Lumbrokinase – A Potent and Stable Fibrin–Specific Plasminogen Activator

Mahendra Kumar Verma[1], KK Pulicherla[2]

[1]*Lecturer, Department of Biotechnology*
[2]*Professor & Head, Department of Biotechnology*
RVR & J C College of Engineering, Chowdavaram, Guntur-522 019, Andhra Pradesh, India
mahendra.sonal1983@gmail.com, pkkanth2006@gmail.com

Abstract

Cardio and cerebrocascular disorders lead to about 26 million deaths every year around the world. Cardio and cerebrovaccular disorders have not only high mortality rate across the globe but also leading to subsequent complication such as thrombolysis could favorably influence the outcome of such life-threatening disorder as myocardial infarction, cerebrovascular thrombosis, and venous thromboembolism. Our healthy system is capable to overcome to these consequences but when there is imbalance of defensive and aggressive factor in our system result come as blood clot in systemic circulation. Now role of thrombolytic agents come in picture as artificial plasminogen activators that convert plasminogen, an inactive form of plasmin to dissolve the clot by converting inactive plasminogen in active plasmin. Plasmin dissolves the fibrin blood clot, but may also degrade normal components of the hemostatic system which can further create another life threatening consequence and death also so there is always need of such an agent who specifically dissolved clot which are in circulation but not others.

Keywords: *Thromboembolic disorders, plasminogen activators, Lumbrokinase, Earthworm Fibrinolytic Enzyme*

1. Introduction

A blood clot (thrombus) develops in the circulatory system can cause vascular blockage leading to serious consequences including death. According to World Health Organization (WHO) report year 2008-2010 numbers of deaths due to cardio and cerbrovascular disorder is comparatively large than any other disorder or disease. A healthy homeostatic system suppresses the development of blood clots in normal circulation, but reacts extensively in the event of vascular injury to prevent blood loss. [1] The consequences of a failed hemostasis include stroke, pulmonary embolism, deep vein thrombosis and acute myocardial infarction. Pathologies involving a failure of hemostasis and the development of clot which requires clinical intervention consisting of administration of artificial thrombolytic agents. Lumbrokinase is one such agent. Other thrombolytic or fibrinolytic agents include streptokinase, urokinase and the tissue type plasminogen activator (t-PA). [2]

A progressive research is going on to find out effective thrombolytic drugs with minimum risk hence numerous trails have been conducted with the various thrombolytic agents but could not active to target. Reason most of them get failed in certain aspect as specificity, side effect or cost factor. Hence there is an urgent need of such an agent which can full fill all

above criteria. A lot of research is going on for the hunt for potent thrombolytic agent. As a result researcher has found lumbrokinase a potent fibrin specific thrombolytic agent to cure the life threatening cardio and cerebrovascular disorder. Recombinant lumborkinase formulations are commercially available in China and Korea since long time as capsule and in other dosage form for the treatment of cardiac thromboembolic disorder [3]. The use of lumborkinase in thrombolytic therapy holds great promise for becoming an important therapeutic adjunct in the treatment of acute vascular occlusions, but such therapy has not reached the stage for general clinical use at global level. Till date proper method of administering thrombolytic agents has not yet been determined, and the processes that bring about lysis of clots are incompletely understood [4]

This review will focus on the biochemical and thrombolytic properties of Lumborkinase, an eukaryotic protein exist in six isoforms (isoenzyme) form tissues and intestinal fluid of earthworm with the fibrin specific plasminogen activator that would appear to have potential thrombolytic agent for treatment of cardio and cerebrovaccular disorder.

Blood Clot Formation

Blood Clot formation is a dynamic process which leads to clot formation and dissolution simultaneously in a controlled fashion. Once clot formed has to be dissolve to make homeostatic balance. In the normal blood circulation blood clotting factor runs in inactive form and form clot when it needed and activates clot dissolving factor to cleanup.

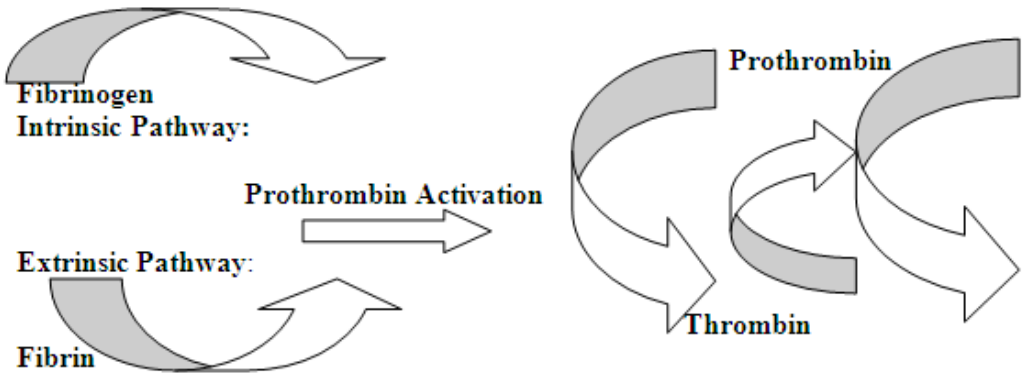

Fig: 1 A Cascade of Blood Plasma Protein Govern Clot Formation

The blood clot formation is defensive mechanism of our system which regulated by various factors and by their cooperative work a blood clot is going to form. There are two mechanism proposed earlier to describe entire mechanism of clot formation, Intrinsic and Extrinsic which involves various blood clotting factor which run in a cascade way to achieve the process. In short 13 factors which generally circulate in inactive form in blood get activated with proper signal and activation of these factors is not random, work in cascade way i.e. one is going to activate other in systemic way and finally result in soluble fibrinogen into insoluble fibrin. After word activation of fibrin dissolving factor which cleanup the blood clot. [5] Hence a healthy system maintains a homeostatic balance in between the defensive and aggressive factor and their availability in the systemic circulation.

