

Bacterial Proteases as Thrombolytics and Fibrinolytics

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Abstract: Proteases regulate important pathophysiological processes in human body such as homeostasis, blood coagulation, fibrinolysis, tumor progression, etc. These biological effects of proteases largely attribute to their applicability as therapeutic agents. Imbalance in blood coagulation and fibrinolysis, two important physiological processes in human body, leads to thrombosis, a leading cause of cardiovascular complications including myocardial infarction, stroke, etc. The enzymes used to dissolve thrombus (blood clot) are known as thrombolytic agents and among them, the enzymes involving hydrolysis of fibrin called fibrinolytic agents. Thrombolytic agents can be classified according to generation, mechanism of action, source and active site of the enzymes. Among the commercially available thrombolytic agents, uPA and tPA are generally safe but are very expensive. On the other hand, the bacterial streptokinase is a relatively cheap thrombolytic agent but causes undesirable side effects such as bleeding complications. For this reason, worldwide research for potent thrombolytic agents to prevent and treat cardiovascular diseases have been continuing. Microbes are considered as a potential source of as well as safe vectors for expressing thrombolytic and fibrinolytic enzymes. *Bacilli* are one of the largest groups for this purpose. They have been collected from different traditional fermented foods or have been produced by solid state fermentation using appropriate nutrient substrates including different agro-industrial wastes such as rice straw, molasses, soybean curd residues, etc. This review focuses on different bacterial proteases reported to have potential thrombolytic and fibrinolytic activities.

Key words: Bacterial proteases, fibrinolysis, thrombolytic agents, thrombosis.

Proteases are among the most diverse families of enzymes that catalyze the breakdown of peptide bonds in a protein. They are also called proteinases, peptidases or proteolytic enzymes.¹ This family of enzyme contains a very large and complex group of hydrolytic enzyme and usually catalyzes hydrolysis of proteins. According to Enzyme Commission (EC) number proposed by International Union of Biochemistry and Molecular Biology (IUBMB), enzymes have been classified into six groups. Proteases belong to EC number 3.4 where EC 3 enzymes are hydrolases enzymes and EC 3.4 are those hydrolases that act on peptide bonds. On the basis of the site of action, they can be divided into exopeptidases and endopeptidases², while on the basis of optimal pH range for their activity, proteases

can be classified as acid proteases (pH optima 2-5), neutral proteases (pH optima 7) and alkaline proteases (pH optima 8-11).³ On the other hand, according to the active site, proteases are classified into seven broad groups: serine proteases, cysteine proteases, threonine proteases, aspartic proteases, glutamic proteases, metalloproteases, and asparagine peptide lyases.⁴ Proteases having fibrinolytic and thrombolytic activities are principally serine proteases and metalloproteases.

Proteases are necessary in every aspect of cellular and biological systems. They are ubiquitous and are found in plants, animals and microbes. Based on their presence in living system, they are classified into two groups: intracellular proteases and extracellular proteases. Extracellular enzymes were originally considered as enzymes and used in industry, while intracellular proteases are considered to regulate biochemical reactions in living system. In

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human body, up-regulation of proteolysis is involved in tumor initiation, progression and metastasis, and causes different types of cancer⁵, while dysregulation of proteolysis is involved in various inflammatory and other diseases.⁶ Besides industrial uses, proteases are used as health care product, in diagnostic kit development, and of course as therapeutics in the

treatment of various diseases.⁷ Since 1978, a number of proteases have been approved by Food and Drug Administration (FDA) for therapeutic applications (Table 1)⁶ and many are now in clinical trial. Microorganisms have been considered as preferred sources of the proteases. Bacterial proteases are more

Table 1. List of FDA approved therapeutic proteases.

