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Crystallization and preliminary crystallographic analysis of the endo-polygalacturonase from *Erwinia carotovora* ssp. *carotovora*

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Crystals of endo-polygalacturonase from *Erwinia carotovora* ssp. *carotovora* have been grown from polyethylene glycol 6000 by the hanging-drop method. Polygalacturonase is important in the virulence of this plant pathogen. The protein crystallizes in space group $C2$ with unit-cell parameters $a = 81.3$, $b = 53.0$, $c = 103.1$ Å, $\beta = 112.6^\circ$ and with a single molecule in the asymmetric unit. The crystals diffract to 1.9 Å.

1. Introduction

Microbial polygalacturonases are important virulence factors in plant disease and plant polygalacturonases are believed to be important in fruit ripening and senescence. It is the polygalacturonase gene that is silenced in the genetically engineered tomato. Polygalacturonases hydrolyse the α-1,4 glycosidic bond in the non-methylated homogalacturonan regions of the pectin polysaccharides found in the plant cell wall. The structures of a number of enzymes that cleave pectins are known: pectate lyase which cleaves the same substrate as polygalacturonase (Yoder *et al.*, 1993; Lietzke *et al.*, 1994; Pickersgill *et al.*, 1994), pectin lyase which cleaves methylated homogalacturonan (Mayans *et al.*, 1997; Vitali *et al.*, 1998) and rhamnogalacturonase A which cleaves the α-1,2 glycosidic linkage between the alternating rhamnose and galacturonic acid residues in rhamnogalacturonan (Petersen *et al.*, 1997). The most pertinent of these for a discussion of the structure and properties of polygalacturonase are rhamnogalacturonase A (RGase A) and pectate lyase. Rhamnogalacturonase belongs to the same family of glycosyl hydrolases as polygalacturonase, family 28 (Henrissat & Bairoch, 1993). The overall architecture of polygalacturonase will therefore be similar to RGase A. It is also expected that the catalytic amino acids will be conserved, since both polygalacturonase and RGase A are inverting glycosyl hydrolases (Biely *et al.*, 1996; Pitson *et al.*, 1998). There is also functional similarity with pectate lyase since both polygalacturonase and pectate lyase bind the same substrate. There is a clear mechanistic difference because pectate lyase cleaves in a calcium-dependent β-elimination reaction, while polygalacturonase cleaves by hydrolysis without the requirement for calcium. The functional similarity is likely to extend to the overall characteristics of the substrate-binding cleft: pectate lyase has a positively charged cleft and the same is anticipated for polygalacturonase. RGase A has several aspartates at its proposed active centre and negative potential in its substrate-binding cleft. Several experiments suggest the importance of a histidine in the activity of polygalacturonase (Rao *et al.*, 1996; Caprari *et al.*, 1996), but it would be a considerable surprise if this histidine were directly involved in catalysis. It is anticipated that the structure will illuminate the extent of the structural similarity with RGase A and the extent of the functional similarity with pectate lyase. X-ray quality crystals of polygalacturonases have been grown previously (Schroter *et al.*, 1994; Yoder & Schell, 1995) but as yet no structure has been reported. The structure of the 376 amino-acid *E. carotovora* ssp. *carotovora* polygalacturonase (Saarilahti *et al.*, 1990), the product of the *pehA* gene (Hemila *et al.*, 1992) after cleavage of the 26 amino-acid signal sequence, has 20% sequence identity with RGase A and will be solved by isomorphous replacement. Its pH optimum is 5.5 (Saarilahti *et al.*, 1990).

2. Production and purification of *E. carotovora* polygalacturonase

Bacillus subtilis strain BRB741 contains the plasmid pKTH1951 and secretes *E. carotovora* ssp. *carotovora* polygalacturonase protein into the culture medium in large quantities (Hemila *et al.*, 1992). The medium (BEM) used for expression had the following composition: 6.7 g l^{-1} yeast nitrogen base without amino acids, 11 m*M* KH_2PO_4, 54 m*M* K_2HPO_4, 5% glucose, 4 ml l^{-1} glycerol, full amino-acid supplement as used in TSSA minimal agar (Cutting & VanderHorn, 1990) and kanamycin at 10 mg l^{-1}. The culture medium was originally developed to produce protein containing selenomethionine for multiple anomalous

Table 1
Quality of the native data measured using the EMBL X31 beamline at the DORIS storage ring, DESY, Hamburg.

