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Crystallization and preliminary X-ray structure determination of *Lupinus luteus* PR10 protein

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The pathogenesis-related protein of the PR10 class from *Lupinus luteus* (yellow lupin), LlPR10.1A, is constitutively expressed in roots. It is also accumulated in leaves treated with a suspension of pathogenic bacteria as a response to stress. Recombinant yellow-lupin LlPR10.1A protein has been overexpressed in *Escherichia coli* as a fusion product with maltose-binding protein. LlPR10.1A crystallizes in the orthorhombic $P2_12_12_1$ space group and the crystals diffract to 2.45 Å resolution. The structure has been solved by molecular replacement, using the structure of a birch-pollen allergen protein as a model.

1. Introduction

Intracellular pathogenesis-related proteins of the PR10 class are ubiquitous in the plant kingdom and represent a class of soluble acidic proteins of molecular mass between 16 and 18 kDa (Awade *et al.*, 1991; Warner *et al.*, 1994). They have been identified after induction under pathological and stress-related conditions (Schmelzer *et al.*, 1989; Somssich *et al.*, 1988; Warner *et al.*, 1992, 1993; Pinto & Ricardo, 1995; Breda *et al.*, 1996). This suggests their involvement in plant defence mechanisms.

Genes encoding PR10 proteins are also developmentally regulated and show organ-specific expression (Iturriaga *et al.*, 1994; Mylona *et al.*, 1994; Sikorski *et al.*, 1996; Legocki *et al.*, 1997). A high amino-acid sequence similarity of PR10 proteins to *Panax ginseng* ribonucleases (Moiseyev *et al.*, 1994, 1997) led to their classification as ribonuclease-like PR proteins (van Loon *et al.*, 1994), but the two *P. ginseng* ribonucleases show very low activity *in vitro*. Proteins of the PR10 class also reveal high sequence similarity to tree-pollen allergens from birch (Breiteneder *et al.*, 1989), hazel (Breiteneder *et al.*, 1993), alder (Breiteneder *et al.*, 1992) and hornbeam (Larsen *et al.*, 1992) and to major food allergens from apple (Vanek-Krebitz *et al.*, 1995), cherry (Scheurer *et al.*, 1997), celery (Breiteneder *et al.*, 1995), carrot (Yamamoto *et al.*, 1997) and parsley (Somssich *et al.*, 1986).

Recently, the first three-dimensional structure of a PR10 class protein, birch-pollen allergen Betv1, was determined by X-ray diffraction and by NMR spectroscopy (Gajhede *et al.*, 1996). The structure is composed of a seven-stranded antiparallel β-sheet wrapped around a 25-amino-acid long C-terminal α-helix. The β-sheet and the C-terminal part of the long helix are separated by two consecutive helices, forming a forked cavity which runs along the structure. In the close vicinity of the cavity, four glycine residues and a lysine form a glycine-rich loop (P-loop) with the G*x*GG*x*G*xx*K motif found in many nucleotide-binding proteins (Saraste *et al.*, 1990). The P-loop motif was found in all PR10 proteins analyzed to date except potato PR10 protein (Matton & Brisson, 1989), where it is shortened to G*x*G.

2. Experimental

2.1. Protein expression and purification

The coding sequence of yellow-lupin LlPR10.1A protein was PCR-amplified from a pET-3a plasmid harbouring the coding region of an Llpr10.1a cDNA clone with mutated internal *Nde*I and *Bam*HI sites (Sikorski, 1997) and subcloned into protein-fusion vector pMal-c2 in frame with the maltose-binding protein (MBP). The fusion protein was overproduced in *Escherichia coli* DH5α cells during a 4 h cell growth. The bacterial lysate was fractionated by 60% ammonium sulfate precipitation. The precipitate was dialyzed against buffer *D* containing 20 m*M* phosphate pH 7.5, 5% glycerol, 10 m*M* β-mercaptoethanol. A protein sample was applied to a DE52 column and fractionated with NaCl. Fractions eluted between 0.1 and 0.5 *M* NaCl were separated by affinity chromatography on amylose resin. The fusion MBP–LlPR10.1A protein was eluted with 10 m*M* maltose in buffer *D*. It was cleaved with 10 µg factor Xa per milligram of protein in buffer *D* containing 2 m*M* $CaCl_2$ and 100 m*M* NaCl during a 4 h incubation at room temperature. The recombinant LlPR10.1A protein was separated from MBP by repeated chromatography on amylose resin. The final purification step was carried out by size-exclusion chromatography on a Superdex 75