2. Thrombolysis or Fibrinolysis

The fibrinolytic enzyme, plasmin, like most other proteases, is capable of acting upon a number of different protein substrates in addition to its physiologic substrate fibrin like proteinaceous clotting-factors like fibrinogen, proaccelerin, antihemophilic factor, prothrombin and Christmas factor, and certain other blood proteins. But in the healthy blood circulation plasmin exist with its inhibitors antiplasmin as Plasmin- Antiplasmin complex which regulates the plasmin activity. Antiplasmin is 30 fold higher than plasmin in plasma concentration act as defensive factor for breakdown of other plasma protein [6]

The complete physiology of the fibrin–clot formation is relatively well understood. A blood clot or thrombus is consists of blood cells occluded in a matrix of the protein fibrin. Thrombolysis or fibrinolysis is enzyme regulated process for dissolution of clot. In mammalian circulation, the enzyme responsible for the fibrinolysis is plasmin which is a trypsin-like serine protease [7]. In the presence of activator the fibrinolytically active plasmin is produced from the inactive protein plasminogen which is present in systemic circulation. The biochemical conversion of the inactive plasminogen to fibrinolytic plasmin involves a limited proteolytic cleavage which is mediated by the various plasminogen activators [8].

Generally two plasminogen activators that occur naturally in blood are the tissue type (t-PA) and the urokinase type (u-PA). The fibrinolytic activity in circulation is regulated by inhibitors of plasminogen activators (e.g., plasminogen activator inhibitor-1, PAI-1, a fast-acting inhibitor of t-PA and u-PA) and plasmin (e.g., a1-antiplasmin, a2 macroglobulin. Recombinant forms of normal human plasminogen activators t-PA and u-PA are used in clinical intervention. Another commonly used plasminogen activator is Lumbrokinase (l-PA), an eukaryotic protein form earthworm that is having fibrin specific thrombolysis too [9]

Apart from Lumbrokinase, t-PA and u-PA do not have a direct fibrinolytic activity and their therapeutic action is via the activation of blood plasminogen to the clot dissolving plasmin. Thus, the plasminogen activating action of Lumbrokinase is fundamentally different from the proteolytic activation brought about by t-PA and u-PA. So plasmin is always present in systemic circulation but in the complex form with its inhibitor Antiplasmin. So it is antiplasmin which protect other blood plasma protein form the degradation by plasmin [10]

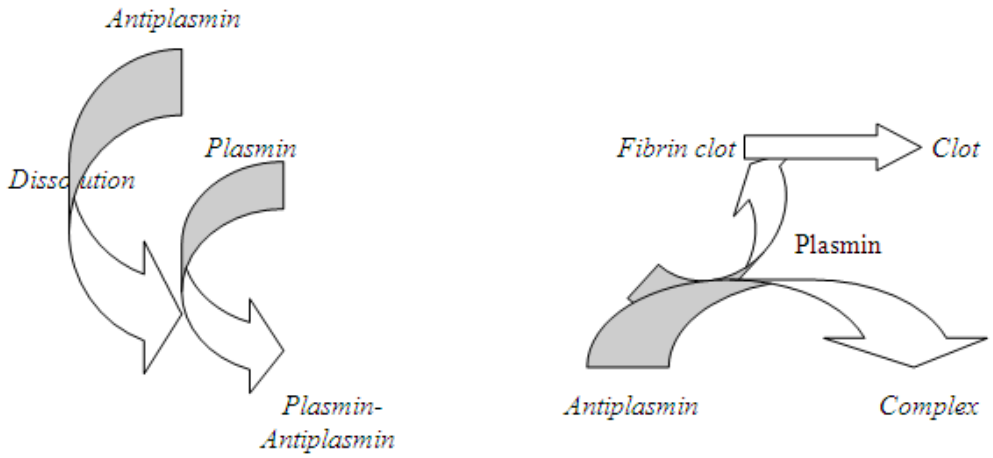

Fig: 2 A Schematic Representation of Role of Antiplasmin (Plasmin Inhibitor) in Clot Dissolution Mechanism.

Current Treatment Regime for Thrombolytic Therapy:

Present scenario of thrombolytic therapy driven by recombinant Plasminogen Activators or plasmin specific thromolyitc agents. Under the current clinical application streptokinase, APSAC (Anisoylated plasminogen streptokinase activator complex- APSAC), Urokinase, scu-PA. Tissue plasminogen activator has been used in last two decades are as t- PA, rt- PA (altepalse), r- PA (reteplase), TNK-rt-PA. Current with the new advancement few third generation agents are as n- PA (Lanoteplase), bat- PA (Desmodus rotundus), Bifunctional molecules conjugating t-PA with monoclonal antibody [11].

Thrombolytic Agents Approved by the Food and Drug Administration

Following thrombolyitc agents has been approved by FDA for the thromolytic therapy and are in clinical use with the better therapy.

- Streptokinase
- Urokinase
- Altepalse
- APSAC (Anisoylated plasminogen streptokinase activator complex- APSAC)
- Reteplase
- TNKase

All of listed above are the plasminogen activators, activating circulating inactive plasminogen into active plasmin which degrade the fibrin clot. All of above listed drug molecules are in clinical use for the thrombolytic therapy with numerous complication and side effects. Reason for thrombolytic therapy complication most of these molecule are not fibrin specific and with fibrin they also degrades other circulating plasma protein which actually a healthy system needed for normal homeostasis. So still there is hunt of a drug which will be fibrin specific and possibly Lumbrokinase will be ones of them as it activates tissue plasminogen activators and also having fibrin specific thrombolysis.

Recombinant forms of normal human plasminogen activators t-PA and u-PA are used in clinical intervention. Both t-PA and u-PA are trypsin-like serine proteases which activate plasminogen directly and having the respective molecular weights of 70 and 55 kDa. Both tPA and uPA are glycoprotein's. u-PA is produced in the kidneys and secreted into the urine [12] whereas tPA is produced by the vascular endothelial cells [13]. Tissue Plasminogen Activator (t-PA) is the choice for therapeutic use is obtained mainly from cultures of recombinant animal cells [14].

Because of its high affinity for the fibrin clot, t-PA activates the clot bound plasminogen 100-fold more effectively than the circulatory plasminogen. Consequently, t-PA is a poor activator of circulatory plasminogen relative to the other available plasminogen activators. But the cost point view t-PA is highly costly as its cloning and expression needs animal cell line as being a eukaryotic protein also larger size is not possible in yeast and bacterial host system due to inability to post translational modification of eukaryotic protein. Due to lack of recognized plasminogen activators, attempts are underway to develop improved recombinant variants of these compounds to achive better results [15].

