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Crystallization and preliminary X-ray analysis of alginate lyases A1-II and A1-II′ from *Sphingomonas* sp. A1

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Alginate lyases depolymerize alginate, a heteropolysaccharide consisting of α-L-guluronate and β-D-mannuronate, through a β-elimination reaction. The alginate lyases A1-II (25 kDa) and A1-II′ (25 kDa) from *Sphingomonas* sp. A1, which belong to polysaccharide lyase family PL-7, exhibit 68% homology in primary structure but have different substrate specificities. To determine clearly the structural basis for substrate recognition in the depolymerization mechanism by alginate lyases, both proteins were crystallized at 293 K using the vapour-diffusion method. A crystal of A1-II belonged to space group $P2_1$ and diffracted to 2.2 Å resolution, with unit-cell parameters a = 51.3, b = 30.1, c = 101.6 Å, β = 100.2°, while a crystal of A1-II′ belonged to space group $P2_12_12_1$ and diffracted to 1.0 Å resolution, with unit-cell parameters a = 34.6, b = 68.5, c = 80.3 Å.

1. Introduction

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; Thomas, 2000). On the other hand, some pathogenic bacteria such as *Pseudomonas aeruginosa* secrete alginate as a component of the capsule-like biofilm responsible for both chronic pulmonary infection and respiratory difficulty in the lungs of patients with cystic fibrosis (Batten & Matthew, 1983; Boat *et al.*, 1989). Alginate is a kind of polysaccharide consisting of β-D-mannuronate and its C5 epimer, α-L-guluronate, arranged in three different blocks: poly-β-D-mannuronate (poly-M), poly-α-L-guluronate (poly-G) and heteropolymeric (poly-MG) regions (Gacesa, 1988). Alginate lyases depolymerize alginate through a β-elimination reaction, resulting in the release of unsaturated saccharides with C=C double bonds at their nonreducing terminal uronate residues (Fig. 1). However, the structural basis for the depolymerization mechanism remains poorly understood. Since alginate oligosaccharides released from alginate through the reaction

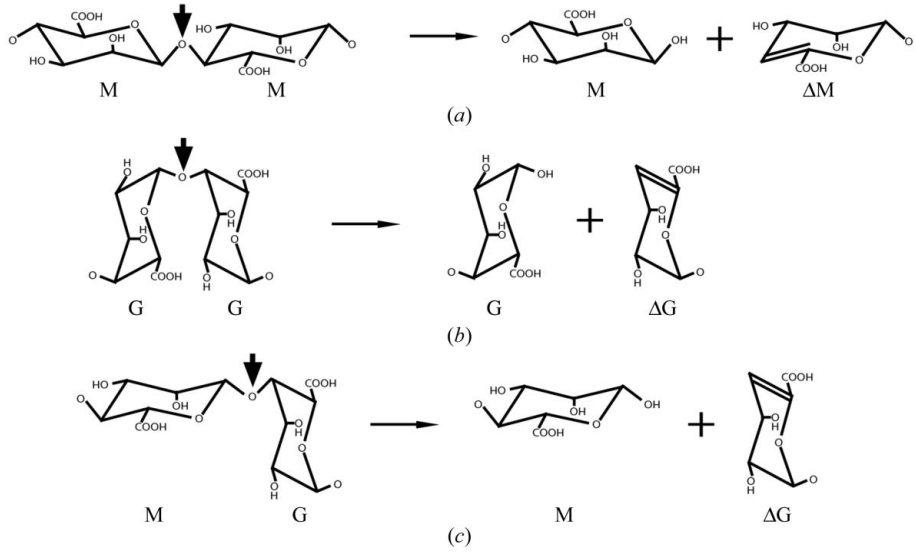
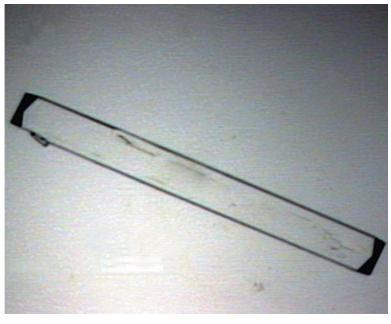


