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Received 5 June 2013
Accepted 18 July 2013

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Crystallization and preliminary X-ray crystallographic analysis of cycloisomaltooligosaccharide glucanotransferase from *Bacillus circulans* T-3040

Bacillus circulans T-3040 cycloisomaltooligosaccharide glucanotransferase (BcCITase) catalyses an intramolecular transglucosylation reaction and produces cycloisomaltooligosaccharides from dextran. BcCITase was over-expressed in *Escherichia coli* in two different forms and crystallized by the sitting-drop vapour-diffusion method. The crystal of BcCITase bearing an N-terminal His₆ tag diffracted to a resolution of 2.3 Å and belonged to space group *P*3₁21, containing a single molecule in the asymmetric unit. The crystal of BcCITase bearing a C-terminal His₆ tag diffracted to a resolution of 1.9 Å and belonged to space group *P*2₁2₁2₁, containing two molecules in the asymmetric unit.

1. Introduction

Cycloisomaltooligosaccharides (CIs) or cyclodextrins are cyclic oligosaccharides of α-1,6-linked glucose residues (Oguma *et al.*, 1993). *Bacillus circulans* strain T-3040 mainly produces CIs with seven to nine glucose units (CI-7 to CI-9) from dextran and releases them into the culture broth. Recently, this bacterium was found to produce CIs with continuous higher degrees of polymerization of up to 17 (CI-17; Funane *et al.*, 2007, 2008). CIs have been reported to strongly inhibit glucanase activity, suggesting that they might have anti-plaque activity (Kobayashi *et al.*, 1995). An inclusion complex-forming ability of CI-10 towards Victoria Blue B has also been reported (Funane *et al.*, 2007). Thus, CIs are expected to be useful functional materials in the food and medical industries.

Cycloisomaltooligosaccharide glucanotransferases (CITases; EC 2.4.1.248) catalyse the intramolecular transglucosylation of dextran to produce CIs (Oguma *et al.*, 1994) and were first isolated from the culture solution of *B. circulans* strain T-3040. The nucleotide sequence of the *B. circulans* T-3040 *cit* gene encoding CITase (BcCITase) shows that it belongs to glycoside hydrolase family 66 (GH66; Oguma *et al.*, 1995) according to the CAZy database (<http://www.cazy.org/>; Henrissat & Bairoch, 1996; Cantarel *et al.*, 2009). Most enzymes in this family are dextranases, which hydrolyse dextran to produce isomaltooligosaccharides. Compared with GH66 dextranases, the amino-acid sequence identity of BcCITase is about 20–30%, and it has a unique 89-amino-acid insertion (Tyr442–Tyr530; the methionine encoded by the translation initiation site of the *cit* gene is numbered residue 1) in the middle part of the enzyme which is absent in most GH66 dextranases (Aoki & Sakano, 1997). The amino-acid sequence shows that this inserted domain belongs to carbohydrate-binding module family 35 (Funane *et al.*, 2011). Besides BcCITase, CITase and dextranase from *Paenibacillus* species possess this inserted domain and these enzymes also show CI-producing activity (Funane *et al.*, 2008; Suzuki, Terasawa *et al.*, 2012; Kim, Yamamoto *et al.*, 2012); however, *Bacteroides thetaiotaomicron* dextranase lacking this inserted domain also has CI-producing activity (Kim, Kiso *et al.*, 2012). Furthermore, the deletion mutants MΔ234 (residues 442–530 deleted) and MΔ23Δ (residues 442–530 and 739–972 deleted), in which this domain was removed from BcCITase, still retained the CI-producing activity and the function of this inserted domain remains unknown (Funane *et al.*, 2011).

We have recently determined the first three-dimensional structure of a GH66 dextranase from *Streptococcus mutans* (SmDex; Suzuki, Kim *et al.*, 2012). The catalytic domain is a $(\beta/\alpha)_8$ -barrel and the β -domains are arranged at both the N- and C-termini. The catalytic residues of GH66 enzymes have been elucidated by structural and biochemical analyses; Asp385 of SmDex is the catalytic nucleophile and Glu453 is the acid/base of the double-displacement mechanism.

Although biochemical approaches have shed light on the enzymatic mechanism of the CI-producing activity of CITases (Funane *et al.*, 2011; Yamamoto *et al.*, 2006; Suzuki, Terasawa *et al.*, 2012), no structural information on CITases is available. In order to develop a CI-producing CITase for industrial applications, information describing the three-dimensional structure of a CITase is indispensable. Here, we report the crystallization and preliminary crystallographic analysis of BcCITase. The three-dimensional structure will aid protein engineering and provide a rationale for the design of CITases. The structure will also help to reveal its associated catalytic and cyclization mechanisms as well as the function of the inserted domain specific to CITases.

