CHAPTER I

Introduction

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1.1 Introduction

The diseases of cardiovascular nature like stroke, myocardial infarction, and venous thrombo-embolic disorders likes pulmonary embolism, deep-vein thrombosis, are responsible for mortality rates up to 29% worldwide of the total rates of mortality [5]. One of the significant factors contributing to the etiology of cardiovascular diseases is the imbalance between fibrin formation and fibrinolysis (hemostatic disorder) that results in intravascular thrombus or in the formation of hemostatic plug in the blood vessels (thrombosis) which poses a clinical challenge in its management [2]. As per the WHO report of 2001, due to the diseases of the cardiovascular nature, 17 million individuals die each year and further it is projected of causing over 25 million deaths per year , by the year 2020 [6]. Higher level of fibrinogen (hyperfibrinogemic state) also stimulates the risk of myocardial infraction via the enhancement of blood coagulation in addition to encouraging the thrombotic events [3].

1.2 Physiology of fibrin formation and its implication in progression of cardiovascular diseases

In the liver a ~340 kDa glycoprotein, fibrinogen is produced. 3 chains of α, β, and γ peptides make up this hexamer [7]. Fibrinogen forms a constituent of the protein cascades involved in the coagulation and is a protein of the acute phase. The thrombin production is the end outcome of the cascade where fibrin is produced via the conversion of fibrinogen (Fig. 1.1).

Fibrinogen is proteolyzes rapidly by thrombin, resulting in the release of A and B fibrinopeptides. The forfeiture of this minor peptide is not ample to allow the ensuing fibrin to be insoluble, but it inclines to form complex combinations with the adjacent molecules fibrinogen and fibrin [8]. Thrombin splits the- fibrinopeptide B peptide resulting in the formation of monomers of fibrin from fibrin that freely polymerize, forming a gel which is insoluble in nature [9]. The fibrin that is polymerized is bound by the forces of electrostatic nature and non-covalent and the transamidation factor XIIIa, the enzyme responsible for its stability factor obtained

via the factor XIII action of thrombin [10] The aggregates of fibrin (clots) and the insoluble amassed platelets cause occlusions of the blood vessels.