Figure 1
Block sites of alginate polymers and alginate lyase reactions. (*a*) MM, (*b*) GG, (*c*) MG block sites. M and G represent β-D-mannuronate and α-L-guluronate, respectively. Vertical arrows indicate the cleavage sites for alginate lyase reactions.



of lyases have been shown to function as oligosaccharins (Darvill *et al.*, 1994), like a bifidus factor (Akiyama *et al.*, 1992) and as an elicitor of plant growth (Yonemoto *et al.*, 1993), the production of various alginate oligosaccharides by the use of lyases is sought for the development of a more functional alginate for future industrial applications. Therefore, it is important to clarify the relationship between the structure and function of alginate lyases, which will be applicable to biochemicals for the molecular design of an edible alginate or the design of drugs for the biofilm-dependent infections caused by *P. aeruginosa*.

Based on their primary structures, alginate lyases are now classified into four groups, polysaccharide lyase (PL) families 5, 7, 14 and 15, which also include some proteins of unknown function (B. Henrissat, P. Coutinho & E. Deleury; http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). Of these families, the basic frames in family PL-5 and PL-7 lyases are generally α/α-barrel and β-sandwich structures, respectively (Yoon *et al.*, 1999; Yamasaki *et al.*, 2004).

Sphingomonas sp. A1 alginate lyase A1-III, which is specific for poly-M, is the only enzyme of the PL-5 proteins that has been structurally analyzed in detail by X-ray crystallography. The flexible motion of a lid loop is important in the reaction mechanism, which involves His192 and Tyr246 as catalytic residues, and arginine residues in the active cleft seem to be important for the binding to the carboxylic acid of mannuronate (Yoon *et al.*, 2001; Mikami *et al.*, 2002). These were important observations in understanding the depolymerization mechanism of alginate lyases. However, PL-5 enzymes have been shown to be similar to each other from the phylogenic tree and all show identical specificity for poly-M (Miyake *et al.*, 2004). Therefore, it is difficult to clarify the relationship between structure and substrate specificity through analysis of PL-5 proteins only. On the other hand, PL-7 enzymes are diverse in primary structure and substrate specificity (Miyake *et al.*, 2004), *i.e.* *Corynebacterium* sp. ALY-1 AlyPG prefers poly-G (Matsubara *et al.*, 1998), *Photobacterium* sp. ATCC 43367 AlxM prefers poly-M (Malissard *et al.*, 1993) and *P. aeruginosa* PA1167 prefers poly-MG (Yamasaki *et al.*, 2004). Structural analyses of PA1167 and AlyPG have indicated that this PL-7 enzyme adopts a β-sandwich fold as a basic frame with no flexible lid loop and has conserved histidine and tyrosine residues in a putative active site (Yamasaki *et al.*, 2004; Osawa *et al.*, 2005). These observations allow comparison with the structure of PL-5 A1-III, but provide no information on substrate specificity.

In this study, we focused on two PL-7 alginate lyases, A1-II and its homologue A1-II′ from *Sphingomonas* sp. A1. Although A1-II and A1-II′ exhibit a high sequence homology of 68%, they show different substrate specificities: A1-II for poly-G and A1-II′ for all substrates. Comparison of their structure–function relationships by X-ray crystallography will clarify how alginate lyases strictly recognize various substrates. We describe here the crystallization and preliminary X-ray crystallographic analysis of A1-II and A1-II′ for further structure determination.

