An Overview of the Fibrinolytic Enzyme from Earthworm

ZHAO Yong-Gang[1], LI Hua[1], XU William[2], LUO Jia[2], XU Rui-An[1*]

[1]*Engineering Research Center of Molecular Medicine, Ministry of Education & Institute of Molecular Medicine, Huaqiao University, Quanzhou 362021,China;*
[2]*Faculty of Science, University of New South Wales, Sydney, NSW 2052, Australia*

[ABSTRACT] Although many fibrinolytic agents have been developed and used for clinical purposes, thromboembolic diseases remain the leading cause of adult morbidity and mortality in the world. Developing effective agents for clinical application from various unusual animal species has received more attention in recent years. A group of fibrinolytic enzymes named earthworm fibrinolytic enzyme (EFE) and secreted by the alimentary tract of earthworm, have exhibited excellent potential in the clinical treatment of blood clotting diseases. It is therefore useful to build up some correlations with the data available in order to better explore the molecular and cellular mechanism of EFE action in the treatment of diseases. The aspects discussed include: the purification, characterization, gene cloning and expression, structure and function as well as clinical application of the EFEs. This review focuses on recent advances in molecular and cellular mechanisms of EFE action and the structure activity relationship.

[KEY WORDS] Earthworm fibrinolytic enzyme (EFE); Thrombolytic agent

[CLC Number] Q55 **[Document code]** A **[Article ID]** 1672-3651(2010)04-0301-08

doi: 10.3724/SP. J. 1009.2010.00301

1 Introduction

Thrombolytic drugs used for clinical applications are categorized into two types: the plasminogen activators, such as tissue-type plasminogen activator (t-PA)[1], urokinase[2], streptokinase[3], APSAC (anisoylated plasminogen streptokinase activator complex)[4] etc, which convert plasminogen into active plasmin to degrade fibrin; and plasmin-like proteins, including alfimeprase[5], microplasmin, TAL6003 etc, which directly act on fibrinogen or fibrin to degrade blood clots. Most of them suffer significant shortcomings including large therapeutic doses, limited efficacy, short half-life and bleeding complications. Earthworm has been used as a fibrinolytic agent for over one thousand years in China, Korea and Japan. Earthworm fibrinolytic enzymes (EFE) are a group of serine proteases with strong fibrinolytic and thrombolytic activity with molecular masses of 23–30 kDa. To date, EFE has been confirmed with excellent potential in the clinical treatment of blood clotting diseases, especially in stroke[6]. The capsules of the extracts of EFEs, commercially referred to as Panford or Boluoke, have been widely used in some Southeast-Asian countries, as well as in North American countries such as Canada and the United States. Because of their pharmacological properties, EFEs have attracted increasing research interest in recent years. However, the details of the relationship between the structure and activity of EFEs remain obscure.

2 Origin of EFEs

Lumbricus rubullus and *Lumbricus bimastus*, are the important resources for fibrinolytic agents in Southeast-Asian countries, such as Japan, Korea and China and so on. Wu *et al*, in 1986, firstly isolated a set of lumbrokinase components from *Eisenia fetida*, with activating plasminogen and degrading fibrin properties. In a recent inventory of EFEs, many direct acting fibrin(ogen)olytic enzymes were described, some also with strong anti-oxidative and anti-bacterial activities[7]. Inspection of the distribution of the fibrinolytic enzymes in the earthworm would contribute to a better understanding of these isozyme functions. Zhao *et al*. have detected that EfP-II and EfP-III-1 are localized in the epithelial cells of the alimentary canal around the clitellum of *E. fetida.*, and suggested that the relatively broad substrate specificity of EfP-II and EfP-III-1 is needed for earthworm to digest different micro-organisms and proteins[8].

Conventional methods for the isolation and purification of EFEs used by most researchers include centrifugation, filtration, ammonium sulphate precipitation and dialysis, ion exchange chromatography and affinity chro-

[Received on] 08-Jan-2010

[Research Funding] This project was supported by the National High Technology Research and Development Program ("863" Program) of China (No. 2008AA02Z135).

[*Corresponding author] XU Rui-An: Prof., Tel: 86-595-22691632, Fax: 86-595-22690952, E-mail: ruianxu@hqu.edu.cn

matography. Such a complicated clarification sequence makes the process scale-up difficult. Aqueous-two phase systems (ATPS) is a simple and easy-to-scale-up method, which allows process integration on account of the simultaneous separation and concentration of the target protein in a clear extract being achieved. The low cost and easy scale-up allow its potential use in large-scale downstream processing of proteins[9]. To date seven fibrinolytic enzymes (EFE-a to EFE-g) have been purified from the earthworm *Eisenia fetida*[10], and eight glycosylated fibrinolytic proteases (EfP-0-1 to EfP-III-2) have been isolated from the earthworm *Eisenia fetida*, among the eight purified trypsin-like glycoproteases, EfP-0-2 and EfP-II-2 are newly isolated isozymes. All the eight proteases are glycoproteins with different carbohydrate contents. It is also confirmed that oligosaccharides are composed of mannose residues by Dot-blotting assay with ConA[11]. Six lumbrokinase fractions (F-Ⅰ-0 to F-Ⅲ-2) with fibrinolytic activities are purified from earthworm *Lumbricus rubellus* lysates using the procedures of autolysis, ammonium sulfate fractionation and column chromatography. Lumbrokinases F-III-1 and F-III-2 are of the highest activity among the six isozymes[12]. In 1999, Sun *et al*. isolated a lumbrokinase (PI239) from *Lumbricus bimastus* with obvious fibrinolytic activity on fibrin plate[13]. The PI239 protein has the same active and substrate sites as those of t-PA and u-PA. The details of the biological and physiological properties of these enzymes are summarized in Table 1.