HiLoad FPLC column in buffer *D* containing 100 m*M* NaCl.

2.2. Crystallization

The recombinant LlPR10.1A protein is soluble in 1 m*M* citrate buffer pH 6.3 to a concentration of 20 mg ml^{-1}. Initial crystallization trials using the hanging-drop vapour-diffusion method and a wide range of conditions (including Hampton Research Screen I and II) failed because of spontaneous precipitation in the form of fine-grained powder. Lowering the protein concentration to 5 mg ml^{-1} reduced the initial precipitation and produced single crystals of dimensions up to 0.3 mm, using ammonium sulfate (40–56%) as precipitant in a wide pH range (7.0–8.5 in 20 m*M* Tris–HCl buffer). Even with lower protein concentration, some initial precipitation was still observed and crystal aggregates, usually in the form of intergrown plates, appeared only later. The single crystal used for diffraction experiments (Fig. 1*a*) was obtained from 50% ammonium sulfate and 20 m*M* Tris–HCl buffer pH 7.5, using a solution of 5 mg ml^{-1} protein in 1 m*M* citrate buffer. The drop volume was 2 + 2 µl. Improved crystallization conditions were subsequently found using the Hampton detergent screen. Good-quality crystals (Fig. 1*b*) grew when the 5.6 mg ml^{-1} protein solution contained either of the detergents sucrose monolaureate or CYMAL-6 (at concentrations of 8 and 22.4 µ*M*, respectively). The reservoir solution contained 54% ammonium sulfate at pH 8.0 (20 m*M* Tris–HCl).

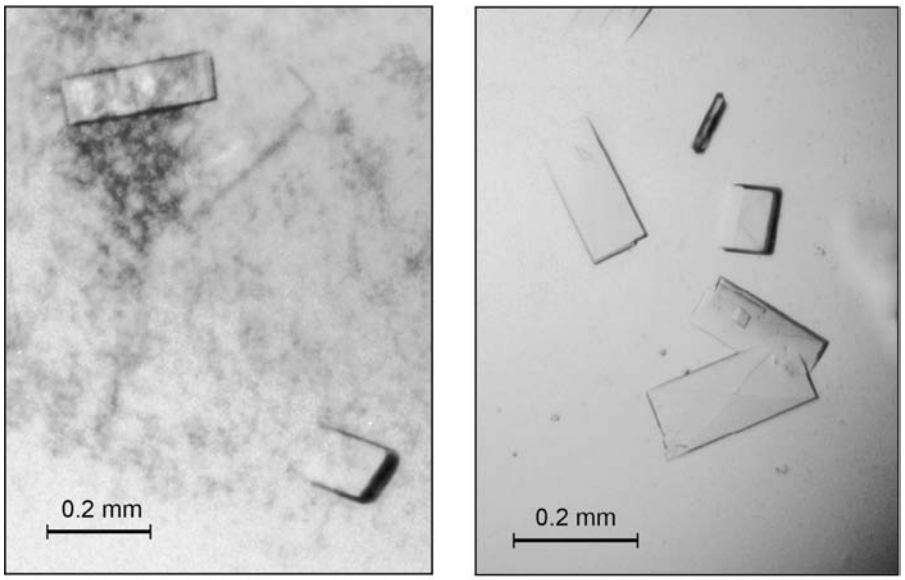

Figure 1
Single crystals of LlPR10.1A obtained in hanging drops (*a*) without detergent and (*b*) with the addition of detergent.

