

## *In situ* localization and substrate specificity of earthworm protease-II and protease-III-1 from *Eisenia fetida*

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### Abstract

Recently, the function in fibrinolysis of earthworm proteases has been studied. In our experiments, earthworm protease-II (*EfP*-II) and earthworm protease-III-1 (*EfP*-III-1) were isolated and purified from *Eisenia fetida*. As shown by the assay of sections of the earthworm on fibrin plates, the enzymic activity was mainly detected around the clitellum. In the presence of anti-*EfP*-II or anti-*EfP*-III-1 serum, the immunological signals of the two isozymes were clearly found in the anterior alimentary mucosa, suggesting that *EfP*-II and -III-1 are localized and expressed in intestinal epithelial cells. The Michaelis–Menten constant ( $K_m$ ) for *EfP*-III-1 reacting with BAEE is smaller ( $1.7 \times 10^{-5}$  M) in comparison with the  $K_m$  values of other substrates such as Chromozym-Try and -TH ( $3.3 - 6.0 \times 10^{-5}$  M). This indicates that *EfP*-III-1 is a trypsin-like protein. *EfP*-II shows a strong trypsin-like, moderate elastase-like and weak chymotrypsin-like serine function. The relative broad substrate specificity of *EfP*-II and *EfP*-III-1 is consistent with their localization in the anterior alimentary canal where different micro-organisms and ingested proteins require to be digested.

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**Keywords:** Earthworm protease; Distribution; Trypsin; Elastase; Fibrinolysis

### 1. Introduction

Several laboratories have carried out research on earthworm proteases (EP) [1–3]. Groups of fibrinolytic isozymes have been isolated from the species *Eisenia fetida* and *Lumbriucus rubellus* [4,5]. From *L. rubellus*, Mihara et al. isolated and studied six fibrinolytic proteases, *LrP*-I-0, -I-1, -I-2, -II, -III-1 and -III-2 [6]. From *E. fetida*, Wang et al. found seven isozymes, *EfP*-a, *EfP*-b, *EfP*-c, *EfP*-d, *EfP*-e, *EfP*-f and *EfP*-g [5].

Although *EfP*-II and *EfP*-III-1 have been studied for several years [5,7–9], the distribution of the proteins in the earthworm

has remained unclear. Some investigators have proposed that earthworm proteases may be localized in the alimentary canal [6]. However, no evidence for this has been reported. Earthworm proteases have been used as an orally administered drug to prevent and treat clotting diseases such as acute myocardial infarction and cerebral embolism [10–13]. Studies have demonstrated that *EfP*-II administered orally can be transported into the blood circulation through the intestinal epithelium and its biological function still be partially maintained [14]. Investigation of the distribution of protease in the earthworm would contribute to understanding of the function of the isozymes.

Among the six *L. rubellus* isozymes, *LrP*-II has a relatively broad substrate specificity and *LrP*-III-1 has the highest activity in fibrinolysis [5,15]. Crystallization and preliminary X-ray analysis of EFEa from *E. fetida* (*EfP*-II) provides the structural information necessary to understand the biological function of EPs [16]. The active site of *EfP*-II is located in a particularly flexible pocket that may form a structural basis for a broad specificity [17–20]. Conformational studies in solution support the view-

**Abbreviations:** EP, earthworm protease; SBTI, soybean trypsin inhibitor; BAEE, *N*- $\alpha$ -benzoyl-L-arginine ethyl ester; ATEE, *N*-acetyl-L-tyrosine ethyl ester; TLCK, *N*- $\alpha$ -*p*-tosyl-L-lysine chloromethyl ketone; TPCK, *N*- $\alpha$ -*p*-tosyl-L-phenylalanine chloromethyl ketone; Ch, Chromozym; DAB, 3,3'-diaminobenzidine; ELISA, enzyme-linked immunological assay

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point that the active site of *EfP-II* is situated in a flexible region [21].

Though a broad substrate specificity of *EfP-II* has been reported, the substrate specificity has not been characterized in detail. Here, we show desired distribution of *EfP-II* and *EfP-III-1* in *E. fetida*, and their specificity towards different substrates and inhibitors.

## 2. Experimental

### 2.1. Materials

Four chromogenic substrates, Chromozym TH (Ch-TH, Car-Val-Gly-Arg-4-pNA, a thrombin-like substrate), Chromozym TRY (Ch-TRY, Tos-Gly-Pro-Arg-4-pNA, a trypsin-like substrate), Chromozym U (Ch-U, Ben- $\beta$ -Ala-Gly-Arg-4-NA, a urokinase-like substrate) and Chromozym ELA (Ch-ELA, Suc-Ala-Ala-Ala-pNA, an elastase-like substrate) were from Roche (Germany). The other two substrates, *N*- $\alpha$ -benzoyl-L-arginine ethyl ester (BAEE, a trypsin-like substrate) and *N*-acetyl-L-tyrosine ethyl ester (ATEE, a chymotrypsin-like substrate), four inhibitors, soybean trypsin inhibitor (SBTI, a specific inhibitor of trypsin), *N*- $\alpha$ -*p*-tosyl-L-lysine chloromethyl ketone (TLCK, a specific inhibitor of trypsin), *N*- $\alpha$ -*p*-tosyl-L-phenylalanine chloromethyl ketone (TPCK, a specific inhibitor of chymotrypsin) and elastinal (a specific inhibitor of elastase), thrombin, skimmed milk, 3,3'-diaminobenzidine (DAB) and bovine serum albumin (BSA) came from Sigma (USA). Sepharose CL-6B was from Pharmacia (USA). Nitrocellulose membrane was from Life-Technologies (USA). Poly-vinylidene difluoride membrane came from Gelman (USA). Goat anti-rabbit IgG conjugated with horseradish peroxidase was from Santa Cruz Biotechnology Inc. (USA). 3,3',5,5'-tetramethylbenzidine was from Promega Inc (USA). The standard earthworm protease (Chinese National pharmacopoeia,  $2.5 \times 10^3$  U/mg) was made by the Institute for the National Control of Pharmacological and Biological Products (China). Other reagents were analytic grade and used without further purification. Mature specimens of the earthworm *E. fetida* were purchased from Beijing Baiao Pharmaceuticals Co. Ltd. Worms were maintained in small plastic boxes containing moist wood pulp, in the dark and at around 20 °C.

