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Research Article

Eisenia fetida Protease-III-1 Functions in Both Fibrinolysis and Fibrogenesis

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The fibrinolytic function of earthworm protease-III-1 (*Ef* P-III-1) has been studied in recent years. Here, we found that *Ef* P-III-1 acted not only in fibrinogenolysis, but also in fibrogenesis. We have used *Ef* P-III-1 to hydrolyze fibrinogen, and to activate plasminogen and prothrombin. Based on the N-terminal sequences of the hydrolytic fragments, *Ef* P-III-1 was showed to specifically recognize the carboxylic sites of arginine and lysine. Analyses by fibrinogenolysis mapping and amino acid sequencing revealed that the isozyme could cleave the alpha, beta, and gamma chains of fibrinogen, showing a high α-fibrinogenase, moderate β-fibrinogenase, and low γ-fibrinogenase activities. Interestingly, *Ef* P-III-1 activated plasminogen and released active plasmin, suggesting a tPA-like function. Furthermore, *Ef* P-III-1 showed a factor Xa-like function on prothrombin, producing alpha-thrombin. The function in both activating prothrombin and catalyzing fibrinogenolysis suggests that *Ef* P-III-1 may play a role in the balance between procoagulation and anticoagulation.

1. INTRODUCTION

Earthworms have been made as a drug (usually in dried powder for oral administration) to improve blood circulation and to treat apoplectic stroke for tens of centuries. Early in 1878, Frédéricq found the alimentary tract of earthworm excreting a type of protease [1]. As described by Charles Darwin in 1883, earthworm digestive fluids can dissolve fibrin (see "The Formation of Vegetable Mould through the Action of Worms"). In the 1980s, groups of fibrinolytic isozymes were isolated from different earthworm species [2–4], such as *Eisenia fetida* proteases (*Ef*P) [5, 6] and *Lumbricus rubellus* proteases (*Lr*P) [1, 7]. The isozymes of *Eisenia fetida* have been purified and made into a drug in capsule to treat clotting diseases. This preparation is stable and has high fibrinolytic activity with a little side effect in hemorrhage [8–10]. Similar to *Lr*P-III-1 [11, 12], *Ef* P-III-1 has the highest fibrinolytic activity among the isozymes and has high stability [13]. Moreover, *Ef* P-III-1 acts as a tissue plasminogen activator (tPA)-like activator and initiates the plasminantithrombus pathway [14–16]. Therefore, the structural and functional characteristics of *Ef* P-III-1 have become interesting and significant [10, 12, 17–20].

Recently, the crystal structure of *Ef* P-III-1 (EFEb) at a resolution of 2.06°A has been solved, and the structural analysis shows that *Ef* P-III-1 should be classified as a trypsin from earthworm [10]. However, the structure of this isozyme is different from other trypsins. As a trypsin-like protease, *Ef* P-III-1 contains two chains: an N-pyroglutamated light chain and an N-glycosylated heavy chain. 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. The crystal structure of *Ef* P-III-1 provides the structural basis for its high level of stability and reveals its complicated posttranslational modifications in the earthworm.

In this laboratory, eight trypsin-like isozymes with fibrinolytic activity were isolated from *Eisenia fetida* through a stepwise-purified procedure: sulfate ammonia precipitation, affinity chromatography with a Sepharose-4B column coupled with soybean trypsin inhibitor (SBTI), and ionic chromatography with a DEAE-cellulose-52 column [21]. Interestingly, all the proteases were glycosylated. In the assay with the substrates of trypsin, chymotrypsin, and elastase

[17], *Ef* P-III-1 was demonstrated as a trypsin-like protease. So far, however, no detailed maps have been provided for the protease involved in fibrinogenolysis and plasminogen activation. Furthermore, the effect of *Ef* P-III-1 on procoagulation has not been investigated yet. This work is concerned with the functions of *Ef* P-III-1 in both fibrinolysis and fibrogenesis.

2. MATERIALS AND METHODS

2.1. Purification and assay of EfP-III-1

Ten mg of crude earthworm proteases, prepared as described [17], was obtained from the ammonium sulfate precipitation of *Eisenia fetida*. *Ef* P-III-1 was purified on a 4-aminobenzamidine dihydrochloride-coupled Sepharose CL-6B affinity column (Pharmacia/ Pfizer Canada) eluted with a gradient of denaturant (from 0.1 to 1M) through a BioRad Gradient Maker [22]. The fractions were assayed by a chromogenic substrate (from Roche Switzerland) as described [23]. The active eluate was pooled and dialyzed against 0.01M Tris-HCl buffer (pH 8.0). Then the purified *Ef* P-III-1 was lyophilized and stored at −20°C before use. To check the purity of the isozyme, the protein sample was resuspensed in 10mM Tris-HCl buffer (pH 8.0) and electrophoresed on 12% SDS-PAGEs, then the gels were stained with Coomassie blue and silver, respectively. The protein concentration was determined by the bicinchoninic acid (BCA) protein assay with bovine serum albumin as the standard (Pierce, Ill, USA).

