# Chapter- II

REVIEW OF LITERATURE

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Introduction:

Thrombotic complication leading to cardiovascular diseases is a main cause of death and disability in the world. Thrombolysis favorably influences the outcome of such life threatening diseases such as myocardial infarction (Collen et al., 1991). Hence calls for aggressive pharmacological treatment, thrombolytic therapy being the obvious rationale. Current clinically approved thrombolytic drugs have significant drawbacks such as reocclusion, bleeding complications, short half life etc. Hence needs to development of more potent and faster acting thrombolytic agents that speedup the clot lysis process and have a lower rethrombosis rate. SAK exhibits better fibrin specificity than t-PA (without having direct affinity for fibrin) and is capable of dissolving platelet-rich clots (Suehiro et al., 1995; Lewis et al., 1951). Many clinical studies on thrombosis shows the superiority of staphylokinase as compared to the clinically approved thrombolytic agents. These natural properties support that SAK should be used as a thrombolytic agent for clinical treatment. In order to improve the thrombolytic potential and antiplatelet activities of staphylokinase, researches are focusing on the mutant of staphylokinase.

The Literature Review of the thesis is divided into three parts.

In the first part of literature review ie., Thrombus, Pathophysiology of thrombosis and its Diseases: This part explains about the thrombus formation, phenomenon of thrombosis, and its pathophysiology, various diseases of thrombosis in
the sub headings of classification of thrombosis based on the type of veins and arteries, Ischaemic heart disease, Myocardial infarction, different types of strokes caused by thrombus.

In the second part, Thrombolytic agents and Its Classification: Enzyme Therapy for treatment of thrombosis based disorders contains the applications of enzymes in medicine, a brief note about the fibrinolytic enzymes, History of Thrombolytic agents, List of sources of Thrombolytic agents, methods of treatment for thrombosis by thrombolytic agents, adverse effects and risks in thrombolytic therapy, Desirable features for an Ideal thrombolytic agent, different types of thrombolytic agents based on its fibrin specificity, generation wise classification of thrombolytic agents ie., first generation thrombolytic agents such as streptokinase, urokinase, second generation thrombolytic agents such as saruplase, anistreplase, alteplase and third generation thrombolytic agents such as monoteplase, tenecteplase, reteplase, lanoteplase, pamiteplase, desmoteplase and staphylokinase and Chemic thrombolytic agents.

The third part of the review ie., Staphylokinase and Genetic engineering or Protein engineering of Staphylokinase include: Introduction about Staphylokinase and its application, about the Staphylococcus aureus and production of staphylokinase, structure of staphylokinase, mechanism of fibrin specificity of staphylokinase, functional properties of staphylokinase, immunogenicity of staphylokinase. Mile stones of staphylokinase explain about the research done on staphylokinase. Protein engineering of Staphylokinase, contains the literature of various mutant staphylokinase that has been constructed by site directed mutations (addition, deletion or substitution).
Part I
Thrombosis, Pathophysiology of Thrombosis and Its Diseases

2.1.0. Thrombosis:

Thrombosis is the formation of a blood clot (thrombus) inside a blood vessel obstructing the flow of blood through the circulatory system. It is the final product of the blood coagulation step in hemostasis. When a blood vessel is injured, the body uses platelets and fibrin to form a blood clot to prevent blood loss (Collen et al., 1998). If the clotting is too severe and the clot breaks free, the traveling clot is now known as an embolus (Furie et al., 2008; Handin et al., 2005).

**Blood Clot Diagram**

![Blood Clot Diagram](image)

**Fig No. 2.1:** Blood clotting in Blood Vessel

2.1.1 Clot formation:

A blood clot results from coagulation of the blood. Blood clots occur at the site of blood vessel damage and involve pooling of the blood. In appropriate activation of the blood clotting process inside a blood vessel results in the formation of a clot known as a thrombus. A thrombus is not normally life threatening, but this depends greatly upon the location of formation and the degree of occlusion of the vessel in which it was formed (Lack et al., 1948). When a clot breaks loose and travels in the body as an embolism this...
is much more serious. An embolus is a blood clot that moves through the bloodstream until it lodges in a narrowed vessel and blocks circulation.

**Fig No. 2.2.:** Blood clot formation in Blood Vessel

### 2.1.2 Pathophysiology of Thrombosis:

The 3 main components of a blood clot are platelets, thrombin, and fibrin; each of these components is a key therapeutic target. During thrombus formation, circulating prothrombin is activated to the active clotting factor, thrombin, by activated platelets. Fibrinogen is activated to fibrin by the newly activated thrombin. Fibrin is then formed into the fibrin matrix. All this takes place while platelets are being adhered and aggregated.

The first step is that an event has occurred that has damaged the delicate endothelial lining, tunica intima, of the blood vessel. Once this has occurred the clotting mechanism begins. Platelets adhere to the rough edges of the damaged vessel wall and rupture. This rupturing releases serotonin (which causes localized vasoconstriction),
adenosine diphosphate (ADP) and thromboxanes. ADP promotes the attraction of additional platelets to the damaged site as well as thromboxanes (a type of prostaglandin), which causes further aggregation and clotting. This is the reason that aspirin, which inhibits prostaglandins, is such an important component in the treatment of an acute myocardial infarction (AMI).

Prothrombin, a naturally occurring substance in the body, is converted into its active form thrombin. The enzyme thrombin then acts on fibrinogen, which is manufactured in the liver, to transform the fibrinogen into fibrin. Fibrin acts like a net to secure the clot in place. From this point, unless the body’s own naturally occurring fibrinolytic (plasmin) can dissolve the clot, or definitive treatment (thrombolytics) takes place, the cycle keeps building on itself, with the help of fatty acids (plasma cholesterol), until the blood vessel becomes occluded. Once the vessel has become occluded, or if perfusion distal to the thrombus is reduced sufficiently, the patient experiences a heart attack and damage results to the myocardium.

Thrombolysis process works best on recently formed thrombi. Older thrombi have extensive fibrin polymerization that makes them more resistant to thrombolysis; hence, the importance of time for thrombolytic therapy. Pathologic thrombosis can occur in any vessel at any location in the body. There are several conditions that predispose to thrombosis, includes: Atherosclerosis (plaque rupture), blood flow changes, metabolic disorders (diabetes mellitus and hyperlipidemia), hypercoagulable states, smoking, trauma and burns.
2.1.3. Diseases of thrombosis:

There are two distinct forms i.e., Venous and Arterial thrombosis, each of which can be presented by several subtypes.

I. Venous Thrombosis:

Venous blood clots often form in areas where blood flow is slow, usually in the legs (Yano et al., 2008). This is again classified as

1. Thrombosis of Deep Vein: Deep vein thrombosis (DVT) is the formation of a blood clot within a deep vein. It mostly affects leg veins, such as the femoral vein.

2. Thrombosis of Portal Vein: Portal vein thrombosis is a form of venous thrombosis affecting the hepatic portal vein, which can lead to portal hypertension and reduction of the blood supply to the liver (Webster et al., 2005).

3. Thrombosis of Renal vein: Renal vein thrombosis is the obstruction of the renal vein by a thrombus. This tends to lead to reduced drainage from the kidney. Anticoagulation therapy is the treatment of choice.

4. Thrombosis of Jugular Vein: Jugular Vein Thrombosis is a condition that may occur due to infection, intravenous drug use or malignancy.

I. Arterial thrombosis:

Arterial thrombosis or atherothrombosis is the formation of a thrombus within an artery (Harmening et al., 2002). The common cause of arterial thrombosis is atrial fibrillation, which causes disturbed blood flow. Later in life, it is clinically manifested as coronary artery disease, stroke, transient ischaemic attack, and peripheral arterial disease.
2.1.4. Coronary Artery Disease (CAD):

Coronary artery disease also atherosclerotic heart disease is the result of the accumulation of atheromatous plaques (this plaque is made up of fat, cholesterol etc) within the walls of the coronary arteries that supply the myocardium (the muscle of the heart) with oxygen and nutrients. The deposition of the plaque in the lumen (free space in the artery for the flow of nutrients, oxygen etc.) of an artery causes narrowing of lumen of the artery by decreasing its diameter. It is sometimes also called coronary heart disease (CHD).

The symptoms and signs of coronary artery disease are noted in the advanced state of disease, most individuals with coronary artery disease show no evidence of disease for decades as the disease progresses before the first onset of symptoms, often a "sudden" heart attack, finally arises. After decades of progression, some of these atheromatous plaques may rupture and (along with the activation of the blood clotting system) start limiting blood flow to the heart muscle.

As the degree of coronary artery disease progresses, there may be near-complete obstruction of the lumen of the coronary artery, severely restricting the flow of oxygen-carrying blood to the myocardium. Individuals with this degree of coronary artery disease typically have suffered from one or more myocardial infarctions (heart attacks), and may have signs and symptoms of chronic coronary ischemia, including symptoms of angina at rest and flash pulmonary edema.
Fig. No. 2.3.: In this figure A shows a normal artery with normal blood flow, figure B shows an artery with plaque buildup.