### 2.2. Purification of *EfP-II* and *EfP-III-1*

Crude earthworm proteases (10 mg), prepared as described previously [14], were obtained from sulfate ammonia precipitation of *E. fetida*. The enzymes were resuspended in 2.0 mL Tris-HCl buffer (0.05 M, pH 7.4) containing NaCl (0.05 M) and loaded onto a Sepharose column (1 cm  $\times$  10 cm) that had been equilibrated in Tris-HCl buffer (0.05 M, pH 8.2) containing NaCl (0.5 mM). The equilibrating buffer was used to wash the sample column further until the absorbance at 280 nm approached to a constant value. The specific elution was then performed with a gradient denaturant (Chinese National Patent: ZL02116747.8) on a Bio-Rad Gradient Maker. The activity of each fraction was assayed with Ch-TH as described previ-

ously [22]. The active eluate was pooled and dialyzed against Tris-HCl buffer (0.01 M, pH 8.0). The purified *EfP-III-1* was then lyophilized and stored at -20 °C before use. The purification of *EfP-II* was performed through a C-18 column on an Agilent 1100-type HPLC apparatus as described previously [21]. The purified isozymes appeared single bands by SDS-PAGE (Fig. 1), and were actively remained after resuspension in Tris-HCl buffer (0.01 M, pH 8.0). The protein concentration was estimated by the Lowry method using BSA (component V) as a control [23].

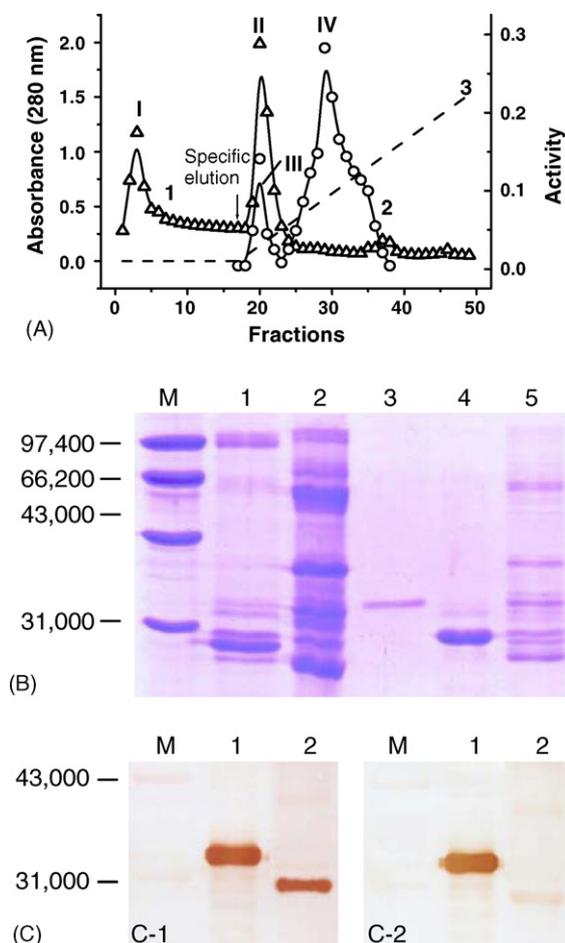


Fig. 1. Purification and identification of *EfP-II* and *EfP-III-1*. (A) The crude preparation of fibrinolytic proteases (10 mg) was purified on an affinity chromatography column. The first 20 mL of elution buffer was 0.05 M Tris-HCl (pH 8.0), containing 0.5 M NaCl, while the following 30 mL was special elution buffer. The left ordinate was the absorbance at 280 nm and the right was the activity in the presence of Chromozym TH. Curves exhibit the flow-through (peak I), specific elution (peak II) and two activity parts (peaks III and IV). Curve 3 represents the gradient of the elution (0–1.0 M). (B) The samples were electrophoresed on 12% SDS-PAGE. M: Low molecular mass proteins marker; lane 1: peak 1; lane 2: peak 2; lane 3: peak 4 (*EfP-III-1*); lane 4: *EfP-II*; lane 5: crude EP. (C) Western blotting of *EfP-III-1* (10  $\mu$ g) was carried out as described previously [30]. M: Low molecular mass standard protein markers; lane 1: *EfP-III-1* and lane 2: *EfP-II*. Samples were stained by Ponceau S Red (panel C-1). For western blotting, anti-*EfP-III-1* serum (1:500) was used as the primary antibody (panel C-2). The membrane was incubated with goat anti-rabbit IgG conjugated with HRP (1:4000) before being visualized using DAB in PBS containing 0.015% hydrogen peroxide.

### 2.3. Measurement of molecular weight

Molecular masses were determined on a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (REFLEX III, Bruker Inc., USA) [24]. A saturated  $\alpha$ -cyano-4-hydroxycinnamic acid, 50% (v/v) solution of acetonitrile and aqueous 0.2% trifluoroacetic acid were used as the matrix in all experiments. Protein samples (0.1 mg/mL, 1  $\mu$ L) were mixed with the saturated solution at a ratio of 1:4 (v/v), applied to the stainless steel sample plate and vacuum dried before measurement in the mass spectrometer.

### 2.4. N-terminal sequencing

The two isozymes were electrophoresed (12% SDS-PAGE) on a Bio-Rad (USA) apparatus. The gel was stained with Coomassie Brilliant Blue R-250 and scanned (HP, China). The protein bands were electroblotted onto a PVDF membrane according to Laemmli [25]. After staining with Coomassie Brilliant Blue R-250 for 10 s, the bands were cut out and inserted into an Applied Biosystem Automated Protein Sequencer (Applied Biosystem Inc., USA) for sequencing.

### 2.5. Antigenic characteristics

Antiserum against *EjP*-III-1 was obtained from an adult New Zealand white male rabbit by subcutaneous injection of the purified enzyme (0.5 mg) in 1.0 mL of 50 mM potassium phosphate buffer (pH 7.2) containing 0.85% NaCl, emulsified in an equal volume of Freund's complete adjuvant. After 40 days, the rabbit was given a booster shot with 0.5 mg of *EjP*-III-1. Whole blood was drawn from a carotid artery 1 week after the booster injection. Antiserum against *EjP*-II with avidity of 10,000 was made by this lab as described previously [14].

