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Crystallization of parasporin-2, a *Bacillus thuringiensis* crystal protein with selective cytocidal activity against human cells

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Bacillus thuringiensis is a valuable source of protein toxins that are specifically effective against certain insects and worms but harmless to mammals. In contrast, a protein toxin obtained from *B. thuringiensis* strain A1547, designated parasporin-2, is not insecticidal but has a strong cytocidal activity against human cells with markedly divergent target specificity. The 37 kDa inactive protein is proteolytically activated to a 30 kDa active form. The active form of the recombinant protein toxin was crystallized in the presence of ethylene glycol and polyethylene glycol 8000 at neutral pH. The crystals belong to the hexagonal space group $P6_1$ or $P6_5$, with unit-cell parameters $a = b = 134.37$, $c = 121.24$ Å. Diffraction data from a native crystal were collected to 2.75 Å resolution using a synchrotron-radiation source.

1. Introduction

The common soil bacterium *Bacillus thuringiensis* (Bt) has been successfully utilized as a biological insecticide for several decades (de Maagd *et al.*, 1999). The bacteria owe this value to a parasporal crystalline inclusion that they produce during sporulation; the inclusion, which is composed of one or more proteins with a large variety in size and composition among Bt strains, often contains proteins that are toxins against insects. Cyt and Cry proteins are two major families of these toxins (Schnepf *et al.*, 1998; de Maagd *et al.*, 2003). The Cyt proteins have a broad cytolytic activity against invertebrate and vertebrate cells including insect cells and mammalian erythrocytes. The Cry protein category consists of phylogenetically heterogeneous proteins. Historically, the Cry proteins have been regarded as toxins that are specifically effective against certain insect species of orders that include Lepidoptera, Diptera and Coleoptera, but the current classification of Bt parasporal proteins groups several non-insecticidal proteins into the Cry proteins based on sequence homology (http://www.biols.susx.ac.uk/Home/Neil_Crickmore/Bt/index.html; Crickmore *et al.*, 1998). Identification of these insecticidal proteins and cloning of their genes have led to the production of genetically modified crop plants expressing these toxins and thereby presenting resistance to harmful insects. Research efforts devoted to these toxins include the crystal structure determination of four Cry proteins (Li *et al.*, 1991; Grochulski *et al.*, 1995; Galitsky *et al.*, 2001; Morse *et al.*, 2001) and one Cyt protein (Li *et al.*, 1996).

Recent screening of non-insecticidal Bt strains for cytocidal activity has identified several strains that produce a group of unique Cry proteins, designated parasporins, with preferential cytotoxicity against particular human carcinoma cells (Mizuki *et al.*, 1999, 2000; Lee *et al.*, 2000, 2001; Kim *et al.*, 2003). Parasporins are distinguished from other Cry proteins by their lack of insecticidal activity and also from Cyt proteins by their lack of broad cytolytic activity such as haemolytic activity. This novel group of protein toxins may have potential for medical applications such as cancer treatment.

Bt strain A1547 (previously designated as strain 90-F-45-14) has been isolated from Japanese soil as one of the strains that are neither insecticidal nor haemolytic but highly cytocidal against human MOLT-4 leukaemia T cells (Mizuki *et al.*, 1999). A protein responsible for the toxicity has been identified and its gene cloned (Ito *et al.*, 2004). The full-length recombinant protein (37 kDa) is not cytocidal and requires proteolytic activation to develop toxicity, as do Cry and Cyt proteins; a 30 kDa active toxin is produced by the removal of 51 amino-acid residues from the N-terminus and a short segment from the C-terminus of the inactive protoxin. The activated form of the protein is strongly cytocidal against human cells with markedly divergent target specificity and preferentially kills liver and colon cancer cells without damaging normal cells in the tumour tissue slices. When exposed to the

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toxin, susceptible cells swell and finally burst into fragments. Hereafter, we will call the protein parasporin-2.

Compared with the three-domain Cry proteins, which make up the largest and the best-characterized subgroup of the insecticidal Cry proteins (de Maagd *et al.*, 2003), parasporin-2 is half their size and lacks the block sequences that are conserved in them (Höfte & Whiteley, 1989). Moreover, the protein does not have an appreciable sequence homology to most of the established Bt toxins. An exception is Cry 15Aa1, which has 23.5% sequence identity to parasporin-2. From the view of sequence homology Cry 15Aa1 itself is an outlier among the Bt toxins, but it is significantly homologous to the mosquitocidal Mtx2 and Mtx3 toxins of *B. sphaericus* (de Maagd *et al.*, 2003). These mosquitocidal toxins show homology to aerolysin from *Aeromonas hydrophyla*, which forms β-barrel-lined membrane pores (Parker *et al.*, 1994).

These comparisons of sequence homology and the observed actions of parasporin-2 on susceptible cells suggest that the protein should be a pore-forming toxin with a novel structure distinct from the known structures of Cry and Cyt proteins. Here, we describe the crystallization of the activated form of parasporin-2 and the crystallographic characterization of the crystals.

