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Crystallization and preliminary X-ray diffraction studies of the catalytic domain of a novel chitinase, a member of GH family 23, from the moderately thermophilic bacterium *Ralstonia* sp. A-471

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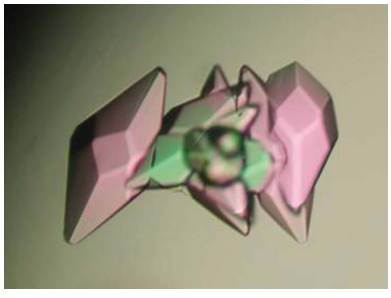
Chitinase from the moderately thermophilic bacterium *Ralstonia* sp. A-471 (Ra-ChiC) is divided into two domains: a chitin-binding domain (residues 36–80) and a catalytic domain (residues 103–252). Although the catalytic domain of Ra-ChiC has homology to goose-type lysozyme, Ra-ChiC does not show lysozyme activity but does show chitinase activity. The catalytic domain with part of an interdomain loop (Ra-ChiC$_{89–252}$) was crystallized under several different conditions using polyethylene glycol as a precipitant. The crystals diffracted to 1.85 Å resolution and belonged to space group $P6_122$ or $P6_522$, with unit-cell parameters $a = b = 100$, $c = 243$ Å. The calculated Matthews coefficient was approximately 3.2, 2.4 or 1.9 Å^3 Da^{-1} assuming the presence of three, four or five Ra-ChiC$_{89–252}$ molecules in the asymmetric unit, respectively.

1. Introduction

Chitin, a β-1,4 polymer of *N*-acetyl-D-glucosamine (GlcNAc), is the second most abundant biopolymer in nature after cellulose. It is easy to obtain from natural resources such as crab and shrimp shell waste. The annual amount of synthesis and degradation of chitin in the biosphere is estimated at 10 gigatons (1×10^{13} kg; Muzzarelli, 1999). *N*-Acetylchitooligosaccharides and chitooligosaccharides, which are generated from the hydrolysis of chitin, are reported to have a variety of biological functions and potential applications (Tokoro *et al.*, 1988; Hirano & Nagao, 1990). Chitin is strongly resistant to chemicals, is highly insoluble and is mass-produced. In nature, chitin is degraded by chitinases (EC 3.2.1.14) that belong to glycoside hydrolase (GH) families 18 and 19 (Henrissat, 1991; Henrissat & Bairoch, 1993). These two families do not share sequence or structural similarities and act *via* different catalytic mechanisms. GH18-family chitinases are found in a wide diversity of organisms. Their catalytic domain has an $(\alpha/\beta)_8$-barrel structure (Davies & Henrissat, 1995) and uses a double-displacement mechanism which leads to retention of anomeric configuration (Tews *et al.*, 1997; Synstad *et al.*, 2004). GH19-family chitinases are mainly found in plants, but have also recently been discovered in bacteria (Ueda *et al.*, 2003; Watanabe *et al.*, 1999). Their tertiary structures show a high α-helical content (Song & Suh, 1996; Kezuka *et al.*, 2006) and have a topology similar to lysozymes from phages (GH19), hen (GH22) and goose (GH23) (Holm & Sander, 1994; Hart *et al.*, 1995; Monzingo *et al.*, 1996). The catalytic mechanism of GH19-family chitinases involves a single-displacement mechanism which leads to inversion of anomeric configuration (Iseli *et al.*, 1996).

We have previously reported the cloning of the goose-type (G-type) lysozyme gene with chitinase activity from *Ralstonia* sp. A-471 (Ra-ChiC; Ueda *et al.*, 2009). Ra-ChiC consists of 252 amino acids including a putative signal peptide (Fig. 1*a*). Mature Ra-ChiC is divided into two domains, a chitin-binding domain (residues 36–80) and a catalytic domain (residues 103–252), based on sequence analysis. The two domains are connected by an interdomain linker (residues 81–102) including seven consecutive glycines and Pro/Thr-



