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

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Single crystals of α-cyclodextrin glucanotransferase isolated from *Bacillus macerans* have been grown with polyethylene glycol 6000 as a precipitating agent by sitting-drop vapour diffusion at room temperature. The crystals were suitable for X-ray analysis and diffracted to at least 2.0 Å (space group $P2_12_12_1$), with unit-cell parameters $a = 66.79$ (2), $b = 79.66$ (1), $c = 141.16$ (1) Å. Assuming the asymmetric cell to be occupied by a monomer of 74 kDa, the unit cell contains 42.6% solvent with a crystal volume per protein mass, V_M, of 2.53 Å^3 Da^{-1}.

1. Introduction

Cyclodextrin glucanotransferase (CGTase; EC 2.4.1.19) is an extracellular enzyme which catalyzes the formation of cyclodextrins (CDs) from starch and related α-1,4-glucans by intramolecular transglycosylation in a Ca^{2+}-dependent manner (Saenger, 1980). CDs are cyclic oligosaccharides consisting of six, seven or eight glucose units (G6, G7, G8) and named α-, β- and γ-CDs, respectively. They are able to form inclusion complexes with many organic and inorganic molecules, thereby changing the physical and chemical properties of the included compounds. α-CDs are particularly useful for food-processing and for pharmaceutical applications owing to their higher solubility compared with β-CDs.

Bacillus CGTases have been classified into two types based on which forms of CDs are produced. One type is the *B. macerans* CGTase, which predominantly produces α-CD. The other type is the β-CD-forming CGTase found in both *B. megaterium* and *B. circulans*. The alkalophilic bacillus *B. stearothermophilus* CGTase, which produces α-CD and β-CD, is considered to be intermediate between these two types (Kitahata & Okada, 1982).

The great interest in CGTases for industrial use has promoted substantial work on the structures of these enzymes. Many X-ray structures of the intermediate or β-type and their mutants have been reported (Klein & Schulz, 1991; Lawson *et al.*, 1994; Knegtel *et al.*, 1995, 1996; Strokopytov *et al.*, 1996; Schmidt *et al.*, 1998; Mosi *et al.*, 1998; Penninga *et al.*, 1995; Wind *et al.*, 1998; Uitdehaag *et al.*, 1999; Ishii *et al.*, 2000).

The structure of the earliest known and biochemically well characterized α-specific CGTase from *B. macerans* (Saenger, 1980; Terada *et al.*, 2001) will considerably improve understanding of the differences between CGTases and their substrate spectra. Here, we report the crystallization of the typically α-CD-producing CGTase from *B. macerans*, which has a molecular weight of 74 kDa as a monomer (687 amino-acid residues).

2. Materials and methods

2.1. Purification of CGTase from *B. macerans*

CGTase was isolated from *B. macerans* strain IFO 3490 according to the modified method of Stavn & Granum (1979). Isolated colonies were aerobically cultivated in a shaking incubator (280 rev min^{-1}) at 310 K in 500 ml medium (1% soluble starch, 0.5% polypeptone, 0.5% yeast extract, 0.1% $KH_2PO_4.H_2O$, 0.02% $MgSO_4.7H_2O$ and 0.03% $CaCl_2.2H_2O$ pH 7.0) for 2 d. Cells were removed by centrifugation (30 min at 10 000*g*; 277 K). Crude extract from the *B. macerans* culture medium was precipitated with 35–60% saturated ammonium sulfate and centrifuged at 20 000 rev min^{-1} for 10 min at 277 K. The pellets were dissolved in 10 m*M* sodium/potassium phosphate buffer pH 7.2 and exhaustively dialyzed against the same buffer before being loaded onto a DEAE-cellulose column (2.6 × 25 cm). The enzyme was eluted with a potassium chloride gradient and dialyzed thoroughly against 10 m*M* Tris–HCl buffer pH 7.2 containing 5 m*M* $CaCl_2$. For further purification, a Superdex G-200 column (1.6 × 95 cm) was used and the active fractions were pooled. The purity was checked by SDS–PAGE.

