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PURIFICATION OF FIBRINOGEN FROM HUMAN PLASMA

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PURIFICATION OF FIBRINOGEN FROM HUMAN PLASMA

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A solvent detergent treated fibrinogen was purified from human plasma by cryoprecipitation (cryo) followed by chemical precipitation using ethanol (EtOH) or ammonium sulfate (AS) as precipitating agents. Amounts of fibronectin (FN), factor XIII A-subunit (FXIIIA), factor XIII b-subunit (FXIIIB), and alpha$_2$-antiplasmin (α_2-AP) in the isolated fibrinogen were quantified. Thromboelastography (TEG) analysis was used to evaluate the clot strength of the isolated fibrinogen and to determine the ability of the ethanol and ammonium sulfate precipitations to eliminate the solvent detergent. Sodium dodecylsulfate-polyacrylamide gel analysis indicated that fibrinogen produced by each of these precipitation methods had similar purity. Quantitative western blot analysis revealed that fibrinogen produced by ammonium sulfate precipitation contained increased amounts of FN, FXIIIB, and α_2-AP. TEG analysis showed that ammonium sulfate precipitated fibrinogen yielded a fibrin clot with the highest maximal strength. In addition, a single ethanol precipitation was sufficient to remove the solvent detergent while a single ammonium sulfate precipitation was not effective in removing the solvent detergent mixture Tri (n-butyl) phosphate (TNBP) and Triton X-100 as judged by the ability of the material to form a clot.

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INTRODUCTION

Fibrinogen

Fibrinogen (FI) is a central molecule in the blood coagulation cascade that is transformed by thrombin into fibrin which polymerizes forming a clot to prevent the loss of blood at the site of vascular injury. Furthermore, because it has multiple binding sites that can react with other proteins, fibrinogen plays important roles in many physiological and pathological processes including blood clotting, fibrinolysis, cell adhesion, inflammation, angiogenesis, atherogenesis, tumorigenesis, and wound healing [1]. Fibrinogen is a 340 kDa glycoprotein consisting of two identical subunits, each of which is made of three polypeptide chains termed Aα, Bβ, and γ [2]. The three chains of fibrinogen differ in terms of their structures and functions [Table 1].

A series of disulfide bonds link fibrinogen polypeptide chains forming elongated 45 nm tri-nodular structures with distinct domains. These domains are arranged into three main structural regions: a central E, two distal D and the αC regions [3-5]. The central E nodule is formed by the N-terminal portions of the six polypeptide chains. The distal D nodules, formed by the C-terminal portions of the Bβ and γ as well as a fraction of the Aα chains, are separated from the E region by coiled-coil regions [6, 7]. The C-terminal region of the Aα-chain extends out from the D domains and is called the αC domains [8].

Table 1: Properties of fibrinogen chains

	α-chain	**β-chain**	**γ-chain**
Molecular Weight [9]	67 KDa	54 KDa	47 KDa
Amino Acids [2]	610	461	411
Fibrinopeptide	FpA	FpB	None
Carbohydrate [10,11]	None	2 oligosaccharide	2 oligosaccharide

Based on the composition of the γ-chain, human fibrinogen can be isolated into two major fractions, fibrinogen-1 and fibrinogen-2 [12, 13]. Fibrinogen-1 possesses two homo-dimeric γ-chains each composed of 411 amino acids while fibrinogen-2 contains two hetero-dimeric molecules one γ-chain and one γ′-chain [12, 14]. The γ′-chain accounts for 15% of plasma fibrinogen and is formed by the alternative processing at the exon 9-exon 10 boundaries of the primary mRNA transcript [15]. The inclusion of intron 9 in the messenger results in the replacement of 4 amino acids (AGDV) of the γ-chain with a 20 amino acid sequence that contains two sulfated tyrosines and several Asp and Glu residues. Therefore, the γ′-chain is longer and contains more anion groups than the γ-chain [16, 14].

Fibrinogen has the ability to react with numerous substances with important physiological consequences. For example, The C-terminal of the Aα-chain (αC domains) of fibrinogen has binding sites for α_2-AP, plasminogen, tissue-type plasminogen activator (tPA), and PAI-2. Table 2 lists binding sites and physiological roles of some fibrinogen-protein interactions.

Table 2: Fibrinogen binding Ligands

Ligands	Binding Site	Function
Fibronectin [17]	Aα-chain	Cell adhesion
FGF-2, bFGF [18]	-	Proliferation of endothelial cells
Lipoprotein (a) [19, 20]	Aα-chain	Inhibit fibrinolysis
Thrombin [21]	γ′-chain	Fibrinopeptide release
Plasminogen [22]	Aα-chain	Enhance fibrinolysis
FXIII [23-25]	γ′-chain	Stabilizing clot
α_2-AP [26]	Aα-chain	Resist fibrinolysis
Integrin [2]	Aα-chain	Cellular interactions
Calcium [27]	Bβ and γ-chains	Promote polymerization

Human fibrinogen heterogeneity results from modification at several different sites both during and after biosynthesis. The heterogeneity arises from alternative splicing [28], alteration of different amino acids by sulfation [29], phosphorylation [30], various degrees of glycosylation [31], proteolysis [32], and some genetic polymorphisms [33]. These mechanisms form over a million species of fibrinogen [34].These heterogeneities can change fibrin matrix properties thus helping to explain the difference that exist between individuals and wound healing.

During coagulation, thrombin converts soluble fibrinogen into fibrin monomers, which then polymerize to form a network of fibrin fibers. This network is stabilized by thrombin activated FXIII to form an insoluble fibrin clot. Formation of fibrin begins

when thrombin binds to a substrate site in fibrinogen and cleaves fibrinopeptides A (FpA) and B (FpB) from the amino termini of the Aα and Bβ chains, respectively. The removal of FpA exposes the polymerization site "A" that contains the N-terminal sequence (Gly-Pro-Arg-Val) of the Aα-chain, while the removal of FpB exposes the polymerization site "B" that contains the N-terminal sequence of the Bβ-chain [35]. The polymerization site "A" interacts with the complementary binding site of the γ-chain, whereas the polymerization site "B" interacts with the complementary binding site of the β chain [36]. These interactions lead to the formation of half-staggered, double-stranded protofibrils that undergo lateral aggregation to form fibers [37]. Thrombin simultaneously converts factor XIII (FXIII) to the active form (FXIIIa) which catalyzes formation of covalent ε-(γ-glutamyl) lysine bonds between adjacent fibrin molecules [37-40]. The cross-linking occurs rapidly between Lys-406 of one γ-chain and Gln-398 of another γ-chain to form γ-dimers [41, 42, 25]. In addition, hetero-dimers cross-linked α-γ have been found in plasma fibrinogen [43].

Different techniques have been developed for the purification of fibrinogen from plasma materials. Cryoprecipitation is the most common method for isolating fibrinogen, which reduces the solubility of fibrinogen at lower temperature to prevent its denaturation. Compared to fresh frozen plasma, cryoprecipitate contains an increased percentage of fibrinogen. Cryoprecipitation involves freezing the citrated plasma at lower temperature, usually -20 °C or less for at least 12 hours. The frozen plasma is slowly thawed at 4 °C followed by centrifugation to isolate the fibrinogen precipitate. The concentration of fibrinogen produced by cryoprecipitation is between 8 to 30 mg/ml and

can be increased to 40 to 60 mg/ml using repeated freeze/thaw cycles [44]. An ultrafiltration procedure has been used to purify fibrinogen where platelet rich plasma is separated using an ultrafiltration chamber with a molecular weight cutoff of 30 kDa. Fibrinogen obtained using this method has a final concentration of 6 mg/ml and lower clottability due to large amount of fibrinogen being denatured [45]. Fibrinogen has also been isolated from human plasma by using chemical precipitation methods in which chemical agents such as ethanol, glycine, β-alanine, ether, or ammonium sulfate are used to precipitate fibrinogen from plasma [46-49].

The clinical use of fibrinogen from pooled human plasma has been previously associated with a high risk of transmission of plasma–borne infectious species such as HIV, HBV, and HCV. The improvements in viral testing and screening have reduced, but not eliminated this risk. Therefore, different techniques have been developed in order to inactivate pathogenic viruses in fibrinogen derived from human plasma. Dry heat treatment at 60-68 °C of plasma cryoprecipitate inactivates HIV but it does not prevent the transmission of HCV [50]. Plasma pathogens have been inactivated by treating fresh plasma with methylene blue followed by exposure to visible light. This approach is less effective versus non-enveloped viruses and results in approximately 20% fibrinogen loss [51]. Solvent detergent (SD) treatment is the most effective procedure for inactivating blood-borne lipid-enveloped viruses. Solvent detergent treatment inactivates viruses by dissolving their lipid envelope but it does not inactivate non-lipid enveloped viruses such as parvovirus or hepatitis A virus [50]. The treatment of cryoprecipitate with a combination of organic solvent, tri (n-buty1) phosphate (TNBP), and detergent (sodium cholate, Tween 80, or Triton X-100) has been shown to inactivate very large quantities of

HBV, HCV, and HIV while preserving the activity of the purified protein. Radosevich et al [52] showed that treating cryoprecipitate with a mixture of 0.3% TNBP and 1% tween 80 at 25 °C for 6 hours inactivated ≥ 5.5 $\log_{10}$ of HIV, ≥ 5 $\log_{10}$ of VSV virus, and ≥ 6.5 $\log_{10}$ of sindbis virus. Horowitz and coworkers [53] revealed that subjecting pooled plasma to a mixture of 1% TNPB and 1% Triton X-100 for 4 hours at 30 °C inactivates ≥ 10^6 CID_{50} of HBV, 10^5 CID_{50} of HCV, and $10^{6.2}$ $TCID_{50}$ of HIV.