Type of protease	Name of protease	Brand name	Manufacturer	Mechanism of action	Therapeutic indication	Year of FDA approval	Source
Serine	u-PA	Abbokinase®	Abbott Labs.	Activates plasminogen	Catheter clearing	1978	Urine or primary cell kidney culture
	t-PA (Alteplase)	Activase®, Cathflo, Activase®	Genentech, Inc.	Activates plasminogen	AMI Stroke Catheter clearing	1987 1996 2002	Recombinant, expressed in CHO cells
	Retepase	Retavase®, Rapilysin	Boehringer Mannheim GmbH	Activates plasminogen	AMI	1996	Recombinant, expressed in <i>E. coli</i>
	TNK-tPA	TNKase™, Metalyse®	Genentech, Inc.	Activates plasminogen	MI	2000	Recombinant, expressed in CHO cells
	FIX	BeneFIX®	Pfizer	Activates FX	Haemophilia B	1990 1997	Human plasma Recombinant, expressed in CHO cells
	FVIIa	NovoSeven®, NovoSeven® RT	Novo Nordisk Pharmaceuticals Inc.	Activates FX and FIX	Haemophilia A and B	1999	Recombinant, expressed in BHK cells
	Topical thrombin in bandages	THROMBIN-JMI	GenTrac	Activates fibrinogen	Bleeding	2006	Bovine
Metallo-protease (zinc)	Thrombin	Recothrom®	ZymoGenetics, Inc.	Activates fibrinogen	Bleeding	2008	Recombinant, expressed in CHO cells
	Activated protein C,	Drotrecogin alfa, Xigris®	Eli Lilly and Company	Activates plasminogen	Sepsis, septic shock	2001 (but withdrawn on Oct. 25, 2011)	Recombinant, expressed in human cell line
	Pancrelipase	Creon®, Zenpep®, Pancreaze®	Abbvie (Abbott) Actavis Ortho-McNeil-Janssen Pharmaceuticals Inc.	Helps in protein digestion	Exocrine Pancreatic Insufficiency	2009	Porcine pancreatic extract
	Botulinum toxin A	Botox®	Allergan	Deactivates Syntaxin and SNAP-25	Various muscle spasms	1989	Bacterial (<i>C. botulinum</i>)
	Botulinum toxin B	Myobloc	Solstice Neurosciences	Deactivates Synaptobrevin	Cervical dystonia	2000	Bacterial (<i>C. botulinum</i>)

FDA=US Food and Drug Administration; u-PA=urokinase type Plasminogen Activator; t-PA=tissue type Plasminogen Activator;TNK-tPA=Tenecteplase; FIX=Factor IX; FVIIa=activated Factor VII;AMI=Acute Myocardial Infarction;MI= Myocardial infarction; CHO=Chinese hamster ovary; BHK= baby hamster kidney

significant than animal or fungal proteases and a large number of bacterial species are known to produce serine-type alkaline proteases.⁸ Proteases are also used as useful agents in the treatment of sepsis, digestive disorders, inflammation, cystic fibrosis, psoriasis, etc. besides having predominant use in treating cardiovascular diseases (CVDs).⁶

Thrombus is a blood clot formed inside the blood vessel by a process called thrombosis. Thrombus formation is one of the major causes of cardiovascular diseases (CVDs), for example, myocardial infarction (commonly known as heart attack), stroke, transient ischemic attack (TIA), venous thromboembolism (VTE) including deep vein thrombosis (DVT), pulmonary embolism (PE), etc. CVDs are the major causes of death globally.⁹ As a result, thrombolytic and fibrinolytic agents are gaining more interests day by day. Among the thrombolytic agents, fibrinolytic proteases are considered as the potent agents to treat and prevent CVDs.¹⁰

Blood clotting is a normal physiological process to prevent hemorrhage. Fibrin is the major component of blood clot which is formed from fibrinogen via proteolysis by thrombin. On the other hand, fibrin clots are hydrolyzed by plasmin to avoid thrombosis in blood vessels. During an unbalanced pathophysiological process, hydrolysis of clots may not occur which lead to thrombosis (formation of blood clot).¹¹

Process of blood clotting or thrombus formation

Hemostasis is a process of cessation of blood loss by forming a clot. An obstructive clot when formed inside a blood vessel is called thrombus formation.¹² Hemostasis can be divided into two types: primary hemostasis (immediate platelet activation) and secondary hemostasis (additional coagulation cascade to form fibrin strands).¹³ When circulating platelets are exposed directly to the collagen of damaged endothelium, they bind to collagen with different surface glycoproteins. It results in activation of platelet integrins which adhere platelets to the site of injury.¹⁴ Activated platelets

change shape from spherical to stellate. They release some chemicals including platelet activating factor (PAF), serotonin, thromboxane A₂, etc to activate additional platelets and to subsequently increase the affinity of platelets to bind fibrinogen.¹⁵

Secondary hemostasis involves sequential activation of different inactive enzyme precursors known as zymogens by proteolytic activity in which each activate enzyme causes activation of another zymogen¹⁵ which lead to coagulation of blood. The coagulation cascade is divided into three pathways: (i) tissue factor pathway (or extrinsic pathway) (ii) contact activation pathway (or intrinsic pathway) and (iii) final common pathway.¹⁶ Active FX and FV form a prothrombinase complex with prothrombin which is a proteolytic enzyme that activates prothrombin zymogen to active thrombin.^{17,18}

During tissue factor pathway, thrombin is formed very rapidly and the amount of FVIIa in blood is higher than other coagulation factors. The main function of thrombin is to convert inactive fibrinogen zymogen to active fibrin monomer which then form hemostatic plug with other constituents. Thrombin also has other functions such as platelet activation, activation of FVIII, FV protein C (in presence of thrombomodulin), FXIII (which helps fibrin polymer to form covalent bond to crosslink) zymogens.¹⁵