3 Identity of EFEs Protein

Although the enzymes are extracted from different species of earthworms, they may have different names. For instance, F-II alias EFEa, and EfP-III-1 alias EFE-b, one enzyme shows high homology to the other; isozyme A shows 36% identity with isozyme C, and both have

Table 1 The biological and physiological properties of different EFEs

Source	Nomination	Activity	Types of proteases	pH, temperature stability or inhibitor	References
Eisenia fetida	EfP-0-1 to EfP-III-2	Relative activity (%): 6.2, 12.8, 25.8, 31.6, 8.8, 2.1, 12.5, 2.3 (the activity of mixture as 100). EfP-0-2 and EfP-II-2 were newly isolated.	trypsin-like fibrinolytic enzymes in glycosylated form.	EfP-II and EfP-III-1 were strongly inhibited by SBTI.	[11]
	Lumbrokinase	fibrinolytic and thrombolytic activity	no date	proteins with isoelectric point in the range 4.6–7.4.	[14]
	EFE-a to EFE-f	EFE-b, EFE-c and EFE-g had relatively higher fibrinolytic activity. EFE-d and EFE-e had average fibrinolytic activity, and EFE-a and EFE-f had relatively lower activity. EFE-a has plasminogen-activating activity and fibrinolytic activity.	EFE-b, EFE-c and EFE-g represent trypsin-like enzymes, EFE-d, EFE-e and EFE-f represent chymotrypsin-like enzymes. EFE-a unknown.	acid enzymes	[15]
	P I and P II(tissue homogenate (G-90) of Eisenia foetida).	All with the esterase activity, but only fraction PI displayed amidase activity. and the majority of activity was represented by P I.	They belong into the tyrosine family.	PMSF at the concentration of 10-4 M inhibited PI (BAEE as substrate).	[16]
	ARSP1	a plasmin and also a plasminogen activator.	Glycoprotein or glycopeptide.	inhibited by PMSF.	[17]
Lumbricus rubellus	Isozymes A to F[18] (formerly named F-III-2, F-III-1, F-II, F-I-2, F-I-1, F-I-0, respectively).	They can acted on elastin, fibrin and actual fibrin clots of whole blood in a rat's vena cava. They also catalyzed the hydrolysis of various esters. Especially A and B have much higher strong caseinolytic and fibrinolytic activities than plasmin. The enzymes had also strong amidolytic activity.	trypsin-like serine proteases with single polypeptide chains and are not glycoproteins. isozymes A, B, D, E, and F represent both trypsin-and chymotrypsin-like activities, but isozyme C also serves as an elastase-like enzyme[19].	The enzymes were stable at below 60°C over a range of pH 2–11 , most active at 55°C, on heating at 80℃ for 30min, the activity completely disappeared they retain full activity for long years at room temperature. high stability toward organic solvents and detergents , The activity of isozymes A, B, and C was inhibited strongly by soybean trypsin inhibitor and aprotinin, but the enzyme activity of D, E, and F was partially inhibited by these inhibitors.	[12]
	F1 to F6	The rank activity orders of proteolytic activities and fibrinolytic activities are F2 > F1 > F5 > F6> F3 > F4 and F6>F2>F5>F3>F1>F4 separately.	All of them are serine protease, F5 and F6 are trypsin-like serine protease. iso-enzymes. F1 is chymotrypsin-like protease.	F1-F4 were completely inhibited by PMSF. F5 and F6 were completely inhibited by aprotinin, TLCK, TPCK, SBTI LBTI, and leupeptin. the six iso-enzymes were stable at pH 4-12.	[20]

high identity (30%–38%) with mammalian serine proteases[18]. The sequence alignment indicates that EFE-a shares 35% identity with F-III-1 or F-III-2[21]. The cDNA sequence of PI239 shows 89.5% homology to F-III-1/2 and EFE-II[7]. It may be explained in the way that enzymes supplied by most of earthworms belong to the same family of earthworm's taxonomy. In contrast, the enzymes from the same earthworm usually have different structures. EFE-a and EFE-b are both extracted from *Eisenia fetida*[15], but they have different structures. Such a phenomenon may be due to the edaphic conditions of different areas varying from each other, the earthworm therefore needs multiple genes to encode different kinds of enzyme to digest the nutrition needed for its survival. Trypsin belonging to the primordial Ser195:TCN /Ser214: TCN / Pro225 linkage (EFE-b also belongs to this linkage[21]) is the ancestor of the chymotrypsin family[22], while EFE-b from the earthworm (a very ancient animal) might be the intermediate stage in the evolution of vertebrate chymotrypsin and most non-digestive trypsin-like proteases (both consisting of two chains) from their ancestor (consisting of one chain). This may be useful for investigators to understand the structures and evolutionary paths of EFEs.

4 Generation of Recombinant EFEs

EFEs capsules have been made from the extract of earthworms. But the extract is still a mixture of different components which can cause side-effects after administration. It is difficult to isolate and prepare only one ingredient by the conventional purification methods. The successful expression of the recombinant EFEs provides a way to obtain single component with fibrinolytic activity.

