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Preliminary X-ray crystallographic analysis of a plant defence protein, the polygalacturonase-inhibiting protein from *Phaseolus vulgaris*

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A leucine-rich repeat plant protein involved in resistance to pathogens, a polygalacturonase-inhibiting protein (PGIP-1) from *Phaseolus vulgaris*, has been crystallized and preliminary X-ray characterization has been performed. The protein contains ten repeats of a short (24 amino-acid) leucine-rich repeat motif. Single crystals of the protein were grown from vapour-diffusion experiments using PEG 2K monomethylether as precipitant; these crystals diffract to at least 2.3 Å resolution. The space group is $P2_1$, with two molecules of PGIP-1 in the asymmetric unit; the crystals contain approximately 38% solvent.

1. Introduction

A rapidly growing number of biologically important proteins from a variety of organisms have been characterized and found to contain a sequence motif termed the leucine-rich repeat (LRR). The functions of these proteins are diverse and include ligand binding and signal transduction, development and enzyme inhibition (Buchanan & Gay, 1996). The motif thus appears to be recruited in a variety of systems where specific protein–protein recognition and interaction is required. In plants, LRR proteins play major roles in both development and defence, where specificity of recognition is a fundamental prerequisite. Several plant proteins exhibit LRRs of the extracellular or extracytoplasmic type, characterized by the consensus sequence L*xx*L*xx*L*x*L*xx*N*x*LT/SG*x*IP*xx*LG*x* (Kajava, 1998). Examples of proteins involved in resistance to pathogens include the polygalacturonase-inhibiting proteins (PGIPs; De Lorenzo & Cervone, 1997), the products of the resistance (R) genes *Cf*$^{-}$ of tomato, which confer resistance to different races of the fungus *Cladosporium fulvum*, and *Xa21* of rice, which confers resistance to *Xanthomonas oryzae* pathovar *oryzae* (Hammond-Kosack & Jones, 1997). In addition to these, there are several LRR-containing orphan receptor kinases involved in arabidopsis development, such as Erecta (Torii *et al.*, 1996), Clavata1 (Clark *et al.*, 1997) and a putative receptor for brassinosteroids (Li & Chory, 1997).

PGIPs interact with fungal endopolygalacturonases (PGs) and *in vitro* inhibit their enzymatic activity (De Lorenzo & Cervone, 1997). The proteins isolated from bean (Desiderio *et al.*, 1997), tomato (Stotz *et al.*, 1994), pear (Stotz *et al.*, 1993) and raspberry (Johnston *et al.*, 1993) have differential inhibition spectra towards a range of PGs from phytopathogenic fungi. Different inhibitory activities against PGs have also been observed in PGIPs from a single plant source (Desiderio *et al.*, 1997), indicating that *pgip* genes undergo diversification during evolution. PGIPs share similarity to the R-gene products Cf^{-} and Xa21 not only in the LRR region but also in regions outside of the LRR domain, suggesting that these proteins are not only structurally but also evolutionarily related (De Lorenzo & Cervone, 1997). R-gene products are thought to function as receptors for pathogen-encoded avirulence (avr) proteins and sequence variation within the LRR domain has been hypothesized to influence recognition specificity: comparison of members of the Cf^{-} family has identified the β-sheet/β-turn region as a 'hypervariable' region, likely to be responsible for their ligand specificity (Parniske *et al.*, 1997). In PGIP, residues determining the recognition specificity reside in the predicted β-sheet/β-turn region of the protein and a single amino-acid variation in this motif can confer to PGIP a new recognition capability (Leckie *et al.*, 1999).

At the present time, no structure of a plant LRR protein has been reported. In this paper, we report the crystallization and preliminary X-ray crystallographic analysis of PGIP-1 from *P. vulgaris* (bean). This 40 kDa glycoprotein has ten tandemly arranged 24-amino-acid leucine-rich repeats and inhibits the polygalacturonase from *Aspergillus niger* (Leckie *et al.*, 1999). X-ray analysis of this protein will help in our understanding of short leucine-rich repeat protein structure and provide a basis for further studies of protein–protein recognition in its inhibition of polygalacturonases and its role in plant disease resistance.