The fibrinolytic activity was measured by the light scattering method as described by Zhou et al. [24]. The isozyme (0.1μM final concentration) was incubated with fibrinogen (0.25mg/mL final concentration) in 0.05M Tris-HCl buffer (pH 7.4) at 25°C for 10 minutes. Then thrombin (6U/mL, Sigma Co., Mo, USA) was added to the mixture, followed by measurement of the Rayleigh-light scattering at 480nm on a fluorescence spectrophotometer (Hitachi F-4500). One enzymatic unit was defined as the amount of enzyme causing the conversion of 1μM of substrate per minute per mg of protein at 25°C. The standard earthworm protease with a specific activity of 2.5×10^3 U (Chinese National Pharmacopoeia) was purchased from the Institute for the National Control of Pharmacological and Biological Products, China.

2.2. Hydrolysis of fibrinogen, plasminogen, and prothrombin by EfP-III-1

Ef P-III-1 (0.1μM final concentration) was incubated with human fibrinogen, plasminogen, or prothrombin (2mg/mL final concentration, Sigma Co., Mo, USA) in 0.05M Tris-HCl (pH 8.3) at 25°C. Aliquots were taken at different time intervals for SDS-PAGE, each of the bands was transferred onto a polyvinylidene difluoride membrane (Gelman, USA) and then each band was cut off from the membrane for the amino acid sequencing [25]. On the basis of the N-terminal sequence of each digested fragment, the cleavage sites were obtained by reference against the complete amino acid sequences of human fibrinogen [26], prothrombin [27], and bovine plasminogen [28]. The immobilized enzyme, prepared as described [20, 29], was also used for the hydrolysis. Fifty microliters of Sepharose CL-6B coupled with *Ef* P-III-1 was put in an eppendorf tube (0.5mL). Fibrinogen (1mg/mL final concentration) was dissolved in Tris-HCl buffer (pH 8.3), added to the eppendorf tube (total volume 200μL), and placed on a shaker at 25°C. Aliquots were taken by spinning down the reaction mixture at different time intervals, and the supernatants (20μL) were subjected to electrophoresis and amino acid sequencing.

2.3. Assays of EfP-III-1, plasmin, and thrombin

The activities of *Ef* P-III-1, plasminogen, and prothrombin were measured by the light scattering intensity on a fluorescence spectrophotometer (Hitachi F-4500). (I) Fibrogenesis: fibrinogen (2.5mg/mL final concentration) was incubated in 0.05M Tris-HCl buffer (pH 7.4) at 25°C for 10 minutes before addition of thrombin (0.2U), followed by the measurement of the Rayleigh-light scattering at 480nm [24]. (II) Assay of *Ef* P-III-1: conditions were as for (I), except that *Ef* P-III-1 (0.1μM as final concentration) and thrombin were both added to fibrinogen. (III) Activation of plasminongen: conditions were referred to (II), except that plasminogen (3U) was incubated with *Ef* P-III-1 at 25°C for 10 minutes, and then added to fibrinogen in the presence of thrombin. (IV) Activation of prothrombin: instead of thrombin, prothrombin (1mg/mL) was incubated with *Ef* P-III-1 at 25°C for 10 minutes and added into fibrinogen under the same conditions. Prothrombin without the incubation was used as control.

2.4. Transmission electron microscopy

Ef P-III-1 (0.1μM final concentration) was incubated with fibrinogen or prothrombin (2mg/mL final concentration) for 30 minutes at 25°C. Aliquots (10μL) were observed by electron microscopy. Samples were adsorbed (0.25–15 minutes) onto 300-mesh Formvar /carbon-coated copper grids. The resultant grids were washed with water, stained with 2% uranyl acetate (1 minute), washed again with water, blotted dry, and viewed in a JEOL JSM-5600 electron microscope operated at 65kV (Japan Electron Optics Laboratory Co., Ltd., Japan). Random images from each sample were captured onlm at 5000- to 50000-fold magnication, digitized, calibrated, and imported into Optimas 6.5.1 for quantitation of filament length and number as described previously [30].

3. RESULTS

3.1. Purification and assay of EfP-III-1

Ef P-III-1 was purified by the affinity chromatography and it showed a single protein band on the gel with an apparent molecular mass of ∼34kd after purification (Figure 1). Light scattering assay showed that the specific activity of the purified enzyme was 33×10^4 U. Sequencing of the

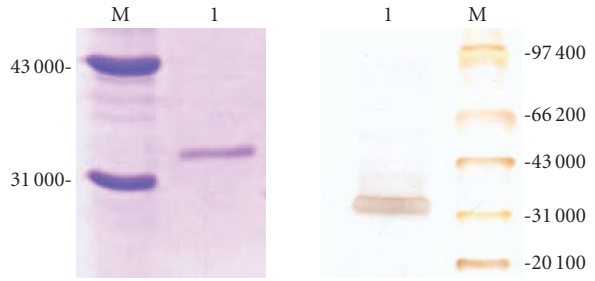

Figure 1: Purification of *Ef*P-III-1. *Ef*P-III-1 (~34kd, lane 1) after purification was electrophoresed on a 12% SDS-PAGE. Then the gel was stained with Coomassie blue (a) or silver (b). M represents the low-molecular-mass protein markers.

N-terminal region of *Ef*P-III-1 revealed amino acid sequence identical to *Lr*P-III-1 (IVGGIEAR...) [12]. The unique sequence also demonstrated the high purity of *Ef*P-III-1.

