I. INTRODUCTION

1.1 Statement and significance of problem

Curtailing operative bleeding has always been a surgical priority. Perioperative hemorrhage increases the need for blood product transfusion, morbidity, mortality and cost although it has been done by skillful surgeons. However, despite excellent surgical skill there may be increased bleeding under a variety of circumstances. These include extensive operations *versus* limited ones, deep dissections *versus* superficial ones, reoperations *versus* primary procedures and the patient's status. Furthermore, other factors over which the surgeon has little or no control may increase surgical hemorrhage, *i.e.* the liver or kidney diseases; recent exposure to medications such as aspirin or anticoagulants; operation requiring extracorporeal circulation; and patient with hemophilia, thrombocytopenia or other coagulopathies (Chiewsilp P, *et al.*, 1983; Green D, *et al.*, 1996; Issarangura P, *et al.*, 1997). So lot of blood and blood components are required, however, complications or the unexpected symptoms may be occurred after blood transfusion (Chiewsilp P, *et al.*, 1981)

An adverse reaction of blood and blood component transfusion is divided into 2 groups (Chiewsilp P, *et al.*, 1981; Chiewsilp P, *et al.*, 1983; Moore SB, 1987).

1. Acute transfusion reactions include hemolysis, febrile reaction, urticaria, blood volume overload, noncardiogenic pulmonary edema, septicemia, hypotension and complications from blood volume overload such as coagulation factor deficiency, hypothermia and abnormal metabolism.

2. Delayed transfusion reactions include hemolysis, red blood cell alloimmunization, hemosiderosis, graft *versus* host disease, petechiae, hepatitis B viral infection, HIV infection, malarial infection and other infections.

Posttransfusion infections especially HIV infection have been reported in Thailand, United States and Japan although these blood and blood components have been tested for VDRL, HBsAg, anti-HIV, HIV-antigen and anti-HCV prior to they were transfused to patients (Tunprasert S, *et al.*, 1991; Cheawsilp P, *et al.*, 1996; Chuansumrit A, *et al.*, 1996; Issarangura P, 1997; Viputsiri O, 1997; Nutchprayoon C, 1998). In Thailand, the posttransfusion HIV infection of anti-HIV negative blood was first reported (Chanarat P, *et al.*, 1990). There were 44 posttransfusion HIV-infected patients since 1991-1996 (Viputsiri O, 1997).

To control blood loss and decrease the risk of infection, physicians have turned to used various topical and systemic agents regarding hemostatic properties. There are a variety of natural and synthetic hemostatic agents including collagent product (*i.e.*, collagen fleece), absorbable gelatin sponges, oxidized cellulose, and synthetic cyanoacrylate-based glues. Many of these agents are believed to be useful in surgical practice but most have not been subjected to rigorous clinical trials. Seaweed extracts (Alginate) were used for capillary hemorrhage arrest. Styptic pencils could diminish bleeding by cauterizing small vessels. Bismuth subgellate, an analogue of ellagic acid, activates factor XII and may be applied to the tonsillar fossae along with epinephrine to stem bleeding. Collagen and microcrystalline collagen (Avitene) enhance platelet adhesion and aggregation, and were widely used in cardiovascular, orthopedic, and plastic surgery. Gelatin sponges and oxidized cellulose can be applied to the bleeding surface, compress severed vessels, absorb fluid, and provide a scaffold for fibrin formation. Russel's viper venom (Steven) very rapidly clots shed blood and has been used topically to staunch bleeding from lacerations in hemophiliacs. Thrombin, usually of bovine origin, is applied alone or with other topical agents to promote hemostasis. However, the most popular use of thrombin is in combination with fibrinogen to form fibrin glue (Gibble JW and Ness PM, 1990; Green D, *et al.*, 1996).

Fibrin glue (fibrin sealant or fibrin tissue adhesive) has been advocated by many surgeons as the material that best approaches the ideal operative sealant. As a naturally occurring and partially human-derived product, the material appears to have no tissue toxicity, promotes a firm seal in seconds to minutes, which is reabsorbed in days to weeks following application, and promote local tissue growth and repair (Gibble JW and Ness PM, 1990 ; Radosevich, 1997). Fibrin glue was first used during the World War I for stop bleeding from parenchymous organs. Plasma was sprayed on serious bleeding wound. Fibrinogen were mixed with bovine thrombin in an attempt to accelerate the formation of fibrin clot on wound during the World War II (Issarangura P, 1997; Radosevich 1997). After that, fibrin glue has found widespread use.

Fibrin glue is a preparation of separate solutions of fibrinogen and thrombin. By taking advantage of the final stages of the clotting cascade, fibrinogen is converted to fibrin monomer which is unstable by thrombin. Factor XIII and calcium chloride are necessary for fibrin monomer to polymerize and stabilize fibrin clot (Thromson DF, *et al.*, 1988; Martinowitz U and Spotnitz WD, 1997). Plasma or cryoprecipitate is used with thrombin and calcium chloride to form fibrin glue but fibrinogen concentration and its amount in plasma and cryoprecipitate is not enough and suitable for use in the surgery. Because fibrin glue depend on a consistent and concentrated source of human fibrinogen, investigation in the United States was largely halted in 1978 when the Food and Drug Administration revoked the license for the clinical use of pooled commercial fibrinogen concentrates. This ban also based on the well-recognized observation of the high risk of hepatitis transmission by fibrinogen, eliminated the importation in the United States of commercially prepared fibrin glue systems (Gibble JW and Ness PM, 1990). So autologous or single donor cryoprecipitate as a source of fibrinogen are considered in the United State. Autologous-source fibrinogen eliminates the risks of virus transmission. Although single-donor and autologous cryoprecipitate methods produce limited amount of fibrinogen concentrate, the yields

are usually significant for most types of operative procedure. However, the use of autologous blood units or samples necessitates preoperative planning, and those products are not generally available for emergency therapy. Single donor cryoprecipitate methods have been more widely applied, as the cryoprecipitate is readily available, and associated risk of viral disease transmission is equivalent to that with transfusion of single-donor blood products.

In July 1996, Uri Martinowitz and Henri Horoszkowski first demonstrated the fibrin glue preparation and the first use of fibrin glue in the operation of patient with hemophilia in Thailand was documented. At present, fibrin glue is prepared by Thai Red Cross Society to serve many hospitals around Thailand. There are two separated components. The first composes of cryoprecipitate and antifibrinolytic agent, and the second composes of thrombin, calcium chloride and antibiotics.

In this study, several preparative methods for fibrinogen and thrombin were studied for developing an appropriate method for single donor preparation which provide a suitable concentration and enough for each operation. Nevertheless, the recommended technique could be performed by local hospital blood bank, which will decrease blood loss, reduce the use of blood and blood components, and could be applied for any hospital around Thailand.

1.2 Literature reviews

1.2.1 Plasma proteins

There are several proteins in plasma can be classified by their functions.

1. **Nutritive.** The amino acids from the metabolism of protein enter the amino acid pool. Some of these amino acids is used for the new protein or other nitrogenous compounds synthesis. The residual amino acid is deaminated to give substances which are either completely catabolised to carbon dioxide and water or used for the formation of glucose (gluconeogenesis) in either case providing part of the body's energy requirement.

2. **Control of body water distribution.** Colloid osmotic pressure or oncotic pressure of plasma protein counteracts with hydrostatic blood pressure to control circulating blood volume.

3. **Buffer.** Protein is a small part in maintaining plasma pH. Protein is negatively charged at normal body pH and act as base except hydrogen ion.

4. **Transporting agent.** Albumin can transport many substances while as the other protein transport only single substance such as transferrin transports iron. The major transport substances are hormone *i.e.* cortisol and thyroxine, lipid, fat-soluble vitamins and metals. The insoluble substances or rapidly excrete substance can reach the organ that they carried and also the toxic substances reduce its toxicity by binding with protein.

5. **Blood coagulation.** Several blood clotting factors involve with coagulation are proteins.

6. **Immunological protection.** Immunoglobulin is antibody in the defense mechanism against infection.

7. **As enzyme.** The most enzymes in plasma are protein, which released from several organs.

Lots of plasma proteins including albumin, fibrinogen and globulin are synthesized in liver, immunoglobulins synthesized by reticuloendothelial system, lymphnode and plasma cell.

1.2.2 **Some physical properties of proteins**

Proteins greatly vary in such physiochemical characteristics as water solubility, buffer capacity, chemical stability, and crystal structure. These physical properties depend on molecular weight, amino acid composition and, above all, the higher-order structure of the protein.

1.2.2.1 **Proteins lose their biological activities when their higher-order structures are destroyed** : Under ordinary conditions, the peptide and disulfide bonds in protein molecules are covalent bonds that are stable. Peptide bonds can be hydrolyzed under mild conditions in the presence of proteolytic enzymes, but in the absence of enzymes they can be cleaved only by heating with strong acids and bases. Disulfide bonds, if present, can be cleaved by reducing or oxidizing agents as shown in figure 1. The noncovalent interactions that maintain the higher-order structure of proteins are far weaker than the covalent bonds. The higher-order structure of proteins can be destroyed by ordinary heating. Temperature between 50 °C and 80 °C can denature most proteins within a few minutes. At these temperatures, neatly folded polypeptide is converted to random coil. The biological function of proteins depend on their higher-order structure. So denaturation brings proteins to a complete loss of their biological function. The physical properties are also changed by denaturation. Water soluble proteins become insoluble. Renaturation is possible for many small proteins. However, larger proteins do not renature. So protein denaturation in general is irreversible.

Protein can be denatured by many treatments other than heating. Many detergents and organic solvents denature proteins by disrupting hydrophobic

interactions. They insinuate themselves between the side chains of hydrophobic amino acids becoming nonpolar. Strong acids and bases denature protein because they change their charge pattern. The protein losses its negative charges in a strong acid and it loses its positive charge in a strong base. This deprives the protein of intramolecular salt bonds that normally help to stabilize its higher-order structure. The counterion that becomes bound to the protein surface under these conditions can also interfere with the higher order structure. The addition of trichloroacetic acid (TCA) is a standard procedure for the removal of proteins from serum or other biological samples. High concentration of some small hydrophilic agent with high hydrogen-bonding potential, such as 2 M urea or 6 M guanidine hydrochloride, also can denature proteins by disrupting the hydrogen bonds between water molecules. Many heavy metal ions, such as lead, cadmium, and mercury can denature proteins by binding to carboxylate groups, and, in particular, to sulfhydryl groups in many proteins. This affinity for functional group in proteins is the basis for their toxicity.

