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Crystallization and preliminary crystallographic analysis of a chitinase from *Clonostachys rosea*

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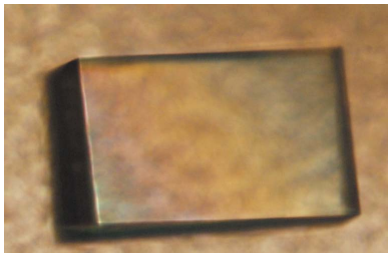

CrChi1 is a chitinase from the nematophagous fungus *Clonostachys rosea* that plays a role in the infection of nematodes. In order to resolve the crystal structure of CrChi1 and to gain a better understanding of its biological functions, recombinant CrChi1 was crystallized at 291 K using PEG 3350 and ammonium dihydrogen phosphate as precipitant and a 1.8 Å resolution X-ray data set was collected from a single flash-cooled crystal (100 K). The crystals belonged to space group $P2_1$, with unit-cell parameters $a = 44.1$, $b = 71.7$, $c = 59.1$ Å, $\alpha = \gamma = 90$, $\beta = 91.3^\circ$. Assuming the presence of one molecule per asymmetric unit, the Matthews coefficient and solvent content were calculated to be 2.45 Å^3 Da^{-1} and 40%, respectively. To our knowledge, this is the first structure determination study of a chitinase from a nematophagous fungus.

1. Introduction

The filamentous fungus *Clonostachys rosea* (syn. *Gliocladium roseum*; teleomorph *Bionectria ochroleuca*) is a common saprophyte with a worldwide distribution. The soil fungus *C. rosea* shows mycoparasitic activity against several plant-pathogenic fungi (Sutton *et al.*, 1997; Li *et al.*, 2004; Luongo *et al.*, 2005). Morandi *et al.* (2003) found that *C. rosea* could suppress sporulation of the plant-pathogenic fungus *Botrytis cinerea* and could be used for the control of botrytis blight. Recently, *C. rosea* has also been reported as a potential agent for the biocontrol of nematodes (Zhao *et al.*, 2005; Li *et al.*, 2006). Penetration of the nematode cuticle or eggshell has been assumed to result from the actions of mechanical forces and hydrolytic enzymes including chitinases (Gan, Yang, Tao, Liang *et al.*, 2007; Yang *et al.*, 2007). We have recently isolated a chitinase (CrChi1) from *C. rosea*, a fungus that is known to be able to degrade the eggs of the root-knot nematode *Meloidogyne incognita* (Gan, Yang, Tao, Yu *et al.*, 2007). At present, much remains unknown about this group of enzymes. For example, no crystal structure is available of any chitinase from nematophagous fungi or mycoparasitic fungi. In this study, we report the expression of the *C. rosea* chitinase CrChi1 in *Escherichia coli*. Purified recombinant CrChi1 protein was crystallized using hanging-drop vapour-diffusion methods and we have performed a preliminary crystallographic analysis.

2. Materials and methods

2.1. Cloning, expression and purification

The partial cDNA sequence for CrChi1 was amplified from *C. rosea* using two primers, the forward primer 5′-AAGT**GAATTC**CGTG-CAACTCCTCGCATG-3′ and the reverse primer 5′-AAATAT**G-CGGCCGC**TTAGGAGAGGCTGTTCTTGA-3′ as described previously (Gan, Yang, Tao, Yu *et al.*, 2007), which encoded the mature peptide of chitinase (amino-acid residues 21–426 of CrChi1; GenBank No. EU000575). The PCR product was digested with *Eco*RI and *Not*I (sites indicated in bold) and ligated into the same restricted sites of the pET28a(+) vector (Novagen, Germany) with a His_6 tag at the N-terminus. The precise sequence of the construct consisted of the mature chitinase peptide and 36 amino-acid residues

