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Crystallization and preliminary X-ray diffraction analysis of a class V chitinase from *Nicotiana tabacum*

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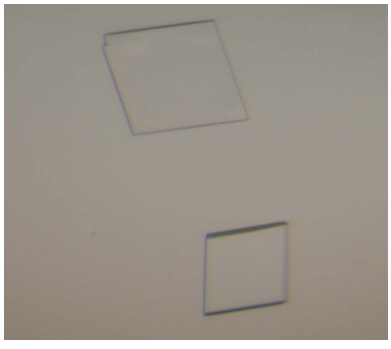

The plant chitinases, which have been implicated in self-defence against pathogens, are divided into at least five classes (classes I, II, III, IV and V). Although the crystal structures of several plant chitinases have been solved, no crystal structure of a class V chitinase has been reported to date. Here, the crystallization of *Nicotiana tabacum* class V chitinase (NtChiV) using the vapour-diffusion method is reported. The NtChiV crystals diffracted to 1.2 Å resolution using synchrotron radiation at the Photon Factory. The crystals belonged to the orthorhombic space group $P2_12_12$, with unit-cell parameters $a = 62.4$, $b = 120.3$, $c = 51.9$ Å. The asymmetric unit of the crystals is expected to contain one molecule.

1. Introduction

Chitin, a β-1,4-linked polysaccharide of *N*-acetylglucosamine (NAG), is hydrolyzed by chitinases (EC 3.2.1.14), which are widely distributed in living organisms and are responsible for self-defence, growth, morphogenesis, cuticle destabilization and stress tolerance (Kasprzewska, 2003; Duo-Chuan, 2006; Bhattacharya *et al.*, 2007; Arakane & Muthukrishnan, 2010; Donnelly & Barnes, 2004). Several plant chitinase genes have been isolated and sequenced and the chitinases have been divided into at least five classes (classes I, II, III, IV and V) based on their deduced amino-acid sequences (Collinge *et al.*, 1993; Melchers *et al.*, 1994). Classes I, II and IV correspond to the GH-19 family and classes III and V to the GH-18 family, according to the CAZy database (Henrissat & Davies, 1997).

The first crystal structure of these enzymes to be reported was that of the class II chitinase from barley seeds (Hart *et al.*, 1993) and was followed by that of the class III chitinase from *Hevea brasiliensis* (Terwisscha van Scheltinga *et al.*, 1994). The class II enzyme is composed of two lobes, which are both rich in α-helical structures, with the substrate-binding cleft between the lobes. In contrast, the class III enzyme exhibits a typical $(\alpha/\beta)_8$-barrel fold and the substrate binds to the top of the barrel. Although the class III and class V chitinases belong to the GH-18 family, the molecular masses of the class V chitinases (about 40 kDa) are higher than those of the class III chitinases (about 30 kDa) and their amino-acid sequence similarity is low. In addition, the physiological role of the class V chitinases appears to differ from that of the class III chitinases (Melchers *et al.*, 1994; Takenaka *et al.*, 2009). Subsequent to these structural reports, the crystal structures of family GH-19 chitinases (classes I, II and IV) from several plants, including jack bean, mustard greens, papaya, Norway spruce and rice, have been reported (Hahn *et al.*, 2000; Ubhayasekera *et al.*, 2007, 2009; Huet *et al.*, 2008; Kezuka *et al.*, 2010). As expected from their amino-acid sequences, the three-dimensional structures of their catalytic domains resemble that of the barley class II chitinase. The structure of class II chitinase complexed with NAG monomers has also been reported and the two NAGs were found to separately bind to subsites −2 and +1, respectively, in the complex structure (Huet *et al.*, 2008). However, no crystal structure of a class V chitinase has been reported to date.

A class V chitinase (NtChiV) was first isolated by Melchers *et al.* (1994) from tobacco leaves inoculated with tobacco mosaic virus. Expression of the chitinase gene (*NtChiV*) is up-regulated by ultraviolet irradiation and wounding in addition to viral attack. The recombinant NtChiV protein exhibited antifungal activity towards *Trichoderma viride*. The enzyme appears to be responsible for tolerance not only to biotic stress but also to abiotic stress. Interestingly, the amino-acid sequence of NtChiV is highly homologous to that of a bacterial chitinase, *Serratia marcescens* chitinase B (Brurberg, 1995). Since the *Serratia* enzyme has been intensively studied with respect to its structure and function (van Aalten *et al.*, 2001; Synstad *et al.*, 2004), the crystal structure of NtChiV would provide important information on the function of a plant class V chitinase by comparison with the *Serratia* enzyme structure.

In this study, we produced recombinant NtChiV protein using an *Escherichia coli* expression system and purified it to homogeneity. The purified NtChiV was employed for crystallization and X-ray diffraction experiments.

