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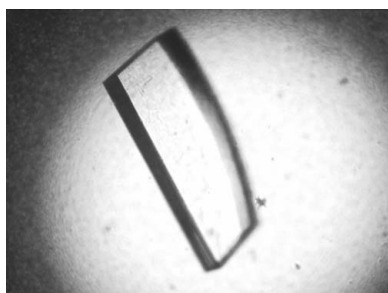
Expression, purification, crystallization and preliminary crystallographic analysis of chitinase A from *Vibrio carchariae*

Chitinase A of *Vibrio carchariae* was expressed in *Escherichia coli* M15 host cells as a 575-amino-acid fragment with full enzymatic activity using the pQE60 expression vector. The yield of the highly purified recombinant protein was approximately 70 mg per litre of bacterial culture. The molecular mass of the expressed protein was determined by HPLC/ESI-MS to be 63 770, including the hexahistidine tag. Crystals of recombinant chitinase A were grown to a suitable size for X-ray structure analysis in a precipitant containing 10% (v/v) PEG 400, 0.1 M sodium acetate pH 4.6 and 0.125 M CaCl₂. The crystals belonged to the tetragonal space group *P*4₂2, with two molecules per asymmetric unit and unit-cell parameters $a = b = 127.64$, $c = 171.42$ Å. A complete diffraction data set was collected to 2.14 Å resolution using a Rigaku/MSR R-Axis IV⁺⁺ detector system mounted on an RU-H3R rotating-anode X-ray generator.

1. Introduction

Chitin, a β -1,4-linked *N*-acetylglucosamine (GlcNAc) polysaccharide, is a major structural component of fungal cell walls and the exoskeletons of invertebrates, including insects and crustaceans. This linear polymer may be degraded through the hydrolytic action of chitinases (EC 3.2.1.14). In correlation with the structural role of chitin, chitinases are important for biochemical and physiological functions in many organisms. In insects, chitinases are essential in the moulting process and may also affect gut physiology through their involvement in peritrophic membrane turnover (Merzendorfer & Zimoch, 2003), whereas plants produce chitinases as part of their defence mechanism against fungal pathogens (Herrera-Estrella & Chet, 1999; Melchers & Stuver, 2000). Chitinases are thought to contribute to a number of morphogenetic processes in filamentous fungi, including spore germination, side-branch formation, differentiation into spores and autolysis (Gooday *et al.*, 1992). Many bacteria express chitinases that enable them to utilize chitin as the sole source of carbon and nitrogen (Yu *et al.*, 1991), whilst mammalian chitinases have been found to regulate the pathophysiological features of an allergic asthma (Wills-Karp & Karp, 2004).

On the basis of amino-acid sequence, chitinases are classified into glycosyl hydrolase families 18 and 19, which are unrelated, differing in structure and mechanism (Henrissat & Bairoch, 1993). Family 18 chitinases are present in a wide range of organisms, including bacteria, fungi, higher plants and humans. All family 18 chitinases share two short sequence motifs which form an $(\alpha/\beta)_8$ TIM-barrel active site. From accumulated structural information, it appears that family 18 enzymes catalyze the hydrolytic reaction by a substrate-assisted mechanism, in which protonation of the glycosidic oxygen leads to distortion of the sugar molecule at the scissile position. The resultant bond cleavage yields an oxazolinium intermediate and retention of anomeric configuration in the products (Papanikolaou *et al.*, 2001; Bortone *et al.*, 2002; Armand *et al.*, 1994; Terwissha van Scheltinga *et al.*, 1995; Sasaki *et al.*, 2002). Family 19 chitinases have only been found in higher plants and in the Gram-positive bacterium *Streptomyces* (Cohen-Kupiec & Chet, 1998; Ohno *et al.*, 1996). In contrast to family 18 chitinases, the catalytic domains of family 19 chitinases have a bilobal $\alpha+\beta$ folding motif with a high α -helical content. The mode of catalytic action of this class of enzymes is a



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single displacement step, which results in an inversion of the anomeric configuration of the products (Brameld & Goddard, 1998; Robertus & Monzingo, 1999).

Chitinase A from a marine bacterium, *Vibrio carchariae*, is a 63 kDa family 18 glycosyl hydrolase (Suginta *et al.*, 2000). This monomeric enzyme acts as an endochitinase and has a broad range of substrate specificity with various chitin oligomers (Suginta *et al.*, 2004, 2005). Kinetic data implied greater affinity of chitinase A towards higher molecular-weight chitooligosaccharides, suggesting that the catalytic cleft of the enzyme comprises an array of binding subsites, most likely comparable to that of CiX1 from *Coccidioides immitis* (Fukamizo *et al.*, 2001; Sasaki *et al.*, 2002). The characteristic multiple binding subsite structure is commonly found in the active sites of hydrolytic enzymes that utilize biopolymers as substrates, such as proteases, lysozyme, cellulases and chitinases. We previously cloned a DNA fragment that encodes the functional chitinase A of *V. carchariae* into the pQE60 expression vector, with the corresponding recombinant protein expressed in *E. coli* M15 host cells (Suginta *et al.*, 2004). In the present study, we describe high-level expression and large-scale purification of the recombinant chitinase A from the same *E. coli* system. We also report the first crystals of chitinase A from marine bacteria and the preliminary analysis of their diffraction data.

