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Purification, crystallization and X-ray analysis of crystals of pectate lyase A from *Erwinia chrysanthemi*

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Pectate lyase A is secreted by *Erwinia chrysanthemi* and is a virulence factor for soft rot diseases in plants. Crystals of pectate lyase A were obtained by vapor-diffusion techniques in the presence of polyethylene glycol. The crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 48.96, b = 148.86, c = 78.61 Å, β = 97.32°. The crystals contain two protein molecules of 38 kDa per asymmetric unit and diffract to 2.4 Å using Cu $K\alpha$ radiation.

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1. Introduction

Erwinia are phytopathogenic bacteria that cause soft rots and other diseases in plants (Barras *et al.*, 1994; Collmer & Keen, 1986). These bacteria secrete large amounts of pectolytic enzymes, resulting in tissue maceration through the cleavage of the glycosidic bonds of pectic polymers. Pectic substances are found in the cell wall and middle lamella of higher plants and are composed primarily of α-1,4-linked galacturonsyl residues. Not only do microbial plant pathogens secrete a wide variety of enzymes to cleave pectic polymers, including pectate lyases, polygalacturonases, pectin lyases and pectin methylesterases, but they often also produce several isoforms of these enzymes. It is unclear why these pathogens have acquired multiple isoforms of pectate-degrading enzymes. The possible explanations include allowing the bacteria to access a wide variety of hosts and environmental conditions, to elevate synthesis of depolymerization enzymes or to handle different physical environments in the heterogeneous cell-wall pectin (Barling *et al.*, 1995). *E. chrysanthemi* secretes five pectate lyase (Pel) isoforms, referred to as PelA, PelB, PelC, PelD and PelE. The *pel* genes, which encode the pectate lyases, are independently regulated, despite their structural homology and tandem clustered organization. One gene cluster, referred to as *pelADE*, exhibits 58–90% amino-acid identity with PelA and PelE, exhibiting 62% identity in strain EC16. Proteins in this family contain one disulfide bond. The second gene cluster, *pelBC*, exhibits 70–85% amino-acid identity. The proteins contain two disulfide bonds. The homology between the pelBC and pelADE families is 27–31% (Keen & Tamaki, 1986).

PelA is significantly different from the other three isoforms, PelB, PelC and PelE, in *E. chrysanthemi* EC16. Whereas PelE, PelC and PelB have basic pIs of >10, >10 and 9, respectively, the pI of PelA is acidic, at 4.6 (Barras *et al.*, 1994). The enzymatic activity of PelA is also different from the other pectate lyases. PelA is about 1000 times less efficient in maceration of plant tissue than is pelE (Tamaki *et al.*, 1988). All *E. chrysanthemi* pectate lyases act by an endolytic cleavage of polygalacturonate. PelB and PelC both cleave polygalacturonate primarily to trigalacturonide endproducts, while PelE endproducts are predominantly disaccharides. In contrast, PelA cleaves the polysaccharide polymer in a more random manner, forming mainly di- and trisaccharides as well as oligomers up to dodecamers (Preston *et al.*, 1992).

The three-dimensional structure of three pectate lyase proteins have been reported, PelC (Yoder *et al.*, 1993; Yoder & Jurnak, 1995*a*) and PelE (Lietzke *et al.*, 1994) from *E. chrysanthemi* EC16 and a pectate lyase from *Bacillus subtilus* (Pickersgill *et al.*, 1994). They all share a novel folding motif, a parallel β-helix (Yoder & Jurnak, 1995*b*; Jenkins *et al.*, 1998). Although PelA is expected to also fold in a parallel β-helix, it is not understood why PelA has significantly different physical and enzymatic properties to its other isoforms. To shed light on this phenomenon, we have crystallized the 361 amino-acid (M_W = 38 756 Da), mature form of PelA from *E. chrysanthemi* EC16 as the first step in the crystal structure determination of this protein.