2. Materials and methods

2.1. Protein preparation

The cDNA encoding the predicted mature protein (NtChiV), consisting of 352 amino-acid residues (26–377), was amplified from first-strand cDNA obtained from the total RNA isolated from leaves of *Nicotiana tabacum* Xanthi using the forward primer 5′-ATGCAAAATGTTAAGGGAGGATACTGGT-3′ and the reverse primer 5′-TTACTTCATCTCTTGAAATGACACTCCCCA-3′ designed from the genomic sequence of NtChiV (Melchers *et al.*, 1994). The PCR products were purified and ligated into the pETBlue-1 vector by TA cloning (pETB-NtChiV). The pETB-NtChiV expression plasmid was introduced into *E. coli* Tuner(DE3) pLacI cells according to the supplier's instructions and the cells were induced with 1 m*M* IPTG (Studier *et al.*, 1990). Protein expression was confirmed from the SDS–PAGE profile (Laemmli, 1970), which exhibited an enhanced protein band at the position of the predicted molecular mass of NtChiV (about 39 kDa; data not shown). *E. coli* Tuner(DE3) pLacI cells transformed with the pETB-NtChiV expression plasmid were grown to an $A_{600\,\mathrm{nm}}$ of 0.6 before induction with 1 m*M* IPTG. After induction, growth was continued for 24 h at 291 K (Studier *et al.*, 1990). The cells were harvested and disrupted by sonication in 10 m*M* sodium phosphate buffer pH 7.5. The supernatant was dialyzed against the same buffer and applied onto an SP Sepharose FF column (1 × 5 cm). The adsorbed proteins were eluted with a linear gradient of NaCl from 0 to 0.3 *M* in the same buffer. The chitinase fractions were further purified by chromatography on a HiPrep 26/60 Sephacryl S-100 HR gel-filtration column. The chitinase fractions exhibiting a single protein band on SDS–PAGE were collected as the purified recombinant protein. The recombinant NtChiV obtained using these procedures was confirmed by N-terminal amino-acid sequence analysis using a Model 492 Procise protein sequencer (Applied Biosystems).

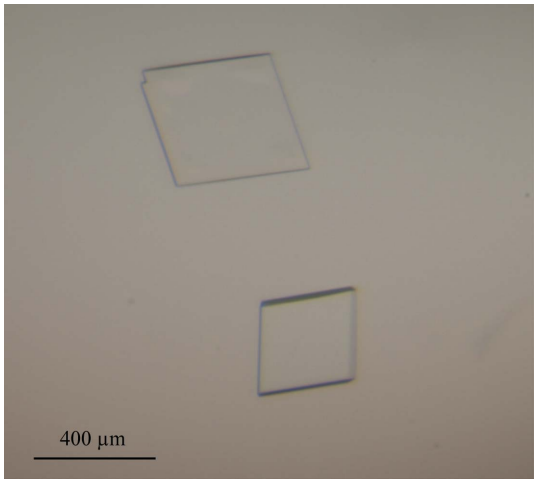

Figure 1
Crystals of NtChiV belonging to space group $P2_12_12$.

Table 1
Data-collection statistics.

Values in parentheses are for the last shell.

Wavelength (Å)	0.98
Space group	$P2_12_12$
Unit-cell parameters (Å, °)	a = 62.4, b = 120.3, c = 51.9, $\alpha = \beta = \gamma$ = 90
Resolution (Å)	50–1.20 (1.22–1.20)
Measured reflections	1632838 (55328)
Unique reflections	120764 (5824)
Redundancy	13.5 (9.5)
Completeness (%)	98.5 (96.6)
$\langle I/\sigma(I)\rangle$	49.5 (9.2)
R_{merge}†	0.075 (0.259)
B factor estimated from Wilson plot (Å^2)	5.6

† $R_{\mathrm{merge}} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity and $\langle I(hkl)\rangle$ is the average intensity over symmetry-equivalent measurements.

2.2. Crystallization

Crystallization conditions were screened by the sitting-drop vapour-diffusion method at 293 K using commercially available crystallization screening kits. Sitting drops were prepared by mixing 1 µl reservoir solution with 1 µl protein solution (5 mg ml^{-1} in water), and were equilibrated against 100 µl reservoir solution. Preliminary NtChiV crystals were obtained using condition No. 48 (100 m*M* Tris–HCl pH 8.5 and 2.0 *M* ammonium dihydrogen phosphate) of Crystal Screen (Hampton Research). The crystallization conditions were then optimized by the hanging-drop vapour-diffusion method using drops prepared by mixing 1 µl sample solution and 1 µl reservoir solution. During optimization, microseeding with small crystal nuclei was employed to facilitate the crystallization of NtChiV. Finally, large single crystals that were suitable for X-ray diffraction experiments were obtained within a month with a reservoir consisting of 100 m*M* Tris–HCl pH 8.5 and 1.7–2.2 *M* ammonium dihydrogen phosphate.

