Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Crystallization, X-ray diffraction analysis and preliminary structure determination of the polygalacturonase PehA from *Agrobacterium vitis*

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Received 12 May 2008
Accepted 29 May 2008

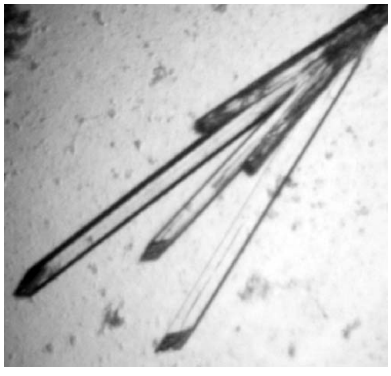
Polygalacturonases are pectate-degrading enzymes that belong to glycoside hydrolase family 28 and hydrolyze the α-1,4 glycosidic bond between neighboring galacturonasyl residues of the homogalacturonan substrate. The acidic polygalacturonase PehA from *Agrobacterium vitis* was overexpressed in *Escherichia coli*, where it accumulated in the periplasmic fraction. It was purified to homogeneity *via* a two-step chromatography procedure and crystallized using the hanging-drop vapour-diffusion technique. PehA crystals belonged to space group $P2_1$, with unit-cell parameters a = 52.387, b = 62.738, c = 149.165 Å, β = 89.98°. Crystals diffracted to 1.59 Å resolution and contained two molecules per asymmetric unit. An initial structure determination by molecular replacement indicated a right-handed parallel β-helix fold.

1. Introduction

The plant cell wall, which primarily consists of cellulose, hemicellulose, pectin and proteins, provides the cell structural support and acts as a physical barrier to prevent disease. Pectins, which are a major component of the primary cell walls of dicots and nongraminaceous monocot cells, are found predominantly in the outer wall regions and the middle lamella, the layer between neighboring cells. Plant pathogenic bacteria and fungi secrete a battery of enzymes to degrade the pectic cell-wall components, including but not limited to polygalacturonases (PGs), pectate lyases, pectin lyases and pectin methylesterases (Brencic & Winans, 2005; D'Ovidio *et al.*, 2004; Hadfield & Bennett, 1998). PGs (EC 3.2.1.15) are inverting glycoside hydrolases that cleave the α-1,4 glycosidic bond in pectate polymers. Based on amino-acid similarity, they are members of glycosidase family 28 (Henrissat, 1991).

The Gram-negative soil bacterium *Agrobacterium vitis*, previously *A. tumefaciens* biovar 3, is the causative agent of crown gall in grapevines, an important disease in viticultural areas (McGuire *et al.*, 1991; Rodriguez-Palenzuela *et al.*, 1991). In addition to its tumorigenic capacity, *A. vitis* also causes grape-specific root decay (Burr *et al.*, 1987) by secreting a single acidic PG named PehA (McGuire *et al.*, 1991). A PG-deficient strain ($PehA^-$) is unable to cause root decay, has decreased attachment in wounding assays and is less tumorigenic than the wild-type strain (Rodriguez-Palenzuela *et al.*, 1991). These results demonstrate the role of PehA as a multifunctional virulence factor.

Of the seven PG structures currently available, five are fungal and two are bacterial. The first reported structure was PehA from the bacterial plant pathogen *Erwinia carotovora* (PDB code 1bhe; Pickersgill *et al.*, 1998). The other bacterial PG is an exopolygalacturonase from *Yersinia enterocolitica* (PDB codes 2uve and 2uvf; Abbott & Boraston, 2007). The fungal PGs are all endopolygalacturonases: PDB entries 1ia5 and 1ib4 from *Aspergillus aculeatus* (Cho *et al.*, 2001), 1hg8 from *Fusarium moniliforme* (Federici *et al.*, 2001), 1k5c, 1kcc and 1kcd from *Stereum purpureum* (Shimizu *et al.*, 2002), 1nhc from *Aspergillus niger* (van Pouderoyen *et al.*, 2003) and 1czf from *A. niger* (van Santen *et al.*, 1999). All of the structures fold in a right-handed parallel β-helix, a common fold among pectolytic enzymes. The six fungal PGs show conservation of molecular size (34.2–37.7 kDa) and are predominantly acidic (pI 6.4 and lower), while the PG from *E. carotovora* is slightly larger (40.1 kDa) and basic (pI 10.2;