2. Methods and results

2.1. Analytical methods

The alginate lyases (A1-II and A1-II′) were assayed as follows. Each enzyme was incubated at 303 K in a reaction mixture comprising 0.05% alginate and 50 m*M* Tris–HCl pH 7.5. The reaction was terminated by immersing the test tubes in boiling water for 5 min. The enzyme activity was determined by measuring the increase in absorbance at 235 nm arising from the double bond formed in the reaction product. 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} = 1.0 corresponds to 1 mg ml^{-1} protein.

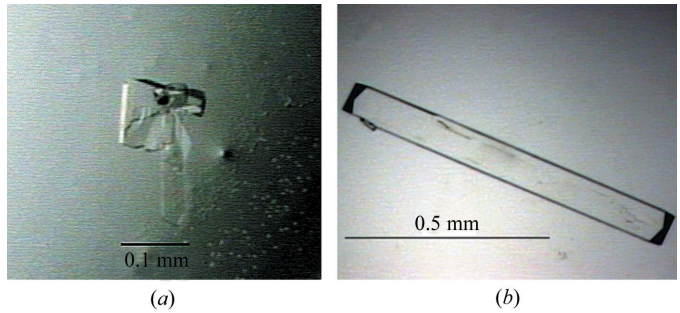
2.2. Protein expression and purification

The expression and purification of A1-II and A1-II′ were performed as described elsewhere (Yoon, Hashimoto, Miyake *et al.*, 2000; Miyake *et al.*, 2004). The purification procedures for A1-II′ were changed somewhat, as described below. Unless otherwise specified, all operations were carried out at 273–277 K. After the growth and ultrasonic disruption of *Escherichia coli* cells producing A1-II′, the cell extract was fractionated with ammonium sulfate. The precipitate (0–15% ammonium sulfate saturation) was removed by centrifugation at 15 000*g* and 277 K for 20 min. After dialysis against 20 m*M* Tris–HCl pH 7.5 (buffer *A*), the supernatant was applied onto a DEAE-Toyopearl 650M column (3 × 6 cm; Tosoh Co., Tokyo, Japan) previously equilibrated with buffer *A*. The enzyme did not bind and was eluted with buffer *A*. The enzyme solution was dialyzed against buffer *B* containing 20 m*M* Tris–HCl pH 7.5, 0.5 *M* NaCl and 10 m*M* imidazole. The solution was applied onto a nickel-bound HisTrap HP column (2 × 5 cm; Amersham Biosciences KK, Tokyo, Japan) previously equilibrated with buffer *B*. The enzyme was eluted with a linear gradient of imidazole (10–500 m*M*) in buffer *B* and the active fractions, which were eluted at 150–250 m*M* imidazole, were combined and dialyzed against buffer *A*. The dialysate was used as purified A1-II′.

The purity of the enzymes (A1-II and A1-II′) was confirmed by SDS–PAGE (Laemmli, 1970). According to the profiles on gel filtration and SDS–PAGE, both A1-II and A1-II′ were in a homogeneous monomeric form with the same molecular weight of 25 kDa. A1-II and A1-II′ were concentrated to 10 mg ml^{-1} by ultrafiltration with a Centriprep tube (Millipore Co., Tokyo, Japan) for crystallization.

2.3. Crystallization

Each alginate lyase (A1-II and A1-II′) purified from *E. coli* cells was crystallized at 293 K by the hanging-drop vapour-diffusion method. Each crystallization droplet was prepared on a siliconized cover slip by mixing 2 µl protein solution (10 mg ml^{-1} protein) with 2 µl precipitant solution. Since a few crystals of each enzyme were initially found on sparse-matrix screening using commercial crystallization kits from Hampton Research (Laguna Niguel, CA, USA), crystallization of the enzymes was attempted under a large number of conditions. After improvement of the conditions, the precipitant solutions most suitable for crystallization were determined to be mixtures of 0.1 *M* Tris–HCl pH 7.5, 0.12 *M* lithium sulfate and 28%



(*a*) (*b*)

Figure 2
Crystals of the family PL-7 alginate lyases. (*a*) A1-II, (*b*) A1-II′.

