

Glycosylated trypsin-like proteases from earthworm *Eisenia fetida*

Jin Xia Wu^a, Xiao Yu Zhao^{a,*}, Rong Pan^b, Rong Qiao He^{b,**}

^a College of Life Science, Hebei University, 180 Wusi East Road, Baoding City 071002, Hebei, China

^b State Key Laboratory of Brain and Cognitive Science, Baiao Pharmaceuticals Beijing C.L., Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Chaoyang District, Beijing 100101, China

Received 12 June 2006; received in revised form 2 October 2006; accepted 2 October 2006

Available online 12 October 2006

Abstract

Although groups of earthworm proteases have been found by several laboratories, it is still unclear how many of the isolated trypsin-like fibrinolytic enzymes are in glycosylated form. Here, eight glycosylated fibrinolytic proteases (*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) were isolated from an earthworm species (*Eisenia fetida*) through a stepwise-purification procedure: ammonium sulfate precipitation, affinity chromatography on a Sepharose-4B column coupled with soybean trypsin inhibitor (SBTI), and ionic chromatography with a DEAE-Cellulose-52 column. Among the eight purified trypsin-like glyco-proteases, *EfP*-0-2 and *EfP*-II-2 were newly isolated isozymes. Glycoprotein staining of the proteases on native-PAGE with a Schiff's reagent (sodium meta-periodate) revealed that the eight proteases were glycoproteins. Measurements of the glycan content with sodium meta-periodate and glycoprotein-test reagent showed that these proteases had different carbohydrate contents. Dot-blotting assay with ConA suggested the oligosaccharides were composed of mannose residues.

© 2006 Elsevier B.V. All rights reserved.

Keywords: Earthworm protease; Isozymes; Glycosylation; Fibrinolysis; Deglycosylation

1. Introduction

In recent years, several fibrinolytic isozymes have been found in two species of earthworms, *Eisenia fetida* and *Lumbricus rubellus* [1–6]. Their biological functions, such as fibrinolysis [7], anticoagulation [8], antioxidation [9], anti-tumorigenesis [10] and triacylglycerolysis [11], have been investigated [12–18]. Nakajima et al. have isolated six fibrinolytic isozymes from *L. rubellus* (*LrP*-I-0, *LrP*-I-1, *LrP*-I-2, *LrP*-II, *LrP*-III-1 and *LrP*-III-2) [1,3], and Wang et al. have identified seven proteases from *E. fetida* (*EfP*s) [19]. Studies with different substrates and inhibitors have shown that these isozymes are alkaline trypsin-like serine proteases [20]. X-ray diffraction

crystallography has shown that one of the *EfP*s, *EfP*-a, has both the trypsin- and chymotrypsin-like characteristics [21,22].

Recently, several fibrinolytic *EfP*s containing glycan chains have been found [23]. Two tyrosine-like serine peptidases, P-I (34 kDa) and P-II (23 kDa), have also been isolated from the earthworm [24]. P-I peptidase has high fibrinolytic activity (completely inhibiting blood clotting at a concentration of 1 µg/ml), and can autocatalytically degrade into P-II. Using *m*-aminophenylboric acid affinity chromatography [25], Li et al. have purified a glycosylated fibrinolytic enzyme from *E. fetida*, which has a different N-terminal sequence from those of the *EfP*s reported previously. Another glycosylated serine protease isolated from *E. fetida* can induce apoptosis [26]. Wang and his colleagues have found that *EfP*-b, a glycosylated isozyme, similarly to *LrP*-III-1, consists of one light and one heavy peptide chain [27]. The light chain is pyroglutamated and the heavy chain is glycosylated at residue Asn-161. Although several glycosylated proteases have been studied, the number of earthworm trypsin-like proteases that are glycosylated still remains unclear. Here, we report at least eight glycosylated trypsin-like proteases from the earthworm *E. fetida*, with a fibrinolytic role.

Abbreviations: *EfP*s, *Eisenia fetida* proteases; *LrP*s, *Lumbricus rubellus* proteases; SBTI, soybean trypsin inhibitor; F-PAGE, fibrin polyacrylamide gel electrophoresis; SOD, superoxide dismutase; PVDF, polyvinylidene fluoride; ConA, concanavalin A; HRP, horseradish peroxidase; DAB, diaminobenzidine

* Corresponding author. Tel.: +86 312 5079735.

** Corresponding author. Tel.: +86 10 6488 9876; fax: +86 10 6485 3625.

E-mail addresses: uyoaix@vahoo.com.cn (X.Y. Zhao), herq@sun5.ibp.ac.cn (R.Q. He).

2. Experimental

2.1. Animal

Two-month-old earthworms (*E. fetida*) 5–8 cm in length were provided by the Research Center of Bioengineering, Hebei Province, China. The earthworms were washed with distilled water and then were bred in the water at ambient temperature for half a day to disgorge the ordure from the alimentary canal.

2.2. Enzymatic assays

(1) *Fibrin plate assay*, modified as described previously [28]. Phosphate buffered saline (PBS, pH 7.2) containing 0.5% agarose, 15 mg fibrinogen (Sigma Company, USA) and thrombin (10 U, Sigma Company, USA) was poured onto a transparent petri dish. Wells (2–3 mm in diameter) were punched on the fibrin plate. Samples were added in the wells, and PBS was used as negative control. The plate was incubated in a humidified box at 37 °C for 3 h and then the enzymic activity was evaluated by measuring the diameter of the fibrinolytic circle against a urokinase standard. (2) *Fibrin polyacrylamide gel electrophoresis (F-PAGE)* [29]. Fibrinogen (final concentration 20 mg/ml) was resuspended in 3.0 M Tris–HCl buffer (pH 8.9) containing 10 U of thrombin, to prepare a 10% resolving gel with 0.1% sodium dodecyl sulfate (SDS). The proteases were electrophoresed and then the gel was rinsed with 1% TritonX-100 (20 ml) in a shaker at room temperature for 1 h. The gel was incubated in 20 ml of phosphate buffer (pH 7.2) for 30 min, followed by Commassie brilliant blue (R-250) staining. (3) *Fibrinogenolysis*. The *EfPs* (final concentration 0.3 µg/µl) were added to fibrinogen (5 mg/ml) and incubated at 37 °C. Aliquots were taken at different time intervals, and were put on ice to terminate the reaction prior to being loaded on 10% SDS-PAGE. The concentration of protein was determined by Lowry method and BSA was used as a control [30].