2.1.5. Ischaemic heart disease:

Ischaemic or ischemic heart disease (IHD), or myocardial ischaemia, is a disease characterized by ischaemia (reduced blood supply) to the heart muscle, usually due to coronary artery disease (atherosclerosis of the coronary arteries). Its risk increases with age, smoking, hypercholesterolaemia, diabetes, and hypertension, and is more common in men and those who have close relatives with ischaemic heart disease. Symptoms of stable ischaemic heart disease include angina (characteristic chest pain on exertion) and decreased exercise tolerance. Unstable IHD presents itself as chest pain or other symptoms at rest, or rapidly worsening angina. Diagnosis of IHD is with an electrocardiogram, blood tests (cardiac markers), cardiac stress testing or a coronary angiogram. Depending on the symptoms and risk, treatment may be with medication, percutaneous coronary intervention (angioplasty) or coronary artery bypass surgery (CABG).
Signs and symptoms:

Ischaemic heart disease may be present with any of the following problems:

- Angina pectoris (chest pain on exertion, in cold weather or emotional situations)
- Acute chest pain: acute coronary syndrome, unstable angina or myocardial infarction.
- Heart failure (difficulty in breathing or swelling of the extremities due to weakness of the heart muscle).

2.1.6. Myocardial Infarction:

Myocardial infarction (MI) or acute myocardial infarction (AMI), commonly known as a heart attack is the interruption of blood supply to part of the heart, causing heart cells to die. This is most commonly due to occlusion of a coronary artery following the rupture of a vulnerable atherosclerotic plaque, which is an unstable collection of lipids and white blood cells (especially macrophages) in the wall of an artery. The resulting ischemia (restriction in blood supply) and oxygen shortage, if left untreated for a sufficient period of time, can cause damage or death of heart muscle tissue (Vandewerf et al., 1999).

Classical symptoms of acute myocardial infarction include sudden chest pain (typically radiating to the left arm or left side of the neck), shortness of breath, nausea, vomiting, palpitations, sweating, and anxiety.
2.1.7. **Thrombosis of cerebral arteries:**

The term “stroke” is used to describe a number of brain disorders with a common feature of a defect in the cerebral vasculature. With a stroke, blood supply to the brain has been partially blocked. As a result, the affected area of the brain cannot function, which might result in an inability to move one or more limbs on one side of the body, inability to understand or formulate speech, or an inability to see one side of the visual field (Donnan et al., 2008). Strokes are classified according to whether they are ischemia, thrombus, embolus or hemorrhage.

2.1.7.a. **Ischemic Stroke:**

Ischemic strokes occur as a result of an obstruction within a blood vessel supplying blood to the brain. The underlying condition for this type of obstruction is the development of fatty deposits lining the vessel walls. This condition is called atherosclerosis. These fatty deposits can cause two types of obstruction:
- Cerebral thrombosis refers to a thrombus (blood clot) that develops at the clogged part of the vessel.

- Cerebral embolism refers generally to a blood clot that forms at another location in the circulatory system, usually the heart and large arteries of the upper chest and neck. A portion of the blood clot breaks loose, enters the bloodstream and travels through the brain’s blood vessels until it reaches vessels too small to let it pass. A second important cause of embolism is an irregular heartbeat, known as atrial fibrillation. It creates conditions where clots can form in the heart, dislodge and travel to the brain.

Silent cerebral infarction (SCI), or “silent stroke,” is a brain injury likely caused by a blood clot interrupting blood flow in the brain. It’s a risk factor for future strokes which could lead to progressive brain damage due to these strokes.

**Fig No. 2.5.: Ischemic Stroke in Brain**

The current approved FDA clinical treatment for all ischemic occlusions is to administer a drug, rt-PA (recombinant tissue plasminogen activator) within three hours of the onset of symptoms. In its nature state, t-PA is produced by vascular endothelium (Magnus et al., 2001). In its native amounts, it is harmless to the body and keeps blood
from pooling and forming clots. However, when a traumatic event occurs, the body’s intrinsic clotting cascade is activated to prevent internal or external hemorrhaging or bleeding.

2.1.7.b. Hemorrhagic Stroke:

A weakened vessel that ruptures and bleeds into the surrounding brain results in hemorrhage. The blood accumulates and compresses the surrounding brain tissue. The two types of hemorrhagic strokes are intracerebral (within the brain) hemorrhage or subarachnoid hemorrhage.

Two types of weakened blood vessels usually cause hemorrhagic stroke: aneurysms and arterio venous malformations (AVMs). An aneurysm is a ballooning of a weakened region of a blood vessel. If left untreated, the aneurysm continues to weaken until it ruptures and bleeds into the brain.

An arteriovenous malformation (AVM) is a cluster of abnormally formed blood vessels. Any one of these vessels can rupture, also causing bleeding into the brain.

Fig No. 2.6.: Hemorrhagic Stroke in Blood vessels of Brain
2.1.7.c. TIA (Transient Ischemic Attack):

Transient ischemic attack (TIA) is often labeled “mini-stroke,” it is more accurately characterized as a “warning stroke”. Transient ischemic attacks result from progressive narrowing of the vessel leading to reduction of blood flow or formation of a transient occlusion by a thrombus. The only difference between a stroke and TIA is that with TIA the blockage is transient (temporary). TIA symptoms occur rapidly and last a relatively short time. Most TIAs last less than five minutes; the average is about a minute. Unlike a stroke, when a TIA is over, usually causes no permanent injury to the brain.

Fig No. 2.7.: Transient Ischemic Attack- narrowing of vessel

2.1.7.d. Thrombotic Stroke:

In thrombotic stroke, a thrombus (blood clot) usually forms around atherosclerotic plaques. Since blockage of the artery is gradual, onset of symptomatic thrombotic strokes is slower. A thrombus itself can lead to an embolic stroke if the thrombus breaks off, at which point it is called an "embolus." Thrombotic stroke can be divided into two categories i.e., large vessel disease and small vessel disease.
• Large vessel disease (LVD) involves the common and internal carotids, vertebral, and the Circle of Willis. Diseases that may form thrombi in the large vessels include: atherosclerosis, vasoconstriction (tightening of the artery), aortic, carotid or vertebral artery dissection.

• Small vessel disease (SVD) involves the smaller arteries inside the brain: branches of the circle of Willis, middle cerebral artery, stem, and arteries arising from the distal vertebral and basilar artery.

2.1.8. Thrombosis of peripheral arteries:

Peripheral arterial disease (P.A.D.) is a disease in which plaque builds up in the arteries that carry blood to your head, organs, and limbs. The plaque causes the arteries to narrow or become blocked. When plaque builds up in the body's arteries, the condition is called atherosclerosis. Over time, plaque can harden and narrow the arteries. This limits the flow of oxygen-rich blood to organs and other parts of body (from heart to head, arms, kidneys, and stomach). A person with PAD also has an increased risk of heart attack, stroke and transient ischemic attack.

2.1.9. Vascular Occlusions of Thrombosis:

Vascular thrombi can occur as a result of both traumatic and non-traumatic events. Serious complications sometimes occur when a thrombus releases emboli that lodge in arteries that supply blood to organs such as the brain or heart. Occlusion of blood vessels results in reduction of blood flow to the affected area. With any instance of complete or partial ischemia, the resupply of blood flow to the affected tissues is a time-
sensitive process- the longer the time that biological tissue is deprived of oxygen, the less chance it will survive or function properly.

The main stay of thrombus management is a regimen of pharmacological therapy that includes thrombolytics to dissolve the clot and anticoagulants to prevent clotting. Streptokinase, urokinase, and tPA are examples of thrombolytics, and heparin is an anticoagulant. The use of these drugs has been shown to improve cardiac function (Gusto 1993). Alternate approaches to vascular recanalization are balloon angioplasty, rotobladeratherectomy, and aspiration devices (Grines et al., 1991). In balloon angioplasty a balloon is inflated inside the vessel to mechanically re-mold the lumen. When all else fails or if the thrombus burden is too large, bypass surgery may be performed.

Part II
Thrombolytic agents and Its Classification

2.2.0. Enzyme Therapy for treatment of thrombosis based disorders:

Medical applications of enzymes are divided into analysis, pharmaceutical and therapeutic applications. Enzymes play a very important role in paraclinical diagnosis, example, estimation of blood sugar level. In the pharmaceutical area, many of the drugs used today are in reality enzymatic inhibitors, such as cytostatics, antibiotics, steroids, etc. After many years of experience in analysis and pharmaceutical areas, it was thought that these powerful enzymes could be used in the therapeutic field, too. This way, a new area of enzymatic therapy began. It was applied to alleviate disturbances in the metabolism, that is, impairments in the functions of the organs and to repair genetic faults.
Enzyme therapy has long been part of the methods of treatment of traditional medicine. Now a days this therapy is developing more in medicine, with many manufacturers targeting their advantages in disease treatment. The concept of the therapeutic enzyme has been around for at least 40 years. For example, a therapeutic enzyme was described as part of replacement therapies for genetic deficiencies in the 1960s (De Duve et al., 1966).

Early observations of Bacillus pyocyaneus revealed that its secretions could destroy anthrax bacilli and protect mice from inoculation with this deadly bacterium. Scientists deduced that the secretions were able to destroy anthrax via enzymatic degradation. This early observation paved the way for the use of enzymes in medicine. Substitution in intestinal enzyme deficiency conditions is a classical treatment, external use of enzymes for impaired wound healing (e.g. in the presence of varicose ulcers) has been part of the armamentarium of medical practitioners for centuries.