2. Materials and methods

2.1. Expression and purification

For the crystallization trials, we constructed two enzyme forms containing His₆ purification tags at either the N-terminus (BcCITase-NH) or the C-terminus (BcCITase-CH). Both constructs contained residues Ser39–Met738 (molecular mass 78 kDa) of the full-length BcCITase protein, which correspond to the protease-insensitive deletion mutant that we constructed for SmDex (Kim *et al.*, 2011; Suzuki *et al.*, 2011). The N-terminal signal peptide (Met1–Ala38) and the C-terminal unconserved region (Met739–Ser972) were deleted from BcCITase-NH and BcCITase-CH. These two constructs have higher stability and have almost the same CI-producing activity as the full-length enzyme (Funane *et al.*, 2011).

Expression and purification of BcCITase-NH were performed as described previously (Funane *et al.*, 2011). Briefly, the protein was expressed in *Escherichia coli* BL21 (DE3) cells and purified by Ni-chelating affinity chromatography. Since the BcCITase-NH protein contains an extra 22 amino acids including the His₆ tag with a thrombin cleavage site at the N-terminus, which might disturb crystallization, the expression plasmid for BcCITase-CH, which did not contain a thrombin cleavage site, was constructed as follows. The required fragment of the *cit* gene was amplified by PCR using Phusion DNA polymerase (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and the two oligonucleotide primers AGT-**CCATGGGCTCAGGCTCTGGCGGCATCG** and AGT**CTCGAG-CATGTAGATCATATCCAGTA** (*Nco*I and *Xho*I sites are shown in bold). The amplified fragment was inserted into the pET-28a vector (Merck–Novagen, Madison, Wisconsin, USA) at the *Nco*I/*Xho*I site using a DNA ligation kit (Takara, Otsu, Japan). The expression plasmid was transformed into *E. coli* BL21 (DE3) cells. Enzyme expression and purification were conducted using the same procedure as used to express and purify the BcCITase-NH enzyme, which has been described previously (Funane *et al.*, 2011).

The His₆ tags were retained on both constructs for crystallization trials. The homogeneity of the purified enzyme was determined by SDS–PAGE and the protein concentration was determined by measuring the absorbance at 280 nm.

2.2. Crystallization

The purified BcCITase-NH enzyme was concentrated to 10 mg ml^{−1} using an Amicon Ultra centrifugal filter device (Merck–Millipore, Billerica, Massachusetts, USA) after dialysis against 10 mM Tris–HCl buffer pH 8.0. Sparse-matrix crystal screening was performed using the following crystallization kits: Crystal Screen HT, Index HT (Hampton Research, Aliso Viejo, California, USA) and Wizard I and II (Emerald BioStructures, Bainbridge Island, Washington, USA). Sitting-drop vapour-diffusion trials were set up using a Thermo Scientific Matrix Hydra II (Thermo Fisher Scientific Inc., Hudson, New Hampshire, USA) automated liquid-handling system in 96-well Intelli-Plates (Art Robbins Instruments, Sunnyvale, California, USA) at 293 K using 50 μ l reservoir solution and with each drop consisting of 0.3 μ l protein solution and 0.3 μ l reservoir solution. Within a few weeks, several crystals were observed using the condition 1.0 M sodium acetate trihydrate, 0.1 M HEPES pH 7.5, 0.05 M cadmium sulfate hydrate (Crystal Screen HT condition No. 82). The quality of the obtained crystals was checked by diffraction images using an R-Axis VII X-ray diffractometer (Rigaku, Tokyo, Japan). Because of the good reproducibility of the crystals as shown in Fig. 1(a), the precipitant conditions 1.0 M sodium acetate trihydrate, 0.1 M HEPES pH 7.5, 0.05 M cadmium sulfate hydrate, 12%(v/v) glycerol and a

Figure 1
Crystals of BcCITase. (a) BcCITase-NH, (b) BcCITase-CH. The scale bars indicate 100 μ m.

Table 1

Data-collection statistics for BcCITase crystals.

Values in parentheses are for the outer shell.

Data set	BcCITase-NH	BcCITase-CH
Diffraction source	PF BL-6A	PF-AR NW12
Wavelength (Å)	0.978	1.000
Temperature (K)	95	95
Detector	ADSC Quantum 4R	ADSC Quantum 210
Space group	$P3_121$	$P2_12_12_1$
Unit-cell parameters (Å)	$a = b = 106.4$, $c = 160.6$	$a = 61.0$, $b = 169.2$, $c = 172.5$
Mosaicity (°)	0.229	0.293
Resolution range (Å)	92.2–2.30 (2.38–2.30)	120.8–1.90 (1.97–1.90)
Total No. of reflections	786631	1032071
No. of unique reflections	47410 (4667)	139159 (13644)
Completeness (%)	100.0 (100.0)	98.5 (97.9)
Multiplicity	16.6 (16.7)	7.4 (7.2)
Average $I/\sigma(I)$	47.1 (10.6)	24.0 (3.8)
R factor†	0.079 (0.423)	0.081 (0.583)
Overall Wilson plot B factor (Å ²)	29.9	24.4

† The R factor was estimated by multiplying the conventional R_{merge} value by the factor $[N/(N-1)]^{1/2}$, where N is the data multiplicity. $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the i th observation of reflection hkl and $\langle I(hkl) \rangle$ is the weighted average intensity for all observations i of reflection hkl .

protein solution with a concentration of 7 mg ml^{−1} in 10 mM Tris–HCl buffer pH 8.0 ($A_{280}^{1\text{ mm}} = 1.4$) were adopted.