The antibody was detected by enzyme-linked immunological assay (ELISA) as described by Sambrook et al. [26]. ELISA plates were coated with 50  $\mu$ L of solution containing 10  $\mu$ g/mL of *EjP*-III-1 in carbonate buffer (0.1 M, pH 8.0). After a 2-h incubation at 37 °C, the plates were washed three times with phosphate-buffered saline (PBS)/0.2% Tween 20 and blocked at 37 °C with 1% BSA in PBS for 1.5 h. The primary antibody antiserum was diluted with PBS/0.2% Tween 20 at ratios of 1:10, 1:100, 1:200, 1:500, 1:1000, 1:2000, 1:5000 and 1:10,000, separately, and incubated at 37 °C for 45 min. The plates were then washed three times with the same buffer as above. Goat anti-rabbit IgG (100  $\mu$ L) conjugated with horseradish peroxidase, which was diluted with PBS/0.2% Tween 20 at 1:5000, was added and the plates were incubated at 37 °C for 2 h. The enzymatic reaction was developed with 10  $\mu$ L 3,3',5,5'-tetramethylbenzidine (6 mg/mL) after washing three times. The reaction was stopped by adding 50  $\mu$ L/well 2.0 M sulfuric acid. Absorbance values were determined with an ELISA reader (Bio-Rad Model 3550, USA) at 450 nm. Each test was carried out in duplicate. Several negative sera were rinsed in each plate to control day-to-day variability in the ELISA assay. In all assays, negative sera showed constant optical density values.

Samples from SDS-PAGE were transferred onto one nitrocellulose membrane and used for immunoblotting [26]. After saturation overnight in PBS containing 5% skimmed milk at 4 °C with constant shaking, the nitrocellulose membrane was incubated with 1 mL anti-*EjP*-II (or anti-*EjP*-III-1) serum diluted with PBS/5% skimmed milk at 1:1000. The membrane was washed three times with PBS/0.2% Tween 20, and incubated with the horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted at 1:4000) at room temperature for 2 h. The blots on the membrane, after washing, were developed using DAB, which generated a brown-colored product. The blots were then placed in a fresh container and covered with 0.015% hydrogen peroxide and DAB solution in the dark for 10–15 min. The reaction was stopped by rinsing the membrane in PBS. The membranes were stained by Ponceau S Red to show all the protein bands. Positive and negative control sera were included in each experiment.

### 2.6. Total relative activity distribution of the earthworm

To investigate the distribution of *EjP*-II and *EjP*-III-1 in the earthworm, we evenly cut the mature earthworm into nine sections from the anterior to the posterior. Each section was put on a fibrin plate and incubated at 37 °C for 12 h. The diameter of the fibrinolysis circle was measured and the enzymic activity contained in each segment was thus estimated.

### 2.7. Immunohistochemical staining

Immunohistochemical staining for light microscopy was performed as described by Sambrook et al. [26] with some modifications. The anti-*EjP*-II (or anti-*EjP*-III-1) serum was used as the primary antibody. In brief, an earthworm was cut into nine pieces and fixed in 4% paraformaldehyde at 4 °C overnight. After fixation, the tissues were transferred into 30% sucrose and allowed to stand at 4 °C till all the tissues were settled down. The tissues were arranged into blocks, and the blocks were filled with O.C.T. embedding medium and then placed on dry ice until frozen. Transverse sections (10  $\mu$ m in thickness) were cut at on a microtome. All the procedures were carried out at room temperature, unless otherwise stated. Before immunostaining, hydrated sections were soaked in 0.3% H<sub>2</sub>O<sub>2</sub> for 10 min to block endogenous peroxidase activity and then they were rinsed in PBS (pH 7.4) for 15 min. The rinsed sections were incubated in normal goat serum for 10 min to eliminate non-specific immunostaining. They were then incubated with the *EjP*-II-antiserum or *EjP*-III-1-antiserum (diluted with PBS at 1:200) for 2 h. Subsequently, the sections were washed in PBS for 5 min (five times) and incubated with goat anti-rabbit IgG conjugated with horseradish peroxidase diluted with PBS at 1:100 in a moist chamber. For the peroxidase reaction, the sections were immersed in a 0.02% DAB in 50 mM Tris-HCl buffer (pH 7.6) that contained 0.005% H<sub>2</sub>O<sub>2</sub>. The sections were then washed in distilled water for 5 min, and dehydrated through an ethanol series, cleared in xylene and mounted. They were examined under an Olympus microscope (IX 71). Sections of earthworm segments, incubated in PBS instead of anti-*EjP*-III-1 or anti-*EjP*-II serum, were used as controls.

## 2.8. Fibrinolytic assays

The fibrinolytic activity was measured using three approaches: (1) monitoring the absorbance at 405 nm during degradation of a chromogenic substrate [22]; (2) detecting light scattering during formation of fibrin [27]; (3) measuring the diameter of the fibrinolytic area on a fibrin plate [28,29]. One enzymic unit was defined as the amount of enzyme causing conversion of 1  $\mu\text{M}$  of substrate per minute per mg of protein at 25 °C. The standard earthworm protease ( $2.5 \times 10^3$  U/mg) was employed as a control.

- (1) Assay with chromogenic substrate: the reaction was carried out in Gly–NaOH buffer (50 mM, pH 9.2, 25 °C). The isozyme (final concentration 10  $\mu\text{M}$ ) was mixed with substrate Ch-TH (or another chromogenic substrate) at different concentrations (1, 5, 10, 20 and 50  $\mu\text{M}$ ), and absorbance was measured at 405 nm. The activity was calculated by the coefficient  $\varepsilon_{405\text{nm}} = 9.75 \text{ mM}^{-1} \times \text{cm}^{-1}$ .
- (2) Light scattering: Rayleigh-light scattering was monitored during the assay of *EjP-II* and *EjP-III-1* (0.25  $\mu\text{M}$  as final concentration) on a fluorescence spectrophotometer (Hitachi F-4500, Japan), as described [27]. The enzyme was incubated with fibrinogen (0.25 mg/mL as final concentration) in Tris–HCl buffer (0.05 M, pH 7.4) at 25 °C for 10 min. Thrombin (6 U/mL) was then added to the mixture, followed by the measurement of light scattering at 480 nm.
- (3) Fibrin plate: the fibrinolytic activity of *EjP-II* or *EjP-III-1* was determined using modified PLg-rich fibrin plates and PLg-free fibrin plates [28,29].

