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Expression, purification, crystallization and X-ray diffraction analysis of ChiL, a chitinase from *Chitiniphilus shinanonensis*

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Chitin, a linear polysaccharide consisting of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc), is widely used because of its biochemical properties. GlcNAc oligomers prepared from chitin have useful biological activities, such as immunostimulation and the induction of plant defence responses. Microbial chitinolytic enzymes have been investigated extensively for their potential use in the eco-friendly enzymatic production of GlcNAc and its oligomers. *Chitiniphilus shinanonensis* SAY3^{T} is a recently found bacterium with a strong chitinolytic activity. The chitinolytic enzymes from this strain are potentially useful for the efficient production of GlcNAc and its oligomers from chitin. ChiL from *C. shinanonensis* is an endo-type chitinase belonging to the family 18 glycoside hydrolases (GH18). To understand the enzymatic reaction mechanism of ChiL and utilize it for further enzyme engineering, the catalytic domain (41–406) of ChiL, the construct for which was carefully designed, was expressed, purified and crystallized by the vapour-diffusion method. The crystal belonged to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 69.19, b = 81.55, c = 130.01 Å, and diffracted to 1.25 Å resolution. The Matthews coefficient (V_M = 2.2 Å^3 Da^{-1}) suggested the presence of two monomers per asymmetric unit with a solvent content of 45%.

1. Introduction

Chitin, a linear polysaccharide consisting of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc), is widely distributed in nature, such as in the exoskeletons of crustaceans and insects and in the cell walls of fungi (Synowiecki & Al-Khateeb, 2003; Tharanathan & Kittur, 2003). Chitin and its deacetylated derivative, chitosan, have attracted considerable interest because of their biological properties, and are widely used in various fields including healthcare, food, agriculture and the chemical and environmental engineering industries (Alves & Mano, 2008; Cohen-Kupiec & Chet, 1998; Kumar *et al.*, 2004; Masotti & Ortaggi, 2009). GlcNAc oligomers prepared from chitin have useful biological activities such as immunostimulation and induction of plant defence responses (Ebel, 1998). In addition, GlcNAc can be utilized as a sweetener and as a nutritional supplement.

A hydrolytic chemical reaction with strong acids has been used for the production of GlcNAc and its oligomers on an industrial scale, but the costs associated with the treatment of waste products to prevent environmental pollution are expensive. Hence, microbial chitinolytic enzymes have been investigated extensively for their potential use in the eco-friendly enzymatic production of GlcNAc and its oligomers.

Table 1
Protein-production information for ChiL from *C. shinanonensis*.

Cleavage sites by BamHI, XhoI and TEV protease in the forward primer, reverse primer and amino-acid sequence, respectively, are underlined. The His_6 tag is shown in italics.

Source organism	*C. shinanonensis* SAY3^T
DNA source	Genomic DNA cloned in a plasmid
Forward primer	AAAACTGAAAACCTGTACTTCCAGGGTAGCGGATCCTACCGCGTCGTCGCCTACTACATC
Reverse primer	CAAGAAAGCTGGGTCTAGATATCTCGAGTTATTAGGTCAGCAATTCGTCGGCGATGAG
Cloning vector	pENTR-TEV-L
Expression vector	pCold I
Expression host	*E. coli* BL21 Star (DE3)
Complete amino-acid sequence of the construct produced	MNHKV*HHHHHH*IEGRHMELGTLEGAPSITSLYKKAGFKTENLYFQGGSGGSYRVVAYYISWGAYGRSYFPSDIDYSKVTHINYAFANIKDGEVVVGDPGVDDGGKNNFTALRKAKKAHPHLRNLISVGGWSWSSGFSDAAATPEARKRFADSAVAFIRKYGFDGVDIDWEYPVEGGAENMKHRPEDKQNYTLLTRSLREALDTAGKADGKYYELTTAVWGNDKFIANTEMDKVSRDFDFINVMSYDFNGTWNKFSGHNAPFVNDPAYDKPGIGKTFNVVSAVEAYLKAGVPADKLVVGVPLYGYSWKGCAAGERNGEYQDCNGKGRGTWEDGNLDFTDIEKNLLNKKGFKRYWNDTAKAAYLYNAETGEFVTYEDPQALKIKLDYIKSKGLGGAMYWEITADRKQTLVNLIADELLT