Hunt for Ideal Thrombolytic Agent

There are numerous drugs and protein which were used for the treatment of cardio and cerebrovascular disorder but results were not up to mark. So researcher are always in the hunt of such an agent which can provide the medication up to level and also satisfy other

parameter like cost factor. Here are some features listed below which supposed to characteristic of thrombolytic agent. Characteristics of the Ideal Thrombolytic Agent:

- Rapid reperfusion.
- Easy Administration.
- Fibrin specific.
- Slow renal clearance
- Chemically stable
- Less first bypass
- Low incidence of systemic bleeding.
- Low incidence of intracranial hemorrhage.
- Resistant to plasminogen activator inhibitor-1 (PAI-1).
- Low reocclusion rate
- No effect on blood pressure.
- No antigenicity
- Reasonable cost

Most of thrombolytic agents what we are using now for the thrombolytic therapy are unable to show all the characteristic. Majority of them are having limitation either for substrate specificity and same time also dissolve necessary blot cloting protein and factor which are essential for normal homeostasis. Hence it is triggers us to search the novel molecule or refinement to the existing one for ideal drug molecule. Hence a lot of progressive research is going on achieve the goal.

3. Lumborkinase

Historical Overview:

Earthworms are the key representatives of the soil fauna and are essential in maintaining soil fertility through their burrowing, ingestion and excretion activities [16]. Around over than 8000 described species worldwide, existing everywhere but in Polar and arid climates [17]. They are increasingly recognized as indicators of agro ecosystem health and eco toxicological sentinel species because they are constantly exposed to contaminants in soil. The following earthworm species (e.g. *Eisenia andrei*, *Eisenia fetida* and *Lumbricus terrestris*) widely used in standardized acute and reproduction toxicity tests belong to the Lumbricidae family (phylum, Annelida; class, Clitellata; subclass Oligochaeta; order, Haplotaxida; super family, Lumbricoidea; family, Lumbricidae.

The fibrinolytic enzymes in the earthworm, *Lumbricus rubellus*, were extracted and named lumbrokinase a collective name for six fibrinolytic iso-enzyme proteins having molecular weights of 25 to 32 KDa. [18] In the year 1992 a study on the crude extract of earthworm shown to have potent thrombolytic effect. Lumbrokinase exist as is iso-enzyme in the intestine and tissue fluid and intestinal fluid of earthworm. The reason why earthworm is having such a potent protease as earthworm feed on the debris of plant and organic matter so digest all these probably it produce Lumbrokinase a serine protease. As research progress LK was isolated from other species of earthworm also and was having fibrinolytic activity. Several investigators purified and further characterized the fibrinolytic enzymes in *L. rubellus* [19] and found the hydrolysis of the plasmogen-rich fibrin and plasmogen-free fibrin. The fibrinolytic enzymes dissolve blood fibrin clots, which are important for clinical application as chemotherapeutic agents [20].

Isolation of Lumbokinase:

Lumbrokinase a collective name for six fibrinolytic iso-enzyme proteins having molecular weights of 25 to 32 kDa. F1 – F6. The fibrinolytic activities of the all six isoforms was found in the following order F6 > F2 > F5 > F3 > F1 > F4. (23) Hwan Cho et al 2003 Six isoform of Lumbrokinase (*Lr*PI- 0, *Lr*P-I-1, *Lr*P-I-2, *Lr*P-II, *Lr*P-III-1, and *Lr*P-III-2) of fibrinolytic enzymes were isolated from *L. rubellus* [3, 4, 23]. The molecular masses of the isozymes measured by ion-spray mass spectrometry are 23,013; 24,196; 24,220; 24,664; 29,667; and 29,662, respectively. Usually in their peptide chains asparagine and aspartic acid residues are comparative less lysine. May be this is the reason as Lumbrokinase have greater stability in organic and inorganic solvent. They have a wide functional acidic range (pH 1.0–11.0) and do not inactivate until 60◦C. The range of enzyme activity (*Lr*P-II and *Lr*P-III-1) is maximally exhibited around pH 9.0 at 50◦C [21].

In the year 1988, Zhou and coworkers isolated least seven components with fibrinolytic activity from earthworm *E. fetida* and found they are stable at pH 5.0– 9.0 and denaturated below pH 2.6. The enzyme a member of serine protease and its molecule weight 45kDa. The two constituting subunits (26k Da and 18kDa) with different fibrinolytic activities are bound by hydrophobic interaction. Further isolation listed eight fibrinolytic enzymes as (*Ef* P-0-1, *Ef* P-0-2, *Ef* P-I-1, *Ef* PI- 2, *Ef* P-II-1, *Ef* P-II-2, *Ef* P-III-1, and *Ef* P-III-2) through a stepwise-purification procedure in 2007 [22].

Mechanism of Action:

The Lumborkinase (LK) group of proteolytic enzymes, extracted from the researched species of earthworm, includes plasminogen activator and fibrin specific serine protease. The mechanism to activate plasminogen in LK is similar to tissue plasminogen activator (t-PA) from other sources, which makes it possible to show the thrombolytic activity only in the presence of fibrin. The mechanisms of action of Lumborkinase include participation in the activation of plasminogen, and direct activity on fibrin itself. Lumbrokinase primarily proteolyzes fibrinogen and fibrin, hardly hydrolyzing other plasma proteins including plasminogen and albumin. The enzymes lumbrokinase have very strong fibrinolytic activity, are stable in a wide pH range, and show great stability against thermal inactivation and degradation. Lumbrokinase are alkaline trypsin-like proteases that are greater than trypsins in their stability and tolerance to organic solvents.

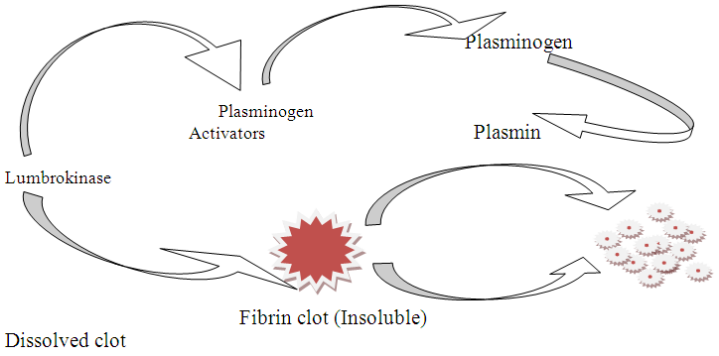

Fig: 3 Diagrammatic View of Lumbrokinase Mediated Thrombolysis. Hence Lumbrokinase is not only plasminogen activators but also fibrin specific thrombolytic agent.

