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Crystallization and preliminary X-ray crystallographic analysis of the amylomaltase from *Corynebacterium glutamicum*

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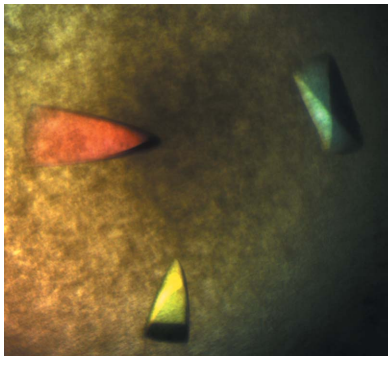

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Amylomaltase (AM; EC 2.4.1.25) belongs to the 4-α-glucanotransferase group of the α-amylase family. The enzyme can produce cycloamylose or large-ring cyclodextrin through intramolecular transglycosylation or cyclization reactions of α-1,4-glucan. Amylomaltase from the mesophilic bacterium *Corynebacterium glutamicum* (*Cg*AM) contains extra residues at the N-terminus for which the three-dimensional structure is not yet known. In this study, *Cg*AM was overexpressed and purified to homogeneity using DEAE FF and Phenyl FF columns. The purified *Cg*AM was crystallized by the vapour-diffusion method. Preliminary X-ray data showed that the *Cg*AM crystal diffracted to 1.7 Å resolution and belonged to space group $P2_12_12_1$, with unit-cell parameters a = 73.28, b = 82.61, c = 118.64 Å. To obtain the initial phases, crystals of selenomethionyl-substituted amylomaltase were produced, and multiple-wavelength anomalous dispersion phasing and structure refinement are now in progress.

1. Introduction

Amylomaltase (EC 2.4.1.25) is a member of the 4-α-glucanotransferase (4αGTase) group of the α-amylase family. Amylomaltase catalyses the hydrolysis and transfer of α-1,4-glucan units to another linear oligosaccharide: the so-called intermolecular transglycosylation or disproportionation reaction. The enzyme also catalyses a unique intramolecular transglycosylation reaction or cyclization reaction within a single α-D-glucan molecule, producing cycloamylose (CA) or large-ring cyclodextrin (LR-CD), a cyclic α-1,4-glucan with a degree of polymerization (DP) of 16 or higher (Takaha & Smith, 1999). LR-CDs can form inclusion complexes with various guest molecules and potentially improve the properties of guest molecules such as their solubility, stability and reactivity (Zheng *et al.*, 2002; Tomono *et al.*, 2002).

Amylomaltases have been found in microorganisms as well as in plants, where they are known as disproportionating enzymes (D-enzymes; Takaha *et al.*, 1996; Kakefuda & Duke, 1989; Lin & Preiss, 1988). In microorganisms, amylomaltase was first identified in *Escherichia coli* as a maltose-inducible enzyme (Monod & Torriani, 1950). Later, amylomaltases from many archaea and bacterial strains, including from *Corynebacterium glutamicum*, were characterized (Kaper *et al.*, 2005, 2007; Terada *et al.*, 1999; Godány *et al.*, 2008; Srisimarat *et al.*, 2011). To date, crystal structures of amylomaltase from *Thermus aquaticus* (PDB entry 1cwy; Przylas, Tomoo *et al.*, 2000), *T. thermophilus* (PDB entry 2owx; Barends *et al.*, 2007), *T. brockianus* (PDB entry 2x1i; Jung *et al.*, 2011), *Aquifex aeolicus* (PDB entry 1tz7; T. R. M. Barends, H. Korf, T. Kaper, M. J. E. C. van der Maarel, L. Dijkhuizen & B. W. Dijkstra, unpublished work), *Thermotoga maritima* (PDB entry 1lwh; Roujeinikova *et al.*, 2002) and *Solanum tuberosum* (PDB entry 1x1n; Imamura *et al.*, 2005) have been determined. However, the amylomaltase from *C. glutamicum* (*Cg*AM) showed low amino-acid sequence similarity (28–32%) to these amylomaltases. *Cg*AM showed a different LR-CD production profile from that of the well characterized *T. aquaticus* enzyme (Terada *et al.*, 1999; Srisimarat *et al.*, 2011). *Cg*AM gave CA with a DP of 19 and higher, while *T. aquaticus* amylomaltase produced CD22 as the smallest product. In addition, the LR-CD production profile of