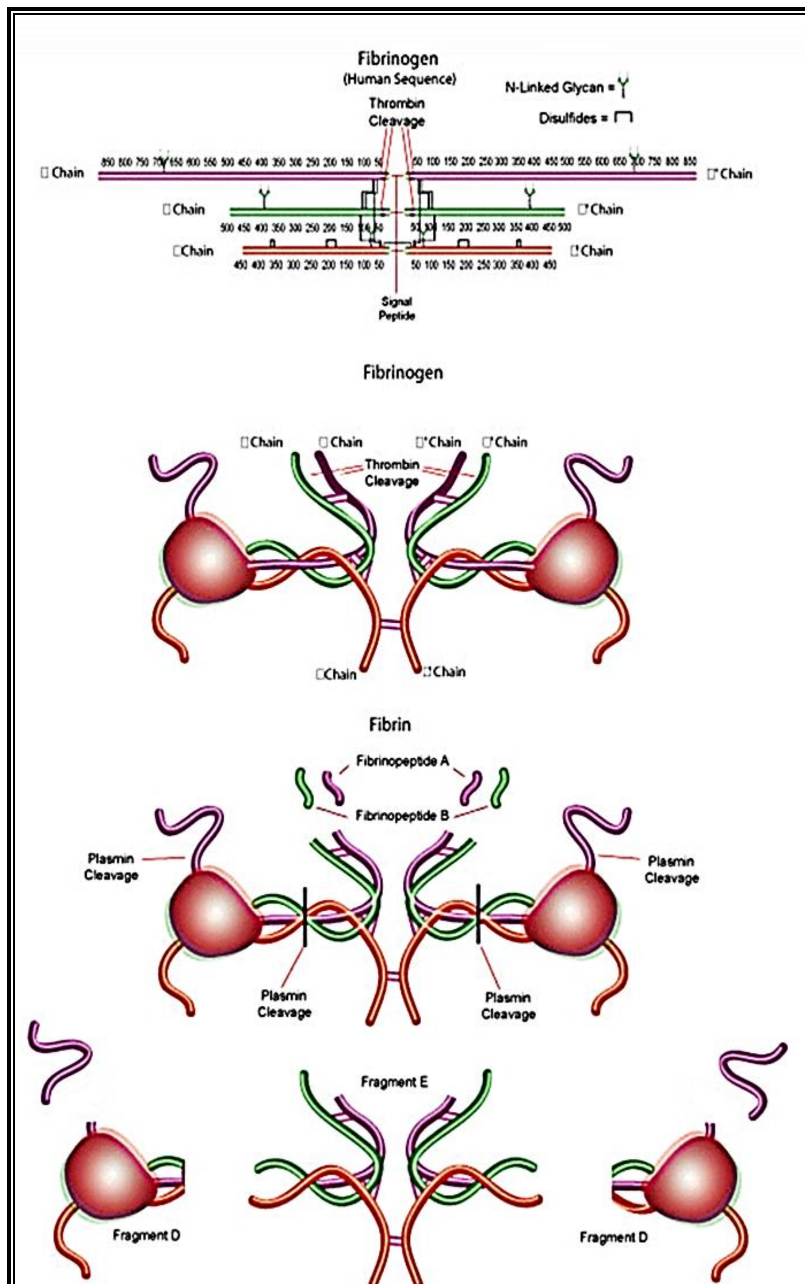

Figure 1.1: Formation of fibrin from fibrinogen

Source: (www.sigmaaldrich.com)

Indeed, a hemostasis imbalance may lead to bleeding excessively or a thrombus forming (an inapt blood clot) that sticks to the blood vessels unbroken walls [11]. Fibrin accumulation in the blood vessels could affect the flow of blood and cause myocardial infarction and other grave cardiovascular illnesses. Only by the removal

of blockages quickly, the tissue will receive oxygen might die or be damaged severely. If the harmed region is enormous in nature, the normal electrical signal conduction via the ventricles would be interrupted, resulting in the heartbeats to be irregular, cardiac arrests, or even mortality [11, 12]. Notably, administration of commercial agents for thrombolysis like the tissue plasminogen activator, streptokinase, and urokinase have been considered as an alternative approach to surgical interventions for removing the blood clots or bypass the heart blockage [13]. However, these cardiovascular drugs have their own limitations because they demonstrate several side effects like gastrointestinal bleeding and allergic reactions. Furthermore, these drugs are expensive and hence out of the reach of common people especially in the developing nations [4]. Commercially available thrombolytic drugs such as streptokinase (SK) and urokinase (UK) have almost displayed an array of demerits in clinical applications like the ones described below:

1.3 Cost effectiveness

In clinical applications, the cost differential of UK and SK (SK costs around INR 3195.5/7500 units/vial: UK, INR 6650/7500 units/vial) are evident during the systematic applications but are comparatively lesser when utilized in low doses by the route selectively [14].

1.4 Side effects

1.4.1 Depletion of fibrinogen level

Despite the equivalent response of SK and UK with equal efficacy, UK has displayed lesser incidences of severe clinical damage than SK in systemic fibrinogenolytic disturbances during their tenure of application as cardiovascular drugs. Although *in vivo* system t-PA resulted in minimal fibrinogenolysis and 10 - 300 times more efficient thrombolysis than UK indicating that the variations seen are mainly due the fibrinogen's effect competitively [14].

1.4.2 Hemorrhagic complications

Hemorrhagic complications are generally encountered due to suppression of fibrinogen levels. It was clinically demonstrated by Califf et al. [15] that large doses systematically of UK or SK normally bring down the levels significantly of the fibrinogen in 24 h, post that the rises could lead to hemorrhages. It is pertinent to

state that frequent hemorrhages were reported by the group at TIMI, after the application of t-PA than SK [14].

