Crystallization and preliminary X-ray crystallographic analysis of a putative feruloyl esterase from *Talaromyces cellulolyticus*

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Feruloyl esterase (FAE; EC 3.1.1.73) catalyzes the cleavage of the ester bond between ferulic acid and polysaccharides in plant cell walls, and thus holds significant potential for the industrial utilization of biomass saccharification. A feruloyl esterase was identified from the genome database of *Talaromyces cellulolyticus* (formerly known as *Acremonium cellulolyticus*). The gene consists of the catalytic domain and a carbohydrate-binding module connected through a serine/threonine-rich linker region. The recombinant enzyme was prepared, purified and crystallized at 293 K using 0.1 M imidazole pH 8.0, 0.2 M calcium acetate, 14% PEG 8000 as the precipitant. The crystal diffracted to 2.6 Å resolution and the crystal system is primitive orthorhombic, with unit-cell parameters $a = 90.9$, $b = 123.4$, $c = 135.4$ Å. Four molecules are assumed to be present per asymmetric unit, corresponding to a Matthews coefficient of $2.50 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 50.88%(v/v).

1. Introduction

Recent research into the production of biofuels, chemicals and composite materials from lignocellulosic biomass has accelerated the development of cellulase-related enzymes for efficient biomass saccharification (van den Brink & de Vries, 2011). Cellulase plays a key role in the degradation of cellulose and hemicellulose, making it one of the most important industrial enzymes for biomass saccharification. Ferulic acid esterase (FAE; also called feruloyl esterase or cinnamoyl esterase; EC 3.1.1.73; <http://www.cazy.org/Carbohydrate-Esterases.html>; CE family 1) cleaves the ester bond between ferulic acid, one of the components of lignin, and hemicellulose in plant cell walls (Wong, 2006). FAE therefore holds significant potential not only as an accessory enzyme for efficient biomass saccharification but also as an application tool (Abokitse *et al.*, 2010; Damásio *et al.*, 2013). Kroon and coworkers were the first to report a fungal feruloyl esterase (FAE) containing a carbohydrate-binding module (CBM), which was isolated from *Penicillium funiculosum* (also called *Talaromyces funiculosus*; Kroon *et al.*, 2000), and they characterized the enzyme at both the gene and protein levels. Several additional studies of FAEs have been reported (Donaghy & McKay, 1997; de Vries & Visser, 1999; Esteban-Torres *et al.*, 2013). Little structural information on fungal FAEs containing a CBM is available, and there are no crystallographic data showing the structural arrangement of the FAE and the CBM. Filamentous fungi produce many different enzymes for degrading lignocellulosic biomass. *T. cellulolyticus* (formerly known as *Acremonium cellulolyticus*) is a high cellulolytic enzyme-producing fungus that was first isolated by Yamanobe *et al.* (1987). We found an FAE homologue gene by searching the draft genome sequence of *T. cellulolyticus* (unpublished data). Here, we describe the cloning, expression and crystallization of recombinant FAE. In the future, we will obtain the X-ray crystallographic structure of the enzyme in order to elucidate the function and mechanism of action of FAE.

Table 1

Macromolecule-production information.

Source organism	<i>T. cellulolyticus</i>
DNA source	<i>T. cellulolyticus</i> CF-2612
Forward primer	ATGGCGATTCCCTGGTCTTGTCT
Reverse primer	AATCCTGCAGGTACAGACTGGGAGTAA
Cloning vector	pUC19
Expression vector	pANC
Expression host	<i>T. cellulolyticus</i> YP-4
Complete amino-acid sequence of the construct produced	MAIPLVLVLAWLLPAVLAASLTQVNNFGDNPGSLQMYIYVPN-TLASKPAVIVAMHPCGGSATEYYGMYDHPADQYGYILI-YPSATRDYNCFDAYSSSLTHNGGSDSLIVNMVKYVIST-YGADSSKVVMTGSSSGAIMTNVLGAYPDVFAAGSAFSGM-PYACLYGAGAADPIMSNQTCSSQGIQHTGQQWAAVYVHNGY-PGYTGRYPRLQMWHTADNVISYADLGQEISQWTTVMGLS-FTGNQNTNPLSGYTKMVGDSQFQAYSAGVGHFVPTDV-SVLDWFGITSGTTTTTTSKTTSTTTSSAPSTGGCT-AAHWAQCGGIGYTGCTACVSPYTCQKSNDDYYSQCL

Table 2

Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	10
Buffer composition of protein solution	20 mM Tris-HCl pH 8.0, 50 mM NaCl
Composition of reservoir solution	14% PEG 8000, 0.1 M imidazole pH 8.0, 0.2 M calcium acetate
Volume and ratio of drop	4 µl (1:1)
Volume of reservoir (ml)	1

2. Materials and methods

2.1. Macromolecule production

The gene encoding FAE was amplified by PCR from *T. cellulolyticus* CF-2612 genomic DNA using the primers indicated in Table 1. The amplified *fae* gene was constructed by introducing the appropriate fragment digested with *HpaI/SbfI* (underlined in Table 1) into the *EcoRV/SbfI* sites of pANC expression vector containing a glucoamylase (*glaA*) promoter and terminator (Inoue *et al.*, 2013). Protoplasts of *T. cellulolyticus* YP-4 were transformed with pANC209 (named Y209) by nonhomologous integration into the host chromosomal DNA (Fujii *et al.*, 2012).

