

Review Article

Earthworm Protease

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The alimentary tract of earthworm secretes a group of proteases with a relative wide substrate specificity. In 1983, six isozymes were isolated from earthworm with fibrinolytic activities and called fibrinolytic enzymes. So far, more isozymes have been found from different earthworm species such as *Lumbricus rubellus* and *Eisenia fetida*. For convenience, the proteases are named on the basis of the earthworm species and the protein function, for instance, *Eisenia fetida* protease (*EfP*). The proteases have the abilities not only to hydrolyze fibrin and other protein, but also activate proenzymes such as plasminogen and prothrombin. In the light of recent studies, eight of the *EfP*s contain oligosaccharides chains which are thought to support the enzyme structure. Interestingly, *EfP*-II has a broader substrate specificity presenting alkaline trypsin, chymotrypsin and elastase activities, but *EfP*-III-1 has a stricter specificity. The protein crystal structures show the characteristics in their specificities. Earthworm proteases have been applied in several areas such as clinical treatment of clotting diseases, anti-tumor study, environmental protection and nutritional production. The current clinical utilizations and some potential new applications of the earthworm protease will be discussed in this paper.

1. Introduction

Earthworm has been recorded with a long history. Five hundred years ago, Shizhen Li compiled the famous medical book *Compendium of Material*, in which the earthworm (Earth dragon) was recorded as a drug prescribed for antipyretic and diuretic purposes in the form of dried powder in clinic. Now the remedy is still used in the folk.

In the end of 19th century, Frédéricq [1] discovered one enzyme secreted from the alimentary tract of earthworm. Then several proteases were separated from the earthworm in 1920 [2]. They could dissolve casein, gelatin, and albumin. This was the preliminary research about the earthworm proteases. Large-scale research about earthworm protease began in 1980. Mihara *et al.* [3] isolated a group of proteases with fibrinolytic activity from the earthworm *Lumbricus rubellus*. Subsequently different purification methods were applied to isolate the enzymes, including gel filtration, affinity chromatography, ion exchanging chromatography, and high-pressure liquid chromatography (HPLC). More proteases have been obtained from different species, such as lumbrokinase [4], earthworm-tissue plasminogen activator

[5], earthworm plasminogen activator [6–11], component A of EFE (EFEa) [12, 13], and biologically active glycolipoprotein complex (G-90) [14–19].

2. Isozymes from Different Species

Earthworms are scientifically classified as animals belonging to the order Oligochaeta, class Chaetopoda, phylum Annelida. *Lumbricidae* is one of the main families in taxonomy [20]. Their native areas are in Europe, America, and Western Asia. *L. rubellus* (humus earthworm) and *E. fetida* (common brandling worm) can be raised and cultivated in large amount.

The earthworm proteases are multicomponent. Because of various living environments, different species of earthworms have different resultant isozymes. The proteases are independently studied in research groups, [3, 21, 22]. Thus, one isozyme may have multiple names. Here, we name the protease after the formal name of earthworm species and the protein function, for example, the protease from *L. rubellus* is called *L. rubellus* protease (*LrP*).

TABLE 1: The molecule mass of the eight isozymes.

Isozyme	<i>EfP</i> -0-1	<i>EfP</i> -0-2	<i>EfP</i> -I-1	<i>EfP</i> -I-2	<i>EfP</i> -II-1	<i>EfP</i> -II-2	<i>EfP</i> -III-1	<i>EfP</i> -III-2
MW (kDa)	22.5	22.4	28.8	28.1	30.6	29.1	34.8	35.0

See Wu *et al.* [26].

2.1. Isozymes from *Lumbricus rubellus*. Six proteases (*LrP*-I-0, *LrP*-I-1, *LrP*-I-2, *LrP*-II, *LrP*-III-1, and *LrP*-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. They are single peptide chains having more asparagine and aspartic acid residues but less lysine. They have a wide functional acidic range (pH 1.0–11.0) and do not inactivate until 60°C. The enzyme activity (*LrP*-II and *LrP*-III-1) is maximally exhibited around pH 9.0 at 50°C [24].

In 2005, Nakajima and colleagues purified an enzyme that catalyzes the hydrolysis of triacylglycerol [25]. The N-terminal amino acid sequence and the catalytic function of the purified enzyme were identical to those of *LrP*-II. The isozyme might act on the hydrolysis of triacylglycerol as well as the protein decomposition.

2.2. Isozymes from *Eisenia fetida*. In 1988, Zhou and coworkers [22] separated at least seven components with fibrinolytic activity from earthworm *E. fetida*. They are stable at pH 5.0–9.0 and denatured below pH 2.6. After that, a plasminogen activator (e-PA) with two subunits was separated from *E. fetida* [10], similar to the results reported previously [6]. This enzyme is considered a serine protease and its molecule mass is 45,000 Da. The two constituting subunits (26,000 Da and 18,000 Da) with different fibrinolytic activities are bound by hydrophobic interaction. Wu and colleagues isolated eight fibrinolytic enzymes (*EfP*-0-1, *EfP*-0-2, *EfP*-I-1, *EfP*-I-2, *EfP*-II-1, *EfP*-II-2, *EfP*-III-1, and *EfP*-III-2) through a stepwise-purification procedure in 2007 [26]. They are all glycoproteins (Table 1). Two of them (*EfP*-0-2 and *EfP*-II-2) are new isozymes and the other six in their primary structures are similar to those purified by Mihara and coworkers.

