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Crystallization and preliminary X-ray diffraction studies of Tyr167His mutant α-cyclodextrin glucanotransferase from *Bacillus macerans*

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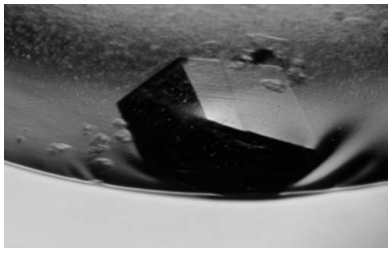

Improving the specificity of α-cyclodextrin glucanotransferase is a significant issue in the field of α-cyclodextrin production. In this study, a constructed Y167H mutant α-cyclodextrin glucanotransferase with enhanced α-cyclodextrin specificity was successfully expressed and purified. Single crystals were grown using PEG 4000 as a precipitating agent by the hanging-drop vapour-diffusion method at 293 K. The crystals exhibited two kinds of morphology in different crystallization conditions. The crystals diffracted to at least 2.2 Å resolution (space group $P2_12_12_1$), with unit-cell parameters a = 65.69, b = 78.70, c = 137.00 Å. Assuming the asymmetric cell to be occupied by a monomer of 75 kDa, the unit cell contains 43.77% solvent with a crystal volume per protein mass, V_M, of 2.19 Å^3 Da^{-1}.

1. Introduction

Cyclodextrin glucanotransferase (EC 2.4.1.19; CGTase) is an enzyme that converts starch into cyclodextrins (CDs). CDs are cyclic α-1,4-glycoside-linked oligosaccharides composed of six, seven and eight glucose residues, known as α-CDs, β-CDs and γ-CDs, respectively (Costa *et al.*, 2012). The CDs can form inclusion complexes with small hydrophobic molecules depending on their hydrophilic rim and their hydrophobic inside cavity (Saenger, 1980). The formation of these complexes alters the chemical, physical and biological properties of these molecules. This fascinating property of CDs leads to their wide functionality in different industries, such as those related to food, cosmetics and pharmaceutics (Del Valle, 2004; Szente & Szejtli, 2004). CGTases can be classified into three groups (α-CGTases, β-CGTases and γ-CGTases) based on their main products (α-CDs, β-CDs and γ-CDs, respectively). However, the bacteria known to produce β-CGTase form a much larger group than those that produce α-CGTase. Moreover, α-CDs possess a smaller cavity and are more soluble than β-CDs, thereby making them more desirable and in demand.

The wild-type strain *Bacillus* sp. 602-1 (stored in our laboratory) has a high α-CD specificity (α-CDs accounted for about 77% of the products). Further study of the site-saturation mutagenesis of Tyr167 at subsite −6 revealed that α-CGTase had a better α-CD specificity when Tyr167 was substituted by histidine: the proportion of α-CDs improved to 88%. Many studies of the crystallization as well as the structure of native and mutant β-CGTases have been carried out, most of which are based on those from *B. circulans* strain 251 (Lawson *et al.*, 1990, 1994; Uitdehaag *et al.*, 2000), *Thermoanaerobacterium thermosulfurigenes* EM1 (Wind *et al.*, 1998; Kelly *et al.*, 2010) and alkalophilic *Bacillus* sp. 1011 (Harata *et al.*, 1996; Ishii *et al.*, 2000). Nevertheless, to our knowledge there has been only one report on α-CGTase crystallization to date, which was performed using that isolated from *B. macerans* strain IFO 3490 (Choe *et al.*, 2003); the crystallization condition used was 20–25%(*w*/*v*) polyethylene glycol 6000, 100 m*M* PIPES buffer, 150 m*M* NaCl, 5 m*M* $CaCl_2$ pH 7.0 and the shape and the preliminary X-ray diffraction data of the crystal were reported.

In the present study, a mutant protein with high α-CD specificity (Y167H) was expressed, purified and crystallized in the presence of imidazole using the hanging-drop vapour-diffusion method. The crystallization condition was optimized and the preliminary X-ray

Table 1
Data-collection statistics for the Y167H mutant α-CGTase.

Values in parentheses are for the outermost resolution shell.