2.3. Purification

One kilogram of earthworms were homogenized in 0.05 M Tris–HCl buffer (pH 7.8) and then centrifuged (8000 rpm, 4 °C, 30 min). The supernatant was salted out with 85% ammonium sulfate at 4 °C overnight; the precipitates (7 g) were resuspended in double-distilled water (300 ml), dialyzed against 0.01 M NaHCO₃ buffer (pH 8.0) at a volume ratio of 1:1000 (4 °C, 3 h, three times), and filtered with a membrane filter of 0.45 µm (Sartorius, Goettingen, Germany) before use:

(1) Soybean trypsin inhibitor (SBTI)-Sepharose-4B affinity chromatography. Sepharose-4B (10 ml) activated by carbonyl-diimidazole (800 mg) [31] was used to couple with SBTI. The supernatant was loaded onto the SBTI-Sepharose-4B column (0.5 cm × 20 cm), and then the column was washed with 0.01 M NaHCO₃ buffer (pH 8.0) until the absorbance at 280 nm approached zero. Urea (6 M) was used to elute the proteases, which were further dialyzed against double-distilled water and were lyophilized before

use. The earthworm proteases purified by the affinity chromatography were named *EfPs* unless stated otherwise.

(2) Purification of the isozymes with DEAE-Cellulose-52 column. The lyophilized *EfPs* were resuspended in 0.01 M phosphate buffer (pH 8.0) and loaded onto a DEAE-Cellulose-52 column (1.0 cm × 20 cm), which had been equilibrated with 0.01 M phosphate buffer (pH 8.0). The column was washed in three volumes of phosphate buffer, and then the isozymes were eluted with a linear gradient of NaCl (0.0–0.4 M) as described previously [32]. The fractions were pooled and chromatographed again until each isozyme was well separated. The eluent was collected and the fibrinolytic activity was detected by fibrin plate assay. Aliquots were subjected to 10% native-PAGE and SDS-PAGE for identifying the purity and the subunits constitution, respectively. The purified isozyme on the SDS-gel was subsequently transferred onto PVDF membrane for N-terminal sequencing by Beijing SaiBaiSheng Biotechnology Corporation and Research Center for Biology, China (Applied Biosystems Procise-PROCISE-HT).

2.4. Hydrolysis of earthworm proteases in the presence of pepsin or trypsin

The *EfPs* (final concentration 1 µg/µl) were dissolved in 0.1 M Gly–HCl buffer (pH 2.2) for pepsin hydrolysis and in 0.1 M Gly–NaOH buffer (pH 9.0) for trypsin hydrolysis, respectively. The samples were incubated at a mass ratio of 50:1 (*EfPs*:[pepsin]) at 37 °C.

Aliquots were taken at different time intervals for 10% native-PAGE. BSA in the presence of trypsin was used as a control.

2.5. Measurement of glycan content

The sugar content of each of the eight isozymes (2.5 mg/ml) was determined using a glycoprotein carbohydrate estimation kit (PIERCE Company, USA). Sodium meta-periodate (2 mg/ml) and the glycoprotein-test reagent (5 mg/ml) were freshly prepared. BSA (2.5 mg/ml), ovalbumin (2.5 mg/ml), human apotransferrin (2.5 mg/ml), fetuin (0.25 mg/ml) and α₁-acid glycoprotein (0.25 mg/ml) were used as references to estimate the sugar content. Glycoprotein was oxidized with sodium meta-periodate and the formation of aldehyde was detected by using the Propriety Glycoprotein Detection Reagent, which produces off purple color on reaction with aldehyde. The maximum absorbance was achieved at 550 nm and the absorbance was proportional to the percentage of the carbohydrate component in each glycoprotein.

2.6. Glycoprotein staining of earthworm proteases on native-PAGE

The *EfPs* (at final concentration of 2.5 µg/µl) were electrophoresed on native-PAGE and then the gel was stained with Schiff's reagent (0.05 M sodium meta-periodate). The proteins on the PAGE stained with Commassie brilliant blue were used as controls.

The purified isozymes (3 μg) such as *EfP*-0-2, *EfP*-I-1, *EfP*-II-1 and *EfP*-III-1 (~0.1 nmol) were employed in Dot-blotting with HRP-ConA [33]. The samples were dotted on a piece of PVDF membrane, and dried at ambient temperature. Then the membrane was rinsed with 0.01 M Tween–phosphate buffered saline (TBS) at pH 7.4 for 15 min, blocked with 1% BSA for 3 h, rinsed with TBS for 15 min, and incubated with HRP-ConA in a shaker at 4 °C overnight. After being rinsed with TBS (15 min), the PVDF membrane was incubated with 0.03% diaminobenzidine (DAB) and H₂O₂. Superoxide dismutase (SOD, ~0.1 nmol)

and ovalbumin (~0.01 nmol) were used as negative and positive controls, respectively.