1.4.3 Immunological responses

UK clinically has serum inhibitors and antibodies while SK's antibody activity is normally very significant and might avert lysis effectively [14]. Its vulnerability for formation of antibodies shrinks in the backdrop of SK's recurrent application [14]. Furthermore, the counter antibodies of SK may be the reason for the sporadic reactions systemically like fevers, pruritus, anaphylaxis, or malaise, which are typically never reported post UK application [14].

1.4.4 Different mechanism of action

The major drawback of SK is that it always acts via formation of an activator complex with the plasminogen, resulting in the formation of plasmin through indirect mechanisms, whereas UK does not follow the same trend of forming activator complexes with plasminogen [14]. As a result, urokinase injections at 10,000 - 48,000 IU/ml will lyse swiftly the thrombi that are large, while SK is incapable of doing the same at a dose of 2500 IU/ml, suggesting the potential benefits of UK application over SK in fibrinolytic therapy [14]. The bottleneck of current day drugs has emphasized the need for exploiting and developing innovative biochemical that is safe and effective fibrinolytic proteases that function directly. To address these issues, several lines of investigations have been pursued for the discovery of superior, cost effective and safe thrombolytic agents from different natural resources. Studies from different laboratories have shown that fermented food-borne microbes are promising candidates for production of thrombolytic agents [16-18].

Till today, therapeutic enzymes are finding more rampant application in the field of medicine compared to any other treatment methods [6]. Therefore, several lines of extensive investigations are being carried out to augment the fibrinolytic therapy's specificity and efficacy [17]. Fibrinolytic proteases from bacteria for example, Nattokinase and Lumbrokinase are potent thrombolytic agents and are derived from fermentation of foods traditionally [17]. Hence, exploration of the metabolomes of fermented food-borne microorganisms as a potential source of safe, non-toxic and

superior thrombolytic agents is worth investigating. Studies have shown that very few attempts have been made to isolate and characterize the fibrin(ogen)olytic enzymes producing microorganisms from the fermented food samples of North-East India, that are thought to be one of the mega-biodiversity zones of the world. Microbial fibrin(ogen)olytic enzymes have been drawing more attention rapidly in past decade in the field of medicine owing to many advantages that have been listed below. Though they are economical, available from easy to find resources, easy to cultivate, and have a maximum yield which can be attained in a shorter duration of time [19].

1.5 Microbial fibrin(ogen)olytic producing resources

Enzymes that are fibrin(ogen)olytic could be widely found in the nature. Often, microorganism and snake venom are widely accepted as the potential sources of fibrinolytic enzymes [6, 20]. In recent days, fibrinolytic enzymes have been discovered from edible and non-edible food sources. Microorganisms especially bacteria residing in non-food and food bases have played a predominant role in the production of fibrinolytic enzymes since ancient times till date [6, 20]. The fibrin(ogen)olytic enzyme(s) were discovered in several microbes, of which the genus *Bacillus* is the pertinent one; especially the *Bacillus subtilis* [21].

The Asian fermented foods like natto, chungkookjang, doen-jang, jeot-gal, and tempeh represent a rich source of fibrinolytic enzyme producing promising bacterial strains. These rousing findings indicate the potential of consuming foods that have undergone fermentation for the prevention of CVD and also explain why the Asians report the lowest rate of clotting disorders [6]. Suzuki et al. (2003) [22] reported that reduction of ECLT (euglobulin clot lysis time) by the dietary supplementation of natto, one could evaluate the complete activities in plasma of the intrinsic fibrinolytic action [23]. Simultaneously, shortened duration of bleeding by the natto extracts in diet, indicated natto as a safe functional food [6].

1.5.1 Classification of fibrinolytic enzymes

The fibrinolytic enzymes are classified in two different ways, either based on their catalytic mechanism or depending on their mode of action [6]. On the basis of the way it functions, enzymes that are fibrinolytic in nature are divided into two classes.

Enzymes like plasmin responsible for the degradation of fibrin which results in the thrombi to dissolve quickly and wholly; for instance the Nattokinase (NK) which represents the first class type. The other type is the tissue-type plasminogen activators (t-PA) that activate the plasminogen activator (PA) [24], streptokinase (SK) [25], and UK [26], which trigger plasminogen to activate plasmin for the fibrin's degradation .