Lumbrokinase shows double thrombolytic effect and that is specific in one way it activates plasminogen activator and start the normal cascade of our own system to dissolve the blood clot and also it is having enormous affinity towards fibrin and specifically dissolved clot.

Vilhardt and Lundin investigated whether earthworm fibrinolytic enzyme III-1 (EFE-III-1) isolated from Lumbricus rubellus is capable of transporting into blood through intestinal epithelium and keeping its biological function in circulation, they raised an antibody against EFE-III-1. The immunological results showed that 10-15% of complete EFE-III-1 was absorbed by the gastrointestinal epithelia [23]. The further evidence for intact absorption of enzyme was concluded by immunohistochemistry approach and the presence in the intestinal epithelial cells. The experiments were conducted on animal model, which concluded the immunoreactive intact EFE-III-1 was found in serum or plasma after intraperitoneal injection of rats. During the following study it was found 10% of the intact enzyme could transport through the intestinal epithelium. The further confirmation for the maximum remaining activity in blood was assayed around 60 min after the intraperitoneal injection [24].

Gene Structure of Lumbrokinase

As per the data form the GenBank there are eight cDNA clones of lumbrokinase are available which are as (GenBank Accession Nos.; AY438622; U25644, AY178854; AY187629; AF304199; AF433650; U25648; and U25643), the coding region of the lumbrokinase cDNA is 852 bp in length and encode 283 amino acids, of which the first 36 amino acids consist of a signal peptide and the last 247 amino acids form the mature protein. (25) The nucleotide sequence of each cDNA clone was analyzed and it was found that there are 13 codons containing the "CG" motifs in the whole sequence, which are relatively rare for mammalians and may not be efficiently translated after being transformed into mammalian cells or tissue [26]. The c DNA of *Eisenia foetida* EFE-3 contained 859 nucleotides, with an open reading frame starting from 112 to 853, encoding a polypeptide of 247 amino acid residues [27] The extracellular enzyme lumbrokinase is isolated by various species of earthworm but are having same mechanism for fibrinolytic action indirectly by activating the circulatory plasminogen and direct on fibrin.

Protein Structure of Lumbrokinase

The protein sequence of various species of earthworm has been analyzed in order to have complete proteomics information. The protein sequences of the isozymes from *L. rubellus* and *E. fetida* have a lot of identical residues. The proteins have distinct predicted secondary structures, for example, β-sheet, α-helix, turn, and coil. The sequence of a isoform of lumbrokinase called as Earthworm fibrinolytic protease II (*Ef* P-II (EFEa)) is highly similar to some related serine proteases with known structures [28-30] or other earthworm serine proteases [31] the catalytic characterization of the earthworm protease is influenced directly by their tertiary structures. The NMR and X ray study shows that *Ef* P-III- 1 (EFE-b) is a trypsin-like protease with two chains (an N terminal, pyroglutamated light chain and an N-glycosylated heavy chain) [32]. The structural features (Figure 4) probably endow *Ef* P-III-1 with high level of stability in resistance to heat, organic solvents, and proteases [33]. The another isoform of Lumbrokianse *Ef* P-II is not only a chymotrypsin-like serine protease but also has an essential S1 pocket of elastase.

Recombinant Lumbrokinase

The isolation and purification of native lumbrokinase is form earthworm is tedious, multistep and hence time consuming and even there will be chance for impurities of other protein. So researcher has started cloning and expression of lumbrokinase gene into prokaryote and eukaryote system form the different species of earthworm and majority of them got success. In the year 2003 Hwan Cho, Eui Sung Choi and Hyung Hoan Lee successfully cloned the lumbrokinase gene form *Lumbricus rubellus into* the bacterial system. The complete c-DNA sequence of the lumbrokinase gene form Lumbricus *rubellus* was amplified using an mRNA template, sequenced and expressed in *E. coli* cells [34].

The most potent isoform of Lumbrokinase for fibrinolysis F6 protease gene consisted of pro- and mature sequences by gene sequence analysis, and the protease was translated and modified into active mature polypeptide by N-terminal amino acid sequence analysis of the F6 protease. The gene structure of F6 protease consisted of the 44 residues from methionine-1 to lysine-44 which is a pro-region and the mature polypeptide which is 239 amino acid residues and one stop codon; 720 bp) which started from isoleucine-45 and continued to the terminal residue. The F6 protease gene was expressed in *E. coli* system as a pro-mature sequence and mature sequence produced inclusion bodies. After the expression into *E coli* system these inclusion bodies were given orally to rats, where thrombus was already generated a drastic reduction in size approximately 60% versus controls. Further these inclusion bodies have shown hemolytic activity when solubilized in pepsin or trypsin solutions. Consequently it was concluded the F6 protease has hemolytic activity, and that it is composed of pro- and mature regions. [35]

Zhe-rong Xu and their team constructed and expressed the mature peptide of LK PI239 in Escherichia coli; we amplified and optimized the gene of LK which was then cloned into the prokaryotic expression vector pET-22b. The engineered construct LK (rLK) was expressed as inclusion bodies and we have developed a purification process of rLK from these inclusion bodies. Further urea concentration strategy was applied to the rLK renaturation process in order to have mature protein. The purified and renatured rLK was successfully analyzed in the animal model and has shown sufficient thromolysis, which again confirms LK as therapeutic agent for thrombotic-associated diseases. [37].

Being a eukaryotic protein it has always problem in the expression in the prokaryotic system as the prokaryotic system lacks post translational modification and protein folding machinery. In this order many Lumbrokinase genes have been cloned, expressed and sequenced but the expression of gene has not achieved up to optima due unknown reason or codon bias. The coding region of the mature lumbrokinase c-DNA is 852 bp in length and which encodes 283 amino acids, of which the first 36 amino acids consist of a signal peptide (pro-peptide) and further last 247 amino acids form the mature protein. The sequence of each cDNA clone was completely analyzed and was found that there are 13 codons containing the "CG" motifs in the whole sequence, which are relatively rare for mammalians and may not be efficiently translated after being transformed into mammalian cells or tissue. Hence those codon were optimized in order to have optimum expression [38].

