Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray diffraction analysis of β-cinnamomin, an elicitin secreted by the phytopathogenic fungus *Phytophthora cinnamomin*

M. Archer,[a] M. L. Rodrigues,[a] M. Aurélio,[b] R. Biemans,[c] A. Cravador[b] and M. A. Carrondo[a]*

[a]Instituto de Tecnologia Química e Biológica (ITQB), Av. Republica, Apt 127, 2781-901 Oeiras, Portugal, [b]Unidade de Ciências e Tecnologias Agrárias, Universidade do Algarve, Campus de Gambelas, 8000-810 Faro, Portugal, and [c]Service de Génétique Appliquée, Université Libre de Bruxelles, 1400 Nivelles, Belgium

Correspondence e-mail: carrondo@itqb.unl.pt

Received 12 October 1999
Accepted 6 January 2000

Cinnamomin (CIN) belongs to a family of 10 kDa proteins designated as elicitins. Some of these proteins induce a hypersensitive response in diverse plant species, leading to resistance against fungal and bacterial plant pathogens. CIN was crystallized by the vapour-diffusion method using either ammonium sulfate or polyethyleneglycol (PEG) as precipitants in solutions buffered at around pH 7. These crystals are isomorphous and belong to the triclinic space group, with unit-cell parameters a = 31.69, b = 36.99, c = 44.09 Å, α = 76.86, β = 84.41, γ = 80.26°. A frozen crystal diffracted X-rays beyond 1.45 Å resolution on a synchrotron-radiation source.

1. Introduction

Cinnamomin is a polypeptide secreted by the oomycetous fungus *P. cinnamomi* Rands, a highly aggressive filamentous soilborne fungus and one of the world's most destructive plant pathogens. Recently, the fungus has been shown to be involved in widespread deaths of cork oaks (*Quercus suber* L.) in Portugal and Spain (Brasier, 1992, 1993; Brasier *et al.*, 1993). Cinnamomin belongs to a group of proteins called elicitins that are produced by members of the genus *Phytophthora* and by *Pythium vexans* (Kamoun *et al.*, 1994; Ricci *et al.*, 1989; Huet *et al.*, 1995). These polypeptides are holoproteins containing 98 residues.

Elicitins can cause a hypersensitive response, including leaf necrosis and cell death, and induce systemic acquired resistance in some plant species (Kamoun *et al.*, 1994; Ricci *et al.*, 1992). According to their toxic activity, elicitins were classified as acidic α-elicitins and basic β-elicitins, the former being less necrotic than the latter (Kamoun *et al.*, 1993). Moreover, β-elicitins have a hydrophilic residue at position 13, usually a lysine, whereas α-elicitins have a hydrophobic valine at this position.

The solution structures of α-capsicein from *P. capsici* (Bouaziz *et al.*, 1994) was determined using multidimensional heteronuclear nuclear magnetic resonance spectroscopy. Furthermore, the three-dimensional structure of β-cryptogein from *P. cryptogea* was solved independently by X-ray crystallography (Boissy *et al.*, 1996) and by NMR spectroscopy (Fefeu *et al.*, 1997). The overall structure reveals a novel fold, consisting of six α-helices and a two-stranded β-sheet facing an Ω loop (Boissy *et al.*, 1996). Recently, the crystal structure of an elicitin–ergosterol complex was determined (Boissy *et al.*, 1999), which is consistent with the hypothesis that elicitins may be sterol carrier proteins, as proposed by Mikes *et al.* (1998). More structural information for these compounds showing elicitor activity is needed for a better understanding of how elicitors bind to plant receptors.

In order to study the role of CIN in the pathogenesis of *P. cinnamomi* and *Q. suber*, a synthetic gene was constructed and expressed in a biologically active form (Duclos *et al.*, 1998). In this work, we report the crystallization and preliminary X-ray diffraction studies of the recombinant β-cinnamomin.