In the past decade, *P. pastoris* has been developed into as an efficient, heterologous gene expression host[23]. With the use of the alcohol oxidase promoter and yeast endogenous signal peptide, or that of the gene of interest, the yields of many recombinant proteins have been fairly high[24]. In 2001, Manabu and Nobuyoshi expressed lumbrokinase F-III-2 for the first time in *P. pastoris* GS115 with the use of the pPICZα(B) vector[25]. EFE PM_{246} (GenBank Accession No. AY178854) was expressed by the recombinant pPIC6aA-PM246 plasmid transformed into *P. pastoris* X-33, and produced about threefold more protein than the flasks in the condition of YPM culture and 96 h suitable methanol induction time. The fibrinolytic activity indicates that purified EFE PM_{246} is not only as a direct fibrinolytic enzyme, but also as a plasminogen activator. High density fermentation broth of recombinant lumbrokinase (rPI239) was developed in the yeast *Pichia pastoris*, using the yeast expression vector pPICZα(A) and the *P. pastoris* KM71 host strain, and the total supernatant protein content is 0.174 $g \cdot L^{-1}$. Both *in vivo* and *in vitro*, this recombinant protein has similar fibrinolytic activity[26]. Lumbrokinase-3, first cloned from the earthworm *Eisenia foetida* by yeast pPIC9K expression vector, was transformed into the *Pichia pastoris* GS115 cells by electroporation. High level expression of LK-3 in yeast cells was confirmed with the different timing courses. The activity of expressed LK-3 was observed in fibrin plates. In addition, the expressed LK-3 protein could also dissolve fibrinogen and bovine serum albumin[23].

Compared to other systems, the bacterial expression system could express recombinant proteins with higher yields in an economical and efficient way. The full-length cDNA of *Lumbricus rubellus* lumbrokinase fraction 6 (F6) protease was amplified by Lee, *et al*. using an mRNA template, sequenced and expressed in *E.coli* cells. The F6 protease gene consists of pro- and mature sequences, and the protease was translated and modified into active mature polypeptide by *N*-terminal amino acid sequence analysis of the F6 protease. F6 protease gene clones having pro-mature sequence and mature sequence produced inclusion bodies in *E. coli* cells. Animal test identifies that the F6 protease has hemolytic activity, and it is composed of pro- and mature regions[27]. The recombinant of the EFE-3 was expressed in *E. coli* as inclusion bodies, and the gene encoding of the native form of EFE-3 was expressed in COS-7 cells in the medium. Both the refolding product of inclusion bodies and the secreted protease with the activities dissolve the artificial fibrin plates[28]. Two cDNA fragments (lrF1 and lrF2), with and without signal peptide coding sequence, representing fibrinolytic enzyme gene of F-III-2, were cloned from earthworm *Lumbricus rubellus*. Both lrF1 and lrF2 proteins were produced as an inclusion body form in *E. coli* BL21 (DE3) pLysE using bacterial expression vector pET28a (+), After protein refolding and purification, the fusion lrF1 and its derivative showed fibrinolytic activity, but the fusion lrF2 and its derivative had no fibrinolytic activity[29], indicating that the *E. coli* expression system could not recognize the endogenous signal peptide of F-III-2, and the effect of the histidine tags at the N-terminus was not significant on the fibrinolytic activity of the expressed protein.

To ascertain whether the product of a genetically modified or newly isolated eukaryotic gene has biological activity, the gene of interest is usually sub-cloned into a mammalian expression vector and then expressed in an *in vitro* system such as in tissue culture. The first research on the expression and characterization of a genetically engineered lumbrokinase (*L. rubellus*) was reported by Hu, *et al.*, two copies of a lumbrokinase cDNA(w) obtained by RT-PCR and a synthesized lumbrokinase cDNA(m) with optimized codons were cloned into a mammary-gland-specific expression vector pIbCP to determine the elements affecting the expression of lumbrokinase. Then pIbCP-LK-LK vector preparations were directly injected into the lactating goat mammary glands, both LK-w

and LK-m were successfully expressed in goat milk. The fibrinolytic activities of the LK-w and LK-m indicated that the codon optimization played an important role in improving the lumbrokinase expression[30]. Shortly, they developed an efficient *in vivo* system by employing a mammary-gland-specific vector, expressing lumbrokinase cDNA variant (LK-m) and the tissue-type plasminogen activator (tPA) cDNA in the lactating-goat mammary glands. High-level expression of the LK-m and tPA was detected, and all of them had fibrinolytic activity tested by fibrinagarose plate assay[31]. This bioreactor system has the potential to be used for the production of thrombolytic proteins in place of transgenic animals.

5 Structural Biology of the EFEs

The structure of EFEs is the basis of their fibrinolytic mechanism, and the determination for their extraordinary substrate specificity. Some of the EFEs isozymes such as EFE-a, EFE-b and isozyme C have been well clarified and can be as representative for comparison in detail.

EFE-a from *E. fetida* was refined to the resolution of 0.23 nm and was detected by multiple isomorphous replacement (MIR). As in almost all other active chymotrypsin-like serine proteases, it also occurs that the α-amino group of the EFE-a *N*-terminal residue (Val16), forms an internal salt bridge with the side-chain carboxyl group of Asp194, which generates an oxyanion hole[32]. EFE-a has a polypeptide fold of chymotrypsin-like serine proteases with essential S1 specific determinants characteristic of elastase. However, a unique four-residue insertion (Ser-Ser-Gly-Leu) after Val217 elongate *β*-strand at the west rim of the S1 specificity pocket, and distal P residues have additional substrate hydrogen binding sites. EFE-a's S1 specific pocket was preferable for elastase-specific small hydrophobic P1 residues, its accommodation of long and/or bulky P1 residues was feasible when enhanced binding of the substrate and induced fit of the S1 pocket were achieved[33]. Two years later, the native crystal structure of it was improved to 0. 18 nm and its complex structure was determined by an inhibitor at a resolution of 0.19 nm[34]. The final structures show that EFE-a possesses multi-substrate-binding sites, and significant conformation adjustment takes place at two loops binding to the *N*-terminal of the substrates, which may enhance the interaction between the enzyme and the substrates. These characteristics make the substrate-specificity of EFE-a less dependent on the property of its S1-pocket, and may endow the enzyme with the ability to hydrolyze chymotrypsin-specific substrates and even trypsin-specific substrates[8]. Although EFE-b was extracted from the same earthworm *Eisenia fetida* as EFE-a, the structure of EFE-b is distinct from EFE-a. It is roughly spherical with a radius of about 2.5 nm. The structural analysis at a resolution of 0.206 nm shows that EFE-b should be classified as a trypsin. However, it is a two-chained protease, including pyroglutamated light chain and an *N*-glycosylated heavy chain with an *N*-terminal, which are distinct from other trypsins. Furthermore, the heavy chain contains a novel structural motif, an eight-membered ring resulting from a disulfide bridge between two neighboring cysteine residues, and a *cis* peptide bond exists between these two cysteine residues[21].