The Position and Optimized Codon for Better Expression of Lumbrokinase:

S. No.	Codon No. Position	Original Codon	Optimized Codon	Amino Acids
1.	8	TCG	TCA	SER
2.	11	GCG	GCA	ALA
3.	57	CCG	CCA	PRO
4.	63	CGA	AGA	ARG
5.	80	CGT	AGT	ARG
6.	109	GCG	GCT	ALA
7.	113	CGT	AGG	ARG
8.	166	CGT	AGA	ARG
9.	188	CGA	AGA	ARG
10.	191	ACG	ACT	THR
11.	216	GCG	GCT	ALA
12.	217	TCG	TCA	SER
13.	267	CGC	AGG	ARG

To achieve better expression and folding of mature Lumbrokinase protein researcher has started cloning and expression in the eukaryotic system. During the lumbrokinase PI239 is expression in *Escherichia coli*, it possesses an extra cysteine residue and is produced as an inclusion body. Hence a renaturation procedure is necessary to obtain a mature protein. In addition, because *E. coli* is a prokaryote system it is unable to perform many eukaryotic posttranslational modifications such as proteolytic processing, folding, and glycosylation etc.

The consequence the recombinant lumbrokinase expressed into *E. coli* system *has* low enzymatic activity even after the denaturation and renaturation procedure. As a result there is need of an eukaryotic system where expression and posttranslational modification will be possible. (39) As a eukaryotic system *Pichia pastoris* has been developed into a highly successful system for the expression of heterologous genes. Majority of fibrinolytic enzymes, including streptokinase and urokinase plasminogen activator, have been expressed in *P. pastoris.* Most of them use GS115 as the expression host and pPIC9K as the expression vector. Recently, an effective system for expression of lumbrokinase (F-III-2) was developed by using *P. pastoris* GS115 as the host strain and pPICZ_-A as the expression vector pPICZ_-A is easier than pPIC9K to manipulate, and selection for the vector in both *P. pastoris* and *E. coli* is based a single small, dominant selectable marker that confers Zeocin resistance [40].

With the successfully complete set of processes for the screening, expression, and fermentation of lumbrokinase in yeast was established. Further with the high density fermentation of engineered yeasts was performed for the first time high density fermentation of yeasts engineered to express lumbrokinase was performed, and a preliminary investigated of the conditions needed for recombinant lumbrokinase expression by high density fermentation was conducted.

4. Recent Advancement of Lumbrokinase:

Crystallization

Even the complete three Dimensional structural information of lumbrokinase is not available. In order to have the information of lumbrokinase protein molecule researcher has started crystallization of lumbrokinase. Hence the initial crystallization studies for lumbrokinase were carried out using the hanging-drop vapour-diffusion method following the sparse-matrix approach (44) (Jancarik & Kim, 1991). Microcrystals were obtained in a system

containing 1.0 M (NH4)2SO4 as precipitant, 2.5% PEG 400 as additive and 0.05 M HEPES buffer pH 7.2 at room temperature. After enormous optimized crystallization conditions, the temperature was adjusted to 288 K and the initial drop solution was adjusted to contain 5 mg mly protein, 0.6 M (NH4)2SO4, 2.5%(v/v) PEG 400 and 0.05 M MOPS buffer pH 7.2, with the reservoir solution containing 2.0 M (NH4)2SO4, 5.0%(v/v) PEG 400 and 0.10 M MOPS buffer pH 7.2 [41].

F0F1-ATPase Micro- mixer

To archive the maximum enzyme activity and efficiency researcher has invent new technology for better utilization of lumbrokinase for the treatment of cerebro and cardiovascular disorders. F0F1-ATPase is a key enzyme in the biological world and one of the most ubiquitous proteins in nature . The Enzyme ATP synthase an universal enzyme that synthesizes ATP, the fuel that powers most of the cellular processes. The enzyme consists of two parts; the F1 motor generates a mechanical torque using the hydrolysis energy of ATP and the F0 motor generates a rotary torque in the opposite direction using a transmembrane proton motive force. In the following design these two motors are connected by flexible coupling, and each motor can be reversed: the F0 motor can drive the F1 motor to synthesize ATP, and the F1 motor can drive the F0 motor to pump protons Ning Tao et al have developed a novel micro-mixer using a biological molecular ATP motor. The micro-mixer was constructed from arrays of chromatophore-embedded F0F1-ATPases, where the d-free F1 part acted as a rotator to mix solutions, and the F0 part was driven by light. The microscopic studies indicated that the micro-mixer did not touch directly on the fibrin labeled with FITC. The force generated by the F0F1-ATPases motor directs drug movement in the solution and accelerated the fibrinolysis process. The study strongly suggests that the micro-mixers generated a nanomechanical force which accelerated the fibrinolysis process in the presence of lower concentrations of lumbrokinase [42].

Immobilization and Chemical Modification

Immobilization and chemical modifications study has been performed to reuse the enzyme in-vivo system which will minimize frequency of dosing Lumbrokinase was immobilized on the surface of polyurethane using maleic anhydride methyl vinyl copolymer as an enzyme carrier [43]. So Immobilized LK polyurethane surface has been shown highly antithrombogenic activity and can reduce surface induced thrombus. Immobilized LK surface may minimize platelet adhesion and activation by preventing fibrinogen from adsorption or by altering the conformation of adsorbed fibrinogen at an early stage of blood contact [44].

Further in order to improve the efficacy and tolerance of thrombolytic agents, its necessary to improve the specificity of the Lumbrokianse on fibrin to avoid the side effects and enhance the resistance to plasminogen activator inhibitor to elongate the half-life. To improve the stability of Lumbrokinase chemical modification has been used to stabilize the native structure of the earthworm protease and decrease the antigenicity during administration. The stabilization of the Lumbrokianse was done by chemical modification of the enzyme with 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide and phenylglyoxal to protect the activity from the autolytic inactivation [45].

