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Expression, crystallization and preliminary X-ray crystallographic studies of *Klebsiella pneumoniae* maltohexaose-producing α-amylase

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A recombinant form of *Klebsiella pneumoniae* maltohexaose-producing α-amylase has been overexpressed in *Escherichia coli* and purified to homogeneity. Crystals were obtained at 293 K by the microbatch technique using 80 m*M* sodium/potassium phosphate buffer pH 6.2 containing 8% polyethylene glycol 3000, 4% polyethylene glycol 3350 and 40 m*M* sodium thiocyanate. Crystals of the overexpressed recombinant enzyme diffracted to better than 2.5 Å resolution at 95 K using a synchrotron-radiation source. The crystals belong to the primitive monoclinic space group $P2_1$, with unit-cell parameters a = 74.8, b = 107.6, c = 82.2 Å, β = 96.2°. Assuming the presence of two molecules per asymmetric unit, the V_M value for the crystal was 2.3 Å^3 Da^{-1}, indicating a solvent content of 47%.

1. Introduction

Several bacterial exo-glycolytic α-amylases that produce α-anomeric α-1,4-linked malto-oligodextrins such as maltotetraose (Zhou *et al.*, 1989), maltopentaose (Okemoto *et al.*, 1986) and maltohexaose (Kainuma *et al.*, 1972; Tsukamoto *et al.*, 1989) from the non-reducing end of starch, amylose and glycogen have been reported. The maltohexaose-producing amylase (EC 3.2.1.98; here abbreviated G6-amylase) of *Klebsiella pneumoniae* is an α-amylase belonging to this category (Kainuma *et al.*, 1972; Nakakuki *et al.*, 1982). The malto-oligodextrins produced by these amylases are expected to be useful in the food and chemical industries as raw materials, although the physiological roles of these α-amylases in bacteria are not yet clearly understood (Boos & Shuman, 1998). The gene for *K. pneumoniae* G6-amylase was cloned and its nucleotide sequence was reported (Momma, 2000) as well as those of the genes of a maltotetraose-producing amylase (G4-amylase) from *Pseudomonas saccharophila* (Zhou *et al.*, 1989), a maltopentaose-producing amylase (G5-amylase) from *Pseudomonas* sp. (Shida *et al.*, 1992) and G6-amylase from *Bacillus* sp. (Tsukamoto *et al.*, 1989). These amylases have been classified into glycosyl hydrolase family GH13 in the CAZy database (Coutinho & Henrissat, 1999; http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). The GH13 family is one of the largest GH families in the CAZy database and contains more than 500 enzymes. Although many three-dimensional structures of GH13-family members have been solved, including that of the G4-amylase of *P. stuzeri* (Morishita *et al.*, 1997), the structures of G5-amylase and G6-amylase have not been determined. The crystal structure of G6-amylase would be expected to have significant structural similarities to that of G4-amylase and other GH13 enzymes, as the consensus-sequence regions for the α-amylase families are well conserved, but the overall sequence similarities between G6-amylase and other maltogenic α-amylases are rather low at 20–30%. Comparison of the three-dimensional structures of these maltogenic α-amylases could provide useful information for understanding the reaction mechanism as well as for the design of mutant enzymes with high activities and stabilities. Here, we report the overexpression, purification, crystallization and preliminary X-ray studies of *K. pneumoniae* G6-amylase.

2. Experimental and results

2.1. Expression and purification

A DNA fragment of *K. pneumoniae* G6-amylase flanked by 5′ *Nde*I and 3′ *Hin*dIII cleavage sequences was amplified by PCR with *Pfu* Turbo DNA polymerase (Stratagene) from the plasmid pG61K (Momma, 2000) containing the complete *K. pneumoniae* G6-amylase gene (DDBJ accession No. AB026384). The primers used for PCR were 5′-AGCTCATATGAACTCGCTGCGCTTGCC-3′ and 5′-AGCTAAGCTTTTACTGCTGCCCGGCCCAG-3′. The fragment was ligated into the *Nde*I/*Hin*dIII-digested pET21a expression vector (Novagen). The resulting vector contained the initiation methionine ATG codon for protein initiation, but did not contain an oligohistidine or other tags. The soluble active form of *K. pneumoniae* G6-amylase was overexpressed in *Escherichia coli* strain B834(DE3). The cells were grown in

LB medium containing 100 µg ml^{-1} ampicillin at 296 K with shaking (200 rev min^{-1}) for 24 h without induction by isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were harvested by centrifugation at 5000g and lysed using 1/100 volume of 10 mg ml^{-1} lysozyme and 1/10 volume of 1% Triton X-100, followed by sonication. The supernatant of the lysate was resuspended in 10 mM Tris–HCl pH 8.0 containing 1 mM EDTA (100 ml). Purification of the expressed G6-amylase was carried out by conventional open-column chromatography using DE-52 (5 × 10 cm column) and then further purified using an ÄKTA system (Amersham Biosciences) with SP Sepharose FF (2.5 × 20 cm) and with Highload Superdex 200 (2.6 × 60 cm) column chromatography. About 20 mg of the soluble active form of *K. pneumoniae* G6-amylase was purified from 1 l culture medium. The active fractions from the final chromatographic procedure were combined and concentrated to an OD_{280} of 20 (10 mg ml^{-1}) using a Centricon YM-10 centrifugal filter (Millipore). Protein purity was checked by SDS–PAGE stained with Coomassie brilliant blue (Fig. 1). The N-terminal amino-acid sequence of the expressed G6-amylase was determined to be Ala-Trp-Thr-Thr-Thr-, which suggested that the leader peptide (residues 1–17) of the recombinant G6-amylase had been processed after the transcription in *E. coli* cells. The molecular weight of the recombinant G6-amylase was estimated to be 67 kDa by SDS–PAGE, which agreed well with the calculated value of 70 kDa deduced from the nucleotide sequence.