Isozyme C, extracted from the earthworm, *Lumbricus rubellus*, shows not only elastase-like activity but also trypsin-like activity. The sequence similarities of isozyme C with porcine elastase[35] and bovine trypsin[36] were 33% and 31% respectively. The catalytic triad of the trypsin family, His, Asp and Ser, was conserved in isozyme C. But the same as in elastase, Asp, which was the primary substrate determinant of trypsin was missing, and one of the two Gly at the entrance of the substrate-binding pocket of trypsin was also replaced by Val, however, the other was replaced by Ser whereas Thr is present in elastase. In addition, the determination of ester-bond hydrolyzing activity of isozyme C was also investigated with esterase-like activity, which was applicable for the synthesis of useful substances[37].

Some of the EFEs have similar function to both tissue plasminogen activator (t-PA) and plasmin[38], which means, they are not only partially activated PLg (plasminogen) to produce plasmin, but also degrade fibrin and fibrinogen directly. The immobilized F-II cleaves human plasminogen at four hydrolytic sites: Lys77-Arg78, Arg342-Met343, Ala444-Ala445 and Arg557–Ile558. The last cleavage site (Arg557–Ile558) is the same as t-PA and u-PA cut PLg[39-40]. EfP-III-1[41] and EFEa[33] also show the same hydrolytic activity. In assay, EFEa cleaved plasminogen at five peptide bonds, three of the P1 residues being hydrophobic and the other two being basic (P*n* and P*n'* denote the *n*th amino acid at the *N* and *C*-terminals of the cleaved peptide bond; S*n* and S*n'* denote the corresponding binding sites of the protease)[42], the cleavage at the peptide bond R557-I558 (plasminogen numbering) led to the generation of active plasmin[33].

EFEs have relatively broad substrate specificities, such as trypsin (cleavage site: Lys-X and Arg-X, X represents an amino acid) and chymotrypsin (cleavage site: Phe-X, Trp-X, Tyr-X and Leu-X)[19]. Fibrinogen is a 45 nm, 340 kDa trinodular protein present at high (2–4 $mg \cdot mL^{-1}$, 6–12 $\mu mol \cdot L^{-1}$) concentrations in plasma, and it consists of two sets each of three different polypeptide chains: Aα, Bβ,and γ, crosslinked by 29 disulfide bridges[43]. EFE-II hydrolyzes Aα-chain of fibrinogen at *C*-terminal region (the same as human neutrophil elastase, HNE)[44] and the site Val21–Glu22 of the *N*-terminal region. Although thrombin can cleave fibrinogen to form fibrin clot, the Gly17–Met51 of the Aα-chain is important *N*-terminal motif for thrombin and the fibrin monomer to recognize each other and associ-

ate into fibrin fibrils. Therefore, EFE-II is different from thrombin, and produces soluble degraded fragments. In the hydrolysis of fibrinogen, plasminogen and prothrombin (Zhao and He, unpublished data), EfP-III-1 cleaves the bonds at alkaline amino acid residues recognized by trypsin (R–X and K–X). EfP-II, however, prefers neutral or hydrophobic amino acid such as V–X, G–X and A–X. This isozyme also recognizes Y–S bonds[40].

It has been speculated that the EFEs directly hydrolyze thrombus *in vitro*. In addition, EFEs also have anti-platelet activity by elevating cAMP level and attenuating the calcium release from calcium stores, resulting in the platelet disaggregating[45]. And the anti-ischemic activity of the EFEs was due to EFEs having anti-apoptotic effect by the activation of JAK1/STAT1 pathway[42]. Anti-coagulation of EFEs can be summarized as Fig. 1.