1.5.1.1 Direct acting fibrinolytic enzymes

Direct acting fibrinolytic enzymes are able to degrade fibrin without activation of plasminogen [27].

1.5.1.1.1 Nattokinase (NK)

NK is a 27.72 kDa single chain of polypeptide, consisting of amino acid residues (275) with a pI 8.6 (Fig.1.6). A significant fibrinolytic action is observed for this enzyme at pH 6 - 12, whilst the activity fibrinolytically decreases with the rise in temperatures above 60 °C. 40 CU (plasmin units)/g wet weight was the derived average activity. NK an enzyme of fibrinolytic nature isolated from bacteria is purified from *B. natto* that induces fibrinolysis by direct as well as indirect mode of action and enhances the fibrinolytic activity [6]. Additionally, the shelf life of the fibrinolytic enzyme in blood was found to be over 3 h, suggesting that NK might be a potent oral thrombolytic candidate from natural sources. Due to limitations of biochemical attributes, they differ in terms of limited functional measurements especially regarding fibrinolytic efficacy [6].

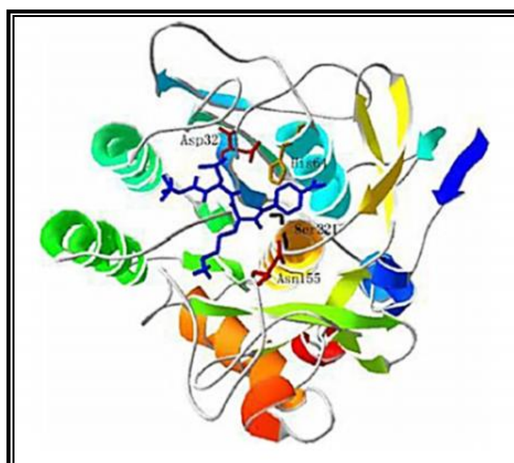

Figure 1.2: Structure of Nattokinase [6]

1.5.1.2 Indirect acting fibrinolytic enzymes

Indirect acting fibrinolytic enzymes are able to degrade fibrin with the activation of plasminogen to plasmin. Presently, most of the agents for thrombolytic action like: prourokinase, urokinase, streptokinase, alteplase (t-PA), reptilase, reteplase (r-PA), anisoylated purified streptokinase activator (APSAC) [28], and brinase complex, represent a class of agents whose fibrinolytic mode of action is indirect. Only in cases of emergency and hospitals, they are normally utilized [28].

1.5.1.2.1 Streptokinase (SK)

SK is derived from beta-hemolytic streptococci, is a single polypeptide chain, associated with multiple structural domains and is responsible for its diverse functional properties (Fig.1.3). However, analysis via calorimetric scanning has indicated SK possessing 2 domains distinctly in its composition. Among them, the domain of the N-terminal was seen to be complementing the PA's domain of protein capacity of the 60–414 amino acid residues [6]. Functionally, it is responsible for the plasminogen's indirect activation via a process of 2-steps, differing from the 1-step way of t-PA and UK [14]. At initial stages, formation of the complex of equimolar of SK with plasmin (or plasminogen), the acylated plasminogen–SK activator complex (APSAC), with the resistance to inactivation by α_2-antiplasmin occurs. Further, this complex is responsible for activating the plasminogen activator and cleaving the peptide bond of the Arg 560 - Val 561. However, only SK can't deliver this as APSAC has an additional half-life which is effective therapeutically in circulation comparative to SK. Owing to the bottlenecks of the present day PAs, efforts are in progress to improve enhanced variants of such recombinant molecules [6].