2. Experimental

2.1. Protein expression and purification

A synthetic gene encoding β-cinnamomin was designed using the previously determined amino-acid sequence (Huet & Pernollet, 1989). The gene was inserted into the methylotrophic yeast *Pichia pastoris* secretion vector pIC9K in frame and downstream to the α-mating factor pre-pro secretion signal sequence of *Saccharomyces cerevisiae*. The *Pi. pastoris* culture supernatant was concentrated in an AMICON stirred cell concentrator using a YM10 membrane (cutoff 10 kDa) at a pressure of 3×10^5 Pa; the ionic strength of this solution was decreased to 20 m*M* sodium acetate pH 4.0. The resulting sample was loaded onto a Macro-Prep S column equilibrated with 20 m*M* sodium acetate pH 4.0. A NaCl gradient was applied from 0 to 100 m*M* NaCl in the working buffer and the CIN fraction eluted from 25 to 50 m*M* NaCl. The eluate was concentrated and applied onto a Superdex 75 FPLC column equilibrated with 20 m*M* potassium phosphate buffered at pH 7.0 and supplemented with 150 m*M* NaCl.

Table 1
Data-collection and processing statistics.

	Crystal *A*	Crystal *B*
X-ray source	Enraf–Nonius, 4.5 kV	ESRF, ID14-EH4 beamline
Wavelength (Å)	1.5418	0.932
Detector	MAR Research IP (30 cm)	ADSC Quantum 4 CCD
Space group	*P*1	*P*1
Unit-cell parameters (Å, °)	a = 31.63, b = 37.13, c = 43.98, α = 76.59, β = 86.09, γ = 80.14	a = 31.69, b = 36.99, c = 44.09, α = 76.86, β = 86.41, γ = 80.26
Total number of reflections	30414	85184
Number of unique reflections	10788	32458
Redundancy	2.8	2.6
Resolution range (Å)	21.7–2.1	20.5–1.45
Completeness† (%)	96.4 (91.6)	95.3 (93.1)
$I/\sigma(I)$†	8.2 (3.3)	13.1 (2.7)
$R_{\rm merge}$† (%)	9.0 (31.3)	8.9 (25.2)

† Values in parentheses refer to the outer resolution shell: 2.18–2.10 Å for crystal *A* and 1.48–1.45 Å for crystal *B*.

2.2. Crystallization

Initial crystallization conditions were screened at 278 and 293 K with the Hampton Research Crystal Screen kit I. The sitting-drop vapour-diffusion method was employed. The droplets contained equal amounts (2 µl) of protein (10 mg ml^{-1} in 10 m*M* Tris–HCl pH 7.5) and reservoir solutions equilibrated against 0.7 ml of reservoir solution in the well. Crystalline material was obtained under different experimental conditions. Small thin plate-like crystals which were prone to forming aggregates were grown at both temperatures from 30% PEG 4K, 0.1 *M* Tris–HCl pH 8.5, 0.2 *M* lithium sulfate or ammonium acetate. Similar crystal forms were obtained in the cold room from 20% PEG 4K, 10% 2-propanol, 0.1 *M* HEPES pH 7.5. Many microcrystals also appeared at room temperature in 1.4 *M* sodium citrate buffered with 0.1 *M* HEPES pH 7.5.

A narrower screening was performed around these conditions, which included variations in the protein concentration, the nature and concentration of precipitants, pH and type of buffer, addition of different salts or organic solvents, drop proportion (protein to mother liquor ratio) and temperature. It was observed that the addition of certain alcohols and ammonium sulfate improved the quality of the crystals. Thicker plates were grown using ammonium sulfate as precipitant.

Finally, two crystallization conditions were optimized at 293 K, yielding crystals suitable for X-ray diffraction analysis. The protein concentration used was 15 mg ml^{-1}. In the first conditions, the initial reservoir solution contained 2.0 *M* ammonium sulfate, 5% MPD, 0.1 *M* HEPES pH 7.0 or 7.5. After 3 d, the precipitant concentration was increased to 2.2 *M*. The nucleation rate was thus decreased, leading to only a few single colourless crystals within 4–5 d (Fig. 1). The second crystallization conditions consisted of 30% PEG 4K, 10% butanol, 0.2 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 7.5; it took about two weeks for crystals to grow. Crystals grown under both conditions showed similar morphologies, with maximum crystal dimensions of around 0.3 × 0.2 × 0.06 mm.

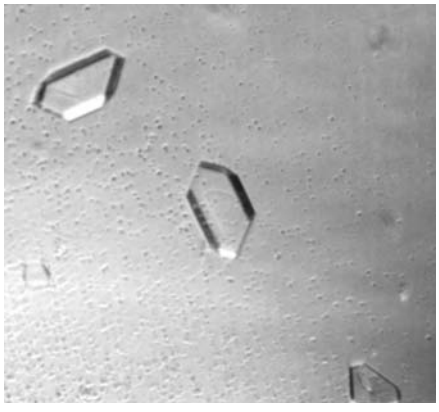

Figure 1
Native crystals of recombinant β-cinnamomin grown from 2.0 *M* ammonium sulfate, 5% MPD, 0.1 *M* HEPES pH 7.0. The maximum dimension is around 0.3 mm. Crystals belong to space group *P*1.