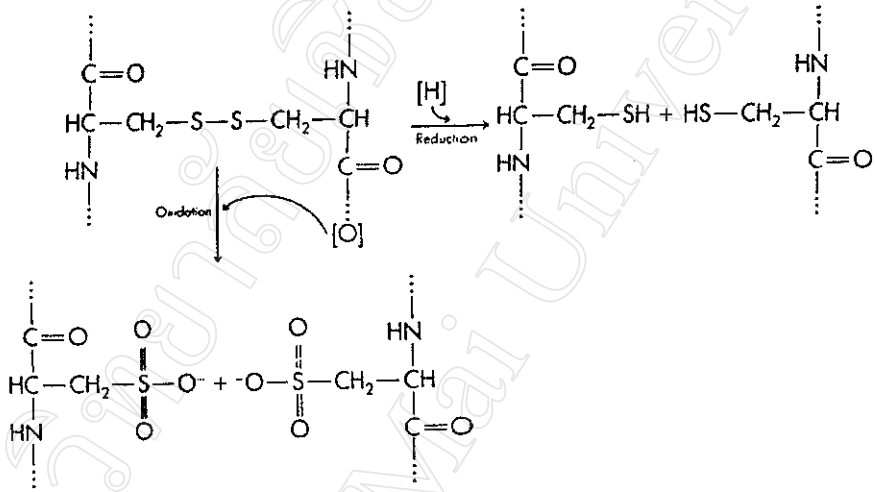


Figure 1 Cleavage of disulfide bonds by reducing or oxidizing agents.

(Meisenberg G and Simmons WH, 1998)

1.2.2.2 **The solubility of protein varies with salt concentration, pH, temperature, ionic strength and dielectric constant.** Fibrous protein and many globular membrane protein are insoluble. However, most globular proteins are water soluble.

The solubility of protein at low ionic strength generally increases with the salt concentration. Salting-in phenomenon occurs as the salt concentration of protein solution increases, the additional counterions more effectively shield the protein molecules' multiple ionic charges and thereby increase the protein's solubility. At high ionic strengths, the solubility of protein decreases. This effect, known as salting-out, is primarily a result of the competition between the added salt ions and the other dissolved solutes for molecules of solvation. At high salt concentration, many of added ions are solvated the amount of bulk available becomes insufficient to dissolve other solutes. Hence, solute-solute interactions become stronger than solute-solvent interactions and the solute precipitates.

Water miscible organic solvents like ethanol or acetone are generally good protein precipitants because their low dielectric constants lower the solvating power of their aqueous solutions for dissolved ions such as protein. This procedure is normally used near 0 $^{\circ}$C or less because, at higher temperatures, organic solvents tend to denature protein. The organic solvents decrease the hydrophobic interaction as well as H-bonding to water, and thereby promoting both unfolding and H-bonding between surface polar groups. Precipitation is different from denaturation. Precipitation is reversible and does not permanently destroy the biological properties of proteins as shown in figure 2A and 2B.

The pH value also affects the water solubility. Solubility of proteins is minimal at their isoelectric point (*pI*) because they has no net charge (number of positive and negative charges are equal). Thus, solubility is decreased by titrating

protein to their *pI* as shown in figure 2C. In solutions of moderate salt concentrations, the solubility of a protein as a function of pH is expected to be a minimum at the protein's *pI* and to increase about this point with respect to pH.

At temperature above 40-50 °C, H-bonds and ionic bonds begin to break, and most proteins denature, allowing their exposed hydrophobic interiors to interact.

Protein may be precipitated selectively by manipulating the pH, ionic strength, and dielectric constant of solvent. Precipitation either at the isoelectric pH (*pI*) or at high salt concentration is preferred because both methods involve the least danger of denaturating proteins.

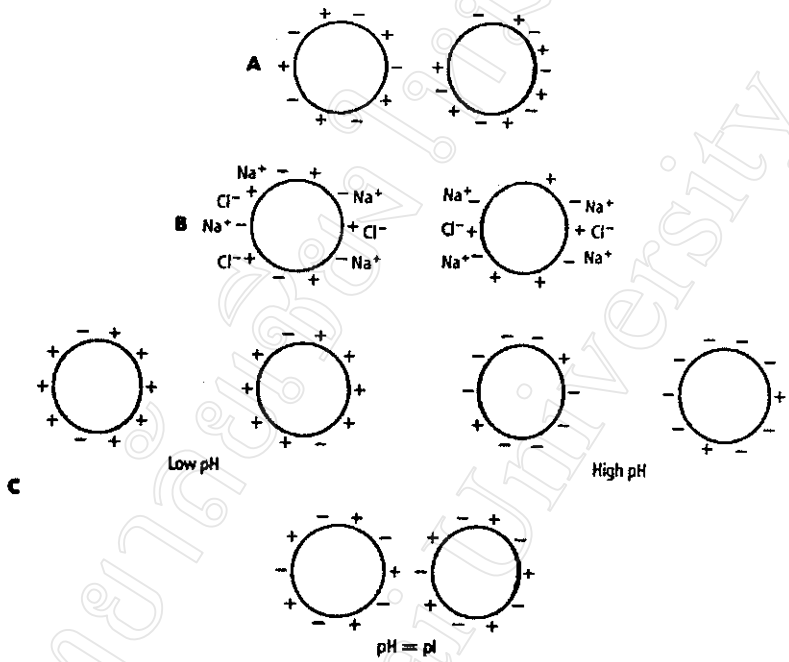


Figure 2 Effects of salt and pH on protein solubility (Meisenberg G and Simmons WH, 1998)

A. Protein in distilled water. Salt bonds between protein molecules cause the molecule to aggregate. The protein becomes insoluble

B. Protein in 5% sodium chloride (NaCl): salt ions bind to the surface charges of the protein molecules, thereby preventing intermolecular salt bonds.

C. The effect of pH on protein solubility: the formation of intermolecular salt bonds is favored at the isoelectric point. At pH values greater or less than the *pI*, the electric interaction between the molecules are mainly repulsive.

1.2.3 **Hemostasis overview**

Hemostasis is a mechanism that occur to arrest bleeding. There are several events involve in hemostasis. The first is vasoconstriction. This is followed by blood platelet aggregation and blood coagulation. The effectiveness of hemostatic mechanism depends on several conditions.

1. The type and degree of injury and the ability of vasoconstriction to occur
2. The ability of platelets and their activity
3. The absence of inhibitors, circulating anticoagulant, and antagonists

Hemostasis can be divided into two stages. Primary hemosatsis includes the platelet and vascular response to vessel injury. Secondary hemostasis includes the coagulation factors response to vessel injury too.

Primary hemostasis : Blood normally flows through the system without any adherence to the walls of the vessels. A thin layer of endothelial cells maintains a thromboresistant surface. When responding to vascular damage, the vascular constriction occurs resulting in immediate reduced blood flow to the site of vascular damage. Platelets exposes to the subendothelial connective tissue components of blood vessels (collagens, microfilaments, basement membranes, von Willebrand factor, and others). It adheres to the exposed surfaces immediately. Reversible primary platelet aggregation takes place during platelets adhere to one another. Platelets also change shape and their organelles become centralized. If there are no further stimulation, platelet may disaggregate. However, with continued stimulation, irreversible secondary aggregation will occurs. Platelets release the content of their dense granules (ADP, ATP, thromboxane A2, ionized calcium, magnesium, epinephrine, phosphate and serotonin) and alpha granule (fibrinogen, platelet derived growth factor, plasminogen activator inhibitor, fibronectin, albumin, β-thromboglobulin,

and factor V absorbed from plasma). ADP promote secondary aggregation and recruits additional platelets to the site of injury. ATP may play a role in limiting the site of platelet plug by inhibiting excessive platelet aggregation. Thromboxane A2 induces and maintains the platelet shape change as well as the platelet release reaction. Serotonin also promotes platelet aggregation and vasoconstriction. During aggregation, phospholipid becomes available on the platelet membrane surface, providing a site for the formation of fibrin clots.

Secondary hemostasis : secondary hemostasis involves the response of the coagulation process to vessel injury that occurs in series of four reactions (Leihmann CA; 1998) : initiation, activation of X, formation of thrombin and formation of an insoluble fibrin clot

The balance between coagulation protein and anticoagulants progresses to coagulation. This secondary phase of hemostasis is the process in which fibrinogen (a soluble plasma protein) is converted to an insoluble fibrin clot. This occurs through a cascade type processes that involves cofactors, and multiple enzymes that are converted from a precursor (zymogen) to an active enzyme (protease).

Initiation : The initiation of clotting begins with the activation of two enzymatic pathways that lead to fibrin formation : the intrinsic and extrinsic coagulation pathways. The intrinsic pathway is initiated by trauma within the vascular system, such as exposed endothelium. This system is slower and more important than the extrinsic pathway. The extrinsic pathway is initiated by an external trauma and quickly occurs by releasing of tissue factor (TF) from injured vessel endothelial cells and subendothelium into the vessel lumen. Intrinsic and extrinsic initiations are shown in figure 3 and 4 respectively.

Activation of X : The activation of factor X is crucial for blood clotting. Once the activation of factor X to Xa occurs by intrinsic or extrinsic pathway, coagulation factors enter the common pathway as shown in figure 5.

Formation of thrombin : Fibrinogen (factor I) is converted by thrombin, a protease enzyme, becoming fibrin monomer, fibrinopeptide A and B. Thrombin then activates factor XIII to factor XIIIa by cleaving a peptide bond from each of two alpha chains; inactive factor XIII along with Ca^{2+} ions enables factor XIII to dissociate into factor XIIIa. If thrombin in circulation is an active form (IIa), uncontrollable clotting will occurs. Generally it circulates in its active form, prothrombin (II).