2. Materials and methods

2.1. Expression and purification

The DNA fragment that encodes chitinase A (amino-acid residues 22–597, without the residue 598–850 C-terminal fragment) was previously cloned into the pQE60 expression vector (Suginta *et al.*, 2004) so as to express the 575-amino-acid fragment with a C-terminal (His)₆ sequence. In this study, high-level expression of this recombinant chitinase A in *E. coli* M15 host cells has been optimized. The cells were grown at 310 K in Luria–Bertani (LB) medium containing 100 µg ml⁻¹ ampicillin and chitinase expression was induced by the addition of isopropyl thio-β-D-galactoside (IPTG) to a final concentration of 0.5 mM when OD₆₀₀ of the cell culture reached 0.6. Cell growth continued at 298 K for 18 h and the cell pellet was collected by centrifugation at 4500g for 30 min. The freshly prepared cell pellet was resuspended in 40 ml lysis buffer [20 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mg ml⁻¹ lysozyme] and then lysed on ice using a Sonopuls Ultrasonic homogenizer with a 6 mm diameter probe (50% duty cycle; amplitude setting, 20%; total time, 30 s; 6–8 cycles). Unbroken cells and cell debris were removed by centrifugation at 12 000g for 1 h. The supernatant was applied onto a Ni–NTA agarose affinity column (1.0 × 10 cm; Qiagen GmbH, Hilden, Germany) and chromatography was carried out gravitationally following the Qiagen protocol (<http://www1.qiagen.com/literature/handbooks/INT/ProteinPurification.aspx>). After loading, the column was washed with 50 ml 20 mM Tris–HCl buffer pH 8.0 containing 5 mM imidazole, followed by another 50 ml of the same buffer containing 10 mM imidazole. 0.5 ml fractions were collected and protein fractions eluted with 250 mM imidazole were concentrated using Vivaspine-20 ultrafiltration membrane concentrators (10 kDa molecular-weight cutoff, Vivascience AG, Hanover, Germany). Further purification was performed using an ÄKTA purifier system (Amersham Biosciences, Piscataway, NJ, USA) on a Superdex 200 HR 10/30 (1.0 × 30 cm) column. The running buffer was 20 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl. A flow rate of 250 µl min⁻¹ was maintained and 0.5 ml fractions were collected and

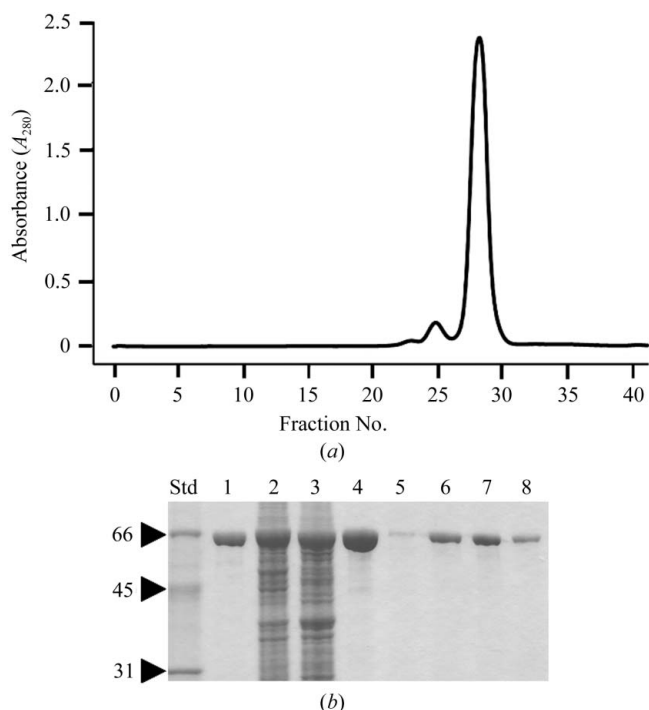
assayed for chitinase activity. Chitinase-containing fractions were pooled and again concentrated using the same type of Vivaspine membrane concentrator. All purification steps were carried out at 277 K, unless otherwise stated. Protein concentrations were determined by Bradford's method (Bradford, 1976) using a standard calibration curve constructed from BSA (0–10 µg). The purity of chitinase A was verified by SDS–PAGE using a Laemmli buffer system (Laemmli, 1970). The accurate molecular mass of the recombinant chitinase A was determined by HPLC/ESI–MS (Thermo Finnigan, Thermo Electron Corporation, San Jose, CA, USA) operated under the conditions given previously (Suginta *et al.*, 2004). Chitinase activity was determined in a 100 µl assay mixture containing protein sample (35 µl), 1 mM *p*NP-(GlcNAc)₂ (25 µl) and 100 mM sodium acetate buffer pH 5.0 (40 µl). The reaction mix was incubated at 303 K for 10 min and the enzymatic reaction was terminated by the addition of 50 µl 1 M NaHCO₃. The amount of *p*-nitrophenol (*p*NP) released was determined spectrophotometrically at 405 nm. One unit of chitinase activity is defined as the amount of chitinase A that produces 1 nmol *p*NP per minute at 303 K.