In 2008, another serine protease was purified from the coelomic fluid of the earthworm *E. fetida* [27]. It has strong antiviral activities against cucumber mosaic virus and tomato mosaic virus. The protease (27,000 Da) is the most active at pH 9.5 and 40–50°C.

2.3. Isozymes from *Lumbricus bimastus*. Three proteins have been isolated from the extraction of earthworm *L. bimastus* by Xu and coworkers [28]. The apparent molecular masses of the proteins are about 30,000, 29,000, and 28,000 Da exhibited on SDS-PAGE, respectively. The fragment encodes a 242-amino-acid protein called PV₂₄₂.

2.4. Isozymes from *Eisenia andrei*. Lee and colleagues have isolated a protease fraction (SPP-501) from the earthworm *E. andrei* [29]. The antithrombotic activity has been investigated in a thrombosis model. SPP-501 shows

both antithrombotic and fibrinolytic activities during oral administration.

Although several groups of isozymes have been studied in the species above, the total number is still not clear. The molecular weights of the proteases are in a relative narrow range (20–35 kDa) and they have activities in a wide pH scope.

3. Localization of the Protease in an Earthworm

*EfP*s are expressed and synthesized in the epithelial cells and mainly localized in the crop and gizzard, particularly in the anterior alimentary regions (Figures 1 and 2) [30]. In these regions, the proteases maybe contribute to digest protein and peptide in food.

4. Activity Assays

There are three methods to measure the activity of the isozymes: fibrin plate, chromophoric procedure, and light scattering (Table 2). Initially, fibrinolytic activity is measured by both plasminogen-rich and plasminogen-free fibrin plates [31, 32]. Individual earthworm is cut into pieces and placed on a plasminogen-rich fibrin plate. The fibrinolytic activity is determined by measuring the diameter of the plaque. Later, an assay using chromophoric substrate has been developed [24]. When the chromophoric substrate Chromozym TH reacts with the proteases, the absorbance at 405 nm will increase. The linear section from 15 to 30 seconds is used to calculate the activity of the protease. The unit is defined as the specific activity required converting 1 μ M substrate/minutes/mg of enzyme.

Another method records the changes of the scattering intensity during the conversion of fibrinogen to fibrin [33]. Changes of the scattering intensity of the thrombin-fibrin colloid follow a sigmoid curve with a relaxation phase and the maximum at 480 nm. The intercept at the maximum slope is directly proportional to the concentration of thrombin when fibrinogen is at a constant concentration. Thus, the intercept is employed to calculate the thrombin activity. The earthworm protease blocks the increase in the light scattering intensity, because the enzyme hydrolyzes both fibrinogen and fibrin. The amount of the protease is inversely proportional to the intensity.

5. Substrates of the Earthworm Protease

The earthworm protease shows different activities in the presence of different substrates. *LrP*-I (*EfP*-I) is considered a chymotrypsin-like protease [4]. *EfP*-II is capable of recognizing the six substrates N- α -benzoyl-L-Arginine ethyl

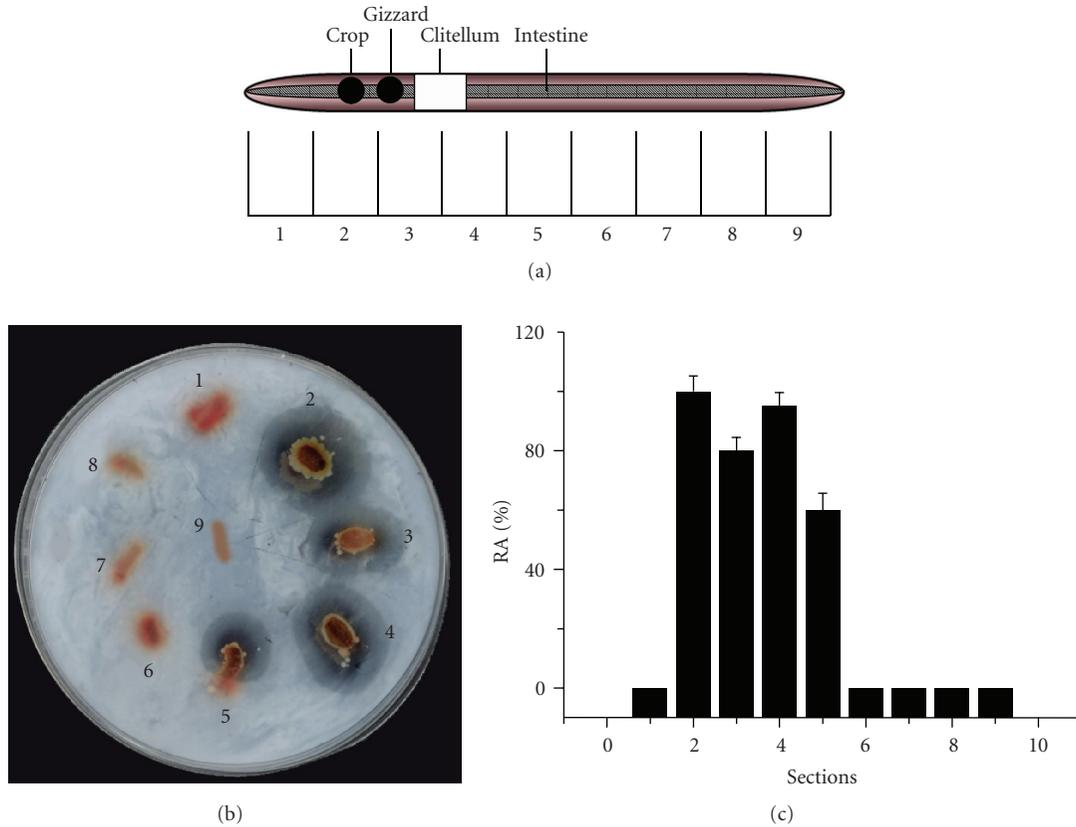


FIGURE 1: Localization and total relative activity of the earthworm segments: (a) nine pieces of the earthworm *E. fetida* (b) fibrin plate assay (c) enzymic activity of each segment (see [30]).