Space group	$P2_12_12_1$
Wavelength (Å)	1.54178
Resolution (Å)	20–2.2 (2.24–2.20)
Total reflections	190950
Unique reflections	36549 (173)
Multiplicity	5.2 (3.8)
Completeness (%)	99.4 (96.6)
$\langle I/\sigma(I)\rangle$	24.8 (4.0)
$R_{\rm merge}$†	0.072 (0.284)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of the reflection intensity and $\langle I(hkl)\rangle$ is the weighted average intensity for multiple and symmetry-related measurements.

diffraction data are reported. We hope that it can promote the determination and analysis of the structure of the mutant α-CGTase with the purpose of shedding further light on the factors affecting the enhancement of α-CD specificity.

2. Materials and methods

2.1. Expression

The Y167H mutant was constructed using site-directed mutagenesis based on the recombinant α-CGTase from *Bacillus* sp. 602-1 (stored in our laboratory). The mutant plasmid was used to transform competent *Escherichia coli* DH5α cells selected on LB medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl) with ampicillin (50 µg ml^{-1}) and verified using DNA sequencing. Mutant enzyme was expressed by transforming the mutant plasmid into the expression host *E. coli* BL21 (DE3) cells. About a 5 ml LB culture of the recombinant *E. coli* BL21(DE3) was grown at 310 K overnight in the presence of ampicillin (50 µg ml^{-1}). The cultures were then transferred into 5 ml TB medium (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol) with 50 µg ml^{-1} ampicillin inoculated at 1%. The TB medium was cooled on ice for 10 min until the OD_{600} value reached 0.6–0.8. Subsequently, the mutant α-CGTase was induced with 0.01 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) at 289 K. 150 m*M* glycine and 20 m*M* $CaCl_2$ were added after 24 h of cultivation. The total induction period lasted for 96 h.

2.2. Purification

The mutant enzyme was harvested and the supernatant obtained by centrifugation at 8000 rev min^{-1} for 30 min at 277 K was used as the crude enzyme. The enzyme was then purified by ammonium sulfate fractionation precipitation (from 25 to 50%). The precipitate from 50% ammonium sulfate precipitation was collected by centrifugation and redissolved in a final volume of 2–3 ml buffer *A* (10 m*M* Na_2HPO_4, 1.8 m*M* KH_2PO_4, 140 m*M* NaCl, 2.7 m*M* KCl, pH 7.0). Centrifugation and ultrafiltration were subsequently performed to remove impurities, after which the resultant supernatant was purified by Ni–NTA agarose column affinity chromatography (GE Healthcare). The nonspecifically binding proteins were removed using 80 ml wash buffer (50 m*M* Na_2HPO_4, 300 m*M* NaCl, 20 m*M* imidazole pH 7.0). The target proteins were then eluted with 10 ml elution buffer (50 m*M* Na_2HPO_4, 300 m*M* NaCl, 200 m*M* imidazole pH 7.0). Fractions of 2 ml were collected in each tube and the enzyme purity was analyzed by SDS–PAGE.

2.3. Crystallization

The fractions containing proteins with high purity were collected and concentrated to 8 mg ml^{-1} using a Millipore Amicon 30 kDa centrifugal device followed by a Millipore Amicon 10 kDa centrifugal device at 277 K. Initial screening was performed with the Crystal