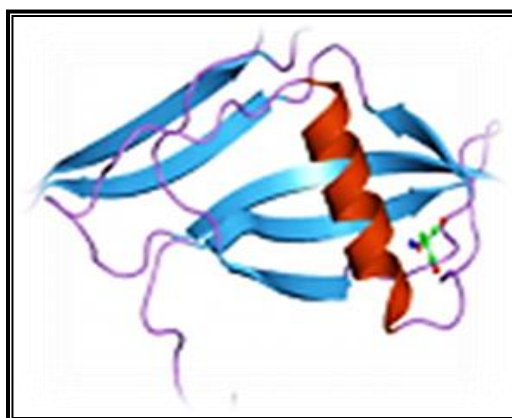

Figure 1.3: Structure of streptokinase [6]

1.5.1.2.2 Staphylokinase (SAK)

SRK is a monomeric chain of polypeptides comprising of amino acids (136) with no linkages of disulfides and is produce by specific *Staphylococcus aureus* strains. Like SK, SRK also forms a 1:1 complex stoichiometrically with plasmin (ogen), as a result of which it non-enzymatically activates other plasminogen molecules. The structure of the complex ternary plasmin–SAK-plasminogen has been reported recently via computer simulation and mutagenesis scanning [29].

1.5.1.2.2.1. Mechanism of fibrin selectivity

In the human plasma SAK addition in the presence of a clot of fibrin, it poorly reacts with the plasminogen. Though, it displays high levels of affinity reaction to plasmin at the surface of the clot [25]. On the surface of the clot, the complex of plasmin–SAK competently stimulates the plasminogen to plasmin. Both uncomplexed plasmin and plasmin SAK are attached to the fibrin and are prevented from being inhibited rapidly by the α_2-antiplasmin, while the unbound complements, released from the clot or formed in the plasma, are quickly repressed by α_2-antiplasmin [25]. Thus, the activation of plasminogen process is limited to the thrombus, inhibiting too much generation of plasmin by α_2- antiplasmin [25].

1.5.1.2.3 Urokinase (UK)

UK similar to the t-PA is a 2-chain polypeptide (Fig.1.4) that facilitates direct activation of plasminogen similar to the SK[14]. In the human urine, it is found at 6IU/ml concentrations and has been also isolated. Recently, by affinity chromatography UK has been isolated and purified from the urine and the cells of the fetal kidney [14]. Also, UK concentrations significantly or a pro-UK recently also have been reported in the human plasma, indicating that UK might too function as fibrinolytic system's part endogenously [14].

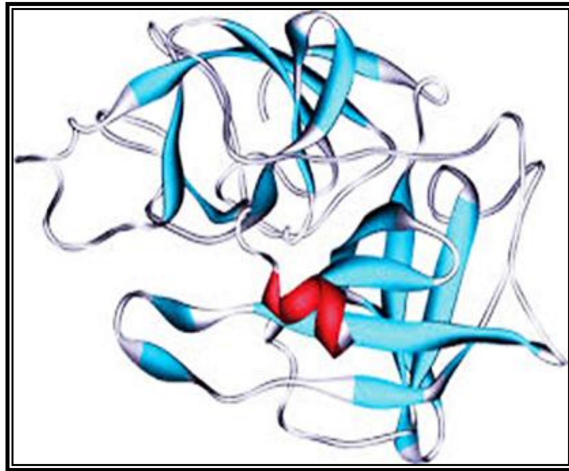

Figure 1.4: Structure of Urokinase [6]

1.5.1.2.4 Tissue plasminogen activator (t-PA).

The blood clots are broken down by the protein Tissue plasminogen activator (t-PA). Precisely, it is found in the cells lining the blood vessels, the endothelial cells (Fig 1.5) and is a serine protease (EC 3.4.21.68) [27]. It catalyzes the plasmin's conversion from plasminogen as an enzyme to plasmin, the key enzyme accountable for breaking down clots. Clinically, it is utilized for its ability to work on the systems of clotting; tPA is utilized for treatment of thrombolytic or embolic strokes only. Its application in head trauma and hemorrhagic strokes is contraindicated [27]. The role of t-PA classically is in the system of clotting. Precisely, t-PA catalyzes the plasmin's conversion from plasminogen. This is done by the cleavage of the plasminogen that is single-chained into 2 chains. A disulfide bond links the 2 chains and the resultant plasmin is obtained [30] .