3.2. Hydrolysis of fibrin and fibrinogen

In order to investigate the fibrinolytic activity of *Ef*P-III-1, we used transmission electron microscopy to observe the morphological changes when fibrin was incubated with the protease. Fibrogenesis was clearly detected when thrombin was incubated with fibrinogen as shown in Figure 2(c). No observable fibrils could be detected after the incubation of fibrin with *Ef*P-III-1 (Figure 2(a)). *Ef*P-III-1 alone as a control also exhibited no fibrils under the electronic microscope (Figure 2(b)). This observation suggests that *Ef*P-III-1 has a high fibrinolytic activity.

Furthermore, we hydrolyzed fibrinogen in the presence of *Ef*P-III-1 to reveal the map of the hydrolysis in which six major distinct fragments with different apparent molecular masses (42, 31, 28, 25, 21, and 13kd, resp.) have been found (Figures 2(d), 2(e)). On the SDS-PAGE, F-b appeared at the initial stage of digestion, followed by the appearance of F-d, F-e, F-a, F-f, and F-g whose band densities in the gel increased with time, and no further degradation could be detected when the reaction was prolonged to over 120 minutes (Figures 2(d), 2(e)). This observation suggests that these five products were stable under the assay conditions. Moreover, another stable band called F-c contains at least two peptides. As exhibited in Table 1, F-d, F-e, and F-f were hydrolytic products from the α-chain of fibrinogen with cleavage sites at R_{252}-G_{253}, R_{19}-V_{20}, and K_{429}-V_{430}, respectively; F-a and F-b came from γ-chain and contained ~410 and ~300 amino acid residues, respectively. It should be noted that α-chain was cleaved so rapidly (lane 3, Figure 2(d)) that the band disappeared within 5 minutes under such conditions. Consequently, hydrolysis of α-chain was further carried out using immobilized *Ef*P-III-1 (Figure 2(f)). The density of α-chain band in the gel gradually decreased during the hydrolysis. As shown in Figure 2(g), four distinct fragments (F-a', F-b', F-c', and F-d') were released, with different apparent molecular masses (33, 31, 28, and 22kd). With the same N-terminus as the intact α-chain [26], F-a' was initially released in 5 minutes during the reaction. The other three fragments had an identical cleavage site at R_{19}-V_{20}. According to the densities of the protein bands on the SDS-PAGE, hydrolysis of α-chain was the fastest, and hydrolysis of β-chain was faster than that of γ-chain. This indicated that *Ef*P-III-1 possesses strong α-fibrinogenase, moderate β-fibrinogenase, and weak γ-fibrinogenase activities.

The light scattering assay was carried out to detect whether *Ef*P-III-1 could cleave fibrinogen. Compared with the changes in the intensity of light scattering of the mixture containing fibrinogen and thrombin (curve 1, Figure 2(h)), the fibrin formation was clearly repressed when *Ef*P-III-1 was added (curve 2, Figure 2(h)). To investigate which is the initial digestion target of the protease, fibrinogen or fibrin, we preincubated fibrinogen with *Ef*P-III-1 for 10 minutes, and then added thrombin. As shown in Figure 2(h), the inhibition of fibrogenesis could be observed in the presence of *Ef*P-III-1, regardless of preincubation with fibrinogen or not. However, the relaxation time was remarkably prolonged (~100 seconds) when the protease was preincubated with fibrinogen (curve 3, Figure 2(h)). The inhibition of fibrin formation could be distinctly delayed, suggesting that *Ef*P-III-1 preferably cleaved fibrinogen under such conditions.

Table 1: Cleavage sites on fibrinogen, plasminogen, and prothrombin by *Ef*P-III-1.

Fragment	*Ef*P-III-1		
	Fibrinogen*	Plasminogen**	Prothrombin***
a	γ-Y_1VATR	D_1LLDD	PR_3 ↓ ANTF
b	γ-Y_1VATR	GR_{557} ↓ IVGG	PR_{158} ↓ SEGS
c	—	—	—
d	α-TR_{252} ↓ GGST	—	—
e	α-PR_{19} ↓ VVER	—	PR_{158} ↓ SEGS
f	α-EK_{429} ↓ VTSG	—	GR_{274} ↓ TATS
g	—	—	ER_{396} ↓ NIEK
h	—	—	PR_{287} ↓ TFGS
i	—	—	PR_3 ↓ ANTF

*, **, and *** indicate that the amino acid sequences are referred to in [25–27], respectively.

3.3. Activation of plasminogen

To check the effect of *Ef*P-III-1 on plasminogen, we used *Ef*P-III-1 to activate plasminogen and observed two resultant fragments P-a and P-b (Figure 3, apparent molecular masses: ~60 and ~30kd, resp.). Cleavage at R_{557}-I_{558} resulted in the release of P-b (Figures 3(a), 3(b)), whose first six amino acids were identical to those of the catalytic domain (or microplasminogen) of activated plasmin [28]. This cleavage site (R_{557}-I_{558}) was also recognized by tPA (Figure 3(c)).