2.3. Diffraction data collection and processing

A crystal grown in the first conditions described above was transferred using a rayon-fibre loop into mother liquor complemented with 10% glycerol (crystal *A* in Table 1) and was placed directly into a nitrogen stream at 115 K (Oxford Cryosystems). X-ray diffraction experiments were carried out using a MAR Research image-plate detector (30 cm) mounted on an Enraf–Nonius rotating-anode generator operating at 4.5 kW with Cu $K\alpha$ radiation. This crystal diffracted to 2.1 Å resolution.

Subsequently, another crystal grown under the same conditions was taken to the ESRF in Grenoble (crystal *B* in Table 1). Immediately prior to the X-ray measurements, the crystal was briefly immersed in a cryo-protectant solution containing 30% PEG 400, 5% MPD buffered in 0.1 *M* HEPES pH 7.0. The crystal was then mounted in a loop and flash-frozen at 110 K in a cold nitrogen stream. Diffraction data were collected at the ID14-EH4 beamline using a ADSC Quantum 4 CCD detector, at a wavelength of 0.932 Å. Since the crystal diffracted beyond 1.45 Å, data collection at the synchrotron followed a two-step protocol. First, X-ray data from the frozen crystal was measured using a crystal-to-detector distance of 120 mm with an oscillation range of 0.5° per film (1 s exposure time) for a total of 360 images. To collect the lower resolution data, oscillation pictures were taken for 0.5 s with a frame width of 1.5° and a crystal-to-detector distance of 180 mm (120 images were measured in total). Data were processed and scaled with the *HKL* suite of programs (Otwinowski & Minor, 1995).

3. Results and discussion

The availability of a reasonable amount of protein with a high degree of purity is an important requirement for crystallization purposes. The methylotrophic yeast *Pi. pastoris* was chosen for the expression of β-cinnamomin as it is able to express and secrete high levels of heterologous proteins (Sreekrishna *et al.*, 1997). The transformants seem to secrete the apparently fully processed protein.

The recombinant protein was first purified by using cation-exchange chromatography. As the ionic strength of the media prevented the correct binding of CIN to the Macroprep S resin, the *Pi. pastoris* culture supernatant was not directly loaded onto this ion-exchange matrix but was first concentrated and dialysed through membranes with a cutoff value of 10 kDa. This ultrafiltration step led to the removal of large quantities of UV-absorbing substances without loss of the CIN. Based on the calculated isoelectric point of the protein (pI 7.55), the elution of the CIN fraction from the Macroprep S column was performed using a salt gradient in sodium acetate buffered at pH 4.0. SDS–PAGE and immunoblotting analysis showed that the fractionated protein was still contaminated by molecules of higher molecular weight and that a further purifi-

cation step was required. Thus, a final passage through a molecular-exclusion column (Superdex75 FPLC) was necessary. The purity of the protein was assessed by SDS–PAGE stained with silver nitrate, which showed the presence of a single band corresponding to a molecular weight $\simeq 10$ kDa, the same as the natural protein. The yield of this purification protocol was about 48 mg of purified recombinant CIN per litre of culture.

After the initial crystallization screening, crystalline material was obtained within a week. However, the optimization of these experimental conditions was a difficult process, owing to the tendency of the protein to form clusters of thin-shaped plates and to the non-reproducibility of the crystallization experimental results. Finally, high-quality single crystals were produced after 3–4 months of trial-and-error crystallization experiments.

Cryo-stabilization of the crystals was first achieved by the addition of glycerol or MPD (to a final concentration of 10–15%) to the crystallization buffers. Since these cryo-protectant solutions sometimes formed ice-rings, different conditions were chosen for the synchrotron data collection, namely a solution containing 30% PEG 400, 5% MPD buffered at pH 7.0.