Table 1
P. vulgaris PGIP-1 X-ray diffraction data-collection statistics.

Figures in parentheses refer to data in the highest resolution bin (2.38–2.30 Å).

Wavelength (Å)	0.87
Temperature (K)	100
Collected at	SRS PX9.6
Space group	$P2_1$
Unit-cell parameters (Å,°)	a = 82.8, b = 47.6, c = 90.9, $\alpha = \gamma = 90$, β = 116.1
Resolution (Å)	40–2.30
Completeness (%)	99.3 (99.3)
$R_{\rm merge}$ (%)	4.1 (9.7)
$\langle I\rangle/\langle\sigma I\rangle$	9.5 (6.2)
Total observations	81469
Independent reflections	25096

2. Materials and methods

2.1. Protein expression and purification

P. vulgaris PGIP-1 was overexpressed in transgenic tomato plants and purified according to the protocol described by Desiderio *et al.* (1997).

2.2. Crystallization and crystal characterization

Protein solution in 20 m*M* sodium acetate pH 5.0 was concentrated to 7–15 mg ml^{-1} by precipitation with 80% ammonium sulfate followed by resuspension in and subsequent extensive dialysis against the same buffer at 277 K. Initial crystallization screening experiments were carried out at 277 and 291 K using the screens of Jancarik & Kim (1991) and Cudney *et al.* (1994), and the hanging-drop vapour-diffusion method. Each experiment utilized a 4 µl drop containing equal volumes of concentrated protein solution and screen solution equilibrated against 700 µl of reservoir solution. Crystalline growth was found only in experiments containing PEG 2K monomethyl ether as precipitant buffered with sodium acetate at 291 K; this was chosen as a starting point for further optimization. Subsequently, various polyethylene glycols (PEG 2K, 4K, 5K and 6K) and polyethylene glycol monomethyl ethers (PEG MME 2K, 5K) were investigated as precipitants in the concentration range 20–30%(*w*/*v*). Attempts were made to improve crystal size and quality by micro- and macroseeding experiments (Stura & Wilson, 1992).

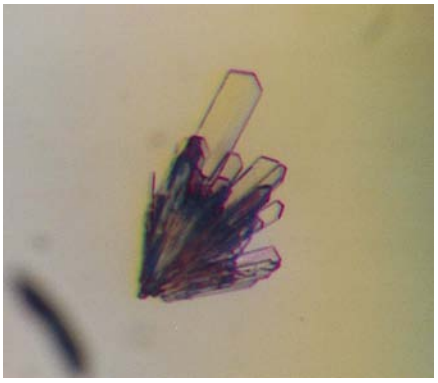

Figure 1
Crystals of native polygalacturonase-inhibiting protein (PGIP-1) from *P. vulgaris*.

2.3. X-ray diffraction data collection

For cryoprotection experiments, crystals were soaked in solutions containing 25%(*v*/*v*) ethylene glycol, 32%(*w*/*v*) PEG MME 2K, 100 m*M* sodium acetate pH 4.7 and 200 m*M* ammonium sulfate. Cryocooling was performed by mounting single crystals in cryoloops (Hampton Research, CA, USA) followed by immediate immersion in a nitrogen-gas stream at 100 K. X-ray diffraction data collection using cryocooled crystals was carried out on station 9.6 at the Daresbury Laboratory (Warrington, England) using an Advanced Detector Systems Corporation Quantum 4 CCD detector. The crystal-to-detector distance was 280 mm and 100 images were collected using 2.0° non-overlapping oscillations. Indexing of images and data processing was performed using a combination of the manufacturer's *DPS* software and programs from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). Calculation of self-rotation functions was performed using the *POLARRFN* program also from the *CCP*4 program suite.

