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Crystallization and preliminary X-ray study of γ-type cyclodextrin glycosyltransferase from *Bacillus clarkii*

Masatake Akita,[a]* Yuji Hatada,[a] Yuko Hidaka,[a] Yukari Ohta,[a] Masayasu Takada,[b] Yoshinori Nakagawa,[b] Koichi Ogawa,[b] Teruo Nakakuki,[b] Susumu Ito[a] and Koki Horikoshi[a]

[a]Japan Marine Science and Technology Center (JAMSTEC), 2-15 Natsushima, Yokosuka 237-0061, Japan, and [b]Nihon Shokuhin Kako Co. Ltd, 30 Tajima, Fuji 417-8530, Japan

Correspondence e-mail: akitam@jamstec.go.jp

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A γ-cyclodextrin glycosyltransferase (EC 2.4.1.19) from *Bacillus clarkii* was crystallized using the hanging-drop vapour-diffusion method at 293 K. X-ray diffraction data were collected to 2.2 Å. The crystal belongs to space group *R*3, with unit-cell parameters $a = b = 211.6$, $c = 52.7$ Å. The asymmetric unit contains one protein molecule, with a corresponding V_M of 3.03 Å^3 Da^{-1} and a solvent content of 59.4%. Molecular replacement was successfully carried out using a homology model based on the three-dimensional structure of the CGTase from *Thermonanaerobacterium thermosulfurigenes* EM1 as a search model.

1. Introduction

Cyclodextrins (CDs), known to occur as α-, β- and γ-CDs comprising six, seven and eight glucosides, respectively, are circular α-1,4-linked oligoglucosides that have a hydrophobic interior and a hydrophilic exterior. They can accommodate various compounds within the cavity of their ring structures, modifying the chemical and physical properties of the guest compounds. Therefore, CDs are widely used in industry for various purposes (Duchêne, 1991).

CDs are synthesized from starch by cyclodextrin glycosyltransferases (CGTases; EC 2.4.1.19) *via* a transglycosylation reaction (Van der Veen *et al.*, 2000). The formation of CD starts with the cleavage of an α-1,4-glycosidic bond in the starch, resulting in the formation of a covalently bound enzyme–substrate intermediate. The reaction is followed by cyclization of the substrate, which precedes the formation of a new intramolecular α-1,4-glycosidic bond (Uitdehaag *et al.*, 2000).

Usually, the products of the various CGTases are mixtures of α-, β- and γ-CDs. α- and β-CDs are the dominant products of CGTases from various bacteria such as *Bacillus macerans* (α-CGTase; Takano *et al.*, 1986), *B. circulans* 251 (β-CGTase; Penninga *et al.*, 1995) and *Bacillus* sp. strain 290–3 (γ/β-CGTase; Englbrecht *et al.*, 1990). Recently, Takada *et al.* (2003) have isolated a CGTase from *B. clarkii* 7364 for the first time and found that it predominantly produces γ-CD. Although the amino-acid sequence similarity is quite high (∼70% identity) among the CGTases from these *bacilli*, their main products are different. The amino-acid sequence alignment suggests that the product specificity of the *B. clarkii* γ-CGTase arises from the absence of several residues at subsite −7 and the deletion of two residues at subsite −3 (Takada *et al.*, 2003; Parsiegla *et al.*, 1998).

The main goal of this study is to clarify the key to the product specificity of CGTases from a three-dimensional structural point of view. Here, we describe the crystallization and preliminary X-ray diffraction analysis of the γ-CGTase from *B. clarkii*.

2. Expression and purification of recombinant γ-CGTase

B. clarkii 7364 was originally isolated by Takada *et al.* (2003). *Escherichia coli* JM-109 harbouring plasmid pGEFT-1 was used as the host for cloning the γ-CGTase gene from *B. clarkii* (Takada *et al.*, 2003). To produce the CGTase extracellularly, the gene in the plasmid was amplified by PCR using LA *Taq* DNA polymerase (TaKaRa Bio, Kyoto, Japan) and then inserted into the cloning site of the expression vector pHSP64 (Sumitomo *et al.*, 1995). The recombinant plasmid was introduced into *B. subtilis* ISW1214 cells. The *Bacillus subtilis* ISW1214 cells harbouring the plasmid were cultured at 303 K for 48 h with shaking in a liquid medium composed of 12%(*w*/*v*) corn steep liquor (Nihon Syokuhin Kako, Shizuoka, Japan), 0.2%(*w*/*v*) Lab-Lemco powder (Oxoid, Hampshire, UK), 0.1%(*w*/*v*) yeast extract (Difco, MD, USA), 0.1%(*w*/*v*) KH_2PO_4, 0.02%(*w*/*v*) $MgSO_4{\cdot}7H_2O$, 0.05%(*w*/*v*) $CaCl_2$, 6%(*w*/*v*) maltose and 15 µg ml^{-1} tetracycline. Cells and culture supernatants were separated by centrifugation at 12 000*g* for 10 min. The supernatant obtained was dialyzed against 10 m*M* Tris–HCl buffer pH 7.5 and then used for enzyme purification. The dialyzed solution was loaded onto a DEAE-Toyopearl 650 M column (Tosoh, Tokyo, Japan) equilibrated in 10 m*M* Tris–HCl buffer pH 7.5 and eluted with a linear gradient of 0–500 m*M* NaCl in 10 m*M* Tris–HCl pH 7.5 buffer. The elution was loaded onto a

Table 1
Crystallographic parameters and data-collection statistics.

Values in parentheses refer to the outer shell.

