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Purification, characterization and crystallization of a group of earthworm fibrinolytic enzymes from *Eisenia fetida*

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Abstract

Seven fibrinolytic enzymes were purified from the earthworm *Eisenia fetida*. The molecular weights of the enzymes were 24 663, 29 516, 29 690, 24 201, 24 170, 23 028 and 29 595, and the respective isoelectric points were 3.46, 3.5, 3.5, 3.68, 3.62, 3.94 and 3.46. All the proteases showed different fibrinolytic activity on fibrin plates. Studies on substrate specificity and inhibition indicated that they belonged to different types of serine proteases. *N*-Terminal sequencing indicated their high homology to those from the earthworm *Lumbricus rubellus.* All the enzymes have been crystallized.

Introduction

Earthworm fibrinolytic enzymes are a group of serine proteases, which have strong fibrinolytic and thrombolytic activities (Cong *et al.* 2000). Compared to the present thrombolytic drugs, earthworm fibrinolytic enzyme is cheap, can be easily stored, and can be administered orally (Mihara *et al.* 1991). As a new drug for thrombosis, it has been widely studied and used for clinical treatment. However, there are still some questions to be answered, such as how many components of fibrinolytic enzymes there are and whether the enzymes have any role as a plasminogen activator. There have been no other systematic reports on earthworm fibrinolytic enzymes other than the work of Mihara *et al.* (1991) and Nakajima *et al.* (1993) who used *Lumbricus rubellus*. There is also no evidence from the 3-dimensional structure of the enzymes to prove the feasibility of developing them into novel fibrinolytic drugs. Thus, we systematically purified different components of the enzyme and studied their biochemical properties. The determination of their 3-dimensional structures is currently in progress.

Methods

Isolation and purification

Using lyophilized crude powder of earthworm fibrinolytic enzymes (provided by the Earthworm Fibrinolytic Enzyme Research Group and Bai'ao Pharmacy Company, Institute of Biophysics, CAS), ATKA Purifier, FPLC, and relative pre-packing chromatography columns, seven fibrinolytic enzymes respectively named EFE-a, EFE-b, EFE-c, EFE-d, EFE-e, EFE-f and EFE-g, were purified to homogeneity following the protocol shown in Figure 1.

Enzyme concentration and fibrinolytic activity

The concentration of each enzyme solution was determined by the method of Lowry using BSA as a standard. Fibrinolytic activity of each solution was measured using both plasminogen-rich and plasminogen-free fibrin plates following the method of Asturp & Mullertz (1952) with urokinase as a standard.

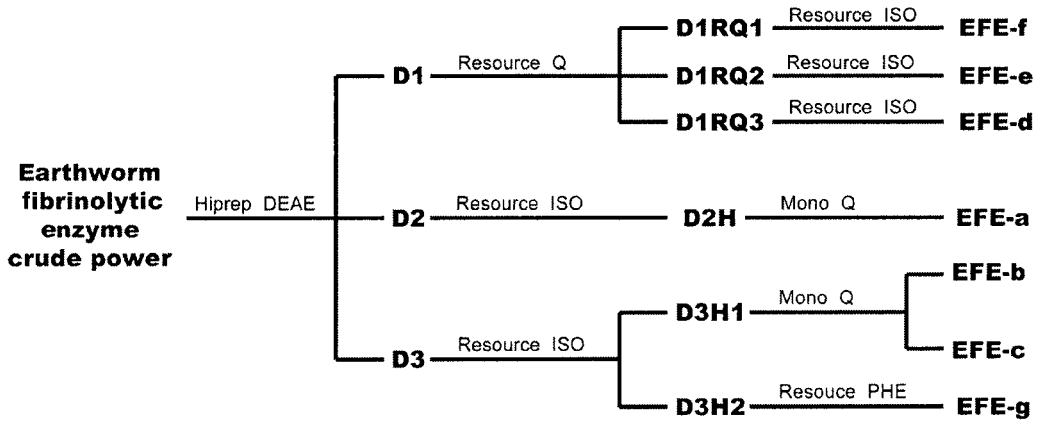

Fig. 1. Flow chart of the purification of seven earthworm fibrinolytic enzymes. The pre-packing chromatography columns used were purchased from Pharmacia Biotech.

Molecular weight measurement and peptide map analysis

Further purification and desalting of each enzyme was done by reversed phase chromatography. The molecular weight of each component was measured by MALDI-TOF. Trypsin-hydrolyzed peptide map analyses of EFE-b, EFE-c and EFE-g were also done.

N-*Terminal amino acid sequencing*

Proteins for *N*-terminal amino acid sequencing were separated by SDS-PAGE and electrotransferred to the PVDF membranes, and then sequenced with Procise Protein Sequencing System, AB.

Measurement of the isoelectric points

Isoelectric-focusing (IEF) was applied to measure the electric point (p*I*) of each enzyme, using Pharmalyte with a narrow pH range from 2.5–5.5 and an IEF Calibration Kit – Low p*I*Kit (pH 2.5–6.5) as markers.