TABLE 2: Three assays of the earthworm protease.

Assays	Fibrin plate	Chromophoric procedure	Light scattering
Materials	Fibrinogen, thrombin, and agar	chromophoric substrates	Fibrinogen, thrombin
Principle	fibrinolytic activity	absorbance at 405 nm	Light scattering at 480 nm
Time	18 ~ 24 hours	~1 minute	~10 minutes
Feature	Fibrinolysis specificity, time consuming	Rapid, reproducible, lower specificity	Rapid, reproducible, lower specificity

ester (BAEE), N-acetyl-L-tyrosine ethyl ester (ATEE), Chromozym TH (Ch-TH, Car-Val-Gly-Arg-4-NA), Chromozym TRY (Ch-TRY, Tos-Gly-Pro-Arg-4-NA), Chromozym U (Ch-U, Ben-β-Ala-Gly-Arg-4-NA), and Chromozy ELA (Ch-ELA, Suc-Ala-Ala-Ala- pNA) and gives relative K_m values as follows: $[K_{mTH}] < [K_{mU}] < [K_{mELA}] < [K_{mBAEE}] < [K_{mTRY}] < [K_{mATEE}]$. This sequence indicates that *EfP-II* acts as a strong thrombin-like, moderate elastase-like, and weak chymotrypsin-like serine protease. On the other hand, *EfP-III-1* reacts with neither Ch-ELA nor ATEE, but reacts with BAEE, Ch-TRY, Ch-U, and Ch-TH, giving relative K_m values as follows: $[K_{mBAEE}] < [K_{mTRY}] < [K_{mU}] < [K_{mTH}]$, characteristic of a trypsin-like protease. Note that the K_m values for these substrates are approximately of the same order of magnitude (10^{-5} M), suggesting a higher substrate specificity for *EfP-III-1* than that for *EfP-II* [34]. The earthworm proteases have the abilities to degrade and digest

various substrates, which may make for their living in a vile environment.

6. Inhibitors of the Earthworm Protease

The activity of the earthworm protease is inhibited by several inhibitors [4, 9, 23, 35, 36]. Diisopropyl fluorophosphate (DFP) completely inhibits the activity of all the isozymes (pH 7.2) at room temperature. *LrP-I-0*, *LrP-I-1*, and *LrP-I-2* are partially inhibited while *LrP-III-1* and *LrP-II* are strongly inhibited by SBTI and aprotinin. However, the activity of the protease is not significantly affected by tosyl-lysyl-chloromethylketose, Tosyl-phenylalanyl chloromethylketose, elastatinal, ε-amino caproic acid, EDTA, or various metal ions [23]. The specificity of substrates and inhibitors gives the evidence that the isozymes are alkaline serine-like proteases.

