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Crystallization of the pectate lyase PelI from *Erwinia chrysanthemi* and SAD phasing of a gold derivative

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The pectate lyase PelI is involved in the degradation of plant tissues by the phytopathogenic bacterium *Erwinia chrysanthemi*. It has been crystallized from a solution containing PEG 550 in the space group $P2_1$, with unit-cell parameters $a = 61.6$, $b = 70.7$, $c = 73.4$ Å, $\beta = 112.8^\circ$. Crystals diffract to 1.45 Å using synchrotron radiation. SAD phases have been computed from a gold-derivative crystal at the wavelength of maximum absorption (L_{III} edge).

1. Introduction

The enterobacterium *Erwinia chrysanthemi* is a causative agent of soft-rot disease in a wide variety of plants. Among a set of pectin-depolymerizing enzymes produced by *Erwinia*, the pectate lyases (Pel) are the major pectinases and play a key role in plant-tissue maceration (Robert-Baudouy *et al.*, 2000). These enzymes catalyse the cleavage of pectate (homogalacturonan) by β-elimination, breaking the α-glycosidic bond between O_1 and C_4 to leave an unsaturated C_4-C_5 bond. This mechanism is metal-ion-dependent: Ca^{2+} is the cofactor of most known Pels, while Co^{2+}, Cu^{2+}, Mn^{2+} or Ni^{2+} are required by some intracellular Pels involved in degradation of pectic oligomers (Shevchik *et al.*, 1999). By sequence analysis, Pels are classed into five distinct families, numbered 1, 2, 3, 9 and 10, of the 13 families of polysaccharide lyases classified in the CAZy database (Coutinho & Henrissat, 1999). The Pels produced by *E. chrysanthemi* are members of families 1 (PelA–PelE and PelZ), 2 (PelW), 3 (PelI) and 9 (PelL and PelX).

The three-dimensional structures of several Pels from families 1, 3 and 10 have been determined (Yoder *et al.*, 1993; Leitzke *et al.*, 1994; Pickersgill *et al.*, 1994; Akita *et al.*, 2001; Charnock *et al.*, 2002; Thomas *et al.*, 2002; Herron *et al.*, 2003). Most of these proteins have a right-handed parallel β-helix fold (Jurnak *et al.*, 1994). This protein fold, which was first reported for *E. chrysanthemi* PelC (Yoder *et al.*, 1993), is shared with many polysaccharide-active enzymes, mostly pectinases (Mayans *et al.*, 1997; Petersen *et al.*, 1997; Pickersgill *et al.*, 1998; Vitali *et al.*, 1998; Jenkins *et al.*, 2001). It is also shared with polysaccharide lyases of families 1, 3 and 9, carbohydrate esterases of family 8 and glycoside hydrolases of family 28 (CAZy). Thus, it was speculated that the β-helix fold could be particularly adapted to polysaccharide binding. However, a new polysaccharide lyase fold with an $(\alpha/\alpha)_3$ barrel has been described in a recent report (Charnock *et al.*, 2002).

The pectate lyase PelI belongs to family 3 and is the only known example from the *E. chrysanthemi* pectinases that consists of two functional modules, named the N-terminal and catalytic domains. These two domains are separated in external media by a specific cleavage produced by *E. chrysanthemi* proteases (Shevchik *et al.*, 1998). The catalytic domain of PelI released by proteolysis seems to possess the same enzymatic properties *in vitro* as the full-length protein. However, contrarily to the full-length PelI, the catalytic domain elicits a necrotic reaction *in planta* associated with an active defence by plants. This type of reaction, called a hypersensitive response (HR), is unusual for Pels, but is characteristic of a set of effector proteins secreted by the type III secretion systems of many plant pathogenic bacteria (Buttner & Bonas, 2003). It should be noted that family 3 of polysaccharide lyases also includes HrpW, the HR elicitor protein without pectate lyase activity, secreted by the type III secretion system (Gaudriault *et al.*, 1998). In contrast, PelI and other pectinases are secreted by the type II (Out) secretion system.