2. Materials and methods

2.1. Preparation of an activated form of recombinant parasporin-2

The active toxin protein was prepared from a recombinant form of N-terminal truncated parasporin-2, which consists of the N-terminal methionine, Asp52–Asn338 of the protoxin and a hexahistidine tag at the C-terminus (Ito *et al.*, 2004). The recombinant protein was expressed in *Escherichia coli* and purified by metal-chelating chromatography. Purified protein (1 mg ml^{-1}) was activated by partial proteolysis with proteinase-K (Merck, Darmstadt, Germany; final concentration 0.1 mg ml^{-1}) at 310 K for 30 min; the reaction was stopped by the addition of 1 m*M* phenylmethylsulfonyl fluoride. The activated protein was separated from the mixture by anion-exchange chromatography using a Q-Sepharose Fast Flow column (Amersham Biosciences, Piscataway, USA). After dialysis against storage solution (20 m*M* Tris–HCl pH 8.0, 50 m*M* Na_2CO_3, 500 m*M* NaCl), the protein was concentrated by ultrafiltration and stored at 277 K until use.

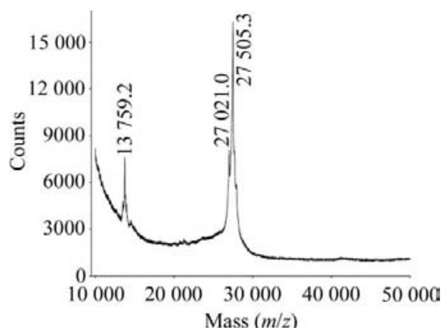

Figure 1
Mass spectrum of the activated parasporin-2 preparation.

2.2. MALDI–TOF mass spectrometry of activated parasporin-2

The activated parasporin-2 solution was mixed with a matrix solution of sinapinic acid saturated in a 1:2 mixture of acetonitrile and water with a TFA content of 0.1%(*v*/*v*). The mixture was analyzed with an Autoflex mass spectrometer (Bruker Daltonics, Billerica, USA). The spectrometer was calibrated using ubiquitin, myoglobin, trypsinogen and bovine serum albumin as molecular-weight standards.

2.3. Crystallization

An aliquot of the protein solution was dialyzed against 100 m*M* 3-(cyclohexylamino)propanesulfonic acid–NaOH (CAPS–NaOH) pH 12.0, 4 m*M* β-mercaptoethanol (β-ME) for 5 h and then against 10 m*M* CAPS–NaOH pH 12.0, 4 m*M* β-ME for more than 5 h. After dialysis, the collected sample solution was filtered through a 0.22 µm Ultrafree-MC filter (Millipore, Billerica, USA).

Initial screening for crystallization conditions was carried out at 298 K by the sitting-drop vapour-diffusion method using standard solutions from commercial kits: Crystal Screen, Crystal Screen 2, Crystal Screen Lite and MembFac (Hampton Research, Laguna Niguel, USA). Drops were prepared by mixing 2 µl protein solution (7.5–10.0 mg ml^{-1}) with an equal volume of reservoir solution and were equilibrated against 100 µl reservoir solution. Similar procedures were used in the optimization of conditions.

2.4. Data collection

The crystals were mounted in a nylon-fibre loop, dipped briefly into reservoir solution supplemented with 15% glycerol and flash-frozen in a nitrogen-gas stream at 95 K. X-ray diffraction data from the frozen crystals were collected on beamline NW12 at PF-AR (KEK, Tsukuba) using a Quantum-210 CCD detector (Area Detector Systems Corporation, Poway, USA). The data were processed with *HKL*2000 (Otwinowski & Minor, 1997) and reduced to structure-factor amplitudes with *TRUNCATE* (French & Wilson, 1978; Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

The final purification status of activated parasporin-2 was characterized by MALDI–TOF mass spectrometry (Fig. 1). The spectrum showed two major peaks. The peak at m/z = 27 505.3 corresponds to the calculated molecular weight of a polypeptide composed of the N-terminal methionine and Asp52–Gln302 of the protoxin (27 516.7 Da); the second peak at m/z = 27 021.0 corresponds to the weight of another derivative containing the N-terminal methionine and Asp52–Leu299 (27 031.2 Da). The low peak at m/z = 13 759.2 is assigned to a doubly charged species with a molecular mass of 27 516.7. Thus, the preparation of activated parasporin-2 contained two major molecular species that differ by three residues in length at the C-terminus; we consider it to be suitable for crystallization. The results also demonstrate that during the activation of the parasporin-2 protoxin proteinase-K removes 36 or 40 residues from the C-terminus of the protoxin in addition to the N-terminal 51 residues.