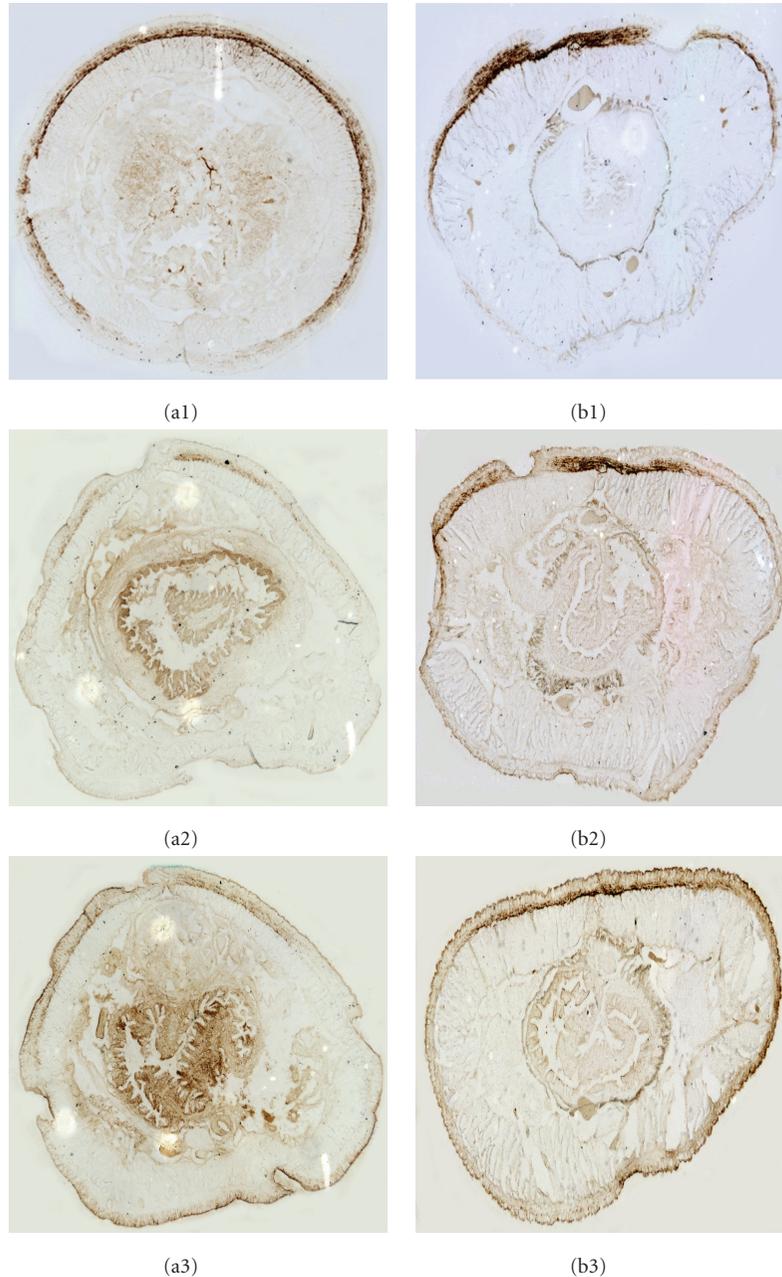


FIGURE 2: *In situ* localization of *EfP-II* and *EfP-III-1* in the intestine of *Eisenia fetida* (a) segment 2 as shown in Figure 1. Anti-*EfP-II* (panel a2) or anti-*EfP-III-1* (panel a3) as primary antibodies was added, without adding the primary antibodies as control (panel a1). (b) Segment 5 (see [30]).

α_2 -Macroglobulin (α_2M), at a high concentration (approx. $2.0 \mu M$) in blood plasma, is an important endogenous inhibitor with the ability to inhibit all four classes of (cysteine, serine, aspartate, and metallo) proteases [37, 38]. The fibrinolytic activity of *LrP-III-1* decreased to 65% when incubated with α_2M , while it decreases to 30% in the plasma under the same conditions [39]. After *LrP-III-1* goes into blood, the enzyme may be firstly inhibited by α_2M because the kinetics of inactivation of *LrP-III-1* with α_2M is similar to that of the initial phase with plasma. α_2M binds to the

enzyme mole by mole equivalently and the interaction may undergo a chelate irreversible inhibition.

7. Protein Structural Features

7.1. Primary Structure. As shown in Table 3, the N-terminal sequences of the isozymes from *L. rubellus* and *E. fetida* have been analyzed [26]. The sequences of the isozymes from *L. rubellus* and *E. fetida* have a lot of identical residues.

TABLE 3: The N-terminal sequences of the isozyme.

<i>EfP</i>	N-Terminal squence	<i>LrP</i>	N-Terminal squence
<i>EfP</i> -0-1	VVGGSDTTIGQYPHQIL	<i>LrP</i> -I-0	VVGGSDTTIGQYPHLSLRVT
<i>EfP</i> -0-2	VVGGSDTTIGQ	—	—
<i>EfP</i> -I-2	IIGGSNASPGEFPWQL	<i>LrP</i> -I-1	IIGGSNASPGEFPWQLSQTRG
<i>EfP</i> -I-2	IIGGSNASPGEFPWQL	<i>LrP</i> -I-2	IIGGSNASPGEFPWQLSQTRG
<i>EfP</i> -II-2	VIGGTNASPGEFPPQ	<i>LrP</i> -II	VIGGTNASPGEIFPWQLSQQRX
<i>EfP</i> -II-2	VIGGTNASPGEFFP	—	—
<i>EfP</i> -III-2	IVGGIEARPYEFP	<i>LrP</i> -III-1	IVGGIEARPYEFPWQVSVRRK
<i>EfP</i> -III-2	IVGXIEARPYEFPWQVSVRRK	<i>LrP</i> -III-2	IVGGIEARPYEFPWQVSVRRK

See Wu *et al.* [26].

The N-terminal sequences of *LrP*-III-1 and *LrP*-III-2 are identical, and so are those of *LrP*-I-1 and *LrP*-I-2; *EfP*-0-1 and *EfP*-0-2; *EfP*-I-1 and *EfP*-I-2; *EfP*-II-1 and *EfP*-II-2; *EfP* -III-1 and *EfP* -III-2, respectively. As shown in Figure 3, so far, they all are thought to belong to the serine protease family, which could be further divided into three groups according to the following sequences: earthworm protease-1 (*EfP*-0), earthworm protease-2 (*EfP*-I, EFEa, and *EfP*-II), and earthworm protease-3 (*EfP*-III-1, EFEb, *LrP*-III-1, and *LrP*-III-2), as shown in the phylogenetic tree.

The glycan chains play a role in the stability, the spatial conformation, and the antigenicity of the protein. Recent studies show that the earthworm proteases are glycosylated [26]. The result of staining on the native-SDS gel with Schiff's reagent shows that the eight isozymes isolated from *Eisenia fetida* by Wu and colleagues are all glycoproteins. In addition, the contents of sugar have been determined with sodium metaperiodate and glycoprotein-test reagent, shown in Table 4. The proteases have different glycan contents and the oligosaccharides are composed of mannose residues.

7.2. Secondary Structure. The secondary structures of *LrP*-III-1 [40], *LrP*-III-2 [41], EFEa [42], EFE-b [43], *EfP*-0 [44], *EfP*-I [45], *EfP*-II [46], and *EfP*-III-1 [47] are predicted on the basis of their primary structures. The proteins have distinct predicted secondary structures, for example, β -sheet, α -helix, turn, and coil, as shown in Table 5. The sequence of *EfP*-II (EFEa) is highly similar to some related serine proteases with known structures [48–54] or other earthworm serine proteases [55] (Table 6).

