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Crystallization and preliminary X-ray analysis of a member of a new family of pectate lyases, PelL from *Erwinia chrysanthemi*

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Abstract

PelL, a pectate lyase (E.C. 4.2.2.9) from *E. chrysanthemi* 3937 that is not homologous to the lyases with known structures, has been purified and crystallized by the hanging-drop method using a variety of organic solvents as precipitant. Elongated lathes grown from poly(ethylene glycol) plus isopropanol belong to the space group $P2_12_12_1$ with cell dimensions a = 55.5, b = 58.2, c = 16.4 Å with a single molecule in the asymmetric unit. Although complete data sets have been collected to 2.3 Å resolution, these crystals diffract to at least 1.9 Å resolution and are suitable for structure determination. Chunky plates grown using other organic solvents as the precipitant diffracted to 3 Å resolution and were partially characterized as a second orthorhombic crystal form with space group $P2_12_12$ and cell dimensions a = 119.1, b = 140.5 and c = 105.4 Å, suggesting four molecules in the asymmetric unit.

1. Introduction

Pectate and pectin lyases (E.C. 4.2.2.9 and E.C. 4.2.2.10, respectively) are produced by bacteria and fungi (Pilnik & Rombouts, 1981), while related genes are found in many plants (Wing *et al.*, 1990). These enzymes catalyse the degradation of pectate or pectin respectively by β-elimination, breaking the α-glycosidic bond between O1 and C4 to leave an unsaturated 4–5 C—C bond.

Analysis of the sequences of these lyases shows that the majority of pectate and pectin lyases fall into the 'extracellular pectate lyase' superfamily (Henrissat *et al.*, 1995). The structures of four lyases from this superfamily have been determined: pectate lyases PelC and PelE from *E. chrysanthemi* (Yoder *et al.*, 1993; Lietzke *et al.*, 1994), the pectate lyase of *Bacillus subtilis* (Pickersgill *et al.*, 1994) and pectin lyase A from *Aspergillus niger* (Mayans *et al.*, 1997).

However, the sequences of several pectate lyases show no recognizable similarity to this superfamily and appear to define five additional families of lyases. These are the 'periplasmic pectate lyases' (Manulis *et al.*, 1988; Hinton *et al.*, 1989), the pectate lyases from *Fusarium solani* (González-Candelas & Kolattukudy, 1992), the oligogalacturonate lyases (Reverchon *et al.*, 1989), pectate lyase PelZ from *E. chrysanthemi* (Pissavim *et al.*, 1996), and a family containing two members, PelX (Brooks *et al.*, 1990) and PelL (Lojkowska *et al.*, 1995) from *E. chrysanthemi*.

Lojkowska *et al.* (1995) point out a string of similarities between PelL and the 'extracellular pectate lyase' superfamily. PelL is a pectate lyase with approximately the same molecular weight (*i.e.* 400 residues compared to 399 for BsPel), requires calcium for activity with a similar pH optimum of 8.5, and has a sequence which suggests an all-β fold and a high content of asparagines, including four pairs of consecutive asparagines and one triplet. Pairs of asparagines are common in the extracellular lyase family at the T2 turns. Thus, PelL and PelX may well have a parallel β-helix fold despite the lack of any definite evidence for the structure of PelL. The alternative is the convergent evolution of a closely related catalytic mechanism. The determination of the three-dimensional structure of PelL will resolve this ambiguity, which at present poses a tantalizing challenge to structure prediction by techniques such as 'threading' (Sippl & Weitckus, 1992).

Lojkowska *et al.* (1995) show that PelL differs from most of the members of the 'extracellular pectate lyase' superfamily in not showing a strong specificity between either moderately methylated pectin (up to 68% of esterification) or demethylated pectin (pectate). Discrimination between pectin and pectate by the extracellular pectate lyases is achieved by a combination of long-range electrostatic interactions and the hydrophobicity of the active site (Mayans *et al.*, 1997). Thus, pectin lyase A from *A. niger* has an active site containing a cage of aromatic residues and a negative electrostatic potential, strongly selecting against the binding of pectate, whilst pectate lyase from *B. subtilis* complexed with calcium (Pickersgill *et al.*, 1994) has a ribbon of positive electrostatic potential in a hydrophillic active site. The broad specificity of PelL and its observed p*I* of 8.2, suggests a new combination of features at the substrate-recognition site.