Factor XIII

Factor XIII, activated in the latter stages of the blood coagulation cascade, is a pro-transglutaminase that exists naturally in an intracellular or plasma form [54, 55]. The plasma form is a tetramer composed of two A-subunits (FXIIIA) and two B-subunits (FXIIIB) with a molecular mass of ~83 and ~80 kDa respectively [56]. Structural investigation of the tetramer indicates that the two A-subunits are globular proteins surrounded by two long flexible B-subunits [57, 58]. There are non-covalent interactions between the two A-chains and appears to be a non-covalent bond between the A and B [59]. The A-subunit contains the active site of the enzyme while the B-subunit serves to transport and protect the catalytic A-subunit until it is required in catalysis [60, 61]. Under normal conditions, the human plasma concentration of FXIII is ~14 to 28 mg/L [62]. Whereas carbohydrate accounts for approximately 8.5% of the total molecular weight of FXIIIB, the FXIIIA contains no carbohydrate [Table 3].

Table 3: Characteristics of FXIII subunits

	A-subunit	**B-subunit**
Molecular weight [56]	83 kDa	80 kDa
Residues [63, 64]	731	641
Carbohydrate [65]	None	~8.5% of the total weight
N-terminal [59]	Acetylated serine	Glutamic acid
Disulfide bonds [63]	None	20
Structure [64]	Globular	Kinked strand
Function [66]	Catalyzes an acyl transfer reaction	Acts as a carrier for A-subunit
Secreted by [66]	Liver, monocytes and megakaryocytes	Liver
Active site [66]	Cys314	None

The primary structure of FXIII subunits has been determined through the aid of amino acid sequence analysis and cDNA cloning. The A-subunit is made of five distinct folded domains: an activation peptide of 37 amino residues that is cleaved off during thrombin activation of the enzyme, a β-sandwich containing residues 38-183, a catalytic core composed of residues 184-515, and two barrels comprised of residues 516-627 and 628-731, respectively [66]. While the catalytic core consists of both α-helices and β-sheets, the β-sandwich and barrel domains contain only β-sheets [Fig 1]. There are several hydrogen bonds and salt bridges between the β-sandwich, the activation peptide, and the catalytic core of the first subunit with the β-barrel and catalytic core of the second subunit [67]. These non-covalent interactions help stabilize the overall structure. Although FXIIIA possesses nine cysteine residues, no disulfide bridges have been detected in the subunit. There are six potential N-glycosylation sites in FXIIIA but no carbohydrate residue has been found in the secondary structure [65].

FXIIIB is a modular protein built from 10 repetitive sushi domains. Each sushi domain contains approximately 60 amino acids held together by two disulfide bridges forming a β-sandwich tertiary structure [63]. Employing electron microscopy and sedimentation techniques, the B-subunit appears as a thin, flexible strand with random kinks whereas unbound FXIIIB appears as a monomeric structure [64].

While plasma FXIII (pdFXIII) circulates as a tetramer, intracellular FXIII is a dimer composed of two A-subunits ($FXIIIA_2$) each containing 730 amino acid residues [68]. This dimeric molecule has a molecular weight of approximately 160 kDa and mainly found in platelets, monocytes, and megakaryocytes [68, 63]. The primary structure of cellular FXIII is identical to plasma FXIIIA and the two molecules share the same immunochemical and electrophoretic properties.

FXIIIA catalyzes an acyl transfer reaction where glutamine residues serve as an acyl donor. This reaction proceeds through acylation and de-acylation intermediate steps to form an amide bond between τ-carbonyl group of glutamine and ε-amino group of lysine [69]. Compared to other transglutaminase family members, FXIIIA has more restricted substrate specificity.

In the final stage of the blood coagulation cascade, the serine protease thrombin activates either form of FXIII in the presence of Ca^{2+}. The activation sequence begins when thrombin hydrolyzes the peptide bond at Arg37-Gly38 releasing the activation peptide from the N-terminal region of the FXIII [69, 70] [Fig 1]. Studies using radio crystallography showed that the released activation peptide did not bind thrombin but

remained attached to the FXIII molecule preventing the access of the substrate to the active site [58]. The activation sequence proceeds when Ca^{2+} binds to either the free activation peptide or the zymogen. This binding induces a conformational change that leads to the dissociation of the activated A-subunits from the B-subunits. As a result, the active site Cys-314 that was originally shielded by the activation peptide becomes unmasked and available for reaction [60, 71].

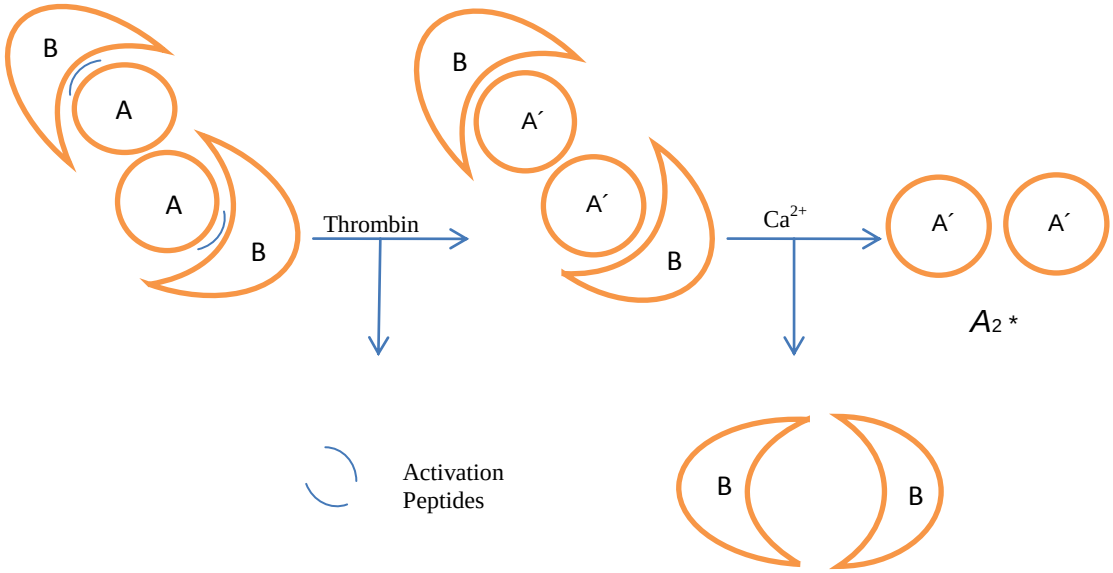


Figure 1: Schematic diagram illustrates FXIII activation in plasma. Removal of the activation peptide by thrombin weakens the binding of B-subunit to the truncated FXIIIA ($A_2{}'B_2$). Conformational change induced by Ca^{2+} causes dissociation of $A_2{}'B_2$ to generate FXIIIa [72].

Except for the dissociation of the B-subunit step, intracellular FXIII activation by thrombin follows the same mechanism as extracellular FXIII. Activation of intracellular FXIII is faster since the B-subunit of plasma FXIII suppresses activation of cellular FXIII

by thrombin thereby down-regulating pdFXIII cross-linking of fibrinogen [73]. The presence of fibrin speeds up the activation of pdFXIII by approximately one hundredfold [71, 74]. This is because the adsorption of thrombin and pdFXIII onto the fibrin (ogen) surface orient these molecules in a way that favors the hydrolysis of the peptide bond at Arg37-Gly38 by thrombin. Therefore, fibrin facilitates the activation of extracellular FXIII while the absence of B-subunit from intracellular FXIII inhibits fibrin catalysis [74].

The B-subunit of FXIII binds to the γ'-chain of fibrinogen thus heterodimeric fibrinogen (fibrinogen 2, γ/γ') serves as a carrier for FXIII in blood and also regulates its activity [75]. The presence of FXIII with fibrinogen in the plasma ensures that the formation of fibrin is associated with FXIII mediated cross-linking to generate a strong clot. FXIII cross-links homo-dimeric fibrinogen 1 molecules (γ/γ) 3.5 times faster than hetero-dimeric fibrinogen 2 molecules (γ/γ') indicating that fibrinogen 2 bound FXIII is essential for inhibiting FXIII mediated cross-linking activity in plasma [74]. Fibrinogen also reduces the amounts of calcium required to activate the truncated FXIIIA dimer (FXIII$A_2{}'$). Besides thrombin, there are several other serine proteases such as endogenous platelet acid and calpain that can also activate FXIII by cleaving the Arg37-Gly38 peptide bond [76, 77].