Chitiniphilus shinanonensis SAY3^T, which was classified as a new genus and species, is a recently found chitin-degrading bacterium with a strong chitinolytic activity which was originally isolated from moat water of Ueda Castle in our local area of Ueda city, Nagano prefecture, Japan (Sato *et al.*, 2009). This original bacterial strain has many newly found chitinolytic enzymes which are potentially useful for the efficient production of GlcNAc and its oligomers from chitin (Huang *et al.*, 2012, 2014). One of these chitinolytic enzymes from *C. shinanonensis*, ChiL (GenBank/DDBJ accession BAK53954), is a chitinase which has one catalytic domain and no chitin-binding domain (Huang *et al.*, 2012). ChiL is classified as an endo-type chitinase belonging to the family 18 glycoside hydrolases (GH18) based on the amino-acid sequence similarity of their catalytic domains (Fukamizo, 2000; Funkhouser & Aronson, 2007; Henrissat & Bairoch, 1993). Crystal structures have been elucidated for GH18 chitinases isolated from various sources, including *Serratia marcescens* (PDB entry 1ctn; Perrakis *et al.*, 1994), *Coccidioides immitis* (PDB entry 1d2k; Hollis *et al.*, 2000), *Aspergillus fumigatus* (PDB entry 1w9p; Rao *et al.*, 2005) and *Bacillus circulans* (PDB entry 1itx; Matsumoto *et al.*, 1999). The catalytic domain of ChiL shows modest similarity (amino-acid sequence identity of 32–34%) to most of these enzymes and moderate similarity (49%) to chitinase A1 from *B. circulans*. Since the three-dimensional structure of ChiL would contribute to understanding its detailed reaction mechanism and further enzyme engineering, we performed expression, purification, crystallization and X-ray diffraction experiments on the catalytic domain of ChiL in this study. This is the first crystallization report of a protein from *C. shinanonensis*.

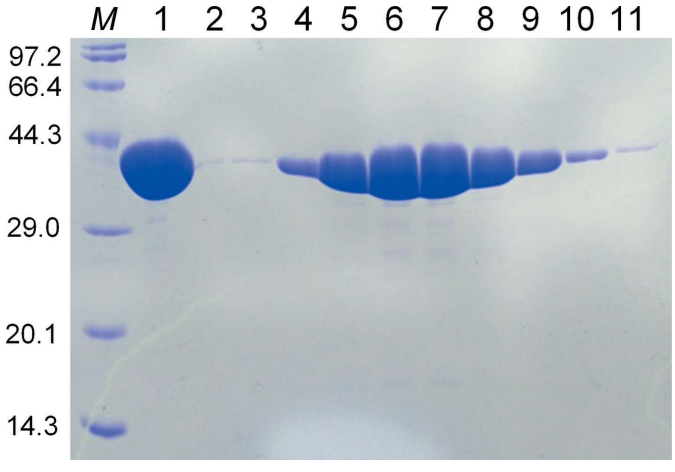

Figure 1
SDS–PAGE analysis of ChiL(41–406) purification by size-exclusion chromatography (SEC). Lane *M*, molecular-mass standards [protein molecular-weight marker (broad), Takara Bio; labelled in kDa]; lane 1, ChiL(41–406) protein sample before SEC; lanes 2–11, eluted peak fractions of ChiL(41–406) by SEC (Supplementary Fig. S5). Proteins were detected by staining with Coomassie Brilliant Blue.

