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Isolation, purification, crystallization and preliminary crystallographic studies of a chitinase from *Crocus vernus*

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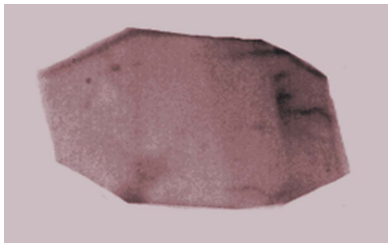

A chitinase has been isolated and purified from *Crocus vernus* corms. N-terminal amino-acid sequence analysis of the approximately 30 kDa protein showed 33% identity to narbonin, a seed protein from *Vicia narbonensis* L. The *C. vernus* chitinase was crystallized by the hanging-drop vapour-diffusion method using PEG 8000 as the main precipitant. The crystal belonged to the monoclinic space group $C2$, with unit-cell parameters $a = 172.3$, $b = 37.1$, $c = 126.4$ Å, $\beta = 127^\circ$ and two molecules per asymmetric unit. Diffraction data were collected to a resolution of 2.1 Å.

1. Introduction

Chitinases catalyze the hydrolysis of chitin, an unbranched homopolymer of 1,4-linked *N*-acetyl-D-glucosamine (GlcNac; Bishop *et al.*, 2000). Chitin is not a component of mammalian cells; it occurs widely elsewhere in nature and is abundant in human pathogens. Chitin is used as a key structural component of the fungal cell wall (Cabib *et al.*, 1992) and insect exoskeletons (Merzendorfer & Zimoch, 2003). It is also found in nematode egg shells (Fuhrman & Piessens, 1985). Chitinases occur in a wide range of organisms, including plants, animals, viruses, bacteria, fungi and insects, and play a variety of roles in these organisms (Brunner *et al.*, 1998; Hoell *et al.*, 2005). Plant chitinases are a structurally diverse group with respect to their physical properties, enzymatic activities and localization (Collinge *et al.*, 1993). Some chitinases, especially in combination with β-1,3 glucanases, inhibit fungal growth *in vitro* (Schlumbaum *et al.*, 1986), substantiating the idea that these enzymes play an important role in plant defence against pathogens. Chitinases can also release elicitors from fungal cell walls (Koga *et al.*, 1992).

Based on amino-acid sequence, plant chitinases have been classified into several classes (Meins *et al.*, 1994); however, in the glycosyl hydrolase classification system they are grouped into two families: families 18 and 19 (Henrissat & Bairoch, 1993; Flach *et al.*, 1992). Family 19 is only known in plants and is comprised of class I, II, IV and V chitinases; their structures have high α-helical contents similar to that of lysozyme. Class III and VI chitinases include all fungal, animal, bacterial and plant chitinases belonging to glycosyl hydrolase family 18 and their catalytic domain possesses a common α/β TIM-barrel fold. Chitinases of family 18 use a substrate-assisted double-displacement mechanism, while those of family 19 use a single-displacement mechanism. This difference in mechanism leads to retention of the configuration of the anomeric C atom for family 18 chitinases and inversion of the configuration of the anomeric C atom in the case of family 19 chitinases (Davies & Henrissat, 1995; Hart *et al.*, 1995; Hollis *et al.*, 2000; Synstad *et al.*, 2004; Monzingo *et al.*, 1996). Upon infection, plants produce several chitinases from both families that differ in localization, activity, chitin-binding properties and catalytic mechanism. This variety of chitinases allows the plant to match many different requirements. Further investigations are needed in order to obtain further information about the substrate specificity of the various chitinases as well as their possible involvement in the control of plant development. Here, we describe the isolation, purification and crystallization of a glycosyl hydrolase family 18 chitinase from the corms of the plant *Crocus vernus* and report the preliminary X-ray diffraction data.