Therapeutic enzymes have a broad variety of specific uses: as oncolytics, thrombolytics or anticoagulants, anti-inflammatories, fibrinolytics, mucolytics, antimicrobials and as replacements for metabolic deficiencies. Additionally, there is a growing group of miscellaneous enzymes of diverse function. Proteolytic enzymes have been widely used as anti-inflammatory agents. Reduction of inflammation and edema is ascribed to the dissolution of soft fibrin to the clearance of proteinaceous debris found in inflammatory exudates.

Development of medical applications for enzymes has been extensively increasing as that of industrial applications. The removal of cytotoxic substances and the
treatment of life-threatening disorders within the blood circulation is among the most successful applications of enzyme therapy.

In 1987, the first recombinant enzyme drug, Activase (alteplase; recombinant human tissue plasminogen activator), was approved by the Food and Drug Administration (FDA). This ‘clot-buster’ enzyme is used for the treatment of heart attacks caused by the blockage of a coronary artery by a clot. This was the second recombinant protein drug to be marketed (the first genetically engineered drug was insulin in 1982). Several other enzymes used as anticoagulant or coagulant agents have since been approved by the FDA.

2.2.1. Fibrinolytic agents:

Enzymes, like their application in medicine, exert their effects in a multitude of ways. One primary focus of enzymatic action is on the protein fibrin. Fibrin is an insoluble protein involved in blood clotting. In the many steps of the clotting cascade, fibrin is the final product. It is derived from its soluble protein precursor, fibrinogen. Fibrin is laid down inside blood vessels that have been compromised by disease or injury. Fibrin forms minuscule strands that eventually dry and harden, capturing blood vessel components effectively.

Certainly, fibrin occupies a vital role in health and healing; however, fibrin may also be responsible for an overzealous propensity to form inappropriate clots in the body. Inappropriate clotting, of course, is a major risk factor for myocardial infarction and stroke. When correctly balanced, deposition and removal of fibrin maintains avoidance of blood loss and adverse viscosity in the vascular system. A balance tipped in favor of fibrin overproduction leads to dangerous clotting.
Essam Kotb (2012) stated: Various types of thrombosis are responsible for an increasing number of deaths each year. In the USA alone, lung blood clots affect an estimated 1,000,000 patients annually (Lopez-Sendon et al., 1995). According to a report published by the World Health Organization (WHO) in 2001, 17 million people die every year of cardiovascular diseases (CVDs). The formation of a blood clot in a blood vessel (intravascular thrombosis) is one of the main causes of CVDs. The major protein component of blood clots, fibrin, is formed from fibrinogen via proteolysis by thrombin. Meanwhile, fibrin clots can be hydrolyzed by plasmin to avoid thrombosis in blood vessels. In an unbalanced situation due to some disorders, the clots are not hydrolyzed, and thus thrombosis occurs (Lopez-Sendon et al., 1995).

So, several investigations are being pursued to enhance the efficacy and specificity of fibrinolytic therapy, and microbial fibrinolytic enzymes have attracted much more medical interest in recent decades (Goldhaber et al., 2001; Tough et al., 2005).

Based on their different working mechanisms, thrombolytic agents are classified into two types. One is plasminogen activators, such as tissue-type plasminogen activator (t-PA) (Collen et al., 2004) and urokinase (Duffy et al., 2002), which activate plasminogen into active plasmin to degrade fibrin. The other type is plasmin-like proteins, which directly degrade fibrin, thereby dissolving thrombi rapidly and completely.

Although plasminogen activators and urokinase are still widely used in thrombolytic therapy today, their expensive prices and undesirable side-effects, such as the risk for internal hemorrhage within the intestinal tract when orally administrated, have prompted researchers to search for cheaper and safer resources (Ismail et al., 1981;
Nakajima et al., 1993; Bode et al., 1996). Therefore, microbial fibrinolytic enzymes have also attracted much more medical interest during recent decades (Bode et al., 1996).

Fibrinolytic enzymes were successively discovered from different microorganisms, the most important among which is the genus Bacillus from traditional fermented foods (Mine et al., 2005).

The physiochemical properties of these enzymes have been characterized, and their effectiveness in thrombolysis in vivo has been further identified. Therefore, microbial fibrinolytic enzymes, especially those from food-grade microorganisms, have potential to be developed as functional food additives and drugs to prevent or cure thrombosis and other related diseases (Ambrus et al., 1979; Sumi et al., 1987; Kim et al., 1996; Hwang et al., 2002).

Fibrinolytic enzymes are mainly proteases. These catalyze total hydrolysis of proteins and specifically act on interior peptide bonds (Bayoudh et al., 2000). All living cells produce different types of proteases, but the majority are produced by microorganisms. Many workers have reported that bacteria are high protease producers (Kalisz et al., 1988). Proteases are grossly subdivided into two major groups, namely exopeptidases and endopeptidases, depending on their site of action. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate (International Union of Biochemistry and Molecular Biology 1992). Based on the functional group present at the active site, proteases are further classified into four families: serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Hartley et al., 1960).
2.2.2. Thrombolytic Therapy (TT):

Thrombolytic Therapy is the use of drugs that dissolves intravascular blood clots and/or fibrin thrombi, digests fibrinogen and other proteins, and recanalizes occluded vessels–usually arteries, to improve circulation. When a blood clot forms in a blood vessel, it may cut off or severely reduce blood flow to parts of the body that are served by that blood vessel. This can cause serious damage to those parts of the body. If the clot forms in an artery that supplies blood to the heart, it can cause a heart attack. A clot that cuts off blood to the brain can cause a stroke.

Thrombolytic therapy is used to dissolve blood clots that could cause serious, and life-threatening, damage if they are not removed. Thrombolytic therapy can prevent or reverse paralysis and other problems in stroke. The enormous success of treating coronary thrombosis with thrombolytic therapy makes the treatment of ischaemic stroke the obvious next step. National Institute of Neurological Disorders and Stroke (NINDS) in the USA reported: a number of large trials have confirmed the benefits of thrombolysis in acute stroke within three hours of development of symptoms (NINDS rt-PA Stroke. 1995).

Administration of a thrombolytic agent such as tissue plasminogen activator, urokinase, or streptokinase to dissolve an arterial clot, such as a clot in a coronary artery in a patient with an acute myocardial infarction. Thrombolytic Therapy also dissolve clots (thrombi) in venous access devices ie., blood clots that form in tubes put into people's bodies for medical treatments, such as dialysis or chemotherapy.
2.2.3. Thrombolytic agent:

Thrombolysis consists of the pharmacological dissolution of a blood clot by administration of thrombolytic agents that activate the fibrinolytic system. Thrombolytic agents are plasminogen activators which convert the proenzyme, plasminogen, into an active enzyme, plasmin, which digests fibrin to soluble degradation products. Inhibition of the fibrinolytic system occurs at the level of the plasminogen activator (mainly by plasminogen activator inhibitor-1, PAI-1) and at the level of plasmin (mainly by α2-antiplasmin).

2.2.4. History of thrombolytic agents:

Although the thrombolytic agents (streptokinase, urokinase, tissue plasminogen activators and their derivatives) are new clinical tools compared with the previous drugs, their history reaches as far back as the 1980s. The history of thrombolytic agents begins in 1861 with the report by Von Brucke on the proteolytic activity of human urine. In 1888, Sahli noted urinary proteolytic activity with some specificity for fibrin. In 1952, Sobel coined the name “Urokinase” after isolation and purification of the fibrinolytic enzyme in urine.

In the early 1930s, William Tillett, a bacteriologist discovered that the beta-hemolytic, group C Streptococci produced a fibrinolytic agent and called it as “fibrinolysin” (Muller et al., 1994). As early as 1933, Tillet and Garner reported a streptococcal substance, streptokinase, that could induce fibrinolysis via activation of plasminogen (Tillet et al., 1933). In 1947, Astrup and Permin showed that animal tissues contain an agent that activates plasminogen (Astrup et al., 1947). This factor, originally termed fibrinokinase, was later also identified in human blood and is now called tissue-
type plasminogen activator (t-PA). In 1948 staphylokinase (Sak), secreted by certain strains of *Staphylococcus aureus*, was shown to have profibrinolytic properties. Macfarlane and Pilling found fibrinolytic activity in urine and Williams demonstrated that this was due to the presence of a plasminogen activator, now called urokinase (urokinase-type plasminogen activator, u-PA).

In the 1970s, Bernik and Nolan observed latent urokinase- like activity in conditioned media of some human cells, now referred to as single-chain u-PA (scu-PA or pro-urokinase). The usefulness of both intravenous SK and u-PA in venous thromboembolism was shown in several studies in the 1960s and 1970s, leading to FDA (Food and Drug Administration) approval of both agents for intravenous use in 1977.

Streptokinase was approved for deep venous thrombosis, pulmonary embolism, thrombosed dialysis fistulas, and arterial thrombosis; u-PA was approved only for pulmonary embolism and thrombosed intravenous catheters. Further FDA approvals for other indications came in 1982 for intracoronary use of both SK and u-PA in treatment of acute myocardial infarction and in 1987 for intravenous use of SK in acute MI (u-PA remains unapproved for intravenous use in acute MI, due to a relative lack of studies). Current commercial SK is still prepared by purification from bacterial cultures. u-PA, currently is produced only by Abbott, and is no longer prepared from human urine but instead by purification from human kidney cell cultures.

