Na^+ than other ions such as Li^+ and K^+. (Di Cera, Page et al. 2007)

Na^+ binding to thrombin requires the cleavage of fibrinogen and promotes cleavage of PAR1, PAR3 and PAR4, but it doesn't induce the cleavage of the protein C. So the Na^+ binding to thrombin enhances the catalytic activity of all substrate except protein C (Di Cera 2008).

The binding of Na^+ obeys the mutation of D189, E217, D222 and Y225. These residues all locate within 5Å from the bound Na^+. D189 can help one of the four water molecules to bind to Na^+ site and provide an important linkage between the Na^+ site and the P1 residue of substrate. E217 can make polar interactions with K224 and T172 which stabilize the 220-loop in the Na^+ site. The primary changes are the formation of the R187-D222 ion pair, which leads to a shift in the backbone O atom of R221a. Also the shift of the side chain of E192 is due to the reorientation of D189 and the k_{cat} value also changes due to the change of the position of the Oγ atom of S195. The H-bond also forms between S195 and E192 when the enzyme is in the Na^+-bound fast form.

The other pathway which is the intrinsic pathway is the primary route for the continued growth of the fibrin clot. The intrinsic pathway starts with the activation of FXI. FXII, high molecular weight kininogen (HMWK) and prekallikrein are included in this reaction and prekallikrein and FXII are converted into kallikrein and FXIIa. The activated FXI forms FXIa which lead to the activation of FIX to FIXa. FIXa activates FX to FXa by acting with co-factor FVIIIa to form tenase complex. This pathway is not very important in trauma-initiated coagulation, so patients who have bleeding disorder have nothing to do with the lack of FXII, HMWK and prekallikrein.

The most important activation is thrombin activation which is explained in the article. Platelet aggregation is mediated by fibrinogen which links adjacent platelets through interactions with the glycoprotein IIb/IIIa complex. At first, platelet is a flowing disc-shaped platelet. With the interaction with the von Willebrand factor via the glycoprotein Ib receptor, it become to a rolling ball-shaped platelet. This interaction is triggered by the releasing signal of platelet which induces the changing to the hemisphere shape. So the surface area is increased so that platelets can interact with the vessel surface and resist the rapid flow of the blood. At last, the hemisphere-shaped platelet changes to the spreading platelet irreversibly.

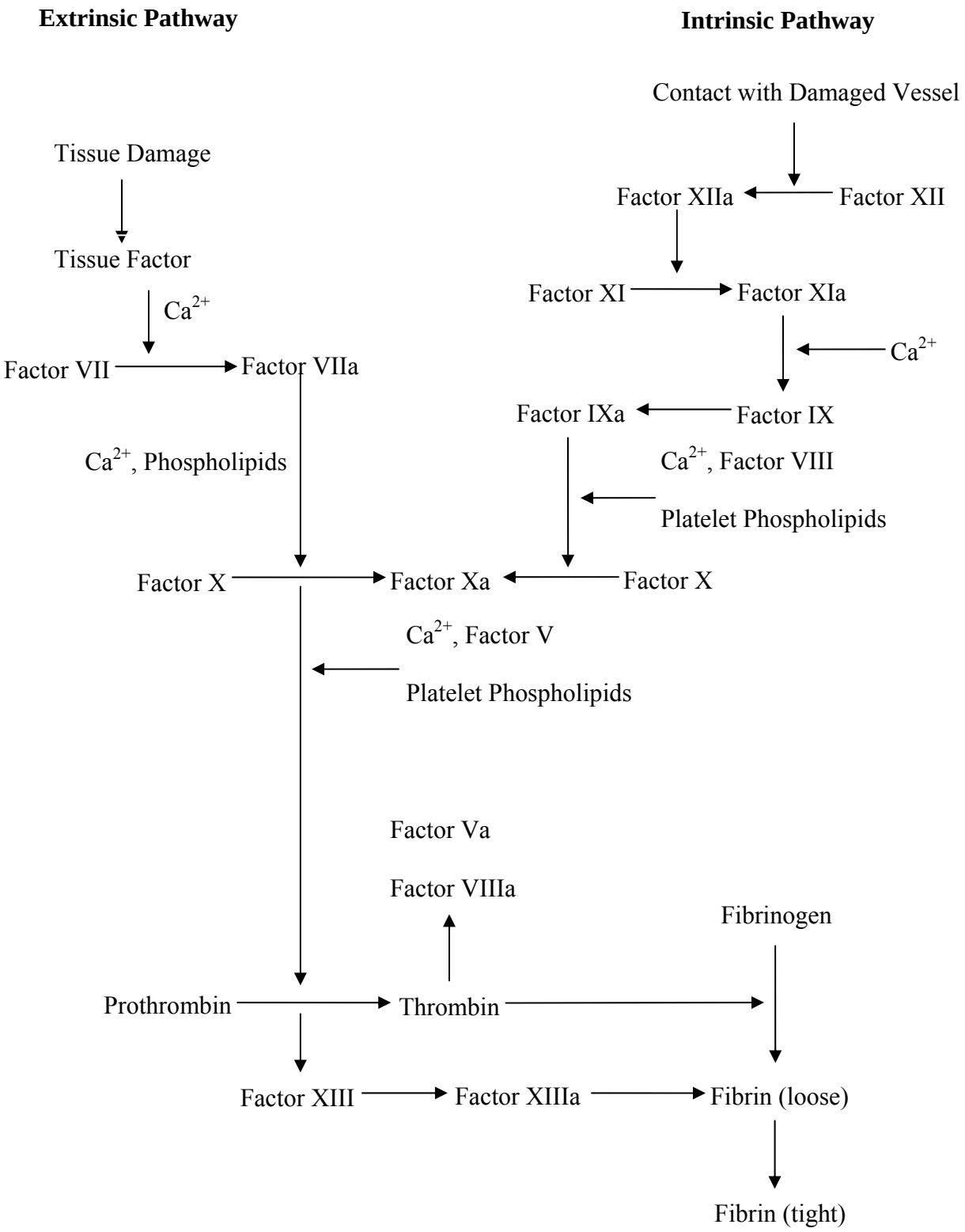


Figure 2 Coagulation Cascade

CHAPTER 2

FIBRINOGEN COAGULATION EVALUATION

2.1 Fibrinogen, Its Structure and Roles in Blood Coagulation

Fibrinogen has an elongated structure which contains two D domains, each connected by a super-coil chain to the central E domain. The molecule consists of Aα, Bβ and γ, which are joined together in the *N*-terminal E domain by five symmetrical disulfide bridges (Huang, Cao et al. 1993). Each fibrinogen Aα-chain contains an *N*-terminal fibrinopeptide A (FPA) sequence, cleavage of which by thrombin initiates fibrin assembly (Nesheim 2003). Thrombin binds to the central E domain of fibrinogen and removes the *N*-terminal peptides of the Aα- and Bβ-chains. Thrombin cleaves the Aα-chains first, removing the N-terminal 16 residue peptide (fibrinopeptide A, FpA) (Mosesson 2005). Removal of FpA exposes a new *N*-terminal sequence GPRVVE (Betts, Merenbloom et al. 2006), known as the "A" site. This "A" site permits the noncovalent association of the central "E" domain with a constitutively exposed "a" pocket in the γ-chain of the D domain of another fibrinogen molecule.

