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Crystallization and preliminary X-ray crystallographic study on a xyloglucan-specific exo-β-glycosidase, oligoxyloglucan reducing-end specific cellobiohydrolase

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A novel xyloglucan-specific exo-β-glycosidase, oligoxyloglucan reducing-end specific cellobiohydrolase (OXG-RCBH), recognizes the reducing end of oligoxyloglucan and releases two glucosyl residue segments from the main chain. OXG-RCBH was crystallized by the hanging-drop vapour-diffusion method with polyethylene glycol 3000 and polyethylene glycol 400 as precipitants. The crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 61.0$, $b = 146.9$, $c = 211.9$ Å. The crystals diffract to a resolution of 2.2 Å and are suitable for X-ray structure analysis.

1. Introduction

Xyloglucan is a major hemicellulose polysaccharide found in plant cell walls. It consists of a cellulose-like backbone chain of β-1,4-glucan with xylosyl side chains attached at the 6-*O* position of the glucosyl residues and associates with cellulose microfibrils by hydrogen bonding, forming a cellulose–xyloglucan network (Hayashi, 1989; Carpita & Gibeaut, 1993; Hayashi *et al.*, 1994). Disassembly of the cellulose–xyloglucan network is required for cell expansion and development. Xyloglucan metabolism is thought to play an important role in cell definition, cell expansion and the regulation of plant growth and development. In the growing plant cell wall, xyloglucan oligosaccharides may provide positive- or negative-feedback control during cell elongation (Takeda *et al.*, 2002). Consequently, compositional analysis of oligosaccharide units in xyloglucan polymers is very important.

Glycosidases are useful tools for analyzing the fine structures of complex carbohydrates and screening and characterization of novel glycosidases is important for ensuring progress in glycotechnology. We previously isolated a novel xyloglucan-specific exo-β-glycosidase, oligoxyloglucan reducing-end specific cellobiohydrolase (OXG-RCBH; EC 3.2.1.–), with a molecular mass of 97 kDa, from *Geotrichum* sp. M128 (Yaoi & Mitsuishi, 2002). It recognizes the reducing end of oligoxyloglucan and releases two glucosyl residue segments from the main chain. This activity is very different from that of known glycosidases. OXG-RCBH should prove to be a powerful tool for identifying the fine structure of oligoxyloglucan and is a suitable target enzyme for close three-dimensional structural examination.

The cDNA encoding OXG-RCBH was cloned and sequenced and was found to have a 2436 bp open reading frame encoding an 812-amino-acid protein including 23 amino acids of a signal peptide. The molecular weight deduced from the amino-acid sequence is 85 kDa, which differs from the molecular weight of the native enzyme (97 kDa) estimated by SDS–PAGE, suggesting the presence of a carbohydrate or other post-translational modification. Based on the homology of amino-acid sequences, OXG-RCBH belongs to glycoside hydrolase family 74. 11 enzymes, including OXG-RCBH, have been shown to belong to this family (http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html), but no three-dimensional structures of enzymes in this family have been determined. Elucidation of the three-dimensional structure of OXG-RCBH will shed light on the molecular mechanism underlying the unique substrate specificity of this enzyme and will also provide information on the folding motif possibly shared between the enzymes of this family. In this paper, we report the expression, purification and crystallization of OXG-RCBH. The crystals obtained allowed us to collect X-ray diffraction data to a resolution of 2.2 Å.

2. Materials and methods

2.1. Expression and purification

The mature region of OXG-RCBH was expressed in *Escherichia coli* cells using a previously described method (Yaoi & Mitsuishi, 2002). The cDNA fragment encoding the mature region of OXG-RCBH (2367 bp, encoding a 789-amino-acid sequence) was cloned into the pET-29a(+) expression vector (Novagen) and transfected into *E. coli* BL21-CodonPlus(DE3) RP cells (Stratagene). The transfected cells were cultured and expression was induced by treatment with 1 m*M* IPTG for 6 h at 310 K. As

described previously (Yaoi & Mitsuishi, 2002), despite the recombinant OXG-RCBH being expressed as an inclusion body, enzymatically active recombinant OXG-RCBH was obtained upon denaturation with 8 *M* urea followed by dialysis. It was then purified to homogeneity by liquid chromatography. It was first loaded onto a POROS 50 HQ anion-exchange perfusion chromatography column (Boehringer Mannheim) equilibrated with 25 m*M* imidazole–HCl buffer pH 7.4. Elution was performed with a linear gradient of NaCl (0–0.5 *M*) in 25 m*M* imidazole–HCl buffer pH 7.4. Fractions containing OXG-RCBH were pooled, dialyzed against 25 m*M* imidazole–HCl buffer pH 7.4 and then loaded on a POROS 50 DEAE anion-exchange perfusion chromatography column (Boehringer Mannheim) equilibrated with 25 m*M* imidazole–HCl buffer pH 7.4 and then eluted with a linear gradient of NaCl (0–0.5 *M*). Finally, OXG-RCBH was gel-filtrated using a HiLoad 26/60 Superdex 200 pg column (Amersham Biosciences) in 50 m*M* sodium acetate pH 4.5 containing 0.5 *M* NaCl. The enzymatic activity and pH and temperature stability of recombinant OXG-RCBH were assayed and confirmed to be very close to those of native OXG-RCBH (Yaoi & Mitsuishi, 2002). Prior to crystallization, purified OXG-RCBH was ultra-filtrated with 50 m*M* sodium acetate pH 4.5 for buffer-exchange and concentrated to 8 mg ml^{-1} using an Ultrafree-15 centrifugal filter device (Millipore).