2. Materials and methods

2.1. Protein purification

The *C. vernus* corms used in this study were obtained from a local supplier. 80 g of nonshelled seeds was ground to a fine powder and suspended in 500 ml 20 m*M* acetate buffer pH 5.0 by 4 h of continuous stirring at 277 K. The yellow-coloured crude extract was centrifuged at 13 000 rev min^{-1} for 30 min and the supernatant was passed through a series of filter papers with pore diameters of 25, 8, 2.5 and finally 0.25 µm. The filtrate was used in cation-exchange chromatography on a pre-packed Mono S 5/50 GL (GE Healthcare) column pre-equilibrated with buffer *A* (20 m*M* acetate pH 5.0). Partially purified protein was eluted with a linear gradient of 0–20% buffer *B* (20 m*M* acetate, 1 *M* NaCl pH 5.0). Eluted fractions were collected and analyzed on 12% SDS–PAGE stained with Coomassie Brilliant Blue G250 dye (Fig. 1). Fractions of the 30 kDa protein contaminated with minor amounts of low molecular-weight proteins were pooled, dialyzed and subjected to size-exclusion chromatography on a Hi Load 16/60 Superdex 75 column. The column was pre-equilibrated with buffer *A* containing 150 m*M* NaCl. A chitinase-activity assay was performed on each fraction eluted from the size-exclusion column using the Chitinase Assay Kit (Sigma; product No. CS0980). Fractions with chitinase activity were pooled and dialyzed extensively against buffer *A* at 277 K using a dialysis membrane (Spectra/P or RC Biotech Membrane, 3500 molecular-weight cutoff, 16 mm flat width, 50 ft). The purity of the chitinase protein was estimated by SDS–PAGE and the purified protein was subjected to dynamic light-scattering (DLS) measurements (Spectroscattter 201; Molecular Dimensions, England) over a suitable period of time. The measurements confirmed the monodispersity and the presence of equal-sized protein molecules in the solution (Fig. 2). The N-terminal amino-acid sequence was determined using an Applied Biosystems 476 Edman sequencer.

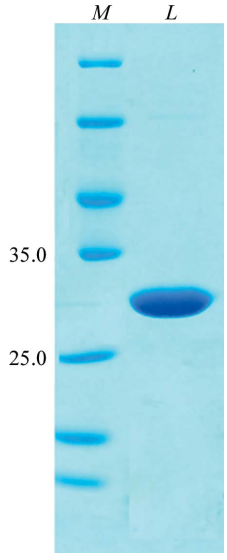

Figure 1
12% SDS–PAGE of the chitinase protein under reducing conditions. Lane *M*: protein markers (kDa); lane *L*, purified protein.

2.2. Crystallization

The PCT Pre-Crystallization Test (Hampton Research) was used to optimize the protein concentration and the purified protein was concentrated to 16 mg ml^{-1} in 20 m*M* acetate pH 5.0 buffer using Amicon Ultra-15 (3000 Da cutoff, Millipore) concentrating tubes. Initial crystallization screening was performed using a Honeybee 961 dispensing robot (Zinsser Analytic GmbH, Frankfurt Germany) applying the sitting-drop vapour-diffusion method at 293 K in 96-well crystallization plates (NeXtal QIA1 µplates, Qiagen). Each potential screening condition consisted of 300 nl protein solution and 300 nl precipitant in the crystallization drop, which was equilibrated against 35 µl reservoir solution. Successful screening conditions were further optimized by applying the hanging-drop vapour-diffusion method in Linbro plates. 2 µl chitinase solution was mixed with 2 µl reservoir solution and equilibrated against 1.0 ml reservoir solution. The plates were kept at a temperature of 293 K. Crystals appeared after 3 d (Fig. 3).