7.3. Tertiary Structure. The catalytic characterization of the earthworm protease is influenced directly by their tertiary structures. The crystal structural study shows that *EfP*-III-1 (EFE-b) is a trypsin-like protease with two chains (an N-terminal, pyroglutamated light chain and an N-glycosylated heavy chain) [56]. The structural features (Figure 4) probably endow *EfP*-III-1 with high level of stability in resistance to heat, organic solvents, and proteases [57].

EfP-II is not only a chymotrypsin-like serine protease but also has an essential S1 pocket of elastase (Figure 4). The S1 specificity pocket is preferable for elastase-specific small hydrophobic substrate, while its accommodation of long and/or bulky substrate is also feasible if enhanced binding

of the substrate and induced fit of the S1 pocket are achieved. Compared with the stable active site of *EfP*-III-1, that of *EfP*-II (EFEa) is more flexible, resulting in a broader substrate specificity [13].

8. Fibrinolytic Mechanism and Cleavage Sites

The earthworm proteases have relatively broad substrate specificities, such as trypsin (cleaving the carboxylic sites of Arg and Lys) and chymotrypsin (cleaving the carboxylic sites of Phe, Trp, Tyr, and Leu) [34, 58]. Furthermore, *EfP*-III-1 specifically recognizes the carboxylic sites of arginine and lysine. The protease cleaves the α chain of fibrinogen at R₂₅₂-G₂₅₃, R₁₉-V₂₀, and K₄₂₉-V₄₃₀, respectively. According to the densities of the protein bands on the SDS-PAGE, hydrolysis of α chain is the fastest, and hydrolysis of β chain is faster than that of γ chain. This indicates that *EfP*-III-1 possesses strong α -fibrinogenase, moderate β -fibrinogenase, and weak γ -fibrinogenase activities. *EfP*-III-1 activates plasminogen cleaving at R₅₅₇-I₅₅₈. This cleavage site is also recognized by tPA. Besides, *EfP*-III-1 has Xa-like function. *EfP*-III-1 recognized peptidyl bonds at R₃-A₄, R₁₅₈-S₁₅₉, R₂₇₄-T₂₇₅, R₃₉₆-N₃₉₇, and R₂₈₇-T₂₈₈. *EfP*-III-1 cleaves prothrombin at R₂₇₄-T₂₇₅, thereby releasing the intermediates fragment 1.2 and prethrombin-2. As mentioned above, *EfP*-III-1 cleaves at R₂₈₇-T₂₈₈ and releases an α -thrombin-like product with a 13-residue deletion at the N-terminus of a chain. Similar to the preference for residue N₃₉₇ by thrombin, which produces the β -thrombin-like fragments [59], *EfP*-III-1 cleaves at residue R₃₉₆. That is to say, *EfP*-III-1 has the ability to hydrolyze fibrinogen and to activate plasminogen and prothrombin, playing a part not only in fibrinogenolysis but also in fibrogenesis. Based on this, the roles of *EfP*-III-1 in procoagulation and anticoagulation can be summarized as follows (Figure 5). The function in both activating prothrombin and catalyzing fibrinogenolysis suggests that *EfP*-III-1 plays a role in the balance between procoagulation and anticoagulation.

9. Oral Administration

Usually the macromolecules cannot permeate the biological membranes. In particular, protein can be degraded by pepsin, trypsinase, and chymotrypsin. The gastric juice has a low pH value and denatures the ordinary proteins.

TABLE 4: The Carbohydrate content of the eight isozymes.

Number	Earthworm protease	Carbohydrate content (%) ^(a)	Relative activity (%) ^(c)
1	<i>EfP</i> -0-1	6.13	6.2
2	<i>EfP</i> -0-2	4.30	12.8
3	<i>EfP</i> -II-1	6.18	25.8
4	<i>EfP</i> -I-2	1.38	31.6
5	<i>EfP</i> -II-1	1.82	8.8
6	<i>EfP</i> -II-2	7.40	2.1
7	<i>EfP</i> -III-1	6.55	12.5
8	<i>EfP</i> -III-2	4.41	2.3
9	Mixture of isozymes ^(b)	4.40	100

^(a) Mass ratio between the carbohydrate content and the glycoprotein,

^(b) Estimated on the fibrin-PAGE by Band-scanned software,

^(c) *EfPs* purified by the SBTI-Sepharose-4B affinity chromatography column. See Wu *et al.* [26].

TABLE 5: Prediction of the secondary structures of the isozymes.

	<i>LrP</i> -III-1	<i>LrP</i> -III-2	EFEa	EFEb	<i>EfP</i> -0	<i>EfP</i> -I	<i>EfP</i> -II	<i>EfP</i> -III-1
Helix (%)	7	4	6	5	7	6	6	4
Sheet (%)	40	41	30	42	35	29	30	44
Turn (%)	15	14	18	13	12	19	17	14
Coil (%)	37	41	46	40	45	46	46	39

By Double Prediction Method (DPM), Antheprot software.

TABLE 6: Comparison of homologous sequences with some serine proteases.

Proteases	Identity (%)
Pocine pancreatic elastase (PPE)	36
Human leukocyte elastase (HLE)	28
Trypsin	35
Chymotrypsin	34
U(t)-PA, Plasmin	32
F-III-1(2)	35
Earthworm chymotrypsinogen	31
Lugworm chymotrypsinogen	44

See Tang *et al.* [13].

Whereas, some therapeutic proteins with specific properties can be absorbed with the intact and active form before being degraded in the alimentary tract, such as β -lactoglobulin, hepatitis-B surface antigen, bromelain, and epoxy- β -carotenes [60].