Examination of substrates specificity

The substrates specificity of each component was examined by a series of chromogenic substrates following the method of Nakajima *et al.* (1993).

Inhibition studies

Enzyme solution with inhibitors was added to the fibrin plates, and at the same time enzyme solution without inhibitors was also added to the fibrin plates as control. The fibrinolytic activity was measured after an 18 h incubation period at 37 °C.

Crystallization

Crystallization trials on each enzyme were carried out using the hanging-drop vapour-diffusion method.

Results and discussion

Purification of the fibrinolytic enzymes

Following the purification protocol shown in Figure 1, 10.2 mg EFE-a, 7.3 mg EFE-b, 11.3 mg EFE-c, 14.2 mg EFE-d, 8.6 mg EFE-e, 5.2 mg EFE-f and 10.9 mg EFE-g could be obtained from 5 g crude enzyme powder, which were available for further characterization and crystallization. SDS-PAGE showed only one band for each enzyme (Figure 2).

Determination of fibrinolytic activity

With urokinase as a standard, the specific activity of each enzyme was determined, as shown in Table 1.

A comparison of all the enzymes showed that EFE-b, EFE-c and EFE-g had relatively higher fibrinolytic activity. EFE-d and EFE-e had average fibrinolytic activity, and EFE-a and EFE-f had relatively lower activity (Figure 3).

Comparison of the fibrinolytic activity between plasminogen-rich and plasminogen-free plates showed that EFE-a had higher activity on plasminogen-rich plates. In contrast, the other enzymes showed no difference. Thus, we assume that in addition to fibrinolytic activity, EFE-a also has plasminogen-activating activity, while the others only have fibrinolytic activity.

Table 1. Fibrinolytic activity of each enzyme on plasminogen-rich fibrin plates.

Enzyme	EFE-a	EFE-b	EFE-c	EFE-d	EFE-e	EFE-f	EFE-g
Specific activity (IU)	250	585	524	432	485	315	644

Fibrinolytic activity was determined using uorkinase as a standard.

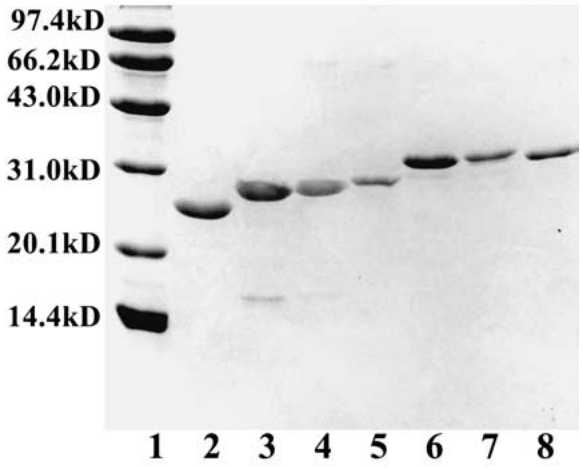

Fig. 2. SDS-PAGE (15%, w/v) of the seven final purified enzymes. (1) Makers, (2) EFE-f, (3) EFE-d, (4) EFE-e, (5) EFE-a, (6) EFE-b, (7) EFE-c and (8) EFE-g. Twenty-five μg for each enzyme was loaded.

Fig. 3. Fibrinolytic activity determination of seven components on plasminogen-rich fibrin plates. All enzymes were dissolved in mixed solution of 100 mmol l^{-1} sodium phosphate buffer, pH 7.7 and 0.9% NaCl (1:17 v/v). The final concentration of each enzyme was 0.2 mg ml^{-1}. Each sample volume was 10 μl and samples were incubated at 37 °C for 12 h. Urokinase (UK) was used as a standard and the concentrations of UK100, UK60, and UK20 were 100 IU ml^{-1}, 60 IU ml^{-1}, and 20 IU ml^{-1}, respectively.

Table 2. Molecular weight and *N*-terminal amino acid sequence of seven components.

Enzyme	Molecular weight	*N*-Terminal amino acid sequence
EFE-a	24663	VIGGTNASPGEFPWQL[Q]
EFE-b	29515	IVGGIEARPYEFP[P]QVSVR
EFE-c	29690	IVGGIEARPYEFP[P]QVSVR
EFE-g	29595	IVGGIEARPYEFP[P]QVSVR
EFE-d	24201	IIGGSNASPGEFPWQL
EFE-e	24170	IIGGSNASPGEFPWQL
EFE-f	23028	VVGGSDTT[K]GQYP

Residues labeled with a frame indicate the difference in *N*-terminal sequences compared with earthworm fibrinolytic enzyme components from *Lumbricus rubellus* (Nakajima *et al.* 1993).

Molecular weight measurement and N*-terminal sequencing*

Molecular weight and *N*-terminal sequencing of each component is shown in Table 2. The *N*-terminal sequences of EFE-b, EFE-c and EFE-g showed no difference and their molecular weights were similar, as were EFE-d and EFE-e.