Thrombolysis and fibrinolysis

The term thrombolysis generally refers to the dissolution of the thrombus while the term fibrinolysis generally refers to the breakdown of fibrin in the blood clot. Fibrin is the end product of the coagulation cascade. Fibrin is dissolved by enzyme plasmin which is a serine protease similar to trypsin. Plasmin is the activated form of plasma zymogen, plasminogen. In human, plasminogen is available as two major glycoforms: type I plasminogen contains two glycosylation molecules and type II plasminogen contains a single glycosylated molecule.¹⁹ When circulating plasminogen binds to cell surface or blood clot, it adopts an open conformation which is cleaved

between Arg-561 and Val-562 to form active plasmin. This cleavage is done by a variety of enzymes like tissue plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), Kallikrein and factor XII.^{20,21,22,23} After plasmin is formed, fibrin is cleaved giving soluble degradation products²⁴ (Figure 1). That means fibrinolysis occurs in two phases: in the first phase, plasminogen is

activated on the fibrin clot surface to form plasmin which dissolved fibrin²⁵ in the second phase, exposing additional binding sites of degraded fibrin leading to amplification of the clot breakdown by plasmin. The primary fibrinolysis is a normal body process, but secondary fibrinolysis occurs due to pharmacological means such as by thrombolytics and fibrinolytics.²⁶

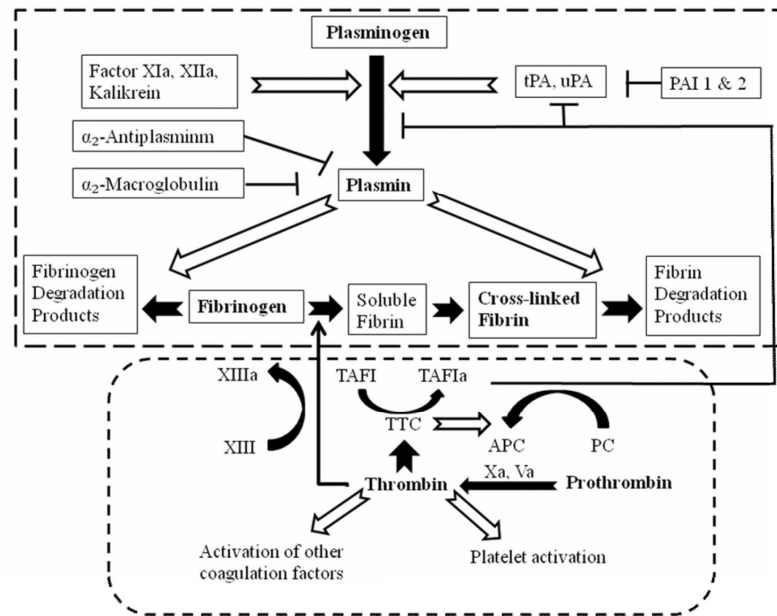


Figure 1. Mechanism of fibrinolysis. tPA= Tissue type plasminogen activator; uPA= Urokinase type plasminogen activator; PAI= Plasminogen activator inhibitor; XI=Plasma thromboplastin, an antecedent serine protease; XII=Hageman factor, a serine protease; XIII=Fibrin stabilising factor, a transglutaminase; TAFI= thrombin-activatable fibrinolysis inhibitor; TTC= Thrombomodulin-thrombin complex; PC= Protein C; APC= Activated protein C, a= Activated form.

Thrombolytic agents: Classification and sources

Thrombolytic agents are the drugs that are used to dissolve blood clots or thrombus formed inside blood vessel, and reopen the vessels (artery or vein). Various CVDs including heart attack, stroke, deep vein thrombosis (clot in a deep leg vein), pulmonary embolism, etc can be treated using thrombolytic agents. Reteplase (rPA or Retavase), alteplase (tPA or Activase), urokinase (Abbokinase), prourokinase, anisoylated purified streptokinase activator complex (APSAC) and streptokinase are available thrombolytic agents (Table 6). On the other hand, all available thrombolytic agents are also classified as 1st, 2nd, 3rd and 4th generation thrombolytic agents.^{27,28} Fibrinolytic agents sometimes are also classified into

two categories: Fibrin specific agents and non-fibrin specific agents²⁹ (Table 2).