Besides fibrin and α_2-antiplasmin, FXIIIA has many other potential substrate such as plasminogen, lipoprotein (a), plasminogen activator inhibitor type 2 (PAI-2), thrombin-activatible fibrinolysis inhibitor (TAFI), fibronectin, and many other proteins [Table 4].

Table 4: FXIII substrates

Substrate	Cross-linking Site	Cross-linking Targets	Function
α_2-antiplasmin [78-80]	Gln2	Lys303 of α-chain	Inhibit fibrinolysis
Fibrin (ogen) α-chain [81-83]	Gln221 and 237 Lys208-Lys606	Itself and γ-chains	Clot stabilization
Fibrin (ogen) γ-chain [23-25]	Gln398, Gln399 and Lys406	Itself and α-chains	Clot stabilization
Fibronectin [84, 85]	Gln3	Itself, fibrin, and collagen	Wound healing; migration of cells into the clot
Collagen [84, 86]	Gln3	Fibronectin, and fibrin	Stabilization of extracellular matrix
Von Willebrand Factor [87, 88]	Gln3	Fibrin, collagen	Platelet adhesion to the clot
Vitronectin [89, 90]	Gln93	Fibrin, collagen	Platelet adhesion to the clot
Factor V [91, 92]	-	-	Increased thrombin generation at the colt surface
Thrombospondin [93]	-	Fibrin	Platelet adhesion to the clot
Actin [94, 95]	-	Fibrin	Clot reaction, stabilization of the platelet cytoskeleton
TAFI [96]	Gln2, Gln5, Gln292	Itself, fibrin	Resistance to fibrinolysis
PAI-2 [97, 98]	-	Lys148, Lys230, Lys413 of fibrin α-chain	Resistance to fibrinolysis

FXIII has important physiological roles in hemostasis, angiogenesis, wound healing, and pregnancy [99, 100]. Plasma FXIII, also known as fibrin stabilizing factor, is essential for preserving hemostasis by performing the following functions: Increasing clot strength by cross-linking fibrin; inhibiting fibrinolysis by cross-linking α_2-antiplasmin to the α-chain of fibrin; inhibiting fibrinolysis by interfering with the adsorption of plasminogen into fibrin surface. FXIII is critical for creating a rigid, strong, and elastic clot needed to stop bleeding; therefore, FXIII deficiency results in severe bleeding [101-104]. FXIII also plays a central role in the events that lead to wound healing such as fibroblast proliferation, attachment of cells to substrate, and in collagen formation [105]. Studies showed that FXIII-deficient individuals have lower fibroblast growth rate than patients with normal FXIII [105, 106]. FXIII activates a series of biochemical reactions that assist in cell adhesion and without FXIII cells lose their regular shape due to cell contact inhibition. In addition, collagen is not produced in the absence of FXIII which delays wound healing [107].

Recombinant FXIIIA (rFXIII), which has similar structure and function as FXIII-A_2, has been expressed in different cell types that include Escherichia coli [108], Sacharomyces cerevisiae [109, 110], and Schizosacharomyces pombe [111]. The numerous rFXIII crystal structures provided a wealth of information on catalytic activity as well as enzyme activation. Moreover, this structural information has been used to explain the consequences of some FXIII mutations [112].

Alpha2-antiplasmin

Human alpha$_2$-antiplasmin is the primary physiological inhibitor of the blood fibrinolytic plasmin. α_2-AP is secreted by the liver as a single-chain glycoprotein of 464 amino acid residues and molecular weight of ~70 kDa with methionine as the N-terminus (Met- α_2AP) [113, 114]. The plasma concentration of α_2-AP is ~70 µg/ml with a half-life of 2.6 days [115-117]. During circulation, Met-α_2AP undergoes proteolytic cleavage between Pro12-Asn13 by circulating anti-plasmin cleaving enzyme (APCE), a proline-specific serine protease [118]. This results in the formation of a 452 amino acid derivative with N-terminal asparagine (Asn-α_2AP). Asn-α_2AP has a molecular weight of ~64 KDa and accounts for 70% of the α_2-AP in plasma while Met- α_2AP accounts for the remaining 30% [113, 114]. Some properties of Met- α_2AP and Asn- α_2AP are listed in table 5. The human α_2-AP gene is located on chromosome 17, specifically 17pter-p12 and 17p13, and contains 10 exons and 9 introns [119]. The complete primary structure of human α_2-AP has been deduced from the sequence of its cDNA.

Human α_2-antiplasmin belongs to the serine protease inhibitor (serpin) family but unlike other serpins, α_2-AP has an extensive C-terminal sequence of approximately 50 amino acids that contains a number of lysine residues that are important for plasmin inhibition and plasmin (ogen) binding [120]. In addition, the C-terminal region of α_2-AP contains an RGD tri-peptide sequence (Arg426-Gly-Asp) that is essential for cell recognition and adhesion [121]. Using the RGD sequence, the C-terminal region of α_2-AP binds to endothelial cell integrins. With the aid of X-ray structure, the C-terminal of

α_2-AP is shown to be positioned in the proximity of the reactive loop and binds strongly to the K1 and K4 domains of plasmin increasing the inhibitory effect of α_2-AP [122-124].

Table 5: Properties of α2-antiplasmin

	Met-α_2AP	Asn-α_2AP
Molecular weight [113, 114]	67 KDa	64 KDa
Amino acids [113, 114]	464	452
N-terminal	Methionine	Asparagine
Percentage [113, 114]	30%	70%
Binding to fibrinogen [118]	Slower	3-13 times faster

Human α_2-AP regulates the fibrinolysis process in three ways [125]:

I. Inactivates plasmin by forming a complex with it [126].
II. Prevents plasminogen adsorption to fibrin surface [127, 128].
III. Increases fibrin resistant to fibrinolysis by cross-linking fibrin with the aid of FXIII [129-131].

In plasma, α_2-AP inactivates plasmin rapidly with a second order reaction rate of $2x10^{-7}$ mol^{-1} s^{-1} [132]. The reaction of Lys448 and Lys464 residues in the carboxyl-terminal of α_2-AP with 1-3 kringles of plasmin (ogen) changes the conformation of the Ser741 of plasmin which then binds to Arg376 of α_2-AP to form the inactive irreversible plasmin-α_2AP complex [133, 134]. Moreover, Lys groups in both α_2-AP and fibrin bind to the same active sites in plasminogen thereby α_2-AP competitively inhibits the binding of plasminogen to fibrin. Free plasmin generated by tissue-type plasminogen activator in plasma has a short half-life (~0.1 sec) due to the rapid inactivation by α_2-AP, while plasmin produced in fibrin surface has a relatively longer half-life (~10 to 100 sec)

because it is only slightly inactivated by α_2-AP [135]. This indicates the importance of prior incorporation of α_2-AP into fibrinogen for effective inhibition of fibrinolysis.

The cross-linking of α_2-AP to fibrinogen by FXIII is the most effective way to inhibit endogenous fibrinolysis concurrent with fibrin formation and caused by activation of plasminogen-bound to fibrin [130, 136]. The cross-linking reaction occurs between either Gln14 of Met-α_2AP or Gln2 of Asn-α_2AP and Lys303 of α-chain of fibrin. FXIII catalyzes transfer of acyl groups donated by α_2-AP to the acceptor amine groups of fibrinogen. The cross-linking reaction of α_2AP with fibrinogen progress rapidly to reaches a maximum plateaus with only 20-30% of the available Lys303 active sites being occupied [137-139]. This limited reactivity suggests that the reaction equilibrium favors dissociation of the cross-linked product or Lys303 active sites are unavailable for cross-linking reaction [140]. Asn-α_2AP cross-links fibrinogen 3-13 times faster than Met-α_2AP. Consequently, the ratio of the two forms of α_2-AP plays a critical role in the regulation of fibrinolytic process [118].

While the molecular mechanism of plasmin inhibition in solution is well understood, the inhibition of plasmin on the fibrin surface still requires further studies. In addition to plasmin inactivation, α_2-AP also inhibits tissue-type plasminogen activator (TPA) and urokinase. The physiological importance of α_2-AP is best characterized by issues encountered by individuals deficient in this inhibitor. These patients experience uncontrolled fibrinolysis and recurrent severe hemorrhagic disorder [141, 142].

Previous studies have shown that the content of α_2-AP in plasma-derived fibrinogen is substantial (1.2-1.8 moles α_2-AP per mole pdFI), and that native FXIII catalyze its cross-linking in circulation [143].

Fibronectin

Fibronectin is the second most abundant protein within the extracellular matrix (ECM) mediating its interactions with variety of cells. Fibronectin is multifunctional glycoprotein exists in a soluble form in plasma, and in an insoluble form in the ECM. Plasma fibronectin (pdFN) is synthesized by hepatocytes and secreted into plasma where it circulates at a concentration of approximately 300-400 µg/ml [144]. Cellular fibronectin (cFN) is composed of covalently cross-linked multimers and synthesized by different cell types including fibroblasts, endothelial cells, chondrocytes, synovial cells, and myocytes [145].