Fibrin is a protein involved in blood clotting. It is a fibrillar protein that is polymerised to form clot over a wound site. The assembly of fibrin improves the intermolecular antiparallel *C*-terminal alignment of γ-chain pairs, which are cross-linked by factor XIII.

The biological functions of fibrinogen and fibrin include inhibiting FXIII to crosslink each other by forming the FXIII A_2B_2 complex (Lorand 2005). This is due to the sub domain which contains fibrinogen becoming cross-linked more slowly than homodimeric fibrinogen molecules. The B subunits can prevent thrombin-independent activation of TFXIII. Thrombin with no substrate can bind to fibrin, which down-regulates thrombin generation. The non-substrate thrombin binding to fibrin is the antithrombin I. Fibrin has two thrombin-binding active

sites called E and D domain. The E domain has lower affinity for thrombin than D domain. A ternary tPA-plasminogen-fibrin complex can be generated from tPA-stimulated plasminogen and fibrin. TPA-stimulated plasminogen activation is strongly promoted by fibrin polymers. When fibrin binds to plasminogen, it is activated and forms a ternary complex. Two sites in fibrin Aα148-160 and γ312-324 are thought to have enhancement on the plasminogen. Interaction with the extracellular matrix can be increased by binding of fibronectin to fibrin.

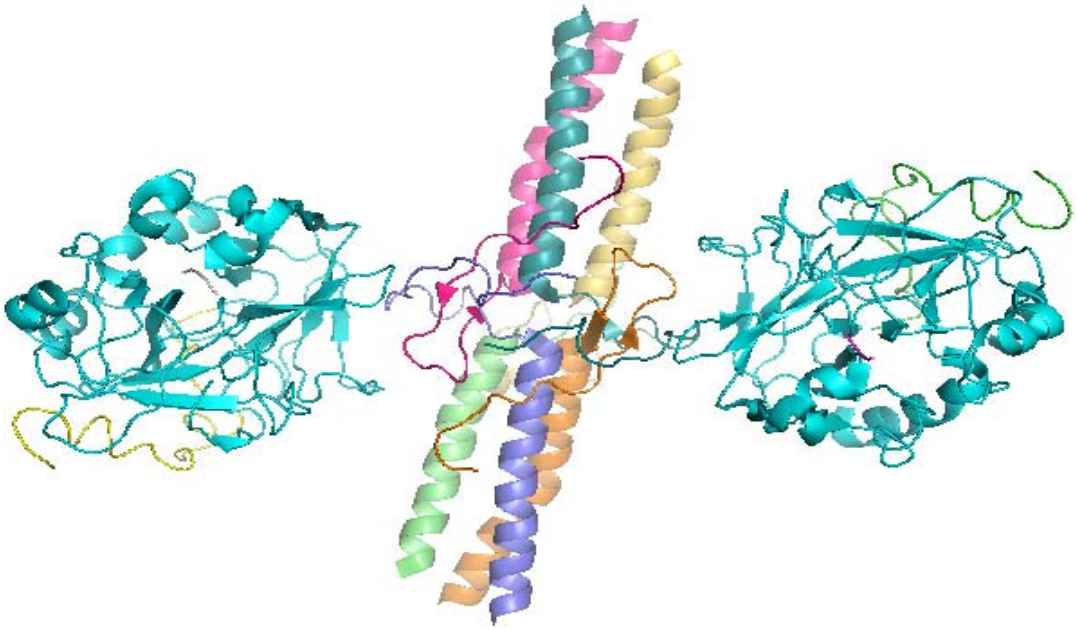


Figure 3 Crystal Structure of the Complex Between Thrombin

and the Central ‘‘E’’ Region of Fibrin（PDB ID: 2A45(Pechik, Madrazo et al. 2004)）

2.2 Importance of Inhibitor Development

2.2.1 General Overview

The thrombin inhibitors are used to inhibit thrombosis formation. Thrombosis is the blood clot formed in the blood vessels. Generally, three abnormalities can cause thrombosis, unbalanced composition of the blood, deteriorated quality of the vessel wall, and abnormal blood flow and stimulation of the aggregation of platelets. These abnormalities can lead to many diseases which are common among human beings. Unbalanced composition of the blood often causes the venous thromboembolism and arterial thrombosis. Venous thrombosis is a blood clot that forms within a vein. Arterial thrombosis is the formation of a thrombus within an artery. These diseases are often believed as the coagulation or aggregation of the fibrinogen and platelet. There are several factors and enzymes that affect the coagulation of the fibrinogen such as factor XIII and thrombin. The final product of the coagulation is fibrin clot which is formed through the intrinsic and/or extrinsic pathway. Aggregation of platelets is another cause of venous thromboembolism. When platelets are exposed to several stimuli, they can undergo a series of interactions to stimulate aggregation. They can adhere to many surfaces, undergo shape change and release the granular contents. They also aggregate to form clump and alter the nature of their surface in a fashion that facilitates blood coagulation. In addition, damage to the vessel wall is also an important factor in thrombosis. When the endothelium on blood vessel is damaged, fibrinogen and platelet will face several factors that they never interact in a good vessel conditions and form clot or aggregation. Since it is not an important factor in the application of our research, it won't be described in this thesis. To treat these diseases which are common in our life, the development of inhibitor in controlling the fibrinogen coagulation and platelet aggregation is important, especially thrombin inhibitor which directly cause the formation of the

fibrin clot. Thrombin is a plasma serine protease that plays a key role in coagulation and hemostasis and other thromboembolic diseases which are known as the major cause of mortality and morbidity in the world. In the market of medicine nowadays, there are many kinds of drugs, which are indirect anticoagulant. Heparin, another type of anticoagulant (Das and Kimball 1995), shows different extent of effect in different patients (Weitz 2003). In the thrombin-fibrinogen system, thrombin is a good target for pharmacological intervention. That's because the critic role it play in blood coagulation. In addition, thrombin also regulates platelet activation and aggregation by acting on protease-activated receptors. With several criteria such as binding to plasma protein, relatively short half-lives, high degree of selectivity, chemically and metabolically stable and nontoxic, thrombin are selected. Because the importance it has in coagulation and hemostasis, thrombin is regarded as an ideal target for anticoagulant therapy. In the pharmaceutical industry, there are several types of thrombin inhibitors. As a serine protease, thrombin is composed of an A chain and a B chain which contains the catalytic amino acids like Asp102, His57 and Ser195. Different from the serine protease, it has specific active sites include S1 pocket, S2 pocket and exosite I. It is commonly considered that direct thrombin inhibitors can be separated to two types. One type is natural peptidic thrombin inhibitors such as hiridin, bothrojaracin, triabin, rhodniin, ornithodorin and petalogastin (Schwienhorst 2006). The other type is designed small-molecule inhibitors (Shen 2001) such as argatroban and melagatran which can bind to the S1 pocket in thrombin. As the DNA drug developed these years, aptamer selected from SELEX method can also recognize the thrombin as a target (Paborsky, McCurdy et al. 1993), bind to the active site and inhibit the activity of the enzyme. To take the foreign DNA as a drug, the specificity and delivery of the drug can be optimized in the human body. In addition, the toxicity of the drug is lower than other synthesized small-molecule drugs.

