

# Crystallization and preliminary crystallographic analysis of earthworm fibrinolytic enzyme component B from *Eisenia fetida*

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Earthworm fibrinolytic enzyme component B (EFE-b) is one of three high-fibrinolytic components found in *Eisenia fetida*. Several crystal forms were obtained by the hanging-drop vapour-diffusion technique. Diffraction data were collected from two well diffracting crystal forms. Crystal form I diffracted to a resolution of 2.25 Å but was merohedrally twinned, with space group  $P2_13$ , unit-cell parameters  $a = b = c = 122.9$  Å and two molecules per asymmetric unit. Crystal form II diffracted to beyond 2.06 Å resolution and belonged to space group  $P6_322$ , with unit-cell parameters  $a = b = 96.4$ ,  $c = 150.8$  Å and one molecule per asymmetric unit. Crystal form II was a true single crystal and is suitable for structure determination.

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## 1. Introduction

Earthworm fibrinolytic enzyme (EFE) is a multi-component enzyme with strong fibrinolytic and thrombolytic activities (Cong *et al.*, 2000). Because it is highly stable, inexpensive and can be administered orally (Mihara *et al.*, 1991), it has been widely studied and used in clinical treatment as a new drug for thrombosis.

Since the discovery of EFE in 1983, many EFE components from different sources have been reported (Lu & Jin, 1986; Mihara *et al.*, 1983; Zhou *et al.*, 1988). Six EFE components from *Lumbricus rubellus* (named F-I-0, F-I-1, F-I-2, F-II-1, F-II-2, F-III-1 and F-III-2) were isolated and characterized by Mihara and coworkers and it was thought that all these components were serine proteases composed of a single peptide chain (Mihara *et al.*, 1991; Nakajima *et al.*, 1993). Of the various components of EFE, F-III-1 and F-III-2 have been investigated intensively (Fan *et al.*, 2001; Nakajima *et al.*, 1996, 1999, 2000; Ryu *et al.*, 1993; Sugimoto & Nakajima, 2001; Wu *et al.*, 2002). It appeared that F-III-2 was non-haemorrhagic, could not induce platelet aggregation and retained potent proteolytic activity for fibrin and fibrinogen to an even greater degree than human plasmin (Nakajima *et al.*, 1996). Intact F-III-1 could be transported through the intestinal epithelium and its thrombolytic activity in blood could still be assayed (Fan *et al.*, 2001).

In order to obtain some structural evidence to determine the feasibility of developing EFE into a novel fibrinolytic drug, we systematically purified seven different components of EFE from the earthworm *Eisenia fetida*, studied their biochemical properties and began to determine their crystal structures (Wang *et al.*,

2003). The structure of earthworm fibrinolytic enzyme component A (EFE-a) determined in our laboratory is the first structure reported of an EFE component and revealed that EFE-a is a unique elastase (Tang *et al.*, 2000, 2002).

Earthworm fibrinolytic enzyme component B (EFE-b) is one of the three components with the highest fibrinolytic activities of the seven characterized (Wang *et al.*, 2003). It has a molecular weight of 29 515 Da (MALDI-TOF result), an isoelectric point of 3.50 and an N-terminal sequence IVGGIEARPYEFPPQ-VSVR. Studies of chromogenic substrates showed that EFE-b can strongly hydrolyze specific substrates for trypsin, human plasmin or t-PA, which indicated that it is a trypsin-like protease but is neither chymotrypsin-like nor elastase-like (Wang *et al.*, 2003). Evidence from molecular weight, isoelectronic point, N-terminal sequence, purification procedure and examination of the fibrin plate and chromogenic substrates all suggested that it was very similar to F-III-1 or F-III-2 isolated from *Lumbricus rubellus* (Mihara *et al.*, 1991; Nakajima *et al.*, 1993). However, no structure has yet been reported for EFE-b, F-III-1 or F-III-2.

