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Isolation, purification, crystallization and preliminary crystallographic studies of chitinase from tamarind (*Tamarindus indica*) seeds

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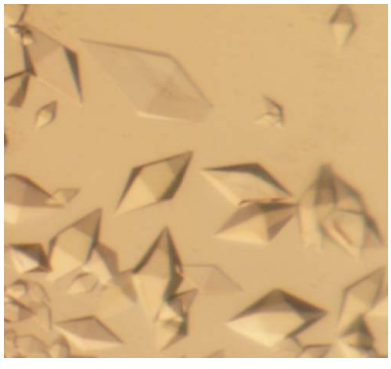

A protein with chitinase activity has been isolated and purified from tamarind (*Tamarindus indica*) seeds. N-terminal amino-acid sequence analysis of this protein confirmed it to be an ∼34 kDa endochitinase which belongs to the acidic class III chitinase family. The protein was crystallized by the vapour-diffusion method using PEG 4000. The crystals belonged to the tetragonal space group $P4_1$, with two molecules per asymmetric unit. Diffraction data were collected to a resolution of 2.6 Å.

1. Introduction

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; Zhu *et al.*, 2004; Hoell *et al.*, 2005). In plants, chitinases play a major role in plant defence by attacking chitin, an unbranched homopolymer of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNac) (Bishop *et al.*, 2000; Collinge *et al.*, 1993). Chitin, which is the second most abundant polymer in nature, is used by many organisms as a structural component of protective cell walls or exoskeletons. It is a major component of fungal cell walls and is also found in insect exoskeletons, crustacean shells, cyst walls of protozoan parasites *etc.* (Arroyo-Begovich *et al.*, 1980; Cohen-Kupiec & Chet, 1998).

Plant chitinases have been classified into seven classes (I–VII) based on sequence similarity. These classes have been further grouped into two families of glycosyl hydrolases: family 18 and family 19 (Collinge *et al.*, 1993; Flach *et al.*, 1992; Neuhaus, 1999). They differ vastly in structure and in their mode of action against substrates. Family 19 is comprised of class I, II, IV and VII chitinases; their structures have high α-helical contents similar to that of lysozyme. Chitinases of family 19 use a single-displacement mechanism which leads to inversion of the configuration of the anomeric carbon. Chitinases of class III, V and VI belong to family 18 and their catalytic domain possess a common $\alpha\beta$-TIM barrel. Family 18 chitinases use a substrate-assisted double-displacement mechanism which leads to retention of the configuration of the anomeric carbon (Davies & Henrissat, 1995; Hart *et al.*, 1995; Hollis *et al.*, 2000; Synstad *et al.*, 2004; Monzingo *et al.*, 1996).

Tamarind (*Tamarindus indica*) belongs to the Leguminosae family and grows naturally in many tropical and subtropical regions of the world. Tamarind is an important food ingredient in many Asian and Latin American dishes. Tamarind kernel powder is used in the food industry as a stabilizer and as an emulsifier (Kumar & Bhattacharya, 2008). A recent study reported that tamarind seeds contain an ∼34 kDa endochitinase which is an acidic class III chitinase (Rao & Gowda, 2008). Plant chitinases notably cause food allergies and are responsible for causing latex–fruit cross-sensitivity syndrome (Hoffmann-Sommergruber, 2002; Wagner & Breiteneder, 2002). In addition to tamarind, other members of the Leguminosae family such as soybean, peanut *etc.* can cause severe allergic reactions (Cirla *et al.*, 1970; Ballmer-Weber & Vieths, 2008; Sicherer & Sampson, 2007). In order to investigate the catalytic mechanism of tamarind chitinase and to study its role as plant pathogenesis-related protein and allergen, we have successfully isolated, purified and crystallized this protein. Here, we report the isolation, purification, crystallization and preliminary X-ray diffraction analysis of a chitinase from *T. indica*.