## 2.9. Determination of enzymic parameters

*EjP-III-1* was assayed using BAEE, Ch-TH, -U or -TRY as substrate. The buffers of  $\text{Na}_2\text{HPO}_4$ –citrate (pH 5.0–7.0), Tris–HCl (pH 8.0–8.9) and Gly–NaOH (pH 9.5–12) each at 50 mM were used to determine the optimal pH range. Activities at different temperatures (18, 25, 37, 45, 55, 65 and 75 °C) were measured with the four substrates at pH 8.3 in 0.05 M Tris–HCl. To determine the Michaelis–Menten constant ( $K_m$ ), the maximum velocity ( $V_m$ ) and the  $k_{\text{cat}}$ , all reactions were carried out in 0.05 M Gly–NaOH buffer (pH 9.2, 25 °C). The enzyme (1  $\mu\text{M}$ ) was mixed with the substrates (BAEE, ATEE, Ch-TRY, -ELA, -TH or -U) at different concentrations (1, 5, 10, 20 and 50  $\mu\text{M}$ ). The  $K_m$ ,  $V_m$  and  $k_{\text{cat}}$  of *EjP-II* or *EjP-III-1* were calculated from Lineweaver and Burk double-reciprocal plots. For hydrolysis of BAEE, the reaction was started by adding *EjP-II* or *EjP-III-1* for each substrate, and the absorbance at 253 nm was recorded at 25 °C. Hydrolysis of ATEE was detected at 237 nm under the same conditions.

## 2.10. Assays in the presence of inhibitors

*EjP-II* or *EjP-III-1* (1  $\mu\text{M}$ ) was incubated with an inhibitor (SBTI, TLCK, TPCK or elastinal) for 30 min, and then the enzymic activity was measured in the presence of the substrates BAEE, Ch-TH, -TRY or -U. The inhibitors were added at differ-

ent concentrations (0.1, 0.5, 1.0, 2.0, 2.5, 5, 10, 20 and 50  $\mu\text{M}$ ).  $I_{50}$  is defined as the concentration of an inhibitor that causes 50% inhibition of an enzyme reaction [30,31].

## 3. Results

### 3.1. Purification and preparation of antiserum

To purify *EjP-III-1*, the earthworm extract was separated into two proteolytic activity parts (peaks III and IV corresponding to fractions from 18 to 22 and 28 to 31, respectively) using affinity

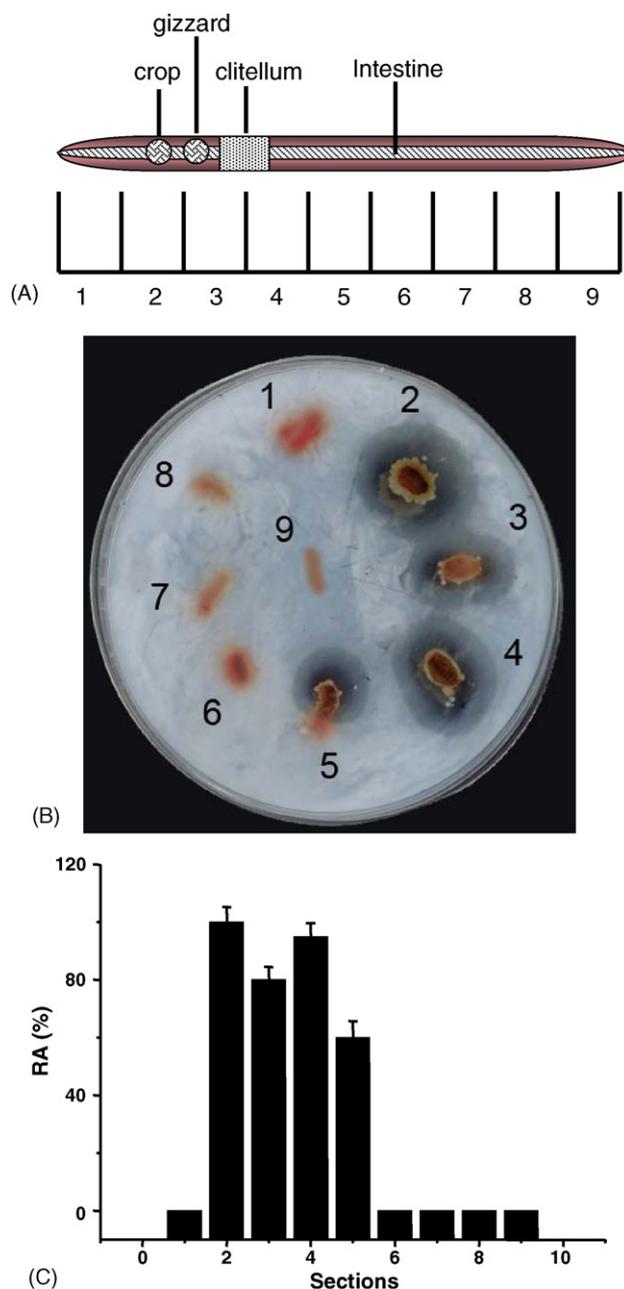


Fig. 2. Localization and total relative activity of the earthworm segments. (A) The earthworm *E. fatida* was evenly sectioned into nine pieces. (B) Each segment was put on the fibrin plate at 37 °C for 12 h. (C) The enzymic activity of each segment. The fibrinolytic activity was calculated by measuring the diameter of the lysis circle.

chromatography (Fig. 1A). The first peak (the main absorption peak at 280 nm) contains multiple protein bands as seen by 12% SDS-PAGE (lane 1, Fig. 1B). Fractions from 28 to 31 (lane 3, Fig. 1B) contain a single protein with an apparent molecular mass of ~34 kDa (29,557 Da, by mass spectrometry), similar to *LrP-III-1*, a homologue from *L. rubbellus* [6]. Sequencing of the N-terminal region reveals an amino acid sequence identical to *LrP-III-1* (IVGGIEAR. . .), indicating the purified protein is *EfP-III-1*. *EfP-II* was isolated as described by Fan et al. [14], and also showed a single protein band on SDS-PAGE (lane 4, Fig. 1B). The molecular mass (24,667 Da) detected by MS and the N-terminal region sequence of *EfP-II* are the same as that of *LrP-II* (VIGGTNAS. . .) [15].

The purified *EfP-III-1* was used to immunize rabbits, yielding an avidity of the immune serum towards *EfP-III-1* of about 10,000 as measured by ELISA. By using anti-*EfP-III-1* serum as the primary antibody in Western blotting, only *EfP-III-1*

(~34 kDa band) was observed (Fig. 1C), suggesting a high immuno-specificity. Further, almost no cross-reaction between *EfP-III-1* and -*II* could be detected in the presence of anti-*EfP-III-1*. The avidity of the immune serum towards *EfP-II* is about 10,000 [14].