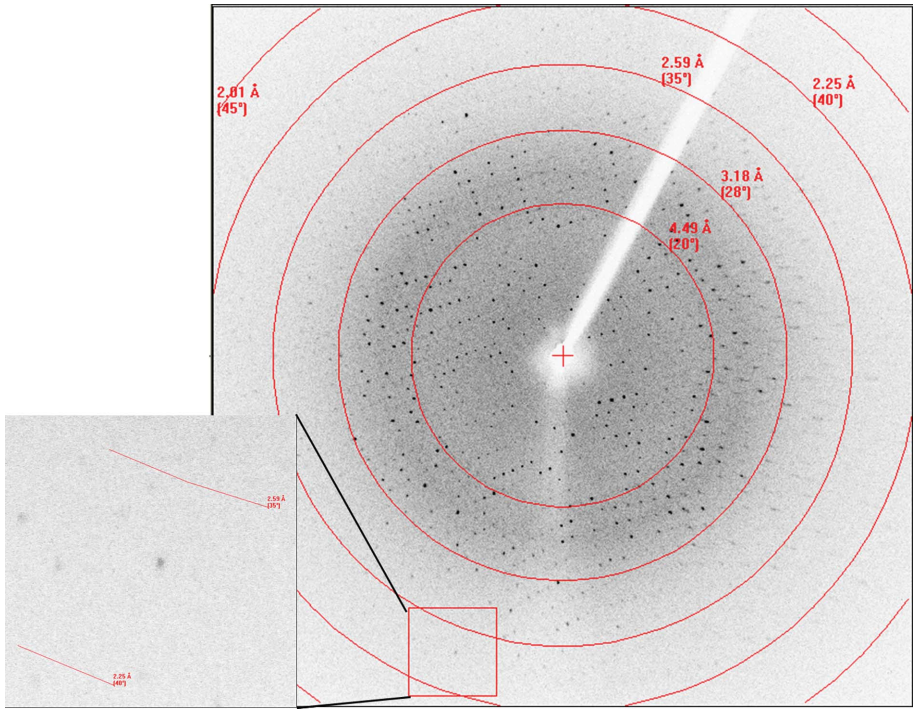

Figure 1
A typical X-ray diffraction image collected from a crystal of the Y167H mutant α-CGTase. The outer circle corresponds to a 2.2 Å resolution limit.

Screen and Crystal Screen 2 kits (Hampton Research) at 293 K using the hanging-drop vapour-diffusion method. 2 µl protein solution (8 mg ml^{-1}) mixed with an equal amount of reservoir solution was equilibrated against 300 µl reservoir solution.

2.4. Crystallographic data collection

For X-ray analysis, the crystals were soaked in a solution consisting of the original reservoir solution supplemented with 20%(*w*/*v*) glycerol and were then flash-cooled to 100 K. The diffraction data were collected in-house using a MicroMax-007 HF rotating-anode X-ray generator operating at 40 kV and 20 mA equipped with an R-AXIS image-plate detector, and the crystal diffracted to at least 2.2 Å resolution (Fig. 1). The data sets were indexed and processed using *HKL*-2000 (Otwinoski & Minor, 1997) and the data-set statistics are summarized in Table 1.

3. Results and discussion

The Y167H mutant enzyme was actively expressed under the experimental conditions and was successfully purified by Ni–NTA affinity chromatography. Based on the SDS–PAGE profiles, the protein exhibited a single band with a molecular weight of about 75 kDa (Fig. 2), which paved the way for subsequent crystallization trials. The molecular weight of this mutant was similar to that of the reported α-CGTase from *B. macerans* strain IFO 3490 (molecular weight of 74 kDa).

On the basis of the results of initial screening, preferable conditions were selected. Initially, the purified enzyme was dialyzed against buffer *A* to remove imidazole introduced during Ni–NTA column chromatography. The sample was concentrated to 8 mg ml^{-1}. Aciform crystals were first observed in 2 weeks using condition No. 22 of Crystal Screen (30% PEG 4000, 0.1 *M* Tris–HCl, 0.2 *M* sodium acetate buffer pH 8.5) at 293 K. Nevertheless, crystallization did not take place at 277 K and only a floccose precipitate appeared in the hanging drop. In addition, the crystal obtained with condition No. 22 of Crystal Screen appeared with a needle shape (typical dimensions of 0.1 × 0.1 × 0.6 mm) and the highest resolution attained was 2.6 Å (Fig. 3*a*). On the other hand, when the purified enzyme did not undergo the dialysis process to remove imidazole, crystals first appeared in 1 week and grew to a larger size under the same crystallization condition. This phenomenon obviously indicates that imidazole can accelerate the crystallization rate of the Y167H mutant enzyme. No crystals appeared in the hanging drop when the concentration of protein was below 8 mg ml^{-1}. However, when the protein concentration reached 10 mg ml^{-1} more crystals were observed with much smaller size. Thus, 8 mg ml^{-1} protein was chosen as the best concentration for crystal growth of the Y167H mutant enzyme. The crystallization was then further optimized using the precipitating agent PEG in the concentration range 15–30%(*w*/*v*), Tris–HCl in the range 0.05–0.1 *M* and sodium acetate buffer in the range 0.1–0.2 *M*. The result indicated that decreasing the concentration of precipitating agent PEG 4000 to 15% helped to form fewer but larger single crystals. Finally, promising single crystals were successfully obtained from the optimal condition (15% PEG 4000, 0.05 *M* Tris–HCl, 0.1 *M* sodium acetate buffer, 25 m*M* Na_2HPO_4, 150 m*M* NaCl, 10 m*M* imidazole pH 8.5) at 293 K with a protein concentration of 8 mg ml^{-1}. It should be noted that the shape of the crystals changed and they grew to a sufficient size (typical dimensions of 0.4 × 0.4 × 0.5 mm) for data collection within 1 week under the optimal condition (Fig. 3*b*). In summary, the crystallization method and the precipitating agent were different from those used to obtain the crystals of α-CGTase from *B. macerans* strain IFO 3490. In this study, two crystal shapes were obtained from the Y167H mutant enzyme under different conditions.