Table 1
Data-collection statistics for crystals of family PL-7 alginate lyases.

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

	A1-II	A1-II′
Wavelength (Å)	1.0	0.7
Resolution (Å)	50–2.2 (2.28–2.20)	50–1.0 (1.04–1.00)
Crystal system	Monoclinic	Orthorhombic
Space group	$P2_1$	$P2_12_12_1$
Unit-cell parameters (Å, °)	a = 51.3, b = 30.1, c = 101.6, β = 100.2	a = 34.6, b = 68.5, c = 80.3
Total observations	58527	920429
Independent reflections	15569	99115
Completeness (%)	98.3 (94.3)	96.0 (76.0)
$I/\sigma(I)$	9.8 (2.8)	9.5 (4.0)
R_{merge} (%)	10.2 (32.4)	5.3 (21.3)
Molecules per AU	1	1

PEG 4000 for A1-II, and 0.1 *M* sodium acetate pH 4.5, 0.2 *M* ammonium sulfate and 27.5% PEG 8000 for A1-II′. Square and long rod-shaped crystals were obtained under these conditions for A1-II and A1-II′, respectively (Fig. 2).

2.4. X-ray analysis

For alginate lyase A1-II, a crystal picked up from a droplet on a mounted nylon loop (Hampton Research, Laguna Niguel, CA, USA) was transferred to a cryoprotectant solution (30% glycerol, 0.1 *M* Tris–HCl pH 7.5, 0.12 *M* lithium sulfate and 28% PEG 4000) 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 the nitrogen-gas stream with 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 180 mm and 1.0° oscillation images were recorded with an exposure time of 15 s. The diffraction data for the crystal were obtained in the resolution range 50–2.2 Å and 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 = 51.3, b = 30.1, c = 101.6 Å, β = 100.2°. The preliminary X-ray crystallographic properties of A1-II are summarized in Table 1.

In the case of an alginate lyase A1-II′ crystal, the cryoprotectant solution was composed of 30% glycerol, 0.1 *M* sodium acetate pH 4.5, 0.2 *M* ammonium sulfate and 27.5% PEG 8000. X-ray diffraction images of the crystal were collected at 100 K with a Jupiter 210 detector and synchrotron radiation of wavelength 0.7 Å at the BL-38B1 station of SPring-8 (Hyogo, Japan). The distance between the crystal and detector was set at 110 mm and 1.0° oscillation images were recorded with an exposure time of 8 s. Diffraction data were obtained in the resolution range 50–1.0 Å. The space group of the crystal was determined to be $P2_12_12_1$ (orthorhombic), with unit-cell parameters a = 34.6, b = 68.5, c = 80.3 Å. The preliminary X-ray crystallographic properties of A1-II′ are summarized in Table 1. Other conditions or procedures for X-ray analysis were the same as those for A1-II described above.

The V_M values (Matthews, 1968) of A1-II and A1-II′ crystals, *i.e.* the crystal volumes per unit of protein molecular weight, were calculated to be 3.1 and 1.9 Å^3 Da^{-1}, respectively, when one molecule of the enzyme was present per asymmetric unit and their solvent contents were 59.7 and 34.9%, respectively. These V_M values and solvent contents lie within the ranges usually found for protein crystals.

It seems to be ideal for the further structural determination of A1-II and A1-II′ that both crystals contain one molecule in the asymmetric unit, compared with the previously prepared crystal of A1-II which contained 16 molecules (Yoon, Hashimoto, Katsuya *et al.*, 2000). The molecular-replacement method using the *CCP*4 program package or *CNS* (Brünger *et al.*, 1998) did not work well with PL-7 alginate lyase PA1167 (PDB code 1vav; Yamasaki *et al.*, 2004) and AlyPG (PDB code 1uai; Osawa *et al.*, 2005) as the model. Therefore, multiple isomorphous replacement (MIR) experiments using these enzymes are now in progress.

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