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Crystallization and preliminary X-ray analysis of earthworm fibrinolytic enzyme component A from *Eisenia fetida*

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Earthworm fibrinolytic enzyme component A, a protein which functions both as a direct fibrinolytic enzyme and a plasminogen activator, was purified from the earthworm *Eisenia fetida*. Diffraction-quality single crystals of the protein were grown by the hanging-drop vapour-diffusion technique with ammonium sulfate as a precipitant. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 40.6$, $b = 127.5$, $c = 129.2$ Å and three molecules per asymmetric unit. The data set reached a resolution of 1.95 Å.

1. Introduction

Thrombosis is one of the most widely occurring diseases in modern life and often results in disability and death. Fibrinolytic enzymes degrade fibrin, the major protein component of blood clots. Medications using fibrinolytic enzymes are the most effective methods being used in the treatment of thrombosis. A variety of fibrinolytic enzymes, such as urokinase, streptokinase, recombinant tissue-type plasminogen activator, staphylokinase and recombinant prourokinase, have been widely studied and used as thrombolytic agents (for a review, see Lijnen & Collen, 1995). However, these agents, which are applied *via* intravenous administration, are expensive and suffer from a number of significant limitations, including fast clearance (Collen *et al.*, 1984), lack of resistance to reocclusion (Lijnen & Collen, 1995) and bleeding complications (Ferres, 1987; Hollander, 1987; Lijnen & Collen, 1995). Owing to the limitations of existing thrombolytic agents, a novel thrombolytic agent having better fibrinolytic efficacy and fewer adverse side effects is widely sought after.

Earthworms have been used as fibrinolytic medicine in East Asia for several thousand years. Therefore, earthworms were investigated to determine the active components used in the traditional prescription as the starting material for developing a new fibrinolytic agent. Earthworm fibrinolytic enzyme (EFE, also known as lumbrokinase; Mihara *et al.*, 1991) is a multi-component fibrinolytic enzyme purified from some earthworm breeds. It was first characterized in 1983 (Mihara *et al.*, 1983). Further studies on the EFE components were reported by the Mihara group (Mihara *et al.*, 1991; Nakajima *et al.*, 1993) and by several groups in China (Lu & Jin, 1986; Lu *et al.*, 1988; Zhou *et al.*, 1988; Li & Zeng, 1993; Yang & Ru, 1997) and in other countries (Park *et al.*, 1998; Hrzenjak *et al.*, 1998). EFE was demonstrated to have significant fibrinolysis efficacy *in vitro* and very low toxic and side effects on experimental animals (Mihara *et al.*, 1983; Lu & Jin, 1986) before clinical trials were undertaken on human subjects. In Korea and China, EFE has been commercially available as a novel orally administered fibrinolytic agent for the prevention and treatment of cardiac and cerebrovascular diseases (Daedo Pharmaceutical Co. Ltd, 1990; Hou, 1995). EFE is stable for long-term storage at room temperature. Oral administration of EFE is also very convenient. Earthworms, the starting material of EFE, can be easily raised, making EFE a relatively inexpensive thrombolytic agent applicable for mass production.

The EFE purified from the earthworm *E. fetida* has been characterized as comprising more than three individual fibrinolytic enzyme components. In this paper, we report the major therapeutically important component, which was identified as earthworm fibrinolytic enzyme component A (EFE component A). It is known that this enzyme belongs to the trypsin-like serine protease family. *In vitro* experiments have shown that this component not only has strong direct fibrinolytic activity, but also functions as a plasminogen activator by converting plasminogen into plasmin, which in turn dissolves fibrin. Therefore, it is a thrombolytic agent having double fibrinolytic activities.

The X-ray crystal structures of the proteins or protein–substrate complexes of several fibrinolytic enzymes have been solved, including tissue-type plasminogen activator (Lambda *et al.*, 1996; Renatus *et al.*, 1997), urokinase (Spraggon *et al.*, 1995), staphylokinase (Rabijns *et al.*, 1997; Parry *et al.*, 1998) and streptokinase (Wang *et al.*, 1998).

Although the EFE component A has been used in thrombolysis therapy, the molecular mechanism of its double fibrinolytic activities still remains unknown. We expect that the crystal structure analysis of this protein will provide important structural information for the understanding of its biological function, especially its double fibrinolytic action, and will also provide a possible model for its structure-based drug modification.