rich repeats (TTP, PPT and PPTT). The catalytic domain of Ra-ChiC is homologous to a lysozyme-like enzyme from *Clostridium beijerincki* (75.1% sequence identity) which belongs to the GH23 family in the CAZy database (http://www.cazy.org/; Cantarel *et al.*, 2008). Ra-ChiC has activity towards ethylene glycol chitin, carboxylmethyl chitin and soluble chitin (50% deacetylated chitin), but not the cell wall of *Micrococcus lysodeikticus*. The activity of Ra-ChiC towards soluble chitins is maintained even if the chitin-binding domain is removed. The enzyme produces the α-anomer by hydrolyzing the β-1,4-glycosidic linkage of the substrate, which indicates that the enzyme catalyzes hydrolysis through an inverting mechanism. Ra-ChiC hydrolyzes *N*-acetylglucosamine hexasaccharide [$(GlcNAc)_6$] and produces $(GlcNAc)_2$ + $(GlcNAc)_4$ and $(GlcNAc)_3$ + $(GlcNAc)_3$, resulting from the second and third glycosidic linkage being split from the nonreducing end, at almost the same concentrations. In contrast, the G-type lysozyme hydrolyzes $(GlcNAc)_6$ in an endo-splitting manner, which predominantly produces $(GlcNAc)_3$ + $(GlcNAc)_3$ as opposed to $(GlcNAc)_2$ + $(GlcNAc)_4$. Thus, Ra-ChiC was found to be a novel enzyme with regard to its structural and functional properties. Here, we describe the crystallization and preliminary X-ray diffraction studies of Ra-ChiC in order to elucidate the structure–function relationships at the atomic level.

2. Methods

2.1. Expression and purification

Mature Ra-ChiC with an N-terminal hexahistidine tag (Ra-ChiC$_{mature}$; Fig. 1*b*) was expressed in *Escherichia coli* as a soluble protein and purified using a HisTrap HP column (Ueda *et al.*, 2009). Ra-ChiC$_{mature}$ was further purified for crystallization. The eluted fraction containing protein from the HisTrap HP column was desalted by gel filtration using a PD10 column (10 × 50 mm; GE Healthcare) equilibrated with 20 m*M* Tris–HCl buffer pH 8.0. The desalted solution containing Ra-ChiC$_{mature}$ was applied onto a RESOURCE S cation-exchange column (1.0 ml; GE Healthcare). After the column had been washed with 5 ml 20 m*M* Tris–HCl buffer pH 8.0, Ra-ChiC$_{mature}$ was eluted with a linear gradient from 20 m*M* Tris–HCl buffer pH 8.0 to the same buffer containing 250 m*M* sodium chloride at a flow rate of 1.0 ml min^{-1} over 30 min. The purity of the recombinant Ra-ChiC$_{mature}$ was verified by SDS–PAGE analysis.

The expression vector for the catalytic domain with Pro/Thr-rich repeats in the interdomain linker, which includes residues 89–252 (Ra-ChiC$_{89–252}$; Fig. 1*c*), was constructed by PCR using an Ra-ChiC DNA clone as template. The forward (5′-CGC **CAT ATG** GGA ACT ACA CCA TCC GAT-3′; the *Nde*I site is shown in bold) and reverse primers (5′-TGC **TCT AGA** ATA TCT GCC GCC ATA GCT CTT-3′; the *Xba*I site is shown in bold) for PCR were synthesized for the regions corresponding to amino-acid residues 89–94 and 246–252 of Ra-ChiC$_{89–252}$, respectively. PCR was conducted according to the method described by Ueda *et al.* (2009). A 0.5 kb DNA fragment obtained by PCR was cloned into pCR II-Blunt-Topo vector (Invitrogen). The nucleotides of the amplified fragments were confirmed by sequencing after ligation. The pCR II-Blunt-Topo vector containing the DNA fragment was treated with *Nde*I and *Xba*I and the fragment was recovered by agarose-gel electrophoresis. The DNA fragment and pCold I vector, which had been digested by *Nde*I and *Xba*I, were mixed and ligated with T4 DNA ligase. Ra-ChiC$_{89–252}$ was expressed and purified in *E. coli* in the same manner as used for Ra-ChiC$_{mature}$.

2.2. Crystallization

Initial crystallization conditions were screened by the sitting-drop vapour-diffusion method using Crystal Screen (CS) and Crystal Screen 2 (CS2) from Hampton Research and Wizard I (WZ I), Wizard II (WZ II) and Precipitant Synergy (PS) from Emerald BioSystems at 293 K. The screens were performed using 96-well Intelli-Plates (Art Robbins Instruments, USA) and a Hydra II Plus One (Thermo Fisher Scientific, USA). Drops were prepared by mixing 0.3 µl reservoir solution with 0.3 µl protein solution and were equilibrated by vapour diffusion against the reservoir solution. The crystallization conditions for diffraction studies were optimized by the hanging-drop vapour-diffusion method, mixing 2.0 µl reservoir solution with 2.0 µl protein solution.