2. Materials and methods

Unless otherwise stated, all materials were purchased from Sigma. Typically, 1.5 l of *Escherichia coli* cells harboring pPEL812, a high-expression plasmid construct containing the *pelA* gene from *E. chrysanthemi* EC16 (Tamaki *et al.*, 1988), were grown at 301 K for 24 h in LB media containing 50 μg ml^{-1} ampicillin and 0.6 mM isopropyl β-thiogalactopyrandoside (IPTG). Cells were

harvested by centrifugation. PelA is localized in the periplasmic space in recombinant *E. coli* cells; periplasmic fractions were prepared from spheroplasts according to the method of Witholt *et al.* (1976). Briefly, this entailed resuspending cells in a Tris–HCl solution with sucrose and EDTA (all buffered to pH 8) and lysozyme, then gently stirring for 5 min at room temperature. After centrifugation, the supernatant (containing the periplasmic fraction) was dialyzed against buffer *A* (10 m*M* Tris–HCl pH 8.0). The dialysate was loaded onto a DEAE-Sepharose ion-exchange column (70 × 15 mm). The column was washed with three column volumes of buffer *A*, then eluted with a linear gradient from 0 to 0.3 *M* KCl in buffer *A* in five column volumes. Fractions containing PelA activity were pooled and prepared for hydrophobic interaction chromatography by addition of ammonium sulfate to sub-precipitation concentrations, typically between 1.3 and 1.6 *M* ammonium sulfate. The protein sample was loaded onto a phenyl-Sepharose CL-4B column pre-equilibrated with buffer *B* (50 m*M* phosphate buffer pH 7.0) and 1.4 *M* ammonium sulfate. The protein was eluted with a linear gradient from 1.4 to 0 *M* ammonium sulfate in buffer *B* in ten column volumes. Fractions containing PelA activity were pooled. The protein was simultaneously concentrated to 12 A_{280} ml^{-1} and the buffer exchanged to 10 m*M* HEPES pH 7.0 using a Collodian Membrane Apparatus with a 10 kDa molecular-weight cutoff (Schleicher & Schuell). Typical yields from 1.5 l culture media were 9 g cells and 10 mg purified PelA. Protein purity was primarily assessed by sodium dodecyl sulfate–polyacrylamide gel electrophoretic analysis.

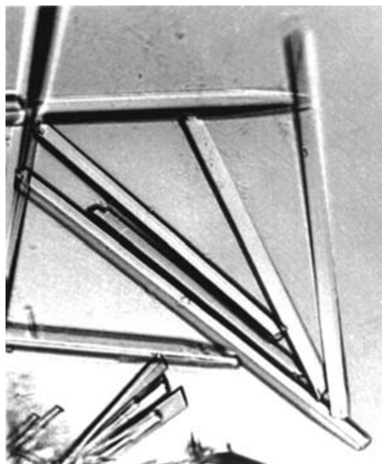

Figure 1
Crystals of PelA from *E. chrysanthemi*. Typical crystal dimensions are 0.8 × 0.10 × 0.05 mm.

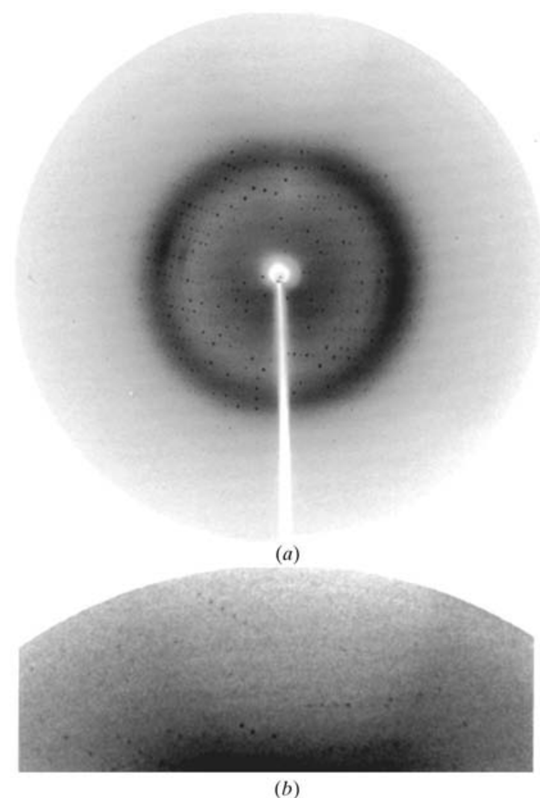

Figure 2
X-ray diffraction image of PelA. The diffraction image was taken from a MAR Research 300 mm detector with a crystal-to-film distance of 150 mm. The oscillation range was 1°, with an exposure time of 1000 s. The crystal was flash-cooled to 149 K. The detector edge corresponds to 2.01 Å resolution. (*a*) One diffraction image, (*b*) an enlargement of the top portion of the image in (*a*).