In 1979, Smith et al., reported the rational design of anistreplase, a combination of SK and its target, plasminogen. This agent, which spontaneously deacylates slowly after injection and thus can be administered as a single bolus, was first used in humans with
acute myocardial infarction by Been et al., and others in 1985, and it was approved for intravenous use in acute myocardial infarction by the FDA in 1989.

In 1947 t-PA was discovered in tissue slices that were shown by Astrup and Permin to lyse fibrin. Pennica et al., reported the cloning of t-PA DNA derived from human melanoma cells and its expression by Escherichia coli in 1983. Clinical use evolved rapidly, leading to FDA approval of intravenous t-PA for acute MI in 1987 and for pulmonary embolism in 1990. Unlike the early t-PAs derived from a variety of human tissues or from melanoma cell culture, current commercial t-PA is prepared from recombinant DNA derived from human melanoma cells and expressed in the Chinese hamster ovary tissue culture system.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Abbreviation</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptokinase (Streptase)</td>
<td>SK</td>
<td>Streptococcal culture</td>
</tr>
<tr>
<td>Urokinase</td>
<td>UK</td>
<td>Renal cell culture</td>
</tr>
<tr>
<td>Alteplase or Tissue plasminogen activator (trade names Retavase, Rapilysin)</td>
<td>t-PA</td>
<td>Recombinant technology</td>
</tr>
<tr>
<td>Anistreplase or Acylated plasminogen: streptokinase activator</td>
<td>APSAC</td>
<td>Streptococcal</td>
</tr>
<tr>
<td>Prourokinase or Single-chain urokinase plasminogen activator</td>
<td>SCU-PA</td>
<td>Renal cell culture</td>
</tr>
</tbody>
</table>

Table No. 2.1.: List of thrombolytic agents from various sources

2.2.5. Adverse effects of thrombolytic agents:

The adverse effects of thrombolytic agents include: major bleeding, cardiac arrhythmias, cholesterol embolus syndrome, anaphylactoid reaction, cerebrovascular accident, intracranial hemorrhage.
2.2.6. Risks of Thrombolysis:

Although thrombolysis can safely and effectively improve blood flow and relieve or eliminate symptoms in many patients without the need for more invasive surgery, it’s not recommended for everyone. Thrombolysis may not be recommended for patients who use blood-thinning medication, herbs, or dietary supplements, or for people with certain conditions associated with an increased risk of bleeding. These conditions include:

- Severe high blood pressure
- Active bleeding or severe blood loss
- Hemorrhagic stroke from bleeding in the brain
- Severe kidney disease
- Recent surgery

Thrombolysis also may be associated with an increased risk of complications in patients who are pregnant or at an advanced age, and in people with other conditions. Patients who undergo thrombolysis have a small risk of infection (less than one in 1,000) as well as a slight risk of an allergic reaction to the contrast dye that may be needed for imaging. Besides risk of serious internal bleeding, other possible risks include:

- Bleeding at the access site
- Damage to the blood vessel
- Migration of the blood clot to another part of vascular system
- Kidney damage in patients with diabetes or other pre-existing kidney disease
The most serious possible complication is intracranial bleeding, which is potentially fatal. But this complication is rare. Bleeding in the brain that causes stroke occurs in less than 1% of patients.

2.2.7. Desirable features for the ideal Thrombolytic Agent:

The characteristics of an ideal thrombolytic agent are summarized below. An ideal drug would provide rapid reperfusion, would have a prolonged half-life that permits single-bolus dosing, facilitating more timely and convenient administration. The agent would also have enhanced fibrin specificity to allow preferential activation of fibrin-bound plasminogen at the clot surface, resulting in increased potency and speed to patency. Furthermore, higher fibrin specificity would limit activation of circulating plasminogen and thus degradation of fibrinogen attributes that would be expected to reduce the risk of bleeding.

Characteristics of the ideal thrombolytic agent are:

- Rapid reperfusion
- Administration as an intravenous bolus
- Fibrin specificity
- Low incidence of systemic bleeding
- Low incidence of intracranial haemorrhage
- Resistant to plasminogen activator inhibitor-1 (PAI-1)
- Low reocclusion rate
- No effect on blood pressure
- No antigenicity
- Reasonable cost
2.2.8. Different types of thrombolytic agents:

Thrombolytic agents can be categorized in several ways. Classification schemes can be devised on the basis of the source of the agent, the propensity for enhanced enzymatic activity on a fibrin or cell surface or the mechanism of action (enzymatic vs. non-enzymatic) or different generation wise (Table No. 2.1). Each of these methods of classification is useful in helping to characterize the diverse nature of plasminogen activators, but regardless of how one defines these agents, they all serve one primary purpose i.e the conversion of plasminogen to plasmin.

Plasminogen activators (PA) are mainly categorized into two types:

1. **Non fibrin specific PA:** Streptokinase and two-chain u-PA (tcu-PA), activate both plasminogen in the circulating blood and fibrin-bound plasminogen. Generated plasmin is rapidly inactivated by α2-antiplasmin and will, after saturation of the inhibitor, degrade other plasma proteins causing the so-called “lytic state”.

2. **Fibrin specific PA:** Fibrin-selective agents, including t-PA, scu-PA and Sak, preferentially activate fibrin-bound plasminogen. Generated plasmin remains associated with fibrin and are protected from rapid inhibition by α2-antiplasmin, because the structures required for this inhibition (active site and lysine binding sites of plasmin) are not available to the inhibitor. This mechanism has triggered interest in using such fibrin-specific agents for thrombolytic therapy.
2.2.9. Classification based on generation:

<table>
<thead>
<tr>
<th>Generation of Thrombolytic Drug</th>
<th>Fibrin Specific</th>
<th>Non Fibrin Specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>First</td>
<td>--</td>
<td>Urokinase*</td>
</tr>
<tr>
<td></td>
<td>--</td>
<td>Streptokinase*</td>
</tr>
<tr>
<td>Second</td>
<td>Recombinant tissue plasminogen activator * (t-PA)</td>
<td>Pro-urokinase (scum-PA)</td>
</tr>
<tr>
<td></td>
<td>Alteplase</td>
<td>SK-plasminogen activating complex* (APSAC)</td>
</tr>
<tr>
<td>Third</td>
<td>Tenecteplase* (TNK-tPA)</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Reteplase*</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Monteplase</td>
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<tr>
<td></td>
<td>Lantoplase</td>
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</tr>
<tr>
<td></td>
<td>Pamiteplase</td>
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<tr>
<td></td>
<td>Staphylokinase</td>
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</tr>
<tr>
<td></td>
<td>Desmoteplase (Bat-PA)</td>
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</tr>
<tr>
<td></td>
<td>Chimeric thrombolytics</td>
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</tr>
</tbody>
</table>

* Approved for clinical use

Table No. 2.2.: Generational Classification of the Thrombolytic Agent

2.2.9.i. The first generation thrombolytic molecules

Initial studies on thrombolytic therapy were primarily focused on whether the treatment was effective in reducing mortality, on dosing regimens, and on the route of administration. Though the thrombolytic therapy for acute myocardial infarction was first attempted in the 1950s, significant advances were not made until the 1980s.

2.2.9.i.a. Streptokinase:

Streptokinase (SK), the oldest and best-known plasminogen activator, is produced by various strains of β-hemolytic Streptococci. Its fibrinolytic activity was first described in 1933 (Sikri et al., 2007). Streptokinase found its initial clinical application in combating fibrinous pleural exudates, hemothorax and tuberculous meningitis. In 1958, streptokinase was first used in patients with acute myocardial infarction and this changed the focus of treatment. Earlier streptokinase produced contradictory results until the GISSI (Gruppo Italiano per la Sperimentazione della Streptochinasi nell'Infarto
Miocardico) trial in 1986, which validated streptokinase as an effective therapy (GISSI, 1986).

Streptokinase is a non-fibrin specific extracellular enzyme that exerts its fibrinolytic action indirectly by activating the circulatory plasminogen. It has a molar weight of 47 kDa and is made up of single-chain polypeptide of 414 amino acid residues (Lakshmi et al., 2003). Streptokinase composed of three distinct domains, denoted as α (residues 1–150), β (residues 151–287), and γ (residues 288–414).

Streptokinase is not an enzyme and therefore does not exhibit plasmin activity by proteolytic cleavage of plasminogen. Instead, it binds non covalently to plasminogen in a 1:1 equimolar fashion and there by confers plasmin activity. The streptokinase-plasminogen complex then acts on other plasminogen molecules to cleave the Arginine-Valine 561 bond and, as a result, generate of plasmin (Anderson et al., 1987). Streptokinase has a plasma half-life of 30 min.

Streptokinase is available in the market as Streptase and Kabikinase. Since it is a bacterial product, its use is normally associated with allergic reactions and antibody-mediated inhibition of plasminogen activation. SK is also associated with bleeding complications due to its non fibrin specificity.