2.7. Deglycosylation

The isozyme *EfP*-I-1 (15 μg) was incubated with 2 μl of PNGase F (0.4 U, from Boehringer Mannheim, Germany) in the deglycosylation buffer: 0.02 M phosphate buffer (pH 7.2) containing 0.2% SDS, 0.1 M mercaptoethanol and 0.75% Triton X-100 at 37 °C. Aliquots were taken at different time intervals

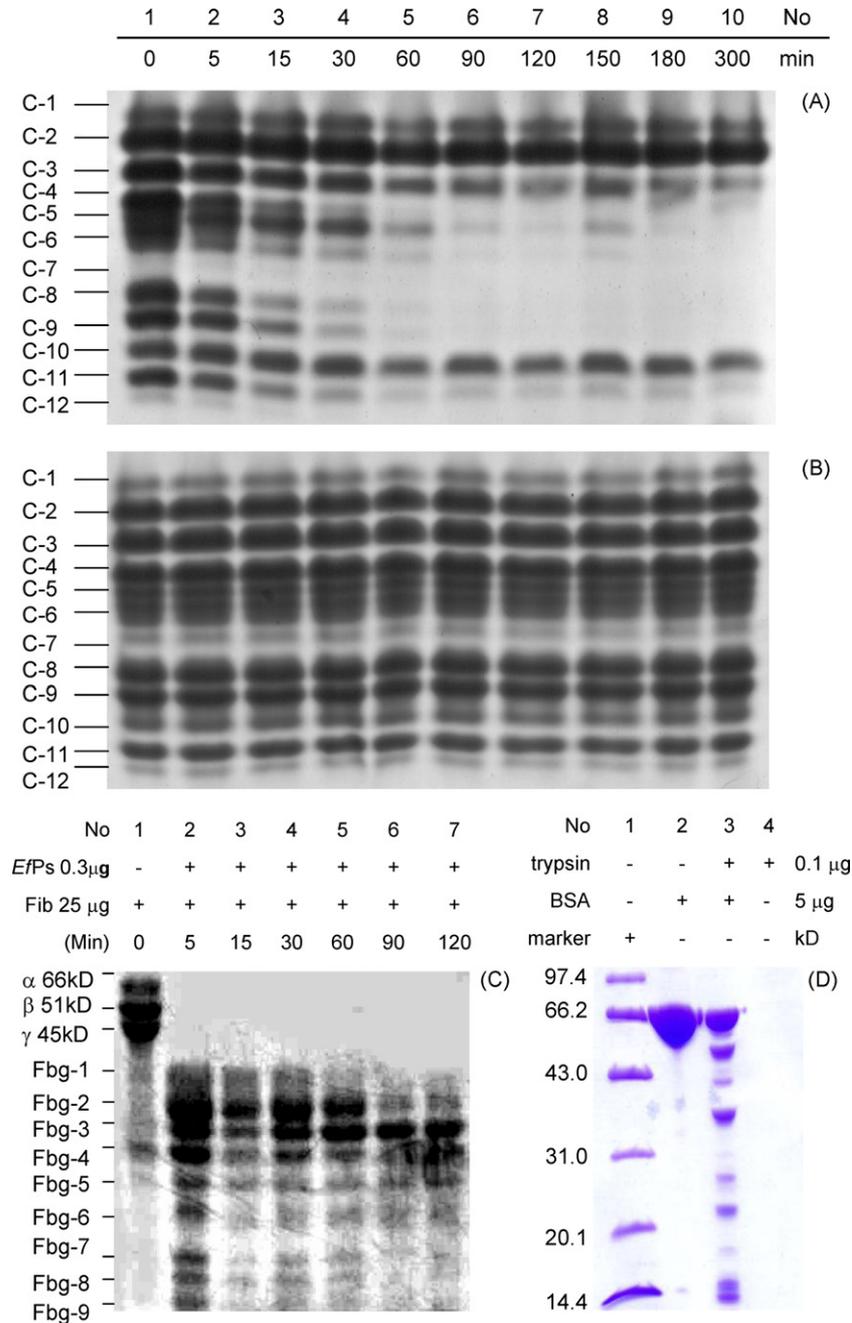


Fig. 1. Fibrinogenolysis of earthworm proteases and hydrolysis in the presence of pepsin and trypsin. The earthworm proteases (*EfPs*) purified by the SBTI-Sepharose-4B affinity chromatography were incubated with pepsin (panel A) and trypsin (panel B), respectively. Aliquots were taken at different time intervals (as indicated) for electrophoresis. Fibrinogen was incubated with *EfPs*, and aliquots thereof were taken for SDS-PAGE (10%) (panel C). BSA in the presence of trypsin was used as a control (panel D, SDS-PAGE).

to be detected on native-PAGE (3 and 7 h). Native glycosylated *EfP*-I-1 and the *EfPs* were used as controls.

3. Results

3.1. Fibrinolytic proteases in the presence of pepsin or trypsin

EfPs purified by the (SBTI)-Sepharose affinity chromatography showed 12 protein bands (from c-1 to c-12) on 10% native-PAGE (panel A, Fig. 1). *EfPs* were added to bovine fibrinogen at 37 °C to analyse their fibrinolytic activity. On SDS-PAGE (panel C, Fig. 1), the three-fibrinogen chains ($\text{A}\alpha$, $\text{B}\beta$ and γ) were digested within 5 min, resulting in about nine fragments (fbg-1 to fbg-9). Among these fragments, fbg-3, fbg-4 and fbg-5 were stable during the incubation.

In order to investigate whether they were stable at different pH values, we incubated these proteases with pepsin at pH 2.2 (panel A, Fig. 1). Observably, components c-1, c-2 and c-10 were stable in the presence of the pepsin; however, c-3, c-4, c-5, c-6, c-7, c-8 and c-9 were hydrolyzed in 60 min. In particular, components c-4, c-6, c-7, c-8 and c-9 were most vulnerable to pepsin, and they were obviously degraded within 30 min. By contrast, hydrolysis of *EfPs* could not be detected in the presence of trypsin under alkaline condition (pH 9.0) (panel B, Fig. 1). BSA, however, as a control, was hydrolyzed into about eight fragments in the presence of trypsin (panel D, Fig. 1). This observation suggests that the earthworm proteases isolated by affinity chromatography are resistant to trypsin, but are vulnerable to pepsin except for components c-1, c-2 and c-10.

