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Crystallization and preliminary X-ray analysis of alginate lyase, a member of family PL-7, from *Pseudomonas aeruginosa*

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Alginate lyase depolymerizes alginate, a heteropolysaccharide consisting of α-L-guluronate and β-D-mannuronate, through a β-elimination reaction. A protein PA1167 with a molecular mass of 25 kDa produced by *Pseudomonas aeruginosa* is an alginate lyase classified into polysaccharide lyase family PL-7. The enzyme was crystallized at 293 K in a drop solution comprising 1.4 *M* sodium chloride, 0.1 *M* potassium sodium phosphate and 0.1 *M* 2-morpholinoethanesulfonate–sodium hydroxide pH 6.5 by means of the vapor-diffusion method. The crystals were monoclinic and belonged to space group $P2_1$, with unit-cell parameters $a = 43.4$, $b = 70.3$, $c = 67.4$ Å, $\beta = 94.5^\circ$. Diffraction data were collected to 2.0 Å from a single crystal.

1. Introduction

There are a large number of polysaccharide lyases, which are classified into 12 families (PL-1 to PL-12) based on their primary structures (B. Henrissat, P. Coutinho and E. Deleury; http://afmb.cnrs-mrs.fr/CAZY/). Polysaccharide lyases recognize uronic acid residues in polysaccharides and catalyze a β-elimination reaction through the release of unsaturated saccharides with C=C double bonds at the non-reducing terminal uronate residues. These characteristics of lyases indicate that they share common structural features determining their uronate-recognition sites and reaction modes (β-elimination reaction). The crystal structures of lyases for pectate (families PL-1, PL-3 and PL-10; Yoder *et al.*, 1993; Akita *et al.*, 2001; Charnock *et al.*, 2002), alginate (family PL-5; Yoon *et al.*, 1999), hyaluronate (family PL-8; Li *et al.*, 2000), chondroitin (families PL-6 and PL-8; Huang *et al.*, 1999; Féthiere *et al.*, 1999) and xanthan (family PL-8; Hashimoto *et al.*, 2003) have been determined and assigned to three folding groups having parallel β-helix, α/α-barrel and α/α-barrel plus antiparallel β-sheets structures as basic frames. However, the three-dimensional structures of no family PL-2, PL-4, PL-7, PL-9, PL-11 or PL-12 lyases have been revealed. Thus, there is little information regarding the structural rules common to polysaccharide lyases.

Alginate is a linear polysaccharide consisting of α-L-guluronate and its C5 epimer, β-D-mannuronate, arranged in three different ways: poly α-L-guluronate (polyG), poly β-D-mannuronate (polyM) and heteropolymeric (polyMG) regions (Gacesa, 1988). Alginate produced by brown seaweed is widely used in the food and pharmaceutical industries owing to its ability to chelate metal ions and to form a highly viscous solution (Onsøyen, 1996), while some pathogenic bacteria such as *P. aeruginosa* produce alginate as a capsule-like biofilm responsible for both chronic pulmonary infections and respiratory difficulties in the lungs of patients with cystic fibrosis (Batten & Matthew, 1983; Boat *et al.*, 1989). The bacterial alginate seems to play a crucial role in the adherence of *P. aeruginosa* to target cells (Ramphal & Pier, 1985). Alginate lyase depolymerizes alginate through a β-elimination reaction. Therefore, it is important to clarify the structure and function of alginate lyase, which is applicable to biochemicals for the molecular design of an edible alginate and is responsible for the biofilm-dependent ecosystem in *P. aeruginosa*.

On the basis of their primary structures, alginate lyases are classified into two families, PL-5 and PL-7. Generally, family PL-5 and PL-7 alginate lyases specifically depolymerize polyM and polyG, respectively (Fig. 1). A family PL-5 alginate lyase (AlgL) of *P. aeruginosa* specific for polyM has been well characterized and documented (Schiller *et al.*, 1993; Wong *et al.*, 2000). Furthermore, we have previously determined the crystal structure of family PL-5 alginate lyase A1-III from *Sphingomonas sp*. A1 (Yoon *et al.*, 1999), and elucidated the catalytic reaction mechanism through structural analysis of the enzyme complexed with an alginate derivative (Yoon *et al.*, 2001). On the other hand, there has been no report of the presence of a family PL-7 alginate lyase in *P. aeruginosa* and the structure–function relationship of family PL-7 alginate lyases specific for polyG has not yet been clarified. However, we recently identified an unknown-function PA1167 protein from

P. aeruginosa as a family PL-7 alginate lyase (Murata, 2003). Structural analysis of family PL-7 alginate lyases together with family PL-5 enzymes is thought to be important for clarification of the complete depolymerization mechanism of alginate involved in the ecosystem in *P. aeruginosa* and the establishment of common structural features and catalytic mechanisms of alginate lyases. Furthermore, family PL-7 alginate lyases show no homology with other family lyases, suggesting that family PL-7 enzymes exhibit novel folding that is distinct from the three types of the lyases so far analyzed.