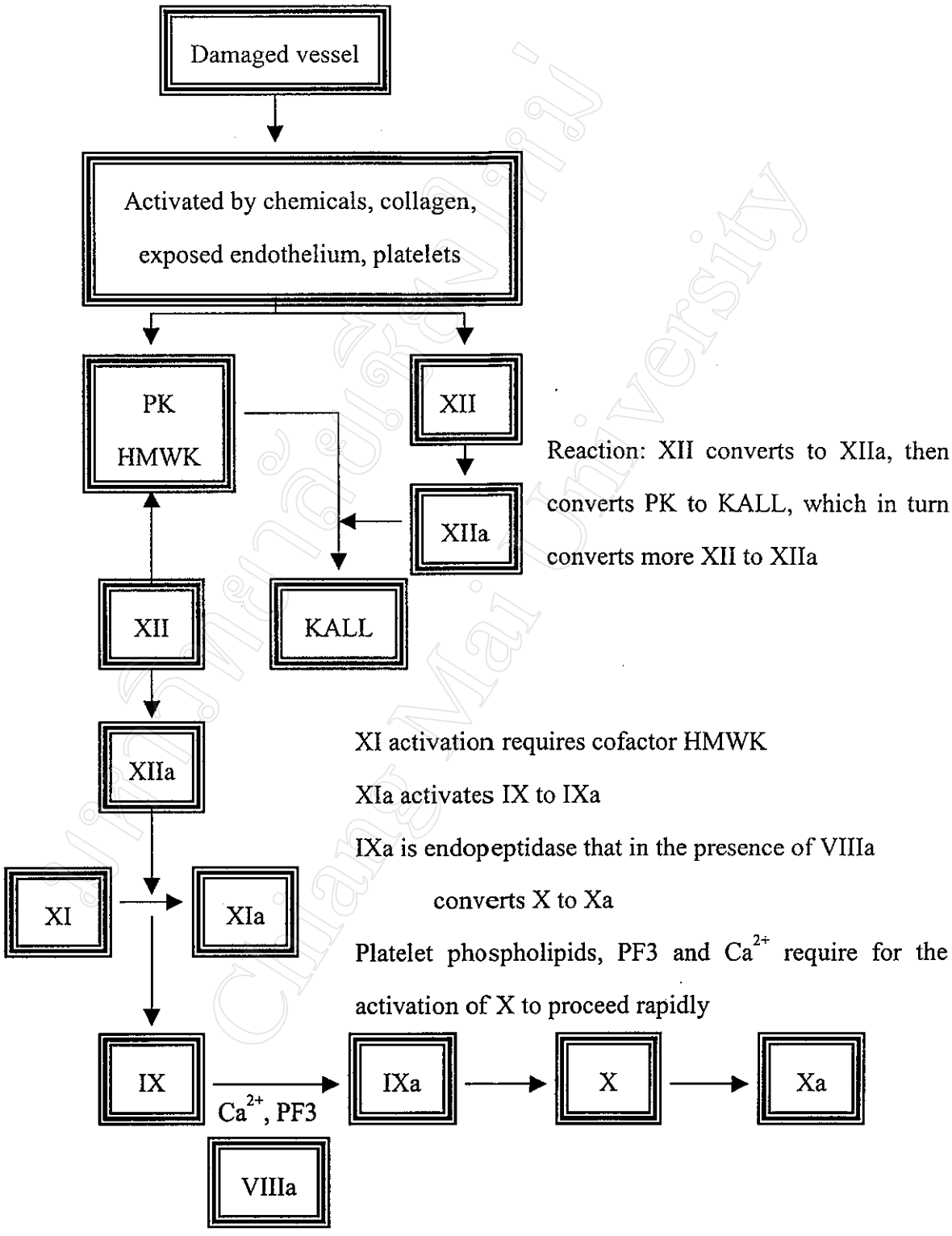


Figure 3 Intrinsic initiation

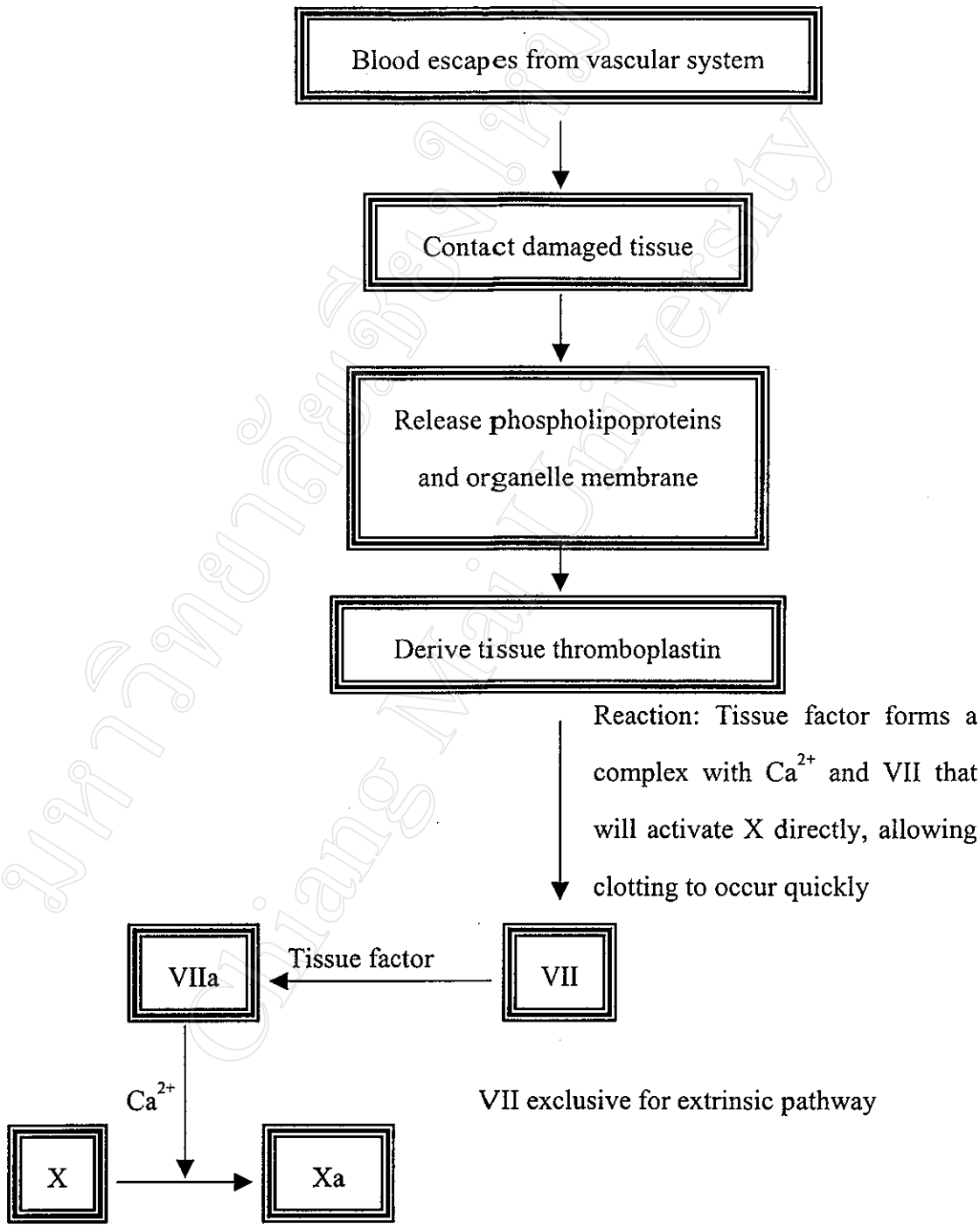


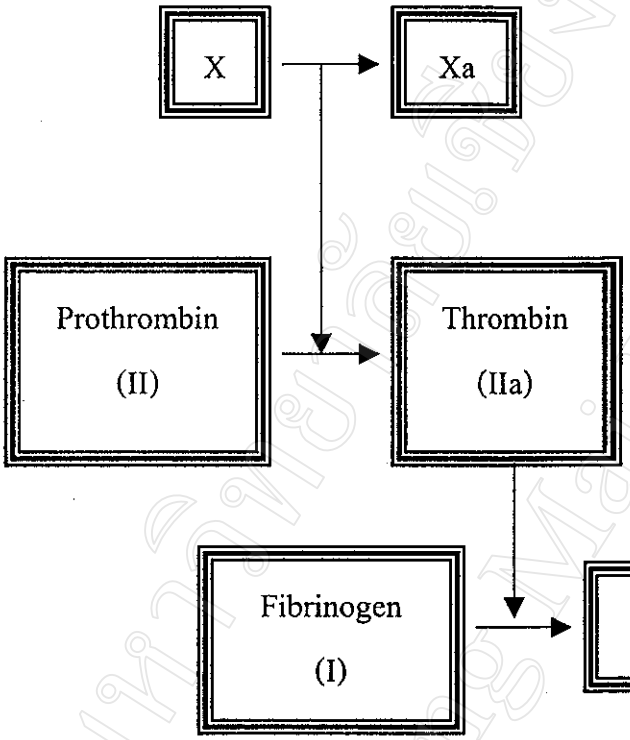
Figure 4 Extrinsic initiation

Formation of an insoluble fibrin clot : Formation of fibrin occurs in three phases include proteolysis, polymerization and stabilization.

1. Proteolysis : Protease enzyme, thrombin, cleaves fibrinogen to fibrin monomer, fibrinopeptides A and B.

2. Polymerization : This occurs spontaneously by end to end polymerization of hydrogen bonding of fibrin monomer.

3. Stabilization : This occurs when the fibrin monomers are linked covalently by factor XIIIa into fibrin polymers, forming an insoluble fibrin clot.



Reaction: Xa in the presence of Va will activate prothrombin (II) to thrombin (IIa). Prothrombin normally circulates in blood as an inactivated form. Prothrombin activation occurs on the surface of platelets, endothelial cells, and smooth muscle cells and is accelerated by factor V and Ca^{2+}.

Factors in common pathway: I, II, V, X

Figure 5 Common pathway

1.2.4 **The coagulation proteins**

Coagulation factors can be classified as substrate, zymogen (enzyme precursor), cofactor and calcium. They involve on coagulation pathway. All coagulation factors except tissue factor are normally present in plasma, tissue factor is found in most organs as well as in large blood vessel, and phospholipid is provided by platelets. High concentration of tissue factors are found in lungs, brain, and placenta. The zymogens are factor II, VII, IX, X, XI, XII, XIII and prekallikrein; the cofactors are factor V and VIII, tissue factor, Ca^{2+}, and high-molecular-weight kininogen (HMWK). Zymogens are substrate that have no biological activity until converted by enzymes to active enzymes. The zymogen factors are converted to enzymes called serine proteases, which have exposed , serine-rich, active enzyme sites except factor XIII. Serine proteases selectively hydrolyze arginine- or lysine-containing peptide bonds of other zymogens, thus converting them to serine proteases. Factor XIII has an active enzyme site containing cysteine rather than serine when converted to its active enzyme form.

Zymogen activation may involve either : (1) a conformational change (*e.g.* twist, turn, or bend) in the zymogen molecule or (2) hydrolytic cleavage of a specific zymogen peptide bond by a serine protease. Initially, coagulation reaction occurs on the injured, exposed endothelial surfaces of blood vessels and consist of zymogen activation by conformational changes. Later, coagulation reactions occur on the phospholipid surfaces of aggregated platelets and involve hydrolytic cleavage of the next sequential zymogen to an active enzyme.

1.2.5 **The coagulation factors**

The nomenclature of coagulation factors are named by Roman numerals assigned by the International Committee on Nomenclature of Blood Coagulation Factors. Each Roman numeral was assigned in order to factor discovery. There are factor I to factor XIII except factor VI. The characteristic of these factors are shown in table 1.

Factor I (Fibrinogen) : Fibrinogen is substrate for thrombin and precursor of fibrin. It is a large glycoprotein. When it is exposed to thrombin, two fibrinopeptides split from fibrinogen molecule. A fibrin monomer is cleaved to form a polymerized clot.

Factor II (Prothrombin or Prethrombin) : Prothrombin is converted to thrombin (IIa) in the presence of Ca^{2+}. Thrombin stimulates platelet aggregation and activates cofactors, protein C, and factor XIII.