2.2.9.i.b. Urokinase:

Urokinase, also called urokinase-type Plasminogen Activator (u-PA), was originally produced from human urine. It is secreted as a 54kDa single-chain molecule (scuPA, prourokinase) which can be converted to a two-chain form (tcu-PA) due to proteolytic cleavage at Lysine at 158-Isoleucine at 159 by plasmin (Husain et al., 1983). Urokinase (u-PA) a serine proteinase of 411 amino acid residues, consist of three
domains: the carboxyl-terminal serine protease domain, the kringle domain and amino terminal the growth factor domain (Qing Huai et al., 2006). The two chain tcu-PA has full enzymatic activity but does not exhibit fibrin affinity while scu-PA has a weak fibrin affinity. In plasma, in the absence of fibrin, scu-PA is stable and does not activate plasminogen; however, in the presence of a fibrin clot, scu-PA induces fibrin specific clot lysis. The initial half-life of scu-PA was 6-9 minutes upon administration to the patients with acute myocardial infarction.

Earlier Urokinase was available in the market in the brand name Abbokinase®, Kinlytic (Microbix Biosystem). It was obtained from human neonatal kidney cells grown in tissue culture. These drugs had been given as intra venously. Side effects were also associated with this as in case of streptokinase drug. In Dec 1998, the FDA removed the Abbokinase® (Abbort Laboratory, Abbott Park, Illions) from the market place due to significant deviation from current good manufacturing process. This has prompted to consider reteplase and alteplase as an alternative.

2.2.9.ii. Second generation thrombolytic molecules:

The search has continued for the ideal thrombolytic therapy. The need for a safer, more effective fibrinolytic drug led to the development of the second generation agents.

2.2.9.ii.a. Saruplase:

Recombinant scu-PA(r-proUK) is known as saruplase which is expressed in *E. coli* and obtained as 45 kDa non glycosyalated molecule (Del Zoppo et al., 1998). Its therapeutic potential was investigated in several clinical studies in patients with Acute Myocardial Infarction (Furlan et al., 2002; Vermeer et al., 1999) or with stroke (Rijken et
al., 1982; Valery et al., 1991). However, the U.S. Food and Drug Agency (FDA) has not approved r-proUK for clinical use and has demanded a new separate study.

2.2.9.ii.b. Anistreplase:

Anistreplase or anisoylated plasminogen-SK activator complex (APSAC) is another form of SK, an equimolar acylated complex of human lysplasminogen and SK. This complex act on plasminogen upon deacylation spontaneously in plasma. Activation of anistreplase to lys-plasminogen-streptokinase occurs by a first order, hydrolytic process, in blood or in the thrombus, with release of the p-anisoyl group by deacylation. The non-enzymatic deacylation process starts immediately after intra venous administration of anistreplase. Since it is a complex, it converts only those molecules of plasminogen to which the streptokinase is bound (it has no effect on endogenous plasminogen) (Mimuro et al., 1992). The plasma half-life of APSAC is 70-120 min. The International Study of Infarct Survival (ISIS)-3 showed a 10.5% mortality rate at 35 days with anistreplase and an intracranial hemorrhage rate of 0.6% (ISIS-3; 1992). Eminase® (Wulfing Pharma GmbH) is the brand name of Anistreplase in the market. It has same side effects as the streptokinase has, but has the advantage of single-bolus administration.

2.2.9.ii.c. Alteplase:

Tissue plasminogen activator (tPA), a serine protease, is a fibrin specific thrombolytic molecule. It is synthesized as a single chain polypeptide of 70 kDa consisting of 527 amino acids (Rijken et al., 1981). Proteolytic cleavage of the Arg 275-Ile 276 (Arginine at 275-Isoleucine at 276) bond by Plasmin, kallikrein, or factor Xa converts this single chain form (sct-PA) into a two-chain species (tctPA). t-PA exhibit its enzymatic activity both in single chain and two chain form (Rijken et al., 1982).
Activase® (Alteplase) is a tissue plasminogen activator produced by recombinant DNA technology. It is synthesized using the cDNA for natural human tissue-type plasminogen activator obtained from a human melanoma cell line (Topol et al., 1982). Alteplase produces TIMI grade 3 flow in 50% to 60% of patients at 90 minutes. In GUSTO-1, (global use of strategies to open occluded coronary arteries) patients who received alteplase had a 6.3% mortality rate at 30 days and a 0.72% incidence of intracranial hemorrhage (GUSTO 1993).

Compared with streptokinase, alteplase resulted in a 1% absolute reduction in death or nonfatal stroke. Activase® (GENENTECH) is a sterile, white to off-white, lyophilized powder for intravenous administration after reconstitution with Sterile Water for Injection. This medication is given by injection directly into the vein within 3 hours after the onset of stroke symptoms. The most common and serious side effect with alteplase is bleeding.

2.2.9.iii. Third generation thrombolytic molecules:

The third-generation thrombolytic agents were developed to improve the efficacy, safety, and ease of administration. The major thrust has been on modifications of rt-PA to develop a drug that restores faster patency and more effective manner as well as being easier to administer. These newer agents are more fibrin specific, that bind to thrombus and predominantly activate clot-bound plasminogen, thus avoiding systemic plasminogen activation. Due to their longer half-life, the newer drugs can often be administered in a single bolus or two boluses.
2.2.9.iii.a. Reteplase:

Reteplase (recombinant plasminogen activator, r-PA) is a single chain deletion variant of alteplase that is expressed in Escherichia coli. It is a non-glycosylated protein of 355 amino acids with 39k Da. It has similar plasminogen activator activity as alteplase in the absence of fibrin, but its binding affinity to fibrin is significantly (5 fold) lower than that of alteplase. The plasma half-life is increased to 14–18 minutes. This allows reteplase to be administered as boluses against as an initial bolus.

The efficiency of reteplase in patients with acute myocardial infarction has been evaluated, in the RAPID I (recombinant plasminogen activator angiographic phase II international dose finding study) trial, where reteplase was administrated as a double bolus (10 plus 10 MU 30 min apart) against the standard dose of alteplase (100 mg over three hours), the patency of reteplase was reached earlier and more frequently than with alteplase. This was confirmed in the RAPID II (reteplase vs alteplase patency investigation during myocardial infarction) trial where the same reteplase dose regimen revealed a higher rates of early reperfusion than accelerated regimen of alteplase (over 1.5 hour) (Bode et al., 1996).

Furthermore, in the GUSTO (global use of strategies to open occluded coronary arteries) III trial mortality after 30 days of patients treated with reteplase was not significantly different from that with alteplase and those treated with alteplase which led to conclusion that reteplase was superior to alteplase in its administration as a double bolus (GUSTO III Investigators 1997). However bleeding and allergic reactions are also associated by using this drug.
2.2.9.iii.b. Tenecteplase:

Tenecteplase is a multiple (three) point mutation of alteplase, rather than a deletion mutant (as reteplase does). It has been specifically bioengineered to have an extended half-life, allowing convenient single bolus dosing, increased fibrin specificity and patency. The substitutions of Asn 117 with Gln (aspargine at 117 substituted with glutamine) delete the glycosylation site in kringle 1 domain while the replacement of Thr 103 with Asn (Threonine at 103 position substituted with Asparagine) reintroduced a new glycosylation site, but at different locus. These modifications prolong its half life (17-20 min.) by reduction in plasma clearance rate. The amino acids Lys 296 –His 297 –Arg 298 –Arg 299 (lysine at 296, Histidine at 297, Arginine at 299, Arginine at 299) are each replaced with Ala which increases resistance by 80 times to inhibition by PA-1 (Guerra et al., 2003). Tenecteplase has a similar ability as native tPA to bind to fibrin with enhanced fibrin specificity 14-fold greater (Keyt et al., 1994).

The efficacy of tenecteplase has been evaluated in several clinical studies. It does not produce the paradoxical procoagulant effect that limits other thrombolytic agents. The long half life (17-20 min.) allows for single bolus administration with 6 to 12 fold greater thrombolytic patency than accelerated tissue type plasminogen activator. It has faster reperfusion, faster clot lysis, longer duration of arterial patency, and lower occurrence of bleeding compared with tissue type plasminogen activator (Benedict et al., 1995). Bleeding and allergic reactions are also associated by using this drug.

2.2.9.iii.c. Lantoplase:

Lantoplase is a deletion and single point mutation of wild-type t-PA. The plasma half life of lanoteplase, that is about 10 times in comparison with alteplase (Nordt et al.,
The long half-life of lantoplase (37 min) allows for single bolus administration in the thrombolytic therapy. In the TIME-1 (intravenous t-PA for treatment of infracting myocardium early) trial of lanoteplase resulted in a higher patency rate of the infarct related coronary artery at 90 minutes than treatment using alteplase in the accelerated regimen (Den Heijer et al., 1998). However, in In TIME 2 trial, it was found that both lantoplase and alteplase have similar efficiency with respect to overall mortality at 30 days. However, the rate of haemorrhagic stroke with lantoplase (1.12%) was significantly higher than that with alteplase (0.64%) [TIME-II 2002]. This increase in the most severe complication of thrombolytic therapy has stopped lantoplase from entering the market to date.

2.2.9.iii.d. Monteplase:

Monteplase is single amino acid point mutant of t-PA. The Cystine 84 has been replaced by Serine (Suzuki et al., 1991). The half life of monoteplase is longer than alteplase (23 min vs. 4 min) (Kawai et al., 1994).