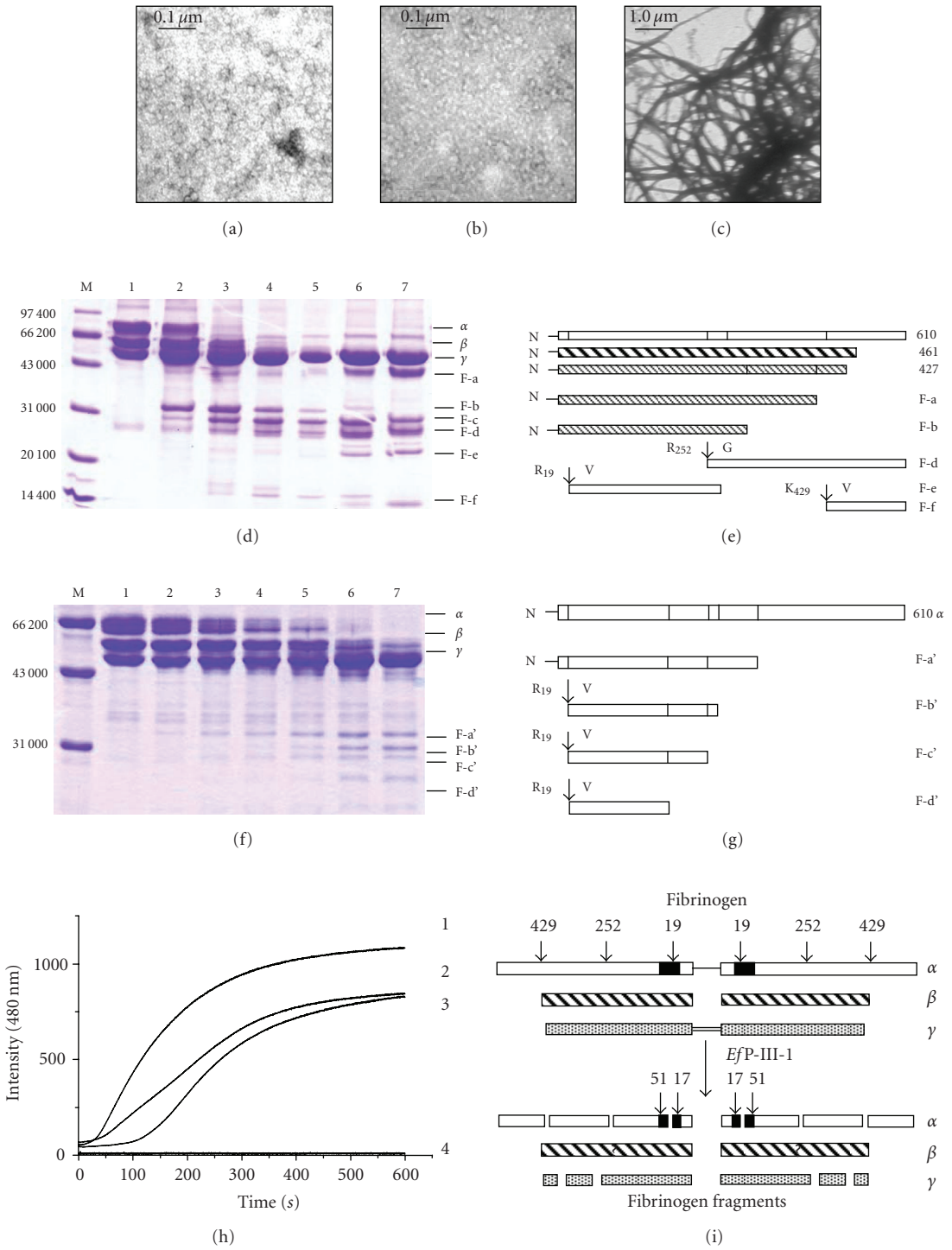

Figure 2: Hydrolysis of fibrinogen by *Ef* P-III-1. *Ef* P-III-1 (0.1μM final concentration) was incubated with human fibrinogen (2mg/mL in 50mM Tris-HCl buffer, pH 7.4) at 25°C. (a–c) Fibrinolysis in the presence of *Ef* P-III-1 (a) was detected by electron microscopy. *Ef* P-III-1 (b) and fibrin alone (c) were used as controls. (d,e) 12% SDS-PAGE showing fibrinolysis in the presence of *Ef* P-III-1 (d). M: low-molecular-mass protein markers; lane 1: fibrinogen which consists of three polypeptide chains: α (66kd), β (54kd), and γ chain (48kd) as control; lanes 2 through 7: aliquots were taken at 0, 5, 15, 30, 60, and 120 minutes during the hydrolysis. (e) The fibrinolytic fragments (F-a to F-f) in the presence of *Ef* P-III-1 (e). (f,g) fibrinolysis in the presence of immobilized *Ef* P-III-1 under the same conditions. α-Fibrinogen fragments were indicated by F-a' to F-d' (g). Fibrinolytic activity (2.5mg/mL, 10 minutes, 25°C) was measured by light scattering assay (h). Thrombin was added to fibrinogen in the absence of *Ef* P-III-1 as a control (curve 1); both *Ef* P-III-1 and thrombin were added to fibrinogen at the same time (curve 2); *Ef* P-III-1 was incubated with fibrinogen at 25°C for 10 minutes before thrombin was added (curve 3), and Tris-HCl buffer as a negative control (curve 4). *Ef* P-III-1 cuts at R_{19}-V_{20} and destroys the N-terminal 35-residue stretch (black) which is the key recognition motif for thrombin (i), according to C. Fuss et al. [31].

Table 2: Activities of fibrinogen, plasminogen, and prothrombin.