This article deals with the crystallization and preliminary X-ray crystallographic analysis of the family PL-7 alginate lyase from *P. aeruginosa*.

2. Methods and results

2.1. Analytical methods

Alginate lyase (PA1167) from *P. aeruginosa* was assayed as follows. The enzyme was incubated at 303 K in 0.2 ml of a reaction mixture comprising 0.025% alginate and 50 m*M* Tris–HCl buffer pH 7.5. The reaction was terminated by immersing the test tubes in boiling water for 5 min and 0.1 ml of the mixture was removed for analysis by the periodate-thiobarbituric acid method (Weissbach & Hurwitz, 1958) (Fig. 1*c*). One unit of enzyme activity was defined as the amount of enzyme required to liberate 1 µmol of β-formylpyruvic acid per minute. The protein content was determined by the method of Bradford (1976), with bovine serum albumin as a standard, or by measuring the absorbance at 280 nm, assuming that E_{280} = 1.64 corresponds to 1 mg ml^{-1} protein. The purity of the enzyme was confirmed by SDS–PAGE (Laemmli, 1970).

2.2. Protein expression and purification

The enzyme was purified from recombinant *Escherichia coli* cells as described in Murata (2003). Unless otherwise specified, all operations were carried out at 273–277 K. *E. coli* cells harboring a plasmid (pET3a-PA1167) were grown in 6 l of LB medium (1.5 l per flask), collected by centrifugation at 6000*g* and 277 K for 5 min, washed with 20 m*M* potassium phosphate buffer pH 6.0 containing 1 m*M* EDTA (buffer *A*) and then resuspended in the same buffer. The cells were ultrasonically disrupted (Insonator Model 201 M, Kubota, Tokyo, Japan) at 273 K and 9 kHz for 20 min and the clear solution obtained on centrifugation at 15 000*g* and 277 K for 20 min was used as the cell extract. The cell extract, after supplementation with 1 m*M* phenylmethylsulfonyl fluoride and 0.1 µ*M* pepstatin A, was fractionated with ammonium sulfate. The precipitate (0–30% saturation) was collected by centrifugation at 15 000*g* and 277 K for 20 min, dissolved in buffer *A* and then applied to a CM-Toyopearl 650M column (2.6 × 11 cm; Tosoh Co., Tokyo, Japan) equilibrated with buffer *A*. The enzyme was eluted with a linear gradient of NaCl (0–0.7 *M*) in bufffer *A* (180 ml), 2 ml fractions being collected every 2 min. The active fractions, which were eluted at around 0.4 *M* NaCl, were combined and concentrated to 6 ml by ultrafiltrataion using a Centriprep (10 kDa molecular-weight cutoff; Millipore Co., MA, USA). The concentrate was applied to a Sephacryl S-200HR column (2.6 × 90 cm; Pharmacia Biotechnology Co., Uppsala, Sweden) previously equilibrated with buffer *A* containing 0.15 *M* NaCl. The enzyme was eluted with the same buffer and 3 ml fractions were collected every 8 min. The active fractions eluted, *i.e.* fraction Nos. 101–105, were combined and dialyzed against buffer *A*. The dialysate was used as the purified enzyme (5.9 mg protein). For crystallization, the purified enzyme was concentrated to 0.6 mg ml^{-1} by ultrafiltration with the Centriprep. The purified enzyme gave a single band corresponding to 25 kDa on SDS–PAGE. Judging from the profiles of the gel filtration (Sephacryl S-200HR) and SDS–PAGE, the enzyme was in a homogeneous monomeric form.