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  1  mtarrflnel adlygvatsy tdykgahiev sddtlvkilr algvnldtsn lpnddaiqrq
 61  ialfhdreft rplppsvvav egdelvfpvh vhdgspadvh ieledgtqrd vsqvenwtap
121  reidgirwge asfkipgdlp lgwhklhlks nersaecgli itparlstad kyldsprsgv
181  maqiysvrst lswgmgdfnd lgnlasvvaq dgadfllinp mhaaeplppt edspylpttr
241  rfinpiyirv edipefnqle idlrddiaem aaefrernlt sdiierndvy aaklqvlrai
301  femprssere anfvsfvqre gqglidfatw cadretaqse svhgtepdrd eltmfymwlq
361  wlcdeqlaaa qkravdagms igimadlavg vhpggadaqn lshvlapdas vgappdgynq
421  qgqdwsqppw hpvrlaeegy ipwrnllrtv lrhsggirvd hvlglfrlfv mprmqspatg
481  tyirfdhnal vgilaleael agavvigedl gtfepwvqda laqrgimgts ilwfehspsq
541  pgprrqeeyr plalttvtth dlpptagyle gehialrerl gvlntdpaae laedlqwqae
601  ildvaasana lpareyvgle rdqrgelael leglhtfvak tpsaltcvcl vdmvgekraq
661  nqpgttrdmy pnwciplcds egnsvliesl renelyhrva kaskrd
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Figure 1
The deduced amino-acid sequence of amylomaltase from *C. glutamicum* ATCC 13032.

*Cg*AM depended on the incubation time and the enzyme concentration. The X-ray crystal structure of *T. aquaticus* amylomaltase illustrated the well conserved catalytic site along with another substrate-binding site, the so-called 'second binding site'. This second binding site was proposed to play a role in LR-CD formation through the hydrophobic interaction of Tyr54 and Tyr101 with substrate. The mutation of these residues altered the hydrolysis activity of *T. aquaticus* amylomaltase, resulting in changes in the cyclization activity of the enzyme (Fujii *et al.*, 2005, 2007). In addition, a recent study of the Y172A mutant, *Cg*AM with an alanine substitution at Tyr172, which corresponds to Tyr54 in *T. aquaticus* amylomaltase, also suggested that Tyr172 of *Cg*AM plays an important role in determination of the LR-CD production profile (Srisimarat *et al.*, 2012).

To date, no crystal structure of *Cg*AM has been reported. Multiple amino-acid sequence alignment of *Cg*AM and other thermostable amylomaltases showed that *Cg*AM contains an extra N-terminal region of approximately 240 amino acids that does not share sequence similarity with other enzymes (Srisimarat *et al.*, 2011). In order to identify the function of this region and understand the basis of the cyclization mechanism of *Cg*AM, the crystallization and preliminary X-ray analysis of full-length *Cg*AM (residues 1–706) are reported in this study.

2. Materials and methods

2.1. Expression and purification

The ORF of *Cg*AM (GenBank BAB99690.1) has 2121 bp and was deduced to encode 706 amino-acid residues (Fig. 1). Full-length *Cg*AM was overexpressed and purified as described previously (Srisimarat *et al.*, 2012). In brief, *E. coli* BL21 (DE3) cells harbouring the pET-*CgAM* plasmid were cultured in Luria–Bertani medium containing 100 µg ml^{-1} ampicillin at 310 K and expression of *Cg*AM was induced by the addition of β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.4 m*M*. After 2 h, the cultured cells were harvested and disrupted by sonication (Bandelin, Germany). Cell debris was removed by centrifugation and the supernatant was applied onto DEAE FF and Phenyl FF (GE Healthcare, UK). The protein fractions containing amylomaltase activity were then collected. The purity of the protein was determined by 7.5% SDS–PAGE.

2.2. Crystallization

The purified protein was dialysed in 10 m*M* phosphate buffer pH 6.0 and concentrated to 5 mg ml^{-1}. The concentration of protein was measured using the BCA Protein Assay Kit (Pierce, USA). Crystallization experiments were carried out at 291 K using the vapour-diffusion method. Crystallization conditions were screened using the Index screen (Hampton Research, USA) in sitting droplets consisting of 0.5 µl protein solution and 0.5 µl precipitant solution in a 96-well plate. After a week, small crystals appeared in a droplet containing precipitant solution consisting of 0.1 *M* bis-tris pH 5.5, 2.0 *M* ammonium sulfate. Diffraction-quality crystals were produced in a VDX plate (Hampton Research, USA) using the hanging-drop vapour-diffusion method.