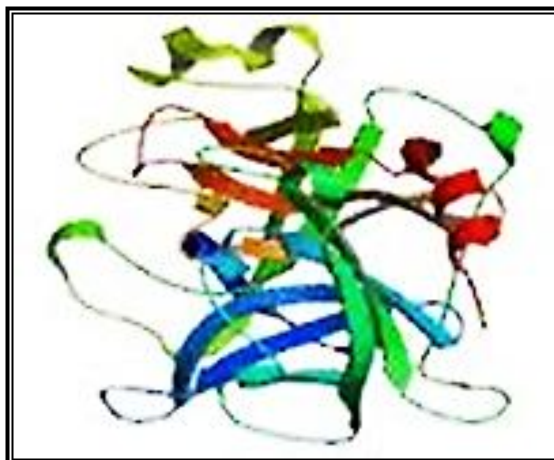

Figure 1.5: Structure of tissue plasminogen activator (t-PA)

Engineered genetically, the recombinant t-PA molecule with a MW of approx. 70 kDa varies from SK or UK in many crucial aspects: t-PA is appreciably activated in the fibrin's presence only; t-PA attaches to fibrin and with plasminogen forming the complex fibrin-t-PA- plasminogen; the plasminogen bound is activated by t-PA forming a plasmin that is bound with fibrin; t-PA has very lesser inclination to form a fibrinolytic state systemically[14]. Due to such facets, t-PA is a promising candidate which might be effective and safer post administration either via systematic or selective routes [31].

1.5.1.3 Anticoagulant

1.5.1.3.1 Warfarin

In the western world and Britain, Warfarin is the most widely utilized anticoagulant [31]. The coagulation is inhibited by the interference with presence of vitamin K into the dependent clotting factors of vitamin K, comprising II, VII, IX and X factors [31]. Considerable amount of variations are seen among the patients in terms of effectiveness which are impacted by race, age, and co-mediations like antibiotics and diet [31].

1.5.1.3.2 Heparin

For rapid anticoagulation requirements, heparin an anticoagulant is intravenously administered when required. In surgeries of open-heart patients, they are treated with heparin during the oxygenation of their blood via the heart-lung machine [31]. Medication is administered post-surgery for reversal of the heparin's action. Glycosaminoglycan is another representative of such an anticoagulant like heparin, that work by their key anticoagulation activity via the inhibition of Factor IIa, Factor Xa, and thrombin in the cascade of coagulation[31] . It is parentally administered due to its short half-life; possibly via by the intravenous infusion continuously. The incidences of osteoporosis, hemorrhage, alopecia, hypersensitivity, and thrombocytopenia have been linked to the use of heparin [31, 32].

1.5.1.4 Antiplatelet

For prevention of formation of clots, or prevent them from becoming larger and preventing occlusion of blood vessels entirely, anti-platelet agents are regularly utilized. The most widely used drug for anti-platelet therapy is Aspirin which is

responsible for preventing platelet aggregation [31]. To prevent infarction, in the cases of prophylaxis and acute myocardial infarction, it is prescribed. Clinical trial doses of antithrombotic agents range from lower than 50 mg/day to above 1200 mg/day. Aspirin's adverse effects are like those of warfarin. Other drugs of anti-platelet therapy like clopidogrel, dipyridamole, and ticlopidine are also well recognized [33]. Ticlopidine and Clopidogrel act by inhibiting the collagen and platelet-activating factors, and are frequently recommended for patients who are allergic to aspirin. Such candidates might be utilized in patients with atherosclerotic condition to avert strokes, coronary artery closure, and heart attacks in patients undertaking angioplasty [34]. Their application though is linked with suppression of bone marrow in specific leucopenia cases. Such medications are often known as "blood thinners," but this word is not really correct. They bar the capacity of the blood to clot, averting clot formation in the vessels and from getting larger [35].