Ried & Collmer, 1986). In contrast, *A. vitis* PehA (gi:1621469) has a mature molecular weight of 54 kDa and a measured pI of ∼4.5 (Rodriguez-Palenzuela *et al.*, 1991). The structure solution of this large and acidic PG is sought in order to further define understanding of the bacterial molecules. The crystallization of the endopolygalacturonase PehA from *A. vitis* and a preliminary account of its structure are described below.

2. Materials and methods

2.1. PehA expression and purification

The construct pCPP2068 (Rodriguez-Palenzuela *et al.*, 1991) containing the gene coding for PehA was kindly provided by A. Collmer (Cornell University, Ithaca, New York, USA). The entire PehA-coding region was amplified using Pfu Turbo (Stratagene) in a polymerase chain reaction with primers designed to include 5′ *Eco*RI and 3′ *Hin*dIII restriction sites (forward, 5′-GGAATTCACCATGCCCGGACCTGTTTTTGCCCGCC-3′; reverse, 5′-CTCTCAAGCTTACTACGACACGATCCGTTCAAAACG-3′). The amplified product was digested with *Eco*RI and *Hin*dIII, ligated into the expression vector pBAD24 (Guzman *et al.*, 1995) prepared with the same enzymes and transformed into *Escherichia coli* strain TOP10. The completed construct, pMDY30.6, contained the PehA-coding sequence driven by the arabinose-inducible promoter P_{BAD}.

E. coli TOP10 cells containing pMDY30.6 were grown at 310 K in 2 l baffled flasks (200 rev min^{-1} orbital shaking) to an A_{600} of 0.6 in LB medium containing 100 µg ml^{-1} ampicillin. Prior to induction, the culture temperature was reduced to 292 K and PehA expression was initiated by the addition of solid arabinose to a final concentration of 0.05%(*w*/*v*). Protein production was continued for a further 18 h at 292 K and cells were then harvested by centrifugation (9700*g*, 5 min). Because the coding region contains a Sec-dependent signal peptide, the expressed protein was purified from the periplasmic fraction following spheroplast formation using the procedure of Witholt *et al.* (1976). Briefly, each 0.5 g of cells was resuspended in 1 ml 0.2 *M* Tris–HCl pH 8, 1 *M* sucrose and 0.01 *M* EDTA. The cell pellet was resuspended by gentle pipet mixing and fresh lysozyme was added to 1 mg ml^{-1} followed by a twofold dilution with water. The solution was incubated on ice for 20 min. The cells were pelleted as above and the soluble component was labelled the periplasmic fraction.

Prior to separation, the periplasmic fraction was dialyzed against 25 m*M* Tris–HCl pH 8.0 using a 30 000 Da molecular-weight cutoff (MWCO) dialysis membrane (Spectra/Por 7, Spectrum Laboratories). The dialyzed material was fractionated by gravity-flow anion-exchange chromatography (Q Sepharose, GE Healthcare) using an NaCl gradient (0–0.3 *M*) in ten column volumes of dialysis buffer. Fractions exhibiting enzymatic activity were combined, dialyzed against 10 m*M* sodium phosphate buffer pH 6.8 and chromatographed on hydroxyapatite resin (BioRad Bio-Gel HT) using ten column volumes of a sodium phosphate gradient (pH 6.8, 0.01–0.2 *M*). Enzymatically active fractions were pooled and buffer-exchanged with 10 m*M* HEPES pH 7.0 using an Amicon ultrafiltration apparatus fitted with a 30 000 Da MWCO membrane. The final concentration of the protein, using a calculated extinction coefficient at A_{280} of 52 580 M^{-1} cm^{-1}, was 6–10 mg ml^{-1}. Its purity was confirmed by SDS–PAGE.