2.2.2 Literature Inhibitors

In the development of direct thrombin inhibitors, people first applied peptidic thrombin inhibitors in thrombin inhibition. A representative thrombin inhibitor is the hirudin which was identified in 1884 and isolated from the salivary gland of medicinal leeches and purified by Markwardt around 1955. Now, hirudin is available through the recombinant DNA technology (Eikelboom, White et al. 2003). As a potent and almost irreversible bivalent inhibitor of thrombin, it can block the active site as well as the substrate recognition exosite which recognize fibrinogen. Due to the problem that it has a half-life of approximately 60 min after intravenous administration, its application in clinic is limited.

In the development based on the inhibition mechanism of hirudin, another representative thrombin inhibitor is synthesized as the bivalirudin. Bivalirudin is a 20-amino acid polypeptide that was developed based on studies of the interactions between hirudin and thrombin. It has a 12-amino acid carboxyl terminal domain which can bind to the fibrinogen recognition exosite 1 in thrombin and a 4-amino acid domain which can bind to the thrombin active site linked by 4 glycine residues (Gladwell 2002). The dPhe-Pro-Arg sequence which belongs to the 12-amino acid domain can mimic the specific binding of human fibrinopeptide A. Compared to hirudin, bivalirudin has the advantages having a shorter half-life to make it more common application in clinic.

The other type of thrombin inhibitor is the small-molecule inhibitors or low molecular weight thrombin inhibitors. A representative compound is argatroban, a synthetic direct thrombin inhibitor derived from L-arginine (Linkins and Weitz 2005). Different from the hirudin, argatroban binds to the thrombin catalytic domain but not exosite 1. It can form a reversible complex with thrombin.

Recently, more potent inhibitors were obtained by replacing the active-site-binding segment by small-molecule inhibitors which is derived from argatroban or NAPAP, and from electrophilic inhibitors such as boronic acid, arginyl methyl ketones and α-ketoamides. For example, argatroban, a direct thrombin inhibitor which is after used as a substitute for heparin, can binds to the catalytic site of thrombin selectively and reversibly (Mureebe 2007), It can be regatded as a competitive inhibitor. In comparing to heparin which is also a thrombin inhibitor, argatroban shows higher inhibition ability. It also stands out of other anticoagulants for the good selectivity for the catalytic site of thrombin and for the ability to bind and inhibit clot-bound thombin. Except for argatroban, some electrophilic compounds can also be used as small-molecule inhibitors. A type of compound which are attached by boronic acid are used in the anticoagulation field as boroArg inhibitors. They can bind to the thrombin catalytic site slowly and tightly.They are often modified by different functional groups for high selectivities to the catalytic sites.

The thrombin inhibitor or the serine protease inhibitor such as AEBSF, APMSF, and PMSF which contain the benzenesulfonyl fluoride functional group will interact with the hydroxyl group to inhibit the enzyme activity. As other serine protease, the most important catalytic site must contain serine which will be involved in the catalytic reaction. It has been proved that aromatic sulfonyl fluorides have higher reactivity than other sulfonyl fluorides by attaching a positively charged benzamidine or pyridinium moiety to the parent benzene ring in the modification of the sulfonyl fluorides. In addition, it has also been proved that benzenesulfonyl fluorides can be made to react with a variety of serine proteases by the addition of amino alkyl or amidine side chains to the benzene ring such as AEBSF and p-APMSF. Take the p-APMSF as an example, a specific, irreversible inhibitor of the class of plasma serine

proteases can cause immediate and complete irreversible inhibition of bovine trypsin and human thrombin. With the functional group which is sulfonyl fluorides, p-APMSF can act by sulfonylation of the active-site serine with formation of an *O*-sulfonylserine. As a result, the enzyme is inactivated by the inhibition of serine in the active site.

2.2.3 Aptamer as Inhibitors

Aptamer are "nucleic acid ligands" that can be generated against amino acids, drugs, proteins and other molecules by employing SELEX. SELEX, which stands for Systematic Evolution of Ligands by Exponential Enrichment, can produce oligonucleotides as aptamers of either single-stranded DNA or RNA that specifically bind to a target ligand (Blank and Blind 2005). Aptamers are isolated from complex libraries of synthetic nucleic acid by an iterative process of adsorption, recovery and re-amplification. They have potential applications in analytical devices, including biosensors, and as therapeutic agents." This concept was defined by William James in the *Encyclopedia of Analytical Chemistry*. The screening potential can be activated when aptamers would bind to proteins and other molecules that do not normally interact with DNA or RNA.

The random oligonucleotides which compose an aptamer library can be made by simply repeatedly duplicating the natural 3'-5' linkage in oligonucleotides easily. When the library is generated, the complexity is determined. For oligonucleotides of N nucleotides in length, generated from y different nucleotides, the complexity = y^N. Such complexity suggests that the pool must contain a few molecules with the correct receptor structure or structures which have catalytic activity (Kulbachinskiy 2007). These molecules can be selected by affinity chromatography or filter binding according to the binding affinity for the target molecules. In this process, the library of nucleotide sequence is exposed to the target molecules in the library

and incubated for a period of time. The molecules which have weak or no affinity for the target will stay in the solution, whereas the molecules with high binding affinity will tend to attach tothe target. With a few of the molecules attached to the beads, the rest unbound molecules remaining in solution can be washed out. Then the target-bound molecules, which have the highest affinity to the target, can be purified away from the target and used for the subsequent steps in the SELEX process (Stoltenburg, Reinemann et al. 2007)