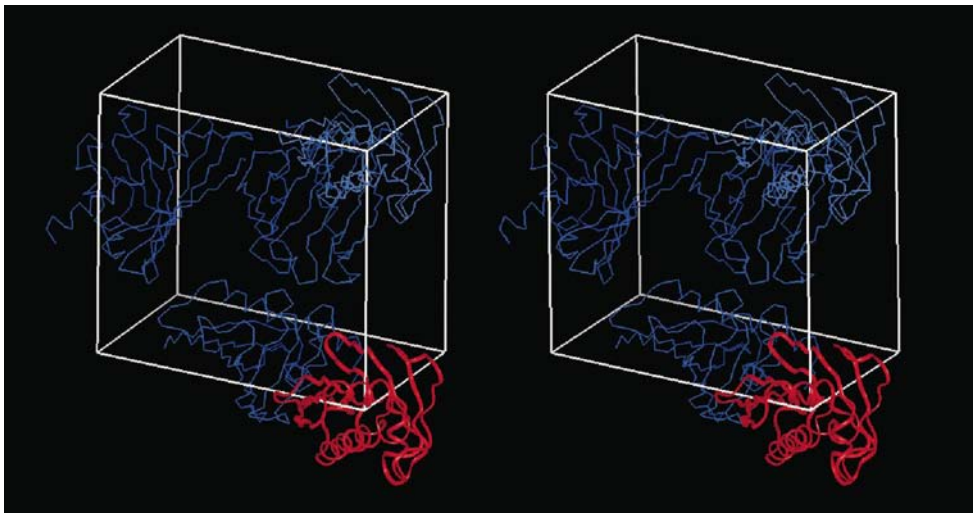

Figure 2
Stereoview of the packing of LlPR10.1A molecules in the orthorhombic unit cell. The principal copy of the protein, shown as a cartoon-type model, illustrates the general fold of the protein, with the extended β-sheet wrapped around the long C-terminal helix. The remaining molecules are represented as a C$^{\alpha}$ trace.

2.3. Data collection and processing

Diffraction data were collected at 291 K from a crystal of dimensions 0.3 × 0.2 × 0.1 mm mounted in a quartz capillary with a small amount of mother liquor, using a 300 mm MAR Research image-plate scanner and Cu $K\alpha$ radiation generated from an SRA2 rotating-anode generator (Siemens) operated at 45 kV and 112 mA. 30 344 reflections were measured to 2.45 Å and reduced to a set of 5166 unique reflections with 97.5% completeness (82.6% in the last resolution shell) characterized by $R_{\rm int}$ = 0.085 and $\langle I/\sigma(I)\rangle$ = 14.8 (0.390 and 2.9, respectively, in the last resolution shell). Indexing and integration of the images was performed using *DENZO* and scaling of the intensity data was performed using *SCALEPACK*; both are from the *HKL* program package (Otwinowski, 1993).

2.4. Structure solution

The structure was solved by molecular replacement, using the program *AMoRe* (Navaza, 1994) with diffraction data in the 15–4 Å resolution range and the structure of birch-pollen allergen protein Betv1 (Gajhede *et al.*, 1996) as a probe. The protein sequences were first aligned using *ClustalW* (Thompson *et al.*, 1994) and the non-conserved residues of the Betv1 model were truncated to the common fragment (usually Ala). Additionally, the disordered residues 60–65 in the PDB model 1bv1 were removed from the molecular-replacement probe. The rotation-function results were not clear cut, with a continuous list of peaks starting with correlation coefficient 0.092. It was only during the translation-search step that the correct orientation could be identified (highest translation-function peak 0.312, next 0.259). It corresponded to the 15th peak on the rotation-function map (80% of the map maximum). After rigid-body refinement, the molecular-replacement solution was characterized by a

correlation coefficient of 0.393 and an R factor of 0.463. Preliminary refinement of the structure, after mutation to the LlPR10.1A sequence and modelling of the missing fragment according to electron density, resulted in a model characterized by $R = 0.250$. The refinement, performed using *X-PLOR* (Brünger, 1992), included a simulated-annealing step.