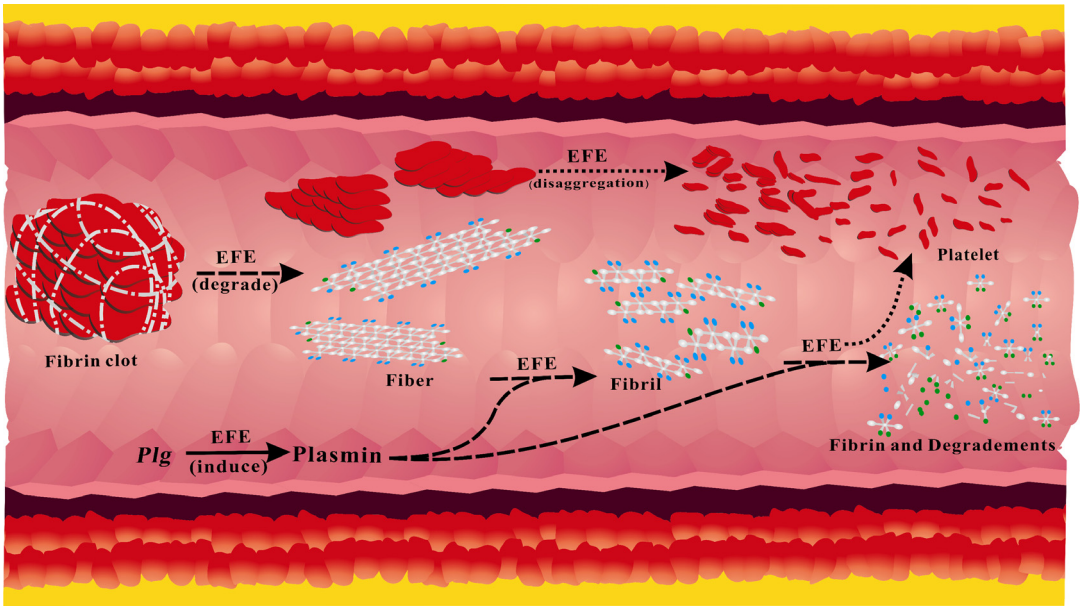

Fig. 1 Diagram of anti-thrombotic of EFEs pathway[46-51]

6 Intestinal Absorption of EFEs

The intestinal absorption of proteins (e.g. fibrinolytic enzymes) remains a controversy[52]. The gastrointestinal tract acts as a barrier against the invasion of exogenous proteins into the body. Such proteins are not easily absorbed from the gastrointestinal tract in an intact form, so it is extremely difficult for them to arrive at certain targeting area[53]. The possibilities that small molecular components of the enzymes can enter the blood stream and act as inducers of the plasminogen activator bound to the endothelium can not be over ruled. In reality, however, the barrier function of the small intestinal epithelium in mammals is incomplete and to a limited extent, macromolecules such as proteins can pass from the lumen to the circulation. The mechanisms of the absorption of macromolecules include paracellular passage and transcellular endocytosis. In the intracellular transport, some proteins bind to receptors on the surface of the intestinal epithelium, which then invaginate to form phagosomes engulfing the proteins. Some proteins fuse to form phagolysosomes (such as lysosomers) in which digestion might occur. The transportation of EFE-III-1 is an intracellular process. Being a strong protease, some of EFEs (like EFE-III-1,-2[52] and EFE-d[55]are resistant to degradation by some cellular enzymes. Thus, they could be discharged in intact form and across the cell membrane by exocytosis. Furthermore, the *N*-terminal sequence of some proteins might also affect protein transportation. EFE-III-1 and -2 are rich in hydrophobic amino acid residues (I-V-G-G-I-E-A-R-P-Y-E-F-P-W-Q-), which may contribute to the membrane transportation[57]. The intestinal absorption of lumbrokinase could also be enhanced by various enhancers. EFE-d is clinically used for the management of cardiovascular diseases. However, this protein drug has a very low (< 0.1%) bioavailability after oral administration[54]. The water-in-oil (*W/O*) micro-emulsions has been explored to enhance the absorption and efficacy for EFE-d. The result shows that intraduodenal bioavailability of EFE-d for *W/O* micro-emulsions was 208-fold higher than that of control solution and the absolute bioavailability was 17.55%. Meanwhile, there was no tissue damage of the rats' intestinal mucosa found. This indicates that the *W/O* micro-emulsion may represent a safe and effective oral delivery system for hydrophilic bioactivity macromolecules.

Some enzymes are degraded rapidly by the clearance system and inactivated by some inhibitors in blood, so these enzymes are present in the blood stream for only a short time. LK Ⅲ-1 was demonstrated to be absorbed into the blood with intact form and the maximum activity found after around 60 min[55-57], and EFE-III-2 lost about 95% of its initial activity in 20 min when incubated with rat plasma

at 37℃, but the modification of it with human serum albumin fragments was significantly more resistant to inactivation by antibody(about 60% of the initial activity[58]). This all indicated that some inhibitors and antibody in plasma possibly inactivated EFE-III-2 when it was absorbed. The effect of α_2M on the activity of EFEs has been investigated, with the results showing that the activity of EFE-III-1 in blood decreased to 65% when α_2M was added. The mechanism of α_2M/EFE-III-1 interaction coincides with a complicated and irreversible inhibition[56].

7 Conclusions and Perspectives

The development of useful clinical agents from various unusual animal species has received a great deal of attention in recent years. Earthworms have been used in antipyretic and diuretic treatments in Chinese traditional medicine for thousands of years. In 1983, Mihara *et al*[59] first isolated fibrinolytic enzymes from the earthworm, and postulated their clinical potential. EFE does not only degrade fibrinogen, elastin and fibrin directly, but also partially converts PLg into active plasmin, which is distinct from UK, t-PA etc. In China and South Korea, the enzymes (EFE-III-1 as a main component) were successfully made into an oral fibrinolytic medicine in 90's of the last century[60]. Earthworm can be easily raised, making EFEs a relatively inexpensive thrombolytic agent applicable for production, and after EFEs are made into oral-capsule, they are stable during a long-term storage at room temperature.

A number of isozymes have been purified and studied in details. Each enzyme may have multi-functions (like G-90[61]) and can contribute to different clinical applications, such as anti-tumor[62], antimicrobial, blood pressure adjuster (hypertension and hypotension), solvent thrombus, protection of cerebral ischemia[63-64], cerebral infarction[65], cardioprotective effect[66], etc. These would be meaningful topics for further investigations.

Even though EFE has some advantages in pharmaceutical application, there are some problems as a clinical fibrinolytic medicine: EFE directly dissolves fibrinogen and fibrin, probably producing plasmin complications; it hydrolyzes some other proteins *in vivo* and the broad specificity may lead to hemorrhage; The half-life of EFE should be prolonged in circulation; The preparation process of the EFE is complex, and the products contain some unwanted components that might be the cause of side-effects (such as vomiting) in clinical application[67]. Pharmacological and clinical studies should be focused on the solutions to these problems.