The strongest fibrinolytic Lumbrokinase has been modified chemically with fragmented human serum albumin (MW, 10,000–30,000). After the chemical modification enzyme lost the antigenicity of the native enzyme. The Lumbrokinase is a non hemorrhagic protein and does not induce platelet aggregation. The Lumbrokinase has been shown the potent

proteolytic activity for fibrin and fibrinogen than that of human plasmin. The Lumbrokianse easily solubilizes in vivo fibrin clots (thrombi) of whole blood induced by thrombin in a rat's vena cava.

5. Clinical Application of Lumbrokinase

A Potent Fibrinolytic Agent

The Lumbrokianse an Earthworm Protease as a Fibrinolytic Agent has been used in China Korea and Japan. The formation of thrombus in the blood causes many complications such as stroke and myocardial infarction. Many of native and recombinant proteolytic enzymes have been used as the thrombolytic agents including urokinase (UK), streptokinase, recombinant tissue-type plasminogen activator, staphylokinase, and recombinant prourokinase [46]. Many of them has shown good result but they also have some limitations such as fast clearance, lack of resistance to reocclusion, bleeding complications, and other adverse effects.

The earthworm protease has dual functions in the fibrinolysis and plasminogen activation, distinct from those enzymes (UK, tissue-type plasminogen activator, etc.) [47-50]. Therefore have been used to treat the thrombosis. As Lumbrokianse during orally experiments both in animals and clinics show significant fibrinolytic efficacy. Further more distinct amelioration is observed in the treatment of blood high viscosity syndrome and thrombocytosis [51]. In addition, the LK are quite stable under the long-term storage at room temperature [52], in the form of oral dosage form. So for Lumbrokianse have been used in as oral dosage to prevent and treat clotting diseases, such as myocardial infarction and cerebral thrombus [53].

Protection of Cerebral Ischemia:

Lumbrokinase has been a suitable drug candidate for ischemic complication. The anti-ischemic activity of LK is due to its anti-platelet activity which raises c-AMP level and lowers the calcium release from calcium stores, the anti-thrombosis action due to inhibiting of ICAM-1 expression, and the anti-apoptotic effect due to the activation of JAK1/STAT1 pathway. At the molecular level Intercellular adhesion molecule-1 (ICAM-1) and Janus Kinase1/Signal Transducers and Activators of Transcription1 (JAK1/STAT1) pathway in protecting brain against ischemic injury by anti-thrombosis and anti-apoptosis [54]

Assistant to Implantation

During and after artificial organ transplantation small thrombus is usually formed on the surface of the graft which may further leads to the more complication as graft rejection. Even after great advancement in medicine and transplantation to improve the blood compatibility to biomaterial. Even though the results, so far, are not satisfactory. During 1994, Lumbrokinase was immobilized on the surface of polyurethane using maleic anhydride methyl vinyl ether copolymer as an enzyme carrier [55]. So Immobilized LK polyurethane surface has been shown highly antithrombogenic activity and can reduce surface induced thrombus. Immobilized LK surface may minimize platelet adhesion and activation by preventing fibrinogen from adsorption or by altering the conformation of adsorbed fibrinogen at an early stage of blood contact [56].

6. Discussion and Conclusion:

Lumbrokinase are becoming more popular now a day in the medical Science for the thrombolytic therapy. Being a eukaryotic protein it has even more scope in the treatment of cardio and cerebrovascular disorder. As advancement in recombinant technology Lumbokinase has been produced in bacterial and eukaryotic system in order to minimize drawback of native Lumbokinase. Further attempt has been made for more effective drug by alteration at gene level, designing suitable host system for enhance expression of recombinant enzyme, Immobilization for the reuse of enzyme and chemical modification to reduce antigenecity with the enhanced activity. In fact always there is progressive research is going on to minimize the complication of thrombolytic therapy. In future it will be suitable drug candidate for the thrombolytic therapy.

References

[1] Collen D. Fibrin-Selective Thrombolytic Therapy for Acute Myocardial Infarction. Circulation.1996; 93:85765

[2] American Heart Association.Dallas Heart Disease and Stroke Statics-2009.

[3] Mihara H, Sumi H, Yoneta T, Mizumoto H, Ikeda R, Seiki M, Maruyama M. Jpn J Physiol 1991;41(3):461-72.

[4] MacFarlane RD. Hemostasis: introduction. Br Med Bull 1977; 33: 183-94.

[5] Alkjaersig, N., Fletcher, A. P., and Sherry, S.: The mechanism of clot dissolution by plasmin, J. Clin. Invest. 38:1086-1095, 1959.

[6] Ambrus, C. M., Back, N., and Ambrus, J. L.: On the mechanism of thrombolysis by plasmin, Circ. Res., 10:161- 165, 1962.

[7] Back, N., Ambrus, J. L., Simpson, C. L., and Shulman, S.: Study on the effect of streptokinase-activated plasmin on clots in various stages of organization, J. Clin. Invest. 37:864-871, 1958.

[8] Castellino FJ. Recent advances in the chemistry of the fibrinolytic system. Chem Rev 1981;81:431 – 446.

[9]. Francis CW, Marder VJ. Fibrinolytic therapy for venous thrombosis. Prog Cardiovasc Dis 1991;34(3):193– 04

[10]. Ambrus, C, M, Back. N and J, L, On the Mechanism of Thrombolysis by plasmin Circ, 10:161-165, 1958

[11] Hyers TM, Agnelli G, Hull RD, et al. Antithrombotic therapy for venous thromboembolic disease. Chest 2001;119:176S-193S

[12] Barlow GH. Urinary and kidney cell plasminogen activator (urokinase). Methods Enzymol 1976;45:239– 44.

[13] Strassburger W, Wollmer A, Pitts JE, Glover ID, Tickle IJ, Blundell TL, et al. Adaptation of plasminogen activator sequences to known protease structures. FEBS Lett 1983;157:219– 23.

[14] Rouf SA, Moo-Young M, Chisti Y. Tissue-type plasminogen activator: characteristics, applications and production technology. Biotechnol Adv 1996;14:239–66.

[15] Nicolini FA, Nichols WW, Mehta JL, Saldeen TGP, Schofield R, Ross M, et al. Sustained reflow in dogs with coronary thrombosis with K2P, a novel mutant of tissue plasminogen activator. J Am Coll Cardiol 1992; 20:228–35.

