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Crystallization and preliminary crystallographic analysis of the catalytic module of endolysin from Cp-7, a phage infecting *Streptococcus pneumoniae*

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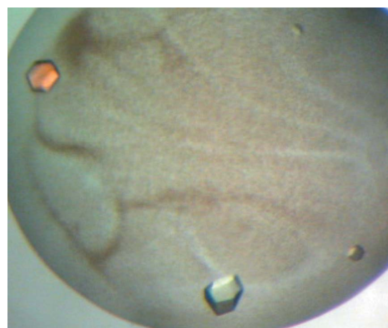
As part of the life cycle of the pneumococcal phage Cp-7, the endolysin Cpl-7 cleaves the glycosidic β1,4 bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine in the pneumococcal cell wall, resulting in bacterial lysis. Recombinant Cpl-7 was overexpressed in *Escherichia coli*, purified and crystallized using the vapour-diffusion method at 291 K. Diffraction-quality tetragonal crystals of the catalytic module of Cpl-7 were obtained from a mixture of PEG 3350 and sodium formate. The crystals belonged to space group *I*422, with unit-cell parameters a = 127.93, b = 127.93, c = 82.07 Å. Diffraction data sets were collected to 2.4 Å resolution using a rotating-anode generator.

1. Introduction

Bacteriophages, or phages, are viruses that infect bacteria. Following replication inside its bacterial host, the phage is faced with a problem: it needs to exit the bacterium to disseminate its progeny. To solve this problem, double-stranded DNA (dsDNA) phages have evolved a lytic system to weaken the bacterial cell wall, resulting in bacterial lysis. Phage lytic enzymes, or endolysins, are highly efficient molecules that have been refined over millions of years for this exact purpose (Fischetti, 2005). The *Streptococcus pneumoniae* bacteriophage Cp-7 (*Podoviridae* family) contains the *cpl-7* gene which encodes the lytic protein Cpl-7 (342 amino-acid residues; ~38.4 kDa; Fig. 1) possessing muramidase (lysozyme) activity (García *et al.*, 1990).

The Cpl-7 lysozyme belongs to the GH25 family of glycosyl hydrolases (http://www.cazy.org/fam/GH25.html); it binds to the bacterial envelope and cleaves the glycosidic β1,4 bonds between the *N*-acetylmuramic acid and *N*-acetylglucosamine moieties of the glycan chains, breaking down the peptidoglycan (PG) backbone.

Most *S. pneumoniae* endolysins display a modular structure. In addition to the catalytic module, they possess a choline-binding module (CBM) which facilitates their anchorage to the choline-containing teichoic acids of the *S. pneumoniae* cell wall (García *et al.*, 1988). In turn, this CBM is formed by the repetition of a basic unit of about 20 amino acids, which is also found in multiple tandem copies in a large family of surface proteins (the choline-binding protein family) from *S. pneumoniae* and its bacteriophages. Interestingly, Cpl-7 does



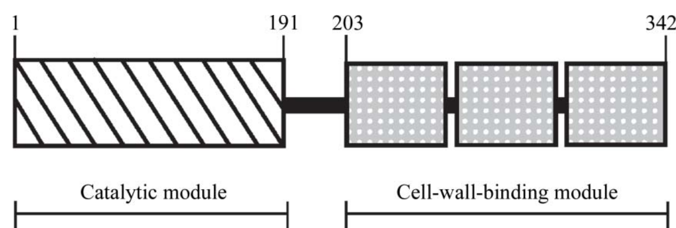


Figure 1
Schematic representation of the modular structure of Cpl-7 endolysin. The catalytic module belongs to glycosyl hydrolase family 25; the linker (black line) and the cell-wall binding module, which is composed of three repeats, are also represented. Residue numbers are indicated at the top.