However, different thrombolytic agents have different working mechanisms. Based on these mechanisms, the thrombolytic agents are classified into two types. i) Plasminogen activators, which activate plasminogen into active plasmin to degrade fibrin. They may present naturally in blood such as tissue-type plasminogen activator (tPA)³⁰ and urokinase-type plasminogen activator³¹, or may be obtained from bacterial sources, e.g. streptokinase. ii) plasmin-like proteins, which directly degrade the fibrin in blood clots, thereby dissolving the thrombi rapidly and completely. Lumbrokinase from earthworm and fibrolase from snake venom are well-

known plasmin-like proteins.^{32,33} On the other hand, based on active site, thrombolytic agents are classified into i) serine protease (NK, subtilisin DFE, and CK) ii) metalloprotease (jeot-gal enzyme, AMMP, and Bacillokinase II), and iii) both serine and metalloprotease (*R. chinensis* 12 and *Streptomyces* sp. Y405).^{34,35} Thrombolytic agents can be obtained from animal cells, plasma, urine or microorganisms.

The microorganisms producing fibrinolytic enzymes include different phyllums and divisions of bacteria, fungi and algae.³⁶ Various sources including *Actinomyces thermovulgaris*³⁷, *Streptomyces* sp. Y405³⁵, *Streptomyces spheroids* M8-2³⁸,

Streptomyces megasporus SD5^{39,40}, *Bacillus subtilis* BK-17⁴¹, *B. subtilis* A1⁴², *B. subtilis* 168⁴³, *B. thuringiensis* IMV B-7324^{44,45}, *Paenibacillus* sp. IND8⁴⁶, *Pseudoalteromonas* sp. IND11⁴⁷, *Serratia* sp. KG-2-1⁴⁸, *Shewanella* sp. IND20⁴⁹, *Aspergillus ochraceus* 513⁵⁰, *Cochliobolus lunatus*⁵¹, *Fusarium oxysporum*^{52,53}, *Penicillium chrysogenum* H9⁵⁴, *Fusarium pallidoroseum*⁵⁵, *Candida guilliermondii*⁵⁶, *Pleurotus ostreatus*⁵⁷, *Rhizopus chinensis* 12⁵⁸, *Mucor substillissimus* UCP1262⁵⁹, *Codium intricatum*⁶⁰ and *Codium latum*⁶¹ have been used to screen and characterize different fibrinolytic enzymes in recent years.

Table 2. Generation-wise classification of thrombolytic agents.

Generation	Thrombolytic agents	Fibrin Specificity	
1 st generation	Streptokinase	Non-specific	
	Urokinase	Non-specific	
2 nd generation	Recombinant tissue plasminogen activator (t-PA)	Specific	
	Saruplase or Prourokinase (scu-PA)	Non-specific	
3 rd generation	Alteplase	Specific	
	Tenecteplase (TNK-tPA)		
	Reteplase		
	Monteplase		
	Lanoteplase		
	Pamiteplase		
	Staphylokinase		
	Desmoteplase (Bat-PA)		
	Chimeric thrombolytics	Staphylokinase chimeric molecule, Prourokinase chimera, Chimeric t-PA/scu-PA	
	Anistreplase		Non-specific
4 th generation	Plasminogen activator inhibitors (PAIs)	Non-specific	

Available thrombolytic agents

A. Tissue plasminogen activator. *Tissue plasminogen activator* (tPA) is a serine protease and catalyzes the conversion of plasminogen to plasmin and helps in dissolving blood clot. Natural tPA are found in endothelial cells of blood vessels lining, while recombinant tPAs are obtained from microbial sources by recombinant DNA technology. They are used intravenously as thrombolytic agents. The usage of tPA in clinical applications is limited due to short

half-life (4-6 min) and high cost of the treatment. tPA is produced as a single chain (sctPA) glycoprotein containing 527 amino acid which is cleaved at Arg 275-Ile276 to a disulphide linked, two chain form (tc-tPA).⁶² Both forms exhibit equivalent activity when fibrin bound.⁶³ The action of tPA on plasminogen is a weak one and the affinity between t-PA and plasminogen is low in absence of fibrin, while is significantly higher in presence of fibrin. t-PA is the major intravascular activator of

plasminogen⁶⁴ and its gene expression is regulated by histamine, bytyrate, retinoids, arterial levels of shear stress and dexamethanosone independently of PAI-1.¹⁹

The three fibrin specific, genetically engineered tissue-type plasminogen activators (r-tPAs) are

alteplase, reteplase, and tenecteplase (TNKase).⁶⁵ Desmoteplase, another r-tPAs are under Phase III clinical trial and the Danish pharmaceutical company, Lundbeck, holds the worldwide rights for this product. Other recombinant tPAs include lanoteplase, saruplase, anistreplase and pamiteplase (Table 3).

Table 3. Different thrombolytic agents and their comparison.

