Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization and preliminary X-ray analysis of an exotype alginate lyase Atu3025 from *Agrobacterium tumefaciens* strain C58, a member of polysaccharide lyase family 15

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Received 17 February 2006
Accepted 20 April 2006

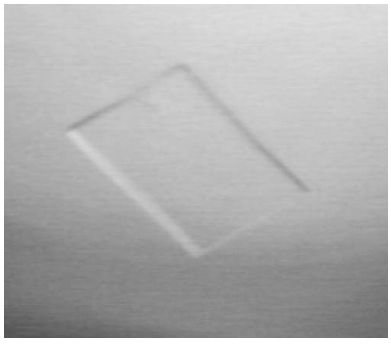
Almost all alginate lyases depolymerize alginate in an endolytical fashion *via* a β-elimination reaction. The alginate lyase Atu3025 from *Agrobacterium tumefaciens* strain C58, consisting of 776 amino-acid residues, is a novel exotype alginate lyase classified into polysaccharide lyase family 15. The enzyme was crystallized at 293 K by sitting-drop vapour diffusion with polyethylene glycol 4000 as a precipitant. Preliminary X-ray analysis showed that the Atu3025 crystal belonged to space group $P2_1$ and diffracted to 2.8 Å resolution, with unit-cell parameters a = 107.7, b = 108.3, c = 149.5 Å, β = 91.5°.

1. Introduction

Alginate produced by brown seaweed is commonly used as a thickener in the food industry and as a gelling agent, emulsifier and chelator of metal ions in the pharmaceutical industry (Thomas, 2000). Enzymatically depolymerized alginate oligosaccharides function as a bifidus factor (Akiyama *et al.*, 1992), an elicitor of plant growth (Darvill *et al.*, 1994), a growth enhancer of human endothelial cells (Kawada *et al.*, 1999) and keratinocytes (Kawada *et al.*, 1997) and an inducer of cytokine production from mouse macrophage cells (Iwamoto *et al.*, 2003) and human mononuclear cells (Iwamoto *et al.*, 2005). The enzymatic production of differently depolymerized alginate oligosaccharides is essential for several medical developments and other industrial uses.

Alginate is a linear polysaccharide composed of α-L-guluronate (G) and its C5 epimer β-D-mannuronate (M). It is arranged in three blocks: poly-α-L-guluronate (polyG), poly-β-D-mannuronate (polyM) and heteropolymeric random sequences (polyMG) (Gacesa, 1988). In the Carbohydrate-Active enZYmes (CAZY) database (http://afmb.cnrs-mrs.fr/CAZY/), alginate lyases are classified into three polysaccharide lyase (PL) families, 5, 7 and 14, based on their amino-acid sequences. Most bacterial alginate lyases belong to families PL-5 and PL-7 (Miyake *et al.*, 2004); nonbacterial enzymes from a *Chlorella* virus (Suda *et al.*, 1999; Sugimoto *et al.*, 2000) and a marine animal (Shimizu *et al.*, 2003) are grouped into family PL-14. Alginate lyases A1-IV and A1-IV′ from *Sphingomonas* sp. strain A1 and Atu3025 from *Agrobacterium tumefaciens* strain C58 have recently been assigned to a novel family, PL-15 (Hashimoto *et al.*, 2005). Almost all alginate lyases endolytically cleave the glycosidic linkage of alginate through a β-elimination reaction and release unsaturated oligouronic acids having 4-deoxy-L-*erythro*-hex-4-enopyranosyluronic acid at the nonreducing end (Wong *et al.*, 2000). We showed that A1-IV and Atu3025 depolymerize alginate in an exolytic fashion, releasing monosaccharides from the nonreducing end of the polymer *via* a β-elimination reaction (Hashimoto *et al.*, 2000; Miyake *et al.*, 2003; Ochiai *et al.*, 2006).