PelI has a 19-residue signal peptide that is cleaved during export. The N-terminal region of PelI (residues 20–119 as defined in the PRODOM domain database; Corpet *et al.*, 2000) exhibits a low sequence similarity to fibronectin type 3 domains, a functionally uncharacterized module present in some glycoside hydrolases and lyases (McKie *et al.*, 2001). The pectate lyase Pel-3 from *E. carotovora*, a close homologue of PelI, presents the same N-terminal extension. The role of this domain is not clear. The catalytic domain of PelI (residues 120–344) shows extensive similarity to the Pels belonging to family 3 of polysaccharide lyases and with the only known structural representative of this family, Pel-15

of *Bacillus* sp. strain KSM-P15 (Akita *et al.*, 2001). This 197-residue protein is folded into an eight-turn parallel β-helix and shows a sequence identity of 26% with PelI, suggesting that the two enzymes share the same fold. The Pel-15 active site is similar to those observed in the family 1 Pels, although the position of its divalent cation, a Ca^{2+}, does not correspond to any of the sites reported for PelC (Scavetta *et al.*, 1999).

Determination of the three-dimensional structure of the pectate lyase PelI should allow a detailed comparison of its overall fold and catalytic site with those of the other Pel families, allowing us to clarify whether these enzymes with parallel β-helix folds have evolved from a common ancestor or have arisen by convergent evolution to a closely related catalytic mechanism. Crystals of the enzyme with bound Ca^{2+} and depleted of its signal peptide have been obtained (*i.e.* PelI 20–344; MW = 35 kDa).

2. Protein expression and purification

A plasmid pTPLI (Shevchik *et al.*, 1997), used for PelI overproduction, was constructed by inserting the *Bgl*II–*Eco*RI 1.8 kbp fragment bearing the *pelI* gene into pT7-6 expression vector (Tabor & Richardson, 1985). *Escherichia coli* BL21(DE3) (Stratagene) cells carrying the pTPLI plasmid were grown at 303 K in Luria broth containing 150 µg ml^{-1} ampicillin. At an OD_{600} of 1.0, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1 m*M* and the cells were grown for an additional 2.5–3 h. Cells were harvested by centrifugation for 10 min at 5000*g* and 277 K and then frozen at 203 K. The overproduced protein was extracted from the cells by two cycles of freeze–thawing (Johnson & Hecht, 1994) using 20 m*M* sodium phosphate, 5 m*M* EDTA pH 7. The extract was diluted with the same volume of 3.4 *M* ammonium sulfate, 80 m*M* sodium phosphate, 5 m*M* EDTA pH 7 and centrifuged for 20 min at 20 000*g* and 277 K. This sample was loaded onto a Phenyl-TSK gel (TosoHaas) column (20 × 2.5 cm), equilibrated with 50 m*M* sodium phosphate, 5 m*M* EDTA (buffer *A*) supplemented with 1.7 *M* $(NH_4)_2SO_4$. The column was washed with 0.2 l of the same buffer and then with 0.1 l of buffer *A* containing 1 *M* $(NH_4)_2SO_4$. The enzyme was eluted with a linear gradient of 1.0–0.5 *M* $(NH_4)_2SO_4$ in buffer *A*. The active fractions were pooled, concentrated in a Centricon 10 (Amicon) and the buffer was exchanged with 20 m*M* sodium acetate, 1 m*M* EDTA pH 5.0 (buffer *B*). The sample was loaded onto a Mono-Q (Pharmacia) HR 10/10 column equilibrated with buffer *B* and PelI was eluted with a 0–0.1 *M* NaCl linear gradient in buffer *B*. The purified enzyme was buffer-exchanged with 10 m*M* Tris–HCl, 0.1 m*M* $CaCl_2$, concentrated to 10 mg ml^{-1} and stored at 253 K.