The enzymatic activity of PelA was measured by monitoring the increase in absorbance at 232 nm, as described by Keen *et al.* (1984). The β-elimination reaction of pectate lyase results in an unsaturated bond between the fourth and fifth carbon of the sugar on the non-reducing end, which can be detected at 232 nm. Activity was measured at room temperature in a 1 cm quartz cuvette. The 1.0 ml assay reaction contained 50 m*M* Tris–HCl pH 8.5, 0.125%(*w*/*v*) polygalacturonic acid, 0.5 m*M* $CaCl_2$ and an appropriate amount of protein.

Initial crystallization conditions were obtained using a sparse-matrix system to screen a wide variety of conditions (Jancarik & Kim, 1991), as implemented with Hampton Research Crystal Kit I and Crystal Kit II. All crystallization experiments used the sitting-drop vapor-phase equilibration method (McPherson, 1998). Usually a 6 µl drop containing the protein and precipitating solution was placed in the depression of a microbridge (Hampton Research) in a 24-well tissue-culture plate. The solution was equilibrated over 0.8 ml of a reservoir containing the precipitating solution only and sealed with clear tape. Crystal screens were duplicated at 295 and at 279 K.

Crystals were mounted in loops (Hampton Research) and flash-cooled in the gas stream from a liquid-nitrogen cryostat (Area Detector Systems, Corp.). X-ray data were collected at 149 K using Cu *Kα* radiation from a rotating-anode X-ray source operated at 50 kV and 100 mA. Immediately prior to flash-cooling, crystals were serially transferred to a cryoprotectant solution containing 6% PEG 8K, 6% PEG 1K and increasing amounts of glycerol from 5 to 30%. Space-group assignment and unit-cell parameters were based on the autoindexing routine of *DENZO* (Otwinowski, 1993).

3. Results and discussion

Several crystal forms of PelA have been observed. These were either too thin for mounting or demonstrated poor diffraction qualities. Optimization of the purification protocol was pivotal in obtaining X-ray quality crystals. The best crystals of PelA were obtained from an initial screen with Hampton Research Crystal Screen II, formulation number 7, while crystals not of diffraction quality or size were obtained with Crystal Screen I, formulation numbers 6, 10, 22 and 28. Optimal crystallization conditions were with polyethylene glycol (PEG) as a precipitant, using a solution containing 6 A_{280} ml^{-1} of PelA, 6%(*w*/*v*) PEG 8K, 6% PEG 1K, 5 m*M* HEPES pH 7.0 equilibrated over a reservoir containing 12% PEG 8K and 12% PEG 1K. Crystals grew in 1–2 d at room temperature or in one week at 279 K.

PelA crystals are monoclinic, with a long thin rod-like morphology and unit-cell parameters $a = 48.96$, $b = 148.86$, $c = 78.61$ Å, $\beta = 97.32°$. Typical crystal dimensions are 0.8 × 0.10 × 0.05 mm and are shown in Fig. 1. The number of molecules per asymmetric unit was estimated based on calculated values of V_m, the ratio of unit-cell volume to protein mass, and estimated values of V_s, the fractional volume occupied by solvent (Matthews, 1968). The fractional solvent content was estimated by $V_s = 1 - (\rho/V_m)$, where ρ is the crystal density. An average value of 1.23 g cm^{-3} was assumed

Table 1
Summary of crystals of pectate lyase A.

Space group	$P2_1$
Crystal morphology	Rods
Unit-cell parameters (Å, °)	a = 48.96, b = 148.86, c = 78.61, β = 97.32
Unit-cell volume (Å^3)	1.465×10^5
V_m (Å^3 Da^{-1})	2.295
Molecules per asymmetric unit	2
Estimated solvent content (%)	46.4
Diffraction limit (Å)	2.4

for ρ. Both two and three molecules per asymmetric unit yield values for V_m that are typical for proteins. In rotation- and translation-function searches for a molecular-replacement solution, two but not three molecules per asymmetric unit could be identified and packed reasonably well in the unit cell. Crystal parameters are listed in Table 1.

A typical diffraction pattern, indicating resolution, is shown in Fig. 2. The crystals diffract to 2.4 Å and weak reflections are observed at higher resolution (Fig. 2*b*). A molecular-replacement solution for the structure, using PelE from *E. chrysanthemi* as a model, is under way. Solutions for both the rotation and translation functions were obtained from *AMoRe* (Navaza, 1994); the polypeptide chain tracing and sequence assignment are under way.

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