Resolution range (Å)	Number of reflections	Completeness (%)	Fraction measured four times (%)	$R_{sym}(I)$
99.00–4.09	3031	91.7	46.5	0.019
4.09–3.25	3060	94.3	45.7	0.024
3.25–2.84	3083	96.2	45.6	0.030
2.84–2.58	3133	97.1	43.6	0.040
2.58–2.39	3117	98.0	43.4	0.051
2.39–2.25	3165	98.4	41.9	0.059
2.25–2.14	3148	99.1	42.4	0.069
2.14–2.05	3169	99.2	40.0	0.078
2.05–1.97	3165	99.5	40.2	0.092
1.97–1.90	3032	95.2	35.9	0.114
All reflections	31103	96.8	42.5	0.038

dispersion experiments. A starter culture of 50 ml of BEM was inoculated from a glycerol stock of BRB741 and the culture grown overnight with shaking at 310 K. This was then added to a further 500 ml of BEM and grown for a further 24 h. Culture supernatant was clarified by centrifugation at 10000 rev min^{-1} for 10 min at 277 K and then filtered through a 0.2 mm filter (Nalgene) with a glass-fibre pre-filter. After addition of acetic acid to lower the pH to 5.0, the filtrate was passed through an SP-Sepharose 1.5 × 13 cm column (Pharmacia) in 250 ml batches, which avoided passing the culture supernatant through FPLC pumps. The column buffer used was 50 m*M* sodium acetate pH 5.0, and protein was eluted with 50 m*M* sodium acetate pH 5.0 containing 400 m*M* sodium chloride. Polygalacturonase activity was detected by adding 250 ml of 0.5% polygalacturonic acid (Sigma) in 100 m*M* sodium acetate at pH 6.0 to an equal volume of sample and incubating at 303 K for 1 h. The production of reducing sugar was then detected using the Somogyi–Nelson method, as described by Wood & Bhat (1988). The desired fractions were then diluted with column buffer to reduce the salt concentration and further purified by FPLC using a 1 ml HiTrap-SP column (Pharmacia), using the same column buffer and a 0–1 *M* sodium chloride gradient. The final yield of polygalacturonase was 40–50 mg per litre of culture and the protein was 99% pure as judged by SDS–PAGE analysis.

3. Crystallization

Polygalacturonase was concentrated in a 10 ml stirred cell (Amicon) using a 10 kDa cut-off membrane (Flowgen) to a final concentration of 5.0 mg ml^{-1} (assuming an OD_{280} of 1.0 corresponds to a protein concentration of 1 mg ml^{-1}). Hampton Crystal Screen Kit I, exploiting the protocol of Jancarik & Kim (1991), gave crystals in 12 of the 50 drops when surveyed 48 h later (solutions 6, 9, 10, 15, 17, 18, 20, 22, 28, 30, 31 and 40). All of these drops contained polyethylene glycol 4000 or 8000 and ten in addition contained either ammonium or lithium sulfate. Crystals grew in the pH range 5.6–8.5. Hampton Crystal Screen Kit II also gave crystals from 30% PEG MME, 30% *t*-butanol and 70% methylpentanediol (solutions: 13, 17, 26 and 35). The best crystals were from 0.2 *M* magnesium acetate, 0.1 *M* sodium cacodylate pH 6.5 and 20% PEG 8000. An improvement in crystal size was obtained by using 4 µl protein plus 2 µl reservoir in place of the original 3 µl protein plus 3 µl reservoir and 18% PEG 6000 in place of 20% PEG 8000. Crystals grew to a maximum size of approximately 0.2 × 0.4 × 1.0 mm. The crystals are stable in mother liquor comprising 0.2 *M* magnesium acetate, 0.1 *M* sodium cacodylate pH 6.5 and 20% PEG 6000.

4. Data collection and reduction

Rapid radiation damage occurred when crystals were irradiated at room temperature.

Preliminary data using cryocooled crystals were collected using a Siemens rotating-anode generator with double mirrors and DIP 1030 image-plate system. Crystals were transferred to a cryo-solution consisting of mother liquor supplemented with 20% glycerol and 10% 2-propanol before being flash frozen in the nitrogen-gas stream of an Oxford Cryosystems Cryostream at 100 K. These data, collected using a crystal of maximum size 0.5 mm, were 96.1 (85.7)% complete to 2.9 Å with $R_{sym}(I)$ of 0.045 (0.061). Figures in parentheses are for the highest resolution shell. The crystals belong to space group *C*2 with unit-cell parameters $a = 81.3$, $b = 53.0$, $c = 103.1$ Å and $\beta = 2.6^\circ$, with a single molecule in the asymmetric unit giving a V_m of 2.7 Å^3 Da^{-1} which is within the acceptable range (Matthews, 1968). The solvent content is approximately 42%. A high-resolution native data set was subsquently collected using beamline X31 at the EMBL Outstation using the DESY storage ring. A larger crystal of longest dimension 1.0 mm was available for this collection. 145 images, each corresponding to a 1° rotation of the crystal, were collected using a crystal-to-detector distance of 150 mm and a wavelength of 1.0 Å. The data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997) and a total of 142703 reflections were used in scaling. The unique data comprises 32121 reflections to 1.9 Å resolution. The overall completeness is 96.8 (95.2)% and the overall $R_{sym}(I)$ is 0.038 (0.114) (Table 1). The overall $\langle I\rangle/\langle\sigma\rangle$ from *TRUNCATE* (Collaborative Computational Project, Number 4, 1994) was 21.45. The structure will be solved by isomorphous replacement.

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