We have previously determined the crystal structures of the family PL-5 alginate lyase A1-III from *Sphingomonas* sp. strain A1 and the family PL-7 alginate lyases A1-II′ from *Sphingomonas* sp. strain A1 and PA1167 from *Pseudomonas aeruginosa*. While A1-III folds into an α_6/α_5-barrel as a basic structural scaffold (Yoon *et al.*, 1999), A1-II′ and PA1167 adopt a β-sandwich fold (Yamasaki *et al.*, 2004, 2005). The substrate specificities of these enzymes are different (A1-III,



polyM; A1-II′, all polysaccharide blocks are catalyzed equally; PA1167, polyG). Subsequent X-ray crystallographic studies have illustrated the structure–function relationships of these alginate lyases (Yamasaki *et al.*, 2005; Yoon *et al.*, 2001). However, there is no structural report on the PL-15 family of these enzymes or on any other exotype alginate lyases. Since family PL-15 enzymes show no homology to polysaccharide lyases from other families, they may constitute a novel folding not observed in polysaccharide lyases analyzed thus far. Structural and functional analyses of the family PL-15 exotype alginate lyase should provide valuable information on the structural factors determining the mode of action.

Atu3025, a member of family PL-15, was identified as an exotype alginate lyase (Ochiai *et al.*, 2006). The molecular weight of Atu3025 is calculated as 87 871 Da from the predicted amino-acid sequence (776 residues). Here, we describe the crystallization and preliminary X-ray crystallographic analysis of Atu3025.

2. Materials and results

2.1. Analytical methods

Alginate lyase was assayed as follows. The enzyme was incubated at 303 K for 5 min in a reaction mixture (1 ml) consisting of 0.05% sodium alginate and 50 m*M* Tris–HCl pH 7.5. Each reaction mixture was heated at 373 K for 5 min to stop the reaction and the amount of unsaturated uronic acids produced in the reaction mixture was determined by the thiobarbituric acid (TBA) method (Hurwitz & Weissbach, 1959). Enzyme activity was determined by measuring the increase in absorbance at 548 nm arising from the condensation of β-formylpyruvic acid with TBA ($\varepsilon = 2.9 \times 10^4\ M^{-1}\ \mathrm{cm}^{-1}$). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of β-formylpyruvic acid per minute at 303 K. Protein content was determined by the method of Bradford (1976), with bovine serum albumin as the standard, or by measuring absorbance at 280 nm, in which an absorbance of 2.06 at 280 nm corresponds to 1 mg ml^{-1} based on the molecular coefficient of Atu3025 obtained using the *ProtParam* tool of the *ExPASy* (*Expert Protein Analysis System*) proteomics server (http://us.expasy.org/).

2.2. Protein expression and purification

Atu3025 was expressed and purified as described elsewhere (Ochiai *et al.*, 2006). Briefly, an overexpression system for Atu3025 was constructed in *Escherichia coli* cells using pET21b as an expression vector (Novagen, Madison, WI, USA). The DNA fragment encoding the complete sequence of the Atu3025 gene (GenBank accession No. AE009232) with the original stop codon was ligated with pET21b. Thus, the polypeptide expressed from the resultant plasmid was not fused to the histidine-tagged sequence. The recombinant Atu3025 expressed in *E. coli* cells was purified by two anion-exchange chromatography steps using DEAE-Toyopearl 650M (Tosoh Co., Tokyo, Japan) and Q Sepharose High Performance (Amersham Biosciences, Uppsala, Sweden) and hydroxyapatite chromatography (Nacalai Tesque, Kyoto, Japan). The purified enzyme was dialyzed against 20 m*M* Tris–HCl pH 7.5 and the protein solution was concentrated by ultrafiltration with a Centriprep (Millipore Co., Tokyo, Japan) to a final concentration of 5 or 18 mg ml^{-1}. The concentrated protein solution containing only 20 m*M* Tris–HCl pH 7.5 as a buffer was used in the subsequent crystallization step.

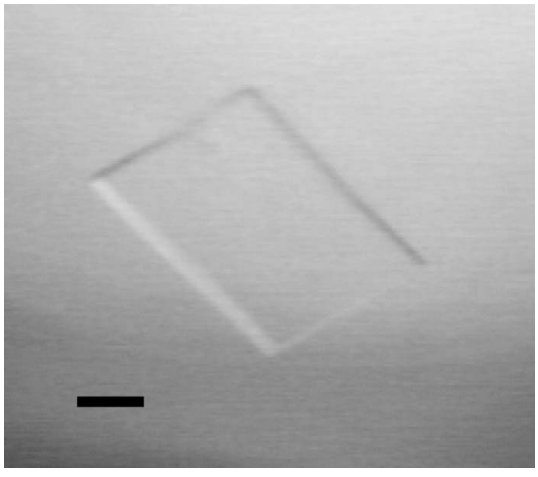


Figure 1
Crystal of Atu3025 from *A. tumefaciens* strain C58. The scale bar is 0.1 mm in length.

