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Crystallization and preliminary X-ray diffraction analysis of a chitin-binding domain of hyperthermophilic chitinase from *Pyrococcus furiosus*

The crystallization and preliminary X-ray diffraction analysis of the chitin-binding domain of chitinase from a hyperthermophilic archaeon, *Pyrococcus furiosus*, are reported. The recombinant protein was prepared using an *Escherichia coli* overexpression system and was crystallized by the hanging-drop vapour-diffusion method. An X-ray diffraction data set was collected to 1.70 Å resolution. The crystal belonged to space group $P4_32_12$ or $P4_12_12$. The unit-cell parameters were determined to be $a = b = 48.8$, $c = 85.0$ Å.

1. Introduction

Chitin is the second most abundant biomass. Thermostable chitinases will enable the effective utilization of chitin as a source of materials of biomass origin. However, in contrast to bacterial, fungal and plant chitinases, only a few structural studies have been reported on archaeal chitinases. Chitinases are composed of a catalytic domain and one or more chitin-binding domains (ChBDs). The chitin-binding domains promote the hydrolysis of chitin by the catalytic domains through accumulation of chitinases onto the surface of chitin and through the disruption of the hydrogen bonds in crystalline chitin (Tomme *et al.*, 1995).

Two adjacent *Pyrococcus furiosus* genes (http://gib.genes.nig.ac.jp; PF1234 and PF1233, separated by 37 bp; Gao *et al.*, 2003) are homologous to the first half and second half of chitinase from *Thermococcus kodakaraensis* (Tk-ChiA), respectively (Tanaka *et al.*, 1999). The stop codon of PF1234 is located in the middle of the putative catalytic domain when compared with Tk-ChiA. When the nucleotide 1169837A (the 1006th adenine from the ORF start position) was deleted from PF1234 and the resultant frame shift was taken into account, the two genes (PF1234 and PF1233) could be combined into a longer gene that best matched the amino-acid sequence of Tk-ChiA (Oku *et al.*, in preparation). From sequence comparison with Tk-ChiA, we found two chitin-binding domains (ChBD1 and ChBD2) and two active (catalytic) domains (AD1 and AD2) in the combined gene (referred to as PF-ChiA). In this study, we focused on ChBD2 (residues 258–358, residues numbered according to the PF1233 amino-acid sequence), which is included in PF1233 (nt1169539–nt1169841 in the genome database of *P. furiosus*) and has homology to the region 616–717 of the Tk-ChiA amino-acid sequence (http://gib.genes.nig.ac.jp; Tk1765).

At present, carbohydrate-binding domains (modules), including the chitin-binding domain, have been classified into more than 40 families, mainly based on amino-acid sequence (CAZy database; http://afmb.cnrs-mrs.fr/CAZY/; Coutinho & Henrissat, 1999). ChBD2 (101 amino acids, 10.8 kDa) is classified into the family 2 cellulose-binding domain group in the CAZy database. Only a few structures are available of domains belonging to this group, *i.e.* the cellulose-binding domain of exo-1,4-β-D-glycanase from *Cellulomonas fimi* (PDB code 1exg; Xu *et al.*, 1995), the internal xylan-binding domain of xylanase D from *C. fimi* (PDB code 2xbd; Simpson *et al.*, 1999) and the C-terminal xylan-binding domain of endo-1,4-β-xylanase 11A from *C. fimi* (PDB code 1heh; Bolam *et al.*, 2001). They all share a relatively low degree of sequence identity (27–30%) with ChBD2. Therefore, we focused on elucidating the tertiary structure and the structure–function relationship of ChBD2 and performed X-ray