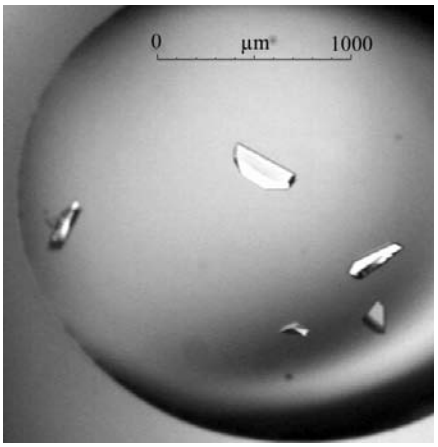

Figure 1
Crystals of PelI from *E. chrysanthemi* obtained at 292 K by the hanging-drop technique. Crystals of dimensions 0.4 × 0.2 × 0.1 mm can be obtained in two weeks.

Table 1
Statistics of data processing.

	In-house native	Synchrotron native	Synchrotron derivative $KAu(CN)_2$ (f''_{max})
X-ray source	Cu $K\alpha$ rotating anode	BM30A, ESRF	BM30A, ESRF
Wavelength (Å)	1.5418	0.9400	1.0400
Space group	$P2_1$	$P2_1$	$P2_1$
Unit-cell parameters (Å,°)	a = 61.6, b = 70.7, c = 73.4, β = 112.8	a = 61.6, b = 70.7, c = 73.4, β = 112.8	a = 61.7, b = 70.7, c = 73.5, β = 112.8
Resolution range (Å)	20.0–1.6 (1.7–1.6)	20.0–1.45 (1.55–1.45)	20.0–1.6 (1.7–1.6)
Total No. of reflections	248150(32655)	379012 (35039)	279614 (41803)
No. of unique reflections	73087 (10209)	102436 (9931)	76466 (12673)
Completeness (%)	98.3 (96.5)	99.5 (99.9)	99.7 (99.9)
Redundancy	3.4 (3.2)	3.7 (3.5)	3.7 (3.3)
$\langle I/\sigma(I)\rangle$	3.7 (2.6)	10.6 (3.4)	16.5 (6.4)
R_{sym}† (%)	9.2 (23.7)	6.5 (33.0)	4.3 (16.3)
$\sigma_{norm}/\sigma_{ano}$‡	—	1.05	1.32
Mean figure of merit (f.o.m.)§	—	—	0.33

† $R_{sym} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i |I_i(hkl)|$, where I_i is the ith measurement of reflection $I(hkl)$. ‡ σ_{norm} and σ_{ano} are mean value of $\sigma(I)$ for acentric reflections assuming reflection $I(hkl) = I(\overline{hkl})$ and $I(hkl) \neq I(\overline{hkl})$, respectively. § f.o.m. = $|F(hkl)_{best}|/F(hkl)$, with $F(hkl)_{best} = \sum_\alpha P(\alpha)F_{hkl}/\sum_\alpha P(\alpha)$.

3. Crystallization

Crystallization trials were achieved using commercial screens from Hampton Research (Crystal Screens I and II, PEG, PEG-Ion and Ammonium Sulfate Screens) and Molecular Dimensions Ltd (Structure Screens I and II) using the hanging-drop vapour-diffusion technique at 277 and 292 K. The reservoirs of Limbro 24-well plates were filled with 700 µl of the crystallization solutions. Hanging drops were obtained by mixing 2 µl protein solution at 10 mg ml^{-1} with 2 µl reservoir solution. Molecular Dimensions Structure Screen II condition No. 27 (0.01 *M* zinc sulfate heptahydrate, 0.1 *M* MES pH 6.5, 25% PEG 550) produced good crystals of dimensions up to 0.4 × 0.2 × 0.1 mm within two weeks at 292 K (Fig. 1). Screening around this condition did not yield better crystals, but did show that the presence of zinc sulfate was necessary for crystallization. Crystals were mounted in a cryoloop and flash-frozen in a 100 K steam of nitrogen gas prior to data collection. X-ray measurements were taken in-house on a 345 mm MAR Research image-plate system using Cu $K\alpha$ radiation from a Nonius FR 591 rotating-anode generator equipped with Osmic mirror optics. They were cryoprotected by adding 0.5 µl ethylene glycol to the 4 µl hanging drop immediately prior to mounting (the resulting solution contains ∼10% ethylene glycol). Crystals belong to a monoclinic space group (Table 1). The calculated V_M value is 2.1 Å^3 Da^{-1} for two molecules in the asymmetric unit, which corresponds to a solvent content of 41% (Matthews, 1968).