2. Material and methods

2.1. Protein purification

Tamarind seeds were obtained from fruit collected from plants growing locally. The fruit pulp was removed manually and the seeds were soaked in buffer *A* (100 m*M* Tris pH 7.4) for ∼6 h at room temperature. A razor blade and a pair of forceps were used to remove the seed coat. The crude extract was prepared by homogenizing the seed kernels thus obtained in buffer *A* at room temperature using a blender. The prepared crude extract was then subjected to centrifugation at 14 000*g* for 20 min at 277 K and the supernatant was collected. The obtained supernatant was applied onto an Affi-Gel Blue matrix (Bio-Rad Laboratories, Hercules, California, USA) pre-equilibrated with buffer *A*. The column was washed with buffer and eluted using a stepwise gradient of NaCl (0.1, 0.2, 0.3, 0.5, 0.8 and 1.0 *M*) in buffer *A*. A chitinase-activity assay was performed on each fraction according to the reported method using Calcofluor dye (Somashekar & Joseph, 1997). Fractions with chitinase activity were pooled, concentrated and dialyzed extensively against buffer *A* at 277 K using a 3.5 kDa dialysis membrane (Pierce, USA). The dialyzed sample was loaded onto a weak cation-exchange matrix [MacroPrep CM (carboxymethyl) support, BioRad Laboratories, Hercules, California, USA] pre-equilibrated with buffer *A*. Chitinase activity was detected in the unbound sample. The unbound sample was subsequently loaded onto a weak anion-exchange matrix [MacroPrep DEAE (diethylaminoethyl), BioRad Laboratories, Hercules, California, USA] pre-equilibrated with buffer *A*. Protein elution was carried out using a stepwise gradient of NaCl (0.05, 0.1, 0.2, 0.3 and 0.5 *M*). Chitinase activity was detected in fractions eluted with 0.1 *M* NaCl. Eluted fractions were analyzed on 15% SDS–PAGE stained with Coomassie Brilliant Blue dye. Highly pure fractions containing chitinase activity were pooled and concentrated to a concentration of 14 mg ml^{-1} using Amicon Ultra-15 (10 000 Da cutoff, Millipore). Protein concentration was determined with the BioRad protein assay kit using BSA as a standard and the protein yield was consequently estimated to be 5 mg per gram of seeds. The N-terminal amino-acid sequence was obtained using the Edman degradation method on a Shimadzu Automated Protein Sequencer (PPSQ-20).

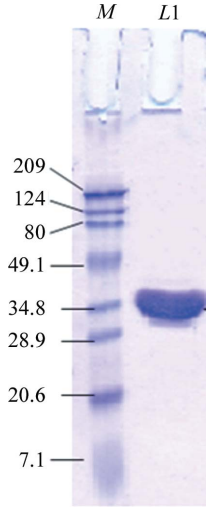

Figure 1
15% SDS–PAGE of tamarind seed chitinase under reducing conditions. Lane *M*, protein markers (kDa); lane *L*1, purified protein.

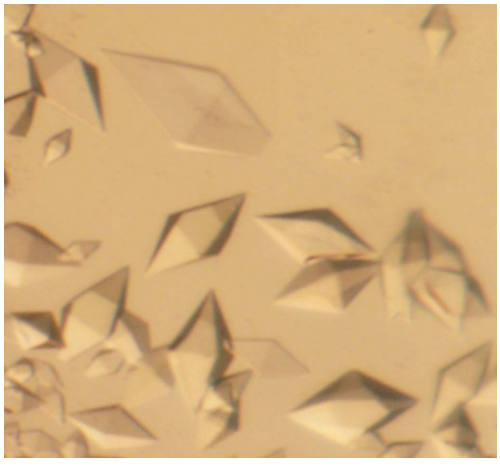

Figure 2
Crystals of chitinase from tamarind seeds. The longest dimension of a typical crystal is between 50 and 100 µm.

2.2. Crystallization

Crystallization was performed by the sitting-drop vapour-diffusion method in 96-well crystallization plates (Hampton Research) at 293 K. Drops were prepared by mixing 2 µl protein solution in buffer *A* with the same volume of reservoir solution. The final protein concentration in the drops was 7 mg ml^{-1}. Initial crystallization conditions were obtained using Crystal Screen (Hampton Research, USA).

2.3. Data collection and analysis

Crystals were mounted in cryoloops (Hampton Research) and flash-cooled by direct immersion into liquid nitrogen prior to X-ray diffraction analysis. Data were collected with a MAR 345 dtb imaging-plate system using Cu $K\alpha$ radiation generated by a Bruker Microstar H rotating-anode generator operated at 45 kV and 60 mA

