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# Preparation and preliminary X-ray analysis of the catalytic module of β-1,3-xylanase from the marine bacterium *Vibrio* sp. AX-4

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β-1,3-xylanase (1,3-β-D-xylan xylanohydrolase; EC 3.2.1.32) is an enzyme capable of hydrolyzing β-1,3-xylan. The newly cloned β-1,3-xylanase from the marine bacterium *Vibrio* sp. AX-4 (XYL4) exhibited a modular structure consisting of three modules: an N-terminal catalytic module belonging to glycoside hydrolase family 26 and two C-terminal xylan-binding modules belonging to carbohydrate-binding module family 31. Despite substantial crystallization screening, crystallization of the recombinant XYL4 was not accomplished. However, the deletion mutant of XYL4, composed of a catalytic module without a xylan-binding module, was crystallized. The crystal belonged to space group $P2_12_12_1$, with unit-cell parameters $a$ = 51.6, $b$ = 75.8, $c$ = 82.0 Å. X-ray diffraction data were collected to 1.44 Å resolution.

## 1. Introduction

β-1,3-Xylan is a homopolysaccharide of β-1,3-linked D-xylose units found mainly in the cell walls of red and green algae (Iriki *et al.*, 1960). β-1,3-Xylanase (1,3-β-D-xylan xylanohydrolase; EC 3.2.1.32) is an enzyme capable of hydrolyzing the β-1,3-xylosidic linkages of β-1,3-xylan to produce β-1,3-xylooligosaccharides with a varying number of xylose units. Enzymatic studies of β-1,3-xylanase have been performed for four bacterial enzymes (Aoki *et al.*, 1988; Yamamura *et al.*, 1990; Araki *et al.*, 1998, 1999) and one fungal enzyme (Chen *et al.*, 1986). The gene encoding β-1,3-xylanase has only been cloned from *Vibrio* sp. XY-214 (Araki *et al.*, 2000) and *Alcaligenes* sp. XY-234 (Okazaki *et al.*, 2002). Both enzymes exhibit a modular architecture consisting of two modules, a catalytic module and a xylan-binding module (XBM), which are assigned to glycoside hydrolase (GH) family 26 (GH26) and carbohydrate-binding module (CBM) family 31 (CBM31) in CAZy (Carbohydrate-Active Enzymes server; http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html; Coutinho & Henrissat, 1999*a*,*b*), respectively. Compared with β-1,4-xylanase, there have been few insights into the structure and the function of β-1,3-xylanase, although the crystal structure of mannanase 26A from *Pseudomonas cellulosa* classified into the same family of β-1,3-xylanases has been determined (Hogg *et al.*, 2001).

Recently, we cloned the gene encoding β-1,3-xylanase from the marine bacterium *Vibrio* sp. AX-4 (XYL4) and expressed it in *Escherichia coli* BL21(DE3)pLysS. From the sequence analysis of XYL4, it was revealed to be a modular enzyme composed of a catalytic module belonging to GH26 and tandem XBMs belonging to CBM31 (Kiyohara *et al.*, 2004). In this report, we describe the preparation and preliminary X-ray analysis of the catalytic module of β-1,3-xylanase from *Vibrio* sp. AX-4.

## 2. Materials and methods

### 2.1. Preparation of expression plasmid and recombinant enzyme

The DNA fragments of the full-length *xyl4* gene (1728 bp open reading frame encoding 576 amino acids) and deletion mutant (catalytic module only, 1050 bp encoding amino acids 1–350) were obtained by PCR using genomic DNA of AX-4 as a template. To express His-tagged fusion protein, PCR fragments were inserted into the *Bam*HI/*Hin*dIII site of pET23a (Novagen). The nucleotide sequence was then confirmed. *E. coli* BL21(DE3)pLysS cells transformed with the plasmid harbouring the full-length *xyl4* gene or the catalytic module were grown at 300 K with shaking in 1 l Luria–Bertani medium containing 100 μg ml$^{-1}$ ampicillin and 34 μg ml$^{-1}$ chloramphenicol until the absorbance at 600 nm reached about 0.5. Isopropyl-1-thio-β-D-galactoside was added to the culture to a final concentration of 0.1 m*M* and cultivation was continued for an additional 6 h. The cells were harvested by centrifugation and suspended in extraction solution (MilliQ water containing 5 μg ml$^{-1}$ leupeptin and pepstatin A) and sonicated for 1 min. Cell debris was removed by centrifugation (8000*g* for 10 min) and the supernatants were loaded onto a HiTrap Q HP column (5 ml, Amersham Bio-