[16] Reynolds JW: The status of earthworm biogeography, diversity, and taxonomy in North America revisited with glimpses into the future. In *Earthworm Ecology* 2nd edition. Edited by: *Edwards CA. CRC Press, Boca Raton, FL, USA*; 2004:63-74.

[17] Reynolds JW: Status of exotic earthworm systematics and biogeography in North America. In *Earthworm Ecology and Biogeography* Edited by: *Hendrix PF. Lewis Publishers, CRC Press, Boca Raton, FL*; 1995:-28.

[18] H. Mihara, H. Sumi, and K. Akazawa, "Fibrinolytic enzyme extracted from the earthworm," *Thrombosis and Haemostasis*, vol. 50, p. 58, 1983.

.[19] Mihara, H., Nakajima, N. and Sumi, H. (1993) Characterization of protein fibrinolytic enzyme in earthworm, *Lumbricus rubellus. Biosci. Biotech. Biochem*. **57**, 1726-1731.

[20] Mihara, H., Sumi, H., Yoneta, T., Mizumoto, H., Ikeda, R., Seikl, M. and Maruyama, M. (1991) A novel fibrinolytic enzyme extracted from the earthworm, *Lumbricus rubellus*. *Jpn. J. Physiol.* **41**, 461-472.

[21] Il Hwan Cho, Eui Sung Choi, Hun Gil Lim and Hyung Hoan Lee Purification and Characterization of Six Fibrinolytic Serine-Proteases from Earthworm *Lumbricus rubellus Journal of Biochemistry and Molecular Biology*, Vol. 37, No. 2, March 2004, pp. 199-205.

[22] Jing Zhao,1, 2 Rong Pan,1, 2 Jian He,1, 2 Ying Liu,1, 2 Dong-Feng Li,3 and Rong-Qiao He1, *Eisenia fetida* Protease-III-1 Functions in Both Fibrinolysis and Fibrogenesis Journal of Biomedicine and Biotechnology Volume 2007, Article ID 97654, 10 pages doi:10.1155/2007/97654.

[23]Vilhardt H. and Lundin S., In vitro intestinal transport of vasopressin and its analogues. Acta Physiol. Scand., 126 (1986b) 601-607.

[24] Cen Wu, Li Li, Jing Zhao, Qiao Fan, Wei-Xi Tian , Rong-Qiao He Effect of a2M on earthworm fibrinolytic enzyme III-1 from Lumbricus Rubellus International Journal of Biological Macromolecules 31 (2002) 71 /77

[25] Il Hwan Cho, Eui Sung Choi and Hyung Hoan Lee Molecular Cloning, Sequencing, and Expression of a Fibrinolytic Serine-protease Gene from the Earthworm *Lumbricus rubellus Journal of Biochemistry and Molecular Biology*, Vol. 37, No. 5, September 2004, pp. 574-581.

[26] Sugimoto M, Nakajima N. Molecular cloning, sequencing, and expression of cDNA encoding serine protease with fibrinolytic activity from earthworm. Biosci Biotechnol Biochem, 2001, 65 (7): 1575–1580.

[27] Mehdi Pirooznia, Ping Gong, Xin Guan , Laura S Inouye , Kuan Yang , Edward J Perkins and Youping Deng Cloning, analysis and functional annotation of expressed sequence tags from the Earthworm *Eisenia fetida BMC Bioinformatics* 2007, 8(Suppl 7):S7

[28]. L. H. Takahashi, R. Radhakrishnan, R. E. Rosenfield Jr., E. F. Meyer Jr., and D. A. Trainor, "Crystal structure of the covalent complex formed by a peptidyl.*α*.,.*α*.-difluoro-.*β*.-keto amide with porcine pancreatic elastase at 1.78.ANG. resolution," *Journal of the American Chemical Society*, vol. 111, pp. 3368–3374, 1989.

[29] A.-Z. Wei, I. Mayra, andW. Bode, "The refined 2.3 A° crystal structure of human leukocyte elastase in a complex with a valine chloromethyl ketone inhibitor," *Federation of the Societies of Biochemistry and Molecular Biology Letter*, vol. 234, no. 2, pp. 367–373, 1988.

[30] M.Marquart, J.Walter, J. Deisenhofer,W. Bode, and R. Huber, "The geometry of the reactive site and of the peptide groups in trypsin, trypsinogen and its complexes with inhibitors," *Acta Crystallographica B*, vol. 39, pp. 480–490, 1983.

[31] M. Sugimoto and N. Nakajima, "Molecular cloning, sequencing, and expression of cDNA encoding serine protease with fibrinolytic activity from earthworm," *Bioscience, Biotechnology and Biochemistry*, vol. 65, no. 7, pp. 1575–1580, 2001.

[32] F. Wang, C. Wang, M. Li, et al., "Crystal structure of earthworm fibrinolytic enzyme component B: a novel, glycosylated two-chained trypsin," *Journal of Molecular Biology*, vol. 348, no. 3, pp. 671–685, 2005.

[33] F. Wang, C. Wang, M. Li, L. Gui, J. Zhang, and W. Chang, "Crystallization and preliminary crystallographic analysis of earthworm fibrinolytic enzyme component B from *Eisenia fetida*," *Acta Crystallographica D*, vol. 60, no. 5, pp. 933–935, 2004.

[34] Il Hwan Cho, Eui Sung Choi, Hun Gil Lim and Hyung Hoan Lee Molecular Cloning, Sequencing, and Expression of a Fibrinolytic Serine-protease Gene from the Earthworm *Lumbricus rubellus Journal of Biochemistry and Molecular Biology, Vol. 37, No. 5, September 2004, pp. 574-581*

[35] Cho IH, Choi ES, Lee H H, Molecular cloning, sequencing, and expression of a ibrinolytic serine-protease gene from the earthworm Lumbricus rubellus. J Biochem Mol Biol. 2004 Sep 30;37(5):574-81.

[36] Zhe-rong Xu a, Yun-mei Yang a,*, Qi-feng Gui a, Li-na Zhang a, Lin Hu bExpression, purification, and characterization of recombinant lumbrokinase PI239 in Escherichia coli Protein Expression and Purification 69 (2010) 198–203.