Aspirin though is not an anticoagulant but has a deep influence on a constituent of the blood known as blood platelet cells, which stick together and result in the formation of clots [33, 34]. Clot formations are also partially resulted from platelets in a coronary artery occurrence, which causes numerous heart attacks. Owing to the aspirin's capacity to prevent clotting on the role of platelets, it is labeled as an antiplatelet and is often recommended in patients recovering from a heart attack to stop clot formations in the vein utilized for the coronary bypass surgery.

Unlike Warfarin and heparin that stop the recurrence and extension of thrombosis, agents that are thrombolytic in action (fibrinolytic enzymes) lyse the pre-existing thrombus. These are streptokinase (bacterial in origin), urokinase (isolated from kidneys), and tissue plasminogen activator (t-PA) which has been engineered genetically. Reports of patients with pulmonary embolism treated with SK and UK are at 3 times more probability to demonstrate resolution of clots than the patients on heparin treatment alone. Such enzymes could also avert some harm if the clots are taken out immediately after their occurrence. SK, a candidate for effective thrombolytic action for the management of pulmonary thromboembolism and acute myocardial infarction is obtained from *Streptococci*. It the body's own fibrinolytic pathways could be potentiated by the conversion of plasminogen to plasmin.

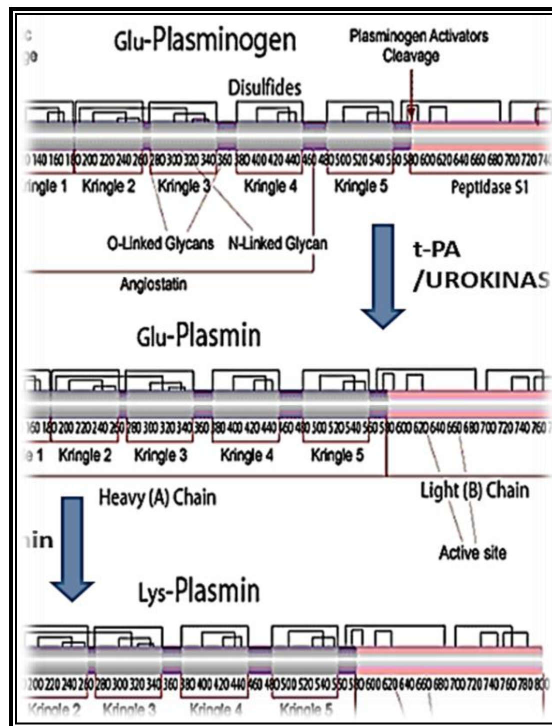

Figure 1.6: Schematic representation of plasmin formation through *in vivo* processing [36]

Native-intact human plasminogen is a 291 amino acid glycoprotein with as many as 24 disulfide bonds. Plasminogen contains a single N-linked sialylated biantennary glycan. The two O-glycans possesses a Gal β-1-3GalNAc core, which is α-2-3 sialylated at the terminal Gal. An additional disialylated form has a second sialic acid residue with an α-2-6 linkage to GalNAc. Mono- and disialylated forms occur at a molar ratio of 80:20 in human plasminogen [37]. The cleavage of plasminogen amidst Arg561 and Val562 leads to the conversion of plasmin. The resultant plasmin, which is activated the form, comprises of 2 chains of polypeptides linked by disulfides. The plasminogen's terminal amino region gives rise to the heavy chain (MW 60 kDa) of the plasmin. The carboxyl terminal is the source for the lighter chain of the plasminogen. The MW of the heavy chain in vivo could range from 63 kDa - 12 kDa based on the extent of proteolysis to the plasminogen from where it was obtained [37]. Plasmin catalyzes hydrolysis of the fibrin by converting itself into its acyl form. It sustains fibrinolysis by forming a complex of fibrin – bound plasmin in the presence of α2–antiplasmin, utilizing judicious estimates of the apparent first –order rate constant for breakage of a single peptide bond in the fibrin plasmin complex, which resulted in a number of peptide bonds cleaving, which suffice the

detachment of the fibrin monomer from the polymeric fibrin (Fig.1.5). This process is known as fibrinolysis [38].