2. Materials and methods

2.1. Protein production

The amino-acid sequence construct for the catalytic domain of the ChiL protein from *C. shinanonensis* was carefully designed using the *XtalPred* web server (http://ffas.burnham.org/XtalPred) for prediction of protein crystallizability (Slabinski *et al.*, 2007). The DNA fragment encoding the catalytic domain (41–406) of ChiL was prepared from the cloned DNA plasmid encoding ChiL from *C. shinanonensis* SAY3^T (Huang *et al.*, 2012) by polymerase chain reaction (PCR) using KOD-Plus-Neo DNA polymerase (Toyobo, Osaka, Japan) and the forward and reverse primers shown in Table 1. The amplified fragment was digested by BamHI and XhoI and cloned into pENTR-TEV-L-*ccd*B (Supplementary Fig. S1*a*), a modified Gateway entry vector with a *Tobacco etch virus* (TEV) protease cleavage site (ENLYFQ|G) derived from pENTR1A (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) between the BamHI and XhoI sites. The expression vector, pCold-ChiL(41–406) (Supplementary Fig. S1*b*), was constructed using the LR reaction of the Gateway Technology (Invitrogen) with pENTR-TEV-L-ChiL(41–406) and pCold I-DEST, a modified pCold I DNA (Takara Bio, Shiga, Japan) expression vector, bearing a cold-shock protein *cspA* promoter, an N-terminal His_6 tag and a Gateway reading frame cassette (Invitrogen). The catalytic domain (41–406) of the ChiL protein with an N-terminal His_6 tag and a TEV protease cleavage site was expressed in *Escherichia coli* BL21 Star (DE3) cells (Invitrogen) harbouring pCold-ChiL(41–406) using LB broth (Lennox) (Nacalai Tesque, Kyoto, Japan) with 50 µg ml^{-1} ampicillin sodium salt at 303 K. At an OD_{600} (optical density at 600 nm) of 0.4–0.6, protein expression was induced by rapidly cooling to 288 K for 30 min and by the addition of 0.1 m*M* isopropyl *β*-D-1-isopropyl thiogalactopyranoside, and the cells were further cultured for ∼24 h at 288 K. The protein was extracted from the harvested cells by

sonication in lysis buffer [50 m*M* Tris–HCl buffer pH 7.5 containing 150 m*M* NaCl, 5 m*M* imidazole and 2 m*M* dithiothreitol (DTT)]. The protein was purified by immobilized metal ion-affinity chromatography (IMAC) with COSMOGEL His-Accept (Nacalai Tesque) and eluted with elution buffer (50 m*M* Tris–HCl buffer pH 7.5 containing 150 m*M* NaCl, 400 m*M* imidazole and 2 m*M* DTT) (Supplementary Fig. S2). The His_6 tag of the protein was cleaved by TEV protease (Kapust *et al.*, 2001) at 277 K for 1 d, and the His_6 tag and the His_6-tagged TEV protease were then removed by IMAC with COSMOGEL His-Accept (Supplementary Fig. S3). The ChiL(41–406) protein was purified further by anion-exchange chromatography (equilibration buffer, 50 m*M* Tris–HCl buffer pH 9.0 containing 1 m*M* DTT; elution buffer, 50 m*M* Tris–HCl buffer pH 9.0 containing 1 *M* NaCl and 1 m*M* DTT) on a RESOURCE Q (6 ml) column (GE Healthcare, Little Chalfont, Buckinghamshire, England) (Supplementary Fig. S4). Moreover, the protein was purified by size-exclusion chromatography (20 m*M* Tris–HCl buffer pH 8.0 containing 100 m*M* NaCl and 1 m*M* DTT) on a HiLoad 16/600 Superdex 75 pg column (GE Healthcare) (Supplementary Fig. S5). The protein purity was confirmed by SDS–PAGE (Fig. 1). The ChiL(41–406) protein construct for crystallization (molecular mass 41.3 kDa) contained a six-amino-acid linker sequence (GGSGGS) at the N-terminus (Table 1).

2.2. Crystallization

Crystallization screening of the ChiL(41–406) catalytic domain was performed at 293 K using the crystallization screens Index HT (Hampton Research, Aliso Viejo, California, USA), PEGRx HT (Hampton Research) and Wizard Classic 1 and 2 (Rigaku Reagents, Bainbridge Island, Washington, USA) by the sitting-drop vapour-diffusion method (1 µl drop volume with a 0.5:0.5 ratio of protein solution:reservoir solution) in 96-well protein crystallization plates (VIOLAMO, AS ONE, Osaka, Japan).