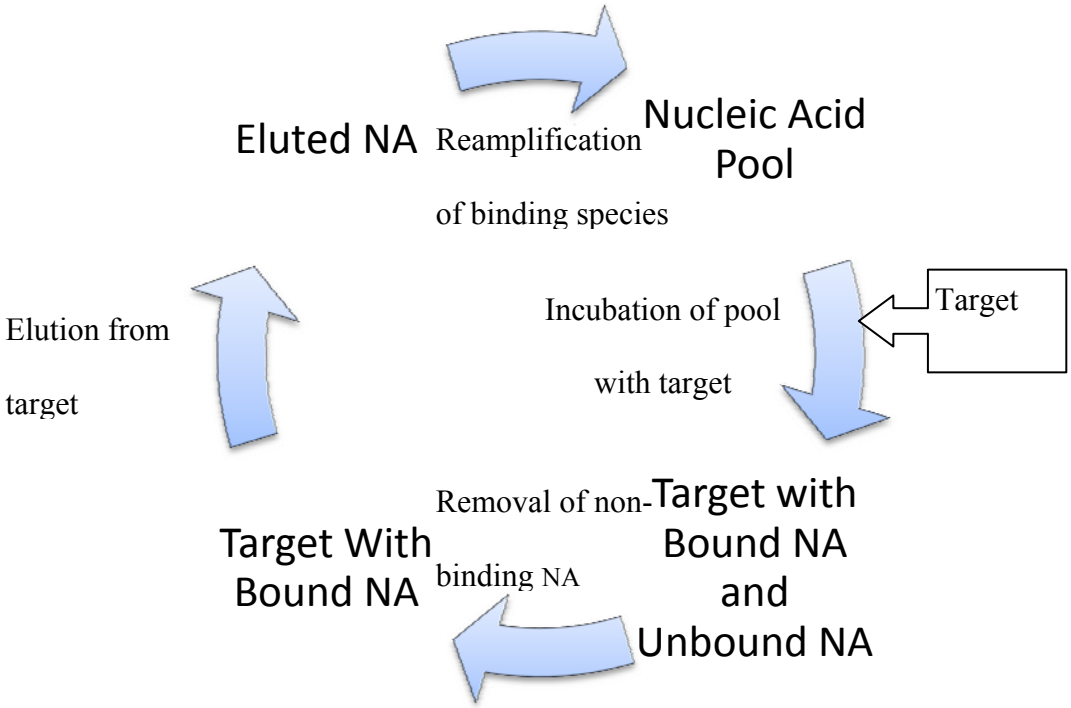


Figure 4 SELEX Process

Because the pool may contain just a few functional molecules, they have to be amplified by polymerase chain reaction (PCR) or in a transcription-based step. This will lead to the generation of a new library of molecules that is enriched with oligonucleotides that can bind to the target. After 5 to 15 rounds of the complete SELEX process, the library of molecules shrinks to a small number that bind tightly to the target. As a result, the nucleotide sequence is

determined. The target binding affinity and specificity of selected sequence are then measured and compared.

2.2.4 Potential Application of Aptamers as Anticoagulant

Aptamers are nucleic acid molecules which are selected to have specific binding to the target molecules. Although the anticoagulant therapy has been developed in the progress as low-molecular-weight heparins and direct thrombin inhibitors, we still need a safer effective anticoagulant because of toxicity and bioavailability. Different from the classical thrombin inhibitors, aptamers have several advantages in specificity, selectivity and so on. Firstly, aptamers show high affinity and specific binding to the target molecules with a low binding constant from high pico-molar to low nanomolar range. Secondly, aptamers are purported to be nonimmunogenic. The relative small sizes of aptamers make them poor antigens, and the aptamer-based anticoagulants are unlikely to induce autoimmune conditions. Thirdly, in the pharmacokinetic and pharmacodynamic properties, aptamers behave well in the PK/PD relationships. Finally, antidotes can be designed to control the pharmacologic activity of aptamers (Nimjee, Rusconi et al. 2005).

According to high binding affinity, aptamers are often studied and applied in pharmaceutical field as antibodies to specific targets. Nowadays, aptamers are used as scientific and biotechnological tools to study specific cellular protein functions and protein-ligand interactions as well as translator or interpreter to demonstrate biologically relevant regulatory circuits or improve understanding of molecular mechanisms of disease process (Rimmele 2003). In details, aptamers can be used as inhibitors in mammalian signal transduction pathways in cells, as ligand-regulated aptamers in gene-specific small-ligand-controlled translation, as tools in antibiotic research which can bind to the bacterial RNA, as antibodies in *in vitro* diagnostics and

in vivo imaging of disease-related targets, as biosensors in specific detection of a large variety of target molecules and also as the sensing part of allosteric ribozymes which have allosteric ability after conjunction of the aptamers to the ribozyme. In addition, aptamer can also be applied as therapeutic agents and potential drugs. In many diseases, aptamers were proved to block specific sites on proteins which is related to diseases. With high specific affinity to targets, aptamers can target growth factors which can stimulate cellular growth, proliferation and cellular differentiation. They can also target intracellular target molecules and factors in autoimmune and inflammatory disease. For application in anticoagulation, aptamers have been developed to bind to several proteins through the blood coagulation process, such as thrombin and FIXa (Ulrich, Trujillo et al. 2006).The development of the studies of aptamer made them from simple research tools to a major technology with commercial potential. In addition, more application aspects are observed by people such as molecular cargoes delivery properties, application in treating HIV and so on. As a result, it is possible that a range of anti-viral aptamers can be generated, and they can bring a new type of therapy in treating diseases related to virus. Obviously, the potential of aptamer technology cannot be ingored by people in the future.

2.3 Literature Methods in Evaluating Fibrinogen Coagulation

Among these methods, one is by using micro-plate reader to measure the absorbance of the solution at 405 nm. In this method, solutions are measured in 96-well plate (Ashour, Gee et al. 1987) to determine the optical density of the fibrin clot. The mechanism of reading the absorbance of the solution or gel can be explained as the light blocking in the process of forming clotting. When fibrinogen is catalysed by thrombin, it forms white gel from transparent solution. As a result, the light go to the gel will be refracted or dispersed which lead to the loss of light when the machine is detecting. As the gel forms more and more, the solutions become less and

less transparent. As a result, the absorbance will increase as more light is blocked. Although it is different from absorption of light in the solution, the results can be regarded as light absorption at the end.

In addition, Roy R. H. et al.(Hantgan and Hermans 1979) used stopped flow light scattering method in measuring the fibrin clot. This method is based on the increase of the scatter when fibrin is formed from fibrinogen.

2.4 Method under Development

2.4.1 Introduction

Serine proteases are a family of enzymes that cut certain peptide bond in proteins. The character of this kind of enzyme is containing serine at the active site. Thrombin is one member of this large family. The catalytic mechanism to cleave the peptide bond is that the electron goes from the nitrogen on imidazole of His to the hydroxyl group to make the oxygen on hydroxyl group to be negatively charged. The electron on oxygen will goes to the carbon which is electrophilic on the ketone of peptide bond to make it cleaved.

In the fibrinogen coagulation system, the fibrinogen will form fibrin which appears as white gel with the catalysis of thrombin. We can use the micro-plate reader to detect the forming of the gel at 405 nm. This method is based on the blocking of the light of the white gel. However, the disadvantage of this method is that the formed gel is not homogeneous so that the detection of absorbance of one spot in a well cannot represent the absorbance of the whole solution in a well. That will make the inaccurate data during the calculation.

To solve this problem, we propose to use a photography method which can be easily observed and measured. This method is to use the photos taken by camera in the dark room to

observe the change of the gel forming in a period of time. The photos which show the changes in 96-well plate can be measured by a software-Image J (Collins 2007) based on the integral of an unit area. Even if the solution is not homogeneous, the intensity we get of each sample is the average intensity in an unit area.

2.4.2 Special Approaches

As we discussed before, the typical method in quantumize the fibrin clot is the determination of absorbance with micro-plate reader. Normally, the fibrinogen coagulation is determined by the micro-plate reader which uses the wavelength at 405 nm to measure the absorbance of the solution. The measurement is processed in the way that each well is read one by one and the unit rounds are repeated every 20 seconds. The reading data are based on the blocking of the light by the coagulation of fibrinogen. The more the gel forms, the less light can pass through the well. As a result, the absorbance will increase as the gel forms. While running, the micro-plate reader emits the one light beam to the well. That means the absorbance it read is from one point of the solution in the well.