sciences). The absorbed protein was washed with 50 m*M* sodium phosphate buffer pH 7.5 (buffer *A*) and eluted with buffer *A* containing 200 m*M* NaCl. The pooled fractions showing $\beta$-1,3-xylanase activity were applied onto a HiTrap Chelating HP column (1 ml, Amersham Biosciences) chelated with $Ni^{2+}$ and pre-equilibrated with 20 m*M* sodium phosphate buffer pH 7.5 containing 150 m*M* NaCl (buffer *B*) and 10 m*M* imidazole. After the column had been washed with the same buffer, the enzyme was eluted from the column with buffer *B* containing 200 m*M* imidazole. The enzyme solution was pooled and applied onto a Superdex 200 HR column (10 × 300 mm, Amersham Biosciences) equilibrated with buffer *B*. The active fractions were pooled and then concentrated using a HiTrap Chelating HP column, as described above. The purified enzyme was dialyzed against 10 m*M* acetate buffer pH 6.0 and used for crystallization. The final yields were 5.5 mg of full-length XYL4 and 6 mg of deletion mutant per litre of culture, respectively.

### 2.2. Crystallization and X-ray data collection

Initial crystallization trials were carried out by the sitting-drop vapour-diffusion method in a 96-well plate at 293 K using a series of crystallization kits purchased from Hampton Research and Emerald BioStructures (deCODE). Each drop was prepared by mixing 1 µl of wild-type or deletion mutant XYL4 solution (10 mg $ml^{-1}$ in 10 m*M* sodium acetate buffer pH 6.0) and the same volume of reservoir solution. Subsequently, in order to obtain a crystal suitable for X-ray analysis, the crystallization condition was scaled up using the hanging-drop vapour-diffusion method in a 24-well plate. Each drop contained 2 µl of protein solution and an equal volume of reservoir solution.

Prior to data collection, the crystal was transferred stepwise into a cryoprotectant buffer consisting of the reservoir solution containing up to 30%(*v*/*v*) glycerol and flash-cooled in a nitrogen-gas stream at 100 K. Data were collected on a Rigaku R-AXIS IV$^{++}$ imaging-plate area-detector system mounted on a Rigaku MicroMax-007 rotating-anode X-ray generator using Cu $K\alpha$ radiation (40 kV, 20 mA). The data were processed with the *HKL*2000 suite (Otwinowski & Minor, 1997). A total of 180 frames were collected with 1° oscillation and 1 min exposures.

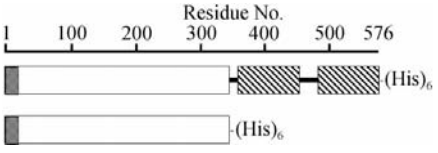


**Figure 1**
Schematic representation of wild-type XYL4 (top) and the deletion mutant (bottom). The modules in XYL4 are as follows: open boxes, catalytic module; shaded boxes, N-terminal signal peptide; hatched boxes, tandem xylan-binding modules. A glycine-rich linker region connects each pair of modules (black bars).

### 2.3. Other methods

Protein determination was performed by the bicinchoninic acid protein assay (Pierce) with bovine serum albumin as the standard. The activity of $\beta$-1,3-xylanase was measured by the following method. A reaction mixture containing 0.5% glycol-$\beta$-1,3-xylan and an appropriate amount of enzyme in 300 µl 50 m*M* sodium phosphate buffer pH 7.5 was incubated at 300 K for 30 min. After incubation, the reaction mixture was centrifuged and 250 µl of the supernatant was recovered. The reducing sugars liberated by hydrolysis of the substrate were determined by the Somogyi–Nelson method (Somogyi, 1952).