Factor III (Tissue factor or tissue thromboplastin) : Tissue factor activates factor VII when blood is exposed to tissue fluids.

Factor IV (Ionized calcium) : This is an active form of calcium. It is needed for thromboplastin activation and for the conversion of prothrombin to thrombin, factor XIII to factor XIIIa, and factor X to factor Xa.

Factor V (Proaccelerin) : This factor is used during clotting and accelerates the transformation of prothrombin to thrombin.

Factor VII (Proconvertin) : It is activated by tissue thromboplastin, which in turn activates factor X.

Factor VIII (Antihemophilic) : It has several functions. Factor VIII has two major parts; VIII:C and vWF. Factor VIII:C involve in the intrinsic pathway while as vWF binds to endothelium for platelet function.

Factor IX (Plasma thromboplastin component) : It influences amount as opposed to rate and is deficient in hemophilia B.

Factor X (Stuart-Prower) : The final common pathway merges to convert prothrombin to thrombin. Its activity is also related to factor VII.

Factor XI (Plasma thromboplastin antecedent) : It is essential to intrinsic thromboplastin generating of the cascade.

Factor XII (Hageman's factor) : It is a surface contact factor that is activated by collagen.

Factor XIII (Fibrin stabilizing factor) : It stabilizes polymerized fibrin monomers in the initial clot in the presence of calcium.

The coagulation factors are divided into three groups : the contact group; the prothrombin or vitamin K-dependent group; and the fibrinogen group. The features common to each group are listed in table 2.

The contact group consists of prekallikrein, HMWK, factor XII and XI. They are activated by contact with a negatively charged surface such as collagen or subendothelium *in vivo* or glass *in vitro*. The role of contact activation in physiologic hemostasis is uncertain.

Table 1 Characteristics of clotting factors. (Jobe MI, 1998)

Factor	Active form	MW ($x10^3$)	Pathway participation	Site of production	Vitamin K dependent?	In Vivo half-life (hrs.)	Plasma concentration (μg/ml)	Minimum hemostasis level (%)*	Chromosome coding for production
I	Fibrin clot	340	Common	Liver	No	72-120	160-415*****	100 mg/dl	4q23-q32
II	Serine protease	72	Common	Liver	Yes	67-106	100	20-40	11
V	Cofactor	330	Common	Liver	No	12-36	7	10-25	1q21-25
VII	Serine protease	63	Extrinsic	Liver	Yes	4-6	0.5	5-10	13
VIII:C	Cofactor	267 (VIII/vWF >1000)	Intrinsic	Uncertain for VIII:C**	No	10-12 (22-40)***	1-8 (VIII/vWF)	25-30	Xq28
IX	Serine protease	55	Intrinsic	Liver	Yes	18-40	5	15-25	X
X	Serine protease	55	Common	Liver	Yes	24-60****	10	10-20	13q32-qter
XI	Serine protease	160	Intrinsic	Liver	No	48-84****	4-6	10-20	4q35
XII	Serine protease	80	Intrinsic	****	No	52-60	30-45	0-5	5q33-qter
XIII	Transglutaminase	320	Common	Liver	No	3-7 days****	25	2-3	XIIIa: 6p24-25 XIIIb: 1q31-q32.1
Prekallikrein	Serine protease	85	Intrinsic	Liver****	No	35****	35-50	****	4
HMWK	Serine protease	120	Intrinsic	Liver****	No	6.5 days****	70-90	****	3q

*: Approximate minimum plasma concentration required for normal coagulation.

**: vWF portion synthesized by endothelial cells and megakaryocytes.

***: 22-40 hours for high-molecular-weight subunit of factor VIII.

****: Insufficient data or significant disagreement exists among published values; for minimum hemostatic level, none reported.

*****: Fibrinogen reported as 160-415 mg/dl.

Table 2 Properties of the coagulation factors. (Jobe MI, 1998)

	Contact	Prothrombin	Fibrinogen
FACTORS	XII, XI, prekallilrein, HMWK	II, VII, IX, X, protein C, protein S	I, V, VIII, XIII
FUNCTION	Serine protease: XII, XI, prekallilrein Cofactor: HMWK	Serine protease: II, VII, IX, X, protein C Cofactor: protein S	Precursor of fibrin: I Cofactor: V, VIII Transamidase: XIII
MW ($X10^3$)	Medium (80-160)	Low (55-70)	High (>250)
STABILITY	Fairly stable	Heat labile: VII, IX, X Well preserved in stored plasma	Heat labile: I, V, VIII Storage labile: V, VIII
VITAMIN K DEPENDENT FOR SYNTHESIS?	No	Yes	No
ADSORBED BY $BaSO_4$, $Al(OH)_3$, AND OTHER SALTS	Partially	Yes	No
CONSUMED IN COAGULATIONS?	Partially	No (except II)	Yes
SITE	Plasma or serum	Plasma or serum (except II is not present in serum)	Plasma
DESTROYED BY PLASMIN OR HIGH CONCENTRATION OF THROMBIN?	No	No	Yes
FOUNDS IN PLATELETS?	No	No	α-granules: I, V, vWF General cytoplasm: XIII Not present: VIII:C
ACUTE-PHASE REACTANTS?	No	No	Yes
PRODUCTION REDUCED BY ORAL ANTICOAGULANTS?	No	Yes	No

The prothrombin (vitamin K-dependent) group includes the vitamin K-dependent coagulation factor II, VII, IX and X, protein C and protein S. Vitamin K is fat soluble vitamin and normally ingested in the diet. It is also produced by the gut flora. Vitamin K is necessary to γ-carboxylate the glutamic acid residues at the N-terminal or amino (NH_2) end of the enzyme precursors. This allows the factor to bind Ca^{2+} and form calcium bridges with the acidic phospholipid surface of activated platelets.

Fibrinogen group contains factor I, V, VIII and XIII. These factors have the highest molecular weights of all the factors. They are the most labile, consumed in the coagulation, and are the only group that act as substrates for the fibrinolytic enzyme, plasmin. Factor I and V are found in platelet α-granules and factor XIII is found in platelet cytoplasm. Factor VIII is not found in platelet. Factor VIII (VIII/vWF) is a large, multimeric molecule that has two major parts as shown in figure 6. One is the coagulation portion (VIII:C) and the other is the von Willebrand portion (vWF). Factor VIII:C acts as a cofactor in the intrinsic coagulation pathway but vWF is important to normal platelet function. Factor VIII:C which is probably produced in the liver, has been called antihemophilic factor because it is defective in patients with hemophilia A. vWF is a large polymer that is synthesized by endothelial cells and megakaryocyte. vWF can be measured by immunological method as vWF:Ag.

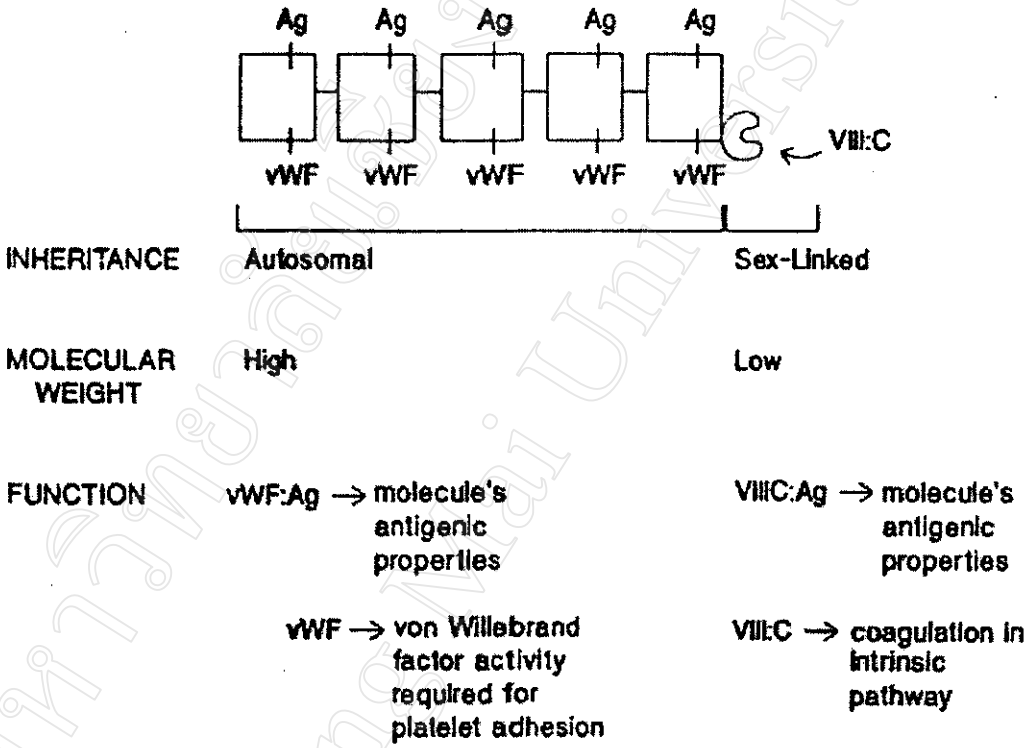


Figure 6 The factor VIII molecule (VIII/vWF) is a polymer with multiple subunits composed of vWF (vWF:Ag) connected to a small coagulation unit known as VIII:C. (Jobe MI, 1998)

1.2.6 **Coagulation cascade**

1.2.6.1 **Intrinsic coagulation pathway** : The initiation of the internal coagulation pathway begins with the contact activation phase of coagulation, which involved all four contact factors.

A. Factor XII activation : Factor XII is a single polypeptide chain zymogen which absorbs to negatively charged surfaces. The complex of prekallikrein and HMWK is also adsorbed to the negatively charged surface with factor XII. Factor XI also complexes with HMWK on the surface. Once the contact group is assembled, factor XII undergoes a conformational change in the presence of kallikrein, with enhancement by HMWK. Factor XIIa is cleaved into fragment (XIIf) by a number of proteolytic enzymes, including plasmin and, probably most importantly, kallikrein. Both factor XIIa and XIIf activate prekallikrein to kallikrein. Factor XIIa and factor XIIf play several roles in hemostasis.

1. In the presence of HMWK, XIIa converts factor XI to the serine protease XIa. So factor XIIa is an initiator of the intrinsic coagulation pathway. While as factor XIIf is a very poor activator of factor XI and XIIf does not activate factor XII.
2. Factor XIIa and XIIf can activate the tissue factor : factor VII (TF:VII) complex to TF:VIIa in the extrinsic coagulation pathway. So factor XIIa and factor XIIf can initiate the extrinsic coagulation pathway.
3. Both factor XIIa and kallikrein form the complex required for conversion of plasminogen to plasmin, which is fibrinolytic. Factor XIIf can also cause conversion of plasminogen to plasmin. So factor XIIa and XIIf initiate fibrinolysis.
4. The conversion of prekallikrein to kallikrein by factor XIIf and HMWK causes the conversion of HMWK to kinin. The plasmin formed as a result

of kallikrein can also initiate the complement system. Factor XIIf can directly activate the first component of the complement system. So factor XIIf initiates the kinin and complement system.