2.3. Data collection and processing

Prior to data collection, the crystal was immersed in reservoir solution supplemented with 30%(*v*/*v*) xylitol for a few seconds and was then flash-cooled in liquid nitrogen. X-ray diffraction data were collected from the crystal using an ADSC Quantum 315r CCD area detector on BL13B1 at the National Synchrotron Radiation Research Center (NSRRC), Taiwan. Data were processed using the *HKL*-2000 software package (Otwinowski & Minor, 1997).

3. Results and discussion

Recombinant *Cg*AM was overexpressed and purified using DEAE and Phenyl FF column chromatography. Purified *Cg*AM shows a single band on SDS–PAGE with an apparent molecular mass of 81 kDa. Crystals suitable for data collection were produced as described in §2. The crystal grew to maximum dimensions of 0.2 × 0.4 × 0.1 mm (Fig. 2) and diffracted to 1.7 Å resolution at a synchrotron source. The crystal belonged to space group $P2_12_12_1$, with unit-cell parameters a = 73.28, b = 82.61, c = 118.64 Å. The diffraction data set

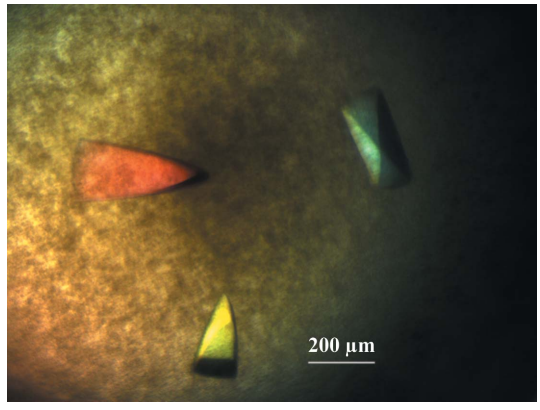

Figure 2
Crystals of *Cg*AM with approximate dimensions of 0.2 × 0.4 × 0.1 mm.

Table 1
X-ray diffraction data and processing statistics.

Values in parentheses are for the outermost resolution shell.

Space group	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 73.28, b = 82.61, c = 118.64, α = 90.0, β = 90.0, γ = 90.0
Volume of the unit cell (Å^3)	718272.3
Solvent content (%)	46
Matthews coefficient† (Å^3 Da^{-1})	2.26
Resolution range (Å)	50.0–1.70 (1.76–1.70)
Total No. of reflections	869461
No. of unique reflections	77144
Completeness (%)	97.3 (94.2)
Average $I/\sigma(I)$	36.0 (6.2)
$R_{\rm merge}$‡ (%)	5.5 (31.2)
Average multiplicity	11.3 (11.5)

† According to Kantardjieff & Rupp (2003). ‡ $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl)\rangle$ is the average intensity from multiple measurements (Diederichs & Karplus, 1997).

has a resolution range of 50–1.7 Å with 97.3% completeness and an $R_{\rm merge}$ of 5.5%. The Matthews coefficient was calculated to be about 2.26 Å^3 Da^{-1}, corresponding to 46% solvent content (Matthews, 1968; Kantardjieff & Rupp, 2003). Diffraction statistics are summarized in Table 1.

Attempts were made to solve the crystal structure of *Cg*AM using the molecular-replacement method. The structures of amylomaltases from PDB entries 1esw (28% sequence similarity; Przylas, Terada *et al.*, 2000) and 1tz7 (32% sequence similarity; T. R. M. Barends, H. Korf, T. Kaper, M. J. E. C. van der Maarel, L. Dijkhuizen & B. W. Dijkstra, unpublished work) were used as search models in *MOLREP* (Winn *et al.*, 2011). However, this method was not successful. This is probably because of conformational differences or low amino-acid sequence similarity. Since *Cg*AM has a high methionine frequency (15 methionine residues in 706 residues), it should be possible to obtain experimental phases by the multi-wavelength anomalous dispersion (MAD) method using selenomethionine-incorporated crystals. As a result, selenomethionine-incorporated *Cg*AM crystals were grown and used for MAD data collection. At present, phase determination and structure refinement of *Cg*AM are in progress.

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