Most of the pectinolytic enzymes of *E. chrysanthemi*, including PelL, are exported *via* the Out system (Pugsley, 1993; Shevchik *et al.*, 1997), which is found in many bacterial pathogens of both plants and animals. It is not known how proteins exported *via* this system are recognized but it has been suggested that the three-dimensional structure of the exported protein may be targeted (He *et al.*, 1991; Palomaki & Saarilahti, 1995). The structures of distantly related or unrelated enzymes exported from *E. chrysanthemi* by this system (*i.e.* PelC, PelE, PelL and CelZ) should enable any shared features of the tertiary structure, such as a common pattern of charges, to be identified.

2. Materials and methods

2.1. *Expression and purification*

A plasmid, pT7L1.6, was constructed for PelL overproduction by inserting the *Nsi*I–*Sal*I 1.5 kb fragment with

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peIL gene from pEL133 (Lojkowska *et al.*, 1995) in pT7–6 (Tabor & Richardson, 1985). The *Escherichia coli* strain used for the overproduction is BL21(DE3) (Stratagene).

BL21(pTL1.6) cells 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 harvested after 2 h of additional growth by centrifugation for 5 min at 5000*g* and 277 K, then frozen at 193 K. The overproduced protein was extracted from the cells by three cycles of freeze-thawing (Johnson & Hecht, 1994).

The PeIL, suspended in the supernatant of a 50% ammonium sulfate precipitate, was concentrated by 90% ammonium sulfate precipitation. The pellet was solubilized in 10 m*M* sodium phosphate buffer containing 1.7 *M* ammonium sulfate and loaded onto a Phenyl–TSK–GEL column, equilibrated with the same buffer. PeIL was eluted with a 1.7–1.0 *M* ammonium sulfate linear gradient. Fractions containing PeIL activity were concentrated in a Centricon 10 (Amicon Co.) and applied to a Mono-Q (Pharmacia) ion-exchange column equilibrated with 20 m*M* Tris–HCl pH 9.0 buffer. PeIL did not bind onto the column and was eluted in the void volume. The protein was loaded onto a Superdex 200 (Pharmacia) gel-filtration column. The PeIL peak fractions were pooled and concentrated in a Centricon 10 (Amicon Co.) in 20 m*M* HEPES–NaOH pH 7.5 buffer. Glycerol was added to 50% and the preparation was stored at 253 K.

2.2. *Protein characterization*

No contaminating protein could be detected in this preparation by SDS–PAGE, loading 20 μg of protein onto a gel. The PeIL sequence contains an N-terminal signal peptide, which is normally cleaved by signal peptidase during passage through the cytoplasmic membrane. PeIL extracted from the *E. coli* periplasm is approximately 2 kDa lower in molecular weight than PeIL retained in *E. coli* cytoplasm (V. Shevchik, unpublished work). As recombinant proteins overproduced in *E. coli* are not always correctly processed, the molecular weight was checked to establish that PeIL extracted and purified from the BL21(pT7L1.6) strain corresponded in molecular weight to the periplasmic form, showing that the purified protein had been processed.

Fig. 1. Rosettes of elongated lathes of PeIL grown from PEG and isopropanol. The largest crystal (at the left) was cut free and used to collect the initial native data. After cutting it free (making the longest dimension shorter) the crystal was approximately 0.6 × 0.1 × 0.02 mm with *b* as the long axis.

2.3. *Crystallization*

To remove buffer and glycerol, the enzyme was placed in a 10 ml stirred cell concentrator (Amicon Co.) fitted with a 10 kDa cut-off polysulfone membrane (Flowgen), repeatedly washed with calcium chloride solution (5 m*M* in the initial screening and 1 m*M* in subsequent trials), and then concentrated. Protein concentrations were estimated using the OD_{280}, assuming an OD_{280} of 1.0 for a 1 mg ml^{-1} solution. Crystallization conditions were surveyed by the protocol of Jancarik & Kim (1991) with screening kits supplied by Hampton Research using the hanging-drop method over 1 ml reservoirs on siliconized cover slips in Linbro trays. A protein concentration of approximately 20–30 mg ml^{-1} was used. For the initial screen, drops of 3 μl of protein plus 3 μl of reservoir were used and drops with 2–4 μl were used subsequently. Crystallization trials were maintained at 291 K.

2.4. *Data collection and processing*

Data collection at LURE (University of Paris-Sud, Orsay, France) used a 30 cm MAR image plate with a wavelength of 0.938 Å on wiggler beamline DW32 and an 18 cm MAR image plate on beamline D41 with a wavelength of 1.385 Å. Data from ELETTRA (Trieste, Italy) used an 18 cm MAR image plate at a wavelength of 1.24 Å. Data collection at BL6A, the Photon Factory (Tsukuba, Japan), used a cylindrical Weissenberg camera with a radius of 430 mm (Sakabe, 1991) and a wavelength of 1.000 Å. Data collection with Cu $K\alpha$ radiation used a MacScience DIP 1030 with double mirrors on a Siemens rotating-anode generator running at 45 kV and 80 mA. All cooling used an Oxford Cryosystems Cryostream except that cooling to 273 K at LURE used locally designed equipment. Images were autoindexed and processed with the program *DENZO* and data were merged and statistics calculated with the program *SCALEPACK* (Otwinowski, 1993).