Complete data sets were collected from single frozen crystals, which diffracted X-rays to about 2.1 Å resolution with a conventional X-ray source (crystal *A* in Table 1) and beyond 1.45 Å with synchrotron radiation (crystal *B* in Table 1). CIN crystals belong to space group *P*1, with unit-cell parameters a = 31.69, b = 36.99, c = 44.09 Å, α = 76.86, β = 84.41, γ = 80.26° for the crystal measured at the ESRF (Table 1). Details of the data-collection and processing statistics from both crystals are presented in Table 1. Assuming that a dimer is present in the asymmetric unit, a V_m of 2.41 Å^3 Da^{-1} is obtained, which corresponds to a solvent content of around 49%. CIN crystals were also formed using PEG as precipitant, but since these crystals are isomorphous to the ones grown from ammonium sulfate and diffract no better, no complete data set was measured for these crystals.

The CIN structure determination by molecular replacement using β-cryptogein (PDB entry 1beo) as a starting model is now in progress.

We acknowledge the opportunity to collect X-ray data at the ESRF synchrotron, Grenoble, France. We are grateful to Carlos Frazão and Pedro Matias for help with data collection. MLR and MA thank Fundação para a Ciência e Tecnologia (FCT) for their PRAXISXXI research grants (BIC/17185/98 and BPD/17265/98). We thank FCT (PRAXIS XXI 3/3.2/FLOR/2112/95) and NATO (SfS PO-CORKOAKS II) for financial support.

References

Boissy, G., de la Fortelle, E., Kahn, R., Huet, J.-C., Bricogne, G., Pernollet, J.-C. & Brunie, S. (1996). *Structure*, **4**, 1429–1439.

Boissy, G., O'Donohue, M., Gaudemer, O., Perez, V., Pernollet, J.-C. & Brunie, S. (1999). *Protein Sci.* **8**, 1191–1199.

Bouaziz, S., van Heijenoort, C., Huet, J.-C., Pernollet, J.-C. & Guittet, E. (1994). *Biochemistry*, **33**, 8188–8197.

Brasier, C. M. (1992). *Nature (London)*, **360**, 539.

Brasier, C. M. (1993). *Proceedings of the International Congress. Recent Advances in Studies on Oak Decline*, edited by N. Luisi, P. Lerario & A. Vannini, pp. 49–57. Bari, Italy: Dipartimento di Patologia Vegetale, Università degli Studi.

Brasier, C. M., Robredo, F. & Ferraz, J. F. P. (1993). *Plant Pathol.* **42**, 140–145.

Duclos, J., Trincão Aurélio, M., Graça, J., Coelho, A. C., Fauconnier, A., Jacquet, A., Bollen, A., Cravador, A., Biemans, R. & Godfroid, E. (1998). *Proceedings of the Twelfth Forum for Applied Biotechnology* pp. 1695–1698. Med. Fac. Landbouw. University of Gent, Belgium.

Fefeu, S., Bouaziz, S., Huet, J.-C., Pernollet, J.-C. & Guittet, E. (1997). *Protein Sci.* **11**, 2279–2284.

Huet, J.-C., Le Caer, J.-P., Nespoulous, C. & Pernollet, J.-C. (1995). *Mol. Plant Microbe Interact.* **8**, 302–310.

Huet, J.-C. & Pernollet, J.-C. (1989). *FEBS Lett.* **257**, 302–306.

Kamoun, S., Young, M., Förster, H., Coffey, M. D. & Tyler, B. M. (1994). *Appl. Environ. Microbiol.* **60**, 1593–1598.

Kamoun, S., Young, M., Glascock, C. B & Tyler, B. M. (1993). *Mol. Plant Microbe Interact.* **6**, 15–25.

Mikes, V., Milat, M. L., Ponchet, M., Panabières, F., Ricci, P. & Blein, J. P. (1998). *Biochem. Biophys. Res. Commun.* **245**, 133–139.

Otwinowski, Z. & Minor, W. (1995). *The HKL Manual.* New Haven, Connecticut, USA: Yale University Press.

Ricci, P., Bonnet, P., Huet, J.-C., Sallantin, M., Beauvais-Cante, F., Bruneteau, M., Billard, V., Michel, G. & Pernollet, J.-C. (1989). *Eur. J. Biochem.* **183**, 555–563.

Ricci, P., Trentin, F., Bonnet, P., Venard, P., Mouton-Perronet, F. & Bruneteau, M. (1992). *Plant Pathol.* **41**, 298–307.

Sreekrishna, K., Brankamp, R. G., Kropp, K. E., Blankenship, D. T., Tsay, J. T., Smith, P. L., Wierschke, J. D., Subramaniam, A. & Birkenberger, L. A. (1997). *Gene*, **190**, 55–62.