Fibronectin is composed of two very similar polypeptides with a molecular weight of 220-250 kDa [146-148]. The polypeptides are linked by inter-chain disulfide bonds located close to the C-terminal region forming a dimer molecule [149, 150]. Using rotary shadowing electron microscopy the polypeptides appear as flexible extended V-shaped molecules or compact globular molecules [151]. Each polypeptide is made of multiple homologous modules called type I, II, and III (F1, F2, and F3) in series [152-154]. There are 12 type I modules, two type II modules, and 15-17 type III modules, which together accounts for ~90% of the fibronectin sequence. Type I modules contain ~45 amino acids held together by two disulfide bonds and located in the amino and

carboxyl termini regions of each subunit. Type II modules are composed of ~60 amino acids linked by two disulfide bonds and localized in the gelatin binding domain of the subunit. Type III modules consists of ~90 amino acids that are clustered together in the middle of the subunit and does not contain disulfide bonds [155]. The N-terminal region of fibronectin consist of five type I modules whereas the C-terminal contains three type I modules.

Fibronectin contains binding sites for different molecules that are localized along the length of the monomer enabling fibronectin to play an important role in many physiological processes [156]. Because it's rich in Arg-Gly-Asp tri-peptide sequences, fibronectin plays important roles in cell migration, growth, and differentiation [157]. Fibronectin incorporated into fibrin by FXIIII mediates interaction of fibrin with cells or platelets during clot formation [158-160]. Bound fibronectin forms a three-dimensional matrix at the wound site that attracts different cell types and extracellular matrix proteins [161]. F1 (1-5) contains binding sites for heparin, fibrin, and cell surface proteins for pathogenic bacteria such as streptococcus dysgalactiae that play a putative mechanism for host by microorganism [162].

PURIFICATION OF FIBRINOGEN FROM HUMAN PLASMA

We are employing a combination of physical and chemical precipitation methods to purify relatively large amount of fibrinogen from human plasma for the purpose of fibrin based bandages and expanding hemostatic foams. Virally inactivated fibrinogen was isolated from human plasma using cryoprecipitation followed by ethanol or ammonium sulfate precipitation. Fig 2 shows the major steps of the fibrinogen production procedure. The aim of this study was to evaluate the ability of ethanol and ammonium sulfate precipitation approaches to eliminate solvent detergent. We also estimated the amount of fibronectin, factor XIIIA, factor XIIIB, and α_2-antiplasmin in plasma fibrinogen purified by the two different chemical precipitation methods.

Materials and Methods

Materials

All reagents of highest purity were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise noted. Human plasma was donated by the U.S. Army Materials Command (Fort Detrick, MD). Human plasma fibrinogen depleted of fibronectin, plasminogen, and von Willebrand Factor was purchased from Enzyme Research Laboratories (South Bend, IN). Recombinant human thrombin (rFIIa) was purchased from ZymoGenetics, Inc. (Seattle, WA). Human FXIII was bought from Enzyme Research Laboratories. Human α_2-AP was obtained from Haematologic Technologies Inc. (Essex Junction, VT). Anti-mouse fibronectin monoclonal IgG_1 antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-human FXIIIB monoclonal antibody was purchased from Green Mountain Antibodies (Burlington, VT). Anti-human FXIIIA polyclonal antibody was bought from US Biologicals. Anti-human α_2AP polyclonal antibody HRP was bought from US Biologicals. Anti-mouse IgG (whole molecule) peroxidase conjugate and anti-sheep IgG peroxidase conjugate were obtained from Sigma Chemical Company. Activated recombinant human factor XIII was purified in our lab. Thromboelastography (TEG) disposable cups and pins were bought from Haemoscope (Niles, IL).

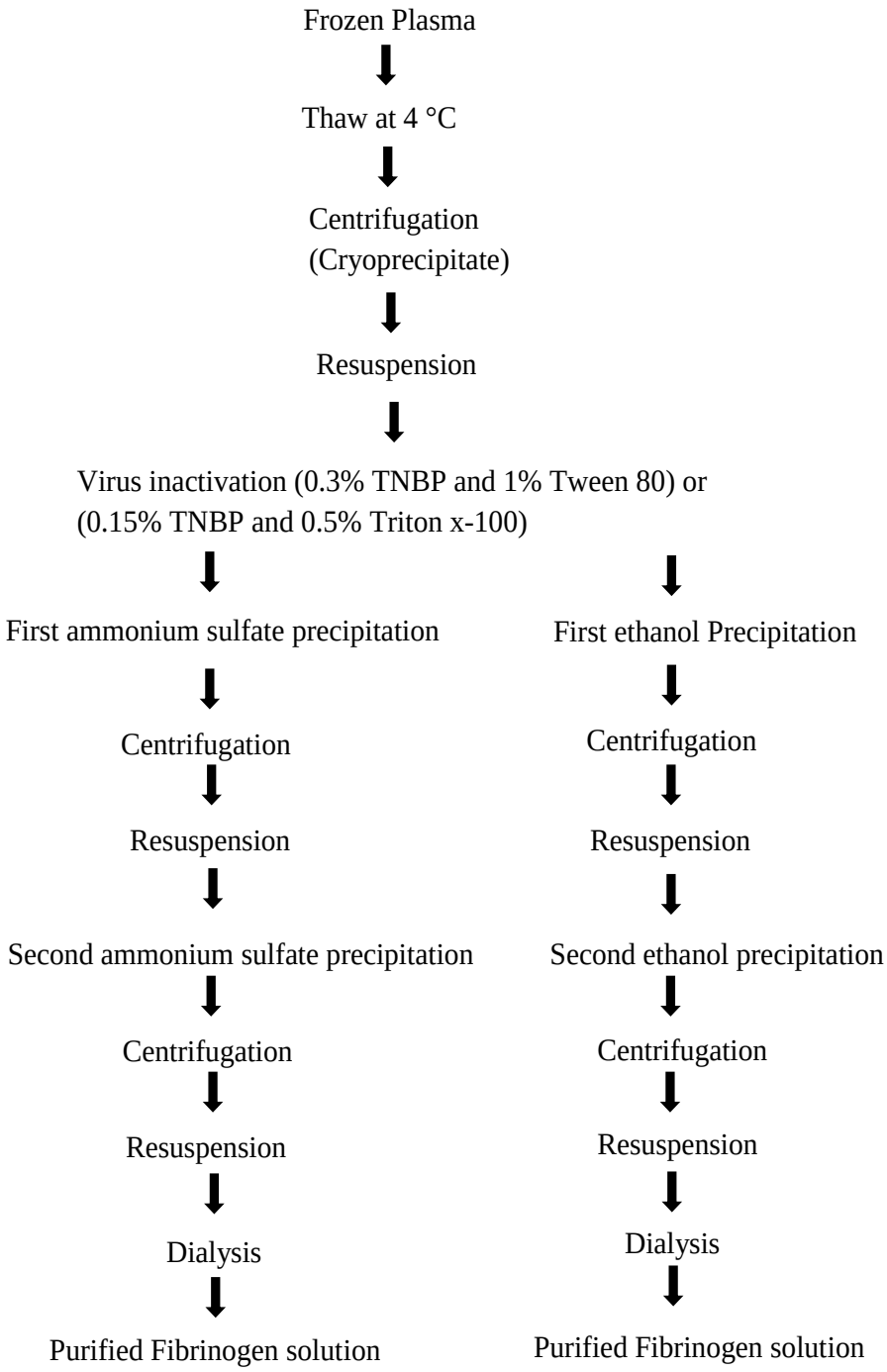


Figure 2: Fibrinogen purification procedure

Preparation of Cryoprecipitate

Fifteen units of human plasma (12.65 liters) frozen at -80 °C were thawed slowly for two days at 4 °C. The resulting mixture was centrifuged in a Sorvall RC-5C Plus Superspeed Centrifuge (Kendro Laboratory, Newtown, CT) at 4000 rpm for 20 min at 4 °C. The supernatant was stored at -80 °C for future fibronectin purification. The fibrinogen-rich cryoprecipitate was re-suspended in a re-suspension buffer containing 20 mM tris-base, 55 mM sodium citrate, 27 mM lysine, PH 6.8. The solution was stirred overnight at room temp. Viral inactivation was carried out using two different types of solvent detergent. Part of the re-suspended cryoprecipitate was adjusted to 0.3% (V/V) TNBP and 1% (V/V) Tween 80 while the other portion was subjected to 0.15% (V/V) TNBP and 0.5% (V/V) Triton X-100 and the two solutions were stirred overnight at room temp.

Ethanol Precipitation

Ethanol precipitation method was conducted by slightly modifying a previously described method [52]. Briefly, the two types of solvent detergent treated solutions were cooled to ~4 °C on ice then adjusted to 10% ethanol. Samples were incubated on ice overnight then centrifuged in a Sorvall RC-5C Plus Superspeed Centrifuge at 4000 rpm at 4 °C for 20 min. The supernatant was discarded and the pellet was solubilized in re-suspension buffer. A second 10% ethanol precipitation step was carried out at ~4 °C for two hours on ice. The resulting pellet was solubilized in re-suspension buffer then dialyzed versus dialysis buffer containing 20 mM sodium citrate, 100 mM sodium

chloride, PH 7.4 at room temp. The dialyzed protein was collected and centrifuged for 20 min at 4000 rpm at 25 °C. Purified fibrinogen was stored in aliquots at - 80 °C.

