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

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

*Instituto de Tecnologia Química e Biológica (ITQB), Av. Republica, Apt 127, 2781-901 Oeiras, Portugal, bUnidade de Ciências e Tecnologias Agrárias, Universidade do Algarve, Campus de Gambelas, 8000-610 Faro, Portugal, and cService de Génétique Appliquée, Université Libre de Bruxelles, 1400 Nivelles, Belgium

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

Abstract

Cinnamomin (CIN) belongs to a family of 10 kDa proteins designated as elicitis. 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 polyethylene-glycol (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. cinnamomoni 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 (Braisier, 1992, 1993; Brasier et al., 1993). Cinnamomin belongs to a group of proteins called elicitis 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, elicitis were classified as acidic α-elicitis and basic β-elicitis, the former being less necrotic than the latter (Kamoun et al., 1993). Moreover, β-elicitis have a hydrophilic residue at position 13, usually a lysine, whereas α-elicitis have a hydrophobic valine at this position.

The solution structures of α-capsicin 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. cryptogeia 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 elicitis 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. cinnamomoni 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 methylophrophic yeast Pichia pastoris secretion vector pIC9K in frame and downstream to the α-mating factor pre-pro secretion signal sequence of Saccharomyces cerevisiae. The P. pastoris culture supernatant was concentrated in an AMICON stirred cell concentrator using a YM10 membrane (cutoff 10 kDa) at a pressure of 3 x 10⁵ Pa; the ionic strength of this solution was decreased to 20 mM sodium acetate pH 4.0. The resulting sample was loaded onto a Macro-Prep S column equilibrated with 20 mM sodium acetate pH 4.0. A NaCl gradient was applied from 0 to 100 mM NaCl in the working buffer and the CIN fraction eluted from 25 to 50 mM NaCl. The eluate was concentrated and applied upon a Superdex 75 FPLC column equilibrated with 20 mM potassium phosphate buffered at pH 7.0 and supplemented with 150 mM NaCl.
2.2. Crystallization

Initial crystallization conditions were screened at 278 and 293 K with the Hampton Research Crystal Screen kit 1. The sitting-drop vapour-diffusion method was employed. The droplets contained equal amounts (2 μl) of protein (10 mg ml⁻¹ in 10 mM 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 ammonium sulfate, 0.1 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. The nucleation rate was second to those of other conditions, with 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.

2.3. Diffraction data collection and processing

A crystal grown in the first conditions described above was transferred using a micro-crystal loop into mother liquor containing 10% glycerol (crystal A) and equilibrated against 0.7 ml of reservoir solution in the well. 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 Kα 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 the 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 ion-pair strength of the protein 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 buffer 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-

Table 1

<table>
<thead>
<tr>
<th>Crystal A</th>
<th>Crystal B</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-ray source</td>
<td>Enraf–Nonius, 4.5 kW</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
<td>1.5418</td>
</tr>
<tr>
<td>Detector</td>
<td>MAR Research IP (30 cm)</td>
</tr>
<tr>
<td>Space group</td>
<td>P1</td>
</tr>
<tr>
<td>Unit-cell parameters (Å)</td>
<td>a = 31.63, b = 37.13, c = 43.98, α = 76.59, β = 86.09, γ = 80.14</td>
</tr>
<tr>
<td>Total number of reflections</td>
<td>30414</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>10786</td>
</tr>
<tr>
<td>Redundancy</td>
<td>2.8</td>
</tr>
<tr>
<td>Resolution range (Å)</td>
<td>21.7±2.1</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>96.4 (91.6)</td>
</tr>
<tr>
<td>Rmerge (I (%)</td>
<td>8.2 (3.3)</td>
</tr>
</tbody>
</table>

1 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.

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 P1.
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 \( \approx 10 \text{ kDa} \), 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 \( P1 \), with unit-cell parameters \( a = 31.69 \), \( b = 36.99 \), \( c = 44.09 \) Å, \( \alpha = 76.86 \), \( \beta = 84.41 \), \( \gamma = 80.26 \)° 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 \( \beta \)-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 financial support.

References