As a typical quantum method in determine the fibrin clot, the advantages of this method is the convenience in operating the experiment and getting data. Because the experiment only requires the working of the micro-plate reader and the whole reacting system can be under a consistent situation. In gathering data, the micro-plate reader output the absorbance by the transformation of the computer. In measuring a homogeneous solution, micro-plate reader after shows the consistent result under the same condition in repeating same experiments. However, in the coagulation process, we observed that gel formation is not homogeneous. It can be clearly seen that the fibrin is formed like a white thin thread in the well which make the solution inhomogeneous. In reading of the micro-plate reader, the absorbance is obtained from one point

of the whole inhomogeneous solution. If the fibrin aggregates a lot at the measure point, the absorbance will be larger than the one with small amount fibrin at the measure point. The error of every well is relatively large and different from each other because different formation of fibrin.

In photography method, the changes are observed by us and the observation results are recorded by the camera. The gel formation is calculated by the software which uses the integral method to calculate the intensity in a unit area.

There are several specific aspects of our method. First of all, the result is easy to observe. The changes can be seen by eyes and recorded by camera. The photos are taken in the dark room in order to make the photos clearer in showing the result. The photos are not taken well by well but all together in one picture. This kind of shooting method not only makes it easier to compare the difference from well to well but also keeps the result showed under a consist situation. There is tiny difference from well to well.

Secondly, the operation of this method is simple and easy. It doesn't require a lot of steps to complete the whole process. After preparation of the solution, they are pipetted to the wells in the 96-well plate. All the rest of work requires the camera to complete.

In addition, the results are more accurate compared to the method with micro-plate reader. With the taking photos method, the whole solution can be seen clearly and the measurement can be taken in an area not a point. As a result, the range of error can be minimized from well to well.

At last, the specific calculation way can show the intensity in a unit area using integral method. If each well can be calculated with the whole area, the amount of formed fibrin can be determined. The higher the intensity means the more gel forms.

2.4.3 Methods and Materials

2.4.3.1 Sample preparation

All stock solutions and buffers prepared are described below. All the reactions were performed in 50 mM Tris-Cl buffer. 1 M HCl was added to adjust the pH to 7.4. 50 ml Tris-HCl buffer was prepared by dissolving 0.30 g Tris base (MW=121.14) into 50 ml deionized water. The $CaCl_2$ stock solution with the concentration of 100mM was prepared in 50 mM Tris-Cl buffer, pH 7.4. For each run, 20 μl $CaCl_2$ solution was required, which was made of 0.555 g $CaCl_2$ (MW=110.99) dissolving in 50 mM Tris-Cl buffer. The fibrinogen stock solution with the concentration of 17.6 μM was prepared in deionized water. The thrombin stock solution with the concentration of 100 unit/mL was prepared in 50 mM Tris-Cl buffer. Three thrombin inhibitors AEBSF, PMSF (Hsia, Ganshaw et al. 1996) and p-APMSF (Laura, Robison et al. 1980) were used to test the fibrinogen coagulation in the presence of thrombin. The AEBSF stock solution with the concentration of 100 mM was prepared in aqueous HCl, pH 5.1. The PMSF stock solution with the concentration of 50 mM was prepared in DMSO. The APMSF stock solution with the concentration of 50 mM was prepared in deionized water.

The Mix A is a mixture of the fibrinogen solution, the $CaCl_2$ solution and Tris-Cl buffer. The final concentration of $CaCl_2$ is fixed at 20mM, while the fibrinogen concentration is 2μM. The negative control solution was prepared by mixing the Mix A with the thrombin stock solution and the final concentration of thrombin was 0.2Iu/mL. The fibrinogen coagulation tests were carried out by mixing the Mix A, the thrombin stock solution and the three inhibitors respectively, in which the final concentration of AEBSF and PMSF were from 0.1 mM to 1.5 mM, the final concentration of APMSF was from 1 μM to 15 μM. These mixtures were called thrombin inhibition solutions.

All of the mixtures were votexed by Vortex-1 Genie Touch Mixer to make the homogeneous solutions. The negative control solution and the thrombin inhibition solutions were incubated in a 96-well plate in 37°C for 30 minutes. Table 2 shows the components and their concentrations for each reaction.

2.4.3.2 Camera photographing

The photos are taken in the dark room. The canon digital camera equipped with Canon EF 17-40mm f/4L USM Lens and Canon EF 50mm f/1.8 II Lens was used for photographing. One photo was taken before adding the thrombin inhibitors. In less than 1 hours after incubation, photos were taken at a time interval of 10 to 20 minutes.

After completing the photographing, the photos were analyzed by utilizing the software Image J (National Institutes of Health), which can calculate the integral in a chosen area. We choose the central area of the well to remove the reflection of the flashlight. At last, the intensity is calculated by integral over area.

2.4.4 Results

In the test of three thrombin inhibitor AEBSF, APMSF and PMSF, I tested the reaction time, relationship between time and amount of formed gel, IC50 of each thrombin inhibitor. For these three thrombin inhibitor, the time to inhibit thrombin is different from each other as seen from the photos.

Figure 5 shows the transparent fibrinogen solution without thrombin inhibitor-AEBSF. Because once I add the thrombin with thrombin inhibitor AEBSF of different concentration, the catalytic reactions start. So this photo can be regarded as it is photographed at 0 minute of this catalytic reaction. From this photo, we can see that there is no difference among all six solutions.

Figure 6 was taken 3 minutes after starting of the reaction. The photo shows that fibrinogen is forming fibrin which is like white gel in the wells. From B9 to C11, the fibrinogen solution are added by thrombin inhibitor with different concentration (1.5mM, 1.0mM, 0.5mM, 0.3mM, 0.1mM, 0.0mM). As we can see, from B9 to C11, the gel formation is different from well to well. The B9 well which has the highest AEBSF concentration forms the smallest amount of fibrin. The C11 well which has the lowest AEBSF concentration forms the largest amount of fibrin. From B9 to C11, the amount of fibrin is increase as the concentration decrease.

Figure 7 was taken 7 minutes after starting of the reaction. This photo shows that gel formation increase as the time period increases. Apparent changes happen among B11, C9, C10 and C11 which means more gel formed in these mixtures. We can see more clearly that different amount of gel formed according to different AEBSF concentration. From B9 to C11, the amount of fibrin is increase as the concentration decrease.

Figure 8 was taken 10 minutes after starting of the reaction. We can still see apparent change of the amount of gel. B9 and B10 almost have no changes in color and B11 to C11 keep changing a lot. It is more clearly to see the different amount of gel formed in different wells.

Figure 9 was taken 20 minutes after starting of the reaction. From the photo, it can be seen that the color of each well changes less than that between 3 and 7 minutes. The catalytic process slows down in this time period.

Figure 10 was taken 30 minutes after starting of the reaction. From this photo we can see that the change in each well become much smaller than before. The coagulation of the fibrinogen is going to complete. As the final photo, the different solutions with different AEBSF concentration have different colors.

