Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray diffraction study of the *endo*-polygalacturonase from *Fusarium moniliforme*

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Received 28 December 1998
Accepted 20 April 1999

Endo-polygalacturonases catalyze the fragmentation and solubilization of the homogalacturonan of the plant cell wall. These enzymes are extracellularly targeted glycoproteins produced by a number of organisms such as fungi, bacteria and plants, and are involved in both pathological and physiological processes. Single crystals of the *endo*-polygalacturonase from the phytopathogenic fungus *Fusarium moniliforme* were obtained by the vapour-diffusion method at 294 K. The starting material as well as the crystal consist of three forms with different degrees of glycosylation. The crystals belong to the orthorhombic space group $P2_12_12_1$ and diffract to 1.9 Å resolution on a synchrotron-radiation source under cryocooling conditions.

1. Introduction

Phytopathogenic fungi produce a wide array of cell-wall degrading enzymes to penetrate and colonize plant tissues during infection. *Endo*-polygalacturonase (PG), an O-glycosyl hydrolase which catalyzes the fragmentation and solubilization of homogalacturonan, is among the first enzymes secreted by fungi; only after its action on the pectic substrate do other cell-wall components become accessible to enzymatic attack. By cleaving the internal glycosidic bonds of homogalacturonan, PG may also release oligogalacturonides, which function as elicitors of plant defence responses (De Lorenzo *et al.*, 1997). The elicitor activity of fungal PGs depends on the presence of a polygalacturonase-inhibiting protein (PGIP) in the plant cell wall, which forms a specific complex with the enzymes and favours the accumulation of elicitor-active oligogalacturonides (Cervone *et al.*, 1987).

A number of structures of microbial pectic enzymes, including pectate and pectin lyases (Yoder *et al.*, 1993; Pickersgill *et al.*, 1994; Lietzke *et al.*, 1994; Vitali *et al.*, 1998), a rhamnogalacturonase (Petersen *et al.*, 1997) and the first polygalacturonase from *Erwinia carotovora* (Pickersgill *et al.*, 1998), have been recently solved by X-ray crystallography. All these proteins share the same central core organization consisting of parallel β-strands forming a large right-handed helix defined as a parallel β-helix (Yoder & Jurnak, 1995). The sequence homology between PG and other pectic enzymes is generally weak (*e.g.* the degree of identity with the polygalacturonase from *E. carotovora* is 22.9%). Different studies have been undertaken to identify the residues of PG responsible for catalysis, substrate binding and interaction with PGIP (Stratilova *et al.*, 1996; Rao *et al.*, 1996; Caprari *et al.*, 1996).

The study of the structure–function relationships of fungal PGs and their plant-derived inhibitors is a main objective of our research; we believe that a successful strategy for improving plant resistance to pathogens may be based on the manipulation of the PGIP–PG interaction. In this context, we have undertaken the structural determination by X-ray diffraction of PG from *Fusarium moniliforme*. The preliminary data from single crystals diffracting to 1.9 Å resolution are reported here.

2. Materials and methods

2.1. Crystallization

A wide range of crystallization conditions were initially tested using the conventional hanging-drop vapour-diffusion method (McPherson, 1990). Single crystals were obtained using PEG 4000 as the precipitating agent under the following conditions: drops produced by mixing equal volumes of protein solution concentrated to 10 mg ml^{-1} and mother liquor consisting of 37–42%(*w*/*v*) PEG 4000 with 0.1 *M* sodium citrate pH 4.7 were allowed to vapour-equilibrate against 1.0 ml of the same mother liquor solution at 294 K. Crystals appeared after 3 d and reached final dimensions after approximately 10 d. Crystals obtained from these first trials were needle-shaped and only reached an appreciable size in one dimension. Better crystals were obtained, under the same conditions, simply by adopting the sitting-drop method and reducing the reservoir volume to 0.5 ml. The maximum dimensions of these crystals were $\sim 1.0 \times 0.3 \times 0.2$ mm.

Table 1
Statistical data-collection parameters for the PG crystal as a function of resolution.

Resolution (Å)	$I > 3\sigma$ (%)	χ^2†	R_{merge}‡	Completeness (%)
20.00–3.25	94.56	0.988	0.072	97.1
3.25–2.58	95.03	1.130	0.088	99.1
2.58–2.25	89.82	1.233	0.120	98.6
2.22–2.05	88.03	1.223	0.152	97.6
2.05–1.90	82.96	1.212	0.127	89.4
Overall	90.32	1.152	0.093	96.4

† $\chi^2 = \sum_h \sum_i (I_{hi} - \langle I_h \rangle)^2 / [\sigma_h^2 N/(N-1)]$, where N is the number of observations. ‡ $R_{merge} = \sum_h \sum_i |I_{hi} - \langle I_h \rangle| / \sum_h \sum_i I_{hi}$, where I_{hi} is the ith observation of the reflection h, while $\langle I_h \rangle$ is the mean intensity of the ith reflection.