Furthermore, the earthworm protease could also be transported into blood through intestinal epithelium and perform its biological functions in the blood [61]. The *in vitro* experimental data show that 15% intact *LrP*-III-1 is absorbed through intestinal epithelium. About 10% full-size enzyme is transported through the peritoneum after the intraperitoneal injection in the rat. The maximum activity in blood is detected around 60 minutes after the injection.

The N-terminal sequences of *LrP*-III-1 and *LrP*-III-2 are similar to protein transduction domain [62]. The sequences are rich in hydrophobic amino acid residues, which may

play a role in the process of the membrane transportation of biological macromolecules.

10. Clinical Application and Medical Research

10.1. The Earthworm Protease as a Fibrinolytic Agent. The formation of thrombus in the blood causes many devastating diseases such as stroke and myocardial infarction. Several enzymes have been used as the thrombolytic agents including urokinase (UK), streptokinase, recombinant tissue-type plasminogen activator, staphylokinase, and recombinant prourokinase [63, 64]. These agents are administered via intravenous injection generally. Some of them are effective, but they also have some limitations such as fast clearance, lack of resistance to reocclusion, bleeding complications, and other adverse effects [63].

The earthworm protease functions in the fibrinolysis and plasminogen activation, distinct from those enzymes (UK, tissue-type plasminogen activator, etc.) [65–67]. Therefore they have been used to treat the thrombosis. The proteases during orally experiments both in animals and clinics show significant fibrinolytic efficacy. A distinct amelioration is observed in the treatment of blood high-viscosity syndrome and thrombocytosis [68]. In addition, the proteases are stable during a long-term storage at room temperature [69], in the form of oral capsule. Earthworm is easily raised, which renders the isozymes into a relatively inexpensive thrombolytic agent. So far, the earthworm proteases have been used as an orally administered fibrinolytic agent to prevent and treat clotting diseases, such as myocardial infarction and cerebral thrombus [70].

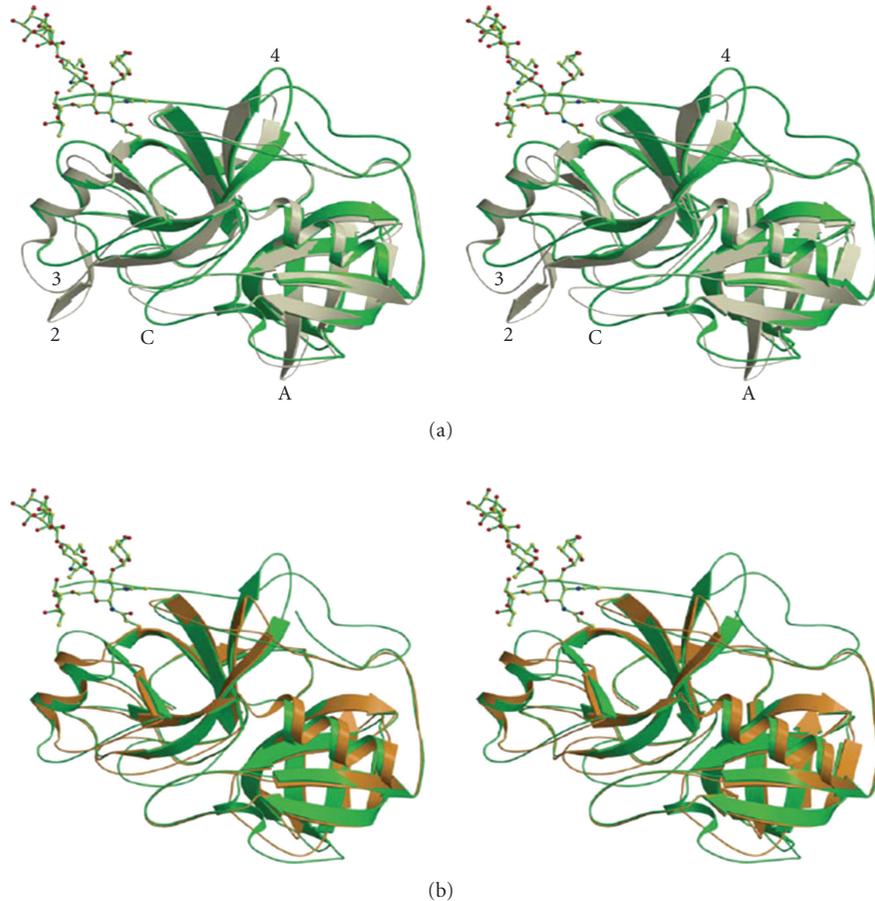


FIGURE 4: Superposition of EFE-b with EFE-a and trypsin. (a) Stereoview of the superposition of EFE-b with EFE-a is illustrated as follows: EFE-b is represented by green and EFE-a by light goldenrod yellow. The glycan is represented by a ball-and-stick model (carbon atoms, green). Some loops in which EFE-b greatly differs from EFE-a are indicated: loop A (34–41); loop C (97–103); loop 2 (217–225); loop 3 (169–174); loop 4 (201–208). (b) Stereoview of the superposition of EFE-b with trypsin is illustrated as follows: EFE-b is represented by green and trypsin (Protein Data Bank, accession number, 1PPE) by orange. The glycan is represented by a ball-and-stick model (carbon atoms, green) (see [56]).