Thrombolytic Agents	t-PA	u-PA	Streptokinase	Staphylokinase	Alteplase	Reteplase
Molecular Weight	70 kDa	32-54 kDa	47 kDa	16.5 kDa	70 kDa	40 kDa
Source	Naturally in blood	Human neonatal kidney cells	β -hemolytic Streptococci	<i>Staphylococcus aureus</i>	Recombinant DNA technology from human melanoma cell line	Single chain deletion variant of alteplase
Proteolytic nature	Protease	Protease	Not protease	Not protease	Protease	Protease
Plasminogen Activation	Direct	Direct	Indirect	Indirect	Direct	Direct
Fibrin Specificity	S	NS	NS	S	S	S
Indication	AIS, APE, STEMI	APE, MI	STEMI APE, DVT	STEMI, Stroke	AIS, APE, STEMI	STEMI
Plasma Half-life	3-4	15	18	6	4-8	11-14
Antigenecity	No	No	Yes	Yes	No	No
Immunogenicity	No	Unknown	No	Yes	No	No
Side effects	Unknown	Yes	Yes	Yes	No	No
Dosage	Unknown	3mU/hr	1.5 mU/hr	15 mg + 15 mg double bolus	15mg bolus+3hr infusion upto85 mg	Double bolus (10U+ 10U, 30 min apart)

Table 3 contd. right side.

Tenecteplase	Lanoteplase	Saruplase	Anistreplase	Desmoteplase	Pamiteplase
70 kDa	54 kDa	47 kDa	131 kDa	52 kDa	Unknown
Multiple (three) point mutation of alteplase	Deletion and single point mutation of wild-type tPA	Recombinant scu-PA	Anisoylated plasminogen-SK activator complex	From the saliva of the vampire bat <i>Desmodus rotundus</i>	Derivative in tPA recombinant CHO cell lines.
Protease	Protease	Protease	Protease	Protease	Protease
Direct	Direct	Direct	Indirect	Direct	Direct
S	S	NS	NS	S	S
STEMI, Stroke	MI	Stroke	MI	Stroke	APE
20	37+11	6-9	90-112	190	30-47
Unknown	Unknown	No	No	No	Unknown
No	Unknown	No	Unknown	Yes	No
No	Yes	Yes	Unknown	Less	Unknown
0.5 mg/ kg single bolus	120, kU/kg single bolus	20 mg bolus + 60 mg/60min	Unknown	0.125mg/k g single bolus	0.1mg /kg single bolus

APE=Acute pulmonary embolism; STEMI=ST-segment elevation myocardial infarction; DVT=Deep vein thrombosis; AIS=Acute ischemic stroke; S=Specific; NS=Non-specific

(a) **Alteplase:** Alteplase is the first recombinant tissue-type plasminogen activator. The US Food and Drug Administration (FDA) in 1996 approved alteplase for treatment of acute ischemic stroke (AIS). This rtPA is administered through intravenous route and is the only thrombolytic agent approved for AIS to date. This enzyme is the first clinically used t-PA molecule invented by Genentech Inc marketed as Activase®. It is the 1st generation tPA and has less fibrin specificity compared to tenecteplase.^{6,66}

(b) **Retepase:** Reteplase is a second generation non-glycosylated r-tPA expressed in *Escherichia coli*, and was approved by FDA in 1996 for the treatment of AMI. It is a truncated form of tPA and contains 357 amino acids of 527 amino acids of original protein. The innovator Boehringer Mannheim marketed this product in the brand name Retavase®. It has reduced affinity for fibrin as well as the rate of clearance, and increased half-life (13-16 minutes) in plasma because it lacks the N-terminal fibronectin finger, the EGF domain and the first kringle domains compared with the native tPA. For this reason, reteplase can be administered as a double bolus rather than as an infusion, reducing the expense of administration and time.^{67,68} Reteplase has similar efficacy compared to the first-generation tPA, but has less fibrin specificity, and can be administered more conveniently than alteplase.⁶

(c) **Tenecteplase:** Tenecteplase, also known as TNK-tPA was approved by the FDA in 2000. It was invented by Genentech Inc and marketed under the brand name of TNKase®. It is a genetically engineered tPA expressed in mammalian cell line, Chinese Hamster Ovary (CHO) cells. TNK-tPA was developed by site-directed mutagenesis of tPA at three sites: a substitution of an asparagines (Asn) residue for threonine (Thr) 103, a substitution of a glutamine residue (Gln) for Asn117 and tetra-alanine substitutions for residues 296-299 in the protease domain. Tenecteplase contains all protein domains present in the first-generation tPA molecule.⁶⁹ However, mutation in Thr103 improves protein solubility and extends the proteases circulation half-life by creating a new N-linked glycosylation site,

while mutation at Asn117 reduces the clearance rate by eliminating a high mannose glycosylation site, and mutation in residues 296-299 causes limitation to interaction with PAI-1.⁷⁰ In humans, the half-life of tenecteplase is 18 minutes^{71,72} and compared to alteplase and reteplase, tenecteplase is more efficacious than alteplase and reteplase and can be administered as a single bolus.⁶