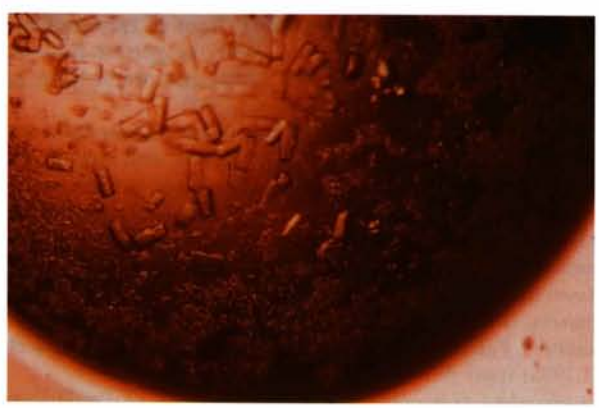

Fig. 2. Chunky plates of PeIL grown from 1,6-hexanediol and isopropanol. The longest dimension of the largest crystals is approximately 0.1 mm.

3. Results

3.1. *Crystallization*

In the initial screen (Jancarik & Kim, 1991), rosettes of elongated lathes grew when PelL, at an estimated protein concentration of 20 mg ml^{-1}, was equilibrated against a reservoir of 20%(*v*/*v*) isopropanol, 20%(*w*/*v*) polyethylene glycol (PEG) 4000 buffered at pH 5.6 by 0.1 *M* sodium citrate after approximately 6 months (Fig. 1). The largest crystals could be cut free from the rosette with approximate dimensions 0.6 × 0.1 × 0.02 mm. The slow growth of these crystals and their habit was reproducible with similar rosettes of lathes appearing under these conditions after approximately 4 months.

As other screen conditions using organic solvents as precipitants had given small crystals, a survey was made using organic solvents as precipitant. Chunky plates, resembling those shown in Fig. 2, grew in one to two weeks from 38 to 50% total concentrations of organic solvents including 2,5-hexanediol, 1,6-hexanediol and 2-methyl-2,4-pentanediol with up to 5% isopropanol. However, nucleation tended to be too rapid, leading to large numbers of small crystals or larger intergrown crystals and only small plates (approximately 0.1 × 0.1 × 0.02 mm) could be mounted. Phase separation was also frequently observed.

Medium-sized single crystals (or near-single crystals that could be cut free of other crystals) with dimensions of approximately 0.2 × 0.2 × 0.075 mm could be grown from 45%(*w*/*v*) 1,6-hexanediol plus 5%isopropanol buffered by 0.1 *M* citrate at pH 5.6 using 4 µl of protein and 2 µl of reservoir in the drop. The protein solution contained 1 m*M* $CaCl_2$. The growth of these crystals was unusual in that a precipitate seemed to form initially followed by the slow growth of crystals from the precipitate.

3.2. *Data collection and processing*

Data from a crystal cut from a rosette of elongated lathes grown from PEG and isopropanol were collected at approximately 273 K using beamline DW32 at LURE. A data set was collected to 2.3 Å resolution using the 18 cm MAR option. Processing of 66 1.0° oscillation images gave 16 980 independent reflections to 2.29 Å resolution from 43 185 observations (after rejecting 76), with an $R_{sym}(I)$ of 6.5% overall (12.8% in the outer 2.37–2.29 Å shell). These data are 96.7% complete to 2.29 Å resolution (90.4% in the outer 2.37–2.29 Å shell). Overall 82.2% of reflections have intensities greater than three standard deviations (70.7% in the outer 2.37–2.29 Å shell). This data set established the space group as $P2_12_12_1$ with cell dimensions a = 55.5, b = 58.2, c = 116.4 Å. This unit cell implies that there is only one molecule of PelL in the asymmetric unit with a V_m = 2.2 Å^3 Da^{-1} (Matthews, 1968). Although only 2.3 Å resolution data has been collected, weak diffraction was observed to 1.9 Å resolution from this crystal.