3.2. Isolation of the active earthworm proteases

To assay the fibrinolysis, the *EfPs* purified through the (SBTI)-Sepharose-4B column were subjected to F-PAGE (panel A-1, Fig. 2) and eight bands were clearly displayed. Note that the eluted proteins contained approximately 12 protein bands on PAGE (panel A-2, Fig. 2). Sequentially, the soluble proteins salted out in the presence of ammonium sulfate (85% saturation) were electrophoresed on F-PAGE (panel A-3, Fig. 2) and on PAGE (panel A-4, Fig. 2). About eight clear bands were exhib-

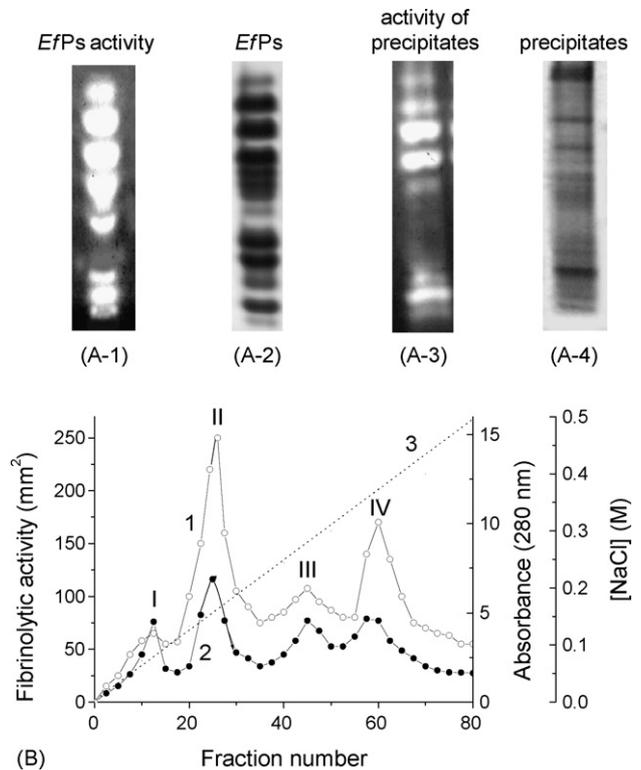


Fig. 2. Purification of earthworm proteases and their activities. *EfPs* (20 μg) were subjected to F-PAGE (panel A-1) and 10% native-PAGE (panel A-2), respectively. The precipitated proteins (20 μg) resulting from 85% saturated ammonium sulfate were subjected to F-PAGE for activity assay (panel A-3) and to PAGE (panel A-4). The precipitates were isolated through SBTI-Sepharose-4B column (0.5 cm \times 20 cm). The isozymes were further purified through a DEAE-Cellulose-52 column (1.0 cm \times 20 cm) [24]. Curves 1–3 represent fibrinolytic activity, protein concentration and NaCl gradient, respectively (panel B).

ited in the fibrin polyacrylamide gel, indicating that at least eight kinds of fibrinolytic proteases contained in the soluble proteins. In other words, the SBTI-specific ligand was associated with the eight kinds of active components that were found in the total soluble proteins from ammonium sulfate precipitation.

The *EfPs* were then purified with an ionic exchange DEAE-Cellulose-52 column with a gradient of NaCl (panel B, Fig. 2). Four eluted peaks (I, II, III, and IV) were displayed. Each peak

Table 1
Carbohydrate contents and activities of the *EfP* isozymes^a

Number	Earthworm proteases	Carbohydrate content (%) ^b	Relative activity (%) ^c	Apparent molecular mass (kDa)
1	<i>EfP</i> -0-1	6.13	6.2	22.5
2	<i>EfP</i> -0-2	4.30	12.8	22.4
3	<i>EfP</i> -I-1	6.18	25.8	28.8
4	<i>EfP</i> -I-2	1.38	31.6	28.1
5	<i>EfP</i> -II-1	1.82	8.8	30.6
6	<i>EfP</i> -II-2	7.40	2.1	29.1
7	<i>EfP</i> -III-1	6.55	12.5	34.8
8	<i>EfP</i> -III-2	4.41	2.3	35.0
<i>EfPs</i>	Mixture of isozymes ^d	4.40	100	–

^a The isozymes purified by the ionic exchange chromatography through DEAE-Cellulose-52 column with a NaCl gradient.

^b Mass ratio between the carbohydrate content and the glycoprotein.

^c Estimated on the fibrin-PAGE by Band-scanned software.

^d *EfPs* purified by the SBTI-Sepharose-4B affinity chromatography column.

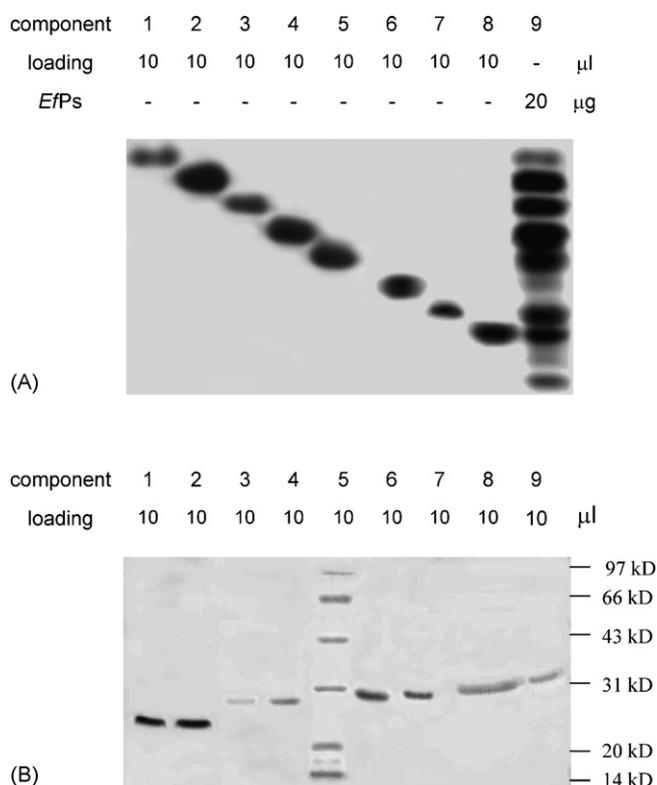


Fig. 3. Components purified by the DEAE-Cellulose-52 column chromatography. Eight components with fibrinolytic activities were separated and electrophoresed on 10% native-PAGE (panel A) and SDS-PAGE (panel B), respectively.