Table 1
Experimental conditions and data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	BL38B1, SPring-8
Wavelength (Å)	1.0
Detector	Jupiter 210cs CCD detector
Temperature (K)	100
Crystal-to-detector distance (mm)	150
Total oscillation angle (°)	180
Rotation angle per frame (°)	1.0
Space group	$P4_32_12$ or $P4_12_12$
Unit-cell parameters (Å)	$a = b = 48.8$, $c = 85.0$
Resolution range (Å)	50–1.70 (1.76–1.70)
$R_{\rm merge}$† (%)	7.6 (23.3)
$\langle I/\sigma(I)\rangle$	14.2 (3.0)
Total reflections	152578
Unique reflections	11986
Completeness (%)	96.8 (80.5)

† $R_{\rm merge} = \sum|I - \langle I\rangle|/\sum I$, where I represents the intensity of the reflection and $\langle I\rangle$ the averaged intensity.

diffraction analysis. In this paper, we describe the expression, purification and preliminary X-ray diffraction studies of a chitin-binding domain of the chitinase.

2. Materials and methods

2.1. Construction of the ChBD2 expression vector

The ChBD2 gene was cloned into pET32 expression vector (pET32_ChBD2) using the LIC method (Novagen) using the following primers: primer 1, 5′-GACGACGACAAGATC**CTGGAAGTTCTGTTCCAGGGGCCC**ACTACCCCTGTCCCAGTCCAGG-3′ and primer 2, 5′-GAGGAGAAGCCCGGTTTAAATTACTTGTCCGTTTATTTCTAGGG-3′. The PF1233 gene from *P. furiosus* genomic DNA was used as a template. The bold region of primer 1 corresponds to the PreScission protease (Amersham Biosciences) recognition sequence (PSsequence). Therefore, ChBD2 has two additional residues (H_2N-Gly-Pro) derived from the PSsequence at its N-terminus. As a result, the expression plasmid coded the fusion protein thioredoxin-His$_6$-tag-PSsequence-ChBD2. *Escherichia coli* Rosetta (DE3) (Novagen) cells harbouring the pET32_ChBD2 plasmid were cultivated in LB medium containing 50 µg ml^{-1} ampicillin at 310 K. When the OD$_{600}$ reached 0.6, expression was induced with 1 m*M* isopropyl-1-thio-β-D-galactopyranoside (IPTG) for 4 h at 310 K. The cells were harvested by centrifugation (6000*g* for 20 min) and stored at 193 K.

2.2. Purification

All the following procedures were carried out at room temperature unless noted. The cell pellet (approximately 20 g) was suspended in buffer *A* (20 m*M* Tris–HCl pH 8.5, 0.5 *M* NaCl; 4 ml buffer per gram of cell pellet) and sonicated. After removal of the cell debris by centrifugation (30 000*g* for 40 min), the supernatant was loaded onto a HiTrap Chelating column (5 ml bed volume; Amersham Biosciences) preloaded with Ni^{2+} and equilibrated with buffer *A*. The column was washed with five column volumes of buffer *A* and the bound protein was eluted with a 100 ml linear gradient of imidazole (0–0.5 *M*) in buffer *A* at a flow rate of 3 ml min^{-1}. The desired protein eluted at around 200–250 m*M* imidazole. The elution peak containing the target protein was dialyzed against buffer *B* (20 m*M* Tris–HCl pH 8.5, 25 m*M* NaCl). To remove the thioredoxin-His$_6$-tag portion, PreScission protease (100 units) was added to the dialysate and the solution was incubated at 277 K for 12 h. The protease digestion produced a mixture of thioredoxin-His$_6$-tag and ChBD2 protein solution. The solution was again applied onto a HiTrap Chelating column (5 ml bed volume) preloaded with Ni^{2+} and equilibrated with buffer *B* and the flowthrough fraction was collected. In this step, the thioredoxin-His$_6$-tag portion was effectively bound to the column and removed from the protease-digested solution. The flowthrough fraction (containing mainly ChBD2) was applied onto a HiTrap Q column (5 ml bed volume; Amersham Biosciences), washed with five column volumes of buffer *B* and eluted with a 100 ml linear gradient of NaCl in buffer *A* at a flow rate of 3 ml min^{-1}. ChBD2 eluted at around 150–200 m*M* NaCl. The fraction containing ChBD2 was collected and concentrated using a 3.5 kDa cutoff filter (Millipore). The protein was dialyzed against 20 m*M* KH_2PO_4/K_2HPO_4 pH 6.5. A 20 mg ml^{-1} sample was used for crystallization trials. The purity of the protein was confirmed by SDS–PAGE, which showed only one band. The protein concentration was estimated using the calculated molar absorption coefficient at 280 nm (ε_{280} = 26 600 M^{-1} cm^{-1}; Edelhoch, 1967).