of the pET28a(+) vector (MGSSHHHHHHSSGLVPRGSHMAS-MTGGQQMGRGSEF) at the N-terminus of the chitinase. The sequence of the insert was verified by sequencing. The recombinant plasmid was transformed into *E. coli* strain BL21 (DE3). Transformed cells were then cultured at 310 K in LB medium containing 50 µg ml^{-1} ampicillin. Overexpression of the protein was induced with 0.1 m*M* IPTG when the absorbance at 600 nm (A_{600}) was 0.5 and the cells were harvested after 14 h incubation at 289 K. Cells were harvested by centrifugation, resuspended in 20 m*M* Tris–HCl buffer pH 8.0 and then homogenized by sonication. The lysate was centrifuged at 12 000*g* for 30 min to remove cell debris. The supernatant was collected and the fusion protein was purified on Ni^{2+}–nitrilotriacetic acid agarose (Amersham, Sweden). The target CrChi1 protein was eluted with 0.1 *M* imidazole buffer. The protein was further purified on a HiTrap Q FF (Amersham, Sweden) ion-exchange chromatography column run in 20 m*M* Tris–HCl buffer pH 8.0, 5 m*M* $CaCl_2$ and developed with a 0–400 m*M* NaCl gradient. The purity of CrChi1 was estimated by SDS–PAGE.

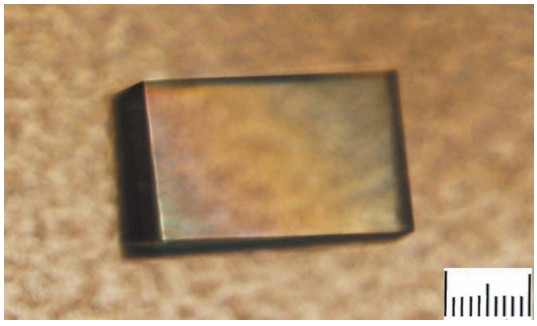

Figure 1
A single crystal of chitinase CrChi1. Crystals were obtained with 0.2 *M* ammonium dihydrogen phosphate and 15%(*w*/*v*) PEG 3350 pH 4.6. Scale bar, 0.1 mm.

2.2. Crystallization

The purified protein was dialyzed against the crystallization buffer (20 m*M* Tris–HCl buffer pH 8.0) and concentrated to 20–25 mg ml^{-1}. Preliminary crystallization experiments were carried out at 291 K in 16-well plates with Crystal Screens I and II (Hampton Research, USA) using the hanging-drop vapour-diffusion method (Jancarik & Kim, 1991). 0.5 µl CrChi1 solution was mixed with 0.5 µl reservoir solution and equilibrated against 0.2 ml reservoir solution at 291 K. Single crystals appeared after 5 d in a solution containing 0.2 *M* ammonium dihydrogen phosphate and 20%(*w*/*v*) PEG 3350 pH 4.6. This condition was further optimized and crystals were obtained reproducibly using 0.2 *M* ammonium dihydrogen phosphate and 15%(*w*/*v*) PEG 3350 pH 4.6; they grew to maximum dimensions of 0.3 × 0.2 × 0.05 mm after 7 d (Fig. 1).

2.3. Data collection and analysis

The crystal was mounted on a nylon loop and flash-cooled in a cold nitrogen-gas stream at 100 K using an Oxford Cryosystems Cryostream. The exposure time was 5 min, the crystal-to-detector distance was 120 mm and the oscillation range per frame was 0.5°. Diffraction data were collected on a MAR345dtb (MAR Research, Hamburg) image-plate detector at 100 K using a Rigaku MM-007 rotating-anode home X-ray generator operating at 40 kV and 20 mA (λ = 1.5418 Å). All intensity data were indexed, integrated and scaled with the *HKL*-2000 package (Otwinowski & Minor, 1997). Diffraction data statistics are given in Table 1.

3. Results and discussion

The *Crchi1* gene from *C. rosea* was successfully cloned and overexpressed in *E. coli* and the recombinant CrChi1 protein was purified; the molecular weight of the protein was found to be 44 kDa.

Initial crystals were obtained from condition No. 79 of the Index Kit (Hampton Research, USA) containing 0.2 *M* ammonium dihy-