Samples	Relative activity (%, mean ± SD)*	Fibrin formation (%, mean ± SD)**
Fibrinogen + thrombin	100 ± 29.9	100 ± 3.5
Fibrinogen + thrombin + *Ef* P-III-1	38.6 ± 1.8	77.8 ± 3.8
Fibrinogen + thrombin + plasminogen	81.1 ± 79.9	69.9 ± 3.6
Fibrinogen + thrombin + *Ef* P-III-1 + plasminogen***	27 ± 9.9	5.3 ± 2.4
Ef P-III-1 + prothrombin + fibrinogen	31.9 ± 1.5	52.9 ± 2.9
Prothrombin + fibrinogen	4.2 ± 2.8	4.9 ± 2.9

*Relative activity was based on the max slope of the reaction curve.
**Fibrin formation was calculated with the intensity of the sample at 10 minutes.
***Refer to Figure 3(d), line 2.

The activation of plasminogen by *Ef* P-III-1 was assayed with the light scattering method (Figure 3(d)). As shown in Table 2, the relative light scattering intensity of the reaction mixture (fibrinogen and thrombin) reached 77.8 ± 3.8 (%) in 10 minutes when *Ef* P-III-1 was added. In particular, the light scattering intensity remarkably decreased (5.3 ± 2.4%) in the presence of both *Ef* P-III-1 and plasminogen, though the intensity increased initially. Although the fibrin formation reached 69.9 ± 3.6 (%) when plasminogen was present alone, the relative fibrogenesis activity (81.1 ± 7.9%) did not markedly decrease under the same conditions. This suggested that *Ef* P-III-1 activated plasminogen and released active plasmin.

3.4. Activation of prothrombin

So far, we have obtained evidence demonstrating the effect of the earthworm protease on fibrinolysis and activation of plasminogen. We further wondered whether *Ef* P-III-1 was involved in procoagulation pathway. By electron microscopy, it is obviously exhibited that some fibrin deposits were formed from fibrinogen in the presence of prothrombin and *Ef* P-III-1 (Figures 4(a), 4(b)). The result of electrophoresis showed that eight major hydrolytic fragments were released after prothrombin was incubated with *Ef* P-III-1 (Figures 4(d), 4(e)). As shown in Figure 4(c), fibrin formation was increased in the presence of both prothrombin and *Ef* P-III-1. However, fibrin was hardly produced when prothrombin alone was added to fibrinogen (curve 4, Figure 4(c)). This suggested that active thrombin was released during the incubation of prothrombin with *Ef* P-III-1.

4. DISCUSSION

The α-chain of fibrinogen plays an important role in fibrin formation during the activation of fibrinogen to fibrin by thrombin (Figure 2(i)). The 35-residue stretch (G_{17} to M_{51}) in the N-terminal region is a key recognition motif for thrombin [32]. Meanwhile, this stretch is essential for fibrin monomers to associate with each other, and to produce fibrin fibrils. We employed the immobilization of *Ef* P-III-1 to hydrolyze the α-chain of fibrinogen (Figure 2(f), 2(g)). Use of immobilized enzyme is based on the following reasons. (1) Hydrolysis of α-chain was a rapid procedure resulting in a release of transient fragments, which could hardly be detected. (2) The immobilization of protease allows us to exclude the effects due to residual activity of *Ef* P-III-1. (3) After immobilization, the activity of *Ef* P-III-1 decreased to 40%–50%, and the enzyme molecule became more resistant to heat, acidic and basic conditions, and denaturants [29]. (4) More importantly, the immobilization of protease enables us to control the reaction easily and to obtain reproducible results. (5) For *Ef* P-III-1, immobilization did not change its substrate specificity (data not shown). As a result, we found that the peptide bond of R_{19}-V_{20} was a cleavage site on α-chain, and the site was close to R_{16}-G_{17} recognized by thrombin [33], it may destroy the recognition stretch and obstruct the polymerization between fibrins.

Besides R_{19}-V_{20}, the α-chain of fibrinogen has been cut by *Ef* P-III-1 at other sites. According to the N-terminal sequencing of F-d and F-f, the cleavage sites were located at R_{252}-G and K_{429}-V. Moreover, the sites were presumably located around R_{334}-P_{335} (F-a'), R_{289}-N_{290} (F-b'), R_{252}-G_{253} (F-c'), and R_{199}-Q_{200} (F-d') based on that *Ef* P-III-1 specifically recognized the carboxylic sites of arginine and lysine which were demonstrated by the N-terminal sequencing of all the detected hydrolytic fragments (Table 1).

In addition, the C-terminal region of the γ-chain of fibrinogen was also cleaved by the protease, producing F-a, F-b, and some small undetectable peptides. Presumably, the hydrolytic sites in the C-terminal region could be located around R_{419}-P_{420} (F-a) and K_{302}-F_{303} (F-b). Hydrolysis at these two predicted sites may also obstruct fibrin formation [34], due to the fact that the C-terminal region of fibrinogen is involved in several functional interactions, including fibrin polymerization [33]. Note that the enzyme digested fibrinogen at multiple hydrolytic sites, leading to a remarkable decrease in fibrinogen concentration. As described by Dempfle et al. [35] and Bovill et al. [36], the decrease of fibrinogen leads to an anticoagulation effect in circulation.