A sequence alignment using the BLAST server (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/>) indicated that EFE-a, the structure of which has been determined previously, has 35% identity to F-III-1 or F-III-2; we therefore supposed that the structure of EFE-b would be very different from that of EFE-a, since it had been indicated that the biochemical properties of EFE-b were very similar to those of F-III-1 or F-III-2. We hope that the structure determination of EFE-b will provide further useful information for the elucidation of the thrombolytic mechanism of EFE in humans.

**Table 1**

Crystallization conditions and data-collection statistics for EFE-b crystals.

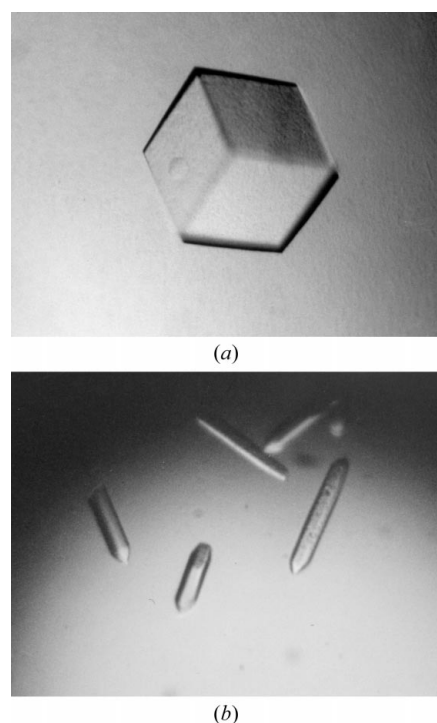
Values in parentheses refer to the outer resolution shell.

	Form I	Form II
Reservoir solution	0.1 M CH <sub>3</sub> COOH pH 4.0, 0.8 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	0.1 M MES pH 6.7, 1.6 M MgSO <sub>4</sub>
Growth temperature (K)	285	285
Cryocooling solution	0.1 M CH <sub>3</sub> COOH pH 4.0, 1.0 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 25% (v/v) glycerol	0.1 M MES pH 6.5, 1.5 M MgSO <sub>4</sub> , 15% (v/v) glycerol
Space group	<i>P</i> 2 <sub>1</sub> 3	<i>P</i> 6 <sub>3</sub> 22
Unit-cell parameters (Å)	<i>a</i> = 122.9, <i>b</i> = 122.9, <i>c</i> = 122.9	<i>a</i> = 96.4, <i>b</i> = 96.4, <i>c</i> = 150.8
Resolution range (Å)	50–2.25 (2.33–2.25)	100–2.06 (2.13–2.06)
All reflections	184357	380577
Unique reflections	28050	26291
Completeness	94.7 (93.4)	100 (100)
Mean <i>I</i> σ( <i>I</i> )	16.40 (3.16)	24.56 (6.48)
<i>R</i> <sub>merge</sub> † (%)	10.7 (49.3)	12.7 (49.2)

†  $R_{\text{merge}} = \sum_h \sum_i |I(h, i) - \langle I(h) \rangle| / \sum_h \sum_i I(h, i)$ , where  $I(h, i)$  is the intensity of the  $i$ th measurement of reflection  $h$  and  $\langle I(h) \rangle$  is the mean value of  $I(h, i)$  for all  $i$  measurements.

## 2. Materials and methods

The isolation and purification of EFE-b was carried out as described previously (Wang *et al.*, 2003). Crystals were grown by the hanging-drop vapour-diffusion method using a 0.8 ml reservoir and a drop typically consisting of 1 µl 10 mg ml<sup>-1</sup> protein solution in water and 1 µl reservoir solution. Initial crystallization conditions were identified using Hampton Research Crystal Screen and Crystal Screen 2 (Jancarik & Kim, 1991) at a temperature of about 288 K



**Figure 1**  
Photographs of EFE-b crystals. (a) Crystal form I, (b) crystal form II.

and were later refined to the conditions indicated in Table 1. Data were collected at 100 K on the synchrotron beamline of the Photon Factory (Tsukuba, Japan). The data were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

## 3. Results and discussion

Searching using Crystal Screen and Crystal Screen 2 from Hampton Research resulted in microcrystals belonging to more than four crystal forms. Two crystal forms that diffracted well were used in further data collection.