Ammonium Sulfate Precipitation

The virally-inactivated cryo solutions were chilled to ~4 °C on ice then adjusted to 1 M ammonium sulfate by adding a 4 M stock solution. Samples were incubated on ice overnight then centrifuged at 4000 rpm at 4 °C for 20 min. The precipitate was re-suspended in re-suspension buffer at room temp then chilled to ~4 °C on ice. A second ammonium sulfate precipitation was done and the pellet was solubilized in re-suspension buffer then dialyzed against dialysis buffer. The isolated protein was centrifuged for 20 min at 4000 rpm at 25 °C then frozen at -80 °C.

BCA Standard and Spectrophotometry

Total protein concentration was determined by spectrophotometric measurement at 280 nm as well as BCA Standard procedure using bovine serum albumin as standard.

Evaluating Purified pdFI by SDS-PAGE

The purity of the isolated fibrinogen was examined by sodium dodecylsulfate-polyacrylamide (SDS-PAGE) gel electrophoresis. Samples were analyzed on 4-12% NuPAGE Bis-Tris Mini Gels (Life Technologies, Carlsbad, CA) under reducing condition. Samples were treated with SDS sample buffer and reducing agent, both obtained from Invitrogen (Carlsbad, CA). After 10 minutes incubation at 74 °C, samples

were loaded onto the gel and run using 2-(N-morpholino) ethanesulfonic acid (MES) for one hour at 200 volts. Gels were stained with colloidal blue (Invitrogen, Carlsbad, CA).

Evaluating Clottability of pdFI by Thromboelastography

Thromboelastography (TEG) Hemostasis system 5000 series (Haemoscope Corporation, Niles, IL) was used to evaluate the ability of purified fibrinogen to form a clot. pdFI (concentrations of 9 or 4.5 mg/ml) were placed in a TEG cup, which is kept at 37^{o}C by the instrument. rFXIIIa (concentration of 0.36 mg/ml) was added. $CaCl_2$ (concentration of 12 mM) and Ringer solution (155 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, and 1 mM $MgCl_2$) were added followed immediately by rFIIa (concentration of 105.6 U/ml) to trigger clot formation. The TEG analyzer collected clot parameters such as the time to reach 2 mm clot strength (R), the time to reach 20 mm clot strength (K), and the maximum clot strength (MA) every five seconds for 30 minutes. The data was collected and analyzed in Microsoft Excel.

Estimating pdFXIIIA, pdFXIIIB, α_2-AP, and pdFN by Western Blot

The contents of pdFXIIIA, pdFXIIIB, α_2-AP, and pdFN on the isolated fibrinogen were estimated by quantitative western blot analysis. Samples were analyzed on 4-12% NuPAGE Bis-Tris Mini Gels (Life Technologies, Carlsbad, CA) under reducing or non-reducing condition. Gels were electroblotted onto polyvinylide fluoride (PVDF) membranes (Millipore, Billerica, MA) for 30 min at 25 volts. Blots were blocked in 50 ml 5% casein in TBST then incubated for 30 minutes with the following antibodies: (a) anti-human FXIIIA polyclonal antibody (US Biologicals, Swampscott, MA), (b) anti-

human FXIIIB monoclonal antibody (Green Mountain Antibodies, Burlington, VT), (c) anti-human α_2AP polyclonal antibody HRP (US Biologicals), (d) anti-mouse fibronectin monoclonal IgG_1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Blot developed with anti-human FXIIIA polyclonal antibody was subsequently detected with anti-sheep IgG peroxidase conjugate (Sigma). Blots developed with anti-human FXIIIB monoclonal antibody and anti-mouse fibronectin monoclonal IgG_1 antibody were probed with anti-mouse IgG peroxidase conjugate (Sigma). Blots were visualized with DAB/Metal concentrate and stable peroxide substrate buffer (Thermo Scientific, Rockford, IL) or chemiluminescence using Immun-Star HRP enhancer and peroxide buffer (Bio-Rad Laboratories, Hercules, CA). The quantity of pdFXIIIA, pdFXIIIB, pdFN, and α_2-AP were determined from the immunoblot of each material using Imagej (National Institute of Health) or Adobe Photoshop Elements (Adobe Systems Incorporated).

Results

Evaluating Purified pdFI by SDS-PAGE

SDS-PAGE analysis [Fig 3] conducted under reducing condition showed that pdFI purified by cryoprecipitation (lane 5), first ethanol precipitation (lane 6), second ethanol precipitation (lane 7), first ammonium sulfate precipitation (lane 8), and second ammonium sulfate precipitation (lane 9) all exhibit three distinct bands with apparent molecular weights of ~67, 54, 47 kDa likely corresponding to the well-established α, β, and γ chains of fibrinogen. Lanes 5, 6, 7, 8, and 9 also revealed a minor band at higher molecular weight of ~220 kDa characteristic of fibronectin monomer. pdFI isolated by

ethanol and ammonium sulfate precipitation contain two faint bands at ~80 and 120 kDa. In addition to the bands at ~80 and 120 kDa, pdFI purified by cryoprecipitation possesses additional faint bands.

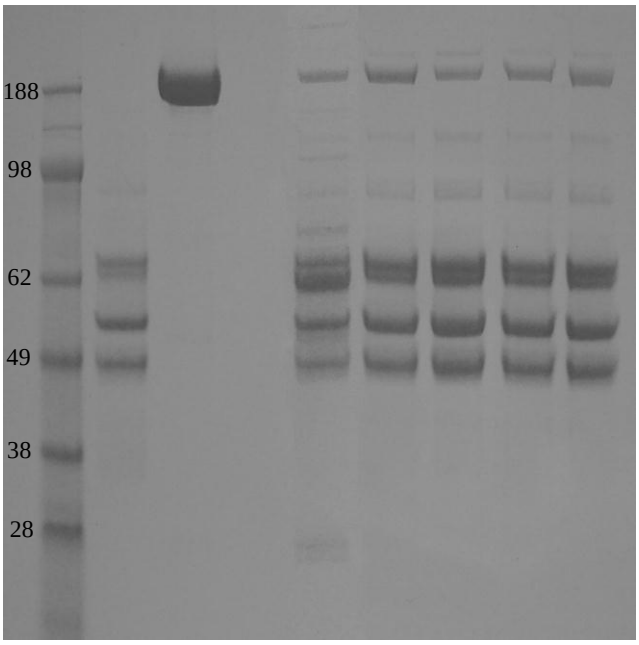


Figure 3: Reduced gel evaluation of purified pdFI. Samples were analyzed on 4-12% SDS-PAGE and stained with colloidal blue. Lane 1 is molecular weight marker. Lane 2 is human fibrinogen (Enzyme Research). Lane 3 is human fibronectin (Enzyme Research). Lane 4 is blank. Lane 5 is pdFI purified by cryoprecipitation. Lanes 6 and 7 are pdFI purified by first and second AS precipitation, respectively. Lanes 8 and 9 are pdFI purified by first and second EtOH precipitation, respectively.

Evaluating Clottability of pdFI by Thromboelastography

The viscoelastic and mechanical properties of clot formed by plasma fibrinogen purified by cryoprecipitation, ethanol, and ammonium sulfate precipitation were evaluated by thromboelastography [Fig 4]. It took 10 seconds to initiate clot formation after rFIIa was added to pdFI isolated by cryoprecipitation, ethanol, and ammonium sulfate precipitation [Table 6]. The maximal strength of clot developed by pdFI purified by the first ethanol precipitation (1x) was 3267.70 ± 9.05 dynes/sec compared to 3278.9 ± 829.58 dynes/sec for clot formed by pdFI purified by the second ethanol precipitation (2x). pdFI isolated by the second ammonium sulfate precipitation (2x) resulted in clot with maximal strength of 5811.7 ± 139.44 dynes/sec whereas pdFI purified by the first ammonium sulfate precipitation (2x) formed clot with maximal strength of 2483.75 ± 167.09 dynes/sec. Clot developed from pdFI purified by the second ammonium sulfate precipitated fibrinogen has the highest maximal strength (5811.7 ± 139.44). Clot formation was inhibited in the solvent detergent (TNBP and Triton X-100) treated pdFI purified by a single ammonium sulfate precipitation (Fig 5).