### 3.2. Localization of the proteases in earthworm

*E. fetida*, a match-sized earthworm, is blood-like in color. To clarify the localization of *EfP-II* and *EfP-III-1*, the worm was evenly cut into nine sections from the anterior to posterior (Fig. 2A). When the nine sections were placed on a fibrin plate, clearing of the plate was observed around the segments 2–5 (Fig. 2B). The highest fibrinolytic activity was detected in segments 2 and 4, whereas very low activity was observed by sections containing the mouth, posterior intestine and anus (Fig. 2C). This suggests that earthworm proteases are mainly

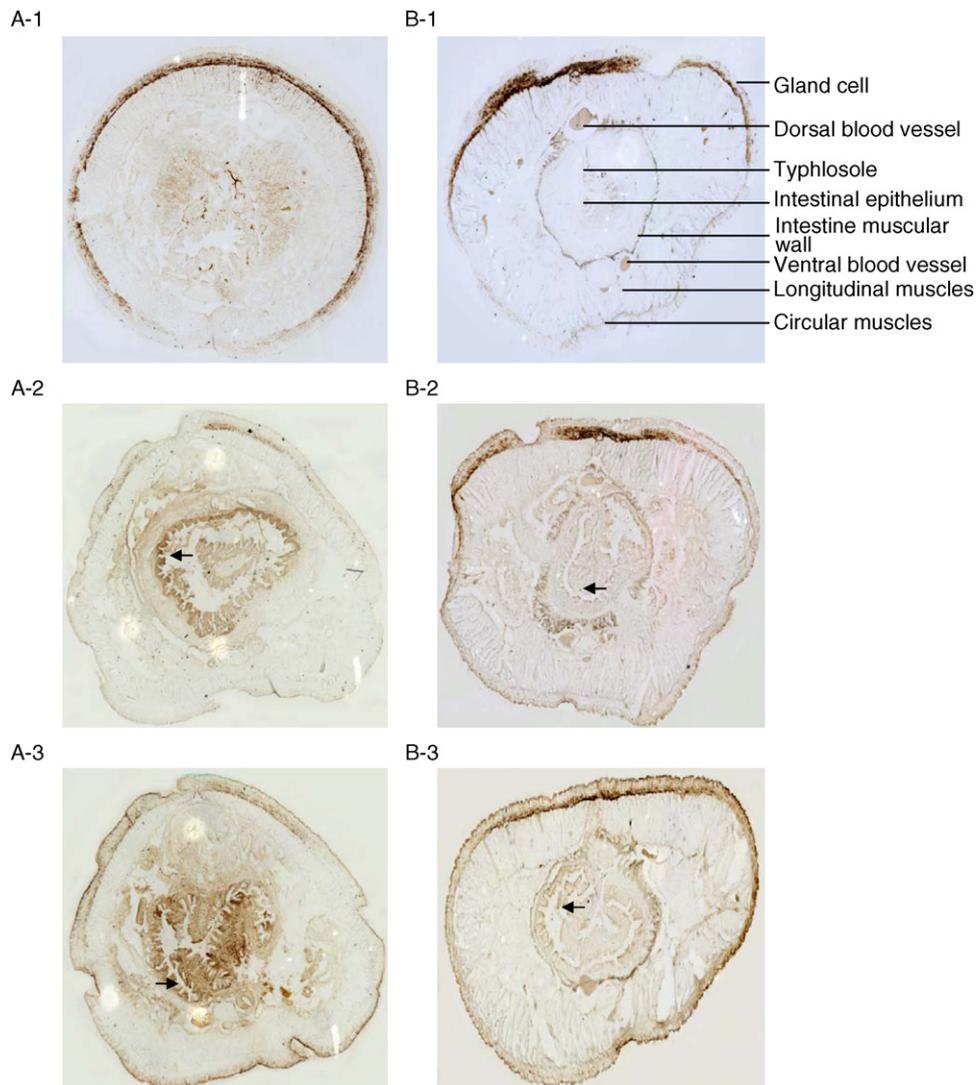


Fig. 3. *In situ* localization of *EfP-II* and *EfP-III-1* in the intestine of *Eisenia fetida*. The protocol for the immunohistochemical visualization of the enzyme on the earthworm transverse sections is described in the Section 2. (A) Sections of segment 2 as shown in Fig. 2. The enzymes were visualized by adding anti-*EfP-II* (panel A-2) or anti-*EfP-III-1* (panel A-3) as primary antibodies, followed by secondary antibody (goat-anti-rabbit IgG-HRP). Sections without adding the primary antibodies were used as controls (panel A-1). (B) The enzymes in the sections of segment 5 were visualized in the presence of anti-*EfP-II* (panel B-2), anti-*EfP-III-1*-serum (panel B-3), and in the absence of the primary antibodies (panel B-1). Arrows indicate the immunological signals.

localized in the crop and gizzard regions, particularly in the anterior alimentary regions. We noted that no activity could be detected in the earthworm extract when the alimentary canal was removed, or washed with buffers (PBS). No enzymic activity of the excreted mucus from the earthworm skin was observed.

Strong immunological staining could be detected in the alimentary wall in the presence of anti-*EfP-II* or anti-*EfP-III-1* serum (Fig. 3A-2 and -3). This indicates that these two isozymes are expressed and synthesized in the epithelial cells. The two isozymes are mainly localized in the anterior alimentary region around the clitellum and appear sparsely only along the posterior intestinal sections (Fig. 3B-2 and -3). The localization of earth-

worm proteases by immunohistochemistry is consistent with the fibrin plate assay.