2.2. Enzyme assays

Polygalacturonase-activity assays monitor the increase of reducing ends following enzymatic cleavage using the previously described parahydroxybenzonic acid hydrazide method (Lever, 1971). Reactions were carried out at 323 K in 50 m*M* potassium acetate buffer pH 5.25 in the presence of 0.5% polygalacturonic acid (USB). Product-formation rates were monitored by UV absorption at 410 nm at 10 s intervals.

2.3. Crystallization

The initial crystallization trials were performed using sparse-matrix sampling methods (Jancarik & Kim, 1991). Crystal Screen, Crystal Screen 2 and Index Screen (Hampton Research) were used in hanging-drop vapor-diffusion experiments in 24-well VDX crystallization plates (Hampton Research). 4 µl drops were equilibrated against 1 ml reservoir solution at 292 K. Drops consisted of a 3:1 protein:reservoir solution ratio, with an initial protein concentration of 6 mg ml^{-1} in 10 m*M* HEPES pH 7.0.

2.4. X-ray data collection and processing

During crystal screening, optimization and cryoprotectant determination, crystals were analyzed using a Rigaku RU200H rotating copper-anode X-ray generator operated at 50 kV and 100 mA with Osmic focusing mirrors and a MAR Research 300 mm phospho-imaging-plate detector. Images were indexed using *HKL*-2000 (Otwinowski & Minor, 1997).

Crystals were cryoprotected prior to the diffraction experiment by rapid soaking in a solution composed of 75% reservoir solution and 25% ethylene glycol. Synchrotron X-ray data were collected from a single crystal cooled to a temperature of 100 K. In anticipation of using bromide anomalous scattering for phasing information, peak, inflection and edge data were collected at wavelengths of 0.9198, 0.9200 and 0.9130 Å, respectively, at the Advanced Photon Source (APS), sector 22-ID, Argonne National Laboratory. X-ray diffraction intensities were recorded on a MAR Research 225 CCD detector in 170 frames using an oscillation range of 0.5°. The crystal-to-detector distance was 125 mm and 4 s exposure times were used. Data were indexed, integrated and scaled using *HKL*-2000 (Otwinowski & Minor, 1997). The program *SOLVE* (Terwilliger & Berendzen, 1999) was used to assess bromide occupancy.

3. Results

3.1. Crystallization

Over the course of eight weeks, the crystallization trials demonstrated that PehA crystallized in numerous conditions, particularly those containing polyethylene glycols or salts, and in several morphologies. Crystals were observed in the following screen conditions: Index Screen Nos. 30, 44, 66, 68–69, 72–75, 78–80, 83–84, 89, 93 and 96, Crystal Screen Nos. 6, 9, 16–17, 22, 28, 38, 40–41 and 45–46 and Crystal Screen 2 Nos. 25–28, 32, 38 and 47. Many of the crystals diffracted to medium resolution (2–2.5 Å) directly from the screening drop. As the crystals indexed to the same unit-cell parameters, a condition containing potassium bromide and common reagents was explored for possible MAD phasing. High-resolution crystals suitable for X-ray diffraction studies (Fig. 1) were obtained after approximately one month at 292 K. X-ray data were collected from crystals grown from an initial 0.004 ml protein-solution drop consisting of 1.5 mg ml^{-1} PehA, 16%(*w*/*v*) PEG 3350, 75 m*M* HEPES pH 7.5 and 120 m*M* potassium bromide equilibrated over a 1 ml 21%(*w*/*v*) PEG 3350, 100 m*M* HEPES pH 7.5 and 160 m*M* potassium bromide. The rod-shaped crystals grew in clusters to typical dimensions of 1.0 × 0.1 × 0.1 mm. Individual rods spontaneously separated from the cluster during cryoprotection.

Table 1
Crystal and data-collection statistics for PehA.

Values in parentheses are for the highest resolution shell.