2.2.9.iii.e. Pamitelase:

Pamitelase is a derivative of t-PA recombinant Chinese hamster ovary cell (CHO) lines. The kringle domain is deleted and there is a substitution of Arg at position 274 at the cleavage site of single chain t-PA which provide resistance to conversion into two chain molecule by plasmin (Ishikawa et al., 1997). The longer half-life and increased potency with distinct fibrin specificity were evaluated in a clinically relevant model of embolic stroke. It has a half life of 30-47 min (Sumii et al., 2001). The thrombolytic effect of a bolus administration of pamitelase was found to be similar with that of wild t-PA (Yui et al., 1996).
2.2.9.iii.f. Desmoteplase:

Desmoteplase is a plasminogen activator derived from the saliva of the vampire bat Desmodus rotundus. It exists as a single chain molecule of 447 amino acids. Its structure is similar to u-PA, consisting of one kringle domain with catalytic domain. It has been produced recombinantly (Kratzschmar et al., 1991). Desmoteplase is more fibrin dependent and fibrin specific than t-PA. Its catalytic efficiency increases thousands times in the presence of fibrin (Gulba et al., 1996).

2.2.9.iii.g. Staphylokinase (SAK):

Staphylokinase (SAK) is a fibrin specific clot dissolving agent for myocardial infarction. It is an extracellular protein secreted by *Staphylococcus aureus* strain after lysogenic conversion with bacteriophages. Matured staphylokinase consists of a single polypeptide chain of 136 amino acids without disulfide bridges. Its molecular weight is approximately 16kDa (Bokarewa et al., 2006). Its mechanism of activation bears similarities to that of streptokinase along with some differences. Like streptokinase, it is not an enzyme and forms a 1:1 stoichiometric complex with plasminogen but unlike the streptokinase-plasminogen complex, the staphylokinase-plasmin complex is inactive and requires for conversion to staphylokinase:Plasmin complex to expose their active site and become a potent plasminogen activator (Collen et al., 1993).

The thrombolytic properties of staphylokinase have been evaluated in several clinical studies. It is highly fibrin specific but lesser fibrinogenolytic properties as compared to streptokinase.

The therapeutic potential of SAK and its derivatives has been studied in the CAPTORS (Collaborative Angiographic Patency Trial of Recombinant Staphylokinase) I
and II trials that support its therapeutic potential (Armstrong et al., 2000; Armstrong et al., 2003). Although clinical experiences with staphylokinase has been encouraging, but limited. Being a heterologous protein, staphylokinase is immunogenic in man who induces antibody formation and resistance to repeated administration (Collen et al., 1992). Furthermore, its potency is also limited by incapability of mediating early reperfusion in 38% of treated patients and by rethrombosis (Collen et al., 1993).

In order to reduce immunogenicity and to prolong the half-life of SAK, a comprehensive site directed mutagenesis program had been done without the substantial loss of their fibrinolytic potency and fibrin specificity. The site directed mutations done for prolong half-life is polyethylene glycol-derivatized cystein-substitution variants of recombinant SAK (Collen et al., 2000; Moreadith et al., 2003).

2.2.10. Chimeric Thrombolytic Agents

Now a day’s several recombinant chimeric fibrinolytic agents are coming with more efficacies. They are either conjugates of plasminogen activators with monoclonal antibodies against fibrin, platelets, or thrombo modulin or hybrids of different plasminogen activator. There are lot of efforts have been done for selection of most potent combination of antibody and effector plasminogen activator agents for recombinant production. Prourokinase and staphylokinase chimera are most effective conjugates with monoclonal antibodies.

2.2.10.i. Staphylokinase chimeric molecule

Staphylokinase chimera are created by its cross linkage with and hirudin to antifibrin and antiplatelet antibodies. Now a days, there is an evidence that activated platelets play a pivotal role in arterial thrombosis and rethrombosis. The platelets
aggregation is closely involved in the reformed secondary clots after thrombolytic therapy (Yasuda et al., 1990). Arg-Gly-Asp(RGD) (Agrinine-Glycine-Aspartic acid) peptide can recognize the platelet membrane glycoprotein IIb/IIIa (GPIIb/IIIa) receptor. It has been also found that the binding of surface glycoprotein GPIIb/IIIa to fibrinogen mediates platelet aggregation and RGD can prevent fibrinogen binding to GPIIb/IIIa on activated platelets, thus inhibiting platelets aggregation (Yamada et al., 1996). Hence, it is interesting to hypothesize that the clot lysis efficacy of SAK can be enhanced with direct active platelet binding ability and at the same time the rethrombosis complication can be minimized with antiplatelet aggregation.

In effort to combine the advantages of staphylokinase with those of thrombin inhibitor and Platelet aggregation inhibitor, for preventing reocclusion, several chimeric proteins have been produced. Hirudin was selected as antithrombin partner due to its compatibility with the secretion system. In one approach, a model fusion protein constructed by joining to the small functional domain hirudin variant (HV1) in both N- and C-terminal configuration, HV1-SAK and SAK-HVI. The rate of plasminogen activation by SAK was not altered by the presence of an additional N- and C-terminal protein sequence. However, cleavage at N-terminal lysines within SAK rendered the N-terminal fusion unstable. Therefore, C-terminal fusions represent stable configuration for rational development of improved thrombolytic agents based on SAK (Szarka et al., 1999).

In an approach, SAK and hirudin were joined together via a pair of engineered coiled sequences that act as the hetero dimerization domain. A lysine rich coiled coil sequence (K-coil) is added to the C-terminal tail of SAK to generate SAK-K coil (SAKK)
and a glutamate rich coiled coil sequence (E. coil) is added to the C-terminal end of hirudin to generate hirudin-E. Coli (HE). These heterodimeric molecule (HE-SAKK) is a superior thrombolytic agent in comparison to staphylokinase as confirmed by in vitro fibrin and plasma clot lysis studies (Lian et al., 2003). The acquisition of RGD sequence is done by site directed mutagenesis in which K 35 (lysine at 35) is substituted with Arg and results in novel SAK variant RGD-SAK which had a much higher affinity with platelets than SAK and thus enriching the thrombolytic activity. It also inhibits ADP-induced platelet aggregation in a dose dependent manner (Chen et al., 2007). PLATSNAK(Platelet-Anti thrombin-Staphylokinase) was designed which include three inhibitory regions RGD sequence, a part of fibrinopeptide A, an inhibitor of thrombin and the C-terminal of hirudin a direct antithrombin (Van Zyl et al., 1997). In an another approach of improvement of the thrombolytic potential of SAK, as well as to introduction of antithrombotic and antiplatelet activity, the recombinant SAK-RGD-K2-Hir was constructed which is more potent and fast acting thrombolytic agent as compared with standard r-SAK (Szemraj et al., 2005; Kowalski et al., 2009).

2.2.10.ii. Prourokinase chimera

Single-chain u-PA (scu-PA) does not bind to fibrin which limits its thrombolytic potential. Targeting of scu-PA to a thrombus by combining the fibrin-specific antibody with its catalytic domain in a single molecule has resulted in a significant increase of both its invitro fibrinolytic and its in vivo thrombolytic potential. This chimera construction has been achieved by chemical conjugation of scu-PA and a monoclonal antibody directed against the NH-terminal region of the ß-chain of fibrin, 59D8 (Bode et al., 1990) or a monoclonal antibody specific for fragment D dimer of cross-linked human fibrin.
(MA-15C5) (Holvoet et al., 1989). A recombinant chimeric PA, rscu-PA-32k/59D8, in which intact 59D8 was fused to scu-PA-32k, yielded a 20-fold higher thrombolytic potency than scu-PA in a rabbit jugular vein thrombosis model (Runge et al., 1991).

This fusion protein specifically targets endothelial cells in vitro and in vivo and provides antigen-specific enhancement of fibrinolytic activity in a mouse model of pulmonary thrombosis, providing evidence that vascular immuno targeting can be used for 32 therapeutic fibrinolysis. It reduces the rate of reocclusion and enhances the speed and efficacy of reperfusion (Bode et al., 1991).

2.2.10.iii. Chimeric t-PA/scu-PA

This chimera plasminogen activator molecule consist of NH-terminal 1-263 amino acids of tPA, which contain the structures responsible for its fibrin affinity, fused to the COOH terminal144-411 amino acids of u-PA, which contain all structures required for the enzymatic and biological properties, characteristic of the intact molecule (Nelles et al., 1987). But it has reduced fibrin affinity as compared with t-PA due to steric interactions between the closely spaced functional domains of the two molecules. Therefore, to overcome this problem, an extended chimeric t-PA/scu-PA protein had been constructed, consisting of amino acids 1-274 of t-PA and 138-411 of Scu-PA, which has an additional sequence of 17 residues in the region joining the two proteins. The chimeric molecule thus contains the finger domain, the growth factor domain, and both kringle domains of t-PA, with additional sequence up to amino acid Phe274 (phenylalanine at 274) which precedes the Arg275-Ile276 (arginine at 275-isoleucine at 276) peptide bond cleaved by plasmin. This region of t-PA is fused to the serine protease part of u-PA starting at Ser138 (serine at 138), 21 residues NH2 terminal to the Lys158–
Ile159 (lysine at 158-isoleucine at 159) peptide bond in scu-PA which is cleaved by plasmin (Lijnen et al., 1988). This chimera showed markedly increased thrombolytic potency when compared with both parent molecules in different animal models.