B. Urokinase or urokinase-type plasminogen activator (uPA). Urokinase is a two chain serine protease and used as a thrombolytic agent in the treatment of pulmonary embolism, coronary artery thrombosis, IV catheter clearance, and venous and arterial blood clots. It is also known as urokinase-type plasminogen activator (uPA) and contains 411 amino acid residues. It is secreted as a single chain glycoprotein named prourokinase (sc-uPA) from endothelial cells, macrophages, renal epithelial cell and some tumor cells. This sc-uPA is converted to a two chain derivative (tc-uPA) following cleavage at Lys158-Ile159 peptide bond by plasmin or kallikrein.^{73,74} uPA has 3 domains: (i) an epidermal growth factor like domain, (ii) a single plasminogen like kringle and (iii) a serine protease domain.⁷⁵ But none of these are responsible for binding tc-uPA to fibrin. As a result, it has a low affinity for fibrin relative to tPA and its effectiveness is not affected by the presence or absence of fibrin.^{76,77} In contrast to the less binding capacity of tc-uPA to fibrin, sc-uPA shows a large binding capacity to fibrin but it has a very low plasminogen activating capacity than that of tc-uPA (<1%).¹⁹ It converts plasminogen to plasmin and has an elimination half life of 12-20 minutes. Initially, urokinase was purified from human urine but now it is produced by tissue culture techniques and recombinant DNA techniques (expressed in *E coli*). It was withheld from the United States market by USFDA in 1998 due to safety issue, but then reintroduced in the market in 2002.

C. Streptokinase. Streptokinase is a first generation thrombolytic agent obtained from *Streptococcus* sp. and is moderately efficacious in practice. It is included in the list of World Health Organization Model List of Essential Medicines.⁷⁸ It

clears approximately 50% of occluded coronary arteries within 90 minutes and reduces mortality upto 25% when administered intravenously.⁷⁹ Streptokinase has a molecular mass of 47 kDa consisting of 414 amino acid (aa) residues.⁸⁰ It was revealed by the crystal structure that SK contains three sequential domains⁸¹, namely α (aa 1 to 150), β (aa 151 to 287), and γ (aa 288 to 411) domains from the amino to the carboxy-termini, linked by flexible loops.³⁵ SK does not have any proteolytic activity as of itself, instead forms a 1:1 stoichiometric complex with a plasminogen or a plasmin molecule.⁸² SK is an indirect plasminogen activator. SK is the drug of choice for thrombolytic therapy, particularly in developing countries, because of its cost effectiveness. As the plasmin, produced through the SK-mediated activation of plasminogen, acts not only on the fibrin network of the thrombus, but also on the SK itself, the in-vivo half-life of SK is limited to about 30 minutes.⁸³

D. *Staphylokinase (SAK)*. Staphylokinase (SAK), is a clot specific plasminogen activator obtained from *Staphylococcus aureus* consisting of 136 amino acid. Even though this specific clot buster found good response in therapy, its use is limited because of its antigenicity and short half-life.⁸³

E. *Nattokinase*. The fibrinolysis mechanism of NK has been explored more extensively than other microbial fibrinolytic enzymes. NK carries out fibrinolytic activity in multiple mechanisms. i) It directly cleaves cross-linked fibrin as its properties largely resemble that of tPA, ii) It activates the production of tPA, which converts inactive plasminogen to active plasmin.^{84,85} Furthermore, NK enhances its fibrinolysis through cleavage and inactivation of the primary inhibitor of fibrinolysis, PAI-1, and regulates total fibrinolytic activity by its relative ratio with tPA.⁸⁶ NK is well-absorbed across the intestinal tract after intraduodenal administration to induce fibrinolysis.⁸⁷

Various characteristics of different thrombolytic agents are compared in Table 3.^{29,88,89}

Biosimilar thrombolytic agents

According to FDA, "A biosimilar product is a biological product that is approved based on a showing that it is highly similar to an FDA-approved biological product, known as a reference product, and has no clinically meaningful differences in terms of safety and effectiveness from the reference product. Only minor differences in clinically inactive components are allowable in biosimilar products." As biosimilars (also known as subsequent entry biologics, biogenerics, or bio-comparables) are not exactly the same as the originator's product, they are not considered as true generics, but rather are only compared to the originator's product biologically and clinically. Biosimilars are intended to be characterized as to demonstrate a high degree of similarity to the reference product rather than demonstrate clinical benefit and are used at the same dose and for the same indication as the reference product like true generics.⁹⁰

Although no biosimilars are approved in Europe, a great boom of biosimilars is developing in Asia to reduce the high cost of biologics and increased financial burden of Asian governments.⁹¹ A large number of biosimilars are also available in other countries like Russia, Canada, etc. Myokinase (company- Biocon) and Shankinase (company - Shantha Biotechnics) are approved biosimilar of streptokinase in India. Shankinase was approved in 2004. Biosimilar of reteplase, MiRel from Reliance Life Sciences was approved in 2009 in India.^{92,93} Elaxim is a biosimilar of tenecteplase from Emcure pharmaceuticals Ltd. which was marketed in 2012 in India. Different marketed thrombolytics and some of their biosimilars are listed in table 4.