2.3. X-ray diffraction and molecular replacement

X-ray diffraction data from a crystal of the catalytic domain of Ra-ChiC were collected in a cold nitrogen-gas stream at 100 K using an MX-225HE system (Rayonix, USA) and synchrotron radiation (1.000 Å wavelength) on beamline BL41XU at SPring-8 (Hyogo, Japan). The oscillation angle was 1.0° and the exposure time was 1.0 s per frame. A total of 180 diffraction images were recorded at a camera distance of 170 mm and were processed using *HKL*-2000 (Otwinowski & Minor, 1997). Molecular-replacement (MR) analysis was performed using the program *Phaser* (McCoy *et al.*, 2007) using the coordinates of goose lysozyme (PDB entry 154l; Weaver *et al.*, 1995), which has 16% sequence identity to Ra-ChiC$_{89–252}$. The partial structure of bovine annexin VI (PDB entry 1avc; Avila-Sakar *et al.*, 1998) and the monozinc carbapenemase CphA from *Aeromonas hydrophila* (PDB entry 1x8g; Garau *et al.*, 2005) were also selected as

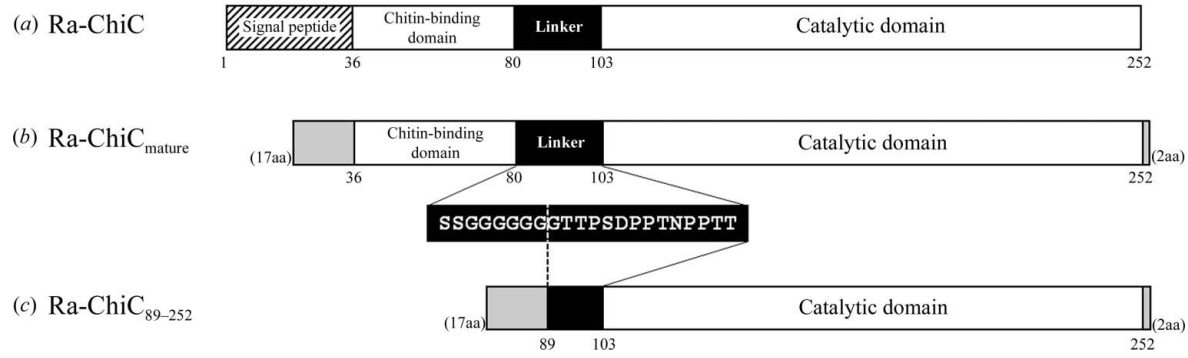
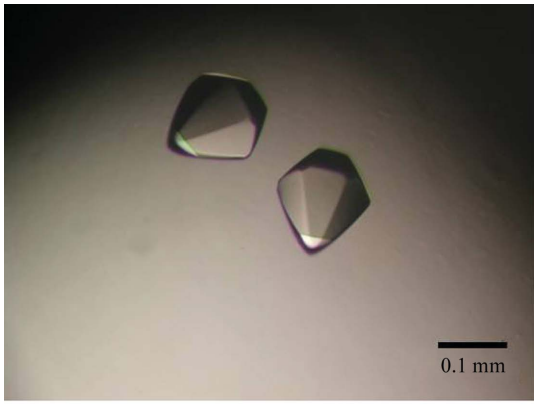


Figure 1
Schematic representation of the Ra-ChiC domain structure. (*b*) and (*c*) are the recombinant constructs used in this study. The pCold I vector-derived amino acids correspond to the grey regions in (*b*) and (*c*).

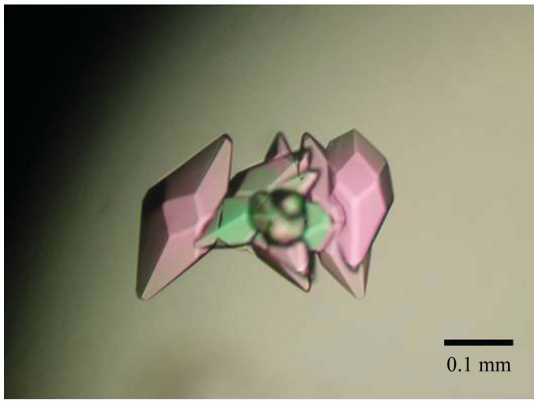
search models for MR analysis from a protein *BLAST* search (Altschul *et al.*, 1997).