Measurement of the isoelectric points

The respective isoelectric points of EFE-a, EFE-b, EFE-c, EFE-d, EFE-e, EFE-f, EFE-g were 3.46, 3.5, 3.50, 3.68, 3.62, 3.94, 3.46, which indicated that all the components were acid enzymes.

Substrates specificity and inhibition

The results shown in the Table 3 indicated that, EFE-b, EFE-c and EFE-g represent trypsin-like enzymes, and that EFE-d, EFE-e and EFE-f represent chymotrypsin-like enzymes. EFE-a could hardly hydrolyze a specific substrate for elastase (S4760, Sigma); only 4.1% activity against elastase was detected. Results showed 16.7% activity against elastase for the substrate for human leukocyte elastase (M4765, Sigma). Thus, EFE-a was not thought to be a trypsin-like enzyme or a chymotrypsin-like enzyme, nor an elastase. The optimum substrate is still unknown.

Table 3. Substrate specificity of purified enzymes (Unit: U mg^{-1}).

	S4760	M4765	S7388	I6886	*B7632	T6140	V6258	Chromozyme t-PA
EFE-a	7	8	22	4	3	12	14	32
EFE-b	0	1	4	1315	1865	2397	3588	7106
EFE-c	0	1	2	1336	1210	2388	3602	7576
EFE-g	0	1	0	942	820	812	1596	4778
EFE-d	0	5	164	24	6	24	42	30
EFE-e	0	4	159	12	4	13	31	10
EFE-f	0	2	2889	4	7	6	30	8
Elastase	171	48	–	6	–	–	–	–
Trypsin	–	–	–	465	572	1080	592	4401
Chymotrypsin	–	–	2171	15	–	–	–	31

The reaction system containing 0.25 mM substrate, 100 mM potassium phosphate buffer (pH 7.2) and 1 μg enzyme ml^{-1} was incubated at 37 °C. The absorbancy at 405 nm was recorded immediately. One activity unit (U) was defined as the amount of enzyme causing the increase of 0.1 absorbancy per min at 37 °C.
−: Not tested.
S4760: *N*-Succinyl-Ala-Ala-Ala-pNA (*p*-nitroanilide), standard substrate for elastase.
M4765: *N*-(Methoxysuccinyl)-Ala-Ala-Pro-Val-pNA, substrate for human leukocyte elastase.
S7388: *N*-Succinyl-Ala-Ala-Pro-Phe-pNA, substrate for chymotrypsin.
I6886: D-Ile-Phe-Lys-pNA, specific substrate for human plasmin.
*B7632: *N*-Benzoyl-Phe-Val-Arg-pNA hydrochloride hydrate, substrate for trysin, thrombin and reptilase (the activity might be low due to its poor solubility in buffer).
T6140: *N*-p-Tosyl-Gly-Pro-Lys-pNA acetate salt, substrate for plasmin.
V6258: D-Val-Leu-Arg-pNA.
Chromozym t-PA: *N*-Methylsulfonyl- D-Phe-Gly-Arg-pNA acetate, substrate for t-PA.
Elastase: E0258, used as a control.
Trypsin: T7409, used as a control.
Chymotrypsin: C3142, used as a control.
Chromozym t-PA was from Roche, Germany; the other reagents were from Sigma, USA.

A comparison of the activity of hydrolyzing specific substrates for human plasmin (I6886, Sigma) and t-PA (Chromozym t-PA, Roche) showed that EFE-b, EFE-c and EFE-g had strong hydrolytic activity, but some nuance still existed. There were more similarities between EFE-b and EFE-c, but EFE-g differed from them. However, EFE-a, EFE-d, EFE-e had very weak hydrolytic activity for the two substrates.

Studies on inhibition indicated that earthworm fibrinolytic enzymes were a group of serine proteases.

Comparison with earthworm fibrinolytic enzymes from Lumbricus rubellus

A comparison of the *N*-terminal amino acid sequence with earthworm fibrinolytic enzyme components isolated from *Lumbricus rubellus* by Mihara *et al.* (1991) showed that EFE-b, EFE-c and EFE-g were quite similar to F-III-1 or F-III-2; EFE-a was almost the same as F-II; EFE-d and EFE-e were quite similar to F-I-1 or F-I-2, and EFE-f was almost the same as F-I-0. Minute differences between them might result from different genera or errors during the sequencing.

However, differences must exist, as there were at least seven different components from *Eisenia fetida* but only six from *Lumbricus rubellus*. Their isoelectric points and hydrolytic activity against series of chromogenic substrate series also showed some difference. Trypsin-hydrolyzed peptide map analyses of EFE-b, EFE-c and EFE-g indicated that they were different from each other. Simulated trypsin-hydrolyzed peptide map analyses of F-III-1 and F-III-2 indicated their differences from EFE-b, EFE-c and EFE-g.

Crystallization

With purified enzymes, crystals were obtained, some of which could be used for X-ray diffraction, indicating that all final products were of high purity. Structure-determination work is currently in processing. Different crystallization behaviors of these components also proved that they were different from one another, which accorded with the results of peptide map analyses and specificity against chromogenic substrates.

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