X-ray source	Synchrotron radiation, sector 22-ID, APS
Wavelength (Å)	0.9198
Detector	CCD, MAR Research 225
Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 52.387, b = 62.738, c = 149.165, β = 89.98
Resolution (Å)	1.59 (1.65–1.59)
Mosaicity (°)	0.17
Matthews coefficient V_M (Å^3 Da^{-1})	2.2
Solvent content (%)	44.50
Molecules per ASU	2
No. of observed reflections	485996 (17626)
No. of unique reflections	122823 (10706)
Average redundancy	4.0 (1.6)
Completeness (%)	94.3 (82.7)
R_{merge}†	0.032 (0.050)
Average $I/\sigma(I)$	24.7 (11.0)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle|/\sum_{hkl}\sum_i I_i(hkl)$, where hkl are the Miller indices of the reflections and $I_i(hkl)$ is the intensity of the ith measurement.

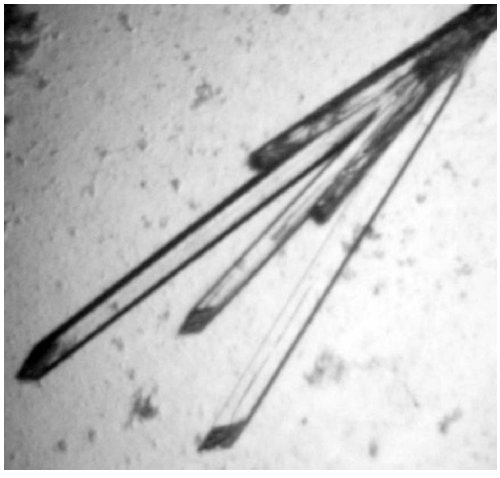


Figure 1
Crystals of PehA. Typical crystals have dimensions of 0.1 × 0.1 × 1.0 mm.

3.2. Data collection and analysis

PehA crystal parameters are summarized in Table 1. Both the V_M and estimated solvent-content values (Matthews, 1968) are typical of protein crystals. Flurorescence spectra prior to data collection and data analysis using the program *SOLVE* (Terwilliger & Berendzen, 1999) indicated minimal bromide occupancy, if any; therefore, the peak data set was treated as a native set for molecular replacement. Processing the peak data as a native set yielded a total of 485 996 reflections containing 122 823 unique data.

An initial problem of space-group ambiguity was resolved using molecular replacement. The mature protein regions of PehA from *Er. carotovora* and *A. vitis* PehA share 33.6% amino-acid identity. Several partial coordinate sets representing the parallel β-helix core of the *Er. carotovora* PG (PDB code 1bhe; Pickersgill *et al.*, 1998) were used as search models in the program *AMoRe* (Navaza, 1994). Unexpectedly, none of the orthorhombic space groups gave an adequate solution. Suspecting an erroneous space-group assignment, the X-ray data were reprocessed to the lower symmetry group $P2$. Using correlation coefficients and R factors from *AMoRe* against X-ray data processed in all possible orthorhombic and monoclinic space groups, the correct space group was assigned as $P2_1$ (Table 2) and solutions for both molecules in the asymmetric unit were easily identified. Analysis of $2F_o - F_c$ maps shows unambiguous density for the parallel β-helix core of both molecules, as well as some loop and termini regions. Manual building of the remaining loops and termini is nearly complete and will be presented elsewhere.

Table 2
AMoRe molecular-replacement results in monoclinic and orthorhombic space groups.

The search model contained residues 56–122 and 135–370 of PDB entry 1bhe.

Space group	Correlation coefficient†	R factor†
$P2_12_12_1$	38.0	53.6
$P222$	35.4	55.5
$P222_1$	37.4	55.0
$P2_12_12$	35.5	55.4
$P2_122$	36.6	55.6
$P22_12$	35.5	55.8
$P2_122_1$	35.8	54.9
$P22_12_1$	36.2	55.1
$P2$	38.4	55.5
$P2_1$	42.0	53.2

† Between observed amplitudes for the crystal and calculated amplitudes for the model.

This work was supported by National Science Foundation grant MCB9983108. Data were collected on the Southeast Regional Collaborative Access Team (SER-CAT) 22-ID beamline at the Advanced Photon Source, Argonne National Laboratory. Supporting institutions may be found at http://www.ser-cat.org/members.html. Use of the Advanced Photon Source was supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract No. W-31-109-Eng-38.

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