3. Results and discussion

Crystallization conditions for Ra-ChiC$_{mature}$ (Fig. 1*b*) were screened using the screening kits at 100 and 50% (CS, CS2, WZ I and WZ II) or 100, 67 and 33% (PS) of the initial concentrations of the precipitating agents. In addition, the protein was prepared at four different concentrations (20, 10, 6.5 and 3.3 mg ml^{-1}) for each crystallization screening. However, Ra-ChiC$_{mature}$ did not crystallize despite the screening of over 2000 crystallization conditions. The difficulty in crystallization of Ra-ChiC$_{mature}$ might derive from the flexibility of the protein, the linker of which includes seven consecutive glycines between the two domains. Therefore, we constructed a deletion mutant, Ra-ChiC$_{89–252}$ (Fig. 1*c*), from which the chitin-binding domain and consecutive glycines were removed, for crystallization. Ra-ChiC$_{89–252}$ eluted as two independent peaks in cation-exchange chromatography. The two eluates were concentrated to 3.6 mg ml^{-1} (sample 1) and 2.8 mg ml^{-1} (sample 2). A total of 384 conditions were screened for crystallization of samples 1 and 2 using CS, CS2, WZ I, WZ II and PS. When using PS the original solution was also diluted to 33% for crystallization screening. After 10 d, small crystals had formed in 22 conditions. There was no significant difference in crystallization between samples 1 and 2. Two crystallization conditions, PS condition No. 44 at 33% concentration and PS condition No. 49 at 33% concentration, were optimized to produce crystals suitable for X-ray analysis. From the results of optimization, bipyramidal crystals with dimensions of over 0.1 mm were obtained using both precipitant solutions (Fig. 2). Crystals *A* and *B* were obtained in 6%(*v*/*v*) PEG 400, 9%(*w*/*v*) PEG 1500, 30 m*M* HEPES pH 7.5 (PS condition No. 44 at 30% concentration) and 1.2%(*v*/*v*) 2-propanol, 7.5%(*w*/*v*) PEG 3350, 30 m*M* calcium chloride, 30 m*M* HEPES pH 7.5 (PS condition No. 49 at 30% concentration), respectively.



(*a*)



(*b*)

Figure 2
Crystals of Ra-ChiC$_{89–252}$. (*a*) Crystal *A* from 6%(*v*/*v*) PEG 400, 9%(*w*/*v*) PEG 1500, 30 m*M* HEPES pH 7.5. (*b*) Crystal *B* from 1.5%(*v*/*v*) 2-propanol, 7.5%(*w*/*v*) PEG 3350, 30 m*M* calcium chloride, 30 m*M* HEPES pH 7.5.

Table 1
Data-collection and reduction statistics for Ra-ChiC$_{89–252}$ crystals.

Values in parentheses are for the highest resolution shell.

	Crystal *A*	Crystal *B*
Space group	$P6_122$ or $P6_522$	$P6_122$ or $P6_522$
Unit-cell parameters (Å)	$a = b = 100.0$, $c = 242.8$	$a = b = 99.7$, $c = 242.5$
Resolution (Å)	35.2–1.90	35.3–1.85
No. of measured reflections	653979	898217
No. of unique reflections	56315	59568
Multiplicity	11.7 (6.7)	15.1 (6.0)
$\langle I/\sigma(I)\rangle$	32.6 (2.7)	47.3 (3.2)
R_{merge}† (%)	8.0 (43.8)	7.7 (40.5)
Completeness (%)	97.8 (89.1)	96.7 (88.0)
Wilson plot *B* factor (Å^2)	24.9	26.3

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

For X-ray diffraction measurements at 100 K, crystals *A* and *B* were soaked in reservoir solutions containing cryoprotectant [PEG 400 for crystal *A* and 2-methyl-2,4-pentanediol (MPD) for crystal *B*]. Crystal *A* was soaked in 28%(*v*/*v*) PEG 400, 9%(*w*/*v*) PEG 1500, 30 m*M* HEPES pH 7.5 and crystal *B* was soaked in 20%(*v*/*v*) MPD, 7.5%(*w*/*v*) PEG 3350, 30 m*M* calcium chloride and 30 m*M* HEPES pH 7.5. Both soaking experiments were carried out by increasing the concentration of the cryoprotectant in a stepwise fashion. Crystals *A* and *B* belonged to space group $P6_122$ or $P6_522$, with unit-cell parameters $a = b = 100$, $c = 243$ Å. The Matthews coefficient (V_M) was 3.2, 2.4 or 1.9 Å^3 Da^{-1}, assuming the presence of three, four or five molecules in the asymmetric unit, respectively. The diffraction data sets from crystals *A* and *B* were integrated and scaled to maximum resolutions of 1.90 and 1.85 Å, respectively. The data-collection statistics are summarized in Table 1. Both crystals had sufficient diffraction quality for continued structure determination to elucidate the structure–function relationship of Ra-ChiC in detail. MR analysis was attempted using several sets of coordinates; however, no search model gave a significant solution for phase determination. Therefore, a search for heavy-atom derivatives for phase determination is under way using the isomorphous replacement and multiple/single anomalous dispersion (MAD/SAD) methods. In addition, selenomethionyl Ra-ChiC$_{89–252}$ is being prepared for SAD/MAD analysis.

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