Figure 5 Observation at Start (Five solutions was prepared with different concentration of AEBSF which are 0.1mM, 0.3mM, 0.5mM, 1.0mM and 1.5mM from B10 to C11. Negative control solution was prepared in B9.The photograph were taken in the dark room at room temperature.)

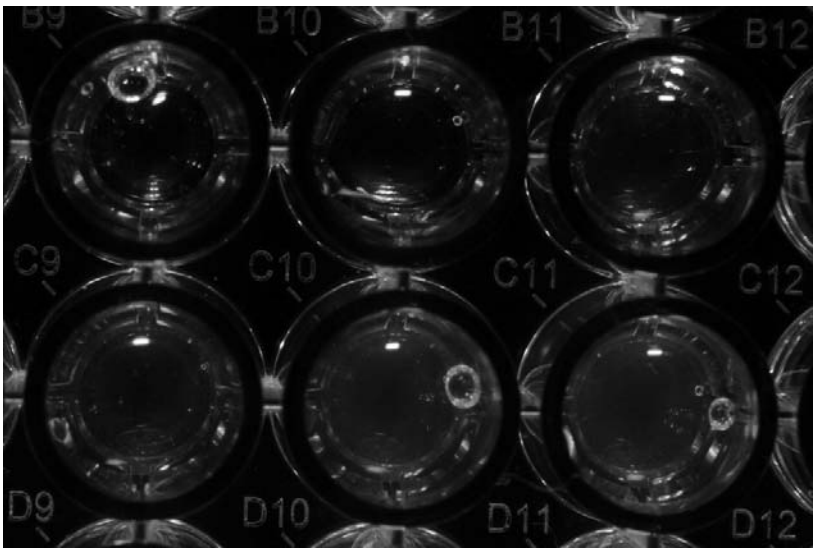


Figure 6 Observation after 3 minutes (Five solutions was prepared with different concentration of AEBSF which are 0.1mM, 0.3mM, 0.5mM, 1.0mM and 1.5mM from B10 to

C11. Negative control solution was prepared in B9.The photograph were taken in the dark room at room temperature.)

Figure 7 Observation after 7 minutes (Five solutions was prepared with different concentration of AEBSF which are 0.1mM, 0.3mM, 0.5mM, 1.0mM and 1.5mM from B10 to C11. Negative control solution was prepared in B9.The photograph were taken in the dark room at room temperature.)

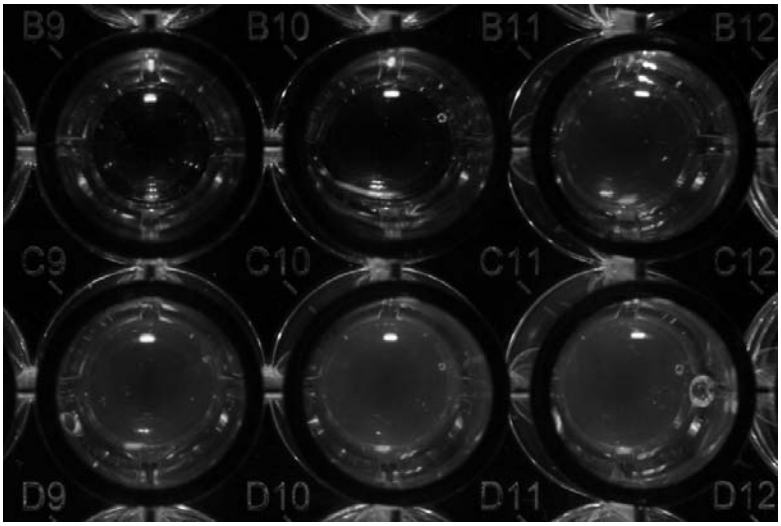


Figure 8 Observation after 10 minutes (Five solutions was prepared with different concentration of AEBSF which are 0.1mM, 0.3mM, 0.5mM, 1.0mM and 1.5mM from B10 to C11. Negative

control solution was prepared in B9.The photograph were taken in the dark room at room temperature.)

Figure 9 Observation after 20 minutes (Five solutions was prepared with different concentration of AEBSF which are 0.1mM, 0.3mM, 0.5mM, 1.0mM and 1.5mM from B10 to C11. Negative control solution was prepared in B9.The photograph were taken in the dark room at room temperature.)

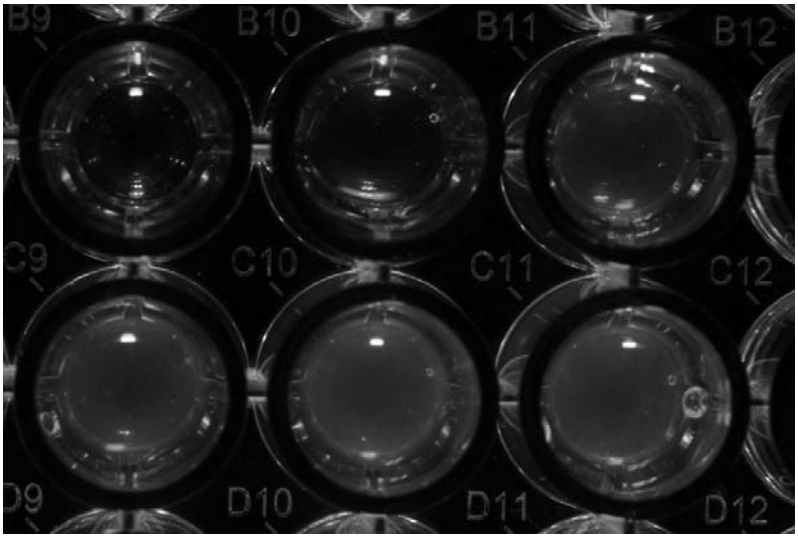


Figure 10 Observation after 30 minutes (Five solutions was prepared with different concentration of AEBSF which are 0.1mM, 0.3mM, 0.5mM, 1.0mM and 1.5mM from B10 to C11. Negative

control solution was prepared in B9.The photograph were taken in the dark room at room temperature.)

After taking the photos, we use ImageJ which is an image processing program to analyze the photos. ImageJ calculate the intensity of the light in a specific area as you choose based on the integral method. After choosing an area without refraction, the light intensity is calculated and exported to the data processing software, such as Microsoft Excel. Unit intensity is treated by using whole intensity value divided by area value. The relationship between time and unit intensity is demonstrated by plots.