Kallikrein plays three roles during contact activation.

1. It perpetuates factor XII activation and its own production.
2. It initiates the kinin system.
3. It initiates the fibrinolytic and complement systems along with factor XIIa.

Plasmin plays three major roles in contact activation.

1. It promotes clot dissolution.
2. If plasmin is not destroyed by plasma antiplasmins, it cleaves factor XIIa to XIIf. So further contact activation is inhibited because factor XIIf is a very poor activator of factor XI.
3. It can activate the kinin and complement systems.

B. Factor XI activation : Factor XI is activated to XIa by factor XIIa. Factor XIa can be activated directly by contact activation like factor XIIa. And factor XIa also activates plasminogen. Both XIa and XIIa are involved in the initiation of the fibrinolytic and complement systems. There must be alternate pathway for factor XI activation because people with factor XII deficiency do not have bleeding abnormalities, while as people with factor XI deficiency may have mild bleeding abnormalities.

C. Factor IX activation : The activation of factor IX to IXa by factor XIa requires Ca^{2+} . Kallikrein is also capable of directly activating factor IX. Factor IXa combines with the factor VIIIa and Ca^{2+} on the platelet phospholipid surface to form the multimolecular IXa-Ca^{2+}-VIIa complex. These complex converts factor X to Xa. The coagulation cascade continues on the platelet surface.

1.2.6.2 **Extrinsic coagulation pathway** : The extrinsic coagulation pathway is much less complex than the intrinsic coagulation pathway. The extrinsic coagulation pathway consists only of tissue factor (TF or factor III), factor VII, and Ca^{2+}.

TF is a receptor protein that present in the plasma membrane of many cell types and has a high affinity for plasma factor VII. The exposure of TF to plasma factor VII allows the formation of a Ca^{2+}-dependent tissue factor : factor VII (TF:VII) complex on the cell surface. Ca^{2+} acts as a bridge between factor VII and TF.

Factor VII is vitamin K dependent and circulates in a form of single-chain glycoprotein. The exact mechanisms causing factor VII activation *in vivo* are uncertain. *In vitro*, substances reported to activate factor VII are kallikrein, thrombin, factor XIIa, factor XIIf, factor IXa and factor Xa. The TF : VIIa-Ca^{2+} complex on a cell surface converts factor X to Xa in the common pathway. TF : VIIa is more enzymatic activity than the factor VII zymogen or TF : VII complex. Large concentration of factor Xa cleaves factor VII into a three-chain molecule that is inactivate in coagulation. So it is a control mechanism for the extrinsic pathway.

1.2.6.3 **Common coagulation pathway** : The activation of factor X to Xa begins the common coagulation pathway. Equal amounts of factor Xa are generated by activation through either intrinsic or extrinsic coagulation pathway. Factor Xa binds to the platelet phospholipid surface and multimolecular complex known as the prothrombinase complex is formed in the common pathway. This complex is Ca^{2+}-phospholipid. The prothrombinase complex converts prothrombin to thrombin in a two-step process. First, peptide bonds in prothrombin are cleaved to produces prothrombin and either prothrombin fragment 1.2 or fragment 1.2.3. Second, a peptide bond is cleaved in prethrombin 2, releasing fully active thrombin that contains two chains, A and B, connected by a disulfide bond. The common pathway reaction is

completed with thrombin activation of fibrinogen to fibrin through a series of steps that stabilize the fibrin clot.

1.2.7 **Fibrinolysis**

There is ample evidence that the blood clotting system is normally in a dynamic steady state in which fibrin clots are constantly being laid down and subsequently dissolved. Fibrinolysis is depend on conversion of the zymogen plasminogen to the enzyme plasmin. Plasmin is a serine protease that specifically cleaves fibrin's triple stranded coiled segment and cut away its covalently cross-linked α chain protuberances as shown in figure 7. Plasmin is not normally present in plasma. Normal plasma contains the inactivation form of plasmin in a precursor (zymogen) called plasminogen (86 kD). Plasminogen is converted to plasmin by the activation of proteolytic enzymes, kinases of plasminogen activators, most notably the 54-kD enzyme urokinase, which is synthesized by the kidney and occurs in the urine, and the homologous 70-kD enzyme tissue plasminogen activator (tPA), which occurs in vascular tissue. In addition, activated Hageman factor, in the presence of prekallikrein and HMK (the contact activation system) activates plasminogen although the physiological significance of the contact activation of the fibrinolytic system has not been determined. These activators activate plasminogen to plasmin then plasmin degrades both fibrinogen and fibrin. In these process, specific fragments are produced called fibrin(ogen) degradation product (FDP) or fibrin(ogen) split products (FSP). These degradation products are removed by the liver, kidney, and reticuloendothelial system. The sequence of reaction in the degradation of fibrin by plasmin result in the four principal products, fragment X, Y, D (D-D dimer) and E as shown in figure 8. Plasmin acts on specific sites of each fragment to create smaller fragments throughout the reaction sequence. Fragments X and Y are referred to as early degradation products; fragment D and E are late degradation products. Fragment

X is the and largest fragment formed (MW 250,000). Fragment is a result of plasmin cleavage of the terminal portions of the alpha chains from a fibrin polymer, leaving isolated fibrin strands. Fragment X is then cleaved by plasmin to form two fragments called Y (YY) and an intermediate complex, DXD. This complex is further cleaved into intermediate complexes DED and DY/YD until finally, fragment E and D (D-D dimer) are formed. A single fragment D has a molecular weight of approximately 90,000, and that of the D-D dimer is approximately 180,000.

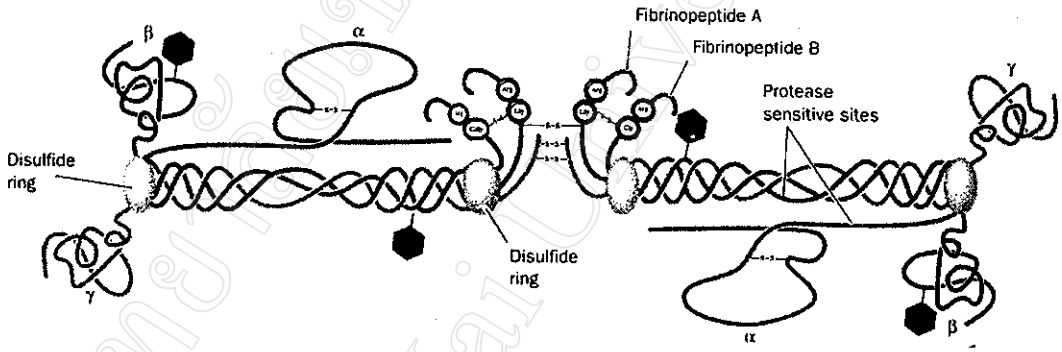


Figure 7 The proposed structure of fibrinogen based on low-resolution structural studies, primary structure determinations, and chain-folding predictions. The so-called disulfide rings are regions containing three disulfide bonds cyclically linking homologous segments of the α, β, and γ chains. N-linked polysaccharides are represented by filled hexagons. The Arg-Gly bonds that are cleaved by thrombin in fibrin activation are indicated. (Voet D and Voet JG, 1995)

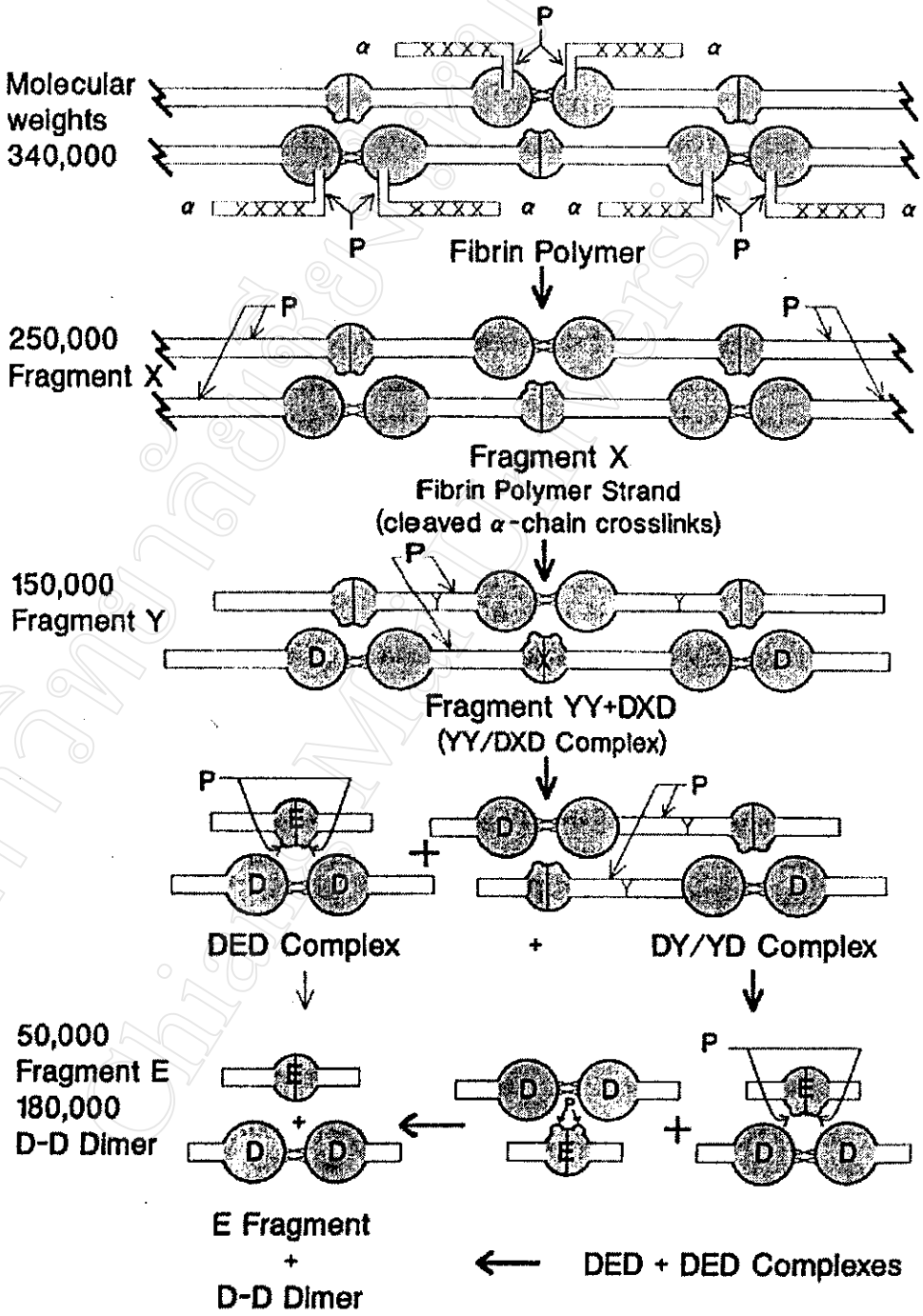


Figure 8 Degradation of fibrin by plasmin. P indicates sites where plasmin cleaves fibrin polymer, fibrin strands, and various complexes. (Jobe MI, 1998)

1.2.8 **Inhibitors of coagulation and fibrinolysis**

A regulatory system must exist within the body to control coagulation and fibrinolysis. This system includes both naturally occurring biochemical inhibitors and physiologic control mechanisms. The counterforces of the both naturally occurring biochemical coagulation and fibrinolytic inhibitors are necessary to give a balance between activated clotting factors and fibrinolytic enzymes. Table 3 summarizes the features of the well-characterized inhibitors of coagulation and fibrinolysis.