### 3.3. Substrate specificity

Interestingly, *EfP-II* and *EfP-III-1* showed different activities in the presence of different substrates. By using the fibrin plate assay, the fibrinolytic activity of *EfP-III-1* is about  $6.6 \times 10^4$  U, three times higher than that of standard earthworm protease. For *EfP-II*, however, some ambiguous fibrinolysis circles on the plate were observed, and the activity could not be clearly monitored. Assay by light scattering [27] showed that fibrinolytic activity

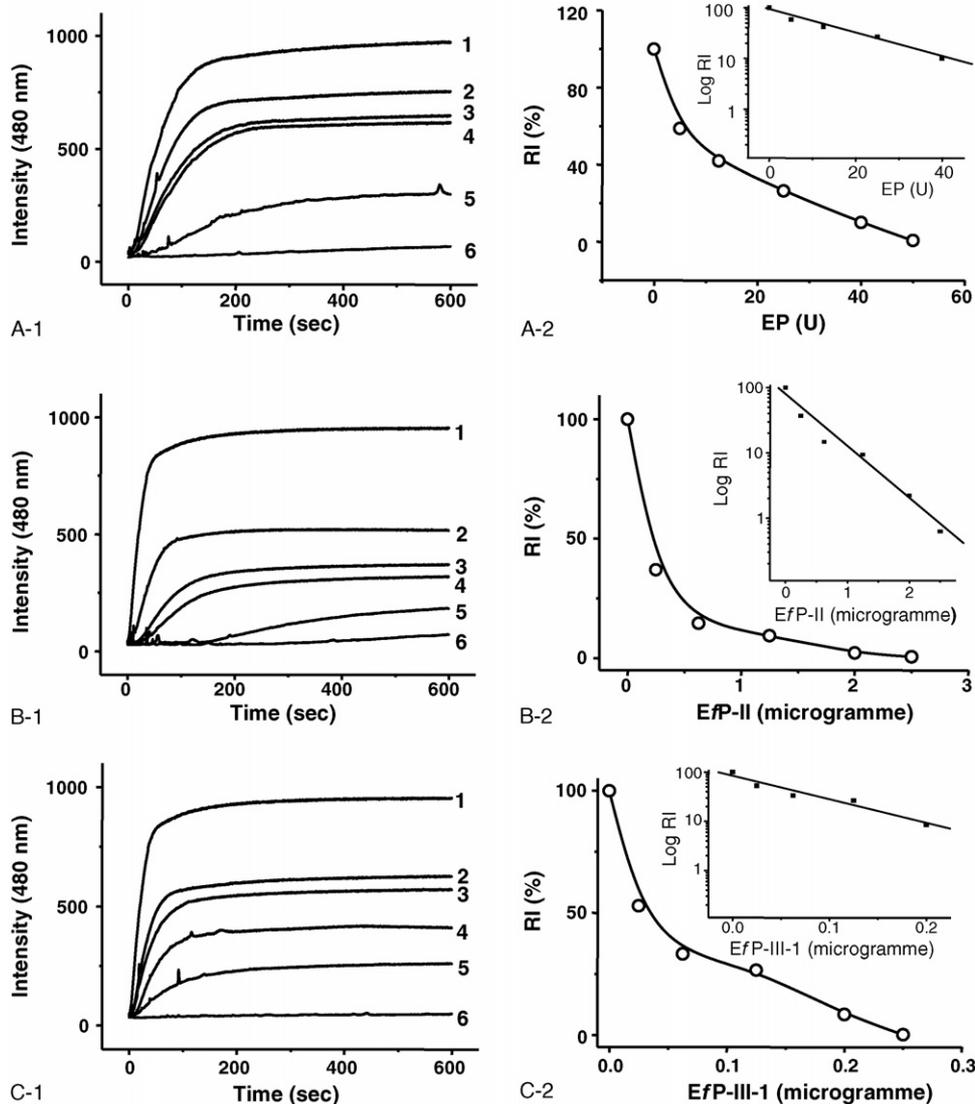


Fig. 4. Activity assay of *EfP-II* and *EfP-III-1* by light scattering. The enzyme was added to the reaction mixture of thrombin and fibrinogen (final concentration 0.25 mg/mL) followed by the measurement of light scattering intensity at 480 nm. (A) Light scattering of the reaction mixture in the presence of the standard earthworm protease. Curves from 1 to 6 (panel A-1) represent different concentrations of the standard EP used for different activities (0, 1, 5, 12.5, 25, 40 and 50 U). The scattering intensity was plotted via the mass of the enzyme as a standard curve to determine the activity of *EfP-II* and *EfP-III-1* in panel A-2. The insert is the same data plotted in semilogarithm. (B) *EfP-II* was added at different concentrations (0, 0.25, 0.625, 1.25, 1.0 and 2.5  $\mu$ g/mL) to the reaction mixture (panel B-1). The relationship between the enzyme concentration and the scattering intensity is shown in panel B-2 for *EfP-II* and panel C-2 for *EfP-III-1*. C. Curves from 1 to 6 denote the final concentrations of *EfP-III-1*: 0, 0.025, 0.0625, 0.125, 0.2 and 0.25  $\mu$ g/mL (C-1). Each line represents the average of three independent experimental measurements.

Table 1  
Activities and parameters of *EfP-II* and *EfP-III-1* in the presence of different substrates and inhibitors

Substrate	Isozyme	Enzymatic parameters			Inhibitor ( $I_{50}$ )		
		$K_m$ (M)	$V_m$ (mol s <sup>-1</sup> )	$k_{cat}$ (s <sup>-1</sup> )	SBTI (M)	TLCK (M)	Elastinal (M)
BAEE	<i>EfP-III-1</i>	$1.7 \times 10^{-5}$	$2.3 \times 10^{-6}$	17.2	$2.0 \times 10^{-7}$	$9.0 \times 10^{-7}$	–
	<i>EfP-II</i>	$3.6 \times 10^{-4}$	$3.3 \times 10^{-6}$	2.1	$5.0 \times 10^{-7}$	–	–
ATEE	<i>EfP-III-1</i>	×	×	×	–	–	–
	<i>EfP-II</i>	$3.3 \times 10^{-3}$	$1.8 \times 10^{-5}$	11.1	–	–	–
Chromozym TH	<i>EfP-III-1</i>	$6.0 \times 10^{-5}$	$1.3 \times 10^{-6}$	99.3	$1.4 \times 10^{-6}$	$1.6 \times 10^{-5}$	–
	<i>EfP-II</i>	$6.4 \times 10^{-6}$	$9.4 \times 10^{-7}$	0.3	$2.5 \times 10^{-6}$	–	–
Chromozym TRY	<i>EfP-III-1</i>	$3.3 \times 10^{-5}$	$6.5 \times 10^{-7}$	48.5	$9.0 \times 10^{-7}$	$1.3 \times 10^{-5}$	–
	<i>EfP-II</i>	$3.9 \times 10^{-4}$	$4.4 \times 10^{-7}$	0.1	$2.0 \times 10^{-6}$	–	–
Chromozym U	<i>EfP-III-1</i>	$3.9 \times 10^{-5}$	$5.7 \times 10^{-6}$	428.4	$6.0 \times 10^{-7}$	$6.6 \times 10^{-6}$	–
	<i>EfP-II</i>	$4.1 \times 10^{-5}$	$7.2 \times 10^{-7}$	0.2	$5.0 \times 10^{-6}$	–	–
Chromozym ELA	<i>EfP-III-1</i>	×	×	×	–	–	–
	<i>EfP-II</i>	$2.5 \times 10^{-4}$	$8.7 \times 10^{-7}$	0.2	$5.0 \times 10^{-6}$	–	$2.5 \times 10^{-6}$

