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Crystallization and preliminary X-ray analysis of a family 19 glycosyl hydrolase from *Carica papaya* latex

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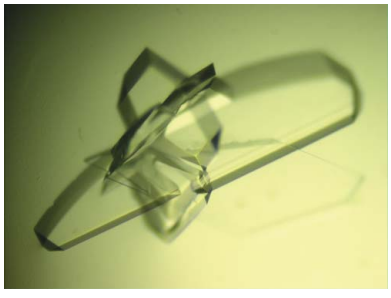


A chitinase isolated from the latex of the tropical species *Carica papaya* has been purified to homogeneity and crystallized. This enzyme belongs to glycosyl hydrolase family 19 and exhibits exceptional resistance to proteolysis. The initially observed crystals, which diffracted to a resolution of 2.0 Å, were improved through modification of the crystallization protocol. Well ordered crystals were subsequently obtained using *N*-acetyl-D-glucosamine, the monomer resulting from the hydrolysis of chitin, as an additive to the crystallization solution. Here, the characterization of a chitinase crystal that belongs to the monoclinic space group $P2_1$, with unit-cell parameters a = 69.08, b = 44.79, c = 76.73 Å, β = 95.33° and two molecules per asymmetric unit, is reported. Diffraction data were collected to a resolution of 1.8 Å. Structure refinement is currently in progress.

1. Introduction

Plants express a variety of defence proteins against pathogens such as viruses, bacteria and fungi. These pathogen-protective proteins exhibit various enzymatic activities and include proteases, β-1,3-glucanases, chitinases and protease inhibitors. Chitinases catalyse the hydrolysis of chitin, a homopolymer of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNac). Chitin is the second most abundant polymer in nature and is found in many organisms, such as the cuticle of insect shells, crustaceans and the cell walls of many fungi. Chitinases have been isolated from bacteria, viruses, plants and animals (Brunner *et al.*, 1998; Zhu *et al.*, 2004; Hoell *et al.*, 2005). They are included in the broad classification of glycosyl hydrolases (Henrissat & Bairoch, 1993; Beintema, 1994), in particular glycosyl hydrolase families 18 and 19 (GH 18 and GH 19).

GH 19 essentially contains plant chitinases, which have a catalytic domain that is rich in α-helical structure. Their catalytic mechanism proceeds with an inversion of the anomeric configuration of the substrate. Five chitinases belonging to GH 19 have been crystallized: three chitinases from plants, those from barley (Hart *et al.*, 1995), jack bean (Hahn *et al.*, 2000) and *Brassica juncea* (Ubhayasekera *et al.*, 2007), and the chitinases from *Streptomyces coelicolor* (Hoell *et al.*, 2006) and *S. griseus* (Kezuka *et al.*, 2006). All these GH 19 chitinases share the same fold with a high α-helical content.

Recently, two chitinases (designated as the major and minor forms based on their relative abundance) were isolated from the latex of *Carica papaya* and characterized (Huet *et al.*, 2006). The major form, which was used for this crystallographic study, has a molecular weight of 26 534 Da as determined by mass spectroscopy and is able to hydrolyse tetra-*N*-acetyl-D-glucosamine, chitin and chitosan. The papaya chitinases were found to be extremely resistant to proteolysis and hence were isolated without any polypeptidic cleavage from an environment containing up to 1 m*M* concentration of fully active cysteine proteinases. Furthermore, the two papaya chitinases were demonstrated in *in vitro* experiments to be resistant to thermolysin, subtilisin and glycyl endopeptidase (Huet *et al.*, 2006).

Our previous studies have also shown an unexpected difference in the secondary-structure content of the papaya chitinases when

compared with other plant GH 19 chitinases (Huet *et al.*, 2006). Infrared spectra and circular dichroism experiments indicated an expected α-helical content of 37–43%, but also an unexpected β-sheet content of 15–23%. This is in contrast to the available X-ray structures of other plant GH 19 chitinases, which only contain 1% β-sheet structure. These observations prompted us to crystallize the papaya chitinase, as described in this paper.