***Bacillus* sp. as a Source of thrombolytic and fibrinolytic proteases**

The thrombolytic agents obtained from bacterial sources are considered safe, and the administration of these fibrinolytic agents upon oral administration could increase fibrinolytic activity in human plasma.⁹⁴ That is why, these enzymes, especially from the genus *Bacillus*, could be useful to develop

potent thrombolytic agents.⁹⁵ *Bacillus* is a genus of Gram-positive rod-shaped endospore forming bacteria which is a member of the division Firmicutes and produce extracellular proteases during post-exponential and stationary phases under most culture conditions.⁸ *Bacilli* have a great diversity of strains. These strains need different nutritional medium that some may grow well in a solution of glucose, ammonium phosphate and a few mineral salts, some need additional growth factors or amino acids, and others require increasingly complex nutrition.⁹⁶ Large-scale production of enzymes such as amylases and proteases is possible because of their ability to excrete these enzymes.⁹⁷ Different traditional fermented foods are important sources of genus

Bacillus that have been found to produce the fibrinolytic enzymes.³⁶ In 1987, *B. natto* was first screened from a traditional Japanese soybean-fermented food named natto which produces nattokinase NK.⁹⁸ After that, some other bacilli from different fermented foods were discovered (Table 5). Moreover, *Bacillus* sp. possessing thrombolytic and fibrinolytic properties have also been isolated from other sources including soils recently. For example, recently extracellular enzymes produced by a mutated form of *Bacillus* sp., *Bacillus licheniformis* EMS-O-1 which was isolated from feather decomposed soil have been reported to have thrombolytic activities.⁹⁹

Table 4. Different marketed thrombolytics and their biosimilars.

Drug names	Originator's brand names	Originator	Indication	Biosimilars version	Biosimilar players
Alteplase	Activase®	Genentech	Catheter thrombosis, Myocardial infarction, Pulmonary embolism, Stroke	Alteplase - Nanogen	Nanogen Biopharmaceutical Co
Retepase	Rapilysin®; Retavase®	Roche; Developer: Allergan; Chiesi USA; EKR Therapeutics	Myocardial infarction, Discontinued embolism	MiRel; R TPR 004 BM 06022; r PA; Rapilysin; Retavase	Reliance Life Sciences Shanghai Fudan-Zhangjiang Bio-Pharmaceutical; Developer: Shandong Ahua Biochemical
				Retepase biosimilar- Nanogen Biopharmaceutical	Nanogen Biopharmaceutical Co
Tenecteplase	Metalyse®; RG3625; TNK; TNK-tPA; TNKase	Genentech; Developer: Boehringer Ingelheim; Genentech; Roche Canada	Myocardial infarction, Discontinued, Catheter thrombosis, Heart arrest	Elaxim	Emcure pharmaceuticals Ltd.
Streptokinase suppository - Heber Biotec	Proctokinase ; rSK suppository - Heber Biotec	Center for Genetic Engineering and Biotechnology; Developer: Heber Biotec	Haemorrhoids	Sedonase	Sedico
				Myokinase; Recombinant streptokinase - Biocon	Biocon
				Prokinase	Emcure pharmaceuticals Ltd.
				Shankinase	Shantha Biotechnics

Table 5. Fibrinolytic enzyme producing *Bacilli* from different traditional foods.