A crystal grown from 1,6-hexanediol was collected at room temperature on beamline D41 at LURE and 64 images were processed to give 15 189 independent reflections from 50 106 observations with an $R_{sym}(I)$ of 7.9% (16.7% in the outer 2.38–2.30 Å shell). This data set was 86.1% complete (72.1% in the outer 2.38–2.30 Å shell). The space group was $P2_12_12_1$ with cell dimensions of a = 55.6, b = 58.4, c = 116.8 Å. Scaling the two data sets gave an overall R factor of 11.2% on F for the 14 653 common reflections, with strong reflections generally scaling to give R factors below 6%.

Crystals grown from the 1,6-hexanediol conditions were soaked in 16 m*M* methyl mercury chloride under the same conditions for 2 h. Data were collected using synchrotron radiation at ELETTRA. A crystal was mounted in a loop and data were collected at 100 K using the soaking solution as cryoprotectant. Processing gave 8464 independent reflections from 35 935 observations with an $R_{sym}(I)$ of 3.5% (4.5% in the outer 2.84–2.74 Å shell). This data set was 83.9% complete (80.6% in the outer 2.84–2.74 Å shell). The space group was $P2_12_12_1$ with cell dimensions a = 55.2, b = 57.7, c = 114.8 Å. Diffraction was observed to 1.8 Å resolution from this crystal. Data collected at room temperature from a crystal that had been soaked under the same conditions were limited by rapid radiation damage.

A crystal grown from 2,5-hexanediol was soaked for 16 h in 19 m*M* potassium gold cyanide [$KAu(CN)_2$] under the 1,6-hexanediol conditions and was used for data collection at room temperature in a capillary on BL6A at the Photon Factory, but suffered rapid radiation damage. An initial 1.0° oscillation showed diffraction to approximately 2.8 Å resolution but the subsequent Weissenberg images showed diffraction to approximately 3.0, 3.5, 4 and 5.5 Å. This crystal was also orthorhombic but with very different cell dimensions, a = 119.1, b = 140.5 and c = 105.4 Å.

Subsequently, crystals grown from MPD and 1,6-hexanediol have been examined with Cu $K\alpha$ radiation and have shown similar cell dimensions. Limited data (23°) were collected from the MPD-grown crystal at 100 K with long exposures. Data could be processed to 3.6 Å resolution only with cell dimensions a = 118.4, b = 137.5 and c = 104.7 Å, merging to give only 28.4% completeness and a rather high $R_{sym}(I)$ of 12.8%. Assuming that these crystals have the same space group, systematic absences imply that this is $P2_12_12$, although clearly a single complete data set is desirable to confirm the space group. This cell suggests that the asymmetric unit contains four molecules giving a V_m = 2.6 Å^3 Da^{-1}.

4. Discussion

Crystals of recombinant PelL from *E. chrysanthemi*, a member of a new family of pectate lyases, have been grown and are suitable for the determination of the structure. Native data have been collected and processed to 2.3 Å resolution, although reflections to higher resolution can be observed. As no homologous structure is available, determination of the structure will require the use of isomorphous heavy-atom derivatives or MAD phasing.

Despite different crystallization conditions and habits, crystals grown using a mixture of PEG and isopropanol as precipitant are closely related to one crystal form grown from 1,6-hexanediol and isopropanol although there may be some non-isomorphism between the data sets.

The mercury data set collected at 100 K is clearly not closely isomorphous with the native data. Detailed analysis of mercury binding requires collection of a native data set at 100 K. The non-isomorphism along the c axis is probably mostly due to freezing. This was checked by irradiating a crystal at room temperature with the same soaking conditions and wavelength. This crystal had cell edges of a = 55.5, b = 58.2 and c = 116.5 Å. Unfortunately, preliminary analysis of

the incomplete (21.3%) room-temperature data set using normal probability analysis (Howell & Smith, 1992) suggests little mercury binding despite the very high concentration of methyl mercury chloride of 16 m*M* and the presence of three cysteines in the PeIL sequence. Preliminary trials have been made with other heavy atoms. Initial attempts to make a platinum derivative with potassium chloroplatinate (K_2PtCl_4) showed a rapid reaction producing yellow poorly ordered crystals while crystals soaked in lanthanides have also diffracted significantly less well than the native crystals.

Characterization of the crystals with the larger orthorhombic cell is still incomplete. The non-isomorphism between the two observed cells, especially along the *b* axis, might be caused by either cooling to 100 K or reaction with gold cyanide. Collection of complete data sets to 3 Å resolution or better will probably require the use of synchrotron radiation at approximately 100 K. Because this cell most probably has four molecules in the asymmetric unit, intercrystal averaging might assist the structure determination if an approximate molecular envelope could be determined and positioned in the two cells.

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