Based on initial screening results, crystallization condition F9 [0.2 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 8.5, 25% polyethylene glycol (PEG) 3350] from Index HT was selected for further optimization by the hanging-drop vapour-diffusion method. We used Falcon 24-well plates (Corning, New York, USA) and 500 µl reservoir solution. The crystal of ChiL(41–406) used for diffraction data collection was obtained in a drop composed of 1 µl 15 mg ml^{-1} protein solution and 1 µl reservoir solution (0.2 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 8.0, 25% PEG 3350) in a few days (Table 2 and Fig. 2).

Figure 2
A crystal (~400 × 500 × 30 µm) of the catalytic domain (41–406) of ChiL from *C. shinanonensis*.

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well plate
Temperature (K)	293
Protein concentration (mg ml^{-1})	15
Buffer composition of protein solution	20 m*M* Tris–HCl pH 8.0, 100 m*M* NaCl, 1 m*M* DTT
Composition of reservoir solution	0.1 *M* Tris–HCl pH 8.0, 0.2 *M* ammonium sulfate, 25% PEG 3350
Volume and ratio of drop	2 µl (1:1 protein:reservoir solution)
Volume of reservoir (µl)	500

2.3. Data collection and processing

X-ray diffraction images were collected on a CCD detector (ADSC Quantum 210r) using synchrotron radiation on the AR-NW12A beamline at KEK Photon Factory, Tsukuba, Ibaraki, Japan (Chavas *et al.*, 2012) at 95 K with reservoir solution added to 25% PEG 400 as a cryoprotectant (Fig. 3). The diffraction data were processed with *HKL*-2000 (Otwinowski & Minor, 1997). Data statistics are shown in Table 3.

3. Results and discussion

Firstly, we carefully designed a construct of the ChiL protein sequence that was promising for crystallization using the

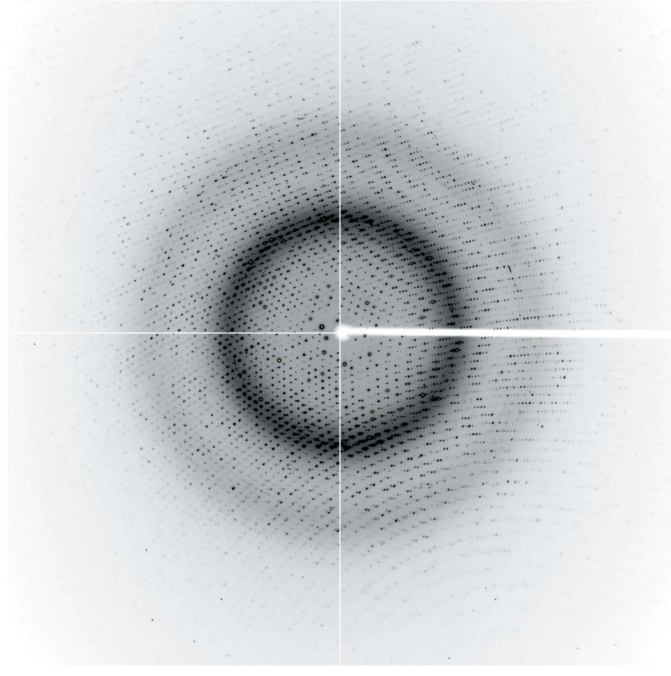

Figure 3
X-ray diffraction image of the ChiL(41–406) crystal. The resolution at the edge and corner of this image is 1.4 and 1.15 Å, respectively.

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	AR-NW12A, KEK Photon Factory
Wavelength (Å)	1.000
Temperature (K)	95
Detector	ADSC Quantum 210r CCD
Crystal-to-detector distance (mm)	117.1
Rotation range per image (°)	0.50
Total rotation range (°)	180
Exposure time per image (s)	1
Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 69.19, b = 81.55, c = 130.01, $\alpha = \beta = \gamma = 90$
Mosaicity (°)	0.69
Resolution range (Å)	50.0–1.25 (1.29–1.25)
Total No. of reflections	1158653
No. of unique reflections	197591 (16344)
Completeness (%)	97.3 (81.3)
Multiplicity	5.9 (2.6)
$\langle I/\sigma(I)\rangle$	25.3 (3.6)
$R_{\rm meas}$†	0.066 (0.348)
$CC_{1/2}$‡	(0.903)
Overall B factor from Wilson plot (Å^2)	11.4

† $R_{\rm meas} = \sum_{hkl}\{N(hkl)/[N(hkl)-1]\}^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| \sum_{hkl}\sum_i I_i(hkl)$.
‡ Pearson's intra-data-set correlation coefficient (Karplus & Diederichs, 2012).