2. Materials and methods

2.1. Preparation and purification of EFE component A

The isolation and purification of EFE component A was carried out as described by Zhou *et al.* (1997). Crude protein was harvested from the homogenate of fresh *E. fetida* by ammonium sulfate precipitation. The target protein was obtained after subjecting the crude protein to successive DEAE–cellulose ion-exchange chromatography, Superdex G-75 gel filtration and Mono-Q ion-exchange chromatography. It is a single polypeptide, having a molecular weight of 24 667 Da (mass spectroscopy result) and an isoelectric point of 3.2. The purified enzyme, which can be directly used for crystallization, was highly active when reacted with its specific substrate (Zhou *et al.*, 1997).

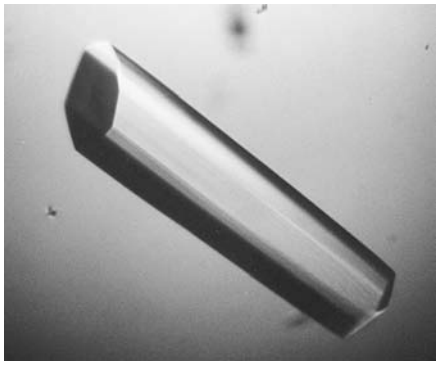

Figure 1
Photograph of EFE component A crystal.

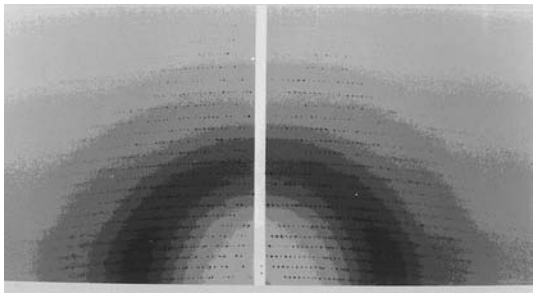

Figure 2
Diffraction pattern of the EFE component A crystal. One of the half frames, with dimensions of 780 × 390 mm, is shown. The crystal-to-detector distance was 572 mm. Reflections at high resolution (about 1.7 Å) were observed at the edge of the frame.

2.2. Crystallization

Initial crystallization trials on the EFE component A were carried out using the hanging-drop vapour-diffusion method following the sparse-matrix approach (Jancarik & Kim, 1991). Microcrystals were obtained in a system containing 1.0 *M* $(NH_4)_2SO_4$ as precipitant, 2.5% PEG 400 as additive and 0.05 *M* HEPES buffer pH 7.2 at room temperature. After extensive optimization of the crystallization conditions, the temperature was adjusted to 288 K and the initial drop solution was adjusted to contain 5 mg ml^{-1} protein, 0.6 *M* $(NH_4)_2SO_4$, 2.5%(*v*/*v*) PEG 400 and 0.05 *M* MOPS buffer pH 7.2, with the reservoir solution containing 2.0 *M* $(NH_4)_2SO_4$, 5.0%(*v*/*v*) PEG 400 and 0.10 *M* MOPS buffer pH 7.2. Diffraction-quality single crystals were readily obtained in 10 d, with maximum dimensions reaching 1.0 × 0.2 × 0.2 mm. A typical crystal is shown in Fig. 1.

2.3. Data collection

Diffraction data were collected at room temperature with a screenless Weissenberg camera (Sakabe, 1991) installed on beamline BL6B (wavelength 1.000 Å) at the Photon Factory (Tsukuba, Japan). The whole data set included 15 IP frames, with a 13° oscillation angle per frame and a 0.5° overlap. Thus, a data set of 187.5° was obtained. Data were processed and scaled using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

Data analysis indicated that the crystal diffracted beyond 1.95 Å, while weak reflections were observed at higher resolution (Fig. 2). The crystal did not seem to decay significantly over the period of time required for data collection. The diffraction data was consistent with the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 40.6, b = 127.5, c = 129.2 Å. Other statistics are shown in Table 1.

Analysis using the method of Matthews (1968) revealed that there are three protein molecules per asymmetric unit, with a V_M of 2.24 Å^3 Da^{-1}, corresponding to a solvent content of 45.0%.

Table 1
Data-collection statistics for EFE component A.

Values in parentheses refer to the outer resolution shell.

Total No. of observations	389782
No. of unique reflections	50208
Multiplicity	7.8
Resolution range (overall) (Å)	20.0–1.95
Resolution range (outer shell) (Å)	2.00–1.95
Completeness (%)	95.1 (88.8)
$I/\sigma(I)$	28.7 (7.8)
$R_{\rm merge}$† (%)	10.2 (42.3)

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

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