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Fibrinolytic Enzymes from Earthworms

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ABSTRACT

The fibrinolytic system is responsible for the proteolytic degradation of fibrin and therefore plays a role in haemostasis and thrombosis. Intravascular thrombosis, a consequence of fibrin aggregation in the arteries, is one of the main causes of cardiovascular disease. Fibrin is the primary protein component of blood clots, which are formed from fibrinogen by thrombin. Formation of fibrin clot and fibrinolysis are normally well balanced in biological systems. However, if fibrin is not hydrolyzed due to some disorder, thrombosis can occur. The most common consequence of such thrombosis is myocardial infarction. Fibrinolytic enzymes are agents which dissolve fibrin clot. Today available agents are mostly plasminogen activator (t-PA), urokinase and streptokinase, which exhibit low specificity for fibrin, have undesired side effect and are also relatively expensive. Therefore, the search for other fibrinolytic agent from various sources continues. The presence of fibrinolytic activity in ceolomic fluid or tissue homogenate from earthworm has been reported previously. Because of that, earthworm tissue homogenate is an attractive source of various physiologically active compounds.

Keywords: blood clotting, coelomic fluid, thrombosis
Abbreviations: t-PA, tissue plasminogen activator; **u-PA**, urokinase plasminogen activator

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INTRODUCTION

Haemostasis is a tightly regulated process of keeping an optimal balance between coagulation and fibrinolysis. Coagulation involves a series of enzymatic reaction, in which inactive plasma proteins are converted into active enzymes in each step of the pathway. The cascade is initiated by the release of tissue factor or damaged collagen underneath the blood vessel endothelium and the formation of fibrin clot. The fibrin clot is formed from fibrinogen by thrombin. This is essential to stop blood loss, after trauma or injury. More than twenty enzymes present in the body assist in clotting of the blood, but only few can break the clot down. One of them is an endogenously produced fibrinolytic enzyme called plasmin, a serine protease activated by tissue plasminogen activator (Sumi *et al*. 1987; Sumi *et al*. 1990; Silverthorn *et al*. 1998), and streptokinase, an extracellular metallo-enzyme. The agents which can dissolve fibrin clots belong to a group of fibrinolytic enzymes. All major fibrinolytic proteins include serine peptidases, their inhibitors, activators and receptors. Under physiological conditions, both coagulation and fibrinolysis are precisely regulated by the measured participation of substrates, activators, cofactors and receptors. Thus, fibrinolysis is a highly regulated system that integrates with the coagulation system through several direct molecular links. Because of that, the fibrinolytic enzymes found an important place in research. The enzymes currently being used for these purposes include urokinase, streptokinase and genetically engineered tissue plasminogen activator, which are expensive and patients may suffer from undesirable side effects. Thus, seeking for available and cheap source of these enzymes is the goal of many researchers.

Earthworms have been used in Chinese traditional medicine to improve blood circulation and to treat apoplectic stroke, as well as antipyretic and diuretic agents for tens of centuries. In the 1980s, groups of fibrinolaytic isoenzymes were isolated from different earthworm species. First enzymes with fibrinolytic and thrombolytic activities, named lumbrokinase, were found in crude extracts from the earthworm *Lumbricus rubellus* (Mihara *et al*. 1983). So far many kinds of earthworm fibrinolytic enzymes from other earthworms, such as *Lumbricus bimastus* (Cheng *et al*. 1996; Xu *et al*. 2002) and *Eisenia foetida* (Hrženjak *et al*. 1998; Li *et al*. 2003; Wang *et al*. 2003) were purified and characterized. Most earthworm fibrinolytic enzymes showed distinctive high stability and strong tolerance to organic solvents and high temperature. Because of their fibrinolytic activity to dissolve fibrin in blood clots, they should be used for the treatment of cardiac and cerebro-vascular diseases. Clinical experiments showed that fibrinolytic enzymes after oral administration to the patients suffering from thrombosis could reduce coagulation of fibrin and blood platelets, without side effect on other functions (Ryu *et al*. 1994; Lijnen *et al*. 1995; Gao *et al*. 1999: Zheng *et al*. 2000). Thus, fibrinolytic enzymes from earthworms could be considered as safe and effective agents in the treatment of thrombosis, cardiac and cerebrovascular clotting diseases. The most remarkable feature of fibrinolytic enzymes was their way of absorption. They could be transported into blood through intestinal epithelium and exerted their biological function in circulation (Fan *et al*. 2001). Other fibrinolytic enzymes such as urokinase and tissue plasminogen activator could be administrated by intraperitoneal injection rather than orally. The

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Table 1 Fibrinolytic enzymes purified from earthworms.