“×” represents no activity for the substrate; “–” means the inhibitor shows no inhibition.  $K_m$ ,  $V_m$  and  $k_{cat}$  of *EfP-II*/*EfP-III-1* were obtained from Lineweaver and Burk double-reciprocal plots [33].  $I_{50}$  is defined as the concentration of an inhibitor causing 50% inhibition of the activity [30,31]. Each result is the average of three independent experimental measurements.

of *EfP-II* ( $\sim 5.0 \times 10^4$  U) is about seven times lower than that of *EfP-III-1* ( $\sim 33 \times 10^4$  U) (see Fig. 4). Although this method is convenient and reproducible, it may not be an efficient method to study the substrate specificity of *EfP-II* and *EfP-III-1*. Therefore, a group of chromogenic oligopeptide substrates for trypsin, chymotrypsin and elastase were used.

As shown in Table 1 *EfP-II* is capable of recognizing the six substrates BAEE, ATEE, Ch-TH, Ch-TRY, Ch-U and Ch-ELA 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 follow:  $[K_{mBAEE}] < [K_{mTRY}] < [K_{mU}] < [K_{mTH}]$ , 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 for *EfP-II*. In addition, *EfP-III-1* (Fig. 5) and *EfP-II* [22] share a similar range of optimal pH (pH 8.0–10) and temperature (45–50 °C).

### 3.4. Inhibition in the presence of different inhibitors

In order to confirm the substrate specificity, the inhibitors SBTI, TLCK, TPCK and elastinal were used (Fig. 6). The inhibition of *EfP-III-1* by SBTI could be observed in the presence of BAEE, Ch-TH, Ch-TRY or Ch-U. Among these substrates, BAEE has the lowest  $I_{50}$  (Table 1), leading to the strongest inhibition of *EfP-III-1*. The degree of inhibition by SBTI is dependent upon the substrate in the following order: BAEE > Ch-U > Ch-TRY > Ch-TH. Another specific trypsin inhibitor, TLCK, a somewhat different pattern of inhibition efficiency for the substrates as follows: BAEE > Ch-TRY > Ch-TH > Ch-U (Fig. 6). It was noted that elastinal does

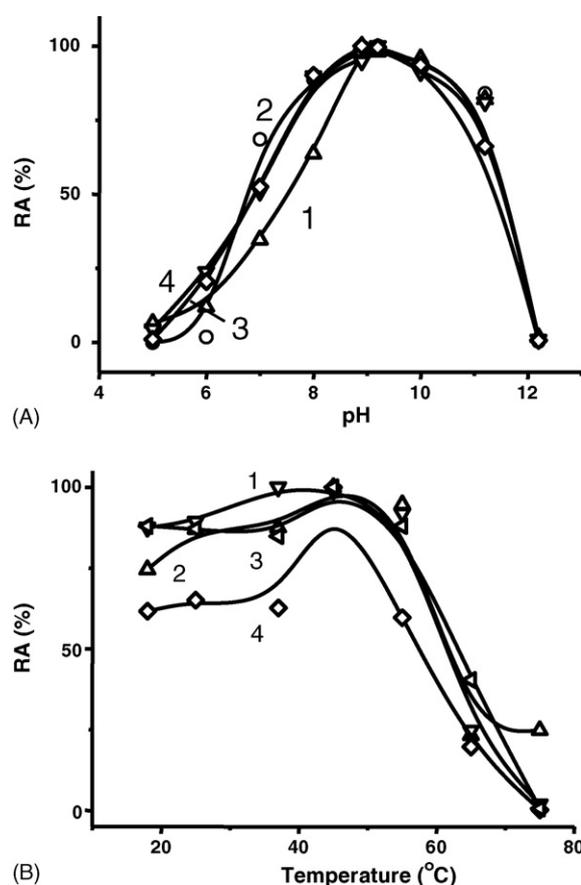


Fig. 5. The optimal pH and temperature of *EfP-III-1*: (A) solutions at different pH values were prepared by using 0.05 M Na<sub>2</sub>HPO<sub>4</sub>-citrate (pH 5.0–7.0), Tris-HCl (pH 8.0–8.9) and Gly-NaOH (pH 9.5–12), respectively. The activity assays of *EfP-III-1* were performed in the presence of Chromozyme-TH (curve 1), Chromozyme-UK (curve 2), BAEE (curve 3), and Chromozyme-TRY (curve 4) in 0.05 M Tris-HCl containing 227 mM NaCl (pH 7.2). (B) The activity of *EfP-III-1* at different temperatures (18, 25, 37, 45, 55, 65 and 75 °C) was measured under the same conditions.