The crystals are orthorhombic, belonging to space group $P2_12_12_1$ with unit-cell parameters a = 65.69, b = 78.70, c = 137.00 Å. The space group of the reported wild-type α-CGTase is the same as this mutant α-CGTase, while the unit-cell parameters of the reported wild-type α-CGTase (a = 66.79, b = 79.66, c = 141.16 Å) are slightly different from the Y167H mutant. A preliminary estimation of the content of

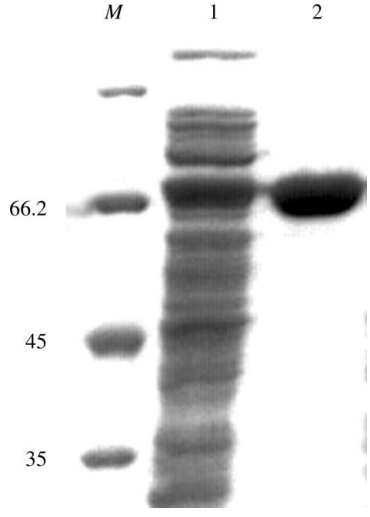

Figure 2
SDS–PAGE analysis of the purified mutant α-CGTase. Lane *M*, molecular-weight standards (labelled in kDa); lane 1, crude enzyme; lane 2, purified enzyme.

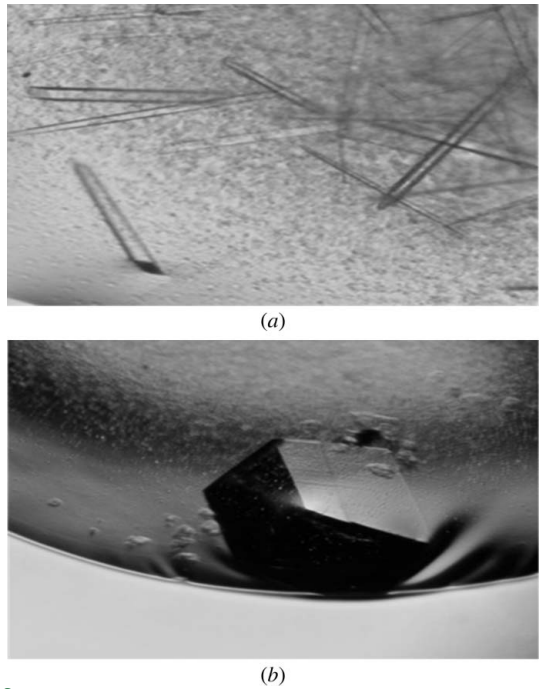

Figure 3
Representative crystals of the Y167H mutant α-CGTase. The crystals grown in 30% PEG 4000, 0.1 *M* Tris–HCl, 0.2 *M* sodium acetate buffer pH 8.5 displayed a needle shape (*a*), while the large single crystals grown in 15% PEG 4000, 0.05 *M* Tris–HCl, 0.1 *M* sodium acetate buffer, 25 m*M* Na_2HPO_4, 150 m*M* NaCl, 10 mM imidazole pH 8.5 had a cube shape (*b*).

the unit cell indicated that there should be one molecule per asymmetric unit, with a crystal volume per protein mass, V_M, of 2.19 Å^3 Da^{-1} (Matthews, 1968).

The structure determination of the Y167H mutant enzyme by molecular replacement is in progress. The data will be used to further investigate the structure of α-CGTase as well as to investigate the mechanism of product specificity of α-CGTase and its mutant enzymes.

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