Source	Fibrinolytic enzyme	Authors
L. rubellus	F-III-1, F-III-2, F-II, F-I-0; F-I-1, F-I-2	Mihara *et al*. 1991 Nakajima *et al*. 1993
E. foetida	PI, PII	Hrženjak *et al*. 1998
E. foetida	EFE a, b, c, d, e, f, g	Wang *et al*. 2003
L. rubellus	F1, F2, F3, F4, F5, F6	Cho *et al*. 2004
E. foetida	P-III-1	Zhao *et al*. 2007

potential use of fibrinolytic enzymes in the prevention and treatment of serious cardiac and cerebro-vascular diseases has been very attractive in medicine and pharmacology. Besides their used as therapeutics, fibrinolytic enzymes could be also used in degradation of organic waste products from the food and livestock industry (Nakajima *et al*. 2000). Also the earthworms are very cheap source.

EARTHWORMS' FIBRINOLYTIC ENZYMES

Several reports are available on isolation and biochemical characterization of fibrinolytic enzymes from different earthworms (**Table 1**). Biochemical studies of fibrinolytic enzymes from *Lumbricus rubellus* have revealed that they are composed of six components: F-I-0, F-I-1, F-I-2, F-II, F-III-1 and F-III-2. These enzymes differed in their substrate and inhibitor's specificity (Mihara *et al*. 1991; Nakajima *et al*, 1993). The enzymes F-II, F-III-1 and F-III-2 were characterized as trypsin-like protease, whereas F-I-0, F-I-1 and F-I-2 were characterized as chymotrypsin-like protease.

Fibrinolytic enzymes have also been purified and characterized from *Eisenia foetida* (Hrženjak *et al*. 1998; Li *et al*. 2003; Wang *et al*. 2003; Zhao *et al*. 2007). From glycolipoprotein mixture (G-90), obtained from tissue homogenate of *Eisenia foetida*, two tyrosine-like serine peptidases (Hrženjak *et al*. 1998) with fibrinolytic activity were isolated. The enzymes were purified using anion exchange chromatography (DEAE Sepharose FF) what resulted in two proteins PI (34 kDa) and PII (24 kDa). After SDS-PAGE analysis, both enzymes were detected as a single band. The enzymes exhibited esterase activity, whereas the amidase activity was shown only with PI. Furthermore, it was shown that enzyme PII is a product of autolysis of PI. The PMSF, a specific inhibitor of serine peptidases, substantially inhibited (100 %) the reaction of PII enzyme with BAEE as substrate. The fibrin clot lyses time in euglobulinic test was significantly shortened by PI and PII, suggesting that these enzymes additionally activated plasminogen. The majority of fibrinolytic activity was belonging to PI, but in combination with PII, the synergistic effect was noticed. That effect could be the result of molecular interaction between PI and PII, which together form a protein of 55 kDa, equal to molecular mass of urokinase plasminogen activators (u-PA) in vertebrates (a and b chain) (Gandolfo *et al*. 1996). However, there could be one another possibility of inhibition of platelet aggregation. Adhesive principles of cell interactions with the surrounding molecules affect the fibrinogen binding GPIIb-IIa integrin receptors on platelets. Many artificial or natural peptides, such as snake venom (echistatin, bitistatin), have recognizable RGD sequences for platelet integrins. When such proteins bind on the integrin receptor, they block fibrinogen linking and platelet aggregation (Phyllips *et al*. 1991). A similar competition could be in the process with PI and PII enzymes. Fibrinolytic activity of PI and PII enzymes was directly proportional to the concentration. The results pointed that isolated peptidases from G-90 express the activity as u-PA. It persists in all body fluids of vertebrates and it has been found in many tissues extracts from invertebrates (Brommer *et al*. 1997). U-PA does not act only on plasminogen, but also on the other proteins of extracellular matrix and basal membrane. It is a key component for cleaning and maintenance of haemostasis (Bugge *et al*. 1996; Brommer *et al*. 1997), including the protection of thrombotic disorders, inflammation, and metastasis of malignant tumors (Zhu *et al*. 1993; Massignon *et al*. 1994; Quax *et al*. 1994; Salgado *et al*. 1994). Very similar enzyme to PI were isolated and characterized from *Eisenia foetida* from other group (Li *et al*. 2003). According molecular mass of isolated enzyme (34.19 kDa), there is some indication that isolated protein is identical to PI. They also found N-glycosylated spot.