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not present such a choline-binding module, but instead contains a putative cell-wall-binding module formed by three nearly identical tandem repeats of 48 amino acids each (Fig. 1). This cell-wall-binding module presents a completely different sequence to those of other known peptidoglycan-binding motifs. Three complete choline-binding proteins (CBP) have been structurally characterized to date, namely Cpl-1 (Hermoso *et al.*, 2003), Pce (Hermoso *et al.*, 2005) and CbpF (Molina *et al.*, 2007), none of which possess an anchor motif similar to that of Cpl-7.

The Cpl-1 lysozyme (which shares a similar catalytic module to that of Cpl-7) and Pal amidase, which are produced by the Cp-1 and Dp-1 phages, respectively, act in a synergistic manner in a murine model (Jado *et al.*, 2003). Furthermore, using this mouse model it has been demonstrated that Cpl-1 can eliminate colonization caused by *S. pneumoniae* and prevent the development of otitis media (McCullers *et al.*, 2007). These results suggest that these active phage lytic enzymes, termed enzybiotics, have a noticeable effect as antimicrobial agents (Hermoso *et al.*, 2007). Hence, knowledge of the structure of these enzymes will be helpful in the establishment of a novel enzybiotic treatment for streptococci-mediated diseases.

2. Experimental

2.1. Protein expression and purification

The full-length Cpl-7-coding gene was amplified by polymerase chain reaction (PCR) from the pCP700 plasmid using sense (5′-G**GAATTC**ATGGTTAAGAAAAATGATTTATTTGTAGACGTTG-3′) and antisense (5′-CCC**AAGCTT**AAAATAGCTAGTAGAAAATTTCTACTAGCTTTTACTTGTTA-3′) primers, in which the nucleotides in bold are digestion sites for the restriction enzymes *Eco*RI and *Hin*dIII, respectively. The resulting purified 1000 kb PCR product was cloned into the expression vector pKLSL$_t$ (Mancheño & Angulo, 2009), rendering plasmid pKLSL$_t$-Cpl-7, which was used to transform competent *Escherichia coli* BL21 (DE3) cells. The expression vector pKLSL$_t$ is based on pET28a(+) (Novagen, Germany) and incorporates the nucleotide sequence coding for LSL$_t$, namely the β-trefoil module of the haemolytic lectin LSL$_a$ from the mushroom *Laetiporus sulphureus* (Mancheño *et al.*, 2005) followed by a sequence coding for a linker stretch (ASSS) and a TEV (tobacco etch virus) endoprotease cleavage site (ENLYFQG; Mancheño & Angulo, 2009). Cloning of the nucleotide sequence coding for LSL$_t$ was performed between the *Nco*I and *Eco*RI restriction sites of pET28a(+). For the production of recombinant LSL$_t$-Cpl-7 fusion protein, the appropriate recombinant strain was cultured in Luria–Bertani broth at 310 K with aeration; when the culture reached an OD$_{600}$ of about 0.8 protein expression was induced with 0.5 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubation was continued for 4 h. After centrifugation, the cleared cell lysate was applied onto a Sepharose 4B affinity column (GE Healthcare). After exhaustive washing with 20 m*M* Tris–HCl pH 8.0 containing 0.1 *M* NaCl buffer and 0.04%(*w*/*v*) sodium azide, the fusion protein LSL$_t$-Cpl-7 was eluted with 200 m*M* lactose in 20 m*M* Tris–HCl pH 8.0 with 0.1 *M* NaCl and 0.04%(*w*/*v*) sodium azide. Fractions containing LSL$_t$-Cpl-7 were pooled and dialyzed against TEV digestion buffer [50 m*M* Tris–HCl pH 8.0 with 0.2 *M* NaCl and 0.04%(*w*/*v*) sodium azide]. The dialyzed sample was concentrated using a 10 kDa cutoff protein concentrator (Amicon YM-10) and digested with TEV protease (kindly provided by Roger Williams at MRC, Cambridge) at a protein:TEV ratio of 80:1(*w*:*w*). Cpl-7 was separated from the LSL$_t$ fusion tag by size-exclusion chromatography on Superdex 75 (GE Healthcare). All purification steps were performed at 277 K. The enzyme was then concentrated at 277 K using a 10 kDa cutoff protein concentrator (Amicon YM-10). The final protein concentration was determined by UV–Vis absorbance measurements with a Nanodrop ND-1000 spectrophotometer (Thermo Scientific), using a theoretical extinction coefficient of 68 425 M^{-1} cm^{-1} at 280 nm.