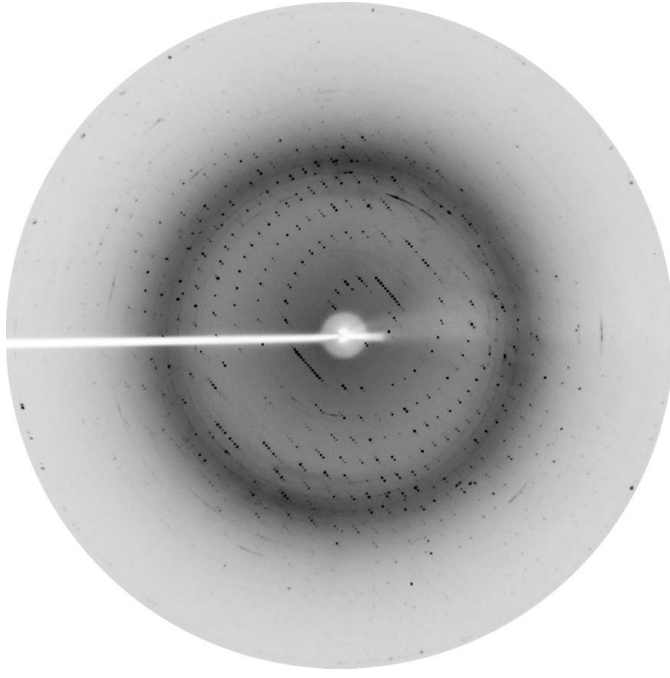

Figure 3
Diffraction of chitinase crystals using in-house radiation at the Macromolecular Crystallographic Facility, Institute Instrumentation Centre. The resolution at the edge of the plate is 2.6 Å.

Table 1
Data-collection statistics for tamarind seed chitinase.

Values in parentheses are for the highest resolution shell.

Space group	$P4_1$
Unit-cell parameters (Å)	$a = b = 67.0$, $c = 173.1$
Resolution range (Å)	50–2.6 (2.69–2.60)
Completeness (%)	98.8 (99.0)
$R_{\rm merge}$† (%)	4.65 (16.34)
Mean $I/\sigma(I)$	7.9 (2.1)
No. of observed reflections	396441 (5653)
No. of unique reflections	25872 (2610)
Redundancy	5.0 (3.6)

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

and equipped with Helios optics. Data were collected as 90 images with a crystal-to-detector distance of 200 mm and 1° oscillation per image and the time of exposure was 5 min. Diffraction data were processed and scaled using the *XDS* suite of programs (Kabsch, 1993).

3. Results and discussion

Tamarind chitinase was purified to homogeneity by a three-step chromatographic procedure involving a combination of affinity and ion-exchange chromatography. The enzyme activity was analyzed after each step of purification by performing chitinase assays as described previously (Park *et al.*, 2004). In the first step, Affi-Gel Blue matrix was used and fractions were eluted using a stepwise gradient of NaCl. Protein with chitinase activity eluted in the 0.5 *M* NaCl fraction. The eluted fraction was dialyzed to remove salt and applied onto CM matrix. The unbound fraction of the CM matrix contained the protein as indicated by the chitinase assay and was loaded onto DEAE matrix. The fraction eluted from the DEAE column with 0.1 *M* NaCl was highly pure and showed chitinase activity. The purity of the sample was confirmed by the presence of a single band on a 15% SDS–PAGE stained with Coomassie Brilliant Blue dye (Fig. 1). The molecular weight of the purified tamarind chitinase was calculated by running protein molecular-weight markers along with the purified protein on SDS–PAGE. The relative mobility of the protein markers was plotted against log molecular weight. The molecular weight obtained was ∼34 000 Da. The purified protein was subjected to automated N-terminal amino-acid sequencing and the first 20 amino-acid residues were determined. The sequence obtained was identical to the recently reported N-terminal sequence of tamarind chitinase (Rao & Gowda, 2008). Purified protein was concentrated to about 14 mg ml^{-1} and immediately used for crystallization.

Diffraction-quality crystals of chitinase were obtained after 15 d using 100 m*M* MES pH 6.0, 10%(*w*/*v*) PEG 4000 (Fig. 2). The crystals belonged to the tetragonal space group $P4_1$ and diffracted to 2.6 Å resolution in-house (Fig. 3). The unit-cell parameters were found to be $a = b = 67.0$, $c = 173.1$ Å with two molecules per asymmetric unit; this corresponds to a crystal volume per unit molecular weight ($V_{\rm M}$) of 2.5 Å^3 Da^{-1}, given the molecular weight of 34 kDa for the protein. This is within the range of the values expected for protein crystals (Matthews, 1968) and corresponds to a solvent content of 51%. The data-collection statistics are summarized in Table 1.

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