On the other hand, Wang *et al*. (2003) have purified seven fibrinolytic enzymes (EFE-a, b, c, d, e, f, g). All enzymes were very similar in size (23 -29.7 kDa) with the isoelectric points in acidic pH range (3.46 – 3.94). Fibrinolytic activity of each enzyme on fibrin plates was different. The enzymes EFE-b, EFE-c and EFE-g showed relatively higher activity in comparison with the enzymes EFE-d and EFE-e. Relatively lower fibrinolytic activity was shown with EFE-a and EFE-f. There also has been some indication that EFE-a, besides fibrinolytic activity, exhibited plasminogen-activating activity. The other enzymes only have fibrinolytic activity. According to substrate specificity, the enzymes EFE-b, EFE-c, EFE-g represent trypsin-like enzymes, and EFE-d, EFE-e, EFE-f a group of chymotrypin-like enzymes. It seems that EFE-a do not belong to trypsin-like or chymotrypsin-like enzymes, nor to the elastases. The optimal substrate for EFE-a has not been found yet. The activity of specific substrates for human plasmin and t-PA showed that EFE-b, EFE-c and EFE-g exhibited strong hydrolytic activity. However, EFE-a, EFE-d and EFE-e had very weak hydrolytic activity. Studies on inhibition indicated that earthworm fibrinolytic enzymes were a group of serine peptidases. The structural analysis of EFE-b (Wang *et al*. 2005) showed that it should be classified as a trypsin from earthworm. However, it is distinct from other trypsins. It is a two-chained protease with an N-terminal, pyroglutamated light chain and N-glycosylated heavy chain. The heavy chain contains a novel structural motif, an eigth-membered ring resulting from a disulfide bridge between two neighbouring cystein residues, and a *cis* peptide bond exists between these two cysteine residues. The crystal structure of EFE-b provides the structural basis for explanation of its high stability. That gets along with using of EFE-b as an oral drug. In earthworm was also found very complicated post-translation modifications, which might contribute in explanation of the origin and evolution of the cymotrypsin family.

The above-mentioned fibrinolytic enzymes could find a place in pharmaceutical industry. They could be used in treatment of deregulated haemostasis, to prevent the formation of blood clots and balanced fibrinolysis. The earthworms are handy everywhere, and the preparation of their extracts is usually very simple (G-90). The main problem of these extract is low concentration of pure enzyme, responsible for fibrinolytic activity. The fibrinolytic enzymes could be purified from earthworms by biochemical approaches. Major limitation of purification in this way is the relatively low yield of the enzymes to be used clinically as therapeutic agents. Recently, certain progress has been made towards production of fibrinolytic enzymes via genetic engineering. The genes encoding F-II-2 (Sugimoto *et al*. 2001), PM 246 (Hu *et al*. 2005) of *Lumbricus rubellus* and F238 (Yuan *et al*. 2006) of *Eisenia foetida* (lumbrokinase 3) have been cloned and expressed in *Pichia pastoris*. Also in bacteria *Escherichia coli*, two cDNA fragments of fibrinolytic enzyme (IrF1 and IrF2), gene F-III-2 (GenBank ABo45719) from earthworm *Lumbricus rubellus*, were cloned into bacterial expression vector pET28a (+) (Li *et al*. 2008). Both, IrF1 and IrF2 proteins were produced as an inclusion body from *E. coli* BL21 (DE3) pLysE. After purification and protein refolding, IrF1 showed a strong fibrinolytic activity, whereas IrF2 had no fibrinolytic activity.

CONCLUSIONS

Earthworm fibrinolytic enzymes represent a group of serine proteases with strong fibrinolytic activity. The preparations

are highly stable, inexpensive and can be administrated as oral drug for prevention and treatment of thrombosis and diseases with disturbed haemostasis. Considering different results described above, a large-scale production of the fibrinolytic enzymes could be perform at low cost in the bacterial expression system. Bacterial system could provide sufficient amount of fibrinolytic protease for therapeutic use and for environmental protection. However, it would be necessary to characterize the difference between the prokaryotic product and the eukaryotic one.

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