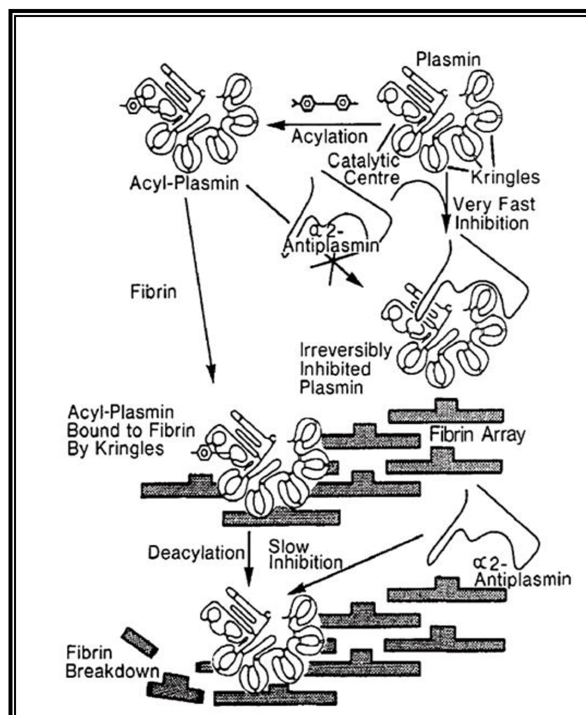

Figure 1.7: Schematic representation of fibrinolysis by plasmin [38]

Nevertheless, with its shorter half-life, it must be continuously infused for maximum effectiveness. Owing to the absence of specificity sites for most of the enzymes that are fibrinolytic in action, effects adversely could be hemorrhages in the gastrointestinal region, with rarely stark anaphylaxis episodes.

1.6 Aims and objectives of current investigation

The objective of the present work as described in the subsequent sections has accounted for the scopes as discussed above. Fibrinolytic protease(s) have gained medical importance owing to their potential application in treatment of cardiovascular relevant disorders. Although, several fibrinolytic enzymes have been reported earlier, however, fragmentary data is available on the non-toxic target specific drugs with their *in vivo* thrombolytic potential. The current phase of industrial biotechnology with the astonishing competences of microbial enzymes together with their diverse specificity and efficiency are perceived as a gold mine for discovering economically feasible and grander products. Enzymes therapeutically, represent the best instance of such endeavors. Especially, the *Bacillus* genus is well

recognized for its application in creating an array of enzymes of prime importance industrially, including the enzymes that are fibrinolytic in nature. The characterization biochemically of majority of the enzymes has wide opened their possibility for the development of drugs with thrombolytic potential [39-41]. It is noteworthy to point out that owing to the large benefits associated with the microorganisms from sources of food for the manufacturing enzymes that are fibrinolytic, these are favored in the pharma sector over others sources. Therefore, it is encouraging to investigate novel fibrinolytic enzymes from the Northeast India, considering the region as one of the mega biodiversity zones of the world. Exploration of microbial diversity of these regions would be initiated by screening the microbes from diverse fermented food samples, commonly used for edible purposes as well as non-toxic sources. The present investigation was undertaken to screen potential promising microbes of NE India which were able to produce fibrino(geno)lytic enzymes, followed by purification and characterization, including detailed investigations on *in vivo* toxicity and thrombolytic capabilities.

The following objectives formed the basis of this investigation:

1. Screening and taxonomic identification of fibrin(ogen)olytic enzyme producing bacterial strains from fermented foods of Northeast India.
2. Purification and characterization of bacterial fibrin(ogen)olytic enzymes produced by potential bacterial strains and comparison with commercial fibrinolytic enzymes.
3. Toxicity assessment of purified bacterial fibrinolytic and fibrin(ogen)olytic enzymes under *in vivo* conditions.
4. Pharmacological application of isolated fibrinolytic/fibrin(ogen)olytic enzymes in the treatment of thrombosis in *in vivo* conditions.