### 3.1. Crystal form I

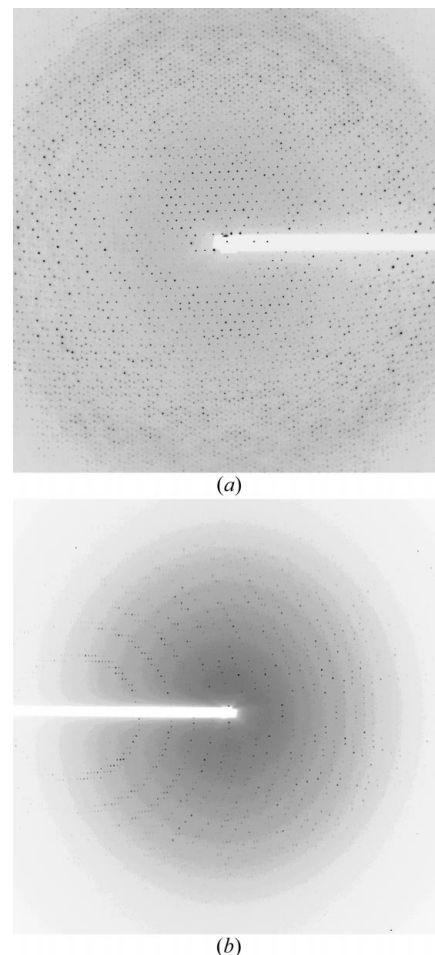
Initially, a precipitate skin was obtained from condition No. 47 of Hampton Research Crystal Screen (2.0 M ammonium sulfate, 0.1 M sodium acetate pH 4.6). When the concentration of ammonium sulfate was decreased to 1.6 M, microcrystals appeared. By decreasing the concentration of ammonium sulfate and changing the pH and temperature to the conditions shown in Table 1 (0.8 M ammonium sulfate pH 4.0, 285 K), cubic shaped crystals suitable for diffraction could be grown (Fig. 1).

A full data set was collected to a resolution of 2.25 Å (Table 1 and Fig. 2). The space group is *P*2<sub>1</sub>3, with two monomers per asymmetric unit. However, tests for merohedral twinning using both *CNS* (Brünger *et al.*, 1998) and *CCP4* (Collaborative Computational Project, Number 4, 1994) programs showed that crystal form I is merohedrally twinned with a twinning fraction of about 0.36.

### 3.2. Crystal form II

Initially, two crystal forms simultaneously occurred in one drop in condition No. 20 of Crystal Screen 2 from Hampton Research (1.6 M magnesium sulfate, 0.1 M MES pH 6.5). One had a regular octahedron shape and the other was a hexagonal prism shape. It was found that temperature had a great effect on the crystal form. When other conditions were the same, at a temperature of 277 K hexagonal prism-shaped crystals (crystal form II) were dominant, while at a temperature of 298 K regular octahedron-shaped crystals were dominant. Different temperatures and pH settings were tried. Finally, suitable crystals of crystal II (Fig. 1) could be harvested in 10–20 d using the conditions shown in Table 1 (pH 6.7, 285 K).

Data analysis indicated that small crystals with dimensions 0.08 × 0.08 × 0.25 mm diffracted well to resolutions beyond 2.06 Å (Fig. 2). The diffraction data was consistent with the hexagonal space group *P*6<sub>3</sub>22, with unit-cell parameters *a* = *b* = 96.4, *c* = 150.8 Å. Detailed statistics are shown in Table 1. No



**Figure 2**  
Diffraction patterns of the EFE-b crystals. (a) Crystal form I, (b) crystal form II.

twinning was detected in this crystal form. Analysis using the method of Matthews (1968) revealed that there is one molecule per asymmetric unit, with a  $V_M$  of  $3.4 \text{ \AA}^3 \text{ Da}^{-1}$ , corresponding to a solvent content of 63.8%. This crystal form is suitable for structure determination and the structure determination is in progress.

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