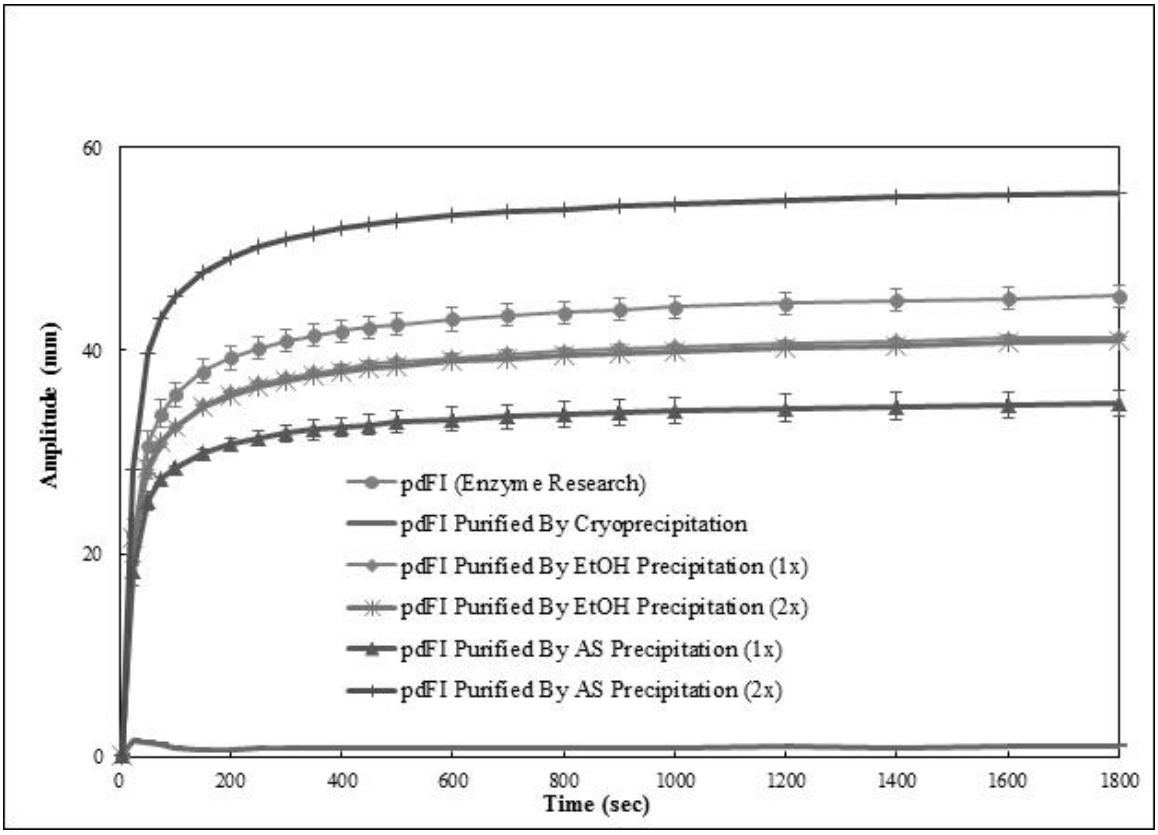


Figure 4: Thromboelastography analysis of the clottability of isolated fibrinogen. Change in maximal strength of clot developed by pdFI (4.5 mg/ml) activated by rFIIa (105.6 U/ml) was recorded over time.

Table 6: Parameters of clot formed by purified fibrinogen

Protein	R (sec)	K (sec)	Angle (deg)	MA (mm)	G (d/sc)
pdFI (Enzyme Research)	10	50	84.2	43.3	3822.53
pdFI Purified By EtOH Precipitation (1x)	10	50	83.7	39.6	3267.70
pdFI Purified By AS Precipitation (1x)	10	50	82.9	33.2	2483.75
pdFI Purified By EtOH Precipitation (2x)	10	50	83.0	39.3	3278.9
pdFI Purified By AS Precipitation (2x)	10	50	85.5	53.8	5811.7
pdFI Purified By Cryoprecipitation	-2825	-225	-26.5	-2.2	-110.6

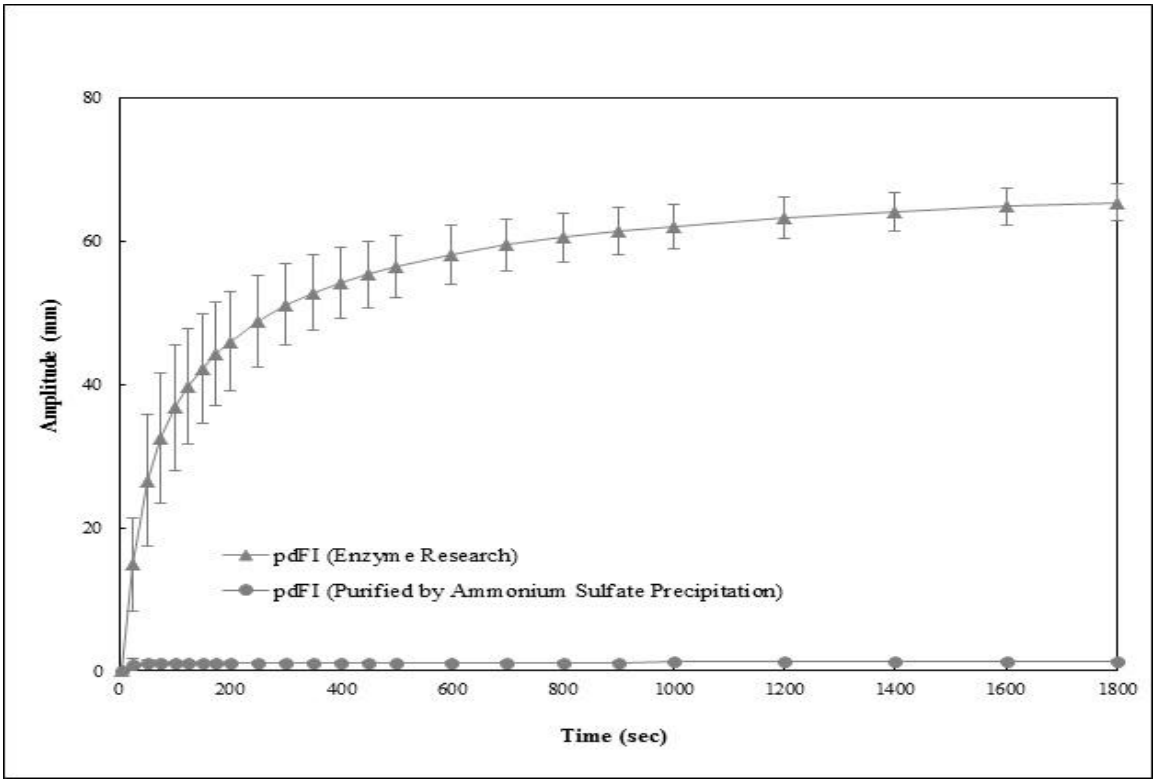


Figure 5: Thromboelastography analysis of the clottability of fibrinogen purified by a single ammonium sulfate precipitation. Clot was formed by treating plasma fibrinogen (9 mg/ml) with thrombin (105.6 U/ml).

Estimating Amounts of pdFXIIIA by Densitometry

Anti-human FXIIIA western blot analysis was conducted to estimate the percentage of FXIIIA in pdFI isolated by the three purification techniques [Fig 6]. pdFI purified by cryoprecipitation (lane 6), ammonium sulfate precipitation (lane 7), and ethanol precipitation (lane 8) show bands corresponding to the known molecular weight of pdFXIIIA. Using densitometry by Imagej, the percentages of FXIIIA in pdFI purified by cryoprecipitation, ethanol, and ammonium sulfate precipitation were 0.652%, 0.781%, and 0.683% respectively.

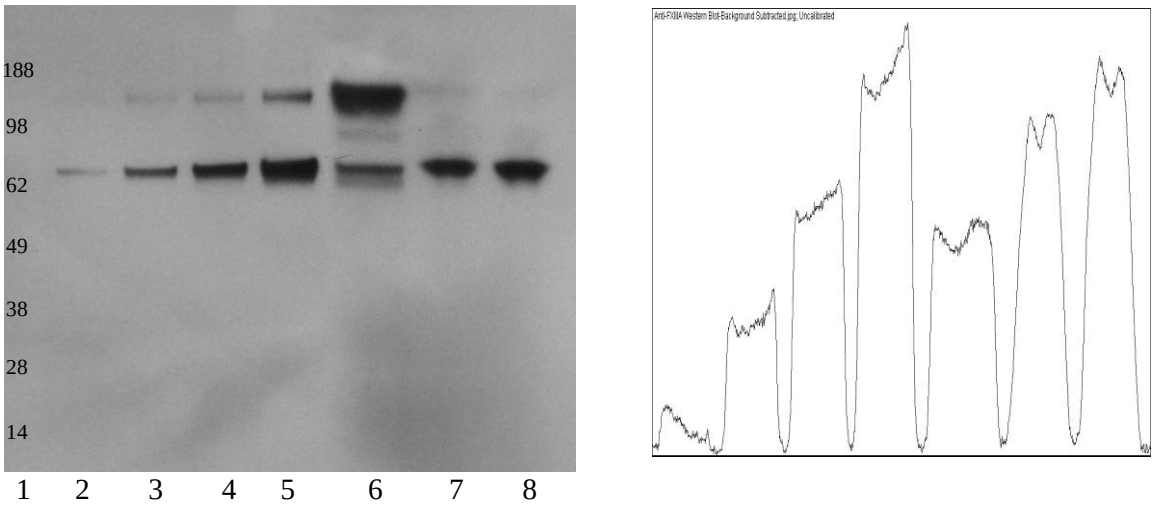
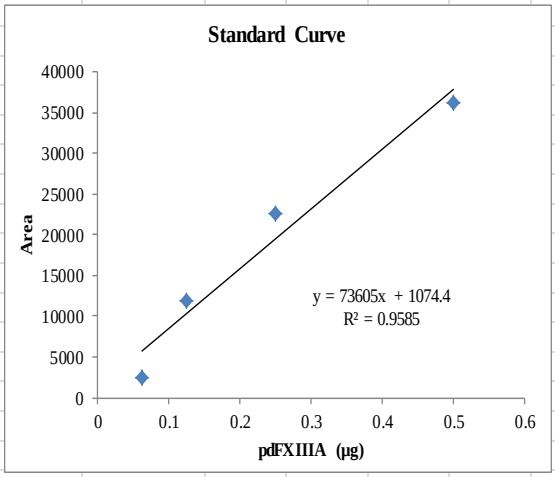