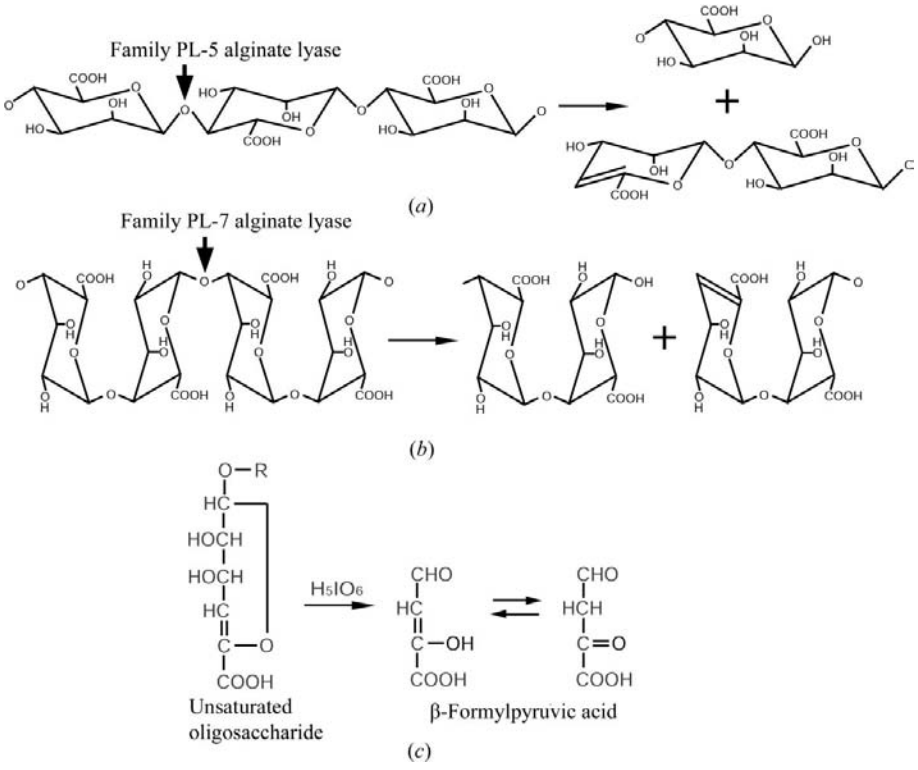

Figure 1
Structures of alginates and alginate lyase reactions. (*a*) polyM, (*b*) polyG and (*c*) β-formylpyruvic acid production from unsaturated oligosaccharides through the periodate oxidation reaction. Thick and thin arrows indicate the cleavage sites for alginate lyases and the lyase reactions, respectively.

2.3. Crystallization

The alginate lyase (PA1167) purified from *E. coli* cells was crystallized at 293 K by the hanging-drop vapor-diffusion method. The solution for a crystallization droplet was prepared on a siliconized cover slip by mixing 3 µl of the protein solution (0.6 mg ml^{-1}) with 3 µl of the precipitant solution. Since few crystals of the enzyme were initially obtained on sparse-matrix screening with commercial crystallization kits (Crystal Screen kits I and II, Hampton Research, Laguna Niguel, CA, USA), crystallization of the enzyme was attempted under a large number of conditions with sodium chloride, potassium sodium tartrate or ammonium sulfate as the major precipitant. Small rod-shaped crystals of the enzyme were found in a droplet consisting of 0.6 mg ml^{-1} enzyme, 1.5 *M* sodium chloride, 0.1 *M* potassium sodium phosphate and 0.1 *M* MES–sodium hydroxide pH 6.5. After

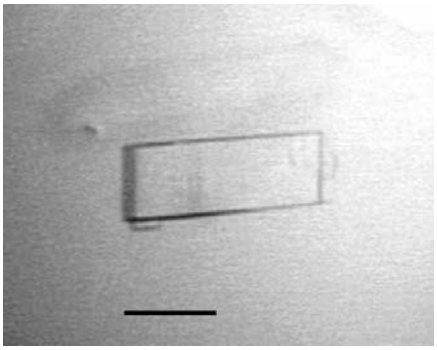

Figure 2
Crystal of the family PL-7 alginate lyase (PA1167). The scale bar is 0.1 mm long.

improvement of the conditions, the solution most suitable for crystallization was determined to be a mixture consisting of 0.6 mg ml^{-1} enzyme, 1.4 *M* sodium chloride, 0.1 *M* potassium sodium phosphate and 0.1 *M* MES–sodium hydroxide pH 6.5. Crystals of the enzyme grew in droplets at 293 K in three weeks (Fig. 2).

2.4. X-ray analysis

A crystal of the enzyme picked up from a droplet in a nylon loop (Hampton Research, Laguna Niguel, CA, USA) was transferred to a cryoprotectant solution (2 *M* sodium chloride, 30% glycerol and 0.1 *M* MES–sodium hydroxide pH 6.5) and then 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 with a MAR CCD detector using synchrotron radiation of wavelength 0.9 Å at the BL-41XU station of SPring-8 (Hyogo, Japan). The distance between the crystal and the detector was set to 150 mm and 1.0° oscillation images were recorded with an exposure time of 2 s. Diffraction data for the crystal were obtained in the resolution range 50–2.0 Å and processed using the *HKL*2000 program package (*DENZO* and *SCALEPACK*; Otwinowski & Minor, 1997). X-ray data statistics are summarized in Table 1. The V_M value (Matthews, 1968), *i.e.* the crystal volume per unit of protein molecular weight, was calculated to be 2.04 Å^3 Da^{-1} assuming two molecules of the enzyme to be present in an asymmetric unit; the solvent content was 39.2%. The V_M value and solvent content lie within the ranges usually found for protein crystals.

Table 1
Data-collection statistics for a crystal of the family PL-7 alginate lyase (PA1167).

Values in parentheses refer to data in the highest resolution shell.

Wavelength (Å)	0.9
Resolution (Å)	50–2.00 (2.07–2.00)
Crystal system	Monoclinic
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 43.4, b = 70.3, c = 67.4, β = 94.5
Total observations	110845
Independent reflections	51382
Completeness (%)	96.2 (87.4)
$I/\sigma(I)$	9.9 (2.6)
R_{merge} (%)	7.7 (36.0)

A selenomethionine derivative of the family PL-7 alginate lyase (PA1167) has already been obtained and crystallized. The phase problem will be solved by means of multiwavelength anomalous diffraction (MAD) experiments and/or the multiple isomorphous replacement (MIR) method, since the enzyme exhibits no homology with proteins of known structure.

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