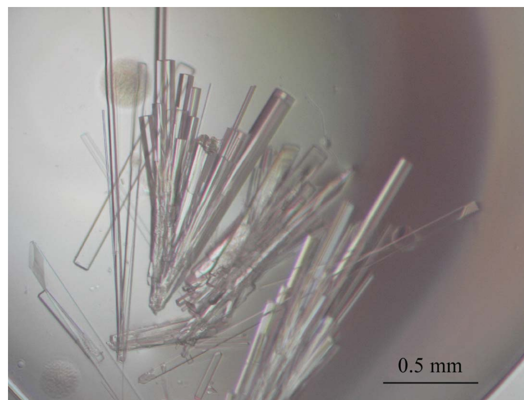

Figure 1
Photograph of CBD2 crystals grown by the hanging-drop vapour-diffusion method. The photograph shows crystals obtained from Crystal Screen solution No. 18.

2.3. Crystallization conditions

Crystal Screen (Hampton Research) was used to screen the crystallization conditions. Crystals were grown by the hanging-drop vapour-diffusion method. 1.5 µl protein sample (20 mg ml^{-1} in 20 m*M* KH_2PO_4/K_2HPO_4 pH 6.5) was mixed with an aliquot of reservoir solution in a 1:1 ratio and left to equilibrate against the reservoir solution at 298 K for about two weeks. Solution No. 18 [0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* magnesium acetate, 20%(*w*/*v*) polyethylene glycol 8000] from Crystal Screen produced a crystal good enough for the following X-ray diffraction experiment.

2.4. Diffraction data collection

For X-ray data collection, a crystal (0.1 × 0.1 × 0.5 mm) was immersed in a solution comprising 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* magnesium acetate and 20%(*w*/*v*) polyethylene glycol 8000 and flash-frozen in a stream of nitrogen gas at 100 K. Diffraction data were collected at BL38B1, SPring-8 (Harima, Japan). The crystal-to-detector distance was 150 mm. The diffraction data were integrated and scaled with the *HKL*2000 program suite (Otwinowski & Minor, 1997). Detailed experimental conditions are summarized in Table 1.

3. Results and discussion

As the expression level of ChBD2 alone was very low (less than 0.1 mg from 1 l culture), we expressed ChBD2 in a thioredoxin-fused

form. Using this thioredoxin-fused system, a sufficient amount of suitably pure ChBD2 was obtained. The yield of the protein was 10 mg from 1 l culture.

Crystals of ChBD2 appeared after a week of vapour diffusion and stopped growing within two weeks (Fig. 1). A single crystal was isolated from the drop and subjected to diffraction data collection. The diffraction data set was collected to 1.70 Å resolution under the conditions described above. The data-collection statistics are summarized in Table 1. Assumption of the presence of one molecule per asymmetric unit gives a crystal volume per protein weight ($V_{\rm M}$; Matthews, 1968) of 2.3 $Å^3\,Da^{-1}$ and a solvent content of 47%. The ChBD2 contains an additional two amino acids (Gly, Pro) at its N-terminus derived from the PSsequence; we used a MW of 11.0 kDa for the $V_{\rm M}$ calculation. Therefore, it is clear that the asymmetric unit contains one molecule. As ChBD2 does not contain methionine residues, we are attempting heavy-atom-derivative preparation for structure determination.

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