T. cellulolyticus Y209 was grown at 30°C in starch-induced medium (Inoue *et al.*, 2013). The supernatant was collected and dialyzed against 20 mM Tris-HCl pH 8.0, 50 mM NaCl buffer and was then applied onto a HiTrap Q HP column (GE Healthcare, Little Chalfont, England) equilibrated with the same buffer. The recombinant protein was eluted using an increasing NaCl gradient. Fractions containing the protein were pooled and dialyzed against 20 mM Tris pH 8.0, 2 M NaCl and applied onto a HiTrap Butyl HP column (GE Healthcare) equilibrated with the same buffer. The FAE protein was eluted using a decreasing NaCl gradient. Finally, the eluted protein was applied onto a Superdex 200 16/60 gel-filtration column (GE Healthcare) equilibrated with 20 mM Tris-HCl pH 8.0, 50 mM NaCl. The purity of the protein was confirmed by SDS-PAGE. The protein concentration was determined using a Pierce BCA Protein Assay Kit (Pierce, Rockford, Illinois, USA) by measuring the absorption at 570 nm.

2.2. Crystallization

The purified protein was dialyzed against 20 mM Tris-HCl pH 8.0, 50 mM NaCl and then concentrated to 10 mg ml⁻¹ using an Amicon Centricon YM-10 (Millipore, Billerica, Massachusetts, USA). Initial crystallization screening was performed manually using Wizard Classic 1 and 2 (Emerald Bio, Bainbridge Island, Washington, USA) by the sitting-drop vapour-diffusion method at 293 K. Each drop comprised 0.5 µl protein solution and 0.5 µl reservoir solution and

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	BL44XU, SPring-8
Wavelength (Å)	0.9
Temperature (K)	100
Detector	Rayonix MX225-HE
Crystal-to-detector distance (mm)	300
Rotation range per image (°)	0.8
Total rotation range (°)	380
Exposure time per image (s)	1.0
Space group	<i>P</i> 2 ₁ 2 ₁ 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	90.9, 123.4, 135.4
α , β , γ (°)	90, 90, 90
Mosaicity (°)	0.3
Resolution range (Å)	50.0–2.6 (2.64–2.60)
Total No. of reflections	159905
No. of unique reflections	41285
Completeness (%)	86.8 (78.2)
Multiplicity	3.9 (2.4)
$\langle I/\sigma(I) \rangle$	5.3 (2.6)
$R_{\text{p.i.m.}}$ (%)	6.8 (20.5)

$\dagger R_{\text{p.i.m.}} = \sum_{hkl} \{1/[N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $N(hkl)$ is the mean redundancy.

was equilibrated against 60 µl reservoir solution. After one week, small crystals were obtained using several conditions, of which No. 46 from Wizard Classic 1 and 2 (10% PEG 8000, 0.1 M imidazole pH 8.0, 0.2 M calcium acetate) was the most promising. The best crystals were obtained with 14% PEG 8000, 0.1 M imidazole pH 8.0, 0.2 M calcium acetate. Detailed information on crystallization is given in Table 2.

2.3. Data collection and processing

Selected crystals were harvested and immersed in cryoprotectant solution consisting of 30%(v/v) glycerol in the mother liquor. The soaked crystal was immediately flash-cooled under a stream of nitrogen gas at 100 K. X-ray diffraction data for the protein crystals were collected on beamline BL44XU at SPring-8, Hyogo, Japan. The data collected from diffraction measurements were merged, indexed, integrated and scaled using programs from the *HKL-2000* software package (Otwinowski & Minor, 1997). Detailed information on data collection and processing is given in Table 3.

3. Results and discussion

A putative feruloyl esterase (TcFAE) gene was found from a draft genome sequence of *T. cellulolyticus* (unpublished data). Sequence alignment identified a catalytic domain containing a typical serine

Figure 1

A photograph of the TcFAE crystals. The length of the largest crystal is 0.8 mm.

esterase motif (SGNH fold) and a carbohydrate-binding module (CBM-1) (Kroon *et al.*, 2000) in the N-terminal and C-terminal domains, respectively. In this study, we crystallized TcFAE in order to characterize the structure and function of this enzyme in future studies.

Needle-like crystals of approximately $0.8 \times 0.1 \times 0.2$ mm grew over a two-week period (Fig. 1) under the crystallization condition described in Table 2. Diffraction data were collected to a resolution limit of 2.60 Å. Data processing revealed that the crystal system was primitive orthorhombic. Furthermore, the high systematic absence value (0.992) estimated by *POINTLESS* (Evans, 2006) from *CCP4* (Winn *et al.*, 2011) suggested that the crystal belonged to space group $P2_12_12_1$, with unit-cell parameters $a = 90.9$, $b = 123.4$, $c = 135.4$ Å. These data give a V_M value of $2.50 \text{ Å}^3 \text{ Da}^{-1}$ (50.8% solvent content) for four 38 kDa molecules in the asymmetric unit (Matthews, 1968). The cause of the low completeness (86.8% overall) seems to be radiation damage during data collection and/or the blind region (Drenth, 1999) arising from accidental coincidence of the crystal c axis and the direction of the oscillation axis. The data-collection statistics are summarized in Table 3. Structural analysis of TcFAE is in progress and details of its structure and function will be reported in the near future.

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