Oral administration of EFE appears to have a relatively slow pharmacological efficacy; therefore it might not be suitable for the treatment of emergency of thrombus. If EFEs are made into injection, it would be meaningful to treat some acute diseases, thromboses in particular. However, the hemorrhage complication and immune response of the enzymes, and the molecular mechanism for its thrombolytic effect *in vivo* remain unclear. These obstacles might be the biggest hindrance to making EFE into an injection agent. Therefore it is necessary to identify and modify EFE's essentially functional groups while reducing its full length in order to avoid hostile response while maintaining the enzyme activity.

References

[1] Collen D, Lijnen H. Tissue-type plasminogen activator: a historical perspective and personal account[J]. *J Thromb Haemost*, 2004, **2**(4): 541-546.

[2] Duffy M. Urokinase plasminogen activator and its inhibitor, PAI-1, as prognostic markers in breast cancer: from pilot to level 1 evidence studies[J]. *Clin Chem*, 2002, **48**(8): 1194-1197.

[3] Banerjee A, Chisti Y, Banerjee U. Streptokinase—a clinically useful thrombolytic agent[J]. *Biotechnol Adv*, 2004, **22**(4): 287-307.

[4] Anderson J, Rothbard RL, Hackworthy RA, *et al*. Multicenter reperfusion trial of intravenous anisoylated plasminogen streptokinase activator complex (APSAC) in acute myocardial infarction: controlled comparison with intracoronary streptokinase[J]. *J Am Coll Cardiol,* 1988, **11**(6): 1153-1163.

[5] Toombs C. Alfimeprase: pharmacology of a novel fibrinolytic metalloproteinase for thrombolysis[J]. *Haemostasis*, 2001, **31**: 141-147.

[6] Zhou XD, Zhao RX, Zheng XR, *et al*. Observation of Boluoke (lumbrokinase) treatment in the recovery of motor function after cerebral infarction [J]. *West China Med Combin*, 1997, **12**: 17.

[7] Tao G, Zhao JS, Shi HF, *et al*. Cloning of thrombolytic enzyme (lumbrokinase) from earthworm and its expression in the yeast *Pichia pastoris*[J]. *Protein Expr Purif*, 2005, **42**(1): 20-28.

[8] Zhao J, Xiao R, He J, *et al*. *In situ* localization and substrate specificity of earthworm protease-II and protease-III-1 from *Eisenia fetida*[J]. *Inter J Biolog Macromol,* 2007, **40**(2): 67-75.

[9] Kula M. Trends and future prospects of aqueous two-phase extraction[J]. *Bioseparation*, 1990, **1**(3-4): 181.

[10] Wang F, Wang C, Li M, *et al*. Purification, characterization and crystallization of a group of earthworm fibrinolytic enzymes from *Eisenia fetida*[J]. *Biotechnol Lett,* 2003, **25**(13): 1105-1109.

[11] Wu JX, Zhao XY, Pan R, *et al*. Glycosylated trypsin-like proteases from earthworm *Eisenia fetida*[J]. *Inter J Biolog Macromol*, 2007. **40**(5): 399-406.

[12] Nakajima N, Mihara H, Sumi H. Characterization of potent fibrinolytic enzymes in earthworm *Lumbricus rubellus*[J]. *Biosci Biotechnol Biochem*, 1993, **57**(10): 1726-1730.

[13] Sun ZJ, Liang GD, Chen F, *et al*. Cloning of cDNA of lumbrokinase and the effect on BHK cell[J]. *Mol Biol*, 2002, **18**(6): 776-779.

[14] Iannucci NB, Camperi SA, Cascone O. Purification of lumbrokinase from *Eisenia fetida* using aqueous two-phase systems and anion-exchange chromatography[J]. *Sep Purifi Technol*, 2008, **64**(1): 131-134.

[15] Wang F, Wang C, Li M, *et al*. Purification, characterization and crystallization of a group of earthworm fibrinolytic enzymes from *Eisenia fetida*[J]. *Biotechnol Lett,* 2003, **25**(13): 1105-1109.

[16] Hrzenjak TM, Popovic M, Tiska-Rudman L. Fibrinolytic activity of earthworms extract (G-90) on lysis of fibrin clots originated from the venous blood of patients with malignant tumors[J]. *Pathol Oncol Res*, 1998, **4**(3): 206-211.

[17] Xie JB, Guo ZQ, Weng N, *et al*. Purification, identification and partial characterization of an apoptosis-related serine protease from earthworm[J]. *Prog Biochem Biophys*, 2003, **30**(3): 453-460.

[18] Nakajima N, Sugimoto M, Ishihara K. Earthworm-serine protease: characterization, molecular cloning, and application of the catalytic functions[J]. *J Mol Catal B-Enzym*, 2003, **23**(2-6): 191-212.

[19] Nakajima N, Sugimoto M, Ishihara K, *et al*. Further characterization of earthworm serine proteases: Cleavage specificity against peptide substrates and on autolysis[J]. *Biosci Biotechnol Biochem*, 1999, **63**(11): 2031-2033.

[20] Cho IH, Choi ES, Lim HG, *et al*. Purification and characterization of six fibrinolytic serine-proteases from earthworm *Lumbricus rubellus*[J]. *J Biochem Mol Biol*, 2004, **37**(2): 199-205.

[21] Wang F, Wang C, Li M, *et al*. Crystal structure of earthworm fibrinolytic enzyme component B: a novel, glycosylated two-chained trypsin[J]. *J Mol Biol*, 2005, **348**(3): 671-685.