2.2. Crystallization

A NanoDrop robot (Innovadyne Technologies Inc.) was used to assay initial crystallization conditions using Crystal Screens I, II, Lite, Index and SaltRx from Hampton Research and PACT Suite and JCSG+ Suite from Qiagen. Initial assays were carried out by the sitting-drop vapour-diffusion method at 291 K in Innovaplate SD-2 microplates (Innovadyne Technologies Inc.), mixing 250 nl protein solution with 250 nl precipitant solution and equilibrating against 80 µl well solution. The stock protein was concentrated to 10 mg ml^{-1} in 20 m*M* Tris–HCl pH 8.0 and 0.1 *M* NaCl.

Figure 2
Crystals of the catalytic module of Cpl-7 endolysin grown at 295 K in 0.2 *M* sodium formate, 20%(*v*/*v*) PEG 3350. The approximate dimensions of the crystals are 0.1 × 0.01 × 0.1 mm.

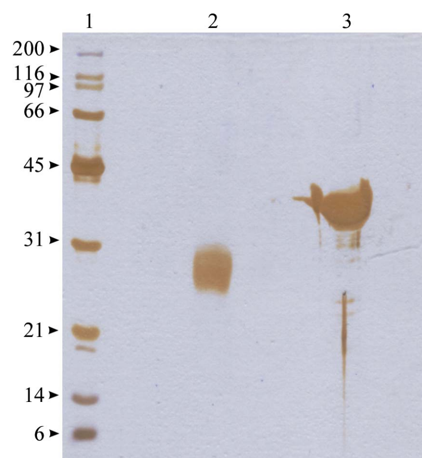

Figure 3
SDS–PAGE analysis of a Cpl-7 crystal. Lane 1, broad-range (BioRad) molecular-weight markers (molecular weights are labelled on the left in kDa); lane 2, dissolved crystal of Cpl-7; lane 3, purified Cpl-7 sample (~38 kDa). The content of the crystal is a partially cleaved Cpl-7 protein compatible with the catalytic module of Cpl-7 (28 kDa). Silver staining was used for protein detection.

2.3. X-ray data collection and processing

X-ray diffraction data were collected at 120 K on an in-house Kappa 2000 Brüker–Nonius CCD detector using Cu $K\alpha$ X-rays generated by an FR591 Brüker–Nonius rotating-anode generator equipped with a double-mirror focusing system and operated at 45 kV and 100 mA. The crystal-to-detector distance was maintained at 200 mm. Crystals were soaked for 10 s in a cryoprotectant solution containing mother liquor and 30%(*v*/*v*) glycerol and were then flash-cooled at 100 K in a nitrogen stream using a cryogenic system controlled by a Cryostream Controller 700 (Oxford Cryosystems). The images were processed and scaled using *DENZO* and *SCALEPACK* from the *HKL*-2000 suite (Otwinowski & Minor, 1997). Intensities were converted to structure-factor amplitudes using *TRUNCATE* (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