3. Results and discussion

3.1. Expression of LlPR10.1A as a fusion protein with MBP in pMal-c2 system

The recombinant LlPR10.1A protein expressed in *E. coli* as a fusion protein was present in the soluble bacterial protein fraction (no inclusion bodies were formed). The final yield of recombinant LlPR10.1A was 15–20 mg per litre of bacterial culture.

3.2. X-ray structure determination

Although LlPR10.1A is highly soluble and can be dissolved even in pure water, it yields good-quality single crystals with difficulty. Under most conditions, only microcrystalline precipitates are formed. The most promising single crystals, as used in this preliminary X-ray analysis, were obtained together with a large amount of precipitate (Fig. 1*a*) using a protein concentration of 5 mg ml^{-1}, 20 m*M* Tris–HCl pH 8.5 and 50% saturated ammonium sulfate. Subsequent use of detergents (sucrose monolaureate and CYMAL-6; Hampton Research detergent screen) improved the crystallization conditions dramatically, leading to a few well formed single crystals in a clear solution (Fig. 1*b*). Further analysis of these new crystals is in progress.

The crystals are stable in the X-ray beam, allowing diffraction data to be collected at room temperature. They are orthorhombic, space group $P2_12_12_1$, with unit-cell parameters $a = 35.9$, $b = 58.7$, $c = 63.3$ Å, and contain one molecule in the asymmetric unit. They are relatively densely packed (Fig. 2), with a Matthews coefficient of 1.99 Å^3 Da^{-1} (Matthews, 1968), which corresponds to a solvent content of 38%. Even though the homology between LlPR10.1A and the birch-pollen allergen protein is modest (45% identity, 65% similarity), Betv1 can be used as a successful probe in molecular-replacement calculations as the general fold of the two molecules is similar. However, the current model indicates that while some secondary-structure elements are highly conserved (the β-sheet), others (most notably the C-terminal helix) may have somewhat different disposition and structural characteristics. The conserved glycine residues at positions 46, 48, 49 and 51 form a glycine-rich loop which connects two β-strands. Although it is exposed on the surface of the molecule, this P-loop is visible in the current electron-density maps.

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References

Awade, A., Metz-Boutique, M. H., Le Ret, M., Genot, G., Amiri, I. & Burkard, G. (1991). *Biochim. Biophys Acta*, **1077**, 241–244.

Breda, C., Sallaud, C., El-Turk, J., Buffard, D., de Kozak, I., Esnault, R. & Kondorosi, A. (1996). *Mol. Plant-Microbe Interact.* **9**, 713–719.

Breiteneder, H., Ferreira, F., Hoffman-Sommergruber, K., Ebner, C., Breitenbach, M., Rumpold, H., Kraft, D. & Scheiner, O. (1993). *Eur. J. Biochem.* **212**, 355–362.

Breiteneder, H., Ferreira, F., Reikerstorfer, A., Duchene, M., Valenta, R., Hoffman-Sommergruber, K., Ebner, C., Breitenbach, M., Kraft, D. & Scheiner, O. (1992). *J. Allergy Clin. Immunol.* **90**, 909–917.

Breiteneder, H., Hoffman-Sommergruber, K., Oriordain, G., Susani, M., Ahorn, H., Ebner, C., Kraft, D. & Scheiner, O. (1995). *Eur. J. Biochem.* **223**, 484–489.

Breiteneder, H., Pettenburger, K., Bito, A., Valenta, R., Kraft, D., Rumpold, H., Scheiner, O. & Breitenbach, M. (1989). *EMBO J.* **8**, 1935–1938.

Brünger, A. T. (1992). *X-PLOR. Version 3.1. A System for X-ray Crystallography and NMR.* New Haven and London: Yale University Press.