2. Material and methods

2.1. Purification

The major form of papaya chitinase was purified as described elsewhere (Huet *et al.*, 2006). Briefly, 4 g of spray-dried papaya latex was dissolved at room temperature in 80 ml H_2O containing 10 m*M* EDTA, 50 m*M* sodium acetate (referring to Na^+ concentration) and 2.5 m*M* dithiothreitol (DTT) at pH 5.0. After activation of the proteinases with DTT for 30 min, 25 m*M* methylmethanethiolsulfonate was added and incubated under stirring for a further 30 min. The reaction mixture was then submitted to exhaustive dialysis against 50 m*M* sodium acetate pH 5.0 prior to centrifugation (30 000*g*, 30 min, 277 K). This provided an aqueous solution containing a total of 1.6 g of various papaya proteins. The papaya chitinase was purified by a succession of cation-exchange and hydrophobic chromatographies. Three pools denoted I, II and III were isolated (see Fig. 1 in Huet *et al.*, 2006). Further purification of the papaya chitinase present in pool II was achieved by chromatography on Fractogel EMD Propyl 650(S) (Merck, Darmstadt, Germany) and on SP-Sepharose-FF (GE Healthcare, Uppsala, Sweden).

For crystallization, purification took place on a Chelating Sepharose column using Cu^{2+} as the ligand. The column (15 × 1.6 cm internal diameter) was pre-equilibrated in 10 m*M* phosphate buffer pH 8.0 and the papaya chitinase was eluted with the same buffer. Following purification to apparent homogeneity, the protein was dialysed against deionized water and concentrated to 14 mg ml^{-1} by ultrafiltration (10 kDa molecular-weight cutoff). The protein concentration was determined spectrophotometrically using an absorption coefficient value of 51 140 M^{-1} cm^{-1} at 280 nm. This value was calculated according to Gill & von Hippel (1989). The protein was stored in pure water at 277 K.

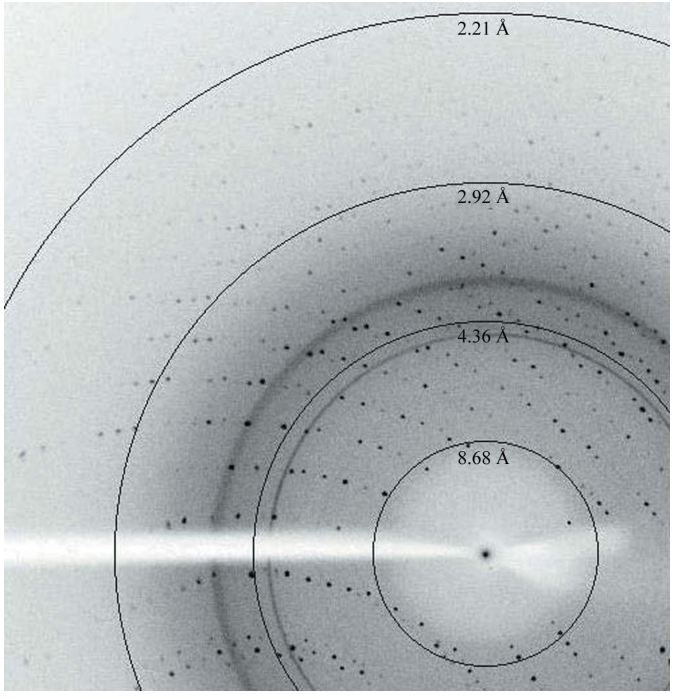


Figure 1
Diffraction pattern of crystal form II.

2.2. Crystallization

The initial protein concentration was 14 mg ml^{-1}. Crystallization conditions were screened by the hanging-drop vapour-diffusion method using commercially available crystallization kits (Cryo kit from Sigma, conditions 1–50; Extension kit from Sigma, conditions 1–50). Drops consisting of 1 µl protein solution and 1 µl precipitant solution were equilibrated against 500 µl precipitant solution at 293 K.