Table 1
Atu3025 crystal data collection.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.0
Resolution (Å)	50–2.8 (2.9–2.8)
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 107.7, b = 108.3, c = 149.5, β = 91.5
Total observations	206527
Independent reflections	84481
Completeness (%)	96.4 (98.5)
$\langle I/\sigma(I)\rangle$	10.4 (3.5)
$R_{\rm merge}$† (%)	9.5 (36.4)

† $R_{\rm merge} = \sum_h \sum_i [|I_i(h) - \langle I(h)\rangle|/\sum_h \sum_i I_i(h)]$, where I_i is the ith measurement and $\langle I(h)\rangle$ is the weighted mean of all measurements of $I(h)$.

2.3. Crystallization

Purified Atu3025 was crystallized by sitting-drop vapour diffusion on Linbro tissue-culture plates. A cover slide was used to seal each well and Micro-Bridges (Hampton Research, Aliso Viejo, CA, USA) were also used for preparing sitting drops. The droplet (6 µl) was prepared by mixing 3 µl protein solution with 3 µl reservoir solution and was equilibrated against 0.5 ml reservoir solution at 293 K. Small square thin crystals of Atu3025 appeared in about a month through sparse-matrix screening using the Crystal Screen Cryo kit from Hampton Research (Aliso Viejo, CA, USA) with a protein concentration of 5 mg ml^{-1}. Crystals were used for subsequent macro-seeding of the droplet under the same conditions using a protein concentration of 18 mg ml^{-1}. Large crystals were obtained after 3–6 months (Fig. 1). The condition most suitable for crystallization was determined to be a mixture of 0.08 *M* Tris–HCl pH 8.5, 24%(*w*/*v*) PEG 4000, 0.16 *M* magnesium chloride and 20%(*v*/*v*) glycerol.

2.4. X-ray analysis

A crystal of Atu3025 was picked up from a droplet in a mounted nylon loop (Hampton Research, Aliso Viejo, CA, USA) and placed directly in a cold nitrogen-gas stream at 100 K. X-ray diffraction images of the crystal were collected at 100 K under a nitrogen-gas stream using a Quantum 4R detector and synchrotron radiation of wavelength 1.0 Å at the BL-38B1 station of SPring-8 (Hyogo, Japan). The distance between the crystal and detector was set at 400 mm and 1.2° oscillation images were recorded at an exposure of 25 s. Diffraction data for the crystal were obtained in the resolution range 50–2.8 Å and were processed using the *HKL*2000 program package

(*DENZO* and *SCALEPACK*; Otwinowski & Minor, 1997). The space group of the crystal was determined to be $P2_1$ (monoclinic), with unit-cell parameters a = 107.7, b = 108.3, c = 149.5 Å, β = 91.5°. Table 1 summarizes the preliminary X-ray crystallographic properties of Atu3025.

The V_M (Matthews, 1968) of the Atu3025 crystal, *i.e.* the crystal volume per unit of protein molecular weight, was calculated to be 2.48 Å^3 Da^{-1} and the solvent content was calculated to be 50.4%, assuming that four molecules of the enzyme were present per asymmetric unit. As the kinetics studies on Atu3025 from *A. tumefaciens* strain C58 indicate that it acts on alginate as a tetramer (Ochiai *et al.*, 2006), the molecular packing in Atu3025 crystals may be strongly correlated with the physiological quaternary structure of this enzyme.

A search for selenomethionine derivatives for phasing using the multiple-wavelength anomalous dispersion (MAD) method is now in progress.

This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) of Japan. We thank Drs H. Sakai and K. Hasegawa of the Japan Synchrotron Radiation Research Institute (JASRI) for their kind help during data collection. X-ray data were collected at BL38B1 of SPring-8 with the approval of the organizing committee of SPring-8 (proposal No. 2005B0315, WH).

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