In our experiments, no fragment from β-chain could be detected in the presence of both native and immobilized *Ef* P-III-1 under the conditions. According to the amino acid sequence, β-fibrinogen is rich in arginine and lysine residues, which are probably vulnerable to *Ef* P-III-1. Thus, it is likely that the β-chain of fibrinogen was hydrolyzed and the degraded bands disappeared within 15 minutes after addition

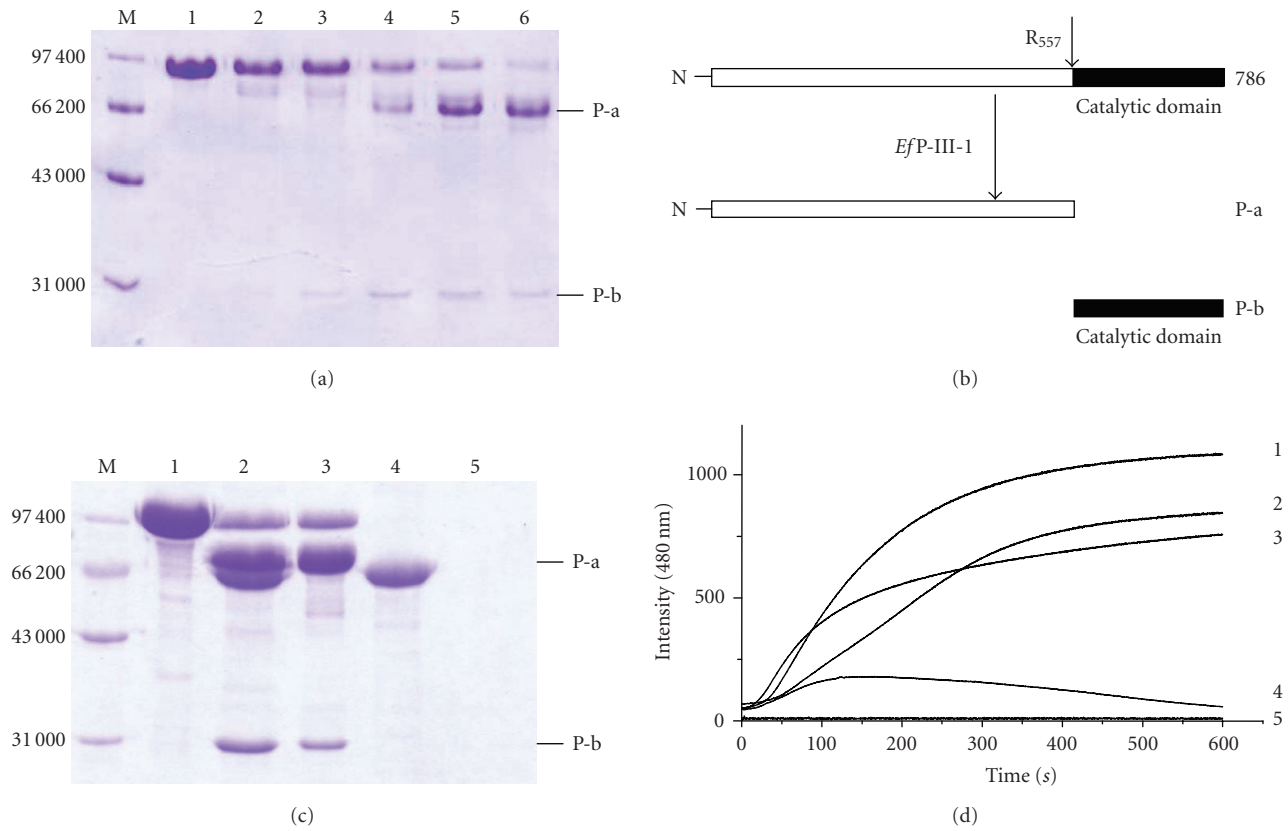

Figure 3: Activation of plasminogen by *Ef* P-III-1. Plasminogen (2mg/mL final concentration) dissolved in 50mM Tris-HCl buffer (pH 7.4) was incubated with *Ef* P-III-1 at 25°C. Aliquots were taken at different time intervals, and loaded on SDS-PAGE (12%) (a). M shows low-molecular-mass protein markers; lane 1: plasminogen as control; lanes 2 through 6 represented the hydrolyzed fragments after 2, 5, 15, 30, and 60 minutes. Cleavage of plasminogen after one-hour incubation with *Ef* P-III-1 at 25°C (b) represented the digested fragments P-a and P-b (catalytic domain or micropalsminogen). Plasminogen in the presence of tPA (0.1μM final concentration) was used as a control (c). Lanes 1 through 3: plasminogen alone, with tPA, or with *Ef* P-III-1; lane 4: tPA alone, and lane 5: *Ef* P-III-1 alone. Enzymatic activity was measured by light scattering assay (d). Thrombin was added to fibrinogen in the presence of *Ef* P-III-1 (curve 4, with plasminogen; curve 2, without plasminogen), and in the absence of *Ef* P-III-1 (curve 3 with plasminogen). Tris-HCl buffer (curve 5) was used as control.

of *Ef* P-III-1. In addition, the protease efficiently cleaves the α-, β-, and γ-chains of fibrinogen at multiple sites in both the N-terminal and C-terminal regions. However, the hydrolyzation of α-chain is faster than that of β-chain, and much faster than that of γ-chain under the same conditions (Figure 2). This indicates that *Ef* P-III-1 acts as a strong α-fibrinogenase, a moderate β-fibrinogenase, and a weak γ-fibrinogenase.

The fibrinolytic system contains a proenzyme plasminogen which is converted to active enzyme plasmin by the action of plasminogen activators. Plasminogen activation by tPA is the most important mechanism in removing fibrin excess [37]. tPA specifically recognizes R_{557}-I_{558} on plasminogen and releases active plasmin. The cleavage site on plasminogen by *Ef* P-III-1 was the same as tPA (Figure 3(a), 3(b)) [28], and no other cleavage sites could be detected under our assay conditions. This indicates that *Ef* P-III-1 is a tPA-like protease.