Space group	*R*3
Unit-cell parameters (Å)	$a = b = 211.6$, $c = 52.7$
Resolution limits (Å)	100.0–2.2 (2.28–2.20)
No. observed reflections	230875
No. unique reflections	42887
Redundancy	5.1 (1.8)
Average $I/\sigma(I)$	20.7 (4.27)
Completeness (%)	95.7 (73.3)
$R_{\rm merge}$ (%)	11.7 (37.3)

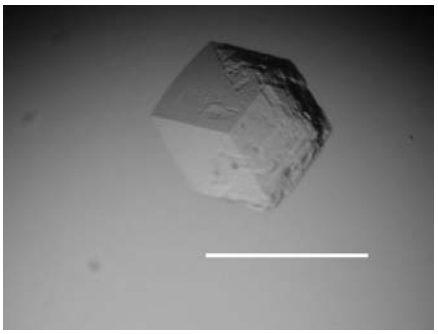

Figure 1
A hexagonal bipyramid crystal of γ-CGTase. The scale bar is 0.2 mm.

hydroxyapatite column (Seikagaku Kogyo) equilibrated in 5 m*M* sodium phosphate buffer pH 7.0 and eluted with a linear gradient of 5–500 m*M* sodium phosphate. The recombinant γ-CGTase-containing fractions were concentrated and loaded onto a Superdex-75 column (Amersham Biosciences, Piscataway, USA) equilibrated in 20 m*M* sodium phosphate buffer pH 7.2 supplemented with 150 m*M* NaCl. The enzyme, which was pure according to SDS–PAGE, was dialyzed against 10 m*M* Tris–HCl buffer pH 7.0 and the retentate was concentrated to 10 mg ml^{-1}. The protein concentration was measured using a Bio-Rad (Hercules, CA, USA) protein-assay kit with bovine serum albumin as a standard.

3. Crystallization and data collection

Crystallization of γ-CGTase was performed at 293 K using the hanging-drop vapour-diffusion method. The initial crystal screening of γ-CGTase was carried out using Hampton Research (Aliso Viejo, CA, USA) Crystal Screens I, II, Lite and Cryo. 2 µl protein solution, prepared as above, was mixed with an equal volume of reservoir solution and equilibrated against 800 µl reservoir solution. After two weeks, square-planar crystals with maximum dimensions of 50 × 50 × 10 µm appeared in condition No. 22 of Crystal Screen I. The conditions were varied in order to obtain larger crystals and hexagonal bipyramidal crystals appeared using 0.1 *M* Tris–HCl buffer pH 8.0, 0.2 *M* sodium acetate and 30%(*w*/*v*) polyethylene glycol 6000 as the precipitant. One crystal reached maximum dimensions of 200 × 200 × 100 µm after 1 month.

The crystal was mounted on a cryoloop and flash-frozen at 100 K by plunging it into liquid nitrogen without added cryoprotectant [30%(*w*/*v*) polyethylene glycol 6000 is a cryoprotectant]. X-ray diffraction data were collected using an R-AXIS VII imaging plate (Rigaku, Tokyo, Japan) on an FR-E rotating-anode X-ray generator (Rigaku) operated at 45 kV and 45 mA with a copper target. The generated X-rays were focused using a confocal mirror. The X-ray wavelength was 1.54 Å. The data set was processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). We calculated the phases by the molecular-replacement method using *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 program package (Collaborative Computational Project, Number 4, 1994).

4. Results and discussion

The best crystallization conditions were 30%(*w*/*v*) polyethylene glycol 6000, 0.2 *M* sodium acetate and 0.1 *M* Tris–HCl buffer pH 8.0 (Fig. 1). The X-ray diffraction data from the γ-CGTase crystal were collected to a resolution of 2.2 Å. The space group was determined to be *R*3. A summary of the data-processing statistics is shown in Table 1. The asymmetric unit of the crystal contains one molecule (75 kDa), with an expected $V_{\rm M}$ of 3.03 Å^3 Da^{-1}, corresponding to a solvent content of 59.4% (Matthews, 1968). The initial phases of the crystal structure were determined using the molecular-replacement method. To construct the search model for molecular replacement, homology modelling was performed using the program package *MOE* v.2002.03 (Chemical Computing Group, Montreal, Canada). The template was the structure of the CGTase from *Thermonanaerobacterium thermosulfurigenes* EM1 (Knegtel *et al.*, 1996; PDB code 1ciu), which showed the highest sequence identity compared with γ-CGTase (57.7% in a 671-amino-acid overlap) of the structurally determined CGTases. The deduced model of the γ-CGTase was refined with an energy minimization implemented in *MOE* and then used for molecular replacement. After determination of the rotation and translation parameters, the correlation coefficient and *R* factor were 0.364 and 44.8%, respectively. One cycle of refinement (rigid-body, simulated annealing, energy minimization and *B*-factor refinement) was then performed using *CNS* v.1.0 (Brünger *et al.*, 1998). After refinement, the *R* factor and $R_{\rm free}$ were 27.2 and 30.2%, respectively. Most amino-acid residues are in good agreement with the calculated $2F_{\rm o} - F_{\rm c}$ electron-density map.

In order to clarify how the γ-CGTase acts on substrate, crystallographic analyses of the mutant γ-CGTase complexed with a variety of different substrate molecules are required. Structural information from these complexes should be useful in designing and/or modifying the functions of CGTases. We are now preparing γ-CGTase mutants and their complexes with various molecules. The γ-CGTase structure will be reported together with these other structures.

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