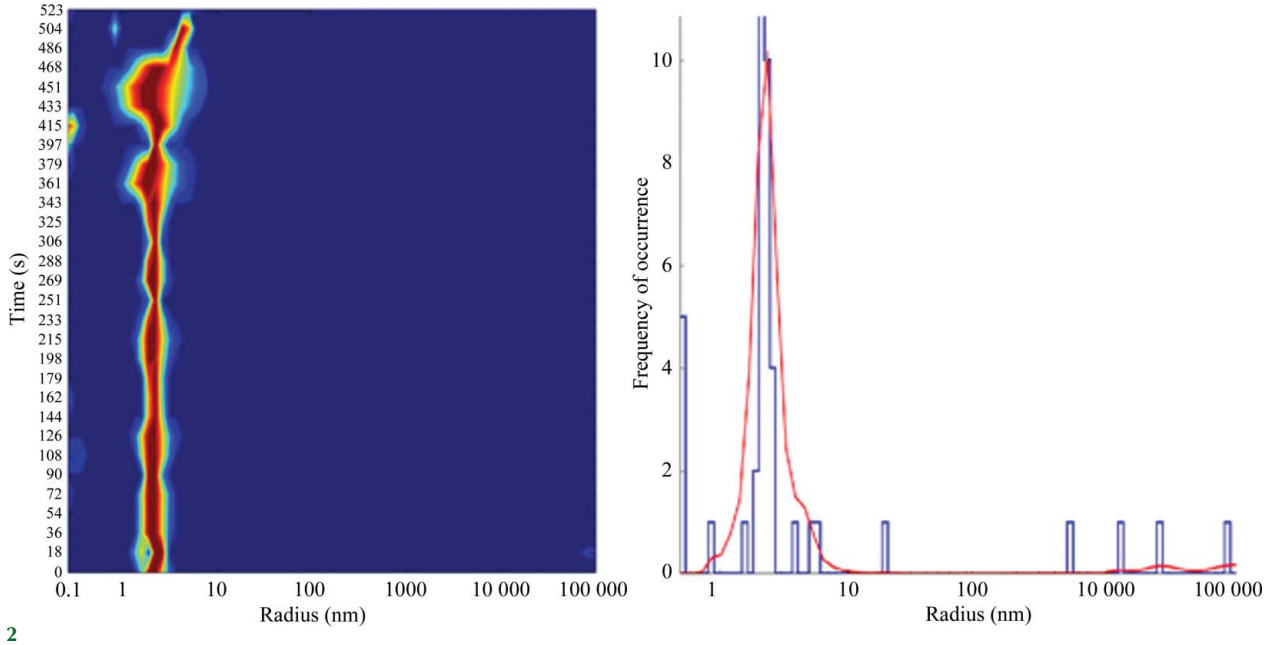

Figure 2
DLS measurement showing the monodispersity of the protein solution. The hydrodynamic radius (R_h) of 2.2 nm confirmed the presence of a monomeric protein in solution.

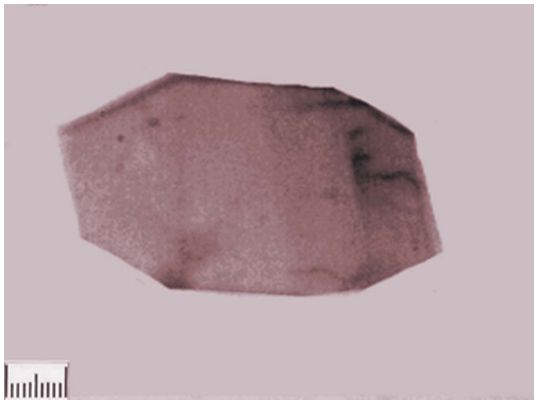

Figure 3
Crystal of chitinase from crocus bulbs. Crystal dimensions are 0.625 × 0.370 × 0.1 mm. The scale bar indicates 0.1 mm.

2.3. Data collection and analysis

Chitinase crystals suitable for X-ray diffraction were mounted within a nylon loop, cryoprotected with mother liquor containing an additional 20% glycerol and flash-cooled in a cold nitrogen-gas stream at 100 K. X-ray diffraction data for structural analysis were collected on the consortium beamline X13 at HASYLAB/DESY with a 165 mm MAR CCD detector using radiation with a wavelength of 0.8123 Å. The exposure time was 30 s, the crystal-to-detector distance was 227.11 mm and the oscillation range per frame was 0.8°. All intensity data were indexed, integrated and scaled with the *HKL*-2000 package (Otwinowski & Minor, 1997). Diffraction data statistics are summarized in Table 1.