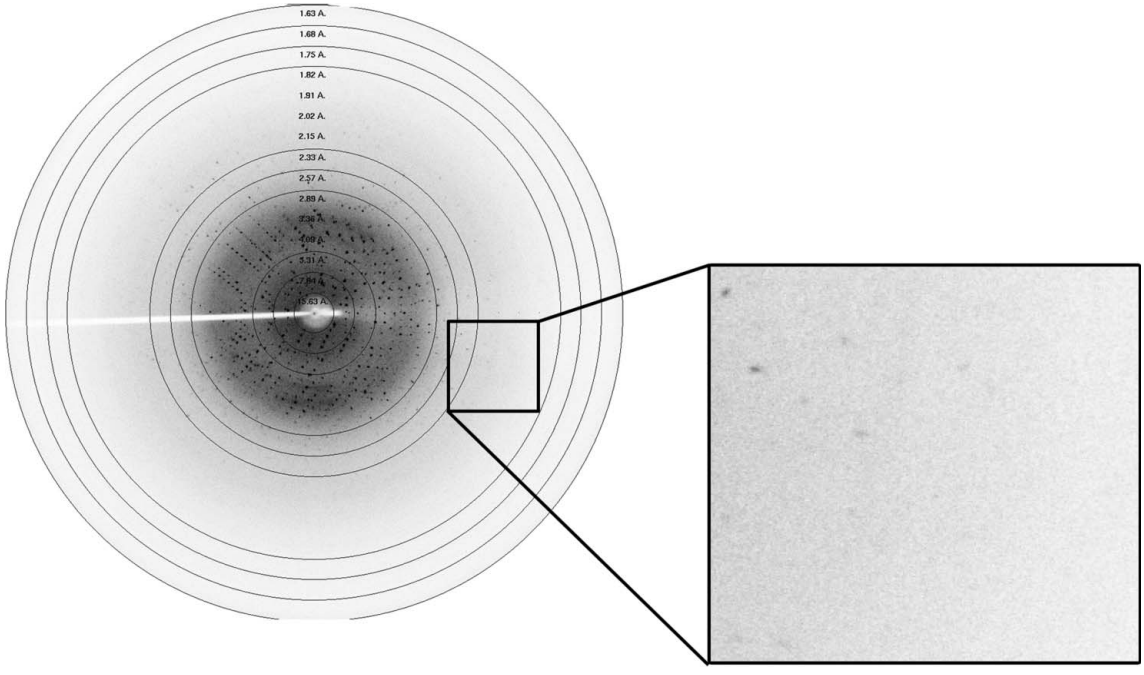

Figure 2
A typical diffraction pattern of the CrChi1 crystal. The diffraction image was collected on a MAR345dtb image-plate detector. An enlarged image is shown on the right.

Table 1
Summary of data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters (Å, °)	a = 44.1, b = 71.7, c = 59.1, $\alpha = \gamma = 90$, $\beta = 91.3$
Resolution range (Å)	50.0–1.8 (1.9–1.8)
Total reflections	126163 (2682)
Unique reflections	34080 (671)
Redundancy	5.2 (4.0)
Average $I/\sigma(I)$	17.7 (2.3)
$R_{\rm merge}$† (%)	8.1 (48.0)
Data completeness (%)	97.7 (92.3)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean intensity of the observations $I_i(hkl)$ of reflection hkl.

drogen phosphate and 20%(*w*/*v*) PEG 3350 pH 4.6. The crystals obtained were twinned, which made them unsuitable for X-ray diffraction. Further crystallization optimization was performed by carefully adjusting the concentration of PEG 3350 and ammonium dihydrogen phosphate together with seeding using a hair to eliminate twinning. As a result, several single crystals were obtained from optimized reservoir solution [0.2 *M* ammonium dihydrogen phosphate and 15%(*w*/*v*) PEG 3350 pH 4.6; Fig. 1] and diffracted to 1.8 Å resolution using a Rigaku MM-007 rotating-anode home X-ray generator (Fig. 2). The crystal belongs to space group $P2_1$, with unit-cell parameters a = 44.1, b = 71.7, c = 59.1 Å, $\alpha = \gamma = 90$, $\beta = 91.3°$. If we assume the presence of one molecule per asymmetric unit, the Matthews coefficient would be 2.45 $Å^3\ Da^{-1}$, with 40% solvent content. Complete data-collection statistics are given in Table 1.

The CrChi1 crystal was found to belong to space group $P2_1$ and the structure was solved by molecular replacement to 1.8 Å resolution employing the crystal structure of a chitinase from *Aspergillus fumigatus* (PDB code 1w9p; Rao *et al.*, 2005) as an initial search model in the program *Phaser* (McCoy *et al.*, 2007). Although CrChi1 and the search model for molecular replacement had relatively high sequence identity and *Phaser* gave a reasonably clear solution (rotation *Z* score 7.2, rotation *Z* score 11.2), the *R* factor was still higher than 45% ($R_{\rm free}$ = 51%). Further refinement is under way. To gain a better understanding of the biological functions of CrChi1, soaking and cocrystallization of CrChi1 with its possible inhibitors and substrate analogues is also under way.

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