Gajhede, M., Osmark, P., Poulsen, F. M., Ipsen, H., Larsen, J. N., van Neerven, R. J. J., Schou, C., Lowenstein, H. & Spangfort, M D. (1996). *Nature Struct. Biol.* **3**, 1040–1045.

Iturriaga, E. A., Leech, M. J., Barratt, D. H. P. & Wang, T. L. (1994). *Plant Mol. Biol.* **24**, 235–240.

Larsen, J. N., Strøman, P. & Ipsen, H. (1992). *Mol. Immunol.* **29**, 703–711.

Legocki, A. B., Karłowski, W. M., Podkowński, J., Sikorski, M. M. & Stępkowski, T. (1997). In *Biological Fixation of Nitrogen for Ecology and Sustainable Agriculture. Proceedings of the Second European Nitrogen Fixation Conference*, edited by A. B. Legocki, H. Bothe & A. Püchler. Berlin, Heidelberg: Springer–Verlag.

Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.

Matton, D. P. & Brisson, N. (1989). *Mol. Plant-Microbe Interact.* **2**, 325–331.

Moiseyev, G. P., Beintema, J. J., Fedoreyeva, L. I. & Yakovlev, G. I. (1994). *Planta*, **193**, 470–472.

Moiseyev, G. P., Larisa, I., Fedoreyeva, L. I., Zhuravlev, Y. N., Yasnetskaya, E., Jekel, P. A. & Beintema, J. J. (1997). *FEBS Lett.* **407**, 207–210.

Mylona, P., Moerman, M., Yang, W.-C., Gloudemans, T., Van De Kerckhove, J., van Kammen, A., Bisseling, T. & Franssen, H. J. (1994). *Plant Mol. Biol.* **26**, 39–50.

Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.

Otwinowski, Z. (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 56–62. Warrington: Daresbury Laboratory.

Pinto, P. M. & Ricardo, C. P. P. (1995). *Plant Physiol.* **109**, 1345–1351.

Saraste, M., Sibbald, P. R. & Wittinghofer, A. (1990). *Trends Biol. Sci.* **15**, 430–434.

Scheurer, S., Metzner, K., Haustein, D. & Vieths, S. (1997). *Mol. Immunol.* **34**, 619–629.

Schmelzer, E., Kruger-Lebus, S. & Hahlbrock, K. (1989). *Plant Cell*, **1**, 993–1001.

Sikorski, M. M. (1997). *Acta Biochim. Pol.* **44**, 565–578.

Sikorski, M. M., Szlagowska, A. E. & Legocki, A. B. (1996). *Plant Physiol.* **110**, 335.

Somssich, I. E., Schmelzer, E., Bollmann, J. & Hahlbrock, K. (1986). *Proc. Natl Acad. Sci. USA*, **83**, 2427–2430.

Somssich, I. E., Schmelzer, E., Kawalleck, P. & Hahlbrock, K. (1988). *Mol. Gen. Genet.* **213**, 93–98.

Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). *Nucleic Acids Res.* **22**, 4673–4680.

Vanek-Krebitz, M., Hoffman-Sommergruber, K., Machado, M. L. D., Susani, M., Ebner, C., Kraft, D., Scheiner, O. & Breiteneder, H. (1995). *Biochem. Biophys. Res. Commun.* **214**, 538–551.

Van Loon, L. C., Pierpoint, W. S., Boller. T. & Conejero, V. (1994). *Plant Mol. Biol. Rep.* **12**, 245–264.

Warner, S. A. J., Gill, A. & Draper, J. (1994). *Plant J.* **6**, 31–43.

Warner, S. A. J., Scott, R. & Draper, J. (1992). *Plant Mol. Biol.* **19**, 555–561.

Warner, S. A. J., Scott, R. & Draper, J. (1993). *Plant J.* **3**, 191–201.

Yamamoto, M., Torikai, S. & Oeda, K. (1997). *Plant Cell Physiol.* **38**, 1080–1086.