3. Results and discussion

Chitinase from *C. vernus* bulbs was successfully purified by a two-step chromatographic procedure involving a combination of ion-exchange and size-exclusion chromatography. The enzyme activity was analyzed during purification using a chitinase assay as described above. The purity of the sample was confirmed by the presence of a single band on 12% SDS–PAGE stained with Coomassie Brilliant Blue G250 dye; the protein showed an approximate molecular weight of 30 000 Da (Fig. 1). MALDI–TOF/TOF mass spectrometry (Bruker, Germany) produced a fragmented amino-acid sequence for the 30 kDa chitinase protein, the gaps in which were filled by addition

Figure 4
Sequence alignment of *C. vernus* chitinase (CVC) and narbonin from *V. narbonensis* L. (nar) showing the catalytic motif.

Table 1
Summary of data-collection statistics.

Values in parentheses are for the highest resolution shell.

Space group	*C*2
Unit-cell parameters (Å, °)	a = 172.3, b = 37.1, c = 126.4, β = 127
V_M (Å^3 Da^{-1})	2.7
Solvent content (%)	54.2
No. of molecules in the asymmetric unit	2
Resolution range (Å)	25.0–2.1 (2.2–2.1)
Total reflections	140335 (20369)
Unique reflections	36468 (5230)
Multiplicity	3.8 (3.9)
Average $I/\sigma(I)$	17.2 (6.8)
R_{merge}† (%)	6.2 (19.4)
Data completeness (%)	96.6 (96.0)

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

of alanines. *BLAST* analysis of this amino-acid sequence indicated 21% identity to narbonin, a seed protein from *Vicia narbonensis* L. (Hennig *et al.*, 1995). The identification of the protein as a glycosyl hydrolase family 18-type chitinase was confirmed by the presence of the D*X*D*X*E sequence motif, which is essential for all family 18 chitinases (Bokma *et al.*, 2002) and is shown in Fig. 4.

The purified protein was concentrated to about 16 mg ml^{-1} (in 20 m*M* acetate pH 5.0 buffer) and used immediately for crystallization. Initial crystals were obtained from condition No. 7 of the JCSG+ Suite (Qiagen, Germany) consisting of 0.1 *M* CHES pH 9.5 and 20%(*w*/*v*) PEG 8000 applying the sitting-drop method. Optimization of the initial condition using Linbro plates provided larger crystals at a pH value of 9.0. A single crystal diffracted to 2.1 Å resolution using synchrotron radiation on the consortium beamline X13. The crystal belonged to space group *C*2, with unit-cell parameters a = 172.3, b = 37.1, c = 126.4 Å, β = 127°. Matthews coefficient (Matthews, 1968) calculations indicated the presence of two molecules per asymmetric unit, which corresponds to a packing parameter V_M of 2.7 Å^3 Da^{-1} and a solvent content of approximately 54.2%. Data-collection statistics are summarized in Table 1.

The phase problem was solved by molecular replacement using the narbonin structure (PDB entry 1nar; Hennig *et al.*, 1995) as a search model and the program *MOLREP* (Vagin & Teplyakov, 2010). *MOLREP* placed two narbonin molecules in the asymmetric unit as indicated by the Matthews coefficient. The *R* factor obtained after *MOLREP* and before refinement was 47%; initial refinement using data in the resolution range up to 2.4 Å lowered the *R* factor to 42%. Although chitinase and narbonin have a relatively low sequence identity (21%), both possess the classical TIM-barrel fold which is conserved in all family 18 chitinases and this is helpful in obtaining phase information. In order to understand the biological function and activity of this chitinase in more detail, soaking and cocrystallization with potential inhibitors is also planned.

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