2.2. Crystallization

Initial crystallization experiments were carried out by the microbatch method in 96-well Impact plates (Hampton Research, Aliso Viejo, CA, USA) filled with 10 µl Al's oil (Hampton Research). For each crystallization drop, 0.5 µl chitinase A (10 mg ml⁻¹ in 20 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl) was added to 0.5 µl of each precipitant from Crystal Screen (Hampton Research) and JB Screen HTS I and HTS II (Jena Bioscience GmbH, Jena, Germany) without mixing. Small crystals were obtained after 4 d incubation at 277 K in condition A1 from JBScreen HTS I [15%(v/v) PEG 400, 0.1 M sodium acetate pH 4.6 and 0.1 M CaCl₂], condition H4 from JBScreen HTS I [30%(w/v) PEG 8000, 0.2 M ammonium sulfate] and condition A10 from Crystal Screen [30%(w/v) PEG 4000, 0.1 M sodium acetate pH 4.6 and 0.2 M ammonium acetate]. Condition A1 was further optimized by the hanging-drop vapour-diffusion method in a 24-well VDX Plate (Hampton Research). A protein drop made up of 1 µl chitinase A solution (10 mg ml⁻¹ in 20 mM Tris–HCl buffer pH 8.0 containing 150 mM NaCl) mixed with 1 µl of various concentrations of precipitants [0–0.5 M CaCl₂ and 10–16%(v/v) PEG 400 in 0.1 M sodium acetate pH 4.6] was equilibrated over 1.0 ml of the respective precipitant. The best single crystals were obtained with 10%(v/v) PEG 400, 0.1 M sodium acetate pH 4.6 and 0.125 M CaCl₂.

2.3. Data collection

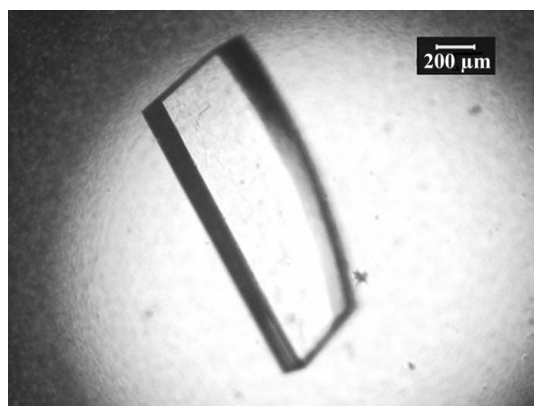
The resultant crystals were immersed in a cryoprotectant solution [20%(v/v) glycerol, 10%(v/v) PEG 400, 0.1 M sodium acetate pH 4.6 and 0.125 M CaCl₂] for roughly 10 s and then picked up with a nylon loop and quickly vitrified in a stream of nitrogen gas at 112 K. A single crystal diffracted X-rays to at least 2.2 Å resolution on a Rigaku/MSR R-AXIS IV⁺⁺ detector mounted on an RU-H3R rotating-anode X-ray generator equipped with Osmic Blue confocal focusing mirrors and 0.3 mm collimator running at 50 kV and 100 mA. The crystal-to-detector distance was set to 190 mm, with all frames collected at 112 K. Diffraction data were recorded over a 65° rotation of the crystal around the φ axis in 260 diffraction images with a width of 0.25° per image. The data were processed with *CrystalClear/d*TREK* (Pflugrath, 1999).