1.2.8.1 **Naturally occurring inhibitors of coagulation**

Antithrombin III (AT-III) is an important coagulation inhibitor found in plasma. It known as heparin cofactor. It is a glycoprotein synthesized in the liver and has a half life about 2.7 days. AT-III inhibits most serine protease including thrombin, XIIa, XIa, Xa and IXa by forming complexes with these activated factors to neutralizing them and preventing their action on other zymogens. Factor VII is the only serine protease that AT-III does not significantly inhibit. AT-III also has an inhibitory effect on plasmin and kallikrein. Thus, AT-III plays a role in monitoring the coagulation, fibrinolytic, kallikrein-kinin, and complement systems.

A heparin-like substance known as heparan sulfate is normally present *in vivo* in cell surfaces. A small amount of plasma AT-III binds to heparan sulfate on the vascular endothelium surface and protects uninjured vessels against thrombus formation by neutralizing serine proteases.

Table 3 Naturally occurring inhibitors of coagulation and fibrinolysis. (Jobe MI, 1998)

Inhibitor	**MW ($x10^3$)**	**Plasma concentration (mg/dl)**	**Rate of inhibition**	**Rate accelerated by heparin?**	**System inhibited**	**Function inhibited**
Antithrombin III	58	21-30	Slow	Yes	Coagulation Fibrinolysis	Thrombin, XIIa, XIa, Xa, IXa and kallikrein Plasmin and Kallikrein
Heparin cofactor II	66	9	Slow	Yes	Coagulation only	Primarily thrombin
α_2-macroglobulin	725	130-325	Variable	No	Coagulation Fibrinolysis	Thrombin and kallikrein Plasmin and kallikrein
α_1-antitrypsin	40-50	245-335	Slow	No	Coagulation Fibrinolysis	Potent inhibitor of XIa; week inhibitor of thrombin Plasmin
C'1 inactivator	105-135	14-130	Slow	No	Coagulation Fibrinolysis	XIIa, XIIf, XIa and kallikrein Plasmin
Protein C-S system	62 (C) 71 (S)	0.2-0.6 1.5 (free)*	Slow	No	Coagulation Fibrinolysis (enhanced)	Va and VIIIa May enhance fibrinolysis by inactivating plasminogen activator inhibitors (PAI)

Table 3 (continued)

Inhibitor	**MW ($x10^3$)**	**Plasma concentration (mg/dl)**	**Rate of inhibition**	**Rate accelerated by heparin?**	**System inhibited**	**Function inhibited**
Protein C inhibitor	67	0.5	?	Yes	Coagulation (enhanced) Coagulation (inhibited)	Activated protein C Xa, thrombin, kallikrein
Plasminogen inactivator inhibitor-1 (PAI-1)	45-50	Trace**	Variable	No	Coagulation Fibrinolytic	Thrombin tPA***, urokinase, activated protein C, plasmin
Tissue factor pathway inhibitor (TFPI)	30-40	0.01	Slow	Yes	Coagulation only	TF:VIIa, Xa
α2-antiplasmin	65-70	5-7	Rapid	No	Fibrinolytic only	Principal inhibitor of plasmin; inhibits tPA***; inactivates urokinase

*: Protein S exists in plasma in both a free, active form and inactive form bound to the complement.

**: PAI-1 plasma concentration (geometric mean) has been reported as 24 ng/ml

***: tPA = tissue plasminogen activators

Naturally occurring heparin is reported to enhance AT-III activity. Heparin attaches to AT-III, causing a conformational change that makes the arginine residue of the AT-III reactive site more accessible to the active site of serine protease. Without heparin, AT-III neutralizes thrombin and factor Xa by forming a 1:1 complex slowly over a period of minutes.

Heparin cofactor II (HCFII) is a plasma antiprotease that inhibits coagulation by a mechanism like that of AT-III. However, its primary structure is quite different. It is primary an inhibitor of thrombin, although it has less affinity for thrombin and reacts more slowly with thrombin than AT-III. HCFII is activated by heparin and other mucopolysaccharides. Unlike AT-III, it does not inhibit plasmin.

α_2-Macroglobulin is a large, naturally occurring plasma glycoprotein. This inhibitor binds with various proteolytic enzymes including thrombin. However, its rate of thrombin inhibition is slower than that of AT-III. α_2-Macroglobulin does not completely inhibit its target enzymes. This is because after binding with an enzyme, α_2-macroglobulin undergoes a conformational change that physically entraps an enzyme so that it may not bind with its substrate, but its active catalytic site remain intact. It also inhibits kallikrein and fibrinolysis.

α_1-Antitrypsin (α_1-antiprotease) is an α globulin that is a potent inhibitor of factor XIa. It may also inactivate thrombin at a slow rate, although this is still uncertain. It is a weak inhibitor of trypsin (a substance that activates factor XII) and of the fibrinolytic system.

C′1 inactivator (C1 esterase inhibitor) is a glycoprotein. It has an affect to fibrinolytic, kinin, and complement systems. In coagulation system, C′1 inactivator is the major inhibitor of the contact system. It inhibits factor XIIa, XIIf, and XIa.

Protein C and Protein S are vitamin K-dependent glycoprotein. Protein S is a cofactor of protein C. Activated protein C complexes with protein S is a potent inhibitor of coagulation as it destroys factors Va and VIIIa. Protein C exist free in the plasma, but protein S exists in both an active free form and an inactive complement-bound form. Protein C activation, which occurs on the endothelial cell surface, requires calcium and thrombin bound to an endothelial cell surface receptor known as thrombomodulin. There are four steps involved in the activation of protein C. First, it is believed that thrombomodulin forms a 1:1 complex with thrombin on the endothelial cell surface. Next, protein C binds to the thrombin-thrombomodulin complex by means of Ca^{2+} bridges and is slowly activated by proteolytic cleavage releasing a peptide (C-activation peptide). Then protein S binds to the complex on the endothelial surface. Finally, activated protein C, in the presence of protein S, proteolytically degrades factors Va and VIIIa. In addition to regulation of clotting, the protein C-S complex may enhance fibrinolysis by neutralizing plasminogen activator inhibitors (PAI).

Protein C inhibitor is a heparin-activated serine protease that inhibits activated protein C, factor Xa, and thrombin. It does not affect plasmin or the plasminogen activator, urokinase.

Tissue factor pathway inhibitor (TFPI) is a single polypeptide that is synthesized by endothelial and other cells. During coagulation, TFPI forms a complex with factor Xa (TFPI:Xa) that inhibits factor Xa and the tissue factor : factor VIIa (TF:VIIa) complex. TFPI is a unique inhibitor since it is bivalent, that is, a single molecule has inhibitory sites for two factors. TFPI generally inhibits factor Xa at about the same rate as AT-III. Heparin induces TFPI normally pooled in the vascular to move into the plasma, causing a several-fold rise in the plasma TFPI concentration and enhanced factor Xa inhibition.

1.2.8.2 **Naturally occurring inhibitors of fibrinolysis**

Plasminogen activator inhibitor-1 (PAI-1) has been found in plasma, platelets, and in many tissues including endothelium. PAI-1 is a single chain glycoprotein that is released from its storage site in platelet α granules upon platelet aggregation. It is also released in response to thrombin formation. It forms a 1:1 complex with both tPA and urokinase, thus inhibiting fibrinolysis and contributing to the stabilization of fibrin. PAI-1 can also inhibit plasmin, activated protein C, and thrombin. On the other hand, PAI-1 is itself inhibited by factor XIa produced during contact activation.

α_2-Antiplasmin is an α_2-glycoprotein and a serine protease inhibitor. It acts as a principal inhibitor of fibrinolysis by binding in a 1:1 irreversible complex to any plasmin that is free in plasma, thus neutralizing plasmin. This prevents plasmin from binding to fibrin and prevents plasmin's premature and uncontrolled digestion of fibrin, fibrinogen, and factors V and VIII by plasmin. It also permits a slow and orderly dissolution of the clot and adequate time for repair of damaged tissues. The complex formed between plasmin and α_2-antiplasmin is similar to the thrombin-AT-III complex. Both inhibitors bind to the active serine site of their respective enzyme targets, thus inactivating the serine protease and preventing its enzymatic action in its usual substrates. In addition, α_2-antiplasmin is crosslinked to fibrin by factor XIIIa, which is assisted in its inhibitory mechanism. α_2-Antiplasmin has been shown to inhibit many serine proteases. Nevertheless, its physiologic role in inhibiting any other than plasmin appears negligible. The conversion of plasminogen to plasmin is also suppressed by α_2-antiplasmin through inhibition of tissue plasminogen activator. α_2-Antiplasmin is the most important naturally occurring inhibitor of fibrinolysis, as it is the first to bind with plasmin in plasma.

α_2-Macroglobulin is a large, naturally occurring plasma glycoprotein that inhibits components in both the fibrinolytic and the coagulation systems. It rapidly inhibits plasmin after α_2-antiplasmin depletion, however, it does not totally eliminate plasmin's function in fibrinolysis.

α_1-Antitrypsin (α_1-protease) is the least significant of the three naturally occurring fibrinolytic system inhibitors. It slowly inactivates plasmin and does not bind plasmin until both α_2-antiplasmin and α_2-macroglobulin are saturated.

1.2.8.3 Synthetic inhibitors of coagulation (anticoagulant drugs)

Heparin blocks the coagulation cascade by promoting the interaction of a circulation inhibitor of thrombin (antithrombin III; AT-III) with activated clotting factor. Low molecular weight heparin (LMWH) is produced from standard heparin by chemical or enzymatic depolymerization. LMWH has a decrease ability to inactivate thrombin because of its smaller molecular size and its inability to form a complex with AT-III and thrombin together. LMWH, however, inactivates Xa by binding to AT-III alone.