[37] Rongliang Hu, Shoufeng Zhang, Huiying Liang, Ning Li,1 and Changchun Tu Codon optimization, expression, and characterization of recombinant lumbrokinase in goat milk Protein Expression and Purification 37 (2004) 83–88.

[38]Xiaoling Yuan, Chenhua Cao, Yajun Shan, Zhenhu Zhao, Jiapei Chen, and Yuwen Cong Expression and Characterization of Earthworm Eisenia foetida Lumbrokinase-3 in Pichia pastoris Preparative Biochemistry & Biotechnology, 36: 273–279, 2006.

[39] Tao Ge, Zhao-Jun Sun, Shi-Hong Fu, Guo-Dong Liang Cloning of thrombolytic enzyme (lumbrokinase) from earthworm and its expression in the yeast *Pichia pastoris* Protein Expression and PuriWcation 42 (2005)

[40] Tao Ge, Shi-Hong Fu, Li-Hong Xu, Qing Tang, Huan-Yu Wang, Kun-Ping Guan, Guo-Dong Liang High density fermentation and activity of a recombinant lumbrokinase (PI239) from *Pichia pastori s*Protein Expression and Purification 52 (2007) 1–7.

[41] Yong Tang,a Jiping Zhang,a Lulu Gui,a Cheng Wu,b Rong Fan,b Wenrui Changa and Dongcai Lianga Crystallization and preliminary X-ray analysis of earthworm fibrinolytic enzyme component A from Eisenia fetida Acta Crystallographica Section D Biological Crystallography Acta Cryst. (2000). D56, 1659±1661.

[42] Ning Tao a,b,1, Jie Cheng a,c,1, Jiachang Yue Using F0F1-ATPase motors as micro-mixers accelerates thrombolysis Biochemical and Biophysical Research Communications 377 (2008) 191–194.

[43] G. H. Ryu, S. Park, M. Kim, D. K. Han, Y. H. Kim, and B. Min, "Antithrombogenicity of lumbrokinase-immobilized polyurethane," *Journal of Biomedical Materials Research*, vol. 28, no. 9, pp. 1069–1077, 1994.

[44] J. Zhao, L. Li, C. Wu, and R.-Q. He, "Hydrolysis of fibrinogen and plasminogen by immobilized earthworm fibrinolytic enzyme II from *Eisenia fetida*," *International Journal of Biological Macromolecules*, vol. 32, no. 3–5, pp. 165–171, 2003.

[45] N. Nakajima, K. Ishihara, M. Sugimoto, H. Sumi, K. Mikuni, and H. Hamada, "Chemical modification of earthworm fibrinolytic enzyme with human serum albumin fragment and characterization of the protease as a therapeutic enzyme," *Bioscience, Biotechnology and Biochemistry*, vol. 60, no. 2, pp. 293–300, 1996.

[46] S. Kasai, H. Arimura, M. Nishida, and T. Suyama, "Proteolytic cleavage of single-chain pro-urokinase induces conformational change which follows activation of the zymogen and reduction of its high affinity for fibrin," *Journal of Biological Chemistry*, vol. 260, pp. 12367–12376, 1985.

[47] M. Verstraete, "Third-generation thrombolytic drugs," *American Journal of Medicine*, vol. 109, no. 1, pp. 52–58, 2000.

[48] J. Zhao and D. Li, "Research progress on anticoagulant protein molucular," *Microbiology*, vol. 29, pp. 102–107, 2002.

[49] S. Kasai, H. Arimura, M. Nishida, and T. Suyama, "Proteolytic cleavage of single-chain pro-urokinase induces conformational change which follows activation of the zymogen and reduction of its high affinity for fibrin," *Journal of Biological Chemistry*, vol. 260, pp. 12367–12376, 1985.

[50] E. L. Madison, G. S. Coombs, and D. R. Corey, "Substrate specificity of tissue type plasminogen activator," *Journal of Biological Chemistry*, vol. 270, no. 13, pp. 7558–7562, 1995.

[51] J. S. Kim, J. K. Kang, H. C. Chang, et al., "The thrombolytic effect of lumbrokinase is not as potent as urokinase in a rabbit cerebral embolism model," *Journal of Korean Medical Science*, vol. 8, no. 2, pp. 117–120, 1993.

[52] Y. Cong, Y. Liu, and J. C. Chen, "Advance in lumbrokinase," *Chinese Journal of Biochemical Pharmaceutics*, vol. 21, pp. 159– 162,2000.

[53] L. Jin, H. Jin, G. Zhang, and G. Xu, "Changes in coagulation and tissue plasminogen activator after the treatment of cerebral infarction with lumbrokinase," *Clinical Hemorheology and Microcirculation*, vol. 23, no. 2–4, pp. 213–218, 2000.

[54] H. L. Sun, J.D. Jiao, Z.W. Pan, D. L. Dong, andB. F. Yang, "The cardioprotective effect and mechanism of lumbrokinase," *Yao Xue Xue Bao*, vol. 41, no. 3, pp. 247–251, 2006.

[55] G. H. Ryu, S. Park, M. Kim, D. K. Han, Y. H. Kim, and B. Min, "Antithrombogenicity of lumbrokinase-immobilized polyurethane," *Journal of Biomedical Materials Research*, vol. 28, no. 9, pp. 1069–1077, 1994.

[56] N. Nakajima, K. Ishihara, M. Sugimoto, T. Nakahara, and H. Tsuji, "Further stabilization of earthworm serine protease by chemical modification and immobilization," *Bioscience, Biotechnology and Biochemistry*, vol. 66, no. 12, pp. 2739–2742, 2002.

Authors

Mahendra Kumar Verma is working as Lecturer at Department of Biotechnology, R.V.R. & J.C. College of Engineering, Andhra Pradesh, India. He is B.Pharma & M.Tech in Biotechnology and currently pursuing PhD as part time. He teaches Molecular Biology Genetic Engineering and Pharmaceutical Biotechnology for undergraduate students.

KK Pulicherla is an Associate Professor and Head of the Department at R.V.R. & J.C. College of Engineering, Andhra Pradesh, India. He researches and teaches in several areas of Genomics, Proteomics, as well as Bioinformatics. As a young researcher he has published 7 papers in Journals and Magazines. He attended and presented 3 papers in Various National and international conferences. He also has a patent to his credits.