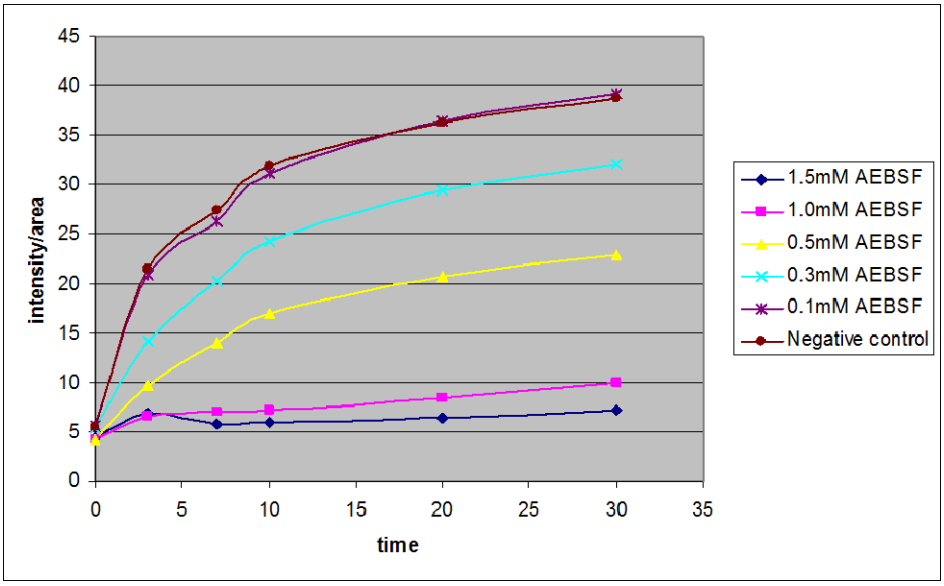


Figure 11 Inhibition Curve of AEBSF (The inhibition curve is calculated by Image J software. The test is processed under 37°C, pH=7.4 Tris HCl buffer.)

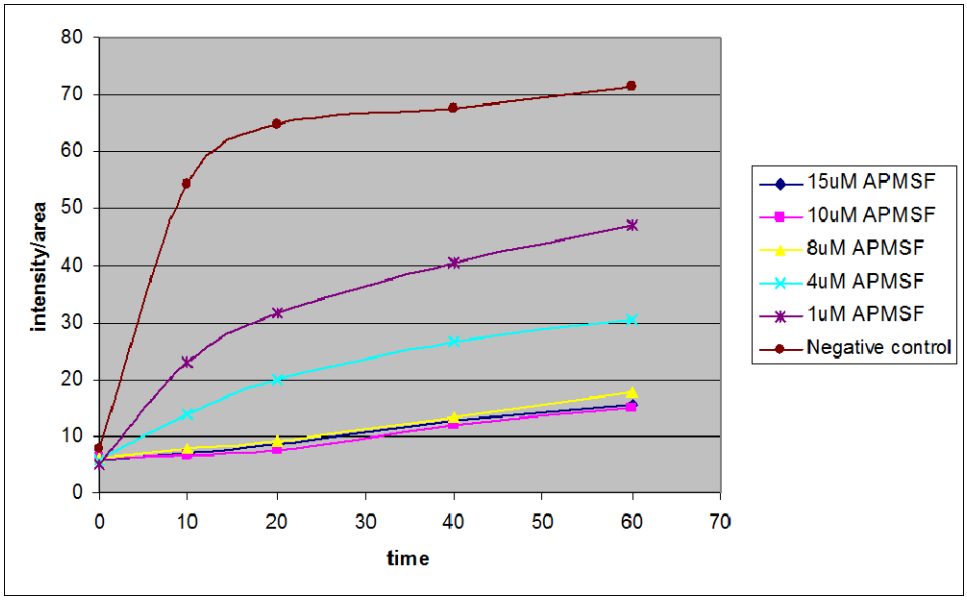


Figure 12 Inhibition Curve of p-APMSF (The inhibition curve is calculated by Image J software. The test is processed under 37°C, pH=7.4 Tris HCl buffer.)

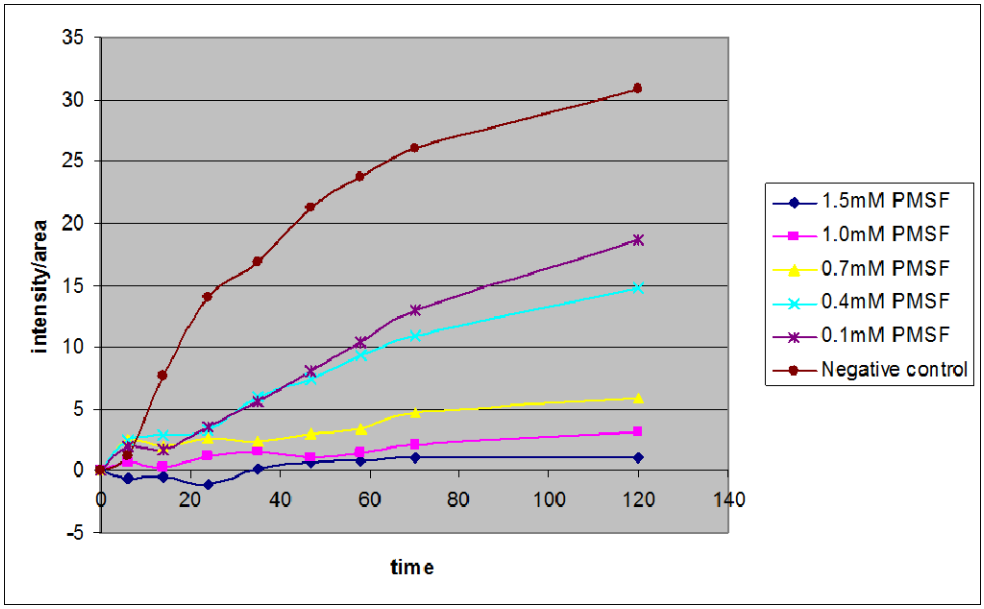


Figure 13 Inhibition Curve of PMSF (The inhibition curve is calculated by Image J software. The test is processed under 37°C, pH=7.4 Tris HCl buffer.)

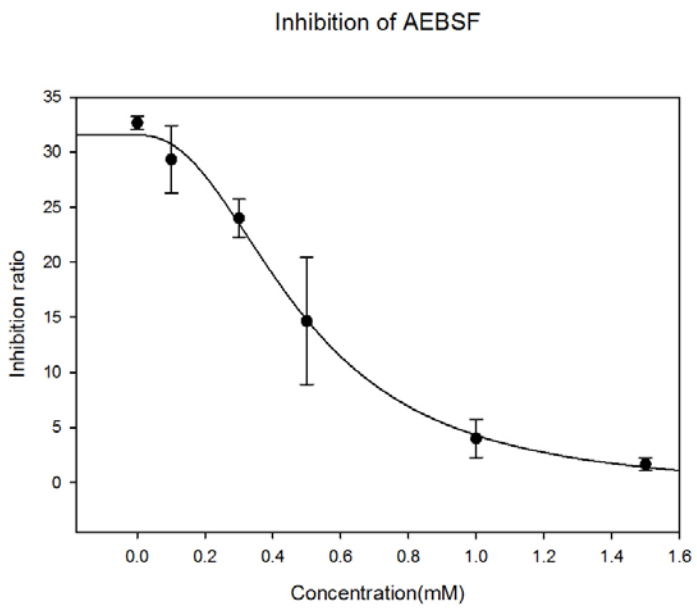


Figure 14 IC50 of AEBSF (The curve is fit by Sigma Plot. IC50 of AEBSF is obtained at 37°C, pH 7.4 buffer system)