Coumarin and warfarin have structure like vitamin K, an important element in the synthesis of a number of clotting factors. Interference in the metabolism of vitamin K in the liver by coumarin derivatives gives rise to clotting factors that are defective and incapable of binding calcium ions.

1.2.8.4 Synthetic inhibitors of fibrinolysis (antifibrinolytic agents)

Aprotinin is a proteolytic enzyme inhibitor obtained from bovine lung. It directly affects fibrinolysis by reducing the conversion of plasminogen to plasmin.

Epsilon (ε)-aminocaproic acid acid (EACA) is a lysine analog which prevents the conversion of plasminogen to plasmin, hence preventing the breakdown of fibrin polymers. It competes with plasminogen attachment to fibrin *via* plasminogen binding sites. This mechanism is similar to natural α_2-antiplasmin.

Tranexamic acid has actions similar to those of EACA, but it is approximately 10 times more potent.

1.2.9 Fibrinogen

Fibronogen is a large plasma glycoprotein (340 kD) and 46 nm long. It is soluble in plasma. It consists of two of each of three non-identical polypeptide chains which are synthesize in liver. All of six polypeptide chains are two Aα (610 residues) chains, two Bβ (461 residues) chains and two γ (411 residues) chains. Thus its structure is $A\alpha_2B\beta_2\gamma_2$. Its forms dimer which is conjugated by disulfide bond near the terminal ends. Bβ chain and γ chain have Asn-linked complex oligosaccharides. Both the Aα and Bβ chain pairs have small fibrinopeptide in their terminal region known as fibrinopeptide A and B (16 and 14 amino acids, respectively) for a total of four fibrinopeptides (two A and two B). The distal of fiber-shape fibrinogen have high negatively charge because of a lot of aspartate and glutamate residue in A portion of Aα chain and B portion of Bβ chain as shown in figure 9. Additional, B portion of Bβ chain also has tyrosine O-sulfate residue which has negative charge. The negative charge end of fibrinogen is not only provide its solubility but it also pushes with the end of the other fibrinogen molecule. Then it prevents the aggregation of fibrinogen. All six amino terminal of fibrinogen molecule is folded in cluster within central domain but the three different carboxyl terminal are on the opposite site . Distal domain is connected with central domain by three stranded coiled coil. The distal domains correspond to fragment D (86 kD each) and the smaller central dimeric

domain to fragment E (45 kD). Amino acid of these proteins could be substituted by the others as the result of point mutation.

The conversion of fibrinogen to fibrin is composed of a number of steps. First, thrombin, a serine protease that composes of two polypeptide chains, cleaves the bond between Arg and X (most of X is glycine) in Aα chain as shown in figures 7 and 9. So the fibrinopeptide A removed from the amino-termini of the Aα chain. These new amino termini with sequence "Gly-Pro-Arg" are centrally located and referred to as "knob" which are able to being with holes on the terminal portion of other fibrinogen molecules. These pair of knobs bridge to the end of fibrin monomer unit which is next to each others. The first phase of polymerization process leads to spontaneous oligomer formation consists of fibrin monomer units which is overlap each other about 10-20 units in solution in the form of fibrin(ogen) or the conjugating of oligomer. The event that occurs with central domain conjugation of these protofibrils is the nearby molecules will rapidly crosslink by factor XIII (which is activated by thrombin). The crosslink occurs specifically between carboxyl-terminal segment of γ chain. In the last phase, the crosslink between carboxyl segment of α chain slowly happened.

Fibrinopeptides B are slowly released from amino-terminal segment of β chain. The releasing may be depend on earlier event surrounding protofibril formation. The new amino-terminals with the sequence Gly-His-Arg are exposed. When fibrinopeptide B is removed, the lateral interaction between protofibrils are occurred.

Mature fiber must be built up in a perfectly uniform way. It has been inferred that the Gly-His-Arg knobs must fit into another set of holes, equivalent to but different from those to which the Gly-Pro-Arg type bind. Recently, the X-ray structure studies of a complex of double-D clearly shows Gly-His-Arg-Pro peptides bound in the β-chain hole.

Clot stabilization requires thrombin, factor XIIIa, and Ca^{2+}. Thrombin activates factor XIII, which then functions as a transamidase, crosslinking adjacent fibrin monomers through formation of stabilizing covalent bonds. Factor XIIIa initially joins the C-terminal segments of adjacent γ chains by forming isopeptide bonds between the side chains of a Gln residue on one γ chain and a Lys residue on another as shown in figure 10. The α chains are similarly crosslinked to on another but at a slow rate.

There would be variant human fibrinogens reflecting dysfunction at each of the steps in fibrin formation. There ought to be mutations that interfere with fibrinopeptide release, others that distort the structure of the knobs, the holes, the end-to-end abutments, the γ-chain crosslinking sites, the association area in the carboxyl regions of α chains, or the crosslinking sites of the α chains. Many variants have been correlated with specific defects. There are variants known in which fibrinopeptide A release is impaired, others where the structure of the α-chain knobs (Gly-Pro-Arg) has been compromised, and still others in which changes in the carboxy-terminal region of the γ chains have given rise to notions of where the hole ought to be. There have also been variants reported that have defective fibrinopeptide B release, but none has been reported in which the structure of the β-chain knob (Gly-His-Arg) has been changed (except for the glycine cleavage site), nor have been any reports of changes in any region where a β-chain knob might conceivably bind. Defective γ-γ crosslinking has been reported for fibrinogen Paris I, however, a situation involving a 15-amino acid residue insert, and also for fibrinogen Asahi, which suffers from excessive glycosylation. There have been reported that α chains have suffered a long terminal deletion and as a result has nothing left to crosslink. (Everse SJ, *et al.*, 1998)

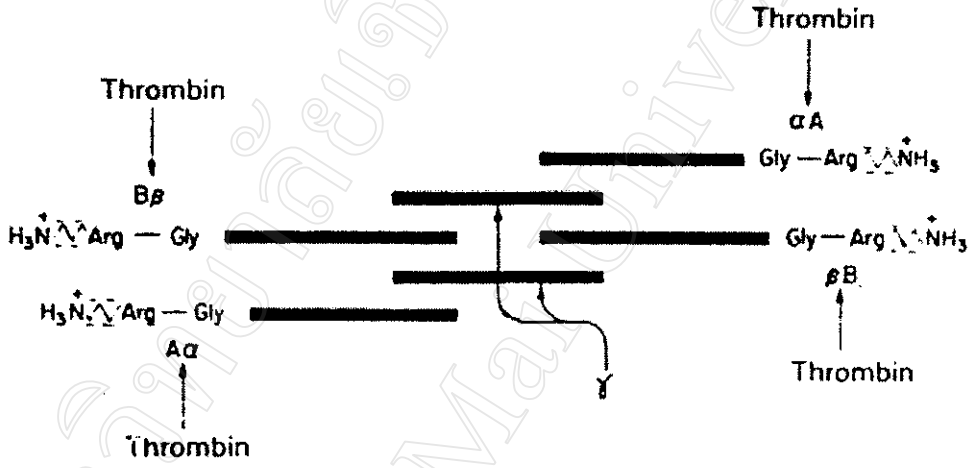


Figure 9 Diagrammatic representation of fibrinogen, its $(A\alpha B\beta\gamma)_2$ structure, charge termini, and the sites of thrombin cleavage (arrows) of four Arg-Gly peptide bonds. (Murray RK, *et al.*, 1988)

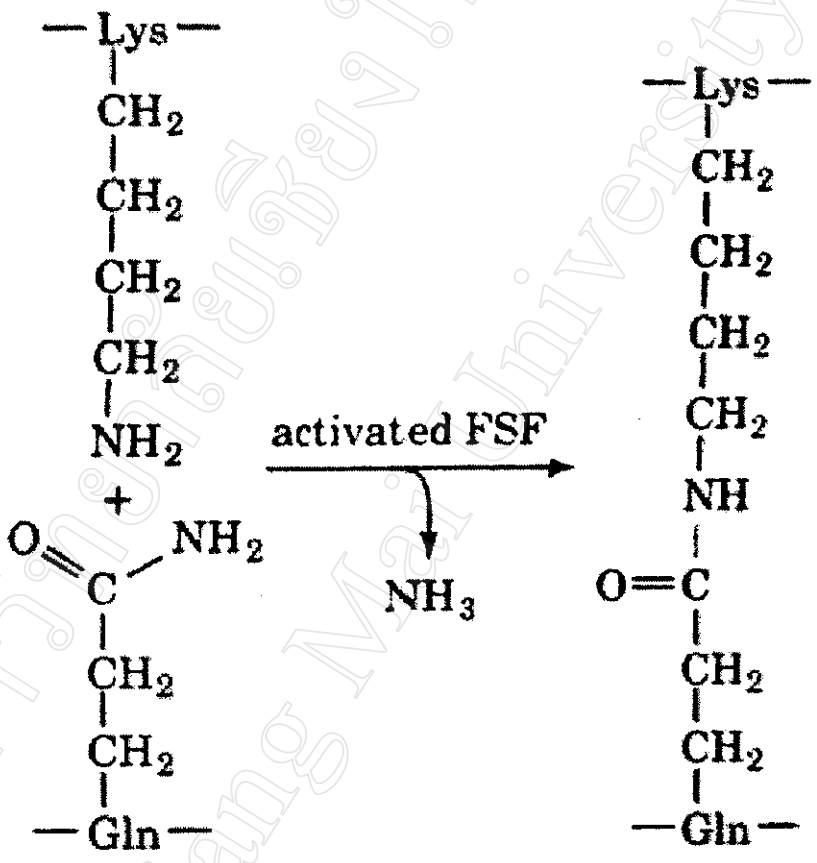


Figure 10 The transamidation reaction forming the isopeptide bonds cross-linking fibrin monomers in "hard" clots as catalyzed by activated fibrin stabilizing factor (FSF, XIIIa). (Voet D and Voet JG, 1995)

1.2.10 **Thrombin**

Thrombin is a 72,000-MW serine protease that consists of two polypeptide chains : in human, a 36-residue A chain and a 259-B chain. Thrombin B chain is homology to trypsin and has similar specificity to trypsin but is far more selective. It cleaves only certain Arg-X and, less frequently, Lys-X bonds with a clear preference for a Pro proceeding the Arg or Lys.

Human thrombin is synthesized in a form of zymogen, prothrombin (II), which is activated by two proteolytic cleavages catalyzed by factor Xa. The cleavage of prothrombin's Arg 271-Thr 272 and Arg 320-Ile 321 bond releases its N-terminal propeptide and separates the A and B chains as shown in figure 11. The last cleavage, which yields active enzyme, results in the formation of an ion pair between Ile 321 and Asp 524. Thrombin then autolytically cleaves its Arg 285-Thr 286 bond, thereby trimming away the N-terminal 13 residues of the A chain to yield α-thrombin. Prothrombin's propeptide consists of three domains : an N-terminal 40-residue Gla domain followed by two 40% identical ~ 115 residue kringle domains. Kringles are crosslinked by three characteristically located disulfide bonds.