4. Data collection and processing

A native data set was collected in-house to 1.6 Å resolution. The crystal-to-detector distance was 108 mm, the oscillation step 1° and the total oscillation range 180°. Data were processed with *MOSFLM* (Leslie, 1992) and were merged, scaled and reduced with *SCALA* and *TRUNCATE* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). Statistics are presented in Table 1. Attempts to solve the structure of PelI by the molecular-replacement method using the program *AMoRe* (Navaza, 2001) and the structure of Pel-15 (Akita *et al.*, 2001) as a model were unsuccessful. Consequently, crystals were soaked for 24–48 h in 6 µl drops of a solution of

mother liquor containing various heavy-atom salts (5–10 m*M* gold, mercury, lead and samarium salts) so as to solve the structure by an isomorphous replacement method. Most crystals were stable in the soaking solutions and their diffraction was tested in-house. The best derivatives were stored at 100 K in a tank of liquid nitrogen prior to synchrotron data collection at the wavelength-tunable beamline FIP-BM30A at ESRF, Grenoble, France.

5. Phasing and model building

The structure of PelI was determined using the anomalous scattering signal of a gold-derivative crystal obtained after a 48 h soak in a mother-liquor solution containing 10 m*M* $KAu(CN)_2$. The unit-cell parameters of the derivative crystal are very close to those of the native (Table 1). A fluorescence scan was taken around the gold L_{III} theoretical absorption edge and diffraction data were collected on a MAR CCD detector at three wavelengths corresponding to the absorption maximum (λ = 1.0400 Å), inflection (λ = 1.0404 Å) and remote (λ = 0.95 Å). For each wavelength, the crystal-to-detector distance was 100 mm, the oscillation step was 1° and the total oscillation range was 180°. The first data set collected at the peak with a maximum resolution of 1.6 Å was sufficient to calculate the phases using the single anomalous dispersion method (SAD). The peak data set was processed with *XDS* and scaled with *XSCALE* (Kabsch, 1993; statistics are given in Table 1) assuming that Friedel's law was broken. A significant contribution by an anomalous scatterer to the intensities was detected by *XSCALE* with a $\sigma_{norm}/\sigma_{ano}$ value equal to 1.3 instead of 1.0 (definition in Table 1) in the resolution range 20–1.6 Å. A total of seven Au atoms in the asymmetric unit were detected by an automated search with the program *SOLVE* (Terwilliger & Berendzen, 1999) and phases were computed; the overall mean figure of merit (f.o.m. definition in Table 1) was 0.33. Density modification with the program *RESOLVE* (Terwilliger, 2002) resulted in an increase in the f.o.m. to 0.52 and in an interpretable map. A model accounting for 65% of the protein was automatically built by *RESOLVE* after the last step of density modification. This model included 308 residues built according to the sequence assignment and 115 residues built without side chains. The C^{α} traces of the two monomers contained in the asymmetric unit were clearly defined, as observed on a graphics station with the program *TURBO-FRODO* (Roussel & Cambillau, 1989). Subsequent attempts to include the edge and the remote data sets for phasing did not improve the quality of the electron-density maps. A rigid-body refinement of this initial structure was performed with *CNS* (Brünger *et al.*, 1998) against the in-house native data set and an *R* factor of 45% was obtained in the resolution range 15–3 Å. A native data set was subsequently collected at the FIP-BM30A beamline to 1.45 Å resolution. The wavelength was 0.94 Å, the crystal-to-detector distance 100 mm, the oscillation step 1° and the total oscillation range 180°. Data processing was performed with *XDS* (statistics in Table 1). Crystallographic refinement is in progress.

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