Eight major hydrolytic fragments were released after prothrombin was incubated with *Ef* P-III-1 (Figures 4(d), 4(e)). The apparent molecular masses of the fragments (from T-a to T-i) were approximately 70, 52, 48, 46, 37, 33, 29, and 25kd, respectively. The N-terminal sequences of the eight fragments indicated that *Ef* P-III-1 recognized peptidyl bonds at R_3-A_4 (T-a, T-g), R_{158}-S_{159} (T-e), R_{274}-T_{275} (T-f), R_{396}-N_{397} (T-g, detectable in both T-e and T-f), and R_{287}-T_{288} (T-h). On the basis of the molecular masses of T-e (~35kd) and T-i (~25kda), we estimated that the cleavage sites at C-terminus were located around R_{493}-P_{494} and R_{243}-N_{244}.

The coagulation cascade culminates in the conversion of prothrombin to active thrombin. This reaction is catalyzed by a multicomponent complex prothrombinase [33, 38]. In the physiological environment, the activation of prothrombin by factor Xa generates the thrombin catalytic sites, and cleavage at R_{274} and R_{320} is required for thrombin formation [27, 39, 40]. In one pathway, human prothrombin is converted relatively slowly to α-thrombin (274–287) in the presence of factor Xa and Ca^{2+} ions by the initial cleavage at residue R_{274} (consequently producing fragment 1.2 and prethrombin 2), followed by cleavage of prethrombin 2 at R_{320} (accordingly generating α-thrombin). In our experiments, *Ef* P-III-1 degraded prothrombin at R_{274} in the absence of Ca^{2+}, and released prethrombin 2 (T-f, Figure 4(e)).