2.3. X-ray data collection

For data collection, the NtChiV crystals were cryoprotected in reservoir solution containing 20% ethylene glycol. The crystals were mounted in a nylon loop and flash-cooled in a nitrogen stream at 95 K. X-ray diffraction data were collected on beamline BL-17A of KEK (Ibaraki, Japan) using an ADSC Q270 CCD detector. Diffraction data were integrated and scaled with *HKL*-2000 (Otwinowski & Minor, 1997). The data-processing statistics are summarized in Table 1.

3. Results and discussion

The NtChiV protein was overexpressed in *E. coli* cells using the pETB-NtChiV expression plasmid and was purified to homogeneity using two purification steps: ion-exchange chromatography on SP-

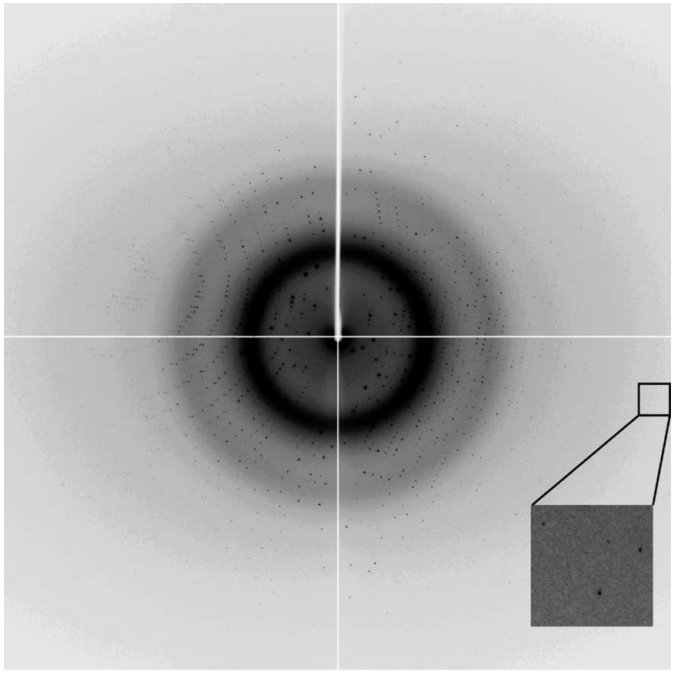

Figure 2
Diffraction pattern of an NtChiV crystal. The edge of the detector corresponds to a resolution of 1.2 Å.

Sepharose FF (GE Healthcare, Tokyo) and gel filtration on Sephacryl S-100 (GE Healthcare, Tokyo). The amount of recombinant NtChiV obtained by this procedure was 29 mg from 0.6 l induced culture. The N-terminal sequence was determined to be Met-Gln-Asn-Val-Lys-Gly-Gly-Tyr-Trp-Phe- by protein sequencing, indicating that the N-terminal methionine residue was not removed in *E. coli*. NtChiV crystals were obtained under reservoir conditions consisting of 100 m*M* Tris–HCl pH 8.5 and 1.7–2.2 *M* ammonium dihydrogen phosphate. Plate-like crystals grew within a month to dimensions of up to 400 × 400 × 50 µm (Fig. 1). The crystals diffracted to a resolution of 1.2 Å (Fig. 2) and belonged to the orthorhombic space group $P2_12_12$, with unit-cell parameters a = 62.4, b = 120.3, c = 51.9 Å. On the basis of the molecular mass of NtChiV (39.0 kDa), the crystals are expected to contain one molecule per asymmetric unit, which corresponds to a solvent content of 50.8% and a Matthews coefficient of 2.50 Å^3 Da^{-1}. We attempted to determine the crystal structure of NtChiV by the molecular-replacement method with the programs *Phaser* (McCoy *et al.*, 2007) and *MOLREP* (Vagin & Teplyakov, 2010), using the coordinates of human chitotriosidase (PDB code 1lg1; Fusetti *et al.*, 2002), which shares 29.8% sequence identity with NtChiV, as a search model. However, all attempts to solve the structure by the molecular-replacement method were unsuccessful. To solve the NtChiV structure by the single-wavelength anomalous dispersion (SAD) method, using selenium as the anomalous scattering atom, we prepared and crystallized selenomethionine-labelled NtChiV protein. More recently, we have succeeded in determining the NtChiV structure by the SAD method. The structural analysis of NtChiV will be discussed elsewhere in the future.

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