A systematic search for crystallization conditions yielded crystals from 0.2 *M* sodium formate and 20% PEG 3350 (condition No. 63 of the JCSG+ Suite). Crystals reached maximum dimensions of 0.1 × 0.01 × 0.1 mm in four months (Fig. 2). Because of the long wait for crystals to appear, we decided to run one of the crystals on SDS–PAGE followed by silver staining (Switzer *et al.*, 1979) to assay the crystal composition (Fig. 3). The gel clearly indicated that the protein inside the crystal was partially cleaved, presenting a molecular weight that was compatible with the catalytic module of Cpl-7 (28 kDa). Despite the small size of the crystals, an X-ray data set was collected to 2.4 Å resolution using an in-house rotating-anode source and presented good-quality patterns (Fig. 4). The crystals belonged to the tetragonal system, space group *I*422, with unit-cell parameters $a = b = 127.93$, $c = 82.07$ Å. X-ray data-collection statistics are summarized in Table 1. Specific volume calculations for the catalytic module of Cpl-7 suggested the presence of one molecule in the asymmetric unit and a solvent content of 59.50% ($V_M = 3.03$ Å^3 Da^{-1}; Matthews, 1968).

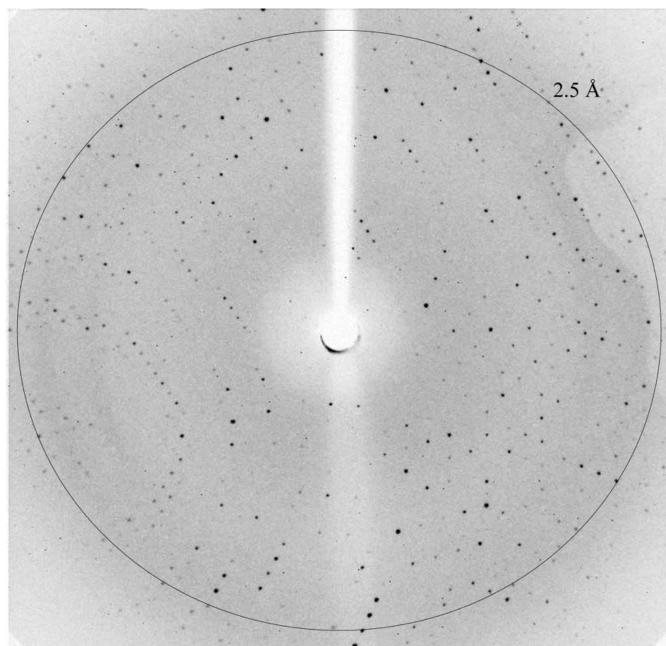

Figure 4
Typical X-ray diffraction pattern of a crystal of the catalytic module of Cpl-7 (oscillation range 1°).

Table 1
Data-collection statistics for the Cpl-7 crystal.

Values in parentheses are for the highest resolution shell.

Crystal data	
Space group	*I*422
Unit-cell parameters (Å)	
a	127.93
b	127.93
c	83.03
Data processing	
Temperature (K)	100
Wavelength (Å)	1.5418
Resolution (Å)	91.28–2.40 (2.49–2.40)
Total reflections	442102
Unique reflections	37549
Redundancy	15.40 (12.00)
Data completeness (%)	99.8 (98.50)
Average $I/\sigma(I)$	20.70 (1.90)
Molecules per asymmetric unit	1
Matthews coefficient (Å^3 Da^{-1})	3.03
Solvent content (%)	59.50
R_{merge}†	0.11 (0.39)

† $R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

Structure solution was initiated with the molecular-replacement method using the structure of the catalytic module of Cpl-1 from bacteriophage Cp-1 (Hermoso *et al.*, 2003; PDB code 1h09) as the initial model. This module shows 84% sequence homology to the catalytic module of Cpl-7 (García *et al.*, 1990). Molecular replacement was performed using the *MOLREP* program (Vagin & Teplyakov, 1998) with reflections in the 15.0–3.5 Å resolution range. A single and unambiguous solution for the rotation and translation functions was obtained, which yielded a final correlation coefficient of 0.64 and an *R* factor of 0.38.

Inspection of the preliminary electron-density map revealed the presence of the complete catalytic module of Cpl-7 but the absence of the cell-wall-binding module. Obviously, the degradation of native Cpl-7 within the crystallization drop, leading to crystallization of the catalytic module, may explain the time that was required for crystal growth. Structural refinement of this module of Cpl-7 is currently in progress.

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