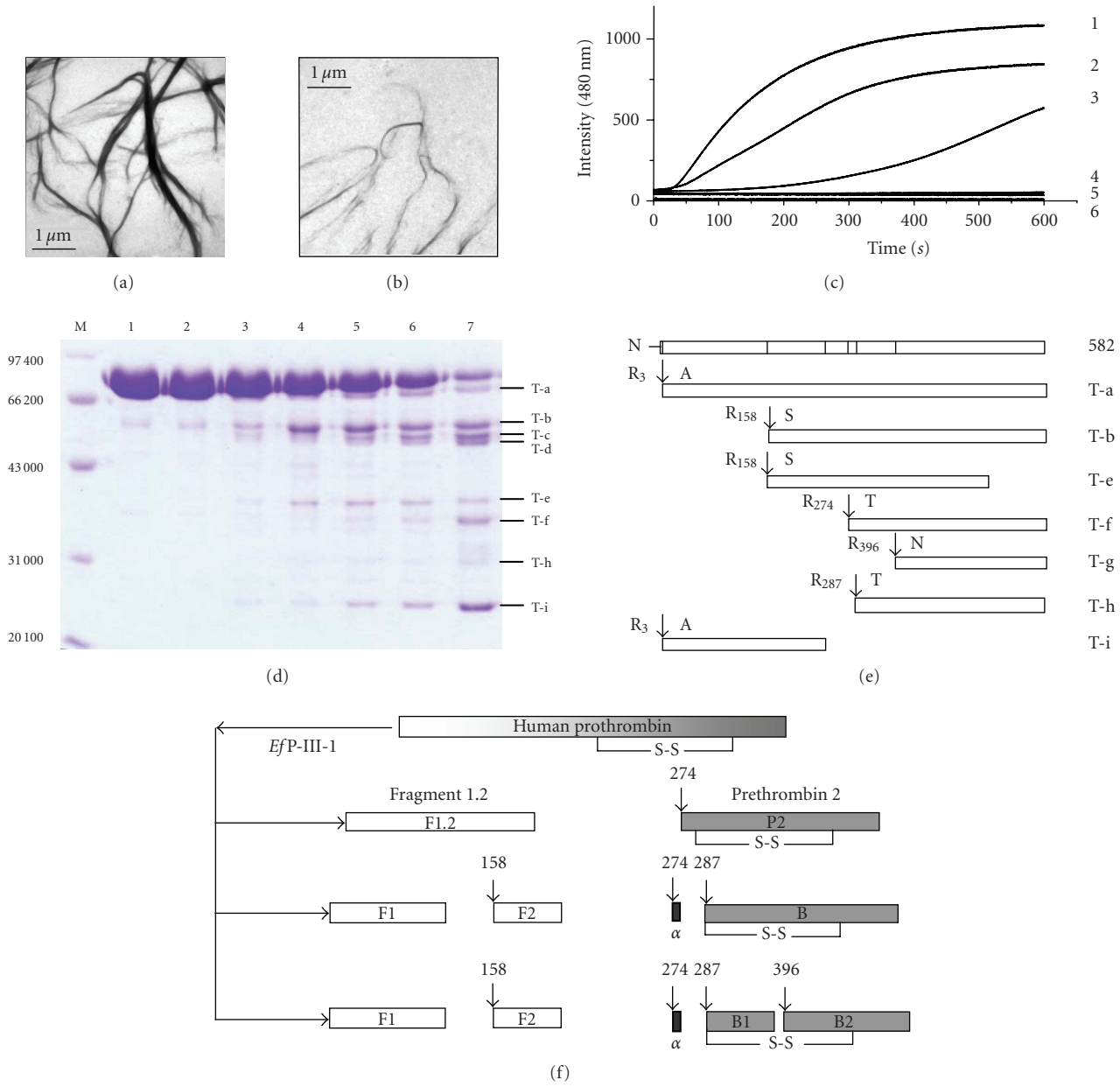

Figure 4: Activation of prothrombin by *Ef*P-III-1. Prothrombin (2mg/mL final concentration) in 50mM Tris-HCl buffer (pH 7.4) was incubated with (a) or without (b) *Ef*P-III-1 at 25°C, and then added into fibrinogen and observed by electron microscopy. Activation of *Ef*P-III-1 on prothrombin was measured by light scattering assay (c). Thrombin was added to fibrinogen in the absence of *Ef*P-III-1 as a control (curve 1); prothrombin (curve 3) or thrombin (curve 2) was incubated with *Ef*P-III-1 before addition to fibrinogen; prothrombin (curve 4) or *Ef*P-III-1 (curve 5) was added to fibrinogen as controls, and Tris-HCl buffer as a negative control (curve 6). Prothrombin in the presence of *Ef*P-III-1 was electrophoresed on 12% SDS-PAGE (d). M: low-molecular-mass protein markers; lane 1: prothrombin alone; lanes 2 through 7: hydrolyzed products after 0, 5, 15, 30, 60, 120 minutes, respectively. T-a to T-i represent the digested fragments of prothrombin in the presence of *Ef*P-III-1 (e). Diagrammatic representation of prothrombin activation products produced by incubation with *Ef*P-III-1 was shown in (f).

Cleavage at R_{287} produced α-thrombin (Figure 4(f)). Similar to the preference for residue N_{397} by thrombin, which produces the β-thrombin-like fragments [41], *Ef*P-III-1 cleaves at residue R_{396}. As shown in Figure 4(c), the activation of prothrombin by *Ef*P-III-1 was time-dependent, and prothrombin was first degraded at R_{274} similar to the activation by factor Xa. Furthermore, cleavage at R_{287} on prethrombin 2 released active α-thrombin for fibrogenesis [41]. Thus, *Ef*P-III-1 had a prothrombinase-like function in activating prothrombin to produce α-thrombin.

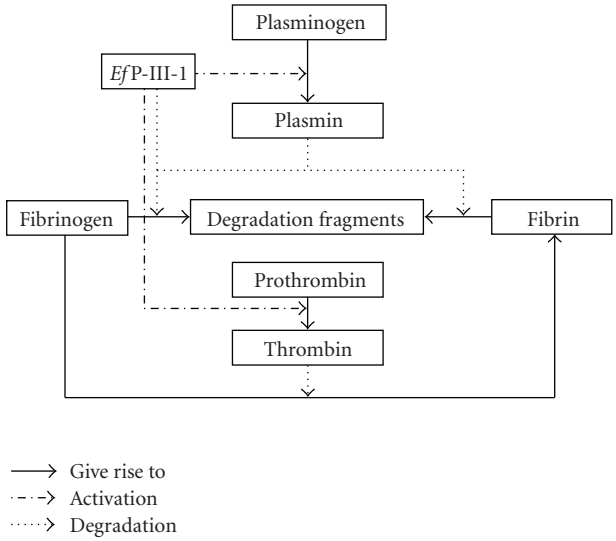

Figure 5: **Roles of *Ef* P-III-1 in procoagulation and anticoagulation.** *Ef* P-III-1 degrades fibrin(ogen) and activates not only plasminogen but also prothrombin. Activation is indicated by dashed lines, and degradation is indicated by dotted lines.

One problem we noted in our experiments was that F-c from fibrinogen was a mixture containing at least two peptides (Figure 2(d)), and so were T-c and T-d from prothrombin (Figure 4(d)). We have run SDS-PAGEs under both reducing and nonreducing conditions, but the mixed peptides could not be separated. There may be two reasons: (1) the molecular masses of the mixed peptides were similar, and/or (2) they were polymerized with each other and could not be separated with SDS, DTT, and β-mercaptoethanol.

Based on our data, the roles of *Ef* P-III-1 in procoagulation and anticoagulation can be summarized as follows (Figure 5): (1) to degrade fibrinogen and fibrin at both the N-terminal and C-terminal regions (Figure 2); (2) to activate PLg and release active plasmin (Figure 3); and (3) to act in an Xa-like manner (Figure 4).

A clinical study of cerebral infarction has showed that earthworm proteases decreased some stroke scores in comparison with control groups [42]. According to clinical observations [38, 42–44], the activated partial thromboplastic time was prolonged, tPA activity and D-dimer levels increased, and the concentration of fibrinogen in blood decreased significantly. In recent years, the mixture of earthworm proteases including *Ef* P-III-1 has been made as an orally administered fibrinolytic agent to prevent and treat clotting diseases [11, 14, 45]. Compared with other drugs such as hirudin [46], tPA [47] and UK [48], "Lumbrokinase" (containing *Ef* P-III-1) has relatively low side effects such as hemorrhage complications (~2%) [42]. *Ef* P-III-1, involving both fibrinolysis and fibrogensis, may play a role in balancing coagulation and anticoagulation in circulation. This may be one explanation for the observed low incidence of hemorrhage complications in clinical applications of earthworm proteases.

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