Initial crystals were obtained using condition No. 17 of the Sigma Cryo kit (0.17 *M* lithium sulfate, 0.085 *M* Tris–HCl pH 8.5, 25.5% PEG 4000, 15% glycerol). Although the crystal diffracted to 3 Å resolution, it was very small. The protein was then concentrated to 26 mg ml^{-1} and crystals appeared in condition Nos. 9 (0.17 *M* ammonium acetate, 0.085 *M* sodium citrate pH 5.6, 25.5% PEG 4000, 15% glycerol), 10 (0.17 *M* ammonium acetate, 0.085 *M* sodium acetate pH 4.6, 25.5% PEG 4000, 15% glycerol) and 17 of the Sigma Cryo kit. Depending on the buffer solutions, the crystals had the shape of fine plates or of bundles of twigs (see Figs. 2*a* and 2*b*). After trying many conditions, varying the buffer pH from 4 to 8.5 and changing the salt composition (sodium acetate, ammonium sulfate and lithium sulfate), different crystals were obtained and tested. These modifications allowed the identification of larger crystals (Fig. 2*b*; here referred to as crystal form I), which diffracted better; despite this, no solutions were found during molecular-replacement attempts.

The addition of *N*-acetyl-D-glucosamine in a large excess to condition Nos. 15 (0.17 *M* ammonium sulfate, 0.085 *M* sodium cacodylate pH 6.5, 25.5% PEG 8000, 15% glycerol) and 17 of the Sigma Cryo kit led to a significant modification of the crystal shape and produced crystals that diffracted to 1.8 Å resolution (Fig. 2*c*; here referred to as crystal form II).

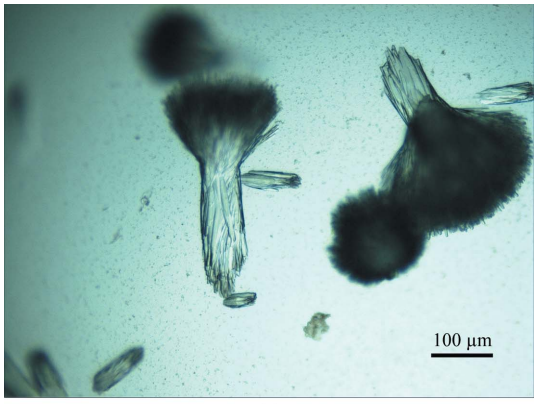
2.3. Data collection and analysis

Crystals were mounted in cryoloops (Hampton Research) and flash-cooled by direct immersion in liquid nitrogen prior to X-ray diffraction analysis. Data were collected with a MAR 345 imaging-plate system using Cu $K\alpha$ radiation generated by a Bruker–Nonius FR591 rotating-anode generator operated at 40 kV and 100 mA and equipped with Osmic mirrors. Data were collected as 246 or 180 images with a crystal-to-detector distance of 150 or 140 mm for crystal forms I and II, respectively, with 1° oscillation per image. The time of exposure was 5 min for crystal form I and 6 min for crystal form II; the crystals diffracted to 2.0 and 1.8 Å resolution, respectively. Diffraction data were processed and scaled using the *XDS* suite of programs (Kabsch, 1993). The data statistics are summarized in Table 1.

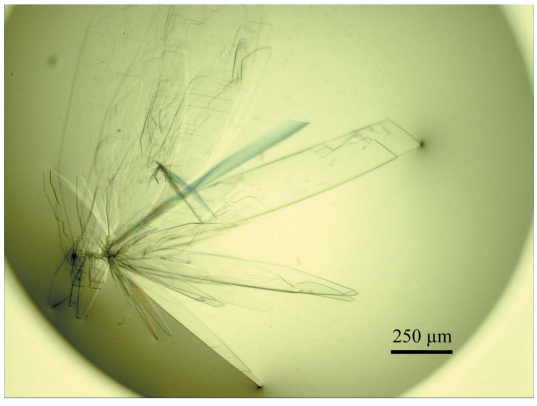
3. Results and discussion

Papaya chitinase was purified using a combination of cation-exchange, hydrophobic and affinity chromatography (Huet *et al.*, 2006). The purity of the sample was confirmed by the presence of a single band in a silver-stained SDS–PAGE and by mass spectroscopy (data not shown).