represented a mixture of two or three components except for peak I, which contained one component. Thus, peaks II, III and IV were further purified by the DEAE-Cellulose-52 chromatography until each component was separated. As shown on 10% native-PAGE (panel A, Fig. 3), the eight components with different fibrinolytic activity were clearly isolated. As shown in Table 1, their apparent molecular masses ranged from 22.5 to 34.8 kDa, as estimated by SDS-PAGE (panel B, Fig. 3). This observation suggests that the earthworm *E. fetida* contains at least eight fibrinolytic components acting as alkaline trypsin-like function.

To clarify whether the protein components were distinct from each other, we analyzed the N-terminal amino acid sequence of

each protease. The sequencing results indicated a unique peptide chain for each component (Table 2). Compared with the N-terminal amino acid sequences reported by Nakajima et al. [3], the eight isozymes could be classified into four groups according to their N-terminal amino acid sequences, molecular masses and mobilities on PAGE [34]. The eight isozymes of *E. fetida* were referred to as *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. Among them, *EfP*-0-2 and *EfP*-II-2 were newly isolated.

3.3. Glycosylation and earthworm proteases

As shown in Table 1, detection with the glycoprotein carbohydrate estimation kit showed that all of the proteases contained oligosaccharide. It was observable that different isozyme had different glycan contents. In particular, *EfP*-0-1, *EfP*-I-1, *EfP*-II-2 and *EfP*-III-1 had relatively high quantities of carbohydrates.

To confirm the glycosylation of the eight isozymes, we used the Schiff's reagent sodium meta-periodate to stain the isolated proteases on PAGE. Similar to the results from carbohydrate analysis (Table 1), the Schiff's reagent could stain all of the components isolated by the affinity chromatography (panel A, Fig. 4). The native *EfP*-III-2 as a sample was also stained under the same conditions. Glucoamylase used as control showed a positive result. No signals could be detected in the case of BSA and SOD, which were used as negative controls. These data suggest once again that the eight proteases isolated from *E. fetida* are glycosylated proteases.

As we know, proteins expressed in prokaryotic cells do not undergo glycosylation. Therefore, we cloned the cDNA of *EfP*-III-2 (GenBank, AF432224) and expressed it in *E. coli*. In the experiments, *EfP*-III-2 was expressed as inclusion bodies and no fibrinolytic activity could be detected, although the N-terminal amino acid residues were identified (panel B, Fig. 4). We used *EfP*-III-2 inclusion bodies to investigate whether the native *EfP*-III-2 was positive for glycosylation on staining. The inclusion body of *EfP*-III-2 did not show any staining signals in the presence of sodium meta-periodate (lane 2, panel A, Fig. 4), but the native isozyme and *EfPs* showed positive results by the glycoprotein staining (lane 3, panel A, Fig. 4). To investigate whether the deglycosylated protease had fibrinolytic activity, we cloned and expressed the other isozyme, *EfP*-I-1 (GenBank, DQ418454). Interestingly, *EfP*-I-1 showed some fibrinolytic activity after

Table 2
The N-terminal sequences of the isozymes

Number	N-terminal sequence of the component	Nomination	Nomination ^a	N-terminal sequences ^b
1	VVGGSDTTIGQYPHQIL	<i>EfP</i> -0-1	<i>LrP</i> -I-0 (F-I-0)	VVGGSDTTIGQYPHQLSLRVT
2	VVGGSDTTIGQ	<i>EfP</i> -0-2	-	-
3	IIGGSNASPGFPPWQL	<i>EfP</i> -I-1	<i>LrP</i> -I-1 (F-I-1)	IIGGSNASPGFPPWQLSQRTRG
4	IIGGSNASPGFPPWQL	<i>EfP</i> -I-2	<i>LrP</i> -I-2 (F-I-2)	IIGGSNASPGFPPWQLSQRTRG
5	VIGGTNASPGFPPQ	<i>EfP</i> -II-1	<i>LrP</i> -II (F-II)	VIGGTNASPGFPPWQLSQRX
6	VIGGTNASPGFPPQ	<i>EfP</i> -II-2	-	-
7	IVGGIEARPYEFP	<i>EfP</i> -III-1	<i>LrP</i> -III-1 (F-III-1)	IVGGIEARPYEFPWQVSVRRK
8	IVGXIEARPYEFPWQVSVRRK	<i>EfP</i> -III-2	<i>LrP</i> -III-2 (F-III-2)	IVGGIEARPYEFPWQVSVRRK

^a Nominated by Nakajima.

^b Sequences provided by Nakajima et al., see reference [3].