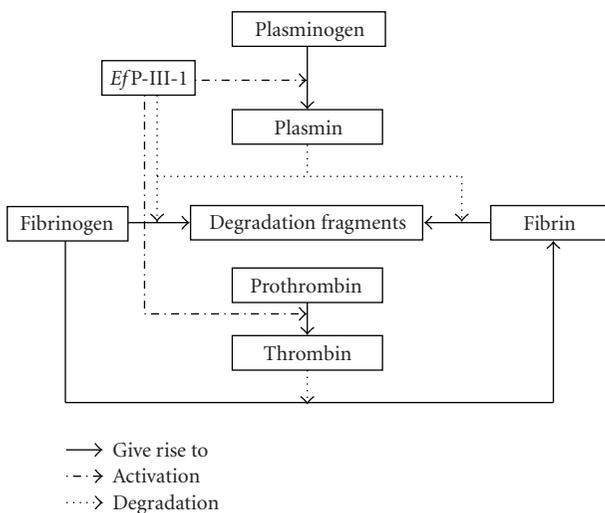


FIGURE 5: Roles of *Efp*-III-1 in procoagulation and anticoagulation. Activation is indicated by dashed lines, and degradation is indicated by dotted lines (see [34]).

10.2. Antitumor. Cancer has a reputation of being an incurable disease. Although some methods such as surgery, chemotherapy, radiation therapy, and immunotherapy are available, they are far from reaching the goal of complete removal of the cancer cells without damage to the rest of the body. It is demonstrated that the earthworm crude extract has the ability to kill the cancer cells directly in vitro [71, 72] and inhibit the occurrence and development of tumor in vivo [73]. Furthermore, it has been proved that the earthworm proteases enhance the curative effects by both radiation therapy and chemotherapy [74, 75].

The most malignant tumors secrete urokinase-type plasminogen activator (u-PA). In order to inhibit the hyperactivity of the u-PA, inhibitors of plasminogen activators are synthesized by the surrounding cells for tissue protection, resulting in a high concentration of fibrin locally. The glycolipoprotein mixture (G-90) was isolated from the homogenate of *E. fetida* [15–17, 19], which is assayed in a euglobulinic test applied to fibrin clot from blood plasma of patients who suffered from malignant tumors. The effect

of G-90 on the fibrinolysis rate is related to not only its concentration, but also to histological type where the malignant tumors invade. The blood with the fibrin clots derived from the dogs with cardiopathies and the dogs with malignant tumors was examined for the time of coagulation and fibrinolysis by adding different substances including G-90. The clotting time in the presence of G-90 shows dogs with malignant tumors > healthy dogs > dogs with cardiopathies [14].

Recently, a glycosylated component is separated from the earthworm *E. fetida* by Xie and coworkers [76], which has relations with apoptosis of tumor cells. It is highly homologous to *LrP-I-1* and *LrP-I-2*. It is identified to be a plasmin and also a plasminogen activator. From the results of the phase-contrast microscopy observation of apoptotic cells and the localization of fluorescent antibodies in cell nucleus, the antitumor activity is observed.

The earthworm protease possesses obvious anti-tumor activity in the hepatoma cells. The proliferation of the hepatoma cell treated with the proteases is inhibited in proportion to the concentration of the proteases. The growth of tumor xenograft in nude mice is significantly suppressed after being fed with the earthworm protease for four weeks. At the same time, it has been found that the earthworm protease can induce apoptosis of hepatoma cells and downregulated the expression of matrix metal protease-2. As described above [77], the earthworm protease is a potential candidate for treating some kind of tumors.

10.3. Assistant to Implantation. After an artificial organ is introduced into a living body, small thrombus is usually formed on the surface of the graft. Many approaches have been tried to improve the blood compatibility to biomaterial. However, the results, so far, are not satisfactory. In 1994, *LrP* was immobilized on the surface of polyurethane using maleic anhydride methylvinyl ether copolymer as an enzyme carrier [78]. So the *LrP*-immobilized polyurethane surface has highly antithrombogenic activity and can reduce surface-induced thrombus. *LrP*-immobilized 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.

LrP has been immobilized in a Korean-type total artificial heart valve by photoreaction, and polyallylamine is used as a photoreactive linker [79–81]. The proteolytic activity on the azocasein of the treated valves is three times higher than that of untreated valves. The *LrP*-treated polyurethane valve leads to decreasing thrombus formation in vivo and their biocompatibility is, therefore, greater than that of untreated valve. This method may be developed and may be useful in clinical application.

10.4. Anti-Ischemia. Recently, the effect of the earthworm protease against myocardial ischemia [82] has been investigated on a rat model with acute myocardial infarction. Meanwhile the L-type calcium current (ICa-L) and intracellular calcium concentration ($[Ca^{2+}]_i$) have been measured. The results indicate that it has protective actions on myocardial

infarction in rats. Decreasing of the ICa-L and $[Ca^{2+}]_i$ in ventricular myocytes is the possible mechanism.

The study has been conducted with 10 patients who had coronary artery disease and stable angina. Stress technetium-99m sestamibi myocardial perfusion imaging has been performed before and at the end of the treatment period. As a result, the angina symptom is ameliorated in 6 out of 10 patients. No adverse reaction such as major or minor bleeding has been observed. That is to say, oral *LrP* improves regional myocardial perfusion in patients with stable angina [83]. In this research, some expectable results have been achieved on patients with coronary artery disease and stable angina. It is in favor of better application of earthworm proteases.

The mechanism of the anti-ischemia function of *LrP* in brain has been also studied. The results show that the anti-ischemic activity of *LrP* was due to its antiplatelet activity by elevating cAMP level and attenuating the calcium release from calcium stores, the antithrombosis action due to inhibiting of ICAM-1 expression, and the antiapoptotic effect due to the activation of JAK1/STAT1 pathway [84].