Figure 1

Purification of chitinase A expressed from *E. coli* M15 cells. (a) Elution profile of recombinant chitinase A obtained from an ÄKTA purifier system with a Superdex 200 HR 10/30 (1.0 × 30 cm) gel-filtration column. The running buffer was 20 mM Tris-HCl buffer pH 8.0 containing 150 mM NaCl. A flow rate of 250 $\mu\text{l min}^{-1}$ was maintained and 0.5 ml fractions were collected. (b) Chitinase A-containing fractions were subjected to SDS-PAGE analysis, followed by Coomassie Blue staining. Lanes: Std, low-molecular-weight standard proteins (serum albumin, ovalbumin and carbonic anhydrase); 1, purified native chitinase A; 2, crude cell lysate after 0.5 mM IPTG induction; 3, clear supernatant; 4, pooled fraction eluted with 250 mM imidazole during Ni-NTA agarose affinity chromatography; 5–8; eluted fractions 26, 27, 28 and 29 from the Superdex 200 HR column, respectively.

3. Results and discussion

In the present study, a *V. carchariae* chitinase A fragment [amino-acid residues 22–597, without the C-terminal sequence 598–850, but with a C-terminally attached (His)₆ tag to permit affinity chromatography on Ni-NTA-agarose] was highly expressed from *E. coli* M15 host cells. Purification of the recombinant chitinase A yielded ~70 mg highly purified protein per litre of bacterial culture. Fig. 1(a) shows an


Figure 2

A crystal of recombinant chitinase A (dimensions 1100 × 400 × 100 μm) obtained from a hanging-drop vapour-diffusion setup using 0.1 M sodium acetate pH 4.6 containing 10% (v/v) PEG 400 and 0.125 M CaCl₂.

Table 1

Statistics for crystallographic data.

Values in parentheses are for the last shell.

Space group	<i>P</i> 422
Unit-cell parameters (Å)	<i>a</i> = <i>b</i> = 127.64, <i>c</i> = 171.42
Solvent content (%)	55.1
Total No. of unique reflections collected	76752
No. of observed reflections	364202
Redundancy	4.75
Wavelength used (Å)	1.5418
Resolution range (Å)	54.16–2.14 (2.22–2.14)
Completeness (%)	97.2 (81.6)
<i>R</i> _{merge} † (%)	7.8 (24.3)
$\langle I/\sigma(I) \rangle$	5.4 (1.7)

† $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where *I_i* is the intensity of the *i*th measurement of an equivalent reflection with indices *hkl*.

elution profile from FPLC on a Superdex 200 HR 10/30 gel-filtration column, representing a single peak corresponding to the band of apparent molecular weight 63 000 Da as shown on SDS-PAGE (Fig. 1b). The molecular mass of the purified protein was confirmed by HPLC/ESI-MS to be 63 770. This value matched, within a limit of 0.05% instrumentation error, the calculated molecular mass of the (His)₆-tagged chitinase A (63 784.23). The recombinant enzyme was fully active, giving a specific activity with pNP-(GlcNAc)₂ substrate of 1.49 nmol $\mu\text{g}^{-1} \text{min}^{-1}$, compared with 1.75 nmol $\mu\text{g}^{-1} \text{min}^{-1}$ for the native enzyme (purified from *V. carchariae*).

With the hanging-drop vapour-diffusion method, the best crystals were obtained with a reservoir solution containing 10% (v/v) PEG 400 and 0.125 M CaCl₂. X-ray diffraction data of a single crystal with dimensions 1100 × 400 × 100 μm (Fig. 2) showed Laue group symmetry of *4/mmm*.

The refined unit-cell parameters are *a* = *b* = 127.64, *c* = 171.42 Å and the crystal is likely to contain two molecules per asymmetric unit, with an estimated Matthews coefficient of 2.74 Å³ Da⁻¹ (Matthews, 1968). Diffraction statistics showed absences that could be characteristic of the tetragonal space groups *P*₄22 or *P*₄322. However, the data were initially scaled and merged in space group *P*422 in order to preserve all the data for subsequent confirmation of the correct space group by molecular-replacement calculations. The data were complete to 2.14 Å resolution; the final statistics for data collection and processing are summarized in Table 1.

A preliminary solution of the structure of *V. carchariae* chitinase was obtained by molecular-replacement calculations using the *AMoRe* (CCP4) program (Navaza, 1994) and the crystal structure of Chi A from *Serratia marcescens* (PDB code 1ctn; 49.3% identical to chitinase A from *V. carchariae*; Perrakis *et al.*, 1994) as the search model. A translation search using all space groups with *4/mmm* symmetry (including *P*₄22 and *P*₄322) showed most compatibility with the *P*422 space group, giving an amplitude correlation coefficient of 32.4% and an *R* factor of 52.6% for the top solution, compared with 12.6–20.6% and 56.0–58.7%, respectively, for all others. Examination of the best solution revealed good crystal packing and no clashes between symmetry-related molecules. This preliminary model is currently being rebuilt and refined.

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Function, Faculty of Science, Mahidol University, Bangkok 10400, Thailand.

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