Microorganism	Source	Origin	Name of enzyme	References
<i>B. natto</i>	Natto	Japan	Nattokinase	Fujita et al., 1993 ¹⁰⁰
<i>Bacillus</i> sp. CK	Chungkook-jang	Korea	CK	Kim et al., 1996 ¹⁰¹
<i>Bacillus</i> sp. KA38	Jeot-gal	Korea	Jeot-gal enzyme	Kim et al., 1997 ¹⁰²
<i>B. subtilis</i> IMR-NK1	Natto	Taiwan	-	Chang et al., 2000 ¹⁰³
<i>Bacillus</i> sp. DJ-4	Doen-jang,	Korea	Subtilisin DJ-4	Kim and Choi, 2000 ¹⁰⁴
<i>B. amyloliquefaciens</i> DC-4	Douchi	China	Subtilisin DFE	Peng et al., 2003 ¹⁰⁵
<i>B. subtilis</i> QK02	Fermented soybean	-	QK-1 and QK-2	Ko et al., 2004 ¹⁰⁶
<i>B. firmus</i> NA-1	Natto	Japan	-	Seo and Lee, 2004 ¹⁰⁷
<i>Bacillus</i> sp. DJ-2	Doen-jang	Korea	bpDJ-2	Choi et al., 2005 ¹⁰⁸
<i>Bacillus</i> sp.	Fermented shrimp paste products	Vietnam	-	Anh et al., 2013 ¹⁰⁹ , Anh et al., 2015 ¹¹⁰
<i>B. coagulans</i>	Terasi and Jambal roti	Indonesia	-	Prihanto et al., 2013 ¹¹¹
<i>Bacillus</i> sp.	Chao Vinh Phong	Vietnam	-	Linh et al., 2013 ¹¹²
-	Fermented fish paste products	Vietnam	-	Uyen et al., 2013 ¹¹³
<i>B. amyloliquefaciens</i>	Traditional soybean-fermented products	Vietnam	-	Huy et al., 2016 ¹¹⁴

Table 6. *Bacilli* from various agro-industrial substrate showing thrombolytic activity.

Microorganisms	Substrate	Specific activity (U/mg)*	Purification fold*	Yield (%)*	Reference
<i>Bacillus subtilis</i>	Soybean curd residues	-	-	-	Zu et al., 2010 ¹¹⁸
<i>Bacillus amyloliquefaciens</i>	Chick peas	-	-	-	Wei et al., 2011 ¹¹⁹
<i>Bacillus subtilis</i>	Red bean	-	-	-	Chang et al., 2012 ⁵⁹
<i>Bacillus altitudinis</i> GVC11	Castor husk	-	-	-	Madhuri et al., 2012 ¹²⁰
<i>Virgibacillus</i> sp. SK 37	Brewery yeast sludge	-	-	-	Lapsongphon et al., 2013 ¹²¹
<i>Bacillus subtilis</i> I-2	Soybean meal	135.75	4.8	10.4	Bajaj et al., 2014 ¹²²
<i>B. amyloliquefaciens</i> FCF-11	Corn husk	23,862.8	443.5	17	Kotb, 2014 ¹²³
<i>Bacillus cereus</i> IND1	Wheat bran	235	3.1	19.9	Vijayaraghavan and Vincent, 2014 ¹²⁴
<i>Bacillus subtilis</i>	Chick peas	115239	38.07	9.08	Xiao et al., 2014 ¹²⁵
<i>Bacillus cereus</i> IND5	cow dung	-	-	-	Biji et al., 2016 ¹²⁶
<i>B. halodurans</i> IND18	Wheat bran	501.8	3.6	20.4	Vijayaraghavan et al., 2016 ¹¹⁵
<i>Bacillus</i> sp. IND12	cow dung	-	-	-	Vijayaraghavan et al., 2017 ¹²⁷

*DEAE cellulose.

Production of bacterial thrombolytic and fibrinolytic enzymes from agro-industrial wastes

Enzymes can be produced by submerged fermentation (SmF) and solid state fermentation (SSF). SSF has many advantages over SmF.⁹⁵ In SSF, fibrinolytic enzymes are produced using many substrates (Table 6). Cost and availability of the substrates, and nutrient composition of the selected

solid waste are the main criteria for the selection of an ideal substrate for SSF. Cow dung medium may be considered as a promise and in expensive substrate for fibrinolytic enzyme production. Normally, 80% moisture content is maintained for the production of fibrinolytic enzyme. In SSF, moisture content is one of the critical factors.¹¹⁵ Cow dung substrate has high moisture-holding capacity, which facilitates the

production of fibrinolytic enzyme from bacterial species. The optimum moisture content for enzyme production may vary depending on the organism and substrate used in SSF process.¹¹⁶ Various carbon and nitrogen sources were used for enhanced production of fibrinolytic enzyme.¹¹⁵ It was revealed that maximum fibrinolytic enzyme production is favored by starch, followed by sucrose. This result was in agreement with the previous study on proteolytic enzymes from *Bacillus* sp.¹¹⁷

CONCLUSION

In this review, protease enzymes with their classifications, therapeutic uses, uses as thrombolytic and fibrinolytic agents have been discussed. Different microbial sources of proteases including bacterial proteases as well as their relative studies and production from traditional fermented foods have also been highlighted. Microbial thrombolytic and fibrinolytic enzymes have attracted much more medical interest in recent decades. Proteases produced by *Bacillus* sp. have been identified to possess thrombolytic and fibrinolytic activities. These proteases are mainly serine proteases such as nattokinase, subtilisin, etc. Exploration of these microbes, specially *Bacillus* sp. may come out with potential sources of enzymes of pharmaceutical and industrial interests.

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