**Part III**

**Staphylokinase and Genetic Engineering or Protein Engineering of Staphylokinase**

2.3.0. Staphylokinase:

Staphylokinase is a protein found in the culture medium after growth of many lysogenic strains of *Staphylococcus aureus* that convert plasminogen into the active proteolytic enzyme plasmin. It is 136 aa long and 16 kDa size extra cellular protein produced during the late exponential growth phase by *Staphylococcus aureus* strains carrying the prophages which contain the SAK gene. It forms a 1:1 stoichiometric complex with plasmin (ogen) that converts other plasminogen molecules to plasmin, a potent enzyme that degrades proteins of the extracellular matrix. The high affinity of the SAK-plasminogen complex for fibrin makes it a promising thrombolytic agent. There is only limited knowledge of the role of SAK in staphylococcal infection. SAK might facilitate *S. aureus* to bind host plasminogen through bacterial cell surface receptors and thereby promote invasion of host tissues.

SAK is highly fibrin-selective and its fibrin selectivity improves therapeutic efficiency by preventing and fibrinogen degradation in plasmin. SAK resulted in high titer of neutralizing specific IgG, which would predict therapeutic refactoriness upon repeated administration. SAK was found to contain three non-overlapping
immunodominent epitopes. Like other currently available thrombolytic agents, SAK also shows significant shortcomings (side effects) including reocclusion and bleeding complication. Several approaches have been developed to overcome these side effects and enhance curative effects such as construction of thrombolytic agents for targeting platelet aggregation.

2.3.1. Applications of Staphylokinase:

- Staphylokinase does not involve more in rethrombosis than that of comparing with streptokinase, because of the small size of the staphylokinase.
- Its properties of lysis of platelet-rich and retarded clots efficiency, and exceptional fibrin specificity help to minimize reoccclusion and bleeding complications.
- The fibrinogen levels in patients treated staphylokinase remain close to 100%, whereas patients treated with tPA have 32% of fibrinogen cleaved and degraded in their plasma (Vanderschueren et al., 1995; Vanderschueren et al., 1997).
- Expression and activity is high, but still activity can be enhanced by protein engineering.

2.3.2. Structure of Staphylokinase:

Staphylokinase consists of 136 amino acids in a single polypeptide chain without disulfide bridges. Structurally, SAK resembles other plasminogen activators, containing a plasminogen-binding site and a serine protease domain (Sakharov et al., 1996). The molecule consists of two folded domains of similar size. The mean distance of the centers of gravity of the domains is 3.7 nm. In solution, the mutual positions of the two domains are variable, suggesting that the molecule is shaped like a flexible dumbbell.
Several molecular forms of staphylokinase have been purified with slightly different molecular weights (Mr; 16,500 to 18,000 on sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) and iso-electric points.

2.3.3. Staphylococcus aureus:

*Staphylococcus aureus* is a facultatively anaerobic, Gram-positive coccus, which appears as grape-like clusters when viewed through a microscope and has large, round, golden-yellow colonies, often with hemolysis, when grown on blood agar plates. *S. aureus* is catalase positive and able to convert hydrogen peroxide (H$_2$O$_2$) to water and oxygen, which makes the catalase test useful to distinguish staphylococci from enterococci and streptococci. A small percentage of *S. aureus* can be differentiated from most other staphylococci by the coagulase test. *S. aureus* is primarily coagulase-positive that causes clot formation while most other *Staphylococcus* species are coagulase-negative. However, while the majority of *S. aureus* are coagulase-positive, some may be atypical in that they do not produce coagulase (the most common organism in patients with nosocomial bacteremia is coagulase-negative *staphylococcus. Incorrect identification of an isolate can impact implementation of effective treatment and/or control measures. It is positively regulated by the "agr" gene regulator. It activates plasminogen to form plasmin, which digest fibrin clots.

2.3.4. Production of Staphylokinase:

*Staphylococcus aureus* is one of the common human pathogens. It permanently colonizes the epithelium of 20% of the population, transiently occurs in more than 60% (Foster et al., 2005). Thus *S. aureus* has a simple access to the host organisms and can occasionally cause both acute and chronic infections. These bacteria are responsible for a
wide range of illnesses: from skin and soft tissue lesions like ulcers and furuncles, through food poisoning, to life threatening infections such as bacteremia followed by arthritis, osteomyelitis or endocarditis and septic shock (Foster et al., 2005; Krut et al., 2003). The “invasive success” of *S. aureus* is associated with the broad spectrum of its virulence factors.

This micro organism expresses a lot of secreted and cell-surface polysaccharides and proteins inclusive of many toxins and enzymes, which promote first the bacterial colonization, then the damage of host tissue, spreading of bacteria through organism, immune evasion and finally lead to fully-symptomatic disease (Foster et al., 2005; Heyer et al., 2002), one of these virulence factors is staphylokinase (SAK), also called fibrinolysin.

SAK is 136 amino acid extra cellular protein produced during the late exponential growth phase by *S. aureus* strains carrying the prophages which contain the SAK gene (Bokarewa et al., 2006; Jin et al., 2004). SAK can be regarded as a very important staphylococcal virulence factor, which leads to tissue damage and improves bacterial invasiveness (Bokarewa et al., 2006).

Jin et al., (2003) and Wieckowska-Szakiel M. et al., (2007) reported that *Staphylococcus aureus* isolates from skin and tissue infections shows more staphylokinase production. They also observed that SAK positive strains were less common among the isolates from patients with lethal bacteremia than among nasal carriage isolates. They also reported an unexpected lack of SAK production in Staphylococci invading internal organs in comparision with the colonizing mucosal
tissue. SAK production was observed in almost all staphylococcal isolates obtained from skin and mucosa (Bokarewa et al., 2006; Jin et al., 2004).

Pulicherla et al., (2011) isolated staphylokinase gene from *Staphylococcus aureus* isolated from wound sample. Subathra Devi et al., (2012) screened staphylokinase producing *Staphylococcus aureus* from environmental samples such as milk, water and sewage. Mohana Srinivasan et al., (2013) screened staphylokinase producing *Staphylococcus aureus* from bovine milk samples and production was carried out in by growing them in Satoh’s medium of pH 6.8 at 30°C, at 100 rpm for 24 hours. Pulicherla et al., (2013) construct a multifunctional recombinant staphylokinase variant SRH (SAK- RGD- Hirulog) with reduced reocclusion which can be produced at economical price.

2.3.5. Functional Properties of Staphylokinase:

2.3.5.i. Interaction with plasminogen:

Staphylokinase is not an enzyme but it forms a 1:1 stoichiometric complex with plasmin(ogen) that activates other plasminogen molecule. Streptokinase and plasminogen produce a complex that exposes the active site in the plasminogen molecule without proteolytic cleavage, whereas generation of plasmin is required for exposure of the active site in the complex with staphylokinase.

The role of staphylokinase during bacterial infection is based on its interaction with the host proteins, α-defensins and plasminogen. α-Defensins are bactericidal peptides originating from human neutrophils. Binding of staphylokinase to α-defensins abolishes their bactericidal properties, which makes staphylokinase a vital tool for staphylococcal resistance to host innate immunity.
2.3.5.ii. Fibrin-Specificity of Staphylokinase:

The fibrin-specificity of staphylokinase in human plasma has been explained by rapid inhibition of generated plasmin. Staphylokinase complex by α2- anti plasmin a more than 100-fold reduced inhibition rate at the fibrin surface, which may allow preferential plasminogen activation at the fibrin clot. However, staphylokinase also dissociates in active form from the plasmin. Staphylokinase complex after neutralization by α2,-antiplasmin, and is recycled to other plasminogen molecule. Thus, extensive systemic plasminogen activation with staphylokinase would be expected in plasma, which is clearly in contradiction with its well-established fibrin-specificity.

To elucidate this apparent paradox, the rate and extent of generation of plasmin, staphylokinase complex in human plasma was monitored, both in the absence and the presence of fibrin. When plasmin-staphylokinase complex is formed in the absence of fibrin but in the presence of excess α2- antiplasmin, it is rapidly neutralized and staphylokinase is recycled to other plasminogen molecules. However, conversion of plasminogen staphylokinase to plasmin-staphylokinase does not occur at a significant rate because it is prevented by α2,-antiplasmin; without plasmin-staphylokinase complex, no significant plasminogen activation occurs. In the presence of fibrin, generation of the plasmin(ogen)-staphylokinase complex is facilitated and inhibition of plasmin. Staphylokinase by α2,-antiplasmin at the clot surface is delayed more than 100-fold.

Recycling of staphylokinase to fibrin-bound plasminogen, after neutralization of the plasmin-staphylokinase complex, will result in more efficient generation of
plasmin(ogen)-staphylokinase complex. This mechanism is mediated via the lysine-binding sites of plasminogen and results in a significantly enhanced plasminogen activation at the fibrin surface. These few regulatory properties of fibrin and α2-antiplasmin allow proposing the mechanism for the fibrin-specificity of staphylokinase in plasma.

2.3.5.iii. Immunogenicity of Staphylokinase:

Staphylokinase shows lower immunogenicity as compared with streptokinase. According to the researchers reports, in the first five patients with acute myocardial infarction receiving an intravenous infusion of 10 mg staphylokinase over 30 minutes, neutralizing antibody titers against staphylokinase were low at baseline and up to 6 days after infusion, but high titers (staphylokinase-neutralizing titers of 12 to 42 pg/mL plasma) of antibodies, which did not cross-react with streptokinase, were consistently demonstrable in plasma at 14 to 35 days.