2.2. Cryofreezing and data collection

Crystals were first measured at room temperature using a Rigaku R-AXIS IIc imaging-plate detector mounted on a Rigaku RU-200 rotating-anode X-ray (Cu $K\alpha$) generator equipped with focusing mirrors and operating at 50 kV and 100 mA. A good diffraction pattern up to 2.5 Å resolution was obtained, but considerable radiation damage was observed as a function of time. In order to overcome this problem, we screened for cryofreezing conditions (Rodgers, 1994). A cryoprotectant solution was obtained by adding 30 µl PEG 200 to 200 µl mother-liquor solution. Crystals were allowed to equilibrate in this solution prior to measurement. New data on one cryo-cooled crystal were collected using a MAR Research 345 imaging-plate detector mounted on the synchrotron-radiation source at ELETTRA (Trieste, Italy). The radiation wavelength was set to 1.0 Å. These data were merged with data collected with our in-house equipment, also under cryo-cooled conditions, with a maximum resolution of 2.06 Å, in order to improve completeness. Data were processed with *DENZO* and scaled with *SCALEPACK* (Otwinowski & Minor, 1996) to a final resolution of 1.9 Å. Data-collection parameters are give in Table 1.

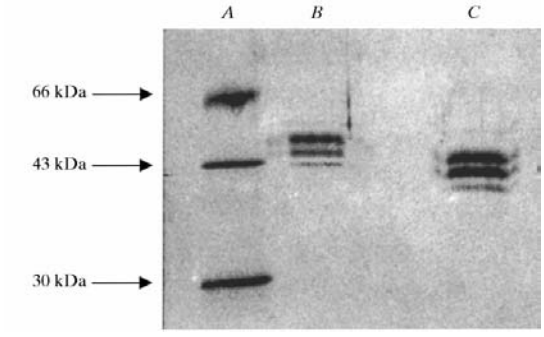

Figure 1
SDS–PAGE analysis. Lane *A*: molecular-weight markers. Lane *B*: protein batch before crystallization. Lane *C*: protein from a washed and dissolved crystal.

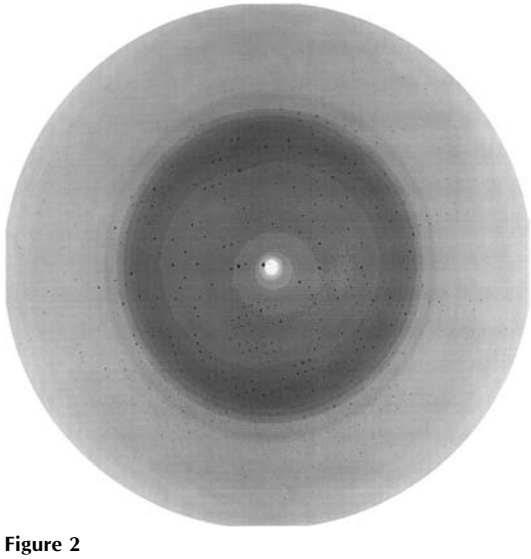

Figure 2
A typical diffraction pattern of a crystal of *endo*-polygalacturonase from *F. monoliforme*. The frame edge is at 1.9 Å.

3. Results and discussion

For the crystallization study presented here, *F. moniliforme* PG heterologously expressed by *Saccharomyces cerevisiae* was used (Caprari *et al.*, 1996). The enzyme was purified as reported (Caprari *et al.*, 1993) and analysed by SDS–PAGE (Laemmli, 1970): it showed three protein bands with molecular masses of 43.25, 46.75 and 50.25 kDa, respectively (lane *B* in Fig. 1), corresponding to different glycoforms of the same polypeptide chain (Caprari *et al.*, 1996). Given the calculated molecular weight of the polypeptide (36.2 kDa), the percentage of carbohydrate varies from 16.3% in the lightest glycoform to 27.9% in the heaviest. Attempts to separate these glycoforms were unsuccessful. SDS–PAGE analysis of the protein from washed and dissolved crystals showed the presence of three bands with molecular masses slightly smaller than those exhibited by the protein prior to crystallization (lane *C* in Fig. 1). The shift in the bands from the crystal compared with those from the protein batch prior to crystallization is likely to arise either from loss of sugars or of peptides from the protein chain.

Highly glycosylated proteins are usually very difficult to crystallize owing to the heterogeneity of their carbohydrate moieties. Different approaches, ranging from enzymatic cleavage to expression in bacterial systems, are often used to overcome this problem (Stura *et al.*, 1992), since protein homogeneity is generally thought to be crucial for successful crystallization attempts (Ducruix & Giegé, 1992). The *F. moniliforme* PG represents an interesting exception: this protein, which consists of at least three different glycoforms, led to crystals retaining the original heterogeneity and exhibiting excellent diffraction quality (Fig. 2).

Analysis of diffraction data shows that PG crystals belong to the orthorhombic space group $P2_12_12_1$ with unit-cell dimensions a = 57.966, b = 61.833, c = 98.535 Å. The overall completeness is 96.4%, while the highest resolution shell is 89.4% complete. The multiplicity is 4.56 and the percentage of data with $I > 3\sigma$ is 90.3%, with an R_{merge} of 9.3% (based on intensities). The average molecular weight of the three protein bands estimated from SDS–PAGE is about 42.2 kDa. Assuming one molecule per asymmetric unit, we obtain a V_m value of 2.08 Å^3 Da^{-1}, corresponding to a solvent volume fraction of about 40.9%, which is in the range usually encountered for protein crystals (Matthews, 1968). Screening for heavy-metal derivatives is in progress.

This work was partially supported by the Institute Pasteur–Fondazione Cenci Bolognetti (Rome), by the Armenise–Harvard Foundation and by the MURST (Progetto Nazionale Biologia Strutturale, 1997) and ASI of Italy.

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