[22] Krem M, Di Cera E. Molecular markers of serine protease evolution[J]. *EMBO J*, 2001, **20**(12): 3036.

[23] Yuan X, Cao C, Shan Y, *et al*. Expression and characterization of earthworm *Eisenia foetida* lumbrokinase-3 in *Pichia pastoris*[J]. *Prep Biochem Biotechnol*, 2006, **36**(3): 273-279.

[24] Cregg J, Vedvick T, Raschke W. Recent advances in the expression of foreign genes in *Pichia pastoris*[J]. *Nat Biotechnol,* 1993, **11**(8): 905-910.

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

[26] Ge T, Fu SH, Xu LH, *et al*. High density fermentation and activity of a recombinant lumbrokinase (PI239) from *Pichia pastoris*[J]. *Protein Expr Purif*, 2007, **52**(1): 1-7.

[27] Cho IH, Choi ES, Lee HH. Molecular cloning, sequencing, and expression of a fibrinolytic serine-protease gene from the earthworm *Lumbricus rubellus*[J]. *J Biochem Mol Biol*, 2004, **37**(5): 574-581.

[28] Dong GQ, Yuan XL, Shan YJ, *et al*. Molecular cloning and characterization of cDNA encoding fibrinolytic enzyme-3 from earthworm *Eisenia foetida*[J]. *Acta Biochim Biophys Sin (Shanghai)*, 2004, **36**(4): 303-308.

[29] Li DH, Tong W, Yang YF. Functional expression of an earthworm fibrinolytic enzyme in *Escherichia coli*[J]. *World J Microbiol Biotechnol*, 2008, **24**(5): 613-618.

[30] Hu R, Zhang S, Liang H, *et al*. Codon optimization, expression, and characterization of recombinant lumbrokinase in goat milk[J]. *Protein Expr Purif*, 2004. **37**(1): 83-88.

[31] Hu R, Zhang S, Xu Q, *et al*. A convenient method for the identification and expression of eukaryotic genes[J]. *Biotechnol Appl Biochem*, 2004, **39**: 307-312.

[32] Robertus JD, Kraut J, Alden RA, *et al*. Subtilisin. Stereochemical mechanism involving transition-state stabilization[J]. *Biochem*, 1972, **11**(23): 4293-4303.

[33] Tang Y, Liang D, Jiang T, *et al*. Crystal structure of earthworm fibrinolytic enzyme component A: revealing the structural determinants of its dual fibrinolytic activity[J]. *J Mol Biol*, 2002, **321**(1): 57-68.

[34] Wang C, Wang F, Li M, *et al*. Structural basis for broad substrate specificity of earthworm fibrinolytic enzyme component A[J]. *Biochem Biophys Res Commun*, 2004, **325**(3): 877-882.

[35] Shotton D, Hartley B. Amino-acid sequence of porcine pancreatic elastase and its homologies with other serine proteinases[J]. *Nature,*1970, **225,** 802 - 806.

[36] HUEROU I. Structural identity within the trypsin family[J]. *Eur J Biochem*, 1990, **193**(3): 767-773.

[37] Sugimoto M, Ishihara K, Nakajima N. Structure and function of an isozyme of earthworm proteases as a new biocatalyst[J].*J Mol Catal B-Enzym*, 2003, **23**(2-6): 405-409.

[38] Mihara H, Sumi H, Yoneta T, *et al*. A novel fibrinolytic enzyme extracted from the earthworm *Lumbricus rubellus*[J]. *Jpn J Physiol*, 1991,**41**(3): 461-472.

[39] Kasai S, Arimura H, Nishida M, *et al*. Proteolytic cleavage of single-chain pro-urokinase induces conformational change which follows activation of the zymogen and reduction of its high affinity for fibrin[J].*J Biolog Chem*, 1985, **260**(22): 12377-12381.

[40] Zhao J, Li L ,Wu C, *et al*. Hydrolysis of fibrinogen and plasminogen by immobilized earthworm fibrinolytic enzyme II from *Eisenia fetida*[J]. *Int J Biolog Macromol,* 2003, **32**(3-5): 165-171.

[41] Zhao J, Pan R, He J, *et al*. *Eisenia fetida* protease-III-1 functions in both fibrinolysis and fibrogenesis[J]. *J Biomed Biotechnol*, 2007, **2007**(5):97654.

[42] Schechter I, Berger A. On the size of the active site in proteases. I. Papain[J]. *Biochem Biophys Res Commun*, 1967, **27**(2): 157.

[43] Wolberg AS. Thrombin generation and fibrin clot structure[J]. *Blood Rev*, 2007, **21**(3): 131-142.

[44] Bach-Gansmo ET, Halvorsen S, Godal HC, *et al*. Impaired coagulation of fibrinogen due to digestion of the *C*-terminal end of the Aα-chain by human neutrophil elastase[J]. *Thromb Res,* 1994, **73**(1): 61-68.

[45] Zhao MM, Li M, Han ZL, *et al*. Cloning and expression of lumbrokinase gene in *Pichia pastoris*[J]. *Acta Microbiol Sin*, 2006, **46**(4): 581-585.

[46] Sun HL, Jiao JD, Pan ZW, *et al*. The cardioprotective effect and mechanism of lumbrokinase[J]. *Acta Pharm Sin*, 2006, **41**(3): 247-251.