Prothrombin is a 72,000-MW single-chain polypeptide and synthesized in the livers in a process that requires an adequate dietary intake of vitamin K. Lack of vitamin K causes the production of an abnormal prothrombin that is activated by factor Xa at only 1-2% of the normal rate. The normal prothrombin contains γ-carboxyglutamate (Gla) residues but the abnormal prothrombin contains Glu in places of these residues.

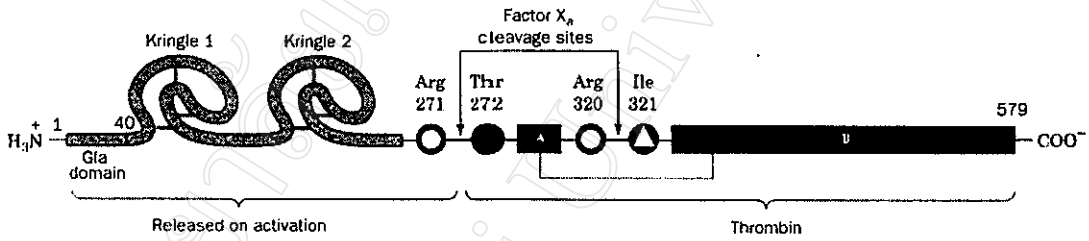


Figure 11 The schematic diagram of human prothrombin showing the peptidebonds that are cleaved by factor Xa to form thrombin. The N-terminal propeptide, which consists of a Gla domain and two tandem kringle domains, is released in this activation process, whereas thrombin's A and B peptides remained linked by one of the protein's several disulfide bonds. Thrombin then automatically excises the A peptide's N-terminal 13 residues. (Voet D and Voet JG, 1995)

1.2.11 **Historical perspective of fibrin glue**

Fibrin glue (also referred to as fibrin sealant, fibrin adhesive, and fibrin tissue adhesive) is probably one of the most complex human plasma derivatives both in terms of composition and clinical application. This product mimics the last step of the coagulation cascade through the activation of fibrinogen by thrombin, leading to the formation of a semirigid fibrin clot. The fibrin clot consolidating and adhering to the application site acts as a fluid tightness agent can hold tissue or materials in a required configuration, while evidencing hemostatic and healing properties.

In 1909, Bergel S first used dry plasma as a source of fibrinogen and fibrin form to establish hemostasis. During World War I, fibrin patches were used to stop bleeding from parenchymatous organs. In 1940, Young JZ and Medawar PB were used fibrin patches in microsurgery for the sutures of the peripheral nerves in an animal model. Later, Sedden HJ and Medawar PB were used it in human. A significant improvement in the development of efficient fibrin glue was made when, in 1944, Tidrick RT and Warner ED mixed fibrinogen with bovine thrombin in an attempt to accelerate the formation of fibrin clot and produce a biological adhesive to human skin grafts. The first commercial multidonor fibrin glue products were made available in Europe in the late 1970s. Most of the current standardized, freeze-dried products are rich in clottable fibrinogen (>80 g/l) and factor XIII (>10 IU/ml); they are solubilized in the presence of an antifibrinolytic agent (usually aprotinin at 3,000 KIU/ml or less), and are then mixed with thrombin (300-1,000 NIH Units/ml), in the presence of $CaCl_2$ (approximately 40-60 mM), which are injected from a separate syringe into the operation site. At present, three commercial fibrin glues, Tisseel and Tissucol (Immuno AG, Vienna, Austria), and Beriplast (Behringwerke AG, Marburg, Germany) are registered in several European countries, Canada and Japan. Only Tisseel is currently licensed in the US by the Food and Drug Administration (FDA),

historically because large-pool fibrinogen concentrates for intravenous use have been associated, prior to the implementation of efficient viral inactivation procedures, with the transmission of hepatitis B virus. License revocation for fibrinogen concentrate was pronounced in 1978. Subsequently, procedures using single-donor or autologous components for the production of fibrin glue were developed. These fibrinogen sources were mixed with commercial bovine thrombin to obtain homemade fibrin glue (Radosevich M, *et al.*, 1997).

A fairly recent improvement in the historical development of commercial fibrin glue is the use of thrombin of human in place of bovine origin. The availability of human thrombin resulted in avoiding two major disadvantages seen with thrombin preparation from animal sources. Firstly, allergic reactions in patients who had developed antibodies to bovine proteins after a first exposure to bovine thrombin also containing additional proteins like factor V. Serious hemorrhagic problems, such as bleeding diathesis or thrombosis, may be encountered in patients developing antibodies against multiple epitopes of bovine factor V or thrombin, respectively. These antibodies may cross-react with the human clotting factors. A correlation between the dose of, or the number of exposures to bovine thrombin and the severity of the coagulation abnormalities has been found but the application site of the fibrin glue and the extent of the treated area may also influence immunogenicity. Secondly, hypothetical risks of transmission of infectious agents, including that causing bovine spongiform encephalitis (classified among prions), to humans when applying thrombin from bovine source.

Currently licensed fibrin glue preparation contain an antifibrinolytic agent, usually aprotinin, to inhibit or slow down the degradation of the fibrin clot by naturally occurring proteolytic enzymes (plasmin) of the fibrinolytic system. Aprotinin is one of the most common and effective exogenous antifibrinolytic agents

used in coagulation therapy and in fibrin glue formulations. It is provide at a concentration of 3,000 KIU or less in the liquid form to solubilize the freeze-dried fibrinogen. Its final concentration is half of the starting solution after mixing fibrinogen and thrombin components. Tranexamic acid, as well as epsilon (ε) aminocaproic acid (EACA) at a concentration of >10 mg/ml, have been use mostly for non-commercial products.

1.2.12 **Production method of alternatives to commercial fibrin glue**

The risk of viral transmission by commercial fibrin glue products is still debated. Theoretically, viruses remaining in these topical preparations could be adsorbed and retained by the patient, which could result in the transmission of hepatitis B, hepatitis C, HIV-1 and 2, and possibly other blood-borne viruses (Gibble JW and Ness PM, 1990). The alternative fibrinogen sources were investigated in the United States. Many of these studies relied on autologous or single donor cryoprecipitate as a source of fibrinogen. Autologous-source fibrinogen eliminates the risks of virus transmission. Although single-donor and autologous cryoprecipitate methods produce limited amounts of fibrinogen concentrate, the yields are usually sufficient for most types of operative procedure. However, the use of autologous blood units or samples necessitates preoperative planing, and those products are not generally available for emergency therapy. Single-donor cryoprecipitate methods have been more widely applied, as the cryoprecipitate is readily available, and the associated risk of viral disease transmission is equivalent to that with transfusion of single-donor blood products.

Modifications of the basic cryoprecipitate method include the addition of a centrifugation step to concentrate further the precipitated fibrinogen. In these studies, centrifugation speeds and times ranged from 1,000 x g for 15 minutes to

6,500 x g for 5 minutes. Wan HL, *et al.* combined a repeat freeze-thaw cycle with centrifugation (4,200 rpm) and complete plasma drainage to enhance fibrinogen yields. Durham LH, *et al.*, Seidentop KH, *et al.*, Weisman RA, *et al.* and Silberstein LE, *et al.* used chemical additives to increase fibrinogen yields. Such methods included ammonium sulfate precipitation or the addition of polyethylene glycol (PEG). Durham LH, *et al.* and Seidentop KH, *et al.* suggested that the safety of ammonium sulfate precipitation methods for *in vivo* use. Weisman RA, *et al.* and Silberstein LE, *et al.* demonstrated the efficacy and safety of PEG methods for topical applications in the middle ear.

Comparative studies have shown that the commercially prepared Tisseel product generally contains the highest concentration of fibrinogen (70-100 mg/ml), and random-donor cryoprecipitate contains the lowest concentration (2.6-25 mg/ml). Cryoprecipitate centrifugation methods result in concentration of fibrinogen ranging from 21.6 to approximately 40 mg/ml. The repeat freeze-thaw centrifugation method of Wan HL, *et al.* results in concentration of fibrinogen ranging from 40 to 58.5 mg/ml.

With the exception of routine cryoprecipitate methods and the centrifugation method described by Sponitz WD, *et al.* (1987), all other methods of concentration rely on preparation in open test tube systems.

To date, the experiment data suggests that the fibrinogen content of the fibrin glue product is critical to the its tensile strength or adhesive strength. While commercially prepared fibrin glue preparation appear to provide uniform, maximum tensile strength as a result of their high fibrinogen content, Wan HL, *et al.* (1989); Seidentop KH, *et al.* (1988); and Silberstein LE, *et al.* (1988) have been able to approximate these results through modifications in cryoprecipitate preparation methods or through the use of precipitation methods.

The thrombin content of fibrin glue affects the rapidity of hemostasis and plays a major role when the hemostatic and sealing properties of the fibrin glue are the most important clinical consideration. Clot formation in less than 5 seconds can induced by thrombin concentration of 500 NIH units. Conversely, the process can be slowed from 30 seconds to several minutes by use of 4 NIH units of thrombin. A dichotomy exists between the sealing and adhesive properties of the fibrin glue. Current evidence suggests that increased thrombin level actually lower the tensile strength of the fibrin glue. Dresdale A, *et al.* (1985) and Durham LH, *et al.* (1987) have suggested that the addition of antifibrinolytic agents is unnecessary.

1.2.13 **Historical perspective of thrombin preparation**

A variety of techniques have been published that are suitable for the isolation of bovine thrombin from starting materials such as commercial topical thrombin, citrate-activated, partially purified prothrombin, or thromboplastin-activated purified bovine prothrombin, purified human prothrombin and human plasma Cohn Fraction III. These techniques have primarily made use of negatively charged ion-exchange resins such as IRC-50, Bio-Rex-70, or sulfoethyl or sulfopropyl Sephadex. In addition, affinity chromatography using p-chlorylbenzamino-ε-aminocaproyl agarose and m-aminobenzylamido-ε-aminocaproyl agarose has also been employed (Mann KG, Lundblad RL, 1987). These techniques were used the expensive equipment and impractical for routine laboratory. The practical method is used *pI* to precipitate prothrombin in bovine plasma and the precipitate was added with $CaCl_2$ to convert prothrombin to thrombin. Thrombin was precipitate again by acetone (Biggs R, 1976). Thrombin by this method cannot use *in vivo* because acetone might be toxic to cells.

1.3 **Objectives**

1. To study and develop the fibrinogen preparation method to give a high concentration, high quality, less interference and simple
2. To define an appropriate ratio of fibrinogen, thrombin, calcium chloride and antifibrinolytic agent that give a high quality and high stability fibrin glue, and low cost
3. To define a suitable method for fibrin glue preparation that can be prepared in routine laboratory