Figure 6: Western blot and densitometry by Imagej analysis of pdFXIIIA. Samples were analyzed under non-reducing condition then developed with anti-FXIIIA Pab and detected with anti-sheep IgG Peroxidase. Lane 1 is molecular weight marker. Lanes 2, 3, 4, and 5 are 0.063, 0.125, 0.25, and 0.5 µg purified pdFXIII respectively. Lane 6 is pdFI purified by cryoprecipitation. Lane 7 is pdFI purified by ammonium sulfate precipitation. Lane 8 is pdFI purified by ethanol precipitation.

	pdFXIIIA (µg)	Area	Std Dev
Standard	0.063	2448.966	25.7
	0.125	11938.687	30.6
	0.25	22682.825	41.5
	0.5	36268.311	32.0
	m =	73605	
	b =	1074.4	



Purification Method	Area	Std Dev	pdFXIIIA (µg)	pdFI Loaded (µg)	pdFXIIIA/pdFI
pdFXIIIA (Cryoprecipitation)	25056.405	35.0	0.32582	50	0.00652
pdFXIIIA (AS Precipitation)	26209.172	25.8	0.34148	50	0.00683
pdFXIIIA (EtOH Precipitation)	29808.28	38.8	0.39038	50	0.00781

Estimating Amounts of pdFXIIIB by Densitometry

The amounts of FXIIIB in pdFI precipitated by the three purification methods were calculated from the anti-human FXIIIB western blot analysis [Fig 7]. pdFI isolated by cryoprecipitation (lane 7), ammonium sulfate precipitation (lane 8), and ethanol precipitation (lane 9) show a band characteristic of the known molecular weight of pdFXIIIB. Densitometric measurement of the bands revealed that cryoprecipitation, ammonium sulfate, and ethanol precipitation pdFI contain 0.329%, 0.809%, and 0.481% respectively.

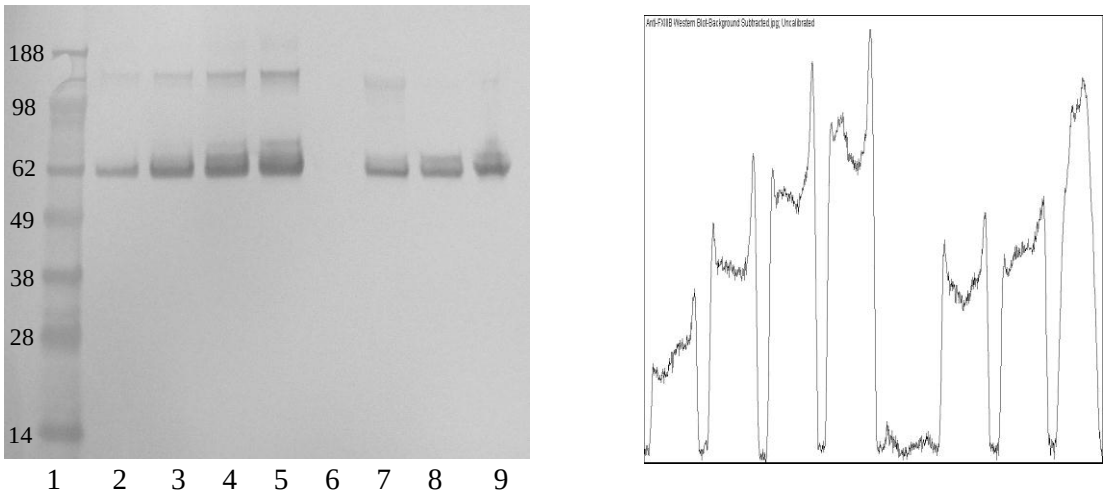
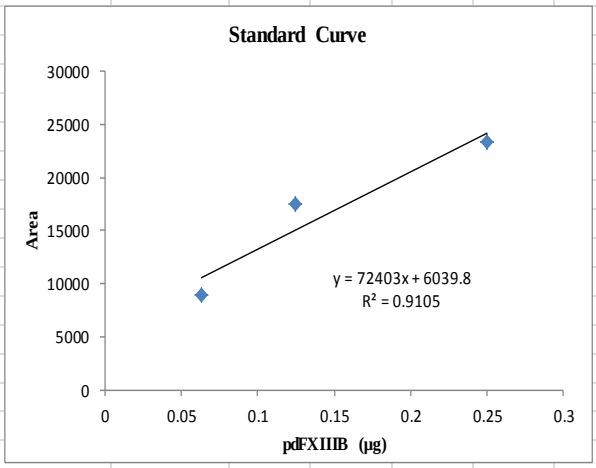


Figure 7: Western blot and densitometry by Imagej analysis of pdFXIIIB. Samples were analyzed under non-reducing condition and developed with anti-FXIIIB Mab then detected with anti-mouse IgG peroxidase. Lane 1 is molecular weight marker. Lanes 2, 3, 4, and 5 are 0.063, 0.125, 0.25, and 0.5 µg purified pdFXIII respectively. Lane 6 is blank. Lane 7 is pdFI purified by cryoprecipitation. Lane 8 is pdFI purified by ammonium sulfate precipitation. Lane 9 is pdFI purified by ethanol precipitation.

	pdFXIIIB (µg)	Area	Std Dev
Standard	0.063	8963.33	12.5
	0.125	17540.29	2.1
	0.25	23328.08	3.3
	m =	72403	
	b =	6039.8	



Purification Method	Area	Std Dev	pdFXIIIB (µg)	pdFI Loaded (µg)	pdFXIIIB/pdFI
pdFN (Cryoprecipitation)	15572.646	38.9	0.13166	40	0.00329
pdFN (AS Precipitation)	17761.428	35.6	0.16189	20	0.00809
pdFN (EtOH Precipitation)	19963.454	61.0	0.19231	40	0.00481

Estimating Amounts of α_2-AP by Densitometry

The content of α_2-AP in the purified pdFI was determined by anti-human α_2AP western blot analysis [Fig 8]. pdFI isolated by cryoprecipitation (lane 6), ammonium sulfate (lane 7), and ethanol (lane 8) precipitation exhibit a minor band at ~62 kDa representing the molecular weight of α_2-AP. By employing densitometry, pdFI purified by cryoprecipitation, ammonium sulfate, and ethanol precipitation contain 0.184%, 0.493%, and 0.254% α_2-AP.

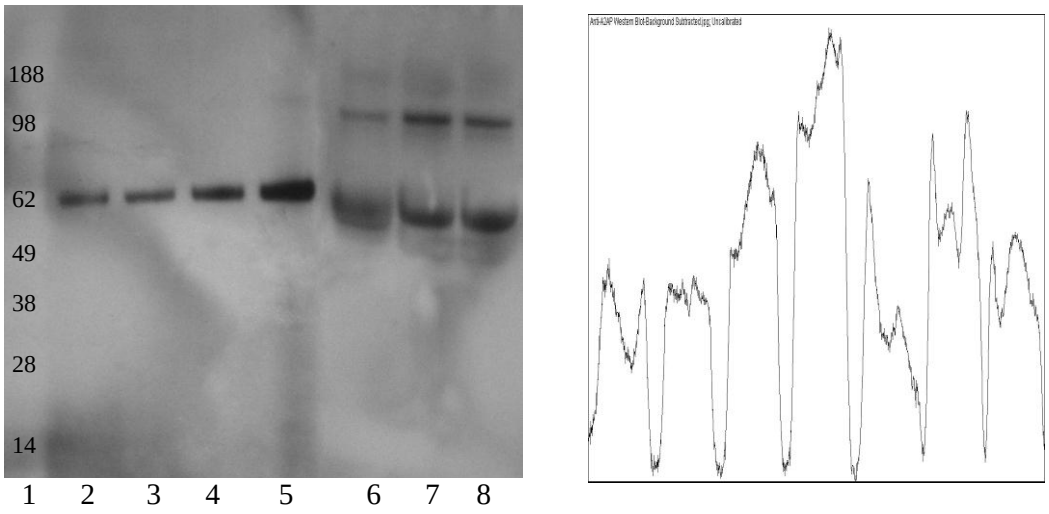
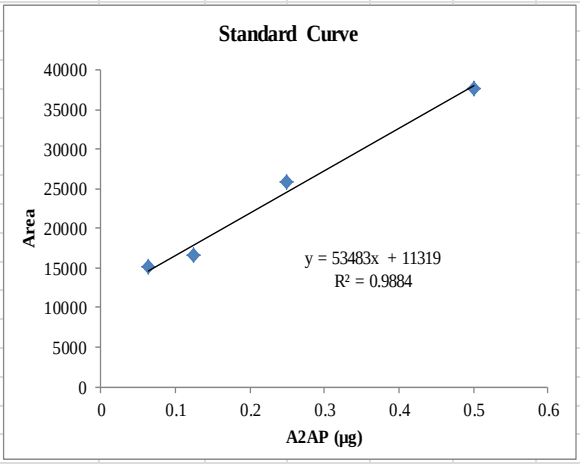


Figure 8: Western blot and densitometry by Imagej analysis of α2-AP. Samples were analyzed under reducing condition and developed with anti- α_2AP Pab HRP. Lane 1 is molecular weight marker. Lanes 2, 3, 4, and 5 are 0.013, 0.025, 0.05, and 0.1 µg purified α_2-AP respectively. Lane 6 is pdFI purified by cryoprecipitation. Lane 7 is pdFI purified by ammonium sulfate precipitation. Lane 8 is pdFI purified by ethanol precipitation.