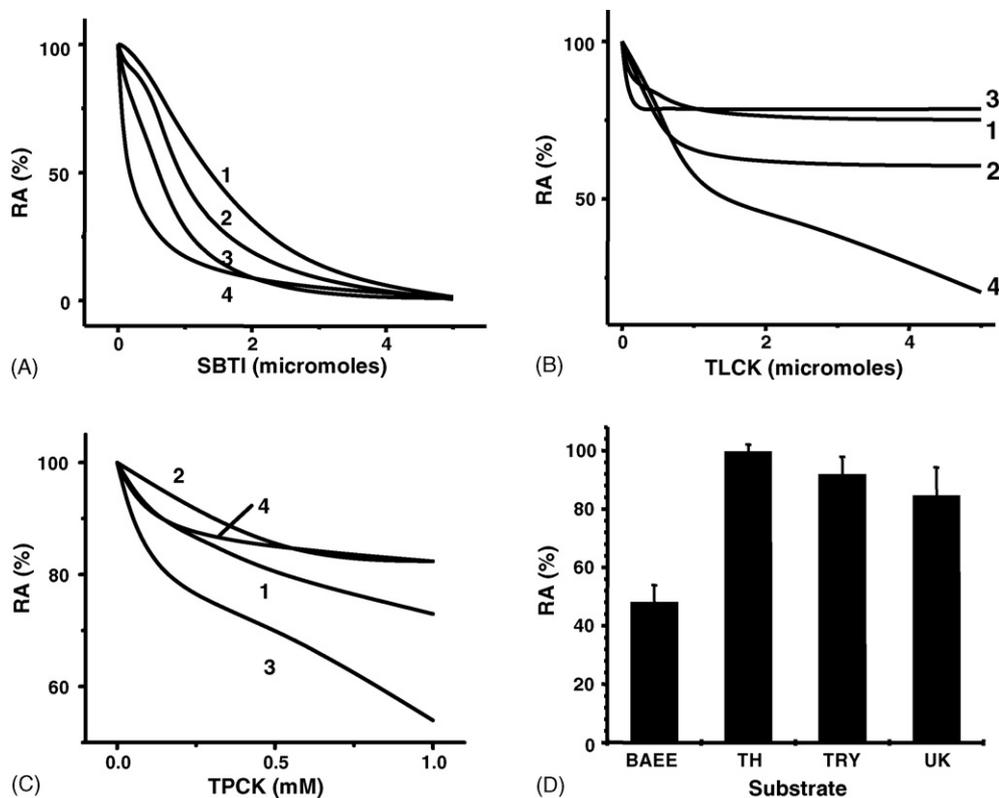


Fig. 6. Effect of inhibitors on *E/P-III-1* with different substrates: *E/P-III-1* (1 μM) was incubated with the inhibitor SBTI (panel A), TLCK (panel B) or TPCK (panel C) in 50 mM Gly–NaOH (pH 9.2, 25 °C) for 30 min before the substrate (50 μM as final concentration) Ch-TH (curve 1), Ch-TRY (curve 2), Ch-U (curve 3) or BAEE (curve 4) was added. The activity was measured in the presence of the different concentrations of the inhibitor: 0, 0.1, 0.5, 1.0, 2.0, 2.5 and 5 μM, respectively. (D) Relative activities are shown of *E/P-III-1* using different substrates (50 μM as final concentration) in the presence of 0.1 μM SBTI. Under these conditions, SBTI shows 50% inhibition of *E/P-III-1* in the presence of BAEE.

not inhibit *E/P-III-1*. For *E/P-II*, the inhibition by elastinal could be observed in the presence of Ch-ELA. The inhibition of *E/P-II* by SBTI could be detected in the presence of Ch-TH or Ch-TRY, but not Ch-ELA.

#### 4. Discussion

The earthworm intestine is a straight tube and is the principal site of digestion and absorption. A dorsal fold of the luminal epithelium called the typhlosole substantially increases the surface area of the intestine. We found in the study that the distribution of *E/P-II* and *E/P-III-1* is almost limited to the alimentary tract. The anterior alimentary region, excluding the mouth and posterior intestine, contains the highest levels of activity, particularly around the clitellum (segments 2–4). This region contains the crop, gizzard and anterior intestine, which are the most important organs to store and digest food (Fig. 2). This indicates that these two enzymes are helpful for the earthworm to digest the proteins in food.

The fact that *E/P-II* is mainly distributed in the crop and gizzard provides the potential to digest a wide range of proteins because it has a broad substrate specificity. Furthermore, the earthworm is hygrophilous and lives in a moist-underground environment containing a broad range of organisms and compounds. In line with its living conditions, the earthworm would require proteases with broad specificities, as has been seen for

*E/P-II* in hydrolysis of different substrates. Similar enzymes are also found in other animals, for instance the collagenolytic serine protease-1 from the crab *Ucapugilator*, which also lives in a complex habitat [32].

*E/P-II* is most strongly inhibited by SBTI and weakly inhibited by elastinal. The inhibition by elastinal was observed only when Ch-ELA was present. However, elastinal cannot inhibit *E/P-II* in the presence of Ch-TH, -TRY, -U or BAEE. The X-ray crystal structure shows that the active site of *E/P-II* is located in a particularly flexible pocket [16,17]. The high flexibility of the pocket provides multiple binding sites that can accommodate diverse amino acid side chains [17,18]. The conformation of the active site of *E/P-II* is flexible and readily induced by the substrate to form a special structure to which the substrate can bind (called “induced fit”) [33,34]. This conformation is also induced in the presence of inhibitors (Table 1). X-ray crystal analysis shows that the locations of some crucial amino acid residues in the active site of *E/P-II* are similar to elastase [16]. However, the smallest  $K_m$  value is obtained in the presence of Ch-TH (Table 1). This suggests that *E/P-II* is a trypsin-, chymotrypsin- and elastase-like protease.

The smallest  $K_m$  value for *E/P-III-1* was observed using BAEE, showing that it is a trypsin alkaline serine-like protease. *E/P-III-1* is most strongly inhibited by SBTI in the presence of BAEE. *E/P-III-1*, favoring the trypsin substrates, does not hydrolyze ATEE and Ch-ELA. Crystal structural analysis shows

that *EfP*-III-1 (also called EFE-b) should be classified as a trypsin [19]. However, it is distinct from other trypsins [20]. This isozyme is a two-chained protease with an N-terminal pyroglutamate light chain and an N-glycosylated heavy chain. The heavy chain contains a novel structural motif and an eight-member ring resulting from a disulfide bridge between two neighboring cysteine residues, with a *cis* peptide bond between these two cysteine residues. The crystal structure of *EfP*-III-1 provides the structural basis for its high level of stability and reveals its complicated post-translational modifications in earthworm.

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, indicating that it has a chymotrypsin-like function and possesses a broader substrate specificity than *EfP*-III-1 [30,35]. As described by Nakajima et al. [36,37], *LrP*-II also recognizes multiple cleavage sites in various proteins such as elastin, hemoglobin,  $\beta$ -amyloid 1–40 and oxidized insulin B-chain. It hydrolyzes the bonds not only at neutral amino acids, but also at aromatic residues.

## 5. Conclusions

This study shows that *EfP*-II and *EfP*-III-1 are localized in the epithelial cells of the alimentary canal around the clitellum of the earthworm *E. fetida*. As shown in the experiments with different substrates and inhibitors, *EfP*-II acts as a strong thrombin-like, moderate elastase-like and weak chymotrypsin-like serine protease. *EfP*-III-1 behaves as a trypsin-like protease. The relatively broad substrate specificity of *EfP*-II and *EfP*-III-1 is needed for the earthworm to digest different micro-organisms and proteins.

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