The initial crystallization lead was obtained using solution No. 37 of Crystal Screen [10%(*w*/*v*) polyethylene glycol (PEG) 8000, 8%(*v*/*v*) ethylene glycol, 100 m*M* HEPES–NaOH pH 7.5] as a reservoir solution; dodecahedral crystals with

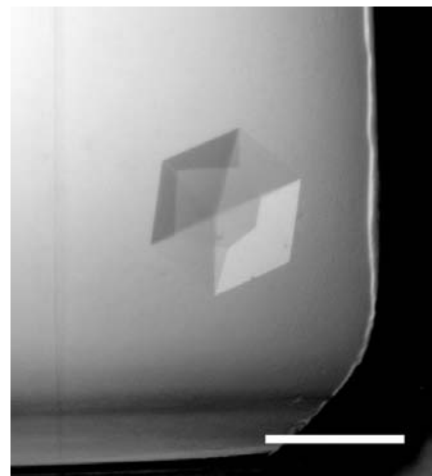

Figure 2
Crystal of activated parasporin-2. The scale bar is 0.3 mm in length.

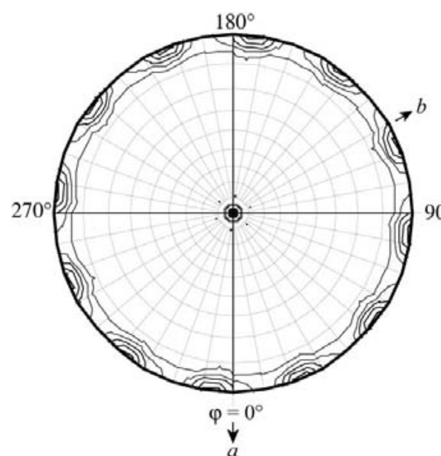

Figure 3
χ = 180° section of the self-rotation function for the activated parasporin-2 crystal. The calculation with *MOLREP* (Vagin & Teplyakov, 1997) used an integration radius of 20 Å and a resolution range of 10–3.5 Å. The section is contoured at 1σ intervals.

sharp edges appeared in 3 d and grew to 0.1 mm in the maximum dimension after a month. The reservoir solution was optimized by changing several parameters including the molecular weight and concentration of the PEG, the concentration and substitution of the ethylene glycol, the use of additives and the variation of the pH.

Crystals suitable for data collection were obtained with reservoir solution containing 16%(*v*/*v*) ethylene glycol, 8%(*w*/*v*) PEG 3350, 1 m*M* $CaCl_2$, 50 m*M* HEPES–NaOH pH 7.0. The incubation temperature was set to 277 K for the first 12 h, 288 K for the next 6 h and then 298 K. Dodecahedral crystals appeared within three weeks and grew slowly over three months to reach 0.3 mm in the maximum dimension (Fig. 2).

Preparative dialysis against a highly alkaline buffer was necessary prior to crystallization; crystallization setups without this step led to a swarm of microcrystals. A tendency for parasporin-2 to form large aggregates is suggested by the observation that the protein formed heavy precipitates two or three weeks after concentration to more than 4 mg ml^{-1} at pH 8.0. Therefore, the highly alkaline conditions presumably dissolve residual aggregates that are probably formed during storage and prepare a monodisperse solution suitable for crystallization. The initial incubation at low temperature was also necessary to reduce excess nucleation.

Table 1
X-ray diffraction data statistics.

Values in parentheses are for the last resolution shell.

Space group	$P6_1$ or $P6_5$
Unit-cell parameters (Å)	
$a = b$	134.37
c	121.24
Resolution range (Å)	25–2.75 (2.9–2.75)
No. observed reflections	364966 (51159)
No. unique reflections	32275 (4690)
Multiplicity	11.3 (10.9)
Completeness (%)	99.7 (100.0)
$\langle I \rangle / \langle \sigma(I) \rangle$	30.0 (4.4)
$R_{\rm merge}$† (%)	6.7 (56.6)
Wilson B factor (Å^2)	75.6

† $R_{\rm merge} = \sum_h \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_h \sum_i I_{h,i}$, where $I_{h,i}$ is the ith observation of reflection h and $\langle I_h \rangle$ is the mean intensity of reflection h.

X-ray diffraction data from a native crystal were collected to 2.75 Å resolution (Table 1). The crystals belong to the hexagonal space group $P6_1$ or $P6_5$, with unit-cell parameters $a = b = 134.37$, $c = 121.24$ Å. To examine the presence of non-crystallographic symmetry, a self-rotation function was calculated. The χ = 180° section of a map (Fig. 3) shows a clear peak of height 5.2σ at polar angles $\theta = 90°$, $\varphi = 22.7°$, which corresponds to a non-crystallographic dyad lying perpendicular to the crystallographic sixfold axis. No distinct peak was found in the χ = 120, 90 or 60° sections. Assuming two, four or six protein molecules each with a molecular weight of 27.5 kDa in the asymmetric unit, the Matthews coefficient ($V_{\rm M}$) was 5.7, 2.9 and 1.9 Å^3 Da^{-1}, respectively (Matthews, 1968). The first value is higher than the normal $V_{\rm M}$ range (1.7–3.5 Å^3 Da^{-1}) but is still acceptable, while the others are within the normal range. Thus, the asymmetric unit contains two, four or six molecules with one non-crystallographic twofold axis. A search for suitable heavy-atom derivatives is in progress.

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