The purified BcCITase-CH enzyme was concentrated to 20 mg ml^{−1} ($A_{280}^{1\text{ mm}} = 4.0$) and crystallization screening was performed in a similar fashion to that used for the BcCITase-NH enzyme. Within a few weeks, several crystals were observed in the condition 1.6 M ammonium sulfate, 0.1 M MES pH 6.5, 10% (v/v) 1,4-dioxane (Crystal Screen HT condition No. 71). BcCITase-CH gave crystal clusters, as shown in Fig. 1(b).

2.3. Data collection and processing

Diffraction data for the BcCITase-NH enzyme were collected on beamline BL-6A of the Photon Factory (PF), High Energy Accelerator Research Organization, Tsukuba, Japan. A single crystal was mounted in quartz glass capillaries of 0.3 mm diameter, the precipitant solution around the crystal was removed using paper wicks and the crystal was flash-cooled in a nitrogen stream at 95 K. Diffraction data were collected with 1° oscillations with 20 s exposure time over a total of 270° at a wavelength of 0.978 Å using a Quantum 4R detector (ADSC, Poway, California, USA).

Diffraction data for the BcCITase-CH enzyme were collected on beamline BL-NW12 of the Photon Factory-Advanced Ring (PF-AR; Chavas *et al.*, 2012). A single crystal of BcCITase-CH was obtained by teasing apart the cluster using a plastic pick. The single crystal was soaked in the reservoir solution containing 10% (v/v) ethylene glycol, immediately scooped into a 0.3 mm nylon loop (Hampton Research) and flash-cooled in a nitrogen stream at 95 K. Diffraction data were collected with 0.5° oscillations with 2 s exposure time over a total of 180° at a wavelength of 1.000 Å using a Quantum 210 CCD detector (ADSC).

The data sets were processed using the programs *DENZO* and *SCALEPACK* from the *HKL-2000* package (Otwinowski & Minor, 1997).

3. Results and discussion

The optimized crystallization conditions for BcCITase-NH gave crystals of dimensions 400 × 100 × 100 µm within a week (Fig. 1a). Using synchrotron radiation, the BcCITase-NH crystals diffracted to

a maximum resolution of 2.3 Å (Fig. 2a). The crystal belonged to the trigonal space group $P3_121$ or $P3_221$, with unit-cell parameters $a = b = 106.4$, $c = 160.6$ Å. The processing statistics of the collected data are summarized in Table 1. The Matthews coefficient was calculated to be 3.28 Å³ Da^{−1} (Matthews, 1968), corresponding to a solvent content of 62.5%, and the asymmetric unit contained one BcCITase-NH molecule.

The optimized crystallization conditions for BcCITase-CH gave crystals of dimensions 150 × 70 × 70 µm within a week (Fig. 1b). The BcCITase-CH crystals diffracted to a maximum resolution of 1.9 Å (Fig. 2b). The crystal belonged to the orthorhombic space group

Figure 2
X-ray diffraction images. (a) BcCITase-NH, (b) BcCITase-CH.

$P2_12_12_1$, with unit-cell parameters $a = 61.0$, $b = 169.2$, $c = 172.5$ Å. The Matthews coefficient was calculated to be $2.81 \text{ Å}^3 \text{ Da}^{-1}$ (Matthews, 1968), corresponding to a solvent content of 56.2%, and the asymmetric unit contained two BcCITase-CH molecules.

The ambiguity of the space group of BcCITase-NH between $P3_121$ and $P3_221$ was resolved by the molecular-replacement method using the SmDex structure (26% amino-acid sequence identity; PDB entry 3vmn; Suzuki, Kim *et al.*, 2012) as a reference model with the program *MOLREP* in the *CCP4* program package (Vagin & Teplyakov, 2010; Winn *et al.*, 2011). Assuming the space group was $P3_121$, a solution was found with a score of 0.326, which was better than the score of 0.283 obtained for space group $P3_221$. The calculated phases from the molecular-replacement method gave an electron-density map for the core structure of BcCITase-NH, but were not sufficient to build the whole structural model. Thus, phase determination will be conducted using the single- or multiple-wavelength anomalous dispersion (SAD or MAD) techniques using selenomethionine-substituted BcCITase.

We would like to thank the beamline researchers and staff at the PF. This work was partially supported by the Program for Promotion of Basic and Applied Research for Innovations in Bio-oriented Industry (BRAIN) and a Grant-in-Aid from JSPS (Japan Society for the Promotion of Science) Fellows relating to the JSPS Postdoctoral Fellowship for Foreign Researchers (17-05467).

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