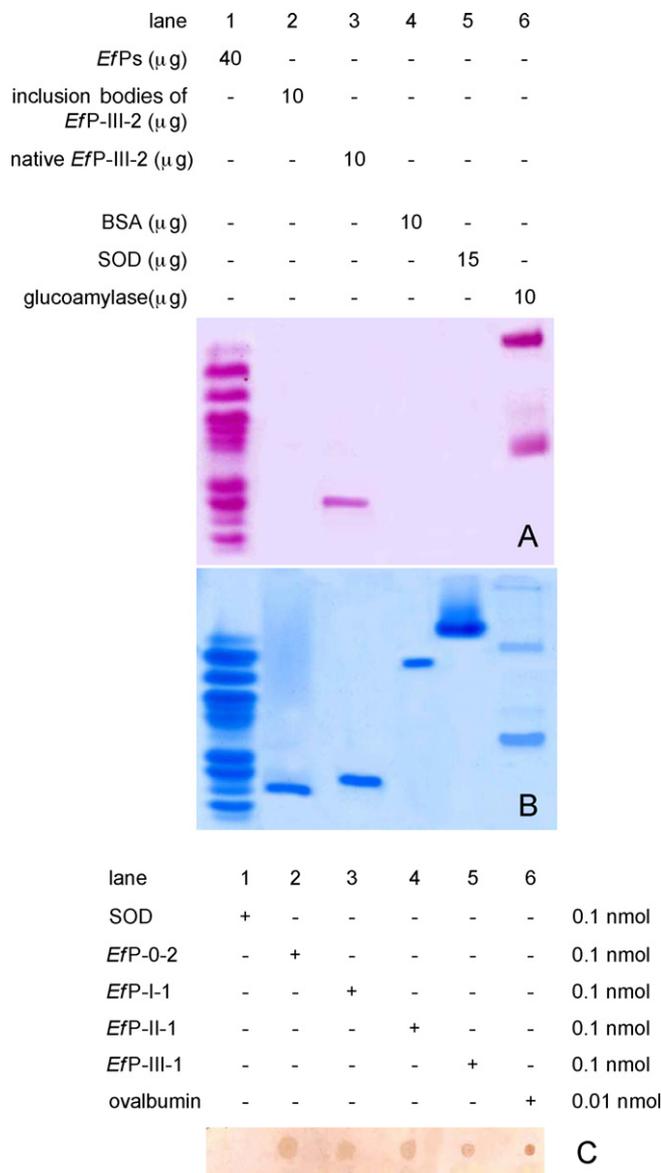


Fig. 4. Glycoprotein staining of earthworm proteases. The *EfPs* (final concentration 2.5 $\mu\text{g}/\mu\text{l}$) were electrophoresed on native-PAGE and then stained with 0.05 M sodium meta-periodate (panel A). BSA, bovine superoxide dismutase (SOD) and the inclusion body (InB) of *EfP*-III-2 produced by *E. coli* were used as negative controls, and glucoamylase as a positive control. The proteins stained with Commassie brilliant blue on the PAGE were also used as controls (panel B). The purified *EfP*-0-2, *EfP*-I-1, *EfP*-II-1 and *EfP*-III-1 were dotted onto PVDF membrane and reacted with HEP-ConA as indicated (panel C). SOD and ovalbumin were used as the negative and positive control, respectively.

expression and purification from *E. coli*. It appeared that the glycan chain was not directly related to the enzymic function under experimental conditions.

ConA, which has an affinity for mannose residues in glycoprotein, is commonly used to identify glycan chain containing mannose residues [35]. Assay of Dot-blotting with HRP-ConA showed that *EfP*-0-2, *EfP*-I-1, *EfP*-II-1 and *EfP*-III-1 contained some mannose residues. Ovalbumin composed of mannose glycan chain was strongly stained; however, SOD without glycan showed no signals under the same conditions (panel C, Fig. 4).

lane	1	2	3	4	
deglycosylation	3	7	-	-	h
deglycosylation <i>EfP</i> -I-1	10	10	-	-	μg
native <i>EfP</i> -I-1	-	-	15	-	μg
<i>EfPs</i>	-	-	-	20	μg

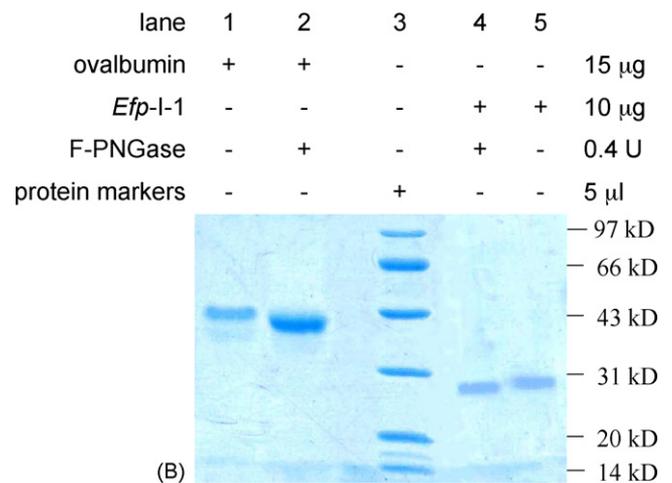
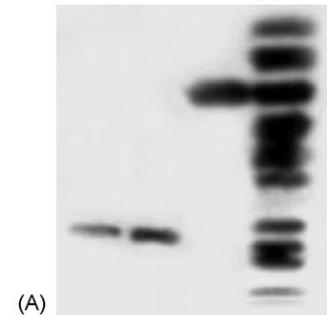


Fig. 5. Deglycosylation of the earthworm protease. *EfP*-I-1 was incubated with 2 μl of N-PNGase-F in the deglycosylation buffer at 37 $^{\circ}\text{C}$. In panel A, aliquots were taken at 3 h (lane 1) and 7 h (lane 2) for native-PAGE. Native glycosylated *EfP*-I-1 (lane 3) and *EfPs* (lane 4) were used as controls. In panel B, deglycosylated *EfP*-I-1 was subjected to SDS-PAGE (lane 4), the native *EfP*-I-1 (lane 5), native ovalbumin and deglycosylated ovalbumin were used as controls (lanes 1 and 2).

EfP-I-1 was deglycosylated by incubation with N-PNGase-F at 37 $^{\circ}\text{C}$. The mobility of native *EfP*-I-1 on native-PAGE was much slower than that of deglycosylated enzyme (panel A, Fig. 5). Nonetheless, the movement of deglycosylated enzyme was slightly faster on SDS-PAGE (panel B, Fig. 5), showing a subtle difference in speed between native and deglycosylated enzyme.