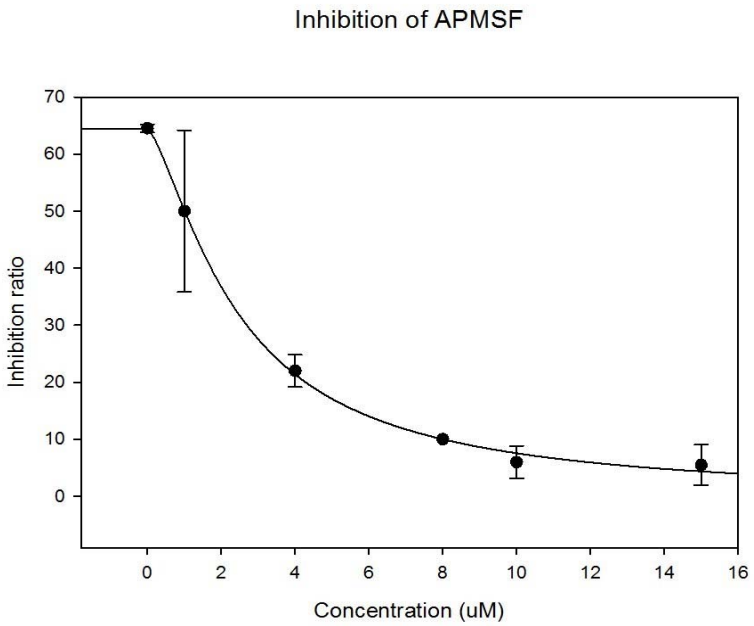


Figure 15 Inhibition of p-APMSF (The curve is fit by Sigma Plot. IC50 of AEBSF is obtained at 37°C, pH 7.4 buffer system)

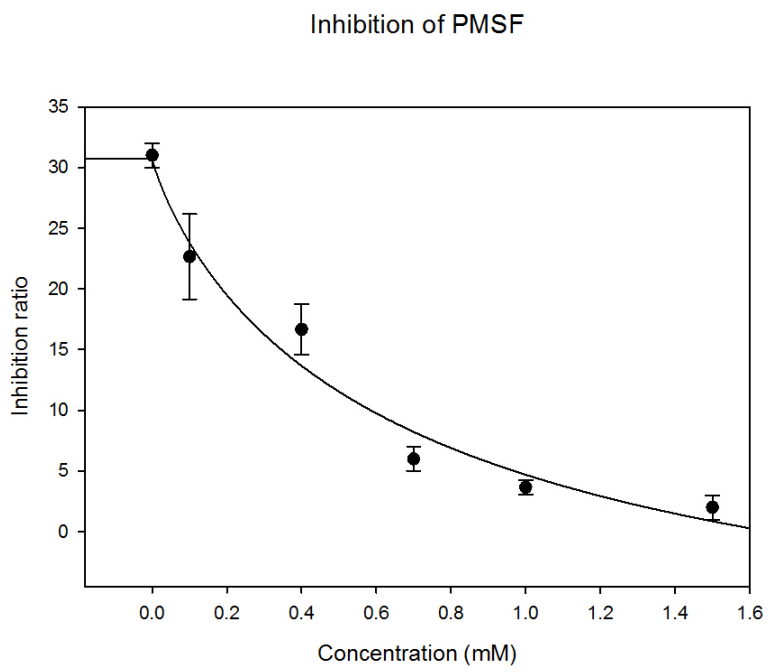
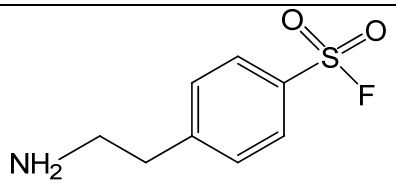
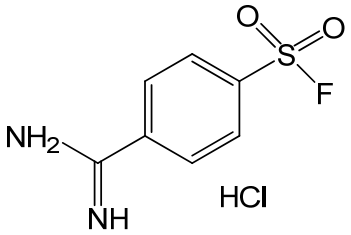
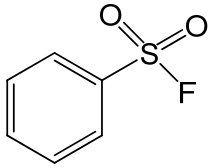


Figure 16 IC50 of PMSF (The curve is fit by Sigma Plot. IC50 of AEBSF is obtained at 37°C, pH 7.4 buffer system)

Figures 7-9 show the concentration-related inhibition and inhibition rate. All inhibitors have concentration dependence during the inhibition. Compare this three inhibition process. PMSF has the longest inhibition time which is about 2 hour while AEBSF has the inhibition time which is about half a hour. The range used for inhibition is also different among these three inhibitors. APMSF can inhibit thrombin in nano-molar range. Compare to APMSF, the concentration range of inhibition of AEBSF and PMSF is much higher than that of APMSF which is in mili-molar range.

Figures 10-12 show the relationship between concentration and inhibition ratio. The calculated IC50 is 0.48 mM for AEBSF, 2.46 μM for APMSF and 0.73 mM for PMSF. The IC50 of APMSF is much lower than the other Inhibitors and shows the lowest IC50 of all three. The IC50 of PMSF is the highest of all. The comparison is showed in the table below:

Table 1 Comparison of Structure, Inhibition Time and IC50 of AEBSF, p-APMSF and PMSF

Inhibitors	Compound Structure	Clotting Time	IC50
AEBSF		30min	0.48 mM
p-APMSF		60min	2.36 μM
PMSF		120min	0.73 mM

2.4.5 Potential Problems

Although it is better to use the photo-taking method to determine the gel formation than to use micro-plate reader method, this photo-taking method still has limitations in application.

Firstly, this method can be used only in the visual experiments such as color changing experiments and gel formation experiments. It is not suitable in the normal colorless solution which just has absorbance changing during the reaction. As a result, it cannot be used in any type of reactions unless it is visual.

Secondly, there are several requirement of environment in this method. The types of lens is required for short distance shooting. For convenience in observing and comparing the results, the photos have to be taken in the dark room. In addition, it requires specific software to analyze those photos. Since the photos show the dark and light color to different extend, they have to be analyzed by a tool which can convert the different color to corresponding quantity. As a result the use of the analyzing tool is limited.

Finally, the reflection effect cannot be ignored during the experimental process. As the photos are taken in the dark room and there is strong flashlight when taking the photos, there must be reflection in the surface of the solution. That will shows as bright color spot in the well. When analyzing the photos, we should choose the areas with equal light present and without the reflection spots.

2.4.6 Conclusion and Discussion

In the analysis of three thrombin inhibitors, AEBSF, p-APMSF and PMSF, they showed different inhibition activity in time- and concentration-dependent manner. In comparing the time of these three inhibitors, AEBSF showed the shortest reaction time whereas PMSF showed the longest reaction time. This may due to the different structure among these compound and also different effects in Tris buffer system. Although three different inhibitors are used in the clotting test, all tests showed concentration dependence of inhibitors and reproducible results. This indicates that the photography system is consistent in our tests. All photograph showed the clotting formation clearly which means the photography methods is well applied in these tests.

There are also some aspects needs to be improved. The standard deviation in these plots is large at several concentrations. This might due to the difference of light intensity when taking the photograph. The reflection is also interference in photos when we are doing the calculation.

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