XtalPred web server (Slabinski *et al.*, 2007) to predict protein crystallizability. Crystallizability predictions of several constructs of ChiL by *XtalPred* suggested that full-length ChiL(1–410) had the least promising crystallizability (Supplementary Fig. S6; expert pool crystallizability class 5; random forest crystallizability class 11), but the truncated ChiL(41–406), *i.e.* only the catalytic domain, has highly promising crystallizability (Supplementary Fig. S7; expert pool crystallizability class 2; random forest crystallizability class 3). The contrasting results were probably caused by the long disordered region (23 amino acids) of full-length ChiL(1–410) predicted by *DISOPRED*2 (Ward *et al.*, 2004). The disordered region was removed in the truncated ChiL(41–406). Therefore, we designed the catalytic domain of ChiL(41–406) with a His_6 tag cleavable by TEV protease as an expression construct suitable for crystallization (Table 1). The ChiL(41–406) protein was highly expressed in *E. coli* and successfully purified by chromatography. The final yield of the purified ChiL(41–406) protein was ∼10 mg per 3 l of culture.

Crystallization screening of the ChiL(41–406) catalytic domain was performed using three sets of commercial crystallization screen kits (Index HT, PEGRx HT and Wizard Classic 1 and 2), and potentially promising crystals were produced in several conditions using PEG as a precipitant (D7, D8, F2, F6, F9, F11, G4 and H3 from Index HT; G4 and G9 from PEGRx HT; A6 and G4 from Wizard) within a few weeks (Supplementary Fig. S8). Condition F9 from Index HT (0.2 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 8.5, 25% PEG 3350) was further optimized, and a diffraction-quality crystal (Fig. 2; ∼400 × 500 × 30 µm) grew in a few days in a hanging drop composed of 1 µl 15 mg ml^{-1} protein solution and 1 µl reservoir solution (0.2 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 8.0, 25% PEG 3350; Table 2). X-ray diffraction data were collected from the crystal using synchrotron radiation on the AR-NW12A beamline at Photon Factory and reached a very high resolution diffraction limit of 1.25 Å (Fig. 3). Data statistics are shown in Table 3. The crystal belonged to orthorhombic space group $P2_12_12_1$, with unit-cell parameters a = 69.19, b = 81.55, c = 130.01 Å, according to *POINTLESS* from the *CCP*4 suite (Winn *et al.*, 2011). Calculation of the Matthews coefficient ($V_{\rm M}$ = 2.2 Å^3 Da^{-1}; Matthews, 1968) suggested the presence of two monomers per asymmetric unit with a solvent content of 45%. For molecular replacement, the structure of chitinase Al from *B. circulars* WL-12 (PDB entry 1itx; Matsumoto *et al.*, 1999) was modified by removing several residues in extra portions according to an amino-acid sequence alignment of the catalytic domains of ChiL and chitinase Al, and the truncated structure was used as a search model. *Phaser* (McCoy *et al.*, 2007) found a solution with two molecules in the asymmetric unit, and the calculated electron density showed a clear map. Model refinement is under way.

In this paper, we first designed a ChiL expression construct suitable for crystallization using *XtalPred* and then performed expression, purification, crystallization and preliminary X-ray diffraction analysis of the catalytic domain (41–406) of ChiL. We obtained several promising crystals for further structural analysis, and one of the crystals diffracted to a very high resolution of 1.25 Å. These results were promising for the structural analysis of ChiL and also imply that the design of expression constructs utilizing *XtalPred* can be a helpful tip for protein crystallization.

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