4. Discussion

Recently, several glycosylated serine proteases have been isolated from different species, such as lobster [36], *Agkistrodon acutus* [37], *Bothrops jararaca* [38] and gila [39]. The bound carbohydrates have some important effects on the structure and function of proteases: (1) Glycosylation stabilizes the spatial structure of a protease. The studies on the crystal structure of

EfP-b indicate the glycosylation of the difucosylated innermost GlcNAc may greatly enhance the rigidity of the glycan and the interactions of the glycan with neighboring protein regions [27], which is a prerequisite for the high stability of thermozymes [40]. The glycosylation of the heavy chain probably endows *EfP*-b with great stability and a high level of resistance to proteolysis [41]. A similar effect has also been demonstrated in *Bothrops* protease A, glycosylation of which has a stabilizing effect on the protein conformation [38]. (2) The oligosaccharides affect the regulation of protease function, for instance the *Agkistrodon actus* serine proteinases-I and -II whose N-linked oligosaccharides collide spatially with some inhibitors and hinder their inhibitory binding [37]. (3) Glycosylation is helpful for a protease to transport cell membrane. Some years ago, Fan et al. and Guo et al. observed that *EfP*-II could be absorbed through the intestinal epithelium when this isozyme was administered orally [13,42]. *EfP*-III-1 was also found to be transported through the cell membrane of several cell lines (HEK-293, HeLa and SY5Y-CH) as visualized by immunohistochemical methods (our unpublished data).

EfP-I-1 had a high fibrinolytic activity and high glycan content (Table 2) as mentioned above. We have cloned the cDNA of *EfP*-I-1, and the amino acid sequence deduced from its nucleotide sequence showed a conservative sequence of N-X-S/T, an N-glycan domain attaching with the asparagine residue. According to Wang et al., *EfP*-III-1 contains a light chain and a heavy chain. The heavy chain is glycosylated on the Asn-161 with an oligosaccharide composed of six monosaccharides [27]. At least one of these monosaccharides is mannose, as shown in dot-blotting assay with HRP-ConA.

So far, we have isolated eight kinds of glycosylated isozymes from *E. fetida*. Among them, *EfP*-0-1, *EfP*-I-1, *EfP*-I-2, *EfP*-II-1, *EfP*-III-1 and *EfP*-III-2 were similar to those reported by Nakajima et al. [3], as determined by comparison of their N-terminal amino acid sequences, molecular masses, enzymic activities and mobilities on PAGE. The latter technique demonstrated *EfP*-0-2 and *EfP*-II-2, two new members of the glycosylated isozymes with fibrinolytic activity.

*EfP*s were localized in the epithelium cells of the earthworm alimentary canal [20]. We homogenized the earthworm and precipitate the soluble proteins with ammonia sulfate, and used SBTI as a specific ligand (inhibitor) to select trypsin-like enzymes, that is, only the trypsin-like earthworm proteases in the ammonium sulfate precipitates could be seized by the affinity chromatographic column. Furthermore, there were more than eight protein bands on the native-PAGE after purification by affinity chromatography (panel A-2, Fig. 2). At least 12 distinct protein bands were observed on the gel, though only eight bands of the proteins showed the fibrinolytic activity on the F-PAGE (panel A-1, Fig. 2). As described in Section 2, we pooled the fractions according to its fibrinolytic activity (panel B, Fig. 2). We have ignored the components that did not hydrolyze fibrin but only some other proteins. Thus, those proteins that bind the SBTI-ligand but have no fibrinolytic activity could not be pooled during the elution. Moreover, we used SBTI as a ligand to capture only the proteases. Probably, some other proteases that could not recognize and associate with the ligand SBTI were washed

off, as SBTI-affinity column was washed with Tris-HCl buffer (pH 8.0). Consequently, only eight isozymes were isolated by the affinity chromatographic column and the following DEAE-Cellulose-52 column. Thus, it is reasonable to predict that the earthworm *E. fetida* has more than eight glycosylated trypsin-like proteases.

5. Conclusion

Experimentally, eight *E. fetida* fibrinolytic proteases were isolated and they were demonstrated as glycoproteins acting as alkaline trypsin-like function. Among the purified proteases, *EfP*-0-2 and *EfP*-II-2 were new isozymes components. However, *EfP*-0-1, *EfP*-0-2 and *EfP*-II-2 functioned in both trypsin-like and chymotrypsin-like enzymic manners. Measurements of the glycan contents with sodium meta-periodate and glycoprotein-test reagent showed that each glycosylated protease had different carbohydrate contents. Dot-blotting assay with ConA indicated the oligosaccharides were composed of mannose residues.

Acknowledgements

This project was supported by Natural Foundation of Sciences, Hebei (C2005000118), Key Subject of Bioengineering, Hebei, and 973-project (2006CB500703). We thank Dr Sarah Perrett for her editing the English language.