2.2. Assay of CGTase functionality

The activity of CGTase was determined by the analysis of the CDs produced using high-

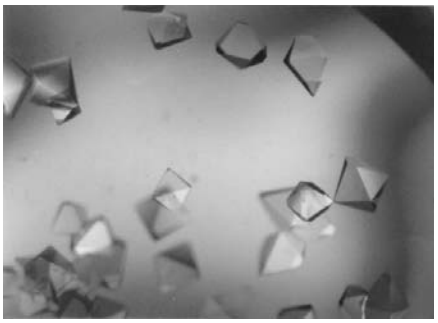

Figure 1
Orthorhombic crystals of CGTase grown with polyethylene glycol 6000. The approximate dimensions of the crystals shown in the figure are 0.3 × 0.3 × 0.4 mm.

performance liquid chromatography (HPLC) as described by Bender (1981). For this, 100 µl of purified enzyme solution (1.0 mg ml^{-1}) was incubated with 500 µl of 3% soluble starch (10 m*M* Tris–HCl, 5 m*M* $CaCl_2$ pH 7.2) for 3 min at 310 K. The reaction was stopped by boiling (5 min at 373 K) and the linear oligoglucoses were separated by methanol precipitation [1:1(*v*/*v*)]. After centrifugation (3 min; 13 000 rev min^{-1}; Eppendorf), the filtrates were analyzed by HPLC (using a Waters µ-Bondapak/carbohydrate column; 7.5 × 30 cm); the eluent was 65% acetonitrile in H_2O (the flow rate was 0.6 ml min^{-1}). The elution peaks were calibrated with pure α-, β- and γ-CD, respectively.

2.3. Crystallization of CGTase from *B. macerans*

For crystallization, the purified enzyme (15–20 mg per 10 l culture medium) was dialyzed against 100 m*M* PIPES buffer pH 7.0 containing 150 m*M* NaCl and 5 m*M* $CaCl_2$. The protein was concentrated to 20 mg ml^{-1} by ultrafiltration using a YM 30 membrane (Amicon). A rapid initial screening of crystallization conditions was conducted using a sparse matrix as described by Jancarik & Kim (1991). For this, 2–4 µl protein droplets (20 mg ml^{-1}) were used with the Hampton Research crystallization kit. Protein solutions were equilibrated against 1 ml of reservoir solution. Large single crystals were grown by the sitting-drop method (droplet size 20 µl).

3. Results and discussions

The purified CGTase from *B. macerans* was highly active (data not shown) and was found to be homogeneous by SDS–PAGE. In four conditions out of the 50 original sparse-matrix screen sets (Hampton Research; Nos. 1, 14, 24 and 46), white precipitation appeared within a day. These sets contained Ca^{2+} ions. They were rescreened by systematically varying the concentration of protein (10–25 mg ml^{-1}), the calcium concentration (5–10 m*M*), the buffer (PIPES, Tris–HCl; 50–100 m*M*) and the precipitating agents (MPD, PEG 4K, 6K, 8K) and their concentrations [15–30%(*w*/*v*)]. To investigate the effect of Ca^{2+} ions, all rescreens were set up with and without Ca^{2+} ions. Ca^{2+} ion-containing sets always displayed white precipitates, whereas in experiments without Ca^{2+} ions the initial white precipitate changed colour to a light yellow over several weeks. In these cases, the protein were found to be denatured.

After stepwise refinement of the crystallization conditions, the best single crystals were obtained by sitting-drop vapour diffusion with 20–25%(*w*/*v*) polyethylene glycol 6000 as a precipitating agent in 100 m*M* PIPES buffer pH 7.0 containing 150 m*M* NaCl and 5 m*M* $CaCl_2$ at room temperature. In the absence of Ca^{2+} ions, crystals were not obtained under any conditions tested. Microcrystals first appeared within a month and continued to grow to their maximum size in about two weeks. Fully grown crystals typically had dimensions of 0.3 × 0.3 × 0.4 mm (Fig. 1).

A single crystal was mounted in a capillary. X-ray data were collected with a MAR Research image-plate scanner using a rotating-anode generator (Enraf–Nonius) operating at 45 kV and 60 mA. The data-collection statistics are shown in Table 1. The crystals are orthorhombic, belonging to the space group $P2_12_12_1$, with unit-cell parameters a = 66.79 (2), b = 79.66 (1), c = 141.16 (1) Å, and diffract to 2.0 Å. They contain one molecule in the asymmetric unit (solvent content 42.6%) and the crystal volume per protein mass, V_M, is 2.53 Å^3 Da^{-1}. These values are within the commonly observed range for globular protein crystals (Matthews, 1968).

The CGTases are known to form a topologically well conserved class of enzymes. Therefore, structure solution by molecular replacement should be possible.

Table 1
Data-collection statistics of CGTase crystals.

Values in parentheses are for the highest resolution shell.

Resolution (Å)	70.7–2.0 (2.06–2.01)
Total reflections	266119 (9056)
Unique reflections	49570 (2817)
Redundancy	5.4 (3.2)
Completeness (%)	98.1 (77.5)
$I/\sigma(I)$	9.1 (5.1)
$R_{\rm merge}$† (%)	5.8 (14.2)

† $R_{\rm merge} = \sum |I - \langle I \rangle| / \sum I$, where $\langle I \rangle$ is mean intensity.

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