3. Results and discussion

Initial crystallization experiments using purified native PGIP-1 gave small clusters of tabular crystals after two months at 291 K in hanging-drop vapour-diffusion experiments using 30%(*w*/*v*) PEG 2K monomethylether as precipitant buffered with 0.1 *M* sodium acetate pH 4.5. Low concentrations of ammonium sulfate were necessary for crystal growth. Optimization of these conditions by finer screening and by various seeding methods did not alter this crystal habit. The optimal crystallization method involved growth by hanging-drop vapour diffusion of a 7 mg ml^{-1} protein solution against a reservoir containing 30%(*w*/*v*) PEG MME 2K, 100 m*M* sodium acetate pH 4.7 and 200 m*M* ammonium sulfate at 291 K to give clusters of single crystals of typical dimensions 100 × 25 × 5 µm, which first appeared after 5 d and grew to maximum size in approximately six weeks (Fig. 1). SDS–PAGE analysis of serially washed crystals showed a doublet of bands of the expected apparent molecular mass (data not shown) corresponding to the glycoforms of the protein known to be present after purification (Desiderio *et al.*, 1997). The complex thus appears to be stable against significant degradation during crystallization, consistent with its stability against proteolytic digestion, and also to crystallize as a mixture of glycoforms.

Diffraction trials of these crystals cryoprotected with 25%(*v*/*v*) ethylene glycol and mounted in cryoloops were carried out on station 9.6 at the SRS, Daresbury Laboratory. Although small, the crystals diffracted to at least 2.3 Å (Table 1). Autoindexing and consideration of systematically absent reflections reveal that the crystals belong to space group $P2_1$, with typical unit-cell parameters a = 82.8, b = 47.6, c = 90.9 Å, $\alpha = \gamma = 90$, β = 116.1°. With the assumption of two molecules of PGIP-1 in the asymmetric unit, a value of the Matthews parameter of 2.01 Å^3 Da^{-1} is obtained, with a corresponding solvent content of approximately 38%.

Self-rotation functions were calculated from the native diffraction data. The κ = 180° section of a map calculated with integration radius 30 Å using data between 8 and 4 Å is presented in Fig. 2. There is evidence of a peak in the map at a height of 6σ at polar angles (φ = 27, ω = 15°) corresponding to a non-crystallographic twofold axis almost parallel to the crystallographic twofold axis.

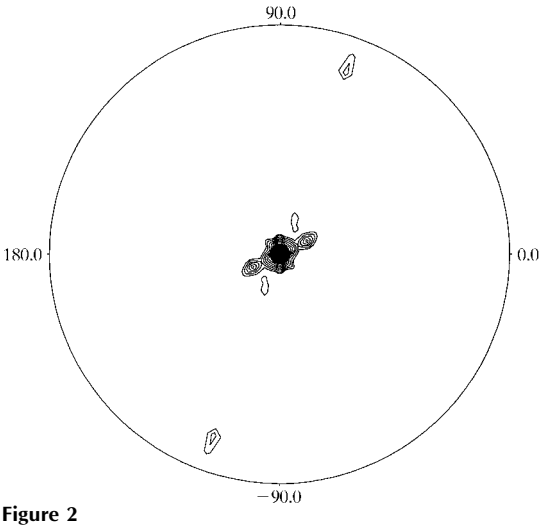

Figure 2
The κ = 180° section of the self-rotation function calculated from the native PGIP-1 diffraction data (integration radius = 30 Å using data between 8 and 4 Å). The section is contoured at 0.5σ intervals from 4σ.

The position and size of this peak was not significantly affected by choice of integration radius and data resolution limits and may correspond to an approximate twofold axis relating the two copies of the protein molecule in the asymmetric unit.

We have subsequently performed trial soaks of these crystals in a variety of heavy-atom salt solutions and found them not to suffer undue deterioration on treatment. Structure solution will proceed by the method of multiple isomorphous replacement.

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