References

- [1] H. Mihara, H. Sumi, T. Yoneta, H. Mizumoto, R. Ikeda, M. Seiki, M. Maruyama, *Jpn. J. Physiol.* 41 (1991) 461–472.
- [2] Y.D. Park, J.W. Kim, B.G. Min, J.W. Seo, J.M. Jeong, *Biotechnol. Lett.* 20 (1998) 169–172.
- [3] N. Nakajima, H. Mihara, H. Sumi, *Biosci. Biotechnol. Biochem.* 57 (1993) 1726–1730.
- [4] B.D. Xing, S.M. Yin, B.G. Ru, *Biochim. Biophys. Acta* 29 (1997) 609–612.
- [5] N. Nakajima, M. Sugimoto, K. Ishihara, K. Nakamura, H. Hamada, *Biosci. Biotechnol. Biochem.* 63 (1999) 2031–2033.
- [6] H.I. Cho, E.S. Choi, H.G. Lim, H.H. Lee, *J. Biochem. Mol. Biol.* 37 (2004) 199–205.
- [7] Y.S. Kim, M.K. Pyo, K.M. Park, B.S. Hahn, K.Y. Yang, H.S. Yun-Choi, *Arch. Pharm. Res.* 21 (1998) 374–377.
- [8] T. Hrzenjak, M. Popovic, T. Bozic, M. Grdisa, D. Kobrehel, L. Tiska-Rudman, *Comp. Biochem. Physiol. B* 119 (1998) 825–832.
- [9] M. Grdisa, M. Popovic, T. Hrzenjakb, *Comp. Biochem. Physiol. A* 128 (2001) 821–825.
- [10] J. Zhao, J.G. Ji, Y.P. Tong, Q. Chen, H. Pu, B.G. Ru, *Biochem. Biophys. Acta* 34 (2002) 576–582.
- [11] N. Nakajima, M. Sugimoto, S. Tsuboi, H. Tsuji, K. Ishihara, *Biosci. Biotechnol. Biochem.* 69 (2005) 2009–2011.
- [12] N. Nakajima, M. Sugimoto, K. Ishihara, *J. Biosci. Bioeng.* 90 (2000) 174–179.
- [13] Q. Fan, C. Wu, L. Li, R. Fan, C. Wu, Q. Hou, R.Q. He, *Biochim. Biophys. Acta* 1526 (2001) 286–292.
- [14] C. Wu, L. Li, J. Zhao, Q. Fan, W.X. Tian, R.Q. He, *Int. J. Biol. Macromol.* 31 (2002) 71–77.
- [15] N. Nakajima, K. Ishihara, M. Sugimoto, H. Sumi, K. Mikuni, H. Hamada, *Biosci. Biotechnol. Biochem.* 60 (1996) 293–300.
- [16] J. Zhao, L. Li, C. Wu, R.Q. He, *Int. J. Biol. Macromol.* 32 (2003) 165–171.
- [17] G.H. Ryu, S. Park, M. Kim, D.K. Han, Y.H. Kim, B. Min, *J. Biomed. Mater. Res.* 28 (1994) 1069–1077.

- [18] M. Sugimoto, N. Nakajima, *Biosci. Biotechnol. Biochem.* 65 (2001) 1575–1580.
- [19] F. Wang, C. Wang, M. Li, L.L. Gui, J.P. Zhang, W.R. Chang, *Biotechnol. Lett.* 25 (2003) 1105–1109.
- [20] J. Zhao, R. Xiao, J. He, R. Pan, R. Fan, C. Wu, X. Liu, Y. Liu, R.Q. He, *Int. J. Biol. Macromol.* 40 (2007) 67–75.
- [21] Y. Tang, D. Liang, T. Jiang, J. Zhang, L.L. Gui, W.R. Chang, *J. Mol. Biol.* 321 (2002) 57–68.
- [22] C. Wang, F. Wang, M. Li, Y. Tang, J.P. Zhang, L.L. Gui, X.M. An, W.R. Chang, *Biochem. Biophys. Res. Commun.* 325 (2004) 877–882.
- [23] X.Y. Zhao, T.Y. Jing, *Chin. J. Biochem. Mol. Biol.* 14 (1998) 407–411.
- [24] T.M. Hrzenjak, M. Popvic, T. Bozic, M. Grdisa, D.J. Kobrehel, *Comp. Biochem. Physiol.* 119B (1998) 825–832.
- [25] L. Li, J. Zhao, R.Q. He, *Prot. Pept. Lett.* 10 (2003) 183–190.
- [26] J.B. Xie, Z.Q. Guo, N. Wang, H.T. Wang, G.Q. Jiang, B.G. Ru, *Prog. Biochem. Biophys.* 30 (2003) 453–459.
- [27] F. Wang, C. Wang, M. Li, J.P. Zhang, L.L. Gui, X.M. An, W.R. Chang, *J. Mol. Biol.* 348 (2005) 671–685.
- [28] S.F. Zhang, R.L. Hu, J. Zhao, M. Li, *Chin. J. Vet. Sci.* 22 (2002) 436–437.
- [29] J.X. Wu, X.Y. Zhao, *Chin. Pharm. J.* 40 (2005) 1656–1659.
- [30] O.H. Lowry, N.J. Rosebrough, A.L. Farr, *J. Biol. Chem.* 163 (1951) 265–275.
- [31] X.Q. Wu, C. Wu, R.Q. He, *Prot. Pept. Lett.* 9 (2002) 75–80.
- [32] X.Y. Zhao, T.Y. Jing, *Prog. Biochem. Biophys.* 28 (2001) 218–221.
- [33] C.K. Ching, J.M. Rhodes, *Gastroenterology* 95 (1988) 137.
- [34] X.Y. Zhao, T.Y. Jing, X.Y. Jiang, M.X. Duan, C.X. Zheng, *Chin. J. Biochem. Mol. Biol.* 18 (2002) 209–212.
- [35] W.C. Boyd, E.L. Shapleigh, *Science* 119 (1954) 419.
- [36] D. Johnston, J.M. Hermans, D. Yellowlees, *Arch. Biochem. Biophys.* 324 (1995) 35–40.
- [37] Z. Zhu, Z. Liang, T. Zhang, Z. Zhu, W. Xu, M. Teng, L. Niu, *J. Biol. Chem.* 280 (2005) 10524–10529.
- [38] N. Murayama, K. Saguchi, R. Mentele, M.T. Assakura, H. Ohi, Y. Fujita, A.C. Camargo, S. Higuchi, S.M. Serrano, *Biochim. Biophys. Acta* 1652 (2003) 1–6.
- [39] P. Utaisincharoen, S.P. Mackessy, R.A. Miller, A.T. Tu, *J. Biol. Chem.* 268 (1993) 21975–21983.
- [40] C. Vieille, G.J. Zeikus, *Microbiol. Mol. Biol. Rev.* 65 (2001) 1–43.
- [41] B. Imperiali, S.E. O'Connor, *Curr. Opin. Chem. Biol.* 3 (1999) 643–649.
- [42] H.F. Guo, Z.Z. He, L. Zhang, *Pharm. Biotechnol.* 9 (2001) 57–60.