2.2. Crystallization and data collection

We attempted initial screening of the crystallization conditions according to the sparse-matrix sampling method (Jancarik & Kim, 1991) using Crystal Screen (Hampton Research, CA, USA) and Wizard and Cryo Screens (DeCODE Genetics, Reykjavik, Iceland). Crystallization was performed using the hanging-drop vapour-diffusion method (McPherson, 1990) at 293 K using a 24-well VDX plate (Hampton Research). The size of the droplet, which consisted of equal volumes of OXG-RCBH and reservoir solution, was 5 µl. The initial conditions under which crystals appeared were refined by varying the pH of the buffer and the concentration of the precipitant. The best crystals of OXG-RCBH were grown from 100 m*M* MES buffer pH 5.8–5.9, 4–5%(*w*/*v*) polyethylene glycol 3000 and 40%(*w*/*v*) polyethylene glycol 400 in about 10 d. They were plate- or prism-shaped crystals of approximately 0.5 × 0.3 × 0.1 mm in size (Fig. 1). The crystals were also grown by the sitting-drop vapour-diffusion method under the same conditions as those used in hanging drop. Crystals obtained by both methods were used for X-ray data collection.

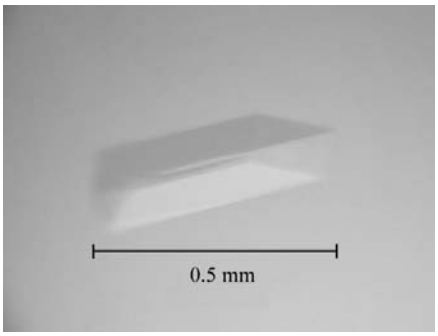

Figure 1
A crystal of recombinant OXG-RCBH of *Geotrichum* sp. M128 expressed from *E. coli*. Typical crystal dimensions are 0.5 × 0.3 × 0.1 mm.

Prior to data collection, the crystal was mounted on a loop made from a nylon fibre of 20 µm in diameter and immersed in liquid nitrogen for freezing. Data from OXG-RCBH were collected at 100 K on beamline 6A at the Photon Factory, KEK, Japan using an ADSC Quantum 4R CCD detector with 0.978 Å radiation and were processed with *HKL*2000 (Otwinowski & Minor, 1997) and the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). The crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 61.0, b = 146.9, c = 211.9 Å. On the basis of the estimated V_M value (Matthews, 1968), the asymmetric unit was assumed to contain two (V_M = 2.8 Å^3 Da^{-1}) or three (V_M = 1.9 Å^3 Da^{-1}) molecules, corresponding to solvent contents of approximately 56 or 34%, respectively. Diffraction intensities from the crystal were collected to 2.2 Å with an R_{merge} of 0.069 and a completeness of 87.5%. Table 1 summarizes the conditions and statistics of the data collection.

Table 1
Data-collection statistics of the native crystal.

Values in parentheses are for the highest resolution shell.

Beamline	PF BL6A
Wavelength (Å)	0.978
Resolution (Å)	2.2
R_{merge}†	0.069 (0.252)
Observed reflections	294393
Independent reflections	85159
Completeness (%)	87.5 (70.9)
Multiplicity	3.5 (3.0)
$\langle I/\sigma(I)\rangle$	9.5 (3.0)

† $R_{merge} = \sum\sum_j |\langle I(h)\rangle - I(h)_j| / \sum\sum_j \langle I(h)\rangle$, where $\langle I(h)\rangle$ is the mean intensity of a set of equivalent reflections.

3. Conclusions

Recombinant OXG-RCBH was expressed, purified and crystallized. The native crystals diffract to a resolution of 2.2 Å and are suitable for X-ray structure analysis. As described above, the amino-acid sequence of OXG-RCBH shows that it belongs to glycoside hydrolase family 74. No structures of members of this family have been determined. Apart from this family of enzymes, there are no known structures that have a similarity in amino-acid sequence to OXG-RCBH. In order to determine the structure of OXG-CBH using the multiple-wavelength anomalous diffraction method, we are now preparing an enzyme in which methionine residues are substituted by selenomethionines.

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