## 3. Results and discussion

As shown in Fig. 1, XYL4 exhibits a modular architecture consisting of three modules connected *via* glycine-rich linker peptides. XYL4 contains a catalytic module classified into GH26 and tandem XBMs classified into CBM31 (Kiyohara *et al.*, 2004). The crystallization of full-length XYL4 (residues 1–576, 63 kDa) was not accomplished, even though extensive crystallization screening and modifications were carried out. In general, GHs such as XYL4 exhibit a modular structure characterized by a catalytic module attached to one or several ancillary non-catalytic modules, which often but not always function in carbohydrate binding. The modules are attached to each other *via* a flexible linker peptide. Thus, attempts to crystallize various GHs as their intact multimodule forms have usually been unsuccessful, mainly because of the flexibility of the linker region between modules.

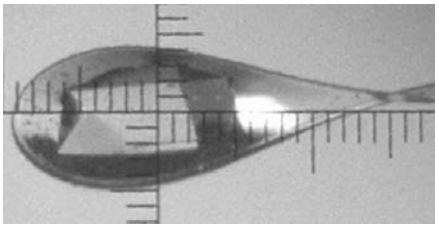

**Figure 2**
Orthorhombic crystal of the deletion mutant of XYL4 in a cryoloop. The size of the crystal was approximately 0.4 × 0.2 × 0.2 mm.

**Table 1**
Summary of crystallographic data.

Values in parentheses refer to the outer resolution shell.

| | |
|---|---|
| Resolution range (Å) | 50–1.44 (1.49–1.44) |
| Space group | $P2_12_12_1$ |
| Unit-cell parameters (Å) | $a$ = 51.6, $b$ = 75.8, $c$ = 82.0 |
| No. unique reflections | 56191 (5297) |
| Completeness | 95.3 (90.6) |
| Redundancy | 7.2 (7.1) |
| $I/\sigma(I)$ | 40.2 (27.8) |
| $R_{merge}$ | 0.041 (0.137) |
| Mosaicity | 0.377 |
| Wilson $B$ factor | 13.2 |

Accordingly, we prepared a truncated form of XYL4 consisting of the catalytic module without XBMs (residues 1–350, 38 kDa; Fig. 1). It was confirmed that the deletion mutant exhibited $\beta$-1,3-xylanase activity to the same extent as that of wild-type XYL4 when soluble glycol-$\beta$-1,3-xylan was used as the substrate (data not shown). As a result of crystallization screening, crystals of the deletion mutant were grown under the following conditions: Nos. 4 and 15 from Crystal Screen I, Nos. 15, 32 and 42 from Crystal Screen II, Nos. 14, 16, 36 and 47 from Wizard I, and Nos. 4, 16 and 31 from Wizard II. Almost all of the crystals exhibited good quality and did not require modifications. Of them, the crystal from condition No. 42 of Crystal Screen II [0.1 *M* Tris–HCl pH 8.5 containing 12%(*v*/*v*) glycerol and 1.5 *M* ammonium sulfate], which grew to dimensions of 0.4 × 0.2 × 0.2 mm within one week, was subjected to X-ray analysis (Fig. 2). The crystal belongs to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a$ = 51.6, $b$ = 75.8, $c$ = 82.0 Å. X-ray diffraction data were collected to 1.44 Å resolution. The data statistics are summarized in Table 1. The asymmetric unit contains one molecule, corresponding to a $V_M$ value of 2.2 $Å^3$ $Da^{-1}$ and a solvent content of 44.2% (Matthews, 1968).

This is the first report of the crystallization and preliminary X-ray analysis of a $\beta$-1,3-xylanase. The preparation of a selenomethionine crystal for use with the multiple-anomalous dispersion (MAD) method is now in progress. It should also be noted that a further trial to crystallize an XBM alone, as well as full-length XYL4, has been conducted extensively.

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