[47] Ji H, Wang L, Bi H, *et al*. Mechanisms of lumbrokinase in protection of cerebral ischemia[J]. *Eur J Pharmacol*, 2008, **590**(1-3): 281-289.
[48] Collen D. On the regulation and control of fibrinolysis. Edward Kowalski Memorial Lecture[J]. *Thromb Haemost,* 1980, **43**(2): 77.
[49] Mihara H, Maruyama M, Sumi H. Novel thrombolytic therapy discovered from traditional oriental medicine using the earthworm[J]. *Southeast Asian J Trop Med Pub Heal*, 1992, **23**(1):131.
[50] Hoylaerts M, Rijken DC, Lijnen HR, *et al*. Kinetics of the activation of plasminogen by human tissue plasminogen activator, Role of fibrin [J]. *J Biolog Chem*, 1982, **257**(6): 2912-2919.
[51] L Standker, R Sillard, KW Bensch, *et al*. *In vivo* degradation of human fibrinogen Aα: detection of cleavage sites and release of antithrombotic peptides[J]. *Biochem Biophys Res Commun*, 1995, **215**(3): 896-902.
[52] Xu R. *Molecular Gene Therapy*[M]. Beijing: Peking University Press & Peking Medical University Press, 2003, **3**: 68-90.
[53] During MJ, Symes CW, Lawlor PA, *et al*. An oral vaccine against NMDAR1 with efficacy in experimental stroke and epilepsy[J]. *Science*, 2000, **287**(5457): 1453-1460.
[54] Cheng MB, Wang JC, Li YH, *et al*. Characterization of water-in-oil microemulsion for oral delivery of earthworm fibrinolytic enzyme[J]. *J Control Rel*, 2008, **129**(1): 41-48.
[55] Sumi C, Ichinose H, Nagatsu T. Characterization of recombinant human aromatic *L*-amino acid decarboxylase expressed in COS cells[J]. *J Neurochem*, 1990, **55**(3): 1075-1078.
[56] Wu C, Li L, Zhao J, *et al*. Effect of alpha M-2 on earthworm fibrinolytic enzyme III-1 from *Lumbricus rubellus*[J]. *Int J Biologl Macromol*, 2002, **31**(1-3): 71-77.
[57] Fan Q, Wu C, Li L, *et al*. Some features of intestinal absorption of intact fibrinolytic enzyme III-1 from *Lumbricus rubellus*[J]. *BBA-Gen Sub*, 2001, **1526**(3): 286-292.
[58] Nakajima N, Ishihara K, Sugimoto M, *et al*. Chemical modification of earthworm fibrinolytic enzyme with human serum albumin fragment and characterization of the protease as a therapeutic enzyme[J]. *Biosci Biotechnol Biochem*. 1996, **60**(2): 293-300.
[59] Mihara H, Sumi H, Akazawa K, *et al*. Fibrinolytic enzyme extracted from the earthworm[J]. *Thromb Haemostas*, 1983, **50**: 258-263.
[60] L Jina, Jinb H, Zhangb G, *et al*. Changes in coagulation and tissue plasminogen activator after the treatment of cerebral infarction with lumbrokinase[J]. *Clin Hemorheol Microcircul*, 2000, **23**(2): 213-218.
[61] Cooper EL, Hrzenjak TM, Grdisa M. Alternative sources of fibrinolytic, anticoagulative, antimicrobial and anticancer molecules[J]. *Int J Immunopathol Pharmacol*, 2004, **17**(3): 237-244.
[62] Hong C, Takahashi S, Imamura M, *et al*. Earthworm fibrinolytic enzyme: anti-tumor activity on human hepatoma cells *in vitro* and *in vivo*[J]. *Chin Med J,* 2007, **120**(10): 898-904.
[63] Ji H, Wang L, Bi H, *et al*. Mechanisms of lumbrokinase in protection of cerebral ischemia[J]. *Eur J Pharmacol*, 2008, **590**(1-3): 281-289.
[64] Zhang X, Zhang J, Kuang P, *et al*. The effect of lumbrokinase on P-selectin and E-selectin in cerebral ischemia model of rat[J]. *J Tradit Chin Med*, 2003, **23**(2): 141-146.
[65] Jin L, Jin H, Zhang G, *et al*. Changes in coagulation and tissue plasminogen activator after the treatment of cerebral infarction with lumbrokinase[J].*Clin Hemorheol Microcircul,* 2000, **23**(2-4): 213-218.
[66] Sun HL, Jiao JD, Pan ZW, *et al*. The cardioprotective effect and mechanism of lumbrokinase[J]. *Acta Pharm Sin*, 2006, **41**(3): 247-251.
[67] Tzamalouka G, Papadakaki M, ElChliaoutakis J. Freight transport and non-driving work duties as predictors of falling asleep at the wheel in urban areas of Crete[J]. *J Safety Res,* 2005, **36**(1): 75-84.

蚯蚓纤溶酶研究概述

赵永刚[1], 李　华[1], 许威廉[2], 罗　佳[2], 许瑞安[1*]

[1] 华侨大学分子药物学研究所分子药物教育部工程研究中心, 泉州 362021
[2] 新南威尔士大学科学学院, 悉尼, 肯辛顿 2052, 澳大利亚

【摘　要】 对具有纤溶活性的物质研究已有多年的发展, 但是血栓性疾病仍然是致人死亡或身体机能障碍的主要原因之一。从具有特殊生理功能的生物体中获取活性物质近来受到广泛关注, 在蚯蚓消化腺的分泌物中纯化得到一组同工酶, 表现出良好的溶栓效果, 并被命名为蚯蚓纤溶酶(earthworm fibrinolytic enzyme, EFE)。本文在多层次上对蚯蚓纤溶酶作一综述, 内容包括: 蚯蚓纤溶酶的纯化、克隆表达, 并详细介绍该酶结构与功能之间的关系以及临床应用等。

【关键词】 蚯蚓纤溶酶; 溶栓药物

【基金项目】 国家高技术研究发展计划“863 计划”(No. 2008AA02Z135)资助项目