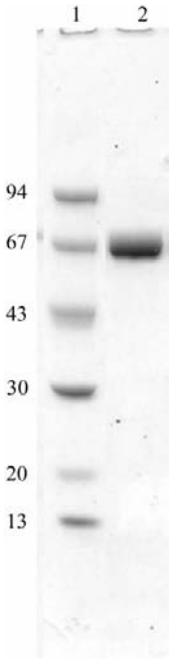

Figure 1
SDS–PAGE of the recombinant *K. pneumoniae* G6-amylase. Lane 1, MW markers (KDa); lane 2, purified recombinant maltohexaose-producing α-amylase.

2.2. Crystallization

Crystallization screening was initially carried out using Crystal Screen (Hampton Research) and Wizard Screens I and II (Emerald Biostructures). All crystallization trials were conducted using the microbatch technique by mixing 2.5 µl protein solution (10 mg ml^{-1}) and an equal volume of precipitating solution and covering with 5 µl Al's oil (Hampton Research). Thin fibrous cluster crystals grew in less than a week using buffer systems containing various polymerization degrees of polyethylene glycol. After refinement of the crystallization conditions, the optimal conditions for crystallization were found to be 80 mM sodium/potassium phosphate pH 6.2 buffer containing 8% polyethylene glycol 3000, 4% polyethylene glycol 3350 and 40 mM sodium thiocyanate. Thin plate-shaped crystals (0.1 × 0.2 × 0.03 mm) were obtained within two weeks at 293 K for *K. pneumoniae* G6-amylase (Fig. 2).

2.3. X-ray data collection and analysis

Diffraction data were collected at beamline BL-NW12 of the Photon Factory Advanced Ring, Tsukuba, Japan. Crystals were soaked in a cryoprotectant solution consisting of a mixture of 20%(w/v) glycerol and the crystallization solution, picked up using a fibre loop and flash-frozen in a stream of nitrogen gas at 95 K. Native data were collected using a Quantum 210 CCD detector (ADSC) in 1.0° oscillation steps over a range of 180°. The data sets were processed and scaled using *DENZO* and *SCALEPACK* from the *HKL*2000 program package (Otwinowski, 1993). The crystals of the recombinant *K. pneumoniae* G6-amylase belonged to space group $P2_1$, with unit-cell parameters a = 74.8, b = 107.6, c = 82.2 Å, β = 96.2°, and diffracted to beyond 2.5 Å resolution (Table 1). Assuming the presence of two molecules per asymmetric unit, the V_M value for the crystal was 2.3 Å^3 Da^{-1}, indicating a solvent content of 47% (Matthews, 1968).

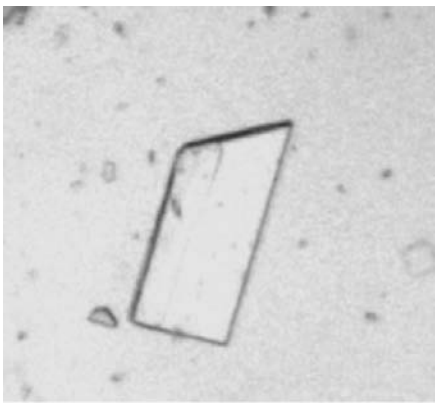

Figure 2
Crystal of the recombinant *K. pneumoniae* G6-amylase obtained by the microbatch method.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

X-ray source	PF BL-NW12
Detector	Quantum 210 CCD
Temperature (K)	95
Wavelength (Å)	1.000
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 74.8, b = 107.6, c = 82.2, β = 96.2
No. reflections	161300
No. unique reflections	44975 (4373)
Resolution (Å)	50–2.5 (2.59–2.50)
R_{merge}	0.090 (0.256)
Average $I/\sigma(I)$	33.9 (2.8)
Multiplicity	3.6 (3.3)
Completeness (%)	96.4 (90.1)

The present *K. pneumoniae* G6-amylase has an N-terminal extension of unknown function containing more than 150 amino-acid residues, which is also observed in the glycoside hydrolases of *Escherichia*, *Salmonella* and *Vibrio*, but does not possess the C-terminal starch-binding domain (SBD) observed in *P. stuteri* G4-amylase. Even if the N-terminal extension and SBD are eliminated from the search sequences, FASTA database searches have given the G6-amylase only 20–30% similarity compared with maltogenic G4-amylase, G5-amylase, another G6-amylase and other related glycoside hydrolases. Because the database homology search did not reveal a suitable model for molecular replacement, we are attempting to obtain selenomethionine-derivative crystals.

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