	A2AP (µg)	Area	Std Dev
Standard	0.063	15139.23	36.8
	0.125	16637.79	66.0
	0.25	25949.74	8.0
	0.5	37714.69	2.4
	m =	53483	
	b =	11319	



Purification Method	Area	Std Dev	A2AP (µg)	pdFI Loaded (µg)	A2AP/pdFI
A2AP (Cryoprecipitation)	17225.438	69.9	0.11044	60	0.00184
A2AP (AS Precipitation)	27128.196	56.1	0.29559	60	0.00493
A2AP (EtOH Precipitation)	19485.310	17.6	0.15269	60	0.00254

Estimating Amounts of pdFN by Densitometry

Anti-human FN western blot analysis was done to estimate the quantity of FN purified with pdFI [Fig 9]. pdFI purified by cryoprecipitation (lane 7), ammonium sulfate (lane 8), and ethanol precipitation (lane 9) show bands at ~220 kDa corresponding to fibronectin monomer. Densitometry by Adobe Photoshop Elements was employed to calculate the percentage of FN in pdFI isolated by cryoprecipitation (3.18%), ammonium sulfate precipitation (8.09%), and ethanol precipitation (3.98%).

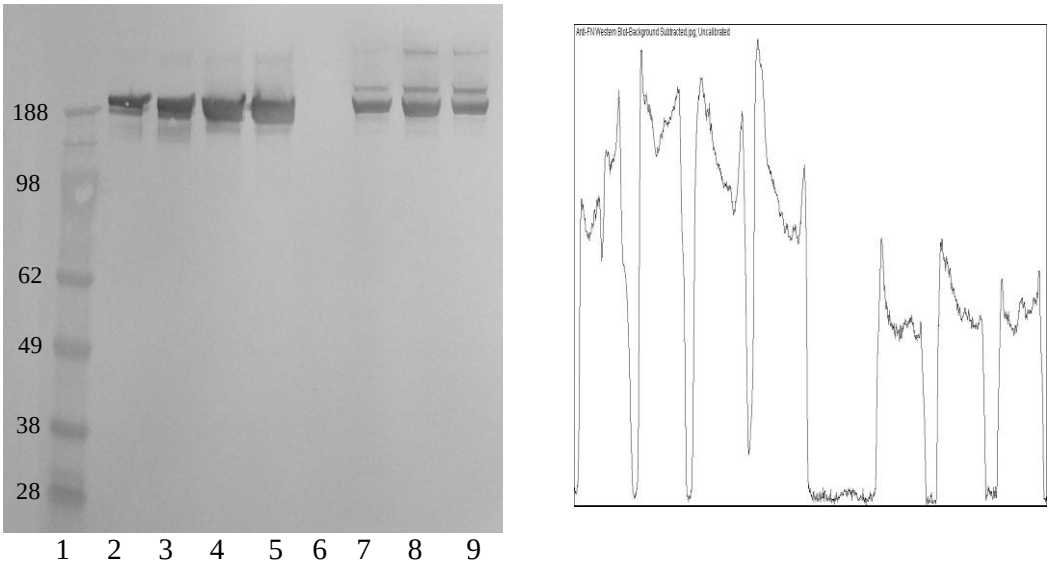
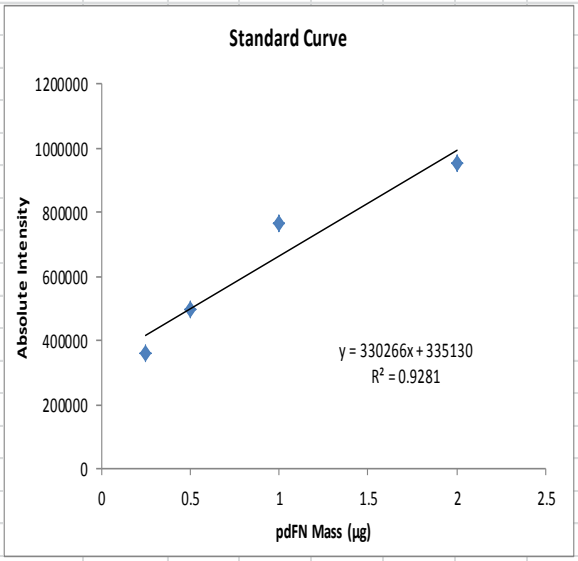


Figure 9: Western blot and densitometry by Imagej analysis of pdFN. Samples were analyzed under reducing condition and developed with anti-mouse fibronectin monoclonal IgG_1 then detected with anti-mouse IgG peroxidase. Lane 1 is molecular weight marker. Lanes 2, 3, 4, and 5 are 0.25, 0.5, 1, and 2 µg purified pdFN respectively. Lane 6 is blank. Lane 7 is pdFI purified by cryoprecipitation. Lane 8 is pdFI purified by ammonium sulfate precipitation. Lane 9 is pdFI purified by ethanol precipitation.

	pdFN (µg)	Mean Intensity	Std Dev	# Pixels	Absolute Intensity
Standard	0.25	175.57	15.2	2057	361147.5
	0.5	169.77	10.0	2940	499123.8
	1	157.4	9.6	4867	766065.8
	2	150.55	12.3	6328	952680.4

m = 330266

b = 335130



Purification Method	Mean Intensity	Std Dev	# Pixels	Absolute Intensity	pdFN (µg)	pdFI Loaded (µg)	pdFN/pdFI
pdFN(Cryoprecipitation)	152.79	10.6	2606	398170.74	0.19088	6	0.03181
pdFN(AS Precipitation)	150.75	8.4	3286	495364.5	0.48517	6	0.08086
pdFN(EtOH Precipitation)	139.39	13	2970	413988.3	0.23877	6	0.03980

DISCUSSION

Various approaches have been developed to isolate fibrinogen from plasma sources. These methods include cryoprecipitation, centrifugation, ultrafiltration, chromatography, and chemical precipitation. We isolated a solvent detergent treated fibrinogen from human plasma using cryoprecipitation followed by ammonium sulfate or ethanol precipitation. Fibrinogen isolated by the three purification methods is highly pure. To determine the effectiveness of purified plasma fibrinogen to form clots, we measured clottability of fibrinogen using thromboelastography. Our study showed that clot formed from plasma fibrinogen produced by the repeated ammonium sulfate precipitation has the highest maximal strength.

Based on the purification method, fibrinogen removed from plasma contains various amounts of fibronectin, factor XIII, alpha$_2$-antiplasmin, plasminogen, and other plasma proteins. We estimated the quantities of fibronectin, factor FXIIIA, factor FXIIIB, and α_2-AP in the plasma fibrinogen isolated by three purification procedures. Plasma fibrinogen obtained by ammonium sulfate precipitation was found to contain increased ratios of FXIIIA, FXIIIB, FN, and α_2-AP to fibrinogen than the starting cryoprecipitate. Plasma fibrinogen from ethanol precipitation also contains higher ratios of FXIIIA, FXIIIB, α_2-AP, and FN to fibrinogen than the original cryoprecipitate. This indicates that chemical precipitation may preserve the binding of FXIIIA, FXIIIB, FN, and α_2-AP to fibrinogen during the purification process.

Viral inactivation was achieved by using two different types of solvent detergent mixtures: (1) 0.3% TNBP and 1% Tween 80, (2) 0.15% TNBP and 0.5% Triton X-100. Since the presence of solvent detergent inhibits clot formation, we utilized thromboelastography to evaluate the effectiveness of ethanol and ammonium sulfate precipitation to remove the solvent detergent. While purifying a solvent detergent treated fibrinogen from human plasma, Burnouf-Radosevich et al [52] applied a second ethanol precipitations to further remove the solvent detergent. However, we showed that clots formed from fibrinogen isolated by first and second ethanol precipitation have similar maximal strength. This indicates that a single ethanol precipitation step is capable of eliminating the solvent detergent. A single ammonium sulfate precipitation partially removed the mixture 0.3% TNBP and 1% Tween 80 but not the mixture 0.15% TNBP and 0.5% Triton x-100. Thus, a second ammonium sulfate precipitation is required for the complete elimination of the solvent detergent mixture TNBP and Triton X-100.

Together the data presented in this thesis indicate that the combination of cryoprecipitation and successive ammonium sulfate precipitations results in the fibrinogen with the strongest clot forming activity. The bases for the increased strength has not been determined but is likely due to the increased ratios of associated proteins, particularly fibronectin in the ammonium sulfate derived material.

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