11. Problems and Potential Solutions of the Earthworm Protease as a Medicine

Though the earthworm protease has good pharmaceutical effect in clinic application, some limitations still exist as a clinical fibrinolytic agent. It hydrolyzes not only the fibrinogen and fibrin but also some other proteins in vivo. Besides, the half-life of the earthworm protease is short in circulation. An ideal fibrinolytic medicine should meet the qualifications such as strong fibrinolytic activity, specificity on fibrinogen and fibrin, low immunogenicity, long half-life in vivo, low reocclusion rate, and reasonable cost [85]. In order to increase the bioavailability and strengthen the drug action, different methods are under trials.

11.1. Drug Delivery. Recently, some other ways of drug delivery have been studied. In Cheng's research, a water-soluble earthworm protease was used in the delivery of the water-in-oil (w/o) microemulsions. The w/o microemulsion comprises of Labrafac CC, Labrasol, Plurol Oleique CC 497, and saline (54/18/18/10% w/w). The characters of conductivity, viscosity, particle size, and in vitro membrane permeability have been studied. The intraduodenal bioavailability of the microemulsion group was 208 folds higher than that of control group. Meanwhile, no tissue damage of the intestinal mucosa has been found after oral multiple-dose administration of the protease microemulsion to rats. Therefore, the w/o microemulsion is a promising oral delivery system for hydrophilic bioactivity macromolecules [86].

Besides, the effect of some absorption enhancers on the intestinal absorption of the earthworm protease has been studied including chitosan, sodium deoxycholate, Na_2EDTA , sodium dodecyl sulfate, sodium caprylate, poloxamer, and HP-beta-CD. The enzyme can be transported into blood

and kept its biological activity across intestinal endothelial membrane after administration via duodenum site, whereas with lower bioavailability. Some of the absorption enhancers have effects on intestinal absorption in vitro and in situ experiments [87]. So the safety enhancer with few side effects is a good choice for drug manufacturing enterprise.

11.2. Parental Routes of Administration. The oral administration of the earthworm proteases has a relatively slow absorption process; hence, it is unsuitable to treat the emergency thrombus such as acute myocardial infarction, acute cerebral thrombosis, peripheral limbs arteriovenous thrombus, and other acute diseases involved in thrombosis. Therefore the injection agent is another choice. In order to fulfill the goal, first, we should analyze all primary structures of the isozymes and identify essential groups and then search the relationship between structure and function that are related to the preparation of injection agent. Second, the antigenic features of the isozymes should be investigated [88]. Third, the structure of the earthworm proteases molecule should be optimized and modified chemically, so that the domains or groups leading to hostile responses could be removed or blocked. Finally the method of cloning and expressing of the recombinant earthworm proteases should be established to investigate the possibility of an injection agent produced by gene engineering.

11.3. Chemically Modified Structure. In order to enhance the efficacy and tolerability of thrombolytic agents, we should improve the specificity of the enzyme on fibrin to decrease the side effects and enhance the resistance to plasminogen activator inhibitor to elongate the half-life.

Chemical modification has been used to stabilize the native structure of the earthworm protease and decrease the antigenicity during administration. The stabilization of the protease is managed by chemical modification of the enzyme with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide and phenylglyoxal to protect the activity from the autolytic inactivation. Stabilization is also possible under acidic conditions, in which the stability of the enzyme was rather low, by immobilization with folded sheet mesoporous material [89]. The strongest fibrinolytic protease *LrP-III-2* has been modified chemically with fragmented human serum albumin (MW, 10,000–30,000) [90]. The modified enzyme lost the antigenicity of the native enzyme. The enzyme is a nonhemorrhagic protein and does not induce platelet aggregation. The enzyme kept potent proteolytic activity for fibrin and fibrinogen than that of human plasmin. The enzyme easily solubilizes actual fibrin clots (thrombi) of whole blood induced by thrombin in a rat's vena cava.

12. Other Potential Utilizations

12.1. Degradation of Proteins in Waste. The proteolytic activity, except for the fibrinolytic activity, of the earthworm protease has been studied using various protein substrates. Both *LrP-III-2* and *LrP-II* are more effective than trypsin

in the production of amino acids from elastin, hemoglobin, casein, and collagen. Thus, the proteases are useful in the field of waste treatment of nondegradable proteins.

12.2. Hydrolyzation of Ester. The earthworm proteases exhibited ester-hydrolyzing ability as well as the proteolytic activity [69]. The earthworm proteases could be used as a biocatalyst for unmasking of the unnecessary acetyl moiety from the building blocks in organic synthesis. For example, the preparation of vinyl p-coumarate from the acetyl p-coumarate vinyl ester in ethanol is enabled using isozymes *LrP-III-1*, *LrP-III-2*, and *LrP-II*. Polylactate film was decomposed to some extent by the enzyme.

12.3. Nutrition for the Microorganisms. The production of the autolysate is considered to be caused mainly by the action of the earthworm's own proteases without the involvement of microbial degradation [69]. Growth of the microorganisms in the medium with the autolysate in place of polypepton and in the original medium without changes in the other ingredients has been compared. The growth of bakers' yeast in the medium containing the same amount of earthworm autolysate as would have been used for polypepton is substantially better than that in the medium containing polypepton. *E. coli* XL1-blue as well as *Bacillus coagulans* IFO 12583 and *B. stearothermophilus* DSM 297 could grow in the media containing the autolysate as well as in those containing polypepton [69].

13. Conclusion

Earthworm proteases are getting more significant in our daily life nowadays. It is applicable in both experiment and production, such as medical usage, environmental protection, and nutritional production. In the near future, more products based on the earthworm protease will reach the market.

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