2.3.6. Research on Staphylokinase / Mile stones of Staphylokinase:

Staphylokinase is a Protein found in the culture medium after growth of many strains of *Staphylococcus aureus* that converts plasminogen into the active proteolytic enzyme plasmin. Soru, Sternberg and Istrati (1959) purified staphylokinase by precipitation first with ammonium sulphate and then with ethanol. Davidson (1960) and Glanville (1963) precipitated protein containing staphylokinase from supernatant fluids of cultures by adjusting the pH to 3.3. Glanville precipitated staphylokinase at 75 percent. The dissolved material was further purified by chromatography on carboxymethyl cellulose (CM-cellulose) columns and one homogeneous component with staphylokinase activity was obtained. This preparation proved to contain no haemolysin, leucocidin,
coagulase or hyaluronatelyase, and the staphylokinase was purified about 44 times. The degree of purity of the kinase was controlled by electrophoresis, chromatography and immune diffusion in gel, and these methods indicated homogeneity of the preparation (Collen et al., 1993).

The fibrinolytic effect of staphylokinase was investigated by Lewis (1964), who used enzyme that had been prepared by precipitation with ethanol. The in-vivo tests were made with dogs, and this preparation of staphylokinase proved too toxic for adequate thrombolytic experiments (Vandewerf et al., 1993). Whether the toxicity was dependent on the kinase itself or on some contaminating protein was not revealed. The amino acid composition of staphylokinase was also investigated. Staphylokinase has been purified by means of isoelectric focusing. Further characterization of the kinase with respect to isoelectric point (PI), pH optimum for activity, influence of metal ions, and heat stability has been reported.

Immuno diffusion tests showed the presence of two or four antigens, depending upon how the material had been eluted from the CM-cellulose columns. With proven clinical significance there is need to produce recombinant endotoxin impurity in finished product. Hence in the present study cloning of engineered staphylokinase in yeast is attempted (Klibanov et al., 1993).

2.3.7. Protein engineering of SAK (Site Directed Mutagenesis):

Site-directed mutagenesis is a technique in which a mutation is created at a defined site in a DNA molecule, usually a circular molecule known as a plasmid. In general, site-directed mutagenesis requires that the wild type gene sequence must be known. This
technique is also known as site-specific mutagenesis or oligonucleotide-directed mutagenesis.

A comprehensive analysis of staphylokinase variants by sequential additive site-directed mutagenesis identified several variants with intact specific activities and fibrin-selective thrombolytic potency, which recognized only one third of the antibodies elicited by treatment with wild-type staphylokinase.

The combination mutants SAK (K35A, E38A, K74A, E75A, R77A) in which Lysin35, Glutamic acid38, Lysine74, Glutamic acid75, and Arginine77, when substituted with Alanine results in neutralizing antibodies elicited in patients and SAK(K74A, E75A, R77A, E80A, D82A) in which Lysine74, Glutamic acid75, Arginine77, Glutamic acid80, and Aspartic acid82 were substituted with Alanine, induced less antibody formation than wild-type SAK in patients with peripheral arterial occlusion. However, their specific activities were only 50% of that of wild-type SAK and they displayed a reduced temperature stability.

Reversal from Ala to the wild-type residue of one or more of the 7 amino acids of SAK listed above (ie, Lys35, Glu38, Lys74, Glu75, Arg77, Glu80, and Asp82) revealed that SAK (K74A), with a single substitution of Lysine74 with Alanine, had a high residual specific activity and a slightly reduced temperature stability. It induced statistically significantly lower antibody levels when administered to patients with peripheral arterial occlusion(Collen et al., 1997; Vanderschueren et al., 1995), providing proof of principle that the antigenicity as well as the immunogenicity of SAK can be reduced by site-directed mutagenesis, without reducing its specific activity.
The SAK (K130T, K135R) (lysine at 130 substituted by Threonine, lysine at 135 substituted by Arginine) variant shows a high specific activity with a moderate reduction (to 90%) of the absorption of antibodies from immunized patient plasma. Addition of either K74R or K74Q (lysine74 substituted with arginine or lysine74 substituted with glutamine) to the template decreased the absorption of antibodies from immunized patient plasma to 76% and 62%, respectively. Combination of E65A or E65Q (Glutamic acid65 substituted with alanine or Glutamic acid65 substituted with glutamine) with K74Q (lysine74 substituted with glutamine) in the SAK (K130T, K135R) (lysine at 130 substituted by Threonine, lysine at 135 substituted by Arginine) template resulted in an absorption of antibodies of 55% and 65%, respectively, without markedly reducing the specific activity.

Addition of K35A (lysine35 substituted with alanine) to SAK (E65Q, K74Q, K130T, K135R) (Glutamic acid65 substituted with glutamine, lysine74 substituted with glutamine, lysine at 130 substituted by Threonine, lysine at 135 substituted by Arginine) reduced the absorption of antibodies to 45%, whereas addition of N95A (Asparagine95 substituted with Alanine) slightly increased the specific activity. Addition of E80A+D82A (glutamic acid80 substituted with alanine + aspartic acid82 substituted with alanine) to the SAK (K130T, K135R) template did not affect the specific activity.

Substitution of K136 with A (lysine 136 with Alanine) reduced the reactivity with human antibodies but also the specific activity. Addition of E65D or E65Q (Glutamic acid65 substituted with alanine or Glutamic acid65 substituted with glutamine) to the SAK(K74Q, E80A, D82A, K130T, K135R) template yielded variants with intact specific
activity (without effecting specific activity) that only bound less than or equal to 45 of the antibodies of pooled immunized patient plasma.

Next, the SAK (E65Q, K74Q, K130T, K135R) (Glutamic acid65 substituted with glutamine, lysine74 substituted with glutamine, lysine at 130 substituted by Threonine, lysine at 135 substituted by Arginine) variant (template) displayed a high specific activity with a significant reduction of absorption (to 65%) of antibodies from pooled immunized patient plasma. Addition of K35A, D82A and S84A, of T90A, E99D,(lysine35 with alanine, aspartic acid82 with alanine and serine84 with alanine, of threonine90 with alanine, glutamic acid99 with aspartic acid) and T101S or of E108A and K109A (threonine101 with serine or of glutamic acid108 with alanine and lysine 109 with alanine) reduced the antibody absorption to around 50%, whereas the combined addition of D82A, S84A, (aspartic acid82 with alanine and serine84 with alanine) and E108A, K109A (glutamic acid108 with alanine and lysine 109 with alanine) reduced to 41%.

Substitution of K136A (lysine146 with alanine) combined with the addition of a Lys at the COOH terminus (▼137K) (added lysine at 137) increased the specific activity in a purified system but not in a plasma and further reduced the absorption of antibodies from pooled patient plasma to 30%. Finally, addition of the K35A (lysine35 with alanine), and T90A (threonine90 with alanine,), E99D (glutamic acid99 with aspartic acid), T101S (threonine101 with serine)substitutions to this template yielded a mutant with intact thrombolytic potency, which only bound 24% of the antibodies of pooled immunized patient plasma.

2.3.8. PEGylation (Polyethylene Glycol) studies:

PEG molecules are covalently attached to proteins by lysine residues. Staphylokinase contains as many as 20 lysine residues, some of which are essential for its activity, but it does not contain cysteine. The plasma clearance and the immunogenicity of heterologous proteins may be reduced by derivatization with polyethylene glycol (PEG). To construct thrombolytic agents targeting platelet aggregation PEGylated SAK was produced by site-directed substitution of selected amino acids with cysteine and derivatization with linear PEG molecules containing thiol-specific functional groups, yielding homogeneous end products with reduced clearances.

Previous investigations have discovered that the adhesion of platelets to vessel walls and the activation and aggregation of platelets are extremely important in blood coagulation. On activated platelets, the binding of surface glycoprotein (GPIIb/IIIa) to fibrinogen mediates platelet aggregation. Substituton of K35 with Arg to constitute a RGD (RGD means Arginine Glycine Aspartic acid) motif, resulting in a novel SAK variant, designated as RGD-SAK, which was supposed to be recognized by the activated GPIIb/IIIa on the surface of platelet membrane. GPIIb/IIIa recognizes a conserved
tripeptide Arg-Gly-Asp (RGD) sequence that can be found in all its ligands (Moreadith et al., 2003). Structural studies have reported that the RGD sequence located at the most exposed top of a mobile loop, joining two strands and protruding from the protein core.

In vitro and in vivo evaluation indicated that mono PEGylation prolongs the circulatory half-life of staphylokinase while maintaining its thrombolytic potency when bolus injected at a reduced dose in patients with AMI (acute myocardial infarction). To study mono PEGylation, Serine in position 3 of staphylokinase when mutagenized to Cystine (Abuchowski et al., 1977). The cysteine-substituted variant derivatized with maleimide-PEG (Mal-PEG) will increases the thrombolytic potency and immunogenicity.

PEGylated SAK had an intact apparent specific activity, maintained fibrinolytic potency and fibrin-selectivity in a human plasma milieu, and had a markedly reduced reactivity with anti-SAK antibodies in pooled immunized patient plasma.