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

Feng Wang, Chao Wang, Mei Li, Lulu Gui, Jiping Zhang and Wenrui Chang*

National Laboratory of Biomacromolecules, Institute of Biophysics, Chinese Academy of Sciences, People's Republic of China

Correspondence e-mail: wrchang@sun5.ibp.ac.cn

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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.

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_3COOH pH 4.0, 0.8 *M* $(NH_4)_2SO_4$	0.1 *M* MES pH 6.7, 1.6 *M* $MgSO_4$
Growth temperature (K)	285	285
Cryocooling solution	0.1 *M* CH_3COOH pH 4.0, 1.0 *M* $(NH_4)_2SO_4$, 25%(*v*/*v*) glycerol	0.1 *M* MES pH 6.5, 1.5 *M* $MgSO_4$, 15%(*v*/*v*) glycerol
Space group	$P2_13$	$P6_322$
Unit-cell parameters (Å)	*a* = 122.9, *b* = 122.9, *c* = 122.9	*a* = 96.4, *b* = 96.4, *c* = 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 $I/\sigma(I)$	16.40 (3.16)	24.56 (6.48)
R_{merge}† (%)	10.7 (49.3)	12.7 (49.2)

† $R_{merge} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_i I(h,i)$, where $I(h,i)$ is the intensity of the ith 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^{-1} 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 and were later refined to the conditions indicated in Table 1. Data were colleted 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).

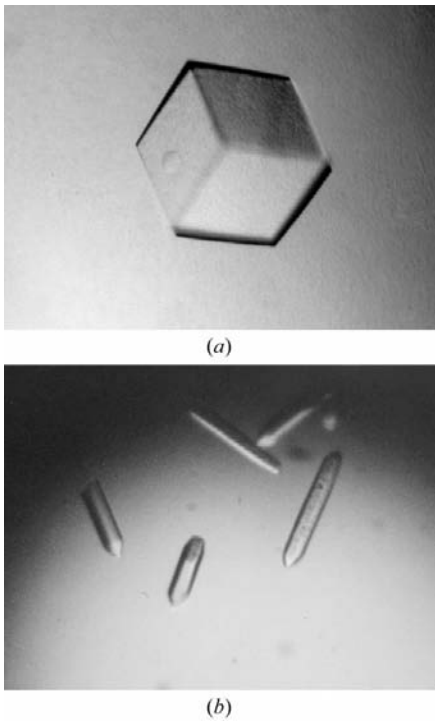

Figure 1
Photographs of EFE-b crystals. (*a*) Crystal form I, (*b*) crystal form II.

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 $P2_13$, with two monomers per asymmetric unit. However, tests for merohedral twinning using both *CNS* (Brünger *et al.*, 1998) and *CCP*4 (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 $P6_322$, with unit-cell parameters $a = b = 96.4$, $c = 150.8$ Å. Detailed statistics are shown in Table 1. No

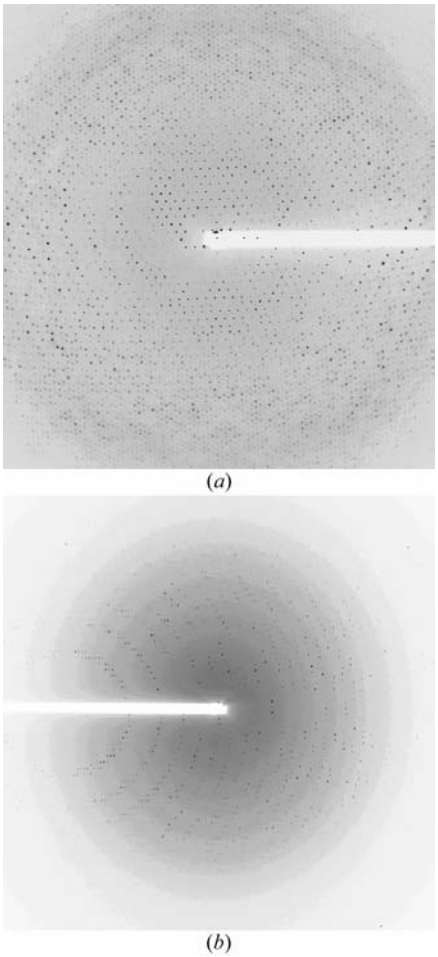

Figure 2
Diffraction patterns of the EFE-b crystals. (*a*) Crystal form I, (*b*) crystal form II.

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 Å^3 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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