The best crystals were obtained after one week using 0.17 *M* ammonium acetate, 100 m*M* sodium citrate pH 5.2, 22% PEG 4000, 15% glycerol (crystal form I; Fig. 2*b*). This chitinase crystal belongs to the triclinic space group *P*1, with unit-cell parameters a = 44.93, b = 72.40, c = 77.51 Å, α = 81.91, β = 89.95, γ = 78.19°, and diffracted at least to 2.0 Å resolution. Based on the molecular weight and space group *P*1, it is assumed that the crystal contains four molecules of chitinase per asymmetric unit. The data-collection statistics are summarized in Table 1. However, this first crystal was found to be refractory to structure determination by molecular replacement.



(*a*)



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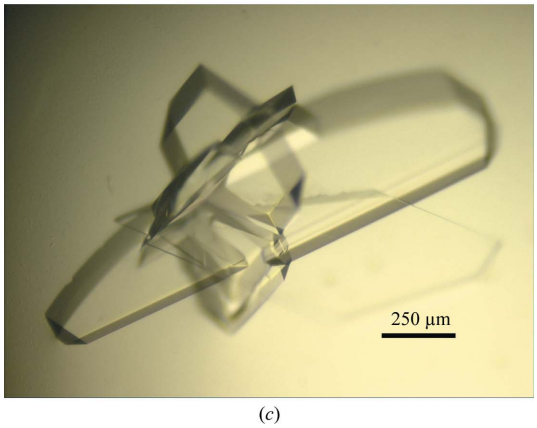

(*c*)

Figure 2
(*a*) A crystal of papaya chitinase obtained using buffer containing 0.17 *M* lithium sulfate, 0.085 *M* Tris–HCl pH 8.5, 25.5% PEG 4000, 15% glycerol. (*b*) Crystal form I obtained using buffer containing 0.17 *M* ammonium acetate, 0.085 *M* sodium citrate pH 5, 25.5% PEG 4000, 15% glycerol. (*c*) Crystal form II obtained using buffer containing 0.17 *M* lithium sulfate, 0.085 *M* Tris–HCl pH 8.5, 25.5% PEG 400, 15% glycerol with *N*-acetyl-D-glucosamine.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Crystal form I	Crystal form II
Detector	MAR 345	MAR 345
Wavelength (Å)	1.54179	1.54179
Resolution range (Å)	19.6–2.00 (2.09–1.98)	19.6–1.80 (1.90–1.80)
Space group	*P*1	$P2_1$
Unit-cell parameters (Å, °)	a = 44.93, b = 72.40, c = 77.51, α = 81.91, β = 89.95, γ = 78.19	a = 69.08, b = 44.79, c = 76.73, α = γ = 90.0, β = 95.33
Content of the ASU	4 monomers	2 monomers
No. of observed reflections	160470 (24236)	155819 (21843)
No. of unique reflections	58340 (9177)	43195 (6557)
Completeness (%)	88.0 (91.5)	98.1 (93.9)
$R_{\rm merge}$† (%)	7.3 (13.7)	6.7 (23.0)
Mean $I/\sigma(I)$	11.12 (6.29)	15.33 (5.71)

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

Observation of the diffraction pattern of crystal form I suggested that the crystal might be affected by crystal defects.

The use of *N*-acetyl-D-glucosamine as an additive to the initial crystallization conditions (condition Nos. 15 and 17 of the Sigma Cryo kit) combined with a protein concentration of 26 mg ml^{-1} yielded thicker crystals without visible defects (crystal form II; Fig. 2*c*). This crystal belonged to the monoclinic space group $P2_1$, with unit-cell parameters a = 69.08, b = 44.79, c = 76.73 Å, β = 95.33°, and diffracted to 1.8 Å resolution. The unit cell is smaller and the Matthews coefficient (Matthews, 1968) is 2.17 Å^3 Da^{-1} for two papaya chitinase molecules per asymmetric unit, with a solvent content of 43.33%.

For molecular replacement, a model was built by comparative modelling with the *MODELLER* program (Šali & Blundell, 1993) based on the three-dimensional structure of barley chitinase (Hart *et al.*, 1995) and the previously reported sequence of papaya chitinase (Huet *et al.*, 2006). A clear solution was obtained with *MOLREP* (Vagin & Teplyakov, 1997) from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) using data in the resolution range 19.6–2.0 Å, in which the first and the second peaks in the translation function showed a relative height (TF/σ